

AROMATIC CATABOLISM BY ESCHERICHIA COLI:

Cloning and Analysis of the 3,4Dihydroxyphenylacetate
Pathway Genes of Escherichia coli C.

John Roland Jenkins BSc.(Hons).

A thesis submitted in accordance with the requirements
of the University of Leicester for the degree of
Doctor of Philosophy.

Department of Biochemistry,

April 1987.

UMI Number: U369181

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U369181

Published by ProQuest LLC 2015. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346



Statement.

The accompanying thesis submitted for the degree of Ph.D, is base on work conducted by the author in the Department of Biochemistry of the University of Leicester during the period between October 1983 and October 1986.

All the work recorded in this thesis is origi nal unless otherwise acknowledged in the text or by references. None of the work has been submitted for any other degree in this or any other university.

Signed:.....

Date:..... 3.4.87.....

Abstract.

Escherichia coli C can utilise 3,4-dihydroxyphenylacetate as a carbon and energy source for growth. The biochemical and genetic characterisation of the chromosomally encoded pathway involved is described in this thesis.

The structural genes have been cloned and found to lie in two operons, *hpcBCDEF* and *hpcGH*, both of which were negatively controlled by the regulatory gene *hpcR*. There also appeared to be an additional level of control acting in a positive manner, mediated by cAMP.

Various subclones isolated in this analysis were useful in the production of pathway intermediates which were identified by a variety of means. The use of the pathway intermediates in the biochemical characterisation of the 3,4DHPA catabolic pathway has lead to the identification of a novel step. The use of both biochemical and genetic techniques in the analysis of the pathway has enabled a greater understanding of the interacting mechanisms of the pathway.

Acknowledgements.

Firstly I would like to thank my supervisor, Dr.R.A.Cooper, for his help and criticisms, especially those of the written work. I am grateful to all the members of the School of Biological Sciences for their advice and for providing the strains and plasmids. I would also like to thank Dr.P.Cullis for his help with the NMR analysis.

I am grateful to every one who has provided moral support and backing, friends, parents, Dave, and especially Nicky.

Finally I acknowledge the SERC for their funding of the studentship.

Table of contents.

Page

No.

1.0 Introduction.

1	Aromatic catabolism.
2	Ring cleavage.
4	Convergence of pathways involved in the catabolism of aromatic compounds.
8	Divergence of microbial evolution.
15	Genes encoding the enzymes for aromatic catabolism.
15	Genetic analysis of aromatic catabolic pathways.
22	Other plasmid-encoded aromatic catabolic pathways.
24	Chromosomally-encoded aromatic pathways.
25	The use of genetic techniques to aid the fine biochemical analysis of aromatic pathways.
28	The use of pathways involved in aromatic metabolism for commercial purposes.
31	The Hydroxyphenylacetate and 3,4Dihydroxyphenylacetate Pathway.
34	Regulation of the HPA pathway.
38	Aims of the project.

2.0 Methods.

39	Bacterial strains and plasmids.
39	Media.
39	Cell-free extracts.
42	Washed-cell suspension for measurement of oxygen consumption.
42	Isolation of intermediates in the 3,4DHPA pathway.

Assays.

44	a) HPA hydroxylase (EC.1.14.13.3).
45	b) 3,4DHPA 2,3dioxygenase assay. (EC 1.13.11.15)
45	c) 3,4DHPA assay.
46	d) CHMS dehydrogenase assay.
46	e) CHM isomerase assay.
47	f) COHED decarboxylase assay.

Page

NO.

47	g) HHDD isomerase assay.
48	h) OHED hydratase assay.
49	i) HHED aldolase assay.
49	j) The formation of pyruvate.
50	Protein purification.
51	FPLC.
51	Nuclear magnetic resonance spectrometry. (NMR)
	<u>Genetic procedures.</u>
51	Strain construction.
52	Phenol extraction of protein from DNA.
53	Ethanol precipitation of DNA.
53	Preparation of chromosomal DNA.
54	Bulk preparation of plasmid DNA.
55	Plasmid mini preparations.
56	Agarose gel electrophoresis.
56	Preparation of λ -phage DNA.
57	Restriction enzyme digestion.
57	Ligation reactions.
58	Phosphatase treatment.
58	Preparation of vectors.
59	Isolation of DNA fragments from agarose gels.
59	Transformation.
60	Tn 1000 mutagenesis.
	Preparation of λ -packaging extracts.
61	a) Sonicated extract from induced strain BHB2690 (prehead donor).
62	b) Freeze/thaw lysate from induced strain BHB2688 (Packaging protein donor).
63	Transfer of nucleic acids from agarose gels to nitrocellulose paper.
63	Screening bacterial colonies by hybridization.
66	Oligo-labelling of DNA by hexadeoxynucleotide primer.
68	Hybridization of radioactive probes to DNA.
69	Mutagenesis using ethylmethanesulphonate(EMS).
70	Conjugal matings.
70	Acrylamide gel electrophoresis.
74	DNA sequencing.
76	Analysis of sequence Data.

3.0 RESULTS.

77 3.1 Isolation and characterisation of pJJ801.

82 Identification of the enzymes encoded by pJJ801.

88 The expression of the *hpc* genes carried by pJJ801 in an
 E.coli K12 strain.

88 Characterisation of pJJ801.

90 a) Subcloning of pJJ801 using the restriction sites
 identified on the plasmid.

90 b) *Tn1000* Mutagenesis.

92 c) Further subcloning of pJJ801.

95 d) The use of pJJ801 to probe a Southern transfer of
 chromosomal DNA.

 Further studies involving pJJ801.

99 a) Characterisation of JJ247 using subclones from
 pJJ801.

101 b) The expression of *E.coli* C *hpc* genes in *P.putida*.

103 c) Additional uses of pJJ801.

106 3.2 Cloning and characterisation of additional *hpc*
 genes.

106 Cosmid cloning.

 Isolation and analysis of new HPA mutants.

110 a) Isolation of JJ200.

111 b) The isolation of JJ210.

113 The isolation of pJJ200 and pJJ210.

 Characterisation of pJJ210 and pJJ200.

115 a) The expression of *hpc* genes from the plasmids pJJ200
 and pJJ210 in *E.coli* strains.

121 b) Subcloning of pJJ210.

 Additional uses of subclones isolated from pJJ200 and
 pJJ210.

126 a) The use of the subclones produced from pJJ210 to
 analyse the mutation in JJ210.

126 b) The expression of the genes encoded on pJJ200 in
 P.putida.

Page

No.

- 129 c) The use of a subclone produced in the
characterisation of pJJ210 to isolate an intermediate
in the 3,4DHPA catabolic pathway.

3.3 Further biochemical characterisation of the
3,4DHPA pathway.

- 130 Isolation and identification of intermediates of the
3,4DHPA catabolic pathway.
- 132 a) 5-Carboxymethyl-2-hydroxymuconate semialdehyde.
(CHMS)
- 132 b) 5-Carboxymethyl-2-hydroxymuconate. (CHM)
- 135 c) 5-Carboxymethyl-2-oxo-hex-3-ene-1,6-dioate. (COHED)
- 135 d) 2-Hydroxyhepta-2,4-diene-1,7-dioate. (HHDD)
- 135 e) 2-Oxo-hept-3-ene-1,7-dioate. (OHED)
- 139 f) 2,4-Dihydroxy-hepta-2,ene-1,7-dioate. (HHED)

Use of isolated intermediates to characterise further
the 3,4DHPA catabolic pathway.

- 143 a) The COHED decarboxylase enzyme.
- 143 b) HHDD isomerase.
- 144 c) OHED hydratase.
- 146 d) HHED aldolase.
- 146 An attempt to purify the 3,4DHPA 2,3dioxygenase.

3.4 Factors affecting the regulation and expression of
the 3,4DHPA pathway.

- 158 Identification of a receptor site on pJJ801 for the
product of *hpcR*.
- 161 Identification of a regulatory gene, *hpcR* on
pJJ200/pJJ210.
- 164 The effect of cAMP on the 3,4DHPA pathway.
- 164 DNA sequencing of the operator-promoter sites of the
hpcBCDEF and *hpcGH* gene clusters.

4.0 DISCUSSION.

- 172 The gene order.
- 178 Biochemical techniques.
- 181 DNA sequencing.

Page

No.

183 Regulation.

187 The expression of *E.coli* C *hpc* genes in other organisms.

189 Future work.

190 Summary.

191 REFERENCES.

Index of figures.

Page

No.

- 3 fig,1.1.1.
Aromatic hydroxylation reactions.
- 5 fig,1.1.2.
Ring cleavage.
- 6 fig,1.1.3.
The catabolism of various aromatic compounds via the
 β -Ketoacid pathway.
- 7 fig,1.1.4.
Convergence of pathways for degradation of aromatic
compounds.
- 20 fig,1.1.5.
The TOL plasmid-specified pathway for the degradation
of toluene and xylenes.
- 23 fig,1.1.6.
The pathway for the catabolism of naphthalene and
salicylate.
- 26 fig,1.1.7.
The catabolic pathway for the degradation of
chlorobiphenyl and biphenyl, also the reported gene
organisation of *bphABC* operon in *P.pseudoalcaligenes*.
- 29 fig,1.1.8.
The PMR spectra of authentic p-cresol.
- 32 fig,1.1.9.
The proposed pathway for Indigo synthesis.
- 33 fig,1.1.10.
The pathway for the catabolism of hydroxyphenylacetate.
- 64 fig,2.1.1.
Construction of apparatus for Southern blot.
- 79 fig,3.1.1.
Determination of the conditions for *Sau3A* partial
digestion of *E.coli* C chromosomal DNA.

83 fig,3.1.2.

A photograph of the restriction digests used to generate the restriction map of pJJ801.

91 fig,3.1.3.

Deletion subclones produced from pJJ801 and the 3,4DHPA catabolic enzymes detected in 5K harbouring the various subclones.

93 fig,3.1.4.

Tn1000 mutagenesis of pJJ801 and the 3,4DHPA catabolic enzymes detected in 5K harbouring the various mutant versions of pJJ801.

95 fig,3.1.5.

An analysis of the subclones produced from partial digestion of pJJ801.

96 fig,3.1.6.

The restriction maps of the subclones produced by *Sau*3a partial digestion of pJJ801 and the 3,4DHPA catabolic enzymes detected in 5K harbouring the various subclones.

97 fig,3.1.7.

A compilation of various subclones used to determine the order of the *hpc* genes carried by pJJ801.

98 fig,3.1.8.

A Southern transfer of various chromosomal digests probed with the 6.5Kb *Bam*H1 fragment from pJJ801.

102 fig,3.1.9.

Restriction maps showing the plasmids pJJS801, pJJS108 and pJJS200.

105 fig,3.1.10.

A Southern transfer of various chromosomal DNA probed with the 6.5Kb fragment from pJJ801.

107 fig,3.2.1.

Determination of the conditions for *Sau*3a partial digestion of *E.coli* C chromosomal DNA.

109 fig,3.2.2.

A Southern transfer of the various cosmids digested with *EcoR*I and probed with the 6.5Kb *Bam*H1 fragment from pJJ801.

116 fig,3.2.3.

A photograph showing examples of the restriction digests used in determining the restriction map of pJJ210.

117 fig,3.2.4.

A photograph showing the restriction digests generated in an attempt to restriction map pJJ300.

118 fig,3.2.5.

An example of the restriction digests used to determine the restriction map of pJJ200 and the subsequent Southern transfer, which was probed with the 4.0Kb *EcoR*I-*Bam*H1 fragment from pJJ801.

122 fig,3.2.6.

The deletion subclones produced from pJJ210 and the 3,4DHPA catabolic enzymes detected in 5K harbouring the various plasmids.

123 fig,3.2.7a.

A Southern transfer of various chromosomal DNA probed with the plasmid, pJJ210.

124 fig,3.2.7b.

A 14 day exposure of the filter to the film showing weak homology with chromosomal DNA other than *E.coli* C.

125 fig,3.2.8.

A comparison of the restriction maps of the three plasmids isolated in this study and the relative positions of the *hpc* genes identified.

133 fig,3.3.1.

A diagram of the spectral scans of CHMS in both acid and alkaline conditions.

134 fig,3.3.2.

A diagram of the spectral scan of CHM showing the spontaneous isomerisation.

136 fig,3.3.3.

A diagram of the difference spectrum of a mixture of CHM and COHED against a fresh sample of CHM.

137 fig,3.3.4.

A diagram of the spectral scan of HHDD showing the spontaneous isomerisation.

138 fig,3.3.5.

A diagram of the NMR spectrum of HHDD.

140 fig,3.3.6.

A diagram of the difference spectrum of a mixture of HHDD and OHED against a fresh sample of HHDD.

141 fig,3.3.7.

A diagram of the spectral scan of HHED.

142 fig,3.3.8.

A diagram of the NMR spectrum of HHED.

150 fig,3.3.9.

The elution profile measured at 280nm of a crude cell extract, from 5K(pJJ002), passing through a DEAE (DE52) cellulose column.

151 fig,3.3.10.

The elution profile measured at 280nm of a partially-purified protein sample, containing the 3,4DHPA dioxygenase, passing through a Superose 12 column.

152 fig,3.3.11.

The elution profile of a protein sample, containing the 3,4DHPA dioxygenase passing through a Pro-reverse phase column.

154 fig,3.3.12.

A photograph of a native polyacrylamide gel.

155 fig,3.3.13.

The elution profile of the 3,4DHPA dioxygenase isolated from a polyacrylamide preparative gel passing through a Pro-reverse phase column.

Page

No.

156 fig,3.3.14a.

A calibration profile showing the relative elution distances of various molecular weight molecules passing through a Superose 12 column.

157 fig,3.3.14b.

A graph showing the calibration curve from the elution profile in fig,3.3.14a.

168 fig,3.4.1.

The strategy used in determining the sequence of the operator-promoter region of the *hpc* operons.

169 fig,3.4.2.

The DNA sequence data of the region encoding the *hpc* operator-promoter.

170 fig,3.4.3.

An interpretation of the DNA sequence data presented in fig,3.4.2.

175 fig,4.1.1.

A diagram showing the relationship between the *hpc* gene order and the reactions of the 3,4DHPA catabolic pathway.

188 fig,4.1.2.

A model for the regulation of the *hpc* genes.

Tables.

16 Table,1.1.1.

Plasmid-encoded degradative pathways.

40 Table,2.1.1.

Strains and plasmids.

80 Table,3.1.1.

Transformation results obtained at various stages in the isolation of pJJ801, using JJ221 as the recipient.

84 Table,3.1.2.

Activities of the 3,4DHPA catabolic enzymes in JJ221(pJJ801).

- 86 Table,3.1.3.
Activities of the 3,4DHPA catabolic enzymes in
JJ247(pJJ801).
- 87 Table,3.1.4.
Activities of the 3,4DHPA catabolic enzymes in
CT001(pJJ801).
- 89 Table,3.1.5.
Activities of the HPA/3,4DHPA catabolic enzymes in
E.coli K12, strain 5K(pJJ801).
- 100 Table,3.1.6.
Activities of certain 3,4DHPA catabolic enzymes in JJ247
harbouring various subclones.
- 104 Table,3.1.7.
Activities of the 3,4DHPA catabolic enzymes of various
P.putida strains harbouring pJJS801/108.
- 112 Table,3.2.1.
Activities of the 3,4DHPA catabolic enzymes detected in
the characterisation of the mutant JJ200.
- 114 Table,3.2.2.
The transformation results using JJ210 as the recipient
and pBR322 as the cloning vehicle.
- 120 Table,3.2.3.
Activities of the HPA/3,4DHPA catabolic enzymes in
5K(pJJ200/pJJ210).
- 128 Table,3.2.4.
Activities of certain 3,4DHPA catabolic enzymes detected
in various *P.putida* strains harbouring pJJS200.
- 131 Table,3.3.1.
Activities of 3,4DHPA catabolic enzymes showing
activities over the HPA induced levels for *E.coli* C.
- 149 Table,3.3.2.
An attempt to purify the 3,4DHPA dioxygenase by ammonium
sulphate precipitation.
- 159 Table,3.4.1.
Activities of the 3,4DHPA catabolic enzymes in 5K
harbouring pJJ210.

Page

No.

163 Table,3.4.2.

Activities of the 3,4DHPA catabolic enzymes in 5K harbouring various plasmids in an attempt to show regulation of the *hpc* genes.

165 Table,3.4.3.

Showing the effect of cAMP on the 3,4DHPA catabolic enzymes in *E.coli* C.

166 Table,3.4.4.

The effect of cAMP on the activities of 3,4DHPA catabolic enzymes in 5K harbouring pJJ210.

Calculations.

81 Calculation,3.1.1.

Calculation of the probability of obtaining a clone carrying the desired genes.

145 Calculation,3.3.1.

The calculation of the molar extinction coefficient for HHDD.

147 Calculation,3.3.2.

Calculation of the molar extinction coefficient for HHED.

AROMATIC CATABOLISM.

The Plant Kingdom produces millions of tonnes of organic compounds annually by the photosynthetic reduction of CO_2 and this represents the major synthesis phase of the carbon cycle. The organic material thus generated is available to serve the nutritional requirements of the Animal Kingdom. There are certain organic compounds produced by plants which cannot be directly metabolised by members of the Animal Kingdom, the most common examples being the biopolymers cellulose and lignin. Cellulose is a linear polymer which is composed of D-glucose units in a $\beta(1-4)$ linkage and lignin is a three dimensional structural polymer which is derived from the enzymic coupling of three aromatic alcohols, *p*-coumaryl, coniferyl and sinapyl alcohol. The oxidative action of microorganisms is the sole process by which the carbon locked in these polymers can be returned to the environment.

The catabolism of the cellulose structural unit, glucose, by microorganisms has been extensively studied. In contrast the microbial degradation of lignin and its structural unit the benzene ring still remains relatively unexplored. The role played by microorganisms in this specialised, but essential, part of the carbon cycle is therefore justification for the study of aromatic catabolism.

Many man-made chemicals such as detergents, pesticides or other synthetic compounds which find their way into the environment contain the aromatic nucleus. Some of these

recalcitrant
(compounds have long term deleterious effects on the environment due to their *toxic* nature. An understanding of microbial aromatic catabolism will allow the determination of which man-made chemicals are safe to use in the environment.

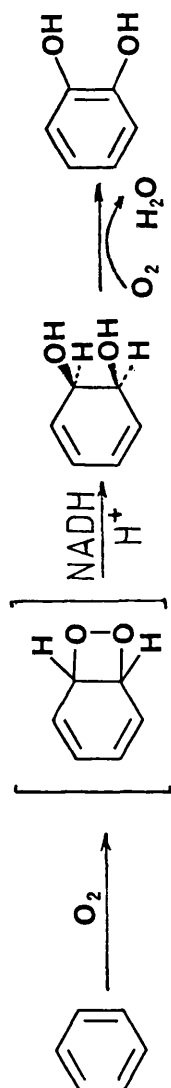
The study of aromatic catabolism is of further value since aromatic catabolic pathways provide an interesting and convenient system for studying functionally related genes.

Ring cleavage.

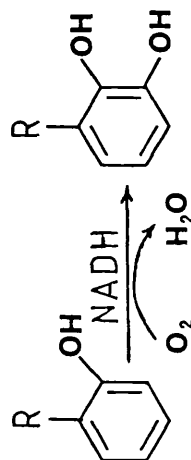
The return of carbon locked within aromatic compounds to the environment relies upon the ability of microorganisms to disrupt the chemically stable structure of the benzene ring. The biological de-stabilization of the benzene ring is achieved by the enzymic insertion of hydroxyl groups (if they are not already present). Dioxygenases (dihydroxylases) catalyse the introduction of two hydroxyl groups, and hydroxylases (mono-oxygenases or mixed function oxidases) catalyse the introduction of a single hydroxyl group into the ring, thus providing a suitable labile conformation for ring cleavage. Fig.1.1.1, shows two examples of such hydroxylation. For a compound to be acceptable as a substrate for ring cleavage two hydroxyls must be present, either *ortho* (1,2-dihydroxy) as in catechol or *para* (1,4-dihydroxy) as in gentisate. Ring cleavage occurs via a dioxygenase which donates two oxygen atoms, one to each of the two carbon atoms on either side of the break. When the ring is *ortho*-dihydroxylated it can undergo two types of cleavage: a 1,2-dioxygenase can cleave the ring between adjacent

Aromatic hydroxylation reactions.

- a) Double hydroxylation of the benzene nucleus, via the 1,2-dioxetane (peroxide) and the cis-1,2-dihydrodiol.



- b) Hydroxylation of a phenolic compound by a mono oxygenase.



hydroxyls (*ortho* cleavage) and a 2,3-dioxygenase can cleave the ring to one side of the two adjacent hydroxyls (*meta* cleavage). If the hydroxyls are opposite each other (*para*) the ring is cleaved between one hydroxyl and a side chain. Fig.1.1.2, shows examples of these types of cleavage.

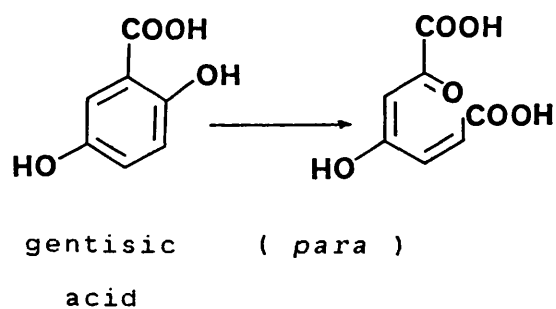
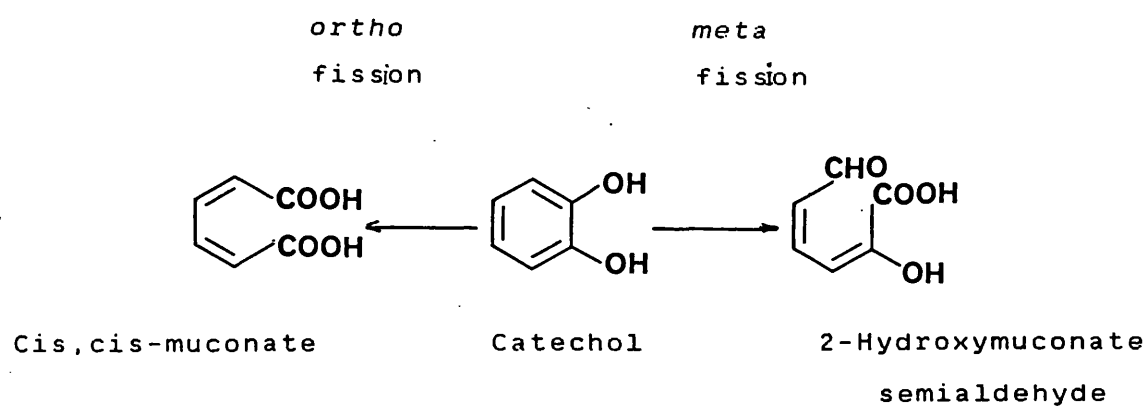
The opened structure can now serve as a substrate for subsequent reactions in which it is converted to central metabolites. These and other aspects of aromatic catabolism have been extensively reviewed by Dagley (1971,1975 and 1978).

Convergence of pathways involved in the catabolism of aromatic compounds.

The convergence of catabolic pathways involved in aromatic degradation has been extensively reviewed (Dagley,1975; Ornston and Yeh,1982; Ribbons and Eaton,1982; Franklin et al,1981). When aromatic compounds are degraded by bacteria the products tend to be channelled through a few compounds which act as substrates for conversion to central metabolites (Ribbons and Eaton, 1982). The most extensively studied of these is the β -ketoadipate pathway (Stanier and Ornston, 1973). Various compounds converge upon either protocatechuate or catechol, for ring cleavage. Fig.1.1.3, shows the cleavage pathways of these two compounds. Both of which undergo *ortho* cleavage and are eventually converted to β -ketoadipate enol lactone and then to β -ketoadipate. This is then converted to the TCA cycle intermediates acetyl-CoA and succinate. The existence of such convergent pathways implies the evolution of a high degree of pathway integration. Fig.1.1.4, shows

Ring cleavage.

Ortho and *meta* fission of catechol by different dioxygenases.

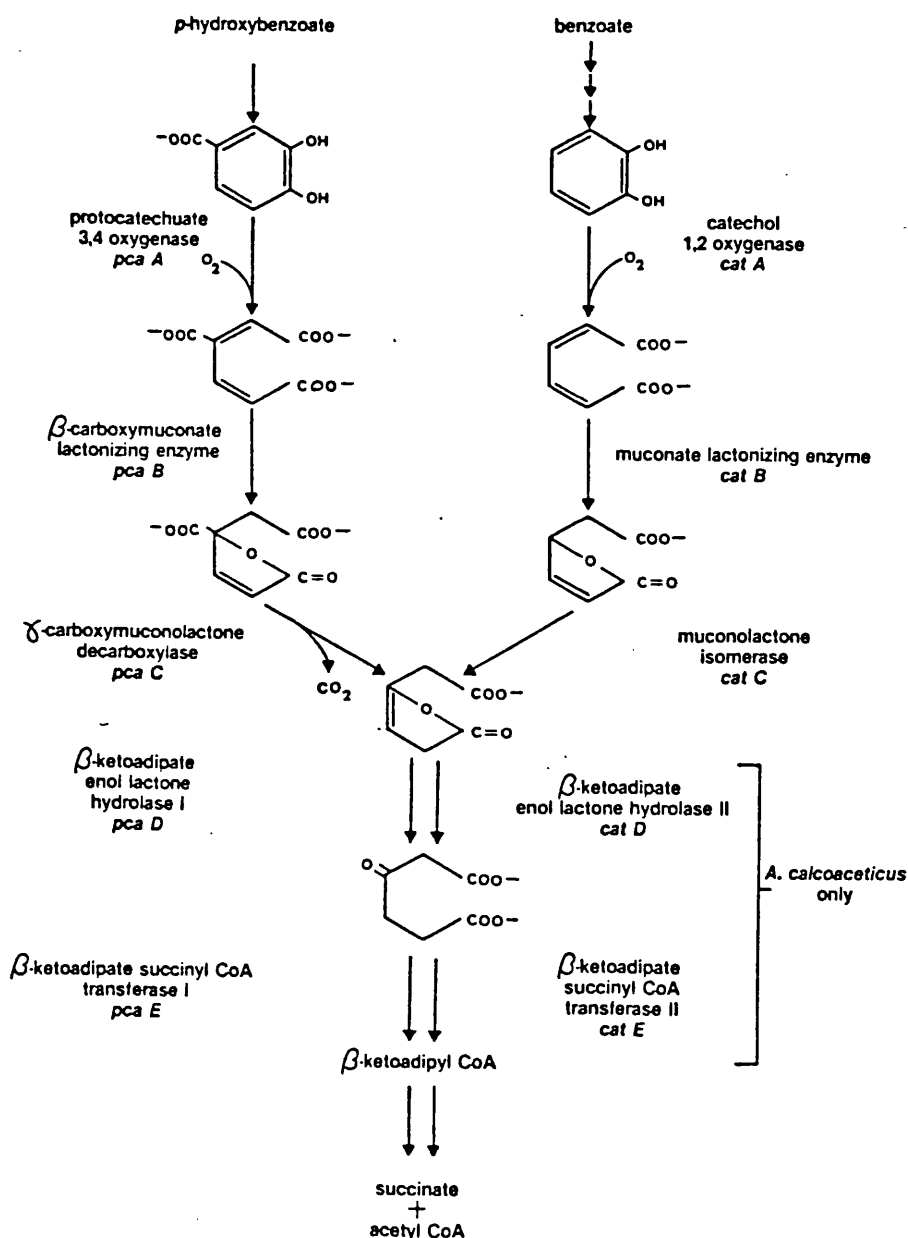


fig, 1.1.3.

The catabolism of various aromatic compounds via the β -Ketoacid pathway.

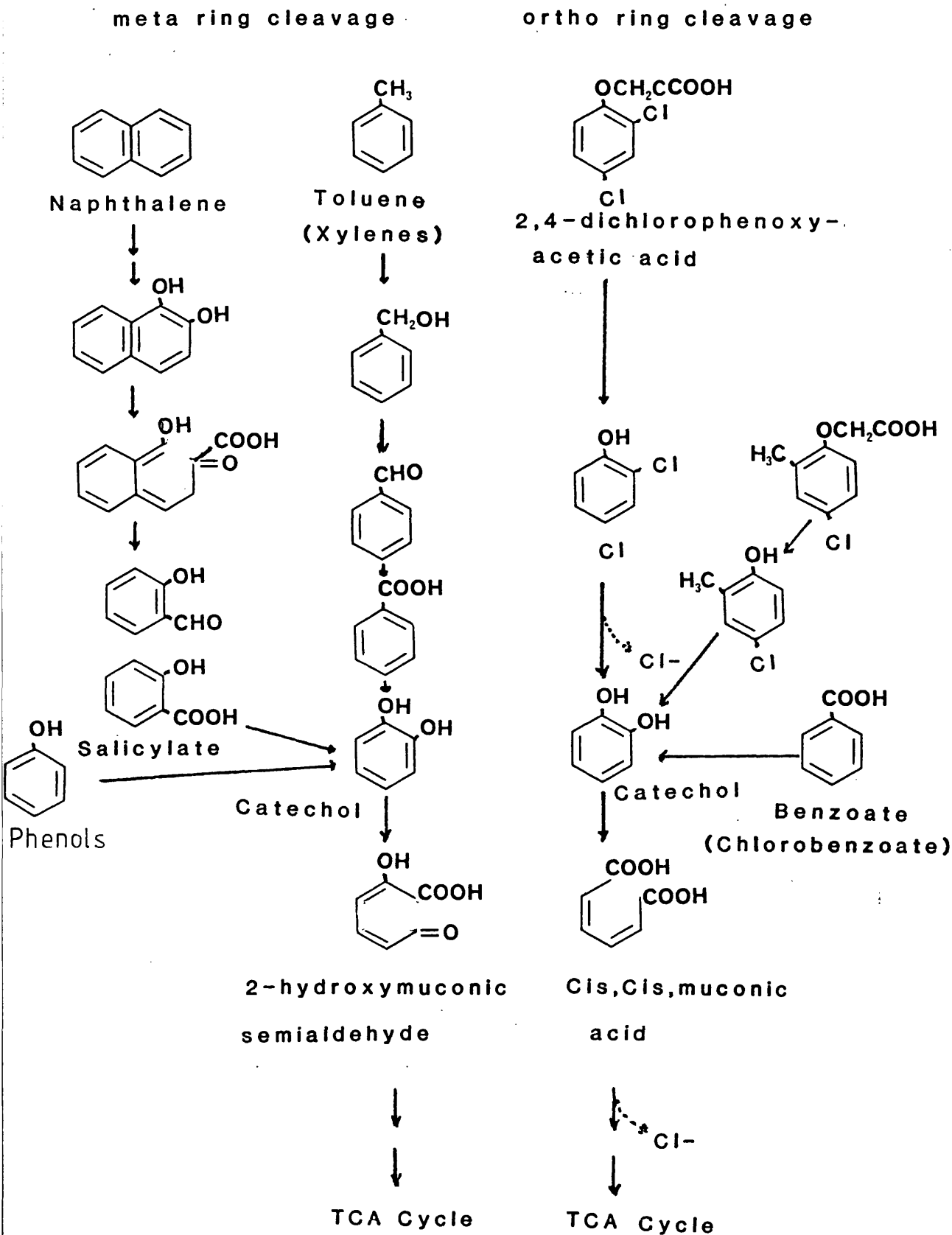
From Shanley et al, (1986).

A. calcoaceticus has two different hydrolases, 1 & 2, and two transferases, 1 & 2, enzymes which are induced by the separate branches of the pathway. *P. putida* only has a single enzyme for each of these functions, induced by both branches of the pathway.



fig,1.1.4.

Convergence of pathways for the
degradation of aromatic compounds.



some examples where compounds are channelled through catechol which is cleaved by both *ortho* and *meta* fission.

Divergence of microbial evolution.

Due to the lack of fossil records the construction of a phylogenetic family tree for bacteria has proved difficult. The apparent restricted metabolic options or nutritional requirements of bacteria have often served as a basis for taxonomic schemes (Stanier et al, 1966). More exacting techniques such as protein or DNA homology are now being employed to determine the relationship between different bacterial species. To date the relatedness of aromatic pathways has concentrated mainly on biosynthetic pathways (Jensen, 1985). Ornston's group are at present investigating the similarity between both the protein (Ornston and Yeh, 1982) and DNA (Shanley et al, 1986) sequences of the enzymes and genes from the β -ketoadipate pathway of *Pseudomonas putida* and *Acinetobacter calcoaceticus*.

An understanding of the possible interactions between different pathways and the clustering of functionally related genes may provide an insight into the evolution of bacterial metabolic pathways. The ability of a bacterium to adapt to novel nutritional conditions has provided the basis for the development of hypotheses concerning the evolution of metabolic pathways. One of the earliest theories to account for evolutionary growth of a metabolic pathway was that of Horowitz (1945). He proposed that pathways developed by a process of "retrograde evolution." This was based on the hypothesis that the first primitive organism could find all

the nutrients it required from the environment. Upon depletion of a required compound, a variant of the organism developed which possessed an additional enzyme that functioned to convert a chemically related compound to the required compound. Only a vague genetic mechanism to support this process was suggested. A more complete evolutionary scheme was put forward by Lewis (1951), who proposed that new genes arose by a two-step process in which new genetic material was created by gene duplication. A new gene thus formed would then be free to undergo "mutation to new functions", whilst the other gene retained its original function. This mechanism was proposed to be responsible for the build up of metabolic pathways. It was also suggested that functionally related but not highly clustered genes also arose by tandem duplication then dispersed later.

There are several arguments against these theories for example, evolution of a new enzyme depends upon the substrates for a particular enzymic reaction being present in high concentrations in order to support the growth of the organism. Not all the intermediates in aromatic pathways are stable or occur naturally in large quantities. However, their presence in the pathways could be explained by a subsequent streamlining of a process once a crude mechanism had been established. A further objection to the gene duplication theory was described by Dagley (1975); 3,4DHPA is degraded to give succinate semialdehyde (SSA) and pyruvate, by a sequence of reactions initiated by a *meta*-cleavage dioxygenase, followed by an NAD-linked dehydrogenase. The next reaction identified by Dagley was a decarboxylation. These reactions occur by very different reaction mechanisms and it is hard to

see how they could evolve from the same gene.

The third and most conclusive argument against tandem gene duplication is the direct comparison of two consecutive enzymes from a pathway. A comparison of the peptide maps for tryptophan synthase A and B from *E.coli* showed no homology (Hegeman and Rosenberg, 1970). No immunological similarities between the muconolactone isomerase and muconate lactonising enzyme of the β -ketoadipate pathway were observed (Stanier et al, 1970).

Another process for generating new genetic material was proposed by Riley and Anilionis (1978). They hypothesised that the *E.coli* chromosome has undergone two duplication events and that this additional DNA had the potential to mutate to serve other reactions.

An alternative mechanism for pathway evolution is enzyme recruitment by the mutation of one enzyme, which can then subsequently participate in another pathway. Four primary mechanisms have been identified which may enable an organism to utilise a previously unmetabolisable compound:

i) Constitutive production of a previously inducible enzyme which possess a slight activity to the novel substrate.

ii) Change in specificity of a preexisting group of enzymes with some activity towards the novel compound.

iii) Decreased sensitivity to a normally toxic compound which could otherwise serve as a substrate.

iv) Acquisition of permeability to a metabolisable compound.

This suggests that genes for a pathway would be scattered around the chromosome. An interesting example to support this theory was demonstrated by Campbell et al (1973). The β -galactosidase (*lacZ*) gene was deleted from a strain of

E.coli. Mutants of this strain with a restored ability to grow on lactose were then isolated. The new gene (*ebg*) was mapped at minute 65 as compared to those of the *lac* operon which normally map at minute 8.

A further example of the mutation and subsequent recruitment of an additional function to a pathway was reported by Hacking et al (1977). They proposed that if sufficient selection pressure was placed on a gene then mutation to a new function would occur. If a duplication event had not taken place then the original function would be subsequently lost. This model was demonstrated by selecting a mutant of *E.coli* that could utilise the previously unmetabolisable compound L-1,2-propanediol, with the resulting loss in the ability to grow on L-fucose.

Enzyme recruitment explains some of the evolution of new or more efficient metabolic pathways, but it does not explain the origin of the 'new' DNA.

Evidence for gene duplication and subsequent mutation enabling recruitment to a new pathway was presented by Ornston and his colleagues (Patel et al, 1973; Ornston et al, 1982). The β -carboxymuconate lactonising enzyme (CML) and muconate lactonising (ML) enzyme from of the β -ketoadipate pathway of *P.putida* were shown to have approximately the same molecular weights, similar subunit structures, crystalline forms and N-terminal amino acid sequences. Close resemblance was found between γ -carboxymuconolactone decarboxylase (CMD) and muconolactone isomerase (MLI). Little similarity, however, was detected between CML and CMD, or ML and MLI which are consecutive reactions in either arm of the β -ketoadipate pathway (see fig.1.1.3, for a diagram of the

pathway).

Hegeman and Rosenberg (1970) reviewed the topic of microbial evolution. They proposed a composite theory in which metabolic pathways originally arose by gene duplication and subsequent mutation. This either provides the next step in the pathway or the DNA source material for enzyme recruitment. It was also suggested that unitary control of these enzymes would prove useful and possible if the genes were brought together in the same operon by translocation.

Various mechanisms for translocation have been well characterised since Hegeman and Rosenberg's proposal. These include viral incorporation into the chromosome followed by transduction, the integration of two insertion elements forming transposon-like structures, F-primes, conjugable and nonconjugable plasmids. Evidence to support the composite theory and enzyme recruitment from duplicated genes in the β -ketoadipate pathway, has previously been discussed. A recent publication by Belfaiza et al, (1986), also has some bearing on the proposed theory. Belfaiza et al conducted a comparative study on the β -cystathionase (*metC*) and cystathionine γ -synthase (*metB*) proteins of the methionine biosynthetic pathway. They showed that although *metB* and *metC* were located at 88 and 65 minutes on the *E.coli* chromosome respectively, there was strong homology (36%) between the *metB* and *metC* proteins. This suggests that they have evolved from a common ancestral gene.

With the various mechanisms of gene translocation it is easy to envisage a situation in which genes for a particular function may be moved around the chromosome. The integration of new genes next to other functionally related genes could

be selected for, since this situation affords an increased regulatory efficiency. If genes are clustered their movement or transfer would be easier. This idea can be developed further to include the action of plasmids. If various genes or groups of genes are only occasionally required they could become integrated into plasmids, which could then act as a bank for these functions within a population. It would also be equally possible that if certain genes became required more frequently they could become integrated into the chromosome. This could lead to the build up and clustering of the genes involved in the metabolic pathways. If this was the case then one may expect to see situations in which pathways were partly chromosomally encoded and partly plasmid encoded.

A similar situation to that just discussed has been observed for the toluene degradative pathway ^{Reviewed by} (Williams and Jeenes, 1981). *P. putida* harbouring the TOL-plasmid has two pathways for the catabolism of benzoate, one chromosomally encoded (*ortho*) and the other encoded by the TOL-plasmid (*meta*). The chromosomally encoded pathway supports a faster rate of growth on benzoate, but these genes are not expressed if the TOL-plasmid is present, so this phenomenon can be utilised in curing experiments. Strains isolated using the benzoate curing method can be shown to have lost the *meta* cleavage pathway, and correspondingly all or part of the TOL-plasmid. What is surprising is that in some circumstances the toluene (*xyl*) genes do not appear to have been lost but have become integrated into the chromosome, where they are no longer functional. These genes also appear to be able to transfer back to a suitable plasmid when the additional functions are required. This has led to the speculation that

the *xyl* genes may be on a transposon-like structure (Sinclair et al, 1986). A further example of this type of evolutionary conservation for the *xyl* genes was demonstrated by Keil et al (1985). They showed that two superficially dissimilar plasmids have very strong regions of homology in the area which carried all the *xyl* genes necessary for the utilisation of toluene.

An example of the involvement of plasmids in the evolution of a pathway for the catabolism of 4-chlorobenzoate (4CB) was demonstrated by Reinecke and Knackmuss (1979). They isolated a strain called *Pseudomonas* B13 that was capable of growth on 3-chlorobenzoate (3CB), but unable to grow on other chlorinated benzoates due to the high specificity of the 3CB-dioxygenase. The TOL-plasmid was introduced into this strain by conjugation, with the intention of supplying a nonspecific benzoate dioxygenase. The initial transconjugants could still not grow on 4CB. For growth a mutation resulting in the loss of the plasmid-encoded *meta* pathway, preventing the diversion of 4CB into lethal products, was required. The toluene pathway will be discussed more fully later.

Experiments have been conducted using broad host range plasmids which demonstrate that genetic exchange between plasmids and the chromosome are not limited to organisms of the same species. As a result of these genetic exchange events all Gram-negative bacteria could be considered in evolutionary terms to be interrelated. Although this has been shown in the laboratory the transfer of genetic information between species occurring in this manner must be limited in Nature, by some mechanism, otherwise the elimination of various species would be expected.

Genes encoding the enzymes for aromatic catabolism.

A great number of enzymes would be required for an organism to catabolise the wide variety of aromatic compounds that are available. If one organism had the capacity to utilise all these substrates it would initially appear as a selective advantage. However, the drain on the cells resources in terms of replicating the additional DNA required would be disadvantageous. Funnelling the aromatic compounds into interconnected pathways may reduce the number of enzymes required, but the most effective way of dealing with the problem is to carry the pathway on a plasmid. Evidence for this is provided by the large number of conjugable plasmids carrying aromatic catabolic pathways. Table 1.1.1, shows several examples of these. These plasmids act a gene bank being retained at low levels in the population until they encounter the specific substrate. Each organism generally carries chromosomal genes for central metabolism and compounds commonly encountered. The plasmid encoded pathways then feed into these reactions increasing the substrate utilising capacity of the organism. These conclusions and other ideas concerning plasmids have been reviewed by Broda (1981) and Williams and Jeenes (1981).

Genetic analysis of aromatic catabolic pathways.

Most studies on the genetics of aromatic pathways have been carried out on naturally occurring plasmids since the genes of interest are already on small easily manipulated fragments of

Table 1.1.1

Plasmid encoded degradative pathways

modified from Franklin et al, 1981

<u>Pathway for degradation</u>	<u>Plasmid</u>	<u>Reference</u>
Toluene/Xylene	TOL	Williams & Murray (1974)
Camphor	CAM	Chakrabarty (1976)
n-Octane	OCT	Fennwald <u>et al</u> (1978)
Salicylate	SAL)	Yen <u>et al</u> (1985)
Naphthalene	NAH)	
2,4 Dichlorophenoxyacetate	pJP4	Don <u>et al</u> (1985a)
3-Chlorobenzoate	pJP4	Don <u>et al</u> (1985b)
4-chlorobiphenyl	pSS50	Shields <u>et al</u> (1985)

DNA. Table, 1.1.1, shows a variety of these plasmids. The most widely studied of these is the TOL-plasmid and its genetic analysis has served as a model for the genetic analysis of the 3,4DHPA pathway reported in this thesis. For this reason a detailed description of the plasmid encoded toluene pathway is given below.

Benzoate was shown to be catabolised via an *ortho*-cleavage pathway (Stanier, 1947) whereas, phenol and methyl substituted benzene are catabolised via benzoate and a *meta*-cleavage pathway (Sala-Trepat and Evans 1971), thus indicating that there were two pathways for benzoate metabolism.

Nakazawa and Yokota (1973) isolated spontaneous mutants of *P.arvilla* which had lost the *meta*-cleavage enzymes but could still grow on benzoate using the *ortho*-pathway. A report by Chakrabarty (1972) had shown that the genes encoding the *meta*-cleavage pathway for salicylate are plasmid encoded. This prompted the suggestion by Nakazawa and Yokota that *P.arvilla* had both pathways for benzoate metabolism and that the genes encoding the *meta*-cleavage pathway were plasmid encoded.

Similar mutants were obtained by Williams and Murray (1974) who showed that the benzoate/toluate *meta*-cleavage pathway genes were carried by a plasmid (TOL). This was demonstrated by mating the plasmid into Tol⁻ mutants thereby restoring their ability to grow on benzoate and toluate by the *meta*-cleavage pathway. Wong and Dunn (1974), independently, demonstrated that the benzoate/toluate *meta*-cleavage pathway was carried on a transmissible plasmid. It was also shown that the TOL-plasmid carried the genes for the catabolism of methyl derivatives of toluene, (*m*-/*p*- xylene), via the

meta-cleavage pathway (Worsey and Williams, 1975).

In an investigation into the nature of *P.putida* strains which had lost the ability to utilise toluene, it was found that some of the 'cured' strains still contained a 48Mdalton plasmid, the wild-type TOL-plasmid being 78Mdaltons. From this it was concluded that a 27Mdalton fragment carried at least part of the toluate pathway (Bayly et al, 1977).

An initial insight into the regulation of the toluene meta-cleavage pathway was shown by the induction of the first series of enzymes, which catalyse the reactions for conversion of the hydrocarbon to the carboxylic acid, by toluene, *m-/p-* xylene (Worsey et al, 1978). A regulatory mutant was isolated in which *m*-xylene or *m*-methylbenzyl alcohol no longer induced the toluate catabolic enzymes. Toluene however, was still able to induce its own degradation. From their results they proposed that the genes were in two regulatory blocks, each controlled by their own regulatory gene.

Restriction maps of the TOL-plasmid (pWW0), (Downing and Broda, 1979) and a 27 Mdalton derivative pWW0-8 (Downing et al, 1979) were determined. Transposon mutagenesis and cloning were used to localise the two gene clusters, one for the upper pathway (hydrocarbon to carboxylic acid) and the other for the lower pathway (carboxylic acid to tricarboxylic acid cycle) (Franklin et al, 1981). The two gene blocks were separated by 14Kb.

The relative positions of the *xyiB, D, E, G* and *F* genes, which encode the benzyl alcohol dehydrogenase, toluene oxygenase, catechol 2,3-dioxygenase, 2-hydroxymuconate semialdehyde dehydrogenase, and 2-hydroxymuconate semialdehyde hydratase,

respectively, were determined by Inouye et al (1981a,b). The enzymes encoded by *xylDEGF* were shown to be non-inducible unless the product of *xylS* was present, indicating positive regulation. The relative positions of the genes from the two regulatory groups and their regulatory genes *xylR* and *xylS* were shown in a map constructed by Lehrbach et al (1983).

A precise genetic map for the lower part of the toluene pathway, showing the position of the eight structural genes, has been determined (Harayama et al, 1984). The gene order found was *xyldLE*, *xylgFJ*, *xyliH* and *xylS*. The first three genes encode the first three consecutive reactions of the lower pathway (toluate to hydroxymuconic semialdehyde [HMSD]). *xylG* and *xylF* encode the enzymes for either side of alternative routes in the benzoate pathway for which HMSD is the substrate. The order of the other genes in general also follow the reaction order as shown by fig,1.1.5. The order of the upper pathway genes was determined using transposon mutagenesis (Harayama et al, 1986) and was found to be *xylC* (benzaldehyde dehydrogenase), *xylA* (xylene oxygenase) and *xylB* (benzyl alcohol dehydrogenase). The order of the genes which encode the enzymes for the upper pathway differs from the reaction order, as can be seen in fig,1.1.5.

A refined model for the regulation of the toluene catabolic pathway, based on complementation analyses, has been described (Inouye et al, 1986). Benzoate (or toluate) is the inducer for the positive regulator *xylS* which acts on the *xylDEGF* operon. It was initially thought that the product of *xylR* (induced by toluene, *m-/p-* xylene) was also a positive regulator acting directly on both *xylCAB* and *xylDEGF*. It has now been suggested that the product of *xylR* acts as an

fig,1.1.5.

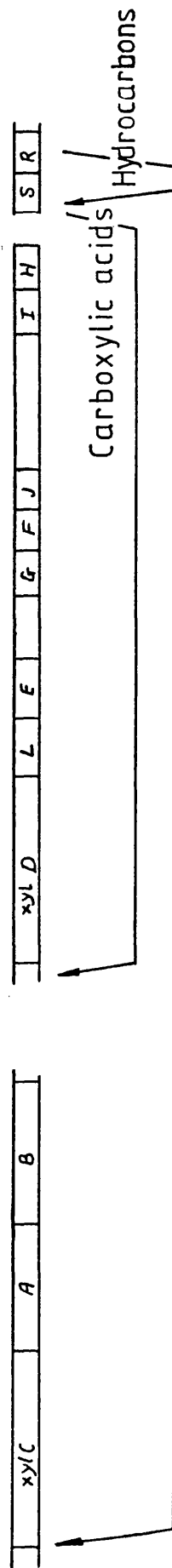
The TOL plasmid-specified pathway for
the degradation of toluene and xylenes.

Derived from Harayama et al, (1984); Harayama et al, (1986) and Spooner et al, (1986).

