STUDY OF AN ARTHROBACTER SP. ESTERASE ABLE TO HYDROLYSE MALATHION

CATHERINE HEDDLE

SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF LEICESTER
ACKNOWLEDGEMENTS

I would like to thank Dr. R.A. Cooper for allowing me to carry out my work in his laboratory and for all his help and guidance throughout my time at Leicester. Also to Zeneca LifeSciences Billingham for their support of this work through a CASE award.

Thanks to the members of Lab 108 for their assistance and companionship, especially Joe Stringfellow and Dr. Steve Hanlon. Also to all other members of the Biochemistry department who have helped me in any way.

Thank you to my parents for their support, encouragement and for moving my belonging to various student houses at regular intervals. Also to Racheal and my brother Jonathan who have had to live with me in the various residences.

Finally I would like to thank Nick for his support and advocating a PMA.
STATEMENT

This thesis, submitted for the degree of Doctor of Philosophy entitled: "Study of an *Arthrobacter* sp. esterase able to hydrolyse malathion", is based on work conducted by the author in the Department of Biochemistry during the period October 1993 to September 1996. All the work recorded in this thesis is original unless otherwise stated. None of this work has been submitted for a degree in this or any other University.
ABSTRACT

Study of an *Arthrobacter* sp. esterase able to hydrolyse malathion

Catherine Heddle

A bacterial isolate tentatively identified as an *Arthrobacter* sp. was isolated from soil using diethylsuccinate as a carbon and energy source. This bacterium was found to have two esterases that were active against diethylsuccinate, malathion and p-nitrophenyl acetate.

One of the esterase genes was studied further by shotgun cloning the gene into pUC18, transforming *Escherichia coli* and identifying the transformant through phenotypic selection. Subcloning identified the region of DNA responsible for encoding the esterase which lead to the partial purification of the protein and the determination of its N-terminal sequence. Information from the N-terminal sequence was then used to construct a degenerate oligodeoxyribonucleotide primer to start the sequencing of the gene.

Sequencing identified the gene encoding the esterase as 1140bp encoding a protein of 380 amino acids which showed sequence identity of between 34-38% with other carboxyl- and aryl-esterases in the databases. These comparisons also identified a putative active site serine within the motif Ser-X-X-Lys and also the partial motif Gly-X-Ser-X-X. Sequence information facilitated the overexpression of the esterase gene in vector pT7-7 which enabled the protein to be purified.

The purified esterase was found to be a monomeric protein of approx. 40kDa. The esterase was found to be inhibited by heavy metals but was stable at pHs in the range 7.4-11 and when stored for long periods at 0 and -20°C. The esterase was found to be exclusively a carboxylesterase and preferentially acted on uncharged esters such as diethylsuccinate rather than the charged monoethylsuccinate the degradation of which appeared to be the rate limiting step in the complete hydrolysis of diethylsuccinate to succinate.
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<td>kDa</td>
<td>kilo Daltons</td>
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<tr>
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<td>Luria broth</td>
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<tr>
<td>M63</td>
<td>M63 minimal media</td>
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<td>mg</td>
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<tr>
<td>Abbreviation</td>
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<td>--------------</td>
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<td>mins</td>
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<td>ml</td>
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<tr>
<td>MOPS</td>
<td>(3-([N\text{-Morpholino}])propane-sulphonic acid)</td>
</tr>
<tr>
<td>NAD(^{+})</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro-blue tetrazolium</td>
</tr>
<tr>
<td>O.D. 680</td>
<td>optical density at 680nm</td>
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<tr>
<td>OPA</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
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<tr>
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<td>polymerase chain reaction</td>
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<tr>
<td>PMS</td>
<td>phenazinemethosulphate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-boric acid EDTA buffer</td>
</tr>
<tr>
<td>(t_d)</td>
<td>Doubling time</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>(N,N,N',N'\text{-tetramethylethlenediamine})</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-(\beta)-D-galactopyranoside</td>
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1. INTRODUCTION

1.1 Background information

Mankind has for many centuries attempted to control his environment. One example of this has been in agriculture where there has been a long running battle with pest infestations. The history of pest control dates back to ancient Greece and Rome where compounds such as sulphur and arsenic were used. Through the following centuries chemical pest control changed very little until the use of Bordeaux mix in the 1890's and the organomercury compounds prior to the First World War. However, there was a dramatic change in the Second World War when the organochloride DDT was developed in Switzerland. At the same time organophosphorus compounds such as tabun and sarin were being developed in Germany for use as nerve gases. These compounds were designed specifically to be toxic in their action as anticholinesterases.

The organochlorides were used widely as pesticides after the Second World War and were highly successful in controlling pests and so improved agricultural output. However, these chemicals became an unacceptable health hazard due to their persistence in the environment leading to biomagnification and toxicity in higher animals (Rosenberg and Alexander, 1979). As a result of this the organophosphorus compounds were developed for use as pesticides after their initial development as nerve gases. These chemicals found widespread use because of their low persistence compared to the organochlorides and their effectiveness at eradicating insects. They conform to the general structure as seen in Fig. 1.1.

The last 50 years has seen a dramatic change in farming practices in North America and Europe. Farms have become larger with fewer crops and reduced crop rotation has led to a monoculture system. These intensive conditions lead to crops being susceptible to pest attack and so increasing the need for pesticides. Various problems arise from the requirement for increasing amounts of pesticides:

- resistance in the target insect population
- natural predator damage
- residues in the environment

When these conditions prevail crops may become a target for resistant insects which, not
General organophosphorus compound structure

R₁ and R₂ alkyl (ethyl or methyl) moiety
R₃ substituted aryl or alkyl moieties

Structure of acetylcholine

Figure 1.1 (A) The general structure of the organophosphorus pesticides, arrows indicate the possible positions of attack by enzymes. (B) the structure of acetyl choline.
affected by the insecticide or natural predators, require the use of another pesticide to control them. This results in an increase in the demand for pesticides to overcome the problem, so leading to a chemically dependent society. Today there are thousands of formulations of these chemicals available for use and millions of tonnes used every year around the world.

The problem of residues left in the environment is of particular importance with regard to exposure of animals and humans. As already mentioned, the first organophosphorus compounds developed included the nerve gases tabun and sarin. These compounds were specifically designed to be toxic in their action as anticholinesterases as their structures are similar to acetylcholine, see Fig.1.1(B). Therefore, these compounds may also act on other organisms that possess acetylcholinesterases such as higher animals and humans. Organophosphorus and carbamate insecticides affect the way in which the signal carried by one nerve is conveyed across the synaptic gap to another nerve or to the system it activates. In vertebrates cholinergic nerve cells emit a new signal by producing acetylcholine at the synapse, see Fig.1.2. Once the signal has been received by the next nerve cell, or the system to be activated, the transmitter must be removed. This is to enable the clearing of the synapse and the receiver for the next signal. Acetylcholine is removed by the action of acetylcholinesterase, which is usually present in excess at the junctions. If the transmitter is not removed, either by sorption, diffusion or destruction, then the following signal will not be recognised by the blocked junction. Constant blockage of the receptor by the signal leads to constant firing of the nerve. This leads to various clinical symptoms including nausea and convulsions, and even death. The action of acetylcholinesterase results in the transient acetylation of the enzyme and the splitting and deactivation of the acetylcholine. The acetylcholine binds to the cholinesterase at two sites, see Fig.1.3(A). The first is thought to be a serine ester-forming site and the second is an anionic site which might contain a glutamic acid residue. The acetylation is carried out by the carbonyl group of the substrate making an electrophilic attack on the hydroxyl group of the serine. This results in the free choline leaving the enzyme, see Fig.1.3(B). The serine ester bond is then rapidly hydrolysed in the enzyme's recovery stage. The enzyme is then able to accept another molecule of acetylcholine. The inhibition caused by organophosphorus compounds is due to them having some structural similarity to acetylcholine. The phosphate
Figure 1.2 The transfer of acetylcholine over the synapse in the nervous system.
Figure 1.3. The binding of acetylcholine to cholinesterase.
- (A) A schematic diagram of the cholinesterase showing the alignment of acetylcholine with the enzyme.
- (B) The acetylcholine is split and the enzyme becomes transiently acetylated.
- (C) The cholinesterase becomes phosphorylated when it hydrolyses an organophosphorus compound.

Ser- Serine
Glu- Glutamic acid
His- Histidine
EB- serine ester bond
Acetylcholine

Ester-forming site

Anionic site

N(CH$_3$)$_3$

Ser - O$^-$ - H$^+$

C - Glu

His

Part of the secondary structure

HO - CH$_2$ - CH$_2$ - N(CH$_3$)$_3$ +

Choline (leaving the surface)

Dephosphorylated residue of the organophosphorus compound

HO - Leaving group

(C)
group is attracted to the serine ester site and the rest of the organophosphorus molecule aligns itself to the active site of the enzyme, see Fig. 1.3(C). The interaction of the acetylcholinesterase with an organophosphorus compound results in the phosphorylation of enzyme rather than acetylation. The resulting serine ester bond is relatively stable to hydrolysis and consequently has a much longer recovery time. The normal turnover of the acetylcholinesterase is approximately $1.4 \times 10^4$ sec$^{-1}$ (Fersht, 1985). For enzyme phosphorylated by an organophosphorus compound it takes approximately 80 min for half of the enzyme molecules to dephosphorylate. This is if the phosphorylating entity has dimethyl attached to it, but the process can be up to six times longer if it is phosphorylated by diethyl phosphate. The formation of the serine ester bond also depends on the stability of the P-X bond in the organophosphorus compound, where X is the 'leaving group’. This stability depends on the electron attracting capability of the group X and is the reason why the various X groups on the organophosphorus compounds are important. These groups are also important as they may influence the way in which the organophosphorus compound may be degraded, or how easy it is for the molecule to get to the active site in the enzyme it is targeting. These mechanisms of action and inhibition of components of the nervous system in both target pests and other organisms highlight the dangers of the organophosphorus compounds. In this respect pesticides are potentially dangerous and their application an example of the intentional pollution of the environment. The contamination of humans, animals, and land can take place in several ways:

- exposure of workers (absorption through skin, inhalation, ingestion)
- foodstuff contamination
- ground water contamination
- industrial effluent
- seepage
- transportation accidents
- residues in containers
- residues on equipment
As our agricultural system is so chemically dependent and there is a constant world-wide demand for crops, the use of pesticides is unlikely to be curbed. However, there are methods by which pollution caused by these chemicals can be reduced.

1.2 Organophosphorus compounds

The organophosphorus insecticides have a general structure as already shown in Fig. 1.1. Variants of the general structure include the phosphates, with four oxygens attached to the central phosphorus; thiophosphates, with three oxygens and one sulphur attached to the phosphorus; dithiophosphates, with two oxygen and two sulphur attached to the phosphorus and the thionophosphates, with a sulphur attached via a double bond. This structural variability of organophosphorus compounds makes them useful in two ways:
- They can be used in a species-specific manner, based on the activity of the enzymes an insect possesses.
- The multiplicity of possible positions to attack in each organophosphorus compound makes the development of tolerance over the whole spectrum of these chemicals difficult, see Fig. 1.1.

Organophosphorus insecticides can be classified into four subgroups with respect to their practical uses:

1. **Low persistence** contact poisons which have a low chemical stability and are hydrolysed by water, e.g. Dichlorvos.

2. **Loco-systemic compounds or persistent** contact poisons, that have variable chemical stability and are lipophilic, e.g. Malathion.

3. **Systemic insecticides**, soluble in lipids and with a higher water solubility than the loco-systemic compounds. This group includes thioesters, e.g. Phorate, and carbamates, e.g. Dimethoate.

4. **Fumigants**, which act in the vapour phase, e.g. Dichlorovos.

Some insecticides such as Chlorfenvinphos can be granulated for safe application and used against soil organisms.
The organophosphorus compounds to be concentrated on in this study are the dithiophosphates malathion and dimethoate which are loco-systemic and systemic insecticides, respectively.

1.3 Pollutant removal

There are various methods of dealing with polluted land. The main options are:-

1. Physical treatment. These methods involve incineration, entrapment and burial of contaminated material. These options are extreme and either completely destroy the contaminated site, as in the case of incineration, or only find a temporary solution as in the case of burial of the waste.

2. Chemical treatment. This involves the application of a chemical to the polluted area which would bring about the destruction of the pollutant. This is a cheap and easy method but can lead to the secondary pollution of the area. Although the first compound may have been converted into a less toxic form, there is still the residual chemical left from the treatment process.

3. Bacterial whole cells. Bacteria can be found in every environment on Earth, they possess the metabolic diversity to adapt, and successfully inhabit every niche available. This metabolic diversity has not only allowed bacteria to conquer the natural environment but has also enabled them to develop the capability to deal with the synthetic compounds with which man has polluted the environment. The metabolic abilities of micro-organisms have long been exploited by man in processes ranging from sewage treatment to the production of alcohol. In more recent times investigations into the capability of bacteria to treat pollution have been undertaken. Chemicals of interest have been those compounds which are a threat to the environment and human health, such as the persistent organochloride pesticides and polychlorinated biphenyls (PCB’s) and dangerous organic chemicals like benzene, xylene and toluene and the poly aromatic hydrocarbons (PAH’s).

One of the richest sources of bacteria capable of degrading a large range of natural and synthetic chemicals is soil. Soil is also used as a source of bacteria with potentially useful enzymes as the soil may have been exposed to some of the chemicals of interest or
similar compounds. With such a diversity of bacteria available it is possible to specifically treat soil with a pollutant to select for bacteria with the desired enzymes for the degradation of that chemical. Degradative abilities of soil bacteria include the plasmid-encoded pathways for the catabolism of toluene and xylene (Williams and Murray, 1974) and 3-chlorobenzoate and \( p \)-chlorobiphenyl (Chaterjee \textit{et al}, 1981). There are also some degradative pathways, such as the one encoded by the \( bph \) gene cluster for the degradation of polychlorinated biphenyl in \textit{Arthrobacter M5}, that are encoded by chromosomal DNA (Peloquin and Greer, 1993). The ability of bacteria to grow at the expense of a polluting compound would enable them to degrade the compound to a non-toxic form. This may then release metabolites that other bacteria could then grow on. However, using whole bacterial cells raises several problems. The bacteria may require other nutrients such as nitrogen and phosphorus sources to be able to degrade the chemical and these might not be readily available in the polluted environment. Another problem may be the availability of the toxic chemical. The pollutant may be deposited on the surface of soil or could be distributed over a wide area including land and water and may cause problems for the bacteria in being able to reach the pollutant. Assuming the bacteria can reach the pollutant there might be problems in the uptake (unless the enzyme(s) are excreted as in the case of \textit{Phanerochaete chrysosporium}) of the chemical for conversion which may involve a series of enzymic reactions. A general concern when using whole cells is the release of viable bacteria into the environment. This is of particular concern if the bacteria are genetically engineered. It is difficult to assess the impact of the release of such organisms on the natural soil floras and once the bacteria are released they are irretrievable.

4. Bacterial enzymes. The final possibility is the use of bacterial enzymes. An enzyme could convert the toxic compound into a less toxic chemical so would do the job of the chemical treatment without the secondary contamination. Using enzymes rather than whole viable cells does not present any of the problems of uptake, nutrition, availability or contamination through non-recoverable bacteria. However, there are drawbacks with this method as with all of the others. The use of bacterial enzymes can be expensive as the enzymes need to be extracted from cells for use. The production of an enzyme for a specific process may only carry out one of the reactions in the degradation of the contaminant and
not the full degradation that might be seen with whole cells. This then requires the reaction that is carried out to be significant in the detoxification of the substrate. The key characteristics for an enzyme that is to be used in a detoxification process are:-
- be able to convert chemicals to a less toxic form.
- no requirement for cofactors (a requirement for cofactors would incur extra cost).
- be obtained in large quantities (be available in bacteria in an over expressed form).
- ideally have a variety of target chemicals so that it could be used as a broad range detoxification tool.
- be stable and able to function under the conditions in the contaminated area.

1.4 Bacterial degradation of organophosphorus compounds

Microbial enzymes able to hydrolyse organophosphorus compounds have been studied in the past. Areas of study have included the potential of enzymes in detoxifying contaminated soil and equipment and hydrolysing the residues left in containers. Other areas of study have been investigations into the phenomenon of ‘problem soils’ in which pesticides are hydrolysed rapidly so decreasing their efficacy. Research has been undertaken on a variety of organophosphorus compounds, the main focus being on the insecticide parathion which has been used extensively against insects world-wide. The hydrolysis of parathion has been studied with respect to whole cells (Munnecke and Hsieh, 1976), in cell-free extracts (Munnecke, 1976) and with regards to the genetic basis of the process (Serdar et al., 1989; Sethunath and Yoshida, 1973). Degradation of parathion by a mixed bacterial culture (Munnecke and Hsieh, 1976) was found to produce p-nitrophenol and diethylthiophosphoric acid as the major metabolites indicating that the hydrolytic cleavage of the P-O bond joining these two components had occurred, see Fig.1.4. On hydrolysis of the parathion to these metabolites the toxicity was reduced by 120 fold. The metabolites that were produced were also found to be water soluble and p-nitrophenol was used by other bacteria as carbon and energy sources (Daughton and Hsieh, 1977). Extract made from the
Figure 1.4 The hydrolysis of parathion to the water soluble metabolites $p$-nitrophenol and diethylthiophosphoric acid (Munnecke et al, 1982)
amino parathion

parathion hydrolase

parathion

parathion hydrolase

paraxon

parathion hydrolase

p-amino phenol + diethylthiophosphoric acid

p-nitrophenol

4-nitrocatechol

hydroquinone + NO₂

Ring cleavage

Ring cleavage
mixed cultures showed hydrolytic activity with several other organophosphorus compounds including paraxon, diazion, dursban, cyanophos, fenitrothion, triazophos, propentamphos, and quinalphos, at rates significantly higher than chemical hydrolysis with sodium hydroxide. The studies of the mixed cultures able to degrade parathion resulted in the isolation of *Pseudomonas diminuta* strain MG. Another parathion hydrolysing bacterium *Flavobacterium* sp. (ATCC 27551) was isolated by Sethunathan and Yoshida (1973). The genetic basis of their ability to degrade parathion was investigated and plasmids of approx. 66kb and 39kb were identified as essential for *Pseudomonas diminuta* and *Flavobacterium* sp. ATCC 27551 respectively, to degrade parathion. Loss of hydrolase activity in both bacteria was correlated with plasmid removal. The gene encoding the parathion hydrolase was termed *opd* (organophosphorus degrading gene). The *opd* genes from *Pseudomonas diminuta* and *Flavobacterium* sp. ATCC 27551 were shown to be similar by restriction mapping and DNA-DNA hybridisation experiments. Hybridisation studies also showed that the plasmids carrying these genes were only similar in the region of the *opd* genes (Mulbry *et al*, 1987). As parathion is not a naturally occurring compound it is possible that the ability to hydrolyse it is due to an enzyme that usually has another role in normal cell metabolism but is able to degrade this compound. The parathion hydrolase encoding gene might also be due to a mutation in a gene encoding a non-specific esterase. Other bacteria have also been isolated that have parathion hydrolase activity (Brown, 1980, Chaudhry *et al*, 1988 and Mulbry and Karns, 1989 a and b). These other enzymes have been identified as parathion hydrolases but were found to be heterogeneous in terms of molecular size, stimulation and inhibition by various compounds and location in the cell. This showed that there were several enzymes that were capable of hydrolysing parathion in nature.

Parathion hydrolase has been tested commercially on contaminated soils and shown to hydrolyse the pesticide at a much greater rate than chemical means of hydrolysis (Munnecke, 1980). The enzyme has also been shown to hydrolyse waste in pesticide containers. In 16hrs 90% of the parathion present as a 48% emulsifiable concentrate was hydrolysed.

Other pesticides such as diazinon, dursban, triazophos, fenitrothion have been studied with regard to their hydrolysis by bacterial cell extracts. Some have been studied in
detail, such as the enzyme able to hydrolyse the herbicide Phenmedipham (Pohlenz et al, 1992), where the enzyme has been purified and the gene encoding it sequenced and analysed.

1.5 Malathion

A member of the dithioate group of organophosphorus insecticides, malathion follows the general formula containing two methyl groups and a diethylsuccinate group attached, via sulphur, to a central phosphate. Figure 1.5 shows the general structure of the organophosphorus pesticides, the dithioate group and malathion.

Malathion is a loco-systemic insecticide and is mainly used in the control of sap-sucking insects such as aphids due to its conversion in vivo to the even more potent acetylcholinesterase inhibitor malaoxon, see Fig.1.6. It has also been used as a mosquitocide and in the treatment of ectoparasites. It is an expensive chemical in comparison with other organophosphorus compounds but still popular with approximately 6x10^7 kg used world-wide in 1984. This widespread use can be attributed to its relatively low persistence and easy application and use against a variety of pests.

Like most chemicals when applied in the correct amount and in the correct manner malathion does not pose a major threat to the environment. It is through incorrect application and accidental spillage that problems arise. These types of problems can be tackled in a variety of ways including chemical treatment, use of whole bacterial cells or through the use of enzymes. However, as already mentioned, some of these methods have drawbacks.

Malathion is known to be inactivated in humans by the hydrolysis of the ester linkage, see Fig.1.6, suggesting that the most suitable enzyme for the detoxification of malathion would be one that would hydrolyse this carboxyl ester bond. It would also be an advantage for the enzyme to have a broad spectrum of activity and be able to hydrolyse several different types of ester bonds. This would then facilitate the use of the enzyme to detoxify other pesticides within the organophosphorus family. Other insecticides belonging to the various groups already mentioned that also contain a carboxyl ester bond including
General organophosphorus structure
$R_1$ and $R_2$ alkyl (ethyl or methyl) moiety
$R_3$ substituted aryl or alkyl moieties

\[
\begin{align*}
R_1 O & \quad P \quad S \\
R_2 O & \quad O \quad R_3 
\end{align*}
\]

General dithioate structure
$R_1$ and $R_2$ alkyl (ethyl or methyl) moiety
$R_3$ substituted aryl or alkyl moieties

\[
\begin{align*}
CH_3 O & \quad P \quad S \\
CH_3 O & \quad S \quad CH_2 COOC_2H_5 \\
& \quad CH_2 COOC_2H_5
\end{align*}
\]

Figure 1.5. (A) Structures of organophosphorus compounds, (B) dithioates and (C) malathion.
Figure 1.6 The conversion of malathion to the potent acetylcholinesterase inhibitor malaoxon by insect metabolism (Hassall, 1990)
Aldicarb; Methiocarb; Carbofuran and Propoxur all of which are carbamate insecticides.

Research into the microbial enzymatic degradation of malathion has not been as extensive as that undertaken for parathion as studies have only identified bacterial isolates with malathion hydrolase activity in their cell extracts. Walker and Stojanovic, (1974), isolated several bacteria from soil that were able to metabolise malathion. Analysis of the metabolites by thin layer chromatography showed that both malathion mono- and dicarboxylic acids were produced as well as, O-dimethyl malathion, potassium dimethyl phosphorodithioate and potassium dimethyl phosphorothioate. The most effective bacterium was found to hydrolyse malathion to both its mono- and di-carboxylic acid forms. Bacteria were isolated from salt marsh environments that had been treated with malathion as part of a mosquito control program, (Bourquin, 1975 and 1977). The bacteria were found to degrade malathion to dicarboxylic acid via the mono ester intermediate when supplied with additional nutrients as carbon and energy sources. There have also been reports that the fungus *Trichoderma viride* and a *Pseudomonas* sp. isolated from soil heavily sprayed with malathion (Matsumura and Boush, 1966) could hydrolyse malathion. The major portion of the products of hydrolysis were found to be the carboxylic acid derivatives of malathion suggesting that hydrolysis was due to the carboxylesterases present in the microorganisms. Another study by Getzin and Rosefield (1971) showed that an enzyme of unknown origin isolated from clay loam was responsible for degrading malathion to its mono ester form. Other soil micro-organisms tested were *Rhizobium* spp. from Egyptian soil that had been exposed to malathion treatment (Mostafa *et al* 1972), again the major metabolites were found to be malathion carboxylic acid derivatives. However, when the mono ester was added to this enzyme system no hydrolysis was seen, suggesting that the formation of the dicarboxylic acid product was due to another enzyme. Paris *et al* (1975) reported the isolation of bacteria from an aquatic system using malathion as a carbon source. Analysis of the metabolites from the bacterial growth media revealed that the major metabolite was β-malathion mono acid with only 1% of the malathion being transformed to the dicarboxylic acid form. A *Pseudomonas* sp. was reported to cometabolise malathion (Singh and Seth, 1989), up to 150ppm in the presence of ethanol. However, the presence of malathion at 200ppm caused a decrease in growth indicating that the malathion was toxic.
at this concentration. On analysis of the hydrolysis products malathion was found to be degraded to only mono ester form. Micro-organisms capable of using malathion as a sole carbon source have also been reported by Barik et al. (1982). The enzymes responsible were characterised with respect to optima of temperature, pH and substrate concentration. However, the enzymes were not purified or studied with respect to any other enzymic capabilities they might have. There has apparently been no attempt to characterise further the process with regard to genetics. From the studies that were carried out on the degradation of malathion a putative pathway of degradation was suggested, see Fig. 1.7

The limitation for using malathion as a growth substrate for the isolation of bacteria is that it has a very low solubility in water of only 145mg/litre. This means that it can not conveniently be used as a sole carbon and energy source to support bacterial growth for the easy analysis of enzymes.

1.6 Dimethoate

Dimethoate is also a dithioate pesticide, see Fig. 1.8, and belongs to the carbamate family of organophosphorus insecticides. The carbamates are structurally and physiologically heterogeneous and are classified on the basis that they are all derivatives of carbamic acid. Some carbamates are insecticides and act as anticholinesterases, others are herbicides and interfere with cell division and there are also classes of carbamates which act as fungicides and nematocides. Dimethoate is a systemic insecticide and is highly solubility in water. The main uses of dimethoate are in aphid control and, because of its high LD$_{50}$, for the maintenance of farm animal hygiene.

As in the case of malathion there appears to have been little research carried out on the degradation of dimethoate. Rosenberg and Alexander (1979) reported that a Pseudomonas sp. was capable of using dimethoate as a sole phosphorus source. The same organism was also reported to be capable of using malathion as a sole phosphorus source. Barik et al., (1982) obtained enzymes capable of degrading malathion, dimethoate and gusathion from two Arthrobacter sp. which were grown on dimethoate and gusathion as sole carbon sources.
Figure 1.7. The proposed pathways for the degradation of malathion (Munneke et al, 1982)
1.7 Use of organophosphorus compounds as sources of phosphorus

The phosphorus content of the bacterial cell is 3% of the dry weight and so could be supplied by the organophosphorus compounds even if they have a low solubility. The use of a low concentration of organophosphorus compound also reduces the problems that might arise trying to isolate bacteria using an organophosphorus compound that is toxic to the micro-organisms. The study by Rosenberg and Alexander, (1979) claimed that extracts from *Pseudomonas* strains grown on media containing diazinon and malathion, but not orthophosphate, caused the degradation of these insecticides, suggesting that the organophosphorus compounds were hydrolysed by an inducible enzyme. However, to utilise the phosphorus present in organophosphorus compounds a variety of bonds may be needed to be hydrolysed which might require the action of more than one enzyme. Research into the hydrolysis of various P-X bonds has been carried out. The parathion hydrolase enzyme from *Flavobacterium* sp. ATCC 27551, a phospho-triesterase, was unable to degrade parathion any further than p-nitrophenol and diethyl phosphoric acid which resisted further hydrolysis. Organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* MG was found to be capable of detoxifying a variety of neurotoxins by hydrolysing the phosphoester bonds, P-O, P-F, P-CN and P-S, albeit at different rates (Lai *et al*., 1995). Another organophosphorus hydrolysing enzyme able to hydrolyse the P-O bond was identified in the *Alteromonas* strain JD6.5, (Cheng *et al*, 1993). This enzyme was able to hydrolyse the P-F bonds in the neurotoxin diisopropylfluorophosphate (DFP) as well as the
P-O bonds in the chromogenic \( p \)-nitrophenyl containing phosphinates such as \( p \)-nitrophenylmethyl phenylphosphinate (NPMPP) and \( p \)-nitrophenylethyl phenylphosphosphinate (NPEPP). These investigations suggest that such enzymes may play a role in releasing the ionic phosphorus moiety even if they, like the \textit{Flavobacterium} sp. parathion hydrolase, cannot degrade this phosphorus containing metabolite.

