The Microbial Transformation of Nitrogen and Sulphur Containing Heterocycles Fused to Aromatic Centres.

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by

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Declaration

This thesis, submitted for the degree of Doctor of Philosophy entitled: The Microbial Transformation of Nitrogen and Sulphur Containing Heterocycles Fused to Aromatic Centres, is based upon work conducted by the author in the Department of Biochemistry at the University of Leicester between October 1996 and October 1999.

All of the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of this work has been submitted for another degree in this or any other University.

Signed: Date:
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Abbreviations

Aldrin 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-exo-1,4-endo-5,8-dimethanonaphthalene

Amp Ampicillin

BIT 1,2-BenzIsOThiazolin-3(2H)-one

BSA Bovine Serum Albumin

Chlordane 1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindane

CIAP Calf Intestinal Alkaline Phosphatase

CMIT ChloroMethylIsoThiazolinone

CTAB CetylTrimethylAmmoniumBromide

DEAE DiEthylAminoEthyl

Dieldrin 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4-endo-5,8-dimethanonaphthalene

dH2O Distilled Water

DTNB 5,5'-DiThiobis(2-NitroBenzoic acid)

EDTA EthyleneDiamineTetra Acetic-acid

EMS Ethyl MethaneSulphonate

FPLC Fast Protein Liquid Chromatography

HPLC High Performance Liquid Chromatography

IAA Iso-Amyl Alcohol

IMS Industrial Methylated Spirits

IPTG IsoPropyl-ThioGalactoside

LB Luria Broth

Mirex 1,1a,2,2,3,3a,4,5,5a,5b,6-Dodecachloro-octahydro-1,3,4-metheno-1H-cyclobuta[c]pentalene

MIT MethylISOThiazolinone
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTA</td>
<td>Nitrilo Tri-acetic Acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>PolyAcrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PCA</td>
<td>ProtoCatechuic Acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>PolyEthyleneGlycol</td>
</tr>
<tr>
<td>PNACL</td>
<td>Protein and Nucleic Acid Chemistry Laboratory</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard Saline Citrate</td>
</tr>
<tr>
<td>STE</td>
<td>Sucrose, Tris, EDTA</td>
</tr>
<tr>
<td>TAE</td>
<td>TrisAcetate, EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>TetraMethylEthyleneDiamine</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
CHAPTER 1 - INTRODUCTION
Chapter 1 - Introduction

1.1. Introduction to Microbial Degradation

Over vast stretches of time microbes have developed the ability to degrade the many different compounds found in nature but the last century has seen an increase in the amounts and types of organic compounds produced by man. Man-made compounds which are very rarely produced naturally are termed xenobiotics, (Greek; xenos - stranger + bios - life). Of particular concern is the recalcitrance of many of these molecules to microbial degradation and their accumulation in the environment. The presence of substituents such as halogen, sulpho or nitro groups can increase the recalcitrance of these molecules. The United States Environmental Protection Agency publishes a list of 12 of the most dangerous Persistent, Bioaccumulative and Toxic (PBT) chemicals (http://www.epa.gov) and these are shown in Table 1.1. The priority PBT chemicals are largely complex halogenated compounds that are persistent in the environment and which have been found to accumulate in plants and animals. An example of this is the pesticide DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane), Figure 1.1. After widespread use this compound was found to accumulate in the food chain which resulted in its use being banned in 1972.

![Figure 1.1 Structure of DDT](image)

The isolation of microorganisms able to degrade xenobiotic compounds has been carried out since early last century. The most common method of isolating bacteria able to carry out degradation of novel compounds is through enrichment culture. This entails inoculating samples of growth media with a variety of microorganisms. Those with the ability to carry out the transformation will quickly outgrow the rest of the population. This allows the isolation of single types or communities of bacteria from the multitude of bacteria present in any environmental sample. The
source of inoculum can have a bearing on the isolation of bacteria. Common sources of inocula are soil samples or samples of activated sludge from sewage works. Using an inoculum which has previously been exposed to the compound of interest can be an advantage. In the environment the interactions between different organisms can be essential to allow the complete mineralisation of certain xenobiotics. Another strategy which has become possible more recently is the construction of useful biodegradative strains. Recently researchers were able to insert genes for aromatic degradation and heavy metal resistance into the radiation resistant organism *Deinococcus radiodurans* to allow clean-up of radioactive sites (Brim et al., 2000).
### Table 1.1. The United States Environmental Protection Agency list of priority PBT chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrin/Dieldrin</td>
<td>Insecticide</td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>Pesticide</td>
</tr>
<tr>
<td>PCBs</td>
<td>Coolants and lubricants</td>
</tr>
<tr>
<td>Chlordane</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Dioxins and Furans</td>
<td>Family of chemicals that includes PCBs</td>
</tr>
<tr>
<td>Mirex</td>
<td>Insecticide</td>
</tr>
<tr>
<td>Octachlorostyrene</td>
<td>By-product of Mg$^{2+}$ production process</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>Combustion product e.g. of wood and petrol</td>
</tr>
<tr>
<td>Alkyl-Lead</td>
<td>Petroleum products</td>
</tr>
<tr>
<td>Mercury and Compounds</td>
<td>By-product of mining and coal burning</td>
</tr>
<tr>
<td>DDT</td>
<td>Insecticide</td>
</tr>
<tr>
<td>Toxaphene</td>
<td>Insecticide</td>
</tr>
<tr>
<td>chlorinated camphene</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1 - Introduction

1.2. Degradation by Microbial Consortia

The degradation of a xenobiotic compound can involve complex metabolic pathways and it can be the case that a single organism lacks some of the enzymes which allow the degradation of a particular compound. The herbicide atrazine is particularly well studied because it has been found to persist in soils for up to 1 year with complete mineralisation being extremely slow (Seiler et al., 1992). The initial steps in the degradation of the molecule are catalysed by three enzymes, AtzA atrazine chlorohydrolase (EC 3.8.1.-) catalyses the hydrolysis of atrazine to hydroxyatrazine. The second step, hydrolytic deamination is catalysed by the product of \textit{atzB} and the third step, also a hydrolytic deamination, is catalysed by the product of \textit{atzC} (EC 3.5.1.-). These genes and their homologues have been found in many of the atrazine degrading cultures investigated so far (Mandelbaum et al., 1993; Yanze-Kontchou and Gschwind, 1994; Rasodevich et al., 1995; Bouquard et al., 1997). Their distribution in a consortium of three bacteria was investigated by De Souza et al. (DeSouza et al., 1998) who found that of the species present in the consortium two bacteria were able to mineralise atrazine between them. The first two steps were carried out by \textit{Clavibacter michiganese} ATZ1 which yielded isopropylamine and N-ethylammelide. \textit{C. michiganese} was able to grow utilising isopropylamine. The second organism \textit{Pseudomonas} sp. strain CN1 was able to catalyse the hydrolytic deamination of N-ethylammelide to give ethylamine and cyanuric acid. Cyanuric acid could subsequently be utilised as a carbon and nitrogen source. Other organisms present in the consortium were able to grow using the ethylamine produced in the reaction. It was suggested that the other organisms also played an important role in that the degradation of ethylamine prevented a change in the pH of the medium.

The relatively recent discovery of co-cultures and single species of bacteria with the ability to degrade atrazine and the widespread nature of the \textit{atzABC} genes in bacteria isolated from a variety of locations could indicate the horizontal transfer of the genes (DeSouza et al., 1998). The spread of genetic information across different bacterial species can occur in several ways. The simplest method is the uptake of naked DNA by competent cells. This relies on the physiological state of the
recipient organism, some species are constantly competent e.g. *Acinetobacter calcoaceticus* (Palmen et al., 1993) whilst others become competent at particular stages in their life e.g. *Bacillus subtilis* (Dubnau, 1991). Another means of gene transfer is the utilisation of mobile elements such as plasmids or transposons. This requires conjugation between bacteria, a trait which can be triggered by genes present on the mobile element. The final method of gene transfer is bacteriophage mediated transfer of DNA. Bacteriophages are able to inject their DNA into target cells which provides a degree of protection for the DNA from environmental damage and viral proteins can also give some protection against recipient restriction endonuclease activity. Studies have shown that the genomes of many bacteria show fairly large percentages of genes which may have been acquired through lateral transfer (Woese, 1987). Adoption of physiological traits does not provide incontrovertible evidence for lateral gene transfer. More compelling evidence can be found through analysis of the genome. The development of high throughput DNA sequencing has allowed the entire sequence of many bacterial genomes to be identified. Factors such as the presence of genes with G/C contents or codon usages which differ from that of the bacterial species as a whole can point to horizontal gene transfer. The presence of transposon integration sites can also be detected in the DNA sequence of an organism.
Figure 1.2 Initial steps in the degradation of atrazine
1.3. Identification of Biodegradative Organisms

The identification of bacteria in the past has been largely based on the biochemical and morphological characteristics of organisms. The idea of using the sequence of DNA, RNA or proteins has been around for several years (Zuckerlandl and Pauling, 1965) particularly with the idea of phylogenetic classification giving more information on the relationships and evolution of bacteria. With the advent of PCR and increasingly automated DNA sequencing the classification and identification of organisms using gene sequences has become common. This method of identification relies on the characterisation of genes which code for functions found throughout the microbiological spectrum and common to all life. The genes encoding the ribosomal RNA are ideal for this purpose. The ribosomes are particularly useful because they are universal molecules with a conserved function but which show enough variability to allow comparisons between different organisms. The 16S rRNA is particularly well studied because unlike the 5S rRNA it is large enough to give a useful amount of information and it is preferred to the 23S rRNA because the complete sequence is more easily obtained due to its smaller size. Early studies utilised nuclease treatment of the ribosome and comparison of the oligonucleotides obtained (Fox et al., 1977). Advances in sequencing methodology allowed the reverse transcription of the ribosomal RNA followed by sequencing of the DNA product (Lane et al., 1985) but the simplest method currently in use is the amplification of the gene encoding the 16S rRNA followed by sequencing of the PCR product. Since the mid 1970's the sequence of the 16S rRNA has been used as a means of grouping microorganisms in order to gain more information about evolutionary relationships amongst microorganisms.

1.4. Thiazolin and Isothiazolin Rings

Compounds containing isothiazolin or thiazolin rings have been found to occur naturally, for instance vitamin B1 contains a thiazolin ring, but the majority are man-made. Some of the most important thiazolin or isothiazolin containing compounds are those with the heterocyclic ring bound to an aromatic centre, Figures 1.3 & 1.4.
Derivatives of benzothiazole such as mercaptobenzothiazole are used in the production of rubber vulcanisation agents that increase the strength and resilience of natural rubber. Wastewaters from rubber plants have been found to contain up to 300 ppm of mercaptobenzothiazole and tyre wear is thought to cause raised levels of benzothiazoles in the run-off water of roads. Some work has been carried out into the microbial degradation of these compounds (Gaja and Knapp, 1997; DeWever et al., 1998) and bacteria have been isolated which can utilise compounds such as benzothiazole and benzothiazole-2-sulphonate as the sole source of carbon, nitrogen and energy though relatively little is known about the details of the metabolism.

An important role of isothiazole type compounds is as biocides. Three compounds commonly used as biocides are benzoisothiazolinone (BIT), methylisothiazolinone (MIT) and chloromethylisothiazolinone (CMIT). These compounds and mixtures of them are sold as preservatives and can be found in a variety of products including paints, industrial cutting oils and chromatography resins. Their mode of action is through interaction with thiol groups within the cell (see Figure 1.5) causing damage to proteins and depletion of glutathione resulting in redox imbalance (Fuller et al., 1985). Little is known about the fate of these compounds in the environment and despite reports of bacteria developing resistance in certain instances none have been isolated which are able to transform these compounds to less biocidal compounds or actually utilise them as a source of nutrients (Brozel and Cloete, 1994; Sondossi et al., 1999). Recent environmental regulations require the fate of all such biocidal molecules released into the environment to be studied.
One of the most familiar benzoisothiazole derivatives is benzoisothiazolinone-1,1-dioxide more commonly known as saccharin. This molecule is used as a non-nutritive sweetener and is found in a variety of products from toothpastes to soft drinks and crisps. To utilise a compound such as benzoisothiazole or its derivatives as a source of carbon, nitrogen and energy, bacteria need to be able to first open the isothiazolin ring and remove any remaining moieties such as sulphonyl groups or amides before being able to utilise the aromatic ring itself.

Figure 1.5. Mode of action of isothiazolin biocides, (Collier et al., 1990).
1.6. Aromatic Degradation

The biodegradation of aromatic ring compounds has been the subject of much research. Originally thought to be relatively inert structures many aromatic ring compounds have been found to be utilised by soil bacteria. The microbial degradation of natural compounds that contain aromatic rings, such as the plant product lignin which contains multiple aromatic centres, is essential for maintaining the flow of carbon in the biosphere. Because of the stability of the aromatic nucleus addition of hydroxyl groups is a prerequisite for aerobic degradation.

Aromatic metabolism generally involves conversion of a particular compound to one of a few common metabolites using enzymes to remove functional groups, such as sulphonyl groups, from the ring structure. These processes normally accompany the addition of hydroxyl groups to the aromatic ring and are carried out by dioxygenase or monooxygenase enzymes. Oxygenases play an important part in the metabolism of aromatic compounds. They catalyse the addition of hydroxyl groups to the stable benzene ring rendering it susceptible to cleavage by the addition of oxygen. The dioxygenases catalyse the addition of two oxygen atoms to the aromatic ring whilst the monooxygenases catalyse addition of one atom of oxygen with the other oxygen atom being reduced to water, which gives rise to the alternative name of mixed function oxygenases. There is also a divide within the dioxygenase enzymes. The dioxygenase enzymes which catalyse the initial hydroxylation of the aromatic ring i.e. benzoate dioxygenase require cofactors such as NADH or NADPH whereas the ring cleavage dioxygenase enzymes such as catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase have no such requirements. Both types of dioxygenase enzyme require an iron cofactor. Ortho cleavage enzymes require Fe³⁺ whilst meta cleavage enzymes require Fe²⁺ or occasionally Mn²⁺ (Que et al., 1981).

Some of the compounds that are central to aromatic metabolism are catechol, protocatechuate, homoprotocatechuate, gentisate, homogentisate, and methyl gallate. These are the molecules where aromatic metabolism converges. Bacteria utilise a series of common pathways for aromatic degradation with the substrate
range being increased by the production of enzymes which convert unusual compounds to one of these common intermediates. The two most common intermediates in microbial aromatic metabolism are protocatechuate and catechol. These compounds both contain hydroxyl groups on adjacent carbons. With the formation of these compounds the metabolism then tends to follow quite well studied pathways. Addition of oxygen by a dioxygenase leads to cleavage of the aromatic ring. The oxygen can be added between the hydroxyl groups resulting in ortho or intradiol cleavage or the cleavage may occur adjacent to the hydroxyl groups giving meta or extradiol cleavage. Following cleavage of the aromatic ring the end products are converted to intermediates of central metabolism such as acetyl CoA or succinate, see Figure 1.6.
Figure 1.6. General scheme for degradation of aromatic compounds.
1.6.1. The Ortho Cleavage Pathway

One of the most well characterised pathways of aromatic metabolism is the ortho cleavage or β-ketoadipate pathway, Figure 1.7. A detailed study of the pathway in *Pseudomonas putida* was published in a set of papers in 1966 (Omston, 1966a; Omston, 1966b; Omston, 1966c; Omston, 1966d). The pathway consists of two parallel sets of reactions which converge at the common intermediate β-ketoadipate enol lactone. The products of the two branches of the pathway differ only in the presence of a carboxyl group yet it has been found that the enzymes of each branch of the pathway are very specific and show no activity against the substrates of the opposite branch.

With one exception to date (Winstanley *et al.*, 1987) the genes of the ortho cleavage pathway are chromosomally encoded. The substrates of the pathway are non-substituted catechols. A related pathway called the modified ortho cleavage pathway is also found that has a wider substrate range which encompasses substituted catechols such as chlorocatechol. In the examples studied so far the modified ortho cleavage pathway is always plasmid-encoded (Mae *et al.*, 1993; Manzoor *et al.*, 1994). Several of the genes of the ortho and modified ortho cleavage pathway share up to 40% amino acid identity which could indicate a common ancestor (Schlömann, 1994). It should be noted however that there are enzymes of the modified ortho pathway such as maleylacetate reductase (EC 1.3.1.32) which have no equivalent in the β-ketoadipate pathway.

The first set of parallel reactions of the β-ketoadipate pathway is the dioxygenation of protocatechuate and catechol by protocatechuate 3,4-dioxygenase (P3,4D) and catechol 1,2-dioxygenase (C1,2D) respectively. These reactions lead to the formation of the products β-carboxy-cis,cis-muconate from protocatechuate and cis,cis-muconate from catechol. P3,4D is composed of an α and a β subunit of mass 23 and 27 kDa respectively. These subunits are further arranged as groups of protomers to form the active protein. The number of protomers varies from 4 – 12 in the range of organisms studied so far (Bull and Ballou, 1981). C1,2D is a more simple homodimeric protein with two subunits in the size range of 32 – 34 kDa (Nakai *et al.*, 1988). The next reaction is the formation of a lactone by β-carboxy-
cis,cis-muconate lactonising enzyme (CMLE) (EC 5.5.1.2) in the protocatechuate branch and cis,cis-muconate lactonising enzyme (MLE) (EC 5.5.1.1) in the catechol branch. These enzymes are structurally unrelated and belong to different families of enzymes. CMLE is a 42 kDa monomeric protein (Patel et al., 1973) whilst MLE is an octamer composed of eight 40 kDa subunits (Meagher and Omston, 1973). The final set of parallel reactions are catalysed by δ-carboxymuconolactone decarboxylase (CMD) (EC 4.1.1.44) and muconolactone isomerase (MI) (EC 5.3.3.4). CMD is a hexameric protein composed of 15 kDa (Parke et al., 1973) subunits whilst the enzyme of the catechol branch, MI, is a decamer composed of 11 kDa subunits (Meagher and Omston, 1973).

The β-ketoacidipate pathway of Pseudomonas putida converges at β-ketoacidipate enol lactone and the remaining steps are carried out by enzymes common to both pathways, enol lactone hydrolase (ELH) (EC 3.1.1.24), β-ketoacidipate;succinyl-CoA transferase (TR) (2.8.3.6) and β-ketoacidipyl-CoA thiolase (TH) (2.3.1.-). Each of the proteins of these steps is coded for by separate genes but the products have a high degree of homology. ELH is a 29 kDa monomeric protein coded for by the genes pcaD and catD (McCorkle et al., 1980). TR is composed of an α and a β subunit of molecular masses 24 kDa and 22 kDa, respectively. The functional protein is composed of two of each of the subunits (Yeh and Omston, 1981). The final enzyme of the pathway, TH, is a tetramer composed of four 43 kDa subunits. Like ELH, TH is related to a variety of proteins which have a wide distribution in nature and catalyse the transfer and thiolytic cleavage of coenzyme A derivatives (Harwood et al., 1994).