Enzyme abbreviations: XO, xylene oxygenase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate dioxygenase; DHCDH, dihydroxycyclohexadiene carboxylate dehydrogenase; C230, catechol 2,3-dioxygenase; HMSH, hydroxymuconic semialdehyde hydrolase; HMSD hydroxymuconic semialdehyde dehydrogenase; 4-OI, 4-oxalocrotonate isomerase; 4-OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 2-oxo-4-hydroxy-pentonate aldolase.

Starting compounds: R,R'=H, toluene; R=H, R'=CH₃, m-xylene; R=CH₃, R'=H, p-xylene.

Genes: xylA-S. xylD encodes TO a multicomponent enzyme whose structural genes are xylX, xylY and xylZ.



inducer for *xylS* not directly on *xylDEGF*. This is also represented in fig,1.1.5.

A molecular analysis of the genes encoding the toluene pathway has also been started. Nakai et al (1983) published the DNA sequence of the *xylE* gene (catechol 2,3dioxygenase) and the position of the open reading frame was identified by comparison with the protein sequence data. The sequences of the promoter regions of *xylABC* and *xylDEGF* (Inouye et al 1984a/b; Mermod et al, 1984) have also been published. These groups identified the transcription initiation sites of *xylABC* and *xylDEGF* by S1 nuclease mapping. A comparison of the operator-promoter regions of the *xylABC* and *xylDEGF* operons identified two regions of homology around -40 and -8 nucleotides (Inouye et al, 1984b). When the operator-promoter regions of wild-type and mutant constitutive *xylDEGF* promoters were compared two regions of homology were again identified. These, however, are centered around -25 and -8 nucleotides and from these results consensus sequences for *P.putida* promoters were proposed. These are A-AGGCT-T and GCTATA or GCAATA respectively (Mermod et al, 1984). A "-25" sequence similar to the proposed consensus sequence can be identified for the *xylABC* operator-promoter region.

The nucleotide sequences of the two *xyl* regulatory genes have also been published, *xylR* (Inouye et al, 1985) and *xylS* (Spooner et al, 1986). The *xylR* transcription start site was determined by S1 nuclease mapping and upstream from this similar sequences to the proposed *P.putida* promoter consensus sequences could be identified. The sequence data presented for *xylS* identifies an open reading frame of 963bp but upstream from this no clear candidate for promoter type

structures can be identified.

Other plasmid encoded aromatic catabolic pathways.

The strategies employed in the study of the TOL-plasmid have been applied to many other aromatic catabolic plasmids. Examples of these are the plasmids carrying the naphthalene and the chloro-phenol/biphenyl pathways. The genes encoding their respective degradation show a noticeable degree of clustering.

The genes encoding the pathway for naphthalene catabolism are carried on the NAH-plasmid and have been analysed by cloning and transposon mutagenesis (Grund and Gunsalus, 1983; Yen and Gunsalus, 1985), see fig. 1.1.6. It was shown that the genes encoding the naphthalene catabolic pathway consisted of two operons, *nahABCDEF* and *nahGHIJK* which are both positively regulated by a single *nahR* gene product. Schell et al (1986) proposed a model for the regulation of the *nah* operons in which the product of *nahR* is always bound to the DNA but in the presence of an inducer it undergoes a conformational change switching on the production of the NAH degradative enzymes. The plasmid encoded enzymes for the degradation of either naphthalene or salicylic acid were unaffected by the presence of glucose or succinate in the medium as the degradative enzymes were still induced and were therefore not subject to catabolite repression (Schell et al, 1986). The studies by Bateman et al (1986) on the transport of naphthalene through the membrane showed that its uptake was independent of a carrier protein, an activated membrane or a requirement for ATP. This implied that there was unlikely to

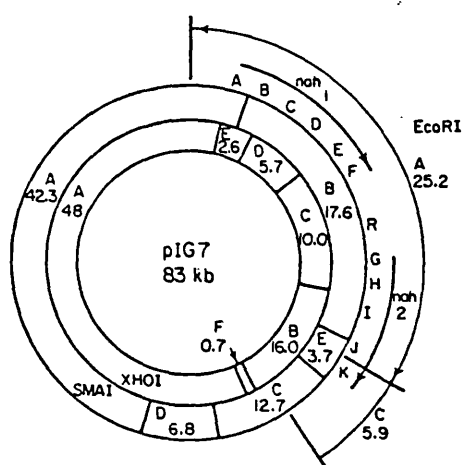
fig, 1.1.6.

The pathway for the catabolism of naphthalene and salicylate.

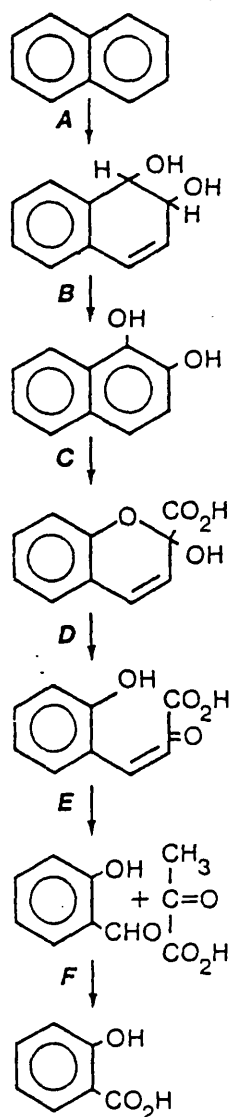
From Grund and Gunsalus, (1983): Yen and Gunsalus, (1986).

The plasmid pIG7 shows the relative positions of the *nah* genes.

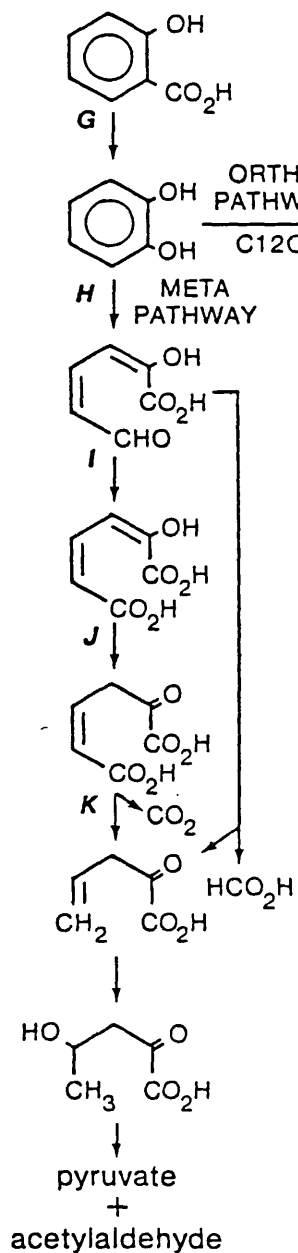
The capital letters indicate genes or gene products: A, naphthalene dioxygenase; B, *cis*-dihydrodiol naphthalene dehydrogenase; C, 1,2-dihydroxynaphthalene dioxygenase; D, 2-hydroxychromene-2-carboxylate isomerase; E, 2-hydroxy- benzalpyruvate aldolase; F, salicylate dehydrogenase; G, salicylate muconate tautomerase; K, 4-oxalcrotonate decarboxylase; L, 2-oxo-4-pentenoate hydratase; M, 2-oxo-4-hydroxypentanoate aldolase.



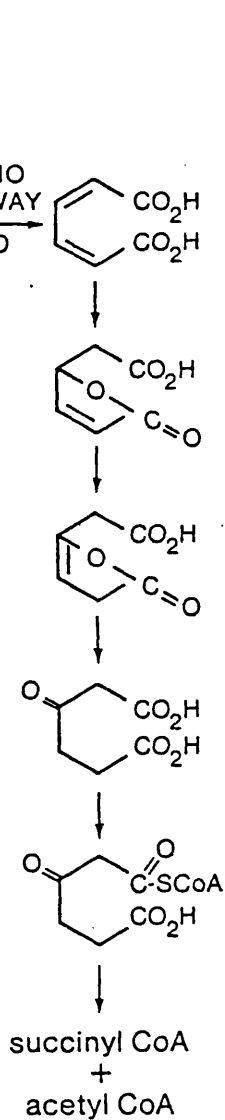
NAPHTHALENE OXIDATION



SALICYLATE OXIDATION



CATECHOL OXIDATION



be a transport protein or a corresponding gene present.

Several potential pathways for the mineralisation of chloro-phenols/biphenyls have been reviewed by Steiert and Crawford (1985). A relationship between the degradation of these types of compounds and the presence of a plasmid has been demonstrated (Shields et al, 1985; Don and Pemberton 1985a). The plasmid pJP4 permits its host *Alcaligenes eutrophus* to degrade and utilise 3-chlorobenzoate and 2,4dichlorophenoxy-acetate. Transposon mutagenesis and DNA cloning techniques were used in the localisation of four genes, *tfdB-E*, which correspond to the second to fifth reactions in the pathway (2,4dichlorophenol hydratase, dichlorocatechol 1,2dioxygenase, chloromuconate cycloisomerase, and chlorodienelactone hydrolase respectively). A fifth gene, *tfdF* was also identified but no function could be assigned to it. The genes were shown to be clustered, but the gene order does not correspond to the reaction order (Don et al, 1985b).

Chromosomally-encoded aromatic pathways.

Two recent reports have dealt with the isolation and genetic characterisation of chromosomally-encoded aromatic pathways. Both reports, however, only describe the cloning of part of the pathway so it would be difficult to draw any conclusions about the evolutionary significance of the organisation of these chromosomal genes. The *catBCDE* genes from *Acinetobacter calcoaceticus* which encode the enzymes for four consecutive reactions of the catechol branch of the β -ketoadipate pathway were isolated by Ornston's group

(Shanley et al, 1986) see fig, 1.1.3, for the pathway. The recombinant plasmid was selected by complementation of a *P. putida* mutant with a lesion in the *catBC* and *pcaDE* genes. The order of the genes was determined by subcloning the plasmid and testing various constructs harboured by *E. coli* HB101 for activities of the β -ketoadipate enzymes. The order identified was *catB* (muconate lactonising enzyme), *catC* (muconate lactone isomerase), *catD* (β -ketoadipate enol-lactone hydrolase) and *catE* (β -ketoadipate succinyl-Coenzyme A transferase).

Furukawa and Miyazaki (1986) have cloned three chromosomally encoded genes from *P. pseudoalcaligenes*, *bphABC*, which correspond to consecutive reactions in the chloro-biphenyl (PCB) pathway. Fig, 1.1.7, shows the gene order in relation to the pathway. It is interesting to note that this is a chromosomally-encoded pathway compared to the previously described plasmid-encoded chloro-biphenyl pathway (Shields et al, 1985; Don and Pemberton, 1985b).

The use of genetic techniques to aid the fine biochemical analysis of aromatic pathways.

The study of a biochemical pathway is an analysis of two interlinking phases, the intermediates and the enzyme catalysed reactions. Using classical biochemical techniques the identification of intermediates and characterisation of the enzymic reactions have been achieved by some of the following methods:

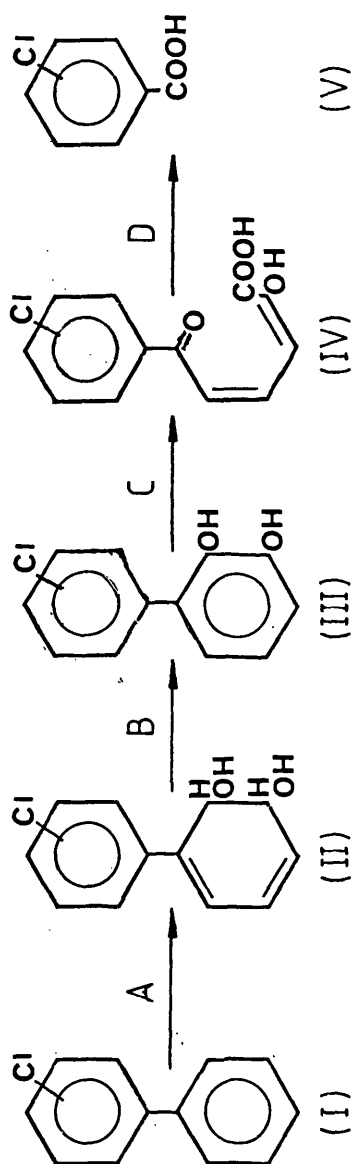
i) Oxidation using whole cells; washed cells are challenged with a potential intermediate and the rate of oxidation

fig,1.1.7.

The catabolic pathway for the degradation of chlorobiphenyl and biphenyl. Also the reported gene organisation of *bphABC* operon in *P.pseudoalcaligenes*.
From Furukawa and Miyazaki (1986).

Compounds: I, biphenyl; II, 2,3-dihydroxy-4-phenylhexa-4,6-diene; III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (meta cleavage compound); V, benzoic acid.

Enzymes: A, biphenyl dioxygenase; B, dihydrodiol dehydrogenase; C, 2,3-dihydroxybiphenyl dioxygenase; D, meta cleavage compound hydrolase (the gene *bphD* had not been cloned).



P/O	bphA	bphB	bphC	bphD
-----	------	------	------	------

measured.

ii) Enzyme assays; using cell free extracts to characterise the enzyme catalysed reactions of a pathway.

iii) Isolation of mutants; identification and use of mutants that are altered for the normal processes involved in the pathway of interest.

iv) Isolation of intermediates; this often relies on blocking the normal series of reactions causing an accumulation of a potential intermediate. The blockage can be either achieved by mutation or in cell-free extract by physical ^{or chemical} treatment.

Intermediates isolated in this way are identified using chromatography or spectrophotometry by comparison with a reference compound. In the study of aromatic pathways the standards for comparison with potential intermediates may not always be available so different more powerful techniques such as NMR or mass spectroscopy are now being employed.

Two examples have recently appeared in the literature in which genetic techniques such as mutagenesis of a particular gene and gene cloning have been used to accumulate intermediates of an aromatic pathway. The compounds were then isolated, purified and analysed. Furukawa and Miyaki (1986) used gene cloning techniques to create a supply of enzymes that could be used to isolate trimethyl derivatives of PCB. Confirmation of these structures was obtained by gas chromatography-mass spectrometry. Whited et al (1986) employed N-methyl-N'-nitro-N-nitroso-guanidine to produce mutants of the toluene pathway, which were blocked so that they accumulated carboxylic acid diols. These were isolated as sodium salts and subjected to NMR analysis in D₂O, which

confirmed their structures as cis-diols. To aid the analysis of the structures authentic p-cresol was used as a standard. An example of the PMR spectra of this compound is shown in fig,1.1.8.

The isolation and identification of intermediates is an essential part of characterising a metabolic pathway. Some of the techniques described above have been used to identify the intermediates of the 3,4DHPA catabolic pathway.

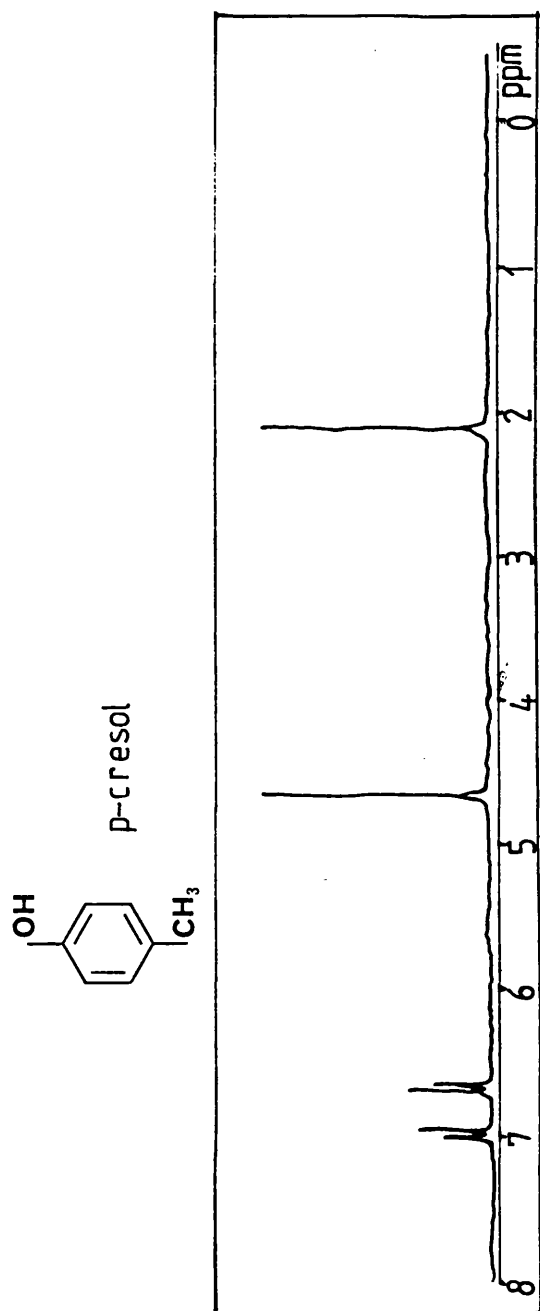
The use of pathways involved in aromatic metabolism for commercial purposes.

As the supply of fossil fuels is exhausted the search for a replacement for this source of organic chemicals has become important. Renewable biomass, in particular plant tissue with a high lignin content, has been proposed as potential candidate for this purpose (Kern, 1981; Muller and Glasser, 1983; Soltes and Lin, 1983).

Microbial attack of wood is normally a slow process. The initial attack is by primary moulds, which are not able to degrade the wood cell walls, but utilise the sapwood or nutrients brought by water movement. This provides the initial penetration into the fibre creating an opening for *white*-rot fungi. These are normally ascomycetes and *fungi imperfecti* that are capable of degrading the lignin containing cell wall by oxidative, extracellular nonspecific enzymes. It is thought that the enzymes do not attack the lignin themselves but act by generating free radicals or active oxygen species. These then destabilise the linkage between the aromatic subunits (Wallace et al 1983). This

fig,1.1.8.

The PMR spectra of authentic p-cresol.



fungal attack, allows the release of low molecular weight aromatic monomers which ~~would be~~ available for bacterial utilisation. To develop this natural process for economic purposes the time scale between initial attack and the release of economically important compounds has to be reduced.

There are several areas which can be developed to harness this natural process. Straw and other plant material which have a high lignin content, are not directly suitable for animal feed. A relatively simple biological process could be developed to upgrade the lignocellulosic material for use as fertiliser or animal feed. The paper manufacturing industry has two major problems which are solvable by biological means. The initial pulping normally involves physical and chemical treatment to extract the cellulose fibres; a bio-delignification process or combination of the two may prove more economic. The resulting lignin containing waste from conventional pulping is a pollutant and expensive to treat. These industrial lignins are an obvious choice for a bio-processing treatment (Wallace et al, 1983).

Most studies involving bacterial aromatic pathways have tended to concentrate on expanding the range of harmful or recalcitrant compounds which they have the ability to mineralise. A few bacterial aromatic pathways, however, have been manipulated to produce products of commercial interest. An example of one of these has been described by Ensley et al (1983). During an investigation of the naphthalene pathway of *P.putida* various cloned *nah* genes were introduced into *E.coli*. Some of the resultant colonies turned blue. It was subsequently shown that the blue colouration was due to the

dye indigo. They proposed that the enzymes produced from the *nah* genes interacted with intermediates from normal *E.coli* metabolism to create a product, which was indigo. Fig,1.1.9, shows the proposed scheme. This discovery has been further developed and is in the process of going into commercial production.

The Hydroxyphenylacetate and 3,4Dihydroxyphenylacetate Pathway.

The 4-hydroxyphenylacetate (HPA) pathway was first described by Sparnins et al (1974) for a species of *Acinetobacter* and two strains of *Pseudomonas*. The pathway for HPA catabolism via 3,4-dihydroxyphenylacetate (3,4DHPA) to succinate semialdehyde (SSA) and pyruvate is shown in fig,1.1.10. Adachi et al(1964) identified 3,4DHPA as an intermediate in the metabolism of HPA by *P.ovalis*. They also showed that 5-carboxymethyl-2-hydroxymuconate semialdehyde (CHMS) was the product of the 3,4DHPA ring fission. These results were confirmed by Sparnins et al (1974) who identified 3,4DHPA, CHMS and other intermediates in the degradation of HPA to carbon dioxide, succinate and pyruvate by *P.putida*. CHMS was identified by comparison of its chromatographic and pH-dependent spectral properties with those of authentic 5-carboxy-methyl-2-hydroxymuconic semialdehyde.

The isolation and characterisation of 5-carboxymethyl-2-hydroxymuconate (CHM) has been described (Sparnins et al 1974). This compound was initially identified by comparison of its absorbance spectrum in acid and alkali to that

The proposed pathway for Indigo synthesis.

From Ensley et al, 1983.

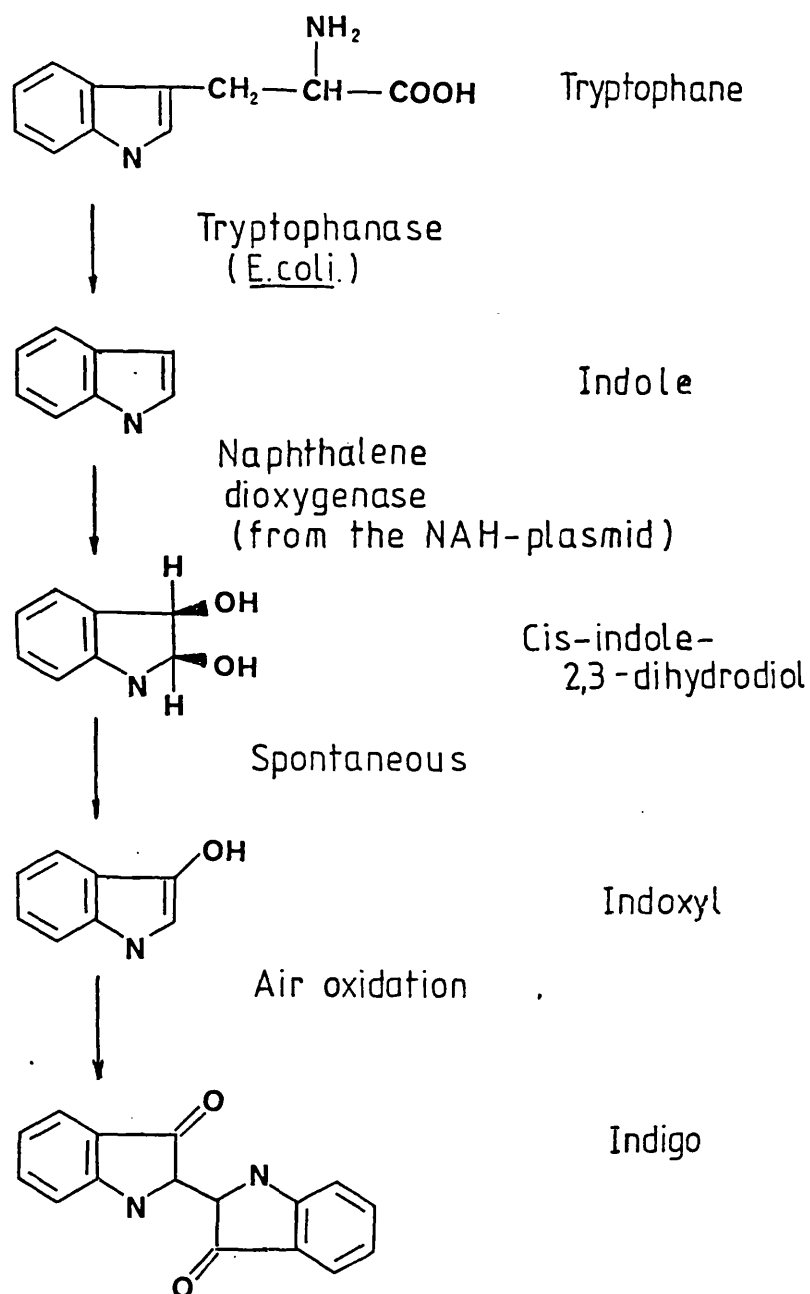


fig.1.1.10.

The pathway for the catabolism of
hydroxyphenylacetate.

Proposed by Dagley, (1973).

HPA hydroxyphenylacetate, hydroxylase.

(I) 3,4DHPA, 3,4-dihydroxyphenylacetate,

a) dioxygenase.

(II) CHMS, 5-carboxymethyl-2-hydroxymuconate
semialdehyde, b) dehydrogenase.

(III) CHM, 5-carboxymethyl-2-hydroxymuconate,
c) isomerase.

(IV) COHED, 5-carboxymethyl-2-oxo-hex-3-
ene-1,6-dioate, d) decarboxylase.

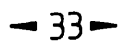
(V) HHDD, 2-hydroxyhepta-2,4-diene-1,7-dioate,
e) isomerase.

(VI) OHED, 2-oxo-hepta-3-ene-1,7-dioate,
f) hydratase.

(VII) HHED, 2,4-dihydroxy-hepta-2-ene-1,7-dioate,
g) isomerase.

(VIII) HKP, 4-hydroxy-2-ketopimilate, h) aldolase.

The stages from III to VIII are arranged to show
the similarities in the mechanisms. Steps V to VII are *still*
only proposals and have not been fully characterised.



observed for 2-hydroxymuconic acid. Mass spectroscopy of its trimethylsilane derivative confirmed its identity as 5-carboxymethyl-2-hydroxymuconic acid. Sparnins et al also observed the formation of an additional compound which had a λ -max at 274nm. Analysis of this compound utilised the shift in λ -max at varying pH and chromatography of the compounds' 2,4-dinitrophenylhydrazone derivative. The conclusion arrived at was that the compound isolated was 2-hydroxyhepta-2,4-diene-1,7-dioate, (HHDD).

A similar pathway for the conversion of HPA to central metabolites was described by Cooper and Skinner (1980) for *E.coli* C. The fact that *E.coli* possesses a pathway for aromatic catabolism may seem unusual however, evidence that enteric organisms come into contact with these types of compounds in the intestinal-fecal environment was presented by Spoelstra (1978). Further examples of the ability of *E.coli* to utilise aromatic compounds have been described by Burlingame and Chapman (1983).

Regulation of the HPA pathway.

Growth of *P.ovalis* on HPA induces the HPA hydroxylase and the 3,4DHPA catabolic enzymes whereas growth on 3,4DHPA induces the 3,4DHPA catabolic enzymes but not the HPA hydroxylase (Adachi et al, 1964). These results implied that the HPA catabolic pathway was divided into at least two regulatory groups. Similar results have been obtained for *P.putida* (Barbour and Bayly, 1977) and *E.coli* C (Skinner, 1981).

When mutants defective in only a single enzyme, the CHMS

dehydrogenase, were grown on glycerol in the presence of 3,4DHPA, the 3,4DHPA catabolic enzymes (except the CHMS dehydrogenase) were shown to be induced. The low specific activities observed were assumed to be due to the accumulation of CHMS affecting the normal growth of the organism. These results were interpreted to mean that 3,4DHPA induces all the enzymes required for its catabolism [*P.putida* (Barbour and Bayly, 1977) and *E.coli* C (Skinner, 1981)].

Both Barbour (1978) and Skinner (1981) isolated constitutive mutants of *P.putida* and *E.coli* C, respectively, using continuous culture techniques with HPA as the carbon source. The mutants obtained were only constitutive for the 3,4DHPA enzymes, indicating that these reactions probably represent the rate limiting stages of the pathway.

Further mutants shown to be defective in both sets of enzymes (HPA to 3,4DHPA and 3,4DHPA to SSA and pyruvate) were obtained from constitutive strains. Revertants of these pleiotropically defective mutants regained the phenotype of the constitutive strain. These results were interpreted as evidence that the HPA and 3,4DHPA enzymes were controlled by the same regulator molecule (Barbour, 1978; Skinner, 1981).

Skinner (1981) obtained additional *E.coli* C HPA negative mutants and combined the observations from these with those already described above for *E.coli* C to propose a model for the regulation of the HPA pathway, which is described in appendix 1 (from Skinner's Thesis). He proposed that the product of a single regulator gene, *hpaR*, controlled both the hydroxyphenylacetate (*hpa*) and 3,4DHPA (homoprotocatechuate, *hpc*) genes. The proposed mechanism by which regulation was

achieved was similar to that observed for the arabinose operon. A brief summary of the evidence is presented below:

i) Transposon mutagenesis of a strain that expresses the 3,4DHPA enzymes constitutively produced a mutant that did not express any of the 3,4DHPA catabolic enzymes. This was explained as the insertional inactivation of ^{a gene encoding} a positive regulatory protein.

ii) This mutant was non-inducible for the HPA hydroxylase and did not express the 3,4DHPA catabolic enzymes. This was presented as evidence for inactivation of a positive regulator molecule which controlled both operons.

iii) Partial diploid experiments, involving the introduction of DNA from a wild-type strain into the constitutive strain by F-mating, were used to demonstrate that the constitutive mutation was recessive to the wild-type regulatory control. This result indicates that the expression of the 3,4DHPA catabolic enzymes was negatively controlled.

If only one uptake system was required to take up both substrates, different interpretations could be placed on some of the results observed for the mutants. Skinner conducted some experiments which indicated that 3,4DHPA could partially (33%) induce HPA uptake. Unfortunately no radiolabelled 3,4DHPA was available so the uptake of 3,4DHPA by HPA-induced cells could not be analysed. The transposon-induced mutant isolated from the constitutive strain, upon which some of the evidence of the regulation of the pathway was based, was not tested to see if its uptake mechanism was defective.

Some useful information concerning the positioning of the *hpa* genes in relation to the *hpc* genes was also demonstrated by Skinner (1981). P1 transduction showed that the two groups

of genes were 90% co-transducible with each other. It was also shown that *E.coli* K-12 could not grow on HPA or 3,4DHPA and that it did not carry any of the *hpa/hpc* genes. This was concluded from the inability of *E.coli* K-12 to repair HPA mutants of *E.coli* C, in F'-mating or P1 transduction experiments. However the ability to introduce the whole HPA/3,4DHPA pathway into *E.coli* K-12 from *E.coli* C by F'-mating and P1 transduction could be demonstrated, showing the close linkage of the genes and making them a possible candidate for cloning.

The HPA catabolic pathway can be considered in terms of three distinct groups of reactions, defined by the induction of the various enzymes, as summarised below:

i) HPA is taken up and hydroxylated at the 3 position on the ring, adjacent to the existing hydroxyl group, converting HPA to 3,4DHPA. The enzymes for these functions are induced by HPA and their respective genes have been designated *hpaA* (permease), *hpaB* (hydroxylase) and *hpaR* (regulatory protein).

ii) 3,4DHPA is converted by a series of further reactions to SSA and pyruvate. The enzymes for this stage are either induced by HPA or 3,4DHPA. This second stage also represents a complete pathway, because 3,4DHPA can be taken up and converted to SSA and pyruvate. 3,4DHPA induces the enzymes for its own catabolism but not those for the conversion of HPA to 3,4DHPA. HPA can therefore be considered as feeding into the 3,4DHPA pathway.

iii) The conversion of SSA to succinate, which is achieved by the NAD-linked SSA dehydrogenase (encoded by the *sad* gene) whose inducer is SSA (Donnelly and Cooper 1981).

Aims of the project.

Most studies on aromatic catabolism have been previously conducted on *Pseudomonas*. However, due to the ease with which DNA manipulations could be achieved in *E.coli* C, a genetic investigation into the 3,4DHPA pathway was initiated in this organism. The information from the *E.coli* C 3,4DHPA pathway could then be used to look for similarities in other species carrying the 3,4DHPA pathway. The catabolism of an aromatic compound by *E.coli* C is an interesting phenomenon and the relationship of the gene order to the biochemical pathway may provide an insight into the evolutionary origins of the pathway.

Utilising the various techniques and ideas described in the Introduction, experiments were designed to gain information about the 3,4DHPA catabolic pathway. These will be interpreted to provide a plausible biochemical and genetic description of the reactions and regulation of the pathway.

The main objectives were:

1. To clone the genes involved in the 3,4DHPA catabolic pathway.
2. To identify the gene order.
3. To reassess the biochemical pathway with the aid of the isolated genes, identifying any new intermediates.
4. To combine both biochemical and genetic data to propose a possible model for the regulation of the pathway.

METHODS.BACTERIAL STRAINS AND PLASMIDS.

The bacterial strains and plasmids used in this study are listed in table, 2.1.1.

MEDIA.

Luria broth and minimal media have been described previously by Miller, (1972) and Harland et al, (1975) respectively. Medium was solidified as appropriate using 1.6% Bacto agar (Difco). Carbon and energy sources were sterilised separately and added to give a final concentration of: HPA (5mM); 3,4DHPA (5mM); and glycerol (20mM). Sodium dithionate (0.05%) was added to 3,4DHPA medium to slow down the spontaneous oxidation. Amino acids were added to give final concentrations of 80µg/ml. Antibiotics at the following final concentrations were incorporated into the media as required; ampicillin (Ap) 100µg/ml; chloramphenicol (Cm) 50µg/ml; and tetracycline (Tc) 10µg/ml. Bacteria were generally grown at 30°C on minimal media and at 37°C on rich media.

CELL-FREE EXTRACTS.

Bacteria from 100ml of medium were harvested in late logarithmic phase of growth and washed with 20ml 0.1M sodium phosphate buffer pH7.5, then resuspended in 4ml of the same buffer. The cells were broken by ultrasonication (2x30sec pulses with a one min. rest between), whilst being cooled on

Table, 2.1.1.

STRAINS AND PLASMIDS.Strains/

<u>plasmid</u>	<u>Genotype/phenotype</u>	<u>Source/reference</u>
----------------	---------------------------	-------------------------

E.coli C strains.

<i>E.coli</i> C	Wild type.	Lab. stock.
MS022	<i>hpcC</i>	Skinner, 1981. EMS mutagenesis.
MS024	pleiotropically defective in the 3,4DHPA catabolic enzymes.	Skinner, 1981. EMS mutagenesis.
CT001	Constitutive expression of the 3,4DHPA catabolic enzymes.	Skinner, 1981. Chemostat selection.
CT101	AS CT001 but defective in the NAD-dependent SSA dehydrogenase (<i>sad</i>).	Skinner, 1981. EMS mutagenesis of CT001.
CT107	As CT101 but noninducible for all 3,4DHPA catabolic enzymes.	Skinner, 1981. Transposon mutagenesis.
JJ221	<i>hpcC recA</i> .	Isolated from MS022, made <i>recA</i> by P1 transduction.
JJ247	<i>hpcB,C, recA</i> .	Isolated from MS024, made <i>recA</i> by P1 transduction.
JJ200	HPA-, 3,4DHPA+ 30°C. HPA-, 3,4DHPA- 37°C.	This study, EMS mutagenesis of <i>E.coli</i> C.
JJ210	HPA- (even with pJJ801 present).	This study, EMS mutagenesis of CT001, followed by transformation with pJJ801.

E.coli K12 strains.

5K	F ⁻ , <i>supE, tonA, hsdR, rpsL, thr-1, leu-B6</i> and <i>thi-1</i> .	Lab. stock.
RB308	F ⁺ , <i>deoC, thyA, lacY recA</i> .	Jones and Holland 1984.

Strains/

<u>plasmid</u>	<u>Genotype/phenotype</u>	<u>Source/reference</u>
----------------	---------------------------	-------------------------

Pseudomonas strains.

2440	r-,m+.	Bagdasarian <i>et al</i> 1981.
PP001	<i>hpcC</i> .	Martin, 1985.
PP003	pleiotropic for the 3,4DHPA catabolic enzymes.	Martin, 1985.

Plasmids.

pBR322	Ap,Tc.	Soberon <i>et al</i> 1980.
pBR328	Ap,Tc,Cm.	Soberon <i>et al</i> 1980.
pLG223	Tc.	Boulnois <i>et al</i> 1985.
pMMB34	Kn. <i>cos</i> λ	Frey <i>et al</i> 1983.
Cos4	Ap. <i>cos</i> λ	Roberts <i>et al</i> 1986.
pJJ801	<i>hpcB-F</i> (pBR322)	This study.
pJJ200	<i>hpcR</i> , <i>hpcGH</i> , <i>hpcBCDEF</i> . (pBR328)	This study.
pJJ210	<i>hpcR</i> , <i>hpcGH</i> , <i>hpcBCDEF</i> . (pBR328)	This study.

The descriptions of the isolation of pJJ801, pJJ200 and pJJ210 are given on pages 77, 110 and 111 respectively.

Subclones produced from pJJ801, pJJ200 and pJJ210 are shown on pages 97, 102 and 122 respectively.

melting ice at 0°C and then centrifuged at 183,600g for 90 min at 4°C. The protein concentration of extracts was measured by the biuret method (Gornall et al 1949), using crystalline bovine serum albumin as standard, and was usually 1-4mg/ml.

WASHED-CELL SUSPENSION FOR MEASUREMENT OF OXYGEN CONSUMPTION.

Cells from logarithmic phase cultures were harvested, washed with 0.1M sodium phosphate buffer pH7.5 (half original volume) and finally suspended in the same buffer to give concentrations of 2-5mg of cells/ml. Oxygen consumption was measured polarographically at 30°C using a Gilson Oxygraph model K-1C, fitted with a Clark oxygen electrode.

ISOLATION OF INTERMEDIATES IN THE 3,4DHPA PATHWAY.

Initially 5-carboxymethyl-2-hydroxymuconic semialdehyde (CHMS) and 5-carboxymethyl-2-hydroxymuconate (CHM) were prepared from 3,4DHPA using heat-treated extracts from HPA grown *P.putida* U according to the method of Sparnins et al (1974). The method relies on the 3,4DHPA catabolic enzymes in the extract to convert 3,4DHPA to the required compound. The reaction is prevented from going to completion by selectively heat inactivating the enzyme after the required stage in the pathway. Enzymes were selectively inactivated by holding the extract at a predetermined temperature for a set time.

Upon identification of the various subclones that encoded particular sequences of the 3,4DHPA catabolic pathway, CHMS

and CHM, as well as additional intermediates were produced using *E.coli* 5K strains harbouring the appropriate subclone, see table,3.3.1, in Results. This process is more advantageous than the previous method because there is no possibility of conversion beyond the product required.

The general method for the production of the intermediates is as follows: 100ml Luria broth Ap culture of strain 5K with the appropriate plasmid was grown overnight, harvested and the cells washed in 0.1M sodium phosphate buffer pH7.5. The cell pellet was resuspended in 4ml of the same buffer and the cells broken as previously described. The reaction mixture consisted of: for the production of CHMS, 20ml 0.1M sodium phosphate buffer pH7.5, 50µmol 3,4DHPA and extract from 5K(pJJ002); for the production of CHM, 25ml 0.1M sodium phosphate buffer pH7.5, 50µmol 3,4DHPA, 67µmol NAD, and extract from 5K(pJRJ003); for the production of HHDD 25ml 0.1M sodium phosphate buffer pH7.5, 50µmol 3,4DHPA, 67µmol NAD and extract from 5K(pJRJ002); and for the production of HHED, 25ml 0.1M sodium phosphate buffer pH7.5, 50µmol 3,4DHPA, 67µmol NAD, 125µmol Mg^{2+} and extract 5K(pJJ212).

Initially 2ml of extract was added to the reaction mixture which was shaken gently in a flask at 30°C until the reaction was complete as determined by measurement of 3,4DHPA. Additional extract was added, if necessary, to complete the reaction.

Concentrated HCl (1ml) was added to the reaction mixture which was then centrifuged to remove the precipitated protein. The resulting supernatant was extracted three times with an equal volume of ethyl acetate, the pooled extracts dried over sodium sulphate and evaporated to dryness under

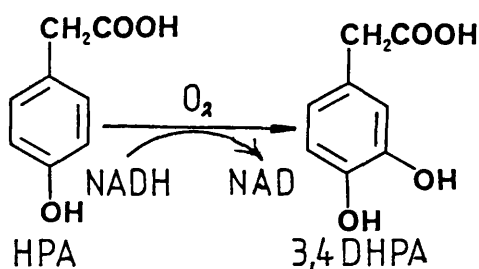
vacuum at 37⁰C in a rotary evaporator. The compound was then dissolved in 0.5ml of water and could be stored at -20⁰C, or freeze dried and kept as the solid.

ASSAYS OF ENZYME ACTIVITIES.

Enzyme assays were carried out in quartz cuvettes (1cm path length) in a total volume of 1ml, at 30⁰C, using a Pye-Unicam SP1800 or a Perkin-Elmer λ -UV 5.

a) HPA HYDROXYLASE (EC.1.14.13.3).

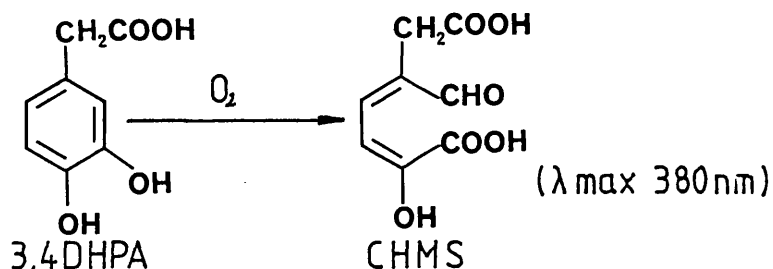
(Skinner and Cooper, 1981)



HPA hydroxylase activity was followed spectrophotometrically at 340nm. The reaction mixture consisted of 0.1M Na-phosphate buffer pH7.5, 0.13 μ mol NADH and 10-200 μ l of an ultracentrifuged cell extract. After measuring the blank rate the reaction was started by the addition of 2.5 μ mol HPA. A molar extinction coefficient of 6,200 dm³ mol⁻¹ cm⁻¹ was assumed for NADH.

b) 3,4DHPA 2,3DIOXYGENASE ASSAY. (EC 1.13.11.15)

(Skinner and Cooper, 1981)



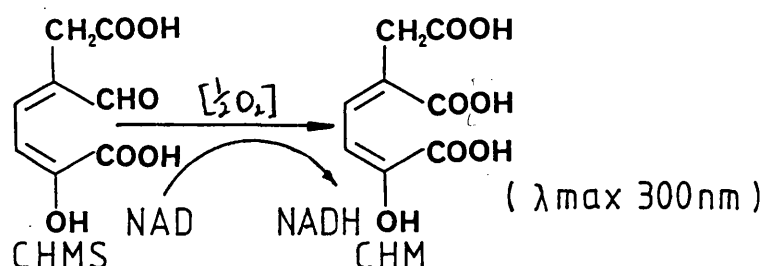
Dioxygenase activity was assayed by following the increase in absorption at 380nm due to the formation of 5-carboxymethyl-2-hydroxymuconate semialdehyde (CHMS). The reaction mixture contained 0.1M Na-phosphate buffer pH7.5, 0.2 μ mol 3,4DHPA and was started by the addition of 5-200 μ l of extract. A molar extinction coefficient of 35,500 dm³ mol⁻¹ cm⁻¹ was assumed for CHMS.

c) 3,4DHPA ASSAY.

This is a variation of the 3,4DHPA dioxygenase assay used to determine whether 3,4DHPA is still present in the reaction mixture when isolating intermediates. The substrate is limiting and the extract containing the dioxygenase is in excess. The reaction mixture consists of 0.1M Na phosphate buffer pH7.5, 200-500 μ l of the intermediate isolation reaction mixture and 50-200 μ l of extract from 5K harbouring a plasmid which constitutively produces the dioxygenase.

d) CHMS DEHYDROGENASE ASSAY.

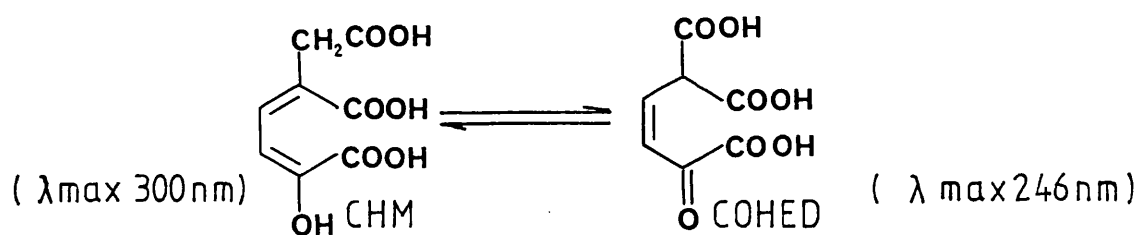
(Skinner and Cooper, 1981)



CHMS dehydrogenase activity was assayed by measuring the decrease in absorbance at 380nm as the CHMS was oxidised to CHM. The reaction mixture consisted of 0.1M Na-phosphate buffer pH7.5, 0.035 μ mol CHMS (giving an absorbance at 380nm of ~0.9 units) and 5-200 μ l extract. The reaction was started by adding 0.2 μ mol NAD. The molar extinction coefficient for CHMS was taken as 35,500 dm³ mol⁻¹ cm⁻¹.

e) CHM ISOMERASE ASSAY.

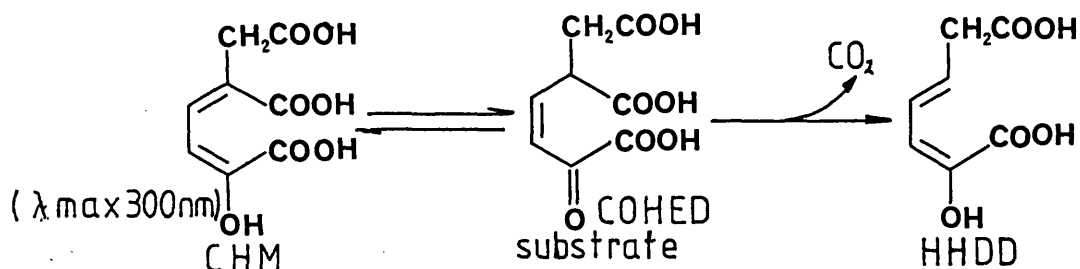
(Garrido-Pertierra and Cooper, 1981)



CHM isomerase activity was assayed by following the decrease in absorbance at 300nm. The assay mixture consisted of 0.1M Na-phosphate buffer pH7.5, 0.05 μ mol CHM (giving an absorbance at 300nm of ~0.9 units). After measuring the blank rate the reaction was started by the addition of 5-100 μ l of extract. The molar extinction coefficient for CHM was taken as 20,000 dm³ mol⁻¹ cm⁻¹.

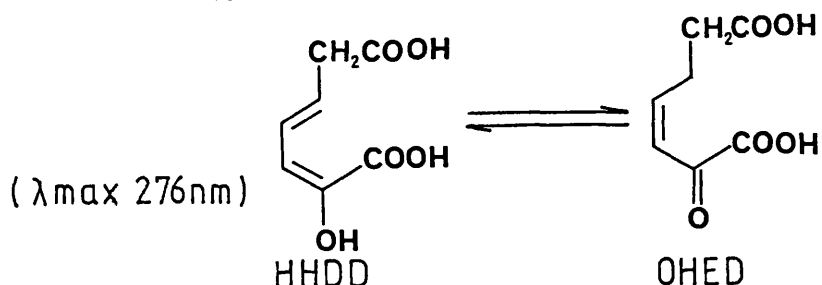
f) COHED DECARBOXYLASE ASSAY.

(Garrido-Pertierra and Cooper, 1981)



To assay the decarboxylase a mixture of CHM and COHED was allowed to form spontaneously in the cuvette. To determine when the spontaneous isomerisation had reached an equilibrium state, thus forming the substrate for the decarboxylase assay, the absorbance at 300nm was monitored. The reaction mixture consists of 0.1 μmol spontaneously isomerised CHM (~0.8 units) in 0.1M Na-phosphate buffer pH7.5 and the reaction was started by the addition of 5-100 μl of extract. The initial rate of reaction was obtained from the decrease in absorbance at 300nm due to the spontaneous conversion of CHM to COHED to restore the equilibrium as the COHED was decarboxylated to form HHDD. The molar extinction coefficient for CHM was taken as 20,000 $\text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}$.

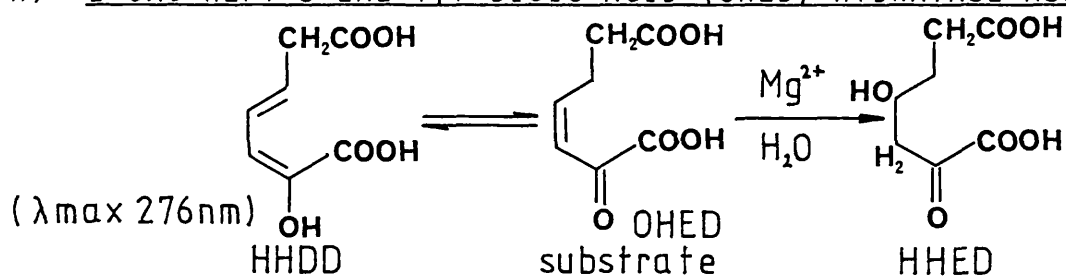
g) HHDD ISOMERASE ASSAY.