Although a single enzyme has not been isolated that is capable of releasing phosphorus, bacteria have been isolated (Cook \textit{et al} 1978) that are able to utilise representatives of ionic phosphorus containing breakdown products of organophosphorus compounds such as diethyl phosphoric acid from parathion as sole phosphorus sources. This process was thought to be plasmid encoded as there was a loss of ability to use the organophosphorus compounds as phosphorus sources after growth on non-selective media. This research on the use of organophosphorus compounds as phosphorus sources suggests that there may be a variety of enzymes involved in the process of releasing the phosphorus for use by the bacterial cells and that this process may be inducible or plasmid encoded.

1.8 The catabolic abilities of \textit{Arthrobacter} sp.

Known \textit{Arthrobacter} sp. have been reported to produce industrially and commercially useful chemicals including glutamic acid, \( \alpha \)-ketoglutaric acid and riboflavin. \textit{Arthrobacter} sp. have also been reported to produce phytohormones, be capable of dinitrogen fixation, lyse pathogenic fungi and degrade pesticides and herbicides as well as a wide range of natural and synthetic molecules. Due to these diverse metabolic activities the \textit{Arthrobacter} sp. are thought to play an important part in the mineralisation process in soil.

Members of the genus \textit{Arthrobacter} are generally nutritionally non-exacting and usually require no addition of supplements to the growth media. A major distinguishing feature of these bacteria is their pleomorphic growth cycle where cells are irregular rods in young culture and are Gram positive, then in older cultures the cells adopt a coccoid appearance and Gram staining can be variable. Although this life cycle is indicative of an \textit{Arthrobacter} sp. the definitive test is the presence of lysine as the cell wall diamino acid.
These bacteria are well adapted for survival in the soil environment as they are extremely resistant to drying. This resistance to desiccation and their nutritional versatility increases their likelihood of survival and ensures that they are one of the common components of the soil flora. *Arthrobacter* sp. have also been isolated from fish, sewage and plants but not from clinical sources. The major routes of catabolism in these bacteria have been shown to be the Embden-Meyerhof-Parnas pathway and, to a lesser extent, the Hexose Monophosphate pathway (Krulwich and Pelliccione, 1979). It has been shown (Sobel *et al*, 1973) that for some *Arthrobacter* sp. there is a need for low concentrations of Krebs cycle intermediates, or pre-growth on these substances, to enable the bacteria to grow on glucose. This is needed to provide the proton motive force to take up glucose because uptake and respiration are coupled.

Bacteria isolated from soil that have been shown to degrade pollutants such as PCBs and pesticides have been identified as *Arthrobacter* sp. *Arthrobacter oxydans* P52 was isolated from soil and found to degrade the phenylcarbamate herbicides phenmedipham and desmedipham cometabolically by hydrolysing their central carbamate linkages (Pohlenz *et al*, 1992). The phenmedipham hydrolase gene, situated on a 41kb plasmid, encoded a 55kDa monomer which showed sequence homology to esterases of eukaryotic origin. Another *Arthrobacter* -like strain able to degrade the fungicide iprodione was isolated from a fast iprodione degrading soil, (Athiel *et al*, 1995). Soil exposed to the herbicide *s*-ethyl-*N*,*N*-dipropylthiocarbamate (EPTC) was used to isolate bacteria that were able to degrade this pesticide. An *Arthrobacter* sp. strain TE1 was subsequently isolated that could grow on EPTC as a sole carbon source. This ability to degrade the herbicide was found to correlate with the presence of a 50.5 megadalton plasmid, (Tam *et al*, 1987). Other carbamate insecticides, carbofuran, carbaryl and bendiocarb, have been shown to be degraded by an *Arthrobacter* sp. isolated from soil, (Ramanand *et al*, 1991). Other pollutants reported to have been degraded by *Arthrobacter* sp. have included, 4-chlorobenzoate (Schmitz *et al*, 1992 and Tsoi *et al*, 1991), polychlorinated biphenyls, (Peloquin and Greer, 1993) and catechol, (Eck and Belter, 1993). Another *Arthrobacter* sp., *Arthrobacter globiformis* SC-6-98-28, was found to be able to hydrolyse the pyrethroid insecticide ethyl chrysanthemate, (Nishizawa *et al*, 1993).
1.9 Carboxylesterase (E3.1.1.1) and related enzymes

Esterases, in the strict sense, catalyse the hydrolysis of a large number of uncharged carboxylic esters (Krisch, 1971). However, Donnelly and Dagley (1980) did report an esterase able to act on the oxalacetic acid 4 methyl ester. Esterase enzymes are widely distributed and are present in vertebrate tissue, insects, plants, bacteria and fungi. From the wide distribution of these enzymes it can be deduced that they are important in many natural processes. In mammalian cells carboxylesterase comprise a family of isozymes that are present in most cells and in particular liver cells. Their role is in the metabolism of ester, thiol ester and amide xenobiotic compounds and so play an important role in the detoxification system of the body (Huang et al, 1993). Esterases in plants are responsible for the production of flavours and aromas so play a part in plant development and reproduction. Bacteria produce carboxylesterase enzymes to enable them to degrade the ester linkages in substrates that they utilise for growth (McKay, 1993).

Bacterial esterases have been studied for a variety of reasons such as their role in the development of flavours, texture and digestibility of foodstuffs (McKay, 1993 and Lambrechts et al, 1995). An esterase from Bacillus subtilis has also been used in the de-esterification of pro-drugs, (Zock et al, 1994 and Chen et al, 1995) where p-nitrobenzyl esters serve as protecting groups on intermediates in the manufacture of clinically important oral antibiotics and de-esterification is required for the synthesis of the final product. It has also been reported that an esterase enzyme has been used for the stereoselective production of pyrethroid insecticides (Nishizawa et al 1993). The esterase from Arthrobacter globiformis SC-6-98-28 was found to stereoselectively hydrolyse (±)-cis, trans-ethylchrysanthemate to produce (+)-trans acid the most effective configuration of the stereoisomers. Studies of bacterial esterases have suggested that a number are general in their action and have the ability to hydrolyse carboxyl ester bonds, thiolester bonds and possibly peptide bonds. Bacterial isolates have also been reported (Lambrechts et al, 1995 and Hong et al, 1991 and Kim et al, 1994) to have a variety of multiple esterases or isoenzymes able to act on one sort of substrate.
The initial aim was to isolate bacteria which exhibited broad esterase activity and were capable of degrading dithioate organophosphorus compounds. The two organophosphorus compounds chosen for study were malathion and dimethoate. Selection of suitable bacteria was carried out by assaying cell extracts of isolates with ester substrates. Once suitable isolates had been identified then further experiments were to be undertaken for which large amounts of enzyme would be required. To facilitate this the gene(s) encoding the enzymes needed to be identified and over expressed. In order to clone the esterase gene(s) for study two approaches were taken:

- Purification of esterase protein(s) from the original isolate for use in N-terminal sequencing. This would then facilitate the construction of a probe to identify the esterase gene in a gene library.
- Construction of a gene library and isolation of the cloned gene(s) by phenotypic change in host organism.

Once the DNA encoding the esterase(s) was identified detailed structural analysis would be carried out on the gene(s). Sequencing would identify the start of the gene and enable the amino acid sequence to be derived and analysed. Sequence information would facilitate the manipulation of the gene to gain over expression of the cloned gene(s). By achieving over expression of the gene the esterase(s) could then be purified and used to characterise enzyme activity.
2. MATERIALS AND METHODS

2.1 Bacterial strains and vectors

Known *Escherichia coli* strains and plasmid vectors used in this study are given in Table 2.1.

2.2 Growth media and conditions

Bacteria were grown on either complex or minimal media. Complex media (Luria Broth [LB]) was as described by Miller (1972) and minimal media was as described by Hareland *et al* (1975). Minimal media for isolation of bacteria from soil contained 10ml of basal salts and 10ml of trace metal salts both 10x concentration (see Table 2.2) per 100ml of distilled water. Minimal media for the growth of *E. coli* strains (minimal medium M63) contained 20ml of basal salts (5x concentration, see Table 2.2) and 1ml of MgSO\(_4\) (0.1M) per 100ml of media. Where appropriate liquid media was solidified by the addition of 1.5%(w/v) Bacteriological agar (Agar No.1, Oxoid). All media were autoclaved at 121°C for 15min.

Cells prepared for the study of enzymes were grown at 30°C whereas those for DNA preparations were grown at 37°C. Liquid cultures were incubated in a Gallenkamp orbital shaker at 200rpm. Carbon sources were sterilised separately by autoclaving or filtration as appropriate. These were then added aseptically to the minimal media to give a final concentration of 60mM carbon, see Table 2.3.

Minimal media and LB flasks were inoculated with a single colony picked with a sterile loop and grown overnight. These overnight cultures were then used to inoculate fresh media.

Ampicillin was added as required to media to give a final concentration of 100µg.ml\(^{-1}\). Isopropyl-β-D-thiogalacto-pyranoside (IPTG, 0.3mM) was incorporated into the media as a β-galactosidase inducer. Host JM109 cells carrying the *lacZΔM15* had the deletion phenotypically complemented by the β-galactosidase α-fragment encoded by the pUC vector. Complementation was indicated by the formation of blue colonies on agar.
<table>
<thead>
<tr>
<th>Strain/Plasmid vector</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> JM109(DE3)</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB+ lacI9 lacZΔM15] (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</td>
<td>Anon. (1989) Promega notes 20 number 2</td>
</tr>
<tr>
<td>pUC18/19</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, lacZ&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Yanisch-Perron <em>et al</em> (1985)</td>
</tr>
<tr>
<td>pT7-7</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, Ø 10</td>
<td>Tabor and Richardson (1985)</td>
</tr>
</tbody>
</table>

**Table 2.1.** Bacterial strains and plasmid vectors used in the course of this study
### Table 2.2. Media composition

<table>
<thead>
<tr>
<th>Media</th>
<th>Content</th>
<th>g/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJC Trace metal (10 times concentration)</td>
<td>Nitrilotriacetic acid MgSO$_4$ FeSO$_4$.7H$_2$O MnSO$_4$.4H$_2$O ZnSO$_4$.H$_2$O CoCl$_2$</td>
<td></td>
</tr>
<tr>
<td>PJC Basal Media (10 times concentration) pH7</td>
<td>K$_2$HPO$_4$.3H$_2$O NaH$_2$PO$_4$.2H$_2$O NH$_4$Cl</td>
<td></td>
</tr>
<tr>
<td>M63 Salts (5 times concentration) Adjusted to pH7 by the addition of KOH</td>
<td>K$_2$HPO$_4$ [NH$_4$]$_2$SO$_4$ FeSO$_4$.7H$_2$O Vitamin B1</td>
<td></td>
</tr>
<tr>
<td>Luria Broth (LB)</td>
<td>Tryptone Yeast extract NaCl</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.3. Carbon sources

<table>
<thead>
<tr>
<th>Carbon Sources</th>
<th>Final concentration (mM)</th>
<th>g/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethylsuccinate</td>
<td>7.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Monoethylsuccinate</td>
<td>10</td>
<td>1.64</td>
</tr>
<tr>
<td>Fructose</td>
<td>10</td>
<td>1.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td>1.8</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20</td>
<td>0.9</td>
</tr>
<tr>
<td>Succinate</td>
<td>15</td>
<td>1.28</td>
</tr>
<tr>
<td>Malathion</td>
<td>0.44</td>
<td>145mg (saturated malathion solution)</td>
</tr>
<tr>
<td>Dimethoate</td>
<td>10</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.57</td>
</tr>
</tbody>
</table>
containing 200μl.100ml⁻¹ (in dimethylformamide) of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) to give a final concentration of 0.03mM. The amino terminal of the α-fragment encoding gene contains the cluster of restriction enzymes sites known as the polylinker. Vector containing DNA ligated into the polylinker causes the disruption of the α-complementation resulting in the formation of white colonies on the indicator agar. This blue/white selection was used to detect the presence of inserts in the multiple cloning sites of pUC vectors.

2.3 Preparation of cell extracts

Extracts were prepared from cells harvested from the mid-to-late exponential phase of growth. Bacteria from liquid culture were harvested by centrifugation at 10,000g for 10 minutes at 4°C. The pellets were washed with 0.2 volumes 0.01M phosphate buffer pH7.4 and resuspended in 0.04 volumes 0.01M Tris HCl buffer containing 9% glycerol and 0.5mM diethiothreitol (DTT) pH7.4. The cells were disrupted by sonication in an MSE Soniprep 150W ultrasonic disintegrator at a peak amplitude of 10 microns at 0°C. Sonication of E.coli was carried out for 2x30sec period with cooling between sonication. For the environmental isolates sonication was for a 2min period with cooling every 30sec. The resulting suspension was ultracentrifuged at 120,000g for 90min at a temperature of 4°C to remove cell membranes.

2.4 Estimation of protein concentration

Protein estimations on cell extracts were carried out using the Folin-Ciocalteu (Lowry et al, 1951) method. A standard curve was constructed using bovine serum albumin (Sigma) in the range 0.05-0.5mg of protein and the absorbance measured at 750nm. Protein estimations for fractions obtained from Fast Protein Liquid Chromatography (FPLC) were made using spectrophotometric reading at 260/280nm (Layne, 1959).
2.5 *Para*-nitrophenyl acetate esterase assay (quantitative)

A general esterase enzyme assay was carried out using *para*-nitrophenyl acetate (*p*-nitrophenyl acetate) as the substrate in an assay adapted from that of Huggins and Lapides (1947). When cleaved by an esterase this chromogenic substrate produces *para*-nitrophenol which can be measured spectrophotometrically at 405nm and pH 8.

\[
\text{\( \begin{array}{c}
\text{OCOCH}_3 \\
\text{O}_2\text{N}
\end{array} \)} + \text{H}_2\text{O} \rightarrow \text{\( \begin{array}{c}
\text{O}^- \\
\text{O}_2\text{N}
\end{array} \)} + \text{CH}_3\text{COOH} + \text{H}^+
\]

*p*-nitrophenyl acetate \hspace{1cm} *p*-nitrophenol \hspace{1cm} acetate

The extinction coefficient was determined as 14.8mM$^{-1}$cm$^{-1}$.

<table>
<thead>
<tr>
<th>TEST(ml)</th>
<th>BLANK(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M phosphate buffer pH 8</td>
<td>0.500</td>
</tr>
<tr>
<td>28mM <em>p</em>-nitrophenyl acetate (in methanol)</td>
<td>0.010</td>
</tr>
<tr>
<td>cell extract</td>
<td>0.025</td>
</tr>
<tr>
<td>Water</td>
<td>0.465</td>
</tr>
</tbody>
</table>

The final concentration of *p*-nitrophenyl acetate in the assay was 0.28mM.

Other *p*-nitrophenyl esters (propionate, butyrate and caproate) were used in assays as esterase substrates. Assay conditions were the same as those for *p*-nitrophenyl acetate and the production of *para*-nitrophenol was measured at 405nm.

2.6 *Para*-nitrophenyl acetate esterase assay (qualitative)

This assay was used as a quick screening method to determine which fractions obtained after FPLC were most active against *p*-nitrophenyl acetate. The assay was carried out using microtitre plates containing 100μl of the following mixture in each well:-

1.50ml 0.1M phosphate buffer pH 8

1.20ml water

30μl *p*-nitrophenyl acetate (28mM in methanol)
To each well 5μl of fraction was added mixing with the pipette tip and then allowing any colour to develop over approximately 10min.

2.7 Diethylsuccinate esterase assay (quantitative)

It is assumed that the hydrolysis of diethylsuccinate involves the removal of one or both of the ethyl groups so degradation can be monitored directly by measuring the production of ethanol, see Fig.2.1. The assay used was adapted from that of Theorell and Bonnichsen (1951) for the detection of ethanol.

<table>
<thead>
<tr>
<th></th>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semicarbazide buffer (pH8.7)*</td>
<td>0.450</td>
<td>0.450</td>
</tr>
<tr>
<td>50mM Diethylsuccinate</td>
<td>0.200</td>
<td>0.000</td>
</tr>
<tr>
<td>0.1 M phosphate buffer pH7.2</td>
<td>0.250</td>
<td>0.000</td>
</tr>
<tr>
<td>24mM NAD⁺</td>
<td>0.030</td>
<td>0.000</td>
</tr>
<tr>
<td>cell extract</td>
<td>0.010</td>
<td>0.000</td>
</tr>
<tr>
<td>ADH (8823 units/ml)</td>
<td>0.020</td>
<td>0.000</td>
</tr>
<tr>
<td>Water</td>
<td>0.040</td>
<td>0.550</td>
</tr>
</tbody>
</table>

(*Semicarbazide buffer contains 75mM pyrophosphate, 7.5mM semicarbazide and 21mM glycine, pH was adjusted with 4M KOH)

Sodium orthophosphate buffer was used to give the desired final pH of pH8. The cell extract was added last and the NADH production was monitored continuously at 340nm. The extinction coefficient for the NADH was 6.2mM⁻¹cm⁻¹. The final concentration of diethylsuccinate in the assay was 10mM.

2.8 Malathion esterase assay (quantitative)

As was described for the diethylsuccinate assay this assay was adapted from that of Theorell and Bonnichsen (1951) for the detection of ethanol, see Fig.2.1.

<table>
<thead>
<tr>
<th></th>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semicarbazide buffer (pH8.7)*</td>
<td>0.450</td>
<td>0.450</td>
</tr>
<tr>
<td>0.44mM Malathion</td>
<td>0.200</td>
<td>0.000</td>
</tr>
<tr>
<td>0.1 M phosphate buffer pH7.2</td>
<td>0.250</td>
<td>0.000</td>
</tr>
<tr>
<td>24mM NAD⁺</td>
<td>0.030</td>
<td>0.000</td>
</tr>
<tr>
<td>cell extract</td>
<td>0.010</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Figure 2.1. Spectrophotometric assay for the detection of the hydrolysis of diethylsuccinate by an esterase.

The NADH produced was measured at 340nm.

ADH  = Alcohol dehydrogenase
NAD+  = Nicotinamide adenine dinucleotide
NADH  = Nicotinamide adenine dinucleotide (reduced)
ADH (8823 units/ml)  0.020  0.000  
Water  0.040  0.550  

(*Semicarbazide buffer contains 75 mM pyrophosphate, 7.5 mM semicarbazide and 21 mM glycine, pH was adjusted with 4M KOH).  
Sodium orthophosphate buffer was used to give the desired pH of pH 8.  
The cell extract was added last and the NADH production was monitored continuously at 340 nm. The extinction coefficient for the NADH was 6.2 mM$^{-1}$cm$^{-1}$. The final concentration of malathion in the assay is 0.088 mM.

2.9 Assay for phosphoesterase activity

Para-nitro phenylphosphate was used as a substrate to assess phosphoesterase activity. The assay was carried out at pH 8 using sodium orthophosphate buffer as used in the p-nitrophenyl acetate assay. The hydrolysis of the phosphoester bond leads to the production of p-nitrophenol which is then measured spectrophotometrically at 405 nm. A 0.28 mM solution (final concentration) of para-nitro phenylphosphate was used to assay enzyme activity against this substrate.

2.10 Thiol detection assay

5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was used to measure thiol containing compound mercaptosuccinic acid at 412 nm (Grassetti and Murray, 1967). To determine the amount of thiol containing compounds in the media the following assay was set up:

<table>
<thead>
<tr>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M phosphate buffer pH 7.5</td>
<td>0.890</td>
</tr>
<tr>
<td>0.01M DTNB</td>
<td>0.100</td>
</tr>
<tr>
<td>sample</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Absorbance was measured at 412 nm. The extinction coefficient for the 2-nitro-5-thiobenzoate formation was 13.6 mM$^{-1}$cm$^{-1}$.
2.11 Amidase assay

The hydrolysis of acetamide was measured using a discontinuous assay measuring the \( \text{NH}_4^+ \) production from acetamide. Esterase enzyme was added to buffer A (citric acid, 0.1M and phosphate buffer 0.2M, pH6) in the wells of a microtitre plate to give a final volume of 0.095ml and then incubated at 37°C for 5min. The assay was then started by the addition of 5\( \mu \)l of acetamide (final concentration 50mM) and incubated again at 37°C. The reaction was then stopped by taking 10\( \mu \)l aliquots into 100\( \mu \)l of phenoxide/acetone reagent (0.675M NaOH; 0.064M phenol/0.136M acetone). The colour was then developed in the dark for 30min and the colour change observed.

2.11.1 D-aminopeptidase assay

The hydrolysis of the D-aminopeptide bond in D-alanine \( p \)-nitroanilide results in the formation of \( p \)-nitroanilide a chromogenic substance which can be monitored spectrophotometrically at 405nm. Assays were carried out using 2mM D-alanine \( p \)-nitroanilide (final concentration):

<table>
<thead>
<tr>
<th></th>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM D-alanine ( p )-nitroanilide (in methanol)</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>100mM phosphate buffer pH8</td>
<td>0.970</td>
<td>0.970</td>
</tr>
<tr>
<td>enzyme</td>
<td>0.010</td>
<td>0.010</td>
</tr>
</tbody>
</table>

2.12 Chloride ion detection assay

Chloride ions were assayed in solution by using ferric ammonium sulphate and mercuric thiocyanate (Bergmann and Sanik, 1957). The following assay was set up:-

<table>
<thead>
<tr>
<th></th>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25M Ferric ammonium sulphate (in 9M nitric acid)</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>Mercuric thiocyanate saturated ethanol</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>Sample</td>
<td>1.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>
A standard curve was constructed using a 1mM NaCl solution in the range of 0.1-1mM NaCl. This was then used to determine the amount of Cl ions present in solution.

2.13 Protein purification

2.13.1 Ammonium sulphate fractionation

Ground ammonium sulphate was used to salt out protein in three stages, 0-40% saturation (22.0g.100ml⁻¹), 40-60% saturation (12.2g.100ml⁻¹) and 60-90% saturation (20.4g.100ml⁻¹). The precipitation was carried out at 0°C using ultracentrifuged cell extract. After each addition of the ammonium sulphate stirring was carried out for 25mins. The mixture was then centrifuged at 12,000g and 4°C for 15 minutes after which the supernatant was removed, measured and treated with the next amount of ammonium sulphate. The pellets from each stage were dissolved in 0.01M phosphate buffer at pH7.2. Protein estimations and enzyme assays were carried out on 50μl samples of the dissolved pellets. Fractionations were also made at saturations of 0-55% saturation (33.1g.100ml⁻¹) and 55-90% saturation (23.8g.100ml⁻¹). These fractionations were carried out under the same conditions as above.

2.13.2 Fast Protein Liquid Chromatography (FPLC)

2.13.2.1 Anion exchange chromatography

For the purification of the esterase protein FPLC was first carried out at room temperature using a Pharmacia Mono Q HR 5/5 anion exchange column. Cell extract was prepared in 20mM Tris HCl buffer at pH7.4 as the usual phosphate buffer could not be used with the Mono Q column. The cell extract was ultracentrifuged and filtered through a 20μm sterile Acrodisc filter before injection into the column. Buffers used were 20mM Tris HCl pH7.4, 9% glycerol, 0.5mM DTT and the same buffer containing 1M NaCl. The buffer without salt was used to equilibrate the column before loading the protein and buffer containing salt was used to provide a gradient for the elution of the proteins. Buffers were filtered and degassed
immediately prior to use. Protein (2.5mg) was loaded onto the column in a volume of 1ml. Gradients were run at 0-1M and 0-0.5M NaCl at a flow rate of 1ml.min⁻¹ and fractions of 1ml were collected.

2.13.2.2 Hydrophobic interaction chromatography

The Pharmacia Phenyl Superose column was used to further purify the protein fractions obtained from the Mono Q column. This was also carried out at room temperature and the buffers used were 20mM Tris HCl (pH7.4), 9% glycerol, 0.5mM DTT and the same buffer containing 1M(NH₄)₂SO₄. The column was equilibrated with filtered and degassed 20mM Tris HCl (pH7.4), 9% glycerol, 0.5mM DTT, containing 1M(NH₄)₂SO₄ prior to use. Protein (0.75mg) containing 1M(NH₄)₂SO₄ was loaded onto the column in a volume of 1ml. Gradients were run at 1M-0M (NH₄)₂SO₄ at a flow rate of 0.5ml.min⁻¹ and fractions of 1ml were collected.

2.13.2.3 Gel filtration

To determine the native size of the purified protein Superose 12 (Pharmacia) gel filtration columns were used. Two columns were set up in series so as to gain better resolution. They were equilibrated with degassed 20mM Tris HCl (pH7.4) containing 0.15M NaCl prior to use. The columns were calibrated with β-amylase 200kDa; ADH 150kDa; Ovotransferin 76kDa; Ovalbumin 42.2kDa; Carbonic anhydrase 29kDa and Cytochrome c 12.4kDa with a buffer flow rate of 0.25ml.min⁻¹. The sample protein 0.2ml (1mg.ml⁻¹), was applied after calibration and passed through the column under the same buffer conditions and the point of elution noted.

2.14.1 SDS Polyacrylamide Gel Electrophoresis PAGE analysis

Samples were run on 12% SDS PAGE gels (Laemmli, 1970) to investigate the proteins present in fractions which had been treated by various of the above methods. Loading
buffer, 5µl, (0.012% bromophenol blue, 40% glycerol, 8% SDS and 0.250M Tris HCl pH6.8) and 1µl mercaptoethanol was added to each of the 20µl samples. The samples were boiled for 2mins before loading onto the gel. The marker used was Sigma Dalton VII-L for SDS gel electrophoresis containing bovine albumin, 66kDa; egg albumin, 45kDa; glyceraldehyde 3-P-dehydrogenase, 36kDa; bovine carbonic anhydrase, 29kDa; bovine pancreas trypsinogen, 24kDa; soybean trypsin inhibitor, 20kDa; bovine milk α-lactalbumin, 14.2kDa. After electrophoresis the gel was stained with Coomassie blue (0.1%w/v Coomassie Brilliant Blue, 45% methanol, 45% water and 10% glacial acetic acid) to visualise the protein bands. The excess stain was removed using a destaining solution of 70% water, 20% methanol and 10% glacial acetic acid.

2.14.2 Native PAGE analysis

Native gels were also run in the same way as the denaturing gels but with the omission of the SDS and without boiling the samples. Gels were run in the cold room at 4°C using buffers that were cooled overnight. This was to reduce the heating of the gel and the possibility of denaturing the proteins during the run. Gels were made the day prior to use and left overnight so that unreacted ammonium persulphate could decompose. The native gels were stained using the simultaneous capture reaction as described by Market and Hunter (1959). This was carried out using α-naphthyl acetate (1% in acetone) as the esterase substrate and Fast Blue RR (diazonium salt) as the coupling reagent, see Fig.2.2. On the addition of the above, dark permanent bands formed within 10mins at the site of esterase locations. Gels were washed with distilled water and stored in 50% ethanol. A second method of gel staining was carried out using diethylsuccinate as the substrate. This method utilised the dye nitroblue tetrazolium which when reduced forms an insoluble product thus locating the site of esterase activity, see Fig.2.3. The reagents used were:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrisHCl 0.5M (pH7.4)</td>
<td>12.5ml</td>
</tr>
<tr>
<td>50mM Diethylsuccinate</td>
<td>22.5ml</td>
</tr>
<tr>
<td>ADH (8823units.ml⁻¹)</td>
<td>2.25ml</td>
</tr>
<tr>
<td>NAD (15mg.ml⁻¹)</td>
<td>3.35ml</td>
</tr>
<tr>
<td>Nitroblue tetrazolium (1mg.ml⁻¹)</td>
<td>12.5ml</td>
</tr>
<tr>
<td>Phenazine methosulphate (1mg.ml⁻¹)</td>
<td>1.25ml</td>
</tr>
</tbody>
</table>
Figure 2.2. Hydrolysis of \(\alpha\)-naphthylacetate to produce \(\alpha\)-naphthol which coupled with the diazonium salt Fast Blue RR forms an insoluble dye product.
Figure 2.3. The hydrolysis of diethylsuccinate by an esterase resulting in the production of ethanol which in turn leads to the formation of an insoluble dye product.

ADH = Alcohol dehydrogenase
NAD = Nicotinamide adenine dinucleotide
NADH = Nicotinamide adenine dinucleotide (reduced)
PMS = Phenazine methosulphate
PMSH$_2$ = Phenazine methosulphate (reduced)
NBT = Nitroblue tetrazolium
NBTH$_2$ = Nitroblue tetrazolium (reduced)
After the gel had been run it was then soaked in ice cold 0.5M TrisHCl (pH7.4) for 15min to ensure that the entire gel was at the appropriate pH for activity staining. After soaking the buffer was discarded and the assay mixture was freshly made and used to incubate the gel at room temperature in the dark until the bands developed. After the development of bands gels were rinsed with water and fixed with 7.5% acetic acid.