Despite the gross similarities in the enzymes of the β-ketoacidipate pathway in the organisms so far studied there are a number of variations on the theme. This is one of the reasons it is considered so important for the study of evolution of metabolic pathways. Amongst others the pathway has been studied in Pseudomonas sp. and Acinetobacter sp. and a number of differences have been found in the organisation and regulation of the genes and expression of the enzymes. In Pseudomonas putida the branches of the pathway converge at β-ketoacidipate enol-lactone whilst in Acinetobacter this convergence occurs at β-ketoacidipate. Another difference is the
expression of enzymes in the latter part of the pathway. *Pseudomonas* sp utilise the same final three enzymes regardless of which branch of the pathway was utilised whilst *Acinetobacter* expresses a different set of proteins for each branch of the pathway right through to the final steps. The compounds which induce the genes of the pathway are also different. In *Pseudomonas* sp. the production of the first enzyme in the protocatechuate branch of the pathway, P3,4D, is induced by its substrate whilst the next five enzymes are all induced by β-ketoacidipate.

The related modified *ortho* pathway, which is usually plasmid-encoded, shares several steps with the β-ketoacidipate pathway but it does utilise some specialised reactions, presumably due to the presence of the halogenated substituents. The initial two reactions catalysed by catechol 1,2-dioxygenase, chlorocatechol 1,2-dioxygenase, muconate cycloisomerase and chloromuconate cycloisomerase (5.5.1.7) are almost identical (Dorn and Knackmuss, 1978; Schmidt and Knackmuss, 1980). The action of chloromuconate cycloisomerase results in the formation of dienelactone whereas the β-ketoacidipate pathway utilises an extra step catalysed by muconolactone isomerase to produce the corresponding enol-lactone. The modified *ortho* pathway then utilises a dienelactone hydrolase (EC 3.1.1.45), which differs markedly from the β-ketoacidipate enol-lactone hydrolase of the β-ketoacidipate pathway, to form maleylacetate (Frantz *et al*., 1987). This is converted to β-ketoacidipate through the action of maleylacetate reductase, an enzyme which has no homologue in the β-ketoacidipate pathway (Kaschabek and Reineke, 1992).
Figure 1.7. The ortho cleavage or β-ketoadipate pathway of aromatic metabolism (Harwood and Parales, 1996).
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Figure 1.8. The modified ortho cleavage pathway (Schlömann, 1994).
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1.6.2. The Meta Cleavage Pathway

The *meta* cleavage pathway has been characterised most closely in the study of the degradative plasmid pWW0, also referred to as TOL, which was isolated from *Pseudomonas putida* selected for growth with toluate. Many of the pathways of *meta* cleavage which have been studied are plasmid-encoded. A striking aspect of the *meta* pathway is the low specificity of the enzymes. This means that an organism containing a plasmid such as TOL gains the ability to grow on a variety of substrates despite being isolated for the ability to grow with a single substrate such as toluene, see Table 1.2.

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Alcohols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>Benzyl alcohol</td>
</tr>
<tr>
<td><em>m</em>-xylene</td>
<td>3-methylbenzyl alcohol</td>
</tr>
<tr>
<td><em>p</em>-xylene</td>
<td>4-methylbenzyl alcohol</td>
</tr>
<tr>
<td>1,2,4-trimethylbenzene</td>
<td>3,4-dimethylbenzyl alcohol</td>
</tr>
<tr>
<td>3-ethyltoluene</td>
<td>3-ethylbenzyl alcohol</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Carboxylic Acids</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>Benzoate</td>
</tr>
<tr>
<td>3-methylbenzaldehyde</td>
<td>3-methylbenzoate</td>
</tr>
<tr>
<td>4-methylbenzaldehyde</td>
<td>4-methylbenzoate</td>
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<td>3,4-dimethylbenzaldehyde</td>
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<tr>
<td>3-ethylbenzaldehyde</td>
<td>3-ethylbenzoate</td>
</tr>
</tbody>
</table>

Because of the location of the degradative pathway on a plasmid the genetic characterisation has outpaced the physical characterisation of the enzymes of the pathway due to the relative ease with which the genes can be located and studied. The genes are arranged in two separately regulated operons which give an upper and a lower pathway. The upper pathway (*xylCMABN*) codes for enzymes which carry
out the conversion of alkylated benzene rings to the corresponding carboxylic acids. The genes of the lower pathway (xylEGFJQKIHI) encode enzymes which then allow the cleavage and utilisation of the carboxylic acid.

1.6.2.1. The Upper TOL Pathway

The first enzyme of the pathway, xylene monooxygenase, has never had its activity measured in-vitro but the genes xylM and xylA which code for the subunits have been sequenced. The enzyme itself is an NADH-dependent monooxygenase with XylM being a membrane associated protein with a molecular mass of approximately 35 kDa (EC 1.14.15.-) whilst xylA codes for a 42 kDa subunit (EC 1.18.1.3) (Suzuki et al., 1991). The next enzyme in the pathway is benzyl alcohol dehydrogenase (EC 1.1.1.90) which catalyses the formation of benzaldehyde (Shaw et al., 1993). This enzyme is coded for by xylB and has a subunit size of 42-43 kDa and it is proposed to be a dimer. The second dehydrogenase of the pathway is benzaldehyde dehydrogenase coded for by xylC (EC 1.2.1.28) (Inoue et al., 1995). This enzyme is also a dimer and the subunit size has been determined as 57 kDa which matches closely the size calculated from the DNA sequence. xylN is known to code for a 47 kDa protein but the function of the protein is unknown. See Figure 1.9.

1.6.2.2. The Lower TOL Pathway

The lower pathway is composed of the genes xylXYZLTEGFJQKIHI. The first enzyme of the lower pathway, toluate-1,2-dioxygenase (EC 1.14.12.-), is coded for by xylXYZ and is made up of three subunits of size 57, 20 and 38 kDa. The next eight genes, xylL, E, G, F, J, K, I and H code for 1,2-dihydroxy-3,5-cyclohexadiene-1-carboxylate dehydrogenase (28 kDa) (EC 1.3.1.55), catechol 2,3-dioxygenase (35 kDa) (EC 1.13.11.2), 2-hydroxymuconic-semialdehyde dehydrogenase (60 kDa) (EC 1.2.1.-) (Inoue et al., 1995), 2-hydroxymuconic-semialdehyde hydrolase (34 kDa) (EC 3.1.1.-), 2-hydroxypent-2,4-dienoate hydratase (28 kDa) (EC 4.2.-.-), 4-hydroxy-2-oxovalerate aldolase (39 kDa) (EC 4.1.3.-), 4-oxalocrotonate decarboxylase (29 kDa) (EC 4.1.1.77) and 4-oxalocrotonate isomerase (7.5 kDa) (EC 5.3.2.-.). The final two genes of the operon have no known function. See Figure 1.10.
1.6.2.3. Regulation of the TOL Pathway

Regulation of the meta pathway of the TOL plasmid involves two regulatory proteins XylR and XylS, the products of xylR and xylS which are located downstream of the lower pathway genes. Induction occurs on interaction of XylR with substrates of the upper pathway. XylR is then able to bind to the upper pathway promoter OP1 and the promoter of xylS, Ps. This results in transcription of the genes of the upper pathway and of xylS. XylS then goes on to induce the genes of the lower pathway through binding the promoter of the lower pathway, OP2. It is also proposed that XylS can interact with the carboxylated substrates of the lower pathway resulting in the initiation of transcription of the genes of the lower pathway.
Figure 1.9. The upper TOL pathway. (Assinder and Williams, 1990)
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Figure 1.10. The lower TOL pathway (Assinder and Williams, 1990).
1.7 Microbial Desulphonation

The complete utilisation of an aromatic ring often requires the removal of moieties such as sulphonyl groups. The degradation of molecules such as benzoisothiazoles will almost certainly require such a step. It has been found that bacteria are able to carry out desulphonative reactions by three general methods; (i) desulphonation through the action of a monooxygenase or dioxygenase, (ii) hydrolytic desulphonation and (iii) an as yet little studied reductive desulphonation.

1.7.1. Desulphonation Through Ring Hydroxylation

One of the best-characterised desulphonation reactions is the 4-sulphobenzoate 3,4-dioxygenase system of *Comamonas testosteroni* which catalyses a step in the metabolism of toluenesulphonate (Locher *et al.*, 1991). This system fits into category (i) and catalyses the conversion of 4-sulphobenzoate to protocatechuate with NADH required as a co-factor. The reaction involves two proteins; reductase C catalyses the transfer of electrons from NADH to the oxygenase component A. Oxygenase A is a dioxygenase which catalyses the addition of two oxygen atoms to 4-sulphobenzoate giving protocatechuate and sulphite. Oxygenase A was characterised and found to be a homodimeric protein with a subunit mass of 50 kDa. In the initial purification the oxygenase was characterised with a reductase B which was later found to belong to a different system. Further work was done on the genetics of the system and the genes are thought to be present as part of an operon called psbAXYCZ in which the products of psbXYZ are as yet unclassified (Cook *et al.*, 1998).

4-Sulphobenzoate 3,4-dioxygenase demonstrates desulphonation prior to ring cleavage but there are examples of desulphonation which accompany ring cleavage (Junker *et al.*, 1994), see Figure 1.12. In this case an *Alcaligenes* sp. was isolated which was able to grow utilising 2-aminobenzenesulphonic acid.
Figure 1.11 Desulphonation of toluenesulphonate via 4-sulphobenzoate to yield protocatechuate (Locher et al., 1991).

Figure 1.12 Desulphonation of 3-sulphocatechol to yield 2-hydroxymuconate. The reactions shown represent a section of the degradation of 2-aminobenzenesulphonate (Junker et al., 1994)
1.7.2. Hydrolytic Desulphonation

Hydrolytic desulphonation has been observed in the degradation of 4-aminobenzenesulphonate via an ortho cleavage pathway (Hammer et al., 1996), see Figure 1.13. The cleavage generates a relatively stable lactone. The lactone then undergoes hydrolysis resulting in spontaneous loss of the sulphonyl group as sulphite. 4-Sulphocatechol is common to the degradative pathways of other compounds such as benzene-1,3-disulphonate, 2-(4-sulphophenyl)butyrate and sulphanilate but the reactions which occur in the conversion of these compounds to 4-sulphocatechol have not yet been characterised.

1.7.3. Reductive Desulphonation

Reductive desulphonation was proposed as another means of degradation of 4-toluenesulphonate. The data was gathered during study of its degradation by a *Pseudomonas* sp. in which 3-methylcatechol was detected by paper chromatography (Focht and Williams, 1970). As yet the transformation has not been observed by other researchers.

1.8. Metabolism of Nitrogen Containing Moieties

The degradation of isothiazolin rings could result in the formation of moieties such as sulphamoyl or amide groups. The degradation of compounds such as benzamide has previously been investigated and amidase enzymes have been isolated which will hydrolyse an aromatic amide to an aromatic carboxylic acid and ammonia (Nawaz et al., 1991). Much of the study of amidase enzymes is focussed on their role in the metabolism of nitrile containing compounds. The enzymatic conversion of acrylonitrile to acrylamide is an important industrial reaction. For instance nitrile hydratase catalyses the formation of benzamide from benzonitrile and amidase catalyses the production of benzoate and ammonia. Organisms have been isolated which express different pathways of nitrile metabolism depending on the induction conditions (Kobayashi et al., 1993). Kobayashi et al. found that *Rhodococcus rhodocrous* can express a high molecular weight nitrile hydratase which will convert nitrile containing compounds directly to the corresponding carboxylic acids.
Figure 1.13 Hydrolytic desulphonation of 4-sulphocatechol to maleylacetate (Hammer et al., 1996).

Figure 1.14. Reductive desulphonation of toluenesulphonate to 3-methylcatechol (Focht and Williams, 1970).
and the organism can also express a low molecular weight nitrile hydratase which converts nitrile containing compounds to carboxylic acids in conjunction with an amidase. The amidase enzyme was found to act preferentially on aromatic amides. Mutation studies carried out by this group found that the residues aspartate 191 and serine 195 which are found as part of an amidase consensus sequence were essential for catalytic activity (Kobayashi et al., 1997).

The metabolism of compounds which contain sulphamoyl moieties has been investigated previously. The microbial degradation of the sweetener cyclamate (Niimura et al., 1974) and the action of the liver enzyme N-sulfoglucosamine sulphohydrolase (Mahuran et al., 1983) are two examples. This topic is discussed further in section 3.1.
1.9. Aims

The aim of this project was to isolate an organism or group of organisms that were able to utilise benzoisothiazole type compounds for growth and then try to identify the mechanisms of the utilisation. Saccharin was chosen as a representative compound.

Based on proposed routes of degradation of the isothiazolin ring the cleavage of the sulphur nitrogen bond was seen as being novel. To gain more insight into this reaction the compound sulphamate was chosen in order to isolate an organism or group of organisms able to utilise sulphur-nitrogen containing compounds.
CHAPTER 2 - MATERIALS AND METHODS
2.1. Growth Media

2.1.1. Nitrogen-Free M63

Nitrogen-free M63 was prepared as a 5 x concentrated stock containing KH$_2$PO$_4$, 68 g/L and FeSO$_4$.7H$_2$O, 2.5 mg/L. The pH was adjusted to 7.0 using KOH. The stock solution was diluted before use and MgSO$_4$ or MgCl$_2$ added to a final concentration of 1 mM before autoclaving (121 °C, 15 minutes). Media was solidified when required by the addition of bacteriological agar to 1.5 % w/v before autoclaving.

2.1.2. Nitrogen-Free PJC

PJC was prepared as a 10 x concentrated basal medium with a supplementary 10 x concentrated trace metal solution.

Basal salts medium:

K$_2$HPO$_4$ 42.5 g/L, NaH$_2$PO$_4$ 10 g/L.

Trace metal concentrate:

Nitrilotriacetic acid 1g/L, MgSO$_4$ 2 g/L, FeSO$_4$.7H$_2$O 120 mg/L, MnSO$_4$.4H$_2$O 30 mg/L, ZnSO$_4$.H$_2$O 30 mg/L, CoCl$_2$ 10 mg/L

The stock solutions were diluted 10 x before use and autoclaved (121 °C, 15 minutes). Media was solidified by the addition of bacteriological agar to 1.5 % w/v before autoclaving.

2.1.3. Luria Broth (LB)

LB contained the following components: yeast extract 10 g/L, tryptone 5 g/L, sodium chloride 10 g/L. Media was solidified when required by the addition of bacteriological agar to 1.5 % w/v before autoclaving.
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2.1.4. NZY⁺ Broth

NZY⁺ broth contained the following components: NZ amine (Sigma) 10 g/L, Yeast extract 5 g/L, NaCl 5 g/L. The pH was adjusted to 7.5 with NaOH then the following ingredients were added 1M MgCl₂ (12.5 ml), 1M MgSO₄ (12.5 ml), 2 M glucose (10 ml) and the media made up to 1L then filter sterilised.

2.2. Glycerol Stocks

Glycerol stocks of organisms were prepared by adding 300 µl of sterile 50% glycerol to 700 µl of media containing bacteria. The sample was then mixed thoroughly by vortexing and then snap frozen in a dry ice/IMS bath. Stocks were stored at −80 °C.

2.3. Genotypes of Bacteria

2.3.1. Epicurian coli XL-10 Gold

TetRΔ(mcrA)183Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 Hte [F' proAB lacIqZAM15 Tn10 (TetR) Amy CamR] α

2.4. Plasmid DNA

The vector pUC18 was utilised for general cloning, see Figure 2.1. The vector is a high copy number plasmid which confers resistance to ampicillin at a concentration of 100 µg/ml. The plasmid also contains lacZ' that codes for a fragment of β-galactosidase that allows blue/white screening for inserted DNA with a host containing a chromosomal fragment lacZΔM15 which codes for the remaining fragment of β-galactosidase. To carry out blue/white screening transformed bacteria were spread on to solidified LB Amp media supplemented with 0.1 mM IPTG and 40 µg/ml X-Gal. The presence of insert DNA disrupts lacZ' which prevents the formation of β-galactosidase and thereby the blue colour from the substrate X-Gal.
Figure 2.1. Restriction map of the general purpose cloning vector pUC18/19.
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2.5. Preparation of Sodium Salt of 2-Sulphobenzamide

2-Sulphobenzamide was prepared by Peter Austin at Zeneca and supplied as an ammonium salt. In order to allow experiments to be carried out the compound was converted to a sodium salt using ion exchange chromatography. A syringe barrel stoppered with glass wool was packed with a slurry of Dowex 50 WX8-200 to give a volume of approximately 6 ml. The column was primed with 1 M NaCl then washed with water. 2-Sulphobenzamide was applied, 100 mg in 1 ml dH₂O, and eluted with dH₂O. The \( A_{270} \) value and ammonia concentration of the eluent was measured. The ammonia concentration of the fractions collected was zero. The solution was then freeze dried.

2.6. Measurement of Sulphite

The concentration of sulphite ions was measured using the reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent or DTNB) with free sulphite (Johnston et al., 1975). To 1 ml of the sample containing up to approximately 0.1 mM sulphite ions, 100 \( \mu l \) of Ellman’s reagent was added (1 mg/ml in 0.1 M potassium phosphate pH7). The solution was mixed and the absorbance at 415 nm was measured after a 2 minute incubation. In order to quantify the amount of colour produced by the reaction of the Ellman’s reagent with possible contaminating thiol compounds an identical sample was mixed with 10 \( \mu l \) of 50 mM formaldehyde before the addition of Ellman’s. This results in the complexing of sulphite ions and any colour is due to thiol groups and can be subtracted from the original value. A standard curve was prepared using sodium sulphite over the range 0-0.1 \( \mu m o l e s \), see Figure 2.2.
Figure 2.2. Example of a standard curve for the measurement of sulphite

\[ y = 8.468x + 0.0047 \]

\[ R^2 = 0.9999 \]
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2.7. Measurement of Sulphate

The concentration of sulphate ions was measured using a turbidimetric method (Sörbo, 1987). Barium chloride reagent was prepared by adding 0.97 g BaCl$_2$ to approximately 80 ml of dH$_2$O followed by 15 g PEG 6000. The volume was made up to 100 ml with dH$_2$O then 200 µl of 50 mM Na$_2$SO$_4$ was added drop-wise with stirring.