HHDD has an absorbance peak at 276nm which decreases rapidly in 0.1M sodium phosphate buffer pH7.5 due to spontaneous isomerisation. However, the rapid spontaneous isomerisation can be slowed by carrying out the assay in

deuterium oxide (D_2O). For this the reaction mixture consisted of 0.97ml D_2O , 20 μ l 0.1M sodium-phosphate buffer pH7.5 and 5-20 μ l of extract. It was assumed that HHDD was converted to pyruvate in equimolar amounts via the enzymes of the 3,4DHPA catabolic pathway, therefore the molar extinction coefficient for HHDD could be calculated from the amount of pyruvate formed. The amount of pyruvate produced from a given quantity of HHDD, with a known absorbance at 275nm, was detected by the NADH linked lactate dehydrogenase assay. The molar extinction coefficient for HHDD at pH7.5 was calculated to be $18,600 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$.

h) 2-OXO-HEPT 3-ENE 1,7 DIOIC ACID (OHED) HYDRATASE ASSAY.



To assay the OHED hydratase an equilibrium mixture of HHDD and OHED was allowed to form in the cuvette, utilising the rapid spontaneous isomerisation rate in 0.1M sodium-phosphate buffer pH7.5. When the decrease in absorbance at 276nm had stopped 5 μ mol of magnesium chloride was added and the reaction started by the addition of 20-50 μ l of extract. The initial rate of reaction is obtained from the decrease in absorbance at 276nm as OHED is converted to HHED with the concomitant isomerisation of HHDD to OHED to maintain the equilibrium.

i) HHED ALDOLASE ASSAY.

The HHED aldolase can be assayed for in two ways; either by following the decrease in absorbance at 260nm upon the addition of a cell extract to the reaction mixture, or by assaying for the product of the aldolase reaction, pyruvate. For the purposes of this study the assay for the product of the aldolase reaction was chosen, as it appeared to be the most reliable method for determining the specific activity of the HHED aldolase.

j) THE FORMATION OF PYRUVATE.

This was used to determine if all the enzymes required for the conversion of a specific substrate to pyruvate were present in the extract being tested. The initial rate of reaction was obtained from the decrease in absorbance at 340nm as NADH was oxidised in the presence of lactate dehydrogenase (LDH) by the pyruvate formed from the substrate being analysed. The reaction was assayed in 0.1M sodium phosphate buffer pH8.0 containing 5 μ mol magnesium chloride, 0.15 μ mol of NADH, 4 units of lactate dehydrogenase and an excess of the substrate.

The formation of pyruvate was also used as a discontinuous assay to determine the molar extinction coefficient for HHDD and HHED. The assay is essentially the same as described above except that the reaction for the conversion of a known amount of intermediate to pyruvate was allowed to continue for 20min before the addition of NADH and LDH. The total

decrease in absorbance at 340nm was measured as NADH was oxidised due to the LDH acting on the pyruvate formed in the reaction.

PROTEIN PURIFICATION.

The initial attempt to purify the 3,4DHPA dioxygenase was by the method of Takeda (1966) who isolated the 3,4DHPA dioxygenase from *P.putida*. This procedure did not prove to be successful for the *E.coli* C enzyme, so an alternative procedure was tried. The protocol subsequently developed for the purification of the 3,4DHPA dioxygenase is described on page 146.

FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC).

The Pharmacia FPLC and columns were used according to the conditions specified in the Pharmacia manual.

NUCLEAR MAGNETIC RESONANCE SPECTROMETRY (NMR).

The analysis and characterisation of the isolated intermediates of the 3,4DHPA catabolic pathway was kindly carried out by Dr.P.Cullis of the Chemistry Dept. Leicester University.

GENETIC PROCEDURES.

STRAIN CONSTRUCTION.

The presence of a *recA* mutation decreases recombination between the plasmid and chromosome where there are homologous DNA sequences. For this reason *recA* mutations were introduced into HPA negative mutants which were used as recipients in cloning experiments.

A strain of *E.coli* K-12 carrying a *recA* mutation with a closely linked Tn10 (tetracycline resistance) transposon inserted into the sorbitol gene, (cotransduction frequency 50% between *recA* and Tn10) was used as the donor for P1 transduction (Miller 1972), the recipients being *E.coli* C HPA negative mutants. In this way an initial screening for *recA* mutants was made by selection for tetracycline resistance.

Further identification was made by testing the tetracycline resistant recipients for sensitivity to irradiation with ultra-violet light (Maniatis et al, 1982).

In generating *recA* strains by this manner the resulting strain is now resistant to tetracycline. Detection of the presence of chromosomal DNA in the *Bam*H1 site of a pBR vector is by insertional inactivation of the tetracycline resistance gene. This means that there could be problems if the strain used in the cloning procedures is already tetracycline resistant. So, the Tn10 carrying the tetracycline resistance must be eliminated. Using the method of Bochner et al (1980) a positive selection for loss of Tn10 can be made by growth on fusaric acid plates. Growth media consisting of nutrient agar, plus a gratuitous inducer of the TET protein and fusaric acid, ensures that only tetracycline sensitive organisms grow.

PHENOL EXTRACTION OF PROTEIN FROM DNA.

Freshly distilled phenol (Fisons) was equilibrated with 0.5M Tris-HCl buffer pH7.4 and made 0.1% with respect to 8-hydroxy-quinoline. The phenol was measured into 50ml aliquots and stored at -20°C .

For the extraction of protein from DNA the Tris-equilibrated phenol was mixed with an equal volume of chloroform/isoamyl alcohol (24:1). An equal volume of this mixture was then added to the DNA solution and the two phases mixed. These extractions were usually carried out in Eppendorf tubes, so the two phases were separated by centrifugation in a microfuge for 1-2 min.. The upper aqueous

phase was removed and any traces of phenol in this fraction were extracted with either chloroform or diethyl ether.

ETHANOL PRECIPITATION OF DNA.

To precipitate DNA from solution 0.1 volume of 3M Na acetate, pH5.6, was added followed by 2-3 volumes ethanol. The mixture was incubated at -70°C for 10-15min. in a dry ice/ethanol bath and then centrifuged for 10min. in a microfuge. The supernatant was poured off carefully and the pellet washed with 70% ethanol. The pellet was then dried under vacuum and resuspended in the desired buffer.

PREPARATION OF CHROMOSOMAL DNA.

High molecular weight chromosomal DNA from *E.coli* and *P.putida* was produced using the method of Chow et al (1977). Cells were grown overnight in 200ml of Luria broth at 37°C . The cells were harvested by centrifugation at 7,000r.p.m. in a 6x250ml rotor using an MSE18 centrifuge for 10 minutes at 4°C , and resuspended in lysis solution (Tris-HCl 10mM, EDTA 1mM, NaCl 100mM pH7.9, 1mg/ml lysozyme) and incubated at 37°C for 10 minutes. Thirty ml of 2% sarcosyl NL97 (in the same buffer) was then added, followed by heat-treated RNase and pronase to final concentrations of 20µg/ml and 1mg/ml respectively. The resulting mixture was then incubated at 42°C for 60 minutes.

The above preparation was extracted three times with phenol, then ethanol-precipitated and dried. The DNA was allowed to redissolve slowly in TE buffer (TE= Tris-HCl 10mM

pH7.9, EDTA 1mM) (for up to three days). Residual traces of phenol were removed by dialysis against three changes of TE buffer.

BULK PREPARATION OF PLASMID DNA.

The Birnboim and Doly Method (1979).

Cells from a 500ml overnight culture were harvested at 9,000r.p.m. in a JA21 rotor using a Beckman J-21B centrifuge for 5 minutes at 4°C, after which they were resuspended in 40ml ice-cold lysis solution (Tris-HCl 10mM, EDTA 1mM, NaCl 100mM, pH7.8, 1mg/ml lysozyme) and incubated on ice for 5 minutes. The spheroplasts were lysed by the addition of 80ml alkaline/SDS (NaOH 0.2M, SDS 1%) followed by incubation on ice for 4 minutes. Then 60ml of ice cold 3M potassium acetate pH5.5 solution was added and the mixture centrifuged at 8,000r.p.m. using a 6x250ml rotor in an MSE18 for 10 minutes at 4°C. The supernatant was mixed with 100ml of isopropanol and centrifuged at 8,000r.p.m. in a 6x250 rotor in an MSE18 centrifuge for 10 minutes at 4°C. The resulting pellet was washed with 70% ethanol and then resuspended in 7.5ml TE buffer. This was prepared for dye-bouyant density equilibrium centrifugation, by the addition of 4ml 5mg/ml ethidium bromide and 1.12g/ml CsCl and centrifuged at 40,000r.p.m. using a Vti65 rotor in a Sorvall OTD65 for 40 hours at 20°C. The plasmid bands were removed and ethidium bromide extracted with isopropanol. The CsCl was removed by dialysis (1 volume of DNA solution to 1000 volumes of TE) against three changes of TE.

PLASMID MINI PREPARATIONS.

The alkaline lysis method (Maniatis et al 1982).

This method was used to determine the average insert size of a potential library and also to analyse plasmid constructions of interest.

A 1.5ml sample of an overnight culture was harvested by centrifugation in an Eppendorf centrifuge for one minute. The medium was removed and the pellet resuspended in 100µl Tris-glucose (glucose 50mM, EDTA 10mM, Tris-HCl 25mM pH8.0) solution and allowed to stand at room temperature for 5 minutes. This was then followed by the addition of 200µl of alkaline SDS solution (1% SDS in 0.2M NaOH) and the contents of the tube mixed. The mixture was then incubated on ice for 5 minutes, followed by the addition of 150µl of ice-cold potassium acetate (3M with respect to potassium and 5M with respect to acetate pH 5.0). The tube was inverted then vortexed for 10 seconds and incubated on ice for 5 minutes. The mixture was then centrifuged, in an Eppendorf centrifuge, for 5 minutes and the supernatant transferred to a fresh tube where it was phenol extracted as described previously. Two volumes of ethanol were added to the supernatant and incubated at room temperature for 2 minutes, followed by a 5 minute centrifugation. The resulting pellet was then vacuum dried and resuspended in TE.

The DNA was then suitable for transformation or restriction endonuclease digestion analysis.

AGAROSE GEL ELECTROPHORESIS.

(Maniatis et al, 1982)

To analyse the molecular weights of most DNA samples 0.8% agarose gels were normally used. However, for high molecular weight DNA 0.5% agarose gels were necessary. The agarose was made up with TAE buffer (0.04M Tris-acetate pH8.0, EDTA 0.001M). Before loading the DNA sample, it was mixed with 0.1 volume loading buffer (0.25% bromophenol blue, 30% glycerol in water). Gels were generally run at 20-50v for 14hr. or 100v for 1-4hr.

Marker fragments of known molecular weight from λ c DNA digested with restriction enzyme(s) were used to determine the size of unknown fragments. The restriction digest normally used was *Hind*III but on some occasions *Xho*I and *Eco*RI were also used.

PREPARATION OF λ -PHAGE DNA.

The method of Blattner et al (1977) was used for the preparation of all types of λ -phage.

Five hundred ml of Luria broth was inoculated with 3 plaques, and the surrounding cells, picked (using a sterile Pasteur pipette) from a freshly prepared plate lysate. This produced the desired ratio of phage to bacterial cells to achieve a titre of at least 10^{10} - 10^{11} phage/ml, which is necessary for further processing.

The phage were precipitated with NaCl (10g/500ml) and PEG 6000 (50g/500ml) and resuspended in 5 ml phage buffer (10mM

Tris, 10mM MgSO_4 , 10mM NaCl and 0.01% gelatine, pH7.5). The phage were then purified by caesium chloride block gradient centrifugation at 40,000r.p.m. for 4 hours at 20°C, in a Beckman AH627 rotor. The purified phage band was isolated from the block gradient then dialysed to remove CsCl. The phage protein coat was removed by digestion with pronase at 1mg/ml for 1 hour at 42°C. The resultant mixture was then extracted with phenol to give protein-free DNA.

RESTRICTION ENZYME DIGESTION.

Restriction digests of DNA were carried out according to the suppliers' recommendations (Bethesda Research Laboratories Inc.).

For partial restriction digestions a series of aliquots were taken from a single large digest at 5 minute intervals and the reaction stopped by the addition of EDTA (to a final concentration of 20mM). The aliquots were then electrophoresed on an 0.8% agarose gel to assay the size of the (partial) digestion products. This allowed suitable digestion conditions to be deduced for a single large digestion, giving DNA fragments around the size desired.

After a restriction digest on any piece of DNA the fragments produced were routinely run out on an agarose gel to ensure the desired products had been achieved cleanly.

LIGATION REACTIONS.

Ligation of the chromosomal DNA and plasmid vector was carried out in 66mM Tris-HCl pH7.6, 6.6mM MgCl_2 , 10mM

dithiothreitol 0.5mM ATP and incubated over night at 10°C with the addition of 1µl T₄, (1 unit) DNA ligase (Boehringer).

PHOSPHATASE TREATMENT.

Calf intestinal alkaline phosphatase(CIP) was obtained from Boehringer and added at 0.1 unit CIP per µg of DNA, after the one hour restriction reaction i.e. in the same buffer. The phosphatase reaction was then allowed to proceed for 15-30 min.. The restriction enzyme and the CIP were removed by phenol extraction and the DNA precipitated with ethanol.

PREPARATION OF VECTORS.

The vectors pBR322 and pBR328 were prepared for the cloning experiments by digesting them with the restriction enzyme *Bam*H1 and then incubated with CIP to remove the terminal phosphate group. The restriction enzyme and the CIP were removed by phenol extraction and the DNA precipitated with ethanol. The vectors were prepared in a similar manner for the subcloning experiments except that different restriction enzymes have been used.

The cosmid vector, Cos4, was prepared by digesting the vector with the restriction enzyme *Bam*H1 then treating it with CIP. The restriction enzyme and the CIP were extracted with phenol and the DNA precipitated using ethanol. The now linear Cos4 was digested with *Pvu*11 to produce the cosmid arms. The *Pvu*11 was extracted with phenol and the DNA precipitated using ethanol.

ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS.

The required fragment of DNA was cut out of an agarose gel and placed in a dialysis bag containing 300 μ l TAE (0.04M Tris-acetate, 0.1M EDTA pH8.0) buffer and electrophoresed for 1-2hr. at 100v. The current was then reversed for 45sec. after which the solution was removed from the dialysis bag and passed through a blue Gilson tip plugged with siliconised glass wool to remove any pieces of agarose gel. The dialysis bag was then washed with a further 300 μ l of TAE and this was also passed through the blue tip. The resulting solution was then extracted with phenol and the DNA precipitated with ethanol.

TRANSFORMATION.

(Kushner, 1978)

This procedure was used to introduce plasmid DNA into a recipient cell, by making the cell more permeable to DNA. The MOPS-rubidium chloride (RbCl) method was used with all plasmids in this study.

The recipient cells were grown to an O.D.₆₈₀ of 0.5 (for *recA* strains) and 0.2 (for non *recA* strains). The cells were then harvested and washed in 1/3 volume MOPS-RbClI (10mM MOPS, 0.01M RbCl pH7.0) solution. The cells were centrifuged and resuspended in 1/3 of the original volume MOPS-RbClII (100mM MOPS, 0.01M RbCl, 50mM CaCl pH6.5) and were then incubated on ice for 30-90 minutes. The cells were centrifuged and resuspended in 1/10 original volume

MOPS-RbClIII. DMSO added to 0.2% and plasmid DNA were then added and the mixture placed on ice for an hour. It was then heat shocked at 55°C for 30 seconds, placed in an ice/water bath, 1ml of Luria broth was added and the cells incubated at 37°C for one hour (non-shaking) to allow them to recover. From this stage they were plated out onto selection plates.

Tn 1000 MUTAGENESIS.

(Guyer et al, 1980)

Tn 1000 mutagenesis relies on a transposition event occurring between an F plasmid carrying Tn1000 and the plasmid of interest, in this case pJJ801.

A strain of *E.coli* K-12, RB308, carrying Tn 1000, was transformed with pJJ801 DNA isolated from 5K. RB308 (F⁺, pJJ801) was then used as the donor in conjugal mating experiments. The recipients were HPA negative mutants, JJ221 (5-carboxymethyl-2-hydroxymuconatesemialdehyde dehydrogenase deficient) and JJ247 (a pleiotropic mutant). The selection for a recipient receiving pJJ801 by conjugation was made on Luria agar containing ampicillin (100µg/ml) or minimal medium containing glycerol and ampicillin (100µg/ml). This selects against the donor which has a thymine requirement and also against any recipients which lack the plasmid, and so are ampicillin sensitive.

PREPARATION OF λ -PACKAGING EXTRACTS.

(Maniatis et al 1982).

a) Sonicated extract from induced strain BHB2690 (prehead donor).

A culture of BHB2690 was grown in 500ml Luria broth to an O.D._{600nm} of 0.3. The lysogen was induced by placing the flask in a water bath at 42°C for 15 minutes with constant shaking. The induced cells were then shaken for a further 2-3 hours at 38-39°C. The cells were harvested by centrifugation at 9,000r.p.m. in a JA21 rotor using a Beckman J21B centrifuge for 5 minutes at 4°C and resuspended in 3.6ml sonication buffer (20mM Tris-HCl 1mM EDTA 5mM β -mercaptoethanol pH8.0). This was followed by 20-30 second sonication bursts, with one minute cooling in between, until the solution had just cleared. Sonicated samples were centrifuged at 12,000r.p.m. in a 8x50ml rotor using an MSE 18 centrifuge for 10 minutes to remove cell debris. An equal volume of sonication buffer and 1/6 volume of freshly prepared packaging buffer (Tris-HCl 60mM pH8.0, spermidine 50mM, putrescine 50mM, $MgCl_2$ 20mM, ATP 30mM, and β -mercaptoethanol 30mM) were added to the supernatant. Aliquots of 15 μ l were then dispensed into precooled Eppendorf tubes and immediately immersed in liquid nitrogen. These were then transferred to -70°C for long term storage.

b) Freeze/thaw lysate from induced strain BHB2688 (Packaging protein donor).

A culture of BHB2688 was grown in 500ml Luria broth to an O.D._{680nm} of 0.3. This was then heat-induced at 45°C with constant shaking for 15 minutes the induced cells were further shaken for 2-3 hours at 38-39°C. The cells were harvested by centrifugation at 9,000r.p.m. in a JA21 rotor using a Beckman J21B centrifuge for 5 minutes at 4°C and resuspended in 3ml of ice-cold sucrose solution (10% sucrose, Tris-HCl 50mM pH 8.0). This was then dispensed into six precooled Eppendorf tubes and 25µl of fresh, ice cold lysozyme solution (2mg/ml lysozyme, Tris-HCl 0.25M pH8.0) was added to each tube, mixed and quickly put into liquid nitrogen. The extracts were thawed on ice and 25µl of packaging buffer added to each tube. Next, the thawed extracts were combined and centrifuged at 40,000r.p.m. in a Ti10x10 rotor using an MSE 65 centrifuge for 60 minutes at 4°C. Aliquots (10µl) of the supernatant were then dispensed into precooled Eppendorf tubes, immersed in liquid nitrogen and then stored at -70°C.

DNA was packaged *in vitro* by combining one aliquot of each of the freeze/thaw and sonicated extracts with approximately one µg of DNA and incubating on ice for one hour.

TRANSFER OF NUCLEIC ACIDS FROM AGAROSE GELS TO
NITROCELLULOSE PAPER. (Southern, 1980)

For some experiments it is necessary to transfer DNA (after electrophoresis in agarose) to nitrocellulose paper. DNA runs as a double-stranded molecule so it must be denatured before transfer. The DNA fragments were denatured by placing the gel on the transilluminator for 5min. and then soaking the gel twice for 15min. in 1.5M NaCl, 0.5M NaOH. The gel was then neutralised by soaking it twice, for 15min in 0.5M Tris-HCl pH7.5, 1.5M NaCl.

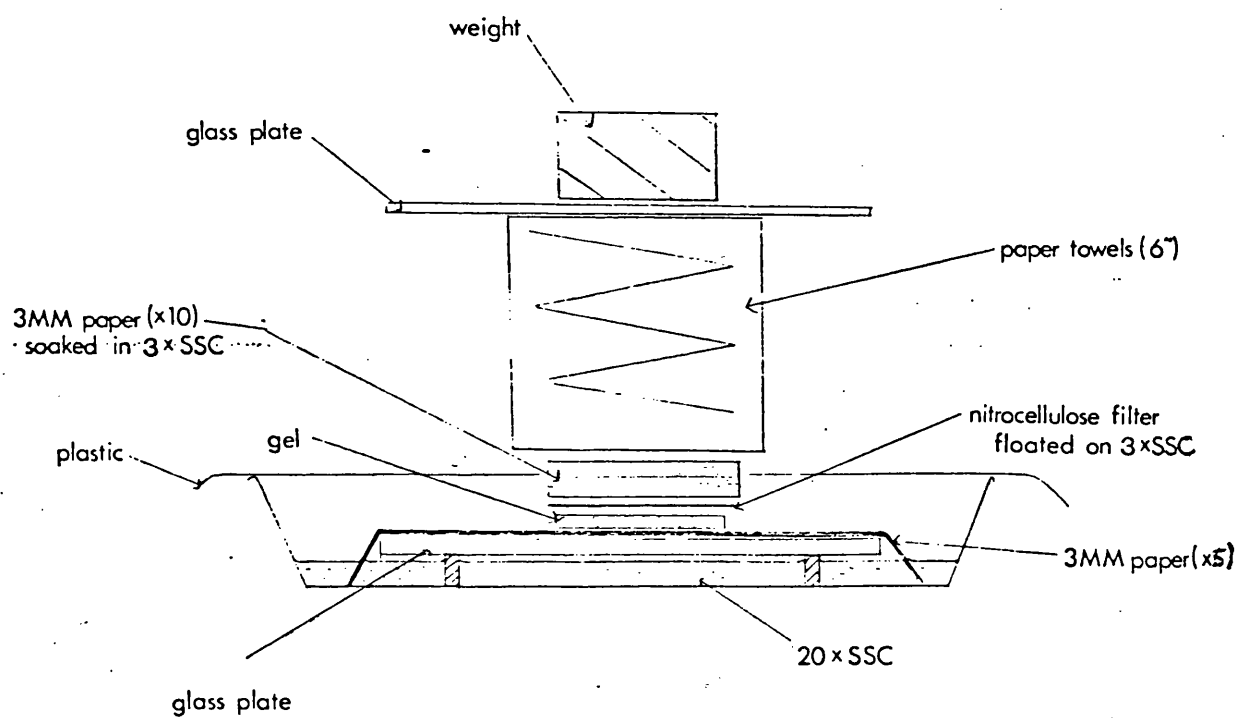
The nitrocellulose paper used as the recipient for the DNA was pre-soaked in 3xSSC (1xSSC= 0.15M NaCl, 0.015M Na-citrate). A construction was then built as shown in fig, 2.1.1. The first pieces of 3MM Whatman paper on top of the gel were soaked in 3xSSC. It is important that there are no air bubbles between the surface of the agarose and the nitrocellulose paper. The transfer was allowed to continue overnight, after which the nitrocellulose filter was baked for 2hr. at 80°C.

SCREENING BACTERIAL COLONIES BY HYBRIDIZATION.

(Grunstein and Hogness, 1975).

Colonies to be screened were patched in duplicate on selection plates. After incubation overnight one of each duplicate was placed at 4°C for storage whilst the other was used for screening. Nitrocellulose filters were then placed on the surface of the plate and left for 2-5 minutes

fig, 2.1.1.



Transfer apparatus for Southern Blots.

(aligning marks were made if necessary) to transfer the colonies. The filters were carefully lifted from the agar surface, and placed on 3mm Whatman paper predampened with 10% SDS. After 5 minutes the filters were placed on a 0.5M NaOH pad for a further 5 minutes after the alkali-treatment the filters were neutralised by being placed on pads wetted with 1.0M Tris-HCl, pH7.5 1.5M NaCl. The filters were then blotted dry and baked for 2 hours at 80°C.

OLIGO-LABELLING OF DNA BY HEXADEOXYNUCLEOTIDE PRIMER.

(Feunberg and Vogelstein, 1983)

Plasmid DNA was cleaved with the appropriate restriction enzyme(s) to give the required fragment for labelling. The digest was then run out on a low gelling temperature agarose gel and the required band excised carefully with the minimum amount of agarose. This was placed in an Eppendorf tube and water added at a ratio of 1.5ml H₂O:1g agarose gel. The tube was then placed in a water bath and boiled for 7 min. to melt the agarose and denature the DNA. The labelling reaction was carried out at room temperature by the addition of the following reagents in the stated order: X μ l H₂O (to a total volume of 25 μ l), 5 μ l OLB buffer, 1 μ l BSA (BRL enzyme grade), X μ l DNA fragment (25ng) upto 16.25 μ l, 1 μ l (³²P) α -dCTP(10 μ Ci/ μ l) and 2 μ l Klenow (large fragment) polymerase 1, 2 units. The reaction was incubated at room temperature for 3hr. and stopped by the addition of 100 μ l Stop buffer. The unincorporated material was removed by passing the reaction mixture through a Sephadex G50 column made in a Pasteur pipette. The fractions with the labelled DNA were

then used in the hybridisation reaction.

Buffers:

TE. buffer.

3mM Tris-HCl

0.2mM EDTA. at pH 7.0.

Solution O.

1.25M Tris-HCl.

0.125 M MgCl_2 at pH 8.0. (stored at 4°C).

dNTP's.

dATP}

dTTP} 0.1M in TE (stored at -20°C).

dGTP}

Solution A.

1000 μl soln. O.

18 μl 2-mercaptoethanol.

5 μl each of the dNTP's.

Solution B.

2M HEPES (titrated to pH 6.6 with 4M NaOH.

(stored at 4°C).

Solution C.

Hexadeoxynucleotides

50 A units suspended in 550 μl TE to give a
concentration of 90 A units/ml.

(stored at -20°C)

OLB buffer.

The solutions A,B,C were mixed together in
the ratio 10:25:15 (50 μl). (stored at -20°C)

Stop buffer.

20mM NaCl, 20mM Tris-HCl pH 7.5, 2mM EDTA,

0.25% SDS and 1mM dCTP (cold).

(stored at -20°C)

HYBRIDIZATION OF RADIOACTIVE PROBES TO DNA.

Nitrocellulose papers impregnated with DNA were prehybridized in degassed, prewarmed ($65-70^{\circ}\text{C}$) 5xSSPE, 5xDenhart's solution and 100 $\mu\text{g}/\text{ml}$ boiled salmon sperm DNA. The prehybridization was carried out in a sealed plastic bag at 65°C for one hour.

For the hybridization, the prehybridization solution was squeezed out of the plastic bag and the hybridization solution added using a Pasteur pipette. The hybridization solution consisted of prewarmed ($65-70^{\circ}\text{C}$), degassed, 4x SSPE 1x Denhart's solution and 100 $\mu\text{g}/\text{ml}$ boiled salmon sperm DNA. The denatured probe DNA was then introduced into the plastic bag which was resealed and incubated overnight at 65°C .

After hybridisation the filter was washed twice for fifteen min. at room temperature in 2xSSPE, 0.1% SDS. Then twice for fifteen min. in 0.1xSSPE, 0.1% SDS. The nitrocellulose filter was blotted dry and autoradiographed.

SSPE: 0.18M NaCl, 10mM NaPO₄ pH7.1 and 1mM EDTA.

Denhart's solution: 0.02% ficol, 0.02% polyvinylpyrrolidone in 3xSSC.

MUTAGENESIS USING ETHYLMETHANESULPHONATE(EMS).

Cultures of *E.coli* were mutagenised in minimal medium with 1.5% EMS for 2hr. at 30°C, as described by Miller (1972). The culture was split into two so that independent mutants might be isolated. The survivors were then allowed to grow to an O.D._{680nm} of 0.4 in non-selective glycerol-minimal media at 30°C.

Mutants unable to grow on a particular carbon source were selected by penicillin treatment. The EMS mutagenised cells were harvested in exponential phase. The cells were then resuspended in a medium that did not allow growth of the desired mutants (HPA), to an OD₆₈₀ of 0.08 and were allowed to grow until the OD₆₈₀ had doubled, at which time penicillin was added to a final concentration of 1-2mg/ml. The cultures were then incubated over night. Cells surviving the penicillin treatment were harvested and resuspended in the same volume of minimal medium.

In the mutagenesis of CT001 the surviving cells were harvested and resuspended in Luria broth where they were treated as the inoculum for a transformation procedure. pJJ801 was then introduced into the mutagenised cells, with the hope that in subsequent selection stages mutations in genes not carried by pJJ801 would be identified. The cells were harvested after the transformation and treated in the same way as the *E.coli* C mutagenised cells, except that ampicillin was always present in the medium.

Various dilutions of the cells were plated onto a medium allowing mutant growth (glycerol). Colonies appearing after

incubation were replica-plated onto HPA and 3,4DHPA plates to identify mutants unable to grow on these substrates. Presumptive mutants identified by their growth patterns were picked off, purified and checked for growth on appropriate selective and nonselective media.

CONJUGAL MATINGS.

In order to transfer cloned *E.coli* C DNA into *P.putida* the desired fragment(s) were subcloned into the shuttle vector pMMB34 which can replicate in both strains. The newly constructed plasmid is then transformed into C600(pLG223). The plasmid pLG223 can co-mobilise the shuttle vector into the recipient. pLG223 is a suicide vector as it cannot replicate in *P.putida*.

Both the donor and the recipient strain were grown (shaking gently) to an OD₆₈₀ of 0.1 and mixed together in a series of ratios: 10:1, 5:1, 1:1, 1:5 and 1:10 which were then incubated at 30°C for 4hr. A sample was then removed, vortexed to disrupt the mating pairs and plated onto the selection plates. The remainder of the culture was allowed to incubate overnight, then a further sample was removed, vortexed and plated onto the selection plates.

ACRYLAMIDE GEL ELECTROPHORESIS.

Various types of polyacrylamide gels were used, dependent upon the type of sample.

Oligonucleotides were resolved on 7.1M urea 20% polyacrylamide gels in 1xTBE buffer. The polyacrylamide/urea

gels were poured in a vertical gel apparatus. The gel was run in 0.5x TBE buffer at approximately 50v/cm for 2-3hr..

For DNA sequencing 6% polyacrylamide/urea gels were used. To increase the amount of useful data obtained from one loading an ionic gradient was made in the lower half of the gel. The gradient was made in a 25ml pipette by drawing up 8ml of a 0.5xTBE buffer acrylamide mix, followed by 12ml of a 2.5xTBE buffer acrylamide solution into the same pipette. The two solutions were mixed at the interface by introducing four air bubbles into the pipette and the resulting mix was poured slowly between the two plates. The top of the gel was filled with 0.5xTBE acrylamide mix. The upper reservoir was then filled with 0.5xTBE buffer and the lower reservoir filled with 1xTBE. The gel was run at about 50v/cm (40w) for 15min. to preheat the gel. The samples were loaded on the gel and run at 50v/cm for 2-3hr.

Proteins were separated on 0.1% SDS/ 7-12.5% polyacrylamide gels, with a 3.5% acrylamide stacking gel. The reservoirs contained 1xTris-glycine 0.2% SDS buffer pH8.9. The loaded gel was run at 500v 40mA on an LKB protein electrophoresis system. Protein samples were mixed with SDS-loading buffer and boiled for 3min. before loading.

Native protein gels were run using the same conditions except the SDS was left out at all the stages. Also a nondenaturing loading buffer was used with the protein samples and they were not boiled.

The protein gels were stained with protein gel staining buffer.

SOLUTIONS:

TBE buffer: (20x)

Tris-base	205g
Boric acid	115g
EDTA	50g
Made up to	1000ml

Acrylamide gel mixes.

20% acrylamide/urea:

38:2g acrylamide/Bis	25ml
(Bis=N,N'-methylene bisacrylamide)	
Urea	21.5g
10xTBE	5ml
Water	to 50ml
10% Ammonium persulphate	0.3ml
TEMED	15µl
(TEMED=Tetramethylethylenediamine)	

6% acrylamide/urea:

0.5xTBE acrylamide urea mix (6%);	
Urea	430g
10xTBE	50ml
38:2 acrylamide/Bis	150ml
water	to 1l

2.5xTBE acrylamide urea mix (6%);

Urea	430g
10xTBE	250ml
38:2 acrylamide/Bis	150ml
Sucrose	50g
Bromophenol blue	50mg
Water	to 1l

12.5% acrylamide/SDS:

	<u>gel</u>	<u>stack</u>
30:0.8 acrylamide/Bis	18.75ml	1.25ml
3M Tris-HCl, pH8.9	5.6ml	-
1M Tris-HCl, pH6.8	-	1.25ml
Water	19.9ml	6.84ml
10% SDS	0.45ml	0.1ml
10% Ammonium persulphate	0.225ml	60µl
TEMED	22.5µl	7µl

SDS-sample buffer:

Glycerol	2ml
SDS	0.4g
0.25M Tris-HCl, pH6.8	5ml
β-mercaptoethanol	1ml
Bromophenol blue	0.01g

Native-sample buffer:

0.18M Tris-HCl, pH6.8	
Glycerol	29%
Bromophenol blue	0.1%

Protein staining:

Kenacid blue	0.2%
Methanol	50%
Acetic acid (glacial)	7.5%

DNA SEQUENCING.

DNA sequencing was carried out by the chain termination method of Sanger et al (1977) using ^{35}S dATP as the radioactive label. Templates for sequencing were prepared by inoculating a 2ml starter culture of JM101 (100 μl of a fresh overnight culture of JM101 in 50ml Luria broth) with 100 μl M13 phage suspension (1 plaque picked into 0.5ml phage buffer). This was incubated, with good aeration, overnight at 37°C , after which 1.5ml of each culture was transferred to an Eppendorf tube and spun in a microfuge for three minutes. Supernatant (1ml) was transferred to a fresh Eppendorf tube and 200 μl of 2.5M NaCl, 10% PEG 6000 was added. The mixture was left at room temperature for 30 min. and then centrifuged for 5min. The supernatants were removed, the tubes respun and the remnants of the supernatants carefully pipetted off. The pellets were resuspended in 100 μl 1.1M sodium acetate, pH7.0 and phenol extracted. The DNA was precipitated by the addition of 250 μl of ethanol and resuspended in 15 μl TE buffer.

For the priming reaction 5 μl of the DNA template solution was mixed with 1 μl TM buffer, 2.5ng of the primer and made up to 10 μl with water. The priming reaction was carried out at 65°C for 30min. and then the reaction was allowed to cool slowly, to room temperature. The primed DNA templates were split into 4x2 μl aliquots to ^{each of} which 2 μl of the appropriate di-deoxy-nucleotide mix was added. After the addition of 2 μl of ^{35}S -dATP/Klenow polymerase mix to each tube the mixture was left at room temperature for 20min.. Then 2 μl of chase

mix was added and the reaction left at room temperature for a further 20 min.. The reaction could then be stored at this stage at -20°C for subsequent analysis or else $2\mu\text{l}$ of sequencing dye was added to each tube and the solution boiled for 3 min.. After the samples had been spun down they were ready for loading onto a polyacrylamide gel. When the gel had run it was immersed in 10% methanol, 10% acetic acid for 15min. The excess fixer solution was drained from the gel, which was blotted onto Whatman 3MM paper and dried down using a gel dryer for 40-60min. The gel was then autoradiographed.

Sequencing mixes.

a) Reaction Mixes.

	<u>T</u>	<u>C</u>	<u>G</u>	<u>A</u>
0.5mM dTTP	25	500	500	500
0.5mM dCTP	500	25	500	500
0.5mM dGTP	500	500	25	500
10mM ddTTP	50	-	-	-
10mM ddCTP	-	8	-	-
10mM ddGTP	-	-	16	-
10mM ddATP	-	-	-	1
TE buffer	1000	1000	1000	1000

(volumes in μl)

b) ^{35}S -dATP Klenow mix:

Sterile H_2O (Q)	60 μl	
^{35}S -dATP (Amersham)	6 μl	(2 μCi)
Klenow polymerase (BRL)	3 μl	(5 units/ μl)

c) dNTP chase mix

0.25mM dATP

0.25mM dCTP

0.25mM dGTP

0.25mM dTTP

d) Sequencing dye mix

De-ionised formamide	100ml
----------------------	-------

Xylene cyanol FF	0.1g
------------------	------

Bromophenol blue	0.1g
------------------	------

0.5M EDTA pH7.5	2ml
-----------------	-----

ANALYSIS OF SEQUENCE DATA.

The data produced was analysed with the aid of DNA data processing packages available on the University mainframe computer.

RESULTS

The biochemical pathway for the catabolism of 3,4DHPA by *E.coli* C has been reported by Cooper and Skinner (1980) see fig.1.1.10. Little is known about the genetic organisation and regulation of this pathway, although Skinner (1981) did attempt some analysis using classical microbial genetic approaches such as phage P1 co-transduction and F-mating. He concluded that the *hpa/hpc* genes were located between minutes 1 and 3 on the *E.coli* C chromosome. The HPA mutations isolated by Skinner were > 90% co-transducible with each other and he also showed that *E.coli* K12 grows neither on HPA, nor carries any *hpa/hpc* genes. P1 transduction of the *hpa/hpc* genes from *E.coli* C to *E.coli* K12 resulted in the *hpa/hpc* genes integrating into at least two positions on the *E.coli* K12 chromosome (at minutes 90to98 and 28to31).

In this section the setting up of cloning systems to identify genes from the 3,4DHPA (*hpc*) pathway is described, along with the characterisation of the plasmids obtained by these procedures.

3.1 ISOLATION AND CHARACTERISATION OF PJJ801

Hybrid plasmids carrying *hpc* genes were sought by their ability to confer upon the recipient HPA negative mutant the ability to grow on HPA. The CHMS dehydrogenase mutant JJ221 (*hpcC*, *recA*) was chosen for the initial cloning experiments because it was the best characterised mutant available and defective in only one gene.

E.coli C chromosomal DNA was prepared according to the

method of Chow et al (1977). Conditions for the partial digestion of chromosomal DNA yielding fragments of about 10Kb were determined by incubating a known amount of DNA with a series of dilutions of the restriction enzyme *Sau3a* for one hour (see fig,3.1.1).

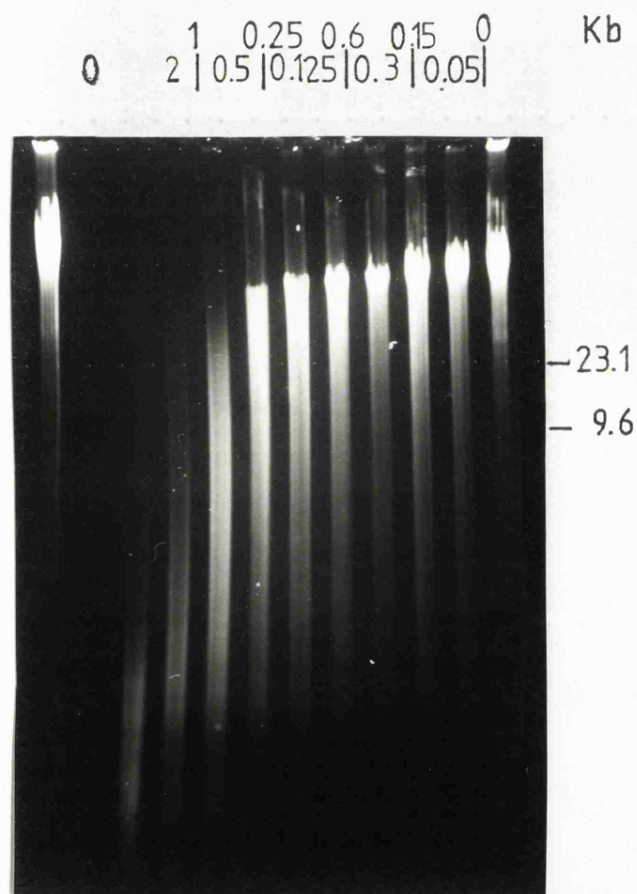
To optimise the conditions of hybrid plasmid production various ratios of pBR328 vector DNA to chromosomal DNA were used in a series of pilot ligations as shown in table,3.1.1. The best results were obtained when a ratio of 1:1 was used, so the ligation reaction for the cloning experiment consisted of 350ng of the vector and 350ng of chromosomal DNA. After the ligation the mixture was used to transform JJ221 and ampicillin-resistant transformants were selected on Luria-Ap agar. The initial selection was on Luria- Ap plates as opposed to minimal-HPA plates because this substantially reduces the number of HPA+ revertant colonies. Of the 6,100 transformants obtained plasmid DNA was isolated from 24, chosen at random, to determine the size of the chromosomal DNA insert. An insert size of approximately 10Kb was initially intended. However, the plasmids analysed showed an insert size range of 3-12Kb, the average being 6Kb. This size was then used in the Clarke and Carbon (1976) equation to calculate the probability of obtaining the desired genes, see calculation, 3.1.1. The probability of obtaining the desired gene from the 6,100 transformants was 99.97%.

All the colonies were then replica plated onto HPA plates and three colonies capable of growth on HPA were identified. To show that these were not simply revertants, plasmid DNA was isolated from each of them and used to transform JJ221. It was shown that each plasmid restored the ability of JJ221

fig,3.1.1.

Determination of the conditions for *Sau*3a partial digestion of *E.coli* C chromosomal DNA.

Units of *Sau*3a/ μ g DNA.



1 μ g of *E.coli* C chromosomal DNA in a total reaction volume of 15 μ l was digested for 1 hour and stopped by the addition of EDTA, then placed on ice. The various samples were then run on an agarose gel, as shown above. From this experiment it was estimated that 0.12 units *Sau*3a/ μ g DNA gave the optimum conditions for producing fragments of approximately 10kb in size.

Table 3.1.1

Transformation results obtained at various stages in the isolation of
pJJ801, using JJ221 as the recipient

Treatment	Number of Transformants (per µg of DNA)	Percentage transformants that grow on LB-tc plates (%)
None (supercoiled pBR 328)	5.5×10^5	100
pBR328 digested with <u>Bam</u> HI, and ligated	5.1×10^5	100
pBR328 digested with <u>Bam</u> HI, treated with calf intestinal phosphatase (CIP) and ligated	1.3×10^3	95
Ratios of pBR328 digested with <u>Bam</u> HI and treated with (CIP): <u>E. coli</u> C chromosomal DNA		
1 : 10	8.7×10^3	50
1 : 2	1.3×10^4	25
1 : 1	1.6×10^4	7
2 : 1	8.5×10^3	51
10 : 1	3.8×10^3	78

Calculation 3.1.1

Calculation of the probability of obtaining a clone carrying the desired genes

Using the Clarke and Carbon (1976) equation:

$$N = \frac{\ln(I - P)}{\ln(I - S)}$$

Where: N = number of colonies

P = probability of obtaining the desired gene

S = size of the insert as a fraction of the E.coli chromosome (4000kb)

$$\text{or } P = 1 - (I - S)^N$$

$$N = 6,100 \times 93\% \quad 93\% = \text{number of tc sensitive transformants}$$

$$S = 6/4000$$

$$\text{So } P = 1 - (1 - 6/4000)^{5673}$$

$$P = 99.97\%$$

to grow on HPA. The three plasmids were then mapped using the restriction enzymes *Bam*H1, *Eco*R1, *Hind*111 and *Sal*1. All three plasmids appeared to be identical and thus were probably siblings. One was chosen for further study and designated pJJ801. A detailed restriction map is shown in fig,3.1.2, this was produced using the restriction enzymes: *Bam*H1*, *Pst*1, *Eco*R1*, *Pvu*11*, *Hind*111, *Sal*1*, *Sph*1, *Pvu*1, *Bcl*1, *Cla*1, *Bgl*11, and *Nco*1, (A * indicates a restriction site within the chromosomal DNA). These restriction enzymes only have one or two restriction sites within the vector. The diagram of pJJ801 shows the plasmid with a 6.5Kb fragment of chromosomal DNA inserted in the *Bam*H1 site of pBR328.

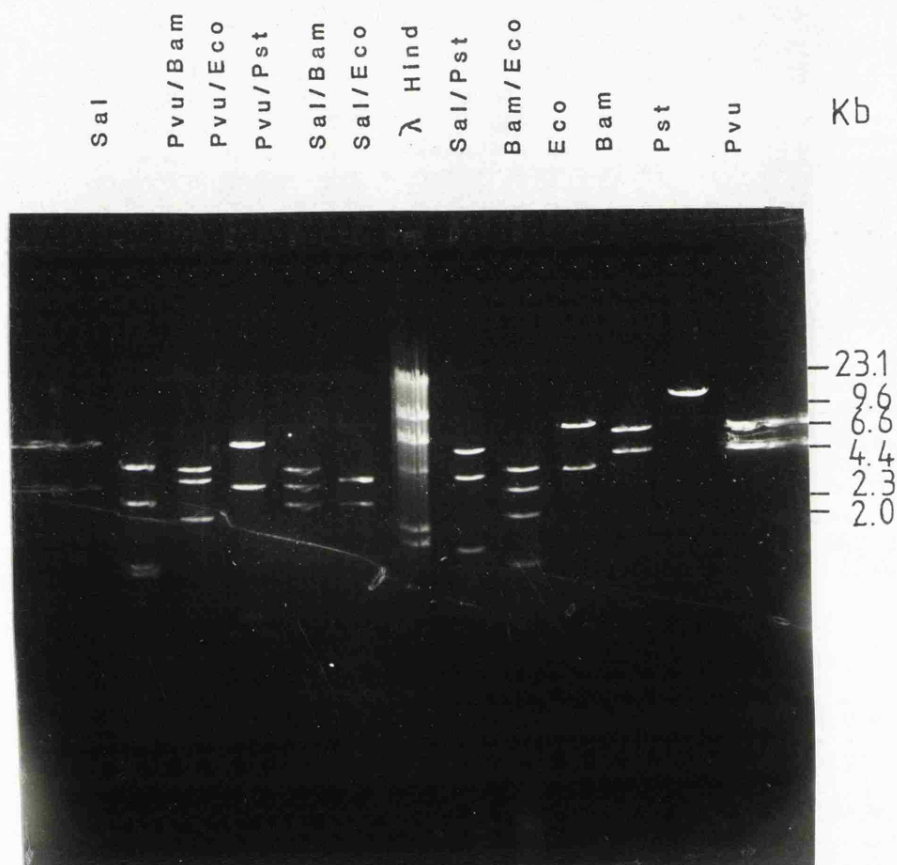
IDENTIFICATION OF THE ENZYMES ENCODED BY pJJ801.

In the previous section the selection of a plasmid, pJJ801, that suppressed the CHMS dehydrogenase mutant JJ221 was described. The ability of pJJ801 to allow growth of JJ221 on HPA suggested that the CHMS dehydrogenase gene (*hpcC*) had been cloned. The enzyme assays carried out on extracts from JJ221(pJJ801) are shown in table,3.1.2. These show that when JJ221(pJJ801) was grown on glycerol there was very little CHMS dehydrogenase activity detectable. However, when JJ221(pJJ801) was grown on HPA high activity for the CHMS dehydrogenase was observed. The CHMS dehydrogenase and other 3,4DHPA catabolic enzymes were found at similar levels to those observed for *E.coli* C grown on HPA. This showed that the CHMS dehydrogenase was encoded by pJJ801 and its expression was inducible in JJ221.

To characterise pJJ801 further it was introduced into a

A photograph showing examples of the restriction digests used to generate the restriction map of pJJ801.

Restriction digests.



The restriction map of pJJ801.

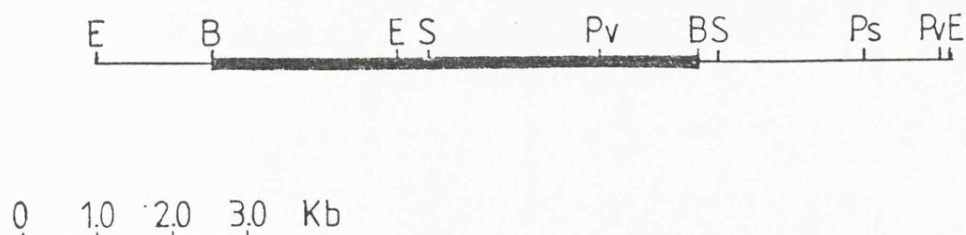


Table 3.1.2

Activities of the 3,4DHPA catabolic enzymes in JJ221 (pJJ801)

Specific activity (nmoles min⁻¹ mg protein⁻¹)

Strain	Growth substrate	3,4DHPA dioxygenase	CHMS dehydrogenase	CHM isomerase	COHED decarboxylase
<u>E. coli</u> C	HPA	180	160	98	43
JJ221	Gly	0	0	1	3
JJ221	Gly/HPA	59	0	45	10
JJ221 (pJJ801)	Gly	0	0.5	2	7
JJ221 (pJJ801)	HPA	190	170	102	56

series of *E.coli* C mutants and the effect of the plasmid observed.