2.15 Blotting of protein for N-terminal sequence analysis

Protein samples were run on a 12% SDS PAGE gel. When the gel was nearly finished running pieces of 3MM paper, cut to the size of the gel, were soaked in transfer buffer (48mM Tris, 39mM glycine, 10% methanol, 0.03% SDS). A gel sized piece of Problot membrane was also soaked completely with methanol and then soaked in transfer buffer until required. When the SDS PAGE finished running a sandwich of 8 sheets of the soaked 3MM, the Problot, the gel and 8 more sheets of 3MM was made in the LKB Semi-dry Blotter ensuring no bubbles were present between the gel and the Problot. The blot was then run for 1hr at 42mA. After blotting the Problot was removed and stained for 5min (Coomassie Blue 0.1%, methanol 50%, water 49.9%). The blot was then destained until the bands were just visible. The Problot was then rinsed with water and allowed to dry.

2.16 N-terminal sequence determination

N-terminal sequencing was carried out on a Applied Biosystems 470A gas phase sequencer by Dr. K. Lilley in the Department of Biochemistry.

2.17 Chromosomal DNA preparation

Chromosomal DNA was prepared using a modified version of the method used by Wilson (1993). *Arthrobacter* sp. cells were harvested from 200ml of culture by centrifugation at 12,000g for 20min. The cells were resuspended in 20ml of lysozyme solution (12ml 0.5M sucrose; 500μl 1M Tris pH8; 5ml 0.1M ethylenediaminetetraacetic acid (EDTA) pH8; 2.5ml
sterile water; lysozyme 5mg.ml\(^{-1}\)) and the mixture was left to incubate at 37°C for 2hrs. EDTA (0.5M, pH8, 4.8ml) was then added and allowed to incubate for a further 10min. Sodium dodecyl sulphate (SDS) (2.8ml of 10%w/v) and Proteinase K (150\(\mu\)l of 2mg.ml\(^{-1}\)) were added, mixed immediately, and incubated at 37°C for another two hours. After incubation 3.6ml of 5M NaCl and 3ml of pre-warmed cetyl trimethylammonium bromide (CTAB) (10%w/v) dissolved in 0.7M NaCl were added. The mixture was incubated at 65°C for 10min. After this final incubation an equal volume of chloroform/iso amyl alcohol (IAA) was added and mixed for 10min by gentle inversion. The two phases were separated by centrifugation at 6,000g for 10min at room temperature. The aqueous phase was transferred to a fresh tube and an equal volume of phenol/chloroform/IAA was added. This time the mixture was inverted gently for 5min and then separated by centrifugation at 12,000g for 15min at room temperature. Again the aqueous phase was removed to a fresh tube and an equal volume of chloroform/IAA was added and gently mixed for 5min. The phases were separated by centrifugation at 6,000g for 10min at room temperature and the aqueous phase was removed. The DNA was then precipitated by the addition of 0.6vol of isopropanol. DNA was removed by spooling it onto a sealed Pasteur pipette, washed in 70% ethanol and dissolved in TE (10mM Tris-Cl; 1mM EDTA pH7.4) buffer. When required the DNA was concentrated by the addition of ice cold ethanol (2vol) and spooled using a sealed Pasteur pipette and dissolved in TE buffer.

2.18 Chromosomal DNA digests

Trial digests were carried out with chromosomal DNA to determine the optimum concentration of enzyme to be used and the optimum digestion time. The enzyme used in the partial digests was Sau3AI as it produces cohesive ends which will re-ligate with BamHI generated cohesive ends. This enabled the fragments from digestion of chromosomal DNA to be ligated into the Bam HI site of the polylinker region of pUC18. Sau3AI was diluted using dilution buffer as described in the information sheet from the manufacturer's (Pharmacia). Trial digests were then set up with varying amounts of enzyme ranging from
0.25 to 2 units and incubated at 37°C for 0, 15, 30, 45, and 60mins. The digests were set up in a total volume of 15μl with:-

- x10 OnePhor All buffer (OPA) 3μl
- chromosomal DNA 1μl (2.5μg)
- Sau3AI xμl
- Sterile water yμl (to make the volume up to 15μl)

The reactions were stopped with 5μl of loading dye (0.01%w/v xylene cynol FF, 0.01%w/v bromophenol blue, 50% glycerol, 40% water, 2% Tris-acetate EDTA (TAE) buffer (40mM Tris-acetate pH7.6; 1mM EDTA) and the samples kept on ice until they could all be analysed on an agarose gel. This reaction was then scaled up to prepare DNA to be used in size fractionation on sucrose gradients.

### 2.19 Sucrose gradient preparation

Sucrose gradients were used to size fractionate the chromosomal DNA. Two sucrose solutions were prepared, one of 20% sucrose and the other 5%.

#### 20% sucrose solution (200ml)

- 5M Sodium chloride 40ml
- 1M Tris-HCl (pH8) 4ml
- 0.5M EDTA (pH8) 2ml
- 50% sucrose 80ml
- Sterile water 74ml

#### 5% sucrose solution (200ml)

- 5M Sodium chloride 40ml
- 1M Tris-HCl (pH8) 4ml
- 0.5M EDTA (pH8) 2ml
- 50% sucrose 20ml
- Sterile water 134ml

The two solutions were autoclaved at 121°C for 15min. A gradient maker was used to set up the sucrose gradients using equal volumes of the two solutions.

After partial digestion with Sau3AI, 50μg of chromosomal DNA was added to each gradient. The gradients were centrifuged in a Beckman SW27 rotor for 24hrs at 110,000g and 15°C. After centrifugation the gradient was removed from the centrifuge tubes using a
peristaltic pump to transfer 1-1.5ml fractions into Eppendorf tubes. Samples of 400µl were taken from every third Eppendorf and precipitated with 2vol of ethanol and 0.1vol of 3M sodium acetate (pH6). The DNA was precipitated on ice for 30min and then centrifuged for 15min at 4°C. The supernatant was removed and the pellet was washed in 70% ethanol, dried under vacuum and dissolved in 10µl of sterile water. The samples were run on a 0.8% agarose gel to determine which fractions contained the DNA fragments of the desired size. The fractions of interest were then pooled and dialysed against 500ml of TE buffer (pH 8) at 4°C for approximately 16hrs with one change of buffer.

After dialysis the DNA was precipitated using 2vol of ethanol and 0.1vol of 3M sodium acetate (pH 6) then centrifugation at 23,000g for 30min at 4°C. The supernatant was removed and the pellet dried under vacuum. The pellet was dissolved in 50µl of TE buffer pH 8.

2.20.1 Plasmid preparation

Cells were harvested from 5ml overnight cultures of LB media containing ampicillin (100µg.ml⁻¹) by centrifugation in a bench top MSE MicroCentaur Microcentrifuge. The supernatant was removed and the pellet resuspended in 100µl of ice-cold GTE buffer (50mM glucose; 25mM Tris HCl (pH8); 10mM EDTA) and allowed to stand at room temperature for 5min. After incubation 200µl of freshly prepared lysis solution (0.2M NaOH; 1%SDS) was added and the contents mixed thoroughly by inversion. The mixture was left on ice for 5min and then 150µl of ice-cold potassium acetate, pH4.8 (3M with respect to potassium, 5M with respect to acetate) was added. The tube contents were mixed again by inversion and then left to incubate on ice for a further 5min. After incubation cell debris was removed by centrifugation for 5min at top speed in a bench top centrifuge. The supernatant was transferred to a fresh tube and extracted with an equal volume of phenol/chloroform/IAA (25:24:1) equilibrated with 1M Tris-Cl pH7.5 to remove contaminating protein. The aqueous phase was then extracted with an equal volume of chloroform/IAA (24:1) to remove any remaining phenol. Plasmid DNA was then precipitated by the addition of 2vol of ice-cold ethanol and incubated in a dry ice/IMS bath.
for 20min. DNA was collected by centrifugation for 10min at 12,000g, washed with 70% ice-cold ethanol and dried under vacuum. The pellet was redissolved in TE buffer containing 20mg.ml\(^{-1}\) Ribonuclease A. The DNA obtained by this method was then used for restriction endonuclease analysis or transformations.

2.20.2 Plasmid preparation for sequencing

Plasmid DNA to be sequenced needed to be of a higher quality than that obtained by the previous method so an alternative method was used. The DNA was prepared by growing \textit{E.coli} JM109 containing the plasmid DNA overnight in LB/ampicillin (100\(\mu\)g.ml\(^{-1}\)). Plasmid DNA was then prepared from 10ml of the overnight culture using the WIZARD\textsuperscript{TM} protocol. This involved the extraction of plasmid from cell extract by adsorption of the DNA to a resin in a column and the rest of the cell lysate passing through the column. The plasmid DNA was then eluted separately. Cells were resuspended in 400\(\mu\)l of cell resuspension solution then mixed by inversion with 400\(\mu\)l of cell lysis solution. Neutralisation solution (400\(\mu\)l) was then added and the mixture inverted several times. The lysate was centrifuged at maximum speed in a bench top centrifuge for 5min. The WIZARD\textsuperscript{TM} resin (1ml) was added to the barrel of a 3ml syringe and the cleared lysate supernatant from each miniprep was then added. The mixture of resin and lysate were then applied to the column attached to each syringe by inserting the plunger into the syringe. After application of the resin/lysate to the column it was washed with 2ml of column wash solution. After washing the column was transferred to an Eppendorf tube and the resin was dried by centrifugation at top speed in the bench top centrifuge for 2min. The column was then transferred to a new Eppendorf tube and the plasmid DNA was eluted by adding 50\(\mu\)l of water, waiting for 1min, then centrifuging for 20sec at top speed in the microcentrifuge. The DNA was stored at -20°C until needed for sequencing.
2.21 Restriction digests

Plasmid DNA was digested with restriction enzymes in accordance with the suppliers guidelines. Digests were carried out at 37°C for 1-2hrs unless stated otherwise. The total volume for each digest was 15µl, containing approx. 0.5µg DNA and approx. 1unit of enzyme and the concentration of buffer advised for that enzyme by the suppliers, the remaining volume was made up using sterile water. Enzymes were purchased from Pharmacia and New England Biolabs.

2.22 Phosphatase treatment of DNA

Where appropriate, after restriction, plasmid DNA was treated with calf intestinal alkaline phosphatase (CIAP) at a concentration of 0.1 units for every 1-10µg of DNA to prevent self ligation. This was carried out at 37°C for 20min, the CIAP was then inactivated by heating at 85°C for 10min. The plasmid was extracted with an equal volume of phenol/chloroform/IAA (25:24:1) to remove the contaminating restriction enzyme and CIAP. The presence of DNA was then checked on a 0.8% agarose gel.

2.23 Agarose gel electrophoresis

Restriction enzyme digests of plasmid and chromosomal DNA were analysed by gel electrophoresis using agarose gels. Gels of 0.8% agarose were made using TAE buffer (40mM Tris-acetate pH7.6; 1mM EDTA) plus ethidium bromide (0.5µg.ml⁻¹). Gels were run at constant voltage of 5-10Vcm⁻¹ in TAE buffer. Samples were prepared by adding 5µl of loading buffer to 15µl of digest [0.25% bromophenol blue (w/v); 0.25% xylene cynol (w/v), 30% glycerol (v/v)] (Sambrook et al 1989). Samples were run until the bromophenol blue dye front had reached the end of the gel and then visualised under U.V. light. Calibration was carried out using a 1kb ladder supplied by Gibco BRL.
2.24 Isolation of DNA fragments from agarose gels

Fragments generated through restriction digests of plasmid DNA were isolated from gels using the JETSORB gel extraction kit (AMS Biotechnology). The DNA was identified by viewing the gel under low power U.V. light and the regions of gel removed and placed in a 1.5ml Eppendorf tube. For each 100mg of gel 300µl of buffer A1 (the ratio of gel slice weight to volumes of buffers A1 and A2 was always 1 to 3 (w/v)) and 10µl of resuspended JETSORB were added. The mixture was vortexed and incubated at 50°C for 15min mixing every 3min during the incubation. After incubation the mixture was centrifuged at 12,000g for 30sec and the supernatant removed. The pellet was washed with 300µl of buffer A1 and then twice with buffer A2. The resulting pellet was dried under vacuum for 5-10min. The DNA was eluted by adding 20µl of TE buffer and incubating at 50°C for 5min flicking once to mix during incubation. The DNA binding matrix was removed by centrifugation at 12,000g for 30sec and the supernatant containing the recovered DNA was removed to a fresh tube. The recovered DNA was then analysed by running on a 0.8% agarose gel.

2.25 Ligation Reactions

Ligation reactions were set up with the following :-

10 x OPA buffer 2µl
ATP (10mM stock) 2µl
T4 ligase (7weiss units.µl⁻¹) 1µl
vector DNA xµl
insert DNA yµl
sterile water zµl(to made total volume up to 15µl)

For the ligation of sticky-ended fragments the ratio of vector to insert was 1:2 and for blunt end ligations 3:1 (Brown, 1991). Ligation reactions were carried out either at 4°C overnight or at room temperature for 4hrs. About half of the ligation reaction mixture was then used to transform competent cells.
2.26 Transformation of competent *E. coli* JM109 cells with plasmid DNA

For the construction of the *Arthrobacter* sp. gene library high efficiency *E. coli* JM109 (Promega) cells were transformed with plasmid DNA according to the suppliers instructions. Transformed cells were spread onto LB/ampicillin plates and incubated at 37°C overnight.

For transformations with plasmids containing subcloned DNA JM109 and JM109 (DE3) cells were made competent by the method of Kushner (1978). The bacteria were grown overnight in 5ml LB tubes and then diluted 50 fold into a fresh tube of LB. These cells were then grown at 37°C until the O.D.680nm was approx. 0.4. The cells were harvested in aliquots of 1.5ml by centrifugation for 30sec, the supernatant was discarded and the pellets resuspended in 0.5ml of MOPS solution A (10mM MOPS pH7.0, 10mM RbCl). The cells were pelleted for a further 20sec, the supernatant removed and the pellets resuspended in 0.5ml of solution B (100mM MOPS pH6.5, 10mM RbCl, 50mM CaCl) and left on ice for 60-90min. Cells were pelleted for 20sec and resuspended in 150µl of solution B and 3µl of DMSO. Plasmid DNA was added and the mixture was left on ice for 60min. After incubation with the plasmid the cells were heat shocked at 55°C for 30sec and then returned to the ice for 1min. LB (850µl) was then added and the mixture was incubated at 37°C for 1hr. The cells were collected by centrifugation, resuspended in 100µl of LB and spread onto LB/ampicillin plates and incubated overnight at 37°C.

2.27 Preparation of subclones

DNA inserted into pUC18 was removed by digestion with restriction enzymes. The fragments of DNA were separated from the plasmid DNA by running on a 0.8% agarose gel. The fragments of interest were viewed under low power U.V. light and cut from the gel. DNA was extracted from the gel using the JETSORB kit as described earlier in section 2.24. Once extracted the DNA was checked on an agarose gel and then ligated into pUC18/19 with suitably prepared cohesive ends.
2.28 Ligation of non-cohesive ends

For the production of some of the subclones restriction digests were carried out that resulted in the production of two cohesive ends that were not compatible. To enable the ligation of these ends and the recircularisation of the plasmid the cohesive ends were filled in using the Klenow Fragment and dNTP's and then blunt end ligated. After digestion with the desired enzymes fragments were run on a 0.8% agarose gel and extracted using the method of Langridge et al (1980) to isolate the DNA fragments from the agarose gel. The procedure for Klenow treatment was followed as per the instruction in the double stranded Nested Deletion Kit (Pharmacia Biotech).

2.29 Nucleotide sequencing

Plasmid DNA was prepared using the WIZARD™ protocol as described in section 2.20.2. Plasmid DNA was sequenced using the chain-termination method of Sanger et al (1977) using a modified T7 DNA polymerase (Tabor and Richardson, 1987). Sequencing kits (T7Sequencing™ Kit) were purchased from Pharmacia Biotech and radio-isotope, $^{35}$S dATP (12.5mCi.ml$^{-1}$) was purchased from Amersham.

The preparation of DNA for sequencing can be divided into five stages, denaturation; annealing; labelling; termination and finally stopping of reactions and the preparation of samples to be loaded on to the sequencing gel.

2.30 Denaturation of plasmid DNA

Plasmid DNA (approximately 5μg) in a total volume of 36μl was denatured by the addition of 4μl of 2M NaOH. This was then vortexed briefly and centrifuged to collect the contents at the bottom of an Eppendorf tube and incubated at room temperature for 5min. After incubation 4μl of 3M sodium acetate (pH4.8) and 90μl of ethanol were added and the mixture placed in a dry ice/IMS bath for 15min. The DNA was collected by centrifugation at
12,000g for 10min and washed with 70% ice-cold ethanol and spun again. The pellet was
dried under vacuum and resuspended in 7µl of sterile water.

2.31 Sequencing reactions

The denatured DNA was prepared as described above and then annealed to the appropriate
primer by the addition of 2µl of annealing buffer and 2µl of the appropriate primer (3ng.µl⁻¹).
The primer was annealed by heating to 65°C for 2min and then allowed to cool slowly at
room temperature to 35°C. Whilst the primer was being annealed 2.5µl aliquots of A,C,G
and T termination mixes were placed in separate tubes and pre-warmed at 37°C. After
cooling the annealed DNA was labelled with the radioisotope through the addition of 3µl of
label mix A, 1µl (12.5µCi.ml⁻¹) of ATP (³⁵S) and 2µl of diluted T7 polymerase
(approximately 1.5 units.µl⁻¹). The contents of the tube were mixed gently with the pipette
tip and collected by brief centrifugation. The reaction was then allowed to proceed for 5min
at room temperature. To terminate the labelling reactions 4µl of the mixture was added to
each of the pre-warmed tubes containing the termination mixes. The termination reactions
were incubated at 37°C for 5min and stopped by the addition of 5µl of stop solution. The
reactions were either loaded onto the sequencing gel immediately or stored at -20°C. Prior
to electrophoresis the reactions were heated to 95°C for 2min.

2.32 Electrophoresis and autoradiography

Sequencing gels were 6% acrylamide, 6.6M urea made up in a 0.5M Tris-borate; EDTA
(2mM) pH 8.3 (TBE) buffer. Gels were pre-run at 2000V for 30-40min to allow the gel to
reach 40-50°C. The volumes of sample loaded per well depended on the comb used. For
the fine comb (36 wells) 1.5µl per well and for the wide comb (24 wells) 4µl per well. To
increase the amount of sequence obtained from each set of reactions two separate loadings of
the samples were run. The first sample was loaded and run until the slower of the xylene
cyanol dye fronts reached the end of the gel, the second sample was then loaded. The
second sample was run until its faster xylene cyanol dye front reached the end of the gel.
After electrophoresis the gel was immersed in 10% methanol 10% glacial acetic acid solution for 20min and then transferred to Whatman 3MM paper and covered in Saran wrap (Dow). The gels were dried onto the paper under vacuum at 80°C for 60-90min. The presence of radio-isotope in the dried gel was checked using a Geiger counter. Autoradiography was carried out for 2-3 days or longer at room temperature using Fuji RX X-ray film.

2.33 Purification of oligonucleotide primers

Oligonucleotide primers were designed from either measured amino acid sequence (Konrad, 1990) or known DNA sequence. Synthesis was carried out using an Applied Biosystems model 380B DNA synthesiser using cyanoethyl-phosphoramidite chemistry. These oligonucleotides were then purified for use in nucleotide sequencing and PCR reactions. Purification was carried out using butanol precipitation (Sawadogo and Van Dyke, 1991).

2.34 Polymerase chain reaction (PCR) to prepare the expression plasmid

For amplification using PCR DNA fragments containing the esterase gene were prepared by the WIZARDTM plasmid preparation protocol. A primer was designed for the 5' end of the gene and the pUC19 forward primer was used as a primer at the 3' end of the DNA fragment to be amplified. The plasmid DNA concentration was calculated by measuring the absorbance of the dissolved DNA at 260 and 280nm. The concentration of the primer DNA was calculated using the equation :-

\[
\text{Concentration} = 100 \times \frac{\text{Absorbance 260nm \times dilution factor of the DNA solution}}{\text{A}} \quad \text{pmoles} \, \mu\text{l}^{-1}
\]

\[
(A = (nG \times 1.17) + (nC \times 0.75) + (nT \times 0.92) + (nA \times 1.54) \text{ where } n \text{ is the number of that base in the oligodeoxyribonucleotide})
\]

The melting temperature of the oligodeoxyribonucleotide was calculated using the equation:-

\[
T_m = 69.3 + [0.41(\%GC) - 650]
\]
Where $L$ is the length of the oligodeoxyribonucleotide.

Using these calculations a series of cycling reactions were determined and the PCR was carried using 100ng of plasmid DNA and 0.5μM of each of the primers. The cycle used was denaturing at 95°C for 1min, primer annealing at 62°C for 1min and synthesis of the complementary strand at 72°C for 2min. This cycle was repeated 30 times. After the cycling reactions were completed a sample of the resulting amplified DNA was checked on a 0.8% agarose gel and then the rest digested with the appropriate restriction endonucleases. The restricted DNA was then used in ligations with appropriately digested vector DNA.

2.35 Computer analysis

Sequence analysis was carried out using the BLAST algorithms (Altschul et al, 1990) to search various databases. These searches compared DNA to DNA sequence and also deduced amino acid sequence to protein sequence. Sequence alignment using the Genetics Computer Group (GCG) package was used to identify regions of similarity between the derived sequence of the esterase and other sequences found in the database searches.

2.36 Identification tests for isolates

2.36.1 Gram stain

A loopful of culture was taken from a single colony and smeared onto a drop of sterile water on a glass slide. The slide was then dried over a Bunsen burner to fix the cells and the slide allowed to cool. Crystal violet (0.5% aqueous solution) was added for 1-2mins and the slide was then flooded with Grams iodine (10g iodine, 20g KI, 1 litre H$_2$O) for a further 1-2mins. This was removed with acetone and washed with water. The slide was then flooded with counter stain (safrin, 0.5% aqueous solution) for 2mins followed by washing with water. Excess water was removed by blotting and the slide was dried over a Bunsen. The stained cells were viewed under a light microscope at x100 magnification with no coverslip using immersion oil and bright field illumination.
2.36.2 Cell morphology

Morphology was first studied under the light microscope. Loopfuls of cells were taken from single colonies grown on minimal media plates containing diethylsuccinate and smeared onto a microscope slide. The bacteria were then viewed under maximum magnification (x100) with oil immersion. Samples of bacteria were taken from plates of different ages and viewed using this method.

Further investigations into the morphology were made using the electron microscope. Again colonies of different ages were taken from plates and viewed by scanning electron microscopy. Two methods of cell preparation were used, the first using 2% ammonium molybdate and the second using 1% uranyl acetate to fix the cells.

2.36.3 Growth temperature

Cells were plated out on minimal media plates containing diethylsuccinate and also on LB plates. The plates were then incubated at various temperatures and their ability to grow at these temperatures was observed.

2.36.4 Acid from glucose

Glucose peptone media (5.0g/l glucose; 10.0g/l peptone; 20ml bromothymol blue (0.2%)) was adjusted to pH 7.6 and autoclaved at 121°C for 15mins. Before inoculation the media was a blue colour (due to the presence of the pH indicator bromothymol blue) indicating an alkaline pH. The flask was inoculated with a loopful of cells from a minimal media plate containing diethylsuccinate. The flask was then incubated at 30°C.

2.36.5 Catalase test

The catalase test was carried out by taking a loopful of cells from a plate containing minimal media and diethylsuccinate. The cells were smeared onto a microscope slide which was
placed inside a petri dish. Using a Pasteur pipette a drop of hydrogen peroxide was placed on the cells and the lid of the petri dish replace quickly to limit the spread of the aerosol of bacteria which might arise from this treatment.

2.36.6 Sporulation test

Bacteria were grown in LB containing 0.04% MnCl₂ as this promotes the formation of spores. Cultures were incubated at 30°C for 1-2 weeks. The presence of spores was checked for by viewing samples of the culture under a light microscope and also by heat treating the cells at 72°C for 15 seconds. Samples of heat treated and untreated cells were then plated out on minimal agar containing diethylsuccinate and incubated at 30°C.

2.36.7 Glycerol stocks

Glycerol stocks were prepared using both liquid cultures and by scraping colonies from agar plates. For liquid culture 0.7ml of culture grown overnight on LB was mixed with 0.3ml of sterile 50% glycerol. For colonies grown on agar 1ml of LB/30% glycerol was used to flood the plate and the colonies were then scraped off using a flamed glass rod. After mixture with the glycerol all stocks were frozen by standing in dry ice/IMS bath then transferred to the -70°C freezer for storage.

2.37 Kinetic analysis

The enzyme kinetic data was analysed via the Michaelis-Menten equation using a custom-written non-linear least squares-fitting algorithm.

2.38 pI determination

The pI of the purified esterase protein was determined both experimentally and using the GCG program. For the experimental determination the protein was suspended in protein
buffer (2% ampholines pH3-10, 300mM NaCl, 1mM EDTA 1mM EGTA (ethylineglycol bis (βamino ethyl ether) tetra acetic acid), 2% triton, 5mM ascorbic acid, 10mM DTT) and was run on isoelectrofocusing rods. The rod gels were made in batches of 10:-

<table>
<thead>
<tr>
<th>Gel component</th>
<th>Quantity for 10 rods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (Electran grade)</td>
<td>3.4g</td>
</tr>
<tr>
<td>Acrylamide (Aristar) 28.38:1.62%</td>
<td>823µl</td>
</tr>
<tr>
<td>Nonidet (10%)</td>
<td>1.237ml</td>
</tr>
<tr>
<td>Q water</td>
<td>1.10ml</td>
</tr>
</tbody>
</table>

Degas for one hour in Buchner flask
- Ampholines pH 3-10 (BDH) 416.5µl
- Ammonium persulphate (10%) 12.6µl
- TEMED 18.55µl

Buffers were 25mM phosphoric acid in the lower tank and 50mM NaOH in the upper tank (degas for 4hrs prior to use). The rods were prepared by sealing one end with two layers of Nescofilm and the gel added to a depth of 10cm using a syringe. The gel was then overlaid with 10µl of 9M urea and allowed to stand for 1hr. After this time the overlay was removed and 20µl of protein buffer with 9M urea solution (60:40%) was put onto the gel followed by 10µl of Q water and allowed to stand for 1-2 hrs. After this the overlayer was removed and replaced with 20µl of protein buffer with 9M urea solution (60:40%) containing coloured markers and the rod filled up with degassed NaOH buffer. The rods were then prefocused at 200V for 15min, 300V for 30min and 400V for 30min. After prefocusing the buffer and overlayer were discarded and the samples were loaded and overlaid with 5µl of protein buffer. The rest of the tube was filled up with NaOH buffer. The rods were then run at 400V for 12hrs and then 1500V for 1hr. The position of the protein was identified by staining with Coomassie Blue stain.
3. ISOLATION AND IDENTIFICATION OF BACTERIA Able to DegradE ORGANOPHOSPHORUS COMPOUNDS

3.1 Introduction

Soil was used as a source for isolating bacteria as it contains a large number of microorganisms from different genera. Soil was also favoured as organophosphorus compounds are used in fields and gardens so exposing the soil flora to them. This may have lead to the development of enzyme systems in the bacteria to enable them to degrade these chemicals. The organophosphorus compounds could then be used as carbon and energy sources or possibly phosphorus and sulphur sources. There have been reports (Rosenberg and Alexander, 1979) to suggest that soil bacteria are capable of degrading organophosphorus compounds and using them for these purposes.

As already mentioned, the soil flora consists of a wide variety of bacteria depending on the conditions within the soil and the immediate environment. Commonly found soil bacteria include the Coryneforms, Actinomycetes, Pseudomonads and Bacillus sp. Arthrobacter sp. have been reported to be capable of degrading many pollutants such as PCB's (Peloquin and Greer, 1993) and pesticides. It has been reported that various members of the genus are capable of degrading organophosphorus compounds such as carbamates (Pohlenz et al, 1992, and Tam et al 1987) as well as other pollutants such as chlorobenzoates, (Tsoi et al, 1991 and Schmitz et al, 1992). Malathion has also been reported to be degraded by an Arthrobacter sp. isolated from a salt marsh environment, (Walker and Stojanovic, 1973 and 1974) and from soil, (Barik et al 1982).