The sample to be measured was centrifuged (5 minutes x 12,000 g) and an aliquot removed and made up to 600 µl with buffer then 200 µl of 0.5 M HCl was added followed by 200 µl of the barium chloride reagent. The reaction was mixed thoroughly and incubated at room temperature for 5 minutes before measuring the turbidity at 600 nm. A standard curve was prepared over the range 0 – 0.5 µmoles using Na$_2$SO$_4$, see Figure 2.3.

![Standard Curve](image)

Figure 2.3 Example of a standard curve for the measurement of sulphate
2.8. Measurement of Ammonia

The method of Muftic (Muftic, 1964) was used to measure the concentration of ammonia in solution. Aliquots of 10 - 200 µl containing up to 0.2 µmoles ammonia were used for measurements. The ammonia concentration was measured by adding the aliquot to 1 ml of phenoxide reagent and vortexing thoroughly. Then 1.5 ml of 0.5% sodium hypochlorite in buffer (0.1 M sodium acetate, 0.08 M HCl, pH 6.0) was added followed by 1.5 ml of sodium nitroprusside (0.01% w/v in buffer). The colour was developed in the dark for at least 30 minutes and the absorbance at 630 nm was then measured. A standard curve was prepared over the range 0 - 0.25 µmoles NH₄Cl, see Figure 2.4.

![Standard Curve](image)

**Figure 2.4 Example of a standard curve for the measurement of ammonia.**
Chapter 2 – Materials and Methods

2.9. Spectrophotometric Assays of Ring Fission Enzymes

2.9.1. Ortho Cleavage of Catechol

Ortho cleavage of catechol was measured by incubating protein (approximately 100 ng) at 30 °C in 0.1 M sodium phosphate buffer pH 7. The production of cis,cis-muconate, on addition of catechol to a concentration of 0.2 mM, was measured using the absorbance at 260 nm. The conversion of 1 μmole of catechol to cis,cis-muconate causes a change in absorbance of 5.6 units (Ngai et al., 1990).

2.9.2. Meta Cleavage of Catechol

Meta cleavage of catechol was measured using the same reaction conditions as ortho degradation but in this case the production of hydroxymuconic semialdehyde is measured using the absorbance at 375 nm. The molar extinction coefficient of the product is 13,800 (Kataeva and Golovleva, 1990).

2.9.3. Ortho Cleavage of Protocatechuic Acid

Ortho cleavage of protocatechuic acid was detected by measuring the disappearance of protocatechuate, as it is converted to β-carboxy-cis,cis-muconate, using the absorbance at 290 nm. Assays were set up as described above and substrate was added to a final concentration of 0.2 mM (Whittaker et al., 1990).

2.9.4. Meta Cleavage of Protocatechuic Acid

Meta cleavage of protocatechuic acid was measured under the same assay conditions but in this case the appearance of the product α-hydroxy-γ-carboxymuconic semialdehyde is measured at 410 nm (Wolgel and Lipscomb, 1990).

2.10. Oxygen Electrode

The consumption of oxygen by oxygenase enzymes was monitored using a Clark type oxygen electrode. Reactions were made up to a final volume of 2 ml and then incubated at 30 °C with constant stirring.
2.11. HPLC Analysis of Growth Medium

Samples of growth medium were analysed using HPLC in order to monitor disappearance of saccharin and identify any intermediates or end-products which might be produced. Samples were first mixed with chloroform to denature proteins present in the growth medium and centrifuged (10 minutes x 4000 \( g \)) to remove denatured protein and whole cells. The upper aqueous layer was removed and heated to 65 °C for 1 hour to ensure removal of any trace amounts of chloroform remaining. The samples were then filtered through nitrocellulose 0.2 \( \mu m \) filters (Sartorius) to remove particles which could damage the HPLC equipment.

Samples were separated using an isocratic elution with a mobile phase containing water, methanol, acetic acid in the ratio 60:40:1. Samples were separated on a Supelcosil 250 x 4.8 mm C18 column with a particle size of 5 \( \mu m \) (Supelco) using a flow rate of 1ml/min and monitored at a wavelength of 270 nm.

2.12. Preparation of Cell-Free Extracts

Cells with sulphamatase activity were harvested by centrifugation (12,000 \( g \) x 30 minutes) washed once with 0.04 volumes of 10 mM phosphate buffer pH 7.2 and centrifuged (3000 \( g \) x 20 minutes). The supernatant was removed and the cell pellets stored at -20 °C until required. Cell extracts were prepared by breaking frozen pellets using a Hughes press (Hughes, 1951). The broken cells were resuspended in 10 volumes of chilled 10 mM phosphate buffer pH 7.2 and then sonicated (3 x 10 seconds, \( \lambda = 10 \) microns) to break the DNA present and reduce the viscosity of the extract. The extract was then centrifuged (20,000 \( g \) x 15 minutes, 0-4 °C) and the supernatant stored at 0-4 °C. In order to prepare a partially purified extract of sulphamatase more quickly the extract could be ultracentrifuged (200,000 \( g \) x 2 hours) making it suitable for application directly on to an FPLC column. During the preparation of cell-free extracts all steps were carried out at 0-4 °C.

2.13. Measurement of Protein Concentration

The concentration of protein was measured using the Bradford assay (Bradford, 1976). Bradford reagent was prepared by dissolving Coomassie Blue G-25 (25mg)
in 12.5 ml of ethanol. Phosphoric acid (25 ml) was added and then the volume made up to 250 ml with dH₂O. The solution was filtered through three layers of 3MM paper (Whatman). Samples were made up to 100 µl and added to 1000 µl of Bradford reagent, colour was developed for 5 minutes and then the absorbance at 595 nm was measured against a reagent blank. A standard curve over the range 2-8 µg bovine serum albumin (BSA) was used, see Figure 2.5.

The protein concentration of the fractions collected during enzyme purification was measured using the absorption at 280/260 nm (Warburg and Christian, 1941). The ratio of the 280/260 value was used to obtain a factor (given in tables) which was used in the equation

\[
\text{Protein conc. (mg/ml)} = A_{280} \times \text{Factor} \times \frac{1}{d}
\]

Where \(d\) = length of light path (cm)

\[
y = 0.0301x + 0.0585 \\
R^2 = 0.9942
\]

Figure 2.5 Example of a standard curve for the Bradford assay.
2.14. DEAE-Cellulose Chromatography

The resin was prepared by washing with 1 M NaOH, then 1 M HCl followed by a final wash in 1 M NaOH. The washed resin was then added to 10 mM PO₄ pH 7.2 buffer, and the pH adjusted to 7.2 using 0.2 M NaH₂PO₄. The column was prepared by pouring the slurry into a 20 ml syringe with a glass wool plug to prevent loss of the resin. The column was equilibrated with approximately 20 column volumes of 10 mM PO₄ pH 7.2 buffer.

Approximately 3 mg of protein for every 1 ml of DEAE-cellulose was applied and washed through with 10 mM PO₄ pH 7.2 buffer. Proteins bind to the column at low ionic strengths and increasing the NaCl concentration of the buffer allows the protein to be eluted.

2.15. Fast Protein Liquid Chromatography (FPLC)

FPLC was carried out using Pharmacia apparatus and Pharmacia Superose 12 HR 10/30 (size exclusion chromatography), Mono Q HR 5/5 (anion exchange chromatography) and Phenyl Superose HR 5/5 (hydrophobic interaction chromatography) columns.

2.16. Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE systems use a stacking gel with a lower percentage of polyacrylamide than the resolving gel. This allows better separation of proteins, with an increased resolution over continuous systems that use one polyacrylamide concentration. The denaturing conditions split proteins into their component subunits and the addition of SDS ensures that all proteins carry the same overall charge to mass ratio so that separation is purely on the basis of molecular mass (Laemmli, 1970).

Protein samples were mixed with gel loading buffer (2.5 mM Tris-Cl pH 6.7, 2 % SDS w/v, 5 % mercaptoethanol v/v, 10 % glycerol, 0.001 % bromophenol blue w/v) in the ratio of 4 parts sample: 1 part loading buffer. Samples were heated at 100 °C
for 2 minutes and allowed to cool before application to the gel. Samples were run for 45 minutes at 200 V with running buffer (0.02 M Tris base, 0.2 M glycine, 0.1 % SDS).

Table 2.1. Preparation of acrylamide gel for SDS-PAGE

<table>
<thead>
<tr>
<th></th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 % acrylamide</td>
<td>4 % acrylamide</td>
</tr>
<tr>
<td>dH₂O</td>
<td>4.35 ml</td>
<td>3.15 ml</td>
</tr>
<tr>
<td>1.5M TrisCl pH 8.8</td>
<td>2.50 ml</td>
<td>----</td>
</tr>
<tr>
<td>0.5M TrisCl pH 6.8</td>
<td>----</td>
<td>1.26 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>40 % Acrylamide/bisacrylamide (37.5:1)</td>
<td>3.00 ml</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>10 % (NH₄)₂S₂O₈</td>
<td>50 μl</td>
<td>25 μl</td>
</tr>
<tr>
<td>Tetramethyl-ethylenediamine (TEMED)</td>
<td>10 μl</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Proteins were visualised by staining overnight with Coomassie blue (0.1 % w/v Coomassie brilliant blue R-250, 45 % methanol, 10 % glacial acetic acid) then removing excess stain by soaking in destain solution (20 % methanol, 10 % glacial acetic acid).

To allow the estimation of the size of separated proteins a series of standard proteins were used, see Table 2.2.
Table 2.2. Molecular weight markers used for SDS-PAGE

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin, Bovine</td>
<td>66,000</td>
</tr>
<tr>
<td>Albumin, Egg</td>
<td>45,000</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P-Dehydrogenase</td>
<td>36,000</td>
</tr>
<tr>
<td>Carbonic Anhydrase, Bovine</td>
<td>29,000</td>
</tr>
<tr>
<td>Trypsinogen, Bovine Pancreas</td>
<td>24,000</td>
</tr>
<tr>
<td>Trypsin Inhibitor, Soybean</td>
<td>20,000</td>
</tr>
<tr>
<td>α-Lactalbumin, Bovine Milk</td>
<td>14,200</td>
</tr>
</tbody>
</table>

2.17. Protein Sequencing

Protein sequencing was carried out by PNACL using an ABI 476 sequencer. Proteins were subjected to Edman degradation then derivatised and separated by reversed-phase HPLC.

2.18. Mass Spectrometry

Electrospray mass spectrometry was carried out by Dr K. Lilley at PNACL using a Micromass Platform single quadrupole spectrometer. Protein samples were dialysed overnight against 50 mM ammonium bicarbonate then lyophilised and resuspended in dH₂O. Samples were resuspended in 50 % acetonitrile with 0.5 % formic acid. Samples were run under the electrospray positive ion mode with a capillary voltage of 2.7 kV, a cone voltage of 30 V and a source temperature of 95°C.

2.19. Preparation of Genomic DNA

Genomic DNA was prepared using a method which took advantage of the protein and polysaccharide binding of cetyltrimethylammonium bromide (CTAB) in a high salt buffer (Wilson, 1990). Cells were grown to an A₆⁸₀ value of about 0.5 and then harvested by centrifugation (30 minutes x 9,000 g). The cells were resuspended in 20 ml of STE buffer (0.3 M sucrose, 25 mM EDTA, 25 mM Tris-HCl pH 8.0) and then centrifuged (15 minutes x 9000 g). The cells were resuspended in 20 ml of STE
buffer containing lysozyme at 5 mg/ml and incubated at 37 °C for 30 minutes. SDS (1 ml of 10 % w/v) and 50 µl of Proteinase K (20 mg/ml) were added and the reaction was incubated for 20 minutes at 50 °C and then 10 minutes at 65 °C. Following this incubation 3.6 ml of 5 M NaCl was added followed by 3.0 ml of 10 % CTAB in 0.7 M NaCl (pre-warmed to 65 °C). The reaction was mixed well and then incubated at 65 °C for 10 minutes. An equal volume of chloroform/IAA (24:1) was added and then the tubes were mixed to give a homogeneous solution. The phases were then separated by centrifugation (10 minutes x 9000 g). The upper aqueous phase was removed and an equal volume of phenol/chloroform/isoamyl alcohol (IAA) (25:24:1) added, again the reaction was mixed until homogeneous and centrifuged (15 minutes x 12,000 g). The upper aqueous phase was removed and an equal volume of chloroform/IAA added, the solution was mixed until homogeneous and then centrifuged (10 minutes x 9000 g). The upper aqueous phase was removed and 0.6 volumes of isopropanol were added and then the solutions were gently mixed. The precipitated DNA was removed by spooling onto a sealed Pasteur pipette heated to give a hook. The spooled DNA was washed in ice cold 70 % ethanol and left to air dry. Once dry the DNA could be redissolved in water overnight at 4 °C, with a 30 minute incubation at 60 °C before use to ensure even dispersion.

2.20. Quantification of DNA

DNA was quantified by measuring the absorbance at 260 nm. At this wavelength an absorbance value of 1 is equal to a DNA concentration of 50 µg/ml. Alternatively the concentration of fragments of DNA could be estimated by comparison of the intensity of the band, obtained after separation by agarose gel electrophoresis and staining with ethidium bromide, with the intensity of the 1.5 kb band of the 1 kb marker which contains 100 ng of DNA /µg of marker applied. To quantify single stranded oligodeoxyribonucleotides the absorbance at 260 nm was measured. In this case an absorbance value of 1 is equal to 33 µg/ml.
2.21. Restriction Enzyme Digestions

Restriction enzyme digests were carried out using enzymes supplied by Pharmacia Biotech. Reactions were set up according to the information supplied with the enzyme. The One-Phor-All buffer system was utilised which uses a single buffer at various concentrations depending on the requirements of the enzyme.

2.22. Partial Restriction Enzyme Digestion

Small scale restriction digests were prepared containing:

20 µg chromosomal DNA  
1.5 units of SauIII Al  
10 µl 10 x One Phor All buffer  
dH₂O to make up to 100 µl

On addition of enzyme, digests were incubated at 37 °C and 20 µl aliquots taken at times 0, 10, 20, 30 and 40 minutes. Enzyme was heat inactivated by incubating at 85 °C for 30 minutes. Aliquots were separated using gel electrophoresis to allow size determination of the fragments produced. Reactions were allowed to cool to room temperature and then the protein removed by Phenol/Chloroform extraction. The DNA was precipitated with ethanol and dissolved in sterile water to give a final volume of 30 µl giving a DNA concentration of approximately 0.7 µg/µl.

2.23. Agarose Gel Electrophoresis

DNA fragments were separated according to size using agarose gel electrophoresis. The agarose gel was prepared by adding agarose to TAE buffer (Tris Acetate 40 mM, EDTA 1 mM) and then heating until dissolved. Typically an agarose concentration of 0.8 % was used. The gel was cooled to 50-60 °C before the addition of ethidium bromide to a final concentration of 0.5 µg/ml. Gels were allowed to solidify then placed into an electrophoresis tank and covered with TAE buffer. DNA samples were mixed with 6 x loading buffer (10 % Ficoll® 400, 0.25 % Bromophenol Blue, 0.25 % Xylene Cyanol FF) before loading onto the gel. Samples were run at 1-5 V/cm for 1-2 hours.
Chapter 2 – Materials and Methods

The marker used for size estimation was the 1 kb ladder (Life Technologies Inc.). Ladder was loaded onto gels (1 µg for 1 cm lane width) and could be used as a means of estimating the amount of DNA present in a sample as the band at 1636 base pairs makes up 10% (100 ng) of the total DNA present in the marker.
Figure 2.6. 1 kb ladder used as a molecular weight marker for agarose electrophoresis. The vector refers to pBR322.
2.24. Gel Purification of DNA Fragments

DNA was extracted from agarose gels using the Qiagen Gel Extraction Kit. This separates DNA from contaminating agarose using an ion-exchange protocol and doesn’t require the use of low melting point agarose.

2.25. Alkaline Phosphatase Treatment

To prevent the cohesive ends of the digested vector annealing to themselves the DNA was dephosphorylated with calf intestinal alkaline phosphatase (CIAP) (Life Technologies Inc.). The reaction was carried out by adding 1 µl (0.1 units) of CIAP to the heat-inactivated restriction enzyme reaction mixture. The reaction was incubated at 37 °C for 30 minutes and then the CIAP inactivated by incubating at 85 °C for 10 minutes. Following this step the protein was removed by extraction with phenol/chloroform and then the DNA was precipitated with ethanol and resuspended in a volume of 10 µl.

2.26. Ligations

T4 DNA ligase purchased from Life Technologies Inc. was used for all ligations. Ligation reactions contained the following:

4 µl ligase buffer (250 mM Tris.HCl, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% PEG 8000).
0.1 Units T4 DNA ligase
dH₂O up to 20 µl

DNA was added in the ratio 1 part vector: 4 parts insert to give a final amount of 0.1-1 µg DNA. Ligation reactions were incubated at room temperature for 2 hours then used to transform *E. coli*.

2.27. Transformation of *E. coli*

Competent *E. coli* XL-10 Gold cells purchased from Stratagene were used for the production of gene libraries. The transformation was carried out by a heat-shock
method as per the suppliers protocol. Following the heat-shock cells were grown in NZY+ broth at 37 °C for 1 hour before being plated onto LB, Amp, X-Gal, IPTG media, see section 2.4.

2.28. End-Labelling DNA Probes.

Oligodeoxyribonucleotide probes were labelled by addition of radioactive phosphate to the 5' end of the molecule through the action of T4 polynucleotide kinase (T4 PNK). Reactions were set up as follows:

- Oligodeoxyribonucleotide: 10 pmoles
- T4 PNK Buffer (10 x): 5 µl
- [γ-32P]ATP: 20 pmoles (3.7 MBq)
- T4 PNK (3U/µl): 1 µl
- dH2O: up to 50 µl

The reaction was incubated at 37 °C for 30 minutes then the enzyme was inactivated by incubating at 65 °C for 5 minutes. Contaminating ATP was removed using Sephadex G-25 microspin columns (Pharmacia) according to the manufacturer's conditions.

