Purification and Sequencing of α-Pinene Monooxygenase from *Pseudomonas fluorescens* NCIMB 11671

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A thesis submitted to the University of London in partial fulfilment of the requirements for the degree of Doctor of Philosophy

September 1994

Centre for Biotechnology

Department of Biochemistry

Imperial College of science, Technology and Medicine

University of London.

To Christos and my parents

ABSTRACT

This thesis describes the purification and sequencing of α–pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671 which appears to be a novel flavin-iron monooxygenase. This enzyme catalyses the NADH linked oxygenation of α–pinene to the corresponding epoxide, is induced by a-pinene and requires FAD and ferrous iron for activity.

α–Pinene monooxygenase has been purified 40-fold by ion exchange chromatography and chromatofocusing and was estimated to be at least 90% pure by electrophoresis. The native molecular weight of the enzyme which consists of two identical subunits is 131,000. It contains 2 moles of FAD/mole of enzyme and is remarkably stable especially when stored in phosphate buffers containing glycerol.

Complementation assays between a genomic library of *P. fluorescens* and mutants that were lacking the monooxygenase activity revealed a cosmid clone which contained the monooxygenase gene. Subcloning of the insert DNA, further complementation and expression procedures showed that the a-pinene monooxygenase gene is located within a 5.3-kb *Sma* I-*Sma* I fragment. A large open reading frame of 1.8 kb, encoding for the monooxygenase, was located within this fragment. No regions of homology to the *E. coli* consensus sequence were found upstream the coding region. However, the promoter region has strong homology to the *nif*-like promoters that have been shown to be regulated by the alternative σ^{54} factor. Possible FAD and NAD binding sites, as well as a Rieske type FeS centre were located at the amino-terminus, the middle, and proximal to the carboxy terminus of the deduced amino acid sequence of the monooxygenase gene respectively. Finally, a DNA sequence with striking similarity to proteins that belong to the NtrC activator family was located downstream the monooxygenase gene, and also two highly conserved NtrC activator binding sites were located just upstream of the promoter region.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. David Leak, for help, guidance and above all allowing me to pursue my own ideas during the course of this work; and the Greek Schrolarship Foundation (IKY) for providing the funding.

I am grateful to Katerina Hadjantonakis, Dr. Chan Kwo Chion (Alain) for all their help in molecular biology techniques, James Mansfield for his help in fermentations, Dr. Mark Woodland for his helpful pieces of advise in protein purification techniques, Dr. K. Saqib for providing me with some mutants, and all the members of the lab 390 for advise and suggestions.

Thanks to the members of the labs 021 and 340 both past and present for letting me use their equipment.

Finally, I would like to thank my fiancé, Christos Andronis, who patiently shared with me all the bad and good times of this project.

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ABBREVIATIONS

4α-FlOOH	4α-Hydroperoxyflavin
Acetyl-CoA	Acetyl-Coenzyme A
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
bp	Base Pairs
BSA	Bovine Serum Albumin
CAPS	3-{Cyclohexylamino}-1-Propansulfonic Acid
CFU	Colony Forming Units
CIP	Calf Intestine Alkaline Phosphatase
dATP	2'-deoxyadenosine 5' triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic Acid
dNTPs	dATP, dCTP, dGTP, dTTP
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylene Diamine Tetraacetic Acid
EPR	Electron Parmagnetic Resonance
EtBr	Ethidium Bromide
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
FPLC	Fast Protein Liquid Chromatography
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Pressure Liquid Chromatography
HSS	High Speed Supernatant
IEF	Isoelectric Focusing
IPTG	Isopropylthio-β–D-Galactoside
kDa	Kilodalton
LB	Luria Bertani
MCS	Multicloning Site
MMO	Methane Monooxygenase
MOPS	3-{N-Morpholino}-Propanesulfonic Acid
NAD(H)	Nicotinamide Adenine Dinucleotide
NADP(H)	Nicotinamide Adenine Dinucleotide Phosphate
NTG	N-methyl-N'-Nitro-N-Nitrosoguanidine
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PAH	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
pMMO	Membrane Bound Methane Monooxygenase
PVDF	Polyvinylidene membrane
RBS	Ribosome Binding Site
RNA	Ribonucleic Acid
sMMO	Soluble Methane Monooxygenase
SMO	Styrene Monooxygenase
TAE	Tris Acetate EDTA buffer
TBE	Tris-Borate EDTA buffer
TCA cycle	Tricarboxylic Acid Cycle
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	Thin Layer Chromatography
X-Gal	5-Bromo-4-Chloro-3-Indocyl-β–Galactoside

CHAPTER 1

Introduction

This thesis describes biochemical as well as genetic studies on the α–pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671. This enzyme catalyses the internal epoxidation of α–pinene to α–pinene oxide (Best *et al.*, 1987). Therefore, it is of interest both as a potential biocatalyst, and for the study of oxygen activation. The first chapter introduces some of the relevant background to put the work into context.

1.1 Biotransformations

Biotransformation is a biological process whereby an organic compound is modified into a recoverable product by simple, chemically defined reactions catalysed by enzymes contained in the cells. Biotransformation differs from fermentation in which the products, such as antibiotics, amino acids, organic compounds and solvents result from the complex biosynthetic or catabolic machinery of primary and secondary metabolism. Microbial, plant and animal cells can supply the enzymes for transformation, but microbes surpass the other two in several respects. Their surface area-volume ratio confers rapid growth and high rates of metabolism leading to efficient transformation of the substrate added. Moreover, the microbial world, rich in species, provides a varied assortment of enzymes for a tremendous variety of reactions on many classes of compounds, and with the facility to adapt to the artificial environment imposed by technical and economic requirements.

Biological systems obey the laws of chemistry. Nevertheless, biotransformations provide the means to carry out reactions that sometimes can

hardly be accomplished by chemical methods and thus, they may be preferred to chemical processes. Biological catalysts have been used not only in the preparation of pharmaceuticals, fine and commodity chemicals and food supplements, but also have provided model systems around which chemists have been able to design new catalysts which mimic the biological ones.

Enzymes are able to catalyse a wide range of organic reactions. They are characterised by high reaction specificity. The catalytic activity is usually restricted to a single reaction type, although in many cases the substrate range of the catalyst is quite broad. The regiospecificity of some enzymes in their reactions involving organic substrates is perhaps their major advantage over their chemical counterparts. The substrate molecule is usually attacked at the same site, even if several groups of equivalent or similar reactivity are present, as a result of specific binding interactions at the active site of the enzyme. An example is the second step in the commercial synthesis of ascorbic acid, where D-sorbitol is converted to L-sorbose by highly selective dehydrogenation with *Acetobacter suboxydans* (Vézina, 1987). Another potential advantage over classical organic synthetic techniques is the chirality of the enzymes. The reactive centre of an enzyme can provide an asymmetrical environment which allows discrimination between the enantiomers of a racemic substrate. Therefore, one of the enantiomers may be attacked preferentially. For example, L-aminoacylase from *Aspergillus oryzae* exclusively hydrolyses acyl-L-amino acids leaving the D-enantiomer of a racemic substrate untouched (Leuenberger, 1981). On the other hand, when the enzyme reaction produces an asymmetric centre, often only one of the enantiomers is formed, or one enantiomer predominates, and the product is optically active, e.g. asymmetric addition of H_2O to the double bond of fumaric acid by *Brevibacterium flavum* yielding L-malic acid. Moreover, reactions can occur at positions of the molecule that ordinarily do not react because of insufficient activation. For example the direct hydroxylation of the 11 position of the steroid nucleus of progesterone by *Rhizobium nigricans* for the production of corticosteroids, very much reduced the

number of steps of an otherwise complex and uneconomical chemical approach (Wang *et al.*, 1979). Finally biological reaction conditions are mild, thus permitting conversion of substances labile to extremes of pH or temperature. However, some features of enzymes, such as their use in aquatic environments, can make them difficult to use in conventional organic synthesis and may necessitate the design of specialised reaction conditions to allow an enzyme catalysed step to be economically incorporated into classical synthesis (Dordick, 1991; Godfresden, 1986; Suckling, 1990).

The substrate to be transformed can be added to growing cells, resting cells, spores, dried cells or to enzymes isolated from these cells either free, or immobilised on a solid support. During biotransformations with growing cells the substrate is usually added at the point of inoculation and the duration of incubation is relatively short, since both growth and biotransformation take place simultaneously. However, there are some advantages in the use of non-growing (resting cells) over the use of proliferating cells. Each step can be optimised individually: the cell density and physiology giving the optimum conversion rate can easily be determined; the system is less susceptible to infection by contaminant microorganisms and product isolation may be easier. Although the use of microbial cells is a cheap and easy way of carrying out a biotransformation procedure, microbial transformations do suffer from disadvantages not found with isolated enzymes. One of the greatest problems is the complex regulatory control of metabolism in living cells which often complicates the effectiveness of simple biotransformations. The conversion of interest may be a single step that lies in the middle of a complex pathway. Thus, significant genetic manipulation of cellular control may be necessary to isolate the product in high yields. On the other hand, the use of immobilized enzymes often offers high operational stability and prolonged operation with high product yields, but involves costly stages of enzyme purification.

By far the greatest commercial use of biotransformations has been in the food and beverage industry. The souring of wine into vinegar was practiced in Babylon by

5000 BC. and several other products, such as cheese, yoghurt, wine, beer, have always been produced through biological processes. Nowadays, microbes are used as chemical reagents for the preparation of key intermediates needed in organic synthesis of fine and medium-priced chemicals, such as pharmaceuticals, steroids, vitamins, flavours and fragrances. Another facet of microbial transformations embraces the degradation of fungicides, insecticides, herbicides and other environmental pollutants into innocuous compounds, in an attempt to improve the environment.

The reactions carried out by microorganisms fall into one of the following six categories: oxidation, reduction, hydrolysis, condensation, isomerisation and formation of C-C bonds or hetero-atom bonds (table 1.1).

One of the most important microbial transformations is hydroxylation, carried out by monooxygenases. These enzymes utilise molecular oxygen and require an electron transport system linked to NAD(P)H-dependent dehydrogenases. Microbial hydroxylations proceed, in some cases, via stereospecific replacement of a hydrogen atom present at the site to be oxidised. Hydroxylases have broad substrate specificity. Much work has been directed towards elucidation of the mechanism of steroid hydroxylations, since the role of microbes in steroid synthesis is central to the synthesis of adrenocortical hormones, corticosterone, cortisone and hydrocortisone, and their therapeutically superior derivatives, such as prednisone, prednisolone and triamcinolone. Four industrial scale transformations of progesterone are used nowadays: the 11α–hydroxylation performed by *Rhizopus arrhizus* and *R. nigricans*, the 11β–hydroxylation performed by *Cunninghamella blakesleeana* and *Curvularia lunata*, the 16α–hydroxylation performed by *Streptomyces argenteolus* and the C-1-hydrogenation performed by *Arthrobacter simplex*, *Septomyxa affinis* and *S. fradiae* (Wang *et al.*, 1979; Vézina, 1987).

Table 1.1: Reaction types mediated by microorganisms (taken from Leuenberger, 1981).

Oxidations	Hydroxylation, epoxidation, dehydrogenation of C-C bonds, oxidation of alcohols and aldehydes, oxidative degradation of alkyl, carboxy alkyl and keto alkyl chains, oxidative removal of substituents, oxidative deamination, oxidation of hetero-functions, oxidative ring fission.
Reductions	Reduction of organic acids, aldehydes ketones, and hydrogenation of C=C bonds, reduction of hetero-functions, dehydroxylation, reductive elimination of substituents.
Hydrolysis	Hydrolysis of esters, amines, amides, lactones, ethers, lactams, etc. Hydration of C=C bonds and epoxides.
Condensation	Dehydration, *O*- and *N*- acylation, glycosidation, esterification, lactonization, amination,.
Isomerisation	Migration of double bonds or oxygen functions, racemisation, rearrangements.
Formation of C-C bonds or hetero atom bonds.	

Bioconversions are valuable in the production of a single desired isomer in cases where the chemical process yields a racemic mixture. For example, epoxidation of the olefin *cis*-propenylphosphonic acid by *Penicillium spinulosum* yields only the (-) form of the antibiotic fosfomycin (figure 1.1), while chemical procedures lead to a mixture of both isomers.

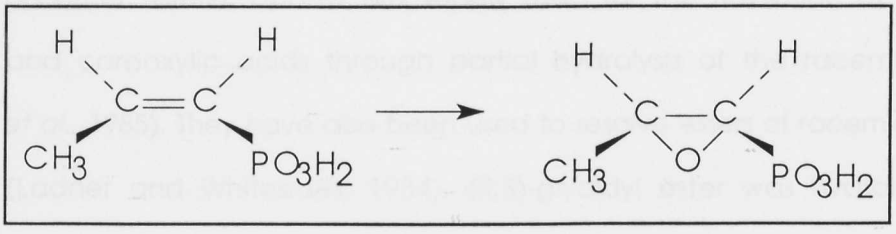


Figure 1.1: Production of fosfomycin by *Penicillium spinulosum* from *cis*-propenylphosphonic acid.

Another example of an enantioselective epoxidation reaction is the production of epichlorohydrin (figure 1.2),a potentially attractive intermediate in the synthesis of β–blockers, by halohydrin epoxidase from *Acinetobacter* sp. AK226 (Yamamoto *et al.*, 1990).

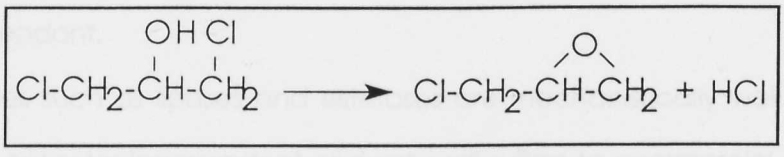


Figure 1.2: Production of epichlorohydrin by halohydrin epoxidase from *Acinetobacter* sp. AK226.

The use of esterases and lipases as catalysts for biotransformations is perhaps one of the most widely studied areas in this field. This is not only a result of their extreme usefulness, but also the fact that these enzymes are easily obtained and very robust, being able to tolerate a wide range of conditions not normally conducive to enzymatic action. Esterases are capable of catalysing the hydrolysis of esters, while lipases are similar to esterases but with a specificity for triglycerides (although they can act on other esters). Lipases are widely used for the resolution of racemic alcohols and carboxylic acids through partial hydrolysis of the racemic esters (Kirchner *et al.*, 1985). They have also been used to resolve esters of racemic epoxy-alcohols (Ladner and Whitesides, 1984). (R,S)-glycidyl ester was hydrolysed by porcine pancreatic lipase to yield a mixture of the chiral epoxyalcohol (R-glycidol) and epoxyester (R-glycidyl ester) with high enantioselectivity. Lipase appeared to be an ideal catalyst for this type of reaction as it was not deactivated through reaction with the epoxide moiety and was active at the organic/water interface thus negating a requirement for the substrate to be solubilised in an aqueous environment. The enantioselectivity of the hydrolysis depends on the structure of the acid components of the ester, being higher when the *n*-alkyl group of the ester is larger. A similar method has also been used to hydrolyse esters of epoxy secondary alcohols as an alternative to the Sharpless chemical oxidative kinetic resolution (Marples and Rogers-Evans, 1989). The enzymatic method produces optically active epoxy secondary alcohols and the enantioselectivity of the hydrolysis was found to be structure dependant.

Enzymes such as lipases and esterases are mechanistically well understood, highly stable, cofactor independent and are well suited to commercial applications. In aqueous media they carry out hydrolysis of their substrates, a reaction with little commercial appeal, except for its enantioselectivity. However, in an organic (low water) environment they can catalyse a wide range of synthetic reactions such as esterification, transesterification and interesterification (acyl-exchange). Lipases in both whole cell preparations or as purified enzymes have been used to carry out

esterifications in nearly anhydrous organic solvents. Suspension of mycelia (containing lipase) from *Rhizopus arrhizus* in octan-1-ol containing palmitic or oleic acid resulted in esterification of the fatty acid (Bell *et al.*, 1978). The water produced by this reaction caused a rise in the aqueous content of the reaction medium and eventually caused the reaction to cease. Removal of water from the system resulted in the production of a highly efficient, long lasting biocatalyst which has potential for use in industrial synthesis.

It is obvious that biotransformations offer a vast repertoire of reactions, a few of which have emerged as important industrial processes. For the industrial production of several organic compounds, the choice between biotransformation and organic synthesis, is purely economic. Newly introduced microbial processes often require important investment to secure the equipment necessary to handle large volumes of biological reagents under aseptic conditions. Low concentrations of substrates and products, and relatively slow reactions are typical of biological processes. Nevertheless, microbial transformations are preferred when they replace several chemical steps, e.g. the 11-hydroxylation of steroids, or when they give abundant access to new complex substrates which defeat organic manipulation.

1.2 Hydrocarbon Degradation by Microorganisms

1.2.1 Introduction

Studies of this field date back to the last century when Miyoshi (1895) first reported the hydrocarbon utilising microorganism *Botrytis cinerea*. It was only during the early 1960s, however, that hydrocarbon biochemistry expanded under the impetus of industrial research, when petroleum companies became interested in topics such as bacterial oil prospecting, refinery waste disposal, or deparaffinization of kerosine and gas oil (Bühler and Shindler, 1984). Since then, the use of inexpensive

petroleum fractions as a carbon source in fermentations, substituting for carbohydrates, has been widely investigated for biomass production as well as for biochemical synthesis of amino acids, fatty acids, vitamins and other substances of commercial interest (Bühler and Shindler, 1984). Gibson and Subramanian (1984) and Dagley (1985) have reviewed the biochemistry, the physiology, and the genetic studies of microbial degradation of aromatic hydrocarbons, and stressed the importance of mineralization of these compounds in the natural carbon cycle. Additionally, a wide range of microorganisms capable of attacking short chain *n*-alkanes and alkenes and/or long chain paraffinic or olefinic hydrocarbons up to C_{44} has been reported (Klug and Markovetz, 1971; Bühler and Shindler, 1984; Hartmans *et al.*, 1989).

The interaction of various organic compounds with microbial cells depends on the structure of the compound and the microbial species used in biotransformation processes. The observations on microbial hydrocarbon degradation, first elaborated by Zobel (1950), and refined by subsequent authors, serves as a general guide to the ability of microorganisms to degrade these hydrocarbons and hence the environmental recalcitrance of these compounds. These observations can be summarised as follows:

(i) Aliphatic paraffins are more readily degraded than aromatic hydrocarbons.

(ii) Many microorganisms preferentially utilise *n*-alkanes with a chain length of 9 to 18 carbon atoms because of their better degradability compared to lower *n*-alkanes. Short chain members show higher toxicity to the cells due to their better solubility in aqueous solutions.

(iii) Saturated compounds are degraded more readily than unsaturated compounds.

(iv) Branched chain compounds are degraded less readily than straight chain compounds, because the former are taken up more slowly into the cell and they are also less susceptible to the degradative enzymes of the β–oxidation pathway.

Aliphatic hydrocarbons in the gaseous, liquid or solid state are utilised by microorganisms. However, their catabolism depends on accessibility to the microbial cells. Both the microbial species and the nature of the substrate play important roles in deciding which mode of interaction is used. These include direct contact through attachment of the cells to large oil droplets; direct contact through accommodation of sub micron oil droplets onto the cell surface; or uptake of hydrocarbon dissolved in the aqueous phase. Gaseous hydrocarbons are soluble to some extent in aqueous solution and their solubility in water decreases as their molecular weight increases. A disadvantage of a poorly water-soluble gaseous substrate is the special attention that needs to be given in order to keep the substrate available to the organisms, but sometimes also to allow removal of gaseous reaction products such as carbon dioxide. Once available, gaseous hydrocarbons are easily transported through the cell membrane by diffusion (Vestal, 1984). On the other hand, the uptake of liquid hydrocarbons by microbial cells occurs predominantly from the solubilised or accommodated form (Reddy *et al.*, 1982). Microorganisms with a high affinity for hydrocarbons utilise both forms of uptake, while microorganisms with low affinity degrade the accommodated sub micron oil droplets more effectively than the oil drop form (Miura, 1978).

The degradation of hydrocarbons by microorganisms is often accompanied by the excretion of emulsifying agents and the formation of hydrocarbon dispersions. Excretion of such compounds is usually induced when the organisms grow on alkanes. Surfactants decrease the interfacial tension and increase the interfacial area of the hydrocarbon improving mass transfer, and thus, enhancing growth. For example, when *Corynebacterium lepus* was grown on glucose as sole carbon source, it synthesised and stored glycolipids intracellularly; whereas, when alkanes were added the glycolipid was excreted into the fermentation medium (Duvnjak and Kosaric, 1981). In addition to extracellular biosurfactants, appropriate structures on the cell surface may participate in stabilising alkane-water emulsions. Käpelli and Fiechter (1977) showed that cells of *Candida tropicalis*, when grown on *n*-alkanes exhibited 25%

higher adsorption of hydrocarbon than those grown on glucose. This increased binding affinity was due to the presence of a polysaccharide fatty acid complex on the cell surface. Finally, externally added surfactants and fatty acids also appear to stimulate the assimilation of alkanes by some species of *Acinetobacter* and *Pseudomonas* (Breuil and Kushner, 1980).

1.2.2 Alkane Oxidation and the Production of Epoxides

The predominant route for the degradation of hydrocarbons by microorganisms is aerobic with initial attack on the substrate requiring a mono- or a di-oxygenase. Oxidation of alkanes generally occurs through initial hydroxylation of the terminal methyl group either monoterminally, leading ultimately to 1-monocarboxylic acids via the corresponding aldehyde, or diterminally, leading to dioic acids. The latter pathway is mainly found in yeasts and to a minor extent in bacteria (Bühler and Shindler, 1984). Except for methane-utilisers, microorganisms oxidising short chain *n*-alkanes have not been extensively studied. However, two distinct groups have been recognised. Representatives of the first group of microorganisms are the obligate methane utilisers, growing only on C_1-compounds such as methane, methanol or methylamine. These compounds are converted to methanol, formaldehyde, formic acid and finally carbon dioxide. The second group includes microorganisms that oxidise the hydrocarbons through various intermediates to acetate, which is then metabolised via the glyoxylate shunt and the tricarboxylic acid cycle (Vestal, 1984).

Methane oxidation has been extensively studied in three strains, *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* and *Methylomonas methanica* (Dalton and Higgins, 1987). The soluble methane monooxygenase (MMO) from *M. capsulatus* consists of three components, a flavoprotein reductase (protein C) which has one FAD molecule and one Fe_2S_2 centre; a small protein of relative molecular mass of 20,000 (protein B) which lacks any prosthetic groups; and a terminal

non-haem iron oxygenase (protein A); and requires NADH for activity. Both *Methylococcus capsulatus* and *Methylosinus trichosporium* appear to produce two forms of MMO, the membrane bound and the soluble form, depending on the availability of copper in the growth medium. In a high-copper medium the organisms preferentially synthesised the particulate MMO, the soluble enzyme being expressed in response to copper "stress" (Leak *et al.*, 1985). The enzyme exhibits broad substrate specificity and is able to catalyse the hydroxylation of primary and secondary C-H bonds (up to hexadecane), the formation of epoxides from terminal and subterminal alkenes, the hydroxylation of the aromatic nucleus, the oxidation of CO to CO_2 and the *N*-oxidation of pyridine. The ability of MMO to catalyse the epoxidation of terminal and subterminal alkenes appeared to be very promising, for the development of a general purpose epoxidation catalyst. However, although the system was able to epoxidise ethene, propene and 1-butene to their corresponding 1,2-epoxyalkanes, with no other oxidation by-products, the epoxides produced were racemic. In addition, in epoxidation reactions of internal alkenes, not only the epoxides but also the corresponding primary alcohols and ketones were produced (Colby *et al.*, 1977; Weijers *et al.*, 1988).

Hydroxylation of higher alkanes is catalysed by one of two different types of monooxygenase, or mixed function oxidase, cytochrome P-450 systems, and non-haem iron monooxygenases. Hydroxylase systems of the cytochrome P-450 type are widely distributed among eukaryotes and prokaryotes. The involvement of cytochrome P-450 in these systems is easily detected by its sensitivity to carbon monoxide and the characteristic spectra of its carbon monoxide inhibited state. The non-haem iron hydroxylation systems reported in several *Pseudomonas* and *Acinetobacter* species are sensitive to cyanide but not to carbon monoxide. In *n*-tetradecane grown *Candida tropicalis* the P-450 linked hydroxylase system is located within the microsomes and consists of cytochrome P-450, an NADPH-cytochrome c reductase and a phospholipid. Growth on alkanes with more than 13 carbon atoms induced the synthesis of the P-450 components and also induced the microsomal

alcohol and aldehyde dehydrogenases (Bertand *et al.*, 1979). The catalytic mechanism of the hydroxylation system from yeast is not fully understood yet, however, it appears to be similar to the microsomal hydroxylase system from liver (Bühler and Shindler, 1984).

Another cytochrome-linked hydroxylation system that catalyses the NADPH-dependent oxidation of *n*-hexadecane to *n*-hexadecanol has been purified from *P. aeruginosa*. The system consists of a non-haem iron oxygenase, a haem protein with spectral characteristics of cytochrome c, and a reductase which is a flavoprotein of low molecular weight (Matsuyama *et al.*, 1981).

Cytochrome P-450 linked monooxygenase systems are usually NADPH-dependent, although some exceptions have been observed. NADH was found to be the cofactor of the alkane monooxygenase system from *Corynebacterium* 7E1C (Cardini and Jurtshuk, 1970) responsible for the oxidation of *n*-octane to *n*-octanol. The system is membrane bound and consists of an NADH-specific flavoprotein reductase, an iron-sulphur protein and a terminal cytochrome P-450 oxygenase. The electron transfer pathway in the *n*-alkane hydroxylase from *Corynebacterium* sp. is shown in figure 1.3., and is characteristic of the bacterial cytochrome P-450 systems.

There is considerably less information on alkane hydroxylation systems that do not contain a cytochrome P-450. The ω–hydroxylase system of *P. oleovorans* var. *putida* originally purified by Coon and co-workers, consists of three components, a NAD(P)H rubredoxin oxidoreductase, with one mole of FAD per mole of enzyme and one Fe_2S_2 centre (Ueda *et al.* 1972), a rubredoxin capable of binding two irons per molecule and serves as an electron carrier (Peterson and Coon, 1968) and a non-haem iron containing ω–hydroxylase which is a membrane associated protein (McKenna and Coon, 1970). The system, in the presence of NAD(P)H and molecular oxygen, can catalyse the hydroxylation of the methyl groups of *n*-alkanes and fatty acids with more than 12 carbon atoms (McKenna and Coon, 1970; Nieder and Subramanian, 1975); epoxidise 1-alkenes to their corresponding 1,2-epoxyalkanes (May and Abbot, 1972a; Abbot and Hou, 1973); and epoxidise terminal dienes to their

corresponding diepoxyalkanes. However, it cannot epoxidise cyclic or internal olefinic groups, aromatic compounds or styrene (May *et al.*, 1975).

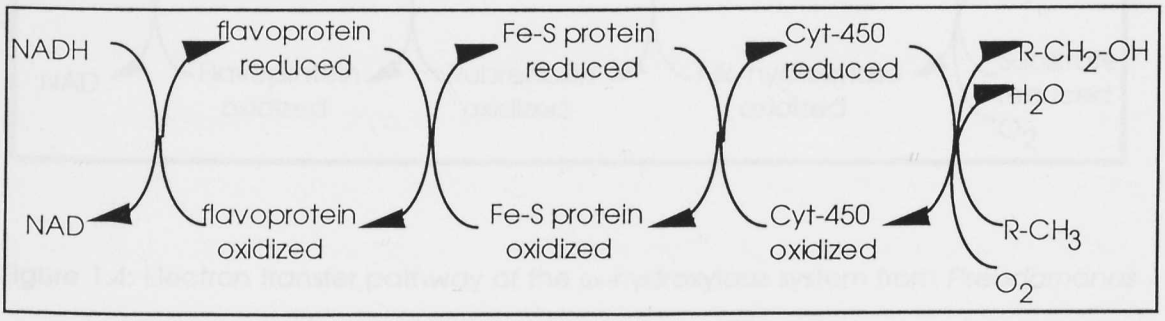


Figure 1.3: Electron transfer pathway of the inducible alkane hydroxylase system from *Corynebacterium* 7EIC (Cardini and Jurtshuk, 1970).

The rates of hydroxylation and epoxidation are similar, and all of the system components plus NADH and molecular oxygen are required for the reaction to occur. In addition, both reactions are inhibited by cyanide, affected similarly by pH, and show same cofactor selectivities. Thus, it was suggested (May and Abbot, 1972b) that epoxidation and hydroxylation proceed via similar mechanisms. The ratio of hydroxylation to epoxidation depends upon the chain length of the alkene. The system showed no epoxidation activity with propene and butene, whereas it showed maximal epoxidation activity when terminal alkenes with 8 carbon atoms were used as substrates. This activity reduced with increasing chain length. However, the methyl group hydroxylation activity of the enzyme is less sensitive to the chain length for short chains, but decreases rapidly as the chain length increases (May *et al.*, 1975). Thus, for example, epoxidation tends to dominate over hydroxylation by 2:1 using 1-decene as a substrate. Figure 1.4 presents the mechanism of electron transport through the ω–hydroxylase system.

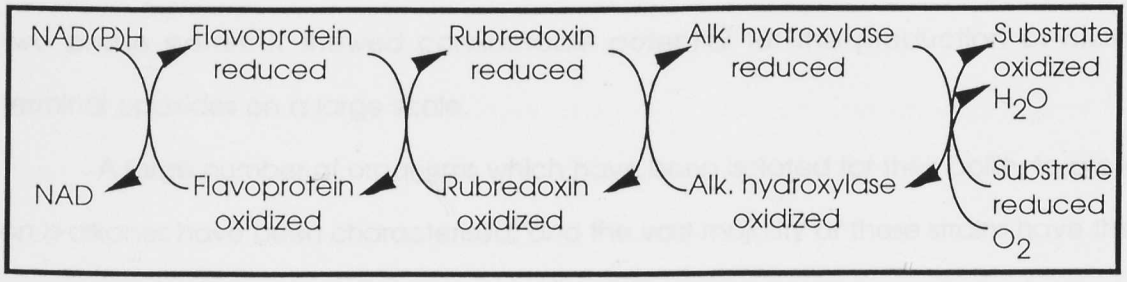


Figure 1.4: Electron transfer pathway of the ω–hydroxylase system from *Pseudomonas oleovorans* var. *putida*.

May and Schwartz (1974) first noted the ability of the ω–hydroxylase system to stereoselectively epoxidise terminal acyclic alkenes, producing (*R*)-(+)-1,2-epoxides together with ω–alkene-1-ols. They found that 1,7-octadiene was preferentially epoxidised to give (*R*)-(+)-7,8-epoxy-oct-1-ene with an optical purity of more than 80% and concluded that if the epoxidation involved the concerted addition of active oxygen to the π electron system of the double bond then the formation of (*R*)-(+)-7,8-epoxy-1-octene must involve attack at the *si-si* face of the prochiral octadiene molecule. Further oxidation gave 1,2-7,8-diepoxyoctane with similar degree of optical purity (May and Schwartz, 1984; Schwartz and McCoy, 1977). Further investigation by May *et al.*, (1977) on the stereochemistry of the reaction revealed that epoxidation of the racemic 7,8-epoxy-1-octene gave a product with poor optical purity. This indicates that the configuration of the epoxide group may have a marked effect on the stereochemistry of the insertion of the second oxygen and this may be one of the reasons for a lessening of the stereoselectivity compared with epoxidation of 1-octene yielding 85% (*R*)-form and 15% (*S*)-form of epoxides. This was also evident from the finding that the terminal methyl group of (*R*)-(+)-1,2-epoxyoctane was hydroxylated faster than that belonging to (*S*)-(+)-1,2-epoxyoctane (de Smet *et al.* 1983). Although the 1,2-epoxyoctane produced by this method was not optically pure,

the enantiomeric excess was far greater than that achieved by any chemical route and therefore in conjunction with the increased epoxide yields from cell renewal in a two phase system it showed considerable potential for the production of chiral terminal epoxides on a large scale.

A large number of organisms which have been isolated for their ability to grow on *n*-alkanes have been characterised, and the vast majority of these strains have the ability to epoxidise terminal double bonds. However, these types of organisms are generally unable to grow on terminal alkenes as they lack the ability to further metabolise the 1,2-epoxyalkanes which have been shown to accumulate extracellularly. It has been concluded that the epoxidation ability resides in an alkane monooxygenase which can hydroxylate terminal methyl groups, and fortuitously epoxidise terminal double bonds.

Subterminal degradation of long chain alkanes has also been observed (Bühler and Shindler, 1984). By this type of oxidation one or more methylene groups of the *n*-alkane are oxidised to secondary alcohols and then to the corresponding ketones, which are further metabolised via esters to (n-1) fatty acids. The initial attack on the *n*-alkane occurs by a similar hydroxylation mechanism to those described above. The alcohols are subsequently converted to the corresponding ketones by a secondary alcohol dehydrogenase. The transformation of the ketones to the corresponding esters is a Bayer-Villiger type reaction. Ester formation requires incorporation of molecular oxygen, and this is catalysed by an NADPH-dependent monooxygenase. *P. cepacia* and *Nocardia* sp. are able to catalyse the oxidation of 2-tridecanone into undecyl acetate which is further split into 1-undecanol and acetate. The formation of an acetate ester intermediate by these organisms has been attributed to a Bayer-Villiger type of monooxygenase (Britton and Markovetz, 1977). The purified enzyme from *P. cepacia* contains FAD, has a molecular mass of 110,000 and is composed of two identical subunits.

1.2.3 Alkene Oxidation and Production of Epoxides

Many hydrocarbons are present in the biosphere, derived from a number of biological and anthropogenic sources. The study of hydrocarbon metabolism by bacteria initially centred around the mineralisation of aliphatic and aromatic hydrocarbons derived from natural sources or spillages and has been covered by a number of reviews (Gibson and Subramanian, 1984; Atlas, 1981). The short chain unsaturated gaseous hydrocarbons are widely distributed in the environment and many of them have been used extensively in the chemical industry. However, until recently the study of microbial degradation of these substrates has received considerably less attention. Two of the major gaseous alkenes, ethene and isoprene are produced naturally. Ethene is a plant hormone and is also produced by some fungi and bacteria. Isoprene, the basic building block of terpenes, is also excreted by many plants (Rasmussen, 1970). The industrial production of ethene on a large scale world wide emphasises its usefulness in many processes. However, leakage of ethene from industrial operations accounts for a relatively small proportion of that present in the environment with 74% derived from biological sources and the majority of the remaining 26% being derived from burning biomass (Sawada and Totsuka, 1986). The remaining gaseous alkenes propene, 1-butene, 2-butene, and butadiene are generally linked to industrial activities with substantial increases in their concentrations in areas where they are being used. The increased interest in the study of organisms degrading alkenes and alkynes is partly due to their contribution in the production of carbon monoxide and involvement in the production and degradation of ozone (Hartmans *et al.*, 1989). The organisms involved may find amplification either in the treatment of waste gases or in the biotechnological production of epoxides.

Unsaturated hydrocarbons may be metabolised by an initial attack on the unsaturated moiety of the molecule, or by an attack at the terminal methyl group. The following reactions may be observed at the unsaturated moiety of the molecule: (i) Haloperoxidases catalyse the formation of α,β- halohydrins from alkenes and α–

halogenated ketones from alkynes in the presence of halide ion and hydrogen peroxide (Geigert *et al.*, 1986). (ii) The hydration of the double bond, which may be carried out stereospecifically, as in *Pseudomonas* sp. which catalyses the formation of the R- form of 10-hydroxystearic acid from oleic acid. (Wallen *et al.*, 1962; Shoepfer, 1966). It is unlikely that such reaction would be a part of an oxidative pathway. Nevertheless, the hydration of 1-hexadecene to hexadecanol by a methanogenic enrichment culture (Shink *et al.*, 1985) is possibly the initial step of the metabolism of this compound, however, it remains one of the few examples to date. (iii) Although reduction of double bonds has been observed in fatty acid synthesis, examples of reductases acting on such bonds are very scarce. One example is the hydrogenation of fatty acids by anaerobic rumen bacteria (Kellens *et al.*, 1986). However, there is no evidence for such a reduction of unsaturated carbon-carbon bonds in the mineralization of unsaturated hydrocarbons. (iv) The oxidation of the double bond by a monooxygenase. The first evidence for the oxidation of the double bond of an alkene was the isolation of 1,2-dihydroxyhexadecane from cultures of *Candida lipolytica* growing on 1-hexadecene (Klug and Markovetz, 1971). Van der Linden (1963) had shown that *Pseudomonas* sp. cells grown on 1-heptene were able to catalyse the formation of 1,2-epoxyheptane, and subsequently many bacteria able to catalyse epoxidations were reported. As stated earlier, alkane grown organisms possess the ability to both hydroxylate terminal methyl groups and epoxidise terminal unsaturated moieties of hydrocarbons. This epoxidation appeared to be fortuitous with the epoxide playing no defined role in the metabolism of terminal alkenes. More recently however a number of examples of organisms which can grow on alkenes but not alkanes have been investigated. These organisms appear to possess a requirement for an unsaturated moiety in their target molecule, the initial oxidative attack forming an epoxide which was then degraded by the organism and was able to serve as the sole source of carbon and energy. The first reports of an epoxidation reaction actually participating in the complete metabolism of an alkene were for the ethene grown *Mycobacterium* E20 (de Bont and Harder, 1978; de Bont *et al.*, 1979).

Epoxides (epoxyalkanes or alkene oxides) are very reactive molecules. In mammals these compounds are formed from alkenes and aromatic compounds through the action of P-450 containing enzymes located on the endoplasmic reticulum. Although the starting materials, (like polycyclic aromatic hydrocarbons (PAH) that are widely distributed in the environment and represent potential health hazards), are themselves biologically inactive, the reactive epoxides may then bind to several tissue components including proteins and nucleic acids and this feature explains the chemical toxicity and carcinogenicity of some drugs, mutagens and carcinogens, containing unsaturated bonds (Weijers *et al.*, 1988; Leak *et al.*, 1992). Epoxides are further degraded in mammals by epoxide hydrases and glutathione S-transferases, resulting in less reactive and consequently less harmful compounds.

The formation and further catabolism of epoxides in microorganisms has also been studied in order to unravel metabolic pathways and provide additional information on the human detoxification process for these compounds. More recently, epoxide metabolism in microorganisms has been studied from the point of view of applied biocatalysis. Epoxides are used as intermediates in organic synthesis as well as bulk chemicals in the manufacture of polymers. They are cyclic ethers, but due to the characteristics of their structure (i.e. the strain inherent of the three-membered ring, combined with the polarity arising from the electronegativity of the oxygen), they possess considerably higher reactivity than normally associated with ethers. Therefore, epoxides can react readily with a large number of reagents, including nucleophiles, electrophiles, acids, bases, reducing agents and some oxidising agents (Leak *et al.*, 1992; Onumonu *et al*, 1994). The chemical production of chiral epoxides has been studied extensively (Scott and Morrison, 1984). Attempts to prepare optically active epoxides via asymmetric oxidation include the use of chiral peracids, chiral phase transfer catalysts, and organometallic complexes with chiral ligands. However, the preparation of non-functionalised chiral epoxides with high enantiomeric purity remains a formidable task, usually requiring tedious resolution of appropriate precursors. Therefore, biological production methods are of particular

interest when the epoxides obtained are in optically pure form. Such chiral epoxides can then serve as synthons for the production of other valuable optically active compounds.

Microorganisms able to grow on gaseous alkenes have been isolated from a range of sources, some of which have been exposed to anthropogenic alkene contamination. The organisms were isolated as pure cultures by growth on minimal medium supplied with the alkene used as sole carbon source at the initial enrichment step. Ethene enrichment cultures always resulted in the isolation of *Mycobacteria* (de Bont, 1976). A ethene-utilizer originally identified as a *Micrococcus* sp. (Mahmoudian and Michael, 1992) has also been shown to be a *Mycobacterium* sp. (Leak, personal communication). Whole cells of ethene-grown *Mycobacterium* E20 were also able to oxidise ethane, but this activity was not present in crude extracts. Ethane grown cells were able to oxidise both substrates and it was assumed that this organism produced two monooxygenases: a soluble alkene monooxygenase after growth with ethene, and a membrane bound, unstable alkane monooxygenase after growth on ethane. Incubation of ethane grown *Mycobacterium* E20 cells with ethane in the presence of fluoroacetate (an inhibitor of the tricarboxylic acid cycle), resulted in accumulation of acetate, while ethene grown cells accumulated epoxyethane. In this way, de Bont and Harder (1978) showed that enzymatic degradation of epoxyethene is dependent upon the presence of NAD^+, acetyl-CoA, FAD and a fourth dissociable cofactor. Further work with *Mycobacterium* E20 confirmed the presence of an inducible alkene specific monooxygenase (de Bont *et al.*, 1979). Enzymatic oxidation of ethene by cell free extracts, prepared from ethene grown cells, depended on the presence of NADH and O_2 (NADPH could replace NADH but with only 25% of the activity). Moreover, resting cell preparations of ethene grown *Mycobacterium* E20 were able to oxidise a wide range of *n*-alkanes and 1-alkenes, but when this activity was investigated in cell free extracts only the terminal alkenes (propene, butene and butadiene) were oxidised to their corresponding 1,2-epoxides.

Many bacteria are capable of oxidising propene to 1,2-epoxypropane (Hou *et al.*, 1983). In contrast to ethene, propene is an asymmetric molecule (with an unsaturated and a saturated carbon-carbon bond), allowing more than one possibility for initial enzymatic attack. *Pseudomonas fluorescens* and *Brevibacterium* sp. catalyse both the hydroxylation and epoxidation of propene to 3-hydroxy-1-propene and 1,2 epoxypropane respectively (Hou *et al.*, 1983). *Mycobacterium* Py1 (de Bont *et al.*, 1983) and *Xanthobacter* Py2 (Van Ginkel and de Bont, 1986) which were isolated from propene enrichment cultures, as well as *Nocardia* By1 and *Xanthobacter* By2 (Van Ginkel *et al.*, 1987) which were enriched with 1-butene, were able to catalyse the oxidation of 1-alkenes to 1,2-epoxyalkanes. A NADH-dependent propene monooxygenase activity was detected in cell free extracts from strains Py1 and Py2, that were able to utilise both 1-propene and 1-butene. Moreover, both strains were able to further degrade the epoxide. Py1 probably degrades it via 1,2-propanediol (de Bont *et al.*, 1983), while Py2 degrades it via acetone (Swaving *et al.*, 1994).

Isoprene (2-methylbutadiene) is a naturally occurring compound whereas butadiene is not formed biologically (Van Ginkel *et al.*, 1987). *Nocardia* TB1 which was isolated from enrichments with butadiene as sole carbon source was able to oxidise the alkadienes butadiene and isoprene in the presence of 1,2-epoxyalkanes as competitive inhibitors of epoxide degradation, resulting in the production of all possible mono- and diepoxides with stereoselectivity (Watkinson and Somerville, 1976). The epoxides were further metabolised by conversion to the corresponding diol and then the acid. The organism was unable to grow on 1 or 2-alkenes but could utilise gaseous alkanes as growth substrates.

Although styrene contains an aromatic nucleus, it can also be classified as a substituted alkene. When styrene concentrations in enrichment cultures were low, styrene utilisers were found to be abundant (Hartmans *et al.*, 1989). In several of these samples, an oxygen- and NADH-dependent styrene degrading enzymatic activity was detected in cell free extracts after growth with styrene. Further metabolism of the

suggested oxidation product, styrene oxide, occurred via phenyacetaldehyde, which was formed by a styrene oxide isomerase, and resulted in the production of 2-phenylethanol or phenylacetic acid. Such a pathway indicated that styrene metabolism involved an initial attack of the ethylenic bond. Biotransformation of styrene to styrene oxide by whole cells was first reported by Higgins *et al.* (1979) by using *Methylosinus trichosporium* OB3b. This oxidation was probably the result of the broad specificity of the methane monooxygenase system in these cells. *Nocardia corallina* B-276 was also reported to catalyse the stereoselective formation of styrene oxide by attacking the unsaturated moiety of the molecule (Furuhashi *et al.*, 1986). Oxidation of styrene to styrene oxide was also reported for *Methylococcus capsulatus* (Bath) and *Brevibacterium* sp. strain CRL 56 (Hou *et al.*, 1983). Finally, Hartmans *et al.* (1990) reported the production of styrene oxide from 14 bacterial isolates, most of which showed a novel FAD-requiring monooxygenase (SMO) activity.

A general pattern has emerged from the isolation of bacteria capable of growth on alkenes. Alkene monooxygenases from a variety of bacteria, have a broad substrate specificity, and thus, are able to catalyse the epoxidation of terminal and sub-terminal alkenes but not the hydroxylation of alkanes (Habets-Crutzen *et al.*, 1984). Moreover, they appear to be more stable in the presence of epoxyalkanes than alkane monooxygenase type enzymes. In these organisms the inability to grow on some alkenes appears to be due to the inability of the epoxide degrading systems to deal with the epoxides formed via the monooxygenase. Thus, epoxides from other terminal alkenes could be accumulated in the extracellular environment. One of the major differences between alkane utilising systems which have a "non-specific" ability to epoxidise alkenes as well as hydroxylate alkanes, and alkene utilising systems which have an absolute requirement for a double bond, is that alkene utilising bacteria can catalyse the stereoselective epoxidation of alkenes whereas generally the alkane utilising systems do not, or produce chiral epoxides at a considerably lower enantiomeric excess. The types of reaction are demonstrated in figure 1.5.

Generally, organisms grown on methane or gaseous *n*-alkanes form racemic mixtures of epoxyalkanes. However the enantiomeric composition of epoxyalkanes produced using an alkene grown bacterium is very dependant both on the type of substrate and the organism used to catalyse the reaction demonstrating the necessity for effective screening programs to detect the desired properties (Weijers *et al.*, 1988; Leak *et al.*, 1992).

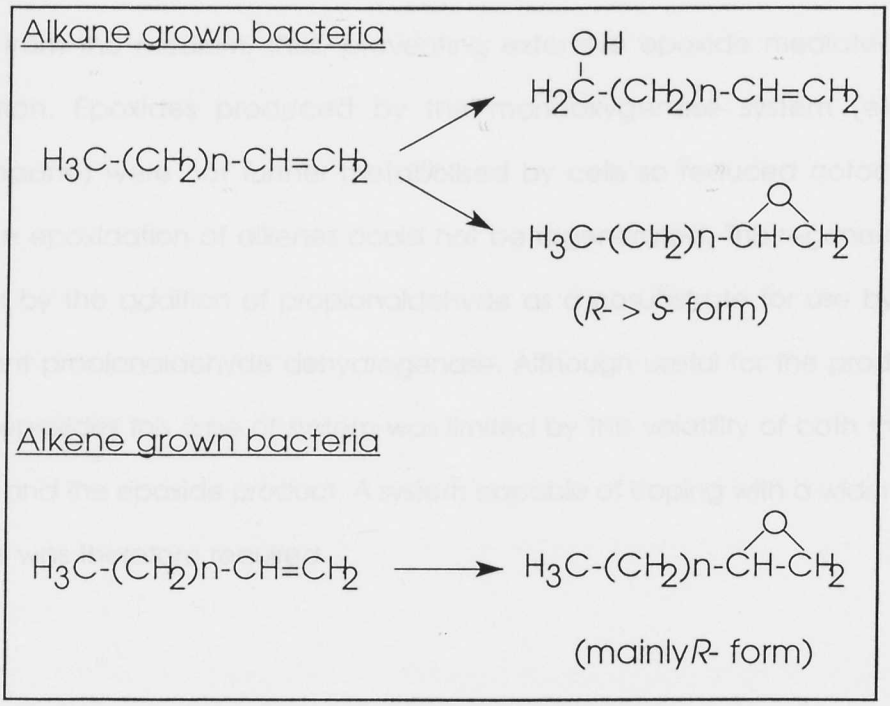


Figure 1.5: Reaction types of alkane and alkene monooxygenases

Alkene utilising bacteria have been employed for producing epoxides which are believed to have a good market as synthons for the preparation of biologically active compounds. The Nippon Mining Company in Japan (now Japan Energy Corporation) was the first to announce the production of chiral epoxides from alkenes through a monooxygenase type reaction using *Nocardia* sp. B276. Later on, a joint venture between Shell and Gist-Brocades announced the production of epoxides

from arylallyl ethers (Johnstone *et al.*, 1986) using *Pseudomonas oleovorans*. Use of whole cells in such processes would have considerable advantages over the use of immobilised enzymes avoiding enzyme isolation procedures and facilitating cofactor regeneration. *Mycobacterium* Py1 has also been studied as an immobilised biocatalyst in a gas/solid bioreactor (de Bont *et al.*, 1983). The gas flow, containing the substrates for the reaction (i.e. oxygen and alkene), as well as the products (i.e. epoxides), was recirculated allowing the system to operate at optimal substrate concentration. Recirculation also allowed for the continuous removal of the toxic product from the medium, thus, preventing extensive epoxide mediated catalyst inactivation. Epoxides produced by the monooxygenase system (except for epoxypropane) were not further metabolised by cells so reduced cofactors used during the epoxidation of alkenes could not be regenerated. This regeneration was achieved by the addition of propionaldehyde as a cosubstrate for use by a NAD^+ dependant propionaldehyde dehydrogenase. Although useful for the production of gaseous epoxides this type of system was limited by the volatility of both the alkene substrate and the epoxide product. A system capable of coping with a wider range of substrates was therefore required.

1.3 Microbial Degradation of Terpenoid Compounds

Terpenoid compounds are formed biologically from C-5 isoprene units and have a structural formula $(C_5H_8)_n$. They occur naturally in plants, as essential oils, in insects, as sex pheromones, and in marine organisms, and encompass a structural diversity that ranges from simple branched-chain hydrocarbons through the monocyclic and bicyclic monoterpenes to complex sesquiterpene structures. In addition to the parent hydrocarbons there are oxygenated derivatives of each class which also occur naturally in many instances, such as alcohols, aldehydes and ketones. The structural complexity of naturally occurring terpenes increases with

chain length. Therefore, the necessary recycling of this organic carbon in nature presents a number of intriguing problems associated with the elimination of side chains, the activation and cleavage of the carbocyclic rings, and the provision of intermediates for central metabolic pathways (Trudgill, 1986).

Microbial transformations of terpenoid compounds have challenging industrial possibilities. They are potentially interesting for practical applications especially in the fragrance and flavour industry, as well as in the pharmaceutical and chemical industry (Krasnobajew, 1984; Best *et al.*, 1987). All microbial transformations of terpenes, reported so far, result in an enzymatic attack at several sites of the molecule, leading to a mixture of metabolites. Therefore, accumulation of a single transformation product is very rare. A large number of enzymes capable of terpene catabolism are no specific in their action (Trudgill, 1984) leading to a cascade of transformations, which in turn result in a great number of products. Thus, although these compounds are widely distributed, few complete degradative pathways have been elucidated to date. The reactions which may occur include hydrogenation, reduction, Bayer-Villiger oxidation, allylic oxidation, epoxidation and hydroxylation. The reaction sites of a terpenoid molecule susceptible to the above degradation procedures are shown in figure 1.6.

Terpenoids pose very specific problems to microorganisms that are able to utilise them. In the case of parent hydrocarbons, there is a requirement to activate a methyl or methylene carbon atom, and this is typically achieved by the action of a monooxygenase that is capable of catalysing the hydroxylation of terminal methyl groups. The related terpene alcohols, aldehydes and ketones already possess a functional group and therefore, they may not need an initial monooxygenase attack. However, this group may not be in a position that readily facilitates further transformation of the molecule and thus, the introduction of an additional hydroxyl group may be necessary. The open chain monoterpenes are typically attacked by oxygenation of the terminal methyl group. Further oxidation of the resulting alcohol leads to the corresponding aldehyde and then to the acid, that finally undergoes β-

oxidation. In the case of mono- and bicyclic monoterpenes the additional problem of ring cleavage is imposed. Studies on the degradation of (+)-camphor by *P. putida* C1B revealed that the terpene molecule is initially attacked by a cytochrome P-450 containing hydroxylase, with the alcohol subsequently converted into the corresponding ketone by the action of an NADH dependent-alcohol dehydrogenase. The following decyclisation reaction appears to be catalysed by a specific type of ketone 1,2-monooxygenase. The enzyme inserts one atom of oxygen into the cyclic ketone and reduces the other atom to water, drawing electrons from NADH. Such a reaction belongs to the biological Bayer-Villiger group of reactions which result in the conversion of stable cyclic ketones into unstable lactones that may subsequently be hydrolysed with the formation of substituted carboxylic acids. This type of oxygen activation is catalysed by simple flavoproteins, devoid of transition metals and consisting of a single polypeptide chain or a multimeric protein composed of a single species of subunit (Donoghue *et al.*, 1976). These enzymes display broad substrate specificity, which may be of ecological significance for the degradation of a wide range of alicyclic compounds of anthropogenic origin accumulated in the environment. Elegant stop-flow studies performed by Ryerson *et al.* (1982) established the mechanism of oxygen insertion into cyclohexanone by 1,2-cyclohexanone monooxygenase. The four-electron reduction of oxygen, at the expense of the two-electron oxidation of cyclohexanone to ε-caprolactone, and the two-electron oxidation of NADPH involves the participation of a 4α–hydroperoxyflavin, the distal oxygen of which reacts as a nucleophilic atom attacking the electrophilic carbonyl carbon in the ketone substrate.

An alternative ring cleavage reaction leading to the formation of a novel aldehyde, through the action of a lyase, has been reported by Best *et al.* (1987) and Griffiths *et al.* (1987a) in the degradation of α–pinene by *P. fluorescens* NCIMB 11671 and *Nocardia* sp. P18.3 respectively. α–Pinene is the most abundant terpene in nature, and is industrially obtained by fractional distillation of turpentine. The chemical industry produces numerous important substances for perfumes from the inexpensive α–

pinene and microbiological approaches for the conversion of α–pinene are also challenging. However, economic production of transformation products by fermentation is restricted by the low conversion rate, the high volatility of the organic substrate and the decomposition of the substrate by autooxidation (Krasnobajew, 1984). Moreover, many terpenes have been reported to possess antibiotic function against diverse microbial flora. However, along with some other soil microorganisms, *Pseudomonas* are very well adapted to play a major role in the degradation of terpenes, since they are ubiquitous in nature and have the metabolic flexibility to grow with a wide range of organic compounds as sole carbon sources (Stanier, 1966).

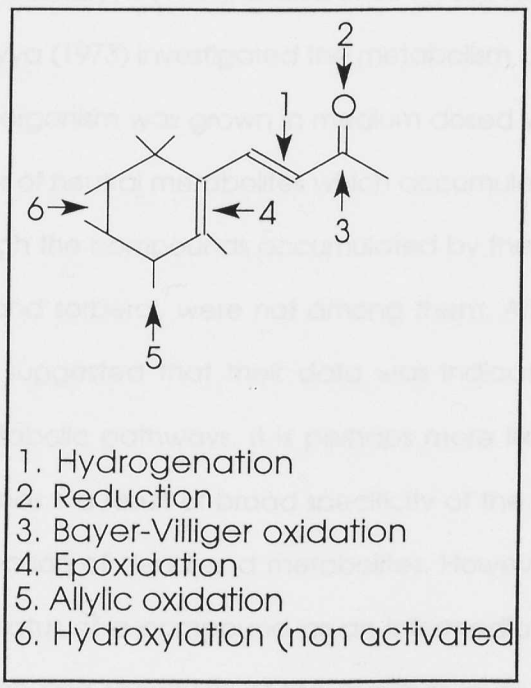


Figure 1.6: Reaction sites of terpenoid substrate molecules which are susceptible to chemical reagents and also easily attacked by corresponding enzyme systems (Krasnobajew, 1984).

Although several groups of workers have studied the metabolism of α– and β–pinenes by a number of microorganisms, including *Pseudomonas* sp.. the routes by which these monoterpenes are degraded to produce central metabolites have not been completely elucidated. The earliest studies on the microbial transformation of α–pinene were performed by Bhattacharyya *et al.* (1960) who reported a strain of *Aspergillus niger* that when grown on yeast extract and corn-steep liquor containing α–pinene, accumulated *cis*-verbenol, verbenone and *trans*-sorberol. The accumulation of such metabolites was indicative of an initial oxygenative attack at the C-4 position of α–pinene. However, two years later, Prema and Battacharyya (1962b) reported that verbenol, verbenone and sorberol were autooxidation products of α–pinene, produced when it was exposed to air, moisture or sunlight.

Shukla and Bhattacharyya (1968), Shukla *et al.* (1968), Hugund *et al.* (1970), and Pugar and Bhattacharyya (1973) investigated the metabolism of α– and β– pinenes by *Pseudomonas* PL. The organism was grown in medium dosed with α–pinene every 24 or 72 h. A large number of neutral metabolites which accumulated in the culture fluids were identified, although the compounds accumulated by the *Aspergilus niger* strain, verbenol, verbenone and sorberol, were not among them. Although Bhattacharyya and co-workers have suggested that their data was indicative of discrete routes leading to central metabolic pathways, it is perhaps more likely that the variety of accumulated compounds is a result of broad specificity of the induced enzymes that could lead to the formation of dead end metabolites. However, the best criteria to support the assigned status of a compound as an intermediate in degradation are knowledge of the nature and specificity of the enzymes involved in such a process, the amount of the accumulated products and the kinetics of their accumulation. Nevertheless, all the evidence pointed toward a prototypic rearrangement of the pinenes to give limonene or 1-*p*-menthene as a monocyclic intermediate. Oxidation of these substrates on the C-7 methyl group to give perillyl alcohol and phellandrol was believed to be the next metabolic step. The alcohols would subsequently be converted to 3-isopropylpimelic acid (figure 1.7) and the formation of this metabolite

was supported by subcellular studies (Shukla and Bhattacharyya, 1968). Although no evidence has been provided for the further oxidation of 3-isopropylpimelic acid to central metabolites, such a pathway giving rise to CoA esters of acetate and succinate proposed by Trudgill (1984) is presented in figure 1.8.

Gibbon and Pirt (1971) isolated a strain of *Pseudomonas* PX1 NCIB 10684 from soil by enrichment with α–pinene as sole carbon source and characterised the metabolites accumulated, as *trans*-carveol, 2-methyl-5-isopropylhexa-2,5-dienoic acid, 2,5,6-trimethylhept-3-enoic acid and 3,4-dimethyl valeric acid. A year later, Gibbon *et al.* (1972) incorporated the above metabolites into a rather complex metabolic pathway, which is shown in figure 1.9. The suggestion that ring cleavage is accomplished by a Bayer-Villiger reaction is interesting, however, it seems unlikely that the lactone proposed would spontaneously rearrange to 2-methyl-5-isopropylhexa–2,5-dienoic acid. Similar results were obtained by Tudroszen *et al.* (1977).

Thus, it appeared that ring cleavage of α–pinene occurred either between carbon atoms 2 and 3 of the cyclohexane nucleus, suggested by the identification of isopropylpimelic acid in fermentation fluids of *Pseudomonas* PL (figure 1.7), or between carbon atoms 3 and 4 of the cyclohexane ring, suggested by the accumulation of 2-methyl-5-isopropyl-2,5-dienoic acid from *Pseudomonas* PX1 (figure 1.9).

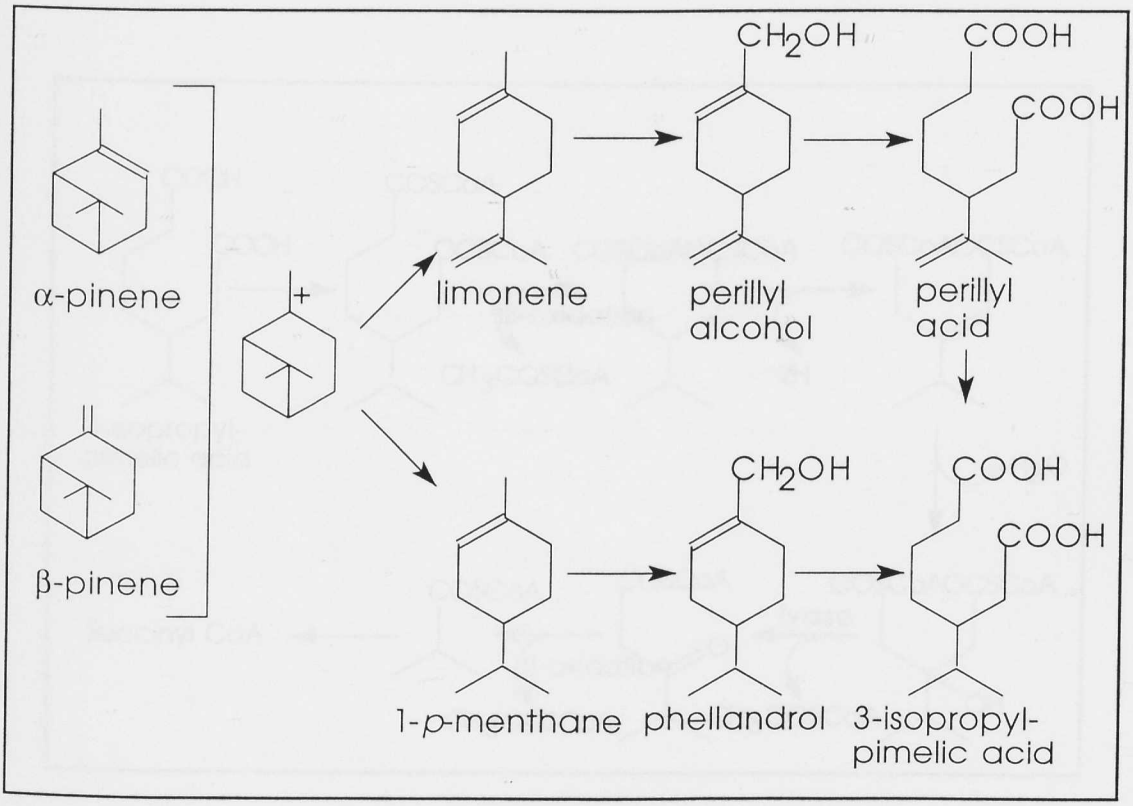


Figure 1.7: Proposed ring cleavage routes for α– and β–pinenes based on identified metabolites accumulated by *Pseudomonas* PL (Shukla and Bhattacharyya, 1968; Shukla *et al.*, 1968).

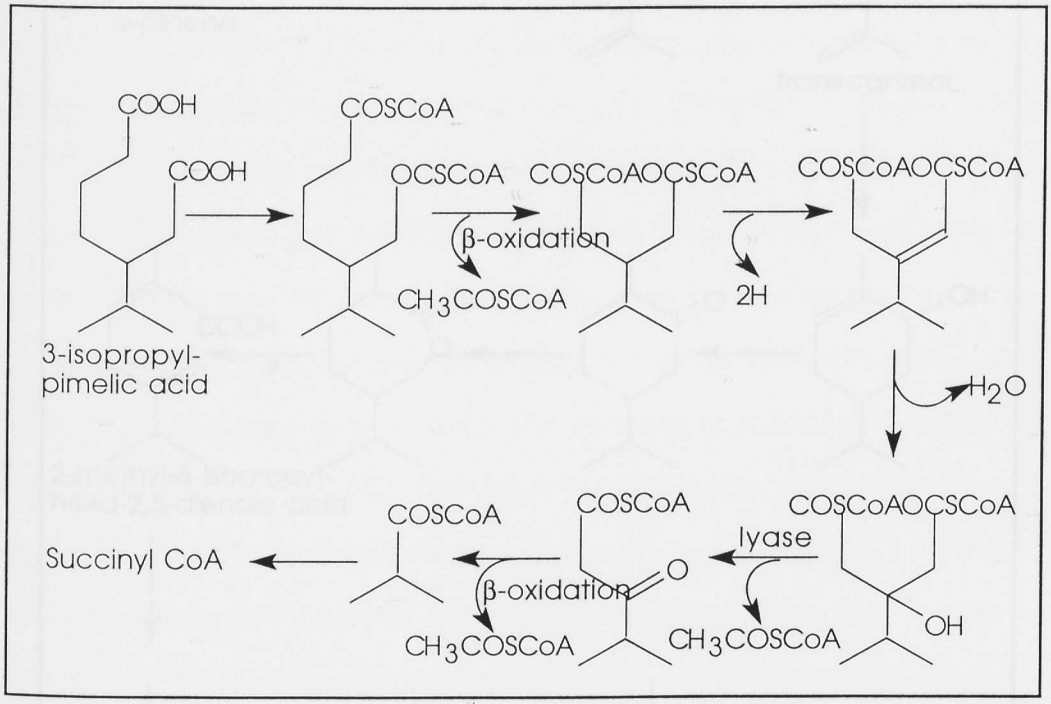


Figure 1.8: Proposed pathway for the degradation of 3-isopropylpimelic acid formed from α–pinene by *Pseudomonas* PL (Trudgill, 1984).

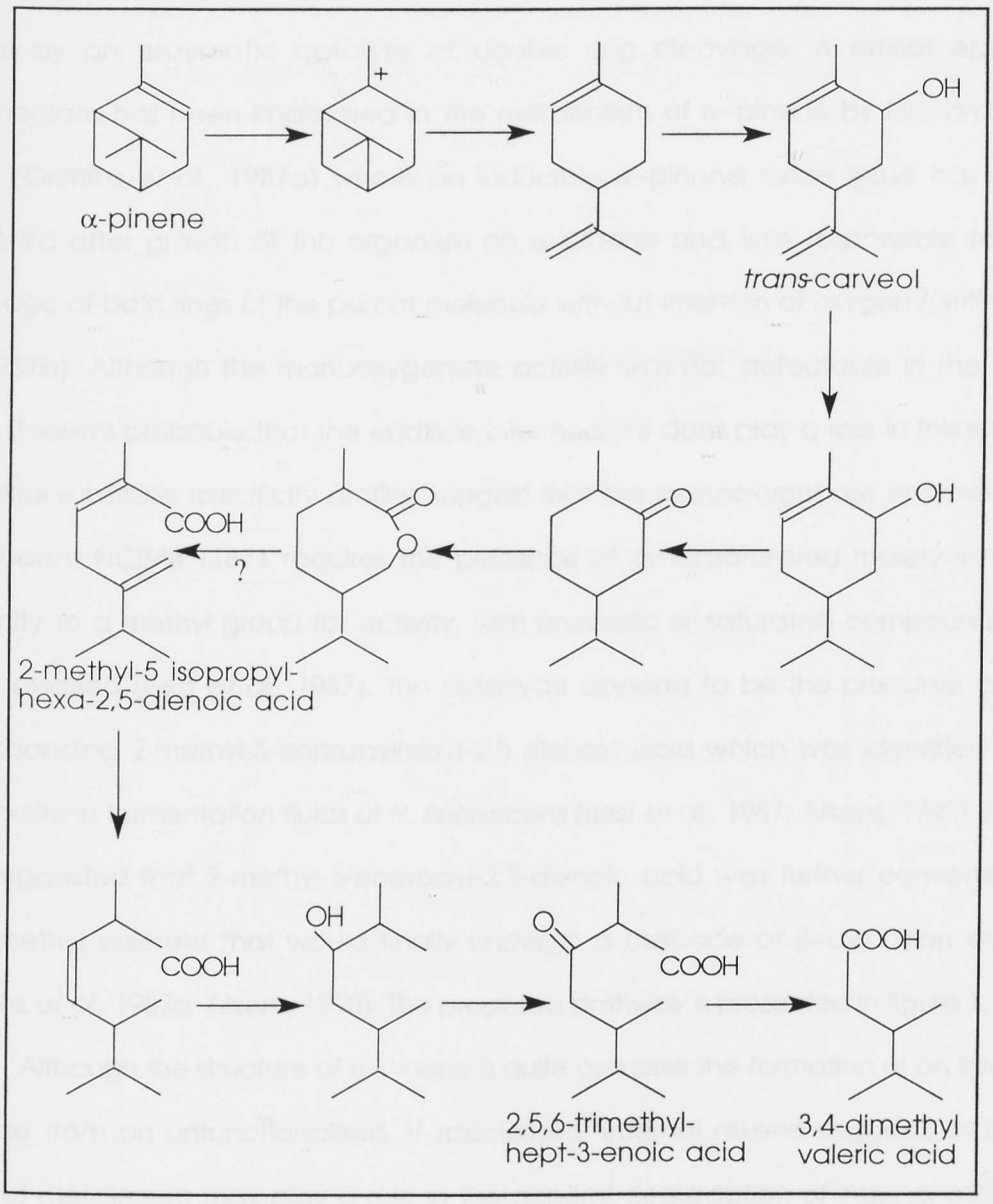


Figure 1.9: Proposed pathway for the degradation of α–pinene by *Pseudomonas* PX1 NCIB 10684 (Gibbon *et al.*, 1972).

A new route for the degradation of α–pinene from *Pseudomonas fluorescens* NCIMB 11671 was proposed by Best *et al.* (1987) and involved the formation of α–pinene oxide from α–pinene through the action of a NADH- dependent monooxygenase. The epoxide is then converted to 2-methyl-5-isopropylhexa-2,5 dienal by an enzymatic catalysis of double ring cleavage. A similar epoxide intermediate has been implicated in the metabolism of α–pinene by *Nocardia* sp. P18.3 (Griffiths *et al.*, 1987a) where an inducible α–pinene oxide lyase has been detected after growth of this organism on α–pinene and was responsible for the breakage of both rings of the parent molecule without insertion of oxygen (Griffiths *et al.*, 1987b). Although the monooxygenase activity was not detectable in the latter strain, it seems probable that the epoxide intermediate does play a role in this system. The initial substrate specificity profiles suggest that the monooxygenase enzyme of *P. fluorescens* NCIMB 11671 requires the presence of an unsaturated moiety in close proximity to a methyl group for activity, with aromatic or saturated compounds not being oxidised (Best *et al.*, 1987). The aldehyde appears to be the precursor of the corresponding 2-methyl-5-isopropylhexa-2,5 dienoic acid which was identified as a metabolite in fermentation fluids of *P. fluorescens* (Best *et al.*, 1987; Aikens, 1990). It was also suggested that 2-methyl-5-isopropyl-2,5-dienoic acid was further converted to 3,4-dimethyl valerate that would finally undergo a cascade of β–oxidation events (Griffiths *et al.*, 1987a; Aikens, 1990). The proposed pathway is presented in figure 1.10.

Although the structure of α–pinene is quite complex the formation of an internal epoxide from an unfunctionalised, if specialised, internal alkene suggests that this route of metabolism may play a role in the general degradation of internal alkenes. Even if the enzyme activity discovered in *P. fluorescens* NCIMB 11671 turns out to be restricted to a small group of structurally related compounds, the ability to exclusively epoxidise alkenes warrants a fundamental study of the enzyme to determine the nature of the active oxygen intermediate used in the epoxidation reaction and the stereoselectivity (if any) imposed on the reaction. The substrate specificity of the enzyme may be open to manipulation through adaptive evolution of the strain or

through genetic manipulation, to create a broad substrate range catalyst for the epoxidation of internal alkenes.

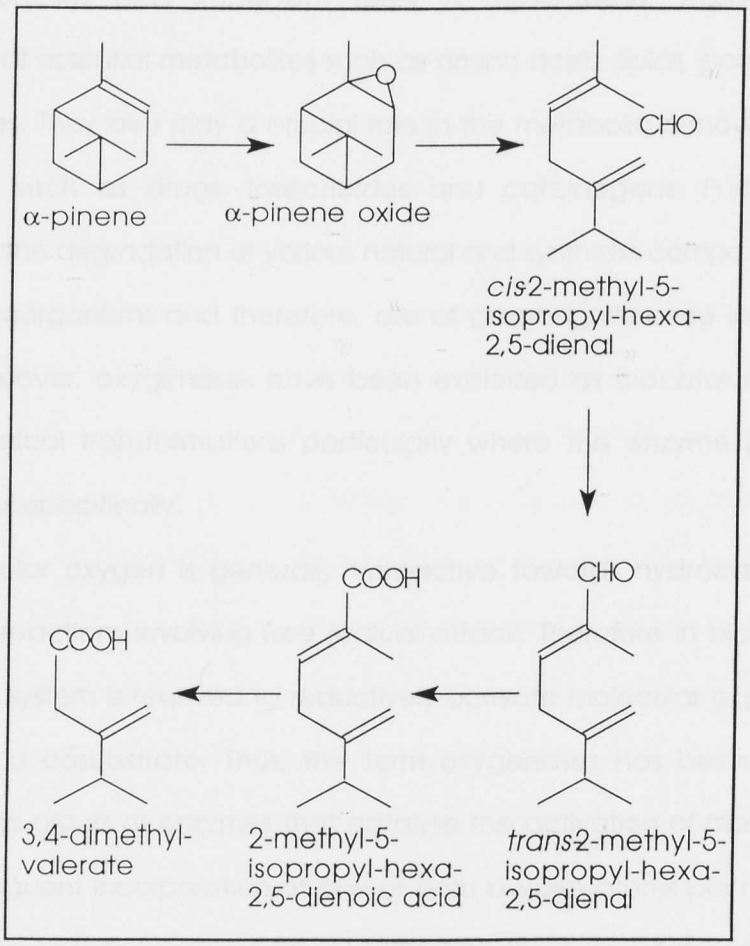


Figure 1.10: Proposed pathway for the degradation of α–pinene by *Pseudomonas fluorescens* NCIMB 11671 and *Nocardia* sp. P18.3 (Best et *al.*, 1987; Griffiths *et al.*, 1987a; Aikens, 1990).

1.4 Oxygenases and the Mechanism of Oxygen Activation

1.4.1 Introduction

Oxygenases play important roles in biosynthesis, transformation and degradation of essential metabolites such as amino acids, lipids, porphyrins, vitamins and hormones. They also play a crucial role in the metabolic removal of xenobiotic compounds, such as drugs, insecticides and carcinogens. Furthermore, they participate in the degradation of various natural and synthetic compounds by soil and airborne microorganisms and therefore, are of great significance in environmental science. Moreover, oxygenases have been exploited as biocatalysts to carry out specific chemical transformations particularly where the enzyme process occurs regio- or stereospecifically.

Molecular oxygen is generally unreactive towards hydrocarbons with the exception of reactions involving free radical attack. Therefore in biological systems an enzymatic system is required to reductively activate molecular oxygen prior to its insertion into a cosubstrate. Thus, the term oxygenases has been appropriately assigned to this group of enzymes that catalyse the activation of molecular oxygen and the subsequent incorporation of one or both oxygen atoms per mole of various substrates. Oxygenases are generally classified on the basis of the stoichiometry of the overall reaction and the cofactors involved. Dioxygenases incorporate two atoms of oxygen into one molecule of the substrate. In contrast, monooxygenases add only one atom of oxygen to the substrate, while the other atom is reduced to water. Therefore, they have been regarded as part oxygenase and part oxidase, and are frequently referred to as mixed-function oxidases. Most oxygenases consist of more than one component, although the flavoprotein monooxygenases are usually single component systems.

A common theme among multicomponent oxygenase systems is the use of short electron transport chains to catalyse electron transfer from an external donor,

usually NAD(P)H, to a terminal transition metal containing oxygenase. Two redox centres commonly a flavin and an iron-sulphur centre are employed in such electron transfer chains. Systems containing three protein components often used two different proteins as redox centres. Thus, toluene dioxygenase (Subramanian *et al.*, 1979; 1981;1985; Gibson *et al.*, 1990; Whited and Gibson, 1991a and b; Yen *et al.*, 1991), pyrazon dioxygenase (Sauber *et al.*, 1977), benzene dioxygenase (Geary *et al.*, 1984; Irie *et al.*, 1987), camphor monooxygenase (Fuzita, 1993), and ω–hydroxylase from *Pseudomonas oleovorans* (McKenna and Coon, 1970; May and Katopodis, 1990) contain two proteins which function in the transfer of electrons to the terminal oxygenase. Two component systems utilise a single protein to maintain the two redox centres. Such systems include phthalate dioxygenase (Batie *et al.*, 1987), benzoate 1,2-dioxygenase (Yamaguchi and Fujisawa, 1978; 1982), and 4-methylbenzoate-*O*-demethylase (Bernhardt *et al.*, 1975; 1978). Methane monooxygenase, although a three component system utilises a single protein to maintain the two redox centres (protein C), whereas the third component (protein B) appears to couple the oxygenase activity of component A to methanol production from methane (Colby *et al.*, 1977; Green and Dalton, 1986; Fox *et al.*, 1989; Pilkington and Dalton, 1991), The short electron transfer chain, whether present in a single or two components, may be envisaged as a $2e^-/1e^-$ transformase taking electrons from a $2e^-$ donor (NAD(P)H) and donating single electrons to the oxygenase. The flavin, which can undergo both $2e^-$ and $1e^-$ oxidoreduction cycles, functions as the initial electron acceptor that transfers electrons to a ferredoxin type of iron-sulphur centre. This second prosthetic group is arranged on the same polypeptide, as the flavin in the two component systems, but on a separate component of most three component systems. In this arrangement, transfer of electron from the reductase to the terminal oxygenase in the presence of NAD(P)H cannot occur in the absence of the iron sulphur centre, as has been shown for naphthalene dioxygenase (Haigler and Gibson, 1991).

1.4.2 Dioxygenases

As stated above, these enzymes catalyse the incorporation of both atoms of molecular oxygen into the substrate. Dioxygenases are involved at several stages in the pathways for the catabolism of aromatic compounds and can be divided into two groups (Mason, 1988):

(i) Enzymes involved in initial ring hydroxylation, such as benzene dioxygenase and benzoate dioxygenase. These ring-hydroxylating dioxygenases produce dihydrodiols by introducing two atoms of oxygen into the aromatic ring. They consist of three or four polypeptides which together form a short electron transfer chain that transfers electrons from NADH to oxygen. They are composed of a flavoprotein with NADH oxidase activity; one or two ferredoxins each with a single (2Fe-S) cluster; and a terminal oxygenase component which also contains a (2Fe-S) cluster and a ferrous iron binding site. The first two components may comprise one polypeptide as in benzoate dioxygenase (Gibson and Subramanian, 1984; Dagley, 1986). The reaction catalysed by these enzymes is shown below (where R is the substrate):

$$R + O_2 + NAD(P)H + H^+ \rightarrow R(OH)_2 + NAD(P)^+$$

(ii) Dioxygenases that catalyze ring fission, such as catechol 1,2-dioxygenase. These enzymes have no cofactor requirement. Usually, ring cleavage enzymes are multimeric proteins consisting of a single polypeptide species, although protocatechuate 3,4-dioxygenases from various *Pseudomonas* species are composed of two different polypeptides (Nozaki, 1979a and b). The meta–fission dioxygenases, which catalyze extradiol cleavage, contain non-haem ferrous irons as prosthetic groups, whereas the ortho-fission enzymes, which catalyze intradiol cleavage, contain non-haem ferric irons (Que *et al.*, 1987). The reaction catalysed by these enzymes is presented below:

$$R + O_2 \rightarrow RO_2$$

However, their mechanisms of action are fundamentally different. Only in the meta-fission enzymes is the iron involved in oxygen binding and redox cycles. In the ortho cleavage enzymes the iron is required for substrate binding with oxygen, interacting directly with the substrate.