3.2 Sources of bacterial isolates

Bacteria were isolated from flower beds, compost heaps and sheep dip contaminated soil as it was thought that the micro-organisms present would have been exposed to organophosphorus compounds. The soil contaminated with sheep dip had been exposed to EctomorTm containing the active ingredient, Propentamphos Tech, at 8%w/v. This organophosphorus compound contains carboxyl ester and phosphoester bonds, see Fig.3.1.
3.3 Attempts to isolate bacteria able to grow on malathion and dimethoate

3.3.1 Malathion

The maximum solubility of malathion in water was 145mg.litre\(^{-1}\). This only resulted in 0.44mM malathion, giving 3.52mM carbon, if the malathion was completely degraded. Although this would possibly support the growth of bacteria it would not provide the amount of carbon and energy required to obtain growth in such quantities that the enzymes of the bacteria could be easily studied. Malathion is also, because it is anticholinesterase in its action, quite a dangerous compound and preferably should not be handled daily. To overcome this problem compounds containing similar target bonds to those in malathion were required as carbon and energy sources. The compounds also needed to be soluble enough to provide carbon and energy to support growth for the study of the enzymes. Hydrolysis of the S-C bonds would lead to the removal of the 'leaving group' of malathion so detoxifying it. It was reported (Hassall, 1990) that the hydrolysis of the carboxyl ester bonds in malathion leads to its detoxification with respect to mammalian toxicity. A potential malathion degradation products and analogue was identified in diethylsuccinate, see Fig.3.2.
Figure 3.2 Diethylsuccinate, the possible degradation products and analogue of malathion.
3.3.2 Dimethoate

Dimethoate is also a dithioate organophosphorus compound and belongs to the carbamate family as it contains an amide group, as already shown in Fig. 1.8. As dimethoate is soluble at 25 g l⁻¹ attempts were made to use this as a carbon and energy source to isolate bacteria. In initial experiments the dimethoate used was Dimethoate 40 BASF, a formulation containing the solvents cyclohexanone and xylene as well as dimethoate. When attempts were made to isolate bacteria capable of degrading dimethoate the results were inconclusive. Colonies isolated on plates containing Dimethoate 40 BASF were also patched onto plates with xylene, cyclohexanone and Dimethoate 40 BASF, respectively, as the carbon and energy sources. This was to identify bacteria that would only grow on the Dimethoate 40 BASF and not on the other constituents of the formulation. The pattern of growth indicated that there were no isolates capable of growing on Dimethoate 40 BASF that did not grow on the other constituents, xylene or cyclohexanone. This suggested that the bacteria isolated could not use the dimethoate as a sole carbon and energy source and were using the other constituents.

Pure dimethoate (stated purity 96%) was obtained from Cheminova and was used as a carbon and energy source in plates and liquid media to try to isolate bacteria able to degrade it. However, this approach also proved unsuccessful.

3.4. Diethylsuccinate

Diethylsuccinate was thought to be a possible intermediate in the degradation of malathion. It is similar in its structure to part of the malathion molecule as it has carboxyl ester groups. It was also an attractive substitute for malathion as it is not toxic and is much more soluble. Bacteria were isolated from soil samples using agar plates containing diethylsuccinate as a carbon and energy source. Growth of the bacteria was then checked in liquid media with diethylsuccinate, see Fig. 3.3, which showed that the bacteria grew on diethylsuccinate. To check this was growth on the diethylsuccinate rather than on the products of spontaneous hydrolysis diethylsuccinate was incubated without bacteria, see Fig. 3.3. No hydrolysis
Figure 3.3 Growth of isolate NSD1 on 7.5mM diethylsuccinate and the production of ethanol

In minimal media at 30°C

- O.D. 680nm
- Ethanol produced in inoculated flask
- Ethanol produced in control flask
was seen without bacteria present showing that the conditions for growth, 30°C and shaking at 200rpm, did not cause the spontaneous hydrolysis of diethylsuccinate and the release of ethanol. Production of ethanol in the inoculated media suggested that the bacteria were capable of hydrolysing the carboxyl ester bonds in the diethylsuccinate to produce ethanol. The rise and fall of ethanol in the media over the time of growth, see Fig.3.3, suggested that the bacteria were using the succinate and ethanol, and that some of the ethanol was being excreted into the media then taken up again and used. This suggested that the bacteria used all of the diethylsuccinate molecule for growth.

The production of ethanol suggested that the desired carboxyl ester bonds were being hydrolysed. Therefore, diethylsuccinate was used to isolate bacteria potentially able to degrade malathion.

3.5 Investigation of the enzyme capabilities of diethylsuccinate utilising isolates

It was assumed that the ability of these bacteria to grow on diethylsuccinate was an indication that they contained enzymes capable of hydrolysing diethylsuccinate. To investigate the enzymes of these isolates several general esterase assays using p-nitrophenyl acetate and α-naphthyl acetate were used. Extracts were made from the isolates and assayed by these methods.

3.5.1 General esterase assay using para-nitrophenyl acetate

The general esterase activity assay used was the hydrolysis of p-nitrophenyl acetate which leads to the production of p-nitrophenol a chromogenic substance. The extinction coefficient of p-nitrophenol was calculated by measuring the absorbance of a given concentration at 405nm at a given pH, see Table 3.1. Calculations were made for the mM extinction coefficient at pH 8 which was the pH of the buffer that the enzyme reaction was to be carried out at. Using these parameters the extinction coefficient was calculated to be
<table>
<thead>
<tr>
<th>pH</th>
<th>Absorbance 405nm (0.1mM)</th>
<th>mM extinction coefficient (mM⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>1.020</td>
<td>10.20</td>
</tr>
<tr>
<td>7.5</td>
<td>1.210</td>
<td>12.10</td>
</tr>
<tr>
<td>8.0</td>
<td>1.480</td>
<td>14.80</td>
</tr>
<tr>
<td>8.5</td>
<td>1.480</td>
<td>14.80</td>
</tr>
</tbody>
</table>

Table 3.1 Measurements made for the extinction coefficient of

*p*-nitrophenol
14.8mM⁻¹cm⁻¹. This information was then used to identify and quantify the activity of esterases against p-nitrophenyl acetate.

3.5.2 Multiplicity of esterases in the bacterial isolates

Extracts prepared from various isolates were analysed by native PAGE and stained for esterase activity using α-naphthylacetate as a substrate. All of the isolates were grown on diethylsuccinate. This showed that the extract of some cells apparently contained no enzyme activity against α-naphthylacetate whereas other extracts showed several bands of activity, see Fig.3.4. The apparent lack of activity in some of the isolate extracts might have been due to the esterases being specific to the diethylsuccinate and not active with α-naphthylacetate. Activity may also have been lost in the process of preparing the extract. These observations were useful as the desired esterase would have to be soluble and also relatively stable if it was to be used in any detoxification process. Any esterases that would not act on α-naphthylacetate would also be of little interest as a broad range esterase would be optimal.

As well as requiring an isolate with general esterase activity it was essential for the bacteria to also have activity against malathion. To test further for an enzyme able to degrade malathion a specific assay for malathion hydrolase was developed.

3.5.3 Malathion esterase assays

The malathion esterase assay was used in the screening process as it was essential for the bacteria that were selected for further analysis to have enzymic activity with malathion. The desired enzyme activity was an esterase able to release ethanol from malathion. The release of ethanol was monitored by measuring the production of NADH at 340nm as described in the Materials and Methods Chapter. The malathion esterase assay showed that the isolates had varying activities against malathion. The malathion esterase and p-nitrophenyl acetate esterase assays were compared, see Table 3.2. Comparison of the activities with these two
Figure 3.4 Extracts from diethylsuccinate positive bacteria assayed with $\alpha$-naphthylacetate

Lane 1- SD12
Lane 2- SD11
Lane 3- SD10
Lane 4- SD9
Lane 5- SD8
Lane 6- SD7
Lane 7- NDS1
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Specific activity with $p$-nitrophenyl acetate ($\mu$mol.min$^{-1}$.mg protein$^{-1}$)</th>
<th>Specific activity with malathion ($\mu$mol.min$^{-1}$.mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>0.09</td>
<td>0.02</td>
</tr>
<tr>
<td>2B</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>BP5</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>CH4</td>
<td>0.16</td>
<td>0.01</td>
</tr>
<tr>
<td>CH42</td>
<td>0.12</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>SD1</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>SD2</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>SD3</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>SD4</td>
<td>0.03</td>
<td>0</td>
</tr>
<tr>
<td>SD5</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>SD6</td>
<td>0.16</td>
<td>0.01</td>
</tr>
<tr>
<td>SD8</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>SD9</td>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td>SD10</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>SD11</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>SD12</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>NSD1</td>
<td>0.19</td>
<td>0.02</td>
</tr>
<tr>
<td>NSD2</td>
<td>0.31</td>
<td>0</td>
</tr>
<tr>
<td>NSD3</td>
<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>NSD4</td>
<td>0.14</td>
<td>0.02</td>
</tr>
<tr>
<td>NSD6</td>
<td>0.13</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.2 Esterase activities of environmental isolates assessed using $p$-nitrophenyl acetate and malathion as substrates
substrates enabled the selection of one isolate, NSD1, which had good general esterase activity and the highest activity against malathion.

3.6 Identification of the environmental isolate NSD1

The isolate, NSD1, chosen through the esterase assay selection methods was identified to genera level by a variety of biochemical, microbiological and microscopic methods, the results of which are shown in Table 3.3. Samples of the bacteria were also sent to The National Collection of Industrial and Marine Bacteria Limited (NCIMB) who carried out similar identification tests. The bacterium was found to be Gram positive mis-shapen rods in young cultures which then showed a tendency to lose the stain and form coccoidal shapes in older cultures. These results showed that the isolate had a distinctive rod/cocci or pleomorphic life cycle. This was further investigated by electron microscopy. Cells were taken from single colonies of different ages that had been grown on plates containing diethylsuccinate as a carbon and energy source. The bacteria were then viewed under the scanning electron microscope, see Fig. 3.5. These showed clearly the change in cell morphology with the age of the bacteria. Also noticeable from these pictures was the exopolysaccharide produced by the bacteria which appeared as a dark background behind the cells. The electron micrographs also showed some possible storage bodies in some of the older cells as indicated in Fig. 3.5.

There was no fermentative metabolism as no acid was produced from growth on peptone media containing glucose. The bacteria were shown to be aerobic as they were catalase positive. The bacteria did not appear to be able to grow above 37°C, showing that they were mesophilic in nature. This was of no surprise as the bacteria were isolated from soil and many soil bacteria are mesophilic. The bacteria were non-motile as assessed by viewing under the light microscope. No spores were formed after prolonged growth on media containing manganese to encourage spore formation. Spore formation was assessed by pasteurisation of the bacteria grown on manganese containing media then plating onto media to monitor the survival. None of the pasteurised bacteria survived whereas control unpasteurised cells were still viable.
<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Results from the NCIMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>Gram positive/Gram variable with age</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Motility</td>
<td>Non-motile</td>
<td>Non-motile</td>
</tr>
<tr>
<td>Spore formation</td>
<td>No spores formed</td>
<td>No spores formed</td>
</tr>
<tr>
<td>Temperature range of growth</td>
<td>23°C, 30°C, 37°C</td>
<td>37°C but not 41°C or 45°C</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>At 48hrs, round, regular, entire, smooth, glossy, opaque, convex. (Pale orange pigment with age)</td>
<td>At 48 hrs, round, regular, entire, smooth, glossy, opaque, convex, 1mm in diameter. (Pale orange pigment with age)</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>Misshapen rods in young cultures, coccoidal in older cultures. Showing pleomorphic life cycle.</td>
<td>N/D</td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxidase</td>
<td>N/D</td>
<td>Negative</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Growth substrates</td>
<td>LB, succinate, diethylsuccinate, fructose, glycerol, glucose.</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Table 3.3 Results of tests carried out to identify the isolate NSD1
(N/D-not done)
Figure 3.5 Electron micrographs of different aged cultures of the *Arthrobacter* sp. isolate grown on plates containing diethylsuccinate as the carbon and energy source

A - 3 day old culture
B - 5 day old culture
C - 7 day old culture
D - 7 day old culture
As the bacteria had a pleomorphic life cycle and were isolated from soil they were tentatively identified as an *Arthrobacter* sp. The tests carried out by the NCIMB identified the bacterium as a Coryneform which might belong to a variety of genera. They suggested that fatty acid analysis or cell wall analysis could be carried out for confirmation of the genus. This is because *Arthrobacter* are the only genus to contain lysine in their cell wall. Although several corynebacteria are reported to exhibit pleomorphic life cycles (Keddie *et al*, 1986) NSD1 was assigned to the *Arthrobacter* as it was isolated from soil on a minimal media, under non-exacting conditions. Jones and Keddie (1992), suggested that isolates with a pleomorphic life cycle obtained under such conditions were most likely be an *Arthrobacter* sp. as this genera is so prevalent in the soil flora and generally require no additional growth factors.

### 3.7 Diethylsuccinate esterase assays

As all of the organisms were isolated using diethylsuccinate as the carbon and energy source it was assumed that all of the extracts must have enzymes capable of hydrolysing diethylsuccinate. After the identification of isolate NSD1, an *Arthrobacter* sp., with the highest esterase activity against malathion and also a good general esterase activity assays were carried out with diethylsuccinate. Diethylsuccinate was used in both spectrophotometric and native gel assays. This gave an insight into the esterase activity with diethylsuccinate and also, using the native gel, the number of enzymes active against this substrate. The *Arthrobacter* sp. was grown on diethylsuccinate and extracts were tested and found to have activity of 0.30µmol.min⁻¹.mg protein⁻¹ with diethylsuccinate in the spectrophotometric assay. Through native gel analysis using diethylsuccinate as the substrate the esterase activity was found to be due to two enzymes, see Fig.3.6. Assays with *Arthrobacter* sp. extract with 0.04mM diethylsuccinate solution showed that the total ethanol released was equivalent to both of the carboxyl ester bonds in the diethylsuccinate being hydrolysed.
Figure 3.6 Extract of *Arthrobacter* sp. run on a native gel and stained with diethylsuccinate showing two bands of activity

Lane 1- empty

Lane 2- extract from *Arthrobacter* sp.

Lane 3- empty
3.8. Growth of *Arthrobacter* sp. isolate on diethylsuccinate

The *Arthrobacter* sp. was found to grow on diethylsuccinate at 30°C with a doubling time of 4.25hrs. To investigate this further the isolate was grown in cultures with succinate alone, succinate plus ethanol, ethanol alone, succinate plus diethylsuccinate and diethylsuccinate alone. These experiments, see Fig.3.7, showed that growth on succinate plus ethanol resulted in a growth yield of the same value of that seen for growth on diethylsuccinate. This suggested that the bacteria used all of the succinate and ethanol for growth.

3.9. Growth on other substrates

The ability to grow on other compounds was tested. The isolate was grown on diethylsuccinate media to mid log phase, cells were harvested and washed and used to inoculate fresh minimal media containing a variety of carbon sources. All carbon sources were added to give a final concentration in the growth media of 60mM carbon. Growth was at 30°C at 200rpm and was monitored at 680nm as shown in Fig.3.8. The isolate was able to grow on succinate (tₜ 9hrs), LB (tₜ 2hrs), glycerol (tₜ 6hrs), fructose (tₜ 3.5hrs), and on glucose (tₜ 26hrs). Growth was, as expected, strongest on the rich LB media. Growth on glucose was poor and suggested that the *Arthrobacter* sp. either did not have enzymes able to transport the glucose into the cell or did not have an efficient enzyme system able to degrade the glucose in the cell. Similar cases of *Arthrobacter* sp. not being able to use glucose have been reported by Sobel *et al* (1973).

3.10 Constitutive esterase enzyme production

Experiments were carried out to find out whether the *Arthrobacter* sp. esterase enzyme activity was induced or constitutive. The isolate was grown on other substrates, succinate, LB, glycerol, fructose and glucose to see if this had any effect on the esterase activity against diethylsuccinate. Extracts from cells grown in this way were assayed using diethylsuccinate as a substrate, see Table 3.4. The results showed that activity was 63-103%
Figure 3.7 Growth of the Arthrobacter sp. on various substrates

Growth in minimal media at 30°C
Figure 3.8 Growth of the *Arthrobacter* sp. on various carbon sources.

Growth was in minimal media at 30°C

- Growth of *Arthrobacter* sp. on succinate (7.5mM)
- Growth of *Arthrobacter* sp. on fructose (10mM)
- Growth of *Arthrobacter* sp. on glycerol (20mM)
- Growth of *Arthrobacter* sp. on glucose (10mM)
- Growth of *Arthrobacter* sp. on diethylsuccinate (7.5mM)
- Growth of *Arthrobacter* sp. on Luria Broth
<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Enzyme activity with diethylsuccinate (µmol.min⁻¹mg protein⁻¹)</th>
<th>% of activity compared to that with diethylsuccinate grown cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>diethylsuccinate</td>
<td>0.30 +/- 0.015</td>
<td>100 +/-5</td>
</tr>
<tr>
<td>succinate</td>
<td>0.19 +/-0.017</td>
<td>63 +/-9</td>
</tr>
<tr>
<td>fructose</td>
<td>0.21 +/- 0.023</td>
<td>70 +/-11</td>
</tr>
<tr>
<td>Luria broth</td>
<td>0.21 +/- 0.012</td>
<td>70 +/-6</td>
</tr>
<tr>
<td>glycerol</td>
<td>0.26 +/- 0.010</td>
<td>87 +/-4</td>
</tr>
<tr>
<td>glucose</td>
<td>0.31 +/- 0.020</td>
<td>103 +/-6</td>
</tr>
</tbody>
</table>

Table 3.4 Cell extracts from *Arthrobacter* sp. grown on various carbon sources assayed with diethylsuccinate to determine whether esterase enzyme was constitutive or inducible.
of that seen with cells grown on diethylsuccinate suggesting that the esterase expression was constitutive.

3.11 Sonication optimum

The optimum conditions for sonication were determined by sonicating a suspension of Arthrobacter sp. cells for different lengths of time and assaying the esterase activity and protein released from the cells. This was carried out to find the point of maximum esterase activity when the cell contents were released before the sonication became detrimental to the enzyme activity. Fig. 3.9. shows the increase of activity and protein with time. The optimum time for sonication was found to be 2mins for Arthrobacter sp., with cooling every 30sec. This was a longer time than was used for the E. coli cells (2x30sec) and this is probably due to the cells being Gram positive.

3.12 Isolation of bacteria able to use organophosphorus compounds as a phosphorus source

Phosphorus constitutes 3% of cell dry weight and is an essential element for growth. There have been reports of bacteria that are able to use organophosphorus compounds as a phosphorus source (Rosenberg and Alexander, 1979). As malathion was thought not to be soluble enough to provide a suitable amount of carbon and energy for growth, attempts were made to use it as a phosphorus source. Attempts were also made to use dimethoate as a phosphorus source as experiments to use it as a carbon and energy source had been unsuccessful. The release of phosphorus or phosphate would require the hydrolysis of several bonds and would likely require the action of more than one enzyme, see, Fig. 3.10.

As all organophosphorus compounds contain a phosphorus atom attached to a variety of different groups enzymes able to hydrolyse these bonds would be useful in detoxifying many different types of organophosphorus compound.
Table 3.9. Assays for the determination of the optimum conditions for sonication of the *Arthrobacter* sp. isolate.

<table>
<thead>
<tr>
<th>Time (seconds)</th>
<th>Enzyme (units.ml⁻¹)</th>
<th>Specific activity (μmol.ml⁻¹.mg protein⁻¹)</th>
<th>Protein (mg.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0.1</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>0.2</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>300</td>
<td>0.3</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>400</td>
<td>0.4</td>
<td>0.4</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 3.9. Assays for the determination of the optimum conditions for sonication of the *Arthrobacter* sp. isolate.

- Enzyme (units.ml⁻¹)
- Specific activity (μmol.ml⁻¹.mg protein⁻¹)
- Protein (mg.ml⁻¹)
3.13.1. Investigating the *Arthrobacter* sp. isolate's ability to use malathion as a phosphorus source

The *Arthrobacter* sp., isolated using diethylsuccinate as a carbon and energy source, was used to inoculate flasks containing malathion as the phosphorus source and diethylsuccinate as the carbon and energy source. The rationale for this was to investigate whether the bacteria could use both compounds. The phosphate buffer which usually supplies 25mM phosphate in the PJC minimal media was replaced by 3-[N-morpholino]propanesulfonic acid (MOPS). It was hoped that this would encourage the bacteria to use the malathion as a phosphorus source as there would be no other phosphorus supplied in the media. The *Arthrobacter* sp. was already known to be capable of degrading part of the malathion molecule by hydrolysing the carboxylester bonds resulting in the release of ethanol. It was hoped that by attempting to use malathion as a phosphorus source then it might be possible for the *Arthrobacter* sp. to degrade the malathion molecule completely. The *Arthrobacter* sp. was used to inoculate MOPS (pH7) containing 5mM (15.6mg of phosphorus/100ml of
media) of orthophosphate buffer as the phosphorus source and diethylsuccinate as the carbon source to check that the bacteria was not inhibited by the MOPS. The flask with the malathion (14.5µl) as the phosphorus source was inoculated and a control was set up with no phosphate. It was assumed that cells require 3mg phosphorus /100ml to produce cell mass at 1mg.ml⁻¹. As malathion is only soluble at 14.5mg.100ml⁻¹ (0.44mM) this would, in theory, only provide sufficient phosphorus (1.45mg.100ml⁻¹) for growth to 0.5mg.ml⁻¹. Flasks were incubated at room temperature and growth was monitored at 680nm the results of the growth are shown in Fig.3.11. The results showed that the Arthrobacter sp. could grow on MOPS buffered media with phosphate (5mM) showing that the MOPS was not inhibitory. However, there was no growth on the MOPS containing the malathion as the phosphate source compared to the growth on the MOPS with no phosphate. From Fig.3.11 the growth seems to have been inhibited by the presence of malathion compared to that seen on PJC minimal media without any phosphorus source. This suggested that the Arthrobacter sp. could not use the malathion as a phosphorus source. Growth without added phosphorus may have been due to phosphorus contaminants in other components of the media. The Arthrobacter sp. was also inoculated into succinate as a carbon and energy source with malathion as a phosphorus source. This time the growth with malathion as the phosphorus source was the same as that seen for no phosphorus source, see Fig.3.12.

3.13.2. Investigating the ability of new isolate to use malathion as a phosphorus source

To find bacteria capable of using malathion as a phosphorus source new isolates were obtained from existing soil samples taken from Propentamphos contaminated soil. Again MOPS buffer was used to replace the usual phosphate buffer, malathion was used as a phosphorus source and diethylsuccinate as a carbon and energy source. Soil suspension was used to inoculate the flasks and loopfuls of the resulting bacterial growth were used to streak out for single colonies. The resulting colonies were found to grow at room temperature and 30°C but not at 37°C. These colonies were used to inoculate fresh flasks with diethylsuccinate as the carbon and energy source and malathion as the phosphorus
Figure 3.11 Growth of *Arthrobacter* sp. on MOPS minimal media with diethylsuccinate as the carbon and energy source and various potential phosphorus sources.

Growth was at room temperature.

- **□** Growth of *Arthrobacter* sp. on diethylsuccinate (7.5mM) in MOPS buffered medium

- **△** Growth of *Arthrobacter* sp. on diethylsuccinate (7.5mM) in MOPS buffered medium + phosphate (5mM)

- **○** Growth of *Arthrobacter* sp. on diethylsuccinate (7.5mM) in MOPS buffered medium + Malathion (0.44mM)
Figure 3.12 *Arthrobacter* sp. growth on MOPS minimal media with succinate as the carbon and energy source and various potential phosphorus sources.

Growth was at room temperature.

- □ Growth of *Arthrobacter* sp. on succinate in MOPS buffered medium with no added phosphate
- △ Growth of *Arthrobacter* sp. on succinate in MOPS buffered medium + 5mM phosphate
- ○ Growth of *Arthrobacter* sp. in MOPS buffered medium + Malathion (0.44mM)
source. Compared to the control flask with no phosphate the malathion containing flask
grew to a higher optical density, see Fig.3.13. The results showed that the new isolate
appeared to use the malathion as a source of phosphorus.

3.14.1 Investigating the *Arthrobacter* sp. isolate's ability to use dimethoate
as a phosphorus source

The organophosphorus compound dimethoate, which had been used unsuccessfully as a
carbon and energy source, was also tried as a phosphorus source. Again MOPS was used
to replace the phosphate buffers in the minimal media and 1mM dimethoate was used as the
phosphorus source. Flasks were inoculated with the *Arthrobacter* sp., which was isolated
on diethlysuccinate and had carboxylesterase activity against diethlysuccinate. Controls
were set up with either no phosphorus or 5mM phosphorus in the form of sodium
dihydrogen orthophosphate and the flasks were incubated at room temperature, see
Fig.3.14. The *Arthrobacter* sp. did not show any growth with the dimethoate as a
phosphorus source compared to the growth where there was no phosphorus in the media.
This was as expected as the *Arthrobacter* sp. had not been able to use malathion as a
phosphorus source.

3.14.2 Investigating the new isolate's, ability to use dimethoate as a
phosphorus source

Flasks were inoculated with the new bacterial isolate and diethlysuccinate (7.5mM) and
dimethoate 1mM. Control flasks were set up with diethlysuccinate as the carbon and energy
source and either no phosphate or with 5mM phosphate buffer for the phosphorus source,
see Fig.3.15. The results suggested that the bacteria could use dimethoate as a phosphorus
source as the growth with dimethoate in the media was comparable to the media with 5mM
phosphate. Cells in diethlysuccinate also went into the death phase after stationary phase
whereas the growth on the media containing dimethoate remained in stationary phase. This
might be due to the bacteria being able to use more of the dimethoate than just the
Figure 3.13 New isolate growth on MOPS minimal media with diethylsuccinate as the carbon and energy source and either malathion as the phosphorus source or no phosphorus source. Growth was at room temperature.
Figure 3.14 Growth of *Arthrobacter* sp. on MOPS minimal media with diethylsuccinate as the carbon and energy source with various phosphorus sources.

Growth was at room temperature.

- Growth of *Arthrobacter* sp. on diethylsuccinate (7.5mM) in MOPS with no phosphorus source
- Growth of *Arthrobacter* sp. on diethylsuccinate (7.5mM) in MOPS with phosphate (5mM)
- Growth of *Arthrobacter* sp. on diethylsuccinate (7.5mM) in MOPS with dimethoate (1mM)
Figure 3.15 Growth of new isolate on MOPS minimal media with diethylsuccinate as the carbon and energy sources and various phosphorus sources.

Growth was at room temperature.

- Growth of new isolate on diethylsuccinate (7.5mM) in MOPS with no phosphorus source
- Growth of new isolate on diethylsuccinate (7.5mM) in MOPS with phosphate (5mM)
- Growth of new isolate on diethylsuccinate (7.5mM) in MOPS with dimethoate (1mM)
phosphorus to maintain the stationary phase.

3.15 Identification of the bacterium able to use malathion and dimethoate as phosphorus sources

This bacterium was sent to the NCIMB for identification tests, the results of which are given in Table 3.5. These tests showed that the isolate was a Gram negative, non fermentative non motile coccobacillus. These characteristics suggested that it could belong to one of several genera, *Alcaligenes*, *Psychrobacter* or *Comamonas*. Further biochemical tests and fatty acid analysis would be required to characterise the isolate and possibly identify the correct genus. Further experiments to investigate enzymes degrading the organophosphorus compounds could not be carried out as there was insufficient time to investigate.