2.29. PAGE of DNA Samples

End-labelled DNA could be tested for presence of label and absence of contaminating radioactive ATP by separation on polyacrylamide gels. The Bio-Rad mini protean II gel system was used to cast and run the gels. Gels were prepared to contain a final concentration of 19% acrylamide (acrylamide/bisacrylamide 19:1), 1 x TBE (90 mM Tris-Borate, 2 mM EDTA) and 7 M Urea. Gels were pre-run at 250 V for 20 minutes prior to addition of sample. The sample was mixed with loading buffer (0.5 x TBE, 80% formamide, 0.1% xylene cyanol FF, 0.1% bromophenol blue) heated to 50 °C for 5 minutes and applied to the gels. Gels were run for 30 minutes at 250 V. The presence of labelled oligodeoxyribonucleotide was detected by exposure to x-ray film.
2.30. Southern Transfers

The transfer of DNA to nylon membranes was carried out according to the method of Southern (Southern, 1975). Prior to capillary transfer the gel was treated by incubating for 10 minutes in depurination buffer (0.125 M HCl) the gel was washed and then incubated in denaturation buffer (0.5M NaOH, 1.5 M NaCl) for 30 minutes. Following the denaturation step the gel was rinsed with dH₂O then incubated for 30 minutes in neutralization buffer (0.5 M Tris.HCl pH 7.5, 1.5 M NaCl). The capillary transfer apparatus consisted of a stack of absorbent sponges soaked with 20 x SSC (3M NaCl, 3M sodium citrate) with a wick of 3MM paper in a tray approximately 6 cm deep. The gel was placed on top of the wick then surrounded with cling film to prevent “short circuiting” of the transfer buffer. Hybond-N nylon membrane (Amersham Pharmacia Biotech) was placed on top of the gel then two sheets of 3MM paper soaked with 20 x SSC were placed on top of the membrane. A stack of paper towels was placed on top of the 3MM paper then a weight of approximately 300 g was applied. The reservoir was filled with 20 x SSC and the transfer of the DNA was allowed to continue for at least 16 hours. Following the transfer the gel was rinsed briefly with 20 x SSC then the DNA was fixed to the membrane using Stratagene UV crosslinking apparatus.

2.31. Hybridisation of Labelled Probe

Prior to hybridisation nylon membranes with bound target DNA were wetted in dH₂O then equilibrated in 2 x SSC. The membrane was prehyridised for a minimum of 30 minutes at the hybridisation temperature in the appropriate buffer (5 x SSC, 5 x Denhardt’s solution, 0.5 % SDS). Denhardt’s solution (100 x) was prepared by adding 1 g of bovine serum albumin, 1 g Ficoll 400 and 1 g polyvinylpyrrolidone to 50 ml dH₂O. A minimum volume of 125 μl of prehybridisation buffer was used for every cm² of membrane. The labelled probe was added directly to the buffer and hybridisation allowed to continue overnight. Excess probe was removed following hybridisation by washing three times with 3 x SSC, 0.1 % SDS for 10 minutes and three times with 2 x SSC, 0.1 % SDS for 10 minutes. Areas of hybridisation were detected by exposing the membrane to x-ray film for up to 7 days.
2.32. Miniprep of Plasmid DNA

An aliquot (1.5 ml) of an overnight culture of *E. coli* in LB Amp was placed into a microfuge tube and centrifuged (1 minute x 12,000 g). The supernatant was removed and the cells resuspended in 100 μl of GTE solution (25 mM Tris.HCl pH 8.0, 10 mM EDTA, 50 mM glucose) and left at room temperature for 5 minutes. Cell lysis solution was added (0.2 N NaOH, 1% SDS; 200 μl) and mixed by inversion then left on ice for 5 minutes. Potassium acetate was added (3M with respect to potassium, 5M with respect to acetate; 150 μl) and then vortexed for 1 second. The reaction was incubated on ice for 5 minutes then centrifuged (5 minutes x 12,000 g). The supernatant was transferred to a fresh tube and the pellet discarded. Phenol/chloroform/IAA (25:24:1; 500 μl) was added and then mixed by vortexing for 1 minute. The phases were separated by centrifugation (5 minutes x 12,000 g) and the upper aqueous phase was transferred to a fresh tube. Chloroform/IAA was added (24:1; 500 μl), mixed by vortexing, and then the phases separated by centrifugation (2 minutes x 12,000 g). The upper aqueous phase was transferred to a fresh tube. The DNA was precipitated by the addition of 2.5 volumes of ice cold absolute ethanol and incubating on ice for 20 minutes. The DNA was pelleted by centrifugation (10 minutes x 12,000 g) and then the pellet was rinsed with 70 % ethanol. The pellet of DNA was dried under vacuum and then dissolved in 50 μl dH2O to give a final concentration of up to 1 μg/μl.

2.33. Polymerase Chain Reaction (PCR)

PCR reactions were set up according to the manufacturers (Promega) protocols, Table 2.3, using the supplied buffers. The cycle conditions are given in the results sections. Because of the proofreading activity of *Pfu* polymerase the enzyme was added last to avoid degradation of primers. All reactions were set up on ice. Reactions were carried out using a Techne Progene thermal cycler.
Table 2.3 Components of PCR reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>dNTP mix (10 mM each nucleotide)</td>
<td>1 μl</td>
</tr>
<tr>
<td>MgCl₂ (Taq only)</td>
<td>0.5 to 3.5 mM</td>
</tr>
<tr>
<td>Primers</td>
<td>20 pmoles of each</td>
</tr>
<tr>
<td>Template DNA</td>
<td>&gt; 0.5 μg</td>
</tr>
<tr>
<td>Polymerase (Pfu or Taq)</td>
<td>1 Unit</td>
</tr>
<tr>
<td>dH₂O</td>
<td>To a final volume of 50 μl</td>
</tr>
</tbody>
</table>

2.34. DNA Sequencing

DNA sequencing was carried out by the Protein and Nucleic Acid Chemistry Laboratory (PNACL) using Big Dye Terminator cycle sequencing reactions and an ABI 377 sequencer.
3.1. Introduction

Sulphamic acid is a strong acid that is of some commercial importance, Figure 3.1. Approximately 60,000 tonnes are produced annually with the majority being used in the manufacture of the non-nutritive sweetener cyclamate, Figure 3.2. In the past sulphamic acid has found uses as a herbicide, as a component of flame retardants and due to its ability to form readily-soluble salts with carbonate- and phosphate-containing deposits sulphamic acid is also used in the removal of limescale from industrial tanks (Metzger, 1994).

Sulphamic acid would appear, potentially, to be a ready source of sulphur and nitrogen for growth of microorganisms. This aspect was originally investigated partly due to the use of sulphamate as a herbicide (Jensen, 1963). Microorganisms able to utilise sulphamate were isolated from leaf mulch and soil samples. This work didn’t go as far as investigating any proteins which might catalyse the degradation of sulphamate. Subsequently there has been interest in the enzymes that cleave sulphur-nitrogen bonds.

An enzyme, cyclamate sulphamatase, EC 3.10.1.2, able to cleave the sulphur nitrogen bond of cyclamate has previously been investigated (Niimura et al., 1974). The enzyme’s action resulted in the formation of sulphate and cyclohexylamine. The enzyme was found to act preferentially on aliphatic sulphamates, with a chain length of 8-10, but showed no action against sulphamic acid. Mahuran et al. (Mahuran et al., 1983) purified the human liver enzyme N-sulfoglucosamine sulphohydrolase, EC 3.10.1.1, which is linked to Sanfilippo A disease, a disorder of glycosaminoglycan metabolism which can result in severe brain damage. This enzyme is involved in the degradation of heparan sulphate and heparin, Figure 3.3.
Chapter 3 – Degradation of Sulphamate

Rein et al. (Rein and Cook, 1999) were able to demonstrate the growth of a mixed culture of microorganisms utilising sulphamate as a source of sulphur. But again this work did not go as far as isolating any of the enzymes involved in the metabolism of sulphamate.

This chapter describes the isolation and identification of a single type of organism able to grow using sulphamate as the sole source of nitrogen and the purification from this organism of an enzyme able to hydrolyse the sulphur-nitrogen bond of sulphamate.

### 3.2. Isolation of Sulphamate-Utilising Bacterium

Bacteria able to utilise sulphamate as the sole source of nitrogen were isolated from soil samples collected from various gardens in and around the University. Samples were used to inoculate flasks containing minimal salts medium PJC which was supplemented with 20 mM succinate as a source of carbon and 10 mM sulphamate as a source of nitrogen. After initial cultivation in liquid medium, cells were spread on to solidified medium to allow the isolation of single types of organism. An initial problem was the ability of cells to grow on solid media where apparently no source of nitrogen was supplied. A possible factor in this phenomenon was the presence of small amounts (0.4 mM) of the chelating agent nitrilotriacetic acid. After this was noted cultivation was carried out using nitrogen-free minimal medium M63. In this manner a single organism capable of utilising sulphamate as the sole source of nitrogen was isolated. The organism grew at 30 °C in medium containing 20 mM succinate and 10 mM sulphamate supplemented with 1 mM MgCl₂. In liquid culture
the bacteria grew as clumps which quickly sank after the cessation of shaking. On solid media the colonies formed were creamy white and approximately 3-4 mM in diameter with irregular edges. When the bacterium was grown with sulphamate replaced by 10 mM NH₄Cl as the source of ammonia for several passages the bacterium still readily utilised sulphamate as the sole source of nitrogen. This indicates that the gene encoding sulphamate hydrolase is likely to be chromosomally encoded.

3.3. Identification of Bacterium

In order to identify the organism isolated, a partial sequence of the 16S rRNA gene was obtained. The first stage was amplification of the gene using PCR. The primers used, which were based on a conserved region of the 16S rRNA gene, were:

FD1 5'-AGA GTT TGA TCC TGG CTC AG-3'
RP1 5'-ACG GHT ACC TTG TTA CGA CTT-3'

Template DNA was obtained by addition of whole cells to the PCR reaction. The reaction was then carried out under the following conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1</strong></td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C 5 minutes</td>
</tr>
<tr>
<td><strong>Step 2</strong></td>
<td>30 cycles</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C 1 minute</td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C 1 minute</td>
</tr>
<tr>
<td>Elongation</td>
<td>74 °C 4 minutes</td>
</tr>
<tr>
<td><strong>Step 3</strong></td>
<td>1 cycle</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>74 °C 10 minutes</td>
</tr>
<tr>
<td><strong>Step 4</strong></td>
<td>1 cycle</td>
</tr>
<tr>
<td>Storage</td>
<td>4 °C up to 12 hours</td>
</tr>
</tbody>
</table>

In order to allow the PCR product to be used as a template for cycle sequencing the primers and other contaminants were removed using the Qiagen PCR Clean Up Kit. Separate sequencing reactions were set up using each of the original primers.
Chapter 3 – Degradation of Sulphamate

The sequence information was used to design internal primers to allow further clarification of the sequence:

**SLF535F**  5’-GTG GTT TGT CGC GTT GTT C-3’

**SLFRev1010**  5’-ACC GAC ATC TCT GCC GGC GTC-3’

The sequence was compared with sequences on the EMBL database, using the FASTA 3 tool (Pearson and Lipman, 1988). The best match obtained was to *Mycobacterium smegmatis*, see Figure 3.4. Over the 1433 bases of the area sequenced there was an identity of 99.7 % with the published sequence (Sander et al., 1996).
Figure 3.4. Comparison of 16S rRNA gene sequence of the sulphamate-utilising organism (Query) with the 16S rRNA gene sequence of *M. smegmatis* (EM_BA1). Primers are underlined.
Chapter 3 – Degradation of Sulphamate

Query_ ACAGGCGGCACACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
EM_BA1 ACAGGCGGCACACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
1060 1070 1080 1090 1100 1110

Query_ TACTGGGCCTAAAGGCTGGGCTAGGTTCCAGCGTTGCTCCCGAAT
EM_BA1 TACTGGGCCTAAAGGCTGGGCTAGGTTCCAGCGTTGCTCCCGAAT
1120 1130 1140 1150 1160 1170

Query_ ACTGCACGCACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
EM_BA1 ACTGCACGCACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
1210 1220 1230

Query_ ACCGCACACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
EM_BA1 ACCGCACACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
1320 1330 1340 1350

Query_ AGTGCACGCACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
EM_BA1 AGTGCACGCACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
1360 1370 1380 1390 1400 1410

Query_ AGTGCACGCACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
EM_BA1 AGTGCACGCACTACGTCGACAGCCGGCCGTAATACGTAGGTTCCAGCGTTGCTCCCGAAT
1420 1430 1440 1450 1460 1470

Query_ TTGCACGCGGCAGCAGCGGAGATGCATCAGCTGTTAGACGCGTTAGAGACGCGGAAAGAAGAA
EM_BA1 TTGCACGCGGCAGCAGCGGAGATGCATCAGCTGTTAGACGCGTTAGAGACGCGGAAAGAAGAA
1480 1490 1500 1510 1520 1530

Query_ CTTACCTGCGCTTGGACATGCACAGGAGGCAGCGGCGGAGATGCATCAGCTGTTAGAGACGCGGAAAGAAGAA
EM_BA1 CTTACCTGCGCTTGGACATGCACAGGAGGCAGCGGCGGAGATGCATCAGCTGTTAGAGACGCGGAAAGAAGAA
1540 1550 1560 1570 1580 1590

59
Chapter 3 – Degradation of Sulphamate

Query_  GTGCAGGTTGTCAGCTGCTGAGACGTGTGGTAAATGCACTCCCGCA
EM_BA1  GTGCAGGTTGTCAGCTGCTGAGACGTGTGGTAAATGCACTCCCGCA

1060 1070 1080 1090 1100 1110
Query_  ACGAGCGCAACCTTGTCTCATGGGAAAGGCTGCGGTACGCTGAGACG
EM_BA1  ACGAGCGCAACCTTGTCTCATGGGAAAGGCTGCGGTACGCTGAGACG

1120 1130 1140 1150 1160 1170
Query_  CCGGGTCAGATCCGAGGAGGTGGGATTGACGTCAGACG
EM_BA1  CCGGGTCAGATCCGAGGAGGTGGGATTGACGTCAGACG

1180 1190 1200 1210 1220 1230
Query_  GGCCTAGTATCGAGTGGACGCTGCTGAGACG
EM_BA1  GGCCTAGTATCGAGTGGACGCTGCTGAGACG

1240 1250 1260 1270 1280 1290
Query_  GGAGCCGGATCGGTAGTTTCCGGCTACCCCGCA
EM_BA1  GGAGCCGGATCGGTAGTTTCCGGCTACCCCGCA

1300 1310 1320 1330 1340 1350
Query_  GTCACAGATCGGCAGACGTTGCGGTAGTTTCCGGCTACCCCGCA
EM_BA1  GTCACAGATCGGCAGACGTTGCGGTAGTTTCCGGCTACCCCGCA

1360 1370 1380 1390 1400 1410
Query_  CCGGCGATCGGTAGTTTCCGGCTACCCCGCA
EM_BA1  CCGGCGATCGGTAGTTTCCGGCTACCCCGCA

1420 1430 1440
Query_  GGAACGGTACGGAAGCTGCGCTAGGACG
EM_BA1  GGAACGGTACGGAAGCTGCGCTAGGACG

60
3.4. Development of an Assay for Sulphamate Hydrolase

The release of ammonium and sulphate ions was anticipated in response to the addition of sulphamate. This was found to be the case with an assay mixture containing 3 mM sulphamate giving rise to a final concentration 2.8 mM ammonia and 2.7 mM sulphate. The equimolar yield of ammonium and sulphate ions indicate that the following reaction was taking place.

\[ \text{SO}_3\text{HNH}_2 + \text{H}_2\text{O} \rightarrow \text{SO}_4\text{H}_2 + \text{NH}_4^+ \]

The production of ammonia from sulphamate was used to measure the activity of sulphamate hydrolase throughout the purification of the enzyme. It was found that addition of EDTA to 1 mM gave an increase in the rate of the reaction of 40% which indicated that divalent cations were not needed for the reaction to proceed. The increase in activity was presumably due to the removal of a contaminating inhibitor by the EDTA. In testing buffers Tris buffer was found to be incompatible with the assay for ammonia which was utilised and increasing the phosphate buffer concentration from 10 mM to 100 mM gave a 20% drop in activity. The final choice for the assay buffer was 10 mM sodium phosphate at pH 7.2. Assays were carried out at 30 °C in a final volume of 1 ml. The reaction typically contained 10 - 30 mM sodium sulphamate as the substrate and 20 μg of purified protein. Reactions were carried out over a 1 hour time period and gave a linear rate.

3.5. Purification of Sulphamate Hydrolase (E.C. 3.10.1.1)

Crude extract was prepared by crushing the frozen cells in a Hughes press and then resuspending in 10 mM sodium phosphate buffer pH 7.2. The extract was sonicated briefly to reduce the viscosity then centrifuged to remove cell debris, see section 2.12. The sulphamate hydrolase was purified until only one band could be detected on an SDS-PAGE gel using Coomassie stain. The first step was to apply the crude extract onto a DEAE-cellulose column. Approximately 27 mg protein was applied on to a 12 ml column. Initially the extract was washed through with 10 mM sodium phosphate buffer pH 7.2 containing 0.2 M NaCl. The eluent was collected in 6 ml fractions until the protein concentration fell to 1/10 of the highest value at which
point the salt concentration was raised to 0.3 M. The higher salt concentration allowed the sulphamate hydrolase to be eluted from the column. The two fractions containing the highest activity were pooled and diluted 2 x to reduce the salt concentration then applied to a Mono-Q HR 5/5 column. The protein was eluted using 10 mM sodium phosphate buffer pH 7.2 with a NaCl gradient from 0 – 1.0 M over 15 ml. The sulphamate hydrolase was eluted at a NaCl concentration of approximately 0.65 M. The 1 ml fraction containing the highest enzyme activity was mixed with an equal volume of 10 mM sodium phosphate buffer pH 7.2 containing 2 M Na₂SO₄. The sample was then applied to a Phenyl Superose column. Protein was eluted using a 15 ml decreasing gradient of 2.0 – 0 M Na₂SO₄ in 10 mM sodium phosphate pH 7.2. The sulphamate hydrolase was eluted at a concentration of approximately 0.45 M Na₂SO₄. Analysis of the sample using SDS-PAGE showed the presence of only one protein. A summary of the purification is shown in Table 3.1.