1.4.3 Monooxygenases

Monooxygenases catalyze the insertion of one atom of oxygen into the substrate, while the other is reduced to water. They are also referred to as mixed function oxidases. In addition, the term hydroxylase, although misleading has also been used in reference to monooxygenases. Monooxygenases may be subdivided into two types on the basis of their electron donors:

(i) Internal monooxygenases that catalyze the incorporation of one atom of oxygen concomitant with the reduction of the other atom by electrons derived from the substrate (R) as in the lactate oxidative decarboxylase from *Mycobacterium phlei* (Hayaishi, 1974). Their reaction stoichiometry is:

$$RH_2 + O_2 \rightarrow RO + H_2O$$

(ii) External monooxygenases require an electron donor (D), such as NAD(P)H. The general stoichiometry is given by the following equation:

$$DH_2 + R + O_2 \rightarrow RO + H_2O + D$$

External monooxygenases are more common and may be further subdivided on the basis of their oxygen binding moieties, and the prosthetic groups involved at the active site. Monooxygenases have a much broader range of cofactor content than

dioxygenases, including redox active transitions metals (Fe or Cu) or conjugated organic cofactors (pterin or flavin coenzymes). These cofactors facilitate the passage of electrons from a two electron reductant (usually NADH or NADPH in biological systems), one electron at a time to molecular oxygen using an oxo-metal or oxygenated cofactor intermediate. On the basis of their prosthetic groups, monooxygenases can be subdivided into the following categories:

(I) Flavin-linked, haem containing monooxygenases, such as P-450 systems. Cytochrome P-450 coupled monooxygenases are broadly distributed in eukaryotic organisms and a few prokaryotes, and thus, they have been extensively studied. They are involved in the oxidative catabolism of steroids, fatty acids, prostaglandins, leukotrienes, biogenic amines, pheromones and plant metabolites. They also metabolise innumerable drugs, chemical carcinogens, mutagens and other environmental contaminants (Unger *et al.*, 1986; Nebert and Gonzalez, 1987 Philpot, 1989). There are two classes of P-450 monooxygenases defined by their electron transport chains. The microbial and mitochondrial systems contain three components: an FAD flavoprotein reductase receives two electrons from NAD(P)H which are subsequently passed via the iron centre of a separate putidaredoxin in bacteria, or an adrenodoxin in mitochondria, to the P-450 haem centre. In the membrane bound microsomal monooxygenases NAD(P)H provides two electrons to the prosthetic group of a more complicated FAD/FMN reductase. Single electrons are subsequently passed either via cytochrome b_5 or directly to clusters of P-450 proteins. In combination with atmospheric oxygen and any of the innumerable possible substrates, the P-450 forms a trimolecular complex and the substrate is oxygenated.

The mechanism of oxygen activation and substrate hydroxylation follows a discrete cycle (Ortiz de Montelano, 1986; Porter and Coon, 1991). Spectrophotometric analyses revealed that the active site of cytochrome P-450 monooxygenases contains ferric (Fe^{+3}) ion which bonds with the pyrrole nitrogen atoms of the photoporphyrin IX. The iron is co-ordinated by five ligands, four nitrogen atoms

derived from the porphyrin ring and a fifth iron-sulphur link to the haem propionate side chains, making up the structure of the enzyme. The sixth co-ordination site remains accessible for binding of the molecular oxygen. Oxidation is initiated by binding of the substrate to the active site of the enzyme complex, which perturbs its spin state equilibrium. This change of the equilibrium facilitates a single electron transfer and thus, the ferric ion is reduced to ferrous (Fe^{+2}) ion. Molecular oxygen is then reversibly bound to the ferrous ion. A second single electron reduction then takes place, and this results in the cleavage of the oxygen-oxygen bond and the formation of water and an oxo-iron complex. The very reactive oxo-iron species carries an overall 3^+ charge and it is responsible for the hydroxylation of the substrate. Several resonance forms are possible for the active oxygen intermediate. Oxygen insertion into the substrate involves hydrogen radical abstraction, followed by radical rebound, resulting in recombination of the transient hydroxyl and carbon radicals to give the product. Depending on the lifetime of the radical intermediates this could result in a loss of stereospecificity. Finally dissociation of the oxidised substrate leaves the ferric iron ready to commence another cycle. The mechanism of action of cytochrome P-450 is presented in figure 1.11.

Perhaps the best characterised cytochrome P-450 is the camphor 5-exohydroxylase system isolated from *Pseudomonas putida* (Yu *et al.*, 1974). The abundance of its purified components enabled Poulos and co-workers (Poulos, 1986) to achieve the three dimensional structure of cytochrome P-450cam to a resolution of 1.7Å. The cytochrome P-450 structure contains three major motifs; an anti parallel helical bundle, a parallel helical bundle and a mixture of helices and anti parallel beta pairs.

(II) Non haem iron monooxygenases, such as the soluble methane monooxygenase (MMO) from *Methylococcus capsulatus* (Bath), and the ω-hydroxylase from *Pseudomonas oleovorans*. The classification of these enzymes as non haem iron type monooxygenases derives from the role of a catalytically active iron which is not co-ordinated within a porphyrin ring complex, within the active site of

the enzyme. The MMO has a wide substrate range and is capable of catalysing the hydroxylation of alkyl groups in alkanes and aromatic compounds, as well as the epoxidation of terminal and subterminal alkenes. The regioselectivity of the oxidative attack appears to depend on the preference of MMO for electron rich groups, such as alkenes and heteroatoms, or in the case of alkane oxidation, for molecules with easily abstracted hydrogen atoms. The reaction mechanism of MMO is similar to that observed in cytochrome P-450 with carbon substrate binding as the first step, followed by reduction, oxygen binding, followed by the formation of an active oxo-iron reagent, and finally, water release with formation of the oxygenated product (Lund and Dalton, 1985; Green and Dalton, 1986). The enzyme is composed of three components; an NADH acceptor reductase which contains one mole of FAD and one Fe_2S_2 as prosthetic groups with a molecular mass of 44,600, a small protein of 17,000 kDa which acts to couple the reaction, and a large oxygenase with three subunits ($\alpha_2\beta_2\gamma_2$) and a molecular mass of 210,000. The oxygenase component contains a binuclear antiferromagnetically coupled 2Fe centre (Woodland and Dalton, 1984; Green and Dalton, 1986).

The ω–hydroxylase system from *Pseudomonas oleovorans* catalyses the hydroxylation of terminal methyl groups of alkanes and fatty acids, as well as the stereospecific epoxidation of terminal olefins, while it is incapable of attacking subterminal bonds (May and Katopodis, 1990). The enzyme is composed of three components; a rubredoxin, an NADH rubredoxin reductase and a monooxygenase which was named by Coon and co-workers ω–hydroxylase, and its mechanism of reaction appears to be similar to that of MMO. Rubredoxin is a red iron-sulphur protein of molecular weight 19,000, which contains no labile sulphide and exhibits an electron paramagnetic resonance spectrum (EPR) characteristic of high-spin ferric in a rhombic field. This small protein is the electron carrier in the system. The reductase is a 55,000 Da flavoprotein. Finally the ω–hydroxylase is a relatively insoluble protein with a molecular weight of 200,000 (α_4). It contains one iron and one cysteine per 41,000 dalton subunit, as well as high concentrations of phospholipid and carbohydrate.

(III) Pterin-linked enzymes such as phenylalanine monooxygenase which is capable of hydroxylating the phenylalanine ring regiospecifically to tyrosine (Dix and Bencovic, 1985). These enzymes are a subclass of the non haem iron monooxygenases, and are involved in aromatic amino acid hydroxylations.

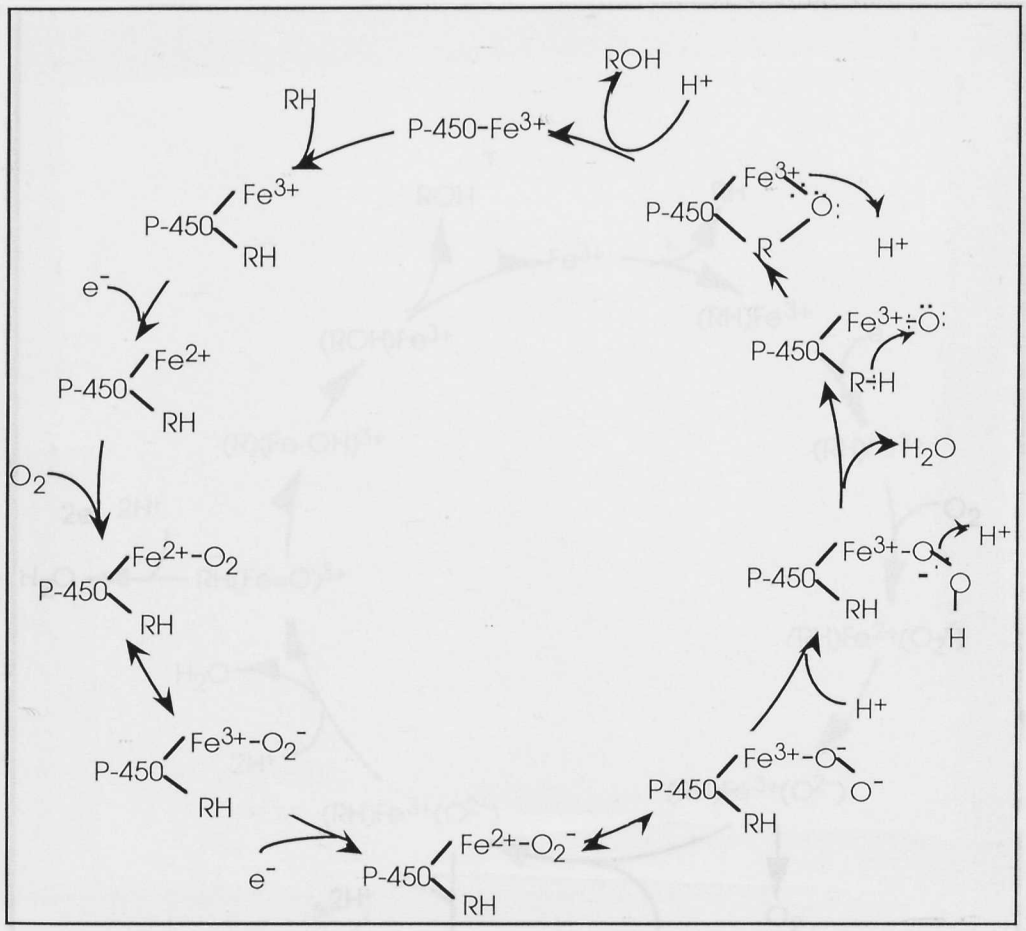


Figure 1.11: Mechanism of action of cytochrome P-450. Fe, represents the haem iron atom at the active site of the complex; RH, the substrate; ROH the monooxygenation product.

(iv) Copper monooxygenases. These enzymes are similar to flavin monooxygenases in that they exhibit selective reactivity. They are best exemplified by dopamine β–hydroxylase (Ingraham and Mayer, 1985) which catalyses the conversion of dopamine to noradrenaline by hydroxylation of the aromatic ring at the *ortho-* position relative to the existing hydroxyl group. The regioselectivity of the hydroxylation is believed to arise from a mechanism whereby both oxygen and substrate are initially bound to the binuclear copper centre, thus restricting other sites of attack. Oxygen insertion occurs only at electron rich centres, although dopamine-β–hydroxylase has been shown to be able to catalyze the epoxidation of the α,β unsaturated substrate analog. However, these enzymes because of their selective reactivity and apparent requirement to bind the substrate to the metal centre, are probably too specific for use as a general purpose epoxidation catalyst.

(v) Flavin monooxygenases. Flavoprotein hydroxylases are generally single component systems with both hydroxylase and reductase sites on a single polypeptide chain, usually bearing one molecule of FAD, or occasionally FMN, at the reactive centre. They may also contain non haem iron, and are commonly found in aromatic metabolism (White-Stevens and Kamin, 1972; Flashner and Massey, 1974; Gunsalus, 1975). These enzymes have been found to be incapable of hydroxylating non activated positions in hydrocarbons and are apparently incapable of epoxidation of isolated double bonds (Walsh and Chen, 1988). However, recently, Best *et al.* (1987) reported the epoxidation of α–pinene by a monooxygenase enzyme from *Pseudomonas fluorescens* NCIMB 11671. The enzyme was capable of catalysing the oxidation of heteroatoms and alkenes but not the hydroxylation of sp^3 carbon centres. Three years later, Aikens (1990), based on partial purification results of this monooxygenase and whole cell biotransformations, suggested that the enzyme requires FAD and ferrous iron for activity. In addition, Hartmans *et al.* (1990), again based on whole cell biotransformations, reported the bacterial degradation of styrene via epoxidation by a novel FAD requiring monooxygenase which did not epoxidise aliphatic alkenes. Flavoprotein monooxygenases also play a major role in

the skeletal disconnection strategy involved in the metabolism of cyclic hydrocarbons. Their ability to form lactones from cyclic ketones in a biological Bayer-Villiger type reaction and the subsequent hydrolysis of that lactone results in decyclisation of the cyclic carbon skeleton. These enzymes are dependent on reduced pyridine nucleotides as electron donors. Most flavin-dependent hydroxylases show a preference for one or other of NADH or NADPH as reductant, but many enzymes can utilise the other pyridine nucleotide as well, although with a less efficient catalytic reactivity (Müller, 1985). All the external flavin-dependent monooxygenases isolated and characterised so far contain FAD as prosthetic group with the exception of luciferase which uses FMN (Takeda *et al.*, 1969; White-Steven and Kamin, 1972; Neujahr and Gaal, 1973; Donoghue *et al.*, 1976; Beadle and Smith, 1982; Liu and Chapman, 1984; Yamada *et al.*, 1985; Davies, 1990; Hartmans *et al.*, 1990; Tu, 1991; Stanton and Jensen, 1993).

The oxygen atom incorporated into the substrate is obtained from molecular oxygen, with the other oxygen atom being reduced to water. These enzymes may be divided in four groups according to their substrate type. Enzymes that attack nucleophilic substrates, such as mammalian microsomal monooxygenases and phenol α–hydroxylases, and enzymes that attack electrophilic substrates, such as bacterial luciferases and cyclic ketone oxygenases (figure 1.12). The mechanism of action of each enzyme diverges as dictated by the nature of the substrate and the effect of each particular apoprotein involved. However, all of the reactions share a common oxygenated enzyme intermediate, the 4α–hydroperoxyflavin (4α-FlOOH) shown in figure 1.13 (Walsh and Chen, 1988). This intermediate is generated by the reaction of the enzyme-bound dihydroflavin with molecular oxygen which occurs by two successive one electron transfers. The process involves the rate-limiting transfer of one electron to yield superoxide anion and a flavin semiquinone radical that is stabilised by resonance. Such enzymes show remarkable versatility as oxygenation reagents. They have an activated 4α–FlOOH bound tightly to their active site, which

delivers either a nucleophilic or electrophilic oxygen equivalent, depending on the electron-poor or electron-rich nature of the bound cosubstrate.

Mammalian microsomal oxygenases (Müller, 1985; Walpole and Wrigglesworth, 1987; Ziegler, 1990) catalyze the oxidation of nucleophilic substrates, such as amines and sulphides to their corresponding *N*-oxides and *S*-oxides. The 4α–hydroperoxyflavin intermediate is unusually stable in these enzymes, particularly in the presence of pyridine nucleotide. The reactions yield the above oxides with stoichiometric formation of 4a–FlOH, and it appears that there is no other intermediate between 4a–FlOOH and 4a–FlOH, which finally breaks down to give water and the flavo-enzyme.

As a general rule, phenol hydroxylases catalyze the hydroxylation of aromatic substrates at the position *ortho*- or *para*- to the already present hydroxyl group. This group of enzymes is best exemplified by *p*-hydroxybenzoate (Howell *et al.*, 1972) orcinol (Otha and Ribbons, 1970) melilolate (Strickland and Massey, 1973) and salicylate (White-Stevens and Kamin, 1972) hydroxylases. Also in this group belongs a phenol hydroxylase isolated from a eukaryote, *Trichosporon crutaneum* (Neujahr and Gaal, 1973). Although the reactivity of these enzymes is similar to the microsomal oxygenases, in that oxygen transfer occurs by nucleophilic attack of the substrate on the 4α–hydroperoxide intermediate, another intermediate, absorbing at a long-wavelength (intermediate II), appears between the 4a–FlOOH and 4a–FlOH (Visser, 1983).

Bacterial luciferases catalyze the oxidation of a long-chain aliphatic aldehyde to the corresponding carboxylic acid. The general reaction scheme is similar to that previously proposed. However, in this reaction type, a novel 4a–hydroxyflavin radical is formed between the 4a-FlOOH and 4a–FlOH species (Tu, 1991).

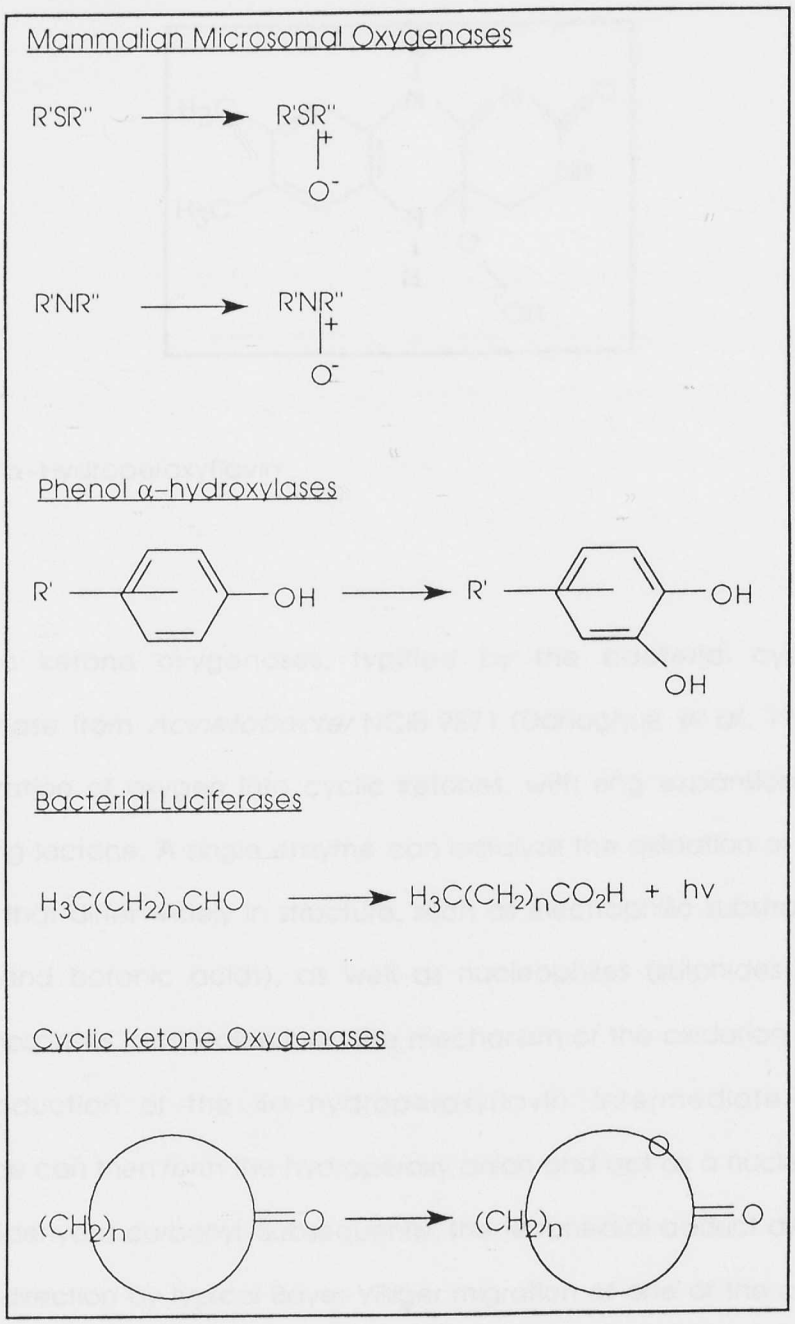


Figure 1.12: Types of reactions catalysed by flavoprotein monooxygenases

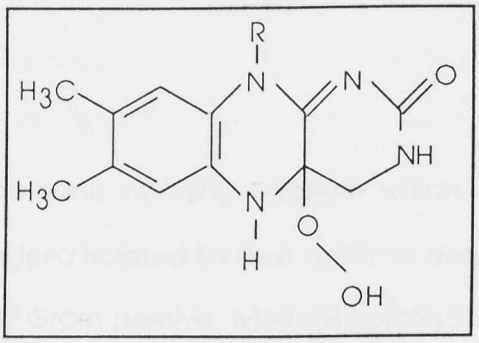


Figure 1.13: 4α–Hydroperoxyflavin

Cyclic ketone oxygenases, typified by the bacterial cyclohexanone monooxygenase from *Acinetobacter* NCIB 9871 (Donoghue *et al.*, 1976), catalyze the incorporation of oxygen into cyclic ketones, with ring expansion to form the corresponding lactone. A single enzyme can catalyze the oxidation of a number of compounds that differ widely in structure, such as electrophilic substrates (ketones, aldehydes, and boronic acids), as well as nucleophiles (sulphides, sulphoxides, selenides, phosphites, and iodine ion). The mechanism of the oxidation reaction also involves production of the 4a–hydroperoxyflavin intermediate. The flavin hydroperoxide can then form the hydroperoxy anion and act as a nucleophile at the ketone (or aldehyde) carbonyl. Subsequently, the tetrahedral adduct decomposes in the forward direction by typical Bayer-Villiger migration of one of the carbon bonds (or the C-H bond for aldehydes) with fragmentation of the weak O-O bond to yield ester (or acid) and 4a–FlOH, which returns to FAD by internal loss of water (Walsh and Chen, 1988).

1.5 Genetics of Hydrocarbon Utilising Bacteria

1.5.1 Introduction

Apart from the methane oxidising bacteria which, as methanotrophs are biochemically distinct, bacteria isolated for their ability to degrade lower alkanes and alkenes are predominantly Gram positive, whereas isolation on higher hydrocarbons isolates predominantly Gram negative species. The latter feature may, however, be an artifact of the isolation techniques used, as it is evident that Gram negative bacteria are more tolerant to organic solvents than Gram positive. When the concentration of the organic substrate is kept low, larger numbers of Gram positive organisms (e.g. *Nocardia* sp.., *Rhodococcus* sp.. and *Corynebacterium* sp..) are isolated. The molecular genetics of Gram negative alkane and aromatic compound utilising bacteria have been extensively investigated the last 10 -15 years. In contrast information about the molecular genetics of Gram positive lower alkane and alkene utilising organisms is virtually non existent.

The majority of the mono- and di-oxygenases from Gram negative organisms reported so far are encoded by genes located on large conjugative plasmids with the exception of methane monooxygenase genes which are chromosomal. These plasmids can transfer laterally into new host bacteria and thereby potentially expand the metabolic potential of other members of an ecosystem. Conjugative plasmids are of great importance for bacteria, since they can bring together genes from different organisms that through mutations can specify new metabolic functions. Thus, in an environment rich in a particular organic compound, a selective pressure exists to acquire and maintain a plasmid that specifies the metabolic pathway for the assimilation of this compound. In some cases, the same catabolic genes are located on a plasmid in one organism and on the chromosome in another. *Pseudomonas* sp.., natural habitants of soil and water, can dissimilate a wide range of organic compounds of both biological and non-biological origin. Their metabolic versatility is

often directed by genes and gene clusters encoding entire or partial catabolic pathways (Franz and Chakrabarty, 1986) borne on plasmids, such as the CAM (Reinwald *et al.*, 1973), NAH (Dunn and Gunsalus, 1973), SAL (Chakrabarty, 1972), XYL Schell, 1990), TOL (Pickup*et al.*, 1983), and OCT (Chakrabarty *et al.*, 1973) plasmids which determine the first and in some instances subsequent steps in the oxidation of camphor, naphthalene, salicylate, *p*- and *m*- xylene and *n*-alkanes, respectively. Although the basic enzymology of many plasmid-encoded pathways has been known for some time, the application of molecular biology and recombinant DNA techniques has only recently begun to elucidate the organisation, regulation and evolution of many degradative genes. Application of rapid DNA sequence determination methods and computerised data base homology searches, has shown that some plasmids contain very similar genes although they are involved in the degradation of apparently dissimilar substrates. For example, plasmid NAH7, encoding metabolism of naphthalene, and plasmid TOL pWWO, encoding toluene degradation, contain the genes *nahH* and *xylE*, both encoding catechol 2,3 dioxygenase that are greater than 80% similar in both DNA and protein sequence (Hayarama *et al.*, 1987). In addition, evidence for a high similarity between plasmid and chromosomal genes responsible for catechol degradation has been also presented (Aldrich *et al.*, 1988). Such results suggest that many degradative genes are related by common ancestry and that substantial genetic exchange and interaction has occurred among various plasmids and the host chromosomes.

1.5.2 Genetics of Alkane Utilising Organisms

n-Alkanes can be utilised by a large number of microorganisms of which *Pseudomonas oleovorans* is a well studied example. The bacterium can grow on intermediate chain length alkanes as well as water soluble substrates such as citrate, pyruvate and malate (Stanier *et al.*, 1966). Growth on alkanes depends on the

presence of the OCT plasmid which contains about 15% of the total genetic information of the bacterium (Chakrabarty *et al.*, 1973). The enzymology of the alkane oxidation system has been elucidated by a number of researchers (McKenna and Coon, 1970; Ueda *et al.*, 1972) who showed that three proteins comprise the alkane hydroxylase, the first oxidation step as described. As stated above (Chapter 1.4) the hydroxylation reaction is carried out by a multimeric protein with 41 kDa membrane bound polypeptide subunits. The reducing equivalents are supplied by the soluble rubredoxin reductase, a 55 kDa flavoprotein that reduces the 19 kDa rubredoxin at its iron-sulphur centre. The latter transfers electrons to the alkane hydroxylase. The alcohol produced is subsequently dehydrogenated by an NAD dependent alcohol dehydrogenase, and the cofactor is regenerated.

Shapiro and co-workers studied the genetics of the system in the late 1970's and showed that these proteins are encoded and regulated by the *alk* system which is located on the OCT plasmid (Benson and Shapiro, 1975; Benson, 1979; Fennewald and Shapiro, 1977;). A number of mutants were obtained which allowed the identification of several structural and regulatory genes. The *alk* regulon consists of two gene clusters: the *alkBFGHJKL* operon that encodes the above enzymatic activities (figure 1.14), and the *alkR* region which encodes the positive regulatory functions required for the expression of the first operon. (Fennewald *et al.*, 1979; Owen *et al.*, 1984, 1986; Eggink *et al.*, 1987*a, b*, 1988, 1990; Kok *et al.*, 1988*a, b*).

The *alkBFGHJKL* genes are organised in a single 7.5 kb operon. Alkanes, alcohols (such as 1-pentanol, 1-, 2- and 3- hexanol and 1-heptanol), as well as ketones (such as 2-heptanone and 2-octanone) are effective inducers of the operon. Subcloning of this DNA fragment and analysis of the peptides encoded in *E. coli* minicells revealed that six polypeptides were formed. The *alkB* gene encodes a 401 amino acid polypeptide, which despite an unusual codon composition can be expressed at high levels in *E. coli* and *Pseudomonas*. This polypeptide is the subunit of the alkane hydroxylase. The translation product, defined from the gene sequence, contains nine hydrophobic sequences of which eight are sufficiently long to be

membrane-spanning segments (Kok *et al.*, 1989*b*). The *alkF* gene encodes for a 132 amino acid polypeptide which corresponds to rubredoxin 1, and the *alkG* gene encodes for a 172 amino acid polypeptide which is rubredoxin 2 (Kok *et al.*, 1989*a*). Genes *alkH* and *alkJ* correspond to aldehyde dehydrogenase and alcohol dehydrogenase respectively (Owen *et al.*, 1984; Kok *et al.*, 1989). Gene *alkK* probably also encodes for an alcohol dehydrogenase (Eggink *et al.*, 1990) and *alkL* encodes for a 230 amino acid polypeptide with unknown function. The *alkR* locus has been found to encode for two proteins. A 99 kDa regulatory protein, *alkS*, which positively regulates the expression of the *alkBFGHJKL* operon and a 48 kDa protein which was shown to be the rubredoxin reductase (Eggink *et al.*, 1990). Comparison of the latter DNA sequence with known sequences of other FAD-containing reductases showed a high degree of similarity with many of them, and especially with the putidaredoxin reductase of the *Pseudomonas putida* cytochrome P-450cam system.

Another example is the methane monooxygenase (MMO) gene cluster from *Methylococcus capsulatus* (Bath). The bacterium possesses two MMOs, one membrane bound (pMMO) which is expressed at high copper concentrations, and the other soluble (sMMO) expressed in copper stress conditions. As already stated, sMMO consists of three components: protein A, the terminal oxygenase ($\alpha_2\beta_2\gamma_2$); Protein C, an iron-sulphur flavoprotein reductase which transfers electrons from NADH to protein A; and protein B that contains no prosthetic groups and is thought to regulate electron transfer (West *et al.*, 1992). Stainthorpe *et al.*, (1989; 1990) using oligonucleotide probes complementary to the N-terminal amino acid sequences of the structural proteins cloned and sequenced the whole gene cluster. All five genes were found to be linked on a 7 kb DNA fragment (figure 1.15) Cell extracts of *Methylococcus* always appeared to contain stoichiometric amounts of protein A polypeptides at high concentration (Woodland and Dalton, 1984) suggesting a co-transcription event. The genes encoding α–, β– and γ– subunits of protein A, *mmoX*, *mmoY* and *mmoZ* respectively, are contiguous on the *Methylococcus* chromosome. Intercistron regions are small, the largest, between *mmoY* and *mmoZ* being 78 bp

long. A second ORF of unknown function separates the structural genes for protein A from the one encoding protein C. Moreover, sequences capable of forming stem loop structures in mRNA were located between *mmoX* and *mmoY*, as well as between *mmoZ* and *orfY*. *MmoX* is preceded by a 5' sequence homologous to the *E. coli* -35 and -10 consensus sequence. Furthermore, a sequence homologous to the -12 and -24 conserved regions of *Pseudomonas* (Deretic *et al.*, 1987) and to the *nif*- and *ntr*- like consensus promoter region (Dixon, 1986) was also found in the A+T rich sequence 5' to *mmoX*. However, the promoters which operate transcription of these highly expressed copper and methane regulated proteins may be entirely novel.

1.5.2 Genetics of Aromatic Compound Utilizing Organisms

A general comparison of the major pathways for the catabolism of aromatic compounds in bacteria has revealed that although the initial conversion steps are carried out by different enzymes the compounds are transformed to a limited number of intermediates such as substituted catechols and protocatechuate (van der Meer *et al.*, 1992). The dihydroxylated intermediates are then channelled into two possible pathways, either a *meta* cleavage pathway or an *ortho* cleavage pathway, that lead to intermediates of central metabolic routes such as the tricarboxylic cycle.

TOL plasmids are self-transmissible and contain two or more operons which encode enzymes required for the degradation of methylbenzenes, such as toluene, xylenes, and 1,2,4-trimethylbenzene via methylbenzoate. On the TOL plasmid pWWO two operons have been located: the *xylCMABN* operon that codes for the "upper pathway" enzymes which oxidise toluene/xylene (methybenzenes) to benzoate/toluates (methylbenzoates), and the meta operon, *xylXYZLEGFJKIH* which codes for the "lower pathway" enzymes that are responsible for the conversion of methylbenzoates to pyruvate, acetaldehyde, and acetate via methyl-catechols (figure 1.16) (Franklin *et al.*, 1981; Harayama and Relik, 1989; Harayama *et al.*, 1991; Horn

et al., 1991). Pu and Pm are the promoters of the upper and meta–cleavage pathway respectively. Transcription from the Pu promoter is positively regulated by the XylR protein which is activated by toluenes/xylenes and their alcohol derivatives. Similarly, transcription from Pm is positively regulated by the XylS protein which is activated by benzoate/toluates (Mermod *et al.*, 1987). Consensus sequences of the -10 region (Pribnow box) and the -35 region (RNA polymerase recognition site) in *E. coli* genes were not found in the region preceding the transcription initiation site of the "upper pathway"(Finette, 1984; Inouye *et al.*, 1984, 1985, 1986; Nakazawa *et al.*, 1990). On the other hand, a striking homology has been observed between the *ntr*-activatable promoters in *E. coli* and the promoter sequence of the *xylABC* operon, raising the possibility that diverse metabolic pathways in Gram negative bacteria may share common regulatory features (Dixon, 1986). In addition, the promoter sequence of *xylS* (Ps) also showed remarkable similarity to the nitrogen-regulated and nitrogen fixation promoters which contain conserved residues at -24(GG) and -12(GC) sites with respect to the transcription initiation site. Dixon (1986) showed that *ntrA* gene, whose product is a specific σ^{54} factor, was required in addition to *xylR* for activation of the Pu promoter in *E. coli*. Moreover, the heterologous regulatory genes *nifA* and *ntrC* could substitute for *xylR* as transcriptional activator of these promoters in the presence of *ntrA*. The promoter sequence (Pr) of the *xylR* gene is homologous to the *E. coli* consensus -35, -10 sequence (Inouye *et al.*, 1985). *xylR* has two characteristic regions that are homologous to the NtrC and NifA sequences. The gene contains a helix-turn-helix motif at the C-terminus, suggesting that XylR is a DNA binding protein; an NtrA-interacting domain, located it the centre of the gene; and an N-terminal region that possibly acts as a receptor for signals transmitted by a sensory NtrB protein (Nakazawa, 1990). Finally, XylR is also involved in the activation of the Pm promoter of the "lower Pathway". However, induction will occur only if a functional XylS protein is also present, suggesting some interaction of these components (Dixon, 1986; Spooner *et al.*, 1986).

The catabolic genes of the NAH7 plasmid which encode the enzymes required for the degradation of naphthalene via salicylate are also clustered in two co-ordinately controlled operons, the *nah* and *sal* operons. As in the previous system, the *nah* operon encodes oxidation of the aromatic hydrocarbon to a direct precursor of catechol, while the *sal* operon encodes for a hydroxylase which converts the precursor to catechol and the enzymes of its further metabolism via the *meta*-cleavage pathway (figure 1.17). The *sal* operon contains similar *meta*-cleavage pathway genes to those present on pWWO, and DNA sequences of these parts of both operons are homologous (Shell, 1986; Assinder and Williams, 1988).

The *nahR* gene encodes a 34 kDa polypeptide which binds to the *nah* and *sal* promoters to activate transcription of the degradation genes in response to the inducer, salicylate. The DNA as well as the deduced amino acid sequence of *nahR* showed approximately 35% identity with the transcriptional activator encoded by the *nodD* gene of *Rhizobium* species, and 32% homology to the *lysR* transcriptional activator gene of *E. coli* (Shell and Sukordhaman, 1989; Shell, 1990). The region of greatest similarity between all three proteins was the probable helix-turn-helix DNA binding motif. All genes that belong to the LysR family of transcriptional activators share the same distinguishing characteristics: (i) they all are of nearly identical size (approximately 300 amino acids), (ii) they all share major amino acid sequence similarities in their N-terminal halves, (iii) they are all transcribed from promoters that are very close to (usually overlapping) and divergent from the promoters of one set of structural genes they control.

It is noteworthy that catechol 2,3 dioxygenases encoded by NAH and TOL plasmids are not only similar to each other, but also show sequence similarities to other extradiol ring cleavage enzymes, such as 1,2-dihydroxynaphthalene dioxygenase encoded by the *nahC* gene in the *nah* operon of plasmid NAH7 (Hayarama and Relik, 1989a).

Other enzymes involved in the initial transformation of aromatic compounds are either dioxygenases or monooxygenases. These enzymes show greater

substrate specificities (i.e. more specific) than the ring cleavage enzymes and have considerable sequence homology and presumed evolutionary relatedness to each other. Multicomponent dioxygenases involved in the NADH-dependent incorporation of two adjacent hydroxyl groups on the aromatic ring consists either of three or two components. The genes for the components of toluene dioxygenase from *P. putida* (Zylstra *et al.*, 1988; Zylstra and Gibson, 1989), benzene dioxygenase from *P. putida* (Irie *et al.*, 1989), biphenyl dioxygenase from *P. pseudoalcaligenes* (Furukawa *et al.*, 1987) and *Pseudomonas* sp. strain LB400 (Erickson and Mondello, 1992) and of naphthalene dioxygenase from *P. putida* NCIB 9816 (Kurkela *et al.*, 1988; Simon *et al.*, 1993) are all clustered but the orders of the equivalent genes may be different. All of these enzymes show high sequence similarities at the ADP binding sites (Wierenga *et al.*, 1983, 1986) as well as at their iron-sulphur centres. In the two component system the electron transport function is fulfilled by a single protein which possesses a ferredoxin-like structure in its N-terminal part and an NADH-ferredoxin reductase-like structure in its C-terminal region. Examples of this group of dioxygenases are the toluate dioxygenase from *P. putida* (Hayarama *et al.*, 1986) and benzoate dioxygenase from *Acinetobacter calcoaceticus* (Neidle *et al.*, 1988, 1991; Harayama *et al.*, 1991). A novel two component dioxygenase, 2-halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS appears to be structurally related to toluate dioxygenase (Fetzner *et al.*, 1992).

Monooxygenases involved in several metabolic steps of aromatic degradation also share both structural and sequence similarities not only between each other, but also with the multicomponent dioxygenases mentioned above. Toluene monooxygenase from *P. mendocina* KR1 is a multicomponent enzyme complex and encoded by five genes, *tmoABCDE* (Yen *et al.*, 1991). The enzyme catalyses the conversion of toluene to *p*-cresol. Comparison of the amino acid sequence deduced from the *tmoC* gene with those of known ferredoxin proteins revealed homology between the *tmoC* gene product and several other ferredoxins functioning in dioxygenase systems. Comparison of the amino acid sequences of the products TmoA, -D, and -E of the toluene-4-monooxygenase from *P. mendocina* KR1

and the gene products DmpL, -M, and -N of the phenol hydroxylases system from *Pseudomonas* sp. strain CF600 (Bartilson and Shingler, 1989; Nordlund *et al.*, 1990; Kivisaar *et al.*, 1991) revealed similarities of 26%, 24%, and 30% respectively. In addition, both monooxygenases contain polypeptides which are structurally related to components of methane monooxygenase (Stainthorpe *et al.*, 1990).

Single component monooxygenases are also found in several pathways, and also share conserved domains. These are simple flavoprotein hydroxylases that are capable of catalysing the hydroxylation of an already activated double bond. The majority of these enzymes are dimers with a monomer size of around 60 to 70 kDa (Nurk *et al.*, 1991). For example salicylate hydroxylase NahG encoded on the NAH7 plasmid (You *et al.*, 1991) showed 25% similarity at the amino acid level with *p*-hydroxybenzoate hydroxylase from *P. fluorescens* (Weijers *et al.*, 1982), which also showed a high degree of similarity with the more recently sequenced *p*-hydroxybenzoate hydroxylase gene from *P. aeruginosa* (Entsch *et al.*, 1988). Higher local sequence similarities occur in specific domains such as the presumed FAD binding region.

Phenol hydroxylase from *P. picketii* (Kukor and Olsen, 1990, 1992) catalyses the conversion of cresols or phenols to the corresponding catechols. The enzyme is a single component flavoprotein with a total molecular mass of 147,000, and it consists of two identical subunits. The absorption spectra of the enzyme suggested that it contains 2 moles of FAD per mole of enzyme. Sequencing of the gene encoding this monooxygenase revealed that the gene did not have significant overall homology with any other sequence in the data base used. However, the N-terminal region as well as the C-terminal region, which contained ADP binding sites, showed high similarities to other FAD-containing hydroxylases. Finally, analysis of the upstream region of the *tbuD* gene, responsible for the oxidation of phenol or cresols to catechol or methylcatechols respectively, revealed a sequence of 27 nucleotides which showed strong homology to positively controlled promoters believed to be transcribed by a *Pseudomonas* σ^{54}-dependent RNA polymerase holoenzyme.

Pentachlorophenol monooxygenase from *Flavobacterium* sp. strain ATCC 39723 is a one component monooxygenase encoded by a 1614 bp ORF (Orser *et al.*, 1993). The enzyme catalyses the dehalogenation of polychlorinated aromatic compounds, but also removes nitro- amino- and cyano- groups from the benzene ring at the *para*- position in relation to the phenol hydroxyl. The transcriptional start was found to be 80 bp upstream of the translational start codon and the transcript was induced in *Flavobacterium* sp., by the presence of pentachlorophenol but was constitutive in an *E. coli pcpB* clone. Protein data base comparisons of the predicted translation product revealed regions of homology with other microbial flavoprotein monooxygenases including phenol-2-monooxygenase from *Trichosporon cutaneum*, and tryptophan-2-monooxygenase from *P. syringae* pv. savastanoi.

Finally, xylene monooxygenase encoded on the TOL pWWO plasmid of *P. putida* consists of two types of polypeptide encoded by XylA and XylM. XylM was shown to have 25% homology with the alkane hydroxylase component AlkB, which catalyses the ω–hydroxylation of fatty acids and the terminal hydroxylation of alkanes. Moreover, the sequence of the first 90 amino acids of the XylA product exhibited a strong similarity to the sequence of chloroplast type ferredoxins, whereas its C-terminus appeared to have high homology with those of ferredoxin-$NADP^+$ reductases (Suzuki *et al.*, 1993). Based on this information the structure and function of xylene monooxygenase was deduced. XylM is probably the catalytic component for the hydroxylation of carbon side chain of xylenes and toluene, and appears to be a membrane bound protein containing ferrous iron as prosthetic group, as is the case with alkane hydroxylase. XylA on the other hand, may have two domains, an N-terminal region similar to the chloroplast-type ferredoxins and a C-terminal region similar to ferredoxin $NADP^+$ reductases. NADH is oxidised at the C-terminus of the XylA protein and then one electron at a time is passed to the N-terminus which subsequently reduces the oxidised form of XylM.

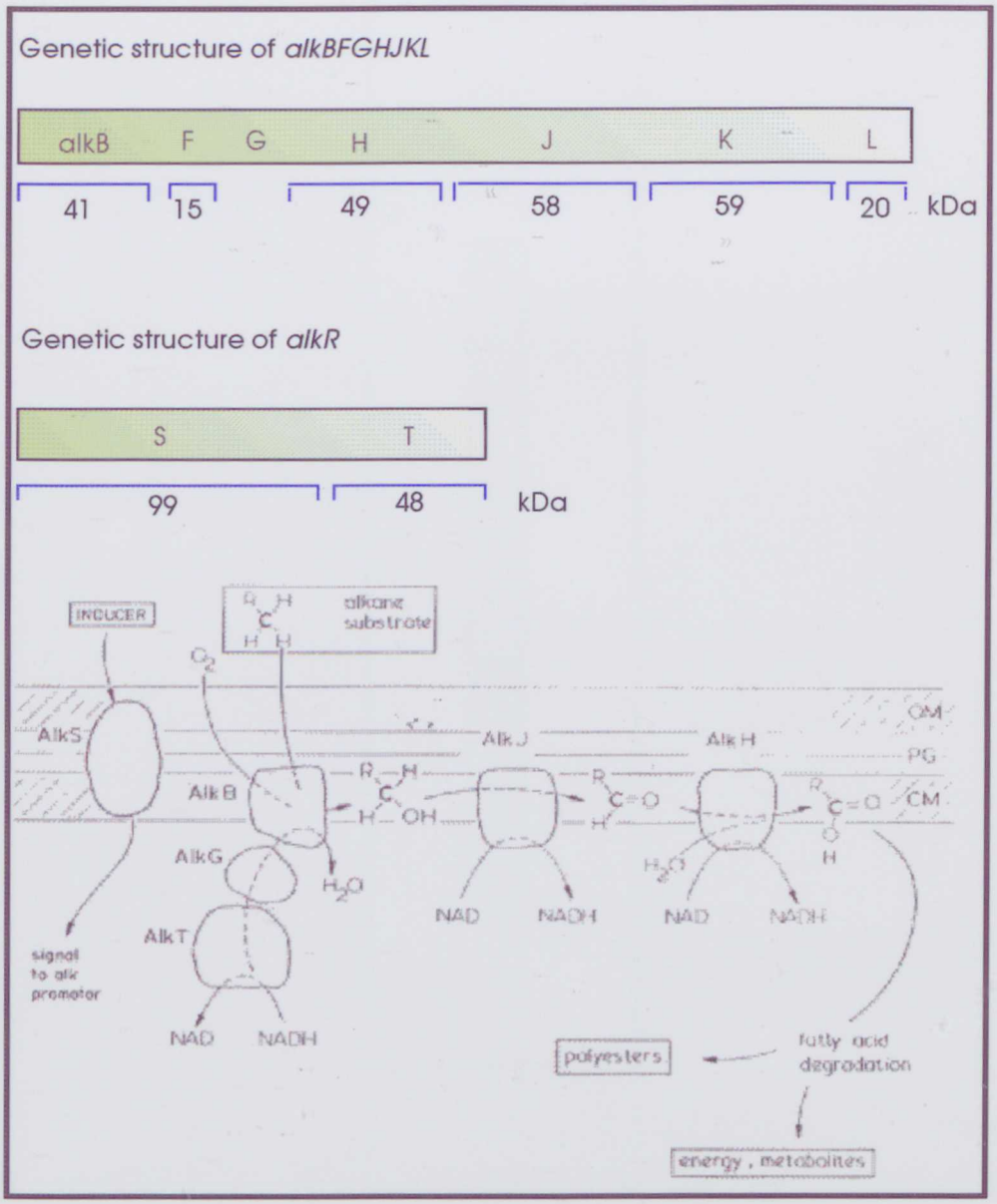


figure 1.14: Genetic structure and nomenclature of the *alkBFGHJKL* operon (a), and model of the cellular organization of the alkane oxidation system (b) (taken from Witholt *et al.*, 1990).

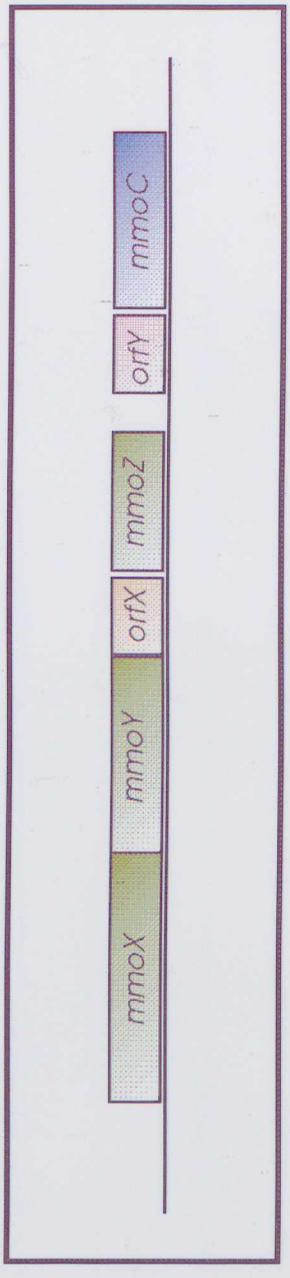


Figure 1.15: Physical map of the methane monooxygenase cluster. Possible promoter sites showing similarities to the -35 and -10 *E. coli* consensus promoter, as well as to the -24 and -12 *ntr*- or *nif*- like promoters were detected upstream of the *mmoX* gene.



Figure 1.16: Toluene degradative pathway encoded in TOL plasmid. Its genes and regulation.

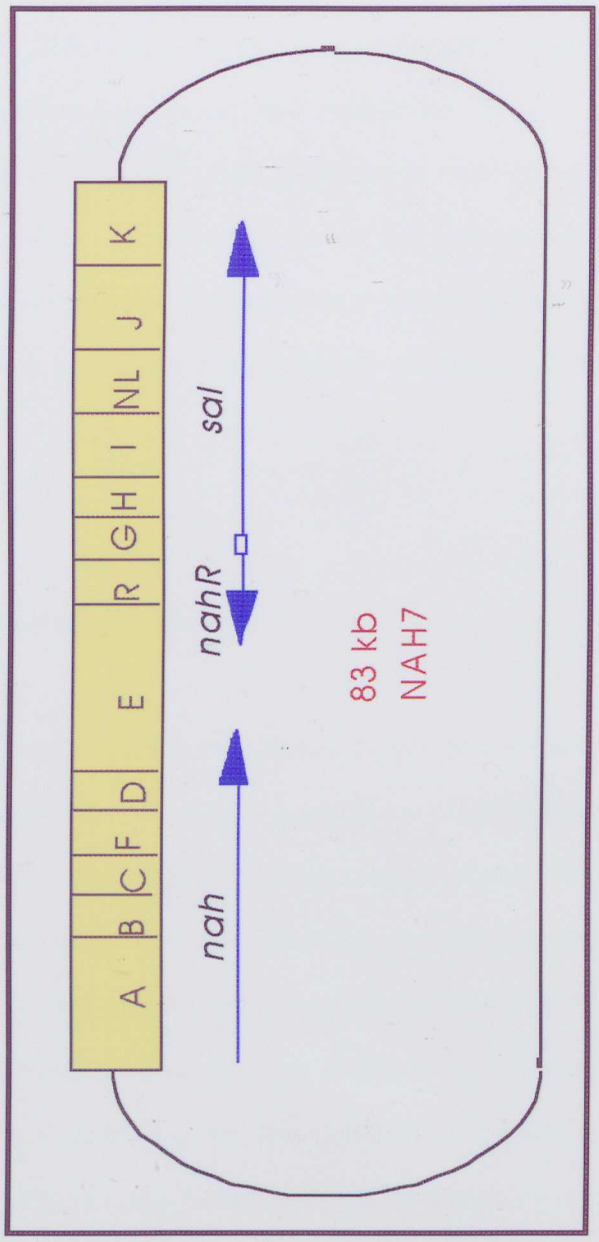


Figure 1.17: Naphthalene degradative pathway encoded in NAH7 plasmid. Its genes and regulation.

1.5.3 Genetics of Alkene Utilizing Organisms

Although microbial epoxidation of 1-alkenes was recognised nearly 30 years ago, when van der Linden (1963) reported the formation of 1,2-epoxyoctane from 1-octene, by octane induced cells of *P. aeruginosa*, little is known about the enzymology and even less about the molecular biology of alkene specific monooxygenases. Much of our understanding is based on the physiology and molecular biology of *P. oleovorans* which has been shown to catalyze the epoxidation of 1-alkenes, and of methane monooxygenase that also has been shown to catalyze the epoxidation of gaseous alkenes to their corresponding 1,2-epoxyalkanes.

1.5.4 Genetics of Terpenoid Metabolism

With the exception of camphor, little is known about the genetics of terpenoid degradation, although the metabolic aspects of degradation of many of these compounds have been elucidated. *Pseudomonas putida* PpG1 ATCC 17453 was originally isolated by enrichment culture with D-camphor as sole carbon source by Bradshaw *et al.* (1959), and was shown to contain a 140-megadalton self transmissible plasmid, termed CAM, which encodes the enzymes for the catabolism of D- and L-camphor. The reaction pathway for the catabolism of camphor to isobutyrate, involves 11 steps with the first step being the most thoroughly investigated. Camphor is first converted to 5-exo-hydroxy-camphor by a monooxygenase system composed of three proteins: an NADH putidaredoxin reductase which is a FAD-containing protein of 48 kDa, encoded by *camA*; an iron-sulphur redox protein (putidaredoxin) of 12 kDa encoded by *camB*; and cytochrome P-450cam, a terminal hydroxylase component of 47 kDa encoded by *camC*. The second step is the dehydrogenation of 5-exo-hydroxy camphor to form 2,5-diketo camphane by F-dehydrogenase encoded by

camD. Genes *camA* to *camD* have been cloned and shown to constitute the *cam* operon (*camDCAB*) (Koga *et al.*, 1989). Their expression is regulated negatively by the *camR* regulatory gene which is located upstream from the *camD* gene and transcribed divergently from the *cam* operon. The *camR* gene is auto repressed. The expression of the operon is negatively regulated through the interaction of CamR with the operator, which is located between *camR* and *camDCAB*, and a marked coordinated induction of the operon is achieved when CamR is inactivated by the inducer camphor (Davies *et al.*, 1990; Fujita *et al.*, 1993). The functional map of the camphor hydroxylase operon is presented in figure 1.18 below.

Putidaredoxin is a small protein of 106 amino acids encoded by *camB* (Koga *et al.*, 1989). Translation of the reductase gene (*camA*) translation is initiated by a GTG codon which is responsible for its low expression, in comparison to its neighbours, cytochrome P-450 gene (*camC*) and putidaredoxin gene (*camB*). Site-directed mutagenesis experiments in putidaredoxin at amino acid residues 58, 65 and 67 have shown a decrease in its association with the reductase component. Most interestingly, a series of mutations at the single tryptophan 106 residue at putidaredoxin's carboxy terminus, suggested that this residue is either involved in the formation of a complex with P-450 and/or with the electron transfer event between the iron sulphur and haem centres (Davies *et al.*, 1990).

The *camC* gene encodes a polypeptide of 412 amino acids which folds to form the haem binding site at the centre. The amino acid side chains contact substrate and oxygen next to the thiolate-ligated haem. The active site as a whole directs the binding and activation of dioxygen, with subsequent use of one atom for the stereospecific hydroxylation of camphor, and the reduction of the other to water. The role of particular peptide residues in this catalytic process has been elucidated using mutational analysis, and confirmed by the crystallographic data obtained by Poulos *et al.* (1987). According to these results, camphor is held at the active site by aliphatic contacts and a hydrogen bond from tyrosine 96 to the carbonyl group of the substrate. Site-directed mutagenesis of these contacts showed that loosening these

contacts resulted in a deterioration of the stereospecificity of the hydroxylation reaction, whereas tightening them gave a slight increase in the stereoselectivity of hydroxylation of smaller camphor analogues. In addition, cytochrome P-450 contains two regions termed HR1 and HR2 with high homology to highly conserved cysteinyl-containing peptides found in other P-450s, obtained from eukaryotic species, that are involved in the haem binding site (Nebert and Gonzalez, 1987). It is believed that cysteine 136 present in the HR1 peptide is the haem binding ligand (Davies *et al.*, 1990).

The camphor hydroxylase operon has provided a beautiful system for the application of state-of-the-art techniques of chemistry, biophysics and recombinant DNA technology. It has become the paradigm for mechanistic studies on iron containing monooxygenases to which most other systems are compared. Nevertheless, despite the wealth of information available for P-450cam, it has still not been possible to confirm the proposed active oxygen intermediate which initiates hydroxylation.



Figure 1.18: Functional map of camphor (*camDCAB*) operon.

1.6 Aims of the Project

The first enzyme of the α–pinene degradation pathway by *Pseudomonas fluorescens* NCIMB 11671 has been shown to be a cytoplasmic monooxygenase enzyme, that catalyses the internal epoxidation of α–pinene. Aikens (1990) suggested that the enzyme was multicomponent, because a non-linear relationship between protein concentration and monooxygenase activity was observed at low protein concentrations . In contrast, Saqib (1992), after complementation of *P. fluorescens* mutants lacking the monooxygenase activity with a genomic library of the wild type bacterium, suggested that the enzyme could be a single component. In addition, the enzyme appeared to be induced by α–pinene, required FAD and ferrous iron (Fe^{2+}) for activity (Aikens, 1990) and was highly specific in terms of substrate utilisation. Whole cells catalysed α–pinene but not limonene conversion to the corresponding epoxide. This indicated that the induced route(s) for α–pinene oxidation used by this isolation had little in common with the degradative pathways of other monoterpenes (Best *et al.*, 1987; Griffiths *et al.*, 1987a).

Therefore, the main aim in this project was to establish the nature of the enzyme. This would be achieved by :

i) Isolation of *P. fluorescens* mutants in the α–pinene degradation pathway and biochemical characterisation of them.

ii) Isolation of active protein and analysis of its properties. This, in the long term, would permit detailed studies on the mechanism of electron transport from NADH to the site of oxygen activation, the molecular interaction between the components of the holoenzyme, and the role of the iron in the activation of molecular oxygen.

iii) Cloning, sequencing and expression of the monooxygenase gene(s). Isolation of the α–pinene monooxygenase gene(e) and subsequent cloning and expression in a different host could be useful for the production of α–pinene epoxide or other internal epoxides, because no downstream degrading enzymes would be present as is the case in the wild type bacterium. In addition, placing the enzyme

downstream of a strong promoter would result in high levels of expression that may not only result in high epoxide production but would also allow further characterisation of the enzyme. Furthermore the availability of the cloned gene will allow subsequent workers to investigate the possibility of extending the substrate range by site directed mutagenesis or directed evolution. A relaxed substrate specificity of the enzyme would not only be useful for the production of chiral synthons that could be the precursors in the synthesis of a variety of chemicals, but could also be exploited to provide further information on the mechanism of oxygen activation. From an academic point of view it would be very interesting to study the mechanism of this enzyme that seems to be highly specific, and be able to determine whether the 2-methyl group of α–pinene or simply a double bond attached to a methyl group is essential for its catalytic activity.

Materials and Methods

2.1 Bacterial Strains and Media

2.1.1 Bacterial Strains

The following *E. coli* strains were used:

Strain	Genotype	Reference
HB101	**F⁻**, *thi*-1, *hsd*S20 (r_B^-,m_B^-), *sup*E44, *rec*A13, *ara*–14, *Leu*B16, *pro*A2, *lac*Y1, *rps*L20(str^r), *xyl*-5, *mtl*-1, *gal*K2, l-	Boyer and Roulland-Dussoix (1969)
HB101 (pRK2013)	**F⁻**, *thi*-1, *hsd*S20 (r_B^-,m_B^-), *sup*E44, *rec*A13, *ara*–14, *Leu*B16, *pro*A2, *lac*Y1, *rps*L20(str^r), *xyl*-5, *mtl*-1, *gal*K2, l-, (Kan^r, Tra$^+$)	Figurski and Helinski (1979)
HB101 (pMMB66)	**F⁻**, *thi*-1, *hsd*S20 (r_B^-,m_B^-), *sup*E44, *rec*A13, *ara*–14, *Leu*B16, *pro*A2, *lac*Y1, *rps*L20(str^r), *xyl*-5, *mtl*-1, *gal*K2, l-, (Amp^r)	Furste *et al.* (1986)
HB101 (pJRD215)	**F⁻**, *thi*-1, *hsd*S20 (r_B^-,m_B^-), *sup*E44, *rec*A13, *ara*–14, *Leu*B16, *pro*A2, *lac*Y1, *rps*L20(str^r), *xyl*-5, *mtl*-1, *gal*K2, l-, (Kan^r, Str^r, Mob$^+$)	Davison *et al.* (1987)
DH5α	**F⁻**, f80d*lacZ*DM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, D(*lacZYA-argF*)U169	Promega (1991)
DH5α (pTZ19U)	**F⁻**, f80d*lacZ*DM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, D(*lacZYA-argF*)U169, (Amp^r)	Mead *et al.* (1986)
DH5α (pBluescript II SK(+))	**F⁻**, f80d*lacZ*DM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, D(*lacZYA-argF*)U169, (Amp^r)	Stratagene (1991)
DH5α (pLAFR5)	**F⁻**, f80d*lacZ*DM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, D(*lacZYA-argF*)U169, (Tc^r, Mob$^+$)	Keen *et al.* (1988)

Pseudomonas fluorescens NCIMB 11671, is a prototroph (Floyd *et al.*,1986; Best *et al.*, 1987). It degrades α–pinene ((5S)-2,6,6-trimethyl bicyclo {3.1.1} hept-2-ene) via epoxidation. Its growth temperature is between 5 and 35°C with an optimum at 30°C. It can utilise glucose, lactate, acetate, (-)-arginine, betaine, (-) and (+) α–pinene and β–pinene as carbon sources. In addition, it is resistant to streptomycin, chloramphenicol, tetracycline and penicillin (Best *et al.*, 1987).

2.1.2 Storage of Bacterial Cultures

Bacterial colonies were maintained for up to 4 weeks in a cold room at 4°C on inverted LB agar plates tightly wrapped with parafilm, with appropriate selection for any plasmid harboured. For long term storage, 750 µl of an overnight culture were placed into a sterile 1.5 ml eppendorf and 250 µl of sterile glycerol were added. The contents were mixed by vigorous shaking and instantly frozen in a dry ice/ethanol bath. Subsequently, these glycerol stocks were stored at -70°C. Cells were recovered by scratching the surface with a sterile loop and streaking onto an appropriate agar plate. The tube was quickly returned into the freezer.

2.1.3 Growth Media

Nutrient broth and **nutrient agar** were prepared according to the manufacturers instructions (DIFCO) and were sterilised by autoclaving. When required, antibiotics were added after the autoclaved medium was cooled down to 50°C.

LB (Luria-Bertani) medium contained 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl per litre of deionised water. The pH was adjusted to 7.4 with 10 N NaOH before autoclaving. For the preparation of LB plates, Bacto-agar at a final concentration of 1.5% (w/v) was added to the broth prior to autoclaving. When

required, antibiotics were added after the autoclaved medium had cooled down to 50°C.

TB (Terrific broth) medium, as well as 2YT, are rich media that lead to extraordinarily high cell densities (Tartof and Hobbs, 1987), and thus, they were routinely used in bacterial cultures for large scale plasmid or cosmid preparations. The medium contained 12 g Bacto-tryptone, 24 g Bacto-yeast extract and 4 ml glycerol per litre of deionised water, and was sterilised by autoclaving. The solution was allowed to cool down to less than 60°C and then 100 ml of a heat sterilised solution of 0.17 M K_2HPO_4 and 0.72 M of KH_2PO_4 were added aseptically.

2YT medium contained 16 g Bacto-tryptone, 10 g Bacto-yeast extract and 5 g NaCl per litre of deionised water. The pH was adjusted to 7.0 with 5 N NaOH and the medium was sterilised by autoclaving.

SOB medium and Ψ (Psi) medium were used for the preparation of competent cells (Hanahan, 1985). The medium contained 2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 10 mM NaCl and 2.5 mM KCl per litre of milli Q (Millipore Corp.) water. The solution was autoclaved for 20 min at 15 lb/in^2. Prior to use, final concentrations of 10 mM $MgCl_2$ and 10 mM $MgSO_4$ were added from filter sterilised 1 M stocks.

Ψ (Psi) medium contained 2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 0.4% (w/v) $MgSO_4$ and 10 mM KCl per litre of milli Q water. The pH was adjusted to 7.6 with 10 M KOH and the medium was sterilised by autoclaving.

SOC medium was used for the outgrowth of transformed cells. This medium was identical to SOB medium but contained 20 mM glucose in addition. Glucose was added prior to use from a filter sterilised stock.

NZY plates were used in the preparation of the genomic library of *P. fluorescens* NCIMB 11671. The medium contained 1% (w/v) NZ amine (casein hydrolysate from ICN pharmaceuticals, UK), 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl, 10 mM $MgSO_4$ and 1.5% (w/v) bacto-agar per litre of deionised water. The pH was adjusted to 7.5 with 2 N NaOH.

Top agar plates were also used in the preparation of the genomic library of *P. fluorescens*. 100 ml of NZY broth were mixed with 0.7 g agarose and the medium sterilised by autoclaving. Melted top agar was added to aliquots of phage λ infected bacteria and spread evenly onto dry, prewarmed (37°C) NZY plates.

X-gal and IPTG plates. 40 µl of X-gal (5-bromo-4-chloro-3-indonyl-β–D-galactoside) and 4 µl of IPTG (isopropylthiolgalactoside) stock solutions were spread on LB plates, containing the appropriate antibiotics. The plates were incubated at 37°C until all fluid had disappeared, (3 to 4 h), before use. The stock solution of X-gal contained 20 mg/ml X-gal in dimethyl-formamide and was stored in dark tubes at -20°C. Stock solution of IPTG contained 200 mg/ml IPTG in milli Q water and was sterilised by filtration though a 0.2 µm filter. Then it was dispensed into 100 µl aliquots and stored at -20°C.

***Pseudomonas* basal minimal medium** (Cohen-Bazire *et al.*, 1957). Solution A, pH 6.8 contained 9.94 g of KH_2PO_4 and 7.8 g of K_2HPO_4 in 300 ml of deionised water. Solution B contained 1 g $(NH_4)_2SO_4$ and 20 ml of Hutner's vitamin free mineral base in 960 ml of deionised water. Solutions A and B were autoclaved separately and after cooling to 55°C, 40 ml of solution A were aseptically added to solution B. Hutner's mineral base contained 5 g nitrilotriacetic acid, 7.34 g $MgSO_4 \cdot 7H_2O$, 1.67 g $CaCl_2 \cdot 2H_2O$, 4.62 mg $(NH_4)_2MoO_4$, 49.5 mg $Fe_2(SO_4)_3 \cdot 7H_2O$ and 10 ml metals solution per 500 ml of deionised water. Metals solution consisted of 250 mg Na_2EDTA, 1.1 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $Fe_2(SO_4)_3 \cdot 7H_2O$, 154 mg $MnSO_4 \cdot H_2O$, 39.2 mg $CoNO_3 \cdot 6H_2O$, and 11.4 mg boric acid in 100 ml of deionised water. For agar plates 1.5% (w/v) noble agar was added to solution B prior to autoclaving.

2.2 Sterilisation

All methods involving the growth of bacteria were carried out aseptically with sterilised media, containers and instruments. Solutions and media were sterilised by

autoclaving for 20 min, at 15 lb/in^2 at 121°C. Small volumes were autoclaved in a domestic pressure cooker, and large volumes in an industrial autoclave. Heat labile solutions and hydrocarbons were filter sterilised by passage though a sterile 0.2 µm pore size "Acrodisc" filter (Gelman Sciences, Northampton, UK).

2.3 Antibiotic Preparations

Ampicillin: 100 mg of ampicillin were dissolved in 1 ml of milli Q water. The solution was sterilised by filtration, dispensed in 100 µl aliquots and stored at -20°C. Working concentrations were 50 to 100 µg/ml.

Kanamycin: 10 mg of kanamycin were dissolved in 1 ml of milli Q water. The solution was sterilised by filtration, dispensed in 100 µl aliquots and stored at -20°C. The working concentration was 25 µg/ml.

Streptomycin: 10 mg of streptomycin were dissolved in 1 ml of milli Q water. The solution was sterilised by filtration, dispensed in 100 µl aliquots and stored at -20°C. The working concentration was 25 µg/ml.

Tetracycline: 5 mg of tetracycline were dissolved in 1 ml of 50% ethanol. The solution was dispensed in 100 µl aliquots and stored at -20°C. The working concentration was 25 µg/ml.

2.4 Buffers and Reagents

2.4.1 Saline

Saline contained 0.9% (w/v) NaCl and was routinely used in all cell serial dilution purposes. Saline was sterilised by autoclaving.

2.4.2 Dilution Buffers

The following buffers were routinely used in cell washing procedures, monooxygenase, lyase, dehydrogenase activity assays and epoxide degradation experiments.

50 mM Sodium phosphate buffer, pH 7.0 was prepared by mixing 39 ml of 0.2 M Na_2HPO_4 with 61 ml of 0.2 M NaH_2PO_4. The final volume was adjusted to 400 ml with deionised water.

Clark and Lubs 50 mM phosphate buffer, pH 7.8 was prepared by mixing 50 ml of 0.2 M K_2HPO_4 with 42.8 ml of 0.2 N NaOH. The final volume was adjusted to 400 ml with deionised water. In α–pinene monooxygenase activity assays the same buffer saturated with α–pinene was used. α–Pinene (99.9%) and the above buffer were mixed vigorously at a volume ratio of 1:1 and the phases were allowed to separate. Subsequently the lower aqueous phase was utilised.

50 mM Glycine-NaOH buffer, pH 9.0 was prepared by mixing 50 ml of 0.2 M glycine with 8.8 ml of 0.2 M NaOH. The final volume was adjusted to 200 ml with deionised water. **25 mM Glycine-NaOH buffer, pH 9.0** was prepared by a two fold dilution of the above buffer.

25 mM Tricine-NaOH buffers, pH 7.4, 8.0 and 8.8 were prepared as follows: 25 ml of 0.2 M tricine were diluted to 120 ml deionised water. The solution was brought to the desired pH by addition of 0.2 M NaOH and the total volume adjusted to 200 ml with deionised water.

2.4.3 Buffers Used in α–Pinene Monooxygenase Purification Procedures

10 mM Tris-HCl buffer, pH 7.8 was prepared by diluting 10 ml of 1 M Tris base solution in 900 ml milli Q water and adjusting the pH to 7.8 with 10% HCl. The final volume

was adjusted to 1 litre with deionised water. This buffer was used in ion exchange chromatography and monooxygenase activity assays.

10 mM P-E-G buffer, pH 8.0 (Whited and Gibson, 1991) was prepared by mixing 50 ml of 0.2 M KH_2PO_4, 46.8 ml of 0.2 N KOH, 100 ml glycerol and 100 ml of 99% ethanol. The final volume was adjusted to 1 litre with milli Q water. The buffer was filtered though a 0.2 µm filter prior to application on the FPLC system. It was routinely used in ion exchange chromatography, for column equilibration and washing off the unbound proteins. The same buffer containing 0.1 to 2 M NaCl was used for the elution of bound proteins from the ion exchange matrix.

25 mM Bis-Tris, iminodiacetic acid buffer, pH 7.1. 25 mM Bis-Tris were brought to pH 7.1 by addition of iminodiacetic acid and the total volume was adjusted to 1 litre with milli Q water. The buffer was filtered though a 0.2 µm filter prior to application on the FPLC system, and stored in a dark bottle at 4°C. It was routinely used for equilibration of the chromatofocusing column.

Polybuffer 74, iminodiacetic acid pH 4.0. A ten fold dilution of polybuffer 74 was brought to pH 4.0 by addition of iminodiacetic acid and the total volume was adjusted to 1 litre with milli Q water. The buffer was filtered though a 0.2 µm filter prior to application on the FPLC system, and stored in a dark bottle at 4°C. It was routinely used for elution purposes during chromatofocusing.

50 mM phosphate buffer, pH 8.0, 0.1 M KCl, was used in gel filtration chromatography. The buffer was prepared by mixing 50 ml of 0.2 M KH_2PO_4 and 40 ml of 0.2 N KOH. 13 g of KCl were added in order to inhibit any binding of the constituents of the protein mixture to the column matrix, and the final volume was adjusted to 200 ml with milli Q water. The buffer was filtered though a 0.2 µm filter prior to application on the FPLC system.

2.4.4 Buffers Used in Protein Electrophoresis and Electroblotting

Acrylamide/Bis stock solution (30% T, 2.67% C) was prepared by dissolving 146 g acrylamide and 4.0 g of N' N' -bis-methylene-acrylamide in a total volume of 500 ml with milli Q water. The solution was filtered though a Whatman no. 2 filter and stored in a dark bottle at 4°C, for not more than 30 days.

5x Electrode (running) buffer, pH 8.3 was prepared by mixing 216 g glycine and 45 g Tris base (final concentrations in the 5x stock solution were 192 mM and 25 mM respectively). The total volume was adjusted to 3 litres with deionised water. In SDS PAGE 0.1% (w/v) SDS was also added. The running buffer was diluted to 1x solution prior to electrophoresis and stored at 4°C.

0.5 M Tris-HCl (stacking gel buffer), pH 6.8 was prepared by adding 6 g Tris base to 60 ml of deionised water. The pH was adjusted to 6.8 with 10 N HCl and the total volume was made up to 100 ml with water. The buffer was stored at 4°C.

1.5 M Tris-HCl (separation gel buffer), pH 8.8 was prepared by adding 54.45 g Tris base to 150 ml deionised water. The pH was adjusted to 8.8 with 10 N HCl and the total volume was made up to 300 ml with water. This buffer was also stored at 4°C.

5x Sample loading buffer was prepared by mixing 1.0 ml of 0.5 M Tris-HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml of 10% (w/v) aqueous SDS solution, 0.4 ml of β–mercaptoethanol, 0.4 ml of 0.05% (w/v) aqueous bromophenol blue solution, and 3.8 ml of deionised water. The sample was diluted 1:4 with sample buffer and heated at 95°C for 5 min prior to loading onto the gel. The sample buffer for native PAGE did not contain SDS and β–mercaptoethanol.

10x Stock electroblotting buffer, pH 11 was prepared by dissolving 22.13 g of CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) in 990 ml of deionised water. The solution was titrated with NaOH to pH 11 and the total volume adjusted to 1 litre with water.

Electroblotting buffer was prepared by adding 200 ml of 10x stock buffer and 200 ml methanol to 1600 ml of deionised water.

2.4.5 Buffers Used in Mutagenesis Procedures

0.1 M Phosphate buffer, pH 7.0 was prepared by dissolving 6.8 g Na_2HPO_4 and 1.16 g NaOH in 500 ml deionized water.

0.1 M Citrate buffer, pH 5.5 was prepared by dissolving 10.5 g citric acid and 4.4 g NaOH in 400 ml deionized water (Miller, 1974).

2.4.6 Buffers and Solutions Used in DNA Manipulations

1 M Tris-HCl, pH 7.5 and pH 8.0 was prepared by adding 121 g Tris base in 900 ml of deionised water. The pH was brought to 7.5 or 8.0 with concentrated HCl and the final volume was adjusted to 1 litre with deionised water. The solution was sterilised by autoclaving and stored at room temperature.

0.5 M Na_2EDTA, pH 8.0 was prepared by adding 186.1 g of $Na_2EDTA{\cdot}2H_2O$ to 800 ml of deionised water. The pH was adjusted to 8.0 with NaOH and the total volume was made up to 1 litre with water. The solution was sterilised by autoclaving and stored at room temperature.

5 M NaCl was prepared by dissolving 292.2 g of NaCl in 800 ml of deionised water. The volume was adjusted to 1 litre with water and the solution was sterilised by autoclaving.

10% (w/v) SDS was prepared by dissolving 100 g SDS in 900 ml of deionised water, at 68°C to assist dissolution. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl and the final volume was made up to 1 litre with water. The solution was dispensed into aliquots and stored at room temperature.

TE buffer was routinely used in DNA manipulations and contained 1 mM sodium-EDTA and 10 mM Tris-HCl, pH 8.0. It was sterilised by autoclaving.

RNAse solution, 10 mg/ml. 10 mg of DNAse free RNAse, prepared as described by Sambrook *et al.* (1989) were dissolved in 1 ml of sterile milli Q water. The resulting

solution was aliquoted in 100 µl fractions and stored at -20°C. The final enzyme concentration used in plasmid purifications was 20 µg/ml.

TE-saturated Phenol/chloroform solution. TE-saturated phenol was mixed with chloroform and isoamyl alcohol at a volume ratio of 25:24:1, to make the phenol/chloroform solution. Additionally, chloroform was mixed with isoamyl alcohol at a volume ratio of 24:1 and the solution was saturated with milli Q water, to make the chloroform solution. Both solutions were routinely used for extraction of DNA preparations.

3 M Na-acetate, pH 5.2 and pH 7.0 was used in the precipitation of nucleic acids. It was prepared by dissolving 408.1 g sodium acetate trihydrate in 800 ml of deionised water. The pH was adjusted to 5.2 with glacial acetic acid, or to 7.0 with 10% (v/v) acetic acid. The final volume was made up to 1 litre with deionised water and the solution was dispensed into aliquots and sterilised by autoclaving.

3 M K-acetate, pH 7.5 was used in the precipitation of nucleic acids. It was prepared by dissolving 9.82 g of potassium acetate in 90 ml of deionised water. The pH was adjusted to 7.5 with 2 M acetic acid and the final volume was made up to 100 ml with pure water. The solution was dispensed into aliquots filter sterilised and stored at -20°C.

The following solutions were used in small and large scale preparation of plasmid DNA by the alkaline lysis method (Sambrook *et al.*, 1989).

Solution I (GTE) was prepared by mixing 9 g glucose, 25 ml of 1 M Tris-HCl solution, pH 8.0 and 20 ml of 0.5 M Na_2EDTA solution, pH 8.0. The final volume was adjusted to 1 litre with deionised water. The working concentrations were 50 mM glucose, 25 mM Tris-HCl and 10 mM Na_2EDTA. The solution was sterilised by autoclaving and stored at room temperature.

Solution II (Lysis solution) was freshly prepared by adding 200 µl of 10 M NaOH, adjusted (stored in plastic bottles), and 1 ml of 10% (w/v) SDS in a total volume of 10 ml with sterile deionised water. The working solution contained 0.2 M NaOH and 1% SDS.

Solution III (K-acetate), pH 5.0 was prepared by adding 11.5 ml glacial acetic acid and 28.5 ml deionised water in 60 ml of 5 M potassium acetate. The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate.

The following solutions were used in small scale preparations of plasmid DNA using the Magic miniprep purification system (Promega, 1991):

Cell Resuspension Solution (CRS) was prepared by mixing 2.5 ml Tris-HCl solution, pH 7.5, 1 ml of 0.5 M Na_2EDTA solution, pH 8.0 and 500 µl of 10 mg/ml RNAse. The final volume was adjusted to 50 ml with deionised water. Thus, the working solution contained 50 mM Tris-HCl and 10 mM Na_2EDTA and 100 µg/ml RNAse, and was stored at room temperature.

Cell Lysis Solution (CLS) was the same as in alkaline lysis. The solution was stored at room temperature.

Neutralisation Solution (NS) contained 2.55 M potassium acetate brought to pH 4.8 with glacial acetic acid. The solution was stored and used at 4°C.

Column Wash Solution (CWS) contained 200 mM NaCl, 20 mM Tris-HCl, pH 7.5 and 5 mM Na_2EDTA in deionised water. The solution was diluted at a volume ratio of 1:1 with absolute ethanol and stored at room temperature.

The following solutions were used in large scale preparations of plasmid DNA by caesium chloride/ ethidium bromide gradient centrifugation (Little, 1988).

Solution Ia was 25% (w/v) glucose, 50 mM Tris-HCl, pH 8.0 and 10 mM Na_2EDTA. The solution was stored and used cold.

Solution IIa was the same as in alkaline lysis, and **Solution IIIa** was the same as in the magic miniprep method.

The following solutions were used in methods for the detection of mega plasmids in *P. fluorescens* NCIMB 11671.

Sarkosyl contained 0.2% (w/v) sarkosyl in TE buffer, pH 8.0. The solution was sterilised by autoclaving and stored at 4°C.

Cell lysis solution was prepared by dissolving 10 mg of lysozyme in 1 ml of 1x TBE buffer, containing 50 µg/ml RNAse. The solution was made fresh whenever it was required, and was filter sterilised.

SDS mixture was prepared by dissolving 20 mg SDS in 10 ml of 1x TBE buffer, containing 10% (w/v) ficoll. The solution was made fresh whenever it was required.

2.4.7 Buffers Used in DNA Electrophoresis

Tris Acetate EDTA buffer (TAE), 50x was routinely used in agarose gel electrophoresis. It was made by dissolving 242 g of Tris base in 800 ml of deionised water. 57.1 ml of glacial acetic acid and 10 ml of 0.5 M of Na_2EDTA, pH8.0 were added and the final volume adjusted to 1 litre. When required, this was diluted to 1x solution with deionised water. The working solution had a final concentration of 40 mM Tris-acetate and 1 mM Na_2EDTA, pH 8.0.

Tris Borate EDTA buffer (TBE), 10x was used in agarose gel electrophoresis of high molecular weight DNA and in DNA sequencing. It was made by dissolving 121 g Tris base, 7.4 g Na_2EDTA and 53.4 g boric acid in deionised water to a final volume of 1 litre. When required, this was diluted to 1x solution with deionised water.

DNA sample loading buffer I contained 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol in TE. The DNA samples were mixed with 1/6 volume of this buffer prior to electrophoresis.

DNA sample loading buffer II contained 2% (w/v) ficoll and 0.2% (w/v) orange G in TE buffer. The DNA samples were mixed with 1/5 volume of this buffer prior to electrophoresis.

Ethidium bromide, 10 mg/ml. 1 g of ethidium bromide was added to 100 ml of deionised water. The suspension was stirred on a magnetic stirrer for several hours to

ensure that the dye was dissolved. Finally the solution was transferred to a dark bottle and stored at 4°C.

2.4.8 Buffers Used in the Preparation of Competent Cells

RF1 was prepared by mixing 10 ml of 1 M RbCl, 5 ml of 1 M $MgCl_2$, 3 ml of 1 M potassium acetate, 1 ml of 1 M $CaCl_2$ and 15 ml glycerol, The total volume was adjusted to 100 ml with milli Q water, and the pH brought to 5.8 with 0.2 N acetic acid. The solution was sterilised by filtration though a 0.2 µm filter and stored at 4°C.