3.16 Discussion

The use of diethylsuccinate as a carbon and energy source was successful in isolating bacteria that had enzymes which were able to hydrolyse malathion. Analysis of the general esterase activity of isolates revealed that many of the diethylsuccinate-utilising bacteria had a multiplicity of esterases. These activities may reflect the variety of roles played by esterases in the cell metabolism such as the degradation of carbon and energy sources and cell wall synthesis. Hydrolysis of the general esterase substrates α-naphthyl acetate and p-nitrophenyl acetate may also be identifying enzymes that are not specific carboxylesterases but do have some activity with these substrates. Such enzymes might be thiol esterases, amidases or peptidases. As well as identifying a diversity of esterases with regard to their mobility on native PAGE there were also variations in specificity. This was demonstrated by the inability of some extracts from diethylsuccinate-isolated cells to hydrolyse malathion. However this lack of activity might also have been explained by instability of the esterase in the preparation process. Alternatively the esterase may have been located in the membrane and hence overlooked as only cell extracts were tested for activity.
<table>
<thead>
<tr>
<th>Test</th>
<th>Result from the NCIMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>Negative</td>
</tr>
<tr>
<td>oxidase</td>
<td>Positive</td>
</tr>
<tr>
<td>catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>growth temperature</td>
<td>Growth at 20°C and 37°C, not at 41°C or 45°C.</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>Negative</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Round, regular, cream, smooth, glossy, opaque, mucoid, convex, 1mm in diameter.</td>
</tr>
<tr>
<td>Spore formation</td>
<td>Negative</td>
</tr>
<tr>
<td>motility</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 3.5 Identification tests on the new isolate, NSDX, able to utilise malathion and dimethoate as a phosphorous sources.
The diethylsuccinate utilising *Arthrobacter* sp. was shown to have two esterases that were active against diethylsuccinate. The esterase activity appeared to be constitutive suggesting that there were no regulatory genes involved in their expression. Assays of esterase activity with a known amount of diethylsuccinate showed that all of the ethanol was released suggesting that both of the carboxyl ester bonds were hydrolysed. This agreed with the observations made when the bacteria were grown on diethylsuccinate as compared to succinate plus ethanol. The use of ethanol as well as succinate as a carbon and energy source was also suggested by the amount of ethanol detected in the growth media as the ethanol concentration only reached a peak of approximately 2mM. If the diethylsuccinate had been hydrolysed and the ethanol released without use a maximum value of 15mM would have been expected. This suggested that the esterases either both hydrolysed the two bonds or acted in series to hydrolyse both bonds.

The use of malathion as a carbon and energy source was limited by its low solubility. Dimethoate was much more soluble and looked to be a more promising candidate. However, tests using dimethoate as a carbon and energy source were unsuccessful. This may have been due to the inability of bacteria to take up the dimethoate. If uptake was not the problem failure to grow may have been due to the bacteria being unable to hydrolyse the dimethoate. It might also be due to the parent compound, or the products of hydrolysis, being toxic to the cell. As dimethoate and malathion were later shown to be able to provide a phosphorus source for growth this suggested that uptake was not impossible. The dimethoate used to provide a phosphorus source was 1mM. However, in experiments where it was used as a carbon and energy source 6-12mM was added. Again this suggested that the parent compound or its degradation products may have been toxic to the cells. Alternatively the compound which might be produced by hydrolysis of the dimethoate may not have been able to be further degraded for use in the cell.

The second isolate, NSDX, appeared to be able to use dimethoate and malathion as phosphorus sources. The amount of malathion used was limited to a final concentration of 0.44mM by its low solubility. Dimethoate was used at 1mM as this would provide enough phosphorus to give 1mg.ml\(^{-1}\) growth. The dimethoate was only used at this low concentration as it was thought that this would reduce any possible toxic effects it might
have on the bacteria or any chance of the bacteria using contaminants in the dimethoate as a phosphorous source. The *Arthrobacter* sp. was also tested on these compounds as phosphorus sources but was unable to grow. This suggested that any contaminating phosphorous in the organophosphorus compounds could not be significant as this would have enabled the *Arthrobacter* sp. to grow to a greater extent compared to growth on media without any phosphorus source. The ability of the isolate NSDX to grow on the organophosphorus compounds may have been due to plasmid encoded enzymes that required the selective pressure of growth in the presence of the compounds. This may be one reason why the *Arthrobacter* sp. was unable to grow on these compounds as it had been kept on plates containing orthophosphate as the phosphorus source.

The identification of the *Arthrobacter* sp. was based on a variety of biochemical tests and morphological observations. These coupled with the method of isolation from soil strongly suggested that the bacterium was an *Arthrobacter* sp. However, for further conclusive evidence on the taxonomy of the bacterium tests on the cell was amino acid composition would be required and analysis of the 16SrRNA. Tests were carried out on NSDX but were not extensive enough to identify the isolate to genera level. Further tests which might be able to determine the genera of the bacteria would be fatty acid and 16SrRNA analysis.
4. CLONING OF THE ESTERASE GENE, ANALYSIS OF SUBCLONES, SEQUENCING OF THE GENE AND SEQUENCE ANALYSIS

4.1 Introduction

At the beginning of this project it was hoped that the gene(s) encoding the esterase(s) from the *Arthrobacter* isolate could be identified from a gene library using a probe. This would first require the purification of the protein(s). The purified protein(s) would then be used to obtain information on the N-terminal sequence(s). The N-terminal sequence(s) would be used to construct a probe (or probes) which would be used to screen a gene library in hybridisation experiments. This would identify the clone(s) carrying either the whole or part of the gene(s) of interest. However, this approach was unsuccessful as the purification of the protein from the original isolate proved more difficult than had been anticipated. To overcome this, an alternative method of identifying the gene was used. This involved the construction of a gene library and the identification of the cloned esterase gene by phenotypic selection. This selection was carried out by using diethylsuccinate as a carbon and energy source.

4.2 Attempts at the separation and purification of the malathion esterase

In the *Arthrobacter* sp., there were two esterases that were active when assayed with diethylsuccinate as a substrate by native PAGE. To enable further study of these enzymes the two proteins needed to be separated and purified. There were two reasons for doing this:-

1. Separation was required to see if both enzymes were able to hydrolyse malathion.
2. Once the activity of the enzyme(s) had been investigated it/they would need to be purified to provide information on the N-terminal sequence. This information would then be used to construct a probe for analysis of a gene library.

Trial attempts at separation and purification were carried out using several methods.
4.2.1 Ammonium sulphate fractionation of *Arthrobacter* sp. cell extract

As a first step in purification ultracentrifuged cell extract from the *Arthrobacter* sp. was fractionated using ammonium sulphate. Fractions made using 0-55% and 55-90% cuts were successful in separating the esterase activities as seen by analysis on a native gel assaying with diethylsuccinate as substrate, see Fig.4.1. Both esterases were also found to be active with malathion, see Table.4.1. However, further attempts at esterase purification from ammonium sulphate fractions by anion exchange chromatography lead to additional loss of activity with no further purification. Other similar standard purification techniques were attempted but all resulted in very little purification. Although there was no purification using these standard methods separation of the enzymes was achieved by either ammonium sulphate fractionation or application to a Phenyl Superose column. The separation of the esterases demonstrated that they were both active with malathion.

4.3 Cloning of the esterase gene from the *Arthrobacter* sp.

4.3.1 JM109 growth characteristics

To assess the suitability of JM109 and other *E. coli* strains such as K10 and NM522 as hosts to construct a gene library they were tested for growth on diethylsuccinate as a carbon and energy source. None of the above strains grew. JM109 was selected as it has other desirable characteristics needed when constructing a gene library such as *recA1*, *endA1*, *hsdM* and *hsdR*. It also has the Δ(*lac-proAB*) *F*[traD36* proAB*+ *lacI*9* lacZ*Δ*M15*] phenotype allowing the blue white selection in conjunction with the pUC plasmid vectors. Growth experiments were also carried out with JM109[pUC18] with no insert on media containing succinate, succinate plus diethylsuccinate, succinate plus ethanol, ethanol alone and diethylsuccinate alone. The bacterium was found to grow on all but the diethylsuccinate alone. Growth on ethanol alone was very poor. However, the presence of the ethanol in the media did not appear to be inhibitory as growth on succinate and ethanol showed a rate of growth comparable to growth on succinate alone.
Figure 4.1. Native PAGE analysis of separation achieved by ammonium sulphate fractionation of the *Arthrobacter* sp. extract

Lane 1- Crude extract
Lane 2- empty
Lane 3- 0-55%
Lane 4- empty
Lane 5- 55-90%
<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total units (μmol·min⁻¹·ml⁻¹)</th>
<th>Specific activity (μmol·min⁻¹·mg protein⁻¹)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>20</td>
<td>66</td>
<td>0.54</td>
<td>0.008</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>0-55% fraction</td>
<td>2.5</td>
<td>8.44</td>
<td>0.188</td>
<td>0.022</td>
<td>35</td>
<td>2.75</td>
</tr>
<tr>
<td>55-90% fraction</td>
<td>2.5</td>
<td>1.15</td>
<td>0.1</td>
<td>0.087</td>
<td>18.5</td>
<td>10.89</td>
</tr>
</tbody>
</table>

Table 4.1 Malathion esterase spectrophotometric assays of fractions from ammonium sulphate fractionation
Growth on succinate and ethanol also showed initial growth on the succinate and then much slower growth on the ethanol which prevented the cells going into stationary phase, see Fig. 4.2. This suggested that the diethylsuccinate and ethanol were not inhibitory to the growth of JM109, so their presence in the media as carbon and energy source or biproducts of growth on diethylsuccinate should not prevent the growth of a transformant with esterase activity.

4.3.2 Construction of a gene library

Chromosomal DNA of the *Arthrobacter* sp. was prepared, as described in the Materials and Methods section 2.17, and this DNA was then partially digested with *Sau3AI* restriction enzyme. Trial digests were carried out varying the amount of enzyme used in the digestion and the length of time the digestion was allowed to continue, see Fig. 4.3. This was to determine the conditions for digestion to produce fragments of approximately 1.5-5kb. Fragments of this size were required as they were thought to be large enough to carry the esterase gene. The optimum enzyme concentration to digest 60µg of chromosomal DNA was found to be 0.25 units for 15min at 37°C to achieve 1.5-5kb fragments. After digestion under these conditions 50µg of the partially digested DNA was size fractionated on a sucrose gradient. Following the size fractionation the DNA was concentrated and the size of the fragments present was checked by running samples on an agarose gel. Fractions between sizes 1.5-6kb were pooled for use in ligation with the vector DNA.

The fragments of chromosomal DNA were ligated into linearized (digested with restriction endonuclease *BamHI*) and dephosphorylated pUC18 DNA. The ligation mixture was used to transform competent *E. coli* JM109 cells (Promega).

4.4 Screening of the gene library for esterase activity

Cells transformed with plasmids were first grown on LB plates containing ampicillin (100µg.ml⁻¹) to improve cell viability and ensure only bacteria with plasmid were selected. Serial dilutions of transformants were made and plated on LB containing ampicillin (100µg.ml⁻¹), X-Gal and IPTG and the blue white selection used to determine how many
Figure 4.2 Growth of JM109 containing pUC18 on various carbon sources.
M63 minimal media was used and ampicillin (100μg.ml-1) at 30°C.

- □ Growth of JM109[pUC18] on 15mM ethanol
- ○ Growth of JM109[pUC18] on 15mM ethanol + 7.5mM succinate
- △ Growth of JM109[pUC18] on 7.5mM succinate
- ✡ Growth of JM109[pUC18] on 15mM succinate
- ❌ Growth of JM109[pUC18] on 7.5mM diethylsuccinate
- ○ Growth of JM109[pUC18] on 7.5mM diethylsuccinate + 7.5mM succinate
Figure 4.3 Digests of *Arthrobacter* sp. chromosomal DNA (2.5μg) at 37°C with 0.25 units of restriction endonuclease *Sau* 3AI at various time points.

Lane 1 1kb Lambda DNA ladder
Lane 2 Undigested *Arthrobacter* chromosomal DNA
Lane 3 Digestion at time = 0min
Lane 4 Digestion at time = 15min
Lane 5 Digestion at time = 30min
Lane 6 Digestion at time = 45min
Lane 7 Digestion at time = 60min
Lane 8 1kb Lambda DNA ladder
transformants contained insert DNA. Using this method approximately 4000 colonies (80% of those plated) were shown to contain inserts. The cells selected by growth on LB/ampicillin plates were transferred to selective plates of M63 minimal media containing diethylsucinate as the carbon and energy source and incubated at 30°C. Several colonies were obtained and these were checked to make sure that the esterase activity was being conferred by pUC18 containing insert DNA. For this plasmid was prepared from these transformants and used to retransform JM109 cells which were then plated onto the media containing diethylsucinate. One colony grew after the retransformation and plate selection. The plasmid conferring this phenotypic change was named pUC18-79.

4.5 Growth of transformant on diethylsucinate

JM109[pUC18-79] were grown overnight on M63 minimal media containing succinate (15mM) and ampicillin (100μg.ml⁻¹). The cells were harvested, washed and used to inoculate fresh minimal media (so that the initial reading at 680nm was approximately 0.100) containing diethylsucinate (7.5mM) as the carbon and energy source. Growth was monitored at 680nm, see Fig.4.4. A control experiment had already been carried out (see Fig.4.2), showing that JM109 with plasmid vector alone would not grow on diethylsucinate. For JM109[pUC18-79] the doubling time was 43hrs and the total change at 680nm was 0.525 absorbance units. From Fig.4.4 the growth on diethylsucinate can be seen to be greater than the growth on 7.5mM succinate suggesting that the cells are using more than just the succinate part of the diethylsucinate. The doubling time of JM109 containing pUC18 grown on succinate was much faster than that seen for growth on diethylsucinate and had a total change at 680nm of 0.426 absorbance units. Growth on the diethylsucinate showed a similar pattern to growth on succinate plus ethanol where after the initial growth on succinate the cells did not go into the stationary phase and subsequent decline but continued to grow steadily. However, growth on diethylsucinate was much slower and the total change in absorbance at 680nm from growth on diethylsucinate was not as much as that seen for JM109[pUC18-79] on succinate (7.5mM) plus ethanol (15mM). This may have been due to the maintenance energy required to maintain the cells
Figure 4.4 Growth of JM109[pUC18-79] on different carbon and energy sources.

M63 minimal media with ampicillin (100μg.ml-1) at 30°C

- Growth of JM109[pUC18-79] on 15mM ethanol
- Growth of JM109[pUC18-79] on 7.5mM diethylsuccinate
- Growth of JM109[pUC18-79] on 7.5mM succinate + 15mM ethanol
- Growth of JM109[pUC18-79] on 7.5mM succinate
housekeeping metabolism during the slow growth on diethylsuccinate. Other limiting factors may have been the uptake of the diethylsuccinate and also the amount of enzyme in the cell able to hydrolyse the diethylsuccinate. Growth on ethanol (15mM), see Fig.4.4, showed that the JM109 did not grow very well on ethanol. This would explain the very slow and relatively small amount of growth on ethanol after growth on succinate. It is unlikely that the reduced growth rate of the JM109[pUC18-79] on diethylsuccinate was due to the presence of the ethanol as this did not greatly effect the growth of the JM109[pUC18-79] on succinate plus ethanol media. To investigate the substrate utilisation during growth on JM109[pUC18-79] on diethylsuccinate the ethanol produced over the time of growth was monitored, see Fig.4.5. This showed that ethanol was present in the growth media over the time of exponential growth and was reduced as the growth slowed down. This suggested that the bacteria were using all of the diethylsuccinate and that some of the ethanol was being excreted by the cell and then taken up and used. Ethanol was not seen to be produced over time when JM109 was incubated with diethylsuccinate showing that there was no spontaneous hydrolysis. No ethanol was seen with growth of JM109[pUC18-79] on succinate showing that ethanol was not produced in the growth media by succinate catabolism.

4.6 Enzyme assays of cell extracts from JM109[pUC18-79]

Extracts were made from JM109[pUC18-79] which had been grown on minimal media containing 15mM succinate and ampicillin (100μg.ml⁻¹). Esterase activity was assayed with p-nitrophenyl acetate, diethylsuccinate and malathion in the spectrophotometric assays, see Table 4.2. Activity was also seen with diethylsuccinate using the native PAGE assay, see Fig.4.6. This native gel assay also showed that JM109 containing pUC18 alone did not have any esterase activity, this was confirmed with spectrophotometric assays. The native gel assay showed only one band of activity in the extract from JM109[pUC18-79] when stained using diethylsuccinate as substrate. This suggested that the cloned DNA only encoded one of the two esterases that had previously been shown to be present in the original isolate NSD1.
Figure 4.5 Incubation of JM109 with diethylsuccinate and growth of JM109[pUC18-79] on diethylsuccinate and succinate and the production of ethanol from these growths.

Growth was at 30°C on M63 minimal media with ampicillin (100μg.ml-1) where appropriate.
<table>
<thead>
<tr>
<th>Transformant</th>
<th>( p)-nitrophenyl acetate esterase (( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg} \text{ protein}^{-1} ))</th>
<th>Diethylsuccinate esterase (( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg} \text{ protein}^{-1} ))</th>
<th>Malathion esterase (( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg} \text{ protein}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109[pUC18-79]</td>
<td>2.24 +/- 0.08</td>
<td>0.715 +/- 0.042</td>
<td>0.052 +/- 0.007</td>
</tr>
</tbody>
</table>

Table 4.2 Enzyme activity of extract from JM109[pUC18-79]. Cells were grown on succinate with ampicillin (100\( \mu \text{g} \cdot \text{ml}^{-1} \)) at 30°C.
Figure 4.6. Native polyacrylamide gel stained using diethylsuccinate as a substrate

Lane 1- Extract (0.135mg of protein) from JM109[pUC18-79]
Lane 2- Extract (0.135mg of protein) from JM109[pUC18-79]
Lane 3- Extract leaked from Lane 2
Lane 4- Extract (0.135mg of protein) from JM109[pUC18]

Arrow 1 indicates the band of esterase activity
Arrow 2 indicates the protein front
4.7 Restriction site map of pUC18-79

The inserted DNA of pUC18-79 conferring esterase activity was characterised by digestion with restriction endonucleases. The initial restriction mapping of the insert DNA was carried out using restriction enzymes that were able to cut the polylinker sites of pUC18, (HindIII, SphI, PstI, SalI, AccI, XbaI, BamHI, XmaI, SmaI, KpnI, SacI, BanII and EcoRI). It was found that the insert DNA could not be removed as a single fragment from pUC18 using BamHI. The enzymes SphI, XbaI, XmaI, SmaI and HindIII had no sites in the insert DNA as digestion of pUC18-79 with these enzymes resulted in one fragment due to cleavage of the appropriate site in the polylinker region. It was found that the insert DNA could be removed from pUC18 by digestion with the combination of XmaI and XbaI as these enzymes had no sites in the insert DNA. Insert DNA removed from the vector by digestion with these enzymes was run on an agarose gel and shown to be approximately 5.7kb in size. Digestion with the combination of XmaI and XbaI was used to transfer the insert DNA into the vector pUC19 to give pUC19-79. This was to determine whether the orientation of the insert with respect to the vector promoter affected expression of the cloned gene. Through restriction analysis some enzymes (BanII, AccI, SalI, PstI) were found to have many sites in the insert making it very difficult to map them. This resulted in only the enzymes EcoRI, and KpnI being used, initially, to map the insert DNA. Further analysis was then carried out using other restriction enzymes, EcoRV, MscI, BgIII and SacII, see Fig.4.7. These enzymes had no sites in either the polylinker region or the rest of the pUC18 DNA so linearisation of the plasmid and the production of fragments on digestion with these enzymes could only be due to the restriction sites within the insert DNA.

4.8 Enzyme assays of cell extracts from JM109[pUC18-79] and JM109[pUC19-79]

JM109 [pUC18-79] and [pUC19-79] respectively were grown on M63 minimal media
Figure 4.7 Restriction map of pUC18-79 using the restriction enzymes with sites in the polylinker and those with no sites in pUC18. The cloned DNA is shown by the thin lines. Thick lines represent the flanking DNA of the vector and the hatched areas indicate the polycloning region. The scale only refers to the cloned DNA.

EI = EcoRI, BI = BamHI, K = KpnI, HI = HindIII, X" = Xba, S* = SalI, P = PstI, S^ = SphI, S' = SacI, X' = Xma, EV = EcoRV, B = BglII, S'' = SacII, M = MscI
containing succinate (15mM) and ampicillin (100μg.ml⁻¹) +/- IPTG (0.3mM) to investigate the effect of the orientation of the insert DNA on the expression of the esterase gene. Extracts of the cells grown under these conditions were made and assayed using p-nitrophenyl acetate, diethylsucinate and malathion as substrates, as shown in Table 4.3. The results showed that neither the orientation of the insert DNA nor the presence of the IPTG had any effect on the expression of the esterase gene. This suggested that the expression of esterase gene was under the control of its own promoter. JM109 containing these clones were grown on diethylsucinate +/- IPTG. This showed that there was little difference in the growth rate depending on orientation of the insert or the presence of IPTG, see Table 4.3.

4.9 Construction of subclones of pUC18-79

The restriction mapping of the insert DNA identified several restriction enzyme sites of use in constructing subclones. These were Kpnl at co-ordinate 3.7kb, MscI at co-ordinates 4kb and 4.7kb, EcoRV at co-ordinates 1kb and 2.4kb and SacII at co-ordinates 0.8kb and 2.2kb, see Fig.4.7. As each of these enzymes had two sites of recognition in the insert DNA (except Kpnl, which has one in the polylinker) restriction digests with these enzymes, followed by ligation, were used to delete pieces of the insert DNA, see Fig.4.8. Digestion with Kpnl resulted in pUC18-791, MscI resulted in pUC18-792, SacII resulted in pUC18-793 and EcoRV resulted in pUC18-794, as shown in Fig.4.8. The newly formed subclones were used to transform JM109. To investigate the effect of insert orientation on esterase gene expression the insert DNA was removed and ligated into the pUC19 vector. These new plasmids, pUC19-791, pUC19-792, pUC19-793, and pUC19-794, respectively, were transformed into JM109. Cell extracts from JM109 containing pUC19-791, pUC19-792, pUC19-793, and pUC19-794 were made and tested for their esterase activity. By doing this it was hoped that it would be possible to identify which part of the insert DNA was important in encoding the esterase gene.
<table>
<thead>
<tr>
<th>Transformant</th>
<th>IPTG</th>
<th>(p)-nitrophenyl acetate esterase ((\mu\text{mol.\text{min}^{-1}.\text{mg protein}^{-1}}))</th>
<th>Diethylsuccinate esterase ((\mu\text{mol.\text{min}^{-1}.\text{mg protein}^{-1}}))</th>
<th>Malathion esterase ((\mu\text{mol.\text{min}^{-1}.\text{mg protein}^{-1}}))</th>
<th>(t_d) (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109[pUC18-79]</td>
<td>-IPTG</td>
<td>2.24 +/- 0.08</td>
<td>0.715 +/- 0.042</td>
<td>0.052 +/- 0.007</td>
<td>42</td>
</tr>
<tr>
<td>JM109[pUC18-79] + IPTG</td>
<td>+IPTG</td>
<td>2.45 +/- 0.16</td>
<td>0.794 +/- 0.017</td>
<td>0.058 +/- 0.007</td>
<td>45</td>
</tr>
<tr>
<td>JM109[pUC19-79]</td>
<td>-IPTG</td>
<td>2.55 +/- 0.10</td>
<td>0.762 +/- 0.016</td>
<td>0.057 +/- 0.003</td>
<td>35</td>
</tr>
<tr>
<td>JM109[pUC19-79] + IPTG</td>
<td>+IPTG</td>
<td>2.51 +/- 0.07</td>
<td>0.866 +/- 0.040</td>
<td>0.061 +/- 0.008</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 4.3 Enzyme activities of extract from JM109[pUC18-79] and JM109[pUC19-79] +/- IPTG grown on LB with ampicillin (100\(\mu\text{g.ml}^{-1}\)) showing representative doubling times for JM109 containing these plasmids.
Figure 4.8 Restriction maps showing the construction of subclones of pUC18-79 using the restriction enzymes with sites in the polylinker and those with no sites in pUC18. The cloned DNA is shown by the thin lines. Thick lines represent the flanking DNA of the vector and the hatched areas indicate the polycloning region. The scale only refers to the cloned DNA.

EI = EcoRI, BI =BamHI, K = KpnI, HI = HindIII, X' = Xba,
S* = Sall, P = PstI, S^ = Sphi, S' = SacI, X' = Xma, EV = EcoRV,
B = BglII, S'' = SacII, M = MscI
4.10 Esterase activity of JM109 containing various subclones

The esterase activity of the cell extracts of JM109, grown on succinate, containing the subclones in both vectors pUC18 and 19 was assayed using malathion, diethyl succinate and p-nitrophenyl acetate, see Table 4.4. Growth of JM109 containing these subclones on diethyl succinate was also monitored, see Fig.4.9. The extracts from cells containing pUC18-793, pUC19-793, pUC18-794 and pUC19-794 showed no activity. This supported the growth experiment where no growth on diethyl succinate was observed with JM109 containing these same subclones. This suggested that removal of the DNA by digestion with either EcoRV or SacII resulted in the removal of part of the DNA encoding the esterase gene. The subclones constructed by digestion with either KpnI or MscI (pUC18-791, pUC19-791, pUC18-792 and pUC19-792) still showed esterase activity suggesting that the DNA removed in these digestions was not involved in encoding the enzyme.

4.11 Further restriction mapping of pUC18-791

It was apparent that the removal of the 2kb fragment by digestion of pUC18-79 with restriction enzyme KpnI (forming pUC18-791) did not effect the esterase activity. As pUC18-791 was the smallest subclone, containing approximately 3.7kb of insert DNA, still exhibiting esterase activity it was chosen for further restriction mapping, see Fig.4.10. This was to try to identify more restriction endonuclease sites to enable the construction of smaller subclones and narrow down further the insert DNA required to encode the esterase gene. The subclones were constructed by the removal of regions of DNA and religating the remaining vector and inserted DNA. In the case of pUC19-798, religation was achieved by filling in the cohesive ends produced by digestion with BstEII and HindIII using dNTPs and Klenow fragment. The subclones pUC19-799, and pUC19-7911 were constructed for use in sequencing, see Fig.4.11.
<table>
<thead>
<tr>
<th>Transformant</th>
<th>IPTG</th>
<th>para-nitrophenyl acetate esterase (μmol.min⁻¹. mg protein⁻¹)</th>
<th>diethylsuccinate esterase (μmol.min⁻¹. mg protein⁻¹)</th>
<th>malathion esterase (μmol.min⁻¹. mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109[pUC18-791]</td>
<td>-IPTG</td>
<td>2.57 +/- 0.06</td>
<td>0.86 +/- 0.012</td>
<td>0.052 +/- 0.004</td>
</tr>
<tr>
<td>JM109[pUC18-791]</td>
<td>+ IPTG</td>
<td>2.00 +/- 0.15</td>
<td>0.70 +/- 0.025</td>
<td>0.046 +/- 0.003</td>
</tr>
<tr>
<td>JM109[pUC19-791]</td>
<td>-IPTG</td>
<td>2.58 +/- 0.06</td>
<td>0.87 +/- 0.029</td>
<td>0.055 +/- 0.005</td>
</tr>
<tr>
<td>JM109[pUC19-791]</td>
<td>+ IPTG</td>
<td>2.42 +/- 0.05</td>
<td>0.85 +/- 0.023</td>
<td>0.051 +/- 0.004</td>
</tr>
<tr>
<td>JM109[pUC18-792]</td>
<td>-IPTG</td>
<td>2.83 +/- 0.06</td>
<td>0.80 +/- 0.012</td>
<td>0.055 +/- 0.004</td>
</tr>
<tr>
<td>JM109[pUC18-792]</td>
<td>+ IPTG</td>
<td>2.54 +/- 0.07</td>
<td>0.77 +/- 0.031</td>
<td>0.053 +/- 0.001</td>
</tr>
<tr>
<td>JM109[pUC19-792]</td>
<td>-IPTG</td>
<td>2.86 +/- 0.06</td>
<td>0.91 +/- 0.036</td>
<td>0.059 +/- 0.002</td>
</tr>
<tr>
<td>JM109[pUC19-792]</td>
<td>+ IPTG</td>
<td>2.46 +/- 0.15</td>
<td>0.83 +/- 0.012</td>
<td>0.058 +/- 0.004</td>
</tr>
<tr>
<td>JM109[pUC18-793]</td>
<td>-IPTG</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>JM109[pUC18-794]</td>
<td>-IPTG</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.4 Enzyme activities of cell extracts from JM109 containing the subclones pUC18-791, pUC19-791, pUC18-792, pUC19-792, pUC18-793 and pUC18-794 +/- IPTG grown on M63 minimal media with succinate and ampicillin (100μl.ml⁻¹)
Figure 4.9 Growth of JM109 containing the subclones of pUC18-79 on diethylsuccinate (7.5mM) M63 minimal media ampicillin (100μg.ml⁻¹) at 30°C.
Figure 4.10 The further restriction mapping of pUC18-791 and the construction of the smaller subclones pUC18-796 and pUC18-798. The cloned DNA is shown by the thin lines. Thick lines represent the flanking DNA of the vector and the hatched areas indicate the polycloning region. The scale only refers to the cloned DNA.