After the initial purification was used to positively identify the band corresponding to sulphamate hydrolase, which was absent when cells were grown with NH₄Cl as a source of nitrogen, a quicker method was developed. The cell-free extract was clarified by ultracentrifugation (120 minutes x 200,000 g) to allow it to be applied directly to a Mono-Q column as described previously. This step and all subsequent steps were carried out at 4 °C which meant that the Phenyl Superose step was omitted due to the insolubility of the Na₂SO₄ at this temperature. Instead the peak Mono-Q fraction was applied to two Superose 12 columns connected in series, see Table 3.2.
Table 3.1. Summary of the purification of sulphamate hydrolase.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Units (μmol/min)</th>
<th>Specific activity (Unit/mg)</th>
<th>Recovery</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>2</td>
<td>9</td>
<td>0.4</td>
<td>0.044</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>DEAE - Cellulose Chromatography</td>
<td>4</td>
<td>1.16</td>
<td>0.18</td>
<td>0.157</td>
<td>45%</td>
<td>3.5</td>
</tr>
<tr>
<td>Mono-Q Chromatography</td>
<td>1</td>
<td>0.47</td>
<td>0.2</td>
<td>0.43</td>
<td>50%</td>
<td>10</td>
</tr>
<tr>
<td>Phenyl Superose Chromatography</td>
<td>1</td>
<td>0.15</td>
<td>0.1</td>
<td>0.7</td>
<td>25%</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 3.2 Summary of the rapid purification method for sulphamate hydrolase

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Protein (mg/ml)</th>
<th>Total Units (μmol/min)</th>
<th>Specific Activity (Unit/mg)</th>
<th>Recovery</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>6.95</td>
<td>0.44</td>
<td>0.06</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>Mono-Q Chromatography</td>
<td>0.5</td>
<td>0.42</td>
<td>0.84</td>
<td>96%</td>
<td>14</td>
</tr>
<tr>
<td>Superose-12 Chromatography</td>
<td>0.075</td>
<td>0.12</td>
<td>1.6</td>
<td>27%</td>
<td>26.7</td>
</tr>
</tbody>
</table>
Figure 3.5. SDS-PAGE showing stages in the purification of sulphamate hydrolase. Lane 1, crude extract. Lane 2, DEAE-cellulose fraction. Lane 3, Mono-Q fraction. Lane 4, Phenyl Superose fraction. Lane 5, SDS-VII marker.
Figure 3.6. SDS-PAGE showing stages in the rapid purification of sulphamate hydrolase. Lane 1, crude extract. Lane 2, Mono-Q fraction. Lane 3, Superose 12 fraction. Lane 4, SDS-VII Markers.
3.6. Properties of Sulphamate Hydrolase

3.6.1. Physical Properties

When sulphamate hydrolase was separated using SDS-PAGE the sub-unit size of the enzyme was calculated as 50.6 kDa. A more accurate estimate of the sub-unit size was obtained by Dr K. Lilley at PNACL, Leicester University, using mass spectrometry. This method gave a sub-unit size of 50 877 Da, ± 8 Da.

The native molecular mass of the enzyme was estimated using gel filtration. Two Pharmacia Superose 12 columns were connected in series in order to increase the resolution of the system. A series of protein standards were eluted in order to calibrate the column.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Mass (kDa)</th>
<th>Elution volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoferritin</td>
<td>400</td>
<td>20.45</td>
</tr>
<tr>
<td>β-amylase</td>
<td>200</td>
<td>22.5</td>
</tr>
<tr>
<td>IgG</td>
<td>160</td>
<td>23.2</td>
</tr>
<tr>
<td>BSA</td>
<td>66</td>
<td>25.35</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>35</td>
<td>27.15</td>
</tr>
</tbody>
</table>

This data allowed the construction of a calibration curve which was then used to estimate the native molecular mass of the sulphamate hydrolase, see Figure 3.7.

The sulphamate hydrolase was eluted at 21.6 ml which indicates a molecular mass of 286 kDa. This value is equal to 5.62 sub-units of 50.88 kDa. The best estimate of the structure of the enzyme based on this data would be of identical sub-units making up a hexameric protein.
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Figure 3.7. Standard curve for Superose 12 gel-filtration columns.

\[ y = -6.0344x + 54.524 \]

\[ R^2 = 0.9993 \]
3.6.2. Kinetic Properties

The $K_m$ of the enzyme was calculated by measuring the activity of the enzyme at nine substrate concentrations in the range 2.5 – 80 mM. Analysis of the data using Grafit (Erithacus software ltd) indicated a relatively high $K_m$ value of $26.36 \times 10^{-3}$ M ± $4.01 \times 10^{-3}$ M. The analysis returned a $V_{\text{max}}$ value of 0.038 μmoles $\text{NH}_4^+$/sec/mg. Using these values and a molecular mass of 286 000 Da for sulphamate hydrolase a $K_{\text{cat}}$ of 10.9 s$^{-1}$ and a catalytic efficiency value ($K_{\text{cat}}/K_m$) of 413 M$^{-1}$s$^{-1}$ were calculated.

3.6.3. Substrate Specificity

Several alternative substrates were tested to see if the enzyme was able to catalyse the hydrolysis of other molecules. The activity of the enzyme was tested against sulphamoyl benzoate, urea, trimethylamine and cyclamate. In each case a substrate concentration of 10 mM was tested and 0.1 mg of enzyme was used. The reactions were incubated at 30 °C for up to 16 hours and the concentration of ammonia was measured except when cyclamate was used where release of sulphate ions was monitored. Under these conditions sulphamate hydrolase showed no activity against any of the alternative substrates chosen.

3.6.4. Partial Amino Acid Sequence

The N-terminal sequence of the purified sulphamate hydrolase was obtained at PNACL at Leicester University and gave the results shown in Table 3.4. Derek Ellison at Medeeva Development in Liverpool kindly attempted to obtain the C-terminal sequence of the sulphamate hydrolase but the data obtained did not allow a sequence to be assigned. Further attempts to assign more amino acid residues were made by Dr K. Lilley. Proteolytic digestion followed by separation and analysis of the peptides was carried out. Of the peptides sequenced, named Peptide 1 and Peptide 2, the two most successful rounds of sequencing gave the sequences shown in Table 3.5.
Figure 3.8. Plot of the reaction rate of sulphamate hydrolase in response to varying substrate concentration.
Table 3.4. N-terminal amino acid sequence of sulphamate hydrolase

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>Identity</th>
<th>Codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proline</td>
<td>C C N</td>
</tr>
<tr>
<td>2</td>
<td>Glutamate</td>
<td>G A R</td>
</tr>
<tr>
<td>3</td>
<td>Lysine</td>
<td>A A R</td>
</tr>
<tr>
<td>4</td>
<td>Proline</td>
<td>C C N</td>
</tr>
<tr>
<td>5</td>
<td>Asparagine</td>
<td>A A Y</td>
</tr>
<tr>
<td>6</td>
<td>Isoleucine</td>
<td>A T H</td>
</tr>
<tr>
<td>7</td>
<td>Valine</td>
<td>G T N</td>
</tr>
<tr>
<td>8</td>
<td>Tyrosine</td>
<td>T A Y</td>
</tr>
<tr>
<td>9</td>
<td>Phenylalanine</td>
<td>T T Y</td>
</tr>
<tr>
<td>10</td>
<td>Histidine</td>
<td>C A Y</td>
</tr>
<tr>
<td>11</td>
<td>Valine</td>
<td>G T N</td>
</tr>
<tr>
<td>12</td>
<td>Aspartic Acid</td>
<td>G A Y</td>
</tr>
<tr>
<td>13</td>
<td>Asparagine</td>
<td>A A Y</td>
</tr>
<tr>
<td>14</td>
<td>Leucine</td>
<td>T T R/C T N</td>
</tr>
<tr>
<td>15</td>
<td>Glycine</td>
<td>G G N</td>
</tr>
</tbody>
</table>
Table 3.5. Amino acid sequences of the peptides obtained through proteolysis of sulphamate hydrolase.

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>Identity</th>
<th>Codon</th>
<th>Residue No.</th>
<th>Identity</th>
<th>Codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Proline</td>
<td>C C N</td>
<td>1</td>
<td>Tyrosine</td>
<td>T A Y</td>
</tr>
<tr>
<td>4</td>
<td>Glutamate</td>
<td>G A R</td>
<td>2</td>
<td>Phenylalanine</td>
<td>T T Y</td>
</tr>
<tr>
<td>5</td>
<td>Isoleucine</td>
<td>A T H</td>
<td>3</td>
<td>Phenylalanine</td>
<td>T T Y</td>
</tr>
<tr>
<td>6</td>
<td>Proline</td>
<td>C C N</td>
<td>4</td>
<td>Glycine</td>
<td>G G N</td>
</tr>
<tr>
<td>7</td>
<td>Glutamine</td>
<td>G A R</td>
<td>5</td>
<td>Alanine/Glycine</td>
<td>G C N/G G N</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>Alanine</td>
<td>G C N</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>Arginine</td>
<td>C G N/A G R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>Lysine</td>
<td>A A R</td>
</tr>
</tbody>
</table>
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3.6. Attempted Cloning of Sulphamate Hydrolase Gene

Attempts were made to isolate the gene encoding the sulphamate hydrolase. Three methods of isolating the gene were attempted; (i) identification in a gene library of a clone encoding sulphamate hydrolase through phenotypic selection (ii) identification through hybridisation to the DNA encoding the N-terminus of the protein and (iii) amplification of the gene using PCR.

3.6.1. Phenotypic Selection

The first stage of this process required identification of a suitable host organism. *E. coli* was tested for its ability to grow with sulphamate as a source of nitrogen. A sample of *E. coli* XL-10 gold was used to inoculate a flask containing minimal salts medium M63 with 20 mM succinate, 10 µg/ml thiamine, 1 mM MgSO₄ and 30 mM sulphamate. These cells showed no growth even after a period of two weeks. Any inhibitory effect of sulphamic acid on the growth of *E. coli* XL-10 gold was tested by growing the organism in the same selective media but with 10 mM NH₄Cl added as a source of nitrogen. These cells showed strong growth within 48 hours.

The gene library was prepared by partially digesting chromosomal DNA with the frequently cutting restriction enzyme *Sau3AI*. Trial digests were set up and aliquots taken at time intervals 0, 10, 20 and 30 minutes and heat inactivated. The optimum digest time was tested for each preparation of chromosomal DNA. Up to 10 identical digests were carried out and after heat inactivation of the enzyme the DNA was precipitated with ethanol in order to reduce the total volume. The resuspended DNA was separated on a 0.8 % agarose gel in order to isolate DNA in the size range 3 – 6 kb. DNA was purified from agarose slices using the Qiagen gel extraction kit. The size fractionated DNA was then ligated into the *BamHI* site of the general purpose cloning vector pUC18. Following ligation the DNA was transformed into *E. coli* XL-10 gold. The transformed cells were allowed to grow in LB overnight and then washed and resuspended in minimal medium M63 supplemented with 20 mM succinate, 1 µg/ml thiamine and 1 mM MgSO₄ with 30 mM sulphamate as the sole source of nitrogen. Cells were incubated in a gyratory shaker at 200 rpm.
and 30 °C for up to 2 weeks but no growth was detected. In this manner approximately 12,000 clones were tested.

An aliquot of the transformed cells was also grown on solidified LB medium with IPTG, X-Gal and ampicillin, a selection of cells were picked and plasmid DNA prepared in order to ascertain the average size of the insert DNA. The plasmid DNA obtained was cut with the restriction enzymes EcoRI and HindIII and then analysed by electrophoresis. Approximately 95% of the clones tested contained insert DNA and the inserts detected ranged in size from 1 – 12 kb with an average size of 3 kb.
Figure 3.9. Partial digest of *Mycobacterium* sp. chromosomal DNA with *Sau*3AI. Lane 1, 1kb marker. Lane 2, 0 minutes. Lane 3, 10 minutes. Lane 4, 20 minutes. Lane 5, 30 minutes. Lane 6, 40 minutes. Lane 7, 1 kb marker.
3.6.2. Detection Through Hybridisation

The N-terminal amino acid sequence allowed the synthesis of a degenerate oligodeoxyribonucleotide to be used as a probe. The probe was designed to anneal to the DNA encoding amino acid residues 1 to 7 and the initiation codon with bases 1 and 2 being thymine and guanidine on the assumption that ATG or GTG was the initiation codon. With the inclusion of the non-hydrogen bonding base inosine in the oligodeoxyribonucleotide when the base could be A,C,T or G the degeneracy of the oligodeoxyribonucleotide was reduced to 24. The melting temperature of the oligodeoxyribonucleotide was estimated to be 54 °C. The final sequence is shown below.

**Slf N-term. 5' TG CCI GAR AAR CCI AAY ATH GT 3'**

Labelling of the oligodeoxyribonucleotide was carried out using T4 polynucleotide kinase to transfer $^{32}$P from $\gamma^{32}$P-ATP to the 5' terminus of the oligodeoxyribonucleotide. Following the labelling reaction unreacted ATP was removed using Pharmacia Sephadex microspin columns. The labelling reaction and subsequent clean up were monitored by separating an aliquot of the reaction by denaturing PAGE then visualising the separated components by autoradiography.

Chromosomal DNA for hybridisation was digested with the restriction enzymes BamHI or PstI and then separated on an agarose gel. The DNA was then transferred to nylon membrane, Hybond-N+, by Southern blotting. The membrane was divided into two sections to allow hybridisation with (i) labelled oligodeoxyribonucleotide **Slf N-term** and (ii) labelled 16S rRNA gene probe **Fd1**, see page 56. Hybridisation with **Fd1** was carried out in order to provide a positive control for the transfer and hybridisation steps. Hybridisation was carried out overnight at 45 °C and then the blots were washed with buffers containing decreasing salt concentrations. No hybridisation was observed with the oligodeoxyribonucleotide **Slf N-term**, even when the temperature was lowered to 40 °C and 35 °C, but the positive control gave a signal suggesting that the methodology was not at fault. An alternative which was tested was the use of DNA prepared using the mini-prep method given in section 2.32 this gave rise to what appeared to be contaminating chromosomal DNA which
again gave no signal when hybridisation was attempted under the same conditions as the chromosomal DNA. This would appear to provide further evidence that the sulphamate hydrolase gene is chromosomally encoded.
Figure 3.10. Denaturing PAGE of labelled oligodeoxyribonucleotide. Lane 1, 1 µl labelling reaction containing oligodeoxyribonucleotide Fd1. Lane 2, 1 µl labelled oligodeoxyribonucleotide after removal of unreacted ATP. Lane 3, 1 µl of labelling reaction containing oligodeoxyribonucleotide S1 f N term. Lane 4, 1 µl labelled oligodeoxyribonucleotide after removal of unreacted ATP.
Figure 3.11. Autoradiograph of *Mycobacterium* sp. chromosomal DNA probed with oligodeoxyribonucleotide Fd1. Lane 1, 1 kb marker. Lane 2, BamHI digested DNA. Lane 3, PstI digested DNA.
3.6.3 PCR Methodology

The primer **Slf N-term** based on the N-terminal amino acid sequence of the sulphamate hydrolase was used for PCR in an attempt to amplify a fragment of the gene encoding the enzyme. By cloning fragments of genomic DNA into the vector pUC18 a primer based on the nucleic acid sequence of pUC18 could then be used as the second primer in the PCR reaction. Because the orientation of the ligated fragment was random, primers from both sides of pUC18 were used, in separate reactions, to increase the chance of amplification. The two primers used are given below.

**M13 FWD** 5'-TGT AAA ACG ACG GCC AGT-3'

**M13 REV** 5'-CAG GAA AC A GCT ATG ACC-3'

Samples of chromosomal DNA were digested using the enzymes *BamHI* or *PstI* and then ligated into pUC18 which had also been cut with the *BamHI* or *PstI* respectively then dephosphorylated. The ligation reaction was used directly in the PCR reaction. The conditions used are shown below.

<table>
<thead>
<tr>
<th>Step 1</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C 1.5 minutes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 2</th>
<th>30 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C 30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>37 °C 30 seconds</td>
</tr>
<tr>
<td>Elongation</td>
<td>72 °C 5 minutes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 3</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Elongation</td>
<td>72 °C 10 minutes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 4</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage</td>
<td>4 °C up to 12 hours</td>
</tr>
</tbody>
</table>

*Taq* polymerase was used for the PCR reactions.

Analysis of the reactions using agarose gel electrophoresis showed amplification but control experiments using just one of the primers in each reaction showed that all of
the amplification was due to the pUC18 primers with the reaction containing just Slf N-term giving no amplification.

Another PCR procedure which was tested utilised the internal sequence obtained through sequencing peptides of sulphamate hydrolase, see Table 3.3. The amino acid sequence of peptide 2 was converted to a degenerate DNA sequence. The degenerate DNA sequence was then reversed and complemented to give a sequence which could be utilised as a PCR primer. This primer was called SLF PEP2.

**SLF PEP2 5' -TTN CKN GCI SCN CCR AAR AAR TA-3'**

Slf N-term was utilised as the second primer in the reaction. The PCR was carried out using Pfu polymerase and genomic DNA from *Mycobacterium* sp.

<table>
<thead>
<tr>
<th>Step 1</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
</tr>
<tr>
<td></td>
<td>1.5 minutes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 2</th>
<th>30 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
</tr>
<tr>
<td>Annealing</td>
<td>40 °C</td>
</tr>
<tr>
<td>Elongation</td>
<td>72 °C</td>
</tr>
<tr>
<td></td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>1 minute</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 3</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Elongation</td>
<td>72 °C</td>
</tr>
<tr>
<td></td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 4</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage</td>
<td>4 °C</td>
</tr>
<tr>
<td></td>
<td>up to 12 hours</td>
</tr>
</tbody>
</table>

Analysis of the PCR reactions by agarose gel electrophoresis showed no product.
3.7. Summary & Discussion

A single type of organism able to utilise sulphamic acid as the sole source of nitrogen was isolated from a soil sample. The organism was identified as a Mycobacterium sp. by comparison of the gene encoding the 16S rRNA. A protein which was able to catalyse the hydrolysis of the sulphur-nitrogen bond giving rise to equimolar amounts of ammonium and sulphate ions was purified. The rate of the reaction was determined by monitoring the build up of the end product ammonia. The protein, termed sulphamate hydrolase, was found by mass spectrometric analysis to have a sub-unit mass of 50 877 Da, ± 8 Da which is only 277 Da (0.5 %) higher than the value obtained from analysis of SDS-PAGE separations. Gel filtration chromatography showed the native molecular mass of the enzyme to be approximately 286 kDa. The most likely structure for the enzyme given this evidence would be a hexamer comprised of identical sub-units. Sulphamate hydrolase was shown to have a relatively high $K_m$ value of 26.3 mM ± 4.01 mM towards sulphamate and the values obtained for $K_{cat}$ (10.9 s$^{-1}$) and the catalytic efficiency (413 M$^{-1}$s$^{-1}$) were relatively low. SDS-PAGE analysis showed that the sulphamate hydrolase made up to 10 % of the total cellular protein in crude extracts. It may be the case that because of its low affinity for sulphamate and its low rate of turnover that sulphamate hydrolase has to be produced in large amounts in order to provide nitrogen at a sufficient rate for the growth of the cells. Sulphamate hydrolase may have been recruited from another function within the cell so may not be ideally suited to catalysing the hydrolysis of the sulphur-nitrogen bond.