JJ247 (*hpcBC, recA,*) was initially thought to be pleiotropically defective in all the 3,4DHPA catabolic enzymes (Skinner, 1981). When pJJ801 was introduced into this strain the ability to grow on HPA was restored. Table,3.1.3, shows the activities of the 3,4DHPA catabolic enzymes detected in JJ247(pJJ801) when grown on the substrates glycerol or HPA. High activities for the 3,4DHPA catabolic enzymes could be detected in extracts from cells grown on either substrate. The values obtained for JJ247(pJJ801) grown on HPA are similar to those observed for *E.coli* C grown on HPA. The specific activities obtained from extracts of JJ247 (pJJ801) grown on glycerol were even higher than those for *E.coli* C grown on HPA. These results indicate that the genes carried by pJJ801 can restore JJ247 to growth on HPA and the enzymes expressed by these genes are produced constitutively in this strain. This suggests that genes other than *hpcC* are present on the clone.

The strain CT001 was isolated by Skinner (1981) as a mutant which produced the 3,4DHPA catabolic enzymes constitutively. The aim of introducing pJJ801 into this strain was to see if the regulated phenotype was restored and so indicate whether a regulatory gene had been cloned. Table,3.1.4, shows the specific activities of CT001 are unaffected by the presence of pJJ801.

A succinate semialdehyde (SSA) dehydrogenase-negative mutant of the constitutive strain, CT101, was transformed with pJJ801 to determine whether the *sad* gene was carried on pJJ801. CT101(pJJ801) failed to grow on HPA, implying that

Table 3.1.3

Activities of the 3,4DHPA catabolic enzymes in JJ247 (pJJ801)

Strain	Growth substrate	Specific activity (nmoles min ⁻¹ mg protein ⁻¹)			
		3,4DHPA dioxygenase	CHMS dehydrogenase	CHM isomerase	COHED decarboxylase
<u>E. coli</u> C	HPA	180	160	98	43
<u>E. coli</u> C	Gly/HPA	120	110	71	27
JJ247	Gly	0	0.5	2	1
JJ247	Gly/HPA	3	2	5	5
JJ247 (pJJ801)	Gly	380	210	250	123
JJ247 (pJJ801)	HPA	190	180	120	52

Table 3.1.4

Activities of the 3,4DHFA catabolic enzymes in CT001 (pJJ801)Specific activity nmoles min⁻¹ mg protein⁻¹

Strain	Growth substrate	3,4DHFA dioxygenase	CHMS dehydrogenase	CHM isomerase	COHED decarboxylase
CT001	Gly	250	210	150	57
CT001	HPA/Gly	310	270	190	63
CT001 (pJJ801)	Gly	260	200	145	53
CT001 (pJJ801)	HPA/Gly	350	283	205	60

pJJ801 does not encode the SSA dehydrogenase gene.

THE EXPRESSION OF THE *hpc* GENES CARRIED BY pJJ801 IN AN *E.coli* K12 STRAIN.

E.coli K-12 strains do not utilise HPA or 3,4DHPA and F-primers from *E.coli* K-12 do not repair *E.coli* C HPA mutants (Skinner, 1981). An *hsdR* derivative of *E.coli* K-12, strain 5K, was transformed with pJJ801. No growth was observed when 5K(pJJ801) was tested for growth on HPA or 3,4DHPA. From this it was concluded that pJJ801 did not encode all the enzymes of the 3,4DHPA catabolic pathway.

Enzyme assays were then carried out on 5K(pJJ801) grown on various substrates. Table 3.1.5, shows that activities for the following 3,4DHPA catabolic enzymes could be detected: 3,4DHPA dioxygenase; CHMS dehydrogenase; CHM isomerase; COHED decarboxylase; and HHDD isomerase. From these results it can be concluded that the genes encoding these enzymes must have been cloned on pJJ801. The 3,4DHPA catabolic enzyme activities could be detected in 5K(pJJ801) when grown on glycerol or Luria broth (non-inducing conditions) which indicates that the enzymes encoded by the plasmid are expressed constitutively in this strain.

CHARACTERISATION OF pJJ801.

The genes that have been cloned on pJJ801 were identified from the activities of the 3,4DHPA catabolic enzymes detected in 5K(pJJ801). This observation was utilised in the ordering of the genes encoded by pJJ801.

Table 3.1.5

Activities of the HPA/3,4DHPA catabolic enzymes in *E. coli* K12, strain 5K
harbouring the plasmid pJJ801

Strain	Growth substrate	HPA hydroxylase	3,4DHPA dioxxygenase	CHMS dehydrogenase	CHM isomerase	COHED decarboxylase	HHDD isomerase
specific activity (nmoles min ⁻¹ mg protein ⁻¹)							
<i>E. coli</i> C	HPA	18	180	160	98	43	1623
5K	Gly/HPA	0	0	0	0	0	0
5K(pJJ801)	Gly/HPA	0	892	389	497	85	NT
5K(pJJ801)	Gly	0	875	400	520	90	3958

NT = not tested

a) Subcloning of pJJ801 using the restriction sites identified on the plasmid.

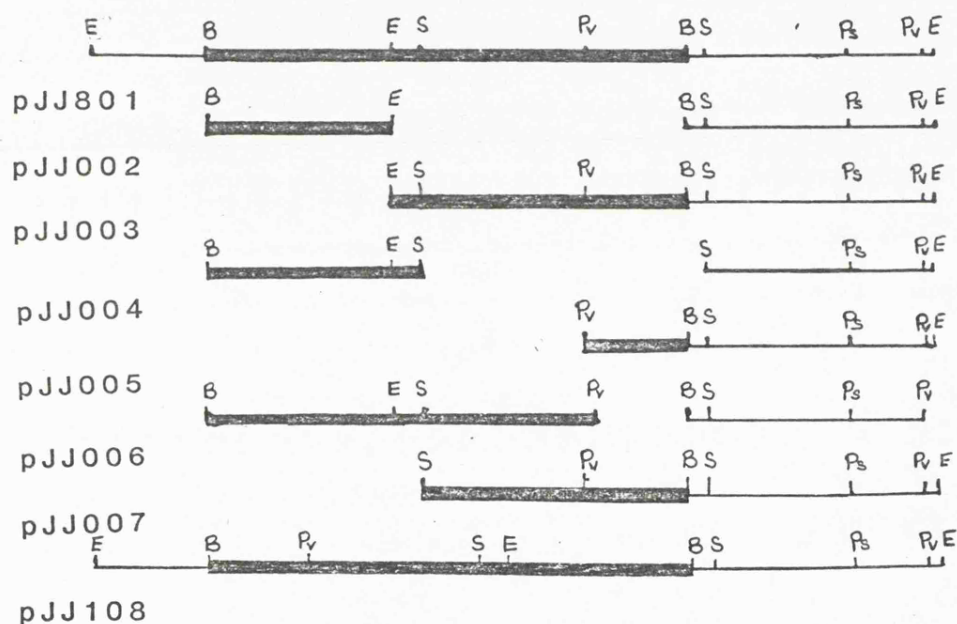
To determine the order of the genes carried by pJJ801 subclones were produced using the restriction sites identified on the cloned DNA. A diagram of the subclones produced along with the enzymes detected in 5K strains carrying these plasmids can be seen in fig,3.1.3. This shows that the 2.5Kb *Bam*H1-*Eco*R1 fragment from pJJ801 contains the first gene (*hpcB*) which encodes the 3,4DHPA dioxygenase, as shown by 5K(pJJ002). The difference in the enzyme activities detected in 5K(pJJ006) and 5K(pJJ801) indicates that pJJ801 carries an additional function, which corresponds to the previously unreported HHDD isomerase (this was designated *hpcF*). When the orientation of the 6.5Kb *Bam*H1 fragment is inverted the 3,4DHPA enzymes are still expressed constitutively in the 5K strain, indicating that pJJ801 carries its own promoter region. If this inverted form of pJJ801 is harboured by JJ221 the regulated phenotype of the 3,4DHPA catabolic enzymes is still retained.

b) Tn1000 Mutagenesis.

Transposon mutagenesis using Tn1000 was used to characterise the plasmid further. Tn1000 mutated forms of pJJ801 were tested in JJ221 and JJ247 to see if they affected growth on HPA. Those that no longer enabled growth on HPA were isolated and then mapped using restriction enzymes to determine the position of the Tn1000. A restriction map for

fig,3.1.3.

Deletion subclones produced from pJJ801 and the
3,4DHPA catabolic enzymes detected in 5K
harbouring the various subclones.



3,4DHPA Dioxygenase	CHMS Dehydrogenase	CHM Isomerase	COHED Decarboxylase	HHDD Isomerase
+	+	+	+	+
+	-	-	-	-
-	-	-	-	-
+	-	-	-	-
-	-	-	-	-
+	+	+	+	-
-	-	-	-	-
+	+	+	+	+

Tn1000 has been produced by Guyer (1978) which shows an offset *Bam*H1 site which can be used in the restriction mapping to determine the position and orientation of the Tn1000 insertion. The insertion sites of the Tn1000 mutated plasmids and the results of assays on 5K harbouring these plasmids can be seen in fig.3.1.4. The isolation and mapping of these mutated plasmids proved rather difficult, so only a few were analysed. From these results only the position of the *hpcB* gene could be confirmed. It was surprising that plasmids containing insertions beyond the *hpcC* gene were not detected the possible reason for this will be discussed later, (see the analysis of JJ247).

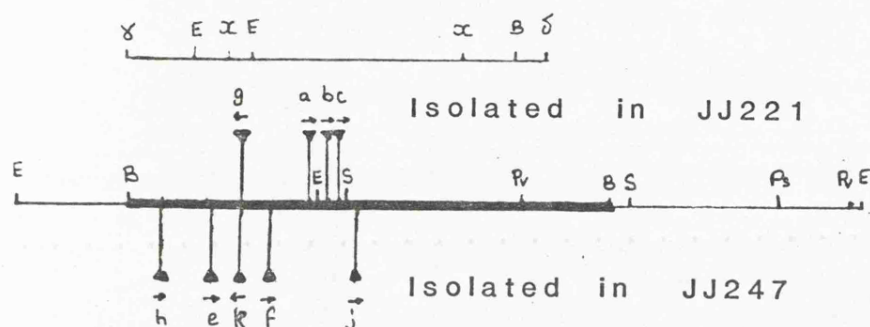
c) Further subcloning of pJJ801.

To complete the ordering of the genes encoded on pJJ801, the 6.5Kb chromosomal insert region bounded by two *Bam*H1 sites was isolated and partially digested with *Sau*3a. The digested fragments were then ligated into pBR328. The ligation mixture was used to transform JJ221 and the resulting ampicillin-resistant transformants tested for growth on HPA. Plasmid DNA was isolated from colonies which grew, ^{due to the selection used each plasmid carried the *hpcC* gene containing an *Eco*R1 site.} To ensure that the subclones were composed of contiguous *Sau*3a fragments the subclones were digested with *Eco*R1 and Southern transfers probed on separate occasions with the ³²P labelled 2.5Kb *Bam*H1-*Eco*R1 and 4.0Kb *Eco*R1-*Bam*H1 fragments of pJJ801, see fig.3.1.5. This shows the ^{that} majority of the subclones ^{constituted} authentic contiguous fragments of DNA. The hybridization of the probe to specific fragments aided the restriction mapping of the subclones. The Southern

fig,3.1.4.

Tn1000 mutagenesis of pJJ801 and the 3,4DHPA catabolic enzymes detected in 5K harbouring the various mutant versions of pJJ801.

Tn1000 insertions

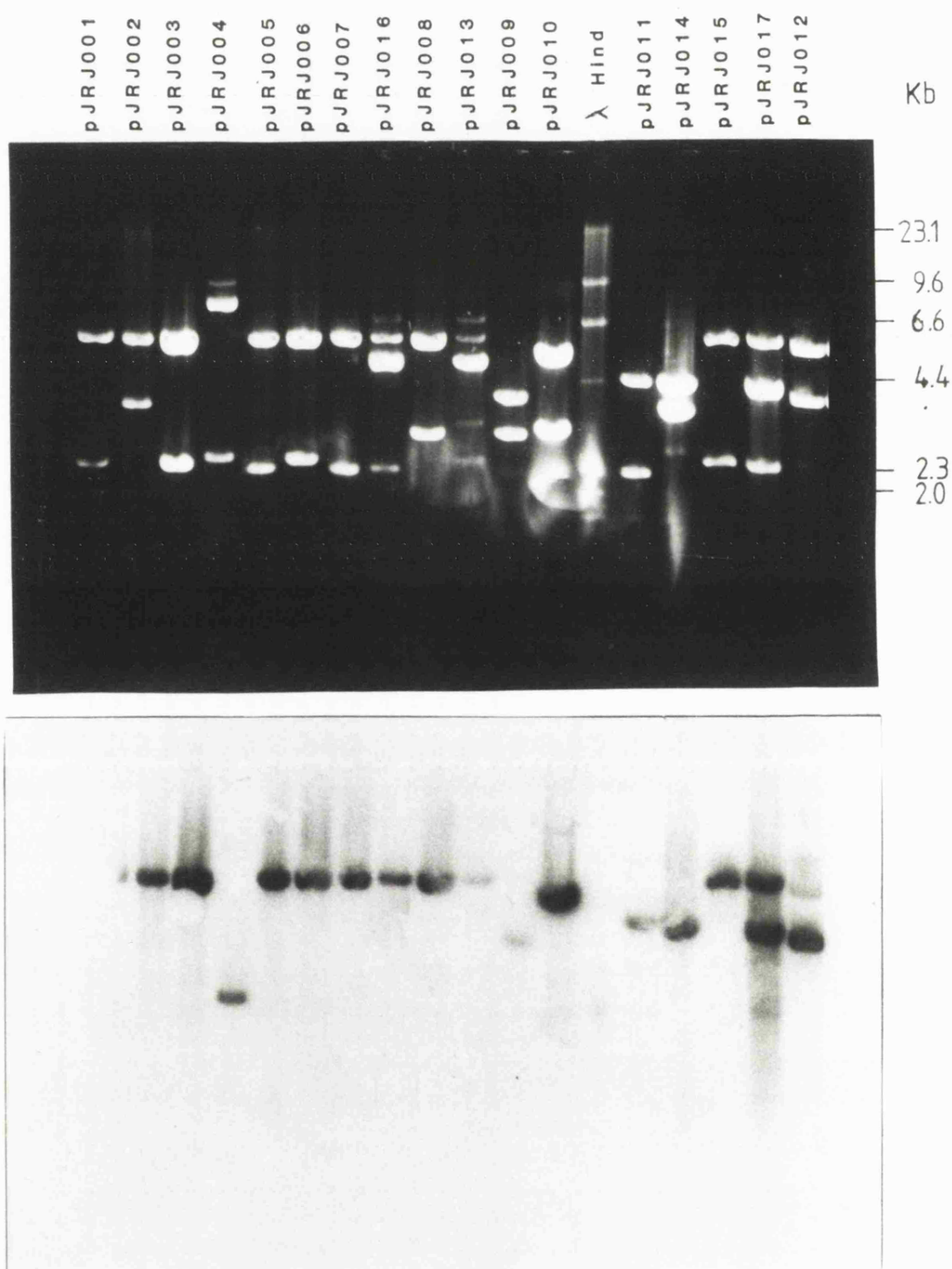


Tn1000 mutant	3,4DHPA Dioxygenase	CHMS Dehydrogenase	CHM Isomerase	COHED Decarboxylase	HHDD Isomerase
a	+	-	-	-	-
b	+	-	-	-	-
c	+	-	-	-	-
e	-	-	-	-	-
f	-	-	-	-	-
g	-	-	-	-	-
h	-	-	-	-	-
j	+	-	-	-	-
k	-	-	-	-	-

fig,3.1.5.

An analysis of the subclones produced from
partial digestion of pJJ801.

EcoR1 restricted subclones.



A Southern transfer of the Sau3a produced
subclones probed with the 2.5Kb BamH1-EcoR1
fragment from pJJ801.

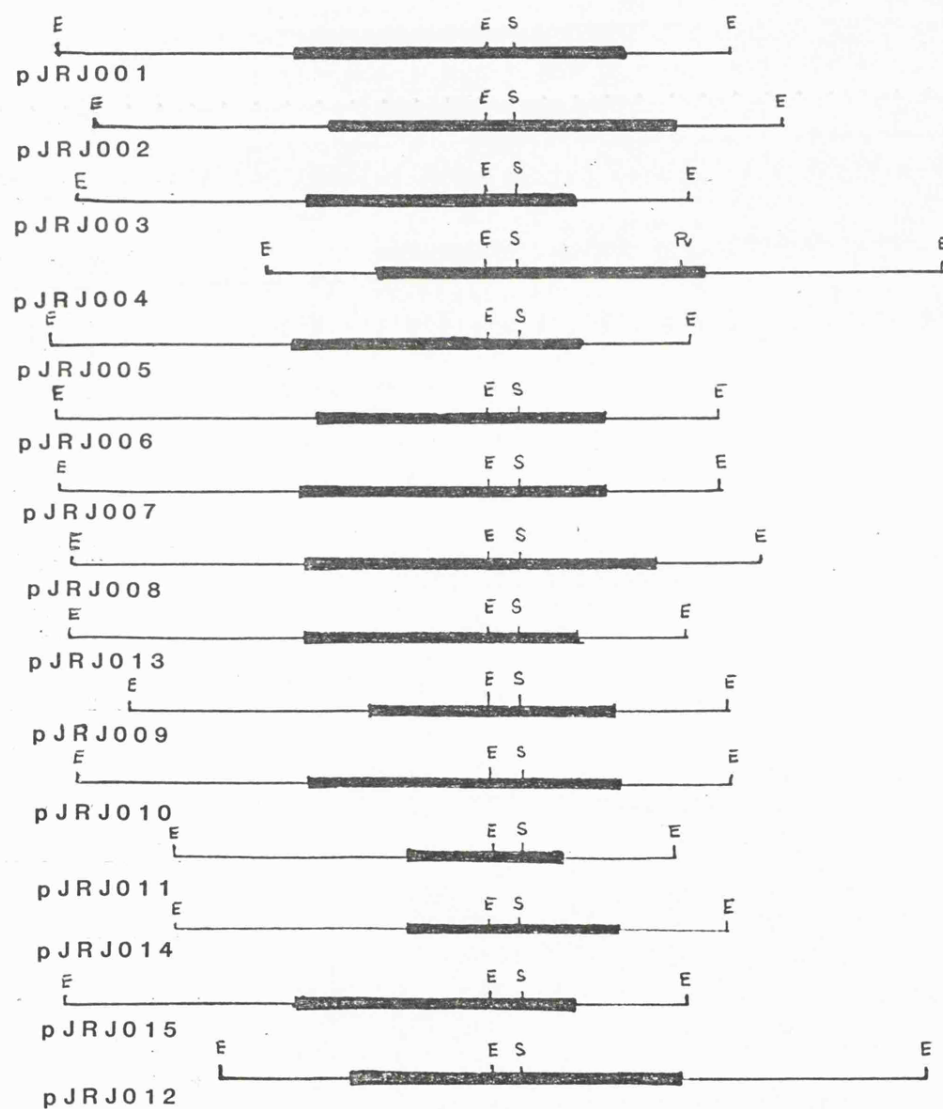
hybridisation identifies the anomalous subclones if more than one restriction fragment hybridises to the probe. The subclones containing contiguous fragments were then transformed into 5K and the resulting strains analysed for the five enzymes of the 3,4DHPA pathway, see fig,3.1.6. The gene order of the *hpc* genes encoding the five 3,4DHPA enzymes, as determined from 5K harbouring various subclones, is shown in fig,3.1.7. This gene order corresponds to the reaction order for the 3,4DHPA catabolic pathway.

d) The use of pJJ801 to probe a Southern transfer of chromosomal DNA.

To ensure that the cloned 6.5Kb *Bam*H1 fragment from pJJ801 has not undergone any rearrangement, *E.coli* C chromosomal DNA was digested with several restriction enzymes. A Southern transfer of the DNA was probed with the ³²P labelled 6.5Kb *Bam*H1 fragment from pJJ801, see fig,3.1.8. It was shown that the structure of the 6.5Kb fragment from pJJ801 corresponds to that of the *E.coli* C chromosome. Two chromosomal bands showed homology when the chromosomal DNA had been digested with *Eco*R1, but only one band hybridised when the chromosomal DNA was digested with either *Bam*H1 or *Pst*I. These results also indicate that there is only one copy of the genes on the chromosome. Little or no homology was detected between pJJ801 and *E.coli* 5K chromosomal DNA when the hybridisation filter was washed at a stringency of 0.1xSSC at 30°C.

fig.3.1.6.

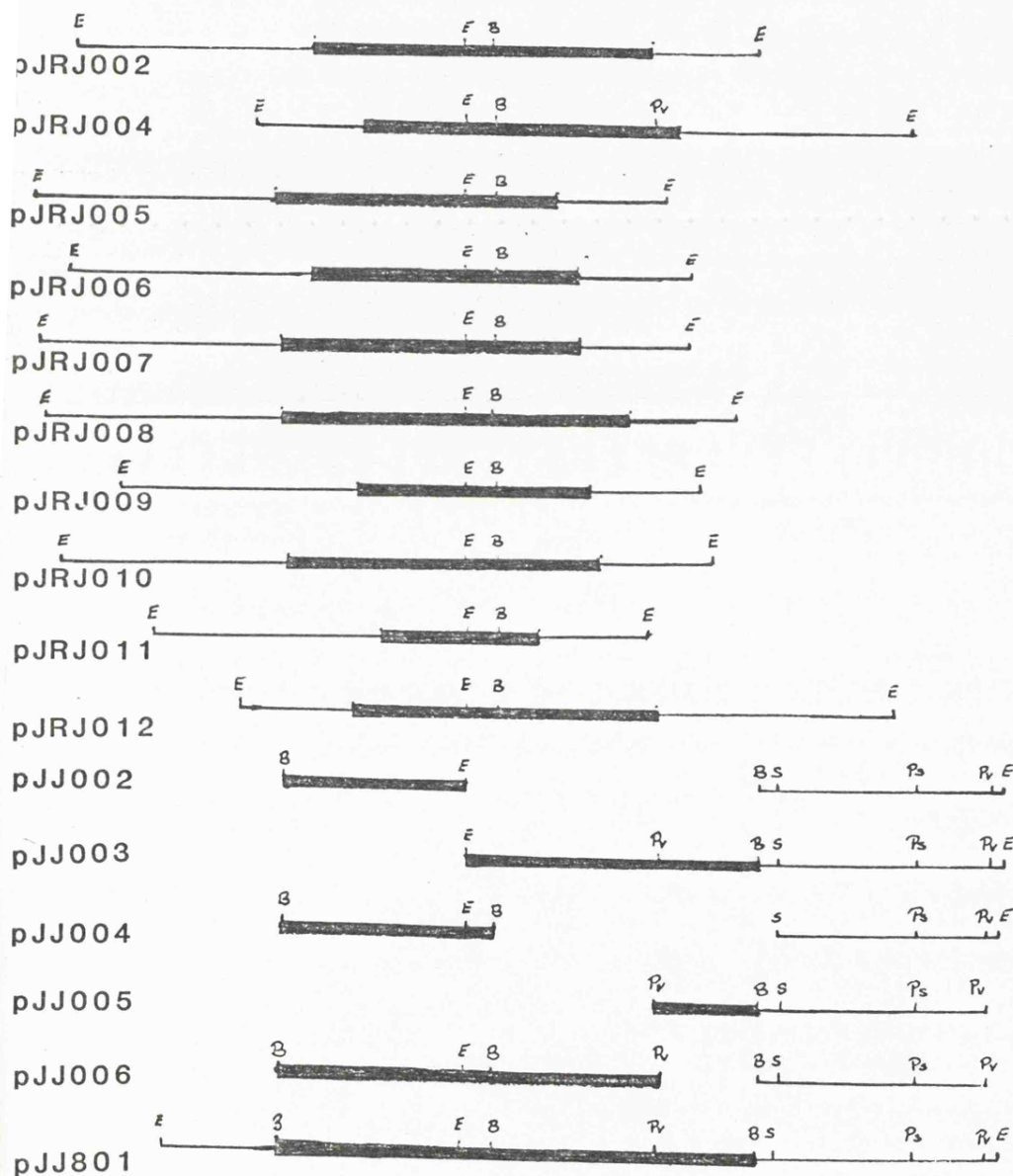
The restriction maps of the subclones produced by
Sau3a partial digestion of pJJ801 and the 3,4DHPA
catabolic enzymes detected in 5K harbouring the
various subclones.



3,4DHPA Dioxygenase	CHMS Dehydrogenase	CHM Isomerase	COHED Decarboxylase	HHDD Isomerase	Inducible in JJ221
+	+	+	-	-	I
+	+	+	+	-	C
+	+	-	-	-	N/T
-	+	+	+	-	C
+	+	-	-	-	I
+	+	-	-	-	C
+	+	-	-	-	I
+	+	+	-	-	I
+	+	-	-	-	N/T
-	+	+	-	-	C
+	+	+	-	-	I
-	+	-	-	-	C
-	+	+	-	-	C
+	+	-	-	-	I
-	+	+	+	-	C



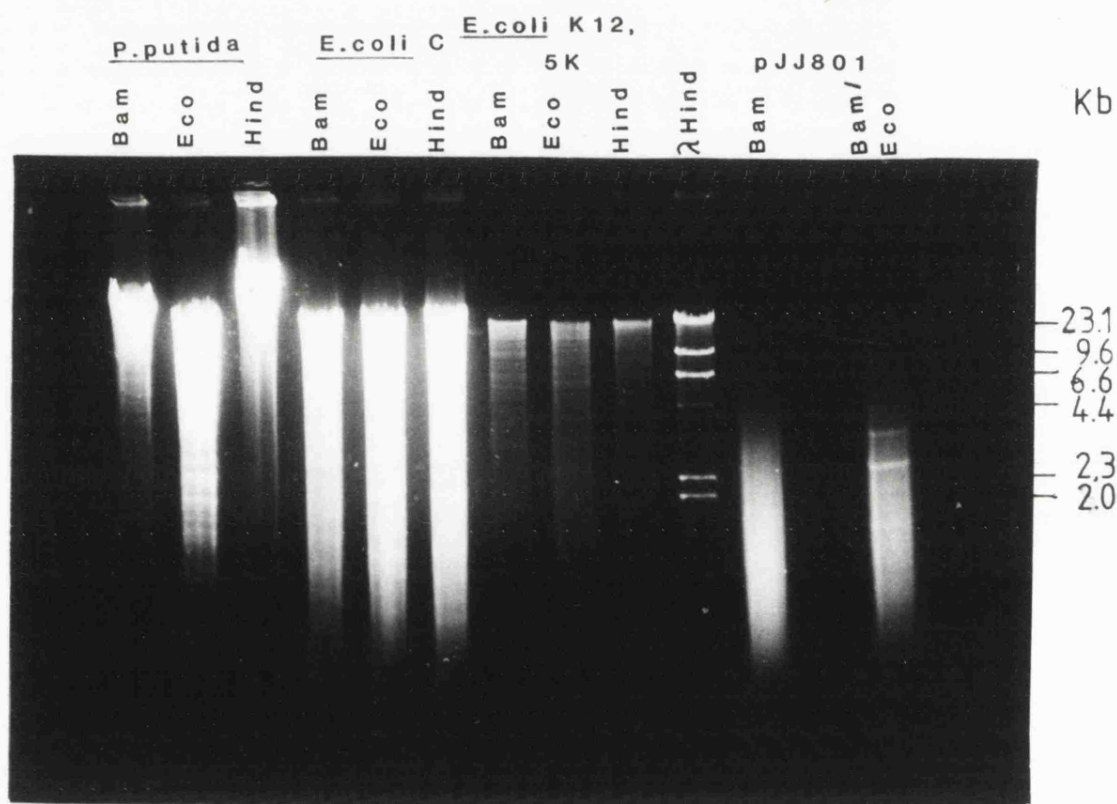
A compilation of various subclones used to determine the order of the *hpc* genes carried by pJJ801.



	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+
3,4DHPA	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dioxygenase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHMS Dehydrogenase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHM Isomerase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
COHED Decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HHDD Isomerase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inducible in JJ221	C	C	I	C	I	I	C	I	I	C	I	I	C	C	C	N _T	N _T	N _T	I

A Southern transfer of various chromosomal digests probed with the 6.5Kb BamH1 fragment from pJJ801.

A photograph of the agarose gel used in the Southern transfer.



A photograph of the autoradiograph (over night exposure).

FURTHER STUDIES INVOLVING pJJ801.

In the process of characterising pJJ801 a range of subclones which contain all or part of the 6.5Kb chromosomal insert fragment were produced. These subclones carry various *hpc* genes and were then used in a series of further experiments.

a) Characterisation of JJ247 using subclones from pJJ801.

The mutant JJ247 is a *recA* derivative of MS024 which was initially thought to be pleiotropically defective for the 3,4DHPA catabolic enzymes (Skinner, 1981). In order to test this, several subclones isolated from pJJ801 were introduced into JJ247 and the resulting strains tested for growth on HPA. As shown in Table 3.1.3. pJJ801 can restore the ability of JJ247 to utilise HPA, although the enzymes are produced constitutively. However, when JJ247 harbours pJJ002 (carrying the 3,4DHPA dioxygenase only) it is unable to grow on HPA, even though it was shown that the 3,4DHPA dioxygenase was being produced constitutively. When JJ247 was transformed with pJRJ003 (carrying the genes corresponding to the 3,4DHPA dioxygenase and the CHMS dehydrogenase) the ability to grow on HPA was restored. The two enzymes encoded by pJRJ003 were shown to be produced constitutively. The results of the assays carried out on JJ247 harbouring the plasmids are shown in table 3.1.6. The other enzymes of the 3,4DHPA catabolic pathway were shown to be inducible. These results suggest that there may be some secondary or weaker promoter allowing

Table 3.1.6

Activities of certain 3,4DHPA catabolic enzymes in JJ247
harbouring various subclones

Specific activity nmoles min⁻¹ mg protein⁻¹

Strain	Growth substrate	3,4DHPA dioxygenase	CHMS dehydrogenase	CHM isomerase	HHED aldolase
JJ247	Gly/HPA	3	2	5	2
JJ247 (pJJ801)	Gly	380	210	250	0
JJ247 (pJJ801)	HPA	190	180	120	25
JJ247 (pJJ002)	Gly	850	0.5	3	0
JJ247 (pJRJ003)	Gly	797	220	7	0
JJ247 (pJRJ003)	HPA	191	254	47	15

the production of the CHM isomerase and subsequent enzymes, so enabling growth. A similar situation was observed by Shanley et al (1986) in the cloning of the β -ketoadipate genes from *Acinetobacter calcoaceticus*. If the normally co-ordinately transcribed *catBCDE* genes have a defective promoter or the promoter region is missing, then under some circumstances the *catCDE* genes may be transcribed from a promoter independent of the promoter that normally governs co-ordinate expression of all four genes.

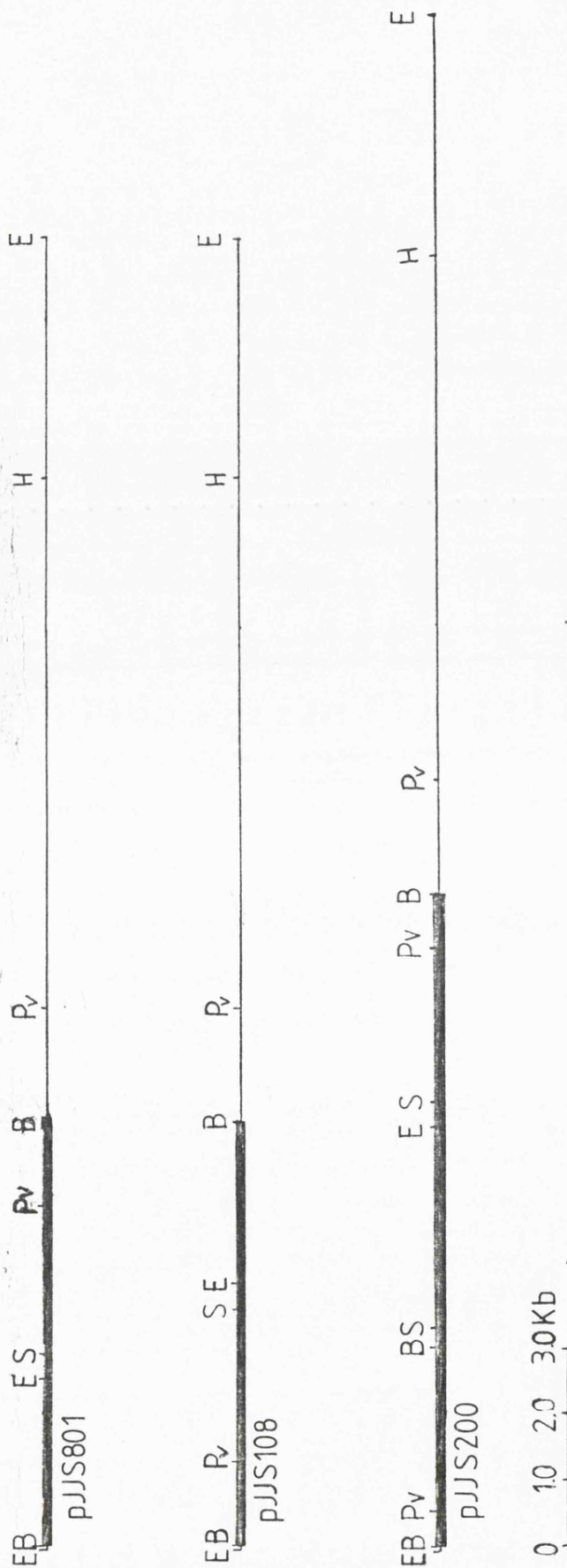
b) The expression of *E.coli* C *hpc* genes in *P.putida*.

The HPA catabolic pathway for *P.putida* has been described by Sparnins et al (1974) and is apparently identical to that of *E.coli* C. To transfer the *E.coli* C genes into *P.putida*, the 6.5Kb *Bam*H1 fragment from pJJ801 was isolated and ligated into the shuttle vector pMMB34. This produced two plasmids, pJJ801 and pJJ8108, which contain the same 6.5Kb fragment but in opposite orientation, see fig,3.1.9. These were then transformed into the *E.coli* K12 strain C600(pLG223) which contains a suicide helper vector, pLG223, for the mobilisation of pJJ801/108 into *P.putida* strains. The two plasmids were then mated into three *P.putida* strains, 2440 (*hsdR*; unable to grow on HPA or 3,4DHPA), PP001 (CHMS dehydrogenase defective) and PP003 (pleiotropically defective for the 3,4DHPA catabolic enzymes). The strains harbouring the plasmids were then tested for growth on HPA and 3,4DHPA. No growth was detected, which was surprising as the two mutants PP001 and PP003 appear to have the same phenotype as JJ221 and JJ247, respectively. A series of enzyme assays were

Restriction maps showing the plasmids pJJS801,

pJJS108 and pJJS200.

The chromosomal insert region from both pJJS801 and pJJS200 have been subcloned into the broad host range vector pMMB34.



carried out on the three strains harbouring the plasmids. There appeared to be no difference between pJJS801 and pJJS108 the results for the three *P.putida* strains are shown in table 3.1.7. The enzymes were found to be produced constitutively but at lower levels than normally found in wild-type *P.putida* induced for the 3,4DHPA catabolic enzymes.

It is interesting to note that when 5K harbours pJJS801 the levels of the 3,4DHPA catabolic enzymes are higher than those detected for the pBR328-based clone pJJ801. This may be due to copy-number or to promoter activity from within the pMMB34 vector. The pJJS108 version was never tested in 5K.

To see if there was any homology between the cloned *E.coli* C *hpc* genes and *P.putida* chromosomal DNA, chromosomal DNA from both strains was digested with the same restriction enzymes. A Southern transfer of the digested DNA was probed with the ^{32}P labelled 6.5Kb region from pJJ801, see fig 3.1.10. This shows that there is only weak homology between the *E.coli* C and the *P.putida* DNA when the filter was washed in 0.1xSSC at 30°C. An exposure of two weeks is required to show faint bands indicating very low homology with something on the *P.putida* chromosome.

c) Additional uses of pJJ801.

The analysis of pJJ801 identified several subclones which only expressed certain 3,4DHPA catabolic enzymes. 5K was then transformed with various versions of these and extracts from the resulting strains were used to isolate intermediates of the 3,4DHPA catabolic pathway. The isolation of the intermediates will be described later.

Table 3.1.7

Activities of the 3,4DHPA catabolic enzymes of various
P. putida strains harbouring pJJS801/108

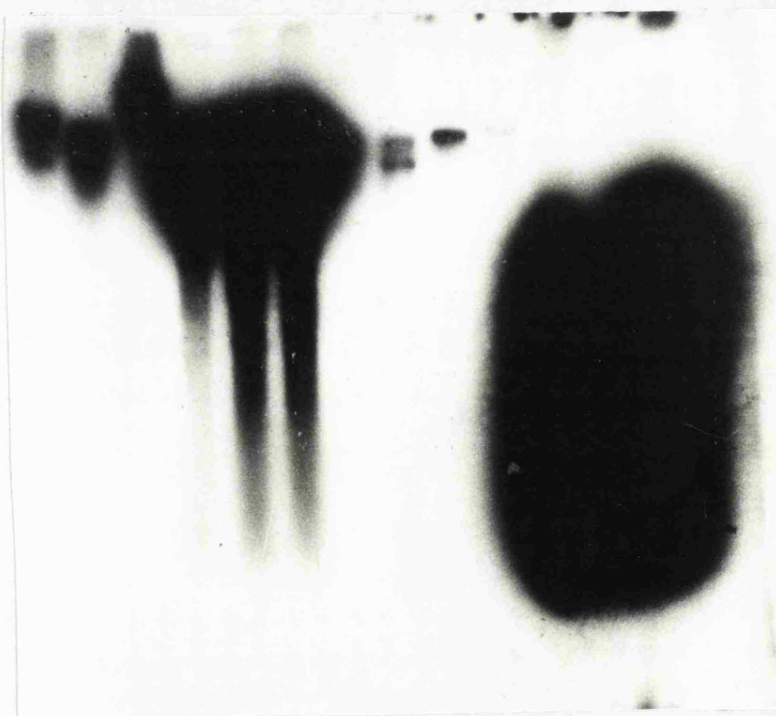
Specificity(nmoles min⁻¹ mg protein⁻¹)

Strain	Growth substrate	3,4DHPA dioxygenase	CHMS dehydrogenase	CHM isomerase	COHED decarboxylase
<u>P.putida</u> U	Gly	0	2	5	9
<u>P.putida</u> U	HPA	388	273	187	96
5K(pJJ801)	Gly	875	400	520	90
5K(pJJS801)	Gly	2,372	1,556	1,800	252
2440 (pJJS801)	Gly	50	80	70	NT
2440 (pJJS801)	Gly/HPA	52	79	74	NT
PP001 (pJJS801)	Gly	65	60	144	36
PP001 (pJJS108)	Gly	63	65	132	33
PP003 (pJJS801)	Gly	70	70	113	34
PP003 (pJJS108)	Gly	72	69	120	36

A Southern transfer of various chromosomal DNA
probed with the 6.5Kb fragment from pJJ801.

An eight day exposure of the filter to the film
 to see if there is any homology with chromosomal
 DNA other than *E.coli* C.

<u>P.putida</u>			<u>E.coli C</u>			<u>E.coli K12,</u>				<u>pJJ801</u>		
						5K						
Bam	Eco	Hind	Bam	Eco	Hind	Bam	Eco	Hind	λ Hind	Bam	bam/	Eco



3.2 CLONING AND CHARACTERISATION OF ADDITIONAL *hpc* GENES

Attempts were made to isolate further clones carrying additional *hpc* genes using the isolation procedure described in section 3.1. Two clones were obtained and these were mapped using restriction enzymes and shown to be smaller than pJJ801. The restriction maps also showed that the cloned DNA did not contain any restriction fragments that were not present on pJJ801. The fact that there were no additional *hpc* genes was also confirmed by an analysis of 5K harbouring these plasmids, where no additional activities for 3,4DHPA catabolic enzymes could be detected. No further work was carried out on these plasmids.

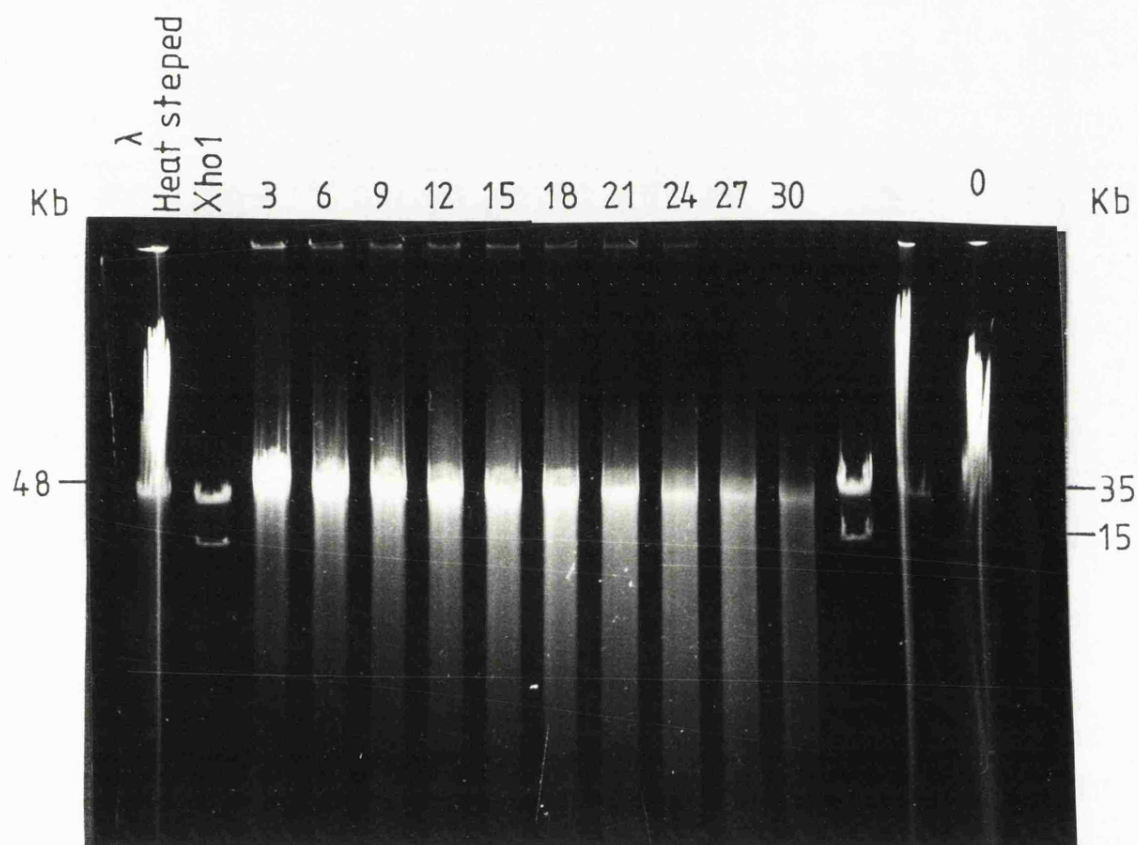
COSMID CLONING.

In order to clone additional *hpc* genes a cosmid library was constructed using JJ221 as the recipient. The cosmid cloning procedure allows DNA fragments of 35-45Kb to be cloned, thereby improving the chances of cloning additional *hpc* genes. 35-45Kb fragments of *E.coli* C chromosomal DNA were generated by *Sau*3a partial digestion. To establish the conditions for partial digestion by *Sau*3a 10µg of chromosomal DNA was digested with 4 units of *Sau*3a and samples removed from the reaction at 3min. intervals, see fig 3.2.1. The 35-45Kb chromosomal fragments were then ligated into the vector Cos4 and the ligation mixture packaged *in vitro*.

It has been shown that the HPA pathway can be transduced into *E.coli* K-12 strains by phage P1 (Skinner 1981).

fig.3.2.1.

Determination of the conditions for *Sau*3a partial digestion of *E.coli* C chromosomal DNA.



The conditions for the partial digestion of *E.coli* C DNA were determined by removing samples at 3 minute time intervals from a restriction digestion reaction containing 10µg DNA and 4units of *Sau*3a.

Therefore 5K was also used as a recipient in an attempt to identify clones encoding the whole pathway.

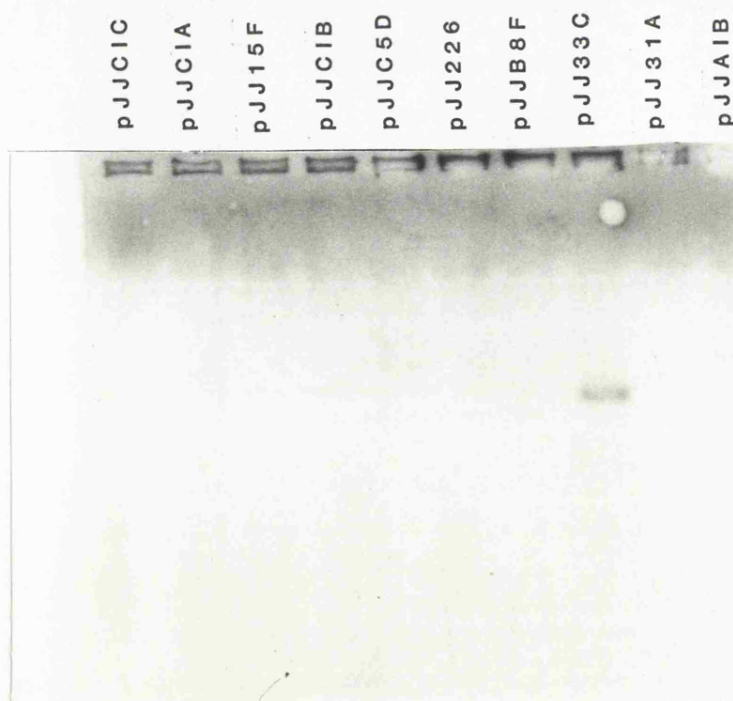
The packaged ligation mix was used to construct libraries in both JJ221 and 5K. Transfectants were selected on Luria-Ap agar and the colonies which grew tested for growth on HPA plates. No plasmids which enabled growth on HPA or 3,4DHPA were isolated in either host. The 576 colonies isolated were then patched onto fresh Luria-AP agar and prepared for colony hybridisation. The ³²P labelled 4.0Kb *EcoR1-BamH1* fragment from pJJ801 was used to probe the library. Ten clones hybridised strongly to the probe. Plasmid DNA was isolated from each of these and used to transform JJ221 and JJ247. None of the ten plasmids in either strain enabled growth on HPA. To determine whether there was any real homology between the 4.0Kb *EcoR1-BamH1* fragment from pJJ801 and the cosmids, each cosmid was digested with *EcoR1* and a Southern transfer probed with the ³²P labelled 6.5Kb *BamH1* fragment from pJJ801, see fig,3.2.2. Only one cosmid still showed homology and this was designated pJJ33C. It was transformed into 5K and the resulting strain tested for 3,4DHPA catabolic enzymes, but none could be detected.

JJ200 (the isolation is described later) an *E.coli* C mutant unable to grow on HPA, was transformed with pJJ33C. JJ200 (pJJ33C) was now able to grow on HPA. However, only a plasmid of 6.2Kb could be isolated from this strain. The new form of the plasmid was used to transform JJ221 and JJ247 where its presence still did not restore the ability to grow on HPA. When the new form of pJJ33C was introduced into 5K and the strain assayed for 3,4DHPA catabolic enzymes the CHM isomerase and COHED decarboxylase could now be detected. As

fig,3.2.2. .

A Southern transfer of the various cosmids
digested with *Eco*R1 and probed with the 6.5Kb
*Bam*H1 fragment from pJJ801.

(The photograph of the agarose gel used in
preparing the Southern transfer is not
presented).



JJ200 is not a *recA* strain pJJ33C may have undergone some rearrangement or deletion allowing the genes to be expressed.

The fact that five *hpc* genes carried by pJJ801 had not been cloned on pJJ33C should have been obvious from the Southern hybridisation, shown in fig,3.2.2. When an *EcoR*I digest of pJJ33C was probed with the 6.5Kb *Bam*H1 from pJJ801 only one band is detectable, whereas when the same probe was used to test an *EcoR*I digest of *E.coli* C chromosomal DNA or pJJ801 two bands of positive hybridisation can be detected. This is because the *hpcC* gene contains an *EcoR*I site as shown in the restriction map, see fig,3.1.7. So, any fragment of DNA containing the five *hpc* genes digested with *EcoR*I should produce two bands on a Southern hybridization.

The cosmid pJJ33C was found to be unstable in most strains so studies on this plasmid were discontinued.

ISOLATION AND ANALYSIS OF NEW MUTANTS.

a) Isolation of JJ200.