RF2 was prepared by mixing 0.5 ml of 0.5 M MOPS (3-(N-morpholino)-propanesulfonic acid) buffer, pH 7.0 with NaOH, 0.25 ml RbCl, 1.88 ml $CaCl_2$ and 3.75 ml glycerol. The final volume was adjusted to 25 ml with milli Q water, the solution was sterilised by filtration though a 0.2 µm filter and stored at 4°C.

2.4.9 Buffers Used in the Construction of the Genomic Library

SM buffer was used for the dilution and storage of *in vitro* packaged phage. 5.8 g NaCl and 2.0 g $MgCl_2$ were added to 995 ml of 50 mM Tris-HCl, pH 7.5. The solution was sterilised by autoclaving and then 2% (w/v) gelatine was added aseptically. The buffer was dispensed into sterile universals and stored at room temperature.

2.4.10 Nucleic Acid Hybridisation Solutions

20 x SSC was prepared by adding 175.3 g NaCl and 88.2 g of trisodium citrate to 900 ml of milli Q water. The pH was adjusted to 7.0 with HCl and the final volume made

up to 1 litre with milli Q water. The solution was autoclaved and stored at room temperature.

50x Denhardt's Solution was prepared by dissolving 5 g ficoll, 5 g polyvinylpyrrolidone and 5 g of BSA (Bovine Serum Albumin) in 300 ml of milli Q water. Once dissolved the total volume was brought up to 500 ml. The solution was filtered though a 0.2 μm filter, dispensed in 25 ml aliquots and stored at -20°C.

Denaturation solution for colony hybridisation contained 1.5 M NaCl and 0.5 M NaOH in milli Q water.

Neutralisation solution for colony hybridisation contained 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5, and 0.001 M EDTA, pH 8.0 in milli Q water.

Acid cut solution was prepared by adding 10.4 ml of concentrated HCl in 500 ml of milli Q water.

Base denaturation solution contained 0.5 N NaOH and 1.5 M NaCl in milli Q water.

Tris neutralisation solution contained 0.5 M Tris-HCl, pH 7.5 and 1.5 M NaCl in milli Q water. The pH of the solution was adjusted to 7.5 with a few drops of concentrated HCl.

Neutralising SSC solution was prepared by adding 10 ml of 20x SSC and 20 ml of Tris-HCl, pH 7.5 in 70 ml of milli Q water.

2.5 Chemicals

α–Pinene, α–pinene oxide, limonene and limonene oxide were purchased from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, England). The majority of other compounds, substrates, media components, antibiotics etc. were obtained from BDH Chemicals, Sigma Chemical Co. Ltd. (both Poole, Dorset, England), Fisons Scientific Apparatus Ltd. (Loughborough, Leics., England). Materials for protein purification were purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Herts, UK), and Pharmacia Fine Chemicals Ltd. (Upsala, Sweden) Bacterial growth media were

obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). Molecular biology reagents and enzymes were purchased from Promega (Southampton, UK), Boehinger Mannheim (East Sussex, UK), Gibco BRL, Pharmacia Fine Chemicals Ltd. (Upsala, Sweden), Northumbria Biologicals Ltd. (Northumberland, UK), International Biotechnology Incorporation Ltd. (Cambridge, UK), United States Biochemical (Cleveland, Ohio, USA), Qiagen gmbh (Chastworth, USA) and Stratagene (San Diego, California, USA).

2.6 Centrifugations

Small scale centrifugations (volume up to 1.5 ml) were carried out using a bench top MSE Micro Centaur centrifuge (MSE scientific instruments, UK) at room temperature, or when required at 4°C. Larger scale centrifugations (2 to 20 ml) were performed using an MSE-18 high speed refrigerated centrifuge (MSE scientific instruments, UK). A Sorvall RC-5 superspeed (Dupont Ltd, UK) with either an SS34 or a GS3 rotor was used for centrifugation of volumes up to 500 ml. Finally, a Sorvall RC8-50 ultracentrifuge fitted with a 70-Ti or VTi-50 rotor was used for preparations of cell free extracts and large scale plasmid DNA isolations.

2.7 Electrophoresis Techniques

2.7.1 Polyacrylamide Gel Electrophoresis of Proteins and Polypeptides

Protein samples were analyzed by both native- and SDS-PAGE as described by Andrews (1990) and Laemmli (1970), using a discontinuous buffer system. The vertical Protean II electrophoresis apparatus from Bio-Rad was routinely used. The glass plates were washed using detergent, water and 70% ethanol and assembled

according to the manufacturers instructions. The separating gel was poured between the plates, covered with a thin layer of water-saturated-2-butanol, and allowed to polymerise for at least 1 h at room temperature. The overlay was poured off and the top of the gel was washed several times with deionised water to remove any unpolymerised acrylamide. Subsequently, the stacking gel was poured onto the surface of the polymerised resolving gel. The teflon comb was inserted immediately without trapping air bubbles and the gel mould was allowed to stand at room temperature for another 30 min for the stacking gel to polymerise. The comb was then removed and the wells washed several times with running buffer prior to loading the sample.

The total monomer concentration used was T=9% or T=12%, whereas the crosslinking monomer concentration was C=2.67%. The recipes used for the preparation of the gels are shown on table 2.1 below. The monomer solution was prepared by combining all reagents except ammonium persulphate and TEMED. The solution was deaerated under vacuum for 15 min and then the two catalysts added prior to casting the gels. In native-PAGE the SDS was replaced by deionised water.

Protein samples and molecular weight standards were diluted at a ratio of 1:4 with 5x sample loading buffer. For SDS-PAGE, the samples were placed in a boiling waterbath for 5 min prior to loading. 5 to 20 µl of samples were loaded in each well and the electrophoresis was carried out at 100 Volts during stacking and 150 Volts during separation, until the dye front reached the bottom of the separation gel. Molecular weight standards were run alongside samples.

The gels were stained with Coomassie blue (0.2% R-250 in 50% methanol, 10% acetic acid), for 60 min and then destained by several washes in 50% (v/v) methanol, 10% (v/v) acetic acid (Andrews, 1990). The gels were finally stored in 7% (v/v) acetic acid.

A silver staining method also used was a modification of the method described by Morissay (1981). The gels were prefixed in 50% (v/v) aqueous methanol containing 10% (v/v) acetic acid, washed in 5% (v/v) aqueous methanol containing

7% (v/v) acetic acid, and fixed in 10% (v/v) aqueous glutaraldehyde using 6 ml solution per ml of gel. Each of these treatments was carried out for 30 min. The gels were soaked in deionised water overnight and then rinsed in fresh water for 30 min or rinsed in several changes of distilled water (one every 10 min) for at least 2 h. Subsequently, the gels were soaked in aqueous dithiothreitol (5 µg/ml), using 12 ml per ml of gel. The gels were rinsed rapidly in a small volume of distilled water followed by a small volume of developer solution consisting of 50 µl of 37% (w/v) formaldehyde in 100 ml of 3% (w/v) sodium carbonate. Then, the gels were soaked in developer solution using 12 ml per ml of gel until the desired level of staining was reached (approximately 5 to 15 min). The staining was stopped by adding 2.3 M citric acid to the developer solution. After 10 min, the background of the gels was destained further with 300 ml of silver destaining solution and finally, the gels were washed several times in distilled water for 30 min and soaked in 0.03% (w/v) sodium carbonate. The method is able to detect 0.1 ng of protein.

Table 2.1: Formulation for SDS-PAGE separation and stacking gels.

	Separation gel (0.375 M Tris, pH8.8)		Stacking gel (0.125 M Tris, pH 6.8)
Monomer Concentration (%T, 2.67%C)	12%	9 %	4 %
Acrylamide/Bis (30% T, 2.67% C)[1]	12 ml	9 ml	1.3 ml
Distilled water	10.05 ml	13 ml	6.1 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	-
0.5 M Tris-HCl, pH 6.8	-	-	2.5 ml
10% (w/v) SDS	300 µl	300 µl	100 µl
10% ammonium persulphate (fresh)	150 µl	150 µl	50 µl
TEMED	15 µl	15 µl	10 µl
TOTAL VOLUME	30 ml	30 ml	10 ml

1: %T = ((g Acrylamide + g Bis-Acrylamide)/Total Volume) x 100; %C = (g Bis-Acrylamide/(g Acrylamide + g Bis-Acrylamide)) x 100.

Silver destaining solution was prepared by dissolving 37 g of sodium chloride and 57.85 g of cupric sulphate pentahydrate in 850 ml of deionised water. 0.88 M of ammonia solution was added until the precipitate disappeared and the volume was adjusted to 1 litre with water.

2.7.2 Electroblotting

Electroblotting was carried out according to the method described by Wilson and Yuan (1989), using a Bio-Rad Protean II electroblotting apparatus. Polyvinylidene membranes (PVDF) were used. The membrane was wetted in methanol for a few seconds (10-20 sec) and then soaked in electroblotting buffer, together with the filter papers and the fibre pads. The gel to be electroblotted was also soaked in the same buffer for 5 min. The "blotting sandwich" was assembled in the following order: perforated plastic cover (black), fibre pad, 3 layers of filter paper, gel, PVDF membrane, 3 layers of filter paper, fibre pad and perforated plastic cover (white), and placed in the apparatus with the black cover facing the anode and the white cover facing the cathode. Electroblotting was performed at 90 Volts, 300 mA for 20 min. The membrane was removed and washed in 3 changes of milli Q water for 5 min each, prior to staining. The blot was stained in 0.2% (w/v) Poinseau S in 1% (v/v) acetic acid and the background destained by several washes with milli Q water. The gel was dried and then the band of interest was excised and forwarded for amino acid sequencing.

2.7.3 Urea-Acrylamide Gel Electrophoresis

Urea-acrylamide gel electrophoresis is a standard method to identify, purify and separate small DNA fragments (Sambrook *et al.*, 1989) such as those generated in sequencing reactions. The IBI STS 45 DNA sequencing apparatus was used. The size of

the gel was approximately 50 cm x 40 cm and contained 64 lanes. The glass plates were cleaned with soap, 70% (v/v) ethanol and acetone and then the short plate was treated with silanising agent (BDH), rinsed with distilled water and 70% (v/v) ethanol, and dried. The plates were arranged for assembly with the silane treated plate facing inward. Wedge spacers, 0.2 mm (top)-0.4 mm (bottom) were inserted, the glass plates were clamped and then taped together. Subsequently, the plates were set at a 45° angle and the polymerising solution was added.

The sequencing gel was prepared by mixing 22.5 ml of (19:1) acrylamide:N-N'-methylene-bis-acrylamide solution (BDH), 15 ml of 10x TBE and 63 g of ultra pure urea in 100 ml of milli Q water. The mixture was placed at 35°C to facilitate dissolution, and then the final volume was adjusted to 150 ml with milli Q water. The solution was filtered though a 0.2 µm filter and deaerated under vacuum. 850 µl of freshly made 10% (w/v) ammonium persulphate and 82.5 µl of TEMED were added, and the mixture was swirled to mix. The solution was poured immediately between the plates taking care to avoid introducing any bubbles. The comb was inserted and the plates were laid in a horizontal position overnight.

The top of the gel was washed several times with running buffer (1x TBE) and was prerun at 60 W constant power for 30 to 45 min. The samples were denatured at 95°C for 5 min and then 3 µl aliquots were loaded into adjacent wells, in sets of four (A, C, G, T). The gel was then electrophoresed at 40 W constant power (resulting in a gel temperature of 50-55°C) until the xylene cyanol dye had reach the bottom of the gel (approximately 3 h) for short runs, or 8 to 9 h for long runs. After electrophoresis the gel was immersed in a solution of 10% (v/v) methanol, 10% (v/v) acetic acid for 1h to remove the urea that can prevent the gel from drying, and then transferred to a sheet of Whatman 3MM filter paper and covered with saran wrap. The gel was then dried at 80°C for 1 h. Subsequently it was placed in a film cassette and a sheet of KODAK X-OMAT AR autoradiography film placed on top of it. The cassette was kept at room temperature overnight and the film was developed automatically in a FUJI film processor.

2.7.4 Agarose Gel Electrophoresis

Agarose gels (0.6% to 1.0% (w/v)) were used to separate DNA restriction fragments. They were prepared by dissolving the appropriate amount of ultra pure agarose (BRL) into 1x TBE or TAE buffer to give the desired concentration, by boiling. After cooling to 50°C ethidium bromide from a 10 mg/ml stock solution was added to a final concentration of 0.5 µg/ml of gel. The agarose was then poured into a prepared tray with the comb in place and allowed to set in an horizontal position for one hour. When set, the gel was placed in an IBI electrophoresis tank. The DNA samples were mixed with 1/6 volumes of loading buffer before loading and then electrophoresed at 20 to 100 Volts depending on the size of the DNA fragments and the application, until the dye had migrated to 3/4 of the gel length. At least one of the samples was a set of molecular weight markers, such as λ (*Hin*d III-*Eco*R I) marker, 1 kb DNA ladder (BRL) or high molecular weight DNA marker (BRL). DNA bands were visualised using a short wave UV transilluminator and photographed using a polaroid MP-4 camera with type 667 film.

2.8 Growth of *Pseudomonas fluorescens* NCIMB 11671 on α–Pinene

2.8.1 Small Scale Cultures

P. fluorescens was grown in a similar fashion to that described by Best *et al.* (1987), although an initial adaptation procedure was not carried out. Therefore, 50 ml universal bottles containing 10 ml LB broth, or nutrient broth were inoculated with a single colony of *P. fluorescens* and incubated overnight at 30°C in a rotary incubator. Routinely, a large inoculum (~10% (v/v)) was used, to inoculate a flask with minimal medium, containing 0.2% (v/v) sodium lactate. The amount of medium in the flasks was only 1/4 of the total flask volume in order to ensure good aeration. Filter sterilised

α–pinene was added to the medium immediately after inoculation at a concentration of 0.2% to 0.5% (v/v). A further 0.2% (v/v) of α–pinene was added to the culture after 20 h of incubation. Various percentages of α–pinene (0.2%, 0.5%, 1%, 3%, 10% (v/v)) or α–pinene oxide (0.2%, 0.5% (v/v)) in a minimal medium without lactate were used for the determination of the optimum substrate concentration and toxicity studies. Moreover, other cosubstrates, such as succinate or pyruvate were used at a concentration of 0.2% (v/v) in order to determine the optimum growth medium. The flasks were incubated for 24 h at 30°C with shaking, and the cells were harvested by centrifugation.

The organism was also grown on minimal medium plates under α–pinene or α–pinene oxide vapour into a self-heated incubator (ENKAB-1 incubator, Elplas Ltd, UK) at 30°C. Colonies approximately 1 mm diameter were produced after 1 to 2 days of incubation. Such plates were wrapped with parafilm and stored at 4°C for short periods.

2.8.2 Large Scale Cultures

Large scale cultivation was achieved in a bioreactor (Chemap, Switzerland) with a working volume of 20 litres. The bioreactor contained 400 ml of Hutner's vitamin free mineral base, 200 ml of 10% (w/v) ammonium sulphate (final N concentration was 0.4 g/Litre), 40 ml sodium lactate (final concentration 0.2% (v/v)) and deionised water to 19 litres. The above medium was sterilised *in situ* at 15 psi, 121°C for 40 min, whereas 800 ml of phosphate buffer, pH 6.8 (31.13 g KH_2PO_4 and 25 g K_2HPO_4 per litre of deionised water) were autoclaved separately and added to the rest of the medium prior to inoculation. The bioreactor was inoculated with 200 ml (1% inoculum, (v/v)) of an overnight culture of *P. fluorescens* in LB broth or nutrient broth and 0.5% (v/v) of α–pinene was added immediately after inoculation. The cultivation conditions were: aeration rate 1 litre air/min agitation 500 rpm; temperature 30°C; pH kept at 7.1 by

automatic addition of 10% (v/v) sulphuric acid. The dissolved oxygen in the bioreactor was monitored using an oxygen electrode (Chemap, Switzerland), whereas the residual oxygen and CO_2 in the off gases were measured using an on-line mass-spectrometer (Gas 200F, VG Gas Analysis Ltd, UK).

The sodium lactate concentration in the medium was measured in the supernatant of 10 ml samples taken from the bioreactor every two hours by HPLC (Waters), on an ion exclusion HPX-87H, 300 x 7.8 mm column (Bio-Rad) at a temperature of 35°C. The mobile phase was 0.01 M sulphuric acid degassed with helium at a flow rate of 0.675 ml/min. 15 µl samples were injected from a WISP 710B auto injector (Waters) and the running time was 19 min. Lactate was detected at 210 nm using an LC spectrophotometer (Waters, model 455). The peak area was recorded on a Hewlett Packard HP 3396A integrator. A standard solution of 20 mM sodium lactate was used to calibrate the system. In order to determine whether the cells were using the D- or L- form of lactate, a commercial kit that measures only L-lactate was used (Sigma, Proc. no 726-UV/826-UV). The measurement was based on the following reaction:

$$Lactate + NAD \xrightarrow{LDH} Pyruvate + NADH$$

Samples were collected every 2 h for a period of 24 h and analysed according to the manufacturers instructions for the presence of L-lactate.

The ammonia concentration in the culture medium was determined by a colorimetric assay using Nessler's reagent (Sigma). Samples were taken from the bioreactor every 4 h and the cells were centrifuged at 15,000 g for 10 min. 10 µl of the clear supernatant were added to 990 µl of milli Q water and the suspension was well mixed. 1 ml of Nessler's reagent was added and the ammonia concentration determined by interpolating the optical density at 430 nm from a standard curve.

Sodium lactate at a concentration of 0.2% (v/v) and ammonium sulphate at a final concentration of 0.2 g/litre were added to the culture as soon as their levels were

dropped to a quarter of their starting amount. The α–Pinene concentration was not easy to determine and thus, 0.5% (v/v) of α–pinene was added empirically to the culture. Usually, the first addition was after 20 h of growth and then every 8-10 h approximately. All additions were performed by sterile transfer though the phosphate buffer inlet.

The contents of the bioreactor were routinely harvested at OD_{600} between 2.5 and 3.0, (approximately the middle of the exponential phase). The cells were harvested by centrifugation at 6,000 g for 30 min at 4°C. The pellets were washed twice in half their original volume of 10 mM phosphate buffer, pH 8.0, containing 10% (v/v) glycerol, resuspended to a slurry with an OD_{600} of 100, drop frozen in liquid nitrogen, and stored at -70°C until needed.

2.9 Preparation of High Speed Supernatants (HSS)

Cultures of *P. fluorescens* or *E. coli* were centrifuged at 6,000 g for 30 min and the pellets washed twice with 10 mM P-E-G buffer, pH 8.0 or 50 mM phosphate buffer, pH 7.0 respectively. The cell pellets were finally resuspended in the same buffers containing 1 mM DTT and 0.002% (w/v) DNAse I to a paste, and lysed by two passages though a French pressure cell at 10,000 lb/in^2 and 4°C. The resulting crude extract was centrifuged at 30,000 g, 4°C to remove debris, and the high speed supernatant was obtained by ultracentrifugation at 115,000 g for another hour at 4°C.

2.10 Estimation of Protein Concentration

The absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to proteins. This phenomenon is used in

the Bradford (1976) protein assay which is available as a protein assay kit (Bio-Rad Laboratories, Richmond, Cal., USA).

Bovine gamma globulin at concentrations 0, 0.288, 0.576, 0.864, 1.152 and 1.44 mg/ml in a total volume of 50 µl was used as a standard for the preparation of a calibration curve. Each standard concentration was placed into a test tube and 2.5 ml of Bio-Rad dye reagent (diluted 1:4 in milli Q water and filtered though Whatman no 2 filter) was added. The tubes were incubated at room temperature for 20 min and then the absorbance at 595 nm was measured against a blank that contained 50 µl of water instead of bovine gamma globulin. The calibration curve was obtained by plotting the absorbance reading, versus the protein concentrations of the standards. The "unknown" protein concentration of other samples was estimated from the calibration curve.

2.11 α–Pinene Monooxygenase Activity Assays

2.11.1 Polarographic Assay

The oxygen uptake activity of the monooxygenase was assayed polarographically with a Clark type oxygen electrode. 100 µl of high speed supernatant or whole cell slurry (containing 0.5 mg of protein) were diluted with 1.9 ml of 50 mM Clark and Lubs phosphate buffer, pH 7.8 and placed in the appropriate compartment of the oxygen electrode. After 30 sec, 100 µl of sonicated α–pinene in the same buffer (10 ml of α–pinene in 10 ml of buffer) and 1 mM of NADH were added and the reaction was continued for one more minute. NADH was not added to whole cell samples. The temperature of the reaction was kept at 35°C by water circulation around the electrode compartment. All measurements were corrected for endogenous oxygen consumption recorded in the absence of the substrate and the monooxygenase activity was determined as oxygen consumption rate.

Flavin containing monooxygenases, especially when supplied with carbon substrate analogs often exhibit substrate-stimulated NADH oxidation but do not form oxidised products. This phenomenon is called uncoupling. The degree of uncoupling was estimated by the method of Uetz *et al.* (1992), using the same oxygen electrode. The reaction was started by the addition of 1 mM NADH as above. When all of the NADH was oxidised, 10 µg of catalase/ml was added and the formation of oxygen was monitored.

2.11.2 Spectrophotometric Assay

Spectrophotometric assays were based on the assumption that α–pinene monooxygenase is an NADH-linked enzyme and that the activation of molecular oxygen requires the oxidation of NADH to NAD which can be measured by the reduction in absorbance at 340 nm. The sample consisted of 800 µl of Clark and Lubs phosphate buffer saturated with α–pinene, pH 7.8, 100 µl high speed supernatant containing 0.5 mg of protein, 1 mM NADH and 0.01 mM of FAD. The sample was incubated in α–pinene saturated buffer and FAD for 10 min and then NADH was added and the enzyme activity determined by monitoring the oxidation of NADH at 340 nm. The temperature of the reaction was 35°C. All measurements were corrected for endogenous NADH consumption recorded in the absence of the substrate.

2.11.3 Electrophoretic Assay

Polyacrylamide discontinuous native gel electrophoresis was carried using a monomer concentration of 9% (% T). After electrophoresis the gels were immersed in Clark and Lubs phosphate buffer, pH 7.8 containing 8.7% (v/v) glycerol, 1 mM NADH and 0.6 mM 4-nitro-blue-tetrazolium chloride and incubated with shaking, at 30°C in the

dark for 60 min. The appearance of purple bands should mark the position of reductases (Hartmans *et al.*, 1991). After staining, the gel was stored in deionised water containing 0.1% (w/v) of sodium azide.

2.11.4 Gas Chromatographic Assay of Product Formation

The formation of α–pinene oxide was assayed by gas chromatography using a Phillips PU 4500 gas chromatograph with an FID detector with separation on a 5% OV-17 column on chromosorb WHP 80/100 mesh and nitrogen as carrier gas. The conditions used were: nitrogen flow rate 40 ml/min; column temperature programme from 100°C to 200°C with the initial temperature held for 2 min and an increase of 12°C/min; detector and injector temperatures 250°C and 180°C respectively. Separations were also done on a 50 M Lipodex C (Mackera-Nagel, Duren, Switzerland) chiral capillary column in a Packard (Chrompack, Netherlands) capillary GC with helium as carrier gas. The conditions used were: helium flow rate 175 ml/min column temperature 120°C; detector and injector temperatures 170°C and 150°C respectively.

GC methods for analysing α–pinene oxide contained whole cell and enzyme incubations. Resting cells were prepared from an overnight culture of *P. fluorescens* in minimal medium containing 0.2% (v/v) sodium lactate and 0.5% (v/v) α–pinene as carbon sources. The culture was centrifuged and the pellet washed twice in Clark and Lubs phosphate buffer, pH 7.8, and resuspended to an OD_{600}=10 in the same buffer. 1 ml aliquots of cell suspension were placed in each of six bijoux bottles, 10 µl of α–pinene was added and the bottles were suba sealed and incubated at 30°C with moderate shaking. One bottle was taken out every 10 min and its contents extracted with 500 µl of dichloromethane. Bottles containing only α–pinene or α–pinene oxide in buffer were also used as controls.

High speed supernatants, or purified α–pinene monooxygenase containing 0.1 to 0.5 mg of protein were placed in a bijoux bottle and incubated with 0.01 mM FAD, 1 mM NADH and Clark and Lubs phosphate buffer, pH 7.8 up to 2 ml. A final concentration of 10 mM $FeSO_4$ was added to purified protein samples only. 10 µl of α–pinene was added and the samples incubated at 35°C with moderate shaking for 15 to 30 min. They were then extracted in 500 µl dichloromethane and analysed by GC or GC-MS analysis. In all assays α–pinene, α–pinene oxide, limonene and limonene oxide purchased from Aldrich Chemical Co. and Sigma were used as standards.

Culture supernatants of both wild type and mutant *P. fluorescens* were also used for the detection of α–pinene oxide, as well as of other metabolites reported in the α–pinene oxidation pathway. Five ml of supernatants from overnight cultures of *P. fluorescens* in minimal medium containing 0.2% (v/v) sodium lactate and 0.5% (v/v) α–pinene as carbon sources were extracted with 2.5 ml dichloromethane. The organic layer was separated, and dried by passage though a sodium sulphate column, and then assayed chromatographically. These assays were used in the characterisation of *P. fluorescens* mutants.

GC-MS analyses were carried out by the ULIST GC-MS Service, on a 25 m x 0.22 mm BP20 capillary column programmed to 100°C (1 min) to 270°C at 8°C per min. Helium was used as carrier gas at a pressure of 13.5 psi.

2.12 α–Pinene Oxide Lyase Activity Assay

2.12.1 Spectrophotometric Assay

The assay was based on the formation of either the *cis*- or *trans*- isomer of 2-methyl-5-isopropylhexα–2,5-dienal, from α–pinene oxide, which absorbs at 235 nm (Griffiths *et al.*, 1987b). For this purpose the following procedure was used: High speed supernatants containing 0.5 mg of protein were mixed with 0.1 M glycine-NaOH buffer,

pH 9.0 so that the total volume was 2 ml. The mixture was incubated at 30°C for 5 min and 1 µmole of α–pinene oxide was added. The absorbance at 235 nm was monitored immediately for a time course of 15 min. All assays were corrected for epoxide breakdown in the absence of enzyme.

2.12.2 Electrophoretic Assay

Polyacrylamide discontinuous native PAGE was carried using a monomer concentration of 9% (%T). After electrophoresis, the gels were immersed in an emulsion of 20 mM α–pinene oxide in 0.1 M glycine-NaOH, pH 9.0 buffer. Precipitation of a white band of the insoluble *trans*-2-methyl-5-isopropylhexα–2,5-dienal in the polyacrylamide gel marked the position of the lyase band (Griffiths *et al.*, 1987b).

2.13 Aldehyde Dehydrogenase Activity Assay

The determination of the activity of this enzyme was based on the assumption that all hydroxylation and epoxidation reactions require the presence of oxygen and NADH and that the cofactor can be regenerated by an NAD-linked alcohol or aldehyde dehydrogenase. 100 µl of high speed supernatant was mixed with 900 µl of 50 mM phosphate buffer, pH 7.0 and 1 µmole of α–pinene oxide was added. The reaction was started by the addition of 1 mM of NAD and the increase in absorbance at 340 nm was monitored over a time course of 10 min. The reaction was carried out at 30°C.

2.14 Purification of α–Pinene Monooxygenase

2.14.1 Buffer Exchange of Protein Samples

The ionic strength of protein samples was adjusted to that of the starting buffer either by dialysis or by passing though a Sephadex PD 10 column (Pharmacia) before application to a chromatography column. The same procedures were also used for removal of salts from a protein sample after elution from a column prior to measuring its activity. Dialysis tubing with a 10 kDa molecular weight cut-off was treated as described by Sambrook *et al.* (1989) before use. The protein sample was placed in the bag and sealed by clipping. The tube was placed in a large volume of the desired buffer (usually 5 litres), and incubated at 4°C for 14 to 16 h. For small sample volumes a PD 10 column was used. The column was washed with 15 ml of the desired buffer, then 2.5 ml of sample were applied and the proteins were eluted with 3.5 ml of the desired buffer.

2.14.2 Ion Exchange Chromatography

All ion exchange chromatography procedures were carried out on an FPLC system at 4°C. Two types of matrix were used: a strong ion exchanger, the Mono Q HR5/5 prepacked column (Pharmacia) and two weak ion exchangers, the MEMSEP 1010 cartridge (Millipore) and the DEAE Sephacel matrix packed into a XK 16/20 column (Pharmacia). The counterions of the columns were changed to phosphate, when necessary, by applying 10 column volumes of 1 M KH_2PO_4. The columns were then equilibrated to 10 mM P-E-G buffer, pH 8.0 by flushing 20 to 50 column volumes though the column, until the pH, as well as the conductivity of the effluent were as those of the starting buffer. The amount of sample applied was usually ≤ 50% of the available bed capacity. The FPLC 1 ml, 2 ml and 10 ml loops were used for application

of the samples. The columns were washed with 5 column volumes of starting buffer. The sample constituents were eluted with 5 to 10 column volumes of a linear gradient (Harris and Angal, 1989; Pharmacia LKB, 1986, 1991a) of 0.25-2 M NaCl and collected in a fraction collector. The samples obtained were dialysed against 10 mM P-E-G buffer, pH 8.0 prior to monooxygenase activity measurements. The tubes containing samples that showed monooxygenase activity were pooled together and their buffer exchanged to the starting buffer of the next column.

2.14.3 Chromatofocusing

All chromatofocusing procedures were carried out on the FPLC system at 4°C. Two chromatofocusing matrices were used: the Mono P HR 5/20 prepacked column (Pharmacia) and the PBE 94 polybuffer exchanger packed into a 50 x 1 cm column. Both columns were equilibrated in 25 mM Bis-Tris buffer, pH 7.1 by applying 10 to 15 column volumes of starting buffer at a flow rate of 1 ml/min. Then the sample (ion exchange eluent showing monooxygenase activity), equilibrated in starting buffer, was applied to the column and the elution started immediately with a 1:10 dilution of polybuffer 74, pH 4.0 at a flow rate of 0.5 ml/min. 10 column volumes of elution buffer were used (Pharmacia LKB, 1988). The samples collected were equilibrated to 50 mM Clark and Lubs phosphate buffer, pH 7.8 and assayed for monooxygenase activity. Active samples were pooled together and drop frozen in liquid nitrogen.

2.14.4 Gel Filtration or Size Exclusion Chromatography

Gel filtration chromatography was carried out on the FPLC system at 4°C. The column used was a Superose 12 HR 10/30 (Pharmacia). The column was equilibrated in 50 mM phosphate buffer, pH 7.8 containing 0.1 M NaCl to avoid non-specific binding

of sample constituents to the column matrix. Blue dextran was applied onto the column in order to define the column void volume. Subsequently, two more mixtures containing proteins with known molecular weights were applied onto the column, in order to obtain a molecular weight calibration curve (Mixture I: Thyroglobulin, Catalase, Albumin, Chymotrypsin; Mixture II: Ferritin, Aldolase, Ovalbumin, Ribonuclease) (Pharmacia LKB, 1991b). 10 mg of protein with monooxygenase activity, obtained by ion exchange chromatography, was equilibrated in the same buffer, applied onto the gel filtration column and then eluted with starting buffer at 0.2 ml/min. The samples collected were dialysed against phosphate buffer without any salt and assayed for α–pinene monooxygenase activity. Samples showing monooxygenase activity were pooled together and drop frozen in liquid nitrogen.

2.14.5 Affinity Chromatography

Affinity chromatography was carried out at room temperature and the columns were run by gravity flow. Two columns were used: a 1 ml Hi-Trap Blue Sepharose column (Pharmacia) and a 1 ml NAD column in 4% beaded agarose (Sigma). Columns were equilibrated in 50 mM phosphate buffer, pH 7.8 as above and then high speed supernatant equilibrated in the same buffer was applied. The column was washed with 5 column volumes of starting buffer and then eluted with 3 column volumes of 2 M NaCl in starting buffer. All samples, effluent, wash and eluent were assayed spectrophotometrically for monooxygenase activity.

2.14.6 Isoelectric Focusing and Titration Curves

Isoelectric focusing at pH 3 to 9 and pH 4 to 6.5 was carried out on the PHAST electrophoresis system (Pharmacia). The sample applied was previously equilibrated

in 0.5 M Tris-HCl, pH 6.8. Loading dye was added to the sample at a ratio of 1:4 and a 3 µl sample was loaded onto the IEF gel that had been prefocused at 2,000 Volts, 2.5 mA 3.5 W, 15°C for 75Vh. The sample was loaded for 15 Vh at 15°C, 200 Volts, 2.5 mA, 3.5 W and then the focusing performed at 2,000 Volts, 2.5 mA, 3.5 W, 15°C for 360Vh. After isoelectric focusing the gel was stained with R250 as described above.

Electrophoretic titration analysis of the ion exchange sample showing monooxygenase activity was also performed on the PHAST electrophoresis system (Pharmacia). An IEF gel from pH 3 to 9 was used. The pH gradient was obtained by prefocusing the ampholytes across the polyacrylamide gel at 2,000 Volts, 2.5 mA, 3.5W, 15°C for 150 Vh. Then 10 µl of sample, prepared as above, were applied, and electrophoresis was carried out perpendicular to the pH gradient at 1,000 Volts, 2.5 mA, 3.5 W, 15°C for 60Vh.

2.14.7 Measuring Enzyme Activity, Specific Activity and Efficiency of Purification

Activity of α–pinene monooxygenase was assayed after each chromatography step and samples showing α–pinene stimulated NADH oxidation activity were concentrated and forwarded to further purification steps. The activity expressed as µmol/min (1 mU), was calculated using a molar extinction coefficient at 340 nm for NADH, e, of 6.2 A_{340}/cm·mol.

The degree of purification of each chromatography step was estimated by comparing the specific activity after each step (S_1) with that of the sample loaded (S_0). The specific activity, expressed in units/mg, was calculated by dividing the enzyme activity by the amount of protein used in the assay.

The percentage of monooxygenase activity recovered was calculated by dividing the total activity at step S_1 (specific activity x mg protein recovered) by the total activity loaded, S_0, and multiplying this by 100.

2.15 Nitrosoguanidine Mutagenesis

2.15.1 Killing Curve for NTG Mutagenesis

The method used was that described by Miller (1972). A single colony of wild type *P. fluorescens* NCIMB 11671 was inoculated in 20 ml nutrient broth and incubated overnight at 30°C with shaking. 6 ml of the overnight culture were used to inoculate 60 ml of nutrient broth and incubated to an OD_{600} of 0.5 as above. The cells were centrifuged at 5,000 g for 20 min and washed twice in 10 ml of 0.1 M sodium citrate buffer, pH 5.5. Finally, the cell pellet was resuspended in 30 ml of the same buffer and 5 ml cell suspension were placed in six test tubes. Aliquots of 1 mg/ml N-methyl, N'-nitro, N-nitrosoguanidine (NTG), freshly prepared in citrate buffer, were added to each bottle so that the final NTG concentrations were 0, 20, 40, 60, 80, 100 µg/ml. The cell suspensions were incubated at 30°C for 90 min.

For a time course experiment, the same concentration of NTG, 50 µg/ml was added to seven bottles prepared as above, and then the cell suspensions were incubated at 30°C for 0, 10, 20, 30, 40, 60 and 90 min. After incubation 1 ml samples of cell suspension were centrifuged at 5,000 g for 10 min. The pellets were washed once in 5 ml of 0.1 M phosphate buffer, pH 7.0 and resuspended in 5 ml of the same buffer. After serial dilutions up to 1 x 10^6 with saline, the cells were spread on nutrient agar plates, incubated at 30°C overnight, and the viable count was determined.

2.15.2 Mutagenesis Procedure

A single colony of *P. fluorescens* NCIMB 11671 was inoculated in 10 ml of nutrient broth and incubated overnight at 30°C with shaking. 1 ml of this culture was used to inoculate 10 ml of fresh nutrient broth and incubated at 30°C to an OD_{600} of 0.5. The culture was centrifuged at 5,000 g for 10 min and the pellet washed twice in sodium

citrate buffer as above, and resuspended in 5 ml of the same buffer. 50 µg/ml of NTG were added and the cell suspension incubated at 30°C for 40 min. Finally, the suspension was centrifuged at 5,000 g for 10 min, washed in phosphate buffer as above, the pellet resuspended in 0.5 ml of phosphate buffer, and after plating for survival counts, the rest was added to 30 ml of nutrient broth, and incubated at 30°C with shaking, for 3 h. Serial dilutions of this culture were spread onto minimal medium agar plates containing 0.3% (v/v) sodium lactate, and incubated overnight at 30°C. Single colonies were picked onto fresh nutrient agar plates to form 4 x 4 grid master plates. These were replica plated onto minimal medium in duplicate and the replica plates incubated at 30°C under α–pinene or α–pinene oxide vapour for 2 to 4 days. Colonies unable to grow on minimal plates were picked from the master plates and streaked on nutrient agar plates. Colonies from the latter plates were tested for reversion and the stable mutants were finally kept.

2.15.3 Reversion Analysis

A single colony of each mutant was inoculated in 10 ml of nutrient broth and incubated overnight at 30°C with shaking. Serial dilutions, up to 1 x 10^9, of these cultures were spread onto minimal plates as well as nutrient agar plates (controls). Minimal plates were incubated under α–pinene or α–pinene oxide vapour at 30°C, and the number of colonies was counted after 2 days of incubation.

2.16 Complementation Studies

2.16.1 Culture Matings

Complementation experiments between the mutants and a genomic library of *P. fluorescens* were carried out in order to identify the recombinant clones that would complement the various mutations. Complementation studies were performed by triparental matings (Figurski and Helinski, 1979; Ditta *et al.*, 1980; Jansenn *et al.*, 1989; Rothmel *et al.*, 1991) using the library clones, *E. coli*, HB101 harbouring the helper plasmid pRK2013, and the mutants (figure 2.1). The following procedure was used: 5 ml LB broth containing the appropriate antibiotic, or no antibiotic at all, were inoculated with a 10% (v/v) inoculum of a library pool or a single colony of HB101/pRK2013, and mutants and incubated overnight at 37°C (*E. coli*) and 30°C (*P. fluorescens*) with shaking. The next day all cultures were centrifuged at 5,000 g for 10 min, washed twice in 5 ml of 50 mM phosphate buffer, pH 7.0, and resuspended in such a volume of the same buffer that their OD at 600 nm would be 0.5. Subsequently, 4 µl of library pool were placed onto a nutrient agar plate with no antibiotics and allowed to dry near the flame. 4 µl of the helper strain were placed onto the library spot, the plate was allowed to dry, and then 4 µl of the mutant under test was placed on top of the other two. The plates were dried near the flame, and then incubated at 30°C overnight. The next day the resulting colonies were mixed with a loop and transferred to minimal plates containing the appropriate antibiotics that were placed at 30°C, under α–pinene or α–pinene oxide vapour as sole carbon source. *Pseudomonas* isolation agar plates, containing kanamycin, were used for the calculation of the transformation efficiency. Growth was examined after two days of incubation and the presence of plasmids in the resulting colonies was confirmed by alkaline lysis. Minimal plates inoculated only with mutants and wild type (WT) were used as controls.

2.16.2 Plate (Patch) Matings

The donor strain (library clones), recipient strains (mutants) and helper strain (HB101(pRK2013)) were grown overnight on LB plates containing the appropriate antibiotics. The next day cells from the donor strain were smeared with a sterile loop onto an LB plate in a small circular area, and then the helper strain cells were mixed with the donor strain. Finally, a loop full of recipient cells was collected and mixed with the donor and helper strains. The plates were incubated overnight at 30°C. The next day, a portion of the mixed cell growth was collected with a sterile loop and streaked on selective medium as above.

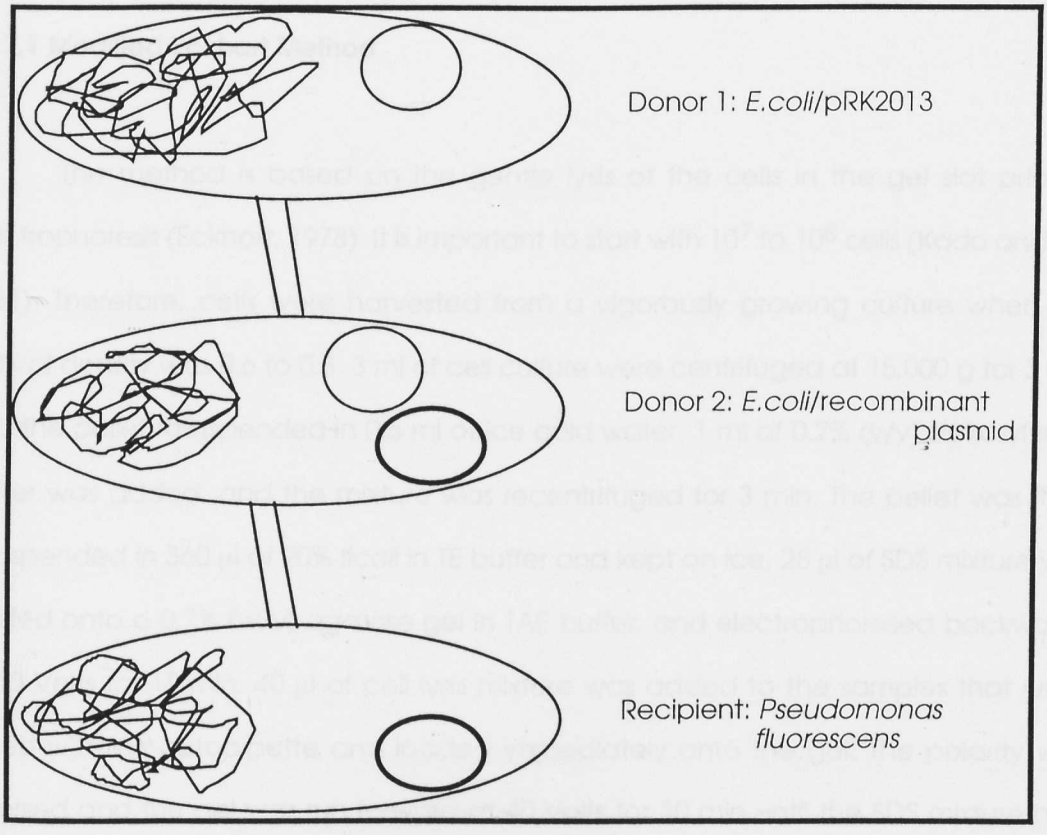


Figure 2.1: Steps involved in triparental matings leading to genetic complementation.

2.16.3 Making Library Pools

For making a library pool a 96 well library plate was removed from the -70°C freezer and allowed to thaw at room temperature for 15 min. The contents of each individual well were mixed with a Gilson pipette and 5 µl aliquots from each well were transferred into a universal bottle containing 5 ml LB broth and 25 µg/ml kanamycin and streptomycin to select for the recombinant plasmids. The pool was incubated at 37°C for 48 h. 800 µl of this culture were transferred into a microfuge tube containing 200 µl of sterile glycerol, and stored at -20°C until needed.

2.17 Large Plasmid Detection in *Pseudomonas fluorescens* NCIMB 11671

2.17.1 Modified Eckhart Method

The method is based on the gentle lysis of the cells in the gel slot prior to electrophoresis (Eckhart, 1978). It is important to start with 10^7 to 10^8 cells (Kado and Liu, 1981). Therefore, cells were harvested from a vigorously growing culture when the optical density was 0.6 to 0.8. 3 ml of cell culture were centrifuged at 15,000 g for 3 min and the pellet resuspended in 0.5 ml of ice cold water. 1 ml of 0.2% (v/v) sarkosyl in TE buffer was added, and the mixture was recentrifuged for 3 min. The pellet was then resuspended in 360 µl of 20% ficoll in TE buffer and kept on ice. 25 µl of SDS mixture was loaded onto a 0.7% (w/v) agarose gel in TAE buffer, and electrophoresed backwards at 60 Volts for 15 min. 40 µl of cell lysis mixture was added to the samples that were mixed with an autopipette and loaded immediately onto the gel. The polarity was reversed and the gel was run forward at 40 Volts for 30 min until the SDS mixture had entered the samples and then 7 V/cm gel length were applied overnight. After electrophoresis the gel was immersed in 5 µg ethidium bromide/ml TAE, for 1 h, and then washed in distilled water for 30 min.

2.17.2 Method of Zunica *et al.* (1981)

A small portion of an overnight colony of *P. fluorescens* was resuspended in 50 µl of 25% (w/v) sucrose in 50 mM Tris-HCl, pH 8. 0.2 µl of 5 mg/ml RNAse in 250 mM Tris-HCl, pH 8.0, 4 µl of 250 mM EDTA and 6 µl of 10 mg/ml lysozyme in 250 mM Tris-HCl, pH8.0 were added. The mixture was incubated at room temperature for 20 min and 4 µl of 20% (w/v) SDS in 10 mM Tris-HCl, pH 8.0 were added. The mixture was incubated at room temperature for a further 2 min and then placed on ice for 1 h. The mixture was subsequently equilibrated at room temperature, mixed well with an autopipette and loaded on a 0.7% (w/v) agarose/TAE gel. Electrophoresis was carried out for 3 h at 10 V/cm gel length and then the gel was stained with ethidium bromide as above. The TOL plasmid and CAM plasmids from *P. putida* that were isolated with the above procedures were used as controls.

2.18 Small Scale Plasmid DNA Isolation (Miniprep)

2.18.1 Alkaline lysis (Dillon *et al.*, 1985; Sambrook *et al.*, 1989)

This method was used for isolation of both plasmid and cosmid DNA from *E. coli* and *P. fluorescens*. A single bacterial colony was inoculated in 2 ml LB broth in a 10 ml universal tube, and incubated overnight at 37°C, or 30°C respectively, with shaking. The culture was centrifuged at 6,000 g for 5 min and the bacterial pellet resuspended in 100 µl ice cold solution I by vigorous vortexing. 200 µl of freshly prepared solution II was added, the contents mixed by inverting the tube rapidly 6 times and the suspension incubated at room temperature for 5 min. Finally 150 µl of ice cold solution III was added, the tube closed and vortexed gently and incubated on ice for 10 min. The bacterial lysate was centrifuged at 30,000 g for 10 min and the clear supernatant transferred into a clean tube. RNAse was added to a final concentration of 20 µg/ml

and the solution incubated at 37°C for 1 h. Subsequently, the DNA was extracted once with phenol/chloroform and twice with chloroform. The double stranded DNA was precipitated with two volumes of dry ice cold absolute ethanol and centrifuged at 30,000 g for 20 min. The DNA pellet was washed with 1 ml of 70% (v/v) ethanol, allowed to dry at room temperature, and then resuspended in 50 to 100 µl TE buffer.

For the isolation of cosmid DNA 10 ml of starter culture were used. The bacterial pellet was resuspended in 200 µl of solution I, lysed with 400 µl of solution II and the proteins precipitated with 300 µl of solution III. The bacterial lysate was subsequently treated as above.

2.18.2 Plasmid DNA Isolation Using the "Magic Mini preps" Purification System

This method was routinely used for the isolation of plasmid DNA, especially for restriction enzyme digestions, or DNA sequencing. 1 to 3 ml of an overnight culture were pelleted by centrifugation at 6,000 g for 5 min and the supernatant removed by aspiration. The cell pellet was resuspended in 200 µl of Cell Resuspension Solution. 200 µl of Cell Lysis Solution were added and the contents mixed by inverting the tube several times until the cell suspension was almost clear. 200 µl of ice cold Neutralisation Solution were added, the suspension mixed by gentle vortexing and the tube placed on ice for 10 min. The lysate was centrifuged at 30,000 g for 10 min and the clear supernatant decanted into a new microcentrifuge tube. 1 ml of Magic Minipreps DNA Purification Resin (Promega) was added and the tubes placed on a rotary wheel at room temperature for 1 to 3 h. For each sample one Magic Minipreps mini column was used, and a 3 ml disposable syringe barrel was attached to the luer-lok extension of each mini column. The resin containing the bound DNA was decanted into the syringe barrel and gently pushed into the column with the syringe plunger. The mini column was washed with 2 ml of Column Wash solution and dried by 1 min centrifugation at 30,000 g. Finally, the plasmid DNA was eluted with 50 µl TE buffer preheated to 65-70°C.

2.19 Large Scale Plasmid DNA isolation (Maxi Prep)

2.19.1 Large Scale Plasmid DNA purification Using PEG (Sambrook *et al.*, 1989)

A single colony of the *E. coli* HB101 or DH5α harbouring the plasmid of interest was inoculated in 5 ml of 2YT and incubated overnight at 37°C. All of the starting culture was used to inoculate 500 ml of terrific broth, that was incubated at 37°C overnight with shaking. The culture was centrifuged at 6,000 g, 4°C, for 20 min and the pellet resuspended in 18 ml of solution I (GTE). 2 ml of a freshly made lysozyme solution (10 mg/ml), and 40 ml of a freshly prepared solution II (SDS/NaOH) were added. The contents were mixed thoroughly but gently by inverting the centrifuge bottle several times, and the suspension stored at room temperature for 10 min. 20 ml of solution III (potassium acetate) were added and the contents mixed very well by shaking the bottle vigorously several times. The suspension was stored on ice for 20 min, and a flocculant white precipitate was formed. The bacterial lysate was centrifuged at 15,000 g, 4°C, for 30 min, and the clear supernatant filtered into a clean centrifuge tube though 4 layers of cheese cloth. 0.6 volumes of isopropanol were added to precipitate the nucleic acids and the bottle was stored at room temperature for 30 min. The nucleic acids were recovered by centrifugation at 30,000 g, room temperature, for 20 min. The pellet was washed with 70% (v/v) ethanol, air dried and resuspended in 3 ml of TE buffer.

The nucleic acids were transferred to a small (50 ml) centrifuge tube and 3 ml of 5 M lithium chloride were added. The contents were mixed well and then centrifuged at 30,000 g, 4°C, for 10 min, to precipitate the high molecular weight DNA. The supernatant was transferred to a clean tube and an equal volume of isopropanol was added. The precipitated nucleic acids were recovered by centrifugation at 30,000 g, room temperature, for 20 min. The pellet was rinsed with 70% (v/v) ethanol, air dried and resuspended in 500 µl of TE buffer, containing RNAse at a final concentration of 20 µg/ml. The solution was transferred to an eppendorf tube and incubated at room

temperature for 30 min. Subsequently, 500 µl of 13% PEG 8000 containing 1.6 M NaCl were added, and the plasmid was recovered by centrifugation at 30,000 g, 4°C, for 5 min. The supernatant was removed by aspiration and the pellet of plasmid DNA was dissolved in 400 µl of TE buffer. The solution was extracted once with phenol, once with phenol/chloroform and once with chloroform. The aqueous phase was transferred to a clean microfuge tube and the DNA precipitated with 100 µl of 10 M ammonium acetate and 2 volumes of absolute ethanol. The DNA was recovered by centrifugation at 30,000 g, 4°C, for 5 min, the pellet washed in 70% (v/v) ethanol, air dried, and dissolved in 500 µl of TE buffer.

2.19.2 Large Scale Plasmid DNA purification Using CsCl-EtBr Density Gradient Centrifugation

This procedure was routinely used for large scale plasmid and cosmid DNA isolation. 5 ml of an overnight culture in LB broth was used to inoculate 500 ml of LB broth. The culture was incubated at 37°C with shaking overnight. The next day the bacterial cells were centrifuged at 6,000 g for 20 min, and the pellet resuspended in 14 ml of solution Ia. Approximately 10 mg of lysozyme were sprinkled over the suspension which then was stored on ice for 10 min. 42 ml of freshly made solution IIa were added, the contents mixed gently and stored at room temperature for a further 20 min. 34 ml of ice cold solution IIIa were added to the bacterial lysate, the contents mixed very well to break up the protein-SDS aggregates, and the tube placed on ice for 30 min. The bacterial lysate was centrifuged at 15,000 g, 4°C for 15 min and the supernatant filtered though 4 layers of cheesecloth into a clean centrifuge tube. The nucleic acids were precipitated by addition of 0.6-0.7 volumes of isopropanol, the bottles placed on dry ice for 30 min and then centrifuged at 15,000 g, 4°C for 30 min. The pellet was dried and then resuspended in 4 ml TE buffer and stored at -20°C.

The nucleic acid solution was transferred into a 12 ml Corex tube, made up to 4.5 ml with TE buffer and then, 1.15 g of CsCl and 450 µl of 10 mg/ml ethidium bromide were added. The solution was placed at 70°C for any remaining proteins to precipitate and then cooled down to room temperature in the dark. The solution was centrifuged at 15,000 g for 10 min. The clear supernatant was transferred slowly into a 5 ml "Quick Seal" Beckman tube. The tubes were balanced by addition of clear paraffin oil, heat sealed and centrifuged at 100,000 g for 16 h at 20°C using a VTi 50 rotor. After centrifugation the DNA bands were visualised under long wave length UV light. The lower band containing covalently closed circular DNA was collected by puncturing the wall of the tube with a syringe, and emptied into a 50 ml falcon tube. The band was extracted twice with 45 ml of water saturated-2-butanol and the DNA phase (lower) was transferred into a clean 12 ml corex tube. The volume of the solution was made up to 7 ml with milli Q sterile water and the DNA precipitated by addition of 5 ml isopropanol. The tube was placed on dry ice for 30 min and the DNA recovered by centrifugation at 30,000 g for 15 min. The DNA pellet was washed with 70% (v/v) ethanol, air dried and resuspended in 500 µl TE buffer.

2.19.3 Large Scale Plasmid DNA purification Using Qiagen Plasmid Purification Protocol

This method was used for large scale isolation of plasmid and cosmid DNA. All buffers used were supplied by the manufacturer. 250 ml of LB broth containing the appropriate antibiotic were inoculated with 2.5 ml of an overnight culture of *E. coli* DH5α carrying the plasmid of interest, and incubated at 37°C overnight. The cells were centrifuged at 6,000 g, 4°C, for 30 min. The bacterial pellet was resuspended in 10 ml of buffer P1. 10 ml of buffer P2 were added and the suspension mixed gently by inverting the tube 5 to 6 times, and incubated at room temperature for 5 min. 10 ml of chilled buffer P3 were added to the suspension which was mixed immediately but gently, by inverting the tube. The suspension was centrifuged at 30,000 g, 4°C for 15 min, the

supernatant removed and recentrifuged as above. The Qiagen-tip 500 was equilibrated with 10 ml of buffer QBT. The clear supernatant from the previous step was applied to the column and allowed to enter the resin by gravity flow. The column was washed with 2 x 30 ml of QC buffer and the DNA eluted with 15 ml of QF buffer. The DNA was precipitated with 0.7 volumes of isopropanol. The precipitate was centrifuged at 30,000 g, room temperature, for 30 min and the supernatant removed carefully. The DNA pellet was washed with 15 ml of 70% (v/v) ethanol, air dried for 5 min and dissolved in 400 µl of TE buffer. All buffers used were supplied by the kit.

2.20 Isolation of High Molecular Weight Chromosomal DNA

A single colony of *P. fluorescens* NCIMB 11671 was inoculated in 10 ml LB broth and incubated overnight at 30°C. The cells were pelleted by centrifugation at 5,000 g, 4°C, for 15 min, the pellet resuspended in 100 µl of 10 mg/ml lysozyme and placed on ice for 10 min. Subsequently, 100 µl of 10 mg/ml proteinase K and 200 µl of 10% SDS were added and the centrifuge tube placed at 59°C for 1 h and then at 65°C for 30 min. 3 ml of 50 mM Tris-HCl containing 20 mM EDTA, pH 8.0 were added to the bacterial lysate. The suspension was mixed gently and then extracted once with phenol, twice with phenol/chloroform and twice with chloroform alone. The nucleic acid layer (supernatant) was removed to a clean tube and the nucleic acids precipitated by addition of 3 M sodium acetate (100 µl for every 1 ml of supernatant) and absolute ethanol (2.5 ml for every 1 ml of supernatant), and centrifugation at 5,000 g for 20 min. The fluffy mass of nucleic acid was removed with a glass rod and washed with 70% (v/v) ethanol. TE buffer (200 µl for every 1 ml of supernatant) was added to the pellet and the tube was placed at 55°C overnight for the nucleic acids to dissolve. RNAse at a final concentration of 20 µg/ml was added to the nucleic acid solution and the tube incubated at 37°C for 1 h. The DNA was extracted once with phenol/chloroform and

twice with chloroform, and then precipitated as above. Finally, the DNA was dissolved in TE at 55°C and checked on a 0.6% (w/v) agarose/TBE gel.

2.21 Large Scale Partial Digestion of Chromosomal DNA

100 µg of *P. fluorescens* high molecular weight DNA were mixed with 10 µl of 10x *Sau* 3A buffer and 0.015 units of *Sau* 3A per µg of DNA. The total volume was adjusted to 100 µl with sterile milli Q water. The mixture was incubated at 37°C for 55 min, then, the tube was placed on ice and a final concentration of 20 mM EDTA was added to stop the reaction. The DNA was extracted twice with phenol/chloroform and precipitated with 2 volumes of absolute ethanol and 1/10 volume of 3 M potassium acetate. The DNA pellet was washed with 70% (v/v) ethanol and then dissolved in 200 µl of TE buffer.

2.22 Fractionation of Partially Digested Chromosomal DNA

A 12 ml linear 10% to 40% (w/v) sucrose gradient was prepared in a Beckman SW27 polyallomer tube. The sucrose solutions were made in buffer containing 10 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0. A 20 ml gradient maker was used: 6 ml of the 40% solution was pipetted into the back reservoir of the gradient maker, while the 10% solution was pipetted into the mixing reservoir containing a magnetic flea. The gradient maker was placed onto the magnetic stirrer and both solutions were allowed to mix in the mixing reservoir while the gradient was collected in the ultracentrifuge tube at a flow rate of 0.3 ml/min. The digested DNA was heated at 65°C for 10 min, placed on top of the gradient and centrifuged in a swing out SW40 rotor at 22,000 rpm for 22 h. After ultracentrifugation the bottom of the tube was punctured and sequential 350 µl DNA samples were collected. 10 µl of every second fraction were mixed with 20 µl water and 5 µl loading dye and analyzed by electrophoresis though a

0.6% (w/v) agarose/TBE gel. Following electrophoresis, gradient fractions containing DNA fragments of 25 to 35 kb were pooled, diluted with TE buffer so that their sucrose concentration would be less than 10% (w/v) and the DNA was then precipitated with 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate. The pellet was washed with 70% (v/v) ethanol, air dried and dissolved in 200 µl of TE buffer.

2.23 Quantitation of DNA

DNA was quantified spectrophotometrically by measuring the absorbance at wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample (an OD of 1 corresponds to approximately 50 µg/ml of double stranded DNA). The ratio of OD_{260}/OD_{280} measurements provided an estimation of the purity of the nucleic acid. This ratio should be between 1.8 and 2.0. Ratios lower than 1.6 indicated significant contamination with protein or phenol and such samples required further purification. Ratios higher than 2.0 indicated the presence of RNA and such samples required RNAse treatment.

2.24 Construction of a genomic Library of *Pseudomonas fluorescens* NCIMB 11671

2.24.1 *In Vitro* Packaging of High Molecular Weight DNA

2 µg of the DNA isolated from the sucrose gradient were ligated to *Bam*H I-*Sca* I digested pLAFR5 at a ratio of 9:1 (vector : insert DNA) in a total volume of 20 µl. A final concentration of 5 mM ATP was added to the ligation mixture to suppress blunt end ligation of the *Sca* I vector ends. The Gigapack II Gold Packaging Extract purchased from Stratagene (San Diego, CA, USA) was used. Each set consisted of 2 tubes

"Freeze/Thaw" extract and "Sonicated" extract and the manufacturers instructions were followed precisely. Two sets of packaging extracts were removed from the -70°C freezer and placed on dry ice. The "Freeze/Thaw" extract was thawed quickly in between the fingers. 4 µl of ligated DNA (2.8 µg) was added to the first extract, and 1 µl (0.2 µg) of control I DNA (supplied in the kit) was added to the second extract. The "Sonicated" extract was also thawed between the fingers and 15 µl were added to the previous extracts, and mixed quickly with the tip of Gilson pipetman. The tubes were centrifuged for 3-5 sec to ensure that all the contents were at the bottom, and the packaging mixtures were incubated at room temperature for 2 h. 0.5 ml of SM buffer was then added and mixed well, followed by 20 µl of chloroform which was mixed gently. Finally, the tubes were subjected to mild centrifugation to sediment debris and the supernatants stored at 4°C until needed.

2.24.2 Titering and Amplification of the Cosmid Library

5 µl aliquots of the packaged cosmid DNA were diluted in 45 µl and 245 µl of SM buffer to give dilutions of 10 and 50 fold respectively. 25 µl of each dilution were mixed with 25 µl *E. coli* DH5α competent cells (OD_{600} of 0.5), prepared by overnight growth from a single colony in LB broth containing 10 mM $MgSO_4$ and 0.2% (w/v) maltose. The two samples were incubated at room temperature for 30 min. 200 µl of LB broth were then added to each sample and the incubation continued at 37°C for one more hour, gently shaking every 15 min, to allow expression of the tetracycline resistance gene. The culture was centrifuged for 1 min at 15,000 g and the pellet resuspended in 50 µl of LB broth. The cells were plated on LB plates containing 25 µg/ml tetracycline and incubated overnight at 37°C. Similarly, 10 µl of a 10^4 dilution of control supernatants were added to 200 µl of competent *E. coli* VCS257 and incubated at 37°C for 15 min. 3 ml of top agar was added to the control sample and quickly poured onto prewarmed LB plates. The control sample was used to estimate the efficiency of packaging.

Amplification of the library was carried out as follows: 6.5 ml of 50x dilution of packaged DNA in SM buffer were mixed with 6.5 ml of competent DH5α cells, and incubated at room temperature for 30 min. 4 volumes of LB broth were added and the cells incubated at 37°C for 1 h with shaking. The culture was centrifuged at 5,000 g for 10 min, the cell pellet resuspended in 1 ml LB broth and spread onto 150 mm LB/Tc plates. The plates were incubated overnight at 37°C. 3 ml LB broth containing tetracycline were added on the plate and the colonies were scraped from the plate with a sterile razor blade. The broth was pipetted into a clean tube and the plate washed with another 3 ml of LB broth. The cells resuspended in LB broth were pooled into the same tube and 15% (v/v) final concentration of glycerol was added. The library was quickly frozen in dry ice and then transferred to a -70°C freezer.

2.25 Restriction Enzyme Digestions

Double stranded DNA (1-20 μg) was mixed with restriction enzymes (1-5 units per mg DNA) in the appropriate buffers in a total volume of 10 to 100 μl adjusted with sterile milli Q water. Reaction mixtures were incubated at 37°C for 1 to 3 h and the reactions stopped by freezing and/or addition of a final concentration of 20 mM EDTA. Double digests were carried out preferentially in the same One For All (OFA) buffer, purchased from Pharmacia, with the enzyme requiring the highest ionic strength used first. When different buffers had to be used the first enzyme reaction was inactivated and precipitated before the second could be incubated in the appropriate buffer.

2.26 Ligation Reactions

Bacteriophage T4 DNA ligase catalyses the formation of phosphodiester bonds between adjacent 3'-hydroxyl and 5'-phosphate termini in DNA. The vector

DNA was usually dephosphorylated prior to ligation. The T4 DNA ligase buffer was used at a final concentration of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 mg/ml BSA. In all ligation reactions three molar ratios of vector to insert DNA were used: (1:1), (1:3) and (3:1). The amount of insert DNA added to each reaction was calculated from the following equation:

$$\frac{ng\ of\ vector\ x\ kb\ of\ insert}{kb\ of\ vector}\ x\ molar\ ratio\ (insert:\ vector) = ng\ of\ insert$$

Unless otherwise stated, the ligation mixture contained 100 ng vector and the appropriate amount of insert DNA in a total volume of 10 µl adjusted with sterile milli Q water. Usually 2 units of T4 DNA ligase were added. The ligation reactions were incubated at 16°C for 16 h. When the ligation buffer contained 50% (w/v) PEG, the reactions were incubated at room temperature for 1 h.

Ligation of dephosphorylated, *Sau* 3A partially digested chromosomal DNA with pLAFR5 digested with *Bam*H I-*Sca* I was carried out at a molar ratio of 1:9 (insert DNA : vector DNA). The amount of DNA added to the reaction was calculated by the formula:

$$\frac{\mu g\ DNA\ x\ 10^6}{635\ x\ bp}\ x\ 6.023\ x\ 10^{23} = DNA\ molecules$$

The reaction had a total volume of 20 µl and contained 5 mM ATP to prevent blunt end ligation of the vector molecules.

2.27 Dephosphorylation

Dephosphorylation was employed to prevent compatible ends in cut DNA from religating. The vector DNA after digestion was extracted with phenol/chloroform

once and with chloroform twice. The DNA was precipitated with absolute ethanol, washed with 70% (v/v) ethanol and dissolved in TE buffer. 1 unit of Calf Intestine Alkaline Phosphatase (CIP) was used for dephosphorylation of plasmid DNA and 4 units for chromosomal DNA. The reaction mixture contained the desired DNA, 10x CIP buffer and CIP, in a total volume of 100 µl adjusted with milli Q water. The reaction was incubated at 37°C for 30 min. At the end of the incubation period SDS and EDTA (pH 8.0) were added at final concentrations of 0.5% (v/v) and 5 mM respectively. The solution was mixed well and then proteinase K was added to a final concentration of 100 µg/ml and the mixture incubated at 56°C for 30 min. The reaction was cooled to room temperature and the dephosphorylated DNA extracted once with phenol alone, once with phenol/chloroform and twice with chloroform. The DNA was precipitated with 1/10 volume of 3 M sodium acetate, pH 7.0 and 2 volumes of absolute ethanol in dry ice for 15 min. The DNA was recovered by centrifugation at 30,000 g for 10 min and the pellet washed with 70% (v/v) ethanol, air dried and redissolved in TE buffer.

2.28 Transformation of *Escherichia coli*

2.28.1 Preparation of Competent Cells

Preparation of electrocompetent cells (Dower *et al.*, 1992): 5 ml of LB broth were inoculated with a single colony of *E. coli* HB101 or DH5α and incubated overnight at 37°C with shaking. All the overnight culture was used to inoculate 500 ml of fresh LB broth and the culture was again incubated at 37°C with shaking to an OD_{600} of 0.5. The growth flask was subsequently chilled on ice for 30 min prior to harvesting the cells. The cells were centrifuged in a cold rotor at 5,000 g, 4°C, for 15 min, the pellet resuspended in 500 ml cold sterile milli Q water, and recentrifuged as above. The new pellet was resuspended in 250 ml of cold sterile milli Q water, centrifuged as above, and resuspended in 10% (v/v) cold glycerol in milli Q water. The suspension was

centrifuged again, and finally, the pellet resuspended in 2-3 ml of 10% (v/v) glycerol, dispensed into aliquots of 100 µl, and stored at -70°C.

Preparation of competent cells (Hanahan, 1985): Several colonies (2-3 mm diameter) were picked from a freshly grown LB plate and dispersed in 1 ml of Ψ (psi) medium. This was used to inoculate 100 ml of Ψ medium in a one litre flask, that was incubated at 37°C with shaking until the OD_{600} was 0.5. The cells were chilled on ice for 30 min prior to harvesting. Then, the cells were pelleted by centrifugation at 5,000 g, 4°C, for 5 min in a prechilled rotor. The pelleted cells were drained thoroughly and all the work was transferred into the cold room (4°C). The pellet was resuspended in 30 ml of ice cold RF1 solution by brief vortexing. The suspension was recentrifuged as above and the pellet resuspended gently in 4 ml of RF2 solution. Then, it was dispensed into 0.2 ml aliquots and stored at -70°C.

2.28.2 Transformation Procedure

Electroporation of *E. coli* (Dower *et al.*, 1992): Electrocompetent cells were allowed to thaw on ice. Meanwhile, the electrode cuvette, and two sterile microfuge tubes were chilled. 40 µl of cell suspension was placed into a microfuge tube and 1 µl of plasmid or cosmid DNA was added. The suspension was mixed with an autopipette and placed on ice for 30 to 60 sec. The mixture was then transferred to the electrode cuvette and placed in the electroporator compartment (Bio-Rad). One pulse of 2,500 V/cm, 25 mFd and 4.8 sec duration was applied. Immediately, 1 ml of cold SOB medium was added, the suspension was transferred to the other microfuge tube, and incubated at 37°C for 1 h. After the outgrowth period serial dilutions up to 10^4 in SOB were made and spread on LB plates containing the appropriate antibiotic. The plates were incubated at 37°C for 24 to 48 h.

Transformation of *E. coli*: Competent cells prepared as above were thawed at room temperature until just liquid and then placed on ice for 5 to 10 min. 1 to 4 ml of plasmid or cosmid DNA were added and the cells were incubated on ice for 1 h. Subsequently, the cells were heat shocked at 42°C for 3 min and allowed to stand on ice for 5 min. 4 volumes of Ψ broth, or SOC medium were added and the suspension incubated at 37°C for 1 h. Serial dilutions of up to 10^6 were spread on LB plates containing the appropriate antibiotics and the plates incubated at 37°C overnight. In all transformations, the ligation mixture and digested vector (with or without dephosphorylation) were used as controls.

2.29 Recovery of DNA from Agarose Gels

2.29.1 Low Melting Point Agarose Method

This method was used for the isolation of DNA fragments that would be used in subcloning procedures. The plasmid DNA was digested with the appropriate enzyme, the reaction loaded on a 0.7% (w/v) low melting point agarose/TAE gel and electrophoresed overnight in the cold room (4°C) at 3 V/cm gel length. The bands were visualised using long wave length UV light and the desired band excised with a sterile razor and placed into a pre weighed microfuge tube. The weight of the band was calculated and then 5 volumes of a solution containing 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0 in milli Q water were added to the gel slice. The tube was incubated at 65°C for 5 min to melt the gel, and then the contents extracted once with phenol alone, once with phenol/chloroform and once with chloroform. The aqueous phase was transferred into a clean tube and 0.2 volumes of 10 M ammonium acetate and 1 volume of isopropanol were added. The mixture was stored at room temperature for 20 min, and the DNA recovered by centrifugation at 30,000 g for 20 min, at 4°C. The pellet was washed in 70% (v/v) ethanol, air dried and dissolved in 20 µl of TE buffer. The

amount of DNA recovered was estimated by gel electrophoresis by comparison with a known amount of λ marker (Sambrook *et al.*, 1989).

2.29.2 "Freeze-Squeeze" Method

The method involves freezing of the agarose band and centrifuging out the liquid including the DNA of interest. The plasmid DNA was digested with the appropriate enzyme and the mixture loaded on a 0.7% (w/v) ultra pure agarose/TAE gel and electrophoresed overnight at 3 V/cm gel length. The bands were visualised as above and the band of interest was excised with a sterile razor. 1 ml of freeze-squeeze buffer (300 mM sodium acetate and 1 mM EDTA in milli Q water), was added to the agarose slice and the tube was stored in the dark for 30 min. The buffer was removed and the slice was placed at -70°C for 30 min. The agarose slab was pushed into a Castor SPIN-X tube using a plastic spatula, and centrifuged for 10 min. The supernatant was made up to 400 µl with TE buffer and then extracted once with phenol alone and once with chloroform. The DNA was then precipitated with 1/10 volume of 3 M sodium acetate, pH 7.0 and 2.5 volumes of absolute ethanol. The DNA pellet was recovered by centrifugation at 30,000 g for 15 min, washed with 70% (v/v) ethanol and dissolved in 15 µl of TE buffer. The amount of DNA recovered was estimated as above.

2.29.3 Geneclean® Kit Method

The plasmid DNA was digested with the appropriate enzyme and the reaction loaded on a 0.7% (w/v) ultra pure agarose/TAE gel and electrophoresed overnight at 3 V/cm gel length. The bands were visualised as above and the band of interest was excised with a sterile razor. The approximate volume of the gel slice was determined, and then, 3 volumes of NaI stock solution (supplied in the kit) was added. The tube was

incubated at 52°C for 5 min with occasional mixing to dissolve the agarose. Subsequently, 5 µl of Glassmilk® suspension were added to the solution containing 5 µg of DNA (an additional 1 µl of glassmilk was added for each 0.5 µg of DNA above 5 µg). The suspension was mixed well and then incubated on ice for 5 min to allow binding of the DNA to the silica matrix, with mixing every 1 min to ensure that glassmilk would stay suspended. Subsequently, the silica matrix with the bound DNA was pelleted by centrifugation at 30,000 g for 5 sec, and the pellet washed 3 times with 700 µl "New solution" (supplied in the kit). The pellet after the third wash was dissolved in 10 µl of sterile milli Q water and the tube placed at 52°C to elute the DNA. The suspension was centrifuged at 30,000 g for 5 sec and the supernatant containing the eluted DNA placed in a clean microfuge tube. The elution was repeated one more time with 5 µl of milli Q water and the two supernatants pooled together. The amount of DNA recovered was estimated as above.

2.30 Erase-a-Base System

2.30.1 Preparation of the Starting DNA

The generation of an ordered set of deletions by this method relies on the uniform digestion rate of Exonuclease III. However, the enzyme also digests from nicks in the starting DNA, creating single stranded gaps. The greater the percentage of nicked molecules in the starting material, the more random the deletions become and more difficult it becomes to screen for the desired deletions among the resulting subclones. Therefore, supercoiled plasmid DNA was purified by CsCl-EtBr gradient centrifugation in order to minimise the amount of nicked and linear molecules in the plasmid preparation. Nicked and linear DNA can be selectively removed from supercoiled DNA by acid-phenol extraction (Zasloff *et al.*, 1978). Phenol saturated with water at pH 4.5 was mixed several times with 50 mM sodium acetate pH 4.0 until the pH

of the aqueous phase was 4.0. The DNA sample consisted of CsCl-EtBr purified plasmid DNA, 50 mM sodium acetate, pH 4.0, and 75 mM sodium chloride in a total volume of 500 µl, using milli Q water throughout. This was extracted 3 times with acid phenol at 4°C, and then the aqueous phase mixed with 0.05 volumes of Tris-HCl, pH 8.6 and 1 volume of chloroform. The aqueous phase was recovered and the DNA precipitated with 1/10 volume of 2 M sodium chloride and 2 volumes of cold absolute ethanol. The pellet was rinsed with 1 ml of 70% (v/v) ethanol and dissolved in TE buffer at a final concentration of 2 µg/ml.

2.30.2 Restriction Digestions for the Production of 5' Overhangs and 3' Protrusions in the Starting DNA

The DNA, cloned in pBluescript II SK(+), was digested with *Xba* I from the T_3 primer site and with *Xho* I from the T_7 primer site. After the digestion 3' protrusions were generated in 3' recessed ends to block exonuclease III digestion of vector sequences, using α–phosphorothioate dNTPs and DNA polymerase I, Klenow fragment. After the digestion reaction the DNA was extracted with phenol, precipitated with 2 M NaCl and absolute ethanol, and dissolved in 88 µl of 1x Klenow buffer. α–Phosphorothioate dNTPs, DTT and the Klenow fragment were added at final concentrations of 40 mM, 1 mM and 50 units/ml respectively. The reaction was incubated at 37°C for 10 min and then at 70°C for another 10 min. The reaction was subsequently extracted once with phenol alone and once with chloroform and the DNA precipitated as above. The pellet was rinsed in 70% (v/v) ethanol and dissolved in 43 µl of milli Q water. The second restriction digestion that would leave 5' overhangs on the insert was carried out using *Bam*H I from the T_3 site and *Cla* I from the T_7 site. The reactions were incubated at 37°C for 2 h and then extracted with phenol as above. The DNA was precipitated and finally dissolved in 60 ml of 1x Exonuclease III buffer.

2.30.3 Exonuclease III Deletions, Ligations, Transformation

The S1 nuclease mix which contained 27 µl of 7.4x S_1 nuclease buffer and 2.4 µl of S1 nuclease (60 units) in a total volume of 200 µl with milli Q water, was prepared in duplicate (for each primer site) and 7.5 µl aliquots were dispensed into each of 30 eppendorfs that were placed on ice. The DNA was warmed to the digestion temperature, 32°C which corresponded to deletions of ~310 bp/min. 2 µl of Exonuclease III (400 units) were added and the reaction incubated at 32°C. 2.5 µl samples were taken out every 40 sec and added to the appropriate S1 tube so that each sample would be on ice at exactly 1 min intervals. 15 samples for each primer site were taken. After all the samples had been taken the tubes were moved to room temperature for 30 min. 1 µl of S1 stop buffer was added and the samples heated at 70°C for 10 min to inactivate the S_1 nuclease. Subsequently, the samples were transferred to 37°C and 1 µl of freshly made Klenow mix (30 µl of 1x Klenow buffer and 3 units of Klenow fragment) and 1 µl of dNTPs mix were added. The reactions were incubated at 37°C for another 5 min, and then transferred to room temperature. 40 µl of ligase mix (100 µl of 10x ligase buffer, 100 µl of 50% PEG, 10 µl of DTT and 5 units of T4 DNA ligase in 1 ml of milli Q water) were added and the reactions incubated at room temperature for 1 h. Finally, transformation was carried out using competent *E. coli* DH5α cells as described above, and screening for the size of inserts in the recombinant clones was carried out by PCR.

2.31 Polymerase Chain Reaction

PCR was used for the amplification of inserts of recombinant clones derived from the above deletion experiment. T_3 (AATTAACCCTCACTAAAGGG) and T_7 (GTAATACGACTCACTATAGGGC) 20-mer primers purchased from the Oswell DNA service (Edinburgh, UK) were used. The reaction mixture for 10 reactions contained 1x

Taq polymerase buffer, 2.5 mM $MgCl_2$, 250 mM of dNTPs and 0.5 mM of each primer in a total volume of 300 µl made up with sterile milli Q water. 39 µl of the above mixture was added to each 1 µl DNA sample, and the solution was mixed by vortexing. The tube was centrifuged to ensure that all the contents were at the bottom, then 1 drop of clear paraffin oil was added, and the samples placed in the compartment of the PCR apparatus (Techne). The DNA samples were denatured at 95°C for 5 min, and then 10 µl of the enzyme mixture (Taq polymerase (Promega), in 100 µl with milli Q water) containing 2.5 units of enzyme, were added when the temperature was around 65°C. The primer annealing step was carried out at 57°C for 30 sec and the extension step at 72°C for 5 min. This first amplification cycle was followed by 30 cycles at 95°C for 30 sec, 57°C for 30 sec and 72°C for 5 min. A final extension step was carried out at 72°C for 12 min and the reactions stored at 4°C prior to electrophoresis though a 0.8% (w/v) agarose/TAE gel.

2.32 DNA Sequencing

2.32.1 Preparation of the double stranded DNA Template

12 µl of plasmid DNA, isolated using the Magic Miniprep Purification System, were denatured with 3 µl of 2 M NaOH. The mixture was incubated at room temperature for 10 min, and then 10.5 µl of sterile milli Q water were added, and the DNA precipitated with 4.5 µl of 3 M sodium acetate, pH 4.8 and 90 µl of dry ice cold absolute ethanol. The tube was placed on dry ice for 30 min and the denatured DNA recovered by centrifugation at 30,000 g for 10 min. The pellet was rinsed with 70% (v/v) ethanol, air dried and dissolved in 10 µl of milli Q water.

2.32.2 Annealing of the primer to the denatured DNA template

The sequencing kits purchased from Pharmacia and United States Biochemical were used for all sequencing procedures. 2 µl of 0.8 mM primer (either synthetic 17-mer primers ordered though Oswell DNA service, or 17-mer Reverse and M_{13} universal primers for pBluescript purchased from Stratagene) and 2 µl of annealing buffer were added to the DNA template prepared as above. The reaction was incubated at 37°C for 20 min and then at room temperature for 10 min.

2.32.3 Labelling Reaction

The T7 DNA polymerase and the labelling mixtures were prepared according to the number of templates to be sequenced, as shown in tables 2.2 and 2.3 during the annealing step.