Analysis of the gene for sulphamate hydrolase may have revealed if the protein had already been assigned a role in the cell, or any similarity to other known proteins could have given clues as to its true role. Despite trying to isolate the gene encoding sulphamate hydrolase, by selection for growth of positive clones, through hybridisation to DNA encoding the N-terminal sequence and by PCR, the gene was not isolated. One aspect of cloning is how many hybrid plasmids must be tested in order to be reasonably confident that a clone can be found. The number of clones
that must be tested to give a reasonable chance of isolating a target sequence can be estimated using the equation,

\[ N = \ln \left( \frac{1-P}{\ln \left[ \frac{1}{1-G} \right]} \right) \]

where \( N \) is the number of clones, \( I \) is the average insert size, \( P \) is the probability and \( G \) is the size of the target genome. The size of the \( M. \text{smegmatis} \) genome is quoted as being \( 4.35 \times 10^9 \) Da, or approximately 6.5 Mbp (Bradley, 1973) and the average insert cloned was approximately 3 kb. With these figures, to give a 99% chance of finding a suitable clone, approximately 10,000 clones would need to be tested. At least this number of clones was tested.

Selection of a clone using growth as an indicator may be problematic although it has proved successful in previous situations. The most obvious problem is the need for an essentially complete copy of the gene encoding sulphamate hydrolase to be present to allow the production of a functional protein. This problem should be overcome by the use of a partial restriction digest which gives rise to a range of fragments not necessarily cut at every restriction site present. Another problem which could arise is the difference in the codon usage between \( \text{Mycobacterium} \) sp. and \( E. \text{coli} \). \( M. \text{smegmatis} \) has a G/C content of approximately 66%, with a marked bias towards codons with G or C at the 3rd position (Dale and Patki, 1990). This difference was thought to present a major problem in expression of Mycobacterial genes in \( E. \text{coli} \) but the cloning and expression of a number of genes has proven successful (Boshoff and Mizrahi, 1998). But this has doubtless been a stumbling block in the expression of some genes. Successful growth selection would require the transcription of the gene for sulphamate hydrolase. A gene cloned into the pUC18 vector which was to make use of the vector’s promoter would have to be sufficiently close and also in the correct reading frame and orientation to allow transcription and translation of a functional protein. A fragment of DNA which contained the sulphamate hydrolase gene and an \( \text{Mycobacterium} \) sp. promoter wouldn’t necessarily support transcription if the sigma factor associated with the \( E. \text{coli} \) RNA polymerase didn’t bind to the Mycobacterial promoter.

A final problem with this method of selection is the manner in which the sulphamate is transported across the cell membrane. It may be the case that
organisms able to utilise sulphamate have a specific transport system, or it may be a case of passive diffusion. The transport may require the presence of a unique protein which would mean that the *E. coli* would only be able to utilise sulphamate if it was able to express a functional sulphamate hydrolase and a functional membrane transport protein. If transport of the sulphamate was a matter of simple diffusion across the membrane then raising the concentration of sulphamate in the medium should result in higher levels inside the cell. This would mean that if a functional protein was being produced by a positive clone, even in low amounts, then the large amounts of sulphamate present could negate the effects of the low amounts of sulphamate hydrolase and the enzymes relatively low affinity for sulphamate. This would allow a positive clone to produce ammonia at a sufficient rate for growth.

A strategy which could have overcome some of the difficulties of phenotypic selection would have been to utilise a different host organism for a gene library prepared using the *Mycobacterium* sp. DNA ligated into a suitable vector. Mutant strains of *M. smegmatis* are available which can be transformed using electroporation at efficiencies of greater than $10^5$ transformants per µg of DNA (Snapper *et al.*, 1990). If a strain of *M. smegmatis* could have been found which was unable to utilise sulphamic acid as a source of nitrogen it could have been ideal. *M. smegmatis* has been adopted as a host strain by many groups cloning genes from the pathogenic species of Mycobacteria due its saprophytic nature and its relatively fast growth. Using *M. smegmatis* as a host organism could have circumvented difficulties with differences in promoters and codon bias between this organism and *E. coli*.

Selection of positive clones through hybridisation with a labelled oligodeoxyribonucleotide should be simpler in terms of identification even though the technical aspects of the work are more demanding. A short oligodeoxyribonucleotide designed using data from the N-terminal amino acid sequence has proven sufficient to identify many genes. None of the difficulties with expression of functional protein or transport of metabolite across the bacterial membrane should apply where hybridisation is utilised. Hybridisation to DNA encoding the N-terminal sequence of the sulphamate hydrolase proved unsuccessful. The use of the probe Fd 1, which hybridises to the ribosomal RNA gene found in all
bacteria, showed that a signal could be obtained by the methodology used and that the template DNA isolated from the *Mycobacterium* sp. was suitable for hybridisation. The question then arises as to why the probe **Slf N-term** gave no hybridisation.

The relatively small amount of N-terminal sequence data meant that the design of the probe was limited. The area chosen gave a probe with a degeneracy of 24 if the neutral base inosine was included in the sequence. Ideally the probe would have a low degeneracy. Any signal obtained would be slightly reduced with only 1/24 of the labelled oligodeoxyribonucleotide present having the correct sequence to bind to the sulphamate hydrolase gene. During experiments highly sensitive double sided X-ray film was used and exposures were carried out at -80 °C for periods of up to 1 week. Although these measures increase the background signal they should have overcome the problem presented by low amounts of hybridised probe. Despite these measures no hybridisation could be detected between the probe **Slf N-term** and the chromosomal DNA prepared from *Mycobacterium* sp. Another approach which could be tried is to design a variety of probes to the N-terminal sequence data. It may be the case that a different choice of probe may anneal more successfully even if the degeneracy of the probe chosen is higher.

In order for hybridisation to occur the sequence of interest must be present. The target DNA was chromosomal DNA isolated from *Mycobacterium* sp. grown in minimal medium M63 with sulphamate present as the sole source of nitrogen. The cells used for inoculation of the liquid cultures were isolated from plates free from contaminating organisms. The possibility that the sulphamate hydrolase might be plasmid-encoded was tested for by treating cells with lysozyme then carrying out a standard alkaline lysis mini-prep. The only DNA obtained appeared to be contaminating high molecular weight chromosomal DNA which again gave no signal in hybridisation experiments. More evidence that the sulphamate hydrolase is likely to be chromosomal was the fact that cells grown with ammonium chloride as the sole source of nitrogen readily utilised sulphamate even after several passages. The loss of a metabolic pathway from an organism when selective pressure is removed can be a sign that the trait is encoded on a plasmid. It may be the case that the sequence chosen isn't suitable for hybridisation and that further N-terminal
sequence data would yield sequence which would allow the design of alternative primers. The internal sequence information obtained from peptides was less reliable than the information obtained by N-terminal sequencing of the full protein and unfortunately the sequence gave rise to primers with high degeneracies.

The final method that was utilised in an attempt to obtain the sequence of the gene encoding sulphamate hydrolase was PCR. This is a sensitive method and was utilised with great success to amplify the gene encoding the 16S rRNA directly from whole cells and genomic DNA. Ideally to amplify the gene encoding sulphamate hydrolase a primer based on the N-terminal and the C-terminal would have been utilised. The attempt to obtain C-terminal sequence was unsuccessful. Two alternative approaches were attempted instead. The first was to ligate fragments of *Mycobacterium* sp. chromosomal DNA into the plasmid pUC18 and utilise primers designed to anneal to the pUC18 sequence. Unfortunately this approach was unsuccessful. The technique attempted has certain drawbacks. Ideally for a PCR procedure of this type a primer with low degeneracy could be found although primers with high degeneracy have been used to amplify genes successfully. Another difficulty is the disparity between the amounts of target sequence for each of the primers. In a standard reaction the target sequence of each primer would be present in equal amounts allowing the addition of equimolar amounts of each primer. It is difficult to judge how the ratio of each primer should be altered to allow a successful amplification. Commercial kits which utilise the same methodology are available which should circumvent some of the difficulties of this method by utilising linkers which are designed specifically for this purpose. It should be noted however that this technique is more successful in allowing PCR to extend from regions of known sequence into regions of unknown sequence i.e. a specific primer is preferable (Siebert et al., 1995). The second PCR strategy attempted was the use of a primer based on the internal amino acid sequence obtained from peptide 2, see Table 3.4, although again this was unsuccessful. With the position of the peptide in the protein being unknown the size of the final product can only be estimated. With a sub-unit size of 50.8 kDa the size of the gene encoding sulphamate hydrolase can be estimated at 1.37 kb (assuming the average mass of an amino acid residue to be
111 Da). This gives a value for the maximum size of the product but the actual size of the PCR product could range from 45 – 1370 bp.

Without any product appearing it is difficult to judge what steps should be taken to improve a PCR reaction. If non-specific priming is occurring a number of steps can be taken such as raising the annealing temperature, lowering the Mg$^{2+}$ concentration to increase specificity or using protocols such as touchdown PCR which starts at a high annealing temperature and then drops each cycle so as to provide a bias towards the required product. Template DNA which has a high G/C content can also be problematic for PCR reactions, again there are steps which can be taken to try and minimise the problem. Dimethyl sulphoxide or betaine can be added to the PCR reaction and are thought to reduce the secondary structure of the DNA allowing better amplification. Alternatively certain polymerases are available which are intended for use with high G/C template DNA. Probably the biggest factor in the failure to obtain amplification in this case is the choice of primer which is limited due to the relatively small amount of sequence data which is available.
CHAPTER 4 – DEGRADATION OF SACCHARIN
4.1. Introduction

Since its discovery in 1879 saccharin has had a somewhat chequered history. It was first synthesised by Constantin Fahlberg who was alerted to its properties by the sweetness of his lunch, presumably due to contamination after working with the compound. On leaving the lab of his supervisor, Ira Remsen, Fahlberg promptly proceeded to patent the compound as a sweetener. This led to a lengthy legal battle between the pair with Fahlberg eventually winning out. The use of saccharin as a sweetener in foods led to the first U.S. federal food law, with the chief chemist of the Department of Agriculture, Harvey Wiley, its sworn enemy. The furore eventually led to the proclamation by President T. Roosevelt that “Anybody who says that saccharin is injurious to health is an idiot”. Roosevelt was being prescribed saccharin by his doctor in the hope of preventing diabetes. Saccharin is still found today in a variety of products from soft drinks to toothpaste but the battle over its safety was very long-winded with the discovery that rats fed saccharin were prone to bladder cancers.

Saccharin is composed of an isothiazolin ring fused to an aromatic ring, see Figure 4.1. It is largely used as a non-calorific sweetener and research has shown that of an oral dose given to mammals 99% is excreted in the urine unchanged with the remaining 1% being excreted as 2-sulphamoyl benzoate, see Figure 4.2 (Zubair and Hassan, 1984). It has been noted that the development of tumours following feeding of sodium saccharin is unique to rats and occurs predominantly in male rats that have been given sodium saccharin from a very early age. The presence of sodium saccharin in the diet of these animals can lead to increased urinary pH, sodium
concentration and volume. There is also an alteration in urothelium proliferation and the tendency of male rats to accumulate urine to use for scent marking may increase their susceptibility to tumours. There is no evidence that saccharin binds to DNA so it is unlikely to have a direct mutagenic effect (Oser, 1985). The National Institute of Environmental Health Sciences of America removed saccharin from their list of potential carcinogens in May 2000.

There is little or no information about the fate of saccharin in the environment. On the surface it would appear to be a ready source of carbon, for growth and energy, nitrogen and sulphur. The first step in its degradation may be the opening of the isothiazolin ring leaving the nitrogen and sulphur atoms open to attack. This would then make the aromatic ring available for degradation via ring hydroxylation. The first question is how the isothiazolin ring is cleaved. Cleavage of the isothiazolin ring could give 2-sulphamoyl benzoate, the product of alkaline hydrolysis of saccharin, or 2-sulphobenzamide which is the product of acid hydrolysis of saccharin. A variety of compounds containing similar functional groups, i.e. benzamide (Nawaz et al., 1992), benzoic acid and sulphobenzoate (Locher et al., 1991), are all known to be degraded by microorganisms but there is no specific reference to the degradation of either 2-sulphobenzamide or 2-sulphamoyl benzoate in the literature. A proposed pathway that shows how the molecule could be degraded is shown in Figure 4.2. The removal of the \( \text{NH}_2 \) group from either molecule would leave 2-sulphobenzoate. Desulphonation could then occur resulting in the release of sulphite.

This chapter describes the isolation of a mixed culture of bacteria able to utilise saccharin as a carbon and nitrogen source. The isolation, from this community, of an organism able to utilise saccharin as the sole source of nitrogen and a second organism able to utilise benzoate and catechol as a source of carbon is described as well as the analysis of the end products of growth.
Chapter 4 — Degradation of Saccharin

Figure 4.2 Possible routes of saccharin metabolism
Chapter 4 – Degradation of Saccharin

4.2. Isolation of Bacteria Able to Utilise Saccharin

To isolate bacteria able to utilise saccharin as a source of carbon and nitrogen flasks containing nitrogen-free minimal salts medium M63 supplemented with 10 mM saccharin and 1 mM MgCl$_2$ were inoculated with soil samples collected from various locations around Leicester University. This eventually yielded a mixed culture that utilised saccharin as a carbon and nitrogen source and contained approximately nine distinguishable types of colony when grown on rich LB medium. When grown in liquid medium at 30 °C the culture had a doubling time of approximately seven days. It was found that supplementing the growth medium with yeast extract to a final concentration of 0.0025 % decreased the doubling time of the culture to a maximum of 24 hours. Spreading samples of culture onto solid media yielded no single colonies able to grow utilising saccharin as the sole source of carbon and nitrogen. This was taken to mean that no single type of bacterium was able to open the isothiazolin ring to yield ammonium and sulphite and then go on to attack the aromatic moiety to give carbon and energy.

4.2.1. Isolation of a Bacterium Able to Utilise Saccharin as Nitrogen Source

Organisms able to carry out the initial steps in the degradation of saccharin were sought by supplementing the minimal medium with 20 mM succinate and inoculating with the mixed population of organisms capable of growth on saccharin. The culture was passaged through several flasks with samples of the initial growth being used to inoculate the next flask. The mixture of cells was then transferred to solid minimal medium for single colony isolation. A single type of colony predominated and this was isolated and initially designated sacc. This bacterium was able to grow as a pure culture in nitrogen-free M63 supplemented with 20 mM succinate, 10 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract. Further investigations of its ability to use various carbon sources for growth showed that the bacterium could also utilise acetate but was unable to grow on benzoate, glucose or glycerol.
4.2.2. Isolation of a Bacterium Able to Utilise Benzoate as a Carbon Source

The mixed population of bacteria isolated for their ability to utilise saccharin as a carbon and nitrogen source showed the ability to utilise benzoate as carbon source if the nitrogen-free M63 was also supplemented with \( \text{NH}_4\text{Cl} \). Cells able to utilise benzoate were spread onto solidified nitrogen-free M63 containing 5 mM benzoate, 5 mM \( \text{NH}_4\text{Cl} \), 1 mM MgSO\(_4\) and 0.0025 % yeast extract and single colonies were picked. The most prominent type of bacterium, designated benz, was one that produced highly spreading growth. These cells showed strong growth when used to inoculate nitrogen-free M63 containing 5 mM benzoate, 5 mM \( \text{NH}_4\text{Cl} \), 1 mM MgSO\(_4\) and 0.0025 % yeast extract. The cells were also able to grow on solidified nitrogen-free M63 containing 5 mM catechol, 5 mM \( \text{NH}_4\text{Cl} \), 1 mM MgSO\(_4\), 0.0025 % yeast extract and 1.8 mM dithionite (included to slow down oxidation of catechol). The cells showed no growth when inoculated into medium containing saccharin as the sole source of carbon.

Having isolated the bacteria "sacc" and "benz" the ability of the two bacteria to grow together with saccharin as the source of carbon and nitrogen was tested. Both organisms were used to inoculate nitrogen-free M63 supplemented with 10 mM saccharin, 1 mM MgCl\(_2\) and 0.0025 % yeast extract. As a co-culture the bacteria showed strong growth utilising saccharin.

4.3. Identification of Bacteria

Identification of the bacteria isolated was carried out by sequencing the 16S rRNA gene of each of the organisms. The primers for the amplification and sequencing were the same primers used to amplify the 16S rRNA gene of the *Mycobacterium* sp. discussed in Chapter 3.

\[
\text{FD1} \ 5'\text{-AGA GTT TGA TCC TGG CTC AG-3'} \\
\text{RP1} \ 5'\text{-ACG GHT ACC TTG TTA CGA CTT-3'}
\]

Template DNA was obtained by addition of whole cells to the PCR reaction. The reaction was then carried out under the following conditions.
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<table>
<thead>
<tr>
<th>Step 1</th>
<th>1 cycle</th>
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<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C 5 minutes</td>
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<tr>
<th>Step 2</th>
<th>30 cycles</th>
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<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C 1 minute</td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C 1 minute</td>
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<tr>
<td>Elongation</td>
<td>74 °C 4 minutes</td>
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<tr>
<th>Step 3</th>
<th>1 cycle</th>
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<tr>
<td>Final Elongation</td>
<td>74 °C 10 minutes</td>
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</table>

<table>
<thead>
<tr>
<th>Step 4</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage</td>
<td>4 °C up to 12 hours</td>
</tr>
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</table>

In order to allow the PCR product to be used as a template for cycle sequencing the primers and other contaminants were removed using the Qiagen PCR Clean Up Kit. Separate sequencing reactions were set up using each of the original primers FD1 and RP1. Following the first round of sequencing further primers were designed to clarify the internal sequence of each organism.

The additional primers used to clarify the internal sequence of sacc were:

- **ALC605F**
  
  5’- TAA CTA CCG AGC TAG AGT GTG TC-3’

- **ALCREV1050**
  
  5’- CAT GCA GCA CCT GTG TTC CG-3’

The additional primer used to clarify the internal sequence of benz was:

- **PSEFD300**
  
  5’- CAG ACT CCT ACG GGA GGC AGC-3’

By comparing the compiled sequences obtained with sequences in the EMBL database using the FASTA 3 tool a best match was found for each organism. The organism able to utilise saccharin as a source of nitrogen, sacc, was identified as an *Alcaligenes* sp. because of its 99.9 % identity with an *Alcaligenes* sp. in the database (Hoffmann *et al.*, 1998). The results of the search are shown in Figure 4.3. The organism isolated for its ability to utilise benzoate as a source of carbon, benz, was found to have a 98.6 % identity with *Pseudomonas pseudoalcaligenes*, see Figure 4.4, so is best classified as a *Pseudomonas* sp. (Moore *et al.*, 1996).
### Figure 4.3 Comparison of the 16S rRNA gene sequence of the saccharin-utilising organism (sacc) with the 16S rRNA gene of *Alcaligenes* sp. (EM_BA1).

Identical bases are marked (\(\cdot\)).