El = EcoRI, BI = BamHI, K = KpnI, HI = HindIII, X'' = Xba,
S* = SalI, P = PstI, S = SphI, S' = SaeI, X' = Xma, EV = EcoRV,
B = BglII, S'' = SacII, M = MscI, B* = BstEII
Clone | Esterase activity
---|---
pUC18-79 | +
pUC18-791 | +
pUC18-796 | +
pUC18-798 | +
Figure 4.11 The further restriction mapping of pUC18-791 and the construction of the subclones pUC18-799 and pUC18-7911 for use in sequencing. The cloned DNA is shown by the thin lines. Thick lines represent the flanking DNA of the vector and the hatched areas indicate the polycloning region. The scale only refers to the cloned DNA.

El = EcoRI, Bl =BamHI, K = KpnI, HI = HindIII, X" = Xba,
S* = SalI, P = PstI, S^ = SphI, S' = SacI, X' = Xma, EV = EcoRV,
B = BglII, S'' = SacII, M = MscI, B* = BstEII
Clone Esterase activity

pUC18-79 +
pUC18-791 +
pUC18-799 -
pUC18-7911 -
4.12 Esterase activity of new subclones

The esterase activities expressed by these new constructs were assayed, see Table 4.5. This narrowed down the DNA responsible for encoding the esterase to 2kb in the construct pUC19-798, as this was the smallest construct which still exhibited esterase activity.

4.13 N-terminal sequencing of esterase expressed from pUC19-798

Extracts were made from JM109[pUC19-798] grown on succinate with ampicillin (100µg.ml⁻¹). When this extract was analysed by SDS PAGE there was a band seen at approximately 40,000 Daltons, which was not present in extracts of JM109 containing pUC19, see Fig.4.12. It was therefore concluded that this protein was the product of the esterase gene being expressed in pUC19-798. The cell extract from JM109[pUC19-798] was ultracentrifuged and run on an SDS polyacrylamide gel for two hours after the dye front had run off the edge of the gel. Separation of the band of 40,000 Daltons from the other proteins in the extract was sufficient that the protein could be blotted and the blot analysed to obtain its N-terminal sequence, see Table 4.6.

Analysis of purified protein from JM109[pUC19-798] enabled the N-terminal sequence to be determined by Edman degradation and information on the first 15 amino acids being obtained. Of these 15 amino acids 13 were clearly defined and two (residues 7 and 15) were ambiguous, see Table 4.6. There was no methionine at the beginning of the amino acid sequence. There would have to be a methionine at the beginning of the gene so it was assumed that the initiating methionine codon must have been removed by the E. coli. As the first six of the amino acids were clearly defined these and a putative initiation methionine were used in the design of the degenerate primer. However, these amino acids would have resulted in the formation of a 21mer with a degeneracy of 768. In the design of oligodeoxyribonucleotide primer OCH1, see Fig.4.13, the final valine was represented by a 'gt'. This resulted in a 20mer having a degeneracy of 192. The primer was used to start sequencing pUC19-798 and to identify the start of the esterase encoding gene.
<table>
<thead>
<tr>
<th>Transformant</th>
<th>IPTG</th>
<th>$p$-nitrophenyl acetate esterase (µmol.min$^{-1}$.mg protein$^{-1}$)</th>
<th>diethylsuccinate esterase (µmol.min$^{-1}$.mg protein$^{-1}$)</th>
<th>malathion esterase (µmol.min$^{-1}$.mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>-IPTG</td>
<td>2.27 +/- 0.12</td>
<td>0.98 +/- 0.20</td>
<td>0.075 +/- 0.007</td>
</tr>
<tr>
<td>[pUC19-796]</td>
<td>+ IPTG</td>
<td>1.82 +/- 0.29</td>
<td>0.99 +/- 0.24</td>
<td>0.08 +/- 0.010</td>
</tr>
<tr>
<td>JM109</td>
<td>-IPTG</td>
<td>3.53 +/- 0.12</td>
<td>0.94 +/- 0.09</td>
<td>0.060 +/- 0.010</td>
</tr>
<tr>
<td>[pUC19-798]</td>
<td>+ IPTG</td>
<td>2.96 +/- 0.25</td>
<td>0.94 +/- 0.05</td>
<td>0.063 +/- 0.012</td>
</tr>
<tr>
<td>JM109</td>
<td>-IPTG</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[pUC18-795]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 Enzyme activities of JM109 containing subclones pUC19-796, pUC19-798, pUC18-795 +/- IPTG, grown on M63 minimal media with succinate (7.5mM) and ampicillin (100µgml$^{-1}$)
Figure 4.12 SDS PAGE showing the presence of a protein band in the cell extract from JM109[pUC19-798] compared to JM109[pUC19]

Lane 1 extract (0.03mg of protein) from JM109[pUC19]

Lane 2 SDS VII marker proteins

Lane 3 extract (0.03mg of protein) from JM109[pUC19-798]
<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Residue</th>
<th>Amount (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>13.48</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>9.80</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>13.10</td>
</tr>
<tr>
<td>4</td>
<td>E</td>
<td>9.09</td>
</tr>
<tr>
<td>5</td>
<td>G</td>
<td>5.51</td>
</tr>
<tr>
<td>6</td>
<td>V</td>
<td>8.59</td>
</tr>
<tr>
<td>7</td>
<td>V/I</td>
<td>7.02/5.48</td>
</tr>
<tr>
<td>8</td>
<td>D</td>
<td>9.51</td>
</tr>
<tr>
<td>9</td>
<td>Q</td>
<td>5.39</td>
</tr>
<tr>
<td>10</td>
<td>R</td>
<td>8.31</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>4.68</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
<td>2.04</td>
</tr>
<tr>
<td>13</td>
<td>G</td>
<td>4.65</td>
</tr>
<tr>
<td>14</td>
<td>L</td>
<td>8.96</td>
</tr>
<tr>
<td>15</td>
<td>K/A</td>
<td>3.96/4.30</td>
</tr>
</tbody>
</table>

Table 4.6 The N-terminal sequence of the purified esterase protein isolated by electrophoresis from the crude extract of JM109[pUC19-798]
Figure 4.13 Design of the degenerate oligodeoxyribonucleotide primer OCH1

<table>
<thead>
<tr>
<th>Measured protein sequence</th>
<th>Met Ala Asn Ile Glu Gly Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide sequence</td>
<td>atg gct aat att gaa ggt gt</td>
</tr>
<tr>
<td></td>
<td>c c c g c</td>
</tr>
<tr>
<td></td>
<td>a a a</td>
</tr>
<tr>
<td></td>
<td>g g</td>
</tr>
</tbody>
</table>

4.14 Nucleotide sequencing of the esterase gene

The degenerate primer, OCH1, was designed to bind at the beginning of the gene. The sequence obtained showed the direction that the gene was being transcribed in as restriction enzymes sites SacII and PstI could be identified and related to the restriction map of pUC19-798, see Fig.4.14. The sequence also showed that there was a BanII site 126bps downstream from the start of the sequence obtained using OCH1 as the primer. The BanII site was identified as a unique site in the insert DNA by restriction endonuclease digest. The only other BanII site present was in the polylinker of pUC. These two sites were used to construct the subclone pUC19-7912 by cutting with BanII and religating the resulting cohesive ends. This removed approx. 1.5kb of insert DNA and resulted in a subclone of 0.5kb, see Fig.4.15. Using pUC19-7912 and the forward primer of pUC19 the complimentary strand to that obtained using the primer OCH1 was sequenced. This identified the sequence of the DNA at the beginning of the gene and confirmed the sequence already obtained using primer OCH1, see Table 4.7.

The rest of the gene was sequenced using custom synthesised oligodeoxyribonucleotide primers. These were designed using sequence as it was obtained, see Table 4.8, and other subclones as already described in Fig.4.11. Using this method primers were designed to obtain the sequence on both strands, see Fig.4.16. By obtaining the whole of the esterase gene sequence this information could then be used to compare with the derived protein sequences in a variety of databases. This would then help to determine whether the sequence encoded a novel enzyme or whether there were similarities to other
Figure 4.14 The sequence obtained using the degenerate oligodeoxyribonucleotide primer OCH1. This locates the restriction enzyme sites *Pst*I and *Sac*II (boxed) which had been identified by restriction mapping.
Ava I Fnu4H I Sau96 I Fnu4H I Sau96 I Hae III Bbv I Ava II  

```
ggccgagttcgcagcagcggtaaggaccgtattcagtctcgccacacttggtgctgcacacttt
ccgggctcaagcgtcgtcgcctctggcataaggtcagctcggctagaacacaggtgtgaa
```

| 241 | 252 | 265 |
| 243 | 255 |
| 255 |
| 256 |
Figure 4.15 The construction of subclone pUC18-7912 for use in sequencing. The cloned DNA is shown by the thin lines. Thick lines represent the flanking DNA of the vector and the hatched areas indicate the polycloning region. The scale only refers to the cloned DNA.

EI = EcoRI, BI = BamHI, K = KpnI, HI = HindIII, X'' = Xba,
S* = SalI, P = PstI, S = SphI, S' = SacI, X' = Xma, EV = EcoRV,
B = BglII, S'' = SacII, M = MscI, B* = BstEII, BII = BanII
Clone | Esterase activity
---|---
pUC18-79 | +
pUC18-791 | +
pUC18-798 | +
pUC18-7912 | -
Table 4.7 Identification of the N-terminal amino acid sequence of the esterase from the nucleotide sequence

<table>
<thead>
<tr>
<th>Actual nucleotide sequence</th>
<th>Translated protein sequence</th>
<th>The measured N-terminal sequence of the esterase protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>gtg</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>gcg</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>aac</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>atc</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>gaa</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>ggc</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>gta</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>tgt</td>
<td>C</td>
<td>V/I</td>
</tr>
<tr>
<td>gac</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>cag</td>
<td>Q</td>
<td>Q</td>
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<tr>
<td>cga</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>ttc</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>tct</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>ggc</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>tgt</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>aag</td>
<td>K</td>
<td>K/A</td>
</tr>
</tbody>
</table>

Table 4.8 Custom designed oligodeoxyribonucleotide primers for sequencing the esterase gene

<table>
<thead>
<tr>
<th>Name of oligodeoxynucleotide</th>
<th>Sequence of oligodeoxynucleotide 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCH2</td>
<td>gtcgcgcacgccccaggg</td>
</tr>
<tr>
<td>OCH3</td>
<td>tggatctcgatgccccgg</td>
</tr>
<tr>
<td>OCH4</td>
<td>aggacgagctcgacccccgc</td>
</tr>
<tr>
<td>OCH5</td>
<td>cgccacgccccggtgccccgc</td>
</tr>
<tr>
<td>OCH6</td>
<td>aggtgacgggtgatccc</td>
</tr>
<tr>
<td>OCH7</td>
<td>gcgccacgccccggtgccccgc</td>
</tr>
<tr>
<td>OCH8</td>
<td>gtgatgaacaagagatgggg</td>
</tr>
<tr>
<td>OCH9</td>
<td>gcaaggatgtgatagccg</td>
</tr>
</tbody>
</table>
Figure 4.16 The use of custom designed oligodeoxyribonucleotide primers and subclones to sequence the entire esterase gene.
enzymes in the databases. The sequence information and the derived amino acid composition also helped in the determination of the subunit molecular weight.

4.15 The nucleotide sequence of the esterase gene

The complete nucleotide sequence of the esterase gene, 1140bp, incorporating sites of restriction endonuclease recognition, is shown in Fig.4.17. There was only one open reading frame. The open reading frame for the esterase gene was 1140bp which encoded a 380 amino acid protein with a predicted molecular weight of 40,679Da. This molecular weight corresponded well with that of approx. 40,000Da predicted for the protein using SDS PAGE analysis. The initiating methionine was found to be encoded by the codon GTG. This is usually a codon for valine unless, as in this case, it is found in the initiation codon position. A ribosome binding site, GGAGG, (Shine and Dalgarno, 1976) was identified 9bp upstream of the initiation codon, see Fig.4.18. Upstream of the initiation codon in many genes there are also -10 and -35 consensus sequences but these were not obvious. This apparent lack of -10 and -35 consensus sequences was also noted by Nishizawa, et al (1995) for the Arthrobacter globiformis carboxylesterase gene.

The percentage G+C content for the esterase gene was 63%, within the range seen for Arthrobacter DNA of 59-66% (Jones and Keddie 1992). Codon usage was as shown in Table 4.9. There was a bias for G or C in the third position as 78.7% of the codons ended with either of these nucleotides. This is characteristic of genes of the Arthrobacter sp. as they are members of the high GC 'actinomycete' branch of the Gram positive eubacteria. The predicted amino acid composition of the esterase gene is shown in Table 4.10. The derived amino acid sequence is shown in Fig.4.18.

Positions of the restriction sites, PstI, SacII, EcoRV, BamHI, and BglII, identified through restriction mapping of pUC18-79 were also seen in the nucleotide sequence of the esterase gene.
Figure 4.17 The nucleotide sequence of the coding region of the gene showing all restriction endonuclease sites
<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Sequence</th>
</tr>
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1040

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Hae III
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ScrF I
Nci I
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Hpa II
Bcm I
Hha I
Mae II
Hae III
Sau96 I
Mbo I
SnaB I
Pnu4HI

Nla III
Spla I
Nla IV
Nla IV
Dpn I
Resa I
Bbv I

| • | || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || || | 1041 1052 1071 1087 1097 1107 1117 1053 1072 1097 1108 1117 1055 1073 1097 1109 1075 1102 1075 1102 1075 1075 1075 1077 1078

Dde I
Hga I
Alu I

cctagacgcacttagctga

1140
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1126 1135 1132
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Table 4.10 The predicted amino acid content of the esterase gene and the predicted molecular weight
Figure 4.18 The derived amino acid sequence from the esterase gene. The
Shine Dalgarno site is underlined and the putative active site, Ser-X-X-Lys,
is double underlined.
4.16 Comparison of the nucleotide and derived amino acid sequence of the esterase gene with other esterase genes

The nucleotide sequence of the esterase gene and its translated protein sequence were compared to other sequences in databases using the BLAST algorithms. Searches were made using the tblastn, blastp, blastx and blastn parameters at the internet site http://www.bio.cam.ac.uk/Seqsrch/blast-all.html. Comparisons of the protein sequences showed an identity of 38% (58% similarity) with the esterase genes estA (McKay et al 1992) and estC (Kim et al 1994) of Pseudomonas fluorescens and a 34% identity (54% similarity) with the carboxylesterase of Arthrobacter globiformis. (Nishizawa et al 1995). The relatedness of the proteins can be seen from the alignments, see Fig.4.19, and also in the dendrogram, see Fig.4.20. The similarity was determined by using the criteria that a Probability value (P value) of below $10^{-4}$ was conclusive of a homologous relationship (Borodovsky et al 1994). This suggests that the esterase is more closely related to the Pseudomonas estA and C than to the Arthrobacter globiformis esterase.

4.17 Further sequencing of pUC19-798

The subclone pUC19-798 contained 2kb of DNA which was more than was required for encoding the esterase gene (1140bp). Approximately 0.5kb of this extra DNA was upstream of the esterase gene. In an attempt to identify a promoter in this region this DNA was sequenced (see Appendix I for whole sequence of the 2kb insert of pUC19-798) and submitted to the databases. However, this did not identify any obvious promoter regions.
Figure 4.19 A Pile Up analysis of the *Arthrobacter* sp. esterase and the closest matching sequences found in the nucleotide and protein sequence databases. Identical regions are shaded in black with white letters and conserved regions are in grey. The Ser-X-X-Lys motif is marked with a ♦ at the Ser. The Gly-X-Ser-X-Gly is marked with a ♦ at the first Gly.

- **A44832** *Pseudomonas* sp. esterase est A
- **S69066** *Pseudomonas fluorescens* esterase III (estC) carboxylesterase est
- **Z49911** *Caenorhabditis elegans* esterase like gene
- **L22516** *Arthrobacter globiformis* carboxyl-esterase protein
SPECIAL NOTE

This item is tightly bound and while every effort has been made to reproduce the centres force would result in damage.
Figure 4.20 A dendrogram showing the relationship between the *Arthrobacter* sp. esterase and the closest matching sequences found in the nucleotide and protein databases.

*Pseudomonas* sp. esterase est A

*Pseudomonas fluorescens* esterase III (estC) carboxylesterase

*Arthrobacter* sp. esterase protein sequence

*Caenorhabditis elegans* esterase like gene

*Arthrobacter globiformis* carboxyl-esterase protein
Pseudomonas sp. estA

Pseudomonas fluorescens estC

Arthrobacter sp. (this study)

Genorhabditis elegans

Arthrobacter globiformis
4.18 Discussion

Ammonium sulphate fractionation showed that both esterases were active with malathion. This information was of use when only one of the esterases was cloned as it was already known to be active with malathion even though its selection had been using diethylsuccinate. JM109 was used as a host organism due to its genotype features, its usefulness in the blue/white selection and its inability to grow or show enzyme activity with diethylsuccinate. Uptake was not considered to pose a problem as the diethylsuccinate was an uncharged molecule and it was thought possible that it would diffuse into the cell even if there was not a specific transport system for its uptake. Uptake was not impossible as the phenotypic selection was successful. The slow growth of JM109[pUC18-79] on diethylsuccinate, compared to that seen on succinate, might have been due to uptake being limiting. Alternatively the amount of esterase produced may have been the limiting factor.

Although JM109 did not appear to grow well on ethanol alone growth of JM109[pUC18-79] on diethylsuccinate suggested that ethanol was being used. The ethanol measured in the media was about 15% of that expected if all the diethylsuccinate been hydrolysed and the ethanol excreted. The use of ethanol was also suggested by the disappearance of the ethanol released which corresponded with a further small increase in growth and delayed decline into stationary phase. These observations suggested that the hydrolysis of the diethylsuccinate and the resulting exposure of the cells to ethanol resulted in the development of enzymes able to use the ethanol more successfully than when exposed to ethanol alone.

Lack of the control of expression of the esterase gene with IPTG suggested that the gene was expressed from its own promoter. However, analysis of the DNA of the coding region and upstream of the start codon did not identify any obvious promoter sequences at -35 or -43. This could be due to them not being similar to the sequences usually seen in E.coli or Arthrobacter sp.

Comparison of the derived protein sequences showed homology with several other bacterial carboxyl- and aryl-esterases. Analysis of the protein sequence also identified the motif Ser-X-X-Lys at Ser68 in the N-terminal region of the protein. This motif has been
identified in a similar N-terminal position in several other enzymes and it has been suggested, through analysis of site directed mutants, that this is a putative active site serine. The other well documented esterase/lipase active site motif, Gly-X-Ser-X-Gly, was not identified in full but as a truncated form, Gly-X-Ser-X-X. In the Pile Up analysis of the proteins this motif was in a similar position, in the C-terminal region, to the full motif seen in the two Pseudomonas sp. esterases. Protein sequences of the homologous esterases from the Pseudomonas sp. contained both of the motifs whereas the Arthrobacter globiformis contained only the Ser-X-X-Lys motif. The Arthrobacter globiformis was also reported to have a weak homology with the C-terminal Lys-Thr/Ser-Gly motif which is found in penicillin recognising enzymes. This was not seen in the Pseudomonas sp. esterases or the malathion esterase. The malathion esterase in this study seemed, through sequence comparison, to be more closely related to the Pseudomonas sp. esterases having 38% identity and two similar putative active sites. The similarity of the esterase to those found in the Pseudomonas sp. may suggest a common ancestry or similar function within the cell. This was also suggested by several areas of the protein sequences which showed strong homology with each other. However, the differences in the two putative active site motifs might suggest divergence from a common esterase to enzymes with different substrate specificities.

It has been reported that the Ser-X-X-Lys motif is found in penicillin recognising enzymes such as those reported by Joris et al (1988) for Streptomyces R61 and by Asano et al (1992) for Ochrobactrum anthropi. It was also shown, by sequence comparison, that many other micro-organisms contained penicillin reactive proteins with the same motif (Brenner 1988). Proteins with this motif were also thought to play a role in peptidoglycan synthesis in bacterial cells. It was suggested by Nishizawa et al (1995) that the Arthrobacter globiformis esterase with a Ser-X-X-Lys motif, which had an identity of 34% with the malathion esterase in this study, might be involved in cell wall peptidoglycan synthesis or an analogous function. The motif has also been seen in the plasmid borne β-lactamases TEM and SHV, Collatz et al (1990), which convey antibiotic resistance in Gram negative bacteria. Collatz et al (1990) suggested that enzymes with this motif might be involved in a defence mechanisms.
As both the esterases were shown to be produced constitutively in the original isolate this would suggest that there was a role for these enzymes in the normal function of *Arthrobacter* cell metabolism. As the *Arthrobacter* sp. was isolated from soil it would have been exposed to a variety of compounds in the environment. Many compounds produced by plants which are responsible for flavours and aromas are esters and it is possible that these compounds might be the natural target of the esterase enzyme. Other compounds encountered in the soil environment might be the antibiotics produced by *Streptomyces*. As this esterase shares some features found in penicillin recognising enzymes conferring antibiotic resistance it might be produced as a defence mechanism to bind similar antibacterial compounds. Alternatively the esterase may be involved in the synthesis of the bacterial cell wall.
5. PURIFICATION AND CHARACTERISATION OF THE ESTERASE

5.1 Introduction

Pure protein was required to characterise the enzyme's activity. This was of interest as reports on similar enzymes (Nishizawa et al 1995, McKay et al 1992 and Kim et al 1994), identified by sequence comparison, claimed to have broad substrate specificity for various carboxyl esters, and also with thiol esters. One of the ideas in the undertaking of this project was to try to find an enzyme, a hydrolase, which might be able to degrade not just one pesticide, in this case malathion, but also a range of other organophosphorus compounds. In characterising the enzyme the aim was to identify other possible substrates and also to identify the optimum conditions of use.

To obtain the purified enzyme in sufficient quantities for study the gene needed to be overexpressed. Knowledge of overexpression would also be needed if the enzyme was to be used in any large scale production.

5.2 Strategy for overexpression of the esterase gene

Experiments on the expression of the original clone and subclones of the esterase gene in JM109 showed that neither the orientation of the insert, or the presence of IPTG, seemed to have any effect on the level of expression. This suggested that the gene was under the control of its own promoter. To obtain overexpression of the esterase encoding gene the start of the gene, and its nucleotide sequence, needed to be identified and any promoter regions removed. This was so that gene expression could be put under the control of a strong promoter.

To obtain overexpression of the gene, the vector pT7-7 (Tabor, 1985 and Anon, 1995), a derivative of the pBR322 family of vectors, was used. This vector uses the T7 RNA polymerase promoter, see Fig.5.1. IPTG is used to induce the expression of the T7 RNA polymerase. This is possible as the host bacteria JM109(DE3) carries the λDE3 lysogen which has the T7 RNA polymerase under the control of the lac system. The pT7-7 vector has a multiple cloning site, as shown in Fig.5.1, which contains a recognition site for
Figure 5.1. Plasmid map of vector pT7-7

bla - Beta lactamase resistance gene

Ø10 - T7 RNA polymerase promoter

rbs - ribosome binding site
the restriction endonuclease \textit{NdeI}. Cleavage of pT7-7 with this enzyme and the ligation of the insert DNA with the appropriate cohesive end results in the formation of an in frame ATG codon. The inserted gene expression is then controlled by T7 RNA polymerase promoter. The plasmid formed by ligation of the appropriate insert DNA in pT7-7 was then used to transform the host JM109(DE3).

\textbf{5.3 Engineering of the esterase gene into the pT7-7 vector}

As already mentioned the design of the pT7-7 vector enables the ligation of a gene into the polylinker at the restriction endonuclease site \textit{NdeI}. This ensures that the gene is in frame and will be overexpressed. To enable this ligation an \textit{NdeI} site must be engineered into the start of the esterase gene. Restriction mapping and sequence analysis of the esterase gene in pUC19-798 revealed that it did not contain any \textit{NdeI} sites. This meant the PCR product (1.4kb) derived from copying the gene from pUC19-798 could be digested with \textit{NdeI} without the gene being restricted. This would enable the removal of the intact gene from the PCR product.

\textbf{5.3.1 Conditions of the PCR}

The gene was copied from pUC19-798 using the PCR and two primers. The primer at the start of the gene (see Fig.5.2) was designed to incorporate an \textit{NdeI} site to place the gene in frame in the pT7-7 vector. The other primer used needed to be situated after the end of the gene to ensure that the whole of the gene would be copied by the PCR. Sequencing of pUC19-798 showed that the esterase gene ended 320bps before the pUC polylinker which contained the \textit{EcoRI} site. As there were no other \textit{EcoRI} sites in the rest of the insert DNA of pUC19-798, this restriction site could be used in conjunction with \textit{NdeI} to remove the gene from the PCR product. To incorporate the \textit{EcoRI} site into the PCR product the forward primer of pUC19, a 17mer, was used as the second PCR primer, see Fig.5.3. Using these two primers the PCR product, of 1.4kb, could be digested with the restriction endonucleases \textit{NdeI} and \textit{EcoRI} and ligated to the corresponding cohesive ends in pT7-7.
Figure 5.2 A PCR primer enabling the insertion of an *NdeI* site at the start of the esterase gene. Nucleotides changed to form the *NdeI* recognition sequence (catatg) are shown in bold.

\[
5' \quad gg \ agg \ ctg \ tca \ gtg \ gcg \ aac \ atc \ gaa \ ggc \ g
3'
\]

Esterase gene original sequence

\[
5' \quad gg \ agg \ ctg \ cat \ atg \ gcg \ aac \ atc \ gaa \ ggc
3'
\]

PCR primer

Figure 5.3 Position for annealing, and direction of transcription, of the primers for the PCR enabling the insertion of an *NdeI* site at the start of the esterase gene and copying of the gene from pUC19-798. The cloned DNA is shown by the thin lines. Thick lines represent the flanking DNA of the vector and the hatched areas indicate the polycloning regions.
The Tm for each of the oligodeoxyribonucleotides was calculated using the equation:

\[ Tm = 69.3 + 0.41(\% G+C) - 650/L \]

Where L = the length of the oligodeoxyribonucleotide (number of nucleotides).

For the oligodeoxyribonucleotide designed to incorporate the NdeI site the Tm was calculated to be 67°C and for the forward primer for pUC19 the Tm was 53°C. The PCR conditions used were based on the methods suggested by Innis and Gelfand (1990) as described in the Materials and Methods section 2.34.

5.3.2 Analysis of the PCR product

The PCR product was run on an agarose gel and was shown to be approximately 1.4kb as expected. The band was excised from the gel and extracted by JETSORB. The extracted PCR product was digested with NdeI and EcoRI and ligated into the pT7-7 vector, which had also been digested with the same enzymes. JM109 cells were transformed with the ligation mixture and transformants were selected on LB plates containing ampicillin (100μg.ml⁻¹). A plasmid preparation was made and restriction digests of this preparation showed that ligation had occurred. The ligated DNA was also checked for correct insertion at the NdeI site by sequencing through this region. This confirmed that the DNA had ligated correctly in frame and created an NdeI restriction site. The pT7-7 vector with the esterase gene insert was named pCH771.

5.4 Induction of the esterase gene in pCH771

5.4.1 Growth and induction on LB

JM109(DE3)[pCH771] were grown overnight on LB media containing ampicillin (100μg.ml⁻¹) at 30°C. These overnight cultures were used to inoculate fresh cultures. The cells were grown to an O.D.680nm of approximately 0.40 and then induced with 0.3mM IPTG and allowed to grow for another 2hrs. Extracts from cells containing pCH771 grown with or without IPTG were assayed using p-nitrophenyl acetate. Assays showed that there
was no increase in the enzyme activity of cells grown in the presence of IPTG, see Table 5.1. Extracts from cells grown +/-IPTG were analysed by SDS PAGE, see Fig 5.4, and both appeared to produce large amounts of the protein of interest. Control JM109(DE3)pT7-7 did not produce the protein. It was thought that cells grown in the presence of IPTG would contain more protein, due to the induction of T7RNA polymerase, and that the protein might be seen as inclusion bodies. However, there appeared to be no difference between the insoluble fractions of cells grown with or without IPTG. These experiments suggested that expression of the esterase gene was not influenced by the addition of IPTG. The ligation of the gene into the vector had been checked by sequencing so this could not be the problem. Cells with only the pT7-7 plasmid did not show any activity with p-nitrophenyl acetate. It was deduced that the protein seen in the extracts of JM109(DE3)[pCH771] with or without IPTG was due to the expression of the inserted esterase gene. A reason for the apparent uncontrolled expression of the esterase gene is that the T7RNA polymerase expression was not tightly controlled by IPTG. This has been suggested to be the case in other studies (Mertens et al, 1995, Makrides, 1996, Anon., 1996) where the T7RNA polymerase is still expressed in the absence of the inducer IPTG. To investigate this further induction experiments were carried out with normal JM109 containing pCH771. Growth and expression of the esterase gene was also carried out using minimal media with succinate for growth.