6 ml of labelling mixture were added to each priming reaction and the samples incubated at room temperature for 5 min.

Table 2.2: T7 DNA Polymerase mixture.

Number of templates	Volume of T7 DNA Polymerase (µl)	Volume of dilution buffer (µl)
1	0.425	1.6
2	0.85	3.2
3	1.275	4.8
4	1.7	6.4
5	2.125	8.0
6	2.55	9.6
7	2.975	11.2
8	3.4	12.8
9	3.825	14.4
10	4.25	16.0
11	4.675	17.6
12	5.1	19.2

2.32.4 Termination Reactions

While the labelling incubation was in progress, 2.5 µl of dideoxy nucleotides were dispensed to the sides of an appropriate number of microfuge tubes, (labelled A, C, G, T for each sample), that were placed at 37°C for at least 1 min. Subsequently, 4.5 µl aliquots of the labelling reaction were transferred to the sides of the four (for each sample) pre-warmed sequencing mixes, using a fresh pipette tip for each transfer. The components were mixed by centrifuging and then incubated at 37°C for 5 min. 5 µl of stop solution was added to each tube immediately afterwards and centrifuged. The reactions were stored at -20°C until electrophoresis. All samples were denatured at 95°C for 5 min, and then 3 ml loaded onto a prewarmed polyacrylamide gel.

Table 2.3: Labelling mixture.

Number of templates	ddH_2O (µl)	dATP labelling mix (µl)	^{35}SdATP (µl)	Diluted T7 DNA Polymerase (µl)
1	1	3	1	2
2	2	6	2	4
3	3	9	3	6
4	4	12	4	8
5	5	15	5	10
6	6	18	6	12
7	7	21	7	14
8	8	24	8	16
9	9	27	9	18
10	10	30	10	20
11	11	33	11	22
12	12	36	12	24

2.33 Nucleic Acid Hybridisation

2.33.1 Preparation of the Membranes for Colony Hybridisation

The genomic library of *P. fluorescens* was plated out on LB plates containing 25 μg/ml tetracycline and then 700 colonies aseptically transferred to 96 well plates containing LB/Tc broth, that were incubated at 37°C overnight. The colonies were replica transferred on to Hybond N^+ membranes (Amersham) placed on LB/Tc plates. The membranes were incubated overnight at 37°C for the bacteria to grow. The next day lysis of the bacteria and binding of the DNA to the membranes was done as follows: Five pieces of 3MM Whatman paper were saturated with the solutions shown in table 2.4 and the membranes placed on each filter paper for various times as indicated on table 2.4.

After bacterial lysis, DNA denaturation and neutralisation treatments, the membranes were placed on dry 3MM filter paper and blotted with another filter paper. Subsequently, they were incubated at 80°C for 10 to 15 min to dry. Finally, the DNA was fixed on the membranes by UV trans-illumination at 312 nm for 3 min.

Table 2.4: Preparation of membranes for colony hybridisation.

3MM paper saturated with the following solution	Membrane-filter contact time
(I) 10% SDS	5 min
(II) Denaturing solution	7 min
(III) Neutralisation solution	3 min
(IV) Neutralisation solution	3 min
(V) 2x SSC	5 min

2.33.2 Southern Blot Procedure

A 0.8% (w/v) agarose/TAE gel loaded with the digested DNA was electrophoresed at 3 V/cm gel length overnight, the bands were visualised using a short wavelength UV length trans-illuminator and photographed. The gel was subsequently placed in a plastic container, covered with acid cut solution and placed on an orbital shaker for 15 min. The first solution was discarded, the gel was covered with base denaturation solution and gently agitated for 30 min. Then it was covered with the neutralisation solution and agitated for a further 30 min. Meanwhile two Hybond N^+ membranes were cut to the size of the gel and soaked in 100 ml of milli Q water for 5 min, and then in 100 ml of 10x SSC until needed. The gel was placed in between the two membranes and 8 sheets of 3 MM paper. In addition, a 3 inch depth of kimwipe paper was also placed on each side of the gel to make a sandwich. A weight was placed at the top and the transfer was carried out for 2 h, until the gel resembled a membrane. Then, the membranes were dipped in a 0.4 N NaOH for 40 sec and into neutralising SSC for 1 min. The blot was dried thoroughly and forwarded to the hybridisation procedure.

2.33.3 Preparation of the Labelled Probe

The Random Primed DNA Labelling Kit from Boehringer-Mannheim was used. The method is based on the hybridisation of a mixture of hexanucleotides to the DNA to be labelled. 100 ng of DNA in a total volume of 12 µl with milli Q water were denatured by heating at 100°C for 10 min. The denatured sample was placed on ice and 3 µl of dNTPs mixture (1:1:1 of dATP, dGTP and dTTP), 2 µl of reaction mixture (supplied in the kit) and 3 µl of ^{32}P-dCTP were added. The mixture was incubated at 37°C for 60 min and the reaction stopped by heating at 65°C for 10 min. The unincorporated nucleotides

were removed by passing the sample down a Quick Spin Sephadex G-50 column (Pharmacia). The probe was collected in 400 µl of TE buffer.

2.33.4 Hybridisation Procedure

Hybridisation was carried out in a hybridisation oven (Techne). The membranes were prehybridised with 25 ml of prehybridisation solution (6x SSC, %x Denhardt's, 0.5% SDS ,containing 250 µl of 10 mg.ml tRNA) at 65°C for 3 h. The DNA probe containing 200,000 cpm was denatured by heating at 100°C for 10 min and immediately added to hybridisation solution (6x SSC, 5x Denhardt's, 0.5% SDS, containing 200 µl of 10 mg/ml tRNA) preheated at 65°C. The prehybridisation solution was poured out and 10 ml of hybridisation solution containing the probe were added. Hybridisation was carried out at 65°C for 16 h. The membranes were finally washed 4 times at 65°C with 25 ml of a solution of 1x SSC and 0.1% (w/v) SDS in milli Q water, and placed into a film cassette with Kodak X-OMAT AR film and incubated at -70°C for 15 min to 3 h.

2.34 Induction of Protein Synthesis Using the P_{tac}-lacI System

A single colony of *E. coli* HB101 or DH5α containing the recombinant plasmid of interest was inoculated in 2YT containing the appropriate antibiotics and incubated at 37°C overnight. 200 µl of this starter culture was used to inoculate 20 ml of 2YT containing the appropriate antibiotics and the culture was incubated at 37°C to OD_{600} of 0.5. The cells were induced with 1 mM IPTG and incubated for a further 4 to 15 h at 37°C. After the induction step the cells were harvested by centrifugation, washed in 50 mM phosphate buffer, pH 7.0 and resuspended in a minimal volume of the same buffer.

CHAPTER 3

Studies on the Growth of *Pseudomonas fluorescens* NCIMB 11671 and Optimisation of the Production of α–Pinene Monooxygenase Activity in Whole Cells and Cell Free Extracts

3.1 Introduction

A large number of organisms able to grow on alkanes possess an alkane monooxygenase which can hydroxylate terminal methyl groups and fortuitously epoxidise terminal double bonds. The epoxides produced are often of low optical purity and therefore, these bacteria are unsuitable for the production of chiral epoxyalkanes. On the other hand, organisms able to grow on alkenes but not alkanes attack the substrate by a stereospecific epoxidation of the terminal double bond. The epoxide is then degraded further by the organism and thus, serves as sole carbon and energy source. Although these bacteria isolated on terminal alkenes also have a capacity to stereospecifically epoxidise subterminal alkenes (Weijers *et al.*, 1988), they are unable to catalyse the epoxidation of truly internal alkenes. α–Pinene monooxygenase from *P. fluorescens* catalyses the internal epoxidation of α–pinene to form α–pinene oxide, and then the cyclohexane and cyclobutane rings are opened in a single enzymatic reaction on α–pinene oxide to form a novel aldehyde, the 2-methyl-5-isopropylhexα–2,5-dienal (Best *et al.*, 1987). The NADH-dependent α–pinene monooxygenase which is responsible for the formation of the epoxide is specific for alkenes with alkanes and aromatic compounds not being oxidised (Best *et al.*, 1987). The enzyme is induced by the presence of α–pinene in the culture medium and requires FAD and ferrous iron for activity (Aikens, 1990).

α–Pinene monooxygenase was chosen as a model system that would provide information about the molecular mechanism of enzymatic epoxidation of internal alkenes. Therefore, further work for the study of this alkene monooxygenase at both the enzymatic and molecular level was initiated. As a precursor to this study optimum growth conditions for *P. fluorescens* on small and large scale had to be established, so that maximum monooxygenase activity could be achieved.

3.2 Small Scale Growth Experiments

3.2.1 Toxicity of α–Pinene and α–Pinene Oxide to *Pseudomonas fluorescens* NCIMB 11671

α–Pinene and α–Pinene oxide could both be potentially toxic to the cells of *P. fluorescens*. In order to determine the optimum concentration of substrate to be used in any growth experiments, 250 ml flasks containing 50 ml minimal medium were inoculated with 0.5 ml of an overnight culture of *P. fluorescens* in LB broth. Various amounts of α–pinene (0.2%, 0.5%, 1%, 3%, and 10%, (v/v)) or α–pinene oxide (0.2% or 0.5% (v/v)) were added to the flasks that were then incubated at 30°C in an orbital shaker for 4 days. Table 3.1 shows that the bacterium can tolerate as high as 10% (v/v) α–pinene, however, the optimal concentration appears to be 0.2%-1% (v/v). Moreover, when α–pinene concentrations higher than 1% (v/v) were used it was impossible to harvest the cells by centrifugation because of the presence of emulsifying agents. Repeated washing of any cell concentrate with 50 mM sodium phosphate buffer pH 7.0 could not remove these emulsifiers. α–Pinene oxide was more toxic to *P. fluorescens* than α–pinene, since no growth was observed at epoxide concentrations higher than 0.2%.

Table 3.1: Toxicity of α–pinene and α–pinene oxide to *Pseudomonas fluorescens* NCIMB 11671 grown on minimal medium at 30°C with shaking.

Incubation time (days)	α–Pinene concentration (v/v)					α–Pinene oxide concentration (v/v)	
	0.2%	0.5%	1 %	3 %	10%	0.2%	0.5%
1	+[a]	+	+	+	-	-[d]	-
2	++[b]	++	++	+	+	+	-
3	+++[c]	+++	+++	++	+	+	-
4	+++	+++	+++	++	++	++	-

a: Absorbance at 600 nm was 0.0-0.5
b: Absorbance at 600 nm was 0.5-1.0
c: Absorbance at 600 nm was higher than 1.0
d: No growth was observed

3.2.2 Optimisation of the Growth Conditions of *Pseudomonas fluorescens* NCIMB 11671 in Minimal Media Supplied with α–Pinene

The procedures described by Best *et al.* (1987) suggested that *P. fluorescens* grew readily in batch fermentations with 0.2% (v/v) α–pinene supplied as sole carbon source. The same system was used for the culture of *P. fluorescens* in a 2 litre flask containing 500 ml medium. However, resting cells and cell free extract preparations from bacteria grown as above showed little α–pinene monooxygenase activity as measured by any of the methods described.

From the work of Aikens (1990) it was thought that the critical aspect in obtaining cell extracts with high monooxygenase activity, would be the amount of α–pinene available to the cells prior to harvesting, so that the enzyme would be fully induced. However, addition of 0.5% and 1% (v/v) α–pinene caused difficulties in harvesting the cells after 24 to 30 h of incubation at 30°C, because of the presence of a biosurfactant. When higher than 1% (v/v) of the hydrocarbon was used it proved impossible to

harvest the cells by centrifugation even when high centrifugal forces, e.g. 30,000 g or repeated washing with 50 mM sodium phosphate buffer, pH 7.0 were used. The induced excretion of this emulsifying agent by higher pinene concentrations probably assists in dispersing the bulk hydrocarbon phase into an emulsion, thus decreasing the interfacial tension and increasing the interfacial area so enhancing the ability of the organism to degrade the hydrocarbon (Bühler and Shindler, 1984).

Although the use of 0.5% (v/v) α–pinene resulted in higher yields (figure 3.1), the presence of the surfactant and thus the potential loss of a proportion of the cells during harvesting, indicated the need for a cosubstrate. This was based on previous observations by Palleroni and Doudoroff (1972), Bühler and Shindler (1984), Aikens (1990) that when a low percentage of lactate or succinate is added to the growth medium, the amount of any emulsifiers produced is very much reduced.

Therefore, sodium salts of succinate, pyruvate and lactate were used as cosubstrates in cultures of *P. fluorescens* supplied with 0.5% (v/v) α–pinene. 2 litre flasks containing 500 ml minimal medium, 0.2% (v/v) of the above cosubstrates and 0.5% of α–pinene were inoculated with 2.5 ml of an overnight culture of *P. fluorescens* in LB broth and incubated at 30°C, with shaking for 24 h. Three flasks for each of the cosubstrates were prepared. In all cases only a small amount of surfactant was produced and the cells were easily harvested by centrifugation at 5,000 g for 20 min. Resting cells were prepared as described and assayed polarographically and by GC for monooxygenase activity. Cells grown on succinate and pyruvate did not show any pinene stimulated oxygen uptake, nor any epoxide production, while cells grown on sodium lactate did. It was assumed that succinate and pyruvate repress the synthesis of α–pinene monooxygenase. Catabolite repression caused by these above two compounds as well as other products of the TCA cycle in *Pseudomonas* spp. has been also reported by other researchers (Lessie and Neidhart, 1967; Smith and Clarke, 1975; Phillips and Mulfinger, 1981; Clarke and Slater, 1986; Zylstra et al., 1989a). Therefore, sodium lactate was selected as the cosubstrate in minimal medium cultures of *P. fluorescens* supplied with 0.5% (v/v) α–pinene.

With use of a cosubstrate, the primary role of α–pinene is to induce the monooxygenase activity. Therefore, it need not be present throughout the growth cycle. In order to establish the optimum time point(s) for addition of α–pinene to the culture, so that high growth rates as well as high monooxygenase activity would be achieved, the following experiment was carried out: 8 flasks with 500 ml minimal medium containing 0.2% (v/v) lactate were inoculated with 2.5 ml of an overnight culture of *P. fluorescens* in LB broth and 0.5% (v/v) α–pinene added at various times after inoculation. The cells were harvested after 24 h of incubation at the late exponential phase (figure 3.1). Table 3.2 shows the optical density at 600 nm of these cultures after 24 h of incubation at 30°C with shaking. The highest optical density as well of the cultures was achieved when α–pinene was added immediately after inoculation and again after 20 h of incubation at 30°C.

Table 3.2: Optical density of cultures of *Pseudomonas fluorescens* NCIMB 11671 in minimal medium containing 0.2% (v/v) sodium lactate, where 0.5% (v/v) α–pinene was added at various times after inoculation.

Time of α–pinene addition	OD at 600 nm
(I) No α–pinene added	1.113
(II) Inoculation	1.243
(III) Inoculation and after 20 h of incubation	1.431
(IV) After 12 h of incubation	0.950
(V) After 12 and 20 h of incubation	1.240
(VI) After 16 h of incubation	0.907
(VII) After 16 and 20 h of incubation	1.487
(VIII) After 20 h of incubation	0.943

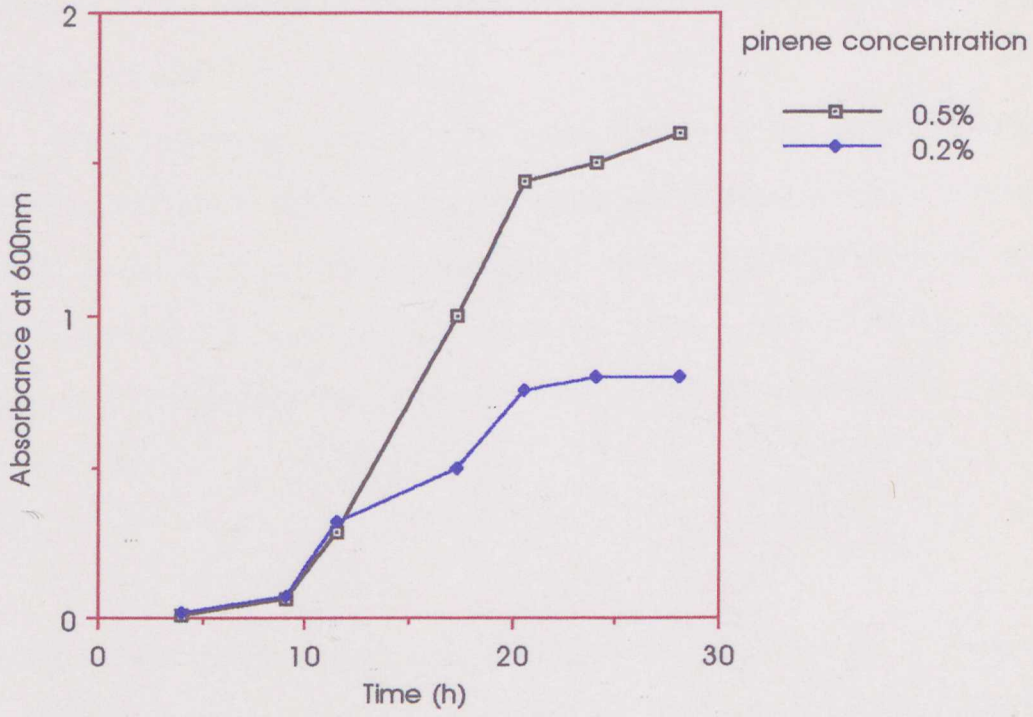


Figure 3.1: Growth of *Pseudomonas fluorescens* NCIMB 11671 in minimal medium supplied with 0.2% and 0.5% (v/v) α-pinene.

Monooxygenase activity of these samples was determined polarographically using cell suspensions, and spectrophotometrically with cell extracts. Table 3.3 shows that sample (III) had the highest monooxygenase activity as well as highest optical density. Therefore, the following conditions were adopted for the culturing of *P. fluorescens*: Minimal medium containing 0.2% (v/v) sodium lactate where 0.5% (v/v) α–pinene was added immediately after inoculation and after 20 h of incubation at 30°C with shaking. Since a good degree of aeration is essential for growth on hydrocarbons, the volume of the culture medium was never greater than 1/4 of the volume of the flask.

PAGE of cell free extracts of the above samples under denaturing and non denaturing conditions did not reveal any significant difference in their polypeptide content. Therefore, it was not possible, based on the data obtained in this study to ascertain whether the polypeptides making up the α–pinene monooxygenase system in *P. fluorescens* were inducible after growth on α–pinene or expressed constitutively.

Table 3.3: Monooxygenase activity (Stimulated - endogenous) of cultures of *Pseudomonas fluorescens* NCIMB 11671 grown in minimal medium containing 0.2% (v/v) sodium lactate, where 0.5% (v/v) α–pinene was added at various times after inoculation.

Time of α–pinene addition	Oxygen uptake (mU/mg protein)	NADH consumed (mU/mg protein)
(I) No α–pinene added	4.37	3.9
(II) Inoculation	6.0	5.6
(III) Inoculation and after 20 h of incubation	7.06	8.65
(IV) After 12 h of incubation	5.8	5.5
(V) After 12 and 20 h of incubation	5.95	6;23
(VI) After 16 h of incubation	5.5	5.15
(VII) After 16 and 20 h of incubation	5.3	4.4
(VIII) After 20 h of incubation	4.0	3.6

The pH variation during growth of *P. fluorescens* under the adopted conditions is shown in figure 3.2. The most rapid growth is characterised by an increase in acidity, followed by a gradual increase in pH as the culture enters the stationary phase. These results are in accordance with those presented Best *et al.* (1987) and Aikens (1990) who demonstrated the transient accumulation of acidic intermediates. Similar findings have been presented by Prema and Battacharyya (1962), Shukla and Battacharyya (1968), Hungund *et al.* (1970), Gibbon and Pirt (1971), Pujar and Battacharyya (1973), Tudroszen *et al.* (1977), Krasnobajew (1981) and Trudgill (1984 and 1986) for other α–pinene utilising *Pseudomonas* spp..

3.2.3 Isolation of Products of the α–Pinene Oxidation Pathway from Culture Fluids and Resting Cells

Data used for the conceptualisation of metabolic pathways for degradation of hydrocarbons has often been obtained, at least in the preliminary stages, from investigation of whole cells. These studies were achieved by either growing the organism at the expense of the hydrocarbon substrate and then analysing the resulting culture fluid, the cells, or both, by GC, TLC, HPLC, or spectrophotometrically. Alternatively, the metabolic route may be revealed by studying washed, non-proliferating suspension of organisms (resting cells), supplied with the appropriate substrate followed by an analysis as above (Klug and Markovetz, 1971; Dagley and Chapman, 1981; Bühler and Shindler, 1984). A compound which accumulates in a liquid growth medium is not necessarily a reaction intermediate in the metabolic pathway. For instance, rapid metabolism resulting from an excess of substrate can result in oxygen limitation and the shunting of intermediates along reducing pathways. On the other hand, when the supply of oxygen does not limit growth, any compounds detected in the culture medium are likely to be those that lie on the metabolic

pathway by which the carbon source is oxidised completely (Dagley and Chapman, 1981). However, many oxidation products may be of a transient nature and thus, they

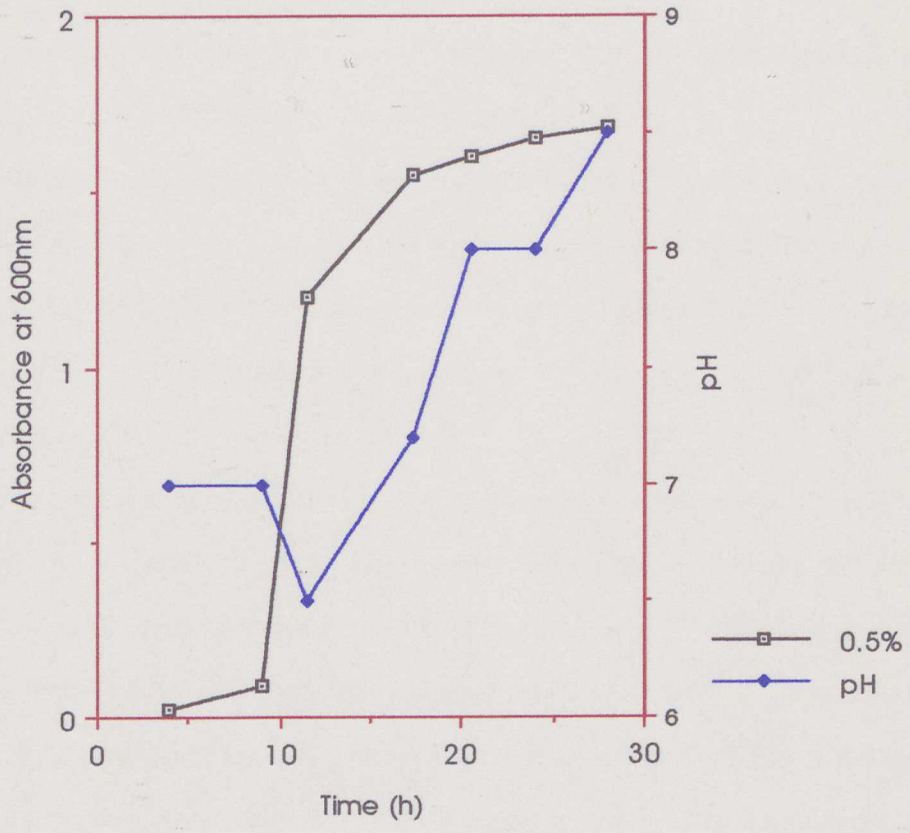


Figure 3.2: Growth curve and variation in pH during growth of *Pseudomonas fluorescens* NCIMB 11671 in minimal medium containing 0.2% (v/v) sodium lactate with 0.5% (v/v) α–pinene added at inoculation and after 20 h of incubation at 30°C, with shaking.

may never appear in detectable amounts during oxidation of the substrate (Klug and Markovetz, 1971).

Supernatants from overnight cultures of *P. fluorescens* grown on minimal medium containing 0.2% (v/v) sodium lactate and 0.5% (v/v) α–pinene were extracted in dichloromethane, as described, and the extracts analysed by GC and GC-MS. Only a small amount of α–pinene was detected in these samples and none of the degradation products reported by Best *et al.*, (1987). It was assumed that the bulk of the α–pinene would have been metabolised by the time of harvesting and the amount of products present, if any, would be undetectable.

Significant accumulation of the epoxide was considered unlikely, as previous assays had showed chemical breakdown at pH values less than pH 9.0 (figure 3.3), a pH at which no cell activity could be demonstrated. However, to avoid acidity in the extracting solvent, dichloromethane was equilibrated at pH 9.0 with 50 mM sodium-glycine buffer, pH 9.0.

Cells from an overnight culture of *P. fluorescens* in the above medium, were harvested in late exponential phase, and resting cell assays carried out as described. GC and GC-MS analysis of these samples detected three compounds, namely, α–pinene, α–pinene oxide and 2-methyl-5-isopropylhexα–2,5-dienal. The mass spectra of the last two compounds as well as their structures are shown in figures 3.4 and 3.5. The spectra obtained were very similar to the ones presented by Best *et al.*, (1987). Moreover, campholene aldehyde and carveol which are reported as decomposition products of the epoxide at pH 7.6, the assay pH used, (Griffiths *et al.*, 1987a) were also detected.

The fall in pH during exponential growth was thought to be a result of the accumulation of acidic metabolites formed from the oxidation of α–pinene, especially as the product resulting from double ring cleavage of α–pinene oxide was 2-methyl-5-isopropylhexα–2,5-dienal the expected precursor to 2-methyl-5-isopropylhexα–2,5-dienoic acid detected by Griffith *et al.* (1987a). However, although the formation of carboxylic acids from the corresponding aldehydes appears to be

thermodynamically more favourable than the formation of the aldehyde (Bühler and Shindler, 1984), and the presence of acids has been confirmed by continuous measurement of the pH in cultures of *P. fluorescens* growing at the expense of α–pinene, no acids were detected in the form of methyl esters from methylated samples of culture supernatants, taken from late exponential phase cultures, or resting cells. This result could be explained by the observation made by Aikens (1990) that during growth of *P. fluorescens* on α–pinene, it was possible to obtain acidic metabolites only during the period of rapid growth, where the pH was seen to fall.

3.2.4 Sensitivity of *Pseudomonas fluorescens* NCIMB 11671 to Antibiotics

An investigation of the antibiotic sensitivity of *P. fluorescens* revealed that growth on nutrient agar plates was not inhibited at all by ampicillin at a concentration of 50 μg/ml, but was slower when 100 μg/ml of the antibiotic were added. On the other hand, it was totally inhibited by both kanamycin and tetracycline at concentrations of 25 μg/ml. The latter result, also observed by Saqib (1992) and Aikens (1990) contradicts that reported by Best *et al.* (1987) who stated that *P. fluorescens* was resistant to the action of tetracycline (concentration unknown). Both kanamycin and tetracycline (25 μg/ml) also totally inhibited the organism's growth in minimal medium plates using α–pinene vapour as sole carbon source.

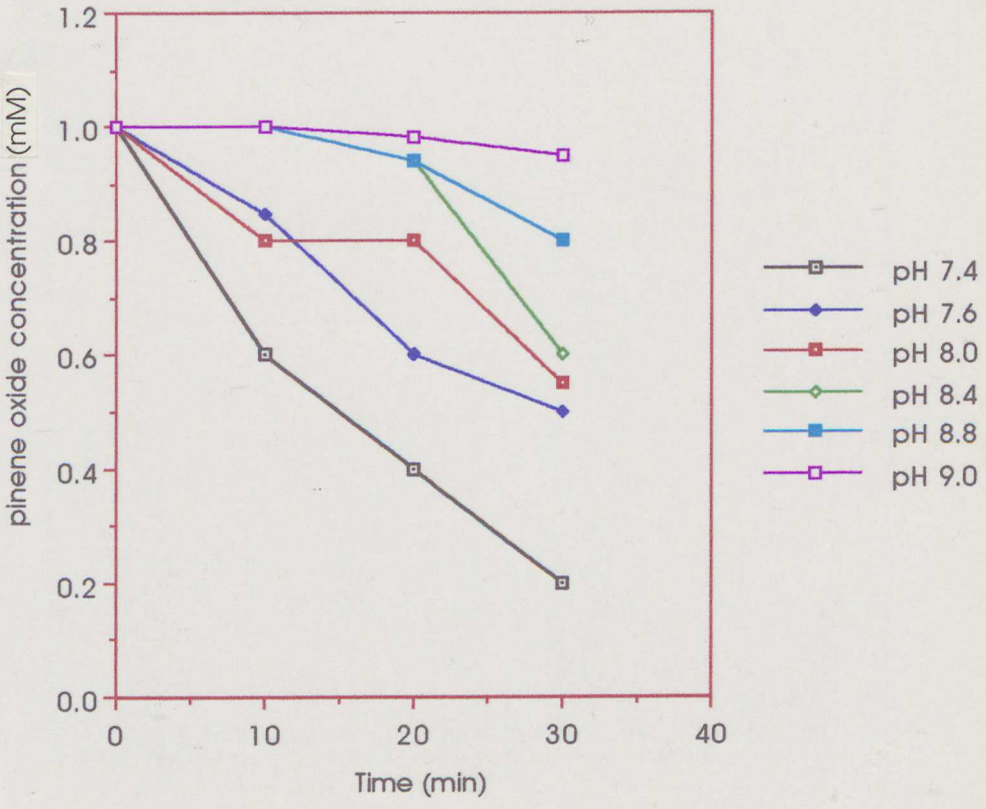


Figure 3.3: Degradation profile of α-pinene oxide incubated in various buffers at 35°C for a period of 30 min.

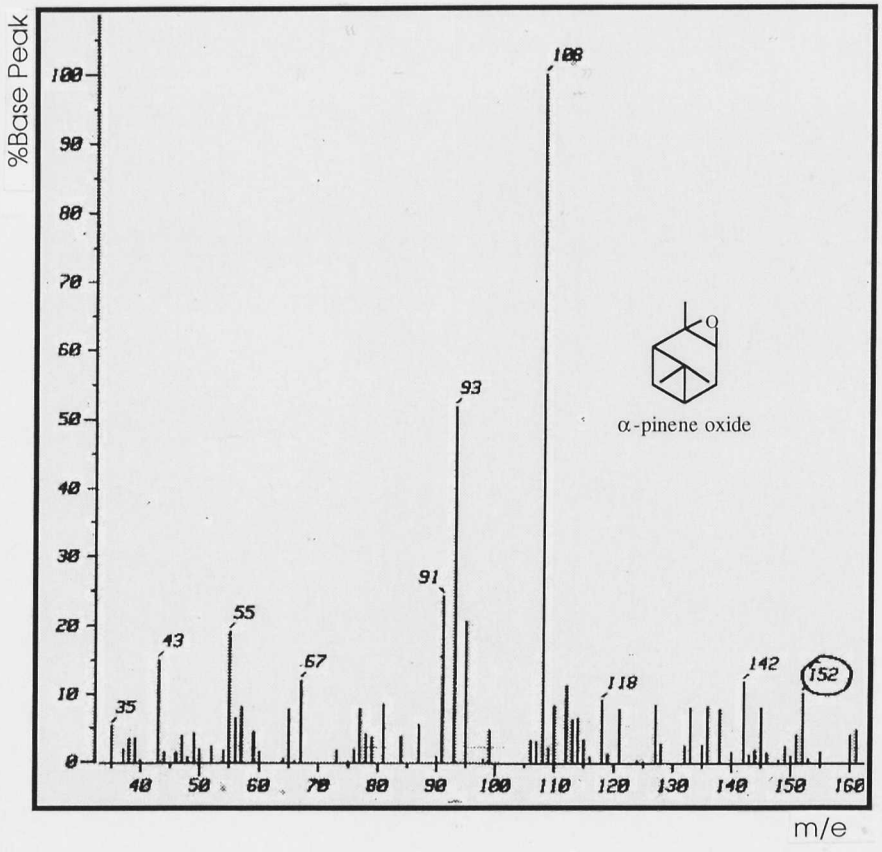


Figure 3.4: Mass spectrum of α–pinene oxide (structure inset) detected in resting cell assays of *Pseudomonas fluorescens* NCIMB 11671.

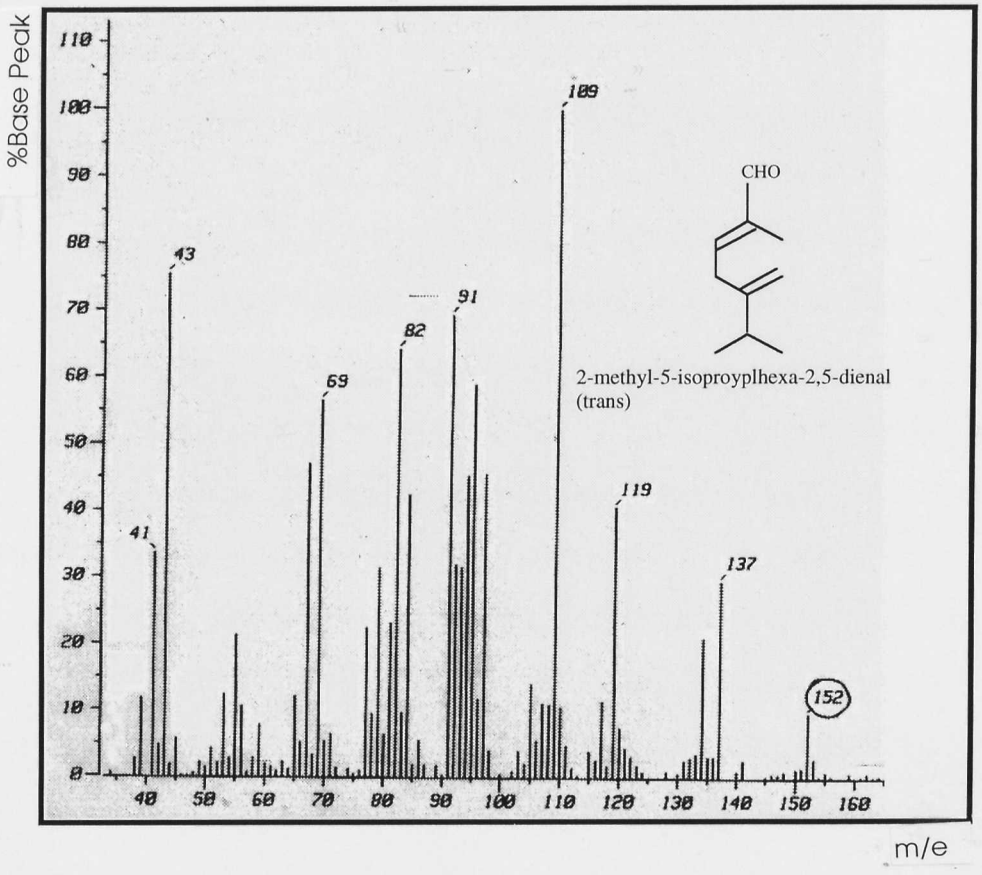


Figure 3.5: Mass spectrum of 2-methyl-5-isopropylhexα–2,5-dienal (structure inset) detected in resting cell assays of *Pseudomonas fluorescens* NCIMB 11671.

3.3 Large Scale Growth Experiments

3.3.1 Batch Culture

The main aim of large scale culture of *P. fluorescens* was to obtain sufficient quantities of cells with high monooxygenase activity so that the α–pinene epoxidation system could be purified by conventional methods of protein chromatography. Based on the previous results, 200 ml of an overnight culture of *P. fluorescens* in LB broth were used to inoculate a 20 litre bioreactor (Chemap, Switzerland) with minimal medium containing 0.2% (v/v) sodium lactate and 0.5% (v/v) α–pinene and the fermentation was carried out at 30°C. The aeration rate was 0.05 vvm and the agitation speed 500 rpm. Both the aeration rate and the agitation speed applied were at the lower suggested limits for fermentations (Stanbury and Whitaker, 1987; Crueger and Crueger, 1990a) because of the highly volatile nature of α–pinene. These conditions would at least minimise the stripping of the organic from the medium, and thus, fewer additions of α–pinene to the culture would be necessary. This would in turn minimise the danger of contamination and also would render the procedure more economical. According to the previous results (figure 3.2) pH control was not necessary until the culture reached the late exponential phase when the pH would become slightly alkaline. Therefore, the pH was maintained at 7.0-7.2 by automatic addition of 10% (v/v) sulphuric acid. The dissolved oxygen level in the culture medium was monitored with an oxygen electrode and the CO_2 concentration in the off gas by an on-line mass-spectrometer. Cell growth was monitored by optical density measurements at 600 nm and the sodium lactate level was determined by HPLC as described. The free ammonia in the culture medium was also determined by a colorimetric assay. Sodium lactate at a concentration of 0.2% (v/v) and ammonium sulphate at a final concentration of 0.2 g/litre were added to the culture medium as soon as they had dropped to 1/4 of their starting concentration. The points at which α–pinene was added were estimated empirically according to the CO_2

concentration in the outlet gasses. When the CO_2 concentration started to plateau, 0.3% (v/v) of α–pinene was added to the culture. Usually the first addition was after 20 h of fermentation and then every 8-10 h approximately. Supplements resulting in a lower percentage of α–pinene than that initially used were chosen in order to avoid any surfactant formation. All additions were performed by sterile transfer though the phosphate buffer inlet (figure 3.6). Some routine results are presented in figures 3.7, 3.8, 3.9 and 3.10.

α–Pinene monooxygenase activity in whole cells was measured polarographically. However, because the cells had very high endogenous activity, the sample preparation was slightly different from that described before: 20 ml of cells were centrifuged at 5,000 g for 10 min, the pellet was washed twice with 10 ml of 50 mM phosphate buffer, pH 7.8, and resuspended in 1 ml of the same buffer. The cell suspension was incubated at room temperature for one hour and then the monooxygenase activity was stimulated by addition of 20 µl α–pinene and incubation for another half hour before measuring activity. Additionally, gas chromatography assays based on the detection of α–pinene oxide, in dichloromethane extracts of culture supernatant were also used for monitoring the monooxygenase activity, however, this method was not always very sensitive and required large quantities of supernatant to be extracted.

In order to determine whether the cells were using the D- or the L- form of sodium lactate,L-lactate dehydrogenase was used, as described. Table 3.4 shows that the L-lactate concentration reduced by a factor of 5.5 in 20 h of fermentation. However, according to the HPLC based estimation the total lactate concentration only reduced by a factor of 3 during the same period. Therefore, it appears that the bacterium preferentially utilises the L-lactate but can also utilise the D-isomer, possibly by converting it to the L- form by means of an isomerase.

The bioreactor was routinely harvested at a culture OD_{600} between 2.5 and 3.0, in approximately the mid-exponential phase, which corresponded to a slight drop in the CO_2 concentration in the outlet gasses (figure 3.9). When harvesting was

attempted at higher optical densities it proved impossible to concentrate the cells by centrifugation because of the presence of a biosurfactant. Due to the fact that enzyme activity appeared to be increased by α–pinene without the need for *de novo* protein synthesis (Aikens, 1990), a stimulatory amount (5 ml) α–pinene (total concentration 0.025%) was injected to the culture 2-4 h prior to harvesting.

Tangential filtration (Bio-Flo Ltd, UK) was used in an attempt to concentrate the cells prior to centrifugation. Unfortunately, this method was totally unsuccessful because of the fact that by reducing the total culture volume the concentration of the biosurfactant present increased, thus, making it impossible to centrifuge the cells. Therefore, the cells were harvested by centrifugation at 5,000 g for 30 min, at 4°C. The pellets were washed twice in half their original volume in 10 mM phosphate buffer, pH 8.0, containing 10% glycerol, resuspended to a slurry with an OD_{600} of 100, drop frozen in liquid nitrogen and stored at -70°C until needed.

Table 3.4: Confirmation that the L- form of lactate is used by *Pseudomonas fluorescens* NCIMB 11671 during fermentation.

Sample	OD at 340nm	Total Lactate concentration (mM)	L-lactate concentration (mM)
L-lactate (standard)	1.4	20	10
Beginning of the fermentation (t=0)	1.18	17	8.5
After 20 h of fermentation	0.22	8.8	1.6

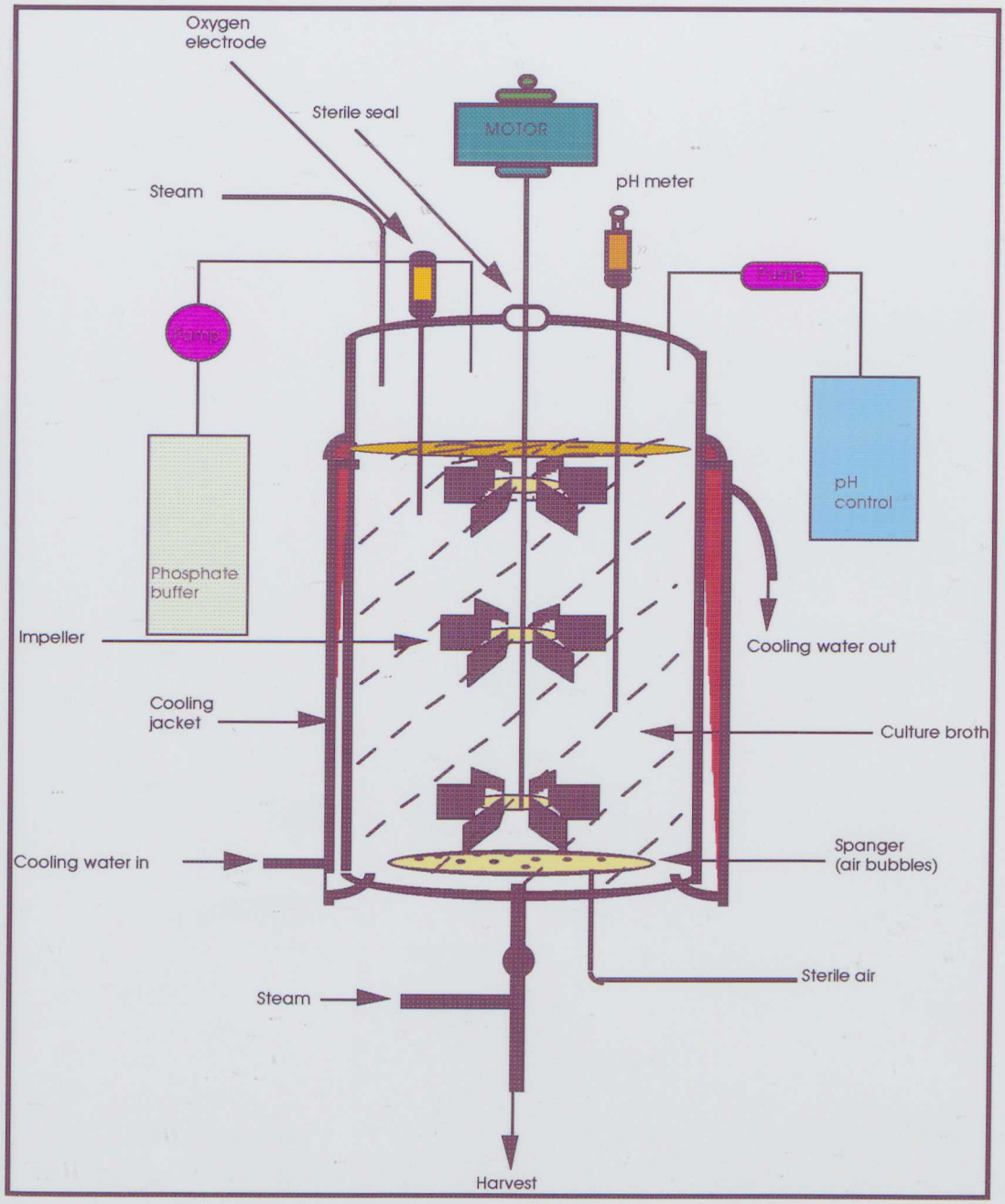


Figure 3.6: Schematic presentation of a bioreactor system similar to the one used for the fermentation of *Pseudomonas fluorescens* NCIMB 11671 on α–pinene.

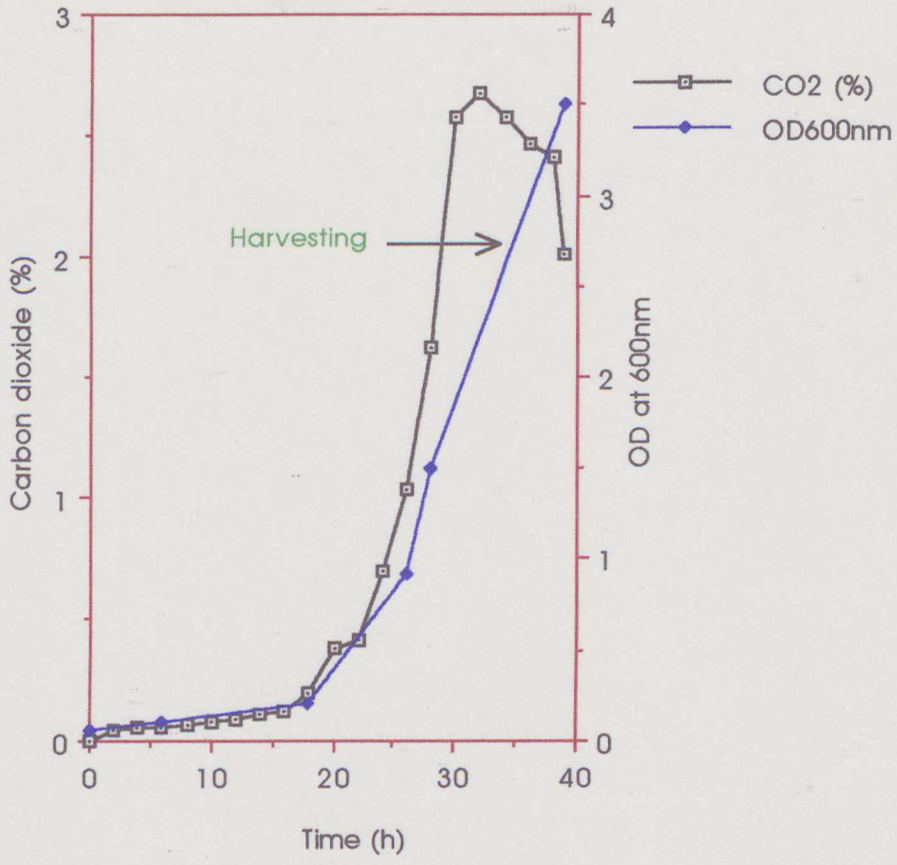


Figure 3.7: Optical density at 600 nm and CO_2 concentration in the outlet gasses of *Pseudomonas fluorescens* NCIMB 11671 during growth in a 20 litre bioreactor.

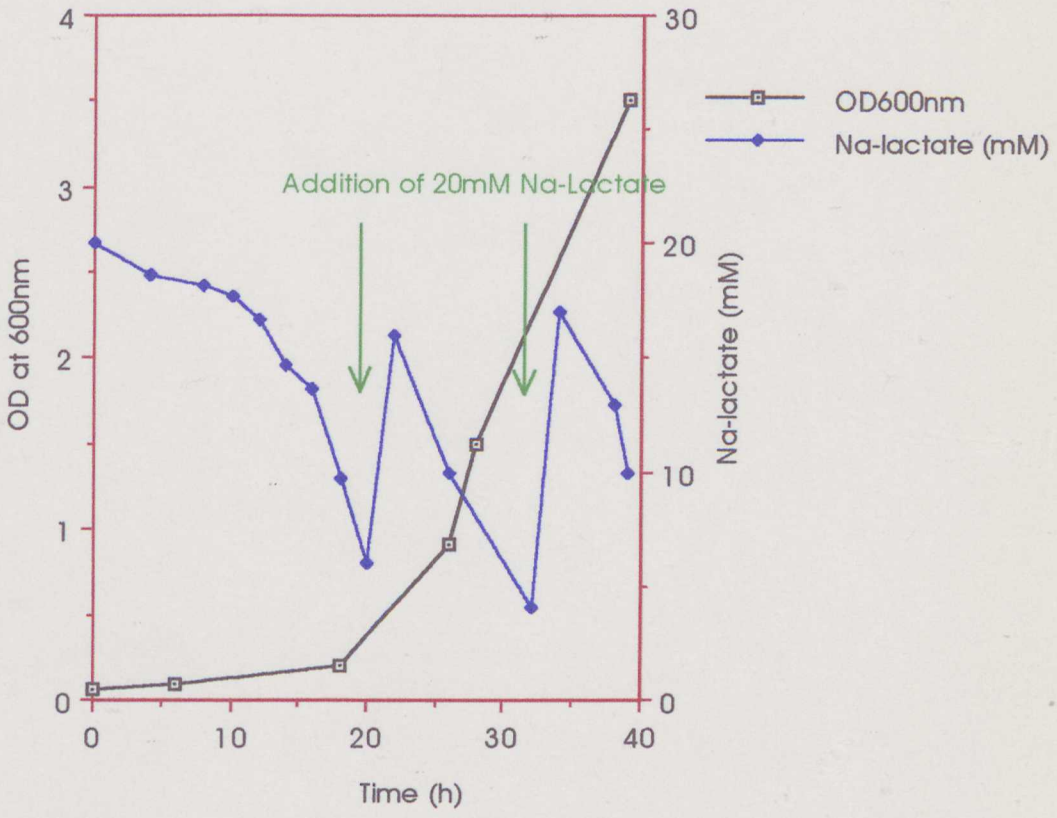


Figure 3.8: Optical density at 600 nm and sodium lactate level in culture fluids of *Pseudomonas fluorescens* NCIMB 11671 during growth in a 20 litre bioreactor.

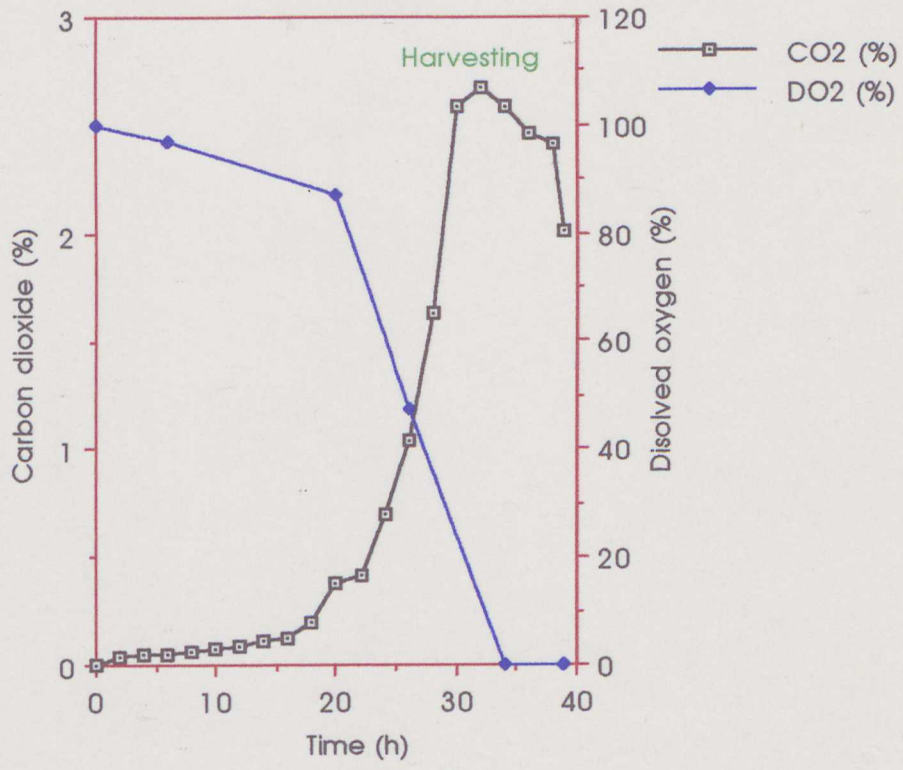


Figure 3.9: Carbon dioxide and Dissolved oxygen levels in culture fluids of *Pseudomonas fluorescens* NCIMB 11671 during growth in a 20 litre bioreactor.

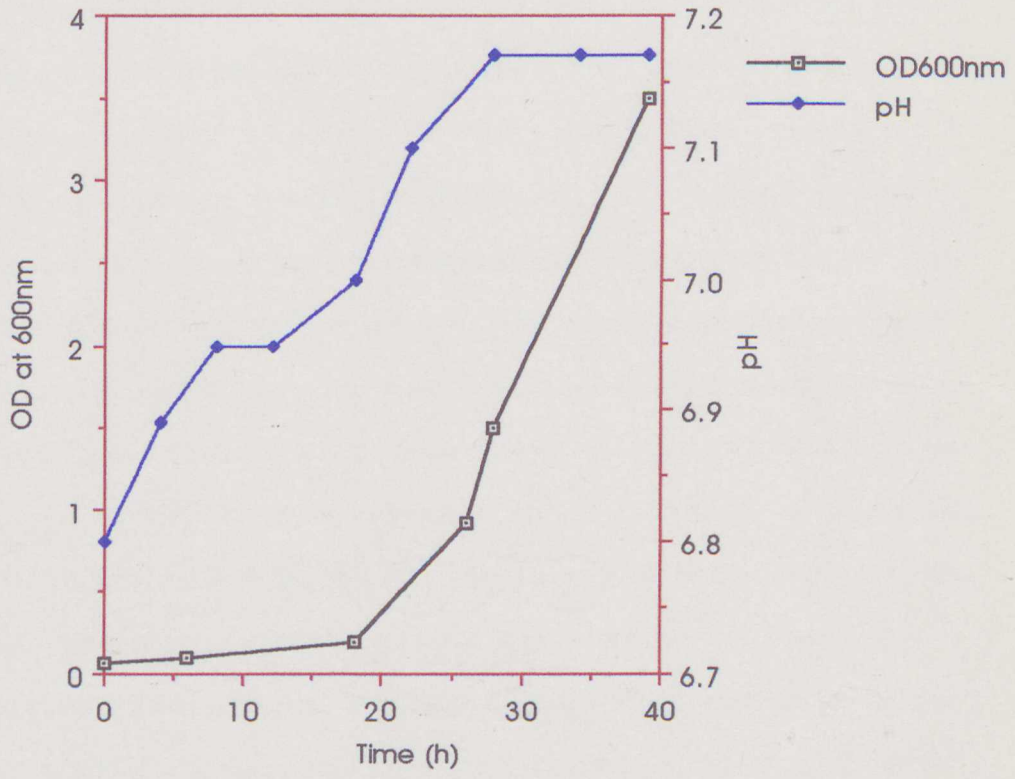


Figure 3.10: Optical density at 600 nm and culture pH during growth of *Pseudomonas fluorescens* NCIMB 11671 in a 20 litre bioreactor.

3.4 Discussion

The detection of the α–pinene monooxygenase activity in cell free extracts produced from *P. fluorescens* grown on α–pinene proved to be somewhat problematical. Best *et al.* (1987) appeared to experience no difficulty in obtaining this enzyme activity in cell free extracts. Studies by Griffiths *et al.* (1987a) on the oxidation of α–pinene grown *Nocardia* P18.3 alluded to the presence of an α–pinene monooxygenase system similar to that described by Best *et al.* (1987). However, this was based on circumstantial evidence as no α–pinene monooxygenase activity could be detected in cell free extracts produced from α–pinene grown *Nocardia* P18.3. The elusive nature of this enzyme system appears to be a common feature encompassing such dissimilar genera as *Nocardia* spp. and *Pseudomonas* spp..

It appeared that the amount of α–pinene present in the growth medium was a major factor in determining whether any α–pinene monooxygenase activity was detectable in cell free extracts produced from α–pinene grown *P. fluorescens*. It has been shown that the organism can tolerate as high as 10% (v/v) α–pinene. However, when more than 1% (v/v) of the organic was added to the growth medium it was impossible to harvest the cells by centrifugation because of the presence of a biosurfactant. Therefore, in small scale cultures, a concentration of 0.5% (v/v) of α–pinene was added at the start of growth and after 20 h of incubation at 30°C to a growth medium containing 0.2% (v/v) of sodium lactate. This system resulted in good growth, minimal production of biosurfactant and high monooxygenase activity in both whole cells and cell free extracts.

Similarly, fermentations were carried out on a 20 litre scale. Reliable estimates of the α–pinene concentration present within the growth medium were impossible to obtain, due to its poor solubility. The stripping of the terpene from the growth medium was dependent both on the aeration rate and the speed of agitation, which therefore had to be kept at the lowest possible levels, 0.05 vvm and 500 rpm respectively. Further additions of α–pinene were made at times estimated empirically according to the

CO_2 percentage in the outlet gasses. It was apparent form this study that the organism preferentially uses L-lactate, with the D- form of lactate being used more slowly, possible after conversion to the L- form by means of an isomerase.

It is common to harvest cells in late exponential phase because of the higher yield of cells obtained, and the absence of toxic metabolites (Mandelstam *et al.*, 1982). All fermentations were harvested approximately before the late exponential phase, as a result of oxygen limitation, when the OD of the culture at 600 nm was between 2.5 and 3.0, and corresponded to a slight fall in the CO_2 concentration in the culture medium. Harvesting at later fermentation times always resulted in high amounts of biosurfactant and thus it was impossible to concentrate the cells by centrifugation.

The only reliable method for the detection of monooxygenase activity during growth was the oxygen electrode measurements, where α–pinene stimulated oxygen consumption was corrected for the endogenous oxygen consumption. Although monooxygenase activity in whole cells was detectable throughout the fermentation procedure, cell extracts showed high monooxygenase activity only when cells were induced by addition of a small amount of α–pinene, 2-4 h prior to harvesting. This kind of reactivation of the enzyme system, also shown previously by Aikens (1990) was very interesting.

Once the growth regime had been optimised, the α–pinene monooxygenase activity as described by Best *et al.* (1987) could easily be detected in cell free extracts. The formation of α–pinene epoxide and 2-methyl-5-isopropylhexα–2,5-dienal was also confirmed in this study. However, although the pH of the culture medium suggested the formation of acidic metabolites in exponential growth, none of them was identified in this study. Best *et al.* (1987) suggested that the aldehyde was the precursor of the corresponding acid, 2-methyl-5-isopropylhexα–2,5-dienoic acid which was also produced during the growth of *Pseudomonas* PX1 (Gibbon and Pirt, 1971) and mutants of *P. putida* PIN11 (Tudroszen *et al.*, 1977) on α–pinene. Although the acid, previously detected by Aikens (1990), was not detected in this study, an

aldehyde dehydrogenase activity was detected in extracts from cells induced by α–pinene.

In summary, this chapter has confirmed the transient nature of the α–pinene monooxygenase activity in *P. fluorescens* and has provided physiological markers, the observation of which could be used to predict the metabolic state of the organism and the probability of producing the enzyme activity in cell free extracts. The necessary condition appeared to be the presence of sufficient but minimal level of α–pinene in the medium; the absence of α–pinene even for a short time resulted in a dramatic reduction in the activity of the system in whole cells and cell free preparations. The activity could be stimulated by the addition of α–pinene to the culture medium prior to harvesting the cells.

CHAPTER 4

Construction and Characterisation of Mutants of *Pseudomonas fluorescens* NCIMB 11671 in the α-Pinene Degradation Pathway

4.1 Introduction

A part of the degradation pathway of α-pinene in *P. fluorescens* has been elucidated (Best *et al.*, 1987; Griffiths *et al.*, 1987a; Aikens, 1990; this study) and limited genetic studies on this pathway, together with preliminary work aimed at cloning the α--pinene monooxygenase, have been performed by Saqib (1992). The isolation and sequencing of the α–pinene monooxygenase gene, should throw light on the mechanism of enzymatic epoxidation of internal alkenes.

In order to identify and isolate the monooxygenase gene one could either probe a genomic library of *P. fluorescens* with an oligonucleotide corresponding to an amino acid sequence derived from the purified enzyme, or carry out complementation procedures between mutant bacteria that lack the enzyme of interest and a genomic library of the wild type bacterium. Some mutants and a genomic library of *P. fluorescens* had been kindly provided by Dr K. Saqib (1992) and thus, the second option was followed.

Biological variation in any genetic study is axiomatic. Most genes have a spontaneous mutation rate which is too low for any variants to be detected simply, unless an extremely effective selection pressure can be applied, such as resistance to antibiotics. In order to obtain mutants of bacterial genes for which selective pressure cannot be applied, it is necessary to increase the mutation rate. This can be done by a variety of physical and chemical agents, only a few of which have stood

the test of time in terms of producing stable mutants at an acceptable frequency (Holloway, 1986a). In general, base substitutions are the most useful mutations, because they can be used to investigate both the *in vivo* activities and the structure-function relationship of the gene product of interest (Foster, 1991). Numerous alkylating agents have mutagenic activity. They attach methyl or ethyl groups at the 6-position on the purine ring, which results in mispairing of the base during subsequent replication. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) is the most commonly used alkylating agent. Because the major mutagenic lesion induced by NG, O^6-methyl guanidine, is not very lethal, high mutation frequencies, as high as 15% of the surviving cells may be produced with this mutagen (Creda-Olmedo and Hanawalt, 1968; Birge, 1991; Foster, 1991). However, NTG-induced mutations tend to cluster at the replication point, giving a high frequency of double mutants. In addition, NTG mutagenesis results in mutations which are almost exclusively GC to AT transitions (Birge, 1991; Foster, 1991).

After mutagenesis, complete characterisation of the mutants should extend our knowledge about the chemistry and enzymology of the α-pinene degradation pathway, and also provide some information regarding its gene organisation and regulation. For that purpose, the mutants produced were tested for activity in the first three enzymes suggested for the pathway (Best *et al.*, 1987; Griffiths *et al.*, 1987a), namely: α-pinene monooxygenase, α-pinene epoxide lyase, and aldehyde dehydrogenase.

4.2 Detection of Large Plasmids in *Pseudomonas fluorescens* NCIMB 11671

Pseudomonas spp. are able to utilise a wide variety of organic compounds as carbon and energy sources. Although the genes encoding for some of the necessary catabolic functions are mapped to chromosomal loci, it is well known that many catabolic functions are encoded on plasmids (Broda, 1979). Oxygenases in pseudomonads are commonly encoded by genes located on large catabolic

plasmids (Witholt, 1990), such as the SAL plasmid which is involved in the degradation of salicylic acid (Chakrabarty, 1972), the NAH plasmid involved in naphthalene degradation (Dunn and Gunsalus, 1973; Shell, 1990), and the TOL plasmid involved in toluene degradation (Worsey and Williams, 1975; Nakazawa *et al.*, 1990).

Therefore, for cloning the α-pinene monooxygenase gene, it was important to establish whether the monooxygenase is encoded by a degradative plasmid or by a chromosomal locus. Such studies were performed using a modification of the Eckhart method (1978) and that of Zunica *et al.* (1981) as described in Materials and Methods but did not reveal the presence of any large plasmid in this bacterium.

4.3 Preparation of a Killing Curve Prior to Mutagenesis of *Pseudomonas fluorescens* NCIMB 11671 with NTG

To generate dose-response curves either the mutagen concentration, or the time of exposure to it, can be varied (Miller, 1972; Foster, 1991). Survival curves using both parameters were prepared in order to establish the appropriate dose of NTG as well as the time of incubation to achieve 50% killing. 50% killing is recommended, because it is believed that such a killing rate would minimise the probability of isolating mutants with double or multiple lesions, while still yielding an acceptable mutation rate (Miller, 1972).

A survival curve with various NTG concentrations, from 0 to 100 μg/ml of cell culture, with a standard incubation time of 90 minutes at 30°C, is presented in figure 4.1. The arrow indicates that 50% killing occurred at a NTG dose of approximately 30 μg/ml. Incubation of cell suspension with a standard NG dose of 50 μg/ml, for 0 to 90 minutes at 30°C showed that 50% killing was achieved with an incubation time of 30 min (figure 4.2). Therefore, in order to achieve high mutation rates, a dose of 50 μg/ml of NTG per ml of cell suspension and an incubation time of 40 min corresponding to 70-80% killing were applied in all subsequent NTG mutagenesis procedures.

4.4 Mutagenesis of *Pseudomonas fluorescens* NCIMB 11671 with NTG

Mutagenesis of *P. fluorescens* was performed with NTG under conditions required for 70-80% killing (50 μg/ml NG and 40 min incubation). NTG acts mainly at the replication fork. Mutation occurs when the methylated base is mispaired in the subsequent replication and yields a complete transition after a further round (Foster, 1991). Therefore, after the NTG treatment, cells washed in phosphate buffer, were incubated in 30 ml nutrient broth at 30°C with good aeration until the optical density of the culture had doubled (approximately 3 h). Using replica plating techniques, the mutagenised culture was replica plated onto minimal agar plates in duplicate, and incubated under α-pinene or α-pinene oxide vapour, at 30°C, for 2 to 4 days. After screening of 6,000 colonies 10 mutants unable to grow on either one, or both of the above substrates were isolated. Table 4.1 presents the phenotypes of the mutants isolated and also of the four mutants kindly provided by Dr K. Saqib. To confirm that these mutants were not auxotrophs they were replicated on minimal lactate medium, saved in 20% (v/v) glucose in LB broth and stored at -70°C.

Three of the mutants AC4, AC5, KS507, were unable to grow on α-pinene when it was supplied as sole carbon source but they were able to grow on α-pinene oxide suggesting that their mutations were in the gene(s) encoding α-pinene monooxygenase. The fact that these pin^-pox^+ mutants grew on α-pinene oxide suggests that the epoxide can either induce the whole α-pinene operon, or that it is the natural inducer of the second enzyme of the pathway, α-pinene oxide lyase. The presence of pin^+pox^- mutants suggests that either there may be more than one metabolic pathways for the degradation of α-pinene in *P. fluorescens* or these mutants could be regulatory mutants that are not induced by the epoxide. Finally, mutants unable to grow on both substrates, pin^-pox^- phenotype, are more likely to be lyase or later pathway mutants.

All mutants isolated had a reversion frequency ranging from 10^{-7} to 10^{-11} per viable cell. In certain cases revertants were not isolated at all. This could be the result of double mutations or deletions generated by NTG (Miller, 1972; Birge, 1991).

Table 4.1: Phenotype of mutants in α-pinene metabolism. Mutants were screened on minimal medium plates at 30°C, under α-pinene or α-pinene oxide vapour as a sole carbon source. Minimal plates containing 0.3% (v/v) lactate or no carbon source were used as controls.

Mutant	α-Pinene as carbon source	α-Pinene oxide as carbon source	Lactate as carbon source	No carbon source	Phenotype
AC1	-	-	+	-	pin^-pox^-[b]
AC2	-	-	+	-	pin^-pox^-
AC3	+	-	+	-	pin^+pox^-
AC4	-	+	+	-	pin^-pox^+
AC5	-	+	+	-	pin^-pox^+
AC6	+	-	+	-	pin^+pox^-
AC7	+	-	+	-	pin^+pox^-
AC8	+	-	+	-	pin^+pox^-
AC9	+	-	+	-	pin^+pox^-
AC10	-	-	+	-	pin^-pox^-
KS505[a]	-	-	+	-	pin^-pox^-
KS507	-	+	+	-	pin^-pox^+
KS509	-	-	+	-	pin^-pox^+
KS519	+	-	+	-	pin^+pox^-

a: All KS-mutants are those provided by Dr K. Saqib.

b: pin or pox (+) or (-) means that these mutants were able or unable to grow on minimal medium containing α-pinene or α-pinene oxide as sole carbon source respectively.

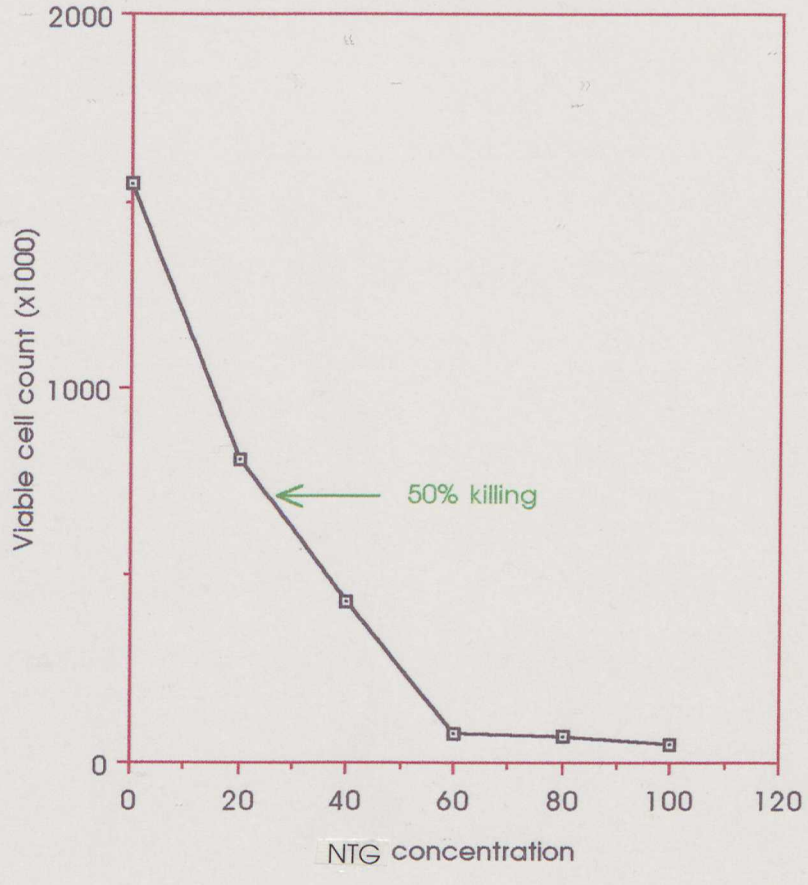


Figure 4.1: Killing curve of *Pseudomonas fluorescens* NCIMB 11671 with 0-100 µg/ml NTG, and a standard incubation time of 90 min at 30°C.

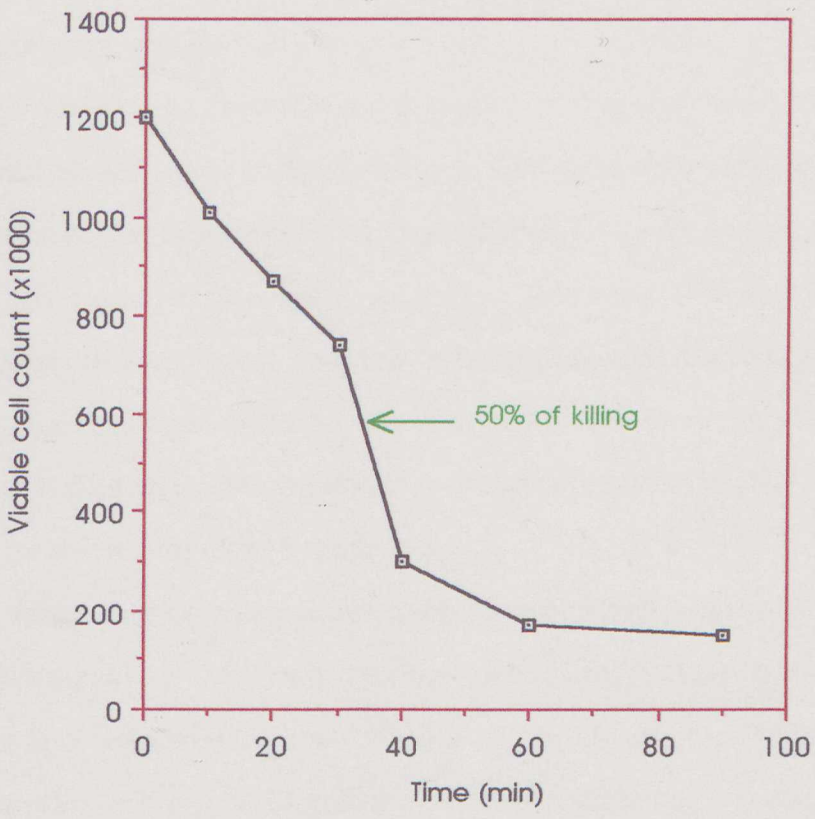


Figure 4.2: Killing curve of *Pseudomonas fluorescens* NCIMB 11671 with 50 µg/ml NTG, and incubation for 0 to 90 minutes at 30°C.

4.5 Characterisation of the Mutants of *Pseudomonas fluorescens* NCIMB 11671

4.5.1 α-Pinene Monooxygenase Activity Assays

Monooxygenase activity was tested by polarographic, spectrophotometric, electrophoretic and gas-chromatographic techniques. Cell free extracts of mutant and wild type bacteria were used in all of the above assays except in polarographic measurements where whole cells were used.

Polarographic methods were based on the α-pinene stimulated oxygen consumption by whole cells of mutants. Wild type cells were used as a positive control, while measurement of the oxygen uptake in the absence of the substrate in all cells, was used as a negative control. Generally, the endogenous oxygen consumption rate of whole cells was high and therefore, it was difficult to determine the monooxygenase activity. However, although these assays were not very sensitive the results obtained were similar to those obtained by the other assays used. These results are presented in table 4.2.

In spectrophotometric assays the enzyme activity was identified by measuring the absorbance at 340 nm in cell free extracts of mutants and wild type in the presence of α-pinene and NADH as cofactor. All cells were grown on minimal medium containing 0.3% (v/v) sodium lactate and 0.2% (v/v) α-pinene as carbon source and/or inducer for 24 h. Wild type cells, that had been grown in the same medium containing 0.2% (v/v) α-pinene, or 0.1% (v/v) α-pinene oxide, or 0.3% (v/v) sodium lactate only, were also used as controls. Measurement of the NADH consumption in assay mixtures where no substrate was added was used to determine the endogenous oxidation rate. The protein concentration of all samples during the assay was 0.5 mg/ml.

Wild type cells that had been grown on minimal medium containing only lactate, or lactate plus epoxide, as carbon source had 1/10th of the monooxygenase activity detected in cells grown in the presence of α-pinene. This suggests that

expression of α–pinene monooxygenase is induced by α-pinene alone, in accordance with previous suggestions (Griffiths *et al.*, 1987a; Aikens, 1990; Saqib, 1992). Five of the mutants showed α-pinene stimulated NADH oxidation activity, namely, AC2, AC3, AC6, AC7, KS519. Although AC8 and AC9 were pin^+pox^- mutants, they did not show any monooxygenase activity. This observation indicates that alternative pathways for the degradation of α-pinene may exist. These results are presented in table 4.2. Mutants AC1, AC4, AC5, AC8, AC9, AC10, KS505, KS507 and KS509 did not show α-pinene stimulated NADH oxidation, and thus, they were considered as monooxygenase minus (MO^-) phenotypes.

In product formation assays, culture supernatants of *P. fluorescens* mutants were extracted in dichloromethane, and analyzed by gas chromatography for the presence of accumulated α-pinene epoxide. The cell paste was also used for resting cell assays and analyzed for the production of the epoxide. However, these resulted in very low epoxide production, which was difficult to detect by GC. On the other hand, epoxide was easily detected as an accumulated product in extracts of culture supernatants. Mass spectra, of supernatant extracts from mutant cultures, revealed the formation of campholenic aldehyde and *cis-* and *trans-* carveol in all samples where small amounts of the epoxide were also detected. Studies on the stability of the epoxide in buffered aqueous solutions at 30°C (Griffiths *et al.*, 1987a and also in this study) (Chapter 3) demonstrated rapid decomposition at pH 7.0 or lower, with the formation of four major metabolites that were identified by GC-MS as campholenic aldehyde, *cis-* and *trans-* carveol and sorberol. Therefore, the presence of the same compounds in the supernatant extracts was taken as evidence of the formation of α-pinene oxide, even in cases where the epoxide itself was not detected. The epoxide was routinely detected in supernatants from AC2, AC3, AC6, AC7, and KS519 supporting findings from the spectrophotometric and polarographic assays mentioned above, which showed that these strains are not α-pinene monooxygenase mutants. Similarly, no epoxide was detected in the other samples, AC1, AC4, AC5, AC8, AC9, AC10, KS505, KS507 and KS519. Furthermore, very little epoxide was detected in samples derived

from cells that were grown on lactate or lactate plus α-pinene oxide and this result enhances the suggestion that only α-pinene is the inducer of the monooxygenase in this bacterium. The results of these assays are also presented in table 4.2.

The electrophoretic approach was based on the assumption that the enzyme has an NADH oxidoreductase activity which could be detected by incubating a native polyacrylamide gel with a tetrazolium dye in the presence of NADH. Cell free extracts from mutant and wild type bacteria were analyzed by non-denaturing electrophoresis (figures 4.3 and 4.4) as described, and the results obtained are presented in table 4.3. Cell free extracts of the wild type bacterium cultured on minimal medium supplied with lactate alone or lactate and α-pinene as carbon sources were used as controls. The activity stained electrophoretic pattern of the wild type bacterium is shown in figure 4.3 (lanes 1, 2, and 3), and comprised three major bands with R_f values 0.35, 0.43 and 0.51. Densitometric analysis of these gels, taking under consideration the amount of protein loaded for each sample, revealed that the first band (R_f value 0.35) had lower intensity, or was absent, in the MO^- mutant profiles compared to that of the WT. The second band (R_f value 0.43) was absent in most of the MO^- mutant profiles but also from KS519 and AC2 which are MO^+ mutants. The third band (R_f value 0.51) was present in all samples in various amounts.

All the above assays showed that monooxygenase production was lower in the wild type sample grown in minimal medium supplied with lactate as sole carbon source, than in the sample grown in the presence of α-pinene. This result indicates that expression of α-pinene monooxygenase is induced by α-pinene, although, there is a baseline level of expression in the absence of α-pinene with cells grown on lactate as carbon source.

4.5.2 α–Pinene Oxide Lyase Activity Assays

In all other α-pinene utilisers reported so far, such as *Pseudomonas* PL, *Pseudomonas* NCIB 10684 and *P. putida* PX1, cleavage of the cyclobutane ring appears to be a broadly distributed transformation, that is accompanied by cleavage of the cyclohexane ring (Shukla and Battacharyya, 1968; Gibbon and Pirt, 1971). On the other hand, *P. fluorescens* was reported to catalyse the NADH-linked oxygenation of α-pinene to form the corresponding epoxide followed by cleavage of the three rings of the epoxide by a decyclase with the formation of a novel aldehyde, 2-methyl-5-isopropylhexa-2,5-dienal (Best *et al.*, 1987; Griffiths *et al.*, 1987a; Aikens, 1990; this study). Epoxide lyase activity in cell extracts of wild type and mutant *P. fluorescens* was routinely assayed by following the formation of the *cis-* or *trans-* isomer of 2-methyl-5-isopropylhexa-2,5-dienal, using both spectrophotometric and electrophoretic techniques.

Cell free extracts of wild type and mutant cultures of *P. fluorescens* grown on minimal medium containing sodium lactate and α-pinene as carbon sources, were assayed spectrophotometrically at 235 nm for α–pinene oxide lyase activity. The initial product of the reaction is *cis*-2-methyl-5-isopropylhexa-2,5-dienal, but assay in glycine buffer at pH 9.0, used to suppress the spontaneous decomposition of the epoxide, results in a rapid isomerisation of the product to the *trans-* form (Griffiths *et al.*, 1987a). Ten of the mutants showed lyase activity, with only AC10, KS507, KS509 and KS519 being inactive; the results are shown in table 4.4. KS507 is a mutant unable to grow on α-pinene but able to grow on α-pinene oxide, however, it does not possess any lyase activity. This observation suggests that alternative pathways for the metabolism of α-pinene may exist in this bacterium. Wild type cells grown on α-pinene showed much higher lyase activity than those grown on lactate or lactate and α-pinene oxide. This suggests that α-pinene acts as the main inducer of all the enzymes of the α-pinene operon/regulon in *P. fluorescens*.