Primers are underlined.
Chapter 4 – Degradation of Saccharin

sacc, CTTGTCAATTGCTACGAAAGGGCACTCTAAATGAGACTGCGGTGAGCAAAACCAGGAGG
EM_BA1 CTTGTCAATTGCTACGAAAGGGCACTCTAAATGAGACTGCGGTGAGCAAAACCAGGAGG

sacc, AAGGTGGGATGACGCTAGTCTACATTGGCTACATCGCACCACACGTACATA
EM_BA1 AAGGTGGGATGACGCTAGTCTACATTGGCTACATCGCACCACACGTACATA

sacc, ATGGTCGGACAGAGGTCGGACACCAGGGAGGGAGACACATCGCACCGATAC
EM_BA1 ATGGTCGGACAGAGGTCGGACACCAGGGAGGGAGACACATCGCACCGATAC

sacc, CTGCACTCGTGTAGCTGGCGGCTTGTCAGAGGTCCTAGGTTGCTACGAGGAGTACGCTACGAGG
EM_BA1 CTGCACTCGTGTAGCTGGCGGCTTGTCAGAGGTCCTAGGTTGCTACGAGGAGTACGCTACGAGG

sacc, CATGACGGGGT
EM_BA1 CATGACGGGGT

sacc, CATGACGGGGT
EM_BA1 CATGACGGGGT
Figure 4.4 Comparison of the 16S rRNA gene sequence of the benzoate-utilising organism (benz) with the 16S rRNA gene of Pseudomonas pseudoalcaligenes. (EM_BA2). Identical bases are indicated by (:), the underlined areas indicate primers.
Chapter 4 – Degradation of Saccharin

480 490 500 510 520 530
benz, CGTGCAGCGACCGCGGTAAATACGAAGGGTGCAAGCGTTAATCAGAAATCTGGGCTAA

EM_BA2 CGTGCAGCGACCGCGGTAAATACGAAGGGTGCAAGCGTTAATCAGAAATCTGGGCTAA

540 550 560 570 580 590
benz, AGGCACGTATAGGTGATTCTTAAAGTTGGAATGGAAGGCCCTCAACTTGGAACCT

EM_BA2 AGGCACGTATAGGTGATTCTTAAAGTTGGAATGGAAGGCCCTCAACTTGGAACCT

600 610 620 630 640 650
benz, CATCCAAAACCTGGCGACCGAGCTAGCAGTACGAGAGGTGTTACGTAGGAATTTCTGTGCTAGCCT

EM_BA2 CATCCAAAACCTGGCGACCGAGCTAGCAGTACGAGAGGTGTTACGTAGGAATTTCTGTGCTAGCCT

660 670 680 690 700 710
benz, AAATGC GT AGAT AT AGGAAGGAAC AC C AGTGGC GAAGGC GAC C AC C TGGAC TGATAC TGA

EM_BA2 AAATGC GT AGAT AT AGGAAGGAAC AC C AGTGGC GAAGGC GAC C AC C TGGAC TGATAC TGA

780 790 800 810 820 830
benz, CATGGCTGTCGTCAGCTCGTGTCGTGAGATGGTTTGTTCAAGTCCTAAGCGCAACC

EM_BA2 CATGGCTGTCGTCAGCTCGTGTCGTGAGATGGTTTGTTCAAGTCCTAAGCGCAACC

900 910 920 930 940 950
benz, CACAAGCCTGAGAGCATGTTTTTACATCACCAAGCCGAGAACGTTACCTTACCTGCACTTG

EM_BA2 CACAAGCCTGAGAGCATGTTTTTACATCACCAAGCCGAGAACGTTACCTTACCTGCACTTG

960 970 980 990 1000 1010
benz, ACATGCTGAGAATCTCCAGATGAGATTGGTGCTCGCAACTCAAGACACAGCTGCTG

EM_BA2 ACATGCTGAGAATCTCCAGATGAGATTGGTGCTCGCAACTCAAGACACAGCTGCTG

1020 1030 1040 1050 1060 1070
benz, CATGGCTTGCTCAGCTGTGGTGCTAGTGATTTTGTTAACTGCTCTGGTAAGCGGAGCACC

EM_BA2 CATGGCTTGCTCAGCTGTGGTGCTAGTGATTTTGTTAACTGCTCTGGTAAGCGGAGCACC

98

Growth of the co-culture containing *Alcaligenes* sp. and *Pseudomonas* sp. in nitrogen-free M63 medium containing 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract was examined. Three lots of media were set up; flask A was inoculated with the *Alcaligenes* sp., flask B was inoculated with the *Pseudomonas* sp. and flask C was inoculated with both organisms. The *Alcaligenes* sp. used for the inoculum were grown in nitrogen-free M63 with 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract and the cells were washed with nitrogen-free M63 before use. The *Pseudomonas* sp. cells used for the inoculum were grown in nitrogen-free M63 with 5 mM benzoate, 5 mM NH$_4$Cl, 1 mM MgSO$_4$ and 0.0025 % yeast extract and these cells were washed with nitrogen-free M63 before use. The media from each flask was assayed for bacteria (using the turbidity at 680 nm as a guide to the number of cells), ammonium ions, sulphate ions, sulphite ions, absorbance at 270 nm and a sample was taken for analysis by HPLC. The absorbance spectrum of saccharin shows a peak at 270 nm (see Figure 4.5), this wavelength was analysed in the growth medium in order to give an indication of the amount of saccharin present. From standard solutions a molar extinction coefficient of 1318 M$^{-1}$cm$^{-1}$ was calculated for a solution of saccharin in nitrogen-free M63, see Figure 4.6.

Initially all of the flasks showed a small amount of growth, this could probably be attributed to the yeast extract present in the growth medium. After this initial growth flask C, inoculated with both organisms, was the only one which showed any significant amount of growth. The culture had a doubling time of approximately 24 hours and a maximum absorbance value of 0.45 was reached after 18 days, see Figure 4.7.

Only when both types of organism were present was there a significant amount of growth utilising saccharin as the source of carbon and nitrogen. The requirement for carbon atoms in biomass is greater than that of sulphur and nitrogen (approximately 30 C: 5 N: 1S) so an accumulation of these atoms could be expected in the growth medium as the carbon atoms of the aromatic ring are utilised. This was indeed the
case. Figure 4.8 shows the increase in the concentration of ammonium ions in flask C. A final value of 7.1 mM ammonia was detected in flask C indicating that 4.4 mM of the original 11.5 mM nitrogen supplied as saccharin was utilised by the cells. It should be noted that a slight accumulation of ammonium ions was also detected in the flask inoculated with *Alcaligenes* sp. (A). This could presumably be attributed to the enzyme activity of the cells in the original inoculum but with no usable source of carbon to allow growth the release of ammonia is minimal.

Figure 4.10 shows the increased presence of sulphite ions when both species were present. Sulphite would be expected if desulphonation of the aromatic ring was occurring to allow the further use of the ring carbon. Sulphite builds up to levels of about 2 mM in flask C but quickly drops to zero, presumably on the oxidation of sulphite to sulphate. A flask containing nitrogen-free M63 supplemented with 11.5 mM saccharin, 1 mM MgCl$_2$, 0.0025% yeast extract and 2 mM Na$_2$SO$_3$ was also set up but not inoculated with bacteria and incubated under the same conditions as flasks A, B and C. Measurement of the concentration of sulphite in this flask showed a drop to undetectable levels within 24 hours. Figure 4.9 shows the increase in the concentration of sulphate ions in flask C. The final concentration of sulphate ions, 12.4 mM, is actually somewhat higher than the original concentration of sulfur supplied as saccharin.

Using the absorbance at 270 nm the original concentration of saccharin in the flasks was calculated at 11.5 mM. In flask C this value dropped to 0.8 mM over the period monitored. Flasks A and B showed no significant change in the saccharin concentration.
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Figure 4.5 Absorbance spectrum of saccharin

Figure 4.6. Standard curve of saccharin absorbance

\[ y = 1.318x + 0.001 \]
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Figure 4.7 Measurement of cell growth by monitoring Abs 680. Flasks contained nitrogen-free M63 supplemented with 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract, flask A was inoculated with *Alcaligenes* sp., flask B was inoculated with *Pseudomonas* sp. and flask C was inoculated with both organisms.

Figure 4.8 Measurement of ammonia concentration present in growth medium. Flasks contained nitrogen-free M63 supplemented with 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract, flask A was inoculated with *Alcaligenes* sp., flask B was inoculated with *Pseudomonas* sp. and flask C was inoculated with both organisms.
Figure 4.9 Measurement of sulphate ion concentration in the growth medium. Flasks contained nitrogen-free M63 supplemented with 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract, flask A was inoculated with *Alcaligenes* sp., flask B was inoculated with *Pseudomonas* sp. and flask C was inoculated with both organisms.

Figure 4.10 Measurement of the sulphite ion concentration in growth medium. Flasks contained nitrogen-free M63 supplemented with 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract, flask A was inoculated with *Alcaligenes* sp., flask B was inoculated with *Pseudomonas* sp. and flask C was inoculated with both organisms.
Figure 4.11 Estimation of the saccharin concentration in the growth medium. Flasks contained nitrogen-free M63 supplemented with 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl₂ and 0.0025 % yeast extract, flask A was inoculated with *Alcaligenes* sp., flask B was inoculated with *Pseudomonas* sp. and flask C was inoculated with both organisms.
4.5. Growth with Saccharin as a Source of Nitrogen

The *Alcaligenes* sp. was able to grow with saccharin as the source of nitrogen if succinate was included as a source of carbon and energy. Growth of the organism was measured by monitoring the increase in absorbance at 680 nm and aliquots of growth media were analysed for the presence of ammonia, sulphate, sulphite and the absorbance at 270 nm in order to provide an indication of the concentration of saccharin present.

The cells showed much quicker growth than was observed when the co-culture was utilising saccharin as the sulphur and nitrogen source, see Figure 4.12. The maximum absorbance value of 0.22 was reached within 2 days. This indicates a doubling time of approximately 4 hours.

Rather surprisingly the concentration of saccharin, as estimated by the absorbance at 270 nm, showed very little change with the growth of the organisms, Figure 4.16. This may be due to the build up of an aromatic ring containing compound which may also show some absorbance at 270 nm thus leading to little change in the value.

Analysis of the concentration of ammonium ions in the media, Figure 4.13, showed an increase in the amount present. This would indicate that the cells were continuing to release nitrogen from saccharin despite there being a build up of excess ammonium in the medium. The same phenomenon was observed with the concentration of sulphite and sulphate in the medium. During growth there was an increase in the concentration of sulphite ions in the medium, Figure 4.15. This peaked at 1.8 mM and then presumably the sulphite was oxidised to sulphate. Figure 4.14 shows the steady increase in the concentration of sulphate ions. The greatest increase in sulphate ion concentration occurs as the concentration of sulphite ions show their greatest decrease.

The most striking occurrence accompanying growth of the *Alcaligenes* sp. was the change in appearance of the growth medium from clear to deep brown, Figure 4.17. This occurred after approximately 4 days of incubation and was presumably due to the end product of saccharin metabolism.
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The release of excess ammonia and sulphite shows that the *Alcaligenes* sp. continues to degrade saccharin despite there being no apparent metabolic pressure to do so. This was further investigated by inoculating the *Alcaligenes* sp. into nitrogen-free M63 containing 20 mM succinate, 0.0025 % yeast extract and 1 mM MgSO₄ supplemented with 11.5 mM saccharin and 10 mM NH₄Cl as the nitrogen source. Despite strong growth there was no formation of the black pigment suggesting that the *Alcaligenes* sp. was utilising NH₄Cl in favour of saccharin,
Figure 4.12. Measurement of the absorbance at 680 nm to monitor growth of *Alcaligenes* sp. utilising saccharin as a source of nitrogen.

Figure 4.13. Measurement of the ammonia concentration of the growth medium of *Alcaligenes* sp. utilising saccharin as a source of nitrogen.
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Figure 4.14. Measurement of the sulphate ion concentration of the growth medium of *Alcaligenes* sp. utilising saccharin as a source of nitrogen

Figure 4.15. Measurement of the sulphite ion concentration of the growth medium of *Alcaligenes* sp. utilising saccharin as a source of nitrogen
Figure 4.16. Estimation of the saccharin concentration of the growth medium of *Alcaligenes* sp. utilising saccharin as a source of nitrogen
Figure 4.17. Photograph of flasks containing nitrogen-free M63, 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl₂, 0.0025 % yeast extract inoculated with *Alcaligenes* sp. The flask on the left demonstrates an example of the medium immediately after inoculation whilst the flask on the right shows the deep brown colour produced after approximately 4 days incubation.
4.6. HPLC Analysis of Growth Medium

Growth medium was analysed using HPLC in order to monitor the use of saccharin and to identify any intermediates or end-products which may have accumulated. Flasks A and B which contained saccharin as the carbon and nitrogen source and were inoculated with *Alcaligenes* sp. and *Pseudomonas* sp. respectively showed no change in their HPLC elution profiles indicating that neither of the organisms was able to transform saccharin. Analysis of the medium from flask C which was inoculated with both organisms showed a large drop in the area of the peak corresponding to saccharin indicating that the saccharin was being utilised and was presumably the source of the ammonia and sulphate which accumulated, see Figure 4.18.

The media from the flask containing *Alcaligenes* sp. utilising saccharin as a source of nitrogen, showed a drop in the amount of saccharin present but not the complete disappearance, Figure 4.19. The appearance of a second peak indicated the presence of an end product which was presumably what eventually caused the medium to turn brown. The compound was found to co-elute with catechol, Figure 4.20. Fractions containing the compound of interest were collected and the absorption spectra was also found to be the same as that of catechol, Figure 4.22. To get further evidence as to the identity of the second peak, aliquots were collected and analysed using mass spectrometry. The results from this indicated that the compound had a molecular weight of 109. A molecular weight of 109 would correspond to the molecular weight of catechol after losing a proton in the bombardment process.

Another clue as to the identity of the unknown metabolite is that on standing, solutions containing catechol become dark brown due to oxidation which eventually results in the formation of melanin like compounds. The formation of melanin like molecules may be what contributes to the brown colour of the medium after growth with saccharin as the source of nitrogen.
Figure 4.18. HPLC elution profile of medium from cells utilising saccharin as a source of carbon and nitrogen. The medium was composed of nitrogen-free M63, 11.5 mM saccharin, 1 mM MgCl₂, 0.0025 % and inoculated with Alcaligenes sp. and Pseudomonas sp. Medium was analysed after (i) 0 days, (ii) 4 days and (iii) 6 days.
Figure 4.19. HPLC elution profile of medium from *Alcaligenes* sp. utilising saccharin as a source of nitrogen. Medium was composed of nitrogen-free M63, 20 mM succinate, 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025 % yeast extract. Medium was analysed after (i) 0 days and (ii) 3 days growth.
Chapter 4 – Degradation of Saccharin

Retention time = 4.995 minutes
Peak area = 1356208 μV*s

Figure 4.20. HPLC elution profile of catechol, 20 ppm

Retention time = 2.75 minutes
Peak area = 2581555 μV*s

Retention time = 5.006 minutes
Peak area = 597992 μV*s

Figure 4.21. HPLC elution profile of catechol and growth medium from Alcaligenes sp. utilising saccharin as a source of nitrogen. Growth medium containing nitrogen-free M63, 20 mM succinate, 11.5 mM saccharin, 1mM MgCl₂, 0.0025 % yeast extract, inoculated with Alcaligenes sp. and sampled after 3 days growth (see Figure 4.19 ii), was mixed with catechol (20 ppm) in a 1:1 ratio.
Figure 4.22. Absorbance spectrum of catechol
4.7. Attempts at Elucidating the Pathway of Saccharin Degradation

The production of ammonia and sulphite in response to growth with saccharin does not provide any details on the mechanisms of saccharin transformation in the cell. Several strategies were used in an attempt to gain more information.

4.7.1. Growth Experiments

The *Alcaligenes* sp. was tested for growth on compounds that were proposed as possible intermediates in the degradation of saccharin. The compounds 2-sulphamoyl benzoate, 2-sulphobenzamide and benzamide were supplied as the nitrogen source in nitrogen-free M63 containing 20 mM succinate, 1 mM MgSO₄ and 0.0025 % yeast extract. The compounds were added to the growth medium at a final concentration of 5 mM. No growth over the levels supported by the yeast extract alone was detected even after periods of up to two weeks.

The identity of the component in the yeast extract which was supporting the increased rate of growth was tested for by substituting a defined mixture of trace elements and vitamins for the yeast extract. The trace element solution contained ZnCl₂ - 70 mg/L, MnCl₂.4H₂O - 100 mg/L, CoCl₂.6H₂O - 200 mg/L, NiCl₂.6H₂O - 100 mg/L, CuCl₂.2H₂O - 20 mg/L, NaMoO₄.2H₂O - 50 mg/L, Na₂SeO₃.5H₂O - 26 mg/L and 1 ml per litre of basal medium was used. The vitamin solution contained biotin - 2 mg/L, nicotinic acid - 20 mg/L, thiamine - 10 mg/L, 4-aminobenzoate - 10 mg/L, pantothenate - 5 mg/L, pyridoxamine - 50 mg/L, cyanocobalamin - 20 mg/L and 2 ml of the vitamin solution was used per litre of basal medium. The growth rate of the *Alcaligenes* sp. in nitrogen-free M63 containing 20 mM succinate, 10 mM saccharin and 1 mM MgSO₄ supplemented with the trace element and vitamin solution was comparable to the low growth rate of the *Alcaligenes* sp. in medium which did not contain yeast extract.

4.7.2. Initial Transformations by Non-Growing Cells

*Alcaligenes* sp. cells grown in nitrogen-free M63 with saccharin present as the source of nitrogen were harvested by centrifugation (10 minutes x 12,000 g) in the mid-logarithmic phase of growth. The cells were resuspended in 10 mM phosphate
buffer pH 7 to give a calculated A$_{680}$ value of 6.0. The cells were divided into two aliquots and saccharin was added to the test sample to a final concentration of 10 mM. The cells were incubated at 30 °C overnight and then each sample was tested for breakdown products of saccharin by the HPLC methodology utilised for analysis of growth media. The HPLC traces of the test and the control samples showed no difference from the initial samples. Analysis of the samples for ammonium and sulphite was also carried out, in this case no difference between the test and control samples was detected.