5.4.2 Investigation of expression of the esterase gene in pCH771 in JM109 cells

JM109[pCH771] was used to inoculate LB containing ampicillin (100μg.ml⁻¹). Induction of expression of pCH771 with IPTG in JM109 was tested. This was to check the expression of the esterase gene in a host that did not contain the λ(DE3) lysogen so did not carry the T7RNA polymerase. Extracts made from these cells were assayed using p-nitrophenyl acetate. The assays showed that there was still some expression of the esterase gene in this host, see Fig.5.5. As expected the expression was not affected by the presence of IPTG because there was no T7RNA polymerase under the control of the lac system in
<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity with p-nitrophenyl acetate (μmol.min⁻¹.mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant + IPTG</td>
<td>25.7 +/- 0.81</td>
</tr>
<tr>
<td>Resuspended pellet + IPTG</td>
<td>0.128 +/- 0.02</td>
</tr>
<tr>
<td>Supernatant - IPTG</td>
<td>23 +/- 0.75</td>
</tr>
<tr>
<td>Resuspended pellet - IPTG</td>
<td>0.12 +/- 0.01</td>
</tr>
</tbody>
</table>

Table 5.1 Enzyme assays of extracts from JM109(DE3)[pCH771] grown on LB with ampicillin (100μg.ml⁻¹), +/- IPTG (0.3mM) at 30°C
Figure 5.4 SDS PAGE analysis of cell extracts from JM109(DE3)[pCH771] and JM109(DE3)[pT7-7] grown on LB with ampicillin (100µg.ml⁻¹), +/- IPTG

Lane 1- Sonicated extract of JM109(DE3)[pCH771] after 1hr +IPTG
Lane 2- Sonicated extract of JM109(DE3)[pCH771] after 1hr -IPTG
Lane 3- Sonicated extract of JM109(DE3)[pT7-7] after 1hr +IPTG
Lane 4- Sonicated extract of JM109(DE3)[pT7-7] after 1hr -IPTG
Lane 5- SDS VII marker
Lane 6- Sonicated extract of JM109(DE3)[pCH771] after 2hrs +IPTG
Lane 7- Sonicated extract of JM109(DE3)[pCH771] after 2hrs -IPTG
Lane 8- Sonicated extract of JM109(DE3)[pT7-7] after 2hrs +IPTG
Lane 9- Sonicated extract of JM109(DE3)[pT7-7] after 2hrs -IPTG
Figure 5.5 Specific activities against p-nitrophenyl acetate of extracts from JM109(DE3)[pCH771] and JM109[pCH771].

JM109(DE3)[pCH771] were grown on M63 minimal media with ampicillin (100μg.ml-1) and LB containing ampicillin (100μg.ml-1).

JM109[pCH771] were grown on LB containing ampicillin (100μg.ml-1).

Specific activity (μmol.min⁻¹mg protein⁻¹)
JM109. However, expression without the presence of the T7 RNA polymerase suggested that the *E. coli* RNA polymerase might be able to bind to the T7 RNA polymerase promoter in pT7-7.

5.4.3 Growth and induction of JM109(DE3)[pCH771] on minimal media

To investigate the effect of the growth media on the expression of the esterase JM109(DE3)[pCH771] were grown in M63 minimal media containing succinate (15mM) as a carbon and energy source. Ampicillin (100μg.ml⁻¹) was added to all cultures and the cells were grown either with or without IPTG. Extracts were made from these induction experiments and assayed using p-nitrophenyl acetate. Assays showed that expression of the esterase gene occurred in the JM109(DE3) host as expected, see Fig.5.5.

These experiments showed that the expression was controlled, to an extent, by IPTG. The cells grown in the presence of IPTG on minimal media showed approximately three times more activity than those grown in its absence. This was not seen with JM109(DE3)[pCH771] on LB. The activity in the extracts of the JM109(DE3)[pCH771] without IPTG suggests that there is leaky expression of the T7 RNA polymerase resulting in expression of the cloned gene (Mertens *et al*, 1995, Anon., 1996). It has been reported that the incomplete repression of the expression system can lead to variable expression results (Mertens *et al* 1995). The difference in expression +/-IPTG in minimal media compared to the cells grown on LB suggested that there may be another factor involved in the uncontrolled expression. This could have been due to the contents of the undefined LB media acting as an inducer of the T7RNA polymerase or due to the faster growth rate on LB media having an effect on the expression of the gene.

Although the expression of the esterase gene did not appear to be completely under control this did not have a detrimental effect as the esterase protein did not appear to be toxic to the cell. Also SDS PAGE analysis showed the overexpressed protein constituted approximately 30% of the total soluble cell protein which was suitable for purification of the esterase.
5.5 Increase in enzyme activity through overexpression

Assays of cell extracts from JM109[pUC 18-79] and JM109(DE3)[pCH771] showed that there had been an increase of approximately ten fold and a hundred fold, respectively, in the enzyme activity. This might be attributed to the copy number of pUC (approximately 200/cell) as the gene did not appear to be under the control of the lac promoter as there was no difference seen with or without IPTG. Expression of the esterase gene in pCH771 appeared to be under the control of the RNA polymerase promoter under certain conditions.

5.6 Protein purification

JM109 (DE3)[pCH771] were grown on LB containing ampicillin (100μg.ml⁻¹) and induced with IPTG (0.3mM) when growth reached an O.D.680nm of approximately 0.40. The cells were then grown for a further 2hrs and harvested. Cell extract, 1ml of 2.5mg.ml⁻¹, was applied to a Mono Q (Pharmacia) column and eluted by a gradient of 0-500mM NaCl at 1ml.min⁻¹. Active fractions, as assayed using the p-nitrophenyl acetate microtitre plate assay, were eluted between 310 and 340mM NaCl. The most active fraction obtained from the Mono Q column, 1ml (0.75mg.ml⁻¹) was then adjusted to contain 1M (NH₄)₂SO₄ and was applied to a Phenyl Superose column (Pharmacia) and eluted by a gradient of 1M-0M (NH₄)₂SO₄. Fractions were collected and the activity, as assessed by p-nitrophenyl acetate microtitre assay, was found to be at 0M (NH₄)₂SO₄ after the gradient had finished.

A homogenous preparation of pure protein was obtained after a three fold purification of the ultracentrifuged extract, see Table 5.2. This agreed with the band seen on the SDS gel, see Fig.5.6, of the ultracentrifuged extract which suggested that the protein was approximately 30% of the cell protein. The protein was shown to be more than 95% pure as assessed by SDS PAGE, see Fig.5.6. Once the conditions had been established for the purification of the esterase the protein was purified for use in experiments to characterise the enzyme.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total units (μmol.min⁻¹)</th>
<th>Specific Activity (units.mg protein⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ultracentrifuged cell extract</td>
<td>1ml</td>
<td>2.5</td>
<td>56.8</td>
<td>22.72</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Mono Q peak fraction</td>
<td>1ml</td>
<td>0.75</td>
<td>46.6</td>
<td>62.2</td>
<td>82</td>
<td>2.7</td>
</tr>
<tr>
<td>Phenyl Superose peak fraction</td>
<td>1ml</td>
<td>0.50</td>
<td>33.2</td>
<td>66</td>
<td>58</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Table 5.2. Purification table for esterase using p-nitrophenyl acetate as the substrate.
Figure 5.6 SDS PAGE analysis of the active fraction from the Mono Q and Phenyl Superose columns

Lane 1  SDS VII marker proteins
Lane 2  Ultracentrifuged crude cell extract (0.015mg)
Lane 3  Active fraction from the Mono Q column (0.015mg)
Lane 4  Active fraction from the Phenyl Superose column (0.015mg)
Lane 5  SDS VII marker proteins
5.7 Determination of the native molecular weight of the esterase enzyme

Purified protein was applied to a tandem arrangement of two Superose 12 (Pharmacia) columns. These columns had previously been calibrated using various molecular weight markers, see Fig.5.7. The elution volume of the protein was measured as 26.9ml and the native molecular weight was calculated by using the equation of the line of the calibration curve already determined, see Fig.5.7. Using this method the native molecular weight was calculated to be 41,677 Daltons. This agreed well with the molecular weight predicted from the DNA sequence of 40,679 Daltons, and that seen on analysis by SDS PAGE of approximately 40,000 Daltons. This showed that the protein was monomeric in its native form.

5.8 p-nitrophenyl acetate $K_m$ and $V_{max}$ determination

The $K_m$ and $V_{max}$ for p-nitrophenyl acetate were determined by measuring the production of para-nitrophenol at 405nm. A 56mM stock solution of p-nitrophenyl acetate was used and the volume of this was varied in the assay so that concentrations of 0.28-4.2mM p-nitrophenyl acetate were used. The enzyme used was from a 1mg.ml$^{-1}$ stock which was then diluted 100 times and 10µl of this was used in the assay. The data obtained from these assays was entered into the KFitSim non-linear line fitting program. The $K_m$ and $V_{max}$ were determined as 0.58mM and 0.02µmol.min$^{-1}$, with a standard deviation of 0.0008 for the fit, as shown in Fig.5.8. The $K_{cat}$ was calculated to be 132sec$^{-1}$. The specificity constant was calculated using $K_{cat}/K_m$ and found to be 2.3x10$^5$ M$^{-1}$sec$^{-1}$.

5.9 Malathion $K_m$ and $V_{max}$ determination

The $K_m$ and $V_{max}$ for malathion were measured using the spectrophotometric assay for the detection of NADH at 340nm. A 0.37mM solution of malathion was used and the volume added to the assay was adjusted to give different concentrations in the range 0.018-
Figure 5.7 The calibration of the Superose 12 gel filtration column and the calculated molecular weight of the esterase protein

\[ y = -0.139x + 8.360 \]

\[ y = (-0.139 \times 26.9) + 8.359 \]
\[ y = 4.6199 \]
Molecular Weight = 41,677 Daltons
Figure 5.8. The $K_m$ and $V_{max}$ for $p$-nitrophenyl acetate using purified esterase.
0.185mM in the assay. The enzymes used was from a 1mg.ml⁻¹ stock which was then
diluted 100 times and 10μl of this was used in the assay. The $K_m$ and $V_{max}$ were found to
be 0.06mM and 0.005μmol.min⁻¹, with a standard deviation of 0.0001 for the fit, as
calculated by the KFitSim program and shown in Fig.5.9. The $K_{cat}$ was calculated to be
33sec⁻¹. The specificity constant was calculated using $K_{cat}/K_m$ and found to be $5.5 \times 10^5$
M⁻¹.sec⁻¹.

5.10 Diethylsuccinate $K_m$ and $V_{max}$ determination

The $K_m$ and $V_{max}$ for diethylsuccinate were determined using the spectrophotometric
assay, measuring the production of NADH at 340nm. A stock solution of 50mM
diethylsuccinate was used and different volumes added to the assay to achieve different
concentrations in the range of 0.25-10mM diethylsuccinate. The enzymes used was from a
1mg.ml⁻¹ stock which was then diluted 100 times and 10μl of this was used in the assay.
The $K_m$ and $V_{max}$ were calculated by the KFitSim program and were 0.29mM and
0.02μmol.min⁻¹, with a standard deviation of 0.0008 for the fit, as shown in Fig.5.10.
The $K_{cat}$ was calculated to be 133sec⁻¹. The specificity constant was calculated using
$K_{cat}/K_m$ and found to be $4.6 \times 10^5$ M⁻¹.sec⁻¹.

5.11 Substrate specificity

The purified esterase enzyme was tested for activity with a variety of substrates. Activity
against substrates, other than carboxylesters, was tested to investigate the esterase’s
potential for use as a general hydrolase. It was hoped that the esterase would have activity
against a range of other bonds such as thiolester, phosphoester and amide. Enzymes
identified by sequence homology to show similarities to this ester have been reported to have
activity against a variety of target bonds. Other workers (Nishizawa et al 1995, McKay et al
1992 and Kim et al 1994) have reported that similar esterase enzymes were capable of
degrading other bonds such as thioesters as well as a variety of carboxyl ester bonds.
Similar esterases were also reported to be able to hydrolyse other esters of the p-nitrophenyl
Figure 5.9. The $K_m$ and $V_{max}$ for malathion using purified esterase

![Graph showing $K_m$ and $V_{max}$ for malathion](image)

Figure 5.10. The $K_m$ and $V_{max}$ for diethylsuccinate using purified esterase

![Graph showing $K_m$ and $V_{max}$ for diethylsuccinate](image)
acetate group with the rate of hydrolysis being dependent on the chain length of the carboxylic acid (Nishizawa et al 1993).

Hydrolysis of different bonds is of interest as there are a variety of bonds found in organophosphorus compounds. Due to problems in obtaining organophosphorus compounds and also the risk involved in handling them it was decided to use model compounds, see Fig.5.11. These chemicals contained similar bonds to those of interest but were easy to assay and routinely available in the laboratory.

5.11.1 Hydrolysis of p-nitrophenyl esters

Hydrolysis of the p-nitrophenyl esters was investigated using p-nitrophenyl acetate, propionate, butyrate and caproate, which vary in the chain length of their carboxylic acid component, see Fig.5.12. The assays were all monitored at 405nm as hydrolysis of the ester bond in all of these substrates results in the production of para-nitrophenol.

Initial assays were set up using the same concentration of substrate (0.28mM final concentration) and a constant concentration of enzyme. The results of these assays, see Table 5.3. suggested that the enzyme preferentially hydrolysed p-nitrophenyl propionate. This substrate was found to be hydrolysed approximately twelve times faster than p-nitrophenyl acetate, as was also reported to be the case for the carboxylesterase studied by Nishizawa et al (1993). To determine whether this faster hydrolysis of the p-nitrophenyl propionate was due to effects of $K_m$ and/or $V_{max}$ these values were determined, see Fig.5.13. This showed that the $K_m$ was 0.11mM and the $V_{max}$ was 0.03μmol.min$^{-1}$. $K_{cat}$ was calculated to be 200sec$^{-1}$ and the specificity constant was 1.8x10$^6$ M$^{-1}$sec$^{-1}$. This was approximately eight times greater than the specificity constant calculated for p-nitrophenyl acetate.
Figure 5.11 Structures of compounds used as model substrates for the hydrolysis of a variety of bonds which might be found in organophosphorus compounds.
Figure 5.12 Structures of the para-nitrophenyl esters with varying carboxylic acid chain length.
Table 5.3 Results from assays to measure the hydrolysis of the \(p\)-nitrophenyl esters using purified esterase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity ((\mu\text{mol.min}^{-1}.\text{mg protein}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(p)-nitrophenyl acetate</td>
<td>66 +/- 3.7</td>
</tr>
<tr>
<td>(p)-nitrophenyl propionate</td>
<td>504.5 +/- 20.6</td>
</tr>
<tr>
<td>(p)-nitrophenyl butyrate</td>
<td>342.3 +/- 25.6</td>
</tr>
<tr>
<td>(p)-nitrophenyl caproate</td>
<td>121.7 +/- 26.8</td>
</tr>
</tbody>
</table>

Figure 5.13 The \(K_m\) and \(V_{max}\) determination for \(p\)-nitrophenyl propionate
5.11.2 Hydrolysis of CoA esters

Possible thiol esterase acitivity of the esterase was investigated using acetyl- (1mM final concentration) and propinyl- CoA (1.42mM final concentration). Enzyme (final concentration 40μg.ml⁻¹) was mixed with these substrates respectively and incubated at room temperature. The production of thiol was monitored at 412nm using the DTNB assay as described in the Materials and Methods, section 2.10. These assays showed that there was very little thiol resulting from the incubation of enzyme with substrate, see Table 5.4. The effect of DTNB on enzyme activity was investigated to see if it would have any inhibitory effect on the enzyme. DTNB was added to the enzyme assay with p-nitrophenyl acetate and was found not to inhibit enzyme activity. As the presence of the DTNB did not appear to be inhibitory the lack of activity against the thiol esters suggested that the esterase did not have any activity with these substrates.

Thiol was detected in these assays but this was also seen in the control. This could be attributed to the presence of unreacted thiol groups in the CoA esters which would be seen immediately or the spontaneous degradation of the thiol ester resulting in some thiol detection over the time of the assay.

5.11.3 Hydrolysis of aminopeptidase bonds and amide bonds

Sequence comparisons of the gene encoding the esterase showed that there was a putative active site at Ser68 in the form of Ser-X-X-Lys. This has been reported (Brenner, 1988) to be found in all penicillin-recognising enzymes. A D-aminopeptidase of Ochrobactrum anthropi also exhibited this Ser-X-X-Lys active site at Ser61 (Asano et al 1992). Study of the O. anthropi enzyme showed that it would hydrolyse peptides and was identified as having peptidase activity using D-alanine-p-nitroanilide as a substrate. To determine the possible aminopeptidase activity of the esterase from the Arthrobacter sp. D-alanine-p-
Table 5.4 Measurement of thiol production on incubation of acetyl CoA and propionyl CoA with esterase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial reading at 412nm</th>
<th>Reading at 412nm after three hours</th>
<th>Change in absorbance at 412nm</th>
<th>Amount of thiol (μmol) present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl CoA + esterase</td>
<td>0</td>
<td>0.076</td>
<td>0.076</td>
<td>0.0100</td>
</tr>
<tr>
<td>Acetyl CoA no enzyme</td>
<td>0</td>
<td>0.074</td>
<td>0.074</td>
<td>0.0100</td>
</tr>
<tr>
<td>Propionyl CoA + enzyme</td>
<td>0.27</td>
<td>0.31</td>
<td>0.04</td>
<td>0.0055</td>
</tr>
<tr>
<td>Propionyl CoA no enzyme</td>
<td>0.24</td>
<td>0.29</td>
<td>0.05</td>
<td>0.0070</td>
</tr>
</tbody>
</table>
nitroanilide was used as a substrate (2mM final concentration). The esterase (40\mu g.ml\(^{-1}\) final concentration) was added to D-alanine-p-nitroanilide and incubated at room temperature and 30\(^\circ\)C and the production of para-nitroaniline monitored at 405nm, as described in the Materials and Methods section 2.11.1. However, no colour was seen in the incubation, even when left overnight. This assay suggested that the enzyme did not have any D-aminopeptidase activity.

Hydrolysis of the acetamide was measured to investigate whether the esterase had any potential use against the carbamate organophosphorus compounds which contain amide groups. This assay was carried out using a discontinuous assay measuring the release of NH\(_4^+\) as described in the Materials and Methods, section 2.11. The buffer that the protein was eluted from the column in was used as a control in the assay for NH\(_4^+\) release. However, this contained Tris which was known to interfere with the assay. The assays were set up in a microtitre plate and the results were judged by eye. No difference was seen between the control with no substrate and the enzyme (40\mu g.ml\(^{-1}\) final concentration) containing wells. The experiment was repeated using enzyme which had been dialysed against a phosphate buffer (1ml of enzyme solution against 1000ml at 4\(^\circ\)C, for 20 hours with two changes of buffer). This was to remove the Tris buffer and any residual (NH\(_4\))\(_2\)SO\(_4\) that might be in the buffer that had been eluted from the FPLC column. However, the results showed that there was no activity as there was no detectable difference between the control and the assay containing the enzyme. The enzyme was still fully active against p-nitrophenyl acetate after dialysis so the treatment had not affected the activity. This experiment suggest that the enzyme did not have any activity against acetamide.

5.11.4 Hydrolysis of phoshoester bond

\textit{Para}-nitrophenyl phosphate was used as a substrate to assay for phosphoesterase activity. On addition of the esterase to this substrate the change in absorbance was monitored at 405nm. However, there was no change seen even when the mixture was incubated overnight. This suggested that the enzyme did not have any phosphoesterase activity.
5.12 Inhibition Studies

For use in environmental detoxification an enzyme should be resistant to inhibition by other commonly found pollutants such as solvents and metals. To investigate the effect of such inhibitors the esterase at 40µg.ml⁻¹, was incubated with them for various times at room temperature. The effect of the inhibitors on enzyme activity (using p-nitrophenyl acetate as a substrate) over time can be seen in Figs.5.14 and 5.15.

Solvents (10% v/v) were added to the diluted enzyme and maintained at room temperature. The results using solvents, Fig.5.14, showed that there was little or no effect with the solvents tested. Of the range of solvents tested, ethanol, acetone, methanol, DMSO and propanol showed no inhibition. However, with butanol there appeared to have been inhibition of 50% after 24hrs incubation, and total inhibition after 72hrs. Information on solvent inhibition may be of use in the formulation of the enzyme in solution for use. Solvents are also used in the formulation of pesticides so might also be an additional contaminant present with the pesticide.

The metals, see Fig.5.15, cobalt (5mM CoSO₄), nickel (5mM NiSO₄) and zinc (5mM ZnCl₂) inhibited activity over a period of time. Zinc took 24hrs to reduce the activity with p-nitrophenyl acetate to 25% of the original. Cobalt and nickel showed similar inhibition to each other reducing activity to 50% of the original over 288hrs. Copper (5mM CuSO₄) and the heavy metals, lead (5mM lead acetate) and mercury (1mM HgCl₂), inhibited activity immediately as no activity was remaining after 4hrs. Calcium (5mM CaCl) did not appear to have any inhibitory effects. The enzyme was also incubated with 1mM EDTA and samples assayed for activity. There was no inhibition with EDTA suggesting that the enzyme did not require a metal ion for activity.

5.13 Temperature stability studies

The application of enzymes to an industrial process, or environmental clean up, requires their stability with regard to temperature. This is important when storing an enzyme in the long term and also for the extent of its usefulness over the time period when it is applied,
Figure 5.14 The effect of solvents on enzyme activity as assessed by monitoring residual activity with \( p \)-nitrophenyl acetate. Samples containing 40\( \mu \)g.ml\(^{-1}\) of enzyme were incubated at room temperature (approximately 21°C).

- ■ no inhibitor
- □ 10% acetone
- □ 10% DMSO
- □ 10% propanol
- ■ 10% butanol
- □ 10% ethanol
- □ 10% methanol

\( \overline{=} \) = error bar
Figure 5.15. The effect of metals on enzyme activity as assessed by monitoring residual enzyme activity with \( p \)-nitrophenyl acetate

- \( \square \) no inhibitor
- \( \square \) 5mM \( \text{CaCl}_2 \)
- \( \square \) 5mM \( \text{PbAc} \)
- \( \square \) 5mM \( \text{NiSO}_4 \)
- \( \square \) 5mM \( \text{CoSO}_4 \)
- \( \square \) 5mM \( \text{CuSO}_4 \)
- \( \square \) 5mM \( \text{ZnCl}_2 \)
- \( \square \) 5mM \( \text{HgCl}_2 \)

\( \text{I} = \) error bar
perhaps in a dilute form. Samples containing 2mg.ml\(^{-1}\) protein were stored at room temperature, on ice and at \(-20^\circ\)C. Samples were taken from these sources and diluted (final concentration 0.2\(\mu\)g.ml\(^{-1}\)) to measure the activity using \(p\)-nitrophenyl acetate. Results from these experiments, see Fig.5.16, showed that when stored in a concentrated form the enzyme was not inhibited by the presence of azide and was stable on ice and at \(-20^\circ\)C for over three months. Storage at room temperature was also stable for up to 3 weeks after which time activity was seen to be slowly reduced to 70\% of the original over the 15 weeks of storage.

In a diluted form enzymes are often less stable. The effect of dilution on the stability was observed by carrying out similar stability assays with diluted enzyme stored at various temperatures at 0.015mg.ml\(^{-1}\). The final assay concentration was 0.15\(\mu\)g.ml\(^{-1}\). The dilution factor had an effect on the stability, with stability being reduced with increasing temperature. At temperatures of more than \(40^\circ\)C the diluted enzyme showed no activity with \(p\)-nitrophenyl acetate after one hour incubation. This suggested that the enzyme would not be of use in processes at these temperatures. When diluted the enzyme was stable for up to 24hrs in a temperature range of 0-37\(^\circ\)C. After this time activity was rapidly reduced to about 50\%, see Fig.5.17. This suggests that if this enzyme was to be used commercially in a diluted form it would only be usefully for overnight or one day of treatment. This coupled with its relative stability when stored on ice or at \(-20^\circ\)C would mean that it could be stored in the freezer and then diluted for use.

5.14 pH stability studies

Enzyme stability in buffers at a variety of pHs was measured. Buffers in the range 6.4-7.4 were made using TrisHCl and in the range 8-12 with KH\(_2\)PO\(_4\). These buffers were made and used to store enzyme at room temperature. On the addition of the enzyme, which was in Tris HCl buffer pH7.4 containing 9\% Glycerol and 0.5mM DTT, to give a final concentration of 40\(\mu\)g.ml\(^{-1}\) the resulting pH was measured. This resulting pH was used to plot against the enzyme activity to determine the effect of the pH on the stability of the
Figure 5.16. Effect of temperature on enzyme stored in a concentrated form (2mg.ml-1).

- Room temperature (approximately 21°C)
- ice
- ice + azide
- -20

= error bars
Figure 5.17 Effect of temperature on enzyme stored in a diluted form (0.015mg.ml⁻¹)

- **ice**
- **30°C**
- Room temperature (approximately 21°C)
- **37°C**
enzyme. Assays for enzyme activity were carried out using p-nitrophenyl acetate as the substrate. Assays were carried out after incubation of the enzyme for periods of time in the appropriate buffer at room temperature. Results, see Fig 5.18 show that the enzyme was stable at room temperature at pHs in the range pH 7.4-11. Assays were carried out to check that the activity seen was not due to the hydrolysis of the p-nitrophenyl acetate caused by the addition of different pH buffers. The same amount of each buffer (10μl) was added to the assay as was used when the enzyme was added. There was no detectable rate of hydrolysis with buffer alone showing that the p-nitrophenol production was due to the action of the esterase.

Experiments to determine the pH optimum for the enzymes action were not carried out as the spontaneous hydrolysis of p-nitrophenyl acetate occurs rapidly at pHs above pH 8.

5.15 pI determination

The pI value of the esterase was measured by using isoelectric focusing rods. Samples of the purified protein were focused overnight and several control rods were run for calibration. The calibration rods were cut into pieces of approximately 3mm and soaked in sterile water. The pH of these soaking rods was measured and used to plot calibration curves, see Fig.5.19. The rod containing the focused protein sample was stained using Coomassie Blue stain and the position of the protein band was measured and used to calculate the pI value from the calibration curves. The pI was found to be between 5.0 and 5.1. The GCG package was used to determine the theoretical pI value from the derived protein sequence and this was calculated to be 4.5.

5.16 Malathion hydrolysis

The hydrolysis of malathion was investigated using the purified enzyme. The extent of malathion hydrolysis was assessed by measuring the production of ethanol. This was to determine whether one or both of the carboxylester bonds, see Fig.5.20, were hydrolysed.
Figure 5.18. Effect of pH on enzyme stability over a prolonged time period.

Enzyme stored at room temperature (approximately 21°C) and activity assayed with p-nitrophenyl acetate at pH8. Buffers pH6.5-7.4 were made with Tris HCl and buffers pH8-12 were made with potassium orthophosphate buffer.

- pH 6.53
- pH 7.10
- pH 7.40
- pH 8.03
- pH 8.8
- pH 9.7
- pH 11.00
- pH 11.92

= error bars
Figure 5.19. Calibration curve for the estimation of the pI value of the esterase.

The band of protein was 19 mm from the acidic end of the rod.