As no further genes were isolated using JJ221 as the recipient in the cloning experiments, it was decided to seek new HPA and 3,4DHPA mutants. *E.coli* C was treated with EMS and mutants defective in the HPA pathway selected by penicillin enrichment. In an attempt to isolate an HPA hydroxylase mutant colonies capable of growing on 3,4DHPA but not HPA were sought. One potential mutant was obtained and designated JJ200. Further analysis revealed it was not a HPA hydroxylase mutant because JJ200 was HPA negative, 3,4DHPA

positive at 30°C and took 24-36 hours to grow but HPA negative, 3,4DHPA negative at 37°C. To try and establish the defect in JJ200 enzyme assays were carried out on the strain, see table, 3.2.1. The information in the table possibly indicates that the defect was in the COHED decarboxylase as it is this and subsequent enzymes that are absent in JJ200 grown on glycerol in the presence of HPA at 30°C but, this is not consistent with the ability to grow on 3,4DHPA.

Despite the difficulties in determining the lesion in JJ200 it was used as the recipient in a cloning experiment. *E. coli* C chromosomal DNA was partially digested with *Sau*3a and ligated into the *Bam*H1 site of pBR328. The ligation mix was then used to transform JJ200 and the transformants selected on Luria-Ap agar. These were then tested on HPA plates and plasmid DNA isolated from the 3 colonies which grew. The resulting hybrid plasmids were mapped with various restriction enzymes and compared to pJJ801. No additional DNA had been isolated but the clones appear to confirm that JJ200 was mutated in the COHED decarboxylase gene because they all carry the *hpcE* gene. It was also shown that the mutation in JJ200 was suppressed by the presence of pJJ801.

In order to clone further *hpc* genes a different mutant was needed as the *hpcE* gene had already been cloned.

b) The isolation of JJ210.

The strain CT001, which is constitutive for the 3,4DHPA catabolic enzymes, was treated with EMS. After the penicillin enrichment stage the cells were transformed with pJJ801, to eliminate in the subsequent selection stage mutants with

Table 3.2.1

Activities of the 3,4DHPA catabolic enzymes detected in the characterisation of the mutant JJ200

Strain	Growth substrate	3,4DHPA dioxxygenase	Specificity (nmoles min ⁻¹ mg protein ⁻¹)					HHDD isomerase	OHED hydratase
			CHMS dehydrogenase	CHM isomerase	COHED decarboxylase				
E.coli C	Succ	0	< 1	5	0		0		0
E.coli C	Succ/HPA	75	102	212	78		+		47
JJ200	Succ	0	< 1	4	0		0		0
JJ200	Succ/HPA	70	47	78	0		0		0

+ = present but specific activity not measured

defects in genes already cloned. The mutagenesis was carried out on the constitutive strain, CT001, to facilitate the analysis of any mutants obtained.

The mutants generated were then screened to see if any were unable to grow on HPA. Using this procedure one stable mutant was isolated. This was cured of pJJ801 and designated JJ210. JJ210 was assayed for enzymes of the 3,4DHPA pathway, but none of the enzymes previously produced constitutively in CT001 were observed, even when JJ210 was grown on glycerol in the presence of HPA. Despite the inability to identify the defect in JJ210, the mutation was not suppressed by pJJ801 so it was useful as a recipient in further cloning experiments.

THE ISOLATION OF pJJ200 AND pJJ210.

It has been shown that if genes expressing membrane proteins (permeases) are cloned on high copy-number vectors the resulting strains may be unstable (Stoker et al, 1982). Since the HPA permease may be linked to the other *hpc* genes sought, gene libraries were constructed in JJ200 and JJ210 using the vector pBR322 which has a lower copy-number than pBR328.

E.coli C chromosomal DNA was partially digested with *Sau*3a, as described for the pBR328 cloning. To optimise the number of recombinant plasmids test ligations of varying amounts of chromosomal and plasmid DNA were set up. The results of using these various ligation mixes to transform JJ210 are shown in table, 3.2.2. The most efficient proportions of chromosomal to plasmid DNA were 2:1, so a ligation reaction consisting of 200ng pBR322 and 400ng partially digested *E.coli* C

Table 3.2.2

The transformation results using JJ210 as the recipient and
pBR322 as the cloning vehicle

Treatment	Number of transformants (per μg of DNA)	Percentage of transformants that grow on LB-tc plates (%)
None (supercoiled pBR322)	6.3×10^5	100
pBR322 digested with <u>Bam</u> HI and ligated	5.5×10^5	100
pBR322 digested with <u>Bam</u> HI, treated with CIP and ligated	1.2×10^3	95
Ratios of pBR322 digested with <u>Bam</u> HI treated with CIP: <u>E. coli</u> C chromosomal DNA		
3 : 1	2.15×10^4	15
2 : 1	2.6×10^4	5
1 : 1	2.3×10^4	10
1 : 2	2.0×10^4	19
1 : 3	1.6×10^4	37

chromosomal DNA was set up for the cloning experiment. Two thirds of the ligation mix was used to transform JJ210 and the transformants selected on Luria-Ap agar. The 2,500 transformants identified in this way were then replica plated onto HPA plates. Two clones were identified which had the ability to restore the growth on HPA. These were designated pJJ210 and pJJ300, and various restriction enzymes were used in an attempt to map them. The restriction map of pJJ210 is shown in fig,3.2.3. However, a restriction map for pJJ300 could not be constructed. In an attempt to aid its mapping, pJJ300 was digested with a series of restriction enzymes and a Southern hybridisation probed with the 32 P-labelled 4.0Kb *EcoR1-BamH1* fragment from pJJ801. These results are shown in fig,3.2.4a/b. From this data it was concluded that pJJ300 contained various fragments which had been ligated together, rather than a contiguous fragment of chromosomal DNA.

The remaining third of the ligation mixture was used to transform JJ200 and from the 800 colonies which grew on Luria-Ap agar only one was capable of growing on HPA. The plasmid DNA was isolated and designated pJJ200. pJJ200 was then mapped using restriction enzymes, see fig,3.2.5.

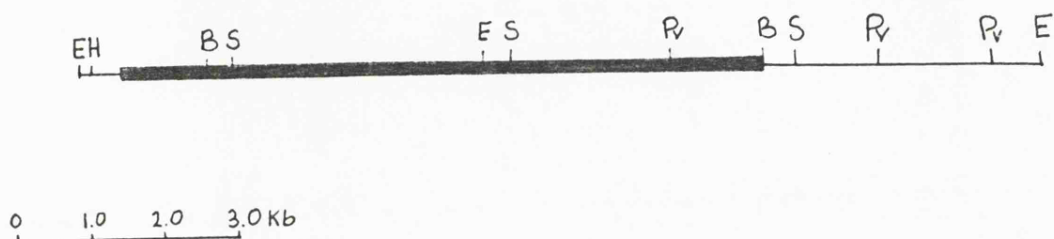
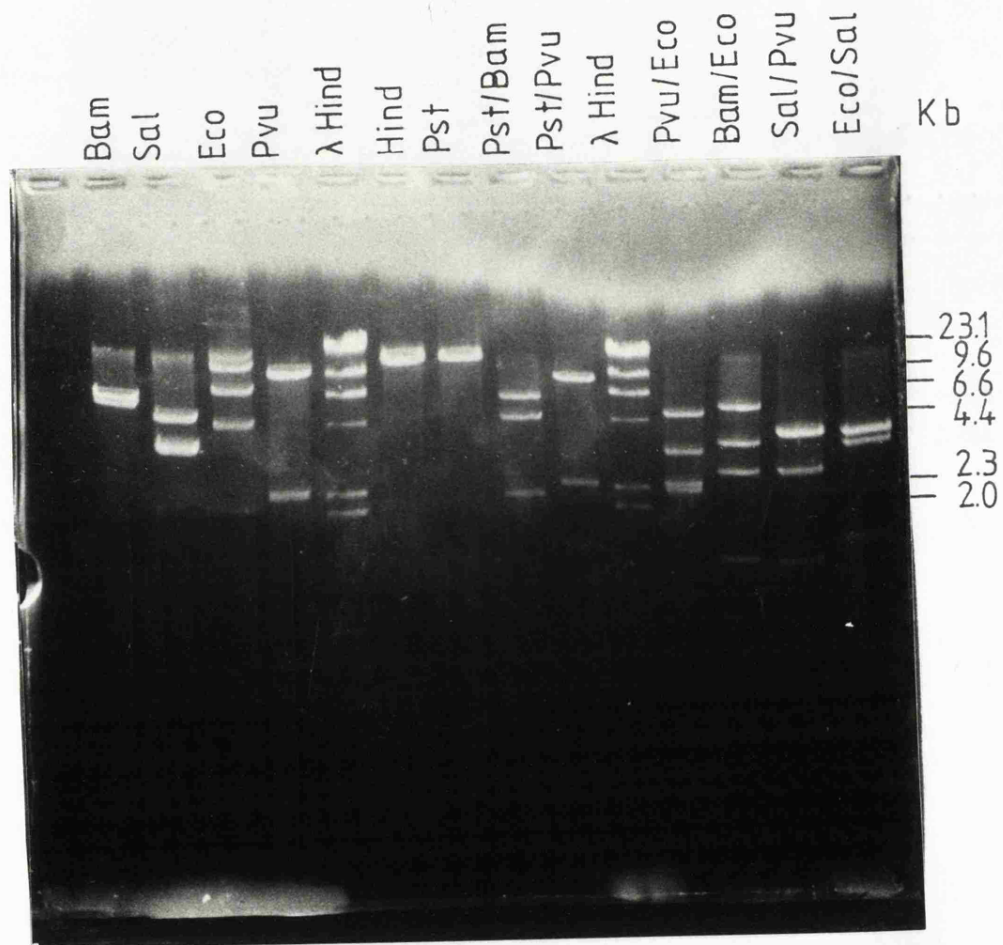
CHARACTERISATION OF pJJ210 AND pJJ200.

a) The expression of *hpc* genes from the plasmids pJJ200 and pJJ210 in *E.coli* strains.

As can be seen from fig,3.2.8, the two plasmids contain similar regions of DNA and as is shown later they also express the same 3,4DHPA catabolic enzymes.

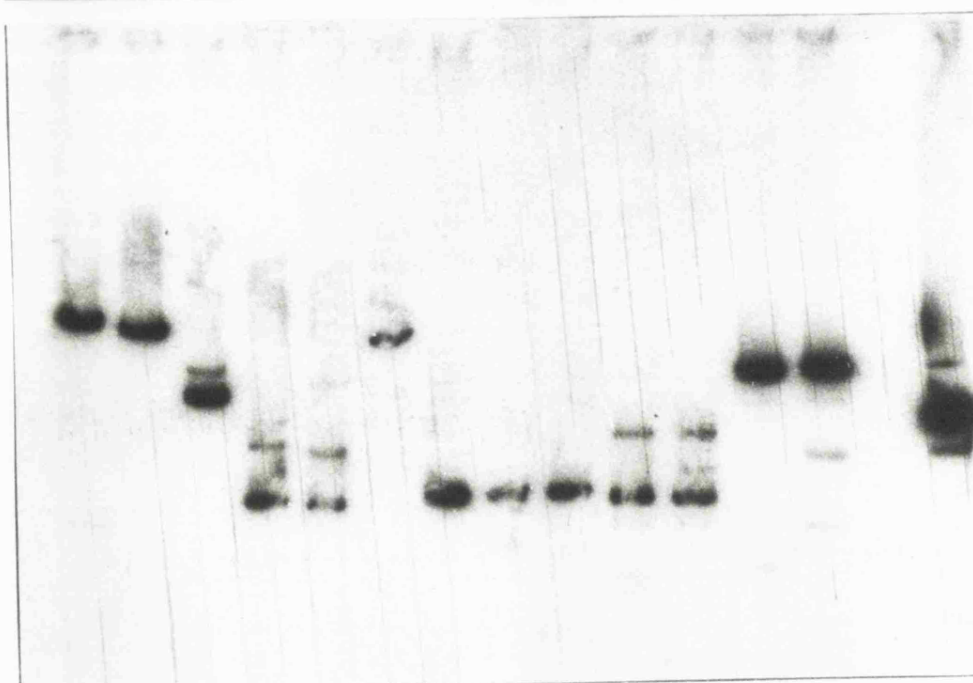
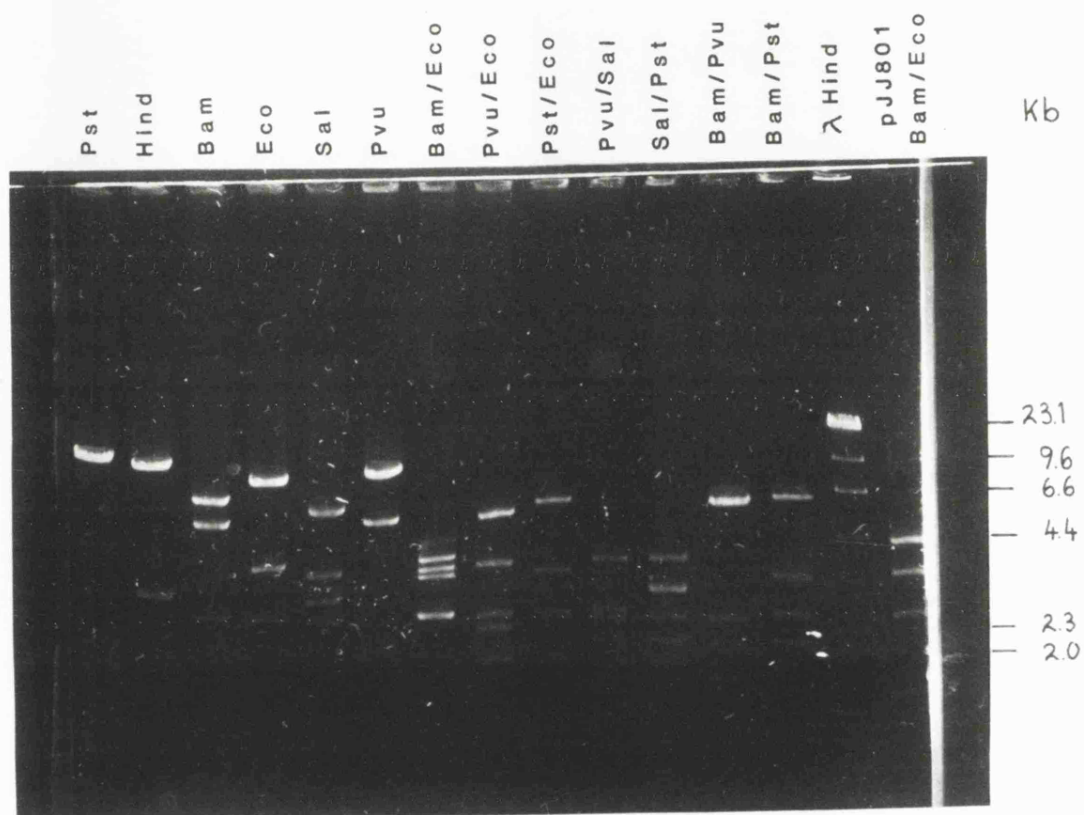
fig,3.2.3.

A photograph showing examples of the restriction digests used in determining the restriction map of pJJ210.



fig,3.2.4.

A photograph showing the restriction digests
generated in an attempt to restriction map
pJJ300.



A Southern transfer of pJJ300 digested with the
above restriction enzymes and probed with the
4.0Kb EcoR1-BamH1 fragment from pJJ801.

Fig. 3.2.4 continued

The sizes of the various restriction fragments of pJJ300

Standard

λ Hind III

Relative mobility (mm)	Size (kb)	A * indicates homology with the 4.0kb EcoRI-BamHI fragment from pJJ801	
28.8	23.1		
32.5	9.6	PstI Size (Kb)	HindIII Size (Kb)
36.0	6.6	13.0 *	10.0 *
41.0	4.4	2.4	3.3
52.0	2.3	2.1	1.7
54.0	1.96		

BamHI Size(Kb)	EcoRI Size(Kb)	SalI Size(Kb)	EcoRI/BamHI Size(Kb)
6.5	8.4	6.2	4.0
5.4 *	3.9	3.65	3.8
2.9	2.8 *	3.1	3.5
		2.4 *	2 x 2.7 **

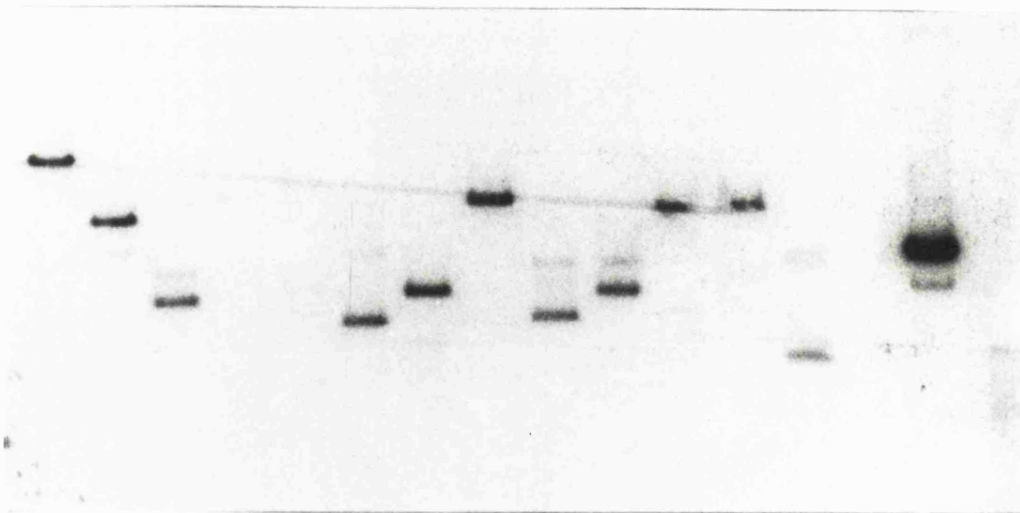
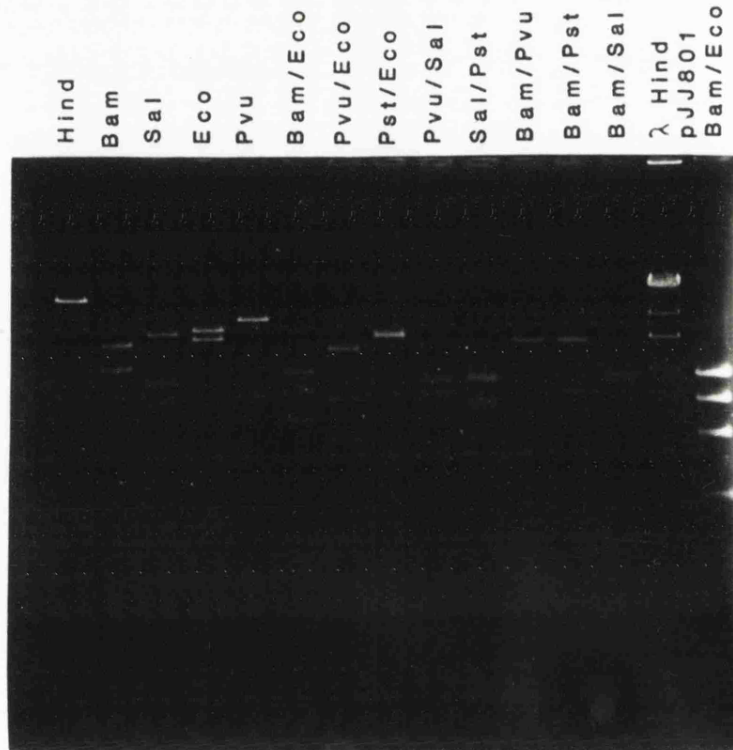
PvuII/EcoRI Size(Kb)	PstI/EcoRI Size(Kb)	PvuII/SalI Size(Kb)
5.8	6.7	3.7 *
3.9	3.65 *	2.8
2.8 *	2.8 *	2.6 *
2.5	2.0	2.3
1.8 *	1.4	1.9
	0.7	1.6
		1.4

BamHI/PvuII Size (Kb)	BamHI/PstI Size(Kb)
2 x 6.0 *	6.4 *
2.5	3.3
1.7	2.5
	2.1
	1.8

Additional bands shown in the autoradiograph occurred due to the impurity of the DNA fragment used to generate the probe.

fig.3.2.5.

An example of the restriction digests used to determine the restriction map of pJJ200 and the subsequent Southern transfer, which was probed with the 4.0Kb *Eco*R1-*Bam*H1 fragment from pJJ801.



A restriction map of pJJ200.



When the two plasmids were introduced, separately, into JJ221 and JJ247 the strains were now able to grow on HPA. The two plasmids were then used to transform 5K (on separate occasions) and the two resulting strains assayed for enzymes of the HPA/3,4DHPA catabolic pathway, see table, 3.2.3. The results indicate that both plasmids appear to carry the same *hpc* genes and that the specific activity of the 3,4DHPA catabolic enzymes was increased by the presence of HPA in the medium. The assays also show that all the enzymes necessary for the conversion of 3,4DHPA to SSA and pyruvate are encoded by genes on the plasmids pJJ210 and pJJ200. 5K(pJJ200/pJJ210) was tested for growth on HPA and 3,4DHPA plates, but none was detected. To ensure that there was not even a small amount of growth, liquid growth experiments were carried out on 5K(pJJ200 or pJJ210). The inoculum was taken from an overnight culture of 5K(pJJ200/pJJ210) grown on glycerol in the presence of HPA. The optical density of the cultures was measured over 10 hours for HPA and 4.5 hours for 3,4DHPA still no growth could be detected. To determine whether HPA or 3,4DHPA was taken up and metabolised whole-cell oxidation experiments with 5K(pJJ200/pJJ210) grown on glycerol plus HPA were carried out. The positive controls for this were *E.coli* C grown on HPA and CT001 grown on glycerol. The results showed that when HPA and 3,4DHPA were added to the *E.coli* C cells in the oxygen monitor an increased rate of oxidation above the basal level could be observed. An increased rate of oxidation could also be observed if 3,4DHPA was added to CT001 cells. No oxidation or partial oxidation of HPA or 3,4DHPA could be observed with 5K(pJJ200) or 5K(pJJ210) cells.

Table 3.2.3

Activities of the HPA/3,4DHPA catabolic enzymes in 5K (pJJ200/pJJ2102)

Strain	Growth substrate	Specific activity (nmoles min ⁻¹ mg protein ⁻¹)					
		HPA hydroxylase	3,4DHPA dioxygenase	CHMS dehydrogenase	CHM isomerase	OHED hydratase	HHED aldolase
<i>E. coli</i> C	Gly	0	0	< 1	5	2	0
<i>E. coli</i> C	HPA	18	180	160	98	82	39
5K(pJJ200)	Gly	0	< 1	3	9	9	0
5K(pJJ200)	Gly/HPA	0	60	50	77	37	+
5K(pJJ210)	Gly	0	< 1	5	10	6	0
5K(pJJ210)	Gly/HPA	0	42	55	103	40	+

+ = present but specific activity not measured

The two plasmids were shown, by an analysis of the restriction maps and their expression in 5K, to encode the same *hpc* genes so only pJJ210 was chosen for further studies.

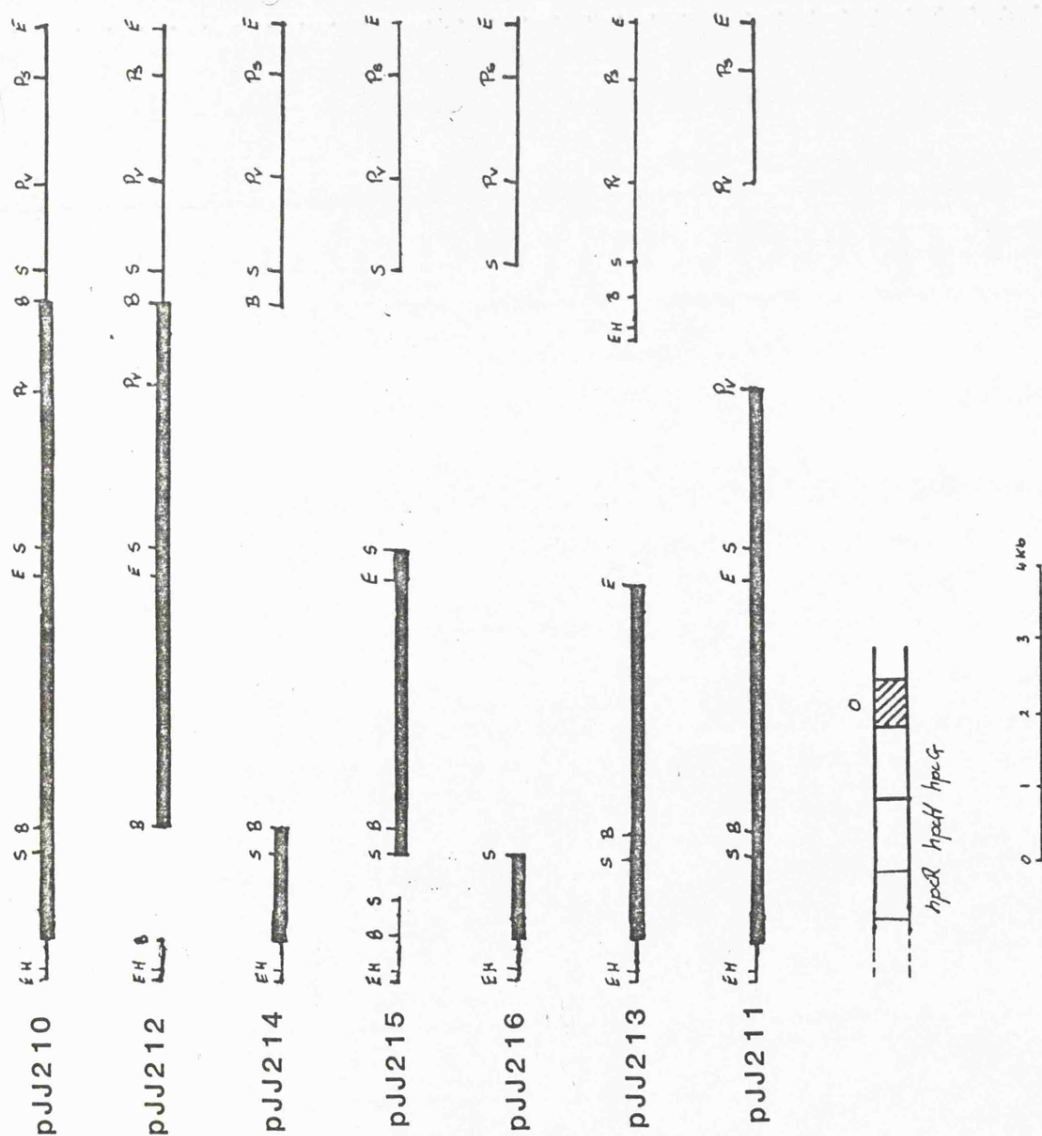
b) Subcloning of pJJ210.

pJJ210 was subcloned using the restriction sites identified on the plasmid. The resulting constructs along with the 3,4DHPA catabolic enzymes detected in 5K harbouring the subclones are shown in fig,3.2.6. From this the relative positions of the genes encoding the OHED hydratase (*hpcG*) and the HHED aldolase (*hpcH*) can be identified. 5K harbouring pJJ801 shows constitutive expression of the 3,4DHPA catabolic enzymes, whereas 5K harbouring pJJ210 appears to show some type of regulation. This feature was used to identify a putative position for the regulatory gene *hpcR*. (see section 3.4 for a discussion of this.)

When the information obtained from the subclones of pJJ210 was combined with that obtained from the analysis of pJJ801, the 3,4DHPA catabolic genes were shown to lie in two separate blocks, *hpcBCDEF* and *hpcGH*, see fig,3.2.8. The *hpcGH* genes precede the *hpcBCDEF* genes. The two clusters are separated by an operator-promoter region from which the respective mRNA's are synthesised in a divergent manner. This gene order was also assumed to be the same in pJJ200 due to a comparison of the restriction maps of the two plasmids. To ensure that there had been no rearrangement of the cloned DNA in the formation of pJJ210 ³²P-labelled pJJ210 was used to probe a Southern transfer of *E.coli* C chromosomal DNA digested with various restriction enzymes. Fig,3.2.7a/b, shows that the

The deletion subclones produced from pJJ210 and the 3,4DHPA catabolic enzymes detected in 5K harbouring the various plasmids.

I	C	NT	C	NT	NT	I	Induced by HPA in 5K
+	-	-	-	-	+	+	HHED Aldolase
+	+	-	+	-	+	+	OHED Hydratase
+	+	-	-	-	-	-	HHDD Isomerase
+	+	-	-	-	-	+	COHED Decarboxylase
+	+	-	-	-	-	+	CHM Isomerase
+	+	-	-	-	-	+	CHMS Dehydrogenase
+	+	-	+	-	+	+	3,4DHPA Dioxygenase



fig,3.2.7a/b.

The restriction digests of the various DNA.

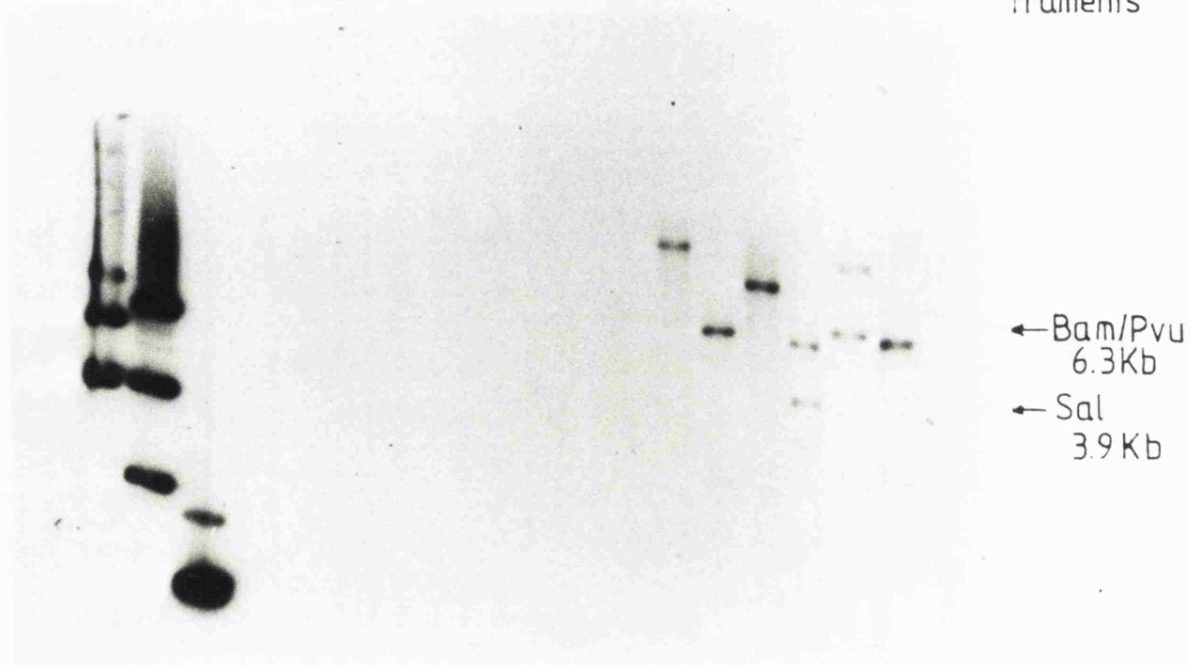
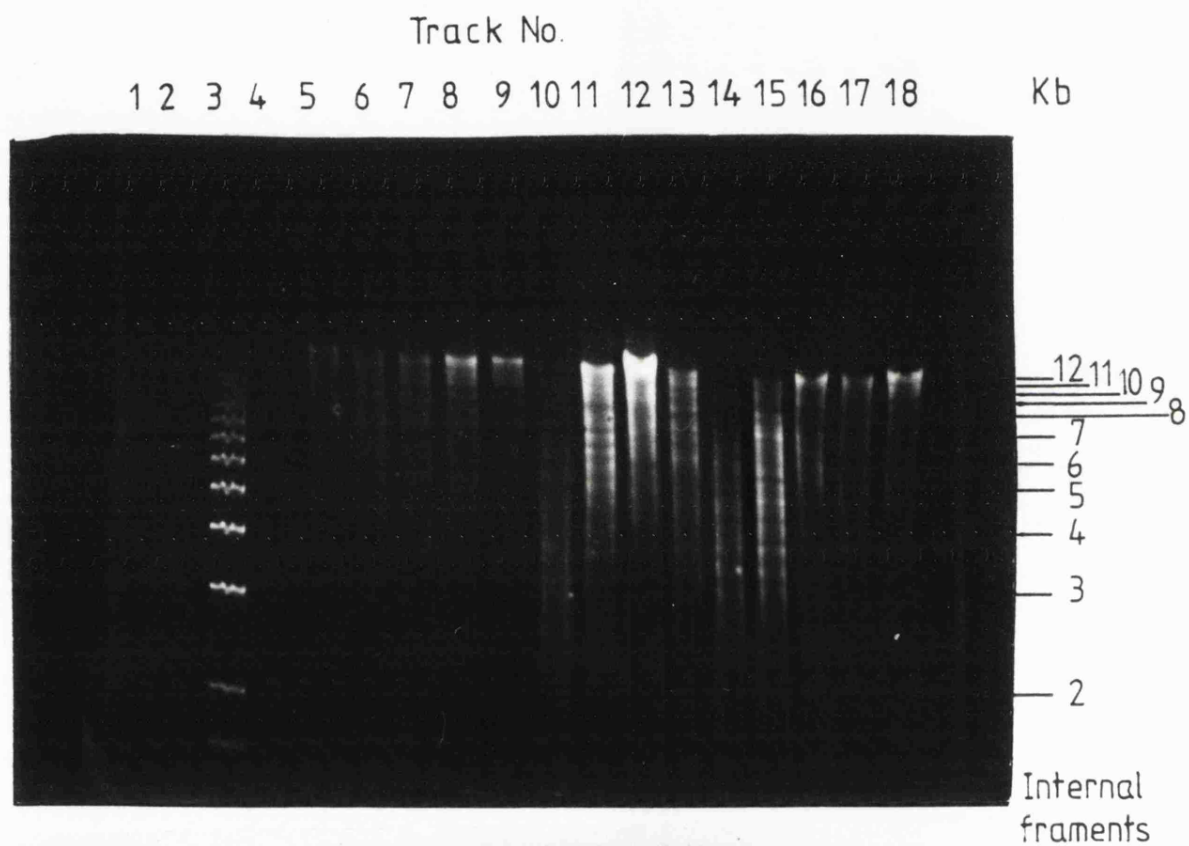
Track No.

1. pJJ210 *Sal*1.
2. pJJ210 *Bam*H1/*Pvu*2.
3. 1Kb ladder.
4. *K.pneumoniae* *Pst*1.
5. *K.pneumoniae* *Eco*R1.
6. *K.pneumoniae* *Bam*H1.
7. *E.coli* K12, 5K *Pst*1.
8. *E.coli* K12, 5K *Eco*R1.
9. *E.coli* K12, 5K *Bam*H1.
10. *P.putida* *Pst*1.
11. *P.putida* *Eco*R1.
12. *P.putida* *Bam*H1.
13. *E.coli* C *Pst*1.
14. *E.coli* C *Bam*H1/*Pvu*2.
15. *E.coli* C *Pvu*2.
16. *E.coli* C *Sal*1.
17. *E.coli* C *Eco*R1.
18. *E.coli* C *Bam*H1.

fig. 3.2.7a.

A Southern transfer of various chromosomal DNA
probed with the plasmid, pJJ210.

A photograph of the agarose gel used in the Southern
transfer.



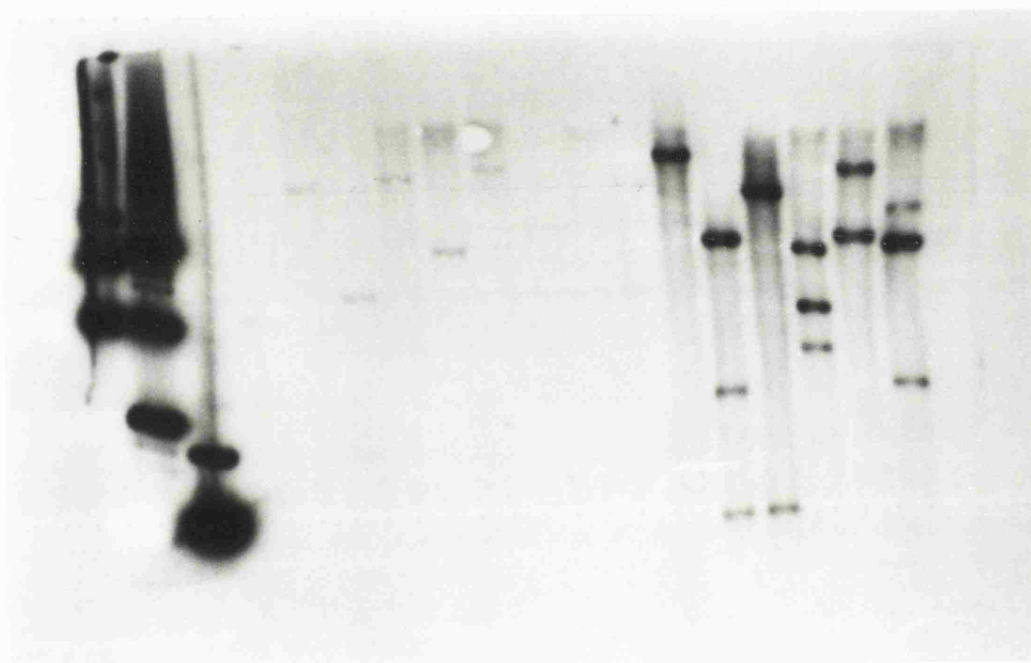
A photograph of the autoradiograph (over night
exposure).

fig,3.2.7b.

A 14 day exposure of the filter to the film
showing weak homology with chromosomal DNA other
than *E.coli* C.

Track No.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



A comparison of the restriction maps of the three plasmids isolated in this study and the relative positions of the *hpc* genes identified.



cloned DNA in pJJ210 corresponds to the same restriction patterns observed for the probed chromosomal DNA. This is shown by the size of hybridised fragments which correspond to internal restriction fragments in pJJ210.

ADDITIONAL USES OF SUBCLONES ISOLATED FROM pJJ200 AND pJJ210.

a) The use of the subclones produced from pJJ210 to analyse the mutation in JJ210.

The various subclones produced from pJJ210 were used to transform JJ210 which was then tested for growth on HPA. Only pJJ210 and pJJ211 restored the ability to grow on HPA, the other plasmids tested, pJJ212, pJJ214, pJJ215, pJJ216 and pJJ213 did not. (See fig,3.2.6, for a list of the *hpc* genes the plasmids encode). This unfortunately does not provide much information about the nature of the mutation in JJ210. The only definite thing that can be stated from these findings is that the mutation is not in the *hpcF* gene (HHDD isomerase), because pJJ211 which lacks this gene can suppress the defect.

b) The expression of the genes encoded on pJJ200 in *P.putida*.

To enable the genes to replicate in *P.putida* the cloned *E.coli* C chromosomal DNA was first transferred to the shuttle vector pMMB34. This was achieved by digesting pJJ200 with *Bam*H1 which gives 3 fragments. The *hpc* genes are carried on

two *Bam*H1 fragments, neither of which contain pBR322 DNA. This digest was then ligated into the *Bam*H1 site of pMMB34 and the ligation mixture was used to transform JJ210. The strain JJ210 was used as the recipient because it has been shown that neither of the two *Bam*H1 fragments on their own can repair the defect. It was therefore assumed that as the whole plasmid repaired the defect the two *Bam*H1 when combined in the correct order would also repair the defect. Transformants were then selected on Luria-Kn agar and tested for growth on HPA. These colonies were then tested on Luria-Ap agar to ensure that the whole of pJJ210 had not been transferred into pMMB34. Plasmid DNA was isolated from the colonies with the correct phenotype and mapped using restriction enzymes to confirm the construction which was designated pJJS200 see fig,3.1.9. pJJS200 was used to transform C600(pLG223) which was then used to mate pJJS200 into the three *P.putida* strains, 2440, PP001 and PP003, which are unable to grow on HPA. Once again it was found that the *P.putida* strains were not able to grow on HPA or 3,4DHPA. Assays for the 3,4DHPA catabolic enzymes were then carried out, see table,3.2.4. The activities detected are very low compared to those observed for *P.putida* grown on HPA, even when the strains harbouring the plasmids were grown on glycerol in the presence of HPA.

Table 3.2.4

Activities of certain 3,4DHPA catabolic enzymes detected in various
P. putida strains harbouring pJJS200

Specific activity nmoles min⁻¹ mg protein⁻¹

Strain	Growth substrate	3,4DHPA dioxygenase	CHMS dehydrogenase	CHM isomerase	OHED hydratase	HHED aldolase
<u>P. putida</u>	Gly	0	5	3	1	0
<u>P. putida</u>	HPA	388	273	187	78	45
PP001	Gly	15	27	37	9	2
PP003	Gly	20	30	50	10	3
2440	Gly	30	41	38	10	3

c) The use of a subclone produced in the characterisation of pJJ210 to isolate an intermediate in the 3,4DHPA catabolic pathway.

Extracts from 5K(pJJ212) were shown to accumulate a compound with a λ -max 260nm. The isolation of this intermediate, HHED, will be discussed later.

3.3 FURTHER BIOCHEMICAL CHARACTERISATION OF THE 3,4DHPA PATHWAY.

ISOLATION AND IDENTIFICATION OF INTERMEDIATES OF THE 3,4DHPA CATABOLIC PATHWAY.

The initial analysis of the enzymes encoded by pJJ801 utilised CHMS and CHM isolated according to the method of Sparnins et al (1974). It was shown that when 5K harboured plasmids carrying *hpc* genes that expressed the 3,4DHPA catabolic enzyme constitutively the specific activities detected in these strains were higher than those detected for *E.coli* C grown on HPA, see table,3.3.1. Extracts from 5K strains, harbouring a specific subclone which only encoded the required enzymes, were used to isolate intermediate compounds of the 3,4DHPA catabolic pathway. The compounds isolated were CHMS, CHM, HHDD and HHED, according to the procedure described in the Methods.

In an attempt to characterise several of the compounds and equilibrium mixes they were sent for analysis by NMR. Initially there were problems due to insufficient quantities or lack of purity. Subsequent samples of sufficient quantity and purity were submitted, some were analysed, but several samples were lost due to technical problems. These ranged from the break down of the machine to the accidental wiping out of the NMR traces stored in the memory bank.

Table 3.3.1

Activities of 3,4DHPA catabolic enzymes showing increased activities over the
HPA induced levels for *E. coli* C

Strain	Growth substrate	Specific activity (nmoles min ⁻¹ mg protein ⁻¹)				
		3,4DHPA dioxxygenase	CHMS dehydrogenase	CHM isomerase	COHED decarboxylase	HHDD isomerase
<i>E. coli</i> C	HPA	180	160	98	35	1623
5K(pJJ002)	Gly	2,350	0	0	0	0
5K(pJRJ003)	Gly	2,527	723	0	0	0
5K(pJRJ002)	Gly	2,300	572	820	180	0

a) 5-Carboxymethyl-2-hydroxymuconate semialdehyde. (CHMS)

CHMS was isolated using extracts from 5K(pJJ002). It showed a λ -max of 380nm in alkali which shifts to a λ -max at 320nm in acid. This is very similar to the results described by Sparnins et al (1974) for CHMS, see fig,3.3.1.

b) 5-Carboxymethyl-2-hydroxymuconate. (CHM)

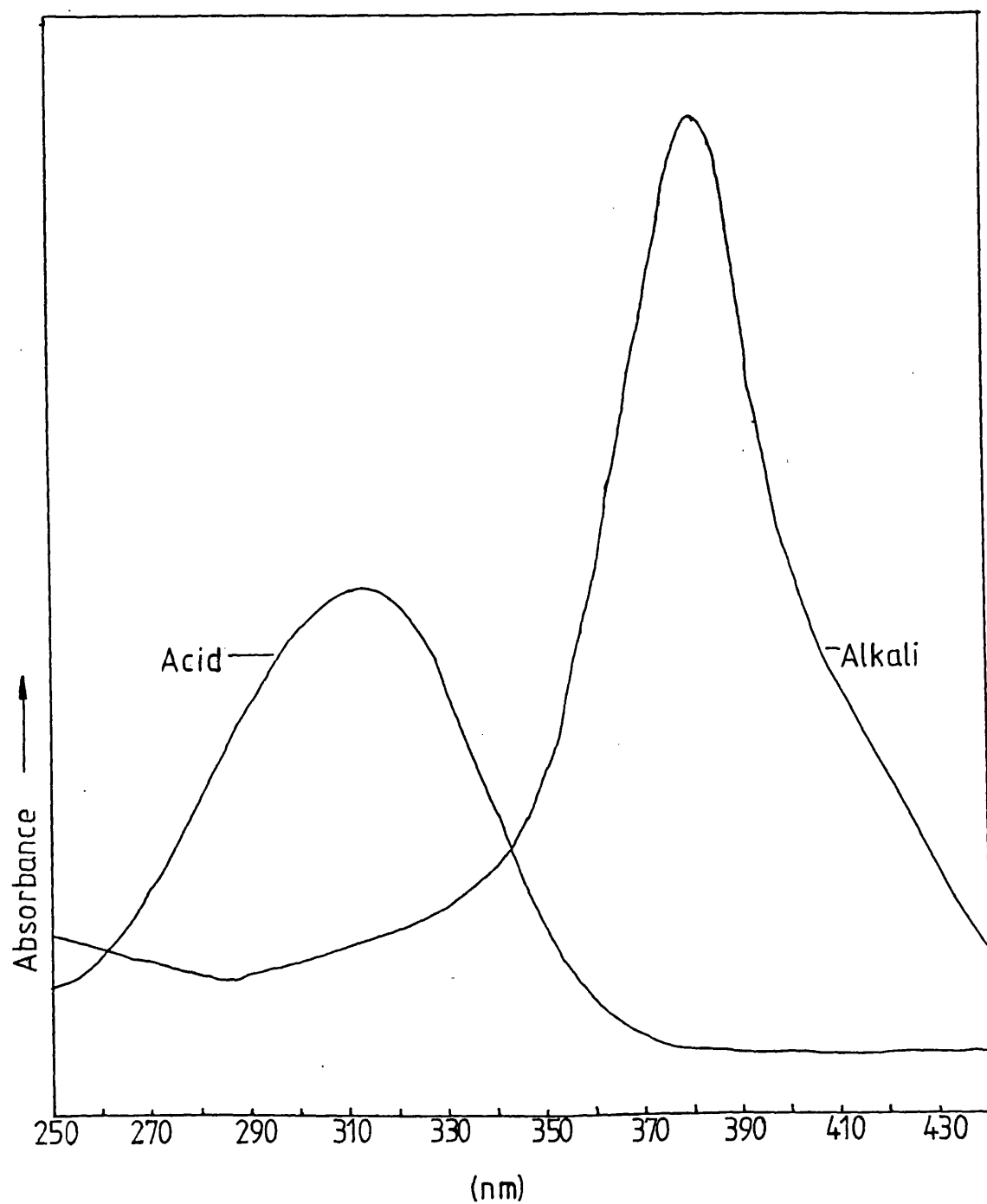
CHM was isolated using extracts from 5K(pJRJ003). This compound was shown to undergo a spontaneous isomerisation, see fig,3.3.2. The spontaneous isomerisation of CHM to COHED has been previously reported by Garrido-Pertierra and Cooper (1981). In an attempt to characterise this compound a lyophilised sample was resuspended in D₂O and submitted unsuccessfully for analysis by NMR. The samples of CHM isolated using heat treated *P.putida* and 5K(pJRJ003) extracts both had the same UV-spectral properties. It was therefore concluded that the CHM isolated using 5K(pJRJ003) extracts was the same as that previously characterised by Sparnins et al (1974).

c) 5-Carboxymethyl-2-oxo-hex-3-ene-1,6-dioate. (COHED)

If a sample of CHM was allowed to isomerise spontaneously an equilibrium mixture of CHM and COHED was obtained. The UV-spectrum of the equilibrium mixture of CHM and COHED can be seen in fig,3.3.2. A sample of the equilibrium mixture was unsuccessfully submitted for NMR analysis. A difference

fig,3.3.1.