Activity staining of non denaturing polyacrylamide gels was also used for the detection of α-pinene oxide lyase activity in cell free extracts of wild type and mutant cultures of *P. fluorescens*. After electrophoresis the gels were immersed in an emulsion of 20 mM α-pinene oxide in 0.1 M sodium glycine buffer, pH 9.0. Precipitation of a white band of the less soluble 2-methyl-5-isopropylhexa-2,5-dienal in the polyacrylamide gel marked the position of the lyase band (figures 4.6 and 4.7). The R_f value of this band was 0.8 and appears to be at the same position as the lyase band detected by Griffiths *et al.* (1987b) in *Nocardia* spp.. The results obtained with this method were in complete agreement with those obtained spectrophotometrically and are presented in table 4.4.

4.5.3 Aldehyde Dehydrogenase Activity Assays

The majority of hydroxylation and epoxidation reactions require the presence of oxygen and NADH, with cofactor regeneration by an NAD-linked alcohol and/or aldehyde dehydrogenase. α–Pinene is converted to α–pinene oxide by an NADH-linked monooxygenase, the epoxide is subsequently converted to the 2-methyl-5-isopropylhexα–2,5-dienal by a lyase and the aldehyde to the corresponding acid by a NAD-linked aldehyde dehydrogenase (Griffiths *et al.*, 1987a).

Incubation of cell free extracts of α–pinene and lactate grown wild type and mutant cultures with α–pinene oxide at 30°C and measurement of the increase in the absorbance at 340 nm, demonstrated the presence of an epoxide stimulated NAD-linked dehydrogenase activity. Seven out of the fourteen mutants showed such activity and the results are presented in table 4.4. Mutants AC2, AC6, AC7, AC9, AC10 KS505 and KS507 did not show any dehydrogenase activity. Extracts from wild type cells grown with α–pinene as carbon source showed significantly higher dehydrogenase activity than those grown with lactate alone.

Table 4.2: Polarographic, spectrophotometric and product formation assays of α-pinene monooxygenase activity in whole cells and cell free extracts of wild type and mutant cultures of *Pseudomonas fluorescens* NCIMB 11671 grown on minimal medium supplied with 0.5% (v/v) sodium lactate only, 0.2% (v/v) sodium lactate and 0.5% (v/v) α-pinene or 0.1% (v/v) α-pinene oxide as carbon sources.

Sample	Carbon source	Polarographic assays	Spectrophoto-metric assays	gas-chromato-graphic assays
WT[a]	lactate	+(low)	+(low)	+(low)
W T	α-pinene oxide	+(low)	+(low)	+(low)
W T	L[c]+α-pinene	+	+	+
AC1(pin^-pox^-)[b]	L[c]+α–pinene	-	-	-
AC2(pin^-pox^-)	L[c]+α–pinene	+	+	+
AC3(pin^+pox^-)	L[c]+α–pinene	+	+	+
AC4(pin^-pox^+)	L[c]+α–pinene	-	-	-
AC5(pin^-pox^+)	L[c]+α–pinene	-	-	-
AC6(pin^+pox^-)	L[c]+α–pinene	+	+	+
AC7(pin^+pox^-)	L[c]+α–pinene	+	+	+
AC8(pin^+pox^-)	L[c]+α–pinene	-	-	-
AC9(pin^+pox^-)	L[c]+α–pinene	-	-	-
AC10(pin^-pox^-)	L[c]+α–pinene	-	-	-
KS505(pin^-pox^-)	L[c]+α–pinene	-	-	-
KS507(pin^-pox^+)	L[c]+α–pinene	-	-	-
KS509(pin^-pox^-)	L[c]+α–pinene	-	-	-
KS519(pin^+pox^-)	L[c]+α–pinene	+	+	+

a: WT means wild type whole cells or cell extracts.

b: pin or pox (+) or (-) means that these mutants were able or unable to grow on minimal medium containing α–pinene or α–pinene oxide as sole carbon source respectively.

c: L means lactate.

Table 4.3: Electrophoretic assays of α–pinene monooxygenase activity in cell free extracts of wild type and mutant cultures of *Pseudomonas fluorescens* NCIMB 11671.

Sample	Bands			Monooxygenase
	R_f=0.35	R_f=0.43	R_f=0.51	activity
WT_L [a]	+(faint)	+	+	+(low)
WT_{PIN} [b]	+	+	+	+
AC1(pin^-pox^-) [c]	+(faint)	-	+	-
AC2(pin^-pox^-)	+	+(faint)	+	+
AC3(pin^+pox^-)	+	+	+	+
AC5(pin^-pox^+)	+(faint)	-	+	-
AC6(pin^+pox^-)	+	+	+	+
AC7(pin^+pox^-)	+	+	+	+
AC8(pin^+pox^-)	+(faint)	+(faint)	+	-
AC9(pin^+pox^-)	+(faint)	-	+	-
AC10(pin^-pox^-)	-	-	+	-
KS505(pin^-pox^-)	+(faint)	-	+	-
KS507(pin^-pox^+)	+(faint)	-	+	-
KS509(pin^-pox^-)	+(faint)	-	+	-
KS519(pin^+pox^-)	+	-	+	+

a: wild type cells grown on minimal medium with lactate as sole carbon source

b: wild type cells grown in minimal medium with lactate and α–pinene as carbon sources

c: pin or pox (+) or (-) means that these mutants were able or unable to grow on minimal medium containing α–pinene or α–pinene oxide as sole carbon source respectively.

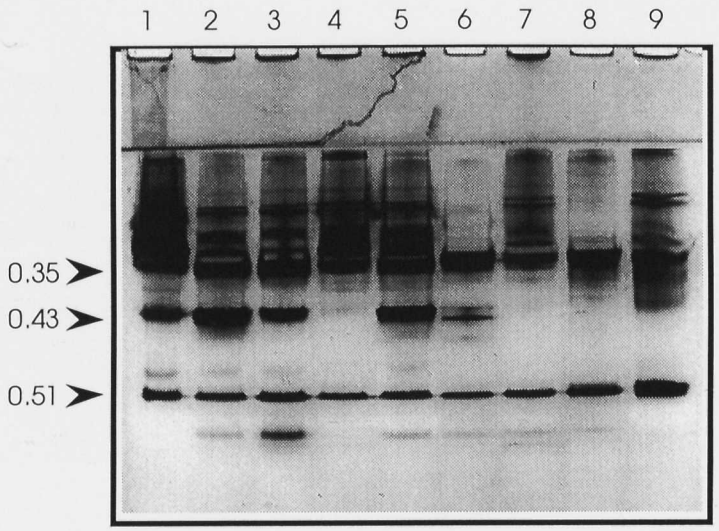


Figure 4.3: Non denaturing PAGE showing NADH oxidoreductase activity stained with 4-nitro blue tetrazolium chloride. Lane 1, WT_L; lane 2, WT_{PIN}; lane 3, WT_{PIN}; lane 4, AC9; lane 5, AC8; lane 6, AC2; lane 7, AC5; lane 8, KS519; lane 9, AC1.

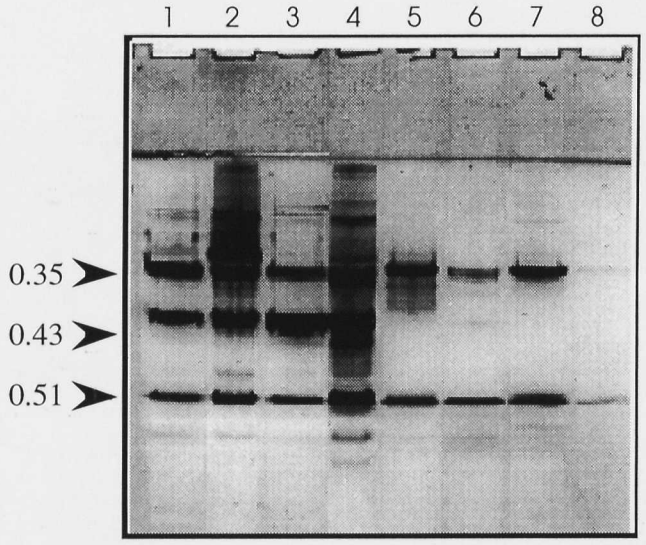


Figure 4.4: Non denaturing PAGE showing NADH oxidoreductase activity stained with 4-nitro blue tetrazolium chloride. Lane 1, AC7; lane 2, AC3; lane 3, AC6; lane 4, WT; lane 5, KS509; lane 6, KS507, lane 7, KS505; lane 9, AC10.

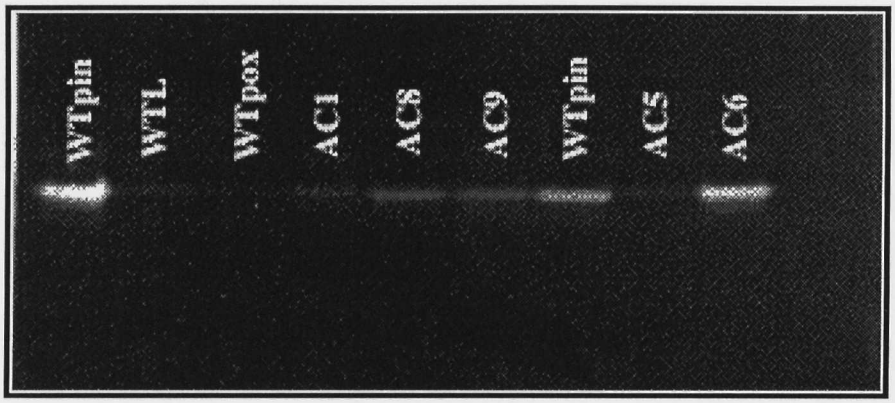


Figure 4.5: Activity stain for α–pinene oxide lyase activity on a non-denaturing gel with cell free extracts from wild type and mutant cultures of *Pseudomonas fluorescens* NCIMB 11671. Lane 1, WT_{PIN}; lane 2, WT_{L}; lane 3, WT_{POX}; lane 4, AC1; lane 5, AC8, lane 6, AC9, lane 7, WT_{PIN}, lane 8, AC5; lane 9, AC6.

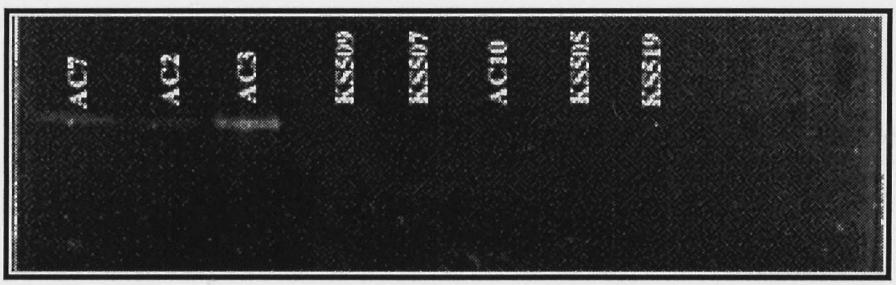


Figure 4.6: Activity stain for α–pinene oxide lyase activity on a non-denaturing gel with cell free extracts from wild type and mutant cultures of *Pseudomonas fluorescens* NCIMB 11671. Lane 1, AC7; lane 2, AC2; lane 3, AC3; lane 4, KS509; lane 5, KS507; lane 6, AC10; lane 7, KS505; lane 8, KS519.

Table 4.4: Monooxygenase, epoxide lyase and aldehyde dehydrogenase activities in cell free extracts of wild type and mutant cultures of *Pseudomonas fluorescens* NCIMB 11671 grown on minimal medium supplied with lactate only, or lactate plus α–pinene or α–pinene oxide as carbon sources.

Sample	Monooxygenase activity	Lyase activity	Aldehyde dehydrogenase activity
WT_L [a]	+(low)	+(low)	+(low)
WT_{PIN} [b]	+	+	+
WT_{POX} [c]	+(low)	+(low)	ND[e]
AC1(pin^-pox^-) [d]	-	+(low)	+
AC2(pin^-pox^-)	+	+	-
AC3(pin^+pox^-)	+	+	+
AC4(pin^-pox^+)	-	+	+
AC5(pin^-pox^+)	-	+(low)	+
AC6(pin^+pox^-)	+	+	-
AC7(pin^+pox^-)	+	+	-
AC8(pin^+pox^-)	-	+	+
AC9(pin^+pox^-)	-	+	-
AC10(pin^-pox^-)	-	-	-
KS505(pin^-pox^-)	-	+(low)	-
KS507(pin^-pox^+)	-	-	-
KS509(pin^-pox^-)	-	-	+
KS519(pin^+pox^-)	+	-	+

a: Extracts from wild type cells grown on minimal medium with lactate as carbon source. **b**: Extracts from wild type cells grown on minimal medium with lactate and α–pinene as carbon source. **c**: Extracts from wild type cells grown on minimal medium with lactate and α–pinene oxide as carbon source. **d**: pin or pox (+) or (-) means that these mutants were able or unable to grow on minimal medium containing α–pinene or α–pinene oxide as sole carbon source respectively. **e**: noT detected

4.5.4 Studies on the α–Pinene Degradation Pathway

Data used for the conceptualisation of metabolic pathways for oxidation of hydrocarbons has been obtained, for the most part, from investigation of whole cells. These studies have taken the form of growing the organism at the expense of the hydrocarbon substrate and then analysing the resulting culture fluid, the cells, or both, by GC, TLC, HPLC, or spectrophotometrically. Alternatively, the metabolic route may be revealed by studying substrate metabolism in washed, non proliferating suspension of organisms (resting cells) (Klug and Markovetz, 1971; Dagley and Chapman, 1981; Bühler and Shindler, 1984; Hartmans *et al.*, 1989; Gibson *et al.*, 1990). A compound which accumulates in a liquid growth medium is not necessarily a reaction intermediate in the metabolic pathway. For example under anaerobic conditions an organism will obtain energy by transferring hydrogen atoms from its growth substrates to other metabolites, and this will give rise to a variety of fermentation products. On the other hand, when the supply of oxygen does not limit growth, any compounds detected in the culture medium are more likely to be those that lie on the metabolic pathway by which the carbon source is oxidised completely (Dagley and Chapman, 1981). However, many oxidation products may be of a transient nature such that they never appear in detectable amounts during oxidation of the substrate (Klug and Markovetz, 1971).

The purpose of this particular study was to characterise the products that accumulated in culture fluids of wild type and mutant *P. fluorescens* and in this way to substantiate the pathway for the degradation of α–pinene proposed from biochemical studies and also to establish any other alternative catabolic pathways present in this bacterium. Thus, wild type and mutant culture supernatants were extracted in dichloromethane previously equilibrated at pH 9.0 and analyzed by GC and GC-MS techniques. All cells were routinely cultured in minimal medium containing 0.3% (v/v) sodium lactate and 0.2% (v/v) α–pinene as carbon sources at 30°C for 24 h. Two samples containing 0.2% (v/v) α–pinene or 0.1% (v/v) α–pinene oxide in the

above medium without any addition of cells were used as controls. Moreover, extracts of culture supernatants of cells grown on minimal medium supplied with lactate as sole carbon source were also used as controls.

Many products were detected after extraction and GC analysis of supernatants from wild type and mutant cultures on minimal medium supplied with α–pinene as carbon source. However, only those that corresponded to the available standards, such as α–pinene, α–pinene oxide, β–pinene, limonene and limonene oxide could be identified by these assays. Therefore, all samples were sent for GC-MS analysis. Mass spectra of these samples revealed the presence of a large number of compounds, most of which were not related to the proposed metabolic pathway. This could be explained by the fact that microbial transformations of terpenoids can involve enzymatic attack at several sites of the molecule leading to mixtures of metabolites. Accumulation of a single transformation product is very rare. After the initial attack a suite of metabolites can be further degraded either completely or partially and this may eventually lead to a cascade of transformation events and sometimes to undesirable partial degradation (Krasnobajew, 1984).

Verbenol, verbenone and sorberol were detected in the control sample (without cells) with α–pinene in minimal medium containing lactate. These compounds were assumed to be autoxidation products of α–pinene. The same compounds have also been suggested as possible autoxidation products of α–pinene by Prema and Battacharyya (1962). Thus, although these products were detected in most of the other samples, they were not considered as metabolites. α–Pinene was always detected in extracts derived from mutant cultures, whereas it was utilised almost completely and therefore, only detected in very small amounts or not at all in wild type extracts. Campholenic aldehyde, *cis*- and *trans*-carveol and sorberol were detected in the control sample (without cells) of α–pinene oxide in minimal medium containing lactate. The same metabolites were also detected by Griffiths *et al.* (1987a) and assumed to be degradation products of the epoxide when the latter is incubated in aqueous buffered solutions at near neutral pH. Small amounts

of α–pinene oxide as well as the metabolites above were detected in cultures of wild type and mutants that expressed monooxygenase activity, such as AC2, AC3, AC6, and AC7. These metabolites were detected in greater overall yield in the extracts of KS519, which is consistent with this mutant lacking epoxide lyase activity. Therefore, all the epoxide made by the action of the monooxygenase would accumulate in the culture supernatants. Myrtenol and myrtenal were detected in almost all the cultures of strains lacking α–pinene monooxygenase activity, such as AC4, AC5, AC9, AC10, KS505, KS509. It is possible that these mutants can utilise a secondary pathway for the degradation of α–pinene. In culture extracts of KS507 mutant, which appears to be a pleiotropic mutant, defective in all three enzyme activities studied, limonene and limonene oxide were detected, suggesting a further pathway for the degradation of α–pinene. Finally in extracts of AC8 and AC1 mutants, which do not express any α–pinene monooxygenase activity, perillyl alcohol and aldehyde were detected, which could result from a hybrid pathway for α–pinene metabolism, incorporating initial limonene production followed by primary allylic hydroxylation and further oxidation as seen in the production of myrtenal. The proposed pathways are presented in figure 4.7.

2-Methyl-5-isopropylhexa-2,5-dienal, the immediate product of the epoxide lyase reaction was not always detected in extracts derived from wild type cells or mutants expressing the monooxygenase and lyase activities. This may be explained by the fact that the formation of carboxylic acids from the corresponding aldehyde is thermodynamically more favourable than the formation of the aldehyde and usually, many difficulties in isolating intermediate aldehydes are encountered (Bühler and Shindler, 1981).

Methylated supernatant extracts were also analyzed for the presence of methyl esters corresponding to any of the acids arising from the main pathway or expected for the secondary pathways. Unfortunately, no acids were detected, which may be partly explained by the observation made by Aikens (1990) that during growth of *P. fluorescens* on α–pinene acid metabolites were produced only during a narrow

growth period where the pH was falling below 7.0. Since the supernatants were collected after 24 H of incubation, when the pH was approximately 8.0, it was not possible to detect any acidic metabolites.

4.6 Discussion

Chemical mutagenesis was carried out in this study in order to isolate mutants defective in the α–pinene degradation pathway and preferably mutants with no α–pinene monooxygenase activity. In order to obtain high mutation rates and minimise double mutations, mutagenesis was performed to give 70% killing with 50 μg NTG per ml of cells. Approximately 6,000 colonies were screened for their ability to grow on α–pinene or α–pinene oxide and finally ten mutants with very low reversion frequencies (10^{-7} to 10^{-11} per viable cell) were isolated. Another four mutants were kindly provided by Dr K. Saqib. These mutants were divided into three categories: unable to grow on α–pinene but able to grow on α–pinene oxide (pin^-pox^+ mutants); able to grow on α–pinene but unable to grow on α–pinene oxide (pin^+pox^- mutants); unable to grow on either substrate (pin^-pox^- mutants). The first group (pin^-pox^+ mutants) consisted of three mutants, AC4, AC5 and KS507 that did not show any α–pinene monooxygenase activity by any of the methods employed. Therefore, in the context of this project, they were the most important group of mutants since they could be used for complementation studies to isolate the monooxygenase gene. The second group consisted (pin^+pox^- mutants) of six mutants, AC3, AC6, AC7, AC8, AC9 and KS519. The existence of such mutants suggests that there are other pathways for the degradation of α–pinene in this bacterium which do not involve formation of α–pinene oxide. The third group (pin^-pox^- mutants) consisted of five mutants, AC1, AC2, AC10 KS505 and KS509 that were unable to grow on either substrate. It was assumed that this phenotype was either the result of a double mutation or the result of a nonsense mutation exerting polar effects on the downstream genes, e.g. because it causes release of the

ribosome from the mRNA molecule, thus, preventing translation of the subsequent polypeptides on the same mRNA (Birge, 1991).

All mutants were characterised not only for their ability to grow on the first two substrates of the α–pinene degradation pathway, but also according to their activities of the first three enzymes suggested for the pathway, namely α–pinene monooxygenase, epoxide lyase and aldehyde dehydrogenase. Mutants AC1, AC4, AC5 and AC8 appeared to be defective only in the monooxygenase activity. AC1 was unable to grow on both α–pinene or α–pinene oxide and thus, although it had lyase and aldehyde dehydrogenase activities, it may possess a second mutation in a later pathway gene. Alternatively it may have lost the ability for exogenous α–pinene oxide to induce the genes necessary for its metabolism while α–pinene still induces the pathway and if this is the case, it may be a regulatory mutant. In addition, AC8, with a phenotype of pin^+pox^- appeared to utilise an alternative pathway for the degradation of α–pinene, and therefore, may also be a double mutant. Mutants KS519 was the only mutant defective in the lyase activity, while mutants AC2, AC6 and AC7 appeared not to express the dehydrogenase activity. Mutants KS509, KS505, and AC9 appeared to be double mutants since they did not express the monooxygenase and lyase (KS509), or monooxygenase and dehydrogenase (KS505, AC9) activities. On the other hand, mutants AC10 and KS507 appeared to have pleitropic mutations since they lacked all three activities. These could result from regulatory or polar nonsense mutations that would result in the entire operon being switched off. Finally, AC3 seems to have a mutation further down the pathway.

Cells grown on lactate or on α–pinene oxide showed much lower activities of all three enzymes studied suggesting that α–pinene is the primary inducer of the genes encoding for the enzymes responsible for its degradation in *P. fluorescens*. Growth on α–pinene oxide is presumed to result from the use of pathway enzymes operating with their background (uninduced) level of activity.

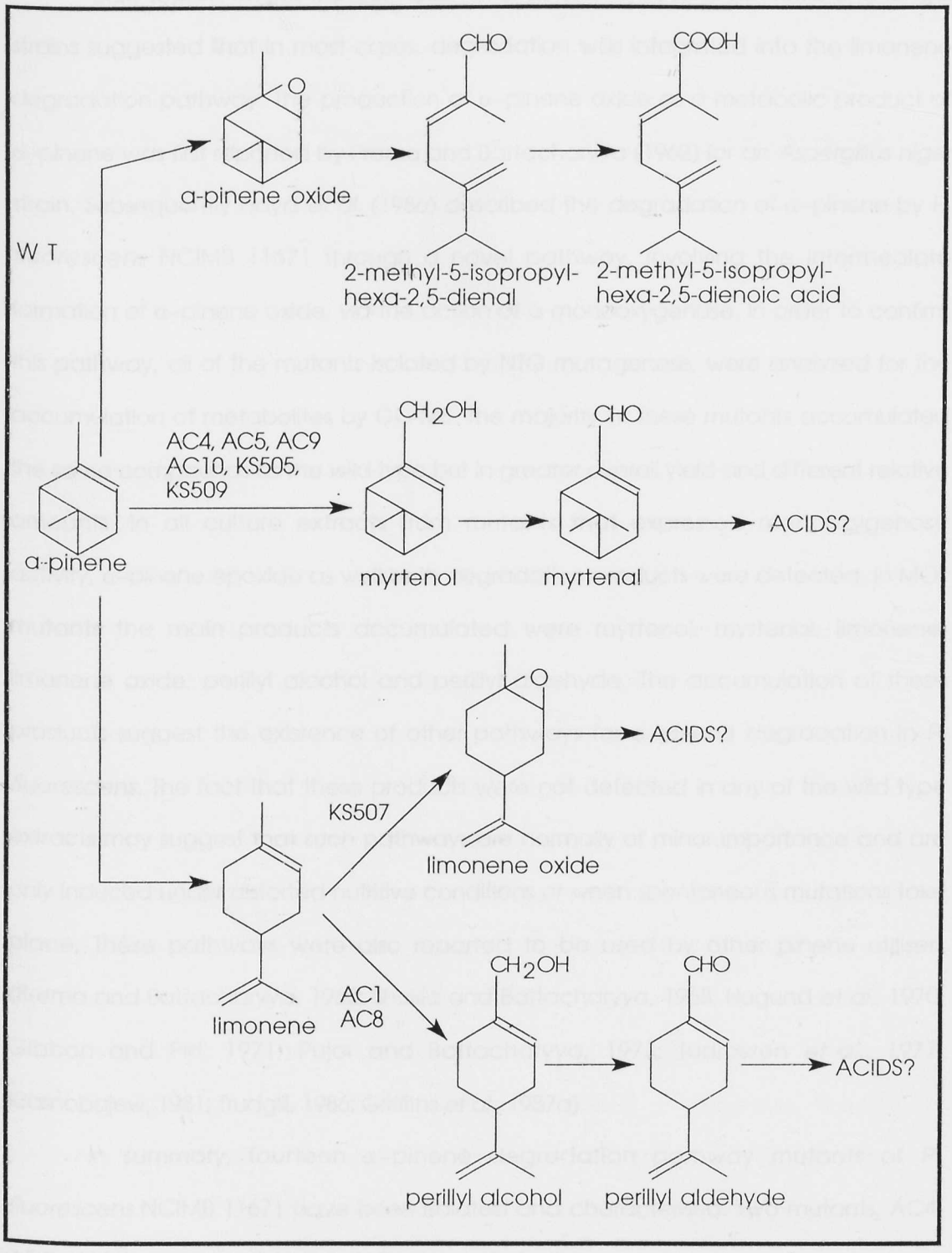


Figure 4.7: Proposed pathways for the degradation of α–pinene by *Pseudomonas fluorescens* NCIMB 11671, according to the products detected in extracts of wild type and mutant culture supernatants.

Early reports on the transformation of α–pinene by several bacterial and fungal strains suggested that in most cases, degradation was integrated into the limonene degradation pathway. The production of α–pinene oxide as a metabolic product of α–pinene was first reported by Prema and Battacharyya (1962) for an *Aspergillus niger* strain. Subsequently Floyd *et al.* (1986) described the degradation of α–pinene by *P. fluorescens* NCIMB 11671 through a novel pathway, involving the intermediate formation of α–pinene oxide, via the action of a monooxygenase. In order to confirm this pathway, all of the mutants isolated by NTG mutagenesis, were analyzed for the accumulation of metabolites by GC-MS. The majority of these mutants accumulated the same compounds as the wild type but in greater overall yield and different relative amounts. In all culture extracts from mutants that expressed monooxygenase activity, α–pinene epoxide as well as its degradation products were detected. In MO^- mutants the main products accumulated were myrtenol, myrtenal, limonene, limonene oxide, perillyl alcohol and perillyl aldehyde. The accumulation of these products suggest the existence of other pathways for α–pinene degradation in *P. fluorescens*. The fact that these products were not detected in any of the wild type extracts may suggest that such pathways are normally of minor importance and are only induced under distorted nutritive conditions or when spontaneous mutations take place. These pathways were also reported to be used by other pinene utilisers (Prema and Battacharyya, 1962; Shukla and Battacharyya, 1968; Hugund *et al.*, 1970; Gibbon and Pirt, 1971; Pujar and Battacharyya, 1973; Tudroszen *et al.*, 1977; Krasnobajew, 1981; Trudgill, 1986; Griffiths *et al.*, 1987a).

In summary, fourteen α–pinene degradation pathway mutants of *P. fluorescens* NCIMB 11671 have been isolated and characterised. Two mutants, AC4, AC5, which appeared to be defective only in the monooxygenase enzyme were used in all subsequent complementation studies for the isolation of the gene which encodes this enzyme.

CHAPTER 5

Complementation Assays between Mutants of *Pseudomonas fluorescens* NCIMB 11671 and its Genomic Library

5.1 Introduction

Although there are many different procedures by which a desired clone can be obtained, they are all variations of two basic strategies: The first involves direct selection for the cloned gene. This means that the cloning experiment is designed in such a way that the only clones obtained are those of the required gene. Almost invariably, selection occurs at the plating-out stage. In general terms direct selection is the preferred method as it is quick and usually unambiguous. However, it is not applicable to all genes (Brown, 1986, 1991). The second strategy involves the identification of the desired clone from a gene library. This procedure entails an initial "shotgun" cloning experiment to produce a clone library ideally representing all of the genes present in the cell, followed by analysis of the individual clones to identify the clone containing the gene of interest. The simplest approach is to directly identify the recombinant DNA molecule. This can be achieved by hybridisation and probing (Lewin, 1990). Another approach is the manipulation of expression libraries by screening with antibodies against a specific antigen. The latter technique is based on the antigen-antibody reaction, and either polyclonal or monoclonal antibodies (Davis and Botstein, 1980) can be used. A third way for the selection of the desired gene can be achieved by detection of the enzymatic activity of its translation product (Brown, 1986, 1991). A procedure based on the latter is conjugal transfer of library clones to mutants lacking the function of the desired gene and detection of wild type phenotype in minimal media. This is commonly known as complementation. For

complementation studies in gram negative bacteria, many of the vectors used are self-transmissible, or can be mobilised from *E. coli* to the gram negative host in *trans*, by triparental mating, e.g. using the plasmid pRK2013 which carries the RP4 *tra* genes (Ditta *et al.*, 1985). Complementation procedures are commonly employed to isolate library clones containing the gene of interest when other detection methods are not applicable (Figurski and Helinski, 1979; Ditta *et al.*, 1980; Harayama *et al.*, 1986; Zylstra *et al.*, 1988; Janssen *et al.*, 1989; Rothmel *et al.*, 1991).

A genomic library of *P. fluorescens* in *E. coli* strain HB101 using the broad host range cosmid pJRD215 was kindly provided by Dr K. Saqib. Moreover, after complementation studies, Saqib (1992) had isolated a recombinant clone pKS73 that complemented the KS505, KS507, and KS509 mutants that were unable to grow on α–pinene. Restriction digestion of this clone with *Hind* III produced three fragments of 1.7, 10.0, and 17.0 kb and the 1.7 kb fragment had been subcloned by Saqib (1992) into the broad-host range cosmid pCP13 to obtain plasmid pKS725. Complementation assays with the latter plasmid and the mutants revealed that it still complemented the pin^- pox^+ mutants but not the pin^-pox^- ones. Although pKS725 could be used for complementation analysis it was not suitable for restriction analysis, or expression and sequencing studies, because the DNA yield from this vector was insufficient. Therefore, the 1.7 kb fragment had been subcloned into pUC18 to produce clone pKS809 that was used for restriction digestion analyses (Saqib, 1992). Moreover, the 1.7 kb fragment had been subcloned into the broad host range expression vector pMMB66 to generate clone pKS902.

Complementation experiments were, therefore, performed between the genomic library of *P. fluorescens* and all the mutants isolated. In addition clone pKS902 was also used. Both methods described in Material and Methods, namely, culture matings and plate matings were employed, although culture matings appeared to be more successful and were used preferentially throughout this study.

5.2 Complementation Studies with the Genomic Library in pJRD215

Recombinant clones in the broad host range cosmid vector pJRD215 should contain inserts with an average insert size of 40 kb (Davison, 1987). According to the mathematical model N = ln (1-p) / (1-f) (Frischauf, 1987) a total of 500 individual recombinant clones, containing 40 kb inserts, should be representative of the complete genome of *P. fluorescens*, assuming a genome size of 4,000 kb. Thus, this number of HB101 colonies, each containing a unique recombinant clone was used in complementation assays. Library pools, each containing 100 clones, 50 clones, and 25 clones, were constructed as described and used to complement the mutants of *P. fluorescens*. For the identification of complementing clones within a library pool, the required number of pools (glycerol stocks) was thawed and 20 µl from each pool was inoculated in 5 ml LB broth containing 25 µg/ml kanamycin and streptomycin. Similarly, *E. coli* HB101(pRK2013) was inoculated in 5 ml LB broth containing 25 µg/ml kanamycin. Both cultures were incubated at 37°C overnight in an orbital shaker. Monooxygenase minus mutants, AC1, AC4, AC5, AC8, AC9, AC10, KS505, KS507, and KS509, were also inoculated in LB broth and incubated with shaking at 30°C overnight. All cultures were pelleted by centrifugation, washed twice with 5 ml of 50 mM phosphate buffer, pH 7.6 to remove any traces of antibiotics and then resuspended in the same buffer to an optical density at 600 nm of 0.5.

Recombinant vector transfer was achieved by triparental mating and double selection conditions for the detection of complementing transformants were applied. Several ratios of donor (D) (HB101(pJRD215)), helper (H) (HB101(pRK2013)) and recipient cells (R) (mutant), were used, in order to establish the optimum conditions for high conjugation efficiency. This conjugation efficiency was calculated using the following formula:

$$\text{Conjugation frequency} = \frac{\text{Conjugants (Km}_+\text{)}}{\text{Recipients}}$$

It was shown, that a cell ratio of 1D:1H:1R routinely resulted in high conjugation frequencies, and thus, these ratios were used in all subsequent complementation procedures. After overnight incubation, the mated cultures were streaked on both minimal plates containing kanamycin that were incubated under α–pinene vapour, and *Pseudomonas* isolation agar plates (Difco) containing kanamycin, which were used to calculate the conjugation efficiency. *P. fluorescens* NCIMB 11671 is very sensitive to kanamycin (Aikens, 1990; this study), and therefore, all mutants able to grow on the latter plates should have acquired the plasmid. Furthermore, transconjugants that could grow on the plates with both selection markers (i.e., antibiotic and α–pinene), should have been complemented by the DNA cloned into the acquired plasmid. However, in order to confirm the acquisition of the recombinant plasmid, plasmids were isolated from the α–pinene grown mutants by the alkaline lysis method (Sambrook *et al.*, 1989). An example of this is shown in figure 5.1. The presence of multiple bands results from different DNA configurations (Sambrook *et al.*, 1989).

Table 5.1 shows the results obtained using pools 1-5. After the first assays using the large library pools, complementation was repeated using subpools and finally individual clones in order to identify the ones which complemented the monooxygenase minus (MO^-) *P. fluorescens* mutants. It is clear from table 5.1 that pool 1 complemented all of the mutants, including those lacking α–pinene monooxygenase activity. Further complementation assays with subpools and finally individual clones revealed that only one clone, pKS73 complemented all of the latter mutants.

The complementation of pin^-pox^+ and pin^-pox^- mutants by a single clone suggests that the genes involved in the degradation of α–pinene could be contiguous, possibly arranged in an operon where a set of structural genes is transcribed as a single transcriptional unit from a common promoter. On the other hand, pKS902 showed complementation to pin^-pox^+ mutants but not pin^-pox^- mutants and this is in agreement with the results obtained by Saqib (1992). However, in all complementation procedures carried out using pKS902, while the transformation

efficiency was high, and this is in accordance with the results obtained by other researchers for the same mobilisable system (Figurski and Helinski, 1979; Ditta *et al.*, 1980; Davison *et al.*, 1990), the complementation efficiency was very low. This result suggests that complementation was the result of recombination rather than complementation in *trans* that would result from the production of a complete polypeptide from the recombinant plasmid.

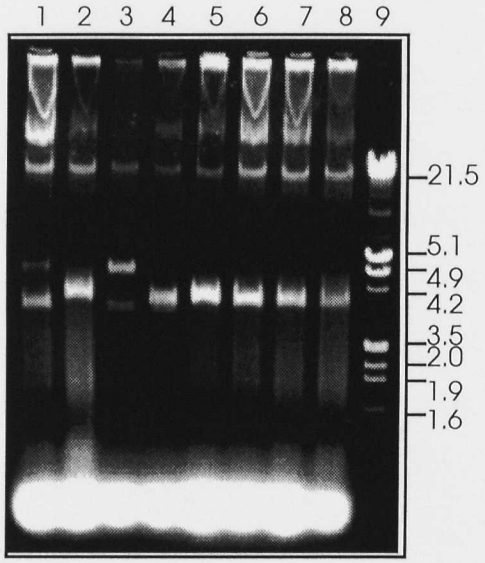


Figure 5.1: Isolation of plasmids from transformed mutants of *Pseudomonas fluorescens* NCIMB 11671 that showed growth on minimal plates containing 25 µg/ml kanamycin and placed under α–pinene vapour as sole carbon source. Lane 1, KS507(pool 1); lane 2, KS509(pool 1); lane 3, AC1(pool 1); lane 4, AC9(pool 1); lane 5, AC4(pool 1); lane 6, AC10(pool 1); lane 7, AC8(pool 1); lane 8, AC5(pool 1); lane 9, λ (*Hind III-EcoRI*) DNA marker.

Based on the observation that pKS73 had an insert of only 19 kb (Saqib, 1992) instead of 40 kb, and also the low transformation and complementation efficiencies obtained from complementation assays with all library pools as well as individual

clones, it was considered worthwhile to check the integrity of the genomic library. Recombinant plasmids were isolated from 200 randomly selected library clones and restricted with *Bam*H I, *Bgl* II, *Hin*d III, *Eco*R I and combinations of them. Restriction digestions were carried out using 3 units of enzyme per µg of cosmid DNA and incubated at 37°C for 3 h. The reaction products were electrophoresed on a 0.6% (w/v) TBE/agarose gel. The sizes of the restricted recombinant clones were compared to the sizes of a High Molecular Weight marker, and a 1 kb DNA ladder (Gibco, BRL). These experiments showed that 40% of the clones had lost their inserts, while the rest of the clones contained inserts of 20 kb or less, instead of the expected 40 kb insert. This high loss of inserts may be due to the fact that during construction of cosmid libraries a common problem is the packaging of polycosmid formations. Even with sized chromosomal DNA fragments, cosmids can be recovered that contain non-contiguous DNA fragments ligated to form a single insert that is usually smaller than it should be, mainly because of vector to vector ligation. This kind of inserts leads to a distorted view of the original DNA organisation (Ish-Horowitz and Burke, 1981). Moreover, pJRD215 was found to be a fairly unstable vector (P. Brown, personal communication). Consequently, this genomic library was considered not to be representative of the *P. fluorescens* genome, and thus, the construction of a new library was necessary.

5.3 Discussion

One of the commonly encountered problems during the construction of a cosmid library is that, even when size-fractionated chromosomal DNA is used, cosmids can be recovered that contain non-contiguous DNA fragments ligated to form a single insert. Although the problem can be partially overcome by dephosphorylating the inserted (target) DNA, this method is very sensitive to the exact ratio of target and vector DNAs and tends to give smaller non-contiguous inserts

because of vector-to-vector ligation. Ish-Horowitz and Burke (1981) proposed that when vectors that contain one *cos* site are used, this problem can be overcome by using a combination of dephosphorylation of the size fractionated target DNA with multiple enzyme digestion procedures involving four separate restriction enzyme digestion steps and two S_1 nuclease treatments. However, in this case, although Saqib (1992) had used size fractionated but non-dephosphorylated chromosomal DNA for the construction of his library, the one *cos* site vector pJRD215 was restricted only with *Bam*H I and dephosphorylated. Therefore, from the arguments above, the presence of smaller inserts may suggest rearrangement of the insert DNA (Ish-Horowitz and Burke, 1981; Bates and Swift, 1983). Furthermore, the high percentage of clones with no inserts may be explained by both the formation of vector concatemers and instability of pJRD215. Such multiple vector formation would also lead to the presence of more than one origin of replication per bacterial cell which also might have increased the plasmid instability (Old and Primrose, 1989; Lewin, 1990). Thus, although fifteen clones from the library were found to complement the various mutants isolated, these were strong arguments against proceeding with detailed mapping and subcloning based on a defective gene library.

Table 5.1: Results from complementation assays between mutants of *Pseudomonas fluorescens* NCIMB 11671 and the genomic library pools (each containing 100 clones).

Mutant	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
AC1 (pin^-pox^-)	+	-	-	+	-
AC4 (pin^-pox^+)	+	-	-	-	+
AC5 (pin^-pox^+)	+	+	+	+	-
AC8 (pin^+pox^-)	+	+	+	+	-
AC9 (pin^+pox^-)	+	+	+	-	+
AC10 (pin^-pox^-)	+	+	-	-	-
KS505 (pin^-pox^-)	+	+	-	+	-
KS507 (pin^-pox^+)	+	-	-	+	-
KS509 (pin^-pox^-)	+	-	-	-	+

From all the clones that complemented the pin^-pox^+ and pin^-pox^- mutants, only recombinant plasmid pKS73 complemented both phenotypes. Assuming that

this clone contains a contiguous sequence, this multiple complementation pattern of pKS73 suggests that the genes encoding α–pinene catabolism may be clustered in an operon. Such an organisation is very common in the *Pseudomonas* genome (Haas, 1983; Holloway, 1986a, b; Holloway, 1990a, b; Drlica and Riley, 1990; Gibson *et al.*, 1990; Rothmel *et al.*, 1991). If this was the case, it would significantly simplify the cloning and analysis of genes involved in α–pinene metabolism. The organisation of these genes into an operon would allow the expression to be coordinately induced or repressed as has been observed in this case, where the low level of constitutive expression of all the enzymes was induced to higher level, specifically by the substrate, α–pinene. Catabolite repression has also been shown for this system, when succinate or pyruvate were used as carbon cosubstrates (Chapter 3).

Finally, when pKS902 was used in complementation assays the transformation efficiency was high, but the complementation efficiency was quite low. If the 1.7 kb insert was expressing the whole monooxygenase enzyme, or even a complete polypeptide, then, conjugant transfer of the plasmid in mutants should result in the wild type phenotype with both transformation and complementation efficiencies being equally high. On the other hand, if the 1.7 kb insert only encoded part of the relevant monooxygenase gene, then the wild type phenotype would be produced only upon homologous recombination between the plasmid insert and the mutated chromosomal DNA (Saunders and Saunders, 1987; Lewin, 1990) which is possible since *P. fluorescens* NCIMB 11671 is a rec$^+$ strain.

CHAPTER 6

Expression of the 1.7 kb Insert of pKS902 in *Pseudomonas fluorescens* and *Escherichia coli*

6.1 Introduction

The biosynthesis of most enzymes is under strict regulatory control so that they are produced in the correct quantities for the optimum viability of the cells. The "housekeeping enzymes", which are involved in metabolic routes such as glycolysis, are produced in large amounts, whereas others are often produced only in small quantities. Consequently, much of the early enzymological studies concentrated upon the easily accessible metabolic enzymes. α–Pinene monooxygenase appears to be one of these enzymes with very low levels of expression, since it constitutes only 0.5% of the soluble cell proteins (Chapter 8). However, recombinant DNA methodology now offers the opportunity of overexpressing these genes from constructs made in *E. coli*. Once the gene is cloned in an expression vector, the gene product may be produced in *E. coli* in a constitutive or inducible manner and thus, the expressed protein can be purified in large quantities to allow biochemical studies. Moreover, sequencing of the cloned gene may give information about its structure and regulatory regions.

For cloning of *Pseudomonas* genes, either the *E. coli* or the *Pseudomonas* host-vector system can be utilised. The *E. coli* cloning system has many advantages: the genetic and functional properties of the host bacteria are well defined, experimental conditions such as those for the isolation of the plasmids and their introduction into the host cells are well established; a variety of well established vectors are available. In addition, the cloned segment on a plasmid is unlikely to be

incorporated into the *E. coli* chromosome by homologous recombination. On the other hand, there are some disadvantages. The major disadvantage is that expression of many *Pseudomonas* genes in *E. coli* is restricted, mainly because transcription from *Pseudomonas* promoters by *E. coli* RNA polymerase is not efficient (Nakazawa and Inouye, 1986; Davison, 1990).

Several vectors are available for the expression of cloned genes in *E. coli*, and they mainly use strong inducible promoters. Four different, strong promoters whose activities can be manipulated by the investigator, namely the promoters of the tryptophan operon, P_{trp}, the lactose operon, P_{lac}, the hybrid (trp-lac) promoter, P_{tac}, and the leftward promoter P_L of bacteriophage λ, have been incorporated into vector molecules close to one or more cloning sites to produce vectors that, under appropriate experimental conditions, provide a high level of expression of genes on cloned DNA fragments (Lewin, 1990; Old and Primrose, 1989).

6.2 Expression of the 1.7 kb Insert of pKS902 in Wild Type and Mutant Cells of *Pseudomonas fluorescens* NCIMB 11671

pMMB66 specifically directs controllable gene expression in a wide range of Gram-negative bacteria (Franklin and Spooner, 1989; Morales *et al.*, 1990; Rothmel *et al.*, 1991). This plasmid contains a polylinker cloning site downstream of the P_{tac} promoter. Expression of cloned sequences is controlled by a *lac* repressor synthesised by the *lac* I^q gene present on the vector. This plasmid contains the origin of vegetative replication and origin of transfer of BHR plasmid R300B, enabling it to be mobilised by IncP or Inc1a helper plasmids. The 1.7 kb fragment which complemented all the pin$^-$pox$^+$ mutants was subcloned into this vector by Saqib (1992) to produce pKS902.

It has been shown that pin$^-$pox$^+$ mutants AC4, AC5 and KS507 were complemented by pKS902 albeit at low frequencies compared with the conjugation

frequency. Therefore, expression of the construct was examined. Wild type cells as well as mutants KS507 and AC5 were transformed with pKS902 by triparental mating and the transconjugants were selected for growth in minimal medium plates containing 100 µg/ml ampicillin with α–pinene vapour supplied as sole carbon source. The presence of plasmids in the resulting colonies was confirmed by alkaline lysis and agarose gel electrophoresis (Sambrook *et al.*, 1989). Single transconjugants were grown overnight in minimal medium, containing ampicillin, with lactate and α–pinene as carbon sources, in a 30°C shaking incubator. The next day a 1% inoculum was used to inoculate fresh minimal medium with 100 µg/ml ampicillin, where the above carbon sources were also added, and induction of protein synthesis using the P_{tac}-lacI system was carried out as described. Three samples were prepared for each transformant: non induced; induced with 1 mM IPTG and incubated for a further 4 h; and induced with IPTG and incubated for a further 15 h. High speed supernatants were prepared for all samples and were assayed spectrophotometrically for any α–pinene stimulated NADH oxidation activity and electrophoresed under non denaturing and SDS electrophoresis conditions.

Spectrophotometric assays confirmed that extracts from the transconjugants expressed monooxygenase activity in contrast to extracts from mutants. Induction with IPTG had no effect on the activity obtained. Native gels were stained with the NADH oxidoreductase activity stain and SDS gels were silver stained. In both gels extracts from α–pinene and lactate grown cells that were not transformed with pKS902 were used as control.

The activity stained native gels are shown in figures 6.1 and 6.2. Densitometric analysis revealed the presence of a band at around 150 kDa (arrowed), which could correspond to the α–pinene monooxygenase in all wild type samples, induced, non-induced and non conjugated, with the same intensity. In contrast, in mutant samples KS507 and AC5, the same band occupied 1/10 and 1/4 of the previous area respectively, whereas wild type level intensity was obtained in all the IPTG induced and non induced transconjugants.

SDS PAGE (T=9%) showed (figures 6.3 and 6.4) that a band with molecular mass of approximately 70,000 appeared in all wild type samples and mutant transconjugants, but not in non conjugated mutants. Densitometric analysis of the gels revealed that the only difference in the polypeptide content between conjugated (with monooxygenase activity) and non conjugated mutants was the above band, and therefore, it was speculated that this polypeptide should be related to the monooxygenase activity. The above band could not be detected in SDS PAGE gels of samples that had undergone induction treatment in LB broth.

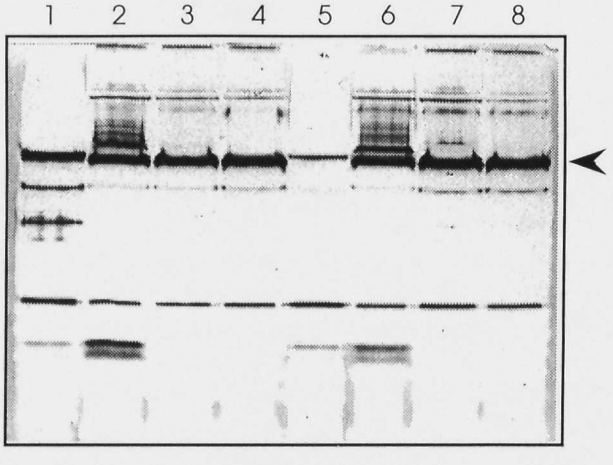


Figure 6.1: Effect of IPTG induction on expression of α–pinene monooxygenase from pKS902, analysed by activity stained native PAGE. Lane 1, wild type (WT); lane 2, WT(pKS902) with no induction; lane 3; WT(pKS902) with 4 h induction; lane 4, WT(pKS902) with 15 h induction; lane 5, KS507; lane 6, KS507(pKS902) with no induction; lane 7, KS507(pKS902) with 4 h induction; lane 8, KS507(pKS902) with 15 h induction.

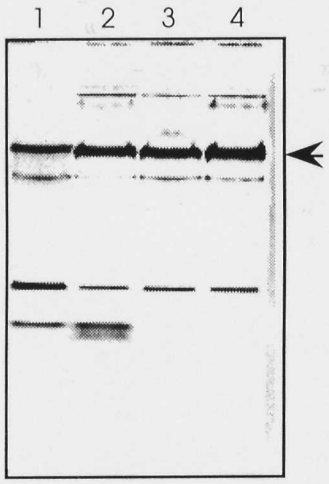


Figure 6.2: Effect of IPTG induction on expression of α–pinene monooxygenase from pKS902, analysed by activity stained native PAGE. Lane 1, AC5; lane 2, AC5(pKS902) with no induction; lane 3; AC5(pKS902) with 4 h induction; lane 4, AC5(pKS902) with 15 h induction.

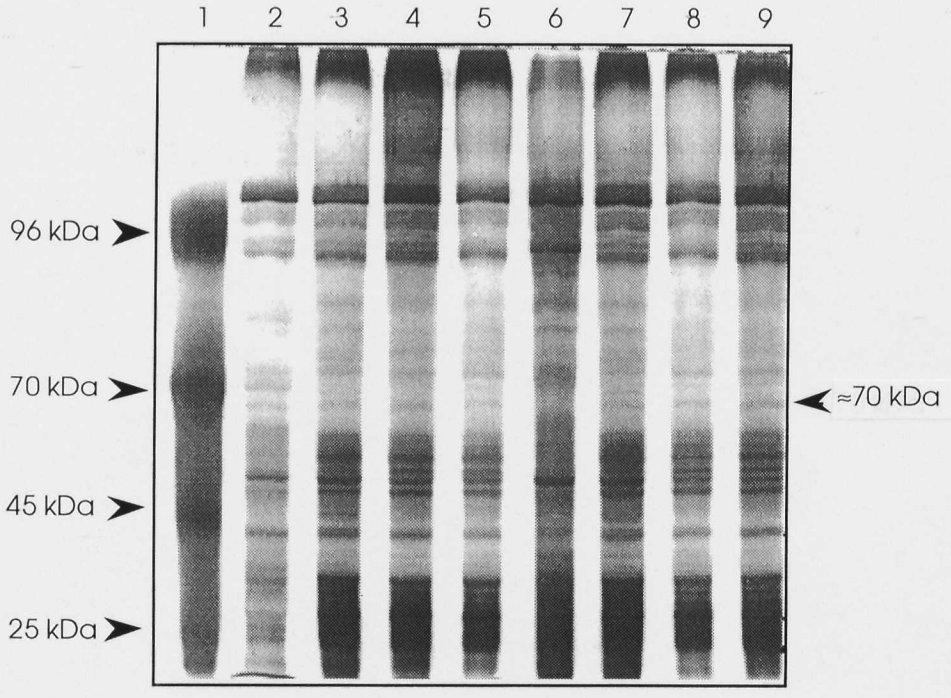


Figure 6.3: Effect of IPTG induction on expression of α–pinene monooxygenase from pKS902, analysed by SDS PAGE. Lane 1, molecular weight markers; lane 2, wild type (WT); lane 3, WT(pKS902) with no induction; lane 4; WT(pKS902) with 4 h induction; lane 5, WT(pKS902) with 15 h induction; lane 6, KS507; lane 7, KS507(pKS902) with no induction; lane 8, KS507(pKS902) with 4 h induction; lane 9, KS507(pKS902) with 15 h induction.
The band with molecular weight of approximately 70 kDa appeared in all wild type samples and mutant transconjugants.

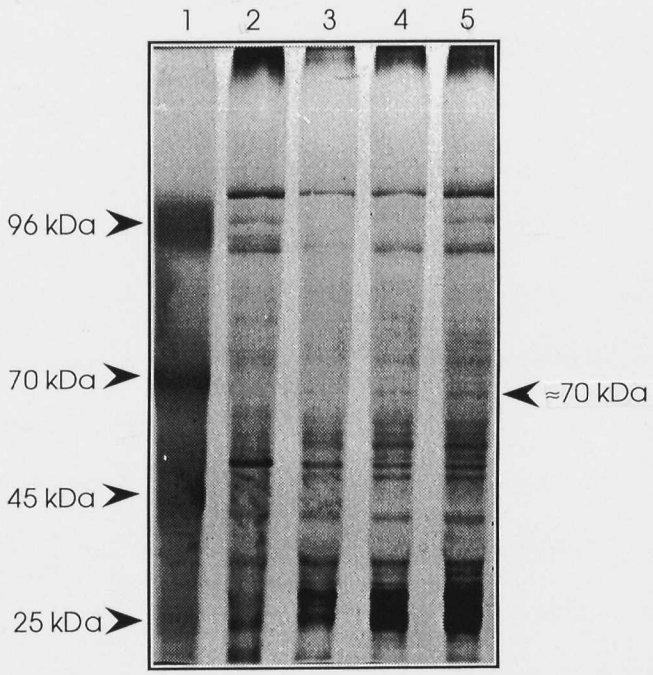


Figure 6.4: Effect of IPTG induction on expression of α–pinene monooxygenase from pKS902, analysed by SDS PAGE. Lane 1, molecular weight markers; lane 2, AC5; lane 3, AC5(pKS902) with no induction; lane 4; AC5(pKS902) with 4 h induction; lane 5, AC5(pKS902) with 15 h induction.
The band with molecular weight of approximately 70 kDa appeared in all mutant transconjugants.

6.3 Expression of the 1.7 kb Insert of pKS902 in *Escherichia coli* Strain HB101

E. coli HB101 cells harbouring the recombinant plasmid pKS902 were also IPTG induced. The cells were induced at an OD at 600 nm of 0.55 and harvested 15 h post-induction. Cell free extracts were prepared as described and assayed spectrophotometrically for α–pinene stimulated NADH oxidation activity and analysed by non denaturing PAGE as above. *E. coli* cells harbouring non recombinant pMMB66 were used as a control.

The activity stained gel in figure 6.5 demonstrates that both IPTG induced and non induced cells harbouring the recombinant plasmid expressed NADH oxidoreductase activity. The band of interest is marked with an arrow. The activity was absent in cells harbouring non recombinant plasmid. Moreover, the activity appears to be the same in both induced and non induced samples.

Spectrophotometric assays for NADH oxidation demonstrated that extracts from cells containing the recombinant plasmid had significant activity while low activity was detected in extracts from HB101(pMMB66) controls. Addition of α–pinene to the assay mixture had no effect in this activity. The results obtained are shown in figure 6.6. However, GC assays failed to reveal epoxide formation with any of the extracts supplied with α–pinene. These results are substantial proof that the 1.7 kb insert of the pKS902 encodes a part of the α–pinene monooxygenase. However, it is unclear at this stage, whether the lack of product formation in "active extracts" implies the requirement for additional polypeptides, typical of multicomponent monooxygenases, or results from the translation of a single α–pinene monooxygenase gene.

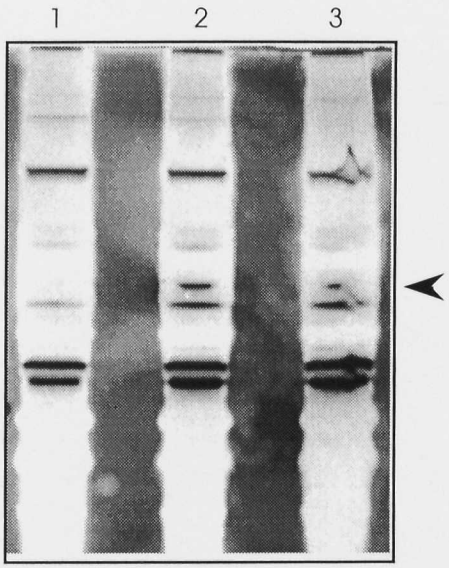


Figure 6.5: Native PAGE of IPTG induced *E. coli* HB101 harbouring recombinant (pKS902), or non recombinant (pMMB66) plasmid. The gel was activity stained. Lane 1, HB101(pMMB66); lane 2, HB101(pKS902) with no induction; lane 3, HB101(pKS902) with 15 h induction.

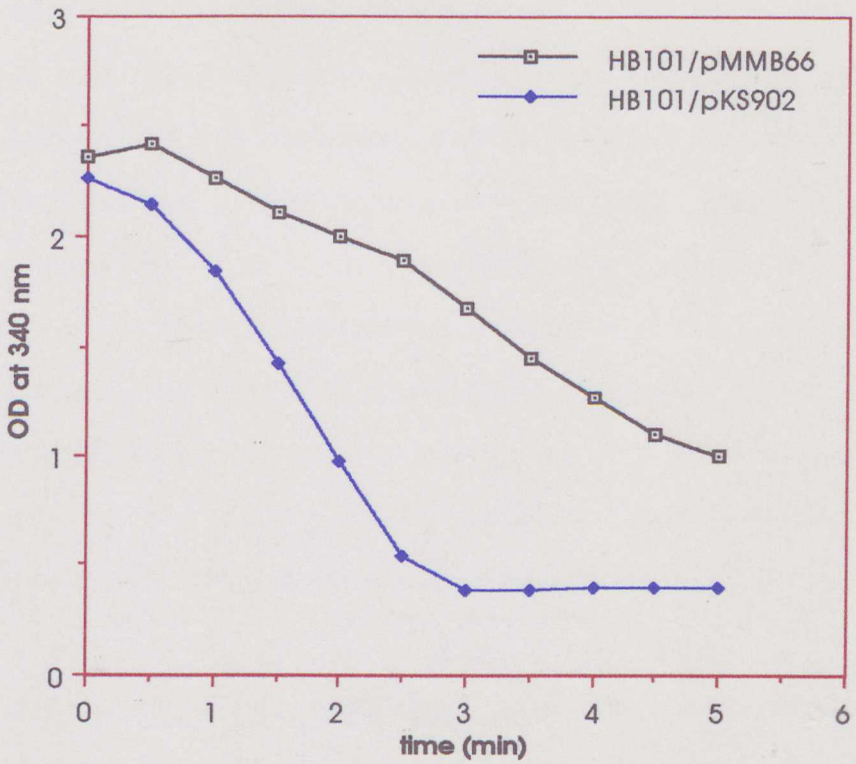


Figure 6.6: NADH oxidation rates of extracts from *E. coli* HB101 harbouring recombinant (pKS902) or non recombinant (pMMB66) plasmid.

6.4 Subcloning of the 1.7 kb Fragment in pTZ19U

The plasmid vector pMMB66 used for the construction of pKS902 is a broad-host range vector and thus, very useful for complementation experiments, and for expression experiments in strains other than *E. coli*. However, pMMB66 is a large, low copy number plasmid (Morales *et al.*, 1990), which could cause difficulties in the detection of protein expression. Furthermore, this vector was not ideal for sequence analysis of the insert. Therefore, the 1.7 kb fragment was transferred to the pTZ series of plasmids. pTZ18 and pTZ19 vectors are multifunctional phagemids, specially designed to permit cloning, DNA sequencing, *in vitro* mutagenesis and transcription in one system. Each of these vectors contains both the pBR322 and f1 origins of replication for generation of double-stranded DNA or large amounts of single stranded DNA upon superinfection with the helper phage M13KO7, respectively. The *lacZ* gene and multiple cloning site (MCS) from pUC18 and pUC19 respectively, are available to facilitate cloning and blue/white screening of recombinants on X-gal plates. The phagemids also carry the highly specific T_7 promoter immediately adjacent to the MCS for *in vitro* synthesis of large amounts of specific mRNAs (Mead *et al.*, 1986).

Plasmid pTZ19U obtained from the Departmental Vector Library, was isolated using a QIAGEN® midi column. 20 µg of this DNA were digested with *Hind* III, the efficiency of digestion checked on a 0.8% (w/v) agarose gel and the digested plasmid dephosphorylated with alkaline phosphatase. The phosphatase was inactivated by heating and the dephosphorylated vector extracted with phenol:chloroform, precipitated with ethanol and resuspended in 200 µl of sterile milli Q water. 10 µl aliquots of the suspension were stored at -20°C until needed. Recombinant plasmid pKS902 was used for the isolation of the 1.7 kb fragment. A maxi DNA preparation followed by purification of the plasmid DNA with PEG was used for the isolation of pKS902 DNA (Sambrook *et al.*, 1989). 40 µg of this DNA was digested with *Hind* III as described previously, and a twentieth of the reaction volume was checked

for complete digestion on a 0.8% (w/v) agarose gel. The rest of the reaction mixture was loaded on a 0.7% (w/v) low melting point agarose gel and electrophoresed at 4°C at 30 V/cm overnight. The 1.7 kb band was then recovered from the gel as described in Materials and Methods and the amount of DNA obtained was estimated by the gel spot method (Sambrook *et al.*, 1989) to be approximately 1.5 µg.

The linearised phosphatased vector pTZ19U and 1.7 kb *Hind* III fragment from pKS902 were ligated overnight at 16°C, in 1:1, 1:3 and 3:1 vector to insert molar ratios, in a total volume of 10 µl to obtain pAC1. Two µl aliquots of these ligation mixtures were used to transform 200 µl *E. coli* DH5α competent cells. Two µl of vector digested with *Hind* III but not dephosphorylated, as well as linearised phosphatased vector alone, and ligation mixture alone were also used as ligation-transformation control mixtures. No colonies appeared on transformation plates of DH5α transformed with ligation mixture alone, whereas only blue colonies appeared in transformation samples with linearised non phosphatased vector. The 1:3 vector to insert molar ratio was the most successful yielding 26 white colonies. Transformation plates of 1:1 and 3:1 samples had 12 and 1 colonies respectively. Plasmids were isolated from all the white colonies using the Promega magic miniprep plasmid purification method, digested with *Hind III* and analysed on an 0.8% (w/v) agarose gel. Twenty out of the 26 plasmids had the right size insert, and their respective colonies stored in LB broth containing 25% (v/v) glycerol at -70°C. The orientation of the inserts with respect to the T_7 promoter of the pTZ19U (left orientation, $pAC1_L$ and right orientation, $pAC1_R$)was defined by restriction mapping based on the results of Saqib (1992). Both orientations were used in subsequent expression experiments.

6.5 Expression of 1.7 kb Insert of pAC1 in *Escherichia coli* DH5α

The monooxygenase gene appeared to be induced by α–pinene alone in complemented *P. fluorescens* mutants only. Expression of the 1.7 kb insert of pAC1 in *E.*

coli, was examined because this vector has a high copy number, and thus, more protein should be produced than with the pMMB66 system. Thus DH5α(pAC1$_L$) was cultured in 2YT broth and incubated at 37°C overnight. 1% (v/v) inoculum was used to inoculate fresh 2YT broth and the culture was induced with 1 mM IPTG for 15 h. The above medium was used because it is believed to result in higher number of vector copies (Sambrook *et al.*, 1989; Qiagen plasmid handbook, 1992). Two samples were prepared; IPTG induced and non induced. In addition, *E. coli* harbouring non recombinant plasmid, with and without IPTG induction, was also used as control.

Whole cell SDS electrophoresis samples were prepared by the method of Laemmli (1970) and analysed on a 12% SDS PAGE gel . Figure 6.7 shows the presence of a distinctive band of approximately 40 kDa in both the IPTG induced and non induced samples of whole cells harbouring the recombinant plasmid. This band which is marked with an arrow, is not present in the samples that contain non recombinant plasmid. The same experiment was repeated with DH5α harbouring pAC1$_R$ containing the insert in the reverse orientation with respect to the T_7 promoter of the pTZ19U and the same results were obtained. The above polypeptide was probably encoded from the 1.7 kb insert of pAC1. The same level of expression was obtained from both insert orientations.

6.6 Discussion

The fact that pin$^-$pox$^+$ mutants were able to grow on α–pinene after complementation with the recombinant plasmid pKS902, suggested that this plasmid contained at least a part of the α–pinene monooxygenase gene. Therefore, this expression vector, based on the hybrid *Tac* promoter, was used in preliminary studies for the optimisation of expression of the polypeptide encoded by its short DNA insert. Cell free extracts from cells carrying the recombinant plasmid which have been induced by IPTG, in either α–pinene medium or LB broth, were assayed for

monooxygenase activity, both spectrophotometrically and electrophoretically. Cell extracts from complemented mutants grown on minimal medium in the presence of α–pinene showed higher activity than those from cells grown on LB broth, and this suggests that the expression of the monooxygenase gene is possibly controlled by the presence of α–pinene in the culture medium. Moreover, upon transformation of the recombinant plasmid in *P. fluorescens* which is a rec$^+$ strain, it is possible that genetic recombination occurs, thus, restoring the activity of an otherwise truncated polypeptide.

The above vector, pMMB66, could be very useful for the manipulation of gene expression in homologous systems (e.g., other *Pseudomonas* strains) so that a variety of objectives, such as the regulation of expression of the cloned gene; the influence of the cellular concentration of its gene product to the activity of related genes; or the influence of the cellular concentration of its product to the cell physiology, could be examined. However, this large, low copy number vector (Morales, 1990) would not be the best choice for expression studies in *E. coli*. Cell extracts from *E. coli* HB101 harbouring pKS902 had shown some NADH oxidation activity by both spectrophotometric and electrophoretic methods, but this activity was very low. Hershfield *et al.* (1974) and Uhlin *et al.* (1979) reported that a significant increase in the level of gene expression is frequently obtained via gene dosage, by cloning the gene into a high copy number vector plasmid. A further increase may be achieved by insertion of the gene in a cloning vector carrying a strong promoter located close to, and oriented towards the cloning site. Therefore, the dual purpose expression and sequencing vector pTZ19U was also used for expression studies in *E. coli*. Whole cell SDS PAGE of recombinant clones showed the presence of a band with a molecular mass of 40,000, that was not present in samples from non recombinant clones suggesting that it was encoded by the insert DNA of clones pKS902 and pAC1. However, the expression of this polypeptide from both recombinant plasmids in *E. coli*, was again low and not induced by the promoter on the vector.

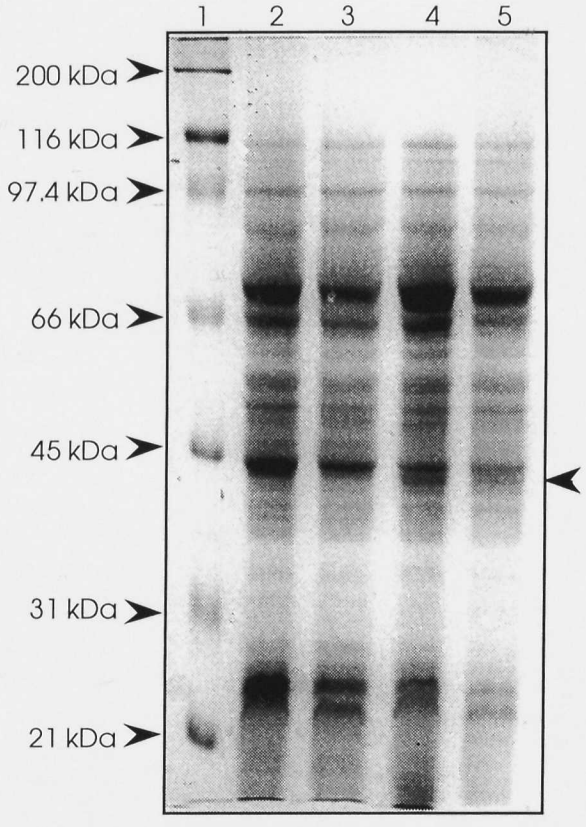


Figure 6.7: Coomassie blue R-250 stained 12% SDS PAGE of IPTG induced, or non induced, *E. coli* DH5α harbouring recombinant (pAC1), or non recombinant (pTZ19U) plasmid. Lane 1, molecular weight markers; lane 2, DH5α(pTZ19U) not induced; lane 3, DH5α(pTZ19U) induced; lane 4, DH5α(pAC1) not induced; lane 5, DH5α(pAC1) induced for 15 h.

In summary, this study has provided evidence that the 1.7 kb insert of the pKS902 plasmid that complemented the pin$^-$pox$^+$ mutants contains a part of the α-pinene monooxygenase gene. This assumption had to be subsequently confirmed by DNA sequencing of the 1.7 kb fragment. In addition, it is possible that the expression of the monooxygenase gene and perhaps all the α-pinene degradation

operon/regulon is positively regulated by the presence of α-pinene in the culture medium.

CHAPTER 7

Sequencing of the 1.7 kb Fragment that Complemented all the α–Pinene Monooxygenase Defective Mutants

7.1 Introduction

Complementation assays between mutants of *P. fluorescens* lacking monooxygenase activity and a genomic library of the above bacterium in *E. coli*, selected a clone, pKS73, that complemented all of the mutants. Subsequent subcloning and expression analyses provided evidence that a 1.7 kb subcloned fragment in pKS902 which also complemented all the monooxygenase defective mutants contained at least a part of the α–pinene monooxygenase gene together with its promoter site. The 1.7 kb fragment was, therefore, sequenced to establish whether it contained an entire open reading frame and recognisable promoter region.

7.2 Sequencing of the 1.7 kb Fragment

In the previous section the 1.7 kb fragment had been transferred into pTZ19U which facilitated sequencing from universal primers. The nucleotide sequence of the 1.7 kb *Hind* III fragment was determined by the dideoxy-chain termination method (Sanger *et al.*, 1977) on double stranded DNA using the Sequenase DNA sequencing kit® (United States Biochemical Corp., Cleveland, Ohio). Both the commercially available Reverse M_{13}, T_7 and M_{13} Universal primers (Stratagene), as well as synthetic primers (provided by Oswell DNA service, Edinburgh, UK) were used for sequencing

reactions. Synthetic primers were designed using the Mac Vector software DNA package (Kodak, UK).

The 1.7 kb *Hind* III fragment was sequenced in both directions (figure 7.1). Based on DNA sequence analysis of the fragment, using the GCG Sequence Analysis Software Package (Devereux, 1984), a region that showed high homology to the *E. coli* -35/-10 consensus promoter (Platt, 1986) was detected at 96 to 101 nucleotides from the start point of the sequencing (figure 7.2). A putative Shine-Dalgarno region (Shine and Dalgarno, 1975) was found 76 nucleotides downstream from the promoter sequence (nucleotide 177). An ATG codon in the same reading frame, at base 197 was followed by a translational termination codon at base 218, resulting in an ORF of only 21 nucleotides. This ORF was followed by a few more small ORFs. A fairly large ORF was, however, detected on the opposite strand, starting at position 1256, that encoded for more than 300 amino acids. Such an ORF would encode a polypeptide of around 35 kDa (Sambrook *et al.*, 1989; Promega protocols, 1991). The ATG codon at position 1256 was preceded by a typical GGAG ribosome binding site (Shine and Dalgarno, 1975) at base 1262. No region with homology to the *E. coli* consensus promoter sequence was found upstream of this ORF. However, a region with striking homology to the -24(TGG) and -12(TTG) consensus sequence of the *ntr*-activatable promoters which are recognised by the *rpo*N alternative sigma factor of *E. coli* (Thomas and Franklin, 1989) was observed only 20 nucleotides upstream of this ATG codon, between bases 1278 and 1290 (figures 7.1, opposite strand, and 7.3, coding strand). Promoters with σ^{54}-like sequences have been reported for a number of *Pseudomonas* genes, including the *xyl* operon, the pilin gene and the carboxypeptidase G2 gene (Deretic *et al.*, 1987).

The nucleotide sequence of the coding region is G+C rich (60%) as has been found for many *Pseudomonas* genes (Enscht *et al.*, 1988; Schell and Shukordhaman, 1989; Zylstra *et al.*, 1989b; Kukor and Olsen, 1992; Shingler *et al.*, 1992, 1993; de Mot *et al.*, 1993). The high G+C content of this DNA fragment resulted in GC compressions (Howe and Ward, 1990) that made the sequence unreadable in these regions, thus, making it

difficult to determine the complete DNA sequence of this ORF. To resolve this problem, sequencing was done utilising the dITP mixture instead of dGTP, which although giving poorer sequencing, resolved many but not all of the compressions.

Nucleotide database comparisons utilising the GenBank and EMBL databases revealed that the first 500 nucleotides proximal to the T_7 promoter were derived from the cosmid pJRD215 (99.7% sequence identity), whereas the rest showed striking similarities, of more than 30% to sequences of other monooxygenases, hydroxylases, dehydrogenases and cytochrome oxidases, among which were the pentachlorophenol monooxygenase from *Flavobacterium*, the tryptophan monooxygenase from *Agrobacterium tumefaciens*, and the 6-aminohexanoate-O-dimer hydrolase from *Flavobacterium*. Therefore, according to the genomic map of this vector (Davison, 1990), it was concluded that the first part of the 1.7 kb *Hin*d III fragment was the *Hin*d III-*Kp*n I region of the multiple cloning site of the pJRD215 vector, whereas the rest of the fragment probably encodes for a part of the α–pinene monooxygenase. The structure of the recombinant pAC1 vector is presented in figure 7.4.

```
1     AATACGACTC ACTATAGGGA AAGCTTATCG ATGATAAGCT GTCAAACATG
51    AGAATTCCCC TGGATTTCAC TCATGACAAT ATCGTCGGAG ATAAATATAA
101   TAATTCCACG ACTATAGACT ATACTAGTAT ACTCCGTCTA CTGTACGATA
151   CACTTCGCTC AGGTCCTTGT CTTTAACGAG GATTGTTACG TACGCTAATG
201   GCGTCAAAAC AAAGACTCTA GACCTAGGCC TTAAGATCAT ATGCATCCGC
251   GGGCCCGGGT TAACGCGTAA TCCATGGATC AAGAGACAGG ATGAGGATCG
301   TTTCGCATGA TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGTGCCTGA
351   ATGAACTCGA GAAAGAGCTG GTAGTTGGCG CAGTGTTCGA AGAACTGCCG
401   ATGTCCAGTA AGATTCTTAC TATGCTGGTT GAACCGGATG CTGGTAAAGC
451   TAGTTGGGTT GCTGCTTCTA CTTATGGTAC CGGGATCGAG GCTGGGACAG
501   TGTCTCAGGT GGGTCTCGGG ATCGGTGCGG GACATGGGCA ATCCTTCTGG
551   CGTGTTCTTC TGGTGCGCTG GTGAATGGCG CAAGTCTACA GGGTAGGCGT
601   AAAAAAATAC CCCGGCATCA CGGCCGCCGT GGCCTGCTGA AACGATCCAC
651   GGGATCACGT GGCGCCTATG CTGACGTATT GGAAAAAATA TTCCACTAAA
701   AATAATTCTG GAATAAATAT TGATTGCGCT CGCCGGACGT TCTAGTCTGC
751   TCCCACCAAG AGCGTGTGCC ATGCCACGGC GGCACACACG CAGGCTGATA
801   AAAATAACAG GAGCCAGCAT GGGCCAGACT CGTTTTGCCA GTGGGCGTCA
851   ATTGGATCTG ATTTGCCTCG GGCGCCTGGG CGTCGACCTT TATGCGCAAC
901   AAGTGGGGGC GCGGCTTGAG GACGTGTCCA GCTTTGCCAA ATACCTCGCG
951   GTTCCTCCGC CAATATCGTT CGGCACGCAC GGCTGGGCCT CAAGTCGGCG
1001  ATGCTGAGCC GGGTAGGGAC GACCACATGG CGTTTCCTGG TCGAATCCCT
1051  GGCTCCGCGA AGGCTGCGAT GTCAGCGGCA TCCAGGTCGA CACCGGAACG
1101  CCTGACCGCC ACATAGAGTG CTGCTGGGCC TCAAGAGCCA CAGCCTTCCC
1151  CCTGGTGTTC TACCGCGAGA ACTGCGCCGA CATGGCGGTG CGCGCCGAAG
1201  ACGTCAGCGA AGCCTTTATC GCCTCCAGCA AGGCGCTGTT GATCACCGGC
1251  ACGCATTTCT CCACCGACGG CGTGTACAAG GCCAGATCCA GGCGCTGACT
1301  ATGCGGCCAG GCACCACGTC AAGCGCGTGC TGGACATCGA CTATCGCCCG
1351  GTGCTGTAGA GAGAGCCTGG CGGGCAAGGC CGATGGCGAA ACGCGGTTTG
1401  TCGCCGACCG GAATGTCAGC CAGCACGTGC AGAACATCCT GCCGCGTTTT
1451  GACCTTGATC GTCGGCACCG AAGAAGAATT TTTGATCGCC GGCGGCAGTG
1501  AGGATTTGCT CAGCGCGCGA AATGGCTTTC CAGGCGAAGG CGTATATGCC
1551  GAAAGCTAGG TGGCCTGTTA TGACGCGCTG ATTGTCATCA CCGAAGGCGA
1601  TTACGCGGAT GGCTATGCTA GTCTCGTCCG AGCGATGACC CTGATCCGCG
1651  TCAAGCTTGC ATGCCTGCAG GTCGACTCTA GAGGATCCCG GG
```

Figure 7.1: Nucleotide sequence of the 1.7 kb insert of pAC1. A putative -35 promoter region (nucleotides -138 to -132) and -10 region (nucleotides -113 to -105) as well as the putative ribosome binding site are marked in red. The putative *ntr*C-like promoter region (nucleotides 1290 to 1278) and ribosome binding site (nucleotides 1262 to 1258) on the complementing strand, as well as the start codon (nucleotides 1256 to 1254) of the polypeptide showing high homology, at the DNA level, to other monooxygenases are marked in blue.

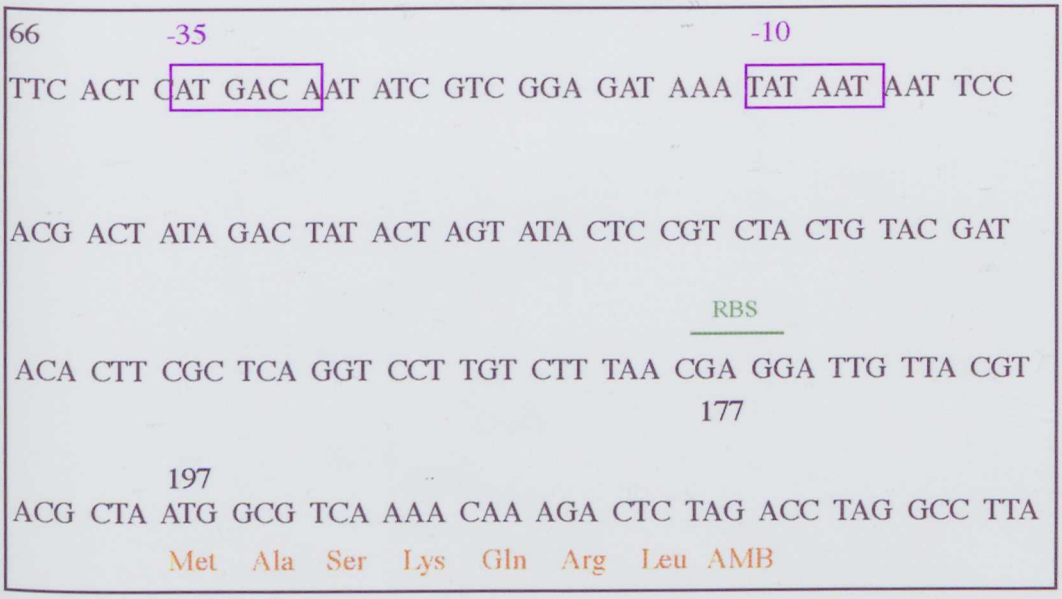


Figure 7.2: Nucleotide sequence of bases 66 to 230 of the 1.7 kb insert of pAC1. A putative -35 promoter region (nucleotides -138 to -132) and -10 region (nucleotides -113 to -105) are boxed. The putative ribosome binding site is marked as RBS.