4.7.3 Enzymatic Activities

4.7.3.1. Amidase or Sulphamate Hydrolase Activity

The presence of ammonium ions in the growth medium suggests that the *Alcaligenes* cells might able to cleave the sulphur-nitrogen bond of the proposed intermediate 2-sulphamoyl benzoate or the carbon-nitrogen bond of the proposed intermediate 2-sulphobenzamide. Cleavage of the sulphur-nitrogen bond of 2-sulphamoyl benzoate would presumably be through the action of a sulphamate hydrolase whilst the cleavage of a carbon-nitrogen bond could be through the action of an amidase enzyme. The end result of either reaction would be the release of ammonium ions. The inability of the *Alcaligenes* sp. to utilise either of these substrates for growth may be due to a problem with the uptake of these compounds. The preparation of cell-free extracts may circumvent this difficulty. Cell extracts were prepared from *Alcaligenes* sp. cells grown to mid logarithmic phase and harvested by centrifugation (10 minutes x 12,000 g). The cells were washed once with a phosphate buffer pH 7 and then if the Hughes press was used to prepare extracts cell pellets were frozen. Alternatively extracts prepared by sonication were resuspended in 10 mM sodium phosphate buffer pH 7. Extracts prepared using the Hughes press were crushed then resuspended in 10 mM sodium phosphate buffer pH 7. The viscosity of the solution was reduced through the addition of approximately 1 mg of DNase and incubated for 1 hour at 4 °C. Cellular debris was removed by centrifugation (10 minutes x 12,000 g). Cell extracts prepared through sonication were sonicated for 4 x 15 seconds at $\lambda = 10$ microns with cooling times of 1 minute. The cellular debris was removed by centrifugation (10 minutes x
Reactions as described in section 2.8 were prepared using the cell-free extracts. Up to 1 mg of protein was added to the final reaction and the substrates saccharin, 2-sulphamoyl benzoate, 2-sulphobenzamide and benzamide were used at final concentrations of 5 mM. The reactions were incubated at 30 °C and aliquots removed periodically and tested for the production of ammonia. Measurements at incubation times of up to 24 hours showed that the extracts produced no more ammonia that reactions incubated without substrate.

4.7.3.2. Desulphonative Activity

The accumulation of sulphite and sulphate ions in the growth media by Alcaligenes sp. utilising saccharin as a source of nitrogen even when sulphate was supplied in the form of MgSO₄ indicates that some sort of desulphonative activity may be present. The action of a desulphonative system was tested for in cell-free extracts prepared using the Hughes press or through sonication in the same manner as the extracts prepared to test for release of ammonia. One difference was that as well as phosphate buffer pH 7 an extract was prepared in Tris.HCl buffer pH 7. Desulphonative activity was tested for by measuring the accumulation of sulphite and sulphate ions in the assay mixture in response to addition of the substrates saccharin and 2-sulphobenzoate to final concentrations of 5 mM. The assay buffer used was the same as the buffer the relevant extract was prepared in. The co-factors NADH and NADPH were included in the reaction to final concentrations of 0.5 mM. The reactions included up to 1 mg of protein and were incubated at 30 °C and allowed to continue for up to 16 hours. The analysis indicated that there was no build up of sulphate or sulphite ions. The same reactions were also carried out using an oxygen electrode to measure any consumption of oxygen. In this case the extract was ultracentrifuged to remove any cell membranes which would give oxygen consumption in the presence of NADH or NADPH. No increase in the rate of oxygen consumption could be measured in response to addition any of the substrates.

4.7.3.3. Dioxygenase Activity

The presence of the ring cleavage enzymes utilised during growth of the mixed culture with saccharin as the source of carbon was tested for in cell-free extracts.
Chapter 4 – Degradation of Saccharin

Cells were prepared by inoculating nitrogen-free M63 containing 10 mM saccharin, 1 mM MgCl₂ and 0.0025 % yeast extract with the Alcaligenes sp. and the Pseudomonas sp. The cells were grown to mid-logarithmic phase then harvested by centrifugation (10 minutes x 12,000 g) then washed once with 10 mM sodium phosphate buffer pH 7 and centrifuged (10 minutes x 12,000 g). The cells were broken by sonication, 8 x 15 seconds at λ = 10 microns with cooling times of 1 minute. The cell-debris was removed by centrifugation (10 minutes x 12,000 g). The resulting cell-free extract was tested for the presence of the ring cleavage enzymes catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, protocatechuate 1,2-dioxygenase and protocatechuate 2,3-dioxygenase using the assays given in section 2.9 (page 38), The presence of catechol 1,2-dioxygenase activity was detected. An initial assessment of the purification of this enzyme was carried out. The extract was centrifuged (120 minutes x 200,000 g) then applied to a Mono-Q column and eluted with a gradient of 0 – 1.0 M NaCl over a volume of 15 ml. The catechol 1,2-dioxygenase activity was found in fractions eluting at a NaCl concentration of approximately 0.45 M. An attempt to further separate the enzyme by applying the Mono-Q fraction to a Phenyl Superose column then eluting with a gradient of 1.0 – 0 M (NH₄)₂SO₄ over 15 ml failed with the activity being spread over the last 5-6 ml of the gradient giving a dilution of the enzyme. Analysis of the Mono-Q and Phenyl Superose fractions by SDS-PAGE did not allow identity of a band corresponding to catechol 1,2-dioxygenase.

When cell-free extracts were prepared using the Pseudomonas sp. grown in nitrogen-free M63 supplemented with 5 mM benzoate, 5 mM NH₄Cl, 1 mM MgCl₂ and 0.0025 % yeast extract the only dioxygenase activity found was catechol 1,2-dioxygenase. When the cell-free extract was fractionated using a Mono-Q column, by the protocol used for the mixed culture grown with saccharin as carbon source, the dioxygenase activity was found in the same fractions. Again further purification using a Phenyl Superose column was unsuccessful.
4.8. Summary and Discussion

A population of bacteria was isolated which was able to utilise saccharin as the sole source of carbon and nitrogen. By using succinate as a source of carbon a single type of bacterium able to utilise saccharin as a source of nitrogen was isolated from the population. After obtaining the sequence of the gene for the 16S rRNA of this organism it was possible to identify the bacterium as an *Alcaligenes* sp. From the original population another bacterium able to utilise benzoate as a source of carbon was isolated and identified as a *Pseudomonas* sp. Together the *Alcaligenes* sp. and the *Pseudomonas* sp. were able to completely degrade saccharin. During growth there was release of ammonia and sulphate with the transient accumulation of sulphite. When the *Alcaligenes* sp. was grown with saccharin as the sole source of nitrogen there was also a build up of a product which resulted in the growth medium turning black after approximately four days of growth. Using HPLC and mass spectrometry the product was identified as catechol.

When the *Alcaligenes* sp. utilised saccharin as a source of nitrogen there was release of excess ammonium ions, sulphite and the end product catechol. These products would seem to indicate that the *Alcaligenes* sp. is able to open the isothiazolin ring and then through a series of reactions cleave an amide bond or a sulphur-nitrogen bond to yield ammonium ions and then continue to alter the molecule in such a way as to remove a carboxyl group and a sulphonyl group resulting in formation of catechol and sulphite. Even when MgSO₄ was included in the growth medium at concentrations of up to 1 mM there was still formation of the black end product indicating that the organism is still carrying out the same reactions despite there being no apparent metabolic pressure upon it to do so. This might indicate that the organism is unable to produce nitrogen from the saccharin molecule without first carrying out the series of reactions which also yield sulphite and catechol. Alternatively the enzymes which carry out the reactions could be constitutively produced or perhaps are all translated as part of the same operon. Growth of the organism in nitrogen-free M63 containing 20 mM succinate, 0.0025 % yeast extract and 1 mM MgCl₂ supplemented with 10 mM saccharin and 10 mM NH₄Cl as the nitrogen source did not give rise to the black colour which would suggest that the
enzymes involved in the degradation of saccharin are not constitutively produced. The measurement of growth, ammonia and sulphite (see Figures 4.12 - 4.14) shows a slight plateau at around days 2 and 3 but then another slight rise which coincides with the formation of the black product. The rise may be due to the effect of the black pigment on the spectrophotometric measurement.

The discovery of catechol 1,2-dioxygenase activity in mixed cultures of the cells growing with saccharin as the source of nitrogen and in the *Pseudomonas* sp. growing with benzoate as the source of carbon suggests that the *Pseudomonas* sp. is carrying out the latter stages of the degradation of saccharin via the β-ketoadipate pathway. The excretion of catechol by the *Alcaligenes* sp. and the ability of the *Pseudomonas* sp. to utilise catechol as a carbon source indicates that this is the point where the metabolism of saccharin changes from the *Alcaligenes* sp. to the *Pseudomonas* sp. The release of catechol and sulphite by the *Alcaligenes* sp. indicates that desulphonation is occurring but the reaction does not accompany ring cleavage (see section 1.7). It seems likely that desulphonation accompanies hydroxylation of the ring by a dioxygenase or a monooxygenase.

The analysis of the medium during growth utilised the absorbance at 270 nm to provide an estimate of the concentration of saccharin. The concentration as estimated by the $A_{270}$ value is subject to interference from other molecules which absorb at this wavelength such as amino acids, nucleotides or end products of metabolism which still contain an aromatic ring. In this case the best example is catechol which is presumably why there was no apparent change in the saccharin concentration as measured at 270 nm when the *Alcaligenes* sp. utilised saccharin as source of nitrogen. The best solution to this problem would probably be to quantify the components of the growth medium using HPLC which would allow the separation of molecules absorbing at 270 nm.

The use of HPLC to separate and identify the unknown metabolite as catechol was successful. It would be useful to further utilise HPLC to specifically quantify the amount of saccharin and catechol at various stages in the growth of the culture. An autosampler would make this aspect easier but the use of an internal standard such
Chapter 4 - Degradation of Saccharin

as benzoic acid to offset slight differences in volume through manual injections could increase the accuracy of the process.

The analysis of the sulphate concentration in the flask containing nitrogen-free M63 11.5 mM saccharin, 1 mM MgCl$_2$ and 0.0025% yeast extract inoculated with the *Alcaligenes* sp. and the *Pseudomonas* sp. indicated that the sulphate concentration reaches levels of approximately 13 mM. This figure is within 10% of the saccharin concentration as estimated using the absorbance at 270 nm but does suggest that the assay may be susceptible to interference from components which are excreted into the growth medium or possibly the cellular debris of growth. It may be useful to investigate deproteination and filtration of the samples with a view to increasing the robustness of the assay.

None of the enzymes that may be involved in the initial steps of the degradation of saccharin were detected. There are a variety of reasons why no activity might be detected. One of the most obvious is inactivation of one or more of the enzymes during the preparation. Also because the reactions which are being investigated are essentially unknown it is difficult to guarantee that the proper parameters are being measured. It might be possible to alter the method by which the cells extracts are prepared. Although the Hughes press method is regarded as a gentle means of preparation it might be the case that it is unsuitable for these cells and although sonication is commonly carried out there is generation of heat which could have deleterious effects on proteins. A variety of methods exist for preparing cell-free extracts and several remain to be tested. One which could be assessed is the preparation of cell extracts using lysozyme and mild detergents to permeabilise cells. This method has the advantage that no mechanical devices are required which could make it possible to carry out the preparation under anaerobic conditions. The method can also be utilised using previously frozen or fresh cells. Oxidation of essential components of the sulphobenzoate dioxygenase was pinpointed as a major problem during the preparation of cell-free extracts from *Comamonas testosteroni* (Locher et al., 1991).

An alternative to isolating the enzymes involved in the degradation of saccharin is to study the genes encoding the proteins. It could be feasible to examine the genes
involved directly through either a gain of metabolic function in an organism such as *E. coli* by transformation with a gene library or through loss of metabolic function in the *Alcaligenes* sp. The advantage of using a gain of metabolic function in screening for genes is the ease of screening transformants. Essentially organisms could be screened for their ability to grow utilising saccharin as a source of nitrogen. The disadvantages of this system have been discussed in chapter 3 and include the requirement for a full copy of the gene to be present and for a functional protein to be produced from the gene. If the reaction required several proteins it would also greatly increase the difficulty of expressing the phenotype in *E. coli*. The use of a cosmid library could be an advantage. The ability to clone approximately 50 kb fragments could circumvent the difficulties associated with the smaller fragments used in plasmid libraries such as the cloning of multiple genes encoding the proteins of a relatively complex metabolic pathway. Another way of gaining information about the pathway without the need for further attempts at preparing proteins could be to try to disrupt the genes coding for the enzymes of the pathway by the use of chemical mutagenesis or transposon insertion mutagenesis. This strategy also has the advantage that the disruption of genes may lead to the build up of intermediates by blocked mutants which could allow their identification giving more insight into the reactions involved in the degradation of saccharin.
CHAPTER 5 - CONCLUDING REMARKS & FUTURE AIMS
5.1. Degradation of Saccharin

The isolation of an organism able to carry out the initial steps in the degradation of saccharin lays the foundation for analysis of the reactions involved in the pathway. The identification of the end products produced by the *Alcaligenes* sp. does answer some questions but leaves many more unanswered. From the identification of catechol as the final product it reveals that the organism is able to open the isothiazolin ring and remove the groups remaining through oxidation. Some sort of amidase or sulphamate hydrolase activity could be assumed due to the build up of ammonia in the medium and a desulphonation reaction is occurring. The presence of catechol rules out the reaction mechanism shown in Figure 1.5 of desulphonation accompanying ring cleavage, it would seem more likely that desulphonation is occurring through the action of an oxygenase enzyme which adds hydroxyl groups to the ring but does not cleave the ring. The lack of success in isolating any of the intermediates of the pathway indicates that a new strategy would seem to be required. The means most likely to yield results is the formation of blocked mutants. The formation of mutants has the advantage that if successful the strategy would allow the identification of pathway intermediates and the mutants could also prove useful in genetic studies. There are several means of creating mutants but the most appealing method would be the use of transposons. Of the types of transposons available a recent innovation is the production of stable complexes of transposable elements bound to transposase protein (Goryshin *et al.*, 2000). This system is said to overcome the need for expression of proteins involved in transposition in target microbes. This strategy has the advantage that mutants could be selected for the acquisition of a trait such as antibiotic resistance and then tested for the disruption of the genes involved in saccharin through metabolic tests such as the loss of the ability to utilise saccharin as the nitrogen source. It may be the case that a mutant which is still able to use saccharin as a source of nitrogen is blocked at a later stage in the pathway, this could result in the loss of formation of catechol and the black pigment which could also be noted relatively easily. The use of a system such as transposon mutagenesis also has the advantage that any useful mutants obtained will
have a specified sequence inserted into their genome which would allow the identification of relevant areas by direct sequencing or through DNA hybridisation.

Another method of disrupting genes is through mutagenesis using mutagens such as ultraviolet light or ethyl methanesulphonate (EMS). This method could provide blocked mutants which might allow the identification of intermediates of the pathway. Chemical mutagens are slightly advantageous to UV mutation because of the lower mortality rate for a given number of mutants. A common procedure is to grow the cells of interest in a selective medium, e.g. nitrogen-free M63 with 20 mM succinate, 10 mM saccharin, 1 mM MgSO$_4$, 0.0025 % yeast extract, then expose them to the mutagen for a number of generations. Following this the cells are once again grown in a selective media with penicillin included in order to select specifically for growing cells. Penicillin affects growing cells by interfering with membrane synthesis. One of the greatest difficulties of screening for a loss of function is finding a suitable growth condition for selection. In this case the obvious choice would be to isolate cells able to grow utilising NH$_4$Cl as the source of nitrogen but unable to utilise saccharin as the source of nitrogen. One of the potential difficulties is the relatively high levels of “background” growth possibly due to the presence of the yeast extract which is a relatively non-defined source of nutrients. Attempts to identify the essential component present in the yeast extract which greatly increases the growth rate were unsuccessful. A variety of vitamins and co-factors were tested.

Mutagenesis makes it more difficult to isolate the genes which have been affected but it could be possible to try and overcome the blocked steps by transforming mutants with a gene library. This could allow the identification of genes by analysing fragments which “rescue” blocked mutants. Another problem to be overcome would be the selection of a plasmid suitable for use in Alcaligenes sp. The parameters for the transformation would also need to be optimised. Electroporation is a relatively robust method and could be the first choice for attempting transformations.
5.2. Degradation of Sulphamate

The isolation of an organism able to cleave the nitrogen-sulphur bond of sulphamate demonstrates that the mechanism could feasibly be present in a pathway for the degradation of isothiazolin rings. The isolation of the *Mycobacterium* sp., closely related to the saprophytic organism *Mycobacterium smegmatis*, and the subsequent study of sulphamate hydrolase showed that the organism was able to use sulphamate as a nitrogen source through the use of an inefficient enzyme as illustrated by the low turnover number (10.9 s⁻¹), high $K_m$ value ($26.36 \times 10^{-3} \text{ M}$) and the low value for catalytic efficiency (413 M⁻¹s⁻¹). The relatively large percentage of the sulphamate hydrolase in comparison to the total cellular protein suggests that the organism has overcome the inefficiency of the enzyme by producing it in large amounts. An interesting future aim would be the study of the gene encoding sulphamate hydrolase in order to gain information as to the identity of the protein. Analysis could give clues as to whether the protein is novel or has another role in the cell from which it has been adapted due to overlap of specificity or possibly through minor mutations.

The approaches utilised to isolate the gene encoding sulphamate hydrolase were unsuccessful. The most promising future approach could be to continue with selection of a clone through the gain of ability to utilise sulphamate but in this case to utilise an alternative host organism to *E. coli*. An organism which had similar codon usage patterns and promoter sequences could overcome the main difficulties associated with expressing genes between different organisms. *Mycobacterium smegmatis* is used in the study of genes from the pathogenic Mycobacteria such as *M. tuberculosis* and *M. leprae* and obtaining a culture to test for the ability to grow utilising sulphamate would be the first step. Strains are available which have mutations which give rise to high electroporation transformation efficiencies and could prove useful in future experiments. Two outcomes can be foreseen, if the *M. smegmatis* strain is unable to utilise sulphamate it would be the ideal host organism for screening a gene library prepared from the organism isolated during this study. If the *M. smegmatis* strain is able to utilise sulphamate as a nitrogen source then the initial step would be to try and characterise any proteins involved in the process by checking sub-unit size, native MW and possibly N-terminal sequence.
to find any similarity between it and sulphamate hydrolase. If the enzymes were similar a new approach would be to try and obtain mutants of the \textit{M. smegmatis} strain which were unable to utilise sulphamate by the approaches outlined in section 5.1, by chemical mutagenesis or transposon mutagenesis, and then use the same approaches to try and isolate the gene or genes affected.
APPENDIX
### IUBMB Degeneracy Codes for Nucleic Acids

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<th>Symbol</th>
<th>Meaning</th>
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<td>A</td>
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<td>G or A</td>
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<td>T or C</td>
<td>PYrimidine</td>
</tr>
<tr>
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<td>A or C</td>
<td>Amino</td>
</tr>
<tr>
<td>K</td>
<td>G or T</td>
<td>Keto</td>
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<tr>
<td>S</td>
<td>G or C</td>
<td>Strong interaction (3 H bonds)</td>
</tr>
<tr>
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<td>Weak interaction (2 H bonds)</td>
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<td>A or C or T</td>
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</tr>
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<td>G or T or C</td>
<td>not-A, B follows A</td>
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