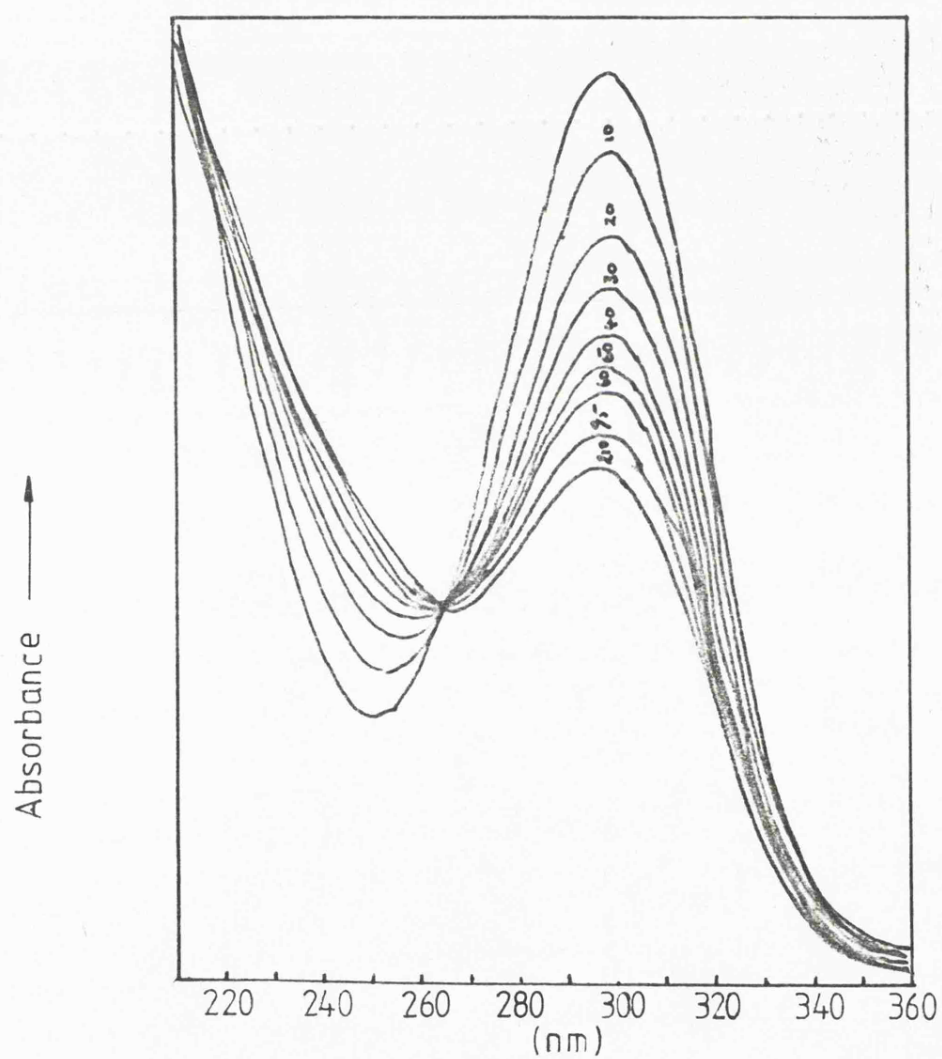
A diagram of the spectral scans of CHMS in both acid and alkaline conditions.



fig,3.3.2.

A diagram of the spectral scan of CHM showing the spontaneous isomerisation.

The time between each scan is shown in minutes.



spectrum of the isomerisation mixture against a fresh sample of CHM is shown in fig,3.3.3. The λ -max for COHED is 246nm. These results are similar to those observed for COHED identified by Garrido-Pertierra and Cooper (1981).

d) 2-Hydroxyhepta-2,4-diene-1,7-dioate. (HHDD)

A compound with a λ -max at 276nm was isolated using an extract of 5K(pJRJ002). It had a similar λ -max to an intermediate of the 3,4DHPA catabolic pathway observed, but not isolated, by Sparnins et al (1974), see fig,3.3.4. A sample of HHDD was lyophilised, dissolved in D₂O and successfully analysed by NMR. The NMR trace which confirms the structure as that of 2-hydroxyhepta-2,4-diene-1,7-dioate is shown in fig,3.3.5.

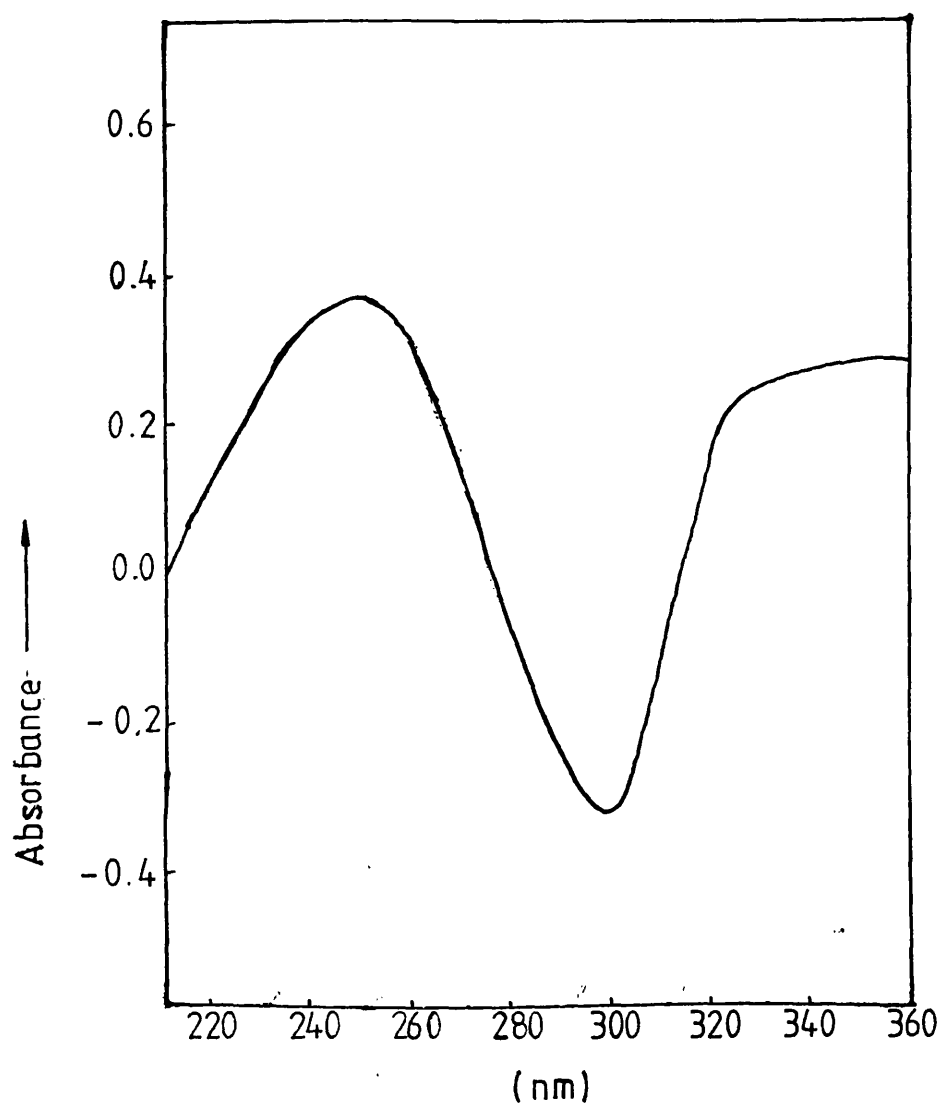
HHDD was shown to undergo a very fast spontaneous isomerisation, in 0.1M Na-phosphate buffer pH7.5, to OHED as shown in fig,3.3.4. This isomerisation is similar to that observed for CHM to COHED shown earlier, fig,3.3.2. This is not too surprising as the chemical structures CHM and HHDD are very similar, the difference only being a carboxyl (COOH) group. The spontaneous rate of isomerisation was greatly reduced when HHDD was dissolved in D₂O, and is the reason for only the HHDD form being identified in the NMR analysis.

e) 2-Oxo-hept-3-ene-1,7-dioate. (OHED)

To analyse OHED an equilibrium mixture of HHDD and OHED was formed in 0.005M Na-phosphate buffer pH7.5, which was then lyophilized, dissolved in D₂O and unsuccessfully submitted

fig,3.3.3.

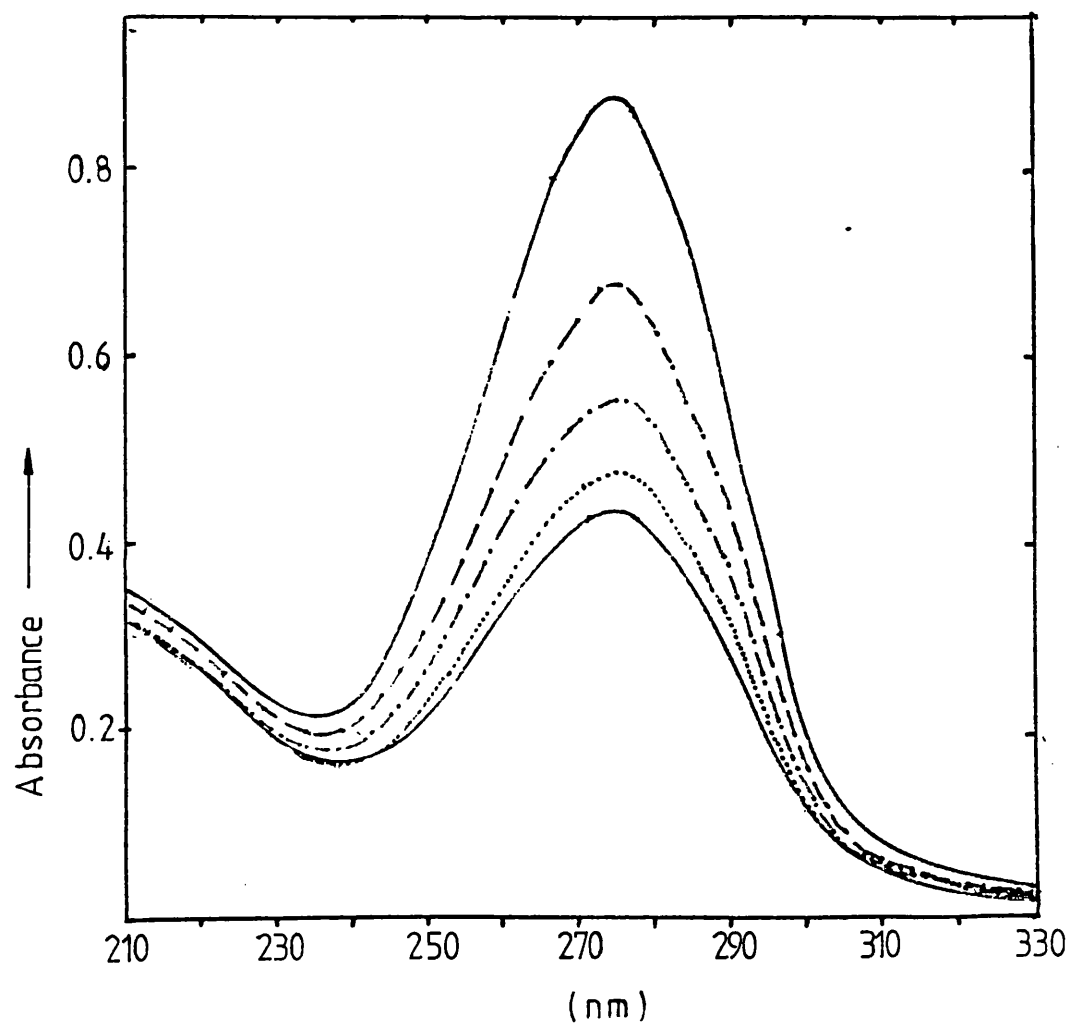
A diagram of the difference spectrum of a mixture of CHM and COHED against a fresh sample of CHM.



fig,3.3.4.

A diagram of the spectral scan of HHDD showing the spontaneous isomeriastion.

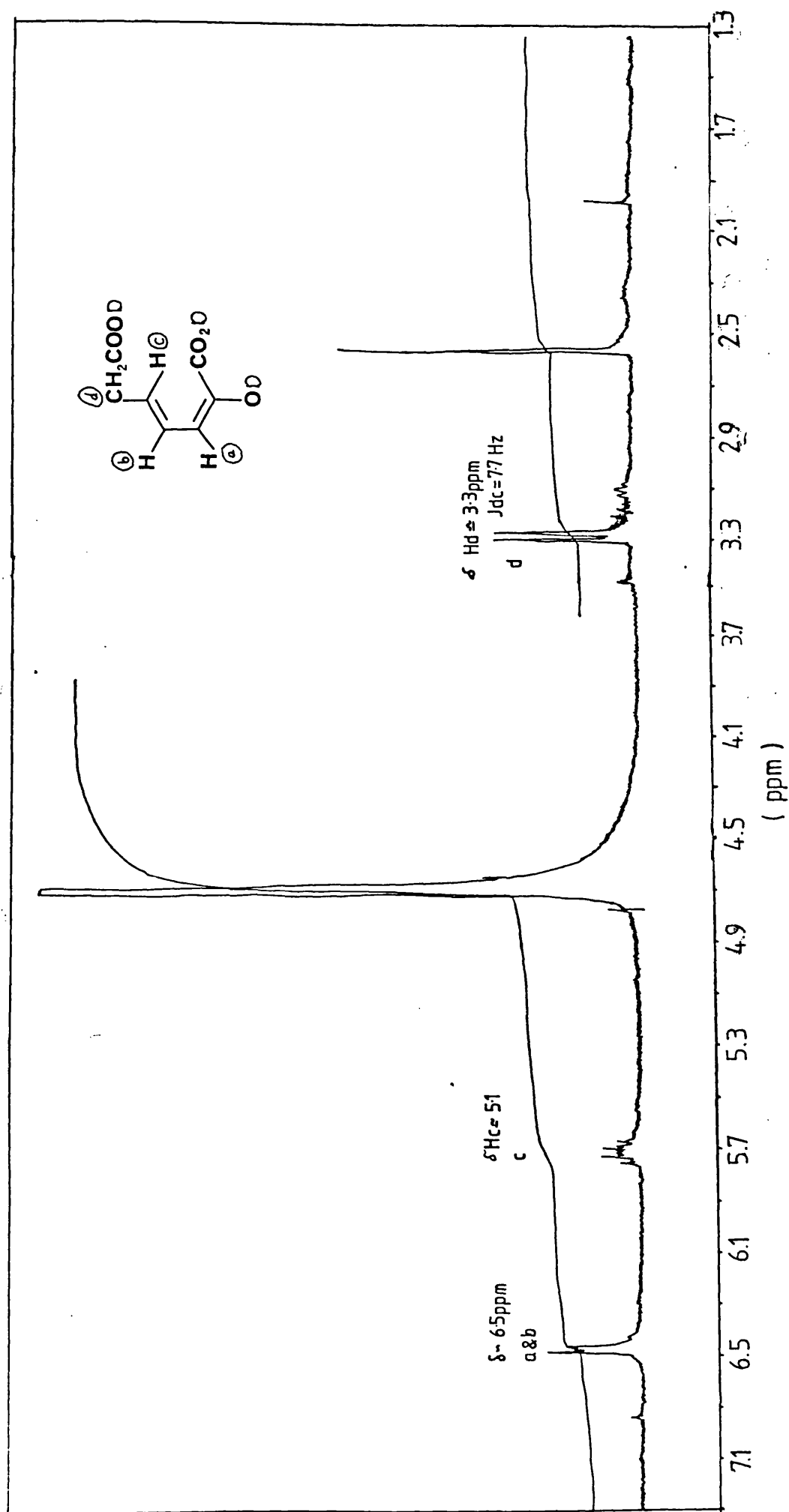
The time between each scan was 30 seconds.



A diagram of the NMR spectrum of HHDD.

fig, 3.3.5.

Determined in D_2O .



for NMR analysis. A difference spectrum of the isomerised mixture against a fresh sample of HHDD, is shown in fig,3.3.6, (conducted at 4⁰C). This shows that OHED appears to have a λ -max of 222nm. It should be remembered that at these low wave lengths the SP1800 is not fully efficient so, a reasonable margin of error must be allowed for. The shift in λ -max as CHM isomerises to COHED is approximately the same as the shift observed as HHDD isomerises to OHED.

f) 2,4-Dihydroxy-hepta-2,ene-1,7-dioate. (HHED)

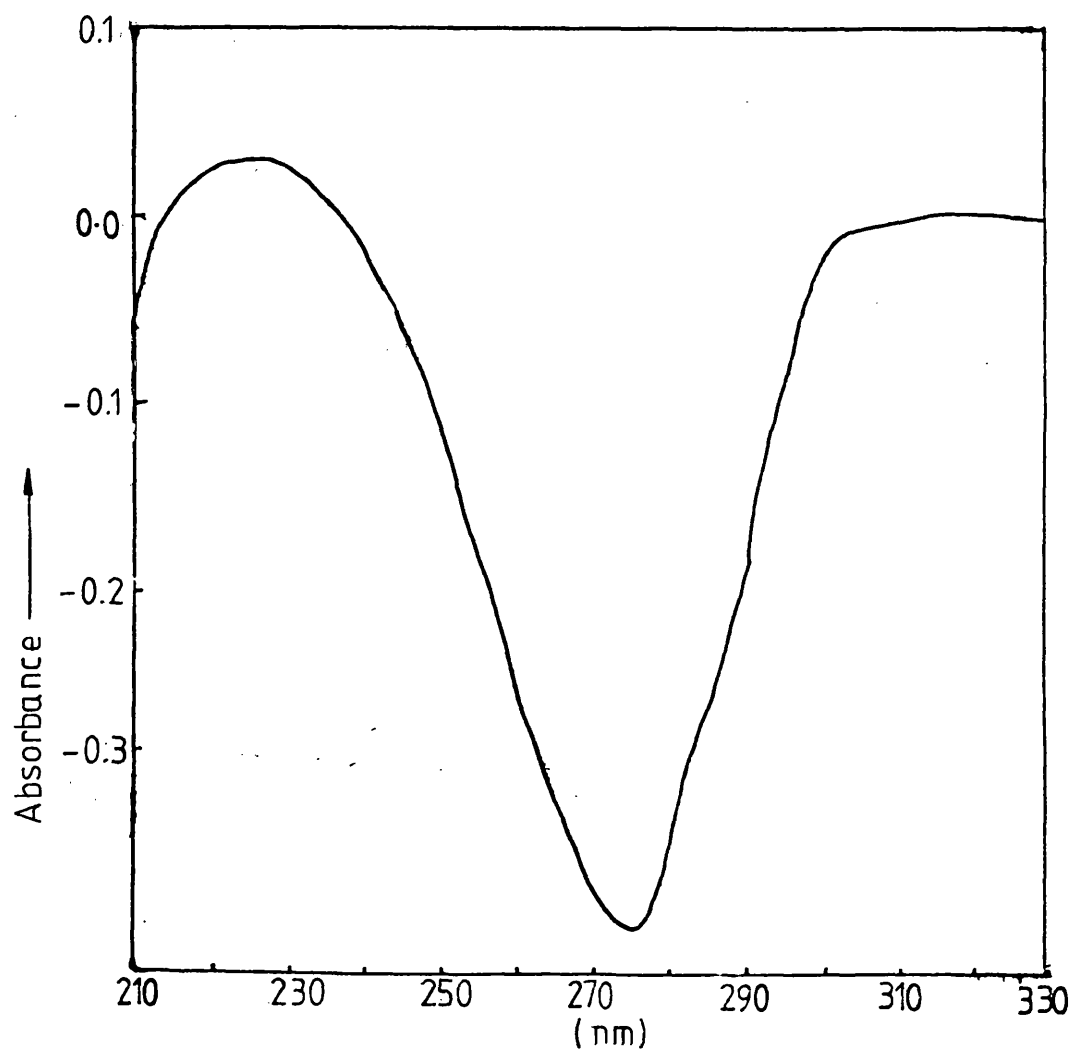
4-Hydroxy-2-ketopimelate (HKP) has been chemically synthesised from SSA and oxaloacetate by Leung et al (1974). This method was also used by a project student D.Burnham (1986) to synthesise chemically HKP, which was used in the study of the the aldolase enzyme from the 3,4DHPA catabolic pathway.

A compound with a similar λ -max (260nm), see fig,3.3.7, was isolated using extracts from 5K(pJJ212). This biologically manufactured compound was successfully subjected to NMR analysis and the trace can be seen in fig,3.3.8. This suggests that the structure is 2,4dihydroxyhepta-2-ene-1,7-dioate (HHED) that is the enol-form of HKP. HHED appears stable and shows no sign of tautomerisation to HKP as proposed in Dagley's (1978) reaction scheme for the 3,4DHPA catabolic pathway. Also no enzyme(s) capable of catalysing this process was encoded by pJJ210, which has been shown to encode all the enzymes necessary for the conversion of 3,4DHPA to pyruvate and SSA. The results from the biologically produced HHED suggest that

fig,3.3.6.

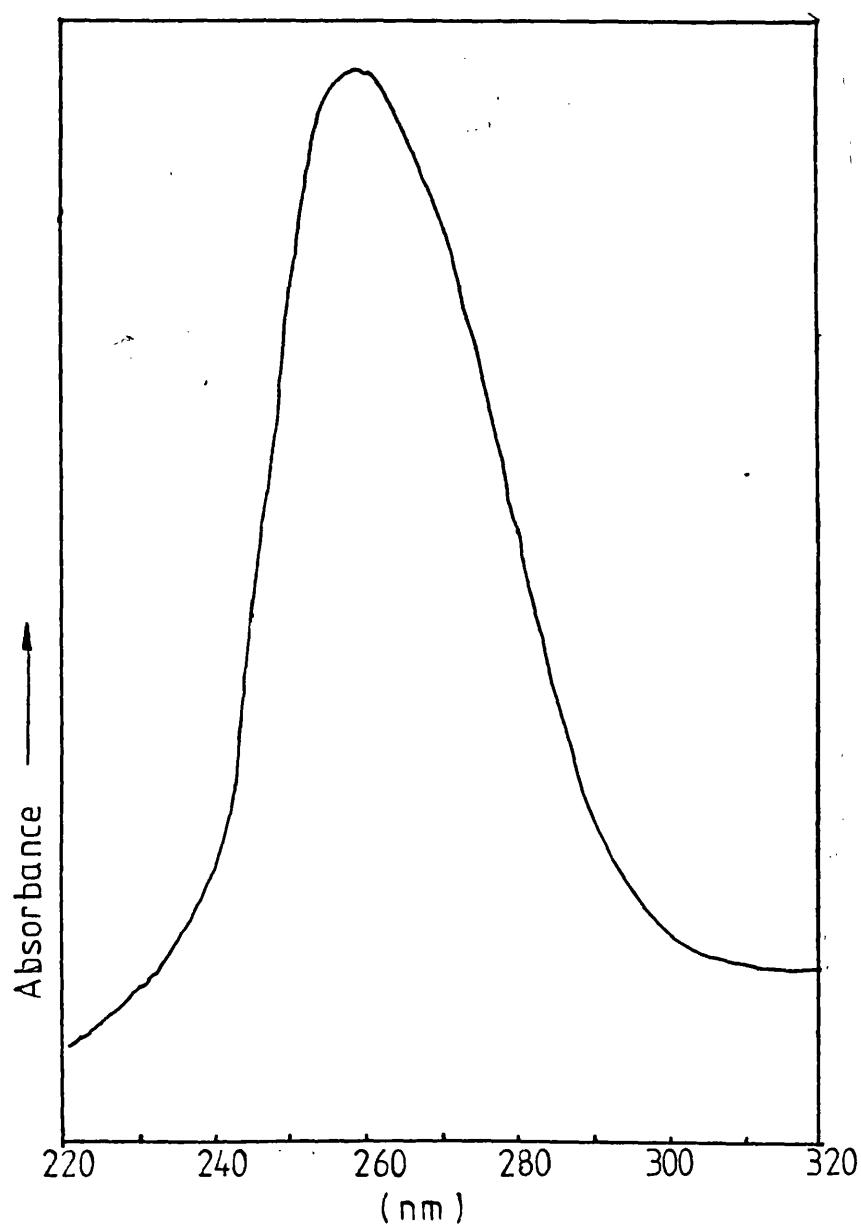
A diagram of the difference spectrum of a mixture of HHDD and OHED against a fresh sample of HHDD.

This shows that OHED appears to have a λ -max of 222nm.

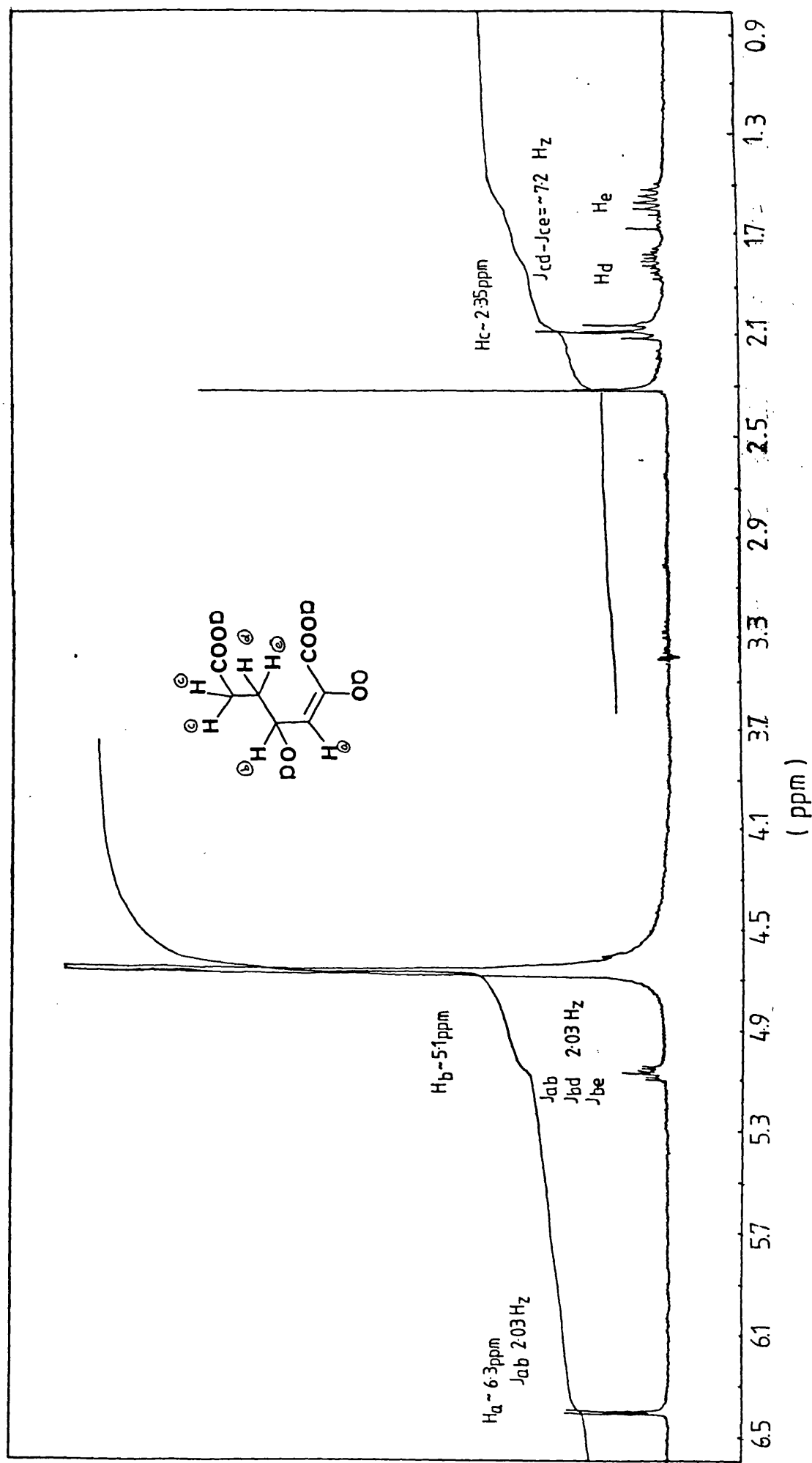


A diagram of the spectral scan of HHED.

HHED has a λ -max of 260nm, at pH7.5, this is the same as the chemically synthesised "HKP".



Determined in D_2O .



it is HHED that is cleaved by the aldolase to produce enol-pyruvate and SSA. The compound called HKP and HHED appear to be the same. Also the chemically synthesised compound serves as a substrate for the cloned aldolase enzyme. From this it would be reasonable to assume that the compound called HKP is HHED.

USE OF ISOLATED INTERMEDIATES TO CHARACTERISE FURTHER THE 3,4DHPA CATABOLIC PATHWAY.

a) The COHED decarboxylase enzyme.

The COHED decarboxylase of *E.coli* B was shown by Garrido-Pertierra and Cooper (1981) to be magnesium-dependent. However, this enzyme from *E.coli* C was shown to be magnesium-independent with the reaction occurring in the presence of EDTA.

b) HHDD isomerase.

As the substrate had not been isolated the HHDD isomerase enzyme had not been detected previously. The enzyme was originally assayed in 0.1M Na-phosphate buffer pH7.5 and detected by an increase in rate over an already fast spontaneous reaction. However, during the NMR analysis it was discovered that D_2O reduces the spontaneous rate by about 5 fold. This reduced blank rate was then utilised in an enzyme assay which allowed a better estimation of the specific activity of the HHDD isomerase since the enzyme catalysed reaction appeared to be unaffected. It was also shown that no

metal ions were required as the reaction is unaffected by the presence of EDTA.

The molar extinction coefficient of HHDD was calculated from the amount of pyruvate enzymically produced from a known quantity of HHDD. The quantity of pyruvate produced from HHDD was calculated using the NADH linked LDH assay. The molar extinction coefficient for HHDD at pH7.5 as determined in Calculation, 3.3.1, is $18,600 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$.

c) OHED hydratase.

The isolation and characterisation of HHDD also enables the subsequent reaction to be analysed. As stated earlier HHDD undergoes a rapid spontaneous isomerisation forming an equilibrium mixture of HHDD and OHED. The equilibrium mixture was then used as the substrate for the OHED hydratase assay. The assay for the hydratase (described in Methods) is a slightly unusual linked assay because it relies on the spontaneous preceding reaction.

The OHED hydratase is the first enzyme encoded by the second *hpc* operon and of the two enzymes in this operon, it is the ~~easier~~ to assay. This fact was used in subsequent studies of factors which affect the regulation of the *hpc* genes.

In the course of the study of this enzyme it was shown that it requires the presence of magnesium ions in the reaction mixture. Until the development of the OHED hydratase assay and the identification of the separate *hpcG* and *hpcH* genes it was uncertain whether this stage required the presence of magnesium ions. This was because this reaction was only

Calculation 3.3.1

The calculation of the molar extinction coefficient for HHDD

It was assumed that HHDD is converted to pyruvate in equi-molar amounts, i.e. 1M of HHDD gives 1M of pyruvate.

Various known amounts of HHDD were enzymically converted to pyruvate. The quantity of pyruvate produced was determined by the change in absorbance at 340 nm which occurs upon the addition of LDH to a reaction mixture containing NADH and pyruvate.

Amount of HHDD	Change at 340nm
10 μ l	0.16
20 μ l	0.31
30 μ l	0.465

$$\begin{aligned}
 \text{Assume } \text{NADH} &\equiv \text{Pyruvate} \\
 1 \text{ } \mu\text{mol NADH} &\equiv A_{340} \quad 6.2 \\
 10 \text{ } \mu\text{l of HHDD} &\equiv 0.16 \\
 &\quad \underline{6.2} \quad \mu\text{mol NADH} \\
 &= 0.0258 \text{ } \mu\text{mol HHDD} \\
 &\quad \text{measured at } 4^\circ\text{C}
 \end{aligned}$$

$$\begin{aligned}
 10 \text{ } \mu\text{l of HHDD} &= A_{275} \quad 0.48 \equiv 0.0258 \text{ } \mu\text{mol} \\
 1 \mu\text{mol} &\quad 1 \quad \times 0.48 \quad = 18.6 \\
 &\quad \underline{\quad} \\
 &\quad 0.0258
 \end{aligned}$$

1 μ mol HHDD in 1ml would have an absorbance of 18.6

Therefore the molar extinction coefficient

$$\begin{aligned}
 &= 18,600 \quad \text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1} \\
 &\quad \underline{\quad}
 \end{aligned}$$

previously measured as part of a combined conversion of COHED to pyruvate.

d) HHED aldolase.

HHED rather than HKP is cleaved by the aldolase to produce SSA and enol-pyruvate since no additional enzyme activities could be detected or proved necessary for this reaction.

The reaction catalysed by the *P.putida* enzyme using the chemically synthesised substrate "HKP" has been shown to require magnesium ions, Leung et al (1974). The *E.coli* C aldolase reaction using the biologically produced HHED as the substrate was also shown to require the presence of magnesium ions.

The molar extinction coefficient for both the chemically synthesised "HKP" and biologically produced HHED at pH7.5 was calculated using the assay for the production of pyruvate, calculation 3.3.2. Both compounds appear to have the same molar extinction coefficient, further evidence for the compounds being the same. The estimated molar extinction coefficient for HHED was $16,400 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$.

AN ATTEMPT TO PURIFY THE 3,4DHAP 2,3 DIOXYGENASE.

The main reason for purifying the dioxygenase from *E.coli* C was to aid the analysis of the DNA sequencing data, see Results 3.4. The N-terminal amino-acid sequencing of the protein would help determine the relative position of the start of the protein on the DNA sequence. The purification of the 3,4DHAP dioxygenase from *P.putida* was described by Takeda

Calculation 3.3.2

Calculation of the molar extinction coefficient for HHED

It was assumed that 1M of HHED was converted to 1M of pyruvate.

$$16 \mu\text{l "HKP"} = 1.0 A_{260} \equiv \text{change at } 340 \text{ nm } 0.365$$

$$54.4 \mu\text{l HHED} = 1.0 A_{260} \equiv \text{change at } 340 \text{ nm } 0.375$$

$$1 \mu\text{mol NADH} \equiv 6.2 A_{340 \text{ nm}}$$

$$54.4 \mu\text{l HHED} \equiv 0.378$$

$$\frac{6.2 \mu\text{mol NADH}}{0.378} =$$

$$16.4 \mu\text{mol HHED}$$

$$\text{so } 1 \mu\text{mol } \frac{1}{0.061} = 16.4$$

Therefore the molar extinction coefficient

$$= \frac{16,400 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}}{1}$$

(1966). Initially it was decided to repeat this process, using 5K(pJJ002) as the source of the dioxygenase. As can be seen in table,3.3.2, the acetone precipitation procedure previously used appears to inactivate the enzyme. However, it was shown that 10% acetone stabilises the enzyme, giving up to 90% of the original activity after 4 days.

Since this method was unsuccessful an alternative approach was tried. The cell-free extract from 5K(pJJ002) was passed through a DEAE-cellulose (DE52) column attached to the FPLC, which was used as a gradient maker. 50mM Na-phosphate buffer pH7.5 was used in the DEAE cellulose column. The 3,4DHPA dioxygenase was eluted from the column using a 0-1M NaCl gradient at a flow rate of 2.5ml/min. The dioxygenase was eluted between 0.35 and 0.45M NaCl. The elution profile shows the analysis of the fractions for protein, as determined by absorbance at 280nm, and dioxygenase activity fig,3.3.9. The peak fractions were collected and concentrated in an minicon concentrator B15. The 10x concentrated protein was then passed down a Superose 12 column; equilibrated against 50mM Na-phosphate buffer pH7.5, at a flow rate of 1ml/min, see fig,3.3.10, for the elution profile. The fraction containing the peak activity eluted at 13.3ml. The peak active fractions from this were then passed down the Pro-reverse phase column which acts in a denaturing manner. A gradient of H_2O TFA (1%) to acetonitrile TFA (1%) was used to elute the protein. The elution profile, fig,3.3.11 shows that the peaks from the Superose 12 column were not pure.

In an alternative strategy fractions were collected from the Superose 12 column and run on a non-denaturing preparative polyacrylamide gel. It was shown that if such a

Table 3.3.2

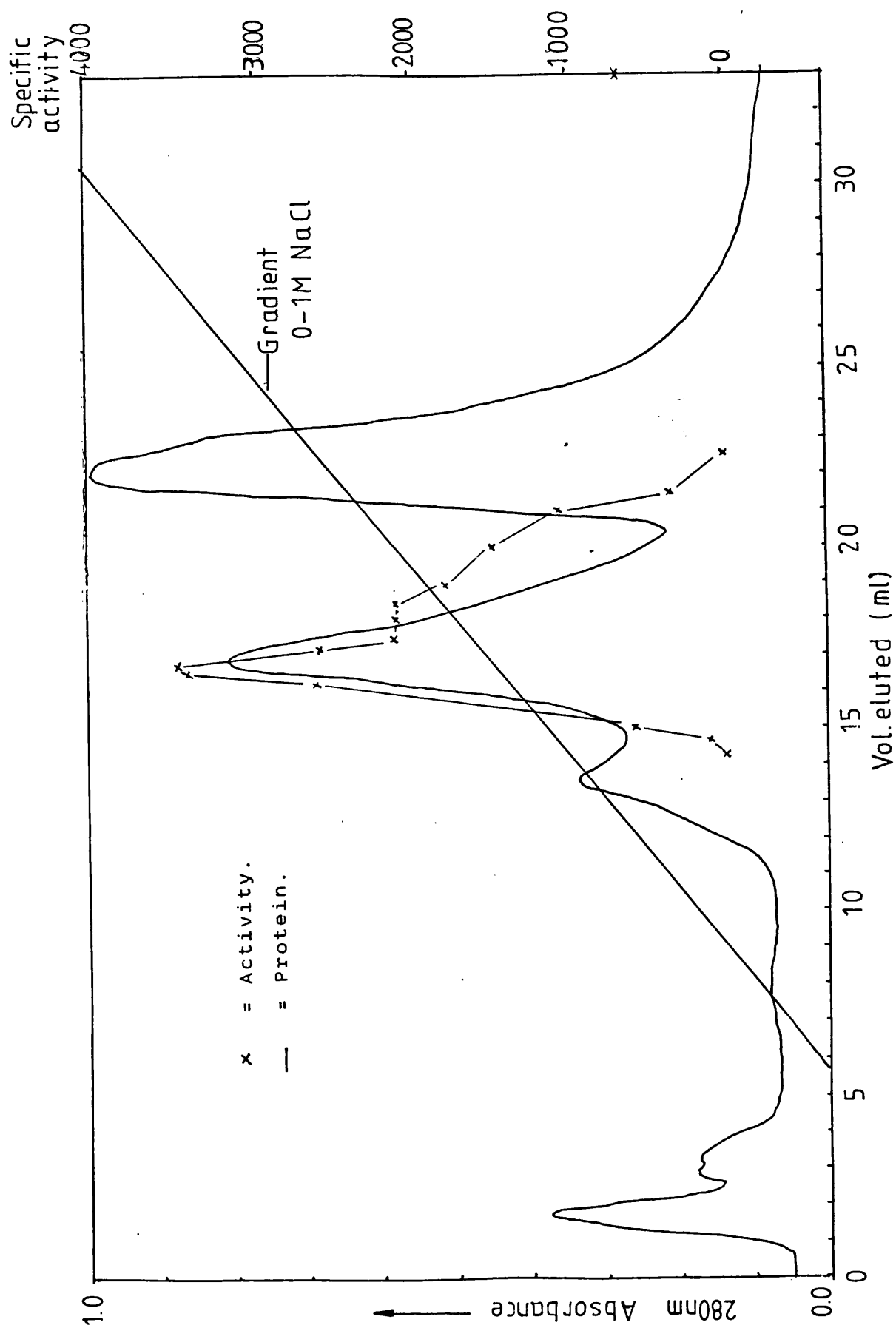
An attempt to purify the 3,4DHPA dioxygenase by ammonium
sulphate precipitation

5K(pJJ002) was used as the source of the enzyme.

Treatment	Specific activity nmole min ⁻¹ mg protein ⁻¹
None (cell free extract) (1 ml)	1835
10% acetone	1838
4 days later	1683
Ammonium sulphate	
0 - 33% (1 ml)	979
Supernatant from (200μl) 33% ppt.	1620
33 - 55% (1 ml)	389
55% + (3.5 ml)	949

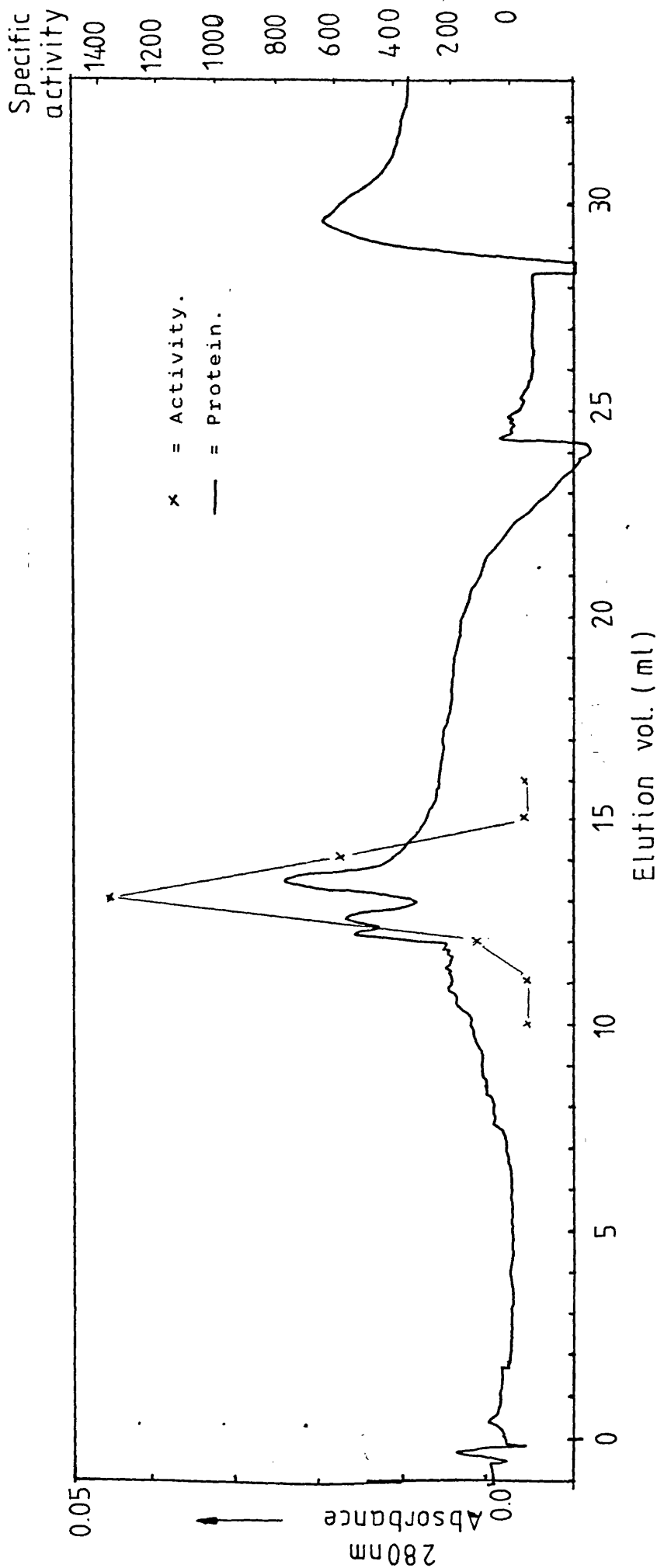
fig.3.3.9.

The elution profile measured at 280nm of a crude cell extract, from 5K(pJJ002), passing through a DEAE (DE52) column. The activity profile of the 3,4DHPA dioxygenase is also shown.



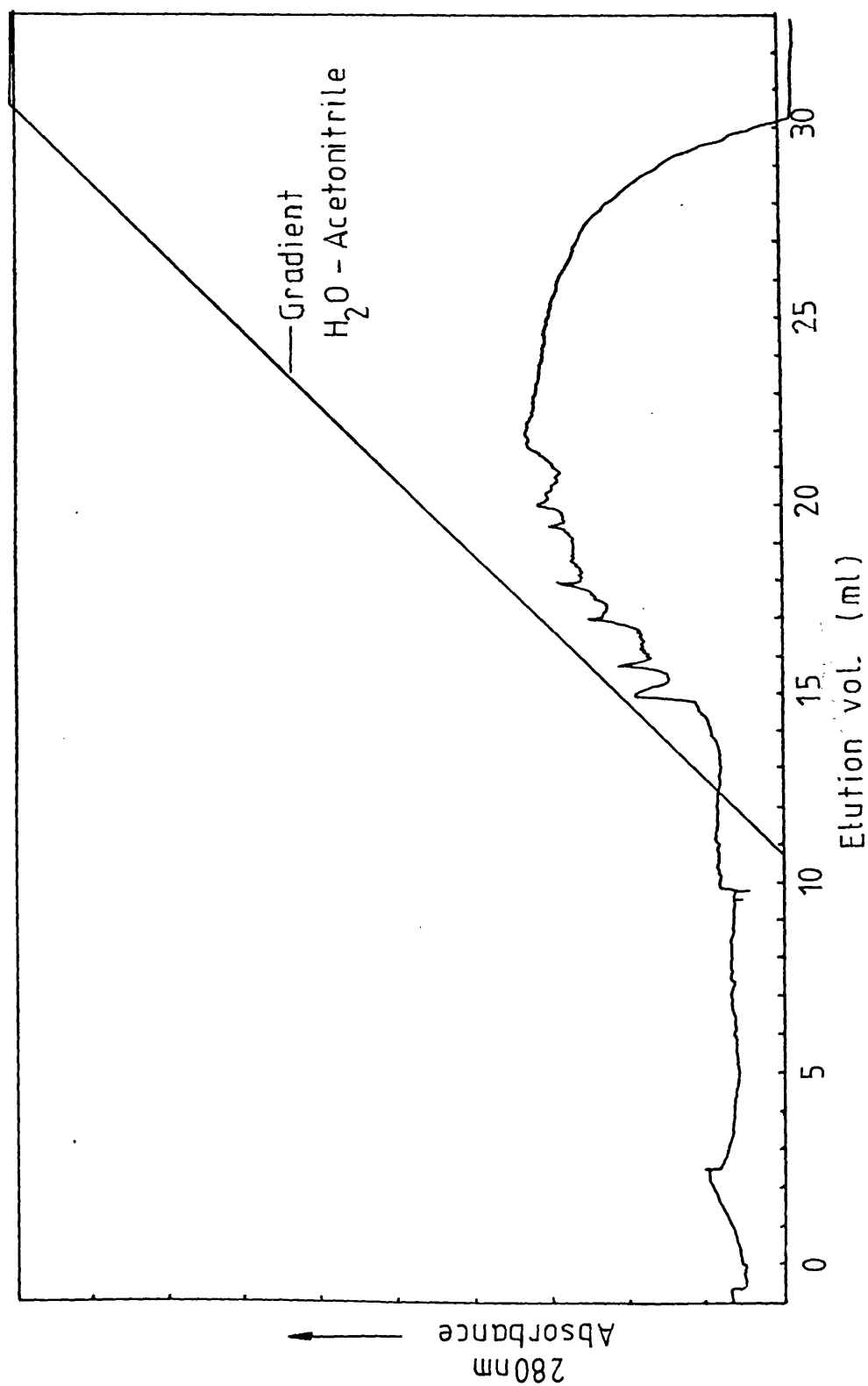
fig,3.3.10.

The elution profile measured at 280nm of a semi-purified protein sample, containing the 3,4DHPA dioxygenase, passing through a Superose 12 column. The activity profile of the 3,4DHPA dioxygenase is also shown.



fig,3.3.11.

The elution profile of a protein sample, containing the 3,4DHPA dioxygenase passing through a Pro-reverse phase column.



gel was flooded with a 0.2M soln. of 3,4DHPA a yellow band of CHMS, presumably corresponding to the position of the dioxygenase could be detected. The fractions from the Superose 12 column had lost a lot of activity by this stage, so to determine the position of the dioxygenase on the gel a sample with very highly dioxygenase activity from the DE52 column was run in a parallel track. The appropriate fragment was cut out see fig,3.3.12, and the protein eluted from the polyacrylamide gel.

The gel-purified protein was then passed down the reverse phase column. The elution profile, fig,3.3.13, shows that there was not enough protein to be detected over the background solvent absorption.

This was unfortunately left at this stage due to lack of time. If this is to succeed in the future a larger preparative gel and more concentrated extract would be needed.