□ pH control rod 1

◊ pH control rod 2
Figure 5.20 Structure of malathion and the assumed hydrolysis pattern
A 0.37mM solution of malathion was made as this was below the maximum solubility of 0.44mM. Calculations were then made to determine the amount of ethanol that would be released on the hydrolysis of one or both of the carboxylester bonds, see Table 5.5. The production of ethanol was then measured by monitoring the production of NADH at 340nm. The results of these assays, also in Table 5.5, showed that only one of the carboxylester bonds was being hydrolysed by the enzyme. This hydrolysis produced ethanol and presumably malathion monocarboxylic acid. Previous reports on the hydrolysis of malathion by bacteria and fungi have shown the monocarboxylic acid to be the major product. This result suggested that there might be some discrimination by the enzyme between the two ester groups. This might be due to one of the ester groups being attached to the α-carbon which is chiral. However, whether a particular linkage was hydrolysed was unclear. Alternatively, the hydrolysis may not be specific for either of the ethyl groups but hydrolysis might depend on the suitable molecule being uncharged. After the hydrolysis of one of the ethyl groups the molecule becomes charged, rather than neutral. As the strict definition of an esterase is an enzyme that catalyses the hydrolysis of uncharged carboxylic esters the resulting charged monocarboxylic ester might then not be recognised as a substrate for the esterase.

5.17 Diethylsuccinate hydrolysis

The hydrolysis of diethylsuccinate was measured using 0.04mM of diethylsuccinate and the purified enzyme. The extent of diethylsuccinate hydrolysis was assessed by measuring the production of ethanol. This was to determine whether one or both of the carboxylester bonds, see Fig.5.21, were hydrolysed. Again calculations of the expected amounts of ethanol release were made for the hydrolysis of one and both of the esters bonds, see Table 5.6. However, when the results of the experiments were compared to these values it appeared that only one of the ester bonds was being hydrolysed, see Table 5.6. The assay procedure was checked by adding more NAD, ADH and semicarbazide to ensure that the reaction was measuring all of the ethanol. This did not seem to make any difference to the result. The assay detection capabilities were also checked with a standard ethanol solution.
<table>
<thead>
<tr>
<th>Volume of 0.37mM malathion solution (μl) reacted</th>
<th>μmole of malathion</th>
<th>Expected change in absorbance at 340nm for hydrolysis of one ethanol bond</th>
<th>Expected change in absorbance at 340nm for hydrolysis of two ethanol bonds</th>
<th>Actual change in absorbance at 340nm</th>
<th>Removal of ethanol (% of maximum possible)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.037</td>
<td>0.23</td>
<td>0.46</td>
<td>0.20</td>
<td>43.5</td>
</tr>
<tr>
<td>200</td>
<td>0.074</td>
<td>0.46</td>
<td>0.92</td>
<td>0.39</td>
<td>41.5</td>
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<tr>
<td>300</td>
<td>0.111</td>
<td>0.69</td>
<td>1.38</td>
<td>0.63</td>
<td>45.0</td>
</tr>
<tr>
<td>400</td>
<td>0.148</td>
<td>0.92</td>
<td>1.84</td>
<td>0.78</td>
<td>42.5</td>
</tr>
</tbody>
</table>

Average = 43% of all ethanol removed

Table 5.5 Expected and actual measured amount of ethanol released on hydrolysis of malathion by the purified esterase.
Figure 5.21 The structure of diethylsuccinate and the assumed hydrolysis pattern.
<table>
<thead>
<tr>
<th>Volume of 0.40mM diethylsuccinate solution (μl) reacted</th>
<th>μmole of diethylsuccinate</th>
<th>Expected change in absorbance at 340nm for hydrolysis of one ester bond</th>
<th>Expected change in absorbance at 340nm for hydrolysis of both ester bonds</th>
<th>Actual change in absorbance at 340nm</th>
<th>Removal of ethanol (% of maximum possible)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.04</td>
<td>0.25</td>
<td>0.50</td>
<td>0.25</td>
<td>50</td>
</tr>
<tr>
<td>200</td>
<td>0.08</td>
<td>0.50</td>
<td>0.99</td>
<td>0.55</td>
<td>55</td>
</tr>
<tr>
<td>300</td>
<td>0.12</td>
<td>0.74</td>
<td>1.49</td>
<td>0.72</td>
<td>48</td>
</tr>
</tbody>
</table>

Average = 51% of both ethanols removed

Table 5.6. The expected and actual amount of ethanol released on hydrolysis of diethylsuccinate by the purified esterase.
This showed that the assay detected all of the ethanol present. The addition of more enzyme to the reaction did not result in the production of more ethanol suggesting that there was no more substrate for the enzyme to act on in the assay. However, when more substrate was added to the reaction there was further production of ethanol. As with the malathion assay these results showed that only one of the ester groups was being hydrolysed in the diethylsuccinate. This suggested that only the uncharged diethylsuccinate or malathion substrate was recognised by the esterase. However, growth of JM109[pUC18-79] on diethylsuccinate suggested that both of the ester groups were hydrolysed and both the succinate and ethanol were being used for growth. To investigate this further other diethyl and also monoethyl compounds were tested as substrates.

5.18 Preparation of monoethylsuccinate

Ethylsuccinyl chloride (stated purity 95%) was purchased from Lancaster. The acid chloride was expected to react with water, and it was postulated that this would result in the formation of monoethylsuccinate, see Fig.5.22. Completion of the reaction was checked by assaying for chloride ions as described in the Materials and Methods section 2.12. A calibration curve for chloride was constructed using a NaCl solution in the range of 0.1-1mM, see Fig.5.23. A solution of ethylsuccinyl chloride was made in water at a concentration of 0.4mM. The absorbance (A) at 460nm for 1ml of the 0.4mM (0.4μmol) ethylsuccinyl chloride solution (carried out in triplicate) was 0.568. This corresponded to a value of 0.420μmol chloride ions from the standard curve. From this it was assumed that all of the ethylsuccinyl chloride had reacted completely with the water to form monoethylsuccinate and HCl.
Figure 5.22 Formation of monoethylsuccinate from ethylsuccinyl chloride

\[
\begin{align*}
\text{CH}_2\text{—COOC}_2\text{H}_5 + \text{H}_2\text{O} & \rightarrow \text{CH}_2\text{—COOC}_2\text{H}_5
\\
\text{CH}_2\text{—COCl} & \rightarrow \text{CH}_2\text{—COOH} + \text{HCl}
\end{align*}
\]

5.19 Esterase activity with monoethylsuccinate

Monoethylsuccinate at a final concentration of 0.04mM was tested as a substrate for the esterase. On the addition of esterase enzyme there was no detectable absorbance change at 340nm indicating that no hydrolysis was occurring. It was thought that the hydrolysis of the monoethylsuccinate might only occur at a very slow rate compared to that seen for the diethylsuccinate. To investigate this the assay was carried out with 10 times the amount of enzyme used normally and assayed for three hours. This still showed no hydrolysis. To check that the assay had not been effected by the presence of the chloride, or any change in the pH resulting from its addition, a known amount of ethanol was added. This assay showed that all the ethanol present was detected and that the coupled reactions were still functioning as expected. The pH of the assay components with monoethylsuccinate as the substrate and containing the released chloride ions was measured and found to be pH8, as expected. Therefore, the esterase should be able to function effectively under these conditions as seen from previous experiments on the stability of the enzyme at different pHs. This experiment suggested that the esterase was unable to act on the monoethylsuccinate which supported the observation that only one of the ester bond in diethylsuccinate was hydrolysed.
Figure 5.23 Calibration curve for the measurement of chloride ions
5.20 Hydrolysis of other diethyl and monoethyl substrates

Diethylmalonate (stated purity 99%) and diethylfumarate (stated purity 98%) were also purchased from Lancaster and tested as substrates for the esterase. The measurement of ethanol released from these substrates on the addition of the esterase showed that they were also substrates, see Table 5.7. However, the esterase only hydrolysed one of the ester bonds, as was the case for diethylsuccinate and malathion. The monoethyl derivatives of malonate (stated purity 98%) and fumarate (stated purity 97%), were also tested as substrates using a final concentration of 0.04mM. These compounds showed no ethanol production on the addition of esterase just as with monoethylsuccinate.

5.21 Growth of JM109 and JM109[pUC18] on monoethylsuccinate

Assays with the purified esterase, using diethylsuccinate as the substrate, showed that the enzyme hydrolysed one of the ester bonds to produce ethanol and presumably monoethylsuccinate. It was then shown that the enzyme did not appear to act on monoethylsuccinate in vitro as a substrate in the spectrophotometric assay. Other substrates, monoethyl and diethyl malonate and fumarate, also showed a similar pattern of results. These results suggested that the esterase was unable to hydrolyse monoethyl substrates. However, despite the lack of extract esterase activity JM109[pUC18-79] cells did grow on monoethylsuccinate. If this was the case, it was thought that the JM109 lacking the cloned esterase must be able to grow on the resulting monoethylsuccinate. To investigate this JM109 alone and JM109[pUC18] were used to inoculate media with monoethylsuccinate as the carbon and energy source. Monoethylsuccinate was added to the M63 minimal media to give a 10mM final concentration (60mM carbon). This resulted in the media being pH6.7 rather than pH7. The pH was adjusted aseptically to pH7 using 2M NaOH. JM109 containing the various constructs were used to inoculate flasks of monoethylsuccinate, the growth was monitored at 680nm, and the production of ethanol was also monitored, see Fig.5.24. No growth was seen initially with the JM109 alone or with pUC18 present. However, growth did occur after approximately 3-4 days. This could
<table>
<thead>
<tr>
<th>Volume of 0.4mM diethylmalonate/diethylfumarate</th>
<th>μmol of diethylmalonate/diethylfumarate</th>
<th>Expected change in absorbance at 340nm for hydrolysis of one of the ester bonds</th>
<th>Expected change in absorbance at 340nm for hydrolysis of both of the ester bonds</th>
<th>Actual change in absorbance at 340nm</th>
<th>Removal of ethanol (% of maximum possible)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethylfumarate 100μl</td>
<td>0.04</td>
<td>0.25</td>
<td>0.50</td>
<td>0.26</td>
<td>51</td>
</tr>
<tr>
<td>Diethylmalonate 100μl</td>
<td>0.04</td>
<td>0.25</td>
<td>0.50</td>
<td>0.29</td>
<td>57</td>
</tr>
</tbody>
</table>

Table 5.7 The calculated and actual ethanol production from diethylmalonate and diethylfumarate.
Figure 5.24 Incubation of JM109 alone on diethylsuccinate. Growth of JM109 and JM109[pUC18] on monoethylsuccinate and the measurement of ethanol produced.

Growth was on M63 minimal media at 30°C with ampicillin (100μg.ml⁻¹) where necessary. Cultures were inoculated using cells that had previously been grown on succinate and washed before use in this experiment.
have been due to the monoethylsuccinate hydrolysing spontaneously and the bacteria growing on the resulting succinate and ethanol. Alternatively, the growth may have been due to the bacteria developing an enzyme able to degrade the monoethylsuccinate. The enzyme may be induced slowly or have undergone a mutation which then enabled it to act on monoethylsuccinate. To investigate the possibility of there being spontaneous hydrolysis monoethylsuccinate was incubated in M63 minimal media at 30°C in the orbital shaker and ethanol measured. This showed that there was some ethanol produced but only after 5-6 days and not in the amounts measured with the JM109 cells growing on monoethylsuccinate.

To investigate this apparent induction or mutation of an enzyme in JM109 that was able to hydrolyse monoethylsuccinate the bacteria were grown on the monoethylsuccinate and extracts were made from these cells and assayed. The assays carried out were with monoethylsuccinate (5mM) showed the specific activity was 0.13μmol.min⁻¹.mg protein⁻¹ and with diethylsuccinate (5mM) 0.025μmol.min⁻¹.mg protein⁻¹. JM109 cells that had been grown on monoethylsuccinate were also used to inoculate fresh media containing monoethylsuccinate and diethylsuccinate respectively. The bacteria grew on monoethylsuccinate without the lag phase that was seen with the initial inoculation. This suggested uptake was not a problem and that the JM109 had not been growing on the degradation products due to the spontaneous hydrolysis of monoethylsuccinate. No growth was seen on diethylsuccinate, suggesting that the enzyme might be specific for monoethylsuccinate.

5.22 Growth of JM109[pUC18-79] on monoethylsuccinate

JM109[pUC18-79] were found to grow on the monoethylsuccinate and produce ethanol, see Fig.5.25, in a similar manner seen for growth on diethylsuccinate. There did not appear to be any lag phase with the JM109[pUC18-79] compared with the JM109 alone or with pUC18. This suggested that the cloned esterase, or a product of the additional DNA in pUC18-79 was responsible for this activity against monoethylsuccinate. A comparison of the growth of pUC18-79 on monoethylsuccinate and diethylsuccinate, see Fig.5.26,
Figure 5.25 Growth of JM109[pUC18] and JM109[pUC18-79] on monoethylsuccinate.

Growth was on M63 minimal media with ampicillin (100μg.ml⁻¹) at 30°C. Flasks were inoculated with washed cells that had been grown on succinate.
Figure 5.26 Growth of JM109[pUC18-79] on mono- and di-ethylsuccinate and the production of ethanol from this growth

Growth was on M63 minimal media at 30°C with amicillin (100μg.ml⁻¹) where necessary. Flasks were inoculated using washed cells that had previously been grown on succinate.

- Growth of JM109[pUC18-79] on 10mM monoethylsuccinate
- Growth of JM109[pUC18-79] on 7.5mM diethylsuccinate
- Ethanol produced by growth of JM109[pUC18-79] on 10mM monoethylsuccinate
- Ethanol produced by growth of JM109[pUC18-79] on 7.5mM diethylsuccinate
diethylsuccinate was faster that seen from growth on monoethylsuccinate. In both cases an increase in ethanol was concomitant with an increase in growth. However, the ethanol measured in the media never reached the maximum possible that could be released from the hydrolysis of mono- and di-ethylsuccinate. This suggested that the bacteria were hydrolysing the mono- and diethylsuccinate then using the succinate and ethanol but that the system for utilizing the ethanol might need some time to be induced resulting in the release and build up of some of the ethanol in the growth medium.

5.23 Growth of JM109(DE3)[pCH771] on mono- and di-ethylsuccinate.

The growth of JM109[pUC18-79] on mono- and di-ethylsuccinate showed similar doubling times. However, the spectrophotometric assays with the purified esterase had only shown activity against diethylsuccinate. Assays with extract from JM109 cells grown on mono-ethylsuccinate suggested that there was an enzyme in JM109 capable of hydrolysing mono-ethylsuccinate. However, this appeared to require several days exposure to enable growth whereas the JM109[pUC18-79] grew on mono-ethylsuccinate at a comparable rate to the growth on diethylsuccinate. The plasmid pUC18-79 contained the esterase gene (1140bp) on a 5.7kb insert so there was a possibility that this extra DNA might be responsible for encoding an enzyme able to hydrolyse the monoethylsuccinate. To investigate this JM109(DE3)[pCH771] were tested for growth on mono- and di-ethylsuccinate. The plasmid pCH771 only contained the esterase gene so that growth on mono- and di-ethylsuccinate seen with bacteria containing this plasmid would have to be due to the esterase gene or the JM109 enzyme.

Growth and the production of ethanol were measured for JM109(DE3)[pCH771] on mono- and di-ethylsuccinate, see Fig. 5.27. The results showed that the rates of growth on mono- and di-ethylsuccinate were similar both with a t½ 15hrs. The growth rate was faster by approximately 20hrs compared to that of JM109[pUC18-79] which could be attributed to there being more esterase present in the JM109(DE3) cells.
Figure 5.27 Growth of JM109(DE3)[pCH771] on succinate, mono- and di-ethylsuccinate respectively.

Growth was on M63 minimal media with ampicillin (100μg.ml⁻¹) at 30°C. Flasks were inoculated with washed cells that had previously been grown on succinate.
The similarities in the growth rates between JM109(DE3) grown on mono- and di-ethylsuccinate and the faster growth rate with a system that produced more of the esterase again suggested that the hydrolysis of monoethylsuccinate was due to the cloned esterase gene product rather than an *E.coli* enzyme. The comparable growth rates on mono- and di-ethylsuccinate suggested that either uptake of the substrates or the action of the cloned esterase on monoethylsuccinate was the rate limiting step.

### 5.24 Further investigation into the hydrolysis of monoethylsuccinate

The previous experiments suggested that the degradation of monoethylsuccinate was the rate limiting step for growth of JM109 containing the cloned esterase on di- and monoethylsuccinate so further experiments were undertaken to investigate this. The $K_m$ and $V_{max}$ for monoethylsuccinate were determined using the purified esterase. The $K_m$ was found to be 2.92mM and the $V_{max}$ 0.0025μmol.min$^{-1}$, with a standard deviation of 0.0012 for the fit, see Fig.5.28. The $K_{cat}$ was calculated to be 13sec$^{-1}$. The specificity constant was calculated to be $4.6\times10^3\text{M}^{-1}\text{sec}^{-1}$ which is a hundred times less than that calculated for diethylsuccinate. This showed that the uncharged monoethylsuccinate was a poor substrate for the esterase and that due to this monoethylsuccinate hydrolysis was likely to be the rate limiting step.

### 5.25 Discussion

The esterase gene was successfully engineered into the pT7-7 vector for expression under the control of the T7RNA polymerase promoter. However, this control was not as tight as had been anticipated. Induction experiments +/-IPTG showed no difference in the amounts of protein produced. This suggested that the T7RNA polymerase was being produced without the need for IPTG. Due to the efficiency of the T7RNA polymerase promoter even a small amount of T7RNA polymerase would be enough to give overexpression. However, leaky expression was also observed in JM109 cells that did not contain the DE3 lysogen so could not encode the T7RNA polymerase. The small amount of induction seen in these cells
Figure 5.28 The $K_m$ and $V_{max}$ for monoethylsuccinate using purified esterase.
was thought to be due to the E.coli RNA polymerase being able to bind to the T7RNA polymerase promoter. The apparent difference in expression seen when cells were grown on LB compared to minimal media might be explained by the faster growth rate of cells on the undefined LB. Faster growth would lead to production of more plasmid and RNA polymerase and therefore more protein expression. The high levels of expression on the minimal media without IPTG might be explained by the leaky expression of the T7RNA polymerase. The leaky expression was not a problem in this system as the protein was not toxic to the cell and was produced in sufficient amount for easy purification.

Through analysis of the native molecular weight the esterase was found to be a monomer of approximately 41kDa. This was expected as several similar esterases, Est A 42kDa, (McKay et al 1992) and est C 41kDa, (Kim et al 1994) both from P. flourescens are monomers of a comparable size.

Characterisation studies showed that heavy metals were inhibitory suggesting reactions with SH groups and changes in the protein structure. Solvents did not appear to have any effect except for butanol. Inhibition of activity with this solvent may have been due to the enzyme being irreversibly extracted into the organic phase.

Temperature stability studies showed that the enzyme was not thermostable. This was as expected as the esterase was isolated from a soil bacterium which was mesophilic and would not grow at 41°C.

Determination of the pl of the protein was carried out both experimentally and by computer analysis of the derived amino acid sequence. These values were different by approximately 0.5 units which may be due to the values being determined by different methods. The computer analysis is based on the linear derived amino acid sequence whereas the experimental analysis was by isoelectric focusing in which the protein may not have been completely linear.

Sequence similarities with other esterases had suggested that the enzyme might have a range of activities. However, analysis of the substrate specificity of the esterase suggested that the enzyme was only active with carboxyl- and aryl-ester substrates. Activities with the carboxyl- and aryl-ester substrates were compared, see Table 5.8. This shows that the esterase had comparable specificity for malathion and diethylsucinate but a much lower one.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (M)</th>
<th>$V_{max}$ (μmol.sec$^{-1}$)</th>
<th>Turnover number (sec$^{-1}$)</th>
<th>Specificity constant (M$^{-1}$sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malathion</td>
<td>$6 \times 10^{-5}$</td>
<td>$8.3 \times 10^{-5}$</td>
<td>33.2</td>
<td>$5.53 \times 10^{5}$</td>
</tr>
<tr>
<td>Diethylsuccinate</td>
<td>$2.9 \times 10^{-4}$</td>
<td>$3.3 \times 10^{-4}$</td>
<td>132</td>
<td>$4.55 \times 10^{5}$</td>
</tr>
<tr>
<td>Monoethylsuccinate</td>
<td>$2.9 \times 10^{-3}$</td>
<td>$3.3 \times 10^{-5}$</td>
<td>13.2</td>
<td>$4.55 \times 10^{3}$</td>
</tr>
<tr>
<td>$p$-nitrophenyl acetate</td>
<td>$5.8 \times 10^{-4}$</td>
<td>$3.3 \times 10^{-4}$</td>
<td>132</td>
<td>$2.27 \times 10^{5}$</td>
</tr>
<tr>
<td>$p$-nitrophenyl propionate</td>
<td>$1.1 \times 10^{-4}$</td>
<td>$5 \times 10^{-4}$</td>
<td>200</td>
<td>$1.82 \times 10^{6}$</td>
</tr>
</tbody>
</table>

Table 5.8 Comparisons of the specificity constants of esterase with different substrates
for monoethylsuccinate. This may support the idea that the hydrolysis of the monoethylsuccinate is the rate limiting step for the growth on both mono- and diethylsuccinate. The action of the esterase may not be effected by there only being one ester group as the highest specificity was seen for p-nitrophenyl propionate which only has one ester group. The reduced specificity with the monoethylsuccinate may be explained by the compound being charged whereas all of the other compounds shown in Table 5.8 were not. The reduced specificity with a charged substrate would agree with the strict definition that esterases as enzymes able to hydrolyse a large number of uncharged carboxylic esters (Krisch, 1971). This may explain why in the past research which has suggested that esterases are responsible for the hydrolysis of malathion has resulted predominantly in the production of the malathion monocarboxylic ester. This might like the monoethylsuccinate be a much poorer substrate for the esterase so remain undegraded or only be degraded very slowly. This would not necessarily be a problem for use of an enzyme in detoxification as hydrolysis of one of the ester bonds is said to prevent the malathion acting as an anticholinesterase (Hassall, 1990). The higher specificity of the esterase for the substrates which are uncharged and release ethanol on hydrolysis may reflect the structure of the natural substrate in the bacterial cell.

The purified esterase did not have a very high specificity for the monoethylsuccinate which may have contributed, in part, to the slow growth of JM109 containing the cloned gene on mono- and di-ethylsuccinate. However, the Arthrobacter sp. grew much faster and with comparable rates on both mono- and di-ethylsuccinate. As the Arthrobacter sp. was observed to have two esterases active with diethylsuccinate and malathion it is possible that each enzyme might be specific for the mono- and di-esters. This would then remove the hydrolysis of monoethylsuccinate as the rate limiting step. Alternatively, the original isolate may have had a separate enzyme which acted on the charged monoester substrates. The third explanation is that the Arthrobacter sp. had an efficient uptake system for these substrates compared to the JM109. This may mean that the slower growth rate in the JM109 was due to a combination of uptake of the substrate and also of the reduced specificity of the esterase for the charged monoethylsuccinate.
The possibility of the second esterase in the *Arthrobacter* sp. being specific for the monoethylsuccinate is feasible as observations of the growth of JM109 on monoethylsuccinate suggest that such an enzyme may have arisen through mutation. JM109 have been shown to be unable to grow on diethylsuccinate when incubated with this substrate for over two weeks. However, a similar experiment with monoethylsuccinate showed that growth took place after approximately three days. When cells from these cultures were transferred to fresh media containing monoethylsuccinate growth was immediate without the three day lag phase. This suggested that a mutation had occurred which enabled the JM109 to use monoethylsuccinate as a carbon and energy source. Initial investigation of the extracts from these cells showed that there was activity with monoethylsuccinate and also some with diethylsuccinate. However, when these cells were used to inoculate media with diethylsuccinate as a carbon and energy source no growth was seen, even when incubated over a two week period. These initial observation suggest that the monoethylsuccinate esterase which has developed in JM109 is specific for the monoester and has a low specificity for diethylsuccinate.
6. FUTURE WORK

6.1 Further investigation of the esterases of the *Arthrobacter* sp. isolate

As only one of the esterases from the original isolate was cloned further cloning experiments could be undertaken to obtain the second esterase gene. From experiments already carried out it is known that this esterase also hydrolyses malathion, diethylsuccinate and p-nitrophenyl acetate. Once cloned, experiments could be carried out to identify the substrate specificity of the second esterase.

6.2 Activity with other organophosphorus compounds

Assays could also be developed with other organophosphorus compounds containing carboxylester bonds to test the diversity of the esterase activity. The ethanol detection assay could be used for substrates such as Aldicarb, Methiocarb, Carbofuran and Propoxur, all of which contain carboxylester bonds that would produce ethanol on hydrolysis. For other compounds with carboxyl ester bonds that did not yield ethanol HPLC or NMR could be used in the analysis of products.

6.3 Mechanism of esterase activity.

As the gene sequence is known and the protein sequence has been deduced this information could be used to study the structure of the protein. As a putative active site serine has been identified by sequence homology this could be investigated further. Site specific mutational studies were carried out at Ser62 of the putative active site motif Ser-X-X-Lys in the aminopeptidase studied by Asano *et al* (1992). These studies identified the Ser61 within the motif as the active site serine. Mutational studies could be made with the esterase in this study where the Ser68 could be changed and the effect of this on enzyme activity could be monitored. The putative second active site motif Gly-X-Ser-X-X could also be investigated by similar methods to determine its significance. As the method of protein purification has been deduced pure protein could also be used in
crystallisation studies. This might also identify a potential active site within the enzyme. Information from crystal structure might also help to determine the mechanism of hydrolysis.

To further characterise the mechanism of hydrolysis by the esterase, studies could be made on the products of hydrolysis. In the case of malathion NMR studies would be able to determine if one of the ester bonds was hydrolysed in preference to the other.

6.4 Investigation of the bacterium able to use malathion and dimethoate as a phosphorus source

The isolate NSDX was shown to grow using malathion and dimethoate as phosphorus sources. Further investigations of this isolate could be undertaken to determine the enzymes responsible for the degradation of the organophosphorus compounds. Extracts of the isolate could be assayed for activity with p-nitrophenyl phosphates to determine their ability to hydrolyse P-O bonds. The pathway for the degradation of the organophosphorus compounds or the ionic phosphonates could be monitored by identifying the products when these compounds are incubated with the extract. For further study of the enzyme(s) involved in the degradation of these compounds attempts could be made to clone the gene(s) which encode them. Cook et al (1978) suggested that the enzymes responsible for degrading the ionic phosphonates were plasmid encoded. This information may provide a starting point for the isolation of the gene(s) by determining whether NSDX has any plasmids and if so do they convey the ability to use organophosphorus compounds to other bacteria.

6.5 Testing the commercial application of the malathion esterase

The commercial application of the enzyme could be tested by trials on point source contaminated land or in the treatment of residues in pesticide containers. Similar trials have been carried out with parathion hydrolase and found to be effective on both on contaminated soil and in containers.
6.6 Further investigation of the monoethylsuccinate esterase of JM109

Initial experiments have suggested that JM109 is able to develop an enzyme that will hydrolyse monoethylsuccinate. Results have suggested that this enzyme may be specific for monoethylsuccinate. Further study of the enzyme may be able to determine its specificity and its origin in the *E.coli* genome.
REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D.J. (1990). Basic Local Alignment Search Tool.


Biochemistry 31: 2316-2328.

App. Env. Microbiol. 61: 3216-3220


TIBS 19 August: 309-313

Bourquin, A. W. (1975). Microbial-malathion interaction in artificial salt-marsh ecosystems,
U.S. Environmental Protection Agency.


Donnelly, M.I., Dagley, S. (1980). Production of methanol from aromatic acids by *Pseudomonas putida* 
J. Bacteriol. 142(3): 916-924

Eck, R., Belter, J. (1993). Cloning and characterisation of a gene coding for the catechol 1,2-dioxygenase of *Arthrobacter* sp. mA3. 


Grassetti, D. R., Murray, J. F. (1967). Determination of sulphydryl groups with 2,2'- or 4,4'- dithiodipyridine. 
Arch. Biochem Biophy. 119: 41-49.


J. Pharm. Pharmacol. 45: 458-465

J. Biol. Chem. 170: 467-482.


Env. Science Technol. **9**:135-138


*Gene* **125**: 35-40.


*Bull. Env. Contam. Toxicol.* **46**: 380-386.


Nucleic Acid Res. 19(3): 674.


Bio/technology 7: 1151-1155.


Proc. Nat. Acad. Sci. USA. 71: 1342-1346


Sobel, M.E., Wolfson, E.B., Krulwich, T.A. (1973) Abolition of crypticity of Arthrobacter pyridinolis toward glucose and α-glucosides by TCA cycle intermediates 
J. Bacteriol. 116: 271-278

Proc. Nat. Acad. Sci. USA 82: 1074-1078


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