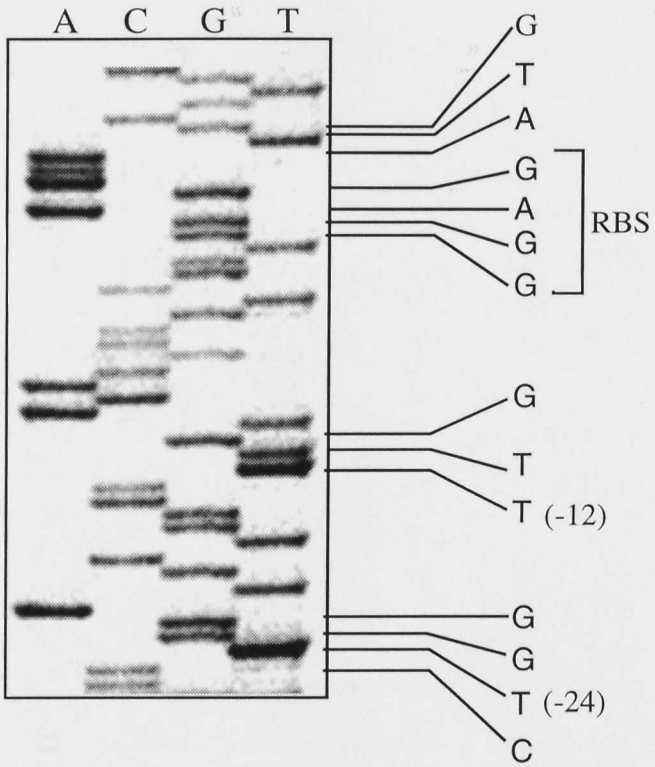


Figure 7.3: Nucleotide sequence of the putative *ntr*-like promoter region, (-24 TGG, -12 TTG) of the large open reading frame (complementary strand). The ribosome binding site is marked as RBS.

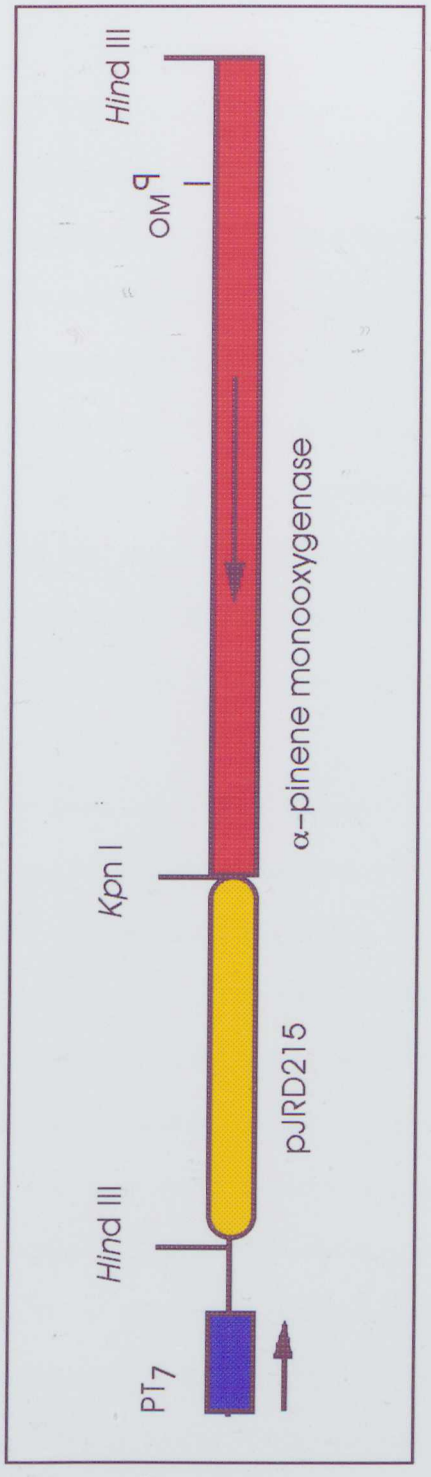


Figure 7.4: Physical map of the pAC1 recombinant plasmid suggested by DNA sequencing. Symbols: PT_7, T_7 promoter; P_{MO}, the putative monooxygenase promoter.

7.3 Discussion

Sequencing of the 1.7 kb *Hind* III insert of pAC1, that had been shown previously to complement all the monooxygenase minus mutants, revealed an ORF following a putative ribosome binding site which potentially encodes for a 35 kDa polypeptide. This was consistent with expression experiments presented above, which showed that recombinant clones containing the 1.7 kb fragment, constitutively expressed in *E. coli* DH5α a polypeptide with a molecular mass of around 40 kDa. Computer database searches at both the DNA and the deduced amino acid levels revealed a high degree of similarity to a number of monooxygenases, hydroxylases, dehydrogenases and cytochrome oxidases. Thus, these results provide substantial evidence that the 1.7 kb fragment encodes for a part of the α-pinene monooxygenase gene(s).

Furthermore, DNA sequencing analysis revealed that the putative promoter of the monooxygenase belongs to a distinct class, the σ^{54} dependent promoters, that have an invariant -24 CTGG, -12 TTGC sequence. Such promoters have little in common with the *E. coli* -35 TTGACA, -10 TATAAT consensus promoter and are recognised by RNA polymerase utilising the alternative σ^{54} factor encoded by *rpoN* or its homologues. They are widespread in nitrogen-fixing bacteria but they are also found in the enteric bacteria such as *E. coli* and *Salmonella* spp., as well as in a number of *Pseudomonas* species (Dixon, 1986; Nordlund *et al.*, 1990; Rothmel *et al.*, 1991; Shingler *et al.*, 1993). All the -24, -12 promoters so far analyzed are regulated by positive activators that usually bind to specific DNA sequences located unusually far (100 to 200 bp) upstream of the promoter. The binding sequences are often inverted repeats that can be moved more than 1 kb away without losing their ability to mediate transcription (Shingler *et al.*, 1993).

This study confirmed that pAC1 contains a part of the monooxygenase gene(s). However, the presence of sequence derived from the cosmid vector pJRD215 in pAC1 indicates that the monooxygenase gene was truncated in the

original cosmid clone and to obtain the full gene it was necessary to go back to the gene bank using the monooxygenase gene as probe. However, it has been demonstrated (chapter 5), that the genomic library of *P. fluorescens* in pJRD215 was characterised by instability and possible DNA rearrangements and was therefore of questionable reliability. Therefore, it was necessary to construct a new library (Chapter 9).

CHAPTER 8

Purification and Properties of α–Pinene Monooxygenase from *Pseudomonas fluorescens* NCIMB 11671.

8.1 Introduction

The complementation studies between the genomic library of *P. fluorescens* and the mutants had shown that all the monooxygenase mutants (pin^-pox^+ phenotype) map to a small region, the 1.7 kb insert of plasmid pKS73. Such result suggests that either the monooxygenase enzyme is a single polypeptide, or that there is a hot spot for NTG mutations within this region. This, apparent simplicity suggested by the complementation studies did not agree with the evidence that the enzyme requires FAD and ferrous iron for activity (Aikens, 1990) which would be more typical of a multicomponent enzyme (Hayaishi, 1974; Wackett *et al.*, 1989; Gibson *et al.*, 1990; Hartmans *et al.*, 1991; Pilkington and Dalton, 1991; Whited and Gibson, 1991; Roelof *et al*, 1992). Hence it was necessary to purify the enzyme to confirm its subunit composition.

It is common in enzyme purification to use ammonium sulphate precipitation as a first step for the separation of the protein of interest from any interfering activities (Harris and Angal, 1989) and many researchers have used this procedure in the purification of monooxygenases (Neujahr and Gaal, 1973; Bernhardt *et al.*, 1975; Donoghue *et al.*, 1976; Patel *et al.*, 1976; Firestone *et al.*, 1978; Yamaguchi and Fujisawa, 1982; Beadle and Smith, 1982; Liu and Chapman, 1984; Uetz *et al.*, 1992). In preliminary studies, Aikens (1990) and Heighway (1991) showed that ammonium sulphate precipitation of cell extracts of *P. fluorescens* resulted in only one fraction at ammonium sulphate concentrations between 1.4 M and 2.0 M showing

monooxygenase activity. The yellow coloration of the cell extracts noted by Best *et al.* (1987) was present in the supernatant of the 1.4-2.0 M ammonium sulphate cut with the precipitated protein being white. On addition of 0.1 mM FAD to the "cut", the α–pinene monooxygenase activity was increased. Dialysis of this ammonium sulphate fraction against buffer containing stabilised ferrous iron, resulted in the loss of α–pinene monooxygenase activity, but retention of the endogenous NADH oxidation activity, while reincubation of the dialysed fraction with FAD and iron reconstituted the enzyme activity (Aikens, 1990). Similar results were obtained by Hartmans *et al.* (1989) for a styrene monooxygenase. These assays suggest that α–pinene monooxygenase is a flavin-containing enzyme because acid ammonium sulphate precipitation often results in loss of the flavin component in flavin-containing monooxygenases and thus, inactivation of these enzymes, which usually can be reactivated by addition of FAD (Donoghue *et al.*, 1976; Hartmans *et al.*, 1989; Locher *et al.*, 1991). However, as this method is obviously disruptive, it was not used in this study in an attempt to retain maximum activity.

8.2 Activity Assays of α–Pinene Monooxygenase from *Pseudomonas fluorescens* NCIMB 11671 in High Speed Supernatants

8.2.1 Polarographic Assays

Polarographic assays were conducted using a Clark type oxygen electrode and were based on the α–pinene stimulated oxygen consumption of HSS, when NADH was also present. The oxygen uptake in the absence of the substrate was used as baseline. Generally, the endogenous oxygen consumption rate in the absence of α–pinene was high and therefore, it was difficult to determine the monooxygenase activity. In order to obtain reasonable levels of activity, it was necessary to incubate the extracts at room temperature in the absence of substrate for at least 30 min to

eliminate the endogenous rate, prior to adding of α–pinene and measuring the oxygen consumption rate. Thus, polarographic assays although in principle easy to use, were time consuming and less sensitive than other methods applied.

8.2.2 Spectrophotometric Assays

Spectrophotometric assays were based on the observation that α–pinene monooxygenase from *P. fluorescens* has an obligatory requirement for NADH (Best *et al.*, 1987; Griffiths *et al.*, 1987a; Aikens, 1990). The monooxygenase activity was identified by measuring the decrease in absorbance at 340 nm in HSS in the presence of α–pinene and NADH as cofactor. Measurement of NADH consumption in samples where no substrate was added was used to determine the endogenous oxidation rate which was subtracted from the rate obtained in the presence of α–pinene, to obtain the α–pinene-dependent NADH oxidation rate. The removal of the membrane fraction from the extracts should have resulted in a lower endogenous NADH oxidation rate and thus, more accurate measurements of the monooxygenase activity. However, despite the fact that HSS were being used, the rates of endogenous NADH oxidation activity were very high. Nevertheless, in contrast to the polarographic measurements, the endogenous NADH oxidation activity and the activity induced by α–pinene could be easily differentiated.

Best *et al.* (1987) suggested the addition of sonicated α–pinene (0.112 μmoles) in Clark and Lubs phosphate buffer, pH 7.8 to the reaction mixture. However, when sonicated α–pinene was added at either the above amount or at any other amount between 0.1 and 2 μmoles, the results obtained were inconsistent. On the other hand, reactions carried out in the buffer saturated with α–pinene gave much more consistent results. Although sonication of α–pinene results in the formation of a micro-emulsion, any sample of it would contain different size micro-droplets which might explain the inconsistency observed.

In order to confirm that measurements of monooxygenase activity in α–pinene saturated buffer were not limited by the substrate concentration, enzyme activities were recorded using a range of protein concentrations. The value of the monooxygenase activities were usually proportional to the protein concentration up to a point above which there was no further increase in activity with increasing protein concentration. At this point the substrate concentration was believed to be the limiting factor. The values of activity below this point were always used in calculations of specific activities.

In multicomponent monooxygenases a non linear relationship between enzyme activity and protein concentration of the sample is often observed, especially when low protein concentrations are used (Fersht *et al.*, 1977; Locher *et al.*, 1991). However, when α–pinene stimulated NADH oxidation rate was plotted against the protein concentration a linear relationship was obtained at protein concentrations increasing from 0.1 mg/ml to 5 mg/ml. This result contradicts the work of Aikens (1990), who suggested that a linear relationship was only achieved at protein concentrations above 1 mg/ml. Similar results to the ones obtained in this study have been obtained for other monooxygenases such as the 4-toluene sulfonate methyl monooxygenase from *Comamonas testosteroni* T-2 (Locher *et al.*, 1991) and acetol monooxygenase from *Mycobacterium* Py1 (Hartmans and de Bont, 1986). These results suggest that α–pinene monooxygenase may be a single component system, with both the monooxygenase and the reductase sites on a single polypeptide chain, or in a single non dissociable complex. This would be consistent with the structure of flavin containing monooxygenases (Gunsalus *et al.*, 1975; Fersht *et al.*, 1977; Mason, 1988).

8.2.3 Electrophoretic Assays

Electrophoretic assays were also based on the NADH oxidation activity of α–pinene monooxygenase. Non-denaturing polyacrylamide gels were incubated with a solution of a tetrazolium dye containing NADH as described in Materials and Methods (Haigler and Gibson, 1990; Hartmans, 1991). Should α–pinene monooxygenase be a multicomponent protein, such a method would only be able to detect the reductase component of the enzyme complex. Assays for multicomponent oxygenases in crude extracts tend to display a non-linear response of oxygenase activity towards the protein concentration, because of the lack of a reductase (Locher *et al.*, 1991). However, according to the results above α–pinene monooxygenase may be a single component system which even if it was composed of more than one polypeptide, it would be expected to run as a single entity on a non-denaturing gel. Therefore, such an electrophoretic method should detect the monooxygenase enzyme itself. Figure 8.1 shows a typical activity profile obtained by this method for HSS derived from lactate and α–pinene grown cells. The activity stained electrophoretic pattern of the bacterium grown on α–pinene consists of three major bands (lane 2) with R_f values of 0.35, 0.43 and 0.51. Although the same protein concentration was loaded on the gel for both samples, the lactate sample showed (by densitometry) much lower activity in the first two bands with R_f values 0.35 and 0.43 than the α–pinene grown sample.

8.2.4 Product Formation Assays

α–Pinene oxide formation was assayed by gas chromatography as described in Materials and Methods. Authentic α–pinene oxide (99% pure) was used as a reference standard. Best *et al.*, (1987) reported that whole cells of *P. fluorescens* NCIMB 11671 were also able to catalyse the epoxidation of limonene to limonene

oxide. Therefore, limonene was also used as a substrate for product formation assays, since limonene epoxide appeared to be more stable under the reaction conditions used. Authentic limonene epoxide (Sigma) was also used as a standard. All GC assays were qualitative in nature, since any accurate estimation of the amount of the epoxide produced was limited by the instability of the epoxide at the pH of the monooxygenase assays. Finally, all results obtained were confirmed by GC-MS analyses performed by the ULIST GC-MS service at King's College, London.

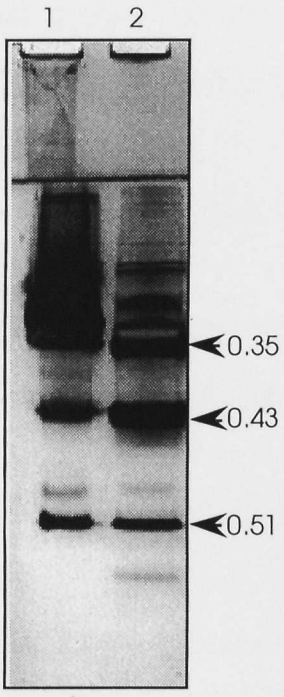


Figure 8.1: Non denaturing PAGE showing NADH stimulated 4-nitro blue tetrazolium reduction activity in High Speed Supernatants derived from *Pseudomonas fluorescens* NCIMB 11671 cells grown on lactate (lane 1) and α–pinene (lane 2).

8.3 Stability of α–Pinene Monooxygenase from *Pseudomonas fluorescens* NCIMB 11671

Determination of the stability of α–pinene monooxygenase was obviously essential before any purification could take place. Cell paste, when frozen at -20°C or -70°C, showed no loss of activity over a period of at least one year. Cell free preparations lost more than half of their original activity when stored in 10 mM potassium phosphate buffer, pH 8.0 at 4°C for 10 days. On the other hand, cell free preparations stored in 10 mM potassium phosphate buffer, pH 8.0 containing 10% glycerol, 10% ethanol and 0.1 mM dithiothreitol (Harris and Angal, 1989; Whited and Gibson, 1991a, b; Yen *et al.*, 1991) were remarkably stable at -20°C. Such preparations retained their original activity for several months and only lost 15% of their activity after 10 days storage at 4°C. However, subsequent tests showed that highly purified preparations were less stable. Similar results have been obtained by Beadle and Smith (1982) for 2,4-dichlorophenol hydroxylase from *Pseudomonas* sp. strain NCIB 9816 and by Tsuji *et al.* (1986) for 4-aminobenzoate hydroxylase from *Agaricus bisporus*.

Enzyme activity in Tris-HCl buffers was almost a quarter of the activity in potassium phosphate buffers. However, when the pH of the Tris-base was adjusted with sulphuric acid at the same pH instead of hydrochloric, almost identical activities were obtained in Tris- and phosphate buffers. Therefore, the difference appeared to be due to the detrimental effect of chloride ions on the enzyme. Storage of HSS at 4°C in 20 mM Tris-HCl buffer, pH 8.0 resulted in loss of monooxygenase activity within three days of storage. Moreover, chloride ions inhibit the reaction to the same degree whether added as sodium or as potassium salts. Similar results were also obtained by Neujahr and Gaal (1973) for the phenol hydroxylase from *Trichosporon cutaneum*, and by Liu and Chapman (1984) for a plasmid encoded 2,4-dichlorophenol hydroxylase.

8.4 Small Scale Purification of α–Pinene Monooxygenase from *Pseudomonas fluorescens* NCIMB 11671

8.4.1 Affinity Chromatography of High Speed Supernatants from *Pseudomonas fluorescens* NCIMB 11671

The interaction of proteins with specific ligands is the basis of one of the most powerful methods of protein purification, affinity chromatography. The ligand is chemically attached to an insoluble and porous solid chromatographic support in such a way that the ligand is available for the protein to bind to it. In the ideal case, only the desired protein binds to the ligand resin and all other proteins pass though the column (Ersson *et al.*, 1989; Creighton, 1993). As α–pinene monooxygenase has an obligatory requirement for NADH, a 1 ml Hi-Trap Blue Sepharose affinity column (Pharmacia) was used. The ligand is a dye, Cibacron Blue 3G-A, which is covalently attached to the highly cross-linked agarose gel via the triazine group. Such columns are recommended for the separation of enzymes requiring adenyl-containing cofactors, including NAD^+ and $NADP^+$. Three samples were collected: the column effluent after application of the sample to the column (with a very pale yellow colour), the wash (with a yellowish colour), and the eluent (yellow). α–Pinene monooxygenase activity in these samples was determined spectrophotometrically using α–pinene saturated buffer, pH 7.8. However, only the wash showed monooxygenase activity (table 8.1), suggesting that the enzyme did not bind to the column. Table 8.1 shows that some separation of the monooxygenase from the endogenous interfering activities had occurred, since the sample eluted with 2M NaCl, after dialysis in starting buffer, showed only endogenous NADH oxidation activity.

Table 8.1: Purification of α–pinene monooxygenase from HSS of *Pseudomonas fluorescens* NCIMB 11671 by Hi-Trap Blue Sepharose (Pharmacia) affinity chromatography.

Sample	Total volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
HSS	1	13	31.2	2.4	100	1
Effluent	1	3.28	0.2	0.06	0.64	0.025
Wash	10	5.96	22.3	3.7	71.5	1.5
Eluent	3	3.264	0	0	0	0

However, the purification factor was very low (Harris and Angal, 1989) and would not be useful for large scale purification of the monooxygenase enzyme. A 1 ml NAD column purchased from Sigma was also used, and similar results were obtained.

8.4.2 Ion Exchange Chromatography of High Speed Supernatants from *Pseudomonas fluorescens* NCIMB 11671

Ion exchange chromatography is the most commonly utilised chromatographic technique, followed by affinity chromatography and gel filtration. Ion exchange of proteins involves their adsorption to the charged groups of a solid support followed by their elution by fractionation and/or concentration in an aqueous buffer of higher ionic strength. Thus, ion exchange of proteins is based on their charge and it is used to resolve proteins which differ only marginally in their charged groups (Harris and Angal, 1989). A weak ion exchanger was used in order to avoid multi-point binding which usually occurs when strong ion exchangers are used. Ion exchange chromatography was carried out, on a small scale, using the Memsep 1010 cartridge

(Millipore) connected to an FPLC system (Pharmacia). 1 ml samples equilibrated in 10 mM potassium phosphate buffer, pH 8.0 were routinely applied to the cartridge. The cartridge was washed with 5 ml of start buffer at a flow rate of 5 ml/min and the proteins were eluted with 200 ml of a linear gradient of 0 to 1 M NaCl in start buffer. The samples that corresponded to each peak of the elution profile (figure 8.2) were pooled, dialysed against Clark and Lubs buffer, pH 7.8, and assayed spectrophotometrically for α–pinene stimulated NADH oxidation activity. Sample 3 ("MO") which eluted at around 200 mM NaCl showed monooxygenase activity, and had a yellowish colour. Table 8.2 shows that ion exchange chromatography resulted in a higher degree of purification and a much higher recovery of the monooxygenase activity than affinity chromatography. Subsequent runs gave similar results and therefore this method was chosen as a first step for the purification of α–pinene monooxygenase from *P. fluorescens*. Moreover, since the enzyme eluted at around 200 mM salt a narrower linear gradient could be used which should enhance the separation of the constituents of the HSS sample (Ersson *et al.*, 1989; Harris and Angal, 1989).

The Memsep 1010 cartridge has the properties of a weak anion exchanger. Since the monooxygenase enzyme was eluted from this Memsep cartridge at fairly low salt concentrations, it was considered reasonable to attempt to purify the enzyme using a strong anion exchanger and for this purpose the mono Q HR 5/5 column (Pharmacia) was chosen. This column is designed for analytical and semi preparative uses with both the FPLC and the HPLC systems. The column was connected to the FPLC system and equilibrated in 10 mM P-E-G buffer, pH 8.0. 1 ml of HSS containing 25 mg of protein was applied. The column was washed with 5 column volumes of starting buffer and the bound proteins eluted with a linear gradient of 0 to 1.0 M KCl in starting buffer. Ten column volumes of gradient were used and the flow rate applied was 1 ml/min. Samples corresponding to individual peaks were pooled together, dialysed against Clark and Lubs buffer and assayed as before. The sample which showed α–pinene monooxygenase activity was again yellowish and eluted at around 300 mM KCl.

Unfortunately, the separation of the protein constituents of the sample by this method was very poor and the monooxygenase activity was spread across a number of eluent samples, possibly as a result of multi point binding to the strong ion exchanger (Harris and Angal, 1989; Pharmacia, 1991). Reduction of the flow rate to 0.2 ml/min and the gradient range to 0 to 0.5 M KCl did not result in any significant improvement in the separation. Therefore, the weak anion exchanger was adopted for large scale purification of α–pinene monooxygenase.

Table 8.2: Purification of α–pinene monooxygenase from HSS of *Pseudomonas fluorescens* NCIMB 11671 by ion exchange chromatography using the Memsep 1010 cartridge (Millipore).

Sample	Total volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
HSS	1	13	236.6[a]	17.2	100	1
Sample 3	7	1.1	198.7[a]	172.5	84	10

a: 0.01 mM FAD was added to the assay mixture prior to measuring the monooxygenase activity.

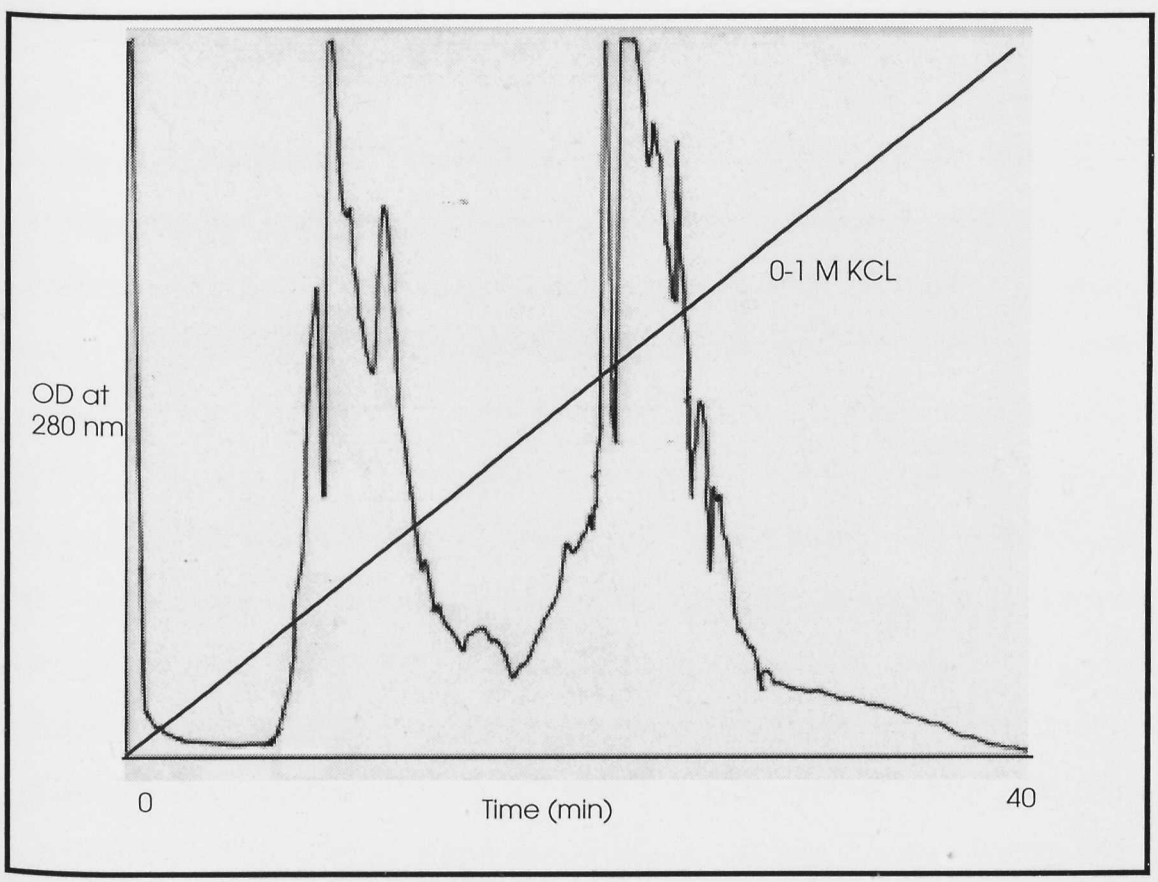


Figure 8.2: Elution profile of the ion exchange protein separation of the HSS from *Pseudomonas fluorescens* NCIMB 11671, using the Memsep 1010 Millipore cartridge on the FPLC system. The elution flow rate was 5 ml/min.

8.5 Large Scale Purification of α–Pinene Monooxygenase from *Pseudomonas fluorescens* NCIMB 11671

8.5.1 Ion Exchange Chromatography of High Speed Supernatants from *Pseudomonas fluorescens* NCIMB 11671

DEAE-Sephacel (Pharmacia) was used in the first step of the preparative purification of α–pinene monooxygenase. This matrix is a beaded cellulose weak anion exchanger recommended for separations over a wide molecular range. It is reported to be ideal for high resolution chromatography of proteins with a recovery close to 100%. The matrix was supplied pre-swollen, as a suspension in 0.5 M NaCl solution containing 25% (v/v) denatured ethanol to prevent microbial growth. As α–pinene monooxygenase appeared to be inhibited by the presence of chloride ions, (Chapter 8.3), the chloride counterions were changed to phosphate as described in Materials and Methods. 50 ml of the matrix were packed in a XK 16/20 (Pharmacia) column and equilibrated in 10 mM potassium phosphate buffer, pH 8.0 containing 10% glycerol by flushing 3 litres of the above buffer through the column at a flow rate of 1 ml/min until the pH and the conductivity of the effluent were the same as those of the starting buffer. The mean column capacity is 30 mg of protein per ml of matrix and thus, up to 1.5 g of protein could be loaded onto the column. Typically, 0.3 to 1.5 g of total protein were loaded. The column was subsequently washed with 2 to 5 column volumes of starting buffer, and the bound proteins were eluted with 5 to 10 column volumes of a linear gradient of 0-0.5 M KCl or 0-0.25 M KCl in starting buffer, at a flow rate of 0.5 ml/min. All DEAE-Sephacel runs were carried out on an FPLC system (Pharmacia) at 4°C. Characteristics of some of the runs are presented below:

Run A: The DEAE-Sephacel column was equilibrated with 10 mM P-E-G buffer, pH 8.0 and 10 ml of HSS containing 680 mg of protein with a total monooxygenase activity of 4.26 U were applied to the column. The column was washed with 3 column

volumes of the same buffer, and bound proteins eluted with 10 column volumes of a linear gradient of 0-0.5 M KCl in starting buffer. The profile obtained is presented in figure 8.3. The tubes corresponding to each peak were pooled and concentrated to 10 ml by ultra filtration using an XP 30 membrane, then dialysed against 50 mM phosphate buffer, pH 7.8 in order to be used for activity assays. Their protein concentration was determined using the Bio-Rad protein assay before assaying spectrophotometrically for α–pinene stimulated NADH oxidation activity. The third peak of the elution profile marked "MO" (tubes 20-30) showed activity and this was confirmed as the monooxygenase by detection of α–pinene epoxide formation by gas chromatography assays. The enzyme eluted from the column at around 170 mM KCl. The total activity of this sample in terms of NADH oxidation was very low, only 0.7 U. However, upon addition of 0.01 mM FAD to the reaction mixture, the activity increased ten times to 7.0 U. Therefore, the enzyme appeared to be losing most of its flavin cofactor either during the ion exchange chromatography or during subsequent dialysis. However, it could be easily reactivated upon addition of this cofactor as observed by Aikens (1990) and Heighway (1991). The purification factor of this step was nearly 10 fold, and more activity was recovered (recovery 159%), than had apparently been loaded. These results are presented in table 8.3.

The activity of the enzyme is only presented on the basis of NADH oxidation rate and not production of the epoxide. The concentration of the epoxide produced was impossible to estimate accurately, due to the lability of the epoxide at the pH of the product formation assays (pH 7.8). As previously observed, the enzyme was not active at pH 9.0 which would have been the pH required to minimise chemical breakdown of the epoxide.

It has been observed that some monooxygenases exhibit substrate-stimulated NADH oxidation but without substrate oxidation. This phenomenon is called uncoupling (Hayaishi, 1974; Mason, 1988) which is a well known property of flavin monooxygenases when substrate analogs are supplied (Hayaishi, 1974; Müller, 1985; Ziegler, 1990; Uetz *et al.*, 1992), whereas, "true substrates" usually exhibit tight coupling

(Firestone *et al.*, 1978). Spectrophotometric assays cannot distinguish between uncoupled oxidation of NADH and that linked to the epoxidation of α–pinene. Therefore, it was essential to measure the degree of uncoupling, if any, before expressing the protein activity as NADH oxidation rate. Thus, after each purification step the degree of uncoupling was determined by the method of Uetz *et al.* (1992), using a Clark type oxygen electrode, as described in Materials and Methods. However, no uncoupling was detected. The monooxygenase fraction was drop-frozen in liquid nitrogen and stored at -70°C until needed.

Figures 8.4 and 8.5 present non-denaturing PAGE of the above samples, with the gels stained with coomassie blue R-250 and activity stained respectively. The sample with monooxygenase activity ("MO"), contained four major proteins named as "a", "b", "c," and "d". On the activity stained gel it is clear that bands "c" and "d" had NADH oxidoreductase activity. Densitometric measurements showed that band "c" had much higher total activity than band "d" and has been enriched compared to the "c" bands of the HSS and the crude extract samples. This was, therefore, a good candidate for the α–pinene monooxygenase.

Table 8.3: Purification of α–pinene monooxygenase from HSS of *Pseudomonas fluorescens* NCIMB 11671 by DEAE-Sephacel ion exchange chromatography. Elution was performed with a linear gradient of 0-0.5 M of KCl.

Sample	Total volume (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
Crude extract	12	800	4.4	5.5	100	1
HSS	10	680	4.26	6.26	96.8	1.13
DEAE Sephacel eluent (MO)	10	123	7.026	57.13	159	10.38

a: 0.01 mM FAD was added to all monooxygenase activity assays.

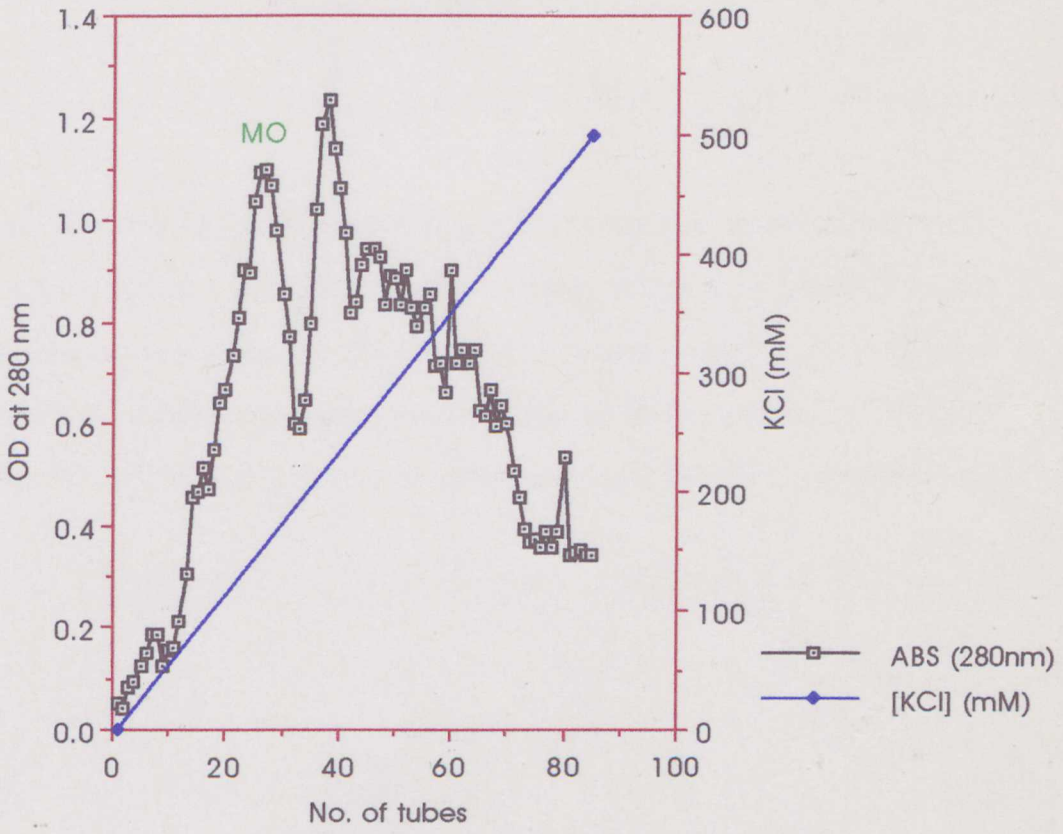


Figure 8.3: Elution profile from DEAE-Sephacel run of HSS from *Pseudomonas fluorescens* NCIMB 11671. Elution was carried out with a linear gradient of 0-0.5 M KCl at a flow rate of 0.5 ml/min. The sample with monooxygenase activity ("MO") corresponds to tubes 20-30.

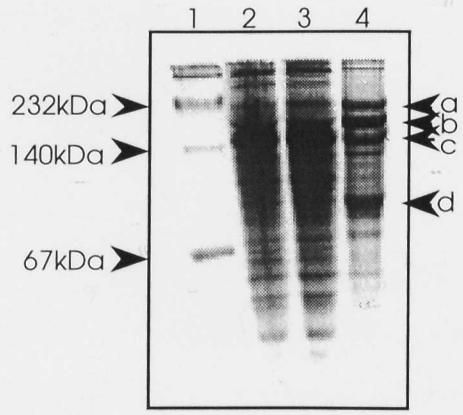


Figure 8.4: Non denaturing PAGE of HSS from *Pseudomonas fluorescens* NCIMB 11671 and the elution fraction from DEAE-Sephacel chromatography which showed monooxygenase activity. Lane 1, Molecular weight markers; lane 2, Crude extract; lane 3, HSS; lane 4, sample "MO" which showed monooxygenase activity with four major bands "a", "b", "c", and "d" (the gel was stained with coomassie blue R-250).

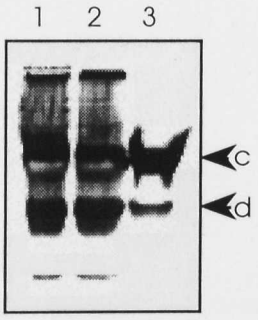


Figure 8.5: Activity stained non denaturing PAGE, of HSS from *Pseudomonas fluorescens* NCIMB 11671 and the elution fraction from DEAE-Sephacel chromatography which showed monooxygenase activity. Lane 1, Crude extract; lane 2, HSS; lane 3, sample "MO" which showed monooxygenase activity. Only bands "c" and "d" (from above) were stained.

Run B: 336 mg of HSS with a total activity of 1.3 U were loaded onto the DEAE-Sephacel column equilibrated in 10 mM P-E-G buffer, pH 8.0. The column was washed with 5 column volumes of starting buffer and then 10 column volumes of a linear gradient of 0-0.25 M KCl in starting buffer was applied. The elution profile is presented in figure 8.6.

Tubes corresponding to individual peaks were pooled together, and concentrated by ultra filtration, using an XP 30 membrane. Their buffer was changed to 50 mM phosphate, pH 7.8 by dialysis, and they were assayed spectrophotometrically for α–pinene monooxygenase activity. Only one sample "MO" (tubes 120-145) which eluted at around 170 mM KCl showed activity. The monooxygenase activity was also confirmed by α–pinene oxide and limonene oxide formation, analyzed by gas chromatography. The total NADH oxidation activity of this sample, after addition of 0.01 mM FAD, was 1.3 U and therefore the recovery (100%) was again very high. Moreover, the purification factor was approximately 2 times higher than in the previous run because of the better resolution achieved by using a narrower concentration ranged salt gradient. These conditions were assumed to be optimal for the separation of the monooxygenase activity from other interfering activities from HSS of *P. fluorescens* NCIMB 11671 by ion exchange chromatography. The results obtained from this run are presented in table 8.4.

A non denaturing PAGE profile of HSS and the eluted active sample is presented in the figure 8.7 below. Sample "MO" has two major protein bands (arrowed) of high and low molecular weight, which are very well separated from each other. Densitometric scanning of these gels revealed that these proteins comprised 35% and 40% respectively, of the total protein contained in the active eluent sample, "MO". The band with the higher molecular weight is at the same position as band "c" from the first run, and may correspond to the monooxygenase enzyme according to the activity stain results obtained (run A). From the native PAGE in figure 8.7 it appeared that the two main protein constituents of the ion exchange eluent (with monooxygenase activity), were very well separated on the basis of their molecular

masses. Therefore, gel filtration of this sample under native conditions could be the next purification step for the isolation of the monooxygenase. The monooxygenase sample was drop frozen in liquid nitrogen and stored at -70°C until that required.

Table 8.4: Purification of α–pinene monooxygenase from HSS of *Pseudomonas fluorescens* NCIMB 11671 by DEAE-Sephacel ion exchange chromatography. Elution was performed with a linear gradient of 0-0.25 M KCl.

Sample	Total protein (mg)	Total activity (U)[a]	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
HSS	336	1.3	3.33	100	1
DEAE-Sephacel eluent (MO)	22.4	1.3	57.99	100	17.41

a: 0.01 mM FAD was added to all monooxygenase activity assays.

8.5.2 Gel Filtration of the Active DEAE-Sephacel Eluent

In order to optimise the purification achieved by gel filtration chromatography, it is necessary to use a matrix which will best resolve the desired protein from the remaining proteins. Accordingly, a preliminary screening was done using Superose 12 (Pharmacia) to estimate the molecular weight of the desired protein and the molecular weights over which the remaining proteins are distributed. 50 mM phosphate buffer, pH 7.8 containing 0.1 M NaCl was used for both equilibration of the column and elution of the proteins, the NaCl being included to minimise the binding of proteins to the matrix by electrostatic or van der Waals interactions (Stellwagen, 1990; Pharmacia, 1993).

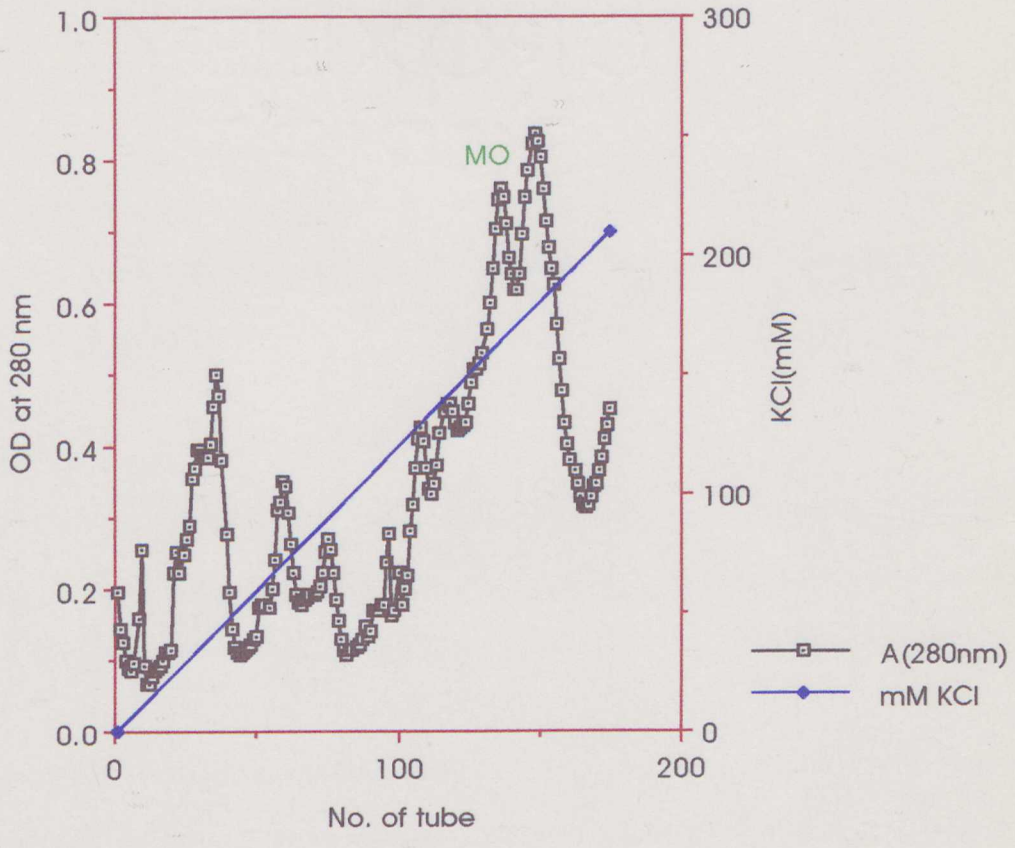


Figure 8.6: Elution profile of proteins from HSS bound to the DEAE-Sephacel. Elution was carried out with a linear gradient of 0-0.25 M KCl. The sample with monooxygenase activity corresponds to tubes 120-145.

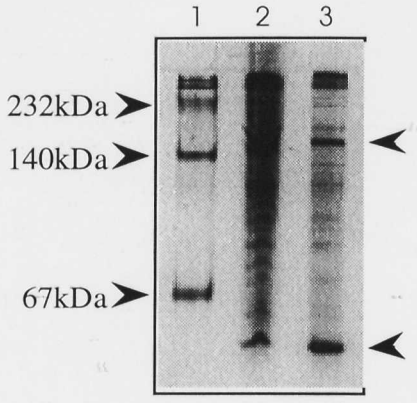


Figure 8.7: Non denaturing PAGE of HSS from *Pseudomonas fluorescens* NCIMB 11671. Lane 1, Molecular weight markers; lane 2, HSS; lane 3, sample "MO", which showed monooxygenase activity.

10 mg of protein (run B) in a total volume of 200 µl with a total monooxygenase activity of 0.65 U were loaded on to the column. The constituent proteins were eluted at a flow rate of 0.2 ml/min, which achieved a separation of the two major proteins (figure 8.8).

The first sample corresponding to the peak marked "MO" had monooxygenase activity as determined by spectrophotometric assays after dialysis against Clark and Lubs phosphate buffer, pH 7.8. The total activity of this sample was 0.195 U giving a recovery of activity from this step of only 30%. However, a 2.3-fold purification factor was obtained in this step giving a 43.7 fold purification in total. These results are presented in table 8.5. From these results, it is clear, that the monooxygenase activity is separated from the major interfering activities by the above method. However, the recovery of activity was not sufficient to proceed to a bulk purification of the ion exchange eluent by gel filtration. Thus, an alternative solution was sought.

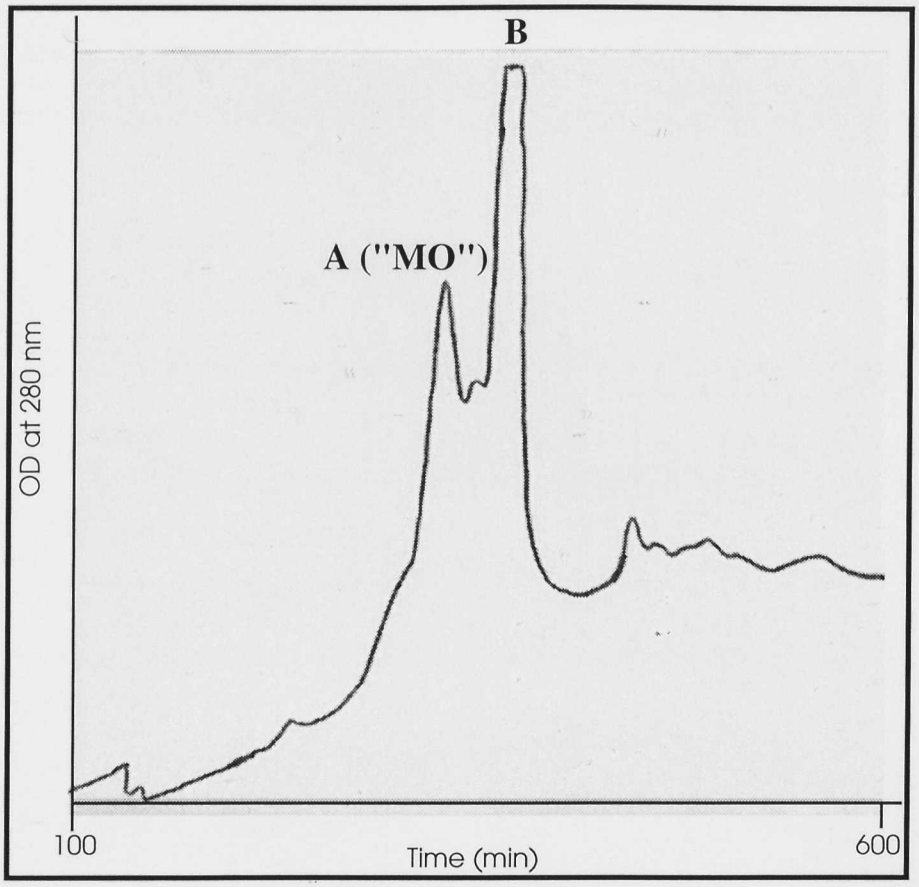


Figure 8.8: Elution profile of the Superose 12 gel filtration column loaded with the active sample from the DEAE Sephacel, run B.

In order to estimate the molecular weight of the monooxygenase, a calibration curve using standard proteins was produced using a gel filtration calibration kit purchased from Pharmacia. The proteins included in this kit and their molecular weights are shown in table 8.6. The calibration kit proteins were divided into two mixtures (table 8.6) before applying to the column, to achieve maximum resolution. Blue Dextran was used for the determination of the column void volume. The calibration curve (figure 8.9) is presented as the molecular weight of the protein standards versus their K_{av} (partition coefficient which represents the fraction of the stationary gel volume that is available for diffusion of a given solute species) (Harris

and Angal, 1989; Andrews, 1991; Pharmacia, calibration kit manual). The use of K_{av} is recommended instead of other elution parameters, because it is less sensitive to errors that may be introduced as a result of variations in column preparation and column dimensions. It does not require determination of the gel internal volume (V_i), which can be unreliable, as required for K_d (distribution coefficient), and it does not require accessory mathematical tables as is required for the calculation of erf^{-1} of K_d. The K_{av} for each protein was calculated by using the following equation and is presented in table 8.6:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume for the protein

V_o = column void volume, or elution volume for Blue Dextran 2000

V_t = total bed volume

The void volume was estimated as 6.3 ml based on the elution time of the blue dextran, and the total volume as 25 ml according to the manufacturers' instructions.

From this calibration curve the molecular weight of the monooxygenase was estimated to be between 130 and 150 kDa.

Although gel filtration has also been used by other researchers as a second step following an ion exchange chromatography step (Fox *et al.*, 1989; Fetzner *et al.*, 1992), in this study it was considered more useful as a relatively late step in the purification procedure, because of the low recovery of monooxygenase activity.

Table 8.5: Purification of α–pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671 by ion exchange (DEAE-Sephacel) and gel filtration (Superose 12) chromatography.

Sample	Total protein (mg)	Total activity (U)[a]	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
HSS	336	1.3	3.33	100	1
DEAE-Sephacel eluent	22.4	1.3	57.99	100	19
Sample applied to Superose 12	10	0.65	57.99	100	19
Superose 12 eluent	1.46	0.195	134.4	30	43.7

a: 0.01 mM FAD was added to all monooxygenase activity assays. Addition of $FeSO_4$ in the assay mixture did not have any effect on the activity obtained.

Table 8.6: Molecular weight calibration curve standards and their K_{av} values

Standard	Molecular Weight (kDa)	K_{av} Values
Blue Dextran	2,000	
Mixture I		
Thyroglobulin	669	0.0734
Catalase	232	0.237
Albumin	67	0.311
Chymotrypsin	25	0.463
Mixture II		
Ferritin	440	0.158
Aldolase	158	0.254
Ovalbumin	43	0.328
Ribonuclease	13.7	0.474

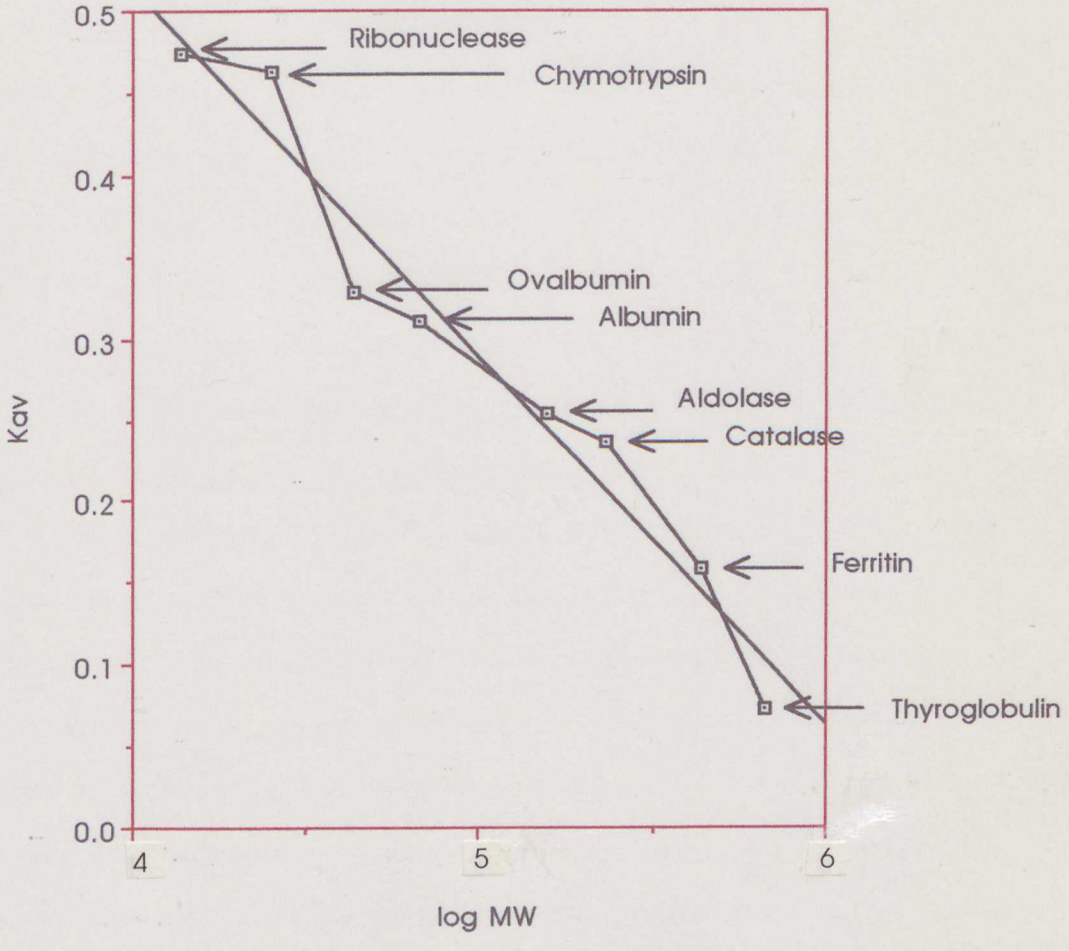


Figure 8.9: Molecular weight (MW) calibration curve for the Superose 12 gel filtration column.

8.5.3 Isoelectric Focusing and Titration Profile of the DEAE-Sephacel Eluent with α–Pinene Monooxygenase Activity

Gel filtration chromatography was not ideal as a second step for the purification of the α–pinene monooxygenase because of the low recovery of monooxygenase activity. Alternative strategies which were considered included a second ion exchange chromatography at a different pH, hydrophobic interaction chromatography, or chromatofocusing (Harris and Angal, 1989). A second ion exchange chromatography step has been used by a number of researchers for the purification of monooxygenases (Fox *et al.*, 1989; Haigler and Gibson, 1990; Whited and Gibson, 1991). whereas others, have used hydrophobic interaction chromatography following an ion exchange step (Schweizer *et al.*, 1987; Harpel and Lipscomb, 1990) for further purification of monooxygenases or dioxygenases. A titration profile of the DEAE-Sephacel eluent was therefore done to indicate which kind of chromatographic procedure would be more applicable in this case.

All molecules with ionisable groups can be titrated. Since biomolecules differ in their composition of buffering ions, each one has its own unique titration curve. The degree of difference between any two titration curves indicates the possibility for these substances to be resolved by ion exchange or chromatofocusing techniques. Each titration curve shows how the charge of a protein changes by increasing the pH. The pH versus net charge curve is a unique characteristic of a molecule and provides the basis for the selectivity of ion exchange and chromatofocusing. In titration curves obtained by electrophoresis the pH at which the curves are furthest apart is the pH at which the net charges of the proteins differ most. These curves are useful to predict how biomolecules are likely to be retained relative to one another in ion exchange, chromatography (Rosengren *et al.*, 1977; Righetti and Gianazza, 1980 and Haff *et al.*, 1983). Therefore, this technique can be a useful guide for choosing the correct media and the optimal pH for ion exchange chromatography and chromatofocusing.

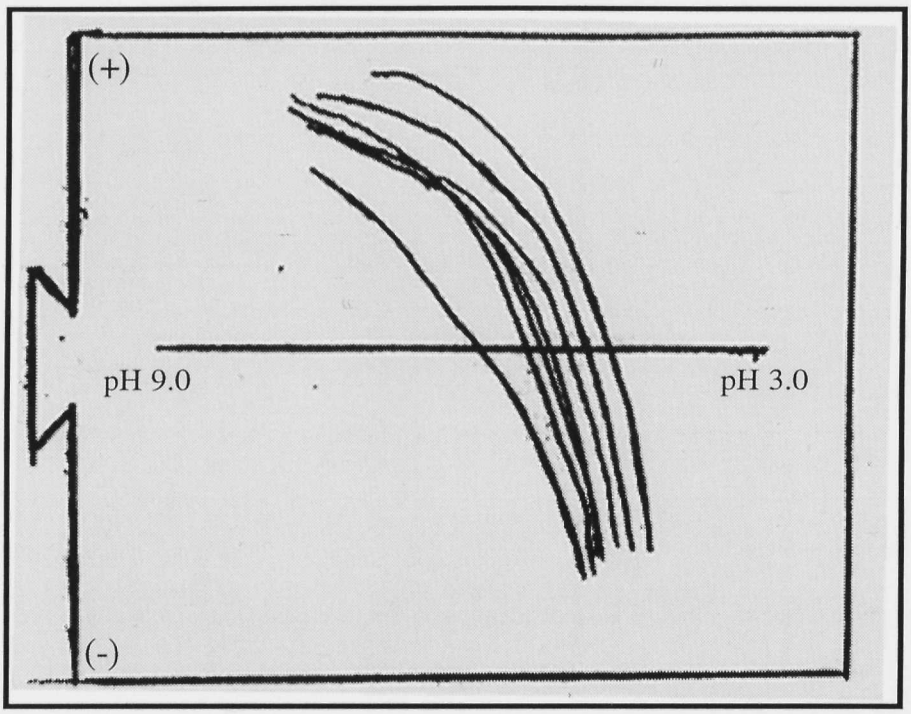


Figure 8.10: Titration profile of the protein components of the DEAE-Sephacel eluent (run A) with α–pinene monooxygenase activity.

The electrophoretic titration curve of the monooxygenase eluent from the DEAE-Sephacel column (run A) was generated by performing electrophoresis perpendicular to a stable pH gradient, on a Pharmacia PHAST electrophoresis system. Once the ampholytes were focused on the gel, electrophoresis was run perpendicular to the pH gradient. The sample components should move towards the anode or cathode depending on their net charge at any particular pH. Figure 8.10 shows the titration profile obtained for a pH gradient between 3.0 and 9.0. The curves of the protein components were nearly parallel, indicating that it was unlikely that an ion exchange chromatography step at any pH between 4.0 and 8.0 would give any better resolution than that already obtained. On the other hand, the bands appeared to be

well separated along the pH axis, between pH 4.0 and 7.0. Thus, a chromatofocusing step with a pH gradient between 4.0 and 7.0 seemed to be more promising.

8.5.4 Chromatofocusing of the DEAE-Sephacel Eluent with α–Pinene Monooxygenase Activity

Chromatofocusing is an analytical technique for separating biomolecules according to their isoelectric points. The essence of chromatofocusing is that the individual molecules initially bound to the column at one pH are eluted by a change in their net charge in a developing pH gradient. The presence of a pH gradient along the length of the column produces a focusing effect. Each component elutes at a point where the pH of the column effluent is approximately the same as the pI of that component (Sluyterman and Elgersma, 1978; Sluyterman and Wijdenes, 1978).

In order to confirm that a pH gradient between 4.0 and 7.0 was suitable, isoelectric focusing (IEF) of the "MO" eluent from the ion exchange run A was carried out with a pH gradient of 4.0-6.5, in the PHAST system. Isoelectric focusing is a high resolution method in which proteins are separated in the presence of an immobilised pH gradient. Under these conditions proteins migrate according to their charge until they reach the pH values at which they have no charge, i.e. their isoelectric point, pI. Figure 8.11 shows the IEF gel obtained, which confirmed that a chromatofocusing step with a pH gradient of 4.0-6.5 should be able to resolve the components of this sample.

A Mono P column was equilibrated with 25 mM Bis-Tris, pH 7.1, iminodiacetic acid buffer and the monooxygenase eluent from the ion exchange run A was also equilibrated in the same buffer by dialysis, prior to its application on the column. This buffer exchange resulted in a 12% reduction in the specific activity of the sample, from 57.13 mU/mg to 50 mU/mg. Then, a sixth of this sample, containing 20 mg of protein with a total activity of 1.0 U, was loaded onto the column and eluted with 46 ml (10 column volumes) of a 1:10 dilution of Polybuffer 74, pH 4.0, iminodiacetic acid, at a

flow rate of 0.5 ml/min. This system should provide a descending pH gradient between 7.0 and 4.0. The elution profile is presented in figure 8.12. As shown in figure 8.12, the monooxygenase sample from ion exchange run A contained four major proteins, and the elution profile obtained from this run also showed four major peaks, although not very well separated. Nevertheless, the tubes corresponding to each peak were pooled together and dialysed against Clark and Lubs phosphate buffer, pH 7.8. All samples were assayed spectrophotometrically for any α–pinene monooxygenase activity. However, none of the samples were active using the standard assay. When 0.01 mM of FAD was added to the reaction mixture only pool corresponding to the first peak marked as "MO" which had eluted at pH 6.1, showed some activity. However, when 0.1 mM $FeSO_4$ was added to the reaction mixture, the activity was 10 fold higher. These results suggest that α–pinene monooxygenase requires both FAD and iron for activity, and are in accordance with the preliminary results of Best *et al.* (1987) and Aikens (1990). In addition, it should be noted that this sample did not show any endogenous NADH oxidation activity which suggested that the monooxygenase enzyme had been successfully separated from all interfering activities.

The results obtained by this purification procedure are presented in table 8.8. It is evident that a remarkably high recovery (100% based on total activity), as well as a high purification efficiency (37.5-fold) were obtained. Therefore, this method could be applied for the large scale purification of α–pinene monooxygenase from *P. fluorescens* NCIMB 11671. The same procedure has been repeated two more times and exactly the same results obtained.

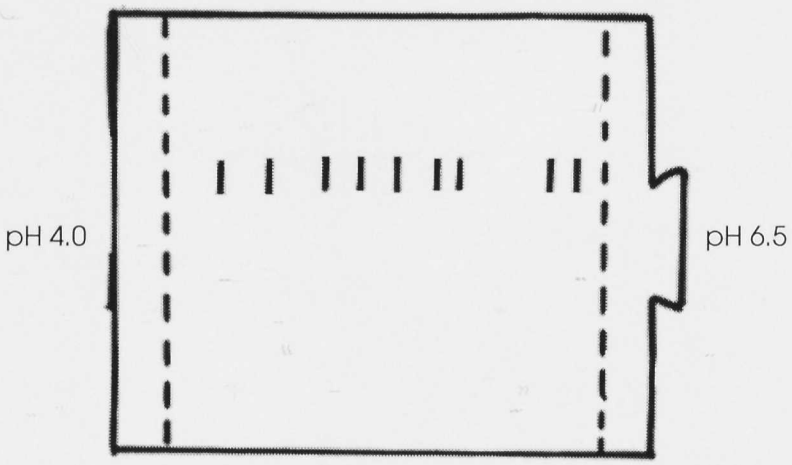


Figure 8.11: Isoelectric focusing of the DEAE-Sephacel eluent (run A) with α–pinene monooxygenase activity on a pH gradient 4.0-6.5.

Table 8.8: Purification of α–pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671 by ion exchange (DEAE-Sephacel, run A) and chromatofocusing (Mono P).

Sample	Total protein (mg)	Total activity (U)[a]	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
Crude extract	800	4.4	5.5	100	1
HSS	680	4.26	6.26	96.8	1.13
DEAE-Sephacel eluent	123	7.06	57.13	159	9.2
Sample applied on Mono P	20 (x 6)	1.0 (x 6)	50.0	159	9.2
Mono P eluent	3.6 (x 6)[b]	0.75 (x 6)[c]	206.15	100	37.9

a: 0.01 mM FAD was added to all monooxygenase activity assays; **b:** These results were multiplied by six, since only one sixth of the DEAE-Sephacel eluent was used per run.; **c:** 0.1 mM $FeSO_4$ was added to these assays.

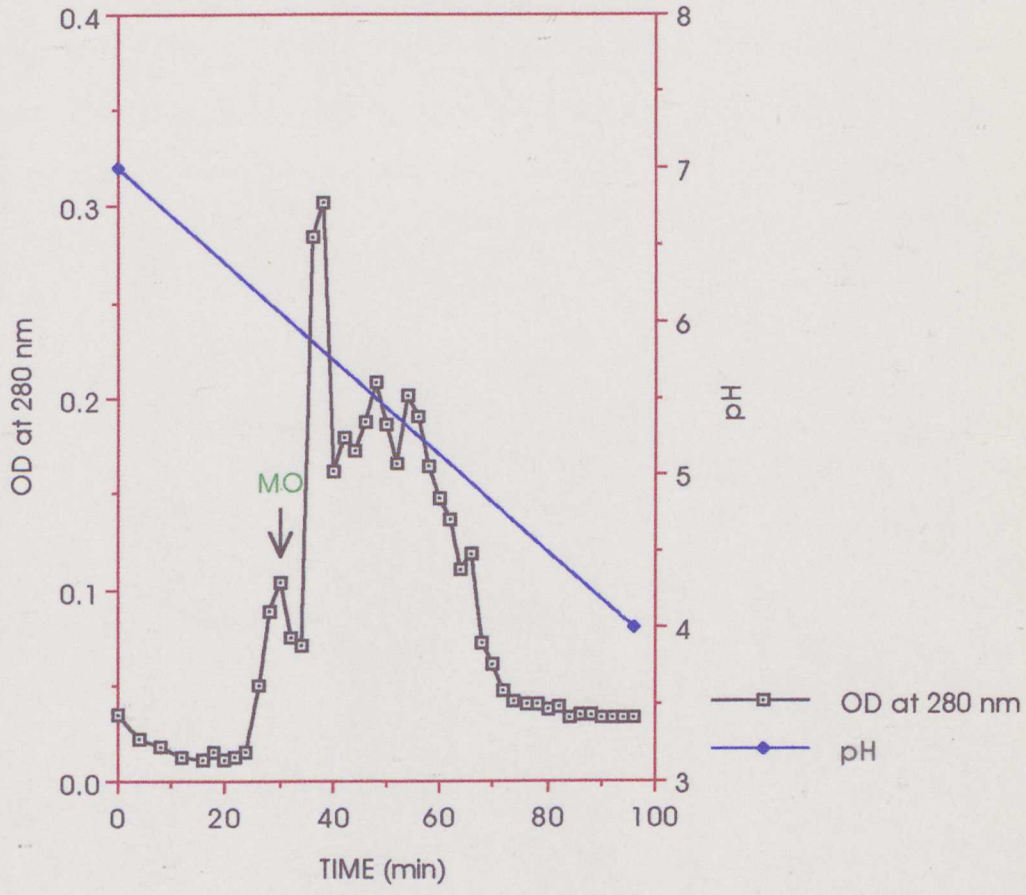


Figure 8.12: Chromatofocusing of the active fraction obtained from ion exchange chromatography (run A) on a Mono P column with a descending pH gradient from pH 7.0 to 4.0

Figure 8.13 shows a non denaturing PAGE profile of the Mono P eluents corresponding to the first two peaks of the elution profile presented above. The second lane corresponds to the "MO" sample, which contains only one major band. Densitometric scanning of stained gels indicated that this component comprised 90% of the total protein present. Thus, it was concluded that at least 80% purification of the monooxygenase enzyme had been achieved by using this procedure. Moreover, this band runs at the same place as band "c" detected in the native PAGE gel of the ion exchange eluent (figure 8.4) that showed monooxygenase activity and reductase activity (figure 8.5). Therefore, it appears that α–pinene monooxygenase is a single component system.

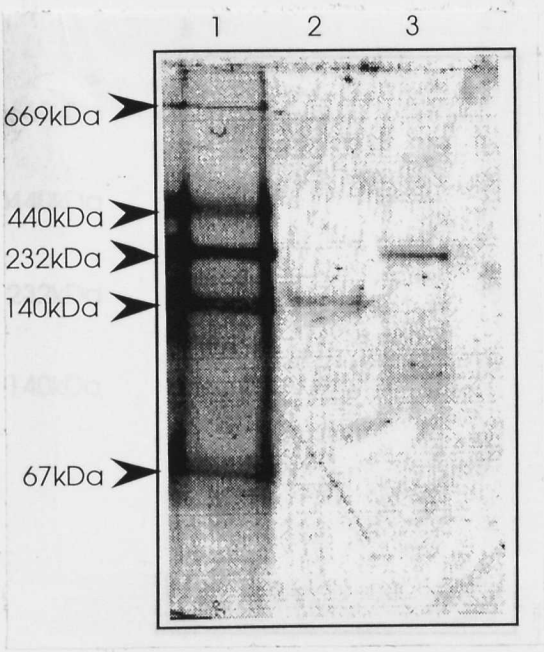


Figure 8.13: Non denaturing PAGE of the Mono P eluents. Lane 1, molecular weight markers; lane 2, sample with monooxygenase activity, "MO"; lane 3, sample corresponding to the third peak of the Mono P elution profile, with no monooxygenase activity. 50 μg of protein was loaded in each lane and the gel was stained with R-250.

It is well known that passage of a macromolecule through a polyacrylamide gel matrix is determined at least in part by its size and thus, it is not surprising that useful fractionation can be achieved on gels of graded porosity. In a linear gel gradient of increasing concentration the migration velocity of a band is inversely related to time and varies exponentially with the distance travelled. Part of the high resolution of such gels results from the fact that throughout the run the leading edge of any particular band is moving through more concentrated gel than the trailing edge, and hence encounters greater resistance, so that there is a band-sharpening effect. Gradient non denaturing gel electrophoresis was performed through a 5-20% polyacrylamide gel, containing 15% (w/v) sucrose as a density stabilising factor, for the estimation of the native molecular weight of the monooxygenase enzyme (Andrews, 1990). The calibration curve obtained by using a Pharmacia High Molecular Weight Protein Standard with proteins ranging from 67 to 660 kDa, is shown in figure 8.14 below. From this gel, it was estimated that the native molecular weight of the α–pinene monooxygenase enzyme is 145,000. This result is in accordance with the one obtained by gel filtration chromatography, where the active monooxygenase fraction was eluted at a molecular weight range of 130-150 kDa.

SDS electrophoresis of the purified monooxygenase sample revealed only one band at approximately 70 kDa (figure 8.15). Therefore, it appears that α–pinene monooxygenase is comprised of two identical subunits of approximately 70 kDa. Most of the flavin-containing monooxygenases studied so far have a subunit size of 60,000 to 80,000, such as 2,4-dichlorophenol hydroxylase from a strain of *Acinetobacter* sp. (Beadle and Smith, 1982), cyclohexanone oxygenase from *Nocardia globerula* and *Acinetobacter* NCIB 9871 (Donoghue *et al.*, 1976), Salicylate hydroxylase from *P. putida* (Yamamoto *et al.*, 1965), orcinol hydroxylase from *P. putida* ORC (Ohta and Ribbons, 1976), 6-aminohexanoate dimer hydrolase from *Pseudomonas* sp. NK87 (Kanagawa *et al.*, 1993) and others.

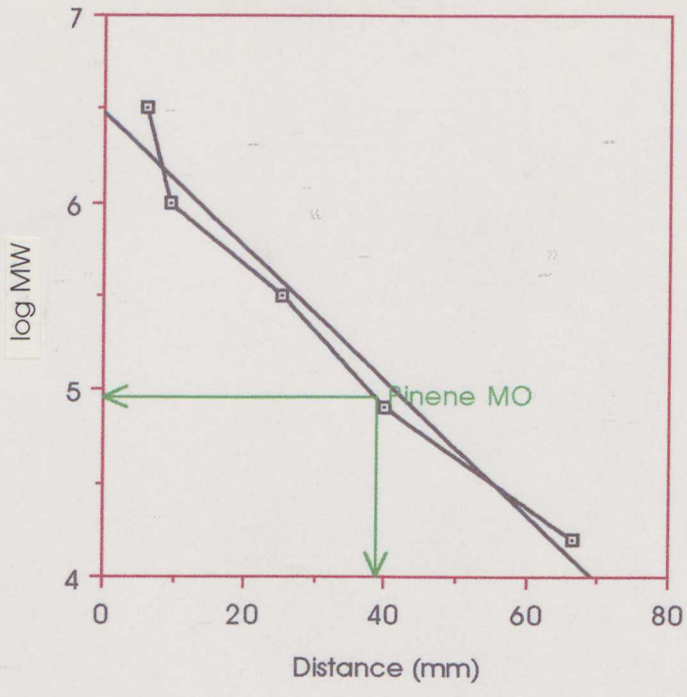


Figure 8.14: Calibration curve of the native gradient gel used for the determination of the native molecular weight of the α-pinene monooxygenase.

The Mono P eluent fraction with monooxygenase activity assayed spectrophotometrically was also tested for the ability to catalyse the epoxidation of α-pinene. 0.2 mg of protein were incubated at 35°C in 50 mM Clark and Lubs phosphate buffer, pH 7.8, with 1 mM of NADH, 0.01 mM FAD, 0.01 mM $FeSO_4$ and 2 μmole of α-pinene in a total volume of 1 ml, for 15 min. A control sample without any protein was also assayed. The reactions were extracted with dichloromethane pre-equilibrated at pH 9.0, and analyzed by capillary GC. A small amount of the epoxide was produced in the test sample but not in the control and the identity of the products was confirmed by GC-MS analysis (figure 8.16). Thus, it was confirmed that the protein

isolated by two protein chromatography steps, an ion exchange and a chromatofocusing, was able to catalyse the epoxidation of α–pinene in the presence of NADH, FAD, and $FeSO_4$

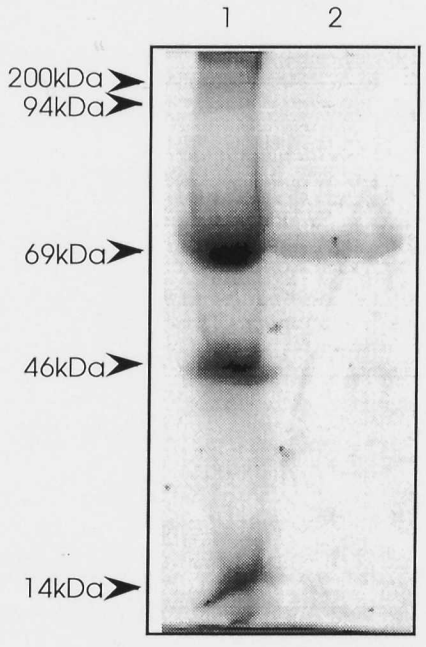


Figure 8.15: SDS-PAGE of the purified monooxygenase after chromatofocusing. Lane 1, molecular weight markers; lane 2, purified monooxygenase (fraction "MO" from Mono P).

It has been shown that the enzyme requires FAD for activity, with the apoenzyme being inactive. In order to determine the absorption spectrum and flavin content of the monooxygenase enzyme, 1 ml of enzyme solution from the Mono P column, containing 0.4 mg of protein was incubated at 4°C in the presence of 0.05 mM FAD for 30 min. Excess FAD was then removed by passing the solution down a PD 10 (Pharmacia) column (Beadle and Smith, 1982). Elution was carried out with 50 mM phosphate buffer, pH 7.8 containing 0.1 mM α–pinene. A control sample containing no

protein was also used. The absorption spectrum of the protein preparation exhibited maxima at 286, 368, and 450 nm (ABS: 0.161, 0.060 and 0.068 respectively). This spectrum is presented in figure 8.17 and it is similar to the spectra of flavin-containing monooxygenases (White-Steven and Kamin, 1972; Neujahr and Gaal, 1973; Donoghue *et al.*, 1976; Beadle and Smith, 1982; Locher *et al.*, 1991). The absorbance ratio at 368 nm/450 nm was 0.88, very similar to that of FAD, 0.82 (Sutton, 1955). The absorption spectrum of the apoenzyme as well as of the control sample, did not show any of these maxima (figure 8.17), The absorbance at 450 nm of FAD dissociated from 1 ml of enzyme solution containing 0.4 mg of protein (2.85 nmoles) was 0.068. Based on the molar absorption coefficient of FAD (e=11,300) (Takeda *et al.*, 1969) the amount of FAD dissociated was determined to be 6.01 nmoles suggesting that 2 moles of FAD are bound per mole of enzyme, and since the enzyme consists of two identical subunits, 1 mole of FAD is bound to each subunit.

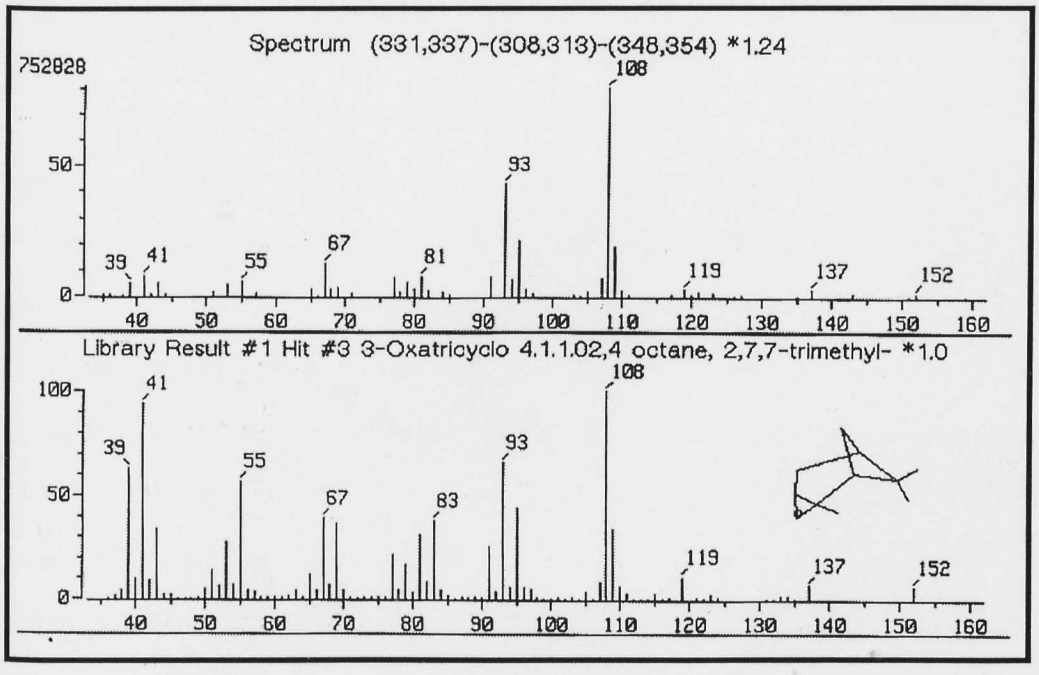


Figure 8.16: GC-MS spectra of α–pinene oxide detected in the reaction mixture of the Mono P eluent when the later was supplied with α–pinene.

Furthermore, the ratio A_{286}/A_{450} for the monooxygenase was 2.4 which is comparable to that reported for highly purified, flavo-iron sulphur proteins, such as methane monooxygenase reductase (Fox *et al.*, 1989), phthalate dioxygenase reductase (Batie *et al.*, 1987), benzoate dioxygenase reductase (Yamamuchi and Fuzisawa, 1978) and putidaredoxin reductase (Bernhardt *et al.*, 1975).

α–Pinene monooxygenase has been purified from interfering activities in a two step procedure, ion exchange chromatography at pH 8.0 followed by chromatofocusing over a pH range 7.0-4.0. However, the chromatofocusing used a small scale procedure. In order to perform larger scale chromatofocusing, a 50 cm x 1 cm column was packed with PBE 94 at 4°C and equilibrated with 25 mM Bis-Tris, pH 7.4, iminodiacetic acid. HSS containing 1.5 g of protein was loaded onto the DEAE Sephacel column and the same conditions as in Run A were applied (Run C). The active fraction, was equilibrated in 25 mM Bis-Tris buffer, pH 7.4, loaded onto the PBE 94 column, and eluted with 10 column volumes of a 1:10 dilution of Polybuffer 74, pH 4.1, iminodiacetic acid at a flow rate of 0.5 ml/min (figure 8.18). The procedure was carried out on a FPLC system at 4°C.