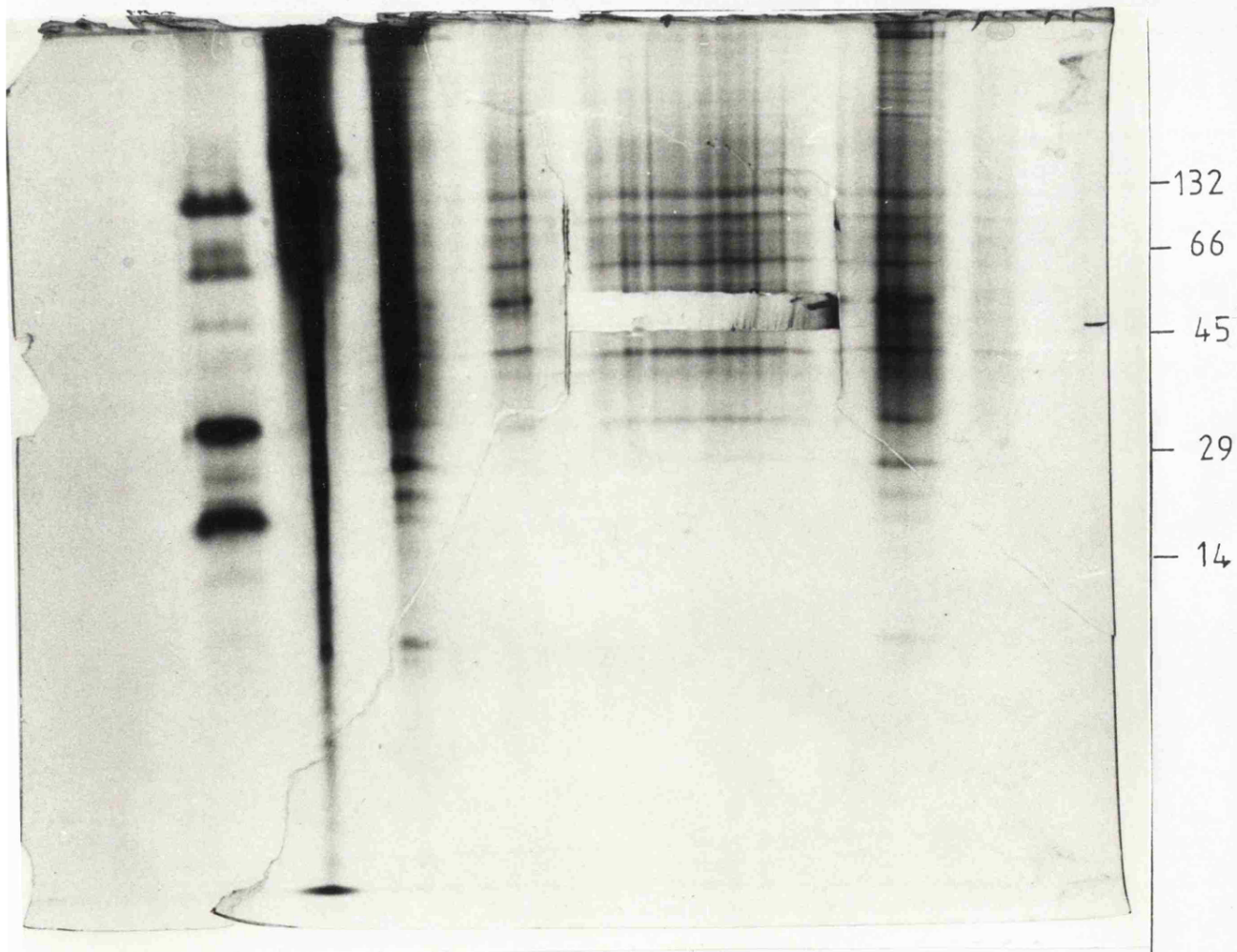
What can be determined from this is the approximate molecular weight of the dioxygenase. During the attempted isolation there were two calibrated stages, the Superose 12 column and the native-gel electrophoresis, at which dioxygenase activity was detected. Fig,3.3.14, shows the calibration curve for standards run on the Superose 12 column and the relative position of the dioxygenase. Fig,3.3.12, shows the photograph of the native-gel. From these a molecular weight of 46,000-48,000 daltons was deduced.

A photograph of a native polyacrylamide gel.

A band corresponding to the 3,4DHPA dioxygenase was cut out of the gel and the protein isolated by electroelution. The photograph shows the replaced gel fragment which was stained with the rest of the gel to show that the protein had been eluted.

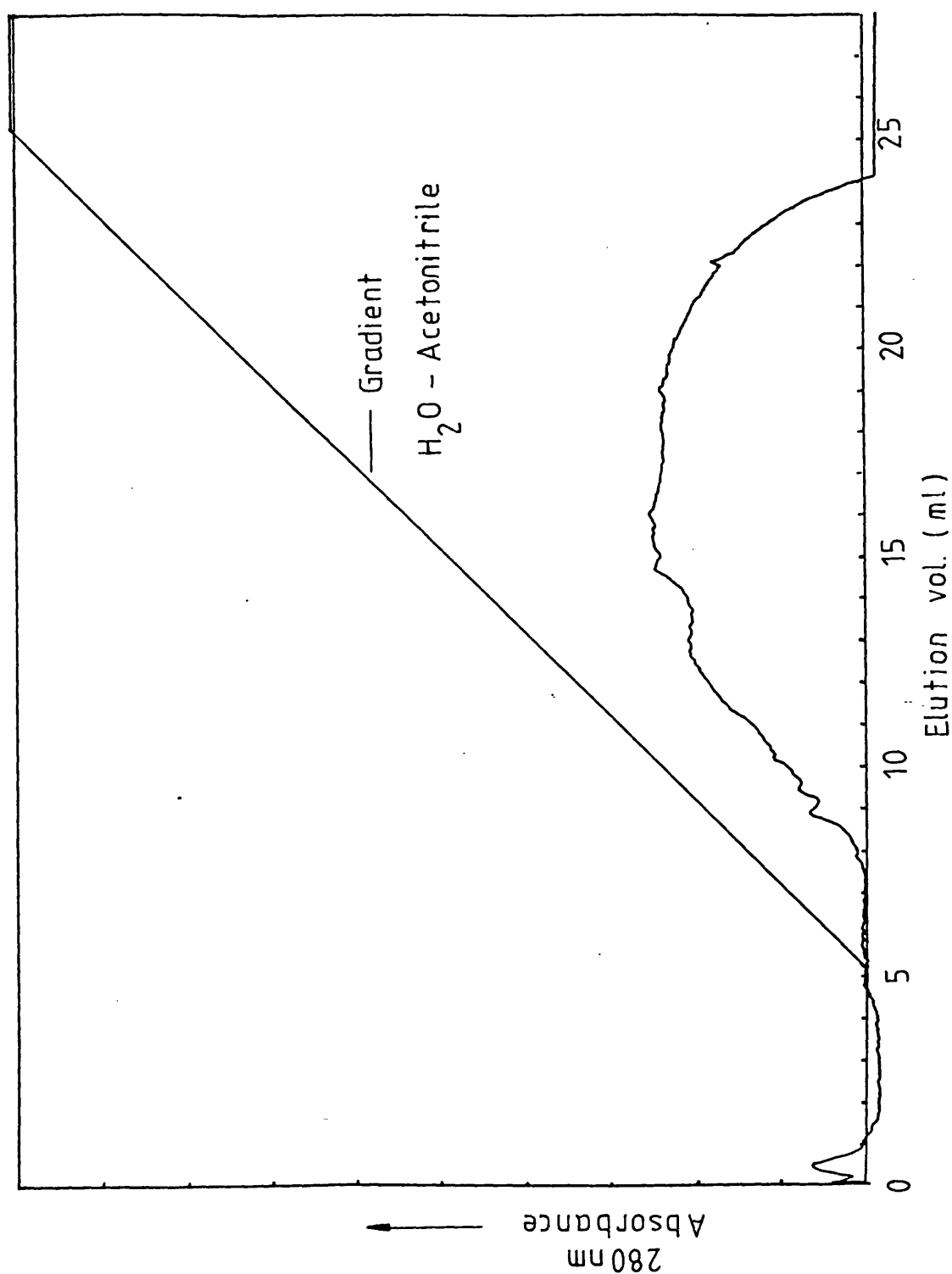
Nature of protein sample

M.Wt marker	Crude extract	1/2 quantity of crude extract	Superose 12	Superose 12 for isolation of 3,4DHPA Dioxygenase	DEAE (DE52)	K daltons
-------------	---------------	----------------------------------	-------------	--	-------------	-----------



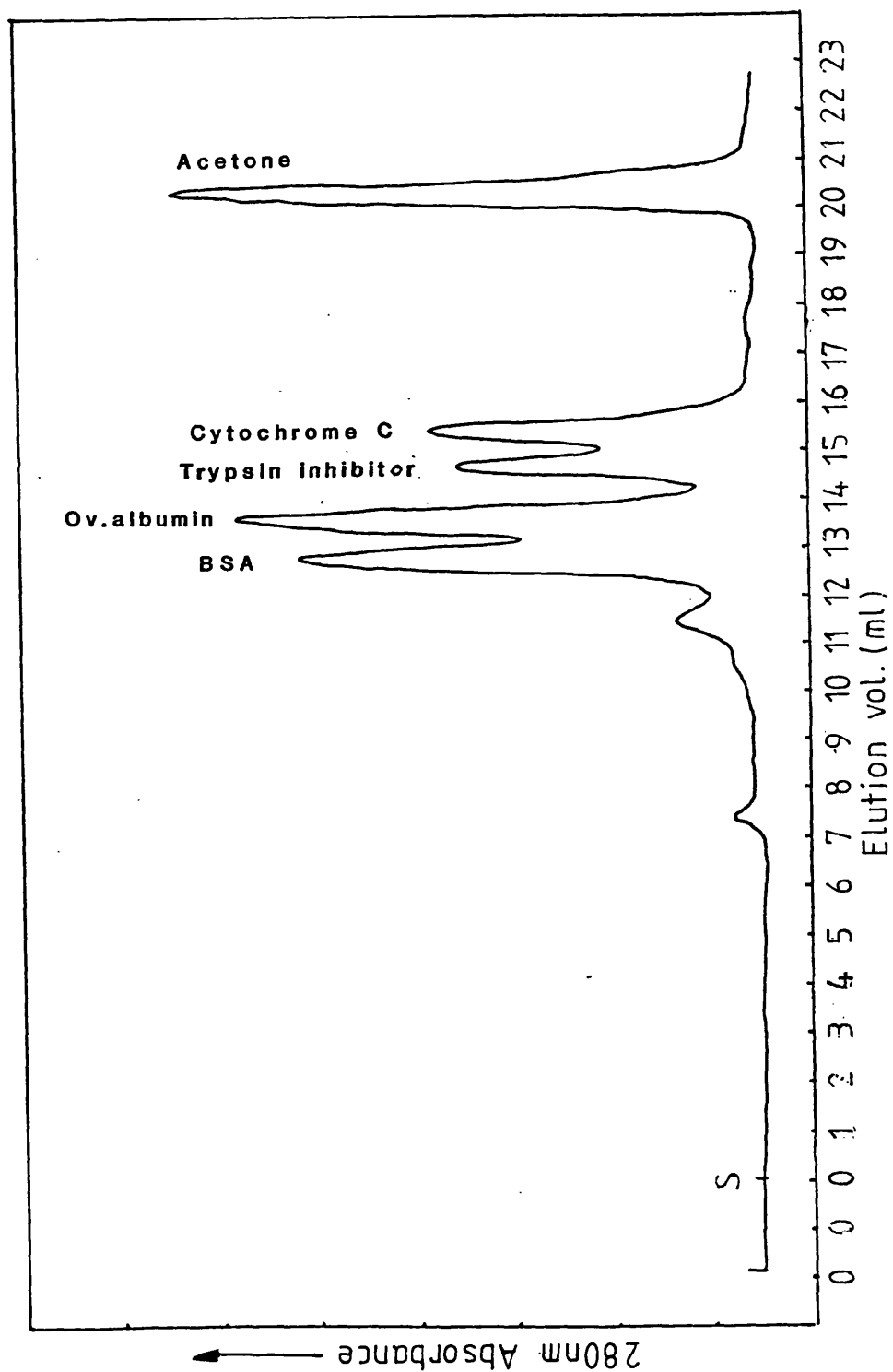
fig,3.3.13.

The elution profile of the 3,4DHPA dioxygenase isolated from a polyacrylamide preparative gel passing through a Pro-reverse phase column.



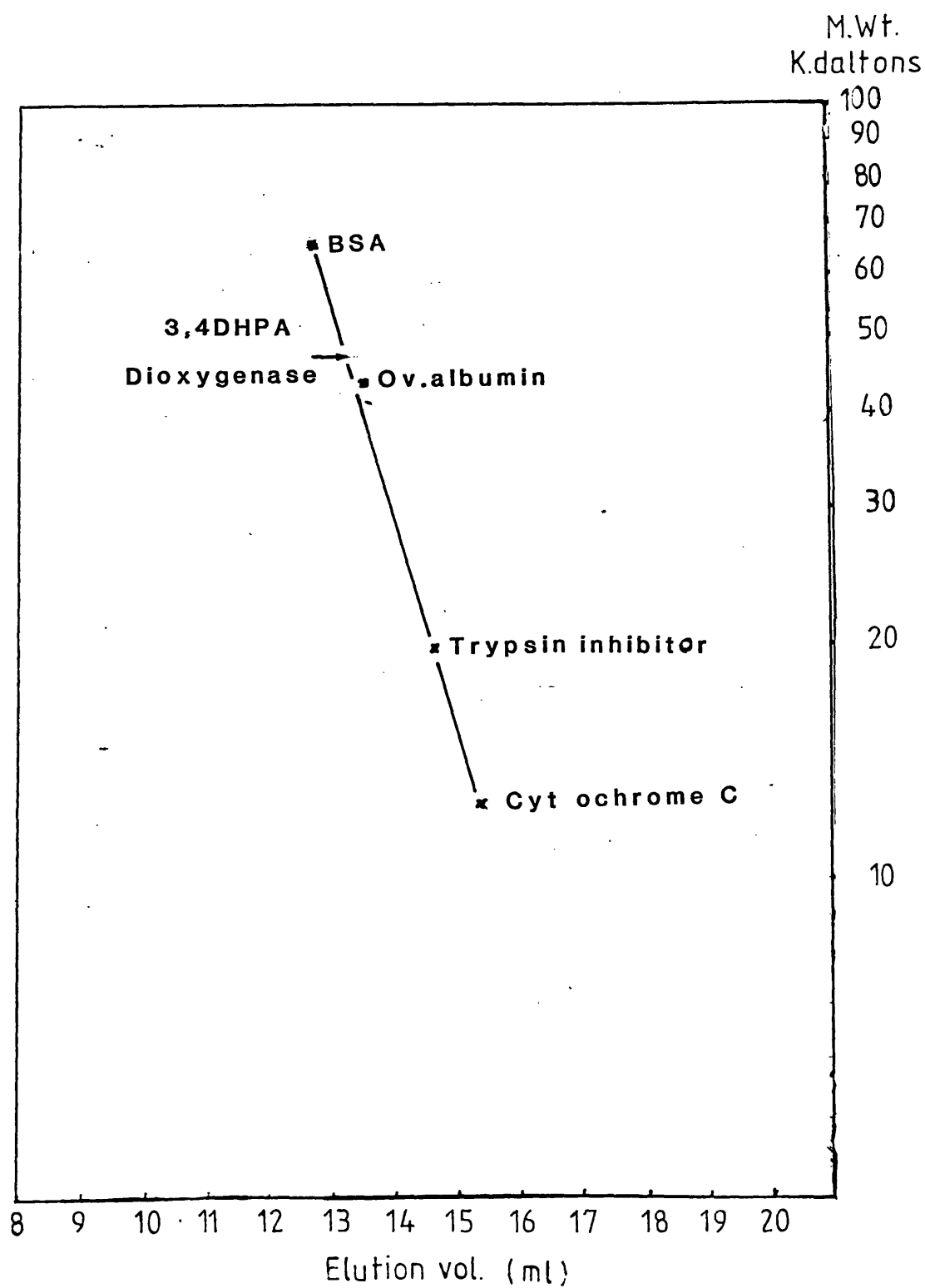
fig,3.3.14a.

A calibration profile showing the relative elution distances of various molecular weight molecules passing through a Superose 12 column.



fig,3.3.14b.

A graph showing the calibration curve from the elution profile in fig.3.3.14a.



3.4 FACTORS AFFECTING THE REGULATION AND EXPRESSION OF THE 3,4DHPA PATHWAY

IDENTIFICATION OF A RECEPTOR SITE ON pJJ801 FOR THE PRODUCT OF *hpcR*.

It was shown (Section 3.1) that if the 6.5Kb *Bam*H1 fragment from pJJ801 was placed in the opposite orientation in pBR328 and the resulting plasmid, pJJ108, introduced into 5K the same 3,4DHPA catabolic enzymes are detectable as for 5K(pJJ801), see table,3.1.5. It was also shown earlier in fig,3.1.6, that the genes encoded by pJJ801 and pJJ108 are inducible in JJ221. These results would indicate that the plasmid pJJ801 carries the operator-promoter region for the *hpc* genes.

When pJJ801 was harboured by 5K five 3,4DHPA catabolic enzymes were expressed constitutively. This indicates that a regulatory gene had not been cloned on pJJ801. This was confirmed with the isolation of pJJ210 and the regulated expression of the *hpc* genes it encoded, see table,3.4.1.

From the regulated expression of the genes of pJJ801 in JJ221 it was concluded that a trans-acting chromosomally encoded protein from JJ221 must be effecting the pJJ801 encoded 3,4DHPA catabolic enzymes. Therefore there should be a receptor site for a regulatory protein on pJJ801. If this is the case it means that the mutation in JJ247 must, at least in part, be due to a defective regulatory gene since the enzymes encoded by pJJ801 are constitutively expressed in

Table 3.4.1

Activities of the 3,4DHPA catabolic enzymes in 5K harbouring pJJ210

Strain	Growth substrate	3,4DHPA dioxxygenase	Specific activity(nmole min ⁻¹ mg protein ⁻¹)			
			CHMS dehydrogenase	CHM isomerase	COHED decarboxylase	OHED hydratase
5K(pJJ801)	Gly	875	400	520	90	0
5K(pJJ210)	Gly	< 1	5	7	< 1	0
5K(pJJ210)	Gly/HPA	42	55	103	13	40
5K(pJJ210)	Gly/3,4DHPA	60	103	145	24	73
						23
						34

JJ247 (Section 3.1).

The position of the regulatory receptor region or operator site was identified by the use of subclones. The subclones produced using the restriction sites identified on pJJ801 indicate that the region of interest was carried on the 2.5Kb *Bam*H1-*Eco*R1 fragment. This is because only in subclones containing this region was there expression of *hpc* genes, i.e. pJJ002, pJJ006 and pJJ801 but not pJJ003, pJJ004, pJJ005, or pJJ007. A more precise location of the operator site came from the subclones produced from the *Sau*3a partial digestion of the 6.5Kb *Bam*H1 fragment of pJJ801. These subclones were isolated using a positive selection for an active CHMS dehydrogenase gene. So it is not surprising that some of the subclones express the CHMS dehydrogenase constitutively even in JJ221 (presumably with promotion occurring from within the vector). There are also some which retain the regulated phenotype in JJ221. If these are compared, a position directly adjacent to the *hpcB* gene is indicated as the operator site, see fig,3.1.7.

The position of the operator region for the *hpcGH* genes appears to be in front of the *hpcG* gene, as shown in fig,3.1.7. The *hpcGH* genes seem to be transcribed from a single promoter. This is inferred by the results of 5K harbouring various subclones of pJJ210. Only the phenotypes: hydratase + (product of *hpcG*) aldolase + (product of *hpcH*); hydratase + aldolase - or hydratase - aldolase - not hydratase - aldolase + can be observed. These phenotypes are shown by 5K harbouring pJJ210, pJJ212 and pJJ801 respectively and no subclone with the last phenotype was isolated, see tables,3.1.7 and 3.2.6.

IDENTIFICATION OF A REGULATORY GENE, *hpcR* ON
pJJ200/pJJ210.

The presence of a regulatory gene was indicated by the low level of expression of the *hpc* genes in 5K(pJJ200/pJJ210) when grown on glycerol compared to the high levels of expression observed when 5K(pJJ801) was grown on glycerol. From their restriction maps it was obvious that the *hpcBCDEF* genes and the operator site were also present on pJJ200/pJJ210, so something must be repressing the constitutive expression of these genes. Further evidence for a regulatory gene on the plasmid affecting the expression of the 3,4DHPA catabolic enzymes was obtained from the increased level of expression of the enzymes when 5K(pJJ210) was grown on glycerol in the presence of HPA or 3,4DHPA (see table,3.4.1). With HPA present in the media the specific activities of the 3,4DHPA catabolic enzymes were about 7-10x those detected for the strain grown on glycerol. The presence of 3,4DHPA increased the levels of the 3,4DHPA catabolic enzymes to about 20x those observed for the strain grown on glycerol. From this it was assumed that a regulatory gene (*hpcR*) had been cloned.

To identify the position of *hpcR* the subclones from pJJ210 were analysed in 5K for the loss of the regulated phenotype, see fig,3.2.6. The results show that if DNA is deleted from the left of either the *Bam*H1 or *Sal*1 site in *hpcH* (as shown in fig,3.2.6) the enzymes are expressed constitutively. This would place the position of the *hpcR* gene to the left of *hpcH* as shown in fig,3.2.6. It is also interesting to note that

hpcR appears to regulate both gene blocks. Fig,3.2.6, shows that when *hpcR* is deleted as in pJJ212 the 3,4DHPA catabolic enzymes of both operons are produced constitutively.

To obtain positive evidence for the position of *hpcR* the 6.5Kb *Bam*H1 fragment from pJJ801 was ligated into the shuttle vector pMMB34 (which has a different antibiotic marker from pBR322). The resulting plasmid was named pJJS801. pJJS801 and pJJ216 (pJJ216 carries only the putative *hpcR* region from pJJ210) were used to transform 5K and transformants containing both plasmids were selected on Luria Ap+Kn agar. 5K(pJJS801,pJJ216) was then grown on glycerol in the presence and absence of HPA to see if the inducible phenotype could be restored. Table,3.4.2, shows that when the *hpc* genes from pJJ801 or pJJ200 are carried by the vector pMMB34 the level of expression of the 3,4DHPA catabolic enzymes is much higher than when they are carried by the pBR-vectors. The regulated phenotype is also lost if the *hpc* genes from pJJ200 are carried by the vector pMMB34, as shown by 5K(pJJS200) table,3.4.2. 5K(pJJS801,pJJ216) grown on glycerol shows lower specific activities for the 3,4DHPA catabolic enzymes than those observed for 5K(pJJS801) grown on glycerol this may be due to the fact that the organism now harbours two types of plasmids. However, when 5K(pJJS801,pJJ216) was grown on glycerol in the presence of HPA the specific activities were increased but not to those observed for 5K(pJJS801) grown on glycerol.

Table 3.4.2

Activities of the 3,4DHPA catabolic enzymes in 5K harbouring various plasmids in an attempt to show regulation of the cloned hpc gene

Specific activity(nmole min⁻¹ mg protein)

Strain	Growth substrate	3,4DHPA dioxygenase	CHMS dehydrogenase	OHED hydratase	HHED aldolase
5K(pJJ801)	Gly	875	400	0	0
5K(pJJS801)	Gly	2,372	1,556	0	0
5K(pJJ210)	Gly/3,4DHPA	60	103	73	63
5K(pJJS200)	Gly	2,437	1,444	20	5
5K(pJJS801, pJJ216)	Gly	1,068	520	0	0
5K(pJJS801, pJJ216)	Gly/HPA	1,502	830	0	0

THE EFFECT OF cAMP ON THE 3,4DHPA PATHWAY.

If *E.coli* C is grown on glucose with HPA present in the medium the 3,4DHPA catabolic enzymes are not induced, table,3.4.3. Table,3.4.3 also shows that if 5 μ mol/ml cAMP was added to the glucose+HPA media the induction of the 3,4DHPA catabolic enzymes was restored to about 40% of that observed for *E.coli* C grown on HPA only. The table indicates that the presence of cAMP affects both operons.

The isolated pathway as encoded by pJJ210, when harboured by 5K, was also affected by the presence of cAMP, as shown in table,3.4.4. 5K(pJJ210) cannot grow on HPA or 3,4DHPA, so to induce the 3,4DHPA catabolic enzymes the strain has to be grown on glycerol in the presence of 3,4DHPA. Table,3.4.4, shows that when 5K(pJJ210) was grown on a medium composed of glycerol, glucose and 3,4DHPA the specific activities of the 3,4DHPA catabolic enzymes were reduced to about 33% of that observed for 5K(pJJ210) grown on glycerol/3,4DHPA. If cAMP was also added to the medium the specific activities of the 3,4DHPA catabolic enzymes were increased. Again one enzyme representing each operon was assayed showing that, as with *E.coli* C, both appeared to be affected by the presence of cAMP.

DNA SEQUENCING OF THE OPERATOR-PROMOTER SITES OF THE *hpcBCDEF* AND *hpcGH* GENE CLUSTERS.

The position of the operator site has been identified as being adjacent to the *hpcB* gene from the analysis of the

Table 3.4.3

Showing the effect of cAMP on the 3,4DHPA
catabolic enzymes in E. coli C.

Specific activity (nmoles min⁻¹ mg protein⁻¹)

Strain	Growth substrate	CHMS dehydrogenase	OHED hydratase
<u>E. coli C</u>	Glucose/HPA	2	1
<u>E. coli C</u>	HPA	160	67
<u>E. coli C</u>	Glucose/HPA cAMP	54	28

One enzyme was assayed representing each gene block

Table 3.4.4

Showing the effect of cAMP on the activities of the
3,4HDPA catabolic enzymes in 5K harbouring pJJ210

Specific activity (nmoles min⁻¹ mg protein⁻¹)

Strain	Growth substrate	CHMS dehydrogenase	OHED hydratase
5K(pJJ210)	Gly	3	6
5K(pJJ210)	Gly/3,4DHPA	60	46
5K(pJJ210)	Gly/3,4DHPA/Glc	16	14
5K(pJJ210)	Gly/3,4DHPA/Glc cAMP	43	35

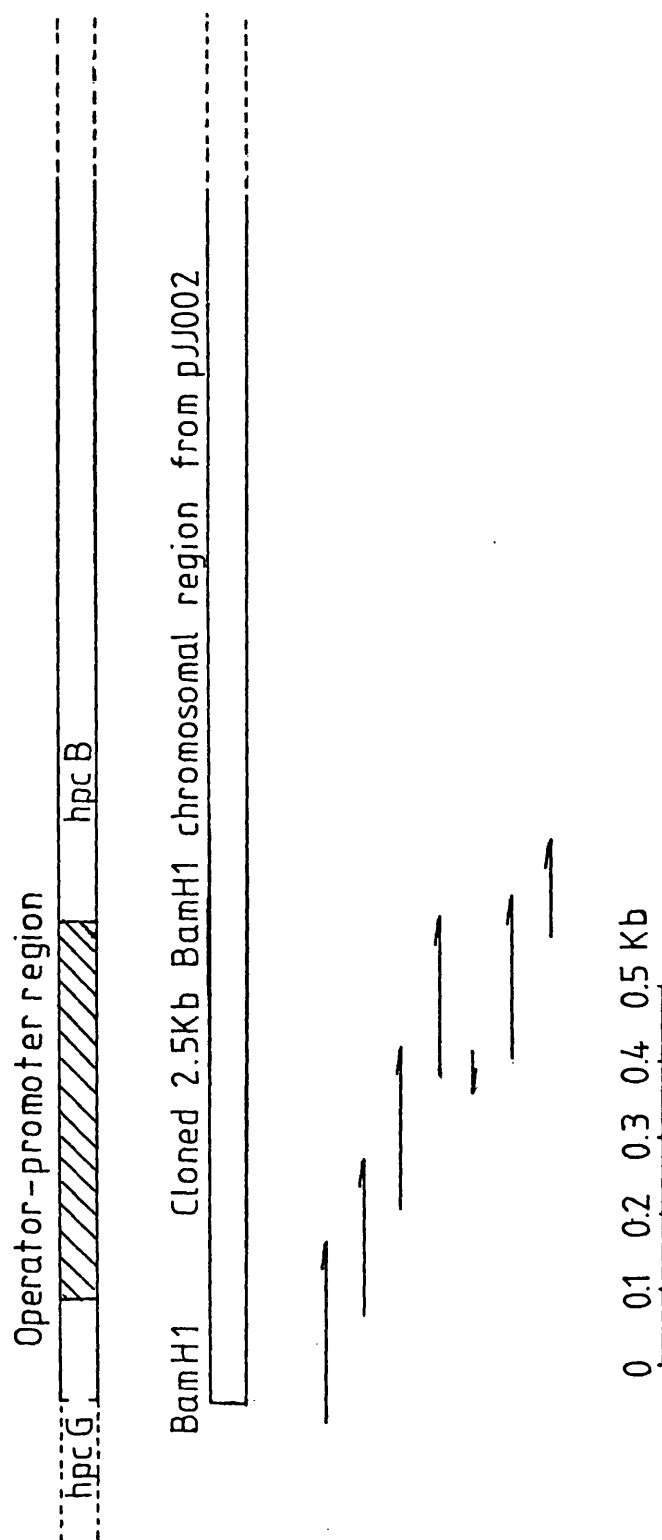
One enzyme assayed representing each of the hpc gene block

regulated expression of the subclones produced from pJJ801 harboured by JJ221. The 2.5Kb *Bam*H1-*Eco*R1 fragment from pJJ801 contains the operator site and the *hpcB* gene. If the restriction maps of pJJ801 and pJJ210 are compared, as shown in fig.3.2.8, the 2.5Kb *Bam*H1-*Eco*R1 fragment from pJJ801^{appears} to contain part of the hydratase (*hpcG*) gene and possibly its promoter, but there is no positive evidence for this. The 2.5Kb *Bam*H1-*Eco*R1 from pJJ801 was ligated into the sequencing vectors MP18 and MP19. Single stranded DNA was isolated from these constructions and used as templates for the dideoxy-sequencing reactions. To extend the sequence data several oligomer primers were used. The specifications for these were determined from the sequencing data. The strategy for sequencing the 2.5Kb *Bam*H1-*Eco*R1 fragment from pJJ801 can be seen in fig 3.4.1. This shows that most of the sequencing data was obtained from the reactions carried out on MP18 carrying the 2.5Kb pJJ801 fragment. Some regions have data obtained from two primers whose extension reactions cover the same region. However, in the time available only one sequencing reaction on the opposite strand (MP19 version) was obtained. Other primers were tried on the MP19 version but for some reason the sequencing reactions did not work.

The sequence data obtained is shown in fig 3.4.2, and the regions of potential interest are high-lighted. Although the sites are marked, no S1 mapping or protein sequence data is available to confirm them. The sequence data was analysed for open reading frames, codon usage and potential start codons, in an attempt to identify regions which may code for the *hpcB* and *hpcG* genes. When suitable candidates for these were identified (those with no stop codons), upstream regions were

fig,3.4.1.

The strategy used in determining the sequence of the operator-promoter region of the *hpc* operons.



The DNA sequence data of the region encoding the
hpc operator-promoter.

← MP18 BamH1 pJJ002 →

↓

```
1  TXTCTCGCAG GTCGACTCTA GAGGATCCCG ATAAAACGGT GATGGATAAC
51  CTTGCCGAAG TAAGCAAGAG GTGATGGTTA ACGGCAAGCC ACGCACGTAT
101 TGGGCTATTT GCAGGACTTT CTGTTCCATC CGAAACGGGC GATGACGCGG
151 TACGTNNCTT TCTGGCGGTG ACGGAANCNT TGCTGCTGGC GCNTTTGTTC
201 TCAAACAAGC AACTTATTGA TTCTTGACGA ACGACAAGAT CTGATGTGCA
251 AAGCTGAACT CTGAAGAACT GATCGANAGC TATTAGGGCN CGGTATTGCT
301 GGTTAGCCAC GATCGTCAGT TTGTCGATAA CACCGTTACA GAATGTTGGA
351 TCTTCGAAGG CGGCGGTAAA ATTGGTCGTT ATGTCGGCGG TTATATGATC
401 GCCCGTGGTC AGCAAGAGCA GTATGTGGTG GTAAACAGCC TCGCGCTGAA
451 AAAAACGAAG AAGCCGCGCA AAAGCAGAAA TGTA AACGC AGCAGTAGCA
501 AACTAAGCTA TAAATTGCAG CGCGAACTGG AGCAGCTACC GACGATTGCT
551 CGAAGATCTG GAGGCGACAG TGGAAGCCCA TCAGACGCAA GTTGGCCGGT
601 ATGCTTCCTT CTTCAATCAG CCGCCTGAGC AGACGCAAAA AGTGCTTGCT
651 GATATGGCTG CTGCAGAGCA GGAGCTGGAG CAAGCCTTTG AACGCAGGSA
701 GTATCTTGAA GCGTTAAAAA ATGGTGGC
```

! Start of interpretation →

An interpretation of the DNA sequence data
presented in fig.3.4.2.

```

-190              -170              -150
51  TTGTCGATAACACCGTTACAGAATGTTGGATCTTCGAAGGCGGCGGTAAAATTGSTCGTT 110
    -----+-----+-----+-----+-----+-----+-----+
AACAGCTATTGTGGCAATGTCTTACAACCTAGAAGCTTCGCGCGCCATTTTAACCAGCAA

-130              -110              -90
111 ATGTCGGCGGTTATATGATCGCCCGTGGTCAGCAAGAGCAGTATGTGGTGGTAAACAGCC 170
    -----+*****-----+-----+-----+-----+-----+
TACAGCGGCCAATATACTAGCGGGCACCAGTCGTTCTCGTCATACACCACCATTGTGCGG

        ← minus 10
        -70              -50              -30
                                Potential CRP binding site
171 TCGCGCTGAAAAAACGAAGAAGCGCGCAAAAAGCAGAAATGTAAAACGCAGCAGTAGCA 230
    -----+-----+-----+-----+*****!*****!*****!=====+
AGCGCGACTTTTTTTGCTTCTTCGCGCGCTTTTCGTCTTTACATTTTGCGTCGTCATCGT

        -10              10              30
        ← minus 10
231 AACTAAGCTATAAATTGCAGCGCGAACTGGAGCAGCTACCGACGATTGCTCGAAGATCTG 290
    -----+*****-----+-----+-----+-----+-----+
TTGATTGATATTTAACGTGCGGCTTGACCTCGTCGATGGCTGCTAACGAGCTTCTAGAC

        50              70              90
RBS Translation start
291 GAGGCGACAGTGGAAAGCCCATCAGACGCAAGTTGGCCGGTATGCTTCCTTCTTCAGTCAG 350
    *****-----+-----+-----+-----+-----+-----+
CTCCGCTGTACCTTCGGGTAGTCTGCGTTCAACCGGCCATACGAAGGAAGAAGTCAGTC
    Met GluAlaHisGlnThrGlnValGlyArgTyrAlaSerPhePheSerGln

        110              130              150
351 CCGCCTGAGCAGACGCAAAAAGTGCTTGCTGATATGGCTGCTGCAGAGCAGGAGCTGGAG 410
    -----+-----+-----+-----+-----+-----+
GGCGGACTCGTCTGCGTTTTTCACGAACGACTATACCGACGACGTCTCGTCCTCGACCTC
    ProProGluGlnThrGlnLysValLeuAlaAspMetAlaAlaAlaGluGlnGluLeuGlu

        170              190
411 CAAGCCTTTGAAAGCAGGGAGTATCTTGAAGCGTTAAAAAATGGTGGC 458
    -----+-----+-----+-----+-----+-----+
GTTGGGAAACTTGGCTCCCTCATAGAAGCTTCGCAATTTTTTACCACCG
    GlnAlaPheGluArgArgGluTyrLeuGluAlaLeuLysAsnGlyGly

```

analysed to try and find certain recognised structures. The best potential candidates for the start region of *hpcB* is shown in fig,3.4.3. This shows a site which is similar to the consensus -10 site (Rosenberg and Court 1979). Down stream from these is a potential GTG start codon.

The data for the start of the *hpcG* gene is not as clear. A potential -10 site is the only recognisable structure, fig,3.4.3. This is one of the areas where future work would be beneficial.

DISCUSSION

In studying the catabolism of 3,4DHPA, by *E.coli* C, a necessary partnership between biochemical and microbial genetic techniques has proved very profitable.

The ultimate aim of this study was to determine the order of the *hpc* genes and to expand the understanding of the regulation of the 3,4DHPA catabolic pathway. To achieve this it was necessary to isolate the functionally active genes on a comparatively small genetic element for easy manipulation of the genes.

THE GENE ORDER.

The cloning of the *hpc* genes was achieved by the complementation of mutants defective in the 3,4DHPA catabolic pathway. A series of clones were produced using pBR-vectors and these were tested to see if they could restore growth of the mutant on HPA. The first clone isolated was pJJ801, which was detected in the CHMS dehydrogenase mutant, JJ221. In attempts to isolate additional clones carrying different *hpc* genes further mutants were generated. JJ200 was isolated and subsequently characterised as a possible COHED decarboxylase mutant. As this gene had already been cloned a new mutagenesis strategy was tried to obtain additional mutants. This involved the EMS mutagenesis of the constitutive strain, CT001, followed by transformation with pJJ801 after the penicillin enrichment stage. pJJ801 carries the *hpcBCDEF* genes, it was therefore hoped that any chromosomal mutations generated in these genes would be complemented by the plasmid. So, in the subsequent selection stages mutations in

additional *hpc* genes would be identified. One stable mutant was isolated and designated JJ210. The use of both new mutants in cloning experiments enabled the isolation of pJJ200 and pJJ210.

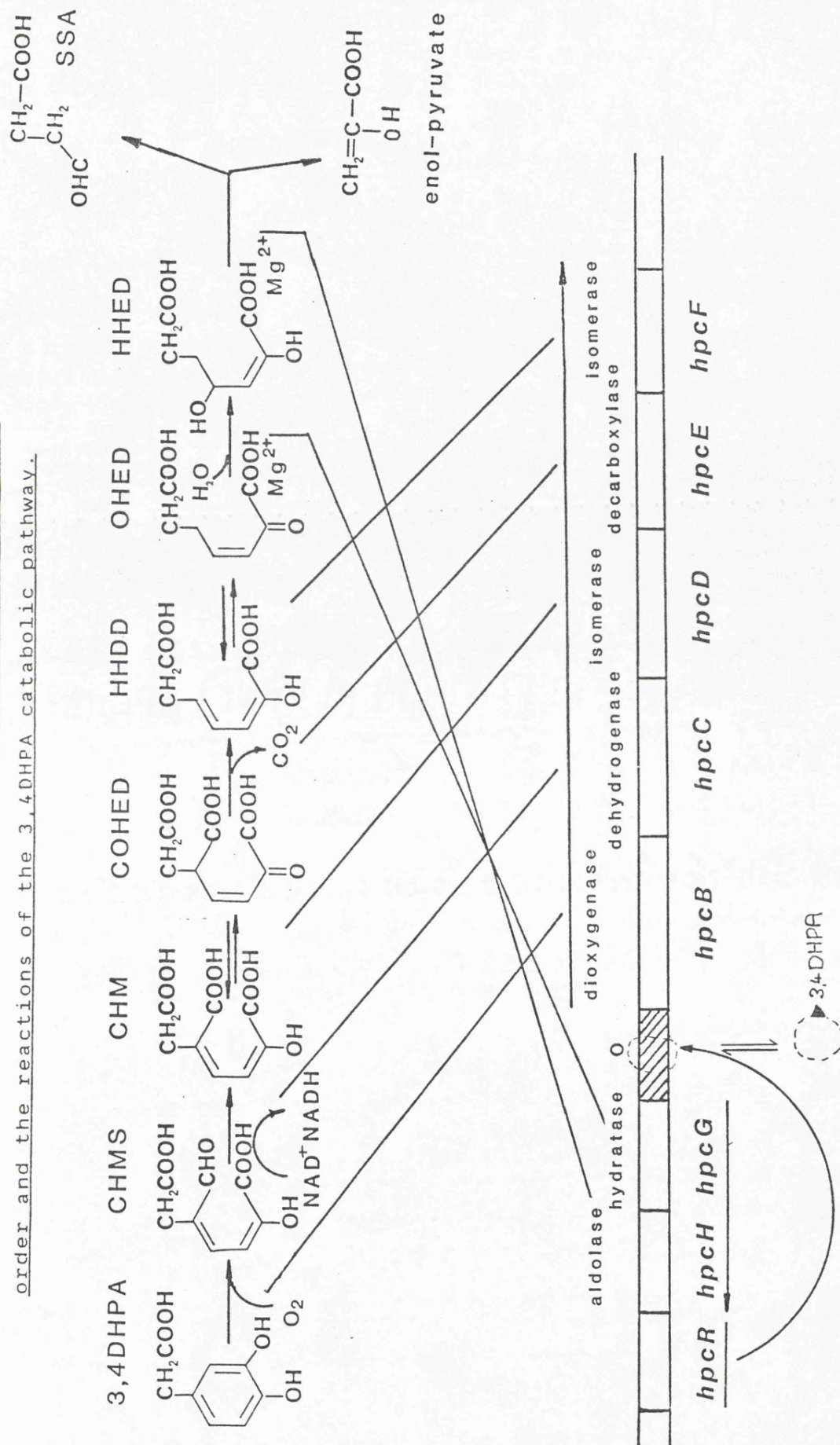
The plasmids were initially characterised by studying the expression of the *hpc* genes they encode in various strains. The presence of pJJ801 in the strains JJ221, JJ247 and JJ200 allowed growth on HPA. The 3,4DHPA catabolic enzymes were shown to be produced inducibly in JJ221 and JJ200 but constitutively in JJ247. The activities of five 3,4DHPA catabolic enzymes: the 3,4DHPA dioxygenase; the CHMS dehydrogenase; the CHM isomerase; the COHED decarboxylase and the HHDD isomerase were found in extracts of 5K(pJJ801). These enzymes were all found to be produced constitutively. pJJ200 and pJJ210 were also used to transform 5K and tested for the presence of 3,4DHPA catabolic enzymes. Both pJJ200 and pJJ210 were shown to behave in a similar manner when harboured by 5K. Oxidation experiments showed that whole cells [5K(pJJ200/pJJ210)], when grown on glycerol in the presence of HPA, were not able to oxidise HPA or 3,4DHPA. When the extracts from these strains were assayed for 3,4DHPA catabolic enzymes all the necessary enzymes for the conversion of 3,4DHPA to SSA and pyruvate were shown to be present. With these two pieces of information and the report that *E.coli* K12 has the NAD-linked SSA dehydrogenase (Skinner and Cooper 1982), it was assumed that the reason for non growth of 5K(pJJ200/pJJ210) on 3,4DHPA was due to the lack of a permease. However, some HPA or 3,4DHPA must enter 5K probably by passive diffusion because the presence of these compounds in the medium significantly increases the levels of

3,4DHPA catabolic enzymes.

The relative position of the *hpc* genes on the three plasmids was determined by subcloning and transposon mutagenesis. Five genes designated *hpcBCDEF* were identified on pJJ801 and these correspond to the first five reactions in the 3,4DHPA catabolic pathway. This block of genes was shown to be preceded by a region which controlled their expression. When JJ221 harbours pJJ801 the *hpc* genes carried by the plasmid are regulated. However, when JJ221 harbours certain subclones produced from pJJ801 the regulated expression was lost. From these results the position of the operator region was determined, see fig,3.1.7. It can be seen from fig,3.1.3,&3.1.4, that Tn insertions or deletions, produced using the restriction sites, in upstream *hpc* genes exert polar effects resulting in the loss of subsequent 3,4DHPA catabolic enzyme activity. The inference from this was that all five *hpcBCDEF* genes were transcribed as a single message from the one promoter. The position of two additional *hpc* structural genes was identified on pJJ200 and pJJ210, see fig,3.2.6. The presence of a regulatory gene on these two plasmids had already been implied by the increase in the levels of 3,4DHPA catabolic enzymes when 5K(pJJ200/pJJ210) was grown in the presence of HPA or 3,4DHPA. Additional information to support this which also enabled the position of *hpcR* to be determined was obtained from subclones of pJJ210 (harboured by 5K) which lacked this gene. In the 5K strains harbouring these plasmids it could be shown that the 3,4DHPA catabolic enzymes were now produced constitutively.

The relative positions of the seven structural *hpc* genes and the regulatory gene are shown in fig,4.1.1. It is interesting

A diagram showing the relationship between the *hpc* gene order and the reactions of the 3,4DHPA catabolic pathway.



to note that the seven genes are separated into two gene blocks *hpcBCDEF* and *hpcGH*. The *hpcBCDEF* genes correspond to the first five reactions of the 3,4DHPA catabolic pathway and the *hpcGH* genes correspond to the last two reactions. This on the surface would seem to support the suggestion of Horowitz (1965) that pathways evolve by a stepwise process as one substrate required by an organism is depleted. The two *hpc* operon however, are facing each other and are transcribed in opposite directions. This means that if the 3,4DHPA catabolic pathway has evolved by a similar mechanism to that proposed by Horowitz there must have been a subsequent rearrangement of the genes. Dagley (1978) has already pointed out that reaction mechanisms of the corresponding enzymes are very different. Sequencing of two consecutive genes in this pathway would provide the most conclusive information as to the relatedness of the genes and whether they have evolved from each other.

The positioning of the two gene blocks and the fact that they both come under the control of *hpcR* would tend to suggest that one of them is a later addition. The positioning next to functionally related genes would agree with Hegman^e and Rosenberg's (1970) proposal that translocation and subsequent unitary control would provide a more efficient pathway. The *hpcBCDEF* genes may have been originally plasmid encoded and donated from another organism leading to a hybrid pathway. This type of event has been demonstrated *in vitro* in the construction of a novel haloaromatic pathway. The additional functions required for the pathway were obtained from the TOL-plasmid (Reineke and Knackmuss, 1979). How the *hpc* genes within the two operons became linked to each other

is open to even more conjecture.

E.coli K12 has been reported not to carry any *hpc* genes (Skinner 1981). To confirm this, DNA was isolated from *E.coli* K12 and a Southern transfer of the DNA was probed with the plasmids pJJ801 and pJJ210. At the same time DNA was isolated from other strains (*E.coli* C, *K.pneumoniae* and *P.putida*) which can grow on HPA and ^{this was also} included in the same experiments. Virtually no homology was observed between pJJ801/pJJ210 and the various chromosomal DNAs tested, other than with the *E.coli* C chromosomal DNA. To visualise any bands which showed homology with other chromosomal DNA required days or weeks of exposure of the film to the filter. This is not a surprising result for *E.coli* K12 as none of the 3,4DHPA catabolic enzymes can be detected in this strain. However, it is strange that as the other two bacteria appear to have the same pathway for HPA catabolism but there is no more homology than for *E.coli* K12 DNA. It must be remembered that fairly high stringencies were used. Even when there was weak homology none of the restriction banding patterns were the same as those observed for *E.coli* C. Only one copy of each gene block was found for the *E.coli* C chromosomal DNA. No ghost bands can be observed, which could indicate duplication of genes on the *E.coli* C chromosome which have subsequently evolved by the process of gene recruitment into *hpc* genes. This leads on to the tentative speculation that *E.coli* C has acquired the 3,4DHPA pathway by horizontal transfer of genetic material, rather than the pathway evolving within this organism. If the *E.coli* chromosome has undergone duplication events as proposed by Riley and Anilionis (1978) one may have expected to detect at least two copies of DNA carrying

hpc type genes. This would of course be dependent on sufficient homology between the subsequently evolved *hpc* genes and more interestingly on the *hpc* genes being present prior to the duplication event. Unfortunately, this type of "dating" the aquisition of genes is purely speculation. Looking for homology with other strains is an idea for the future and may provide an insight into the origins of the *E.coli* C *hpc* genes.

BIOCHEMICAL TECHNIQUES.

In determining the *hpc* gene order it was necessary to combine both genetic and biochemical techniques. The gene order was shown by identifying the gene product of various subclones harboured by 5K. This could only be achieved when there was a suitable assay system for each reaction in the 3,4DHPA catabolic pathway. The assays developed to analyse the 3,4DHPA catabolic enzymes were all continuous and relied on absorption change monitored spectrophotometrically. Apart from 3,4DHPA all the intermediates in the 3,4DHPA catabolic pathway (to act as substrates for the assays) had to be prepared. The first two, CHMS and CHM were initially isolated according to the method of Sparnins et al (1974). However, as shown in the Results these and the other intermediates were isolated more efficiently using extracts of 5K harbouring the relevant plasmid-encoded 3,4DHPA catabolic enzymes. The CHMS, CHM, HHDD and HHED isolated in this way were shown to behave in a similar manner to their counterparts isolated and characterised by Sparnins et al (1974) and Leung et al (1974) for *P.putida*.

HHDD was isolated using cell-free extracts of 5K(pJRJ002) the compound has a λ -max at 276nm and was characterised by NMR analysis. This compound can be shown to be present prior to a rapid spontaneous isomerisation. This same reaction can also be demonstrated for a very rapid enzyme catalysed reaction, encoded by *hpcF*. These results suggest that HHDD is one of the intermediates in the 3,4DHPA catabolic pathway. The acidification stage of the preparation of HHDD appears to affect the equilibrium between HHDD and OHED resulting in only the isolation of HHDD. The availability of HHDD as a substrate enabled an assay to be developed to show that there was an enzyme catalysed reaction responsible for the isomerisation of HHDD to OHED. The reaction did not require any cofactors and was not impaired by the presence of EDTA. The reaction mechanism for the isomerisation of HHDD to OHED is assumed to be similar to that described by Garrido-Pertierra and Cooper (1981) for the isomerisation of CHM to COHED.

In an attempt to characterise further the equilibrium mixture, a spectrum of the mixture against a freshly prepared solution of HHDD was run (at 4°C), see fig,3.3.6. This shows that OHED has a λ -max at 222nm. A shift in λ -max of 54nm occurs as CHM is isomerised to COHED a shift of the same extent is observed for the isomerisation of HHDD to OHED.

The hydratase enzyme which catalyses the conversion of OHED to HHED was assayed at 276nm using a spontaneously formed equilibrium mixture of HHDD and OHED. This reaction has only been previously measured as part of the overall conversion of CHM to pyruvate, as determined by the LDH-linked assay. Using the new assay the hydratase was shown to require the addition

of magnesium ions to enable the reaction to proceed. It was necessary to develop separate assays for all the pathway reactions, as the previous assay system relied on the presence of all the required 3,4DHPA catabolic enzymes necessary to convert the test substrate to pyruvate. This could have caused problems when analysing the subclones, if the gene order did not correspond to the reaction order.

The final intermediate in the 3,4DHPA catabolic pathway is HHED. This was isolated using a subclone of pJJ210, (pJJ212) which carries all the *hpc* genes apart from *hpcR* and *hpcH*. HHED was characterised by NMR analysis. Leung et al (1974) proposed that 4-hydroxy-2-keto pimelate (HKP) was the last intermediate in the 3,4DHPA catabolic pathway, produced by a tautomerisation of HHED. This group chemically synthesised what they thought was HKP and showed that extracts of *P.putida*, induced for the 3,4DHPA catabolic enzymes converted it to pyruvate. This compound has been synthesised in Leicester by a project student D.Burnham (1985). It has been shown that enzymes from *E.coli* C or 5K harbouring clones act on this compound. The chemically synthesised "HKP" and HHED were measured to have the same molar extinction coefficient. Spectral scans of the two compounds also appear to be the same. Unfortunately there was not enough of the chemically synthesised "HKP" for analysis by NMR. It seems a reasonable assumption that the two compounds are the same. There is no evidence for the isomerase hypothesised by Dagley (1978). This can be concluded from the analysis of the plasmid pJJ210 which encodes all the enzymes necessary to convert 3,4DHPA to SSA and pyruvate and the failure to detect any spontaneous spectral changes for HHED. The analysis of HHED is fairly

conclusive and it would therefore be reasonable to state that "HKP" is actually HHED. The aldolase which cleaves HHED to SSA and pyruvate was shown to be magnesium ion dependent.

The subsequent conversion of SSA to succinate, by the NAD-linked dehydrogenase has been discussed by Skinner and Cooper (1982).

The 3,4DHPA catabolic pathway as determined by this study is shown in fig,4.1.1.

DNA SEQUENCING.

In an attempt to find out more about the *hpc* genes the operator regions identified as being responsible for their regulation were subjected to DNA sequence analysis. Both the operator region for *hpcBCDEF* and *hpcGH* appear to be within a 800bp region as shown by a comparison of the restriction maps of pJJ801 and pJJ210, see Results. The strategy for sequencing this area is also shown in the Results fig,3.4.1.

The DNA sequencing data is not complete due to time limitations but the results obtained do show some potential sites of interest. An interpretation of the data is shown in fig,3.4.3.

Two features of *E.coli* promoters are known to be important for transcriptional activity, the sequences centered around position -35 and -10 and the distance between them. The most frequently occurring or consensus sequences at these positions are TTGACA and TATAAT, respectively (Rosenberg and Court, 1979). An additional consensus sequence which can function instead of the -35 ^{sequence} is that for the cAMP receptor protein (CRP) binding site (Busby 1986). A sequence very similar to

that proposed for the CRP^{binding} consensus can be identified in the sequence data presented in fig.3.4.3. This is particularly interesting if combined with the results from the cAMP counter repression experiments. These showed that if glucose was also present in a medium containing 3,4DHPA then the 3,4DHPA catabolic enzymes were not expressed. The repression could be partly overcome if cAMP was also added to the medium. i.e. further evidence for the involvement of CRP in regulating HPA/3,4DHPA catabolism.

DNA sequences with homology to the 3'-terminus of the 16S rRNA have been proposed as the sites of ribosome binding, so allowing initiation of translation (Gold et al, 1981) A similar sequence to this can be identified downstream from the putative -10 region. Also 6bp down from this potential ribosome binding site is a GTG codon which can act as an initiation codon (Looman and Knippenberg, 1986).

Potential sites for -35/CRP-binding site, -10 region, ribosome binding site and potential GTG initiation codon, all with reasonable spacing, can be shown for the region preceding the *hpcBCDEF* operon. Very little could be identified in terms of recognisable structures for the *hpcGH* operon, although there is a potential candidate for a -10 region.

It should be re-emphasised that the structures highlighted and subsequent interpretations are only regions of potential interest as identified by computer analysis. There is no S1 nuclease or protein sequence data to support these interpretations.

REGULATION.

Some previous ideas concerning the regulation of the HPA/3,4DHAP catabolic pathway have been described in the Introduction. These proposals were based on the interpretation of experiments using phage P-1 transduction, F-mating and mutants. The cloning and subsequent analysis of plasmids carrying *hpc* genes has provided a greater insight into their regulation.

pJJ801 was isolated using the CHMS dehydrogenase mutant, JJ221. Both the plasmid and chromosomal 3,4DHPA catabolic enzymes were shown to be regulated when pJJ801 was present in this strain this indicates that the genes on the plasmid were being regulated in some manner. When pJJ801 was introduced into ^{strain} 5K the 3,4DHPA catabolic enzymes encoded by the plasmid were found to be expressed constitutively. The constitutive expression of the enzymes shows that a functional regulatory gene had not been cloned. The presence of pJJ200/pJJ210 in 5K shows that the genes can be regulated in this strain so the situation observed with 5K(pJJ801) is not an artifact of cloning.

If 5K(pJJ200/pJJ210) is grown in the presence of 3,4DHPA or HPA the extracts can be shown to contain raised levels of 3,4DHPA catabolic enzymes, corresponding to both *hpc* operons. Further evidence for a regulatory gene, *hpcR*, which affects both gene blocks was seen using a subclone of pJJ210. When 5K harbours pJJ212, which has lost *hpcR* and part of *hpcH*, all the enzymes encoded by the plasmid are produced constitutively. The absence of a region of DNA corresponding

to *hpcR* results in constitutive expression of the 3,4DHPA catabolic enzymes, as demonstrated by 5K harbouring pJJ212, pJJ215 and pJJ801. This suggests that the regulation is negative, similar to the lactose operon (Miller and Reznikoff, 1978).

To demonstrate that *hpcR* regulated the *hpc* structural genes in a negative manner the two plasmids pJJ216 and pJJS801 were introduced into 5K. The isolated *hpcR* gene is present on pJJ216. The operator region and five (*hpcBCDEF*) structural genes are carried by pJJS801. If only pJJS801 is present in 5K then the 3,4DHPA catabolic enzymes are expressed at very high levels. When both pJJS801 and pJJ216 are present in 5K the levels of the 3,4DHPA catabolic enzymes decrease to about half that observed for 5K(pJJS801). A possible reason for the level of the 3,4DHPA catabolic enzymes is due to an inherent effect of the vector, pMMB34, which carries the structural genes. It has already been shown that when the same 6.5Kb *Bam*HI fragment from pJJ801, carrying the five structural genes *hpcBCDEF*, was inserted into both pMMB34 and pBR328 higher specific activities for the 3,4DHPA catabolic enzymes were observed when pMMB34 was used. It would therefore seem plausible that if insufficient *hpcR* gene product was produced, because it was carried by the vector pBR322, then it could not totally repress the expression of the *hpcBCDEF* genes carried by pMMB34. Increased levels of the 3,4DHPA catabolic enzymes can be demonstrated in 5K(pJJS801, pJJ216) if the strain is grown in the presence of HPA. The levels of the 3,4DHPA catabolic enzymes are not as high as those detected for 5K(pJJS801). This may be due to the reduced copy number as the 5K(pJJ216,pJJS801) strain now carries two

plasmids.

It was previously suggested (Skinner,1981) that the regulation of the 3,4DHPA catabolic pathway was both positive and negative in a manner similar to the arabinose operon. This type of regulation requires the regulatory protein to be bound at all times (Dunn et al 1984). The absence of the regulatory gene does not result in constitutive production of the catabolic enzymes. It has already been shown that the 3,4DHPA catabolic enzymes are expressed constitutively in the absence of *hpcR*. Skinner's (1981) evidence for positive regulation was based on an interpretation of the results observed for a transposon-produced mutation in the constitutive strain. The mutant was noninducible for both the HPA and 3,4DHPA catabolic enzymes. This strain was the result of many selection schemes: selection for constitutivity; mutagenesis by EMS to produce a SSA dehydrogenase mutant and finally transposon mutagenesis. The interpretations based on this mutant have to take into account its rather complex origin and with the hindsight of the results presented in this thesis, more complicated explanations to those proposed by Skinner (1981) would be required. If only the 3,4DHPA catabolic enzymes were lost then the results could be explained as insertional inactivation of the operator region. The mutant is unfortunately not available any more, so no analysis of it using the plasmids was possible.

It has been shown that 3,4DHPA induces the enzymes responsible for its degradation (Barbour,1978: Skinner,1981). It has also been demonstrated that if the cloned pathway, e.g. pJJ210, is harboured by 5K both HPA and 3,4DHPA can be shown to induce the 3,4DHPA catabolic enzymes. It is not too

surprising that HPA also induces the enzymes because of the structural similarity to 3,4DHPA, but it is assumed that it is acting as a gratuitous inducer.

From the results presented in this study a model for the regulation of the two gene blocks, *hpcBCDEF* and *hpcGH*, has been suggested, see fig,4.1.1. It is proposed that in the absence of an inducer the product of *hpcR* binds to the operator sites of *hpcBCDEF* and *hpcGH*. (Due to the close proximity of these two operator/promoter regions there may only be one binding site, this could be determined by DNA foot printing analysis.) The bound repressor would prevent the transcription of the two *hpc* mRNAs. If, however, an inducer, 3,4DHPA, was present it would interact with the repressor preventing binding to the operator-promoter site and so allow transcription.

An additional level of control has also been implicated, by the DNA sequence data and more strongly from the experiments which show that the repression of the 3,4DHPA catabolic enzymes due to the presence of glucose in the medium can be overcome if cAMP is also added to the medium. This refinement to control comes into effect if both an inducer and an other preferential substrate, resulting in low levels of internal cAMP, is present in the medium. The inducer still prevents the repressor molecule binding to the operator regions, but the presence of the preferential substrate (^{leading to} low levels of cAMP) prevents expression of the 3,4DHPA catabolic enzymes. The control is presumably mediated by the levels of cAMP in the cell, the presence of high cAMP levels acting positively with CRP and in conjunction with the inducer allowing expression of the 3,4DHPA catabolic enzymes. A representation

of this is shown in fig.4.1.2.

The conversion of 3,4DHPA to central metabolites requires the production of a large number of enzymes, which will be a drain on the cells' resources. It would make economic sense if 3,4DHPA was part of an order of substrate usage as proposed by Busby (1986). The proposed method of regulating the order of substrate usage was by cAMP levels within the cells this also ties in with the results observed for the 3,4DHPA catabolic pathway.

THE EXPRESSION OF *E.coli* C *hpc* GENES IN OTHER ORGANISMS.

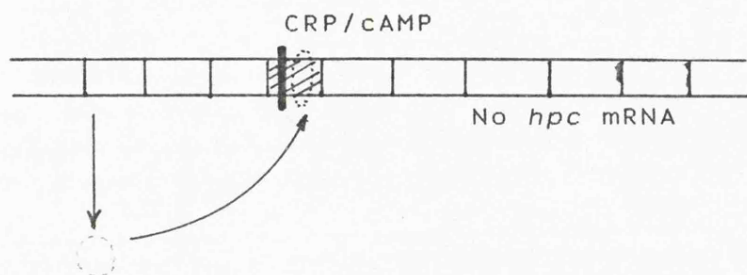
The expression of the *hpc* genes in an *E.coli* K12 strain has already been discussed and used to great advantage. Although the enzyme activities for all the 3,4DHPA catabolic enzymes could be demonstrated in 5K(pJJ210), no growth on 3,4DHPA could be observed. It was assumed that the inability to utilise the substrate was due to the lack of a permease, which resulted in insufficient substrate being present within the cell to support growth.

To enable the replication of the *E.coli* C *hpa* genes in *P.putida* strains a fragment of DNA carrying the genes was subcloned into the shuttle vector pMMB34. Three *Pseudomonas* strains were available for this study, PP001 a CHMS dehydrogenase mutant, PP003 a possible pleiotropic mutant with a similar phenotype to JJ247 and 2440 an *hsdR* mutant that also cannot grow on HPA for unknown reasons. If the *E.coli* C *hpc* genes are introduced into the three *Pseudomonas* strains the 3,4DHPA catabolic enzymes are expressed constitutively. However, the specific activities measured are

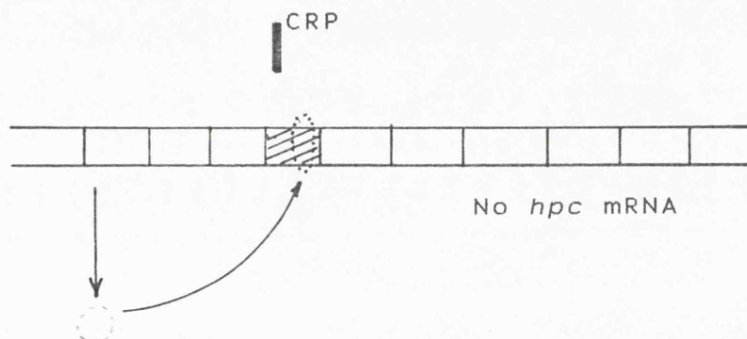
fig,4.1.2.

A model for the regulation of the *hpc* genes.

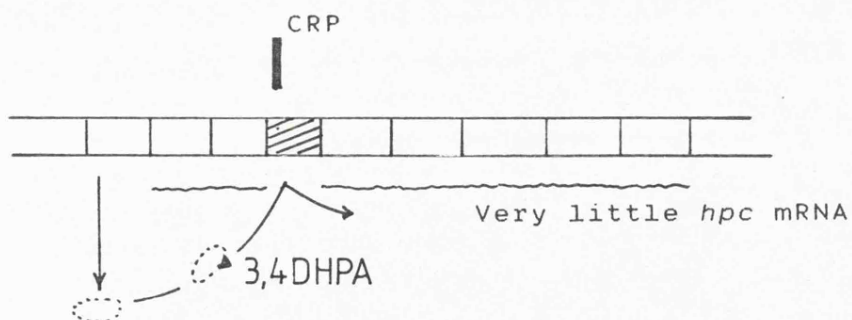
a) No glucose; cAMP high; No 3,4DHPA.



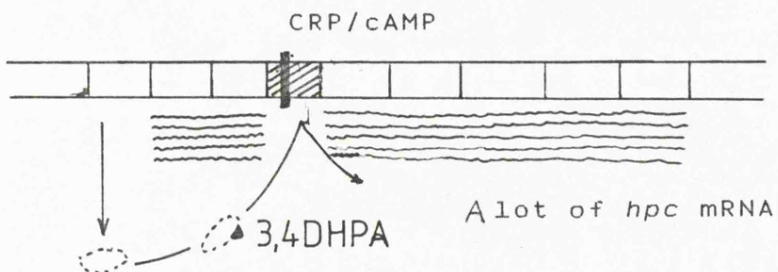
b) Glucose; cAMP low; No 3,4DHPA.



c) Glucose; cAMP low; 3,4DHPA.



d) No glucose; cAMP high; 3,4DHPA.



much lower than those normally observed for *Pseudomonas* and *E.coli* strains induced by growth on HPA. The CHMS dehydrogenase is detectable in extracts produced from PP001 (pJJS801/pJJS200) it is therefore surprising that these strains cannot grow on HPA. It may be that the level of expression from the *E.coli* C *hpcC* gene is not sufficient to support growth. The defects in the other two *Pseudomonas* strains have not been fully characterised so there are many possible reasons for these strains not being able to grow on HPA with the *E.coli* C *hpa* genes present.

FUTURE WORK.

There are several areas discussed in this study which would benefit from further development, to increase the understanding of the 3,4DHPA catabolic pathway:

* The model proposed for regulation provides a basis for further experiments to test the hypothesis: This would require the subcloning of the *hpcR* gene and receptor sites for the gene product onto separate plasmids but of similar origins ^{of replication} to eliminate any copy number problems. Further proof of the involvement of cAMP in the regulation of the pathway could be demonstrated by gel binding studies with the CRP protein as described by Busby (1986).

If the product of *hpcR* could be isolated then some interesting foot-printing experiments could be undertaken to show where and under what conditions it bound to the operator sites of the *hpc* structural genes.

* The sequencing data from both promotor regions could be improved: The interpretation of the data could then be

enhanced by S1 nuclease mapping to determine the mRNA start point or N-terminal amino acid sequencing of the relevant protein.

* The origins of the *E.coli* C *hpc* genes: This would require the screening of other organisms, capable of using 3,4DHPA, for homology with the *E.coli* C *hpc* genes.

* HPA feeds into the 3,4DHPA catabolic pathway: The genes responsible for the uptake and hydroxylation of HPA could be cloned. The problems of whether *hpcR* regulates these genes as well as the *hpc* structural genes could be determined. Also whether there is one or two uptake system(s) for the uptake of HPA and 3,4DHPA.

SUMMARY.

The 3,4DHPA catabolic pathway has been characterised both biochemically and genetically. The intermediates have been isolated and identified. Using these intermediates assay systems have been developed to enable the determination of the gene order. There are seven structural genes which are divided into two operons *hpcBCDEF* and *hpcGH*. Both operons are regulated negatively by the product of *hpcR*. The induction of the 3,4DHPA catabolic enzymes is also affected by the presence of cAMP. This information is shown in the form of a diagram fig,4.1.1, and fig,4.1.2, which also shows the 3,4DHPA catabolic pathway as determined by this study.

The initial cloning and development of the biochemical techniques involved in this study will hopefully provide the basis for further research into the 3,4DHPA catabolic pathway.

REFERENCES:

- Adachi K, Takeda Y, Senoh S and Kita H 1964. Metabolism of *p*-hydroxyphenylacetate in *Pseudomonas ovalis*. *Biochem.Biophys.Acta.* 93:483-493.
- Bagdasarian M, Lutz R, Franklin FCH, Bagdasarian MM Frey J and Timmis KN 1981. Specific-purpose plasmid cloning vectors. II Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* 16:237-247.
- Barbour MG and Bayly RC 1977. Regulation of the meta-cleavage of 4-hydroxyphenylacetate by *Pseudomonas putida*. *Biochem.Biophys.Res.Comm.* 76:565-571.
- Barbour MG 1978. Regulation of synthesis of the 4-hydroxyphenylacetate meta-cleavage pathway. Ph.D thesis. Dept. Microbiology, Monash University Clayton, Victoria, Australia.
- Bateman JN, Speer B, Feduik L and Hartline RA 1986. Naphthalene association and uptake in *Pseudomonas putida*. *J.Bacteriol* 166:155-161.
- Bayly RC, Wigmore GJ and McKenzie DI 1977. Regulation of the enzymes of the meta-cleavage pathway of *Pseudomonas putida*: The regulation is composed of two operons. *J.Gen.Microbiol.* 100:71-79.
- Belfaiza J, Parsot C, Martel A, de la Tour CB, Margarita D, Cohen GN and Saint-Girons I 1986. Evolution in biosynthetic pathways: Two enzymes catalysing

consecutive steps in methionine biosynthesis originate from a common ancestor and possess a similar regulatory region. Proc.Natl.Acad.Sci. 83:867-871.

Birnboim HC and Doyl J 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic.Acids.Res. 7:1513-1523.

Blattner FR, Williams BG, Blechl AE, Denston-Thompson K, Faber HE, Furlong LA, Grunwald DJ, Kieter DO, More DD, Schumm JM, Sheldon EL and Smithies O 1977. Charon phage: Safer derivatives of bacteriophage lambda for DNA cloning. Science 196:161-169.

Bochner BR, Huang HC, Schieven GL and Ames BN 1980. Positive selection for loss of tetracycline resistance. J.Bacteriol. 143:926-933.

Boulnois GJ, Varley JM, Sharpe GS and Franklin FCH 1985. Transposon donor plasmids, based on Col1b-P9, for use in *Pseudomonas putida* and a variety of other Gram negative bacteria. Mol.Gen.Genet. 200:65-67.

Broda P 1979. Plasmids. Oxford: Freeman

Burlingame R and Chapman PJ 1983. Catabolism of Phenylpropionic acid and its 3-Hydroxyderivative by *Escherichia coli*. J.Bacteriol. 155:113-121.

Burnham D 1986. Project student, produced the "HKP" according to the method of Leung et al, 1974.

Busby SJW 1986. Positive regulation and gene expression. In: Regulation of gene expression - 25 years on. 51-78. SGM Symp.39 Eds Booth IR and Higgins CF. Cambridge University Press.

- Campbell JH, Lengyl JA and Langridge J 1973. Evolution of a second gene for β -galactosidase in *Escherichia coli*. Proc.Nat.Acad.Sci. USA 70:1841-1845.
- Chakrabarty AM 1972. Genetic basis of the biodegradation of salicylate in *Pseudomonas*. J.Bacteriol. 112:815-823.
- Chakrabarty AM 1976. Plasmids in *Pseudomonas*. Ann.Rev.Genet. 10:7-30.
- Chow LT, Kahmann R and Kamp D.(1977) Electron microscopy characterization of DNAs of bacteriophage Mu. J.Mol.Biol. 113:591-609
- Clarke L and Carbon J 1976. A colony bank containing synthetic Col E1 hybrid plasmids representing the entire *E.coli* genome. Cell 9:91-99.
- Cooper,R.A. and Skinner,M.A.1980. Catabolism of 3- and 4-hydroxyphenylacetate by the 3,4dihydroxyphenylacetate pathway in *Escherichia coli* C. J.Bacteriol.143:302-306.
- Dagley S 1971. Catabolism of aromatic compounds by micro-organisms. Adv.Micro.Phys. 6:1-45.
- Dagley S 1975. A biochemical approach to some problems of environmental pollution. Essays in biochemistry 11:81-138.
- Dagley S 1978. Microbial catabolism, the carbon cycle and environmental pollution. Die Natur wissenschaften 65:85-95.
- Don RH and Pemberton JM 1985a. Genetic and physical map of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pJP4. J.Bacteriol. 161:466-486.
- Don RH, Weighman AJ, Knackmuss H-J and Timmis KN 1985b.

Transposon mutagenesis and cloning analysis of the pathway for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J.Bacteriol. 161:85-90.

Donnelly MI and Cooper RA 1981. Two succinate semialdehyde dehydrogenases are induced when *Escherichia coli* K12 is grown on γ -aminobutyrate. J.Bacteriol. 145:1425-1427.

Downing RG and Broda P 1979. A cleavage map of the TOL plasmid of *Pseudomonas putida* mt-2. Mol.Gen.Genet. 177:189-191.

Dunn TM Hahn S Ogden S and Schleif RF 1984. An operator at -280 base pairs that is required for repression of the BAD operon promoter. Proc.Nat.Acad.Sci. USA 81:5017-5020.

Englesberg,E. Squires,C and Meronk,F. 1969. The L-arabinose operon in *Escherichia coli* B/r: A genetic demonstration of two functional states of the product of a regulator gene. Proc.Nat.Acad.Sci. USA 62:1100-1107.

Ensley BD, Ratzkin BJ, Osslund TD, Simon MJ, Wackett LP and Gibson DT 1983. Expression of naphthalene oxidation genes in *Escherichia coli*. Nuc.Acid.Res. 11:2927-2941.

Fennewald M, Prevatt W, Meyer R and Shapiro J 1978. Isolation of Inc P-2 plasmid DNA from *Pseudomonas aeruginosa*. Plasmid. 1:164-173.

Feunberg AP and Vogelstein B 1983. A technique for radiolabeling DNA restriction endonuclease

fragments to high specificity. Analytical
Biochemistry 132:6-13.

Franklin FCH, Bagdasarian M, Bagdasarian MM and Timmis KN
1981. Molecular and functional analysis of the
Tol plasmid pWW0 from *Pseudomonas putida* and
cloning of the genes for the entire regulated
aromatic meta cleavage pathway.

Proc.Natl.Acad.Sci. USA 78:7458-7462.

Franklin FCH, Bagdasarian M and Timmis KN 1982.

Manipulation of degradative genes of soil
bacteria. 109-137. In. Microbial degradation of
xenobiotics and recalcitrant compounds. Eds.
Leisinger T, Cook AM, Hutter H and Nuesch J.
Academic Press. London.

Frey J, Bagdasarian M, Feiss D, Franklin FCH and
Deshausses J 1983. Stable cosmid vectors that
enable the introduction of DNA into Gram-negative
bacteria. Gene 24:299-308.

Furukawa K and Miyazaki T 1986 Cloning of a gene
cluster encoding biphenyl and chlorobiphenyl
degradation in *Pseudomonas pseudoalcaligenes*.
J.Bacteriol. 166:392-398.

Garrido-Pertierra A and Cooper RA 1981 Identification and
purification of distinct isomerase and
decarboxylase enzymes involved in the
4-Hydroxyphenylacetate Catabolic Pathway of
Escherichia coli. Eur.J.Biochem 117:581-584.

Gold L, Priknow D, Scheinder T, Shinedling S, Singer BS
and Stormo G 1981. Translational initiation in
prokaryotes. Ann.Rev.Microbiol. 35:365-403.

- Gornall AC, Bardawill CS and David MM 1949. Determination of serum proteins by means of the biuret reaction. *J.Biol.Chem.* 177:751-766.
- Grund AD and Gunsalus IC 1983. Cloning of genes for naphthalene metabolism in *Pseudomonas putida*. *J.Bacteriol.* 154:676-685.
- Grunstein M and Hogness DS 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc.Nat.Acad.Sci USA* 72:8123-8136.
- Guyer,M.S. 1978. $\gamma\delta$ the sequence of F is an insertion sequence. *J.Mol.Biol.* 126:347-365.
- Hacking AJ Linn ECC Regulatory changes in the Fucose system associated with the evolution of a catabolic pathway for propanediol in *Escherichia coli* 1977. *J.Bacteriol.* 130:832-838.
- Harayama S, Lehrbach PR and Timmis KN 1984. Transposon mutagenesis analysis of meta-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. *J.Bacteriol.* 160:251-255.
- Harayama S, Leppik RA, Rekik M, Mermod N, Lehrbach PR, Reineke W and Timmis KN 1986. Gene order of the TOL catabolic plasmid upper pathway operon and oxidation of both toluene and benzyl alcohol by the xylA product. *J.Bacteriol.* 167:455-461.
- Harland WA, Crawford RL, Chapman PJ and Dagley S 1975. Metabolic function and properties of 4-HPA-1-hydroxylase from *Pseudomonas acidovorans*. *J.Bacteriol.* 121:275-285.
- Hegeman GD and Rosenberg SL 1970. The evolution of

bacterial enzyme systems. Ann.Rev.Microbiol.
24:429-462.

Holloway BW and Morgan AF 1986. Genome organisation in
Pseudomonas. Ann.Rev.Microbiol. 40:79-107.

Horowitz NH 1945. On the evolution of biochemical
synthesis. Proc.Nat.Acad.Sci. US 31:153-157.

Horowitz NH 1965. The evolution of biochemical
syntheses-retrospective and prospective. In:
Evolving genes and proteins. Eds. Bryson V and
Vogel HJ. New York: Academic Press. 15-23.

Inouye S, Nakazawa A and Nakazawa T 1981a. Molecular
cloning of the TOL genes *xylB* and *xylE* in
Escherichia coli. J.Bacteriol. 145:1137-1143.

Inouye S, Nakazawa A and Nakazawa T 1981b. Molecular
cloning of gene *xylS* of the TOL plasmid: Evidence
for positive regulation of the *xylDEGF* operon by
xylS. J.Bacteriol. 148:413-318.

Inouye S, Nakazawa A and Nakazawa T 1983. Molecular
cloning of regulatory gene *xylR* and operator-
promotor regions of the *xylABC* and *xylDEGF*
operons of the Tol plasmid. J.Bacteriol.
155:1192-1199.

Inouye S, Yousuke E, Nakazawa A and Nakazawa T 1984a.
Nucleotide sequence surrounding transcription
initiation site of *xylABC* operon on TOL plasmid
of *Pseudomonas putida*. Proc.Natl.Acad.Sci. USA
81:1688-1691.

Inouye S, Nakazawa A and Nakazawa T 1984b. Nucleotide
sequence of the promoter region of the *xylDEGF*
operon on TOL plasmid of *Pseudomonas putida*.

Gene 29:323-330.

Inouye S, Nakazawa A and Nakazawa T 1985. Determination of the transcription initiation site and identification of the protein product of the regulatory gene *xyIR* for *xyI* operons on the TOL plasmid. J.Bacteriol. 163:863-869.

Inouye S 1986. Personal communication. (Geneva 1986 *Pseudomonas* Work Shop).

Jensen RA 1985. Biochemical pathways in prokaryotes can be traced backward through evolutionary time. Mol.Biol.Evol. 2(2):92-108.

Jones,C and Holland,B. 1984. Inactivation of essential division genes, *ftsA*, *ftsZ*, suppresses mutations at *sfiR* a locus mediating division initiation during the SOS response in *Escherichia coli*. E M B O J. 3:1181-1186.

Kemp MB and Hegerman GD 1968. Genetic control of the β -ketoadipate pathway in *Pseudomonas aeruginosa*. J.Bacteriol. 96:1488-1499.

Keil H, Keil S, Pickup RW and Williams PA 1985. Evolutionary conservation of genes coding for meta pathway enzymes within TOL plasmids pWWO and pWW53. J.Bacteriol. 164:887-895.

Kern HW 1981. Microbial degradation of lignosulfonates. 299-370 In: Microbial degradation of xenobiotic and recalcitrant compounds. Eds. Leisinger T, Cook AM, Hutter H and Nuesch J.

Kushner SR 1978. An Improved Method for Transformation of *Escherichia coli* with ColEI-derived plasmids. In: Boyer HB and Nicosia S (eds) Genetic

Engineering. Amsterdam: Elsevier North-Holland,
p17-23.

Lehrbach PR Jeenes DJ and Broda P 1983. Characterisation
by molecular cloning of insertion mutants in the
TOL catabolic functions. *Plasmid* 9:112-125.

Leung P-K, Chapman PJ and Dagley S 1974. Purification
and properties of 4-Hydroxy-2-Ketopimelate
aldolase from *Acinetobacter* J.Bacteriol.
120:168-172.

Lewis EB 1951. Pseudoallelism and gene evolution. Cold
Spring Harbor Symp. Quant. Biol. 16:159-174.

Looman AC and van Knippenberg PH 1986. Effects of GUG
and AUG initiation codons on the expression of
lacZ in *Escherichia coli*. *FEBS* 197:315-320.

Maniatis T, Fritsch EF and Sambrook J. 1982. (eds) In:
Molecular Cloning a Laboratory Manual. Cold
Spring Harbor Laboratory, Cold Spring Harbor, New
York.

Martin M 1985. Visiting research fellow. Isolation of
HPA⁻ mutants.

Mermod N, Lehrbach PR, Reineke W and Timmis KN 1984.
Transcription of the TOL plasmid toluate
catabolic pathway operon of *Pseudomonas putida*
is determined by a pair of coordinately and
positively regulated overlapping promoters.
EMBOJ. 3:2461-2466.

Miller JH 1972 (ed) In: Experiments in Molecular
Genetics. Cold Spring Harbor Laboratory, Cold
Spring Harbor, New York.

Miller JH and Reznikoff WS 1978. (Eds.) The operon.

Cold Spring Harbor Laboratory, New York.

Murray K, Duggleby CJ, Sala-Trepat JM and Williams PA

1972. The metabolism of benzoate and methyl-benzoates via the *meta*-cleavage pathway by *Pseudomonas arvilla* mt-2. Eur.J.Biochem. 28:301-310.

Muller PC and Glasser WG 1983. Lignins' potential

contribution to the feasibility of biomass conversion to ethanol. Biotech.Bio.Eng.Symp. 13:481-494.

Nakai C, Kagamiyama H, Nozaki M, Nakazawa T, Inouye S,

Ebina Y and Nakazawa A 1983. Complete nucleotide sequence of the Metapyrocatechase gene on the TOL plasmid of *Pseudomonas putida* mt-2. J.Biol.Chem. 258:2923-2928.

Nakazawa T and Yokota T 1973. Benzoate metabolism in

Pseudomonas putida (*arvilla*) mt-2: demonstration of two benzoate pathways. J.Bacteriol. 115:262-267.

Ornston LN and Yeh W-K 1982. In: Biodegradation and

detoxification of environmental pollutants. Ch.5 Recurring themes and repeated sequences in metabolic evolution. 105-126. Ed. Chakrabarty AM. CRC Press, Boca Raton, Fla.

Owen DJ, Eggink G, Hauer B, Koc M, McBeth DL, Yang YI and

Shapiro JA 1984. Physical structure, genetic content and expression of the *alkBAC* operon. Mol.Gen.Genet. 197:373-383.

Patel RN, Meagher RB and Ornston LN 1973. Relationships

among enzymes of the β -Ketoacid pathway. II

- Properties of crystalline β -carboxy-*cis,cis*-muconate lactonizing enzymes from *Pseudomonas putida* (arvilla) mt-2. *Biochemistry* 12:3531-3537.
- Reineke W and Knackmuss H-J 1979. Construction of haloaromatic utilising bacteria. *Nature* 227:385-386.
- Ribbons DW and Eaton RW 1982. Chemical transformations of aromatic hydrocarbons that support the growth of microorganisms. 59-84. In: *Biodegradation and detoxification of environmental pollutants*. Ed. Chakrabarty AM. CRC Press, Boca Raton, Fla.
- Ribbons DW and Williams PA 1982. Genetic engineering on microorganisms for chemicals: diversity of genetic and biochemical traits of *Pseudomonas*. 211-232. In: *Genetic engineering of microorganisms for chemicals*. Eds. Hollaender A, DeMoss RD, Kaplan S, Konisky J, Savage D and Wolfe RS. Plenum Publishing Corp., New York.
- Riley M and Anilionis A 1978. Evolution of the bacterial genome. *Ann.Rev.Microbiol.* 32:519-560.
- Roberts I, Mountford R, High NJ, Bitter-Suermann D, Jann K, Timmis K and Boulnois G 1986. Molecular cloning and analysis of genes for production of K5, K7, K12 and K92 capsular polysaccharides in *Escherichia coli*. *J.Bacteriol.* 168:1228-1233.
- Rosenberg M and Court D 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. *Ann.Rev.Genet.* 13:319-353.
- Sala-Trepat JM and Evans WC 1971. The *meta* cleavage of catechol by *Azotobacter* species using the

4-oxalocronate pathway. Eur.J.Biochem.

20:400-413.

Sanger F, Nicklen S and Coulson AR 1977. DNA sequencing with chain-termination inhibitors.

Proc.Nat.Acad.Sci. USA 74:5463-5467.

Schell MA and Wender PE 1986. Identification of the *nahR* gene product and nucleotide sequences required for its activation of the *sal* operon.

J.Bacteriol. 166:9-14.

Shanley MS, Neidle EL, Ehrlich Parales R and Ornston LN 1986. Cloning and expression of *Acinetobacter calaceticus* *catBCDE* genes in *Pseudomonas putida* and *Escherichia coli*. J.Bacteriol. 165:557-563.

Shields MS, Hooper SW and Sayler GS 1985. Plasmid-mediated mineralisation of 4-chlorobiphenyl.

J.Bacteriol. 163:882-889.

Sinclair MI, Maxwell PC, Lyon BR and Holloway BW 1986.

Chromosomal location of TOL plasmid DNA in *Pseudomonas putida*. J.Bacteriol. 168:1302-1308.

Skinner MA 1981. Aromatic catabolism by *Escherichia coli*.

Ph.D. Thesis, Leicester University, England.

Skinner M A and Cooper R A 1982. An *Escherichia coli* mutant defective in the NAD-Dependant Succinate Semialdehyde Dehydrogenase. Arch.Microbiol 132:270-275.

Soberon X, Covarubias L and Bolivar F 1980. Construction and characterisation of new cloning vehicles. IV Deletion derivatives of pBR322 and pBR325. Gene 9:287-305.

Soltes EJ and Lin S C-H 1983. Vehicular fuels and

- oxychemicals from biomass thermochemical tars.
 Biotech.Bio.Eng.Symp.13:53-64.
- Southern EM 1980. Gel electrophoresis of restriction fragments. Methods in Enzymol. 68:152-176.
- Spornins V L, Chapman P J and Dagley S 1974. Bacterial degradation of 4-Hydroxyphenylacetic acid and Homoproteo-catechuic acid. J.Bacteriol 120:159-167.
- Spoelstra SF 1978. Degradation of tyrosine in anaerobically stored piggery wastes and in piggery wastes and pig faeces. Appl.Environ.Microbiol. 36:631-638.
- Spooner RA, Lindsay K and Franklin FCH 1986. Genetic and functional sequence analysis of the *xylR* and *xylS* regulatory genes of the T01 plasmid pWW0. J.Gen.Microbiol. 132:1347-1358.
- Stanier RY 1947. Simultaneous adaptation: a new technique for the study of metabolic pathways. J.Bacteriol. 54:339:348.
- Stanier RY, Palleroni NJ and Doudoroff M 1966. The aerobic *Pseudomonas* a taxonomic study. J.Gen.Microbiol. 43:159-60.
- Stanier RY and Ornston LN 1973. β -Ketoadipate pathway. Adv.Microbial.Phys. 9:89-151.
- Steiert JG and Crawford RL 1985. Microbial degradation of chlorinated phenols. Trends in biotechnology 3:300-305.
- Stoker NG, Fairweather NF and Spratt BG 1982. Versatile Low-copy-number plasmid vectors for cloning in *Escherichia coli*. Gene 18:335-341.

- Takeda Y 1966. Crystallization and some properties of dioxygenases related to tyrosine metabolism in bacteria. In: Biological and chemical aspects of oxygenases. 373-377. Eds. Bloch K and Hayaishi O. Maruzen Company, Ltd., Tokyo.
- Wallace L, Peterson A, McCarthy A, Raeder U, Ramsey L, MacDonald M, Haylock R and Broda P 1983. The problem of lignin biodegradation. Biochem. Soc. Symp. 48:87-95.
- Whited GM, McCombie WR, Kwart LD and Gibson DT 1986. Identification of cis-diols as intermediates in the oxidation of aromatic acids by a strain of *Pseudomonas putida* that contains a TOL plasmid. J. Bacteriol. 166:1028-1039.
- Williams PA and Murray K 1974. Metabolism of benzoate and methyl benzoate by *Pseudomonas putida* (arvilla) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. 120:416-423.
- Williams PA and Jeenes DJ 1981. Origins of catabolic plasmids. Microbiology-1981. 144-147.
- Wong CL and Dunn NW 1974. Transmissible plasmid coding for the degradation of benzoate and *m*-toluate in *Pseudomonas arvilla* mt-2. Genet. Res. 23:227-232.
- Worsey MJ and Williams PA 1975. Metabolism of toluene and the xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. 124:7-13.
- Worsey MJ, Franklin FCH and Williams PA 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWW0) from *Pseudomonas putida*

mt-2. J.Bacteriol. 134:757-764.

Yen K-M and Gunsalus IC 1985. Regulation of naphthalene
catabolic genes of plasmid NAH7. J.Bacteriol.
162:1008-1013.

Appendix 1

From Skinner 1981 (Ph.D Thesis).

A Model for the Regulation of the HPA Catabolic enzymes.

4.5.1 Nature of the Tn5-induced mutation in CT107.

The Tn5-induced mutation in CT107 (sad, HPA-noninducible) resulted in loss of all the HPA catabolic activities and of the HPA uptake activity. The mutation caused the same results in the SSA dehydrogenase positive background of E. coli C. The E. coli C derivative (CT110: hpa::Tn5) was unable to grow on HPA or 3,4-dHPA but reverted on HPA to grow on 3,4-dHPA as well. The revertant (CT111) also regained

the 3,4-dHPA constitutive phenotype present in the parental strain (CT101: 3,4-dHPA constitutive, sad) of the original mutant (CT107). Various explanations for such a wide-reaching pleiotropic phenotype may be considered:

- 1) The Tn5 insertion in an HPA gene causes polar inactivation of the other genes,
- 2) The insertion inactivates the substrate uptake system thereby preventing induction of the catabolic enzymes,
- 3) The insertion causes a change in a regulatory gene which prevents induction of the HPA catabolic enzymes and the permease(s).

The first of these possibilities is rendered unlikely by the fact that the HPA-hydroxylase obviously exists in an operon which is distinct from the other operon(s) for the 3,4-dHPA catabolic enzymes. The second possibility requires that the Tn5 insertion inactivates one uptake system that takes up both HPA and 3,4-dHPA.

The second possibility, that the pleiotropic lesion in CT107 was caused by the insertion of Tn5 in a gene encoding a common permease, seems ^{un-}likely in view of the fact that no activity was detectable, for any of the HPA catabolic enzymes, in CT107 (not even at the normal uninduced levels). It is difficult to see how a permease mutation could result in the reduction of the basal enzyme activities that are normally

seen in wild-type cells not exposed to HPA.

As the HPA catabolic sequence consists of at least 2 units (not including the SSA dehydrogenase), the regulatory gene affected cannot be an initiator/promoter/operator sequence, as each operon will possess such a sequence. The affected gene must therefore code for a trans-acting regulatory protein. Insertion of Tn5 into a gene is likely to cause an extensive change (by chain termination) in the structure of the gene product. It is unlikely, therefore, that the insertion would cause the relatively specific change needed to bring about a noninducible phenotype in a negatively controlled system (the insertion would have to prevent binding of the inducer whilst not affecting binding of the repressor to the operator). A more likely situation would be where the transposon inserts in a gene coding for a positive regulator. Total loss of regulator function would result in a noninducible phenotype. Thus the most likely explanation for the phenotype of CT107 is that Tn5 has inserted in the gene coding for a regulator protein (which probably acts as a positive regulator) that controls the HPA uptake/hydroxylase operon and the 3,4-dHPA catabolic operon(s).

In proposing a model for the regulation of synthesis of the HPA catabolic enzymes, three pieces of information have to be considered, namely:

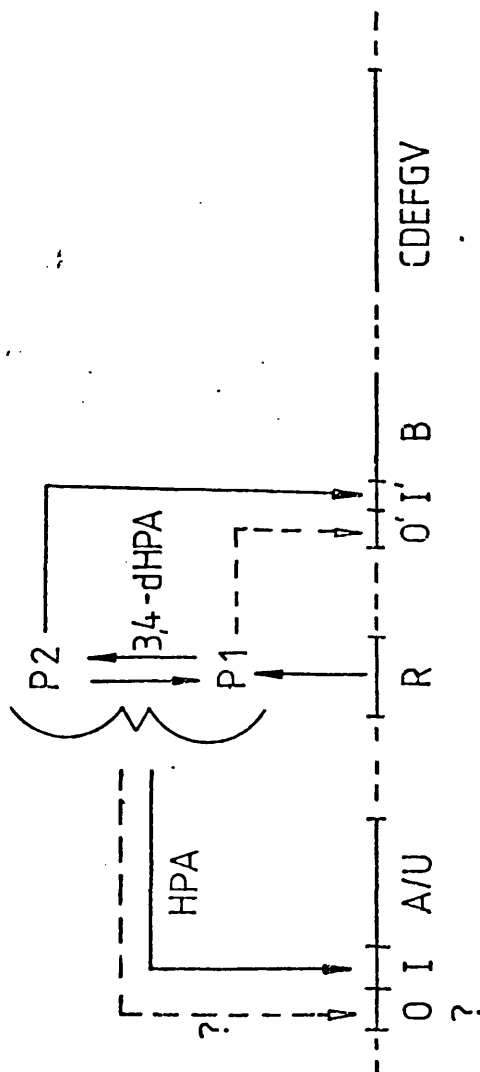
- 1) the mutation in CT107 (non-inducible for all the HPA catabolic enzymes), caused by the insertion of Tn5, was most likely caused by insertional inactivation of a positive regulatory protein (similar to araC⁻) rather than by insertional modification of a negative regulatory protein (to give a lacI^S-type molecule).
- 2) the fact, that the non-inducible mutation in CT107 overcame the 3,4-dHPA dioxygenase constitutive mutation in the parental strain, is most satisfactorily explained by the insertional inactivation of a positive regulatory protein (which has been modified in the parental strain to cause constitutive expression, as does araC^C);
- 3) the apparent dominance of the wild-type regulatory allele over the constitutive allele in the partial diploid experiment, a result which indicates that the expression of the 3,4-dHPA dioxygenase is negatively controlled.

Two lines of evidence, therefore, point to positive regulation of the HPA hydroxylase operon and of the 3,4-dHPA catabolic enzymes, but the other line points to negative regulation of the 3,4-dHPA catabolic enzymes. Superficially, these results are irreconcilable but a similar situation was observed for the arabinose operon (Englesberg, 1971). Mutants lacking, or defective in, the gene encoding the regulator

(araC⁻) were pleiotropically non-inducible . The araC⁻ mutants were recessive to the wild-type, inducible allele (araC⁺) and to the allele which caused constitutive expression (araC^C). These results all indicated that the araC product was a positive regulator. The positive regulation model predicts that the araC constitutive allele would be dominant over the wild-type allele. However, the inverse relationship proved to be true, a characteristic of negative control. Thus a dual control system was proposed where araC produces a protein, P1, that acts as a repressor, binding to the operator (araO) to prevent expression of the operon. In the presence of inducer (L-arabinose), P1 undergoes a conformational change to P2 and is released from the operator. This relief of repression, however, does not, in itself, allow expression of the operon. For expression to occur, the P2/inducer complex must also bind to the initiator (araI) to act as a positive regulator (activator). AraC constitutive mutant genes produce mutant regulators (P3,P4...Pn) that are unable to bind to the operator (and so are unable to act as repressors) but are able to bind to the initiator, activating transcription, even in the absence of inducer. In partial diploids of the type araC^C/araC⁺, P1 produced by the wild-type allele binds to the operator (in the absence of inducer), repressing expression of the operon. In the presence of inducer, repression ceases and so the partial diploid is inducible for expression of the operon; thus the constitutive allele is recessive to the wild-type allele.

The arabinose model, with suitable modifications to allow for induction of the HPA hydroxylase operon by HPA and induction of the 3,4-dHPA catabolic enzymes by 3,4-dHPA, is consistent with the results obtained for regulation of synthesis of the HPA catabolic enzymes. In the HPA model (Fig. 21), the hpaR gene produces a protein, P1, that binds at the operator(s) controlling the expression of hpaCDEFGV (which encode the 3,4-dHPA permease and catabolic enzymes) so repressing the expression of those genes. In the presence of 3,4-dHPA, P1 changes conformation to P2 and is released from the operator. The P2/3,4-dHPA complex then binds at the initiator(s), which control the expression of hpaCDEFGV, thus activating transcription of the operon(s). The same regulatory protein, in the presence of HPA, binds to the initiator of the HPA hydroxylase operon to activate expression of the HPA hydroxylase and uptake genes. It is not possible to say which conformation of the regulatory protein (P1, P2 or some other conformation) is responsible for forming a complex with HPA, nor is it possible to say whether regulation of the HPA hydroxylase operon also involves a negative regulation aspect. The gene arrangement depicted in Fig. 21 is not meant to represent the actual gene order and no attempt has been made to indicate how many operons are involved (except that there must be two or more, there being two blocks of coincident, if not coordinate, regulation).

Fig.21. A model for the regulation of expression of the hpa genes in E. coli.



KEY:

hpa genes: HPA: hydroxylase (A); 3,4-dHPA: dioxxygenase (B); CHMS: dehydrogenase (C); CHM isomerase (D); CHM decarboxylase (E); HHDD: hydratase (F); HKP: aldolase (G); HPA permease (U); 3,4-dHPA permease (V); regulator (R); operator (O) and initiator (I).
P1 represents the repressor state of the regulator, P2 represents the activator state.

Abstract.

Escherichia coli C can utilise 3,4-dihydroxyphenylacetate as a carbon and energy source for growth. The biochemical and genetic characterisation of the chromosomally encoded pathway involved is described in this thesis.

The structural genes have been cloned and found to lie in two operons, *hpcBCDEF* and *hpcGH*, both of which were negatively controlled by the regulatory gene *hpcR*. There also appeared to be an additional level of control acting in a positive manner, mediated by cAMP.

Various subclones isolated in this analysis were useful in the production of pathway intermediates which were identified by a variety of means. The use of the pathway intermediates in the biochemical characterisation of the 3,4DHPA catabolic pathway has lead to the identification of a novel step. The use of both biochemical and genetic techniques in the analysis of the pathway has enabled a greater understanding of the interacting mechanisms of the pathway.