Unfortunately, the resolution was quite poor in this chromatographic procedure. However, tubes corresponding to each peak were pooled and assayed spectrophotometrically for α–pinene monooxygenase activity. The first peak, marked as "MO", eluted at pH 5.8 (tubes 30-40), showed monooxygenase activity after addition of FAD and $FeSO_4$, and again no endogenous activity was detected. The activity was confirmed by the production of both α–pinene oxide and limonene oxide detected by GC and GC-MS. The monooxygenase enzyme had eluted this time at a slightly lower pH than in the previous run. A second peak eluting at pH 5.6 also showed low monooxygenase activity suggesting distribution of the activity over a wide area, and thus the resolution was poorer with this run than with the previous one. The results of this purification procedure are presented in table 8.9 below.

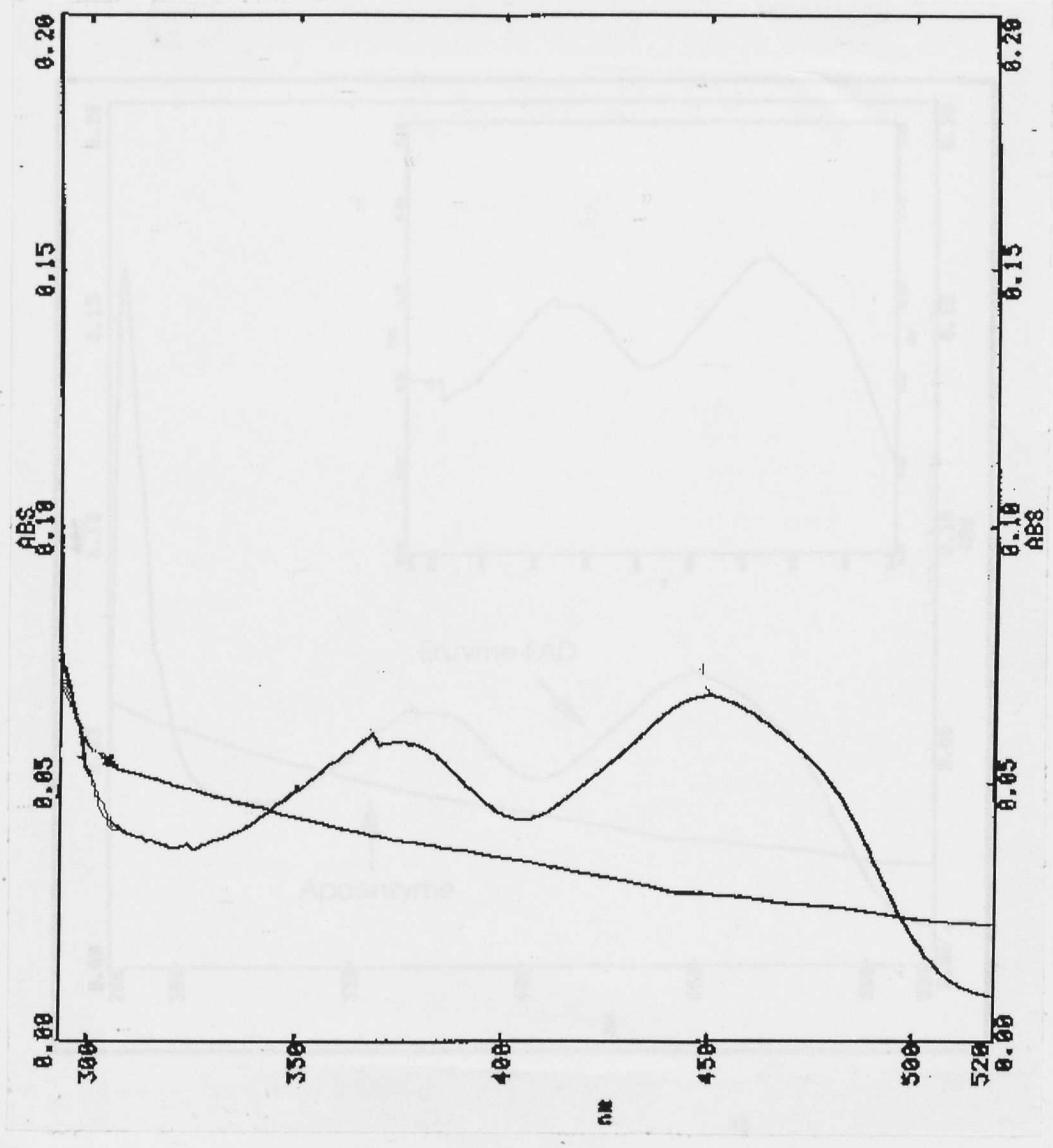


Figure 8.17: Absorption spectra of purified α–pinene monooxygenase containing FAD (absorption maxima at 286, 368, and 450 nm), and of the apoenzyme (no absorption maxima).

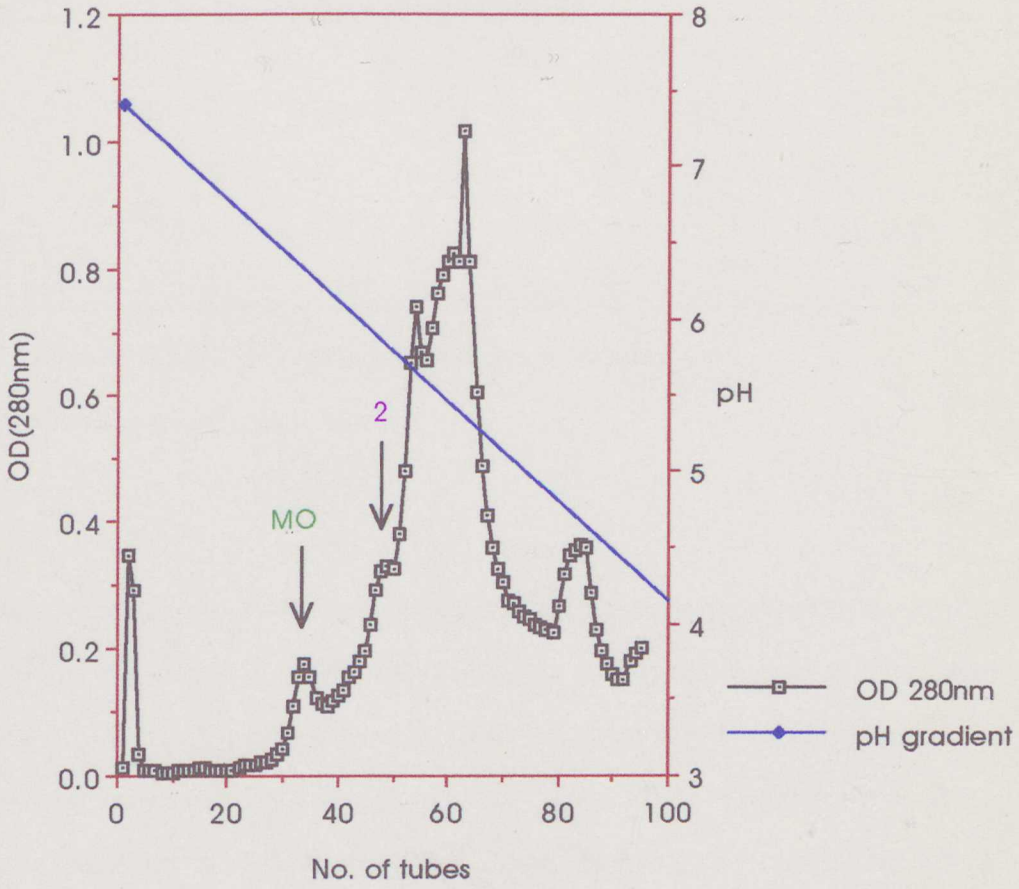


Figure 8.18: Chromatofocusing of the active fraction obtained from ion exchange chromatography (Run C), on a PBE 94 chromatofocusing column, with a descending pH gradient from pH 7.0 to pH 4.0.

Table 8.9: Preparative scale purification of α–pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671 by ion exchange (DEAE-Sephacel) and chromatofocusing (PBE 94) chromatography.

Sample	Total protein (mg)	Total activity (U)[a]	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
HSS	1,500	8	5.33	100	1
DEAE-Sephacel eluent	142	5.9	41.6	73.75	7.8
Sample applied to the PBE 74	96	4.0	41.6	73.75	7.8
PBE 74 eluent	9	1.53[b]	170.3	19.1	31.9

a: 0.01 mM FAD was added to all monooxygenase activity samples.

b: 0.1 mM $FeSO_4$ was added to these assays.

Unfortunately, both the recovery of activity and purification efficiency were not as high as in the previous example, probably because both columns were overloaded resulting in the loss of some protein. Additionally, the resolution of the chromatofocusing column used was not as high as the Mono P column used in the previous procedure. Moreover, the monooxygenase eluted at a lower pH than in all previous experiments. It has been reported that when working with large amounts of sample there may be a tendency for some proteins to elute slightly later than their normal elution pH (Pharmacia, 1988). This may be due to the protein precipitating at its isoelectric point as the gradient migrates down the column, and redissolving as the pH reaches a lower value. At this point it will be repelled from the matrix and will migrate down the column with the eluent, until it starts to precipitate again. The elution pH will thus be slightly lower than the pI of the protein.

Figure 8.19 presents a non denaturing PAGE of the PBE eluents that correspond to the first and second peak of the elution profile (figure 8.18). Densitometry of the gel

and integration of the peak areas showed that at least 80% purification had been achieved in the first band. Figure 8.20 shows an SDS-PAGE of the same samples. Again the monooxygenase reveals one major band at around 70 kDa, confirming that the enzyme is composed of two identical subunits. The second lane, corresponding to the second PBE sample with low monooxygenase activity contained one major band with a molecular weight of around 30 kDa and a number of minor bands. Both of these PBE samples as well as a HSS supernatant sample were incubated at 35°C in 50 mM phosphate buffer, pH 7.8 containing 0.01 mM of FAD, 0.1 mM of $FeSO_4$, 1 mM of NADH and 3 μmole of α–pinene for 15 min. A control sample that did not contain any protein was also prepared. The reaction mixtures were extracted in dichloromethane pre-equilibrated at pH 9.0 and analyzed by GC-MS. α–Pinene oxide was detected in the monooxygenase sample, but not in any of the other samples, including the control one. On the other hand, 6,6-dimethylbicyclo(3.1.1)-hept-2-ene-2-carboxaldehyde (myrtenal) (figure 8.21) was detected in both the PBE-2 sample and the HSS sample, but not in the control or the monooxygenase sample. This, as already proposed (Chapter 4), may be a product of an alternative pathway for α–pinene degradation catalysed by another monooxygenase or a damaged α–pinene monooxygenase. The fact that this product also accumulated in the HSS sample may suggest that the turnover number of the enzymes that are able to further metabolise it is very low and thus, such a pathway of α–pinene metabolism should be a secondary one.

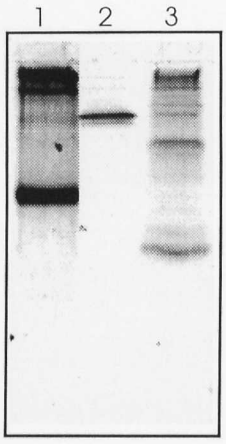


Figure 8.19: Native PAGE of the two active fractions obtained from chromatofocusing on a PBE 94 column with a descending pH gradient from pH 7.0 to 4.0. Lane 1, molecular weight markers; lane 2, sample with α–pinene monooxygenase activity, "MO"; lane 3, sample with low α–pinene monooxygenase activity (2).

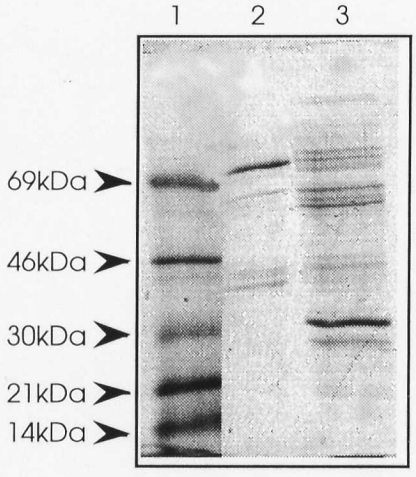


Figure 8.20: SDS-PAGE of the two active fractions obtained from chromatofocusing on a PBE 94 column with a descending pH gradient from pH 7.0 to 4.0. Lane 1, molecular weight markers; lane 2, sample with α–pinene monooxygenase activity, "MO"; lane 3, sample with low α–pinene monooxygenase activity (2).

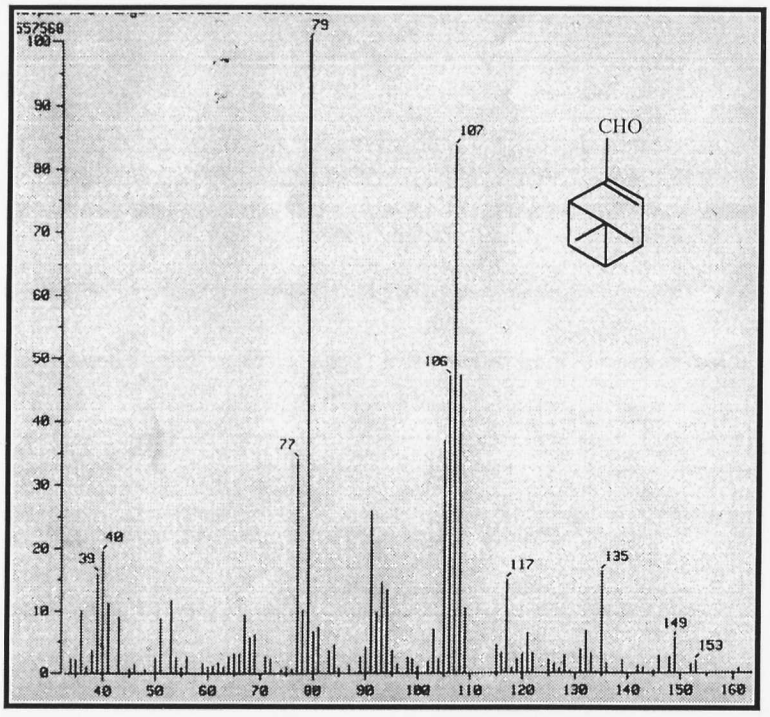
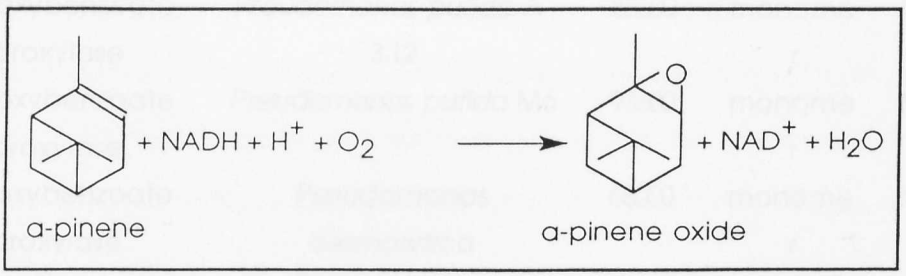


Figure 8.21: GC-MS spectra (structure inset) of the compound produced from PBE-2 sample (low monooxygenase activity) obtained from chromatofocusing on a PBE 94 column with a descending pH gradient from pH 7.0 to 4.0.

8.6 Discussion

α–Pinene monooxygenase is the first enzyme involved in the catabolism of α–pinene by *P. fluorescens* NCIMB 11671 (Best *et al.*, 1987; Griffiths *et al.*, 1987a; Aikens, 1990). The monooxygenase enzyme catalyses the conversion of α–pinene to α–pinene oxide and the reaction proceeds with equimolar consumption of oxygen and NADH as shown by Best *et al.* (1987), Aikens (1990) and also in this study (Chapter 3). The observed stoichiometry is consistent with the following equation:



α–Pinene monooxygenase has been purified to homogeneity in this study by a two step procedure, ion exchange chromatography and chromatofocusing. The recovery of the monooxygenase activity employing the DEAE-Sephacel and the Mono-P system was remarkably high (100%) and the purification factor was nearly 40-fold. Such high recovery of activity may reflect to the removal of inhibitors during the purification procedure. Approximately the same degree of purification was achieved by using the second system, DEAE-Sephacel and PBE-94, but this time the recovery of the monooxygenase activity was much lower, only 20% and this may be attributed to the overloading of the columns and the resulting distribution of the monooxygenase activity in more than one peaks.

Table 8.10: Comparison of flavin-containing aromatic monooxygenases of microbial origin (Beadle and Smith, 1982; Tsuji *et al.*, 1986; Walsh and Chen, 1988).

Enzyme	Organism	M W	Subunit composition	Electron donor
2,4-Dichlorophenol hydroxylase	*Acinetobacter* sp.	240000	tetramer	NADPH
3-Hydroxybenzoate 4-hydroxylase	*Pseudomonas testosteroni*	145000	dimer	NADPH
4-Hydroxybenzoate 6-hydroxylase	*Pseudomonas aeruginosa*	85000	monomer	NADH
4-Hydroxybenzoate hydroxylase	*Pseudomonas putida* A-3.12	83600	monomer	NADPH
4-Hydroxybenzoate hydroxylase	*Pseudomonas putida* M6	93600	monomer	NADPH
4-Hydroxybenzoate hydroxylase	*Pseudomonas desmolytica*	68000	monomer	NADPH
4-Hydroxybenzoate hydroxylase	*Pseudomonas fluorescens*	90000	dimer	NADPH
4-Hydroxyisophthalate hydroxylase	*Pseudomonas putida*	103000	dimer	NADPH
Melilolate hydroxylase	*Acinetobacter* sp.	65000	monomer	NADH
Melilolate hydroxylase	*Pseudomonas* sp.	250000	tetramer	NADH
Orcinol hydroxylase	*Pseudomonas putida* 01	65000	monomer	NADH
Orcinol hydroxylase	*Pseudomonas putida* ORC	70000	monomer	NAD(P)H
Phenol hydroxylase	*Trichosporon cutaneum*	148000	dimer	NADPH
Resorcinol hydroxylase	*Pseudomonas putida* ORC	70000	monomer	NAD(P)H
Salicylate hydroxylase	*Pseudomonas putida*	57200	monomer	NADH
Salicylate hydroxylase	*Pseudomonas* sp.	91000	dimer	NADH
4-Amino benzoate hydroxylase	*Agaricus bisporus*	49000	monomer	NAD(P)H

After 40-fold purification, α–pinene monooxygenase was shown by gel electrophoresis and densitometry of the gels to be 90% pure. The enzyme accounts for an estimated 0.5% of the total cytoplasmic cell protein (This is calculated by dividing the amount of protein recovered after the Mono P run by the starting amount of protein, assuming 100% recovery of the monooxygenase activity). The very low expression of this enzyme in conjunction with its high catalytic activity, detected in this study, may be explained by the established instability of α–pinene oxide in aqueous solution at near neutral pH because of the chemical reactivity of the three-member epoxide ring (Griffiths *et al.*, 1987b). Thus, a low level of monooxygenase expression suggests that production of α–pinene oxide would be the rate limiting step ensuring its complete conversion to 2-methyl-5-isopropylhexa-2,5-dienal. Moreover, the low expression of the monooxygenase enzyme may also explain the lack of apparent difference in the polypeptide content of cells grown on lactate or α–pinene as carbon source (Chapter 3).

Determinations of the relative molecular mass carried out under non denaturing conditions by both gel filtration and gradient PAGE and denaturing conditions by SDS -PAGE, suggest that the enzyme is a dimer with a monomer relative molecular mass of 70,000. This is characteristic of flavin-containing aromatic monooxygenases which have a relative molecular mass in the range of 60,000 to 90,000 (table 8.10). Several of the enzymes exist as dimers although a few are tetrameric, with a monomer relative molecular mass of 65,000.

After two steps of purification and subsequent dialysis the enzyme required addition of both FAD and $FeSO_4$ for reconstitution of its activity in terms of both NADH oxidation and α–pinene epoxidation. The absorption spectrum of the enzyme, shown in figure 8.17, is typical of a flavin-containing monooxygenase (Maki *et al.*, 1969; Takeda *et al.*, 1969; White-Steven and Kamin, 1972; Neujahr and Gaal, 1973; Donoghue *et al.*, 1976; Beadle and Smith, 1982; Liu and Chapman, 1984; Tsuji *et al.*, 1986; Haigler and Gibson, 1990; Locher *et al.*, 1991; Uetz *et al.*, 1992). The prosthetic group is FAD since FAD alone reconstituted active enzyme from apoenzyme, whereas other cofactors such

as FMN or riboflavin were unable to reconstitute the activity of the enzyme. It was proven in this study and also observed previously by Aikens (1990), that the FAD moiety is tightly bound to the enzyme at neutral pH and low ionic strength, but tends to dissociate at higher ionic strength even at pH 7.0. Similar results have also been obtained for other flavin-containing monooxygenases by other researchers (Maki *et al.*, 1969; Takeda *et al.*, 1969; Neujahr and Gaal, 1973; Donoghue *et al.*, 1976; Beadle and Smith, 1982). The molar ratio of FAD:protein calculated from the flavin absorbance at 450 nm was approximately 2:1. Therefore, since α–pinene monooxygenase is composed of two subunits, there is one molecule of FAD per enzyme subunit.

Aikens (1990) showed that ammonium sulphate precipitation of crude extracts from *P. fluorescens* resulted in a fraction obtained at ammonium sulphate concentrations of 1.4-2.0 M that showed low monooxygenase activity. Dialysis of this fraction against a 50 mM potassium phosphate buffer, pH 8.0 resulted in a totally inactive enzyme. However, after addition of FAD the enzyme regained its NADH oxidation activity but not its α–pinene epoxidation activity. On the other hand, dialysis of this fraction against the above buffer containing both FAD and ferrous iron resulted in an enzyme with complete reconstitution of the monooxygenase activity. Similar results were also obtained in this study. The enzyme obtained after two stages of purification had very low α–pinene monooxygenase activity. However, fully active enzyme was obtained after addition of FAD and $FeSO_4$ to this sample. It appeared however that the ferrous iron was not involved in a cytochrome P_{450} type prosthetic group. The system was not inhibited by low levels of CO (Aikens, 1990) and did not exhibit a spectrum associated with a typical reduced CO difference spectrum usually found with cytochrome P_{450} systems. Moreover, as it is discussed above, the ratio A_{286}/A_{450} for the purified monooxygenase enzyme is 2.4 which is comparable to that reported for highly purified, flavo-iron sulphur proteins (Bernhardt *et al.*, 1975; Yamamuchi and Fuzisawa, 1978; Batie *et al.*, 1987; Fox *et al.*, 1989). Therefore, it appears that α–pinene monooxygenase may be a flavo-iron sulphur protein. This suggestion could be further enhanced by the inhibition studies carried out by Best *et al.* (1987).

Addition of sulfydryl group and iron binding agents such as *p*-chloromerculiphenylsulfonate, 5,5-dithiobis-(2-nitro)-benzoate and 2,2'-bipyridyl in cell-free extracts resulted in 70 to 95% inhibition of α–pinene oxidation activity.

The presence of Fe^{2+} as a cofactor necessary for the activity of the system was not surprising based on the assumptions of Walsh and Chen (1988) who suggested the necessity of an iron centre in monooxygenases able to catalyse olefin epoxidations. The properties of the Fe^{2+} ion in the α–pinene monooxygenase system appear to be very similar to those observed in ω-hydroxylase. That is, that the ω-hydroxylase also lacks any distinctive absorption spectrum for oxidised or reduced forms, so making any elucidation of the role of the ferrous ion in the production of an active oxygen species difficult. However, the ferrous ion is required for the system to be able to catalyse its oxidative reactions, while removal of it causes loss of both the NADH oxidising and epoxidation catalytic properties of the enzyme. The Fe^{2+} ion can be removed by dialysis and the addition of Fe^{2+} to an apoenzyme preparation results in reconstitution of the activity (May and Katopodis, 1990). The fact that both FAD and Fe^{2+} are necessary for α–pinene monooxygenase activity, means that the FAD is almost certainly acting as a redox intermediate. If this were the case then, by analogy with other non haem iron monooxygenases, one might predict the presence of an iron sulphur centre involved in electron transfer from FAD to iron. However, a more detailed examination of their relationship in the activation of molecular oxygen would be required before any theories on the nature of the active oxygen species involved in the monooxygenase reaction could be advanced.

α–Pinene monooxygenase appears to be a remarkably stable enzyme, with cell paste showing no loss of activity when stored at -20 or -70°C for a period of at least one year. The stability of the enzyme appears to decrease with the purification. However, addition of dithiothreitol and glycerol to highly purified preparations protects against inactivation. The protection afforded by dithiothreitol, that was added to the enzyme storage buffer, against inactivation indicates that as with other monooxygenases (Neujahr and Gaal, 1973; Beadle and Smith, 1982), sulfydryl groups

are important for enzyme activity, and this suggestion is supported by the inhibition assays, carried out by Best *et al.*, (1987).

The substrate specificity of α–pinene monooxygenase appears to be very limited. It cannot oxidise saturated hydrocarbons or attack aromatic rings (Best *et al.*, 1987; Aikens, 1990). It can only attack substrates with a double bond in proximity to a methyl group. The requirement for a methyl substituent on the double bond may have a number of implications for both the substrate specificity and the mechanism of reaction employed by α–pinene monooxygenase. The methyl group may have been necessary to bind the substrate to the active site of the monooxygenase and to correctly orientate the double bond in relation to the active oxygen species. From a mechanistic point of view the methyl group may be necessary to stabilise a carbocation formed as part of a non-concerted reaction mechanism. The presence of the methyl substitution would stabilise, by donation of electrons, a tertiary carbocation on the methyl substituted methylene carbon. A secondary carbocation, lacking the methyl substitution, would probably not have sufficient stability to allow for the prolonged existence of the intermediate.

In summary, α–pinene monooxygenase is a cytoplasmic enzyme, that has an absorption spectrum similar to the spectra of many flavin-containing monooxygenases and appears to contain iron as a prosthetic group. It is a remarkably stable enzyme, especially when stored in potassium buffers containing glycerol and dithiothreitol. The enzyme has been purified 40-fold by ion exchange chromatography and chromatofocusing, and estimated to be more than 80% pure by gel electrophoresis. It is a dimer with a native molecular weight of 148,000, and contains 2 mole of FAD per mole of enzyme. Until it has been demonstrated whether the iron or the flavin is the oxygen activating component it is not possible to speculate on the unique reactivity of this enzyme. However, all monooxygenases studied to date, which involve iron as the oxygen activating cofactor are multicomponent, while, oxygenases which use flavins as the oxygen activating component are not

known to carry out epoxidation. Therefore, α–pinene monooxygenase could be a novel type of monooxygenase.

CHAPTER 9

Construction of a Genomic Library of *Pseudomonas fluorescens* NCIMB 11671

9.1 Introduction

A library of genomic DNA is a complete set of cloned fragments of the total DNA of an organism. Genomic libraries have traditionally been constructed in phage lambda or cosmid vectors introduced into *E. coli*. The λ DNA in a phage particle is a linear duplex molecule of about 49 kb. At each end of this molecule there are 12-nucleotide, protruding, single stranded cohesive termini, known as the *cos* sites. During normal replication of λ phage linear concatemers are formed containing tandem arrays of the λ genome, with *cos* sites spaced at approximately 49 kb intervals. These sites are essential for efficient packaging into λ heads *in vivo* and *in vitro*. The λ bacteriophage system selects DNA molecules about the size of λ genome, namely, 37 to 52 kb. The shortest λ DNA molecules that produce plaques of nearly normal size are 25% deleted. Therefore, λ vectors can accommodate foreign DNA inserts of about 20 kb. Cosmids are plasmids containing the cos sites necessary for packaging into λ heads, but no other λ-related components. In addition, they contain a drug resistance marker, a plasmid origin of replication and one or more unique restriction sites for cloning. Thus, the cosmid cloning system makes use of the *cos* sites but replaces the λ genome with the desired insert DNA. In this way, an average sized cosmid can use all of the packaging capacity of bacteriophage λ particle to accommodate inserts up to 45 kb. Therefore, cosmids are commonly used in the cloning of large genomes. Several advantages of cloning large DNA segments are that large genes can be isolated on a single recombinant clone; several linked genes can be isolated on the same recombinant molecule; fewer colonies need to

be screened to isolate the clone of interest; and finally, larger segments of the genome can be covered during "chromosome walking" experiments, thus, facilitating the analysis of gene linkage (Bates and Swift, 1983; Dilella and Woo, 1987; Frischauf, 1991).

However, there are several problems associated with cosmid cloning. Three main problems which have been encountered are vector concatemerisation resulting in cosmids lacking inserted DNA; recombinant rearrangement caused by multiple inserts ligated into a single cosmid; and differential growth of cosmid clones causing misinterpretation of sequences in amplified cosmid libraries. A cloning system which overcomes the first two problems has been described by Ish-Horowitz and Burke (1981). Concatemerisation of the vector, and insert DNA rearrangements can be avoided by a multiple enzyme digestion procedure, involving four separate restriction enzyme digestions and two S_1 nuclease digestions to prepare two cosmid arms, as well as phosphatase treatment of the DNA to be cloned, prior to its ligation to the vector. However, this technique is tedious, time consuming and the final yield of cosmid arms is disappointingly low.

The construction of vectors with two *cos* elements separated by a blunt-end restriction enzyme site considerably simplified the construction of cosmid DNA libraries. The use of two *cos* sites on a single plasmid eliminates the need to separately prepare two cosmid arms, each of which contains a single cohesive end. After cleavage at the blunt-end restriction site, cosmid concatemerisation does not occur during the subsequent ligation step when high ATP concentrations are employed (Bates and Swift, 1983). Therefore, vector pLAFR5 (Keen *et al.*, 1988) which contains tandem *cos* sequences and can accommodate DNA inserts of approximately 30 kb was used for the construction of a new genomic library of *P. fluorescens*. It is a broad host range vector that can be transferred to *Pseudomonas* spp. and many other gram negative bacteria by conjugation. Furthermore, Keen *et al.* (1988) observed that inserts in this vector were considerably more stable after introduction into *P. syringae* pv. *glycinea* than DNA maintained in cosmid vectors based on the

host range replicons RSF1010 or pRK2. The physical map of this vector is presented in figure 9.1.

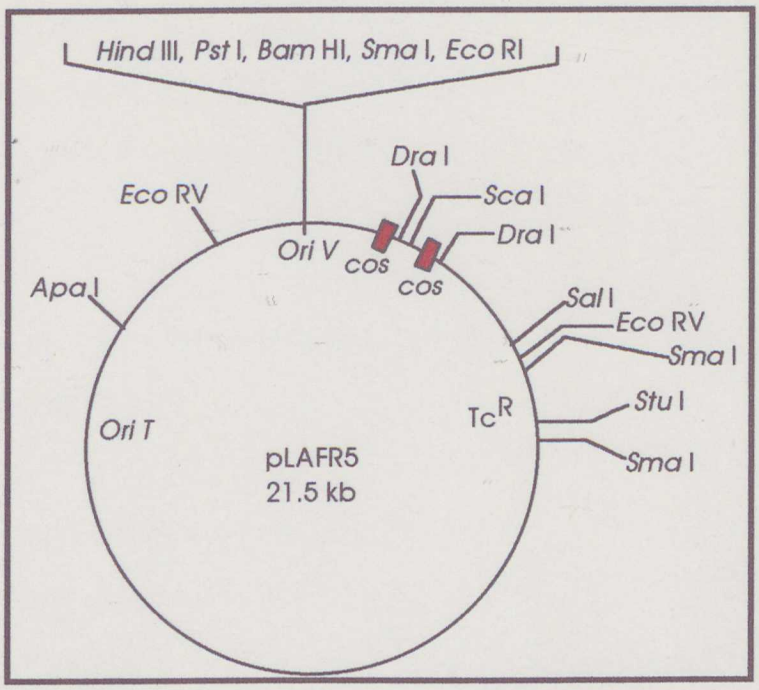


Figure 9.1: Physical map of the cosmid vector pLAFR5 (Keen *et al.*, 1988).

An ideal genomic library should represent the whole genome of the organism of interest. This task can be achieved by cloning of random DNA fragments of a large size (>20 kb). Since the DNA is randomly fragmented, there will be no systematic exclusion of any sequence. Furthermore, clones should overlap one another, giving an opportunity to "walk" from one clone to an adjacent one. The probability (P) of the presence of any given sequence in a library of N clones with an average insert size of f (as a fraction of the genome) is given by the following equation (Old and Primrose, 1989; Frischauf, 1991):

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

Therefore, in a genomic library of 613 clones with 30 kb chromosomal DNA inserts *P. fluorescens*, a given unique sequence will be present with a probability of 99%, assuming a chromosomal DNA size of 4,000 kb (Pemberton, 1974).

9.2 Isolation of High Molecular Weight Chromosomal DNA from *Pseudomonas fluorescens* NCIMB 11671

Since large fragments of DNA are required for cloning with the cosmid system, the *P. fluorescens* chromosomal DNA should be very large (>80 kb) before digestion. This requirement is one of the most critical steps in the cosmid cloning procedure and therefore, precautions were taken not to shear the genomic DNA. Any mixing or sampling of the DNA solution was performed by gentle inversion or gentle pipetting through a wide-tip pipette. The method used was a combination of these described by Dilella and Woo (1987) and Poustka and Lehrach (1988). Lysis of the cells was achieved after incubation with lysozyme and proteinase K. The resulting DNA pellet after RNAse treatment was slowly resuspended in TE buffer, by overnight incubation at 55°C. The purity of the DNA was determined spectrophotometrically (Sambrook *et al.*, 1989) by measuring the absorbance at 260 and 280 nm, and was found to be satisfactory since the ratio of OD_{260} versus OD_{280} was 1.83. The quality of the genomic DNA was also checked by electrophoresis through a 0.6% (w/v) agarose/TBE gel at 1 V/cm gel length. The gel was run at room temperature using TBE electrophoresis buffer. The results obtained are presented in figure 9.2. It is clear that the DNA isolated was of high molecular weight. A total of 1 mg of chromosomal DNA was isolated and stored at 4°C until needed.

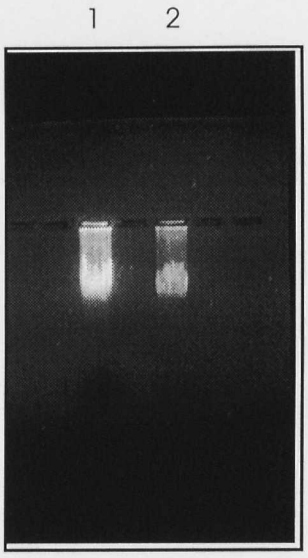


Figure 9.2: Chromosomal DNA from *Pseudomonas fluorescens* NCIMB 11671. Lane 1, chromosomal DNA from *P. fluorescens*; lane 2, intact λ DNA of 49 kb.

9.3 Large Scale Digestion and Preparation of Partially Digested Chromosomal DNA for Genomic Cloning

As mentioned above, an ideal library should contain cloned random DNA fragments representing the whole genome of the organism of interest. Such random fragments can be produced by partial restriction digestion of the chromosomal DNA with *Sau*3A or *Mbo* I. These enzymes have tetra- nucleotide recognition sites, which therefore occur frequently in the target DNA. The restriction is not allowed to go to completion, so that the bulk of fragments are relatively large, in the range of 10-30 kb. These are effectively a random set of overlapping fragments, carrying cohesive ends (Old and Primrose, 1989; Sambrook *et al.*, 1989).

In order to establish the optimum conditions for partial digestion of the chromosomal DNA, the latter was digested with different concentrations of *Sau*3A, for

a fixed incubation time of 1 hour at 37°C. Concentrations of enzyme ranging from 0.00375 to 4 units per µg DNA were utilised, and the reactions were terminated by chilling at 4°C and adding EDTA to a final concentration of 20 mM. The extent of restriction digestion was tested by electrophoresis through a 0.6% (w/v) agarose/TBE gel at 1 V/cm gel length. The gel was run at room temperature for 48 h using TBE electrophoresis buffer which was changed after 24 h of electrophoresis. Figure 9.3 presents the results obtained after restriction of 1 µg of the target DNA with 0.00375 to 0.03 units of *Sau*3A. The samples appear as smears of decreasing size range with increasing amounts of enzyme used. According to this, the optimum concentration of *Sau*3A for the production of DNA fragments with an average size of approximately 30 kb, was 0.00375 units/µg DNA. Sambrook *et al.* (1989) suggests that once the optimum concentration of enzyme for the production of appropriate DNA fragments is established in a small scale experiment, then, half the number of units should be used for large scale digestion of the target DNA.

Nevertheless, in order to confirm the results obtained, a fixed concentration of *Sau*3A of 0.0015 units/µg of target DNA was used to digest 1 µg of *P. fluorescens* DNA for a series of increasing times. The reactions were terminated as described above and examined on a 0.6% (w/v) agarose gel. The optimum results were obtained at incubation times of 50-60 min (figure 9.4). Therefore, for large scale digestion the genomic DNA should be incubated at 37°C for a period of 50-60 min with 0.0015 units of *Sau*3A per µg of DNA (Sambrook *et al.*, 1989).

200 µg of high molecular weight *P. fluorescens* DNA were digested using identical conditions to those in the small scale partial digestions. An aliquot of the digested DNA was analyzed by electrophoresis as before, and the rest was extracted with phenol/chloroform, precipitated with ethanol and dissolved in 200 µl of TE buffer. This DNA was stored at -20°C until needed.

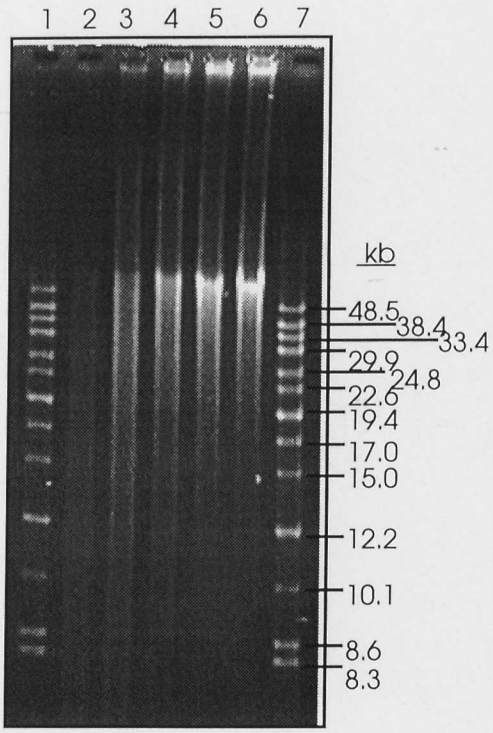


Figure 9.3: Establishing conditions for partial digestion of high-molecular-weight DNA. In this experiment 1 µg of *Pseudomonas fluorescens* NCIMB 11671 genomic DNA was digested with varying amounts of *Sau*3A at 37°C for 1 hr. Lane 1, high molecular weight DNA markers (Gibco, BRL); lane *P. fluorescens* chromosomal DNA digested with 2, 0.03 u/mg; lane 3, 0.015 u/mg; lane 4, 0.0075 u/mg; lane 5, 0.00375 u/mg; lane 6, uncut DNA; lane 7, high molecular weight DNA markers.

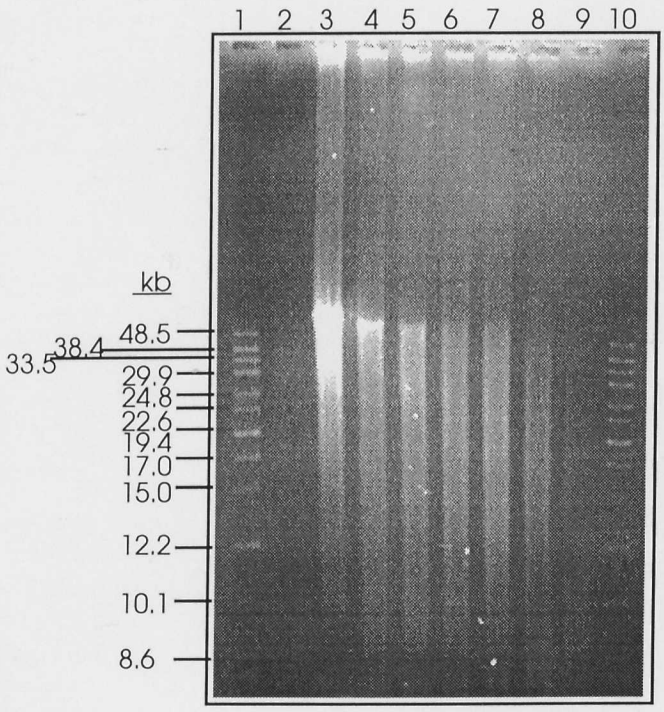


Figure 9.4: Establishing conditions for partial digestion of high-molecular-weight DNA. In this experiment 1 µg of *Pseudomonas fluorescens* NCIMB 11671 genomic DNA was digested with 0.0015 units of *Sau*3A at 37°C for a series of increasing times. Lane 1, high molecular weight DNA markers (Gibco, BRL); lane 2, uncut *P. fluorescens* chromosomal DNA; lane 3, chromosomal DNA digested using 30 min incubation; lane 4, 40 min incubation; lane 5, 50 min incubation; lane 6, 60 min incubation; lane 7, 70 min incubation; lane 8, 80 min incubation; lane 9, 90 min incubation; lane 10, high molecular weight DNA markers.

9.4 Size-Fractionation and Alkaline Phosphatase Treatment of Partially Digested Chromosomal DNA

In preparing a DNA library it is very important to avoid random co-ligation of insert DNA fragments since this would lead to cloned sequences that have no counterpart in the genome. There are two ways of preventing such ligation events; size-fractionation and alkaline phosphatase treatment of the insert DNA (Frischauf, 1991). Since phage λ has a strict size requirement for packaging DNA into phage heads, only molecules less than 105% the length of wild type phage can be packaged. Thus, if the insert DNA has been size-fractionated on a gradient or a gel, so that the ligation of two insert molecules and the required vector components produces a recombinant cosmid molecule that exceeds the λ packaging capacity, these molecules will not be packaged and therefore, not transferred into bacterial cells. Additionally, dephosporylation of the insert DNA will result in molecules unable to ligate to each other, but still able to ligate to vector DNA.

Both of the above procedures were used in order to minimise the risks of co-ligation of inserts that would result in a distorted view of the *P. fluorescens* genomic DNA. The partially digested DNA was size-fractionated in a 10-40% sucrose gradient by ultracentrifugation in a SW40 swing out rotor at 22,000 rpm and room temperature for 22 h. Following centrifugation, the bottom of the tube was punctured and fractions of 350 µl were collected. 10 µl of every third fraction were mixed with gel loading dye (Sambrook *et al.*, 1989) and analyzed by electrophoresis through a 0.6% (w/v) agarose/TBE gel at 1 V/cm gel length. The gel was run using TBE electrophoresis buffer, at room temperature, for 48 h. Figure 9.5 shows that fractions 15, 16 and 17 contain the optimum size DNA fragments, of approximately 30 kb. The three fractions were pooled together and diluted with TE so that their sucrose concentration would be less than 10% (w/v), and then precipitated with ethanol. Dephosphorylation of this DNA was carried out with 2 units of alkaline phosphatase at 37°C for 30 min. Proteinase K was then used to remove the phosphatase enzyme and the DNA preparation was

subsequently extracted with phenol/chloroform, precipitated with ethanol and finally dissolved in 20 ml of TE buffer.

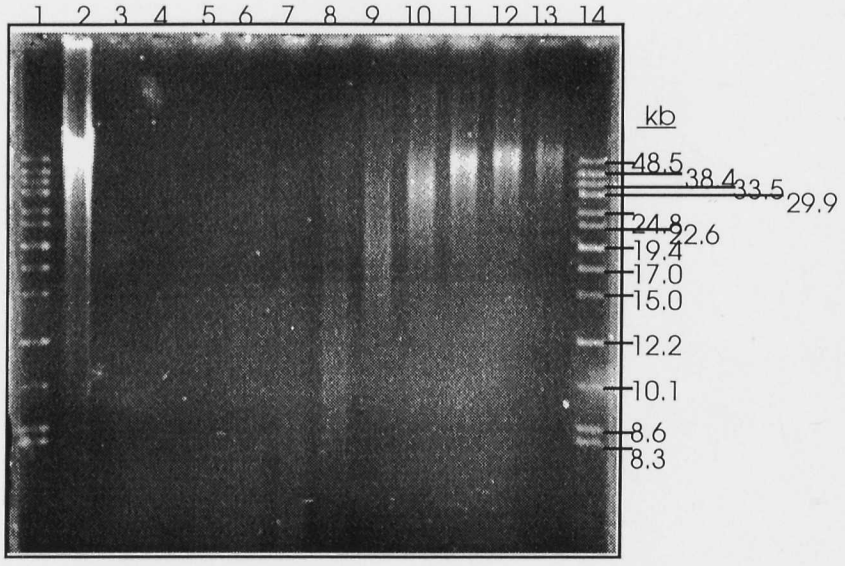


Figure 9.5: Size-fractionation of partially digested chromosomal DNA from *Pseudomonas fluorescens* NCIMB 11671 on a 10-40% sucrose gradient. Lane 1, high molecular weight DNA markers; lane 2, *P. fluorescens* chromosomal DNA uncut; lane 3, fraction 1; lane 4, fraction 3; lane 5, fraction 5; lane 6, fraction 7; lane 7, fraction 9; lane 8, fraction 11; lane 9, fraction 13; lane 10, fraction 15; lane 11, fraction 17; lane 12, fraction 19; lane 13, fraction 21; lane 14, high molecular weight marker.

9.5 Preparation of Cosmid pLAFR5 for Cloning

DNA of the cosmid vector pLAFR5 was kindly provided by Dr N.T. Keen (Department of Plant Pathology, University of California, USA). This DNA was electroporated into *E. coli* DH5α cells. The DH5α strain was used because it releases less carbohydrate during cell lysis than *E. coli* HB101 and also has lower endonuclease

activity, resulting in higher quality plasmid DNA preparations than HB101 (Sambrook *et al.*, 1989). pLAFR5 was then isolated from DH5α using the Qiagen purification procedure with a maxi-tip 500 yielding 200 µg of vector DNA.

Bam HI digestion of pLAFR5 produces complementary ends to *Sau*3 A fragments and was therefore, used for cleavage at the cloning site of the vector. The two *cos* sites of the vector were separated by *Sca* I which produces blunt ends. Thus, 50 µg of cosmid were digested first with *Bam* HI and then with *Sca* I. One unit of each enzyme per µg of DNA was used and the reactions were incubated at 37°C for 3 to 4 h. Following each digestion the reaction mixture was extracted with phenol/chloroform and the DNA precipitated with ethanol and potassium acetate. The digestions were analysed by electrophoresis through a 0.8% (w/v) agarose/TAE gel (figure 9.6). The double digested DNA was dissolved in 20 µl milli Q water and stored at -20°C until needed.

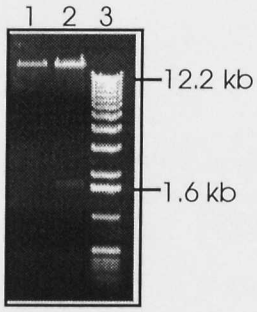


Figure 9.6: Restriction digestion of pLAFR5 with *Bam* HI and *Sca* I. Lane 1, pLAFR5/*Bam* HI; lane 2, pLAFR5/*Bam* HI-*Sca* I; lane 3, 1 kb DNA ladder (Gibco, BRL).

9.6 Ligation of Insert DNA to the Vector

The best yield of recombinant clones for a given amount of DNA is obtained in the presence of a large excess of vector (Frischauf, 1991). In practice, background

from linear cosmid vector molecules limits the vector excess that is desirable. In order to determine the appropriate amount of vector it is necessary to estimate the molar amount of insert DNA. The latter was calculated from the formula:

$$\frac{\mu gDNA \times 10^{-6}}{635 \times bp} \times 6.023 \times 10^{23}\ DNA\ molecules$$

Pilot reactions with various ratios of vector to insert DNA were set up, and it was found that the optimum vector:insert DNA ratio was 9:1. Therefore, this was used for the large scale ligation reaction. Ligations were conducted in 5 mM ATP to suppress blunt end ligation, so that vectors lacking inserts would not be packaged in phage λ heads (Keen *et al.*, 1988). Moreover, the total concentration of DNA in the ligation reaction was greater than 200 μg/ml in order to favour mixed concatemers between the arms of the cosmid vector and the *P. fluorescens* genomic DNA (Sambrook *et al.*, 1989). Two units of T_4 DNA ligase were added to the ligation mixture and the reactions incubated at 16°C for 18 h. A tenth of the ligation reaction mixture was analysed by electrophoresis though a 0.4% (w/v) agarose/TAE gel. Digestion of pLAFR5 with *Bam* HI and *Sca* I should result in the production of two fragments of 1.7 and 19.8 kb (figure 9.7). Since ligation was carried out at 5 mM ATP, blunt end ligation should not occur. Therefore, if the ligation reaction had been successful, the chromosomal DNA should be converted to high molecular weight concatemers that would appear as a smear on the agarose gel, and the arms of the cosmid DNA should be converted to homo- and heterodimers, that would appear as distinct bands on the gel. It is important to see ligation of both vector and chromosomal DNA and indeed that was the case. The following formations of cosmid arms appeared: (i), 39.8 kb (two large arms); (ii), 21.5 kb(a large and a small arm); and (iii), 3.0 kb (two small arms). This result suggested success of the ligation reaction. Products (ii) and (iii) are too small to be packaged, while product (i) consisting of two long arms, which is about the right size for being packaged in phage heads, would not survive in *E. coli* because the *oriV* region is present in the small arm of the cosmid.

9.7 *In Vitro* Packaging

In vitro packaging was carried out as described in Materials and Methods using the commercially-available Gigapack II XL Gold extracts (Stratagene). According to the manufacturers instructions these extracts preferentially size select extra large DNA inserts, with high packaging efficiency. Four µl of the ligation mixture containing 2.8 µg DNA were used. The efficiency of the packaging extracts depends not only on the extracts, but also on the DNA concentration, the individual DNA preparation, the order of addition of the extracts and the duration of the packaging reaction. This parameter should be tested with every new extract (Frischauf, 1991). Thus, the efficiency of packaging was calculated according to the manufacturers instructions, using *E. coli* strain VCS257 and wild type λ DNA-cI857Sam7 (both supplied). The efficiency was calculated from the formula below. According to the instruction manual 400 plaques on the 10^{-4} dilution corresponding to an efficiency of 60% should normally be obtained; 300 plaques were obtained in this case giving an efficiency of packaging of 50%.

$$Efficiency\ of\ packaging = \frac{(no.\ plaques) \times 10^{-4}}{(no.\mu g\ DNA) \times (no.\mu l\ plated)}$$

Before plating the library, the titre of the packaged material was determined using the batch of cells that was also used for the preparative plating. Therefore, 25 µl of 10 and 50 fold dilutions of the *in vitro* packaged extracts were mixed with the same amount of *E. coli* DH5α cells that had previously been treated with magnesium sulphate. The number of colony forming units (CFU) was calculated from the following formula:

$$CFU = \frac{no.\ of\ colonies\ on\ the\ plate}{\mu g\ of\ genomic\ DNA\ used}$$

Titres usually range from 100,000 to 800,000 transformants per microgram of size-fractionated chromosomal DNA (Dilella and Woo, 1987). The titre obtained from the 50 fold dilution was 800,000 and thus the remaining ligation mixture was packaged and titred in the same way.

9.8 Amplification of the Genomic Library

Lambda and cosmid libraries can either be amplified or screened directly. The advantage of amplification is that it provides an almost inexhaustible supply of library that is viable for very long periods if properly stored. The main disadvantage of amplification is that it can severely distort the concentration of each independent recombinant in the mixture because of different amplification of different clones. Therefore, as a matter of principle, it is always preferable to use a primary library. Primary cosmid libraries can be stored at -70°C on filters soaked with the appropriate freezing medium for very long times. However, the limiting factor in the use of primary libraries is often the quality of the filters that are being hybridised over and over again, with eventual loss of signal. Therefore, it may be more convenient to amplify a library and then to plate it out whenever it has to be screened with a set of probes (Frischauf, 1991).

Thus, 6.5 ml of 1/50 fold dilution of packaging extracts were mixed with the same amount of competent DH5α cells, with an optical density at 600 nm of 0.5, and the amplification step was carried out as described in Materials and Methods. The library was divided into three fractions that were amplified independently by keeping the products of 23 x 23 cm plates separate. This would prevent over-amplified clones from appearing at a high frequency in the whole library, should restrict different over-amplified clones to different fractions, and would ensure that independent clones would be isolated if another fraction was screened (Frischauf, 1991). The titre obtained after the amplification step was the same as above, and the amplification factor was

$1.44x10^6$. The library was stored as three separate fractions in LB broth containing 15% (v/v) glycerol and 25 µg/ml tetracycline at -70°C, and was plated out when needed.

9.9 Testing the Genomic Library for the Presence and the Size of Inserts

According to Keen *et al.* (1988), the use of size-fractionated DNA in the construction of genomic libraries with pLAFR5 should result in less than 1% of clones lacking suitable inserts. Therefore, the genomic library of *P. fluorescens* NCIMB 11671 was tested for the presence and the size of inserts before screening. Insets cloned into pLAFR5 disrupt the expression of β-galactosidase (Kenn *et al.*, 1988) and therefore, the existence of inserts could be confirmed by plating the library on LB agar plates containing X-gal and IPTG. Only 5 out of 500 clones in the 10^{-6} dilution were blue and thus, 1% of the clones did not contain any insert. Cosmid DNA was isolated from fifty of the plated clones, using the alkaline lysis method, restricted with *Eco* RI and analysed by gel electrophoresis. This revealed that 4% of the library clones contained inserts of approximately 15 kb instead of the expected 30 kb, while the rest contained 27 to 34 kb inserts.

9.9 Discussion

A genomic library of *P. fluorescens* NCIMB 11671 in *E. coli* DH5α has been constructed using the broad host range cosmid vector pLAFR5. This vector was chosen because it is very stable in *Pseudomonas* spp. (Keen *et al.*, 1988) and thus, could be used directly for complementation purposes. The amplified library had a total CFU number of 800,000 and an amplification factor of $1.44x10^6$. Furthermore, only 4% of the clones had inserts smaller than expected, and 1% of clones had no insert at all.

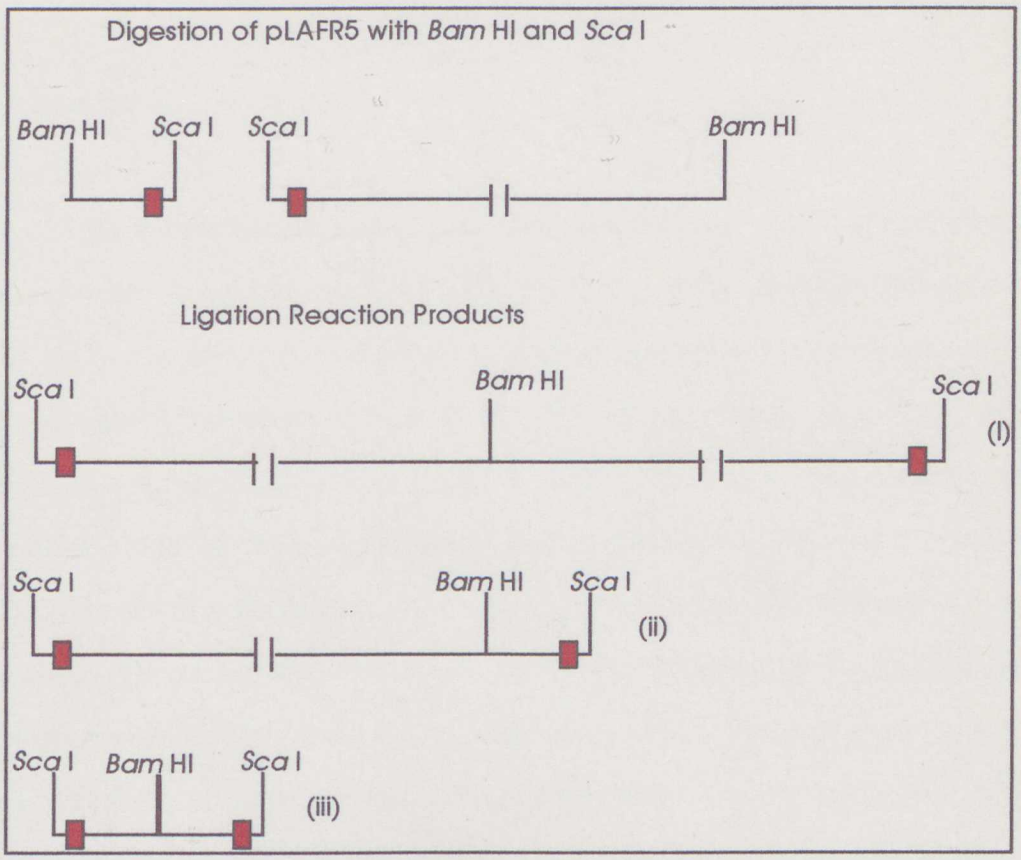


Figure 9.7: Expected products from the ligation reaction of vector DNA converted to homo- and heterodimers on an agarose gel. The red square represents the *cos* sites.

CHAPTER 10

Screening of the Genomic Library of *Pseudomonas fluorescens* NCIMB 11671 with the 1.7 kb Fragment

10.1 Introduction

One of the principle uses of cloning technology is to isolate specific genes directly from the genome. However, any particular gene represents only a very small part of the bacterial chromosome. In order to identify such a small proportion, an appropriate screening procedure should be followed. Generally, genetic, immunochemical, south-western, nucleic acid hybridisation, and recombinational methods could be followed. Genetic methods involve expression of the inserted foreign gene in the background of an appropriate mutant so that its function can be detected by complementation. Immunochemical detection of clones synthesising a foreign protein is also based on the expression of the inserted gene. A particular advantage of the latter method is that genes that do not confer any selectable property to the host can be selected. However, it requires that a specific antibody is available. Nucleic acid hybridisation is a very powerful approach that uses a highly labelled radioactive probe of RNA or DNA whose hybridisation with the gene is assayed by autoradiography. Finally, the recombinational method is based upon homologous recombination of a phage λ clone carrying the desired sequence, with a probe sequence which inserted into a specially constructed plasmid vector pVX in the *E. coli* host (Old and Primrose, 1989).

Screening of cosmid libraries is a relatively easy process, since cosmids accommodate large DNA fragments and thus, only a small number of clones need to be screened. The genomic library of *P. fluorescens* was screened to identify the small

proportion of cosmids harbouring the α–pinene monooxygenase gene. In order to achieve 99% probability of the presence of any given sequence assuming an insert size of 30 kb, 613 clones should be screened (Frischauf, 1991). This could readily be accomplished by DNA hybridisation using the 1.7 kb insert of pAC1 as a probe in a colony hybridisation procedure. An overview of the technique is illustrated in figure 10.1. Bacterial colonies carrying chimeric vectors are lysed on nitro-cellulose or nylon filters, then their DNA is denatured *in situ* and fixed on the filter. Subsequently, the filter is hybridised with a radioactively labelled probe based on the desired sequence, and any colonies containing homologous sequences are identified by autoradiography. Then the corresponding clones can be recovered from the original reference library.

10.2 Preparation of the Probe

The 1.7 kb insert from pAC1 was used for screening the genomic library of *P. fluorescens* in pLAFR5 and isolation of cosmids containing the α–pinene monooxygenase gene. Therefore, pAC1 DNA was isolated by the large scale plasmid purification procedure using CsCl/Et-Br gradient centrifugation, and 20 µg of the plasmid digested with 10 units of *Hind* III at 37°C for 3 h. An aliquot of the reaction mixture was removed and analysed for complete digestion by electrophoresis through a 0.8% (w/v) agarose/TAE gel, and the rest was loaded on a 0.7% (w/v) ultra pure agarose (BRL) gel and electrophoresed overnight in TAE buffer at 2 V/cm gel length. The gel was visualised under a long wave length ultraviolet light and the 1.7 kb band was excised from the gel with a sterile razor blade. The DNA was recovered from the agarose slice by the "freeze-squeeze" method. This involved freezing the agarose band and centrifuging out the liquid containing the DNA. The ethidium bromide was removed by phenol/chloroform extraction and the DNA precipitated with absolute ethanol and recovered in a small volume of TE buffer. The amount of

DNA obtained by this method was calculated by the gel spot method (Sambrook *et al.*, 1989) to be 8 µg.

The probe was labelled with ^{32}P using the "random primed" DNA labelling method developed by Feinberg and Vogelstein (1984). The method is based on the hybridisation of a mixture of all possible hexanucleotides to the DNA to be labelled. The complementary strand is synthesised from the 3'OH termini of the random hexanucleotide primers using the Klenow enzyme. Neither the amount or the length of the DNA fragment to be labelled influence the reaction, and since high activities are obtained by this method only minimal amounts of DNA are required (Feinberg and Vogelstein, 1984). The Random Primed DNA Labelling kit from Boehringer-Mannheim was used for the labelling of 100 ng of DNA. The reaction was terminated by heating at 60°C for 10 min, and the unincorporated radiolabel was removed by passing the DNA down a quick spin Sephadex G-50 column. The probe was obtained in 400 µl TE buffer and calculated to have 2,850 cpm/µl.

10.3 Preparation of the Hybond N$^+$ Nylon Filters

The genomic library of *P. fluorescens* was plated out on 2YT agar plates containing 25 µg/ml tetracycline. 700 single colonies were aseptically transferred with a toothpick to 96 well microtitre plates containing 2YT broth and tetracycline (25µg/ml). The plates were incubated overnight at 37°C and then replica transferred in duplicate onto Hybond N$^+$ membranes placed on 2YT plates containing tetracycline and incubated at 37°C overnight. The next day, the resulting colonies were lysed with SDS and Tris-HCl, pH 7.4 and NaOH, neutralised with NaCl, and the denatured DNA was fixed on the membrane by exposure under UV light of 312 nm for 3 min.

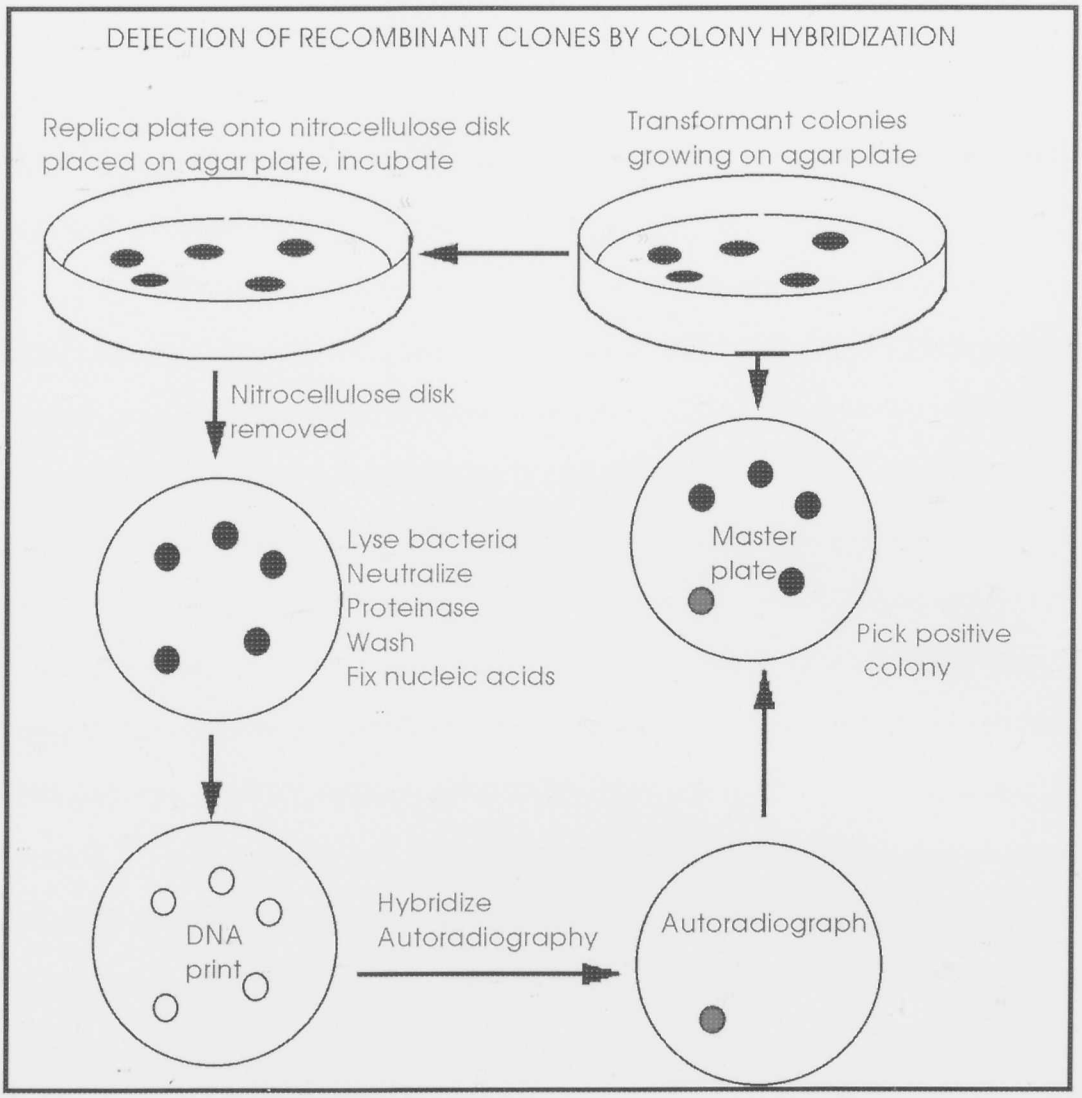


Figure 10.1: Overview of colony hybridisation procedure, for the detection of the recombinant vector carrying the gene of interest.

10.4 Hybridisation of the Library Clones with the 1.7 kb Probe

Factors that affect the rate and efficiency nucleic acid hybridisation of a nucleic acid probe to a target sequence immobilised on a solid support such as a nylon membrane include hybridisation time, temperature, concentration of the target sequence, and concentration of the probe. Other variables that usually improve the hybridisation reaction include the use of rate enhancers and blocking agents to suppress non-specific hybridisation.

Nucleic acid molecules can tolerate a certain number of mismatched base pairs and still form stable duplexes, provided a significant number of base pairs do match and form bonds. The degree of mismatch which can be tolerated in a hybridisation reaction and still maintain a double stranded molecule depends on the environmental conditions. Conditions favouring considerable mismatch, namely low stringency, are low temperature, low concentrations of formamide and high salt concentrations. On the other hand, under conditions of high stringency, high temperature and low salt concentrations, only exact matches of DNA will anneal (Old and Primrose, 1989). Therefore, since more than 800 bp of the 1.7 kb probe would provide an exact match with insert DNA that contains the monooxygenase gene, conditions of high stringency were applied. Additionally, tRNA from *E. coli* supplied from Boehringer-Mannheim was used as a blocking agent to eliminate non-specific binding.

A prehybridisation step should greatly reduce non-specific probe hybridisation. The prehybridisation and hybridisation solutions should be identical for long DNA probes such as the one used in this study. Thus, the Hybond N^+ membranes containing the denatured DNA were prehybridised at 65°C with 25 ml of hybridisation solution for 3 h. Then, 70 µl of the double stranded DNA probe, containing 200,000 cpm, denatured by boiling for 5 min, was added to 10 ml of fresh, hybridisation solution prewarmed at 65°C. The membranes, were hybridised in the latter solution at 65°C for 16 h, in a Techne hybridisation oven, followed by four high stringency washes at 65°C,

for 15 min each as described in Materials and Methods. The membranes were exposed to autoradiography film at -80°C and a strong signal was obtained after 30 min of exposure. The pAC1 recombinant plasmid was used as positive control and the pLAFR5 cosmid vector as negative control. As expected, the probe bound to the positive but not to the negative control DNA. Four of the library colonies exhibited hybridisation in duplicate tests, and thus, were picked from the master plates and inoculated into fresh 2YT broth containing 25 µg/ml tetracycline for subsequent studies.

In order to confirm that the library clones isolated by colony hybridisation contained a part or all of the 1.7 kb fragment, cosmids were isolated by alkaline lysis (Sambrook *et al.*, 1989), named as pAC3, pAC5, pAC6 and pAC7 and digested with *Eco*R I, *Dra* I and *Eco*R I/*Sca* I, *Dra* I/*Pvu* II. One unit of each enzyme per µg of cosmid DNA was used and the reactions incubated at 37°C for 3 h. An aliquot of the reaction was analysed for complete digestion by electrophoresis through a 0.7% (w/v) agarose/TAE gel and the rest was loaded on a 0.7% (w/v) agarose/TAE gel and electrophoresed overnight at 2 V/cm gel length. The next day the gel was blotted onto two Hybond N$^+$ membranes as described in Materials and Methods. One of the membranes was hybridised with the 1.7 kb probe under exactly the same conditions as above. The autoradiography film was developed after 30 min of exposure and showed that all four cosmids hybridised with the 1.7 kb fragment. Again, pLAFR5 was used as negative control which did not show any hybridisation. Therefore, these four clones were presumed to contain all or a part of the monooxygenase gene(s).

10.5 Complementation of the Monooxygenase Minus Mutants with pAC3, pAC5, pAC6 and pAC7

E.coli colonies containing the recombinant plasmids pAC3, pAC5, pAC6 and pAC7 were conjugated with the monooxygenase minus mutants, AC4, AC5 and KS507

using the plate (patch) matings method. After the triparental matings the cultures were mixed with a loop and streaked onto minimal plates containing 25 µg/ml tetracycline, and incubated at 30°C under α–pinene vapour, for 4 days. Non conjugated mutants were streaked on nutrient agar plates containing tetracycline, on minimal plates, as well as minimal plates containing tetracycline as controls. Minimal plates were incubated under α–pinene vapour as above. Table 10.1 shows that non conjugated mutants were unable to grow on α–pinene in the presence of tetracycline, whereas conjugated mutants were able to grow under both the above conditions.

Table 10.1: Complementation studies between mutants lacking α–pinene monooxygenase activity and the four library clones isolated after colony hybridisation of the *P. fluorescens* NCIMB 11671 genomic library with the 1.7 kb probe (pAC3, pAC5, pAC6 and pAC7).

Mutant	NA[1]	NA/tc	Minimal medium /α–pinene	Minimal medium /Tc /α–pinene
AC4 (pin^-pox^+)	+	-	-	-
AC5 (pin^-pox^+)	+	-	-	-
AC10 (pin^-pox^+)	+	-	-	-
KS507 (pin^-pox^+)	+	-	-	-
AC4(pAC3,5,6,7)[2]	+	+	+	+
AC5(pAC3,5,6,7)	+	+	+	+
AC10(pAC3,5,6,7)	+	+	+	+
KS507(pAC3,5,6,7)	+	+	+	+

1: NA is nutrient agar.
2: mutant containing one of the pAC3, pAC5, pAC6 or pAC7 recombinant plasmids.

10.6 Restriction Mapping of pAC3, pAC5, pAC6 and pAC7

The ultimate aim in characterising a gene is to determine its nucleotide sequence and to extend the sequence into neighbouring genes on either side. Having isolated a segment of DNA a crucial step before obtaining its sequence is to map the nucleic acid at the molecular level.

Restriction mapping of the recombinant cosmids isolated by colony hybridisation would assist in the identification of the shortest DNA fragment required for subcloning and sequencing of the α–pinene monooxygenase gene. To achieve this, the recombinant cosmids were isolated on a large scale on a CsCl/Et-Br gradient. 1.5 mg of each cosmid was isolated. Single and double digestions of these cosmids were carried out using various restriction endonucleases as shown in table 10.2. In single digestions the 10x enzyme buffer supplied by the manufacturer was used, while in double digestions the one-for-all buffer (OFA) (Pharmacia) at the appropriate dilution was used. When digestion with different concentration of buffer were required in double digestions, the enzyme that required the higher buffer concentration was used first and then the reaction was diluted and the second enzyme was added. Routinely, each digestion was performed using 20 μg of cosmid DNA and 2 units of enzyme per μg of DNA. The reactions were incubated at 37°C, except for *Sma* I where incubation was at 25°C, for 3 h. The reactions were terminated by chilling on ice and the DNA fragments were analyzed by electrophoresis through a 0.7% (w/v) agarose/TAE gel, at 4 V/cm gel length overnight. Restriction mapping revealed that pAC3 had an insert of 25 kb, pAC5 had an insert of 27 kb, pAC6 had an insert of 30 kb and pAC7 had an insert of 28 kb. The restriction maps obtained are presented in figures 10.2 and 10.3. The common regions among the four plasmids are underlined with a red arrow. It was observed that all common regions lay in the *Eco*R I-*Eco*R I fragment of pAC3 (figure 10.2) and therefore, it was evident that the 1.7 kb probe should hybridise within this DNA region. Thus, this plasmid was chosen for further subcloning procedures.

Table 10.2: Restriction endonucleases used in single and double digestions of pAC3, pAC5, pAC6 and pAC7.

Single digestions	Buffer	Double digestions	Buffer (OFA*)
*Eco*R I	React 3 (NBL)	*Eco*R I/*Pst* I	2X/1X
Pst I	H (Boehringer)	*Bam*H I/*Ssp* I	2X/2X
*Bam*H I	E	*Pst* I/*Hind* III	1X/1X
Ssp I	E	*Pvu* II/*Hind* III	1X/1X
Hind III	B	*Dra* I/*Hind* III	1X/1X
*Eco*R V	D	*Bam*H I/*Hind* III	2X/1X
Pvu II	B	*Eco*R I/*Hind* III	2X/1X
Dra I	B	*Eco*R V/*Hind* III	2X/1X
Sal I	C	*Sal* I/*Hind* III	2X/1X
Sma I	React 4 (NBL)	*Kpn* I/*Hind* III	1X/1X
Kpn I	A	*Sca* I/*Hind* III	2X/1X
Sac I	A	*Sma* I/*Hind* III	1X/1X
Ava I	B	*Sac* I/*Hind* III	1X/1X
Xba I	D	*Ava* I/*Hind* III	2X/1X
Sca I	H	*Xba* I/*Hind* III	1X/1X
Hpa I	A	*Hpa* I/*Hind* III	1X/1X
Bgl II	D	*Bgl* II/*Hind* III	1X/1X
Cla I	C	*Cla* I/*Hind* III	1X/1X
*Bst*E II	D	*Bst*E II/*Hind* III	2X/1X

*: OFA is the One For All buffer from Pharmacia.

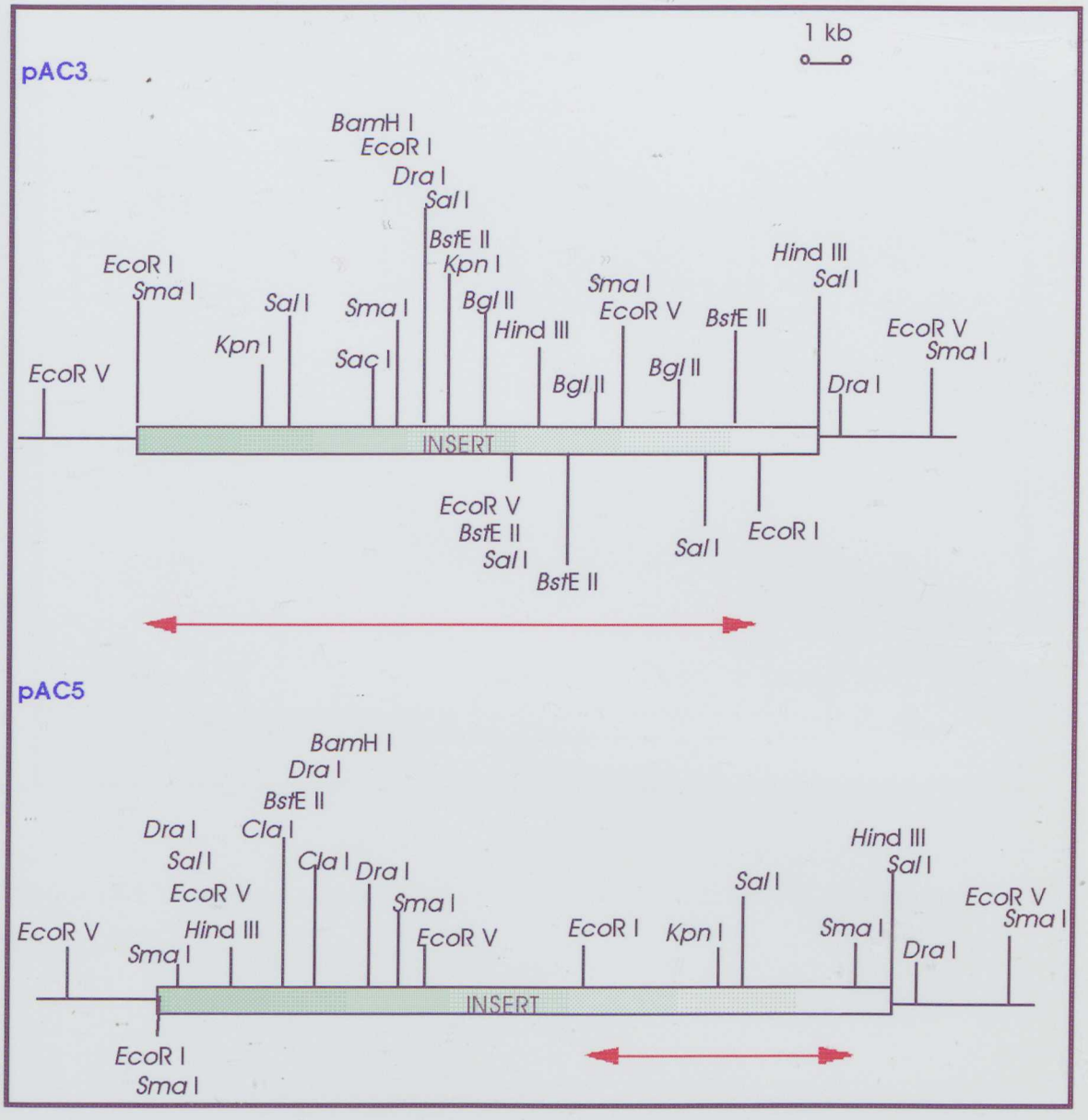


Figure 10.2: Restriction maps of pAC3 and pAC5. The red arrows correspond to the common regions among the four clones.

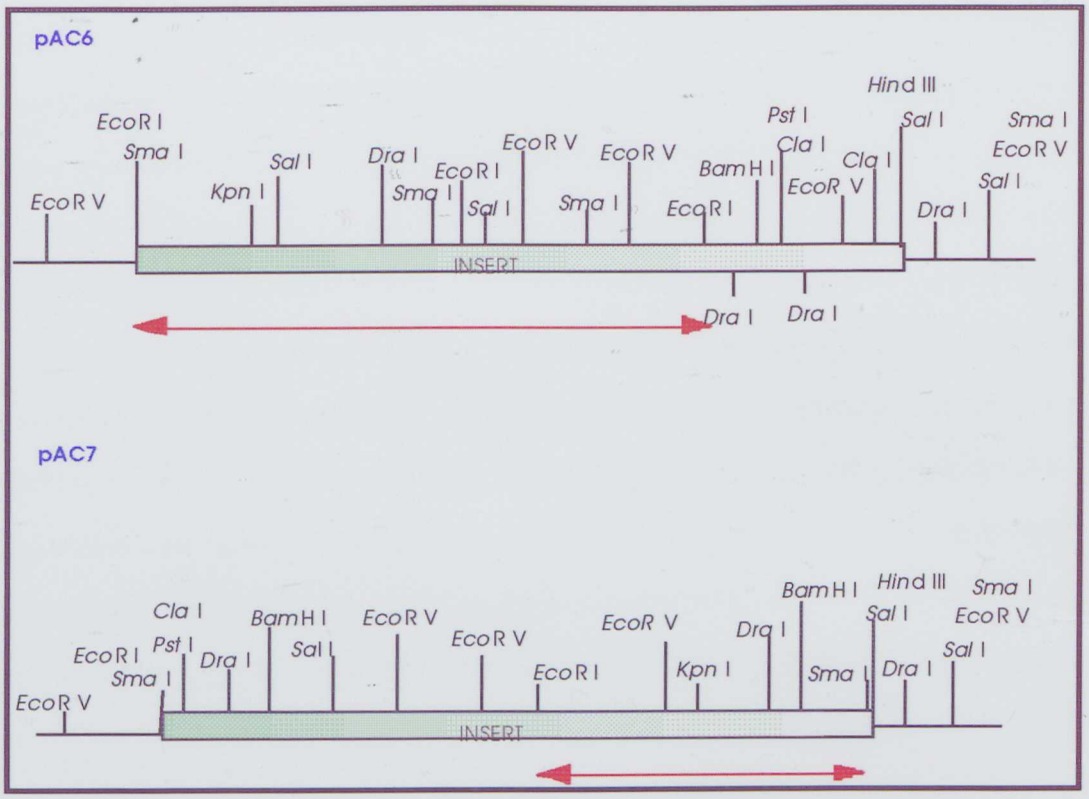


Figure 10.3: Restriction maps of pAC6 and pAC7. The red arrows correspond to the common regions among the four clones.

10.7 Subcloning of the 5.3 kb *Sma* I Fragments of pAC3

In order to identify the most suitable pAC3 restriction fragment(s) for subcloning, 10 µg of pAC3 were digested as described above with the following restriction enzymes: pAC3/*Pvu* II; pAC3/*Sma* I; pAC3/*Eco*R V; pAC3/*Eco*R I; pAC3/*Pst* I; pAC3/*Kpn* I; pAC3/*Eco*R I-*Hin*d III; and pAC3 *Bam*H I-*Hin*d III. The reaction products were electrophoresed through a 0.7% (w/v) agarose/TAE gel at 4 V/cm gel length overnight. The next day the gel was blotted onto Hybond N^+ nylon membrane. The DNA transfer was carried out for 3 h and the DNA fixed on the gel by crosslinking at 312 nm for 3 min. The membrane was subsequently hybridised with the 1.7 kb probe under the same conditions as above, and then exposed on an autoradiography film at -80°C. The film was developed after 10 min of exposure (figure 10.4) and showed that the probe was hybridising with the 2.7 kb *Pvu* II fragment; the 5.3 kb *Sma* I fragment; the ≈ 9 kb *Eco*R V fragment; the ≈ 5 kb *Eco*R I fragment; the 0.7 kb *Pst* I fragment; the 6.7 kb *Kpn* I fragment; the 9 kb *Eco*R V-*Hin*d III fragment and the 27 kb *Bam*H I-*Hin*d III fragment. pLAFR5 was again used as negative control. As illustrated in figure 10.5 the common region for all these fragments which presumably contains the hybridisation site of the probe is within the *Sma* I-*Kpn* I restriction fragment (blue arrow). Therefore, since the orientation of the probe was not known the 5.3 kb *Sma* I-*Sma* I, the 6.2 kb *Sma* I-*Sma* I and the 9 kb *Eco*R V-*Eco*R V fragments (red arrows) were subcloned, in order to be certain of obtaining all of the monooxygenase gene for sequencing.

20 µg of pAC3 DNA were digested with *Sma* I and another 20 µg with *Eco*R V. Two units of enzyme per µg of DNA were used and the reactions were incubated at 25°C for *Sma* I and 37°C for *Eco*R V for 3 h. The reactions were terminated by chilling on ice and analysed for complete digestion by electrophoresis through a 0.7% (w/v) agarose/TAE gel. The rest of the reaction was loaded on an 0.7% (w/v) ultra pure agarose gel and electrophoresed overnight at 4 V/cm gel length in TAE buffer. The next day the gel was visualised at long UV light and the bands of interest were excised from the gel with a sterile razor blade and purified by the Geneclean procedure as

described in Materials and Methods. Approximately 800 ng of each fragment were obtained and stored at -20°C until needed.

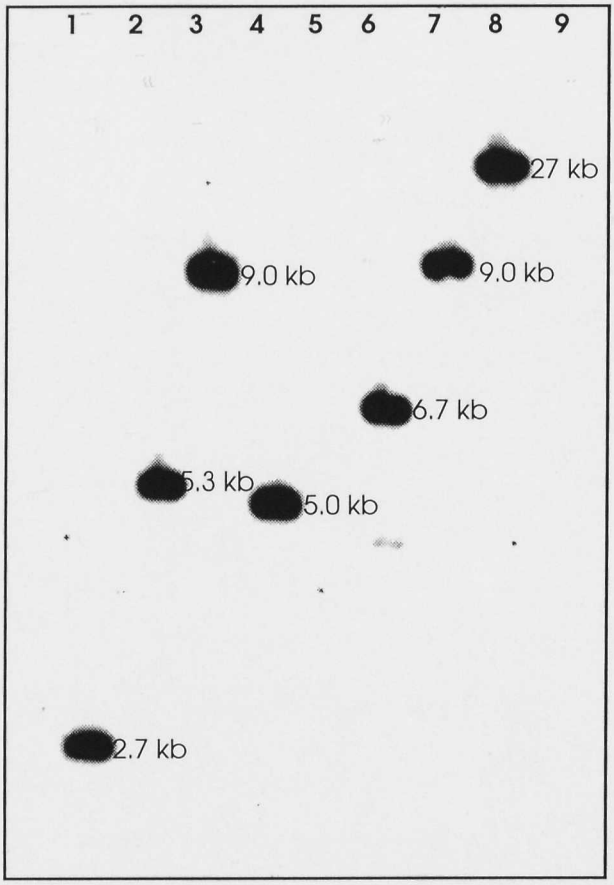


Figure 10.4: Southern blot of pAC3 restriction digestion reactions hybridised with the 1.7 kb probe. Lane 1, pAC3/*Pvu* II; lane 2, pAC3/*Sma* I; lane 3, pAC3/*Eco*R V; lane 4, pAC3/*Eco*R I; lane 5, pAC3/*Pst* I; lane 6, pAC3/*Kpn* I; lane 7, pAC3/*Eco*R I-*Hin*d III; lane 8, pAC3 *Bam*H I-*Hin*d III; lane 9, pLAFR5.

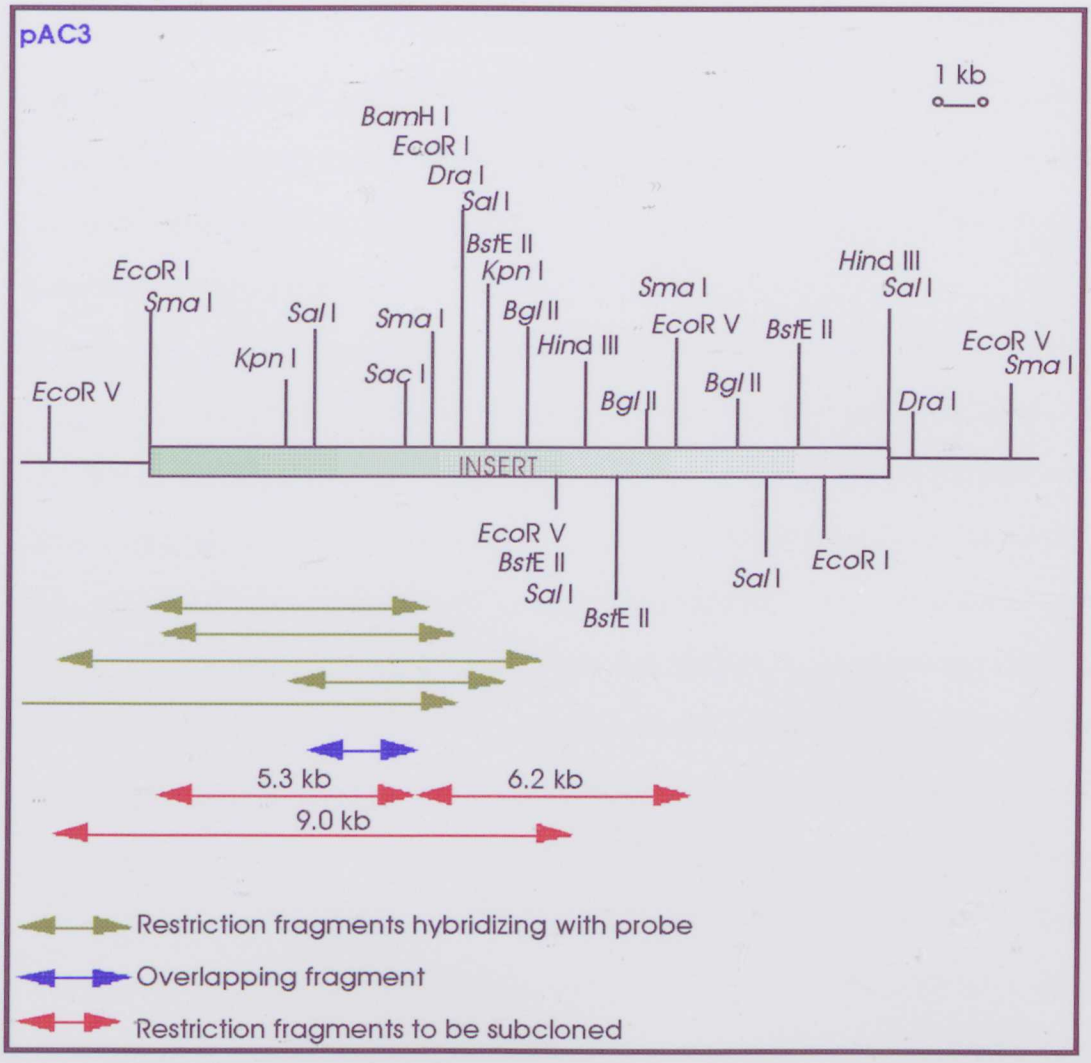


Figure 10.5: Illustration of the hybridisation results of pAC3 with the 1.7 kb probe as well as of the subcloning procedure to be followed.

The pBluescript II SK(+) vector from Stratagene was used for the subcloning of the fragments because it is a small, (2.9 kb) very high copy number vector which was designed for sequencing. It also contains the β-galactosidase gene associated with its multicloning site, thus allowing for blue-white colony screening on X-gal/IPTG plates. pBluescript II SK (+) DNA was kindly provided by K. Hantzandonakis and electroporated into *E. coli* DH5α electrocompetent cells. Subsequently, the vector was isolated on a large scale on a CsCl/Et-Br density gradient and 20 μg restricted with 20 units of *Eco*R V at 37°C for 2 h. Following the digestion the vector was dephosphorylated and stored in -20°C until needed.

Both *Eco*R V and *Sma* I restriction digestions produce blunt ended DNA fragments and therefore, all pAC3 fragments isolated above could be ligated to the *Eco*R V restricted pBluescript II SK (+) (Sambrook *et al.*, 1989). The 5.3 kb and the 6.2 kb *Sma* I fragments and the 9 kb *Eco*R V fragment were ligated overnight at 16°C to pBluescript II SK (+), at a ratio of 3:1 (insert DNA:vector DNA), to produce plasmids pAC8, pAC9 and pAC10 respectively. After the ligation, 4 μl of each reaction mixture were transformed into *E. coli* DH5α competent cells. Ligation mixture with no DNA, as well as restricted and dephosphorylated vector were also used to transform DH5α, as transformation controls. Following growth at 37°C in SOC medium, the cells were plated on X-gal/IPTG plates containing 100 μg/ml ampicillin for blue-white colony selection. Plasmids were isolated from 20 white colonies arising from each ligation, using the Promega magic mini columns and restricted with *Xba* I, which would digest the vector but not the insert DNA, in order to estimate the size of their inserts. All the colonies tested appeared to have inserts of the right size. In order to further confirm this result, two colonies from each ligation were grown overnight in LB broth and the plasmids were isolated using the magic mini columns method. The plasmids were once again restricted with *Xba* I and electrophoresed through a 0.8% (w/v) agarose/TAE gel at 8 V/cm gel length. The gel was blotted onto a Hybond N^+ membrane and hybridised with the 1.7 kb probe at 65°C as above. Autoradiography showed that pAC8 (containing the 5.3 kb *Sma* I-*Sma* I fragment) and pAC10 (containing the 9 kb *Eco*R V-

*Eco*R V fragment) produced a signal, whereas pAC9 (containing the 6.2 kb *Sma* I-*Sma* I fragment) did not. Therefore, the respective clones were saved in LB broth containing 15%(v/v) glycerol and stored at -70°C.

10.8 Expression Studies of the α–Pinene Monooxygenase using the pAC3, pAC8 and pAC9 Plasmids

An expression experiment was attempted using the plasmids pAC3, pAC8, and pAC9 in order to acquire some information on which plasmid is likely to contain the α–pinene monooxygenase gene. Therefore, flasks with 100 ml of 2YT containing the appropriate antibiotics were inoculated with 1 ml aliquots of overnight cultures of DH5α, DH5α(pBluescript II SK (+)), DH5α(pLAFR5), DH5α(pAC3), DH5α(pAC8) and DH5a(pAC9). The flasks were placed on a rotary incubator at 30°C. Three flasks for each sample were prepared. After approximately 3 h, when the optical density of the cultures was 0.5, they were induced with 1 mM IPTG, or 2 mM α–pinene, or not induced at all (control). The cultures were allowed to grow for another 15 h and then the cells were harvested by centrifugation at 4,000 g for 30 min. After two successive washes with 50 mM potassium phosphate buffer, pH 7.8 the cells were resuspended in 10 ml of the same buffer. One ml of these suspension was then used for the preparation of samples for SDS electrophoresis. The rest of the suspension was placed on ice and sonicated at 16 mA for 10 min. The sonicated samples were centrifuged at 30,000 g for 30 min and the supernatant kept for spectrophotometric as well as GC monooxygenase assays. 200 μl of extracts were used in these assays.

SDS electrophoresis of the above samples through 9% and 12% polyacrylamide gels did not show any difference in the electrophoretic profile between non induced and induced samples, or between *E. coli* containing non recombinant plasmids and *E. coli* containing recombinant plasmids. On the other hand, pAC3 and pAC8, induced by α–pinene, showed a low α–pinene stimulated

NADH oxidation activity when assayed spectrophotometrically (table 10.3) , but no product formation was detected by GC, or GC-MS assays. The fact that the putative promoter of α–pinene monooxygenase is a σ^{54}-like promoter, as well as the possible absence of a complete activator gene, may explain the low expression rate of the enzyme in the above vectors. Therefore, based on the spectrophotometric and hybridisation results, pAC8 was selected for further study.

Table 10.3: Spectrophotometric assays for α–pinene monooxygenase activity in crude extracts of DH5α(pAC3), DH5α(pAC8) and DH5α(pAC9).

Sample	Induction	Monooxygenase activity
DH5α	no	-
DH5α	IPTG	-
DH5α(pBS II SK (+))	no	-
DH5α(pBS II SK (+))	IPTG	-
DH5α(pLAFR5)	no	-
DH5α(pLAFR5)	IPTG	-
DH5α(pAC3)	no	-
DH5α(pAC3)	IPTG	-
DH5α(pAC3)	α–pinene	+
DH5α(pAC8)	no	-
DH5α(pAC8)	IPTG	-
DH5α(pAC8)	α–pinene	+
DH5α(pAC9)	no	-
DH5α(pAC9)	IPTG	-
DH5α(pAC9)	α–pinene	-

10.9 Discussion

700 clones of the *P. fluorescens* genomic library were hybridised with the 1.7 kb insert of the pAC1 that had been shown to encode part of the α–pinene monooxygenase. Four clones, pAC3, pAC5, pAC6 and pAC7 were isolated. All four plasmids were shown to complement the monooxygenase minus mutants. Full restriction mapping of the inserts established the overlapping regions and the single common region used to locate the 1.7 kb fragment hybridisation site. This lay in the middle of the insert of plasmid pAC3. Thus, pAC3 should contain the whole of the monooxygenase gene, and therefore, this insert DNA was subcloned. It has been shown in chapter 8 that the monomer size of the α–pinene monooxygenase is approximately 70 kDa. Such a polypeptide should be encoded by approximately 2.0 kb DNA (Sambrook *et al.*, 1989; Promega protocols, 1991). Three restriction fragments of pAC3 were subcloned into the pBluescript II SK (+) sequencing vector to give plasmids pAC8 (*Sma* I-*Sma* I, 5.3 kb fragment), pAC9 (*Sma* I-*Sma* I, 6.2 kb fragment) and pAC10 (*EcoR* V-*Eco*R V, 9 kb fragment). Plasmid pAC10 should contain the whole gene, since the hybridisation site of the probe lies at its centre. However, it is a rather large plasmid for further manipulations, and a high copy number vector such as pBluescript may be unstable with such a large insert. On the other hand, plasmid pAC8 also contained the probe hybridisation region and depending on the orientation of this region, either towards the *Eco*R I and *Sma* I sites of the multicloning site of pLAFR5 (figure 9.1), or in the opposite direction, it might contain the whole monooxygenase gene. However, if not, then plasmid pAC9 should contain the rest of the monooxygenase gene. In an attempt to determine which clone was likely to contain the gene of interest, expression studies were carried out, which revealed that crude extracts of DH5α(pAC8) had a low level of monooxygenase activity when assayed spectrophotometrically. From the above results it was assumed that the orientation of the α–pinene gene was towards the *Eco*R I site of the vector, and therefore, pAC8 plasmid should contain the entire gene.

CHAPTER 11

Sequencing of the pAC8 Recombinant Plasmid

11.1 Introduction

The ultimate characterisation of a cloned fragment depends upon determining its primary DNA sequence. It is possible by analysis of the sequence to identify specific regions such as promoters, ribosome binding sites, transcription terminators, and open reading frames. Furthermore, sequence analysis of protein coding regions permits determination of the frequency a particular species uses the available codons, as well as prediction of characteristics such as cofactor requirement from the identification of typical binding motifs.

The two procedures commonly used for DNA sequencing are the chemical method of Maxam and Gilbert (1977) and the chain termination method of Sanger *et al.* (1980). The former method, or chemical DNA sequencing relies on the use of base specific modification and cleavage reactions. Sanger sequencing or controlled enzymatic DNA replication, uses dideoxynucleotide analogs in primer-directed DNA extension to produce discrete DNA fragments. Maxam-Gilbert sequencing requires a prior knowledge of the restriction map of the target sequence, because it is necessary to cleave the DNA into manageable sizes for sequencing. In contrast the Sanger method requires no prior knowledge of the restriction map of the sequence, because primers can be complementary to the cloning vectors. Furthermore, it is possible to read more sequence information per gel with the Sanger than with the Maxam-Gilbert sequencing method because of better band resolution. However, Sanger sequencing can present problems in sequencing regions with strong DNA secondary structure, which is not a problem with Maxam-Gilbert sequencing

(Saunders and Saunders, 1987; Hillis *et al.*, 1990). Because of reasons given, the Sanger method is the most widely used for sequencing and it was used throughout this study.

An efficient way to sequence large DNA fragments is to generate a nested set of deletions in the target DNA effectively moving the priming site closer to the sequence of interest (Entsch *et al.*, 1988; Shiller *et al.*, 1993; Shingler *et al.*, 1993). The system is based on the procedure developed by Henikoff (1987) in which exonuclease III (Exo III) is used to specifically digest insert DNA from a 5' protruding or blunt end restriction. The adjacent sequencing primer binding site is protected from digestion by a 4-base 3' overhang restriction site or by a phosphorothioate filled end. The uniform rate of digestion by the enzyme allows deletions of predetermined lengths to be made simply by removing aliquots from the reaction at various times. Therefore, the DNA fragment of interest is first cloned into the multicloning site of an appropriate vector, e.g. pBluescript, so that at least two restriction sites lie between the insert DNA and the sequencing primer binding site. The enzyme that cuts closest to the priming site must leave 4-base 3' overhangs which are resistant to Exo III digestion. The enzyme that cuts closest or even within the insert must leave 5' overhangs or blunt ends, susceptible to Exo III digestion. Subsequently, Exo III digestion is carried out and samples are removed from the reaction tube at timed intervals to tubes containing S_1 nuclease which removes the remaining single stranded tails. After neutralisation and heat inactivation of the S_1 nuclease, Klenow DNA polymerase is added to flush the ends which are then ligated to re-circularise the partially deleted constructs. The ligation mixtures are then used to transform competent cells. Therefore, each successive time point yields a collection of subclones containing clustered deletions extending further into the original insert.

11.2 Preparation of Plasmids Carrying a Nested Set of Deletions Using the Erase-a-Base System®

The preparation of plasmids carrying a nested set of deletions was performed by using the erase-a-base system® supplied by Promega which is based on the action of Exo III and S_1 nuclease. The generation of an ordered set of deletions by this method relies on the uniform digestion rate of Exo III. However, this enzyme also digests from nicks in the starting double stranded DNA molecules, creating single stranded gaps. Therefore, the presence of random nicks in the starting DNA results in the randomising of the deletions obtained, making it difficult to screen for the desired deletions among the resulting subclones. Thus, it is important to minimise the proportion of nicked molecules in the starting DNA preparation. For this purpose, the pAC8 DNA, purified by equilibrium sedimentation in CsCl/Et-Br gradient was subsequently extracted with acid phenol at 4°C according to the method of Zasloff *et al.* (1978). This method selectively removes nicked and linear DNA molecules from supercoiled DNA.

The insert DNA of pAC8 had been cloned in the *Eco*R V site of pBluescript II SK (+) which lies at approximately the middle of its multicloning site (figure 11.1). In order to allow sequencing of both strands of the insert DNA, digestions with Exo III were carried out in both directions, namely from T_3 and T_7 primer sites. Before Exo III treatment, the insert DNA should be digested with two enzymes from each site, as described above. The optimal enzymes that would cut closest to the priming site and leave 4-base 3' overhangs would be *Sac* I from the T_3 site and *Kpn* I from the T_7 site. Unfortunately, both of these enzymes also had sites within the insert DNA, therefore, *Xba* I and *Xho* I that leave 5' overhangs were used. After the initial digestion 3' protrusions were generated in 3' recessed ends, to block the Exo III digestion of vector sequences using α–phosphorothioate dideoxynucleotides and the Klenow fragment. This procedure generates protective sites for the primers and was followed by phenol/chloroform extraction and DNA precipitation. Subsequently, the second

digestion to generate 5' overhangs on the fragment containing the insert DNA was performed with *Bam*H I from the T_3 site or *Cla* I from the T_7 site. This restriction digestion was also followed by phenol/chloroform extraction and ethanol precipitation of the DNA.

The Exo III treatment was carried out as described at 32°C and samples were removed at 1 min intervals to tubes containing S_1 nuclease to remove the single stranded tails. According to the manufacturer's instructions such conditions should correspond to deletions of 310 bp/min incubation. Fifteen time points were used for each priming site, corresponding to a predicted deletion of 4.7 kb from each site. The Klenow polymerase was added to flush the ends and then the deletion-containing vectors were religated at room temperature for 1 h, with ligation buffer containing PEG (50%). The 30 ligation mixtures were then used to transform competent DH5α cells and after overnight incubation of the transformants, plasmids from 5 colonies from each time point were isolated using the alkaline lysis procedure (Sambrook *et al.*, 1989) and analysed for insert size and integrity of the primer sites by PCR.

DNA polymerase carries out the synthesis of a complementary strand of DNA in the 5' to 3' direction using a single stranded template but starting from a double stranded region, the primer site. PCR uses the same principle but employs two primers each complementary to opposite strands, of the region of DNA to be amplified. The primers are annealed to a single stranded template obtained by heat denaturation and are arranged in such a way, that each primer extension reaction directs the synthesis of DNA towards the other primer. This results in the production of extension products that are complementary to and capable of binding more primer, and thus, each successive cycle essentially doubles the amount of DNA synthesised in the previous cycle. Therefore, exponential accumulation of a specific target fragment occurs (Saiki *et al.*, 1988; Innis and Gelfand, 1990; Taylor, 1992). The 20-mer T_3 and T_7 primers (Kretz *et al.*, 1993) were used in all PCR reactions as they have higher Tm temperatures and this results in less mispriming and accumulation of non specific products. PCR reactions were routinely carried out with the method described

(chapter 2) which was optimised for amplification of large DNA fragments. A prolonged DNA denaturing step at 95°C for 5 min was employed as a first step of any PCR amplification procedure, since the *P. fluorescens* genome is 60% G+C rich (chapter 7). In order to avoid heat denaturation of the Taq polymerase the enzyme was added to the reaction mixtures after the first DNA denaturation step, when the temperature was around 65°C and then annealing was performed at 57°C for 30 sec. Usually extension of the primers is carried out at 72°C giving an extension rate of 1 kb of template per min. Therefore, since some of the DNA fragments to be amplified would be fairly large, (≈5.0 kb), the extension in each cycle was carried out for 5 min. Also, a final extension step of 12 min was used (Innis and Gelfand, 1990; Clackson *et al.*, 1992; Ledbetter and Nelson, 1992; Taylor, 1992). After PCR the reactions were kept at 4°C before electrophoresis through a 0.7% (w/v) agarose/TAE gel at 8 V/cm gel length. All 150 clones isolated after erase-a-base procedure were analysed by PRC for the size of their inserts. Some of the PCR results are presented in figure 11.2. PCR amplification procedures revealed that deletions occurred only in the insert DNA, as expected, leaving the vector primer binding sites intact. Thus, 20 clones containing deletions from each primer site, estimated to represent the whole original 5.3 kb *Sma* I-*Sma* I insert DNA with a high degree of overlap, were chosen for sequencing.

11.3 Sequencing of the Exo III-S1 Nuclease Treated Clones

Forty deletion clones, pAC8-11 to pAC8-51, covering the entire insert of the pAC8 plasmid, were sequenced. DNA was isolated using the magic mini plasmid purification procedure and stored in -20°C until needed. The nucleotide sequence of the inserts of all these plasmids was determined by the dideoxy-chain termination method of Sanger *et al.* (1977) on double stranded DNA using the T_7Sequencing Kit™ supplied by Pharmacia. Both the commercially available 17-mer Reverse M_{13}

(AAACAGCTATGACCATG) and Universal M_{13} primers (GTAAAACGACGGCCAGT) (Stratagene) were used.

The inserts of the clones isolated were sequenced in both orientations, and the sequences obtained were assembled using the Assembly Lign commercial DNA software package (Kodak, UK). The complete nucleotide sequence of the α–pinene monooxygenase along with the translation of the open reading frame (ORF) is presented in figure 11.3. The sequence reveals a long ORF of 1774 bp capable of encoding 591 amino acids. The ORF includes the initiation codon ATG at position 1 and the termination codon TGA at position 1774. As observed previously, the ATG codon was preceded by a putative ribosome binding site, GGAG (Shine and Dalgarno, 1975) which is indicated by solid horizontal lines above the nucleotide sequence (figure 11.3). The estimated molecular weight of the complete polypeptide encoded by this sequence is 65.5 kDa, which is close to the 70 kDa obtained by SDS-PAGE calculations. As has been found for other pseudomonads genes the coding region is 62% G+C rich as a result of preferential usage of codons with G or C at the third position (Enscht *et al.*, 1988; Shell and Sukordhaman, 1989; Zylstra *et al.*, 1989; Nordlund *et al.*, 1990; Kukor and Olsen, 1992; de Mot *et al.*, 1993; Shingler *et al.*, 1992; Shingler *et al.*, 1993). The codon usage is shown in table 11.1 below.

As previously discussed in chapter 7, a region with strong homology to the *xyl*CAB and *xyl*S promoters of the TOL plasmid pWW0 (Inouye *et al.*, 1984; Köhler *et al.*, 1989) is present 20 nucleotides upstream of the ATG codon. This promoter sequence belongs to a distinct class of σ^{54} (*rpo*N) dependent promoters, that have an invariant -24CTGG, -12TTGC sequence and often require upstream regulatory elements (Shingler *et al.*, 1993). In this complete sequence, two possible high-affinity binding sites for a *ntr*C-like regulatory gene, GCAC-GTGC (Stelitz-Mörsdorf *et al.*, 1993) were also identified upstream from the promoter site (figure 11.3). This suggests that the expression of α–pinene monooxygenase may be positively regulated by an activator gene homologous to the *ntr*C gene product, as has been observed with other σ^{54} dependent promoters.

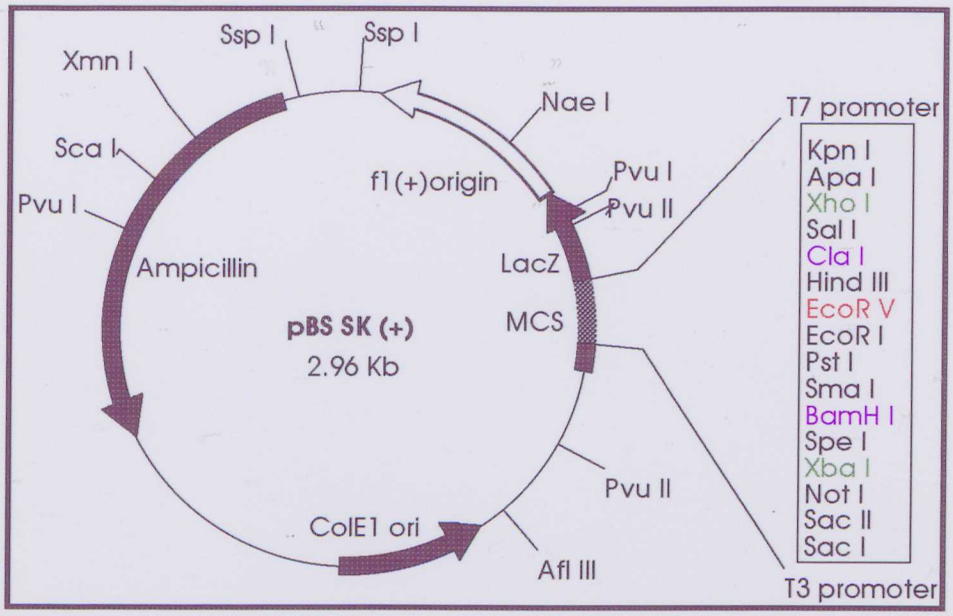


Figure 11.1: Physical map of the pBluescript II SK(+) plasmid (taken from the Stratagene catalogue, 1993).

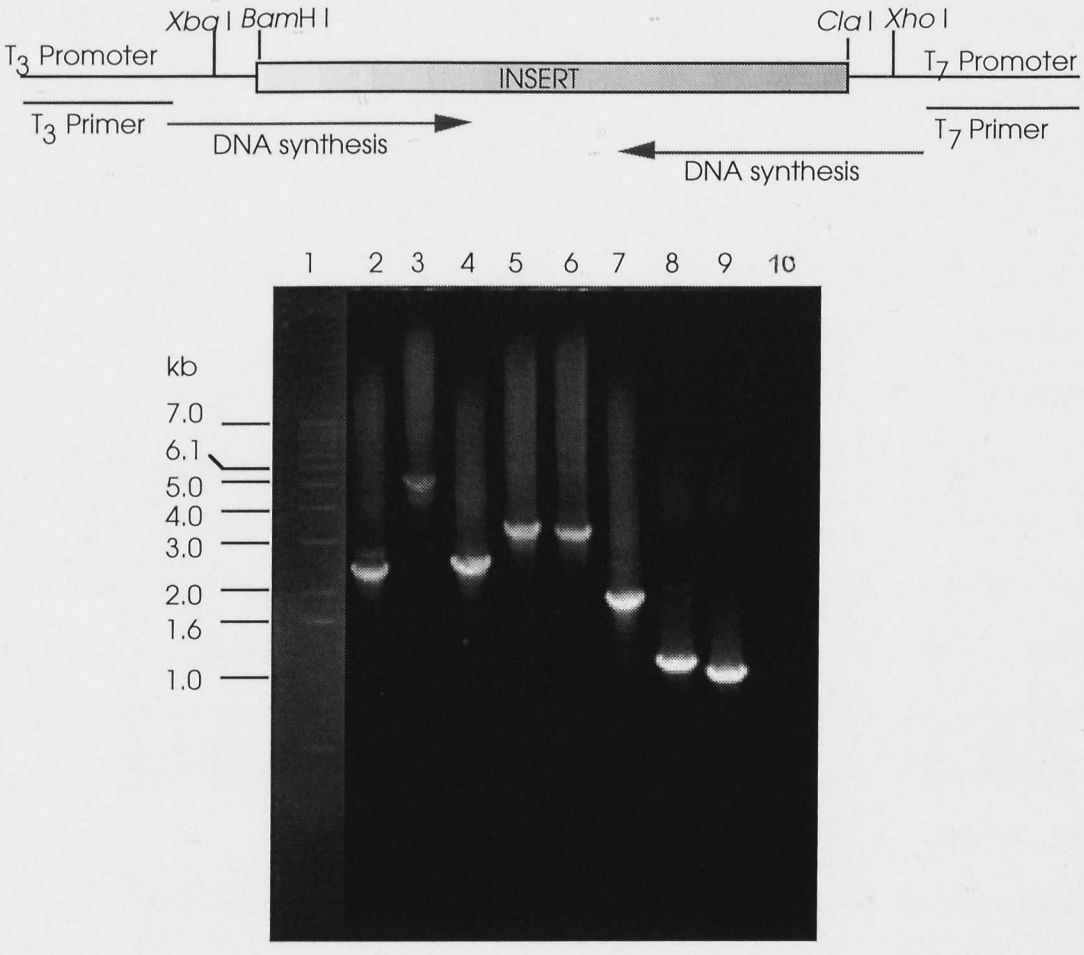


Figure 11.2: PCR amplification of the insert DNA of Exo III-S_1 nuclease treated pAC8 plasmids. Lane 1, low molecular weight DNA marker (1 kb ladder, Gibco BRL); lane 2, pAC8-32; lane 3, pAC8-35; lane 4, pAC8-51; lane 5, pAC8-52; lane 6, pAC8-55; lane 7, pAC8-91; lane 8, pAC8-122; lane 9, 8-133; lane 10, pAC8-23.

Enzymatic analysis of α–pinene monooxygenase has revealed that the enzyme contains 2 mole FAD/mole protein which accepts reducing equivalents from NADH. Therefore, the enzyme should belong to a class of enzymes that contain two ADP binding sites: one is usually located at the N terminus and is involved in FAD binding, while the second site is located in the middle of the sequence and is involved in the binding of NAD (Eggink *et al.*, 1990). The precise location of these sites can be determined on the basis of a consensus sequence (fingerprint) involved in ADP binding (Wierenga *et al.*, 1986). This amino acid fingerprint is derived from known structures of cofactor binding enzymes and predicts whether a particular amino acid sequence can fold into a βαβ-unit with binding properties for the ADP moiety of NAD(P) or FAD. It consists of rules for a set of 11 amino acids that should occur at conserved positions in an amino acid stretch (figure 11.4). The length of the peptides found to date varies from 29 to 31 residues, because of the fact that the loop between the α–helix and the second β-strand is variable. The three glycine residues as well as the acid residue at the end of the fingerprint are strictly conserved in NAD or FAD binding βαβ-units. Wierenga *et al.* (1986) concluded from their survey that all fingerprints with a score of 11 (all 11 conserved amino acids are at the appropriate positions following the βαβ unit structure), and most likely all fingerprints with a score of 10 as well, fold as ADP binding βαβ-units. A score of 9 out of 11 gives less certainty about the existence of an ADP binding βαβ-fold. However, if the two deviating amino acid residues are not replaced by charged residues, a peptide with a score of 9 could be a candidate for an ADP binding βαβ-folding unit (Neidle *et al.*, 1991; Altenschmidt *et al.*, 1992; Kukor and Olsen, 1992;). The potential ADP binding sites, in α–pinene monooxygenase and other enzymes are presented in figure 11.4. As depicted in this figure, the sequence G-X-G-X-X-G, together with other fingerprint residues in the same region, has been implicated in forming a βαβ-fold responsible for binding the ADP moiety of this prosthetic group in many but not all (Porter and Kasper, 1985) flavin containing monooxygenases.

Table 11.1: Codon usage for the α–pinene monooxygenase gene from *Pseudomonas fluorescens* NCIMB 11671.

TTT phe F	6	TCT ser S	2	TAT tyr Y	4	TGT cys C	6
TTC phe F	10	TCC ser S	6	TAC tyr Y	3	TGC cys C	14
TTA leu L	5	TCA ser S	11	TAA OCH	-	TGA OPA	1
TTG leu L	5	TCG ser S	8	TAG AMB	-	TGG trp W	17
CTT leu L	11	CCT pro P	15	CAT his H	10	CGT arg R	12
CTC leu L	5	CCC pro P	4	CAC his H	14	CGC arg R	24
CTA leu L	5	CCA pro P	19	CAA gln Q	16	CGA arg R	18
CTG leu L	12	CCG pro P	11	CAG gln Q	19	CGG arg R	14
ATT ile I	3	ACT thr T	4	AAT asn N	4	AGT ser S	2
ATC ile I	6	ACC thr T	9	AAC asn N	5	AGC ser S	10
ATA ile I	4	ACA thr T	3	AAA lys K	7	AGA arg R	6
ATG met M	8	ACG thr T	8	AAG lys K	6	AGG arg R	8
GTT val V	7	GCT ala A	6	GAT asp D	11	GGT gly G	12
GTC val V	12	GCC ala A	34	GAC asp D	10	GGC gly G	21
GTA val V	3	GCA ala A	18	GAA glu E	8	GGA gly G	6
GTG val V	9	GCG ala A	19	GAG glu E	11	GGG gly G	3

```
1     ATCAGGGTC  ATCGCTCGG  ACGAGACTA  GCATAGCCA  TCCGCGTAA
46    TCGCCTTCG  GTGATGACA  ATCAGCGCG  TCATAACAG  GCCACCTAG
91    CTTTCGGCA  TATACGCCT  TCGCCTGGA  AAGCCATTT  CGCGCGCTG
136   AGCAAATCC  TCACTGCCG  CCGGCGATC  AAAAATTCT  TCTTCGGTG
181   CCGACGATC  AAGGTCAAA  ACGCGGCAG  GATGTTCTG  CACGTGCTG
226   GCTGACATT  CCGGTCGGC  GACAAACCG  CGTTTCGCC  ATCGCGCTT
271   GCCCGCCAG  GCCCCACAG  CACCGGGCG  ATAGTCGAT  GTCCAGCAC
315   GCGCTTGAC  GTGGTGCCT  GGCCGCATA  GTCCAGCGC  CTGGATGCT
361   GGCCTTGTA  CACGCCGTC  GGTGGAGAA  ATGCGTGCC  GGTGATCAA
                                     M  R  A    G  D  Q

406   CAGCGCCTT  GCTGGAGGC  GATAAAGGC  TTCGCTGAC  GTCTTCGGC
      Q  R  L    A  G  G    D  K  G    F  A  D    V  F  G

451   GCGCACCGC  CATGTCGGC  GCAGTTCTC  GCGGTAGAA  CACCAGGGG
      A  H  R    H  V  G    A  V  L    A  V  E    H  Q  G

496   AAGGTTTCC  GCGGTCCTT  GAGGCCCAG  CAGCACCAG  GGCGGTGAG
      K  V  S    A  V  L    E  A  Q    Q  H  Q    G  G  E

541   GCGTTCCGG  GTCGACCTG  GATGCCGCT  GACATCGCA  GCCTTCGCG
      A  F  R    V  D  L    D  A  A    D  I  A    A  F  A

586   GCCAGGGAT  TCGACCAGG  AAACGCCCC  ATGTGGTCG  TCCCCTACC
      A  R  D    S  T  R    K  R  P    M  W  S    S  P  T

631   CCGGCTCAG  CATCGCCGA  CTTGAGGCC  CAGCGTGCG  TGCCGAACG
      P  A  Q    H  R  R    L  E  A    Q  R  A    C  R  T

675   ATATTGGCG  GAGGAACCG  AGGTATTGG  CGGAGGAAC  CGAGGTATT
      I  L  A    E  E  P    R  Y  W    R  R  N    R  G  I

721   TGGCAAAGC  CGGACACGT  GTCAAGCGC  GATAAAGGT  CGACGCCCA
      W  Q  S    R  T  R    V  K  R    D  K  G    R  R  P

766   GGCGCCCGA  GGCAAATCA  GATCCAATT  GACGCCCAC  TGGCAAAAC
      G  A  R    G  K  S    D  P  I    D  A  H    T  Q  N

811   GAGTCTGGC  CCATGCTGG  CTCCTGTTA  TTTTTATCA  GCCTGCGTT
      E  S  G    P  C  W    L  L  L    F  L  S    A  C  V

856   GTGCCGCCG  TGGCATGGC  ACACGCTCT  TGGTGGGAG  CAGACTAGA
      V  P  P    W  H  G    T  R  S    W  W  E    Q  T  R

901   ACGTCCGGC  GAGCGCAAT  CAATATTTA  TTCCAGAAT  TATTTTAGT
      T  S  G    E  R  N    Q  Y  L    F  Q  N    Y  F  S

946   GGAATATTT  TTTCCAATA  CGTCAGCAT  AGGCGCCAC  GTGCTCCCG
      G  I  F    F  P  I    R  Q  H    R  R  H    V  L  P

991   TGGATCGTT  TCAGCAGGC  CACGGCGGC  CGTGATGCC  GGGGTATTT
      W  I  V    S  A  G    H  G  G    R  D  A    G  V  F

1036  TTTTTACGC  CTACCCTGT  AGACTTGCG  CCATTCACC  AGCGCACCA
      F  L  R    L  P  C    R  L  A    P  F  T    S  A  P

1081  GAAGAACAC  GCCAGAAGG  ATTGCCCAT  GTCCCGCAC  CGATCCCGA
      E  E  H    A  R  R    I  A  H    V  P  H    R  S  R

1126  GACCCACCT  GGAAGACAC  TGTCCCAGC  CCTCCGATC  AATGCCGAA
      D  P  P    G  R  H    C  P  S    P  P  I    N  A  E

1171  CGCCTGTTG  CAGCTGATG  ACCGACGAA  TACGAAAGC  CTGCGCGCC
      R  L  L    Q  L  M    T  D  E    Y  E  S    L  R  A

1216  AACTCAAGC  GCATGCAGC  TACATAGCA  GCAGAGCCA  ACGTCACGC
      N  S  S    A  C  S    Y  I  A    A  E  P    T  S  R
```

```
1261 ATGAAGTGT  ACTTTGCAC  ACCATGCTT  CCTGCCTGG  GCCGTGGCA
       M K C     T L H     T M L     P A W     A V A

1306 ACGCTGGTG  ACGGCGACC  CTGGTGGTC  GGCTCGGTC  AGCAGTGTG
       T L V     T A T     L V V     G S V     S S V

1351 GTCGGCGGT  GGCGTGCAA  GCCGGTGCC  AGCGTGGCT  TCCGGTGCG
       V G G     G V Q     A G A     S A A     S G A

1396 CCAGCGGGA  TCAGCCAGG  CGGCGGGCA  GCATGGCCA  AGGGGCCCA
       P A G     S A R     R R A     A W P     R G P

1441 CGGCGAGGA  CTTCGGTTA  CTACGTCGA  CAGCCTGTT  CCGCGACGA
       R R G     L R L     L R R     Q P V     P R R

1486 CCGCCCTGC  AGCCGTCAG  CGATCATGC  GGTGCACGG  TGTCGTTGC
       P P C     S R Q     R S C     G A R     C R C

1531 GCGCATCTT  CGCCCGGAC  CTTGAGCAA  CGACGGCCA  ACTCGCCGC
       A H L     R P D     L E Q     R R P     T R R

1576 CGAAGACCG  TGCGTACCT  TGCGCAGTT  GATCAGCCA  GCGCACCAA
       R R P     C V P     C A V     D Q P     A H Q

1621 CCTCAGCCA  GTCCGAGGC  CGAAGCGCG  TATCGACAA  GGTCTACGC
       P Q P     V R R     R S A     Y R Q     G L R

1666 GACGCCCGT  AAAGCCATC  GAAGACGCC  AAGCTCAAA  GCCAAGCAA
       D A R     K A I     E D A     K L K     A K Q

1711 GCCGCCGAG  ACCGCCGCC  AAAGTCGCC  GCCTGGACT  TCGCTGTGG
       A A E     T A A     K V A     A W T     S L W

1756 ACATTCGTG  GCGCTGCTG  ATCGGTGCC  TTCTTCGCC  ACGTTCGCA
       T F V     A L LL    I G A     F F A     T F A

1801 GCACTTCGG  CGGTCGTCG  TCGGGACGC  GGTCTACAA  CCTGCAGCA
       A L R     R S S     S G R     G L Q     P A A

1846 CACCAACAA  GCGCATCCA  CCTGATCTC  CGGCCTGGG  CGCACGTAC
       H Q Q     A H P     P D L     R P G     R T Y

1891 CGCGAGCAG  ATGCGCACG  TGCCGGCCA  ATGACCTGT  GATCGCCAT
       R E Q     M R T     C R P     M T C     D R H

1936 CAGCTTCAC  GCCCTATGG  CAAAGAAAC  CCAGCACTG  CCTGCGCAT
       Q L H     A L W     Q R N     P A L     P A H

1981 CGCCCAGCA  TCACCAGGC  CAAGACCCT  GATCATCAC  CGACAGCAA
       R P A     S P G     Q D P     D H H     R Q Q

2026 CCTGTCGCC  CCTGGCCAA  GCGGGCAAC  GCGTGCTGC  TGGTCAATG
       P V A     P G Q     A G N     A C C     W S M

2071 AAGGTTCTT  CGTTCGCCT  TCCGCTCGC  TCAGCCACC  TTGTGCCTG
       K V L     R S P     S A R     S A T     L C L

2116 TGCCAGCGT  TGTTCATTG  CGGTGGCGT  ATCGGTTGG  AATTGAAGG
       C Q R     C S L     R W R     I Q W     N OPA

2161 TGGATGAGA  TTCACGAGC  AGGTCGGCT  TCGACGATG  ACCGCACCC
2206 AAAAGCAAA  AAACGGGAG  GGGTTTGCC  CTCCATATC  GGACCGAGC
2251 GACGCCCGG  AATCAGTGC  ATGAAATCG  CGATCAGGA  TGATGAGCG
2296 GATCGCACA  CCGAGGAAC  ACAGCAGTA  ATGAGCGCA  TGATGTTCT
2341 CTTGAATTA
```

Activator binding site
Promoter
Ribosome binding site
FAD/NAD binding sites

Figure 11.3: DNA sequence of α-pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671.

Two potential ADP binding sites are evident in the sequence of α–pinene monooxygenase. The putative FAD binding domain with a score of 9, is located near the NH_2-terminus (residues 11 to 39). It has a slightly different structure from the one proposed above (figure 11.4). However, as the two deviating amino acids are not charged this region may be assumed to be candidate for an ADP binding site according to the criteria of Wierenga *et al.* (1988). An alternative ADP binding domain which centres around GGDLG codons (residues 16-20) has also been identified as an FAD binding site in protein C, component of methane monooxygenase (MMO) from *Methylococcus capsulatus* (Bath) and in cytochrome b_5 reductase (Stainthorpe *et al.*, 1990). The second site, the putative NAD binding domain with a score of 7 (figure 11.4) is located internally (residues 207 to 214) in the α–pinene monooxygenase sequence. Although this score is low according to the previous suggestions, it is similar to the scores of the ADP sites from all the other enzymes presented in figure 11.4.

The nucleotide sequence was analyzed using the GCG Sequence Analysis Software Package (Devereux, 1984; Henicoff *et al.*,, 1990) available through Daresbury. The programs used were SEQED for sequence loading, MAP for restriction site identification, TRANSLATE for amino acid sequence determination, FASTA for data base searching and GAP and BESTFIT for sequence alignments. The EMBL and GenBank sequence data banks were searched for sequences similar to the ORF of α–pinene monooxygenase. This search identified quite extensive similarities, at the DNA level, with 6-aminohexanoate-dimer-hydrolase from *Flavobacterium* (Tsuchiya *et al.*, 1989), mannose dehydrogenase from *P. aeruginosa*, chlorobiphenyl dioxygenase from *P. pseudoalkaligenes* (Taira *et al.*, 1992), toluate 1,2-dioxygenase (*xylX*) from *P. putida* plasmid pWW0 (Harayama *et al.*, 1991), catechol 1,2-dioxygenase from *Pseudomonas* sp. (Van der Meer *et al.*, 1991), chlorobenzene dioxygenase from *Pseudomonas* sp. transposable element Tn5280 (Van der Meer *et al.*, 1991) and cytochrome bc_1, FeS subunit from *Paracoccus denitrificans* (Kurowski and Ludwig, 1987).

In contrast, the amino acid identities were not distributed randomly throughout the sequence but showed distinct clustering. The proposed NADH binding domain and its surrounding region had 25 to 40 % amino acid identity to several cytochrome P-450 type reductases, indicating that the electron transfer part of α–pinene monooxygenase has a functional and probably an evolutionary relationship to NADH-cytochrome P-450 reductase. Similar results were obtained for xylene monooxygenase from the pWW0 plasmid of *P. putida* (Suzuki *et al.*, 1991).

Furthermore, the amino acid sequence near the C-Terminus of the enzyme showed striking similarities (amino acid identities in the range of 25 to 30%) to Rieske-type-iron-sulphur centres found in ferredoxins of either bacteria or chloroplasts. Figure 11.5 presents a comparison of amino acid sequences from pea, spinach, *Synecocystis* 6803, *Synechococcus* 7002 and α–pinene monooxygenase. From this figure it is evident that many of the conserved amino acids in the above organisms are also conserved in α–pinene monooxygenase.

Just after the C-terminus of the protein a potential region capable of forming a stem-loop structure was detected in the nucleotide sequence (figure 11.6). Although this loop is very close to the protein-coding region, it may act as a transcription terminator. There are only a few sequences published for chromosomal genes of *Pseudomonas* spp. which also should contain termination signals. They show no obvious consensus for the termination of transcription. For example in the gene for exotoxin A (Gray *et al.*, 1984) and similarly for the pobA gene encoding p-hydroxybenzoate hydroxylase (Entsch *et al.*, 1988), a loop past the C-terminus of the protein has been proposed. For the pilin genes (Johnson *et al.*, 1986), a loop centred 180 bp beyond the C-terminus has been postulated. However, none of these suggestions appear to correspond to the simple terminator structures for bacteria, namely, a region of G+C-rich dyad symmetry followed by a stretch of AT residues near the 3' terminus of the transcript as described by Galloway and Platt (1986) and Platt (1986).

	xy-y-G-G--G---y--y (loop) y-y-z			
Pin MO	QQRLAG=GDKGFADVFGA		VLAVD	
	11	28	35	39
Pin MO	WIVSAGHGGRDAGVFFLRLP		TSAPE	
	207	219	226	230
AlkT	AIVVVGAGTAGVNAAFWL		IFSRE	
	2	19	30	34
CamA	NVVIVGTGLAGVEVAFGL		RLVGD	
	6	23	33	37
AlkT	SVVVLGGGVIGLEVASAA		VTVIE	
	144	161	168	172
TodA	RLLIVGGGLIGCEVATTA		VTILE	
	144	161	168	172
NahG	RIGIVGGGISGVALALEL		VQLFE	
	8	25	33	37
TodA	HVAIIGNGVGGFTTAQAL		SLIGD	
	3	20	30	34
PHBH	QVAIIGAGPSGLLLGQLL		NVILE	
	4	21	28	32
TfdB	DVLVVGTGPAGASAGALL		TMLIN	
	8	25	32	36

Figure 11.4: Homology between the proposed ADP binding regions of α-pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671 with other amino acid sequences involved in ADP binding. Abbreviations: (x) K,R,H,S,T,Q,N; (y) A,I,L,V,M,C; (z) D,E; AlkT, rubredoxin reductase; CamA putidaredoxin reductase (Eggink *et al.*, 1990); PHBH, *p*-hydroxybenzoate hydroxylase (Wierenga *et al*, 1983); NahG, salicylate hydroxylase; TfdB, 2,4-dichlorophenol hydroxylase; TodA, toluene reductase (Zylstra and Gibson, 1989); (-) refers to any amino acid; (=) refers to no amino acid.



Figure 11.5: Comparison of amino acid sequences of Rieske FeS proteins from pea, spinach and cyanobacteria with the region close to the C-terminal sequence of α-pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671. Pea, spinach and cyanobacteria sequences were taken from Salter *et al.* (1992). Highly conserved amino acids are in red, identical amino acids are in green and similar amino acids in blue.

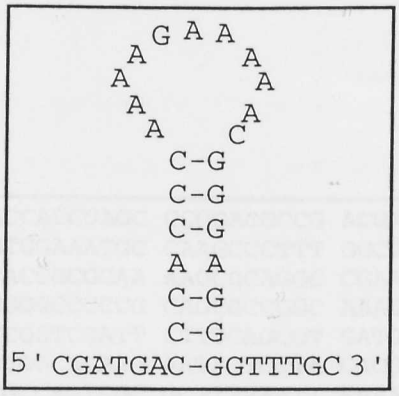


Figure 11.6: Proposed stem loop structure after the TAG codon of the α–pinene monooxygenase gene from *Pseudomonas fluorescens* NCIMB 11671.

The region downstream of the monooxygenase gene did not show sequence similarities to any known enzymes. However, the DNA sequence of the final 1 kb region (figure 11.7) of pAC8, proximal to the *Eco*R I site of the vector had a remarkable 55% homology to the *ntr*C gene from *Azospirillium brasilense* Sp7 (Liang *et al.*, 1993), as well as to other transcriptional activator genes that are subject to phosphorylation. Moreover, the deduced amino acid sequence also had a striking 30% identity to the *ntr*C transcription activator gene from *Rhizobium meliloti* (Szeto *et al.*, 1987). The product of such genes can activate σ^{54}-like promoters providing further evidence that the monooxygenase promoter that has been proposed, belongs to this class of *ntr*-like promoters, and may be positively regulated by a *ntr*C-like activator protein.

```
1     TGGCGATATA ACCACCGAGC CCGGACGCCG ACGATCTGGG TGAACGCCAG
51    CCAGATAATG GTGGAAATGC CAAGCCCTTT GGCGCTGATG CCACTGTCGG
101   CCCACGCGGA CACCGCGCAA AAGCCCAGGC CGAAGCCCAG TAACACCAGA
151   ATCAGCGAGA CGGGCCGCCG CAGCGCCGGC AAAGATCGCA CCCCAGGAAA
201   CCCGGAGCGC GCGCTCGATT CTTGCAGCGT GATGACGATG TGATCATTGT
251   TGTTGTGCTC CCGGCAGAAA AATAGTGTTA CACGTCTCAA AAGGGACAGT
301   GCAGCCGGCG TGCCAGTCGC GCTTTAAATT AAAATCCTTA CATTTCAATG
351   TGTTAAGTAA ATTGAACTTA CTCGATCCTT GCAACTTGCA AGAAATGACC
401   GCTGACGGCG TGCGTTATGC ATTCCCAGGG CGAACGCGCA TGAAAGCTGT
451   GTCAGTTTTG CCAACATGAA GTTTTATTTA GGAAGCGTTC AGGTATTTCT
501   TGGCAAAATG CCTCCAATTC CGTAGCAGCC GGCTTCGAGC TCGCGGTCTT
551   CCAGTGTGAA TCGCTCACCA GGTAACCCTA TGACCCGCAT TTTGACCATC
601   GAGGATGACG CCGTAACGCG CCGCGAGATC GTCGCCGAGC TGAGTAGCCA
651   TGGACTGGAC GTAGATTGGG TGGACAACGG CCGCGAAGCG TGGTCCGCCG
701   CCGTCAGTGG TGATTACGAC CTGATCACCC TCGACCGCAT GCTGCCGGAA
751   CTCGATGGCC TGGCCAGTCT CACCACCTTG CGCACCATCG GCGTGTCGAG
801   AGCTCCTGAT GATCAGCGCC CTCTCTGACG TAGACGAACG CGTGCGCGGC
851   CTGCGCGGCG GCGACGATTA CCTGACCAAG CCGTTCGCCT CCGACGAAAT
901   GGCCGCCCGC GTCGAAGTGC TGCTGCGGCG CAAAAGCACG GTCAAGGAAT
951   TCGAAACCGC CCTGCGCGTG GCCGACCTGG AGCTGAACCT GATCAGCCGC
1001  GAACGCAGCC GCGCCGATCA GCCGCTGAGC CTGTTGCCCA CCGAATACAA
1051  ACTGCTGGAG TTCCTGATGC GCAACACCGG GCAGATCCTG TCGCGGATGA
1101  TGATCTTCGA GGAAGTCTGG GGTTATCACT TCGACCCCGG CACCAACCTG
1151  ATCGACGTGC ACATCGGTCG CCTGGCCAAG AAGATCGATC CCCATCGAAT
1201  TCTTTGATGC TGAGTAAGAA ATGATCGACA TCGAAATGTT TCCACACTGC
1251  TCACATCATA TGTCACTCAG TGTGATCTGC AGCCGGGATC ACATGTCTAG
1301  AGC
```

Figure 11.8: DNA sequence of the region downstream the α–pinene monooxygenase gene with homology to the NtrC activator genes. Since this sequence does not represent a complete gene, the six frame amino acid sequence, used in the database search, is not shown here.

11.4 Discussion

The nucleotide sequence of the DNA region encoding α–pinene monooxygenase of *P. fluorescens* NCIMB 11671 has been determined and a large ORF of 1774 bp identified. The base composition of the 1.8 kb ORF, which from the overlap with the previous sequence obtained, probably encodes for α-pinene monooxygenase, was found to be 62% G+C, which is similar to that reported previously for other genes from *Pseudomonas* spp. (Enscht *et al.*, 1988; Shell and Sukordhaman, 1989; Zylstra *et al.*, 1989; Nordlund *et al.*, 1990; Kukor and Olsen, 1992; de Mot *et al.*, 1993; Shingler *et al.*, 1992; Shingler *et al.*, 1993). This high G+C value is due in large part to the codon usage preference found in the coding region for the α–pinene monooxygenase gene, where there is a some bias toward guanine- or cytosine-terminated codons. The bias toward guanine and cytosine also extended to the variable first-position codons for leucine and arginine. This codon usage bias has been reported for many other pseudomonas chromosomal genes, with the notable exception of the pilin genes of *P. aeruginosa*, which are only about 50% G+C (Johnson *et al.*, 1986).

Analysis of the region upstream of the translational start of the monooxygenase gene revealed a sequence of 12 nucleotides which showed strong homology to a set of promoters proposed to be transcribed by *Pseudomonas* spp. σ^{54}-like RNA polymerase holoenzyme. Although the existence of multiple σ factors in *Bacillus subtilis* has been recognised for some time (Helman and Chamberlin, 1988), it was originally believed that only one species of RNA polymerase, and hence only one species of σ factor, was involved in transcription in *E. coli* and related bacteria. However, recent studies on the regulation of gene expression in this organism have revealed the existence of other σ factors which allow this organism to switch on a subset of specific genes under certain physiological conditions. RpoD, the predominant σ factor in *E. coli*, recognises DNA sequences mainly in regions situated 35 and 10 nucleotides upstream of the transcription start site. Two additional σ factors

which recognise different sets of promoter sequences have recently been identified: These are the products of *rpoH* gene (RpoH) and of the *rpoN* gene (RpoN). The RpoH σ factor, responsible for the expression of genes involved in the so-called heat shock response, recognises promoters of the heat shock genes, whose consensus sequence differs from that recognised by RpoD (Cowing *et al.*, 1985). The RpoN protein which was originally discovered as an indispensable factor for the expression of the *ntr* (nitrogen regulation) and *nif* (nitrogen fixation) metabolic operons, recognises promoter sequences containing invariant GG and GC sequences located 24 and 12 nucleotides, respectively, upstream of the transcription start point (Dixon, 1986). Recent observations have suggested that the RpoN σ factor may be required not only for the regulation of nitrogen metabolism but also for the activation of further metabolic functions, such as expression of the *fdhF* gene, which encodes the anaerobically inducible formate hydrogen lyase in *E. coli*, (Birkmann *et al.*, 1987) the C4-dicarboxylic acid transport genes in *Rhizobium meliloti*, (Ronson *et al.*, 1987) and the catabolic genes responsible for the degradation of xylenes and toluene encoded by the TOL plasmid of *P. putida* (Dixon, 1986, Mermod *et al.*,, 1987). These observations suggest that the physiological function of the RpoN σ factor is not limited to the nitrogen regulation system (Köhler *et al.*, 1989).

All -24, -12 promoters analysed so far are regulated by positive transcriptional activators. Three potential positive regulator genes may be involved in their control. Two of them, *ntr*C and *nif*A, are almost completely interchangeable and seem to encode *bona fide* activators. The function of the third gene *ntr*A, is not well understood. Promoters that can be activated by the *nif*A or *ntr*C products have almost the same nucleotide sequence between positions -11 and -17 (Raibaud and Schwartz, 1984). A DNA sequence showing high homology to the *ntr*C gene was discovered downstream of the monooxygenase gene. In addition, two possible high-affinity binding sites for *ntr*C-like gene product were also identified upstream the monooxygenase gene promoter. Therefore, it seems probable, that the expression of α–pinene monooxygenase is positively regulated by a protein which belongs to

the NtrC family of transcriptional activators. This type of activator contain three domains. A conserved central domain is required for activation of transcription by σ^{54} RNA polymerase, as well as the hydrolysis of ATP to allow formation of open transcriptional complexes. The carboxyl-terminal domain contains a DNA-binding sequence, with a typical helix-turn-helix motif. Except for NifA and XylR, these proteins also contain hydrophobic sequence motifs at the amino termini that are conserved in a larger family of response regulatory proteins. Effector molecules most probably interact through the amino terminal region to activate the regulatory activity of these proteins ((Henicoff *et al.*, 1988; Ramakrishnan and Newton, 1990; Shingler *et al.*, 1993).

The nucleotide sequence of the α–pinene monooxygenase gene showed little significant overall homology with other sequences in the GenBank or EMBL databases, as already stated. However, when the deduced amino acid sequence of the monooxygenase was compared with the sequences in the SwissProt protein database, a conserved region between residues 11 to 39 and 207 to 214 showed significant similarity (figure 11.5) to sequences in some monooxygenases, oxidoreductases and dehydrogenases with ADP binding domains.

One additional region of the deduced monooxygenase amino acid sequence was found to resemble chloroplast type ferredoxins, containing a Rieske FeS centre. Residues important for ferredoxin structure and function were conserved in α–pinene monooxygenase, spinach, pea, *Synechococcus* and *Synecosystis* amino acid sequences. Plant-type ferredoxins have four symmetrically placed cysteine sulphurs co-ordinating to the (2Fe-2S) core of the centre (Taira *et al.*, 1992).

Conclusions and Future Work

12.1 Conclusions

At the outset of this project some preliminary aspects of the biochemistry of α–pinene monooxygenase had been established (Best *et al*, 1987; Aikens, 1990;). α–Pinene monooxygenase is an external monooxygenase which catalyses the oxidation of α–pinene to α–pinene oxide and requires NADH and oxygen as co-substrates (figure 12.1).

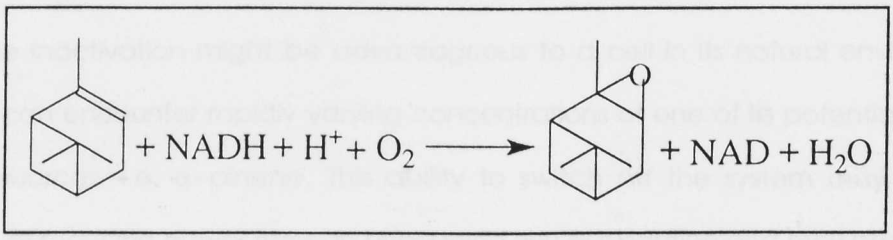


Figure 12.1: α–Pinene monooxygenase catalyses the oxidation of α–pinene to α–pinene oxide.

The enzyme appears to have remarkable substrate specificity since it can only attack substrates with a double bond in proximity to a methyl group. Moreover, it had been established that the enzyme required FAD and ferrous iron for full activity. In the absence of ferrous iron the enzyme appeared to have an NADH oxidase activity (Aikens, 1990). However, preliminary enzymological (Aikens, 1990) and molecular genetic studies (Saqib, 1992) gave conflicting evidence as to the complexity of the

enzyme, with enzymological studies suggesting it was multicomponent, while genetic studies suggested only a single gene product was involved.

Griffiths *et al.* (1987b) noted the presence of an inducible α-pinene oxide lyase in α-pinene grown *Pseudomonas* PX1 and *Nocardia* P18.3. Although they were unable to locate any α-pinene monooxygenase activity in cell free extracts of the strains tested, the circumstantial evidence obtained from a number of sources appears to suggest a unified route for the metabolism of α-pinene via α-pinene oxide to novel branched acids not encountered in the established routes for the degradation of other mono or bicyclic monoterpenes. Aikens (1990) provided evidence that the α–pinene monooxygenase system from *P. fluorescens* NCIMB 11671 could be present in either active or inactive forms and that the reactivation can take place rapidly without the need for *de novo* protein synthesis. This observation, confirmed in this study, could underlie the problems in detecting activity encountered by Griffiths *et al.* (1987). Reversible inactivation might be advantageous to a cell in its natural environment where it can encounter rapidly varying concentrations of one of its potentially useful carbon sources, i.e. α–pinene. This ability to switch off the system may prevent wastage of reducing power through uncoupled NADH oxidation and may prevent the formation of potentially damaging non-metabolisable epoxides, or even other by-products from α–pinene analogues.

This study is the first report of the purification and sequencing of α–pinene monooxygenase from *P. fluorescens* NCIMB 11671 which appears to be a novel flavin-iron containing monooxygenase.

Biochemical and genetic studies revealed that α–pinene monooxygenase is a single component system, which constitutes 0.5% of the total soluble cell protein in induced wild type cells. The enzyme was purified 40 fold (based on specific activity measurements) by a two step procedure, an ion exchange chromatography step and a chromatofocusing step and was shown to be at least 85% homogeneous by SDS electrophoresis. It has a native molecular mass of approximately 131,000 as determined by DNA sequencing, with slightly higher values given by gradient native

PAGE and gel filtration experiments. The enzyme is composed of two identical subunits and contains 1 mole of FAD per subunit. Putative FAD and NAD binding sites were located at the NH_2-terminus and the middle of the deduced amino acid sequence. Moreover, sequence comparisons with available databases suggested that the enzyme should contain a Rieske type FeS centre, which is located near the C-terminus of the deduced amino acid sequence.

As well as recognising the substrate, the major function of a monooxygenase is to activate dioxygen producing a sufficiently reactive intermediate to insert one atom into the substrate, the other oxygen being converted into water. In comparison with other monooxygenases, α–pinene monooxygenase appears to be somewhat enigmatic. In terms of polypeptide composition the enzyme appears to be a simple homodimer typical of flavoprotein monooxygenases which activate oxygen via a flavin 4a-hydroperoxide intermediate. However, the requirement for ferrous iron and evidence for a FeS centre belie this simplicity. At the other end of the scale it would be surprising to discover that the ferrous iron was involved in oxygen activation, given the molecular complexity of the other non-haem iron monooxygenases and the exquisite reaction specificity of α–pinene monooxygenase. One intriguing possibility is that this enzyme is similar to the pteridine dependent monooxygenases most of which also require ferrous iron for activity. These are believed to activate oxygen initially by formation of 4a-hydroperoxy-tetrahydropterin which subsequently reacts with the ferrous iron to produce the active oxidant. It is feasible that the proposed FeS binding site is in fact a simple iron binding site (and this would conveniently circumvent the need to propose a role for the FeS centre). However, confirmation of these proposals awaits detailed biochemical study.

Therefore, by analogy with the flavin system (Visser, 1983; Schreuder *et al.* 1988; Müller, 1985; Ziegler, 1990) and the pteridine dependent reactions (Dix and Bencovic, 1985) it could be proposed that the reduced FAD reacts rapidly with dioxygen to form a flavin 4a-hydroxyperoxide intermediate (4a-OOH), which decays to form the active oxidant. According to this mechanism, iron (II) would react with the peroxidated

cofactor, rather than with dioxygen itself, forming an iron (II) peroxyflavin (Fe-OOH) adduct. Flavins have no significant affinity for metal ions except for a few such as Ag, Cu, Mo, Ru and Fe. Model investigations of metal-flavin interactions are very limited. However, it is believed that metal-flavin complexes are probably due to metal-flavin charge transfer (Shinkai, 1990). Heterolytic decomposition of this intermediate could yield 4a-OH and a ferryl species that would be the active oxidant. Oxygenated product (SO) could then be formed by nucleophilic attack of the oxygenatable substrate (S) on the terminal oxygen of the enzyme bound ferryl species. This would be followed by release of water and oxidised pyridine nucleotide. Reduction of flavin and addition of oxygen would then complete the catalytic cycle by generating the oxygenating intermediate. The proposed mechanism is presented in figure 12.2.

An alternative possibility proposed by Feig and Lippard (1994) in their recent review on the reactions of non-haem iron (II) centres with dioxygen is that the reaction between metal and dioxygen precedes formation of the 4a-OOH intermediate, in pteridine-dependent reactions. Such a suggestion is in agreement with the results obtained with the Undenfriend biomimetic reaction which appears to be similar to many mononuclear monooxygenases. However, all the above speculations remain to be established by studies on EPR spectroscopy redox titrations as well as stopped flow studies of the purified enzyme.

The gene encoding for α–pinene monooxygenase is probably expressed under the control of a σ^{54}-like promoter which according to the preliminary expression experiments performed in this study, appears to be positively activated, possibly by an activator protein (PinR) that belongs to the NtrC family of activators, as suggested by DNA sequencing. It has been shown that there is a background expression of α–pinene monooxygenase when wild type cells are grown in the absence of α–pinene. However, expression of this enzyme is increased at least 10-fold by growth in the presence of the inducer α–pinene. A wild type level of NADH oxidase expression was also seen in *E. coli* using constructs in which it is apparent that the NtrC-like protein was absent or truncated. Possible activator binding sites were identified upstream of the

promoter region. The structure of this part of the *P. fluorescens* chromosomal DNA, as suggested by DNA sequencing of pAC8 is presented in figure 12.3. The presence of a positive activator of the α–pinene monooxygenase promoter, as well as activator binding sites, remain to be confirmed.

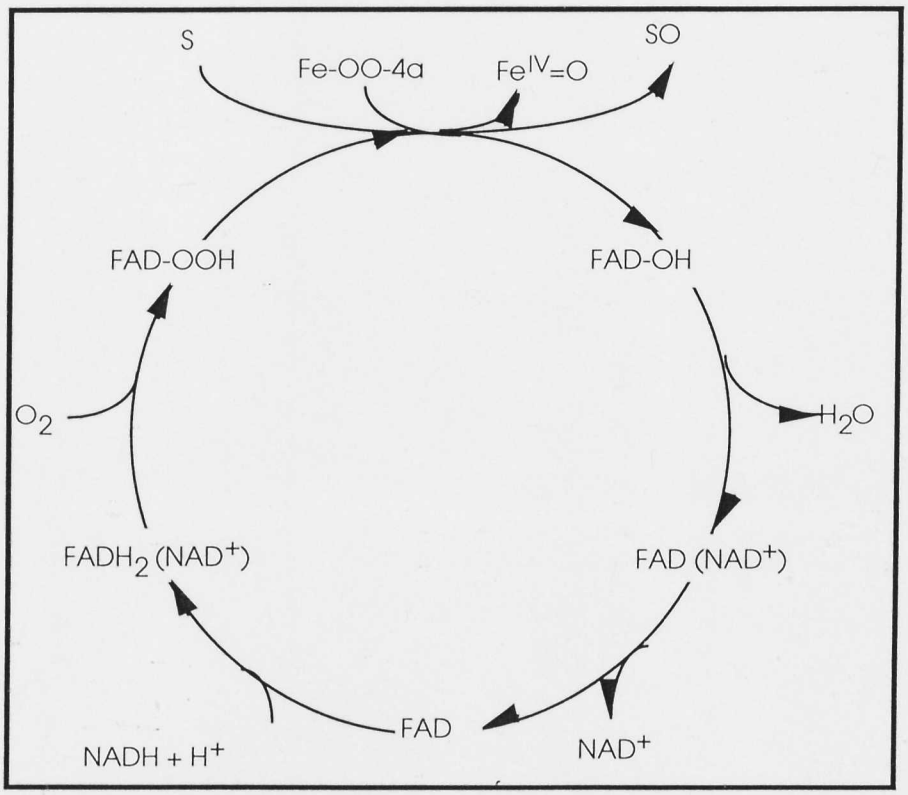


Figure 12.2: Proposed reaction mechanism of α–pinene monooxygenase from *Pseudomonas fluorescens* NCIMB 11671.

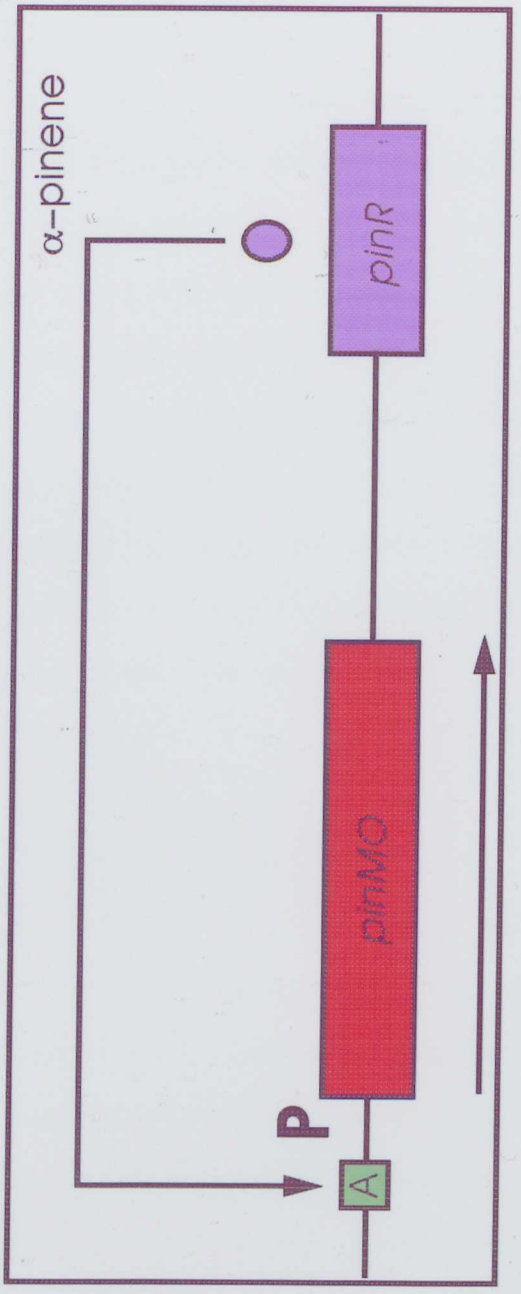


Figure 12.3: Proposed structure and function of the α–pinene degradation operon in the area surrounding the α–pinene monooxygenase gene. Symbols: A, activator binding site; P, promoter; *pinMO*, α–pinene monooxygenase gene; *pinR*, activator gene.

In addition, it is noteworthy that α–pinene monooxygenase does not appear to be a part of an operon. This of course raises the question of how the downstream genes are induced. The evidence presented in this study suggests that α–pinene oxide is not the inducer of these genes, although experiments on how well it is taken into the cells have not been performed. It could be that PinR is also involved in induction of a second set of downstream genes possibly by activating another regulatory gene, as is the case in the TOL operon, where the XylR protein in the presence of toluenes and xylenes positively controls both the Pu promoter which drives transcription of the *xylCMABN* genes, as well as the XylS protein, which in turn activates the Pm promoter for the transcription of the *xylXYZLEFJKIH* genes (Gibson and Zylstra, 1990; Franklin *et al.*, 1981; Harayama *et al.*, 1989; Harayama and Relik, 1989; Horn *et al.*, 1991; Inouye *et al.*, 1985; Spooner *et al.*, 1986).

12.2 Future Work

This study revealed that the α–pinene monooxygenase appears to be a novel flavo-iron protein, although its mechanism of catalysis still remains to be established by studies on EPR spectroscopy redox titration, as well as stopped flow studies of the purified enzyme. However, the gene encoding for the monooxygenase naturally has a very low level of expression, and thus, the amount of enzyme purified from *P. fluorescens* NCIMB 11671 was insufficient for further detailed characterisation of the enzyme. In order to acquire enough purified enzyme for spectroscopic and crystallography studies it will be essential to overexpress the monooxygenase gene. In order to achieve high expression three factors should be considered: the gene should be placed downstream of an efficient promoter, where a Shine-Dalgarno sequence along with an appropriately spaced initiating methionine codon should be present, and where the sequence following the methionine codon should be free of any secondary structures (Yansura and Simmons, 1992).

However, since the α–pinene monooxygenase gene is expressed from a *ntr*-like promoter which is activated by an activator gene that belongs to the NtrC family of activators, another approach would be the use of a suitable *E. coli* strain. Ntr-mediated activation of gene transcription can be demonstrated in strains which constitutively synthesise the *Klebsiella pneumoniae nif*A or *ntr*C gene products (Dixon, 1986). Therefore, the monooxygenase gene could be cloned in an *E. coli* strain where the NtrC or the NifA activator proteins are produced constitutively, and this would provide a suitable system for the expression of the gene of interest. In these systems the conserved central domain of the activator, *ntr*C, gene interacts with NtrA and/or RNA polymerase. A plausible model for transcriptional activation of this unique class of promoters involves initial recognition of the -12 and -24 regions by RNA polymerase interacting with *ntr*A and subsequent interaction of the activator either with the promoter or with RNA polymerase to initiate transcription. It is not clear at present why this mode of transcriptional activation is employed to regulate such diverse metabolic functions in Gram-negative bacteria as nitrogen assimilation and hydrocarbon catabolism. Possibly, the transcription complex formed after initiation at these promoters has unique properties with respect to elongation, pausing and termination which would be particularly advantageous for gene expression under specific growth conditions (Dixon, 1986).

Finally, the presence of a positive activator of the α–pinene monooxygenase promoter, as well as its binding sites, still remains to be confirmed. The most common factor indicating that a gene can be positively controlled is the finding that mutations located outside this gene prevent or greatly decrease its expression. Mutations that may identify a regulator gene comprise deletions insertions or nonsense mutations, which should only affect the initiation of transcription of the gene of interest rather than any other step in its expression. Techniques for the characterisation of potential regulatory mutations include gene fusion, S_1 nuclease or reverse transcriptase treatment. In gene fusion techniques, the promoter of the gene of interest is placed in front of an easily assayable gene, such as the genes encoding for β–galactosidase,

galactokinase, chloramphenicol transacetylase, or amylomaltase. Mutations in the regulatory gene must affect the expression of such hybrid operons in the same way as they affect the expression of the gene of interest. S_1 nuclease and reverse transcriptase experiments assay the mRNA molecules produced *in vitro* and *in vivo*. Thus, the effect of a regulatory mutation on transcription events at one given nucleotide can be studied (Raibaud and Scwhartz, 1984). Similarly, approaches to identify activator binding sites include searching for sequence homology in promoters controlled by the same activator, mapping mutations that alter the function of positively controlled promoter when present in *cis*, and using *in vitro* techniques. Defining an activator binding site by genetic means has proved difficult, mainly because a mutation in this site usually leads to a phenotype that at least superficially is identical to that resulting from mutations in the regulatory gene itself. On the other hand, biochemical identification of activator binding sites can be achieved by footprinting experiments, which determine which portion of DNA is protected against the action of a nuclease or of a chemical reagent by an activator *in vitro*.

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