NUCLEOTIDE SEQUENCE DETERMINATION, SUBCLONING, EXPRESSION AND CHARACTERIZATION OF THE xylLT REGION OF THE

Pseudomonas putida TOL PLASMID pDK1

DISSERTATION

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements For the Degree of

DOCTOR OF PHILOSOPHY

BY

Ronald F. Baker, B.G.S., B.S.
Denton, Texas
December, 1992
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The lower operon of TOL plasmids encodes enzymes for conversion of a family of aromatic acids to catechols and subsequently to Kreb's cycle intermediates via the meta-cleavage pathway. Initial attack, mediated by toluate-1,2-dioxygenase (TO, xylXYZ), gives carboxyl substituted diols which are in turn acted upon by 1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase (DHCDH, xylL) to give the corresponding catechols. These serve as ring-cleavage substrates for catechol-2,3-dioxygenase (C230, xylE).

The complete nucleotide sequence of the region encoding the DHCDH function of the pDK1 lower operon was determined. DNA analysis has shown the presence of two open reading frames, one gene consisting of 777 nucleotides encoding a polypeptide of 27.85 kDa and another gene of 303 nucleotides encoding a polypeptide of 11.13 kDa.

The results of enzymatic expression studies suggest that DHCDH activity is associated only with xylL. However although the addition of xylT cell-free extracts to xylL
cell-free extracts does not produce an increase in DHCDH activity, subclones carrying both xylL and xylT exhibit 300-400% more DHCDH activity than subclones carrying only xylL. Correspondingly the xylTE dioxygenase shows an 8-fold increase in activity over that of the xyle dioxygenase. Furthermore, the xylT gene exerts its regulatory function at the transcriptional (DNA) level rather than at the protein level. This is in support of DNA sequence analysis that shows the presence of two large A/T rich intergenic regions of 115 bp between the xylZ and the xylL genes and 139 bp between the xylL and the xylT genes. Both these intergenic sequences contain palindromic sequences that form stem and loop structures. Finally, the xylT gene product is a ferrodoxin protein in nature. The mechanism by which the xylT gene exerts its regulatory effects on the xylL and the xyle genes if any, has yet to be determined.
I like to express my thanks and gratitude to my major professor, Dr. Robert Benjamin for his support and advice in both my research and career decisions. I would also like to thank Dr. Daniel Kunz for his advice on metabolic pathways and the use of his laboratory for the many enzymatic assays carried out during the course of this study. Many thanks to Dr. Gerard O'Donovan for both scientific input and his support and advice regarding my future career decisions. My thanks also go to Dr. Art Goven and Dr. John Knesek for their time, effort and patience in serving as committee members. The help of my fellow students in Dr. Robert Benjamin's lab shall never be forgotten.

Most of all, I would like to thank my parents, Mr. and Mrs. Baker, my wife Elizabeth and my sister Audrey together with my brother Brian and his family for their support and unfailing belief in me.
# TABLE OF CONTENTS

LIST OF TABLES ................................................................. vii

LIST OF ILLUSTRATIONS ..................................................... viii

LIST OF ABBREVIATIONS ..................................................... xi

CHAPTERS

I. INTRODUCTION ............................................................. 1

TOL Plasmids
Organization of TOL Pathway Genes
The Ortho-cleavage Pathway
Regulation of TOL Pathway Genes
Structural and Physical Properties of TOL Plasmids
The xylL Region Encoding 1,2-dihydroxycyclohexa-3,5-diene Carboxylate Dehydrogenase
Significance of Research Project

II. MATERIALS AND METHODS .............................................. 41

Bacterial Strains and Plasmids
Media and Culture Conditions
Media for Enzyme Expression in the Production of dihydrodiols
Long Term Storage of Bacterial Strains
Tanaka and Weisblum Large Scale Plasmid Isolation from E. coli Strains
Digesting DNA with Restriction Endonucleases
Bacterial Alkaline Phosphatase Treatment of Vector DNA
Phenol Extraction of Nucleic Acids
Qualitative and Quantitative Identification of DNA
Ligation of DNA for Cloning into Plasmids and Phages
Preparation of Competent Cells
Transformation of E. coli DH5αF' and DH5α with recombinant pUC and M13 molecules
Screening of Recombinant Plasmids and Phage
Rapid Analytical Scale Isolation of Plasmid DNA by the Alkaline Lysis Technique
Agarose Minigel Electrophoresis
Preparative Scale Isolation of Cloned DNA Fragments (Restriction Endonuclease Digestion of Recombinant Plasmids)
Vertical Agarose Gel Electrophoresis
Detection of DNA by Ultraviolet Shadowing
Recovery of DNA from Agarose Gels by Electroelution
Non-denaturing Polyacrylamide Gel Electrophoresis
Polyacrylamide Sequencing Gels
Preparation and Electrophoresis of Polyacrylamide Sequencing Gels
Autoradiography of Sequencing Gels
Gel Photography
Dideoxy Sequencing (Sanger Method)
Preparation of Single Stranded Recombinant M13 Phage
Sanger Dideoxyribonucleotide DNA Sequencing of Single Stranded templates
Sequencing Double-Stranded DNA Templates by the Dideoxyribonucleotide Method
Rapid Preparation of Double-Stranded DNA Sequencing Templates
Preparation of Dihydrodiols
Preparation of Cell-Free Extracts
Modified Lowry Procedure for Determination of Protein Concentration
Enzyme Assays

III. RESULTS........................................... 136

Nucleotide Sequence Determination
DNA Sequence Analysis
Subcloning of the xylLT Region
Verification of Subclones
Enzyme Assays

IV. DISCUSSION.........................................................207

BIBLIOGRAPHY..........................................................229
# LIST OF TABLES

1. Plasmids that contribute to the metabolic diversity of the Pseudomonads 4
2. Growth characteristics of some *Pseudomonas putida* HS1 strains 26
3. Comparison of restriction endonuclease maps of three TOL plasmids pWW0, pWW53 29
4. Dihydroxycyclohexadiene substituted products resulting from the oxidation of aromatic compounds 36
5. Utilization of the dihydrodiol products of the xylLT gene 37
6. Comparison of the xylL and the xylT structural genes 151
7. Comparison of the putative ribosome binding sites of the thirteen structural genes of the pDK1 lower TOL operon 152
8. Comparison of intergenic regions of the TOL lower operon 162
9. DHCDH activities of selected xylLT region subclones 205
10. C230 activities of selected xylLTE region subclones 206
LIST OF ILLUSTRATIONS

1. Genetic map of the *Pseudomonas putida* HS1 TOL plasmid 8
2. Metabolic pathway for the degradation of aromatics by *Pseudomonas putida* HS1 carrying the TOL plasmid pDK1 10
3. Bifurcation of the TOL pathway at catechol 12
4. Regulation of the *Pseudomonas putida* HS1 TOL plasmid 19
5. Comparison of the organization of the TOL regions of three TOL plasmids 23
6. Mode of action of the *xylD* and the *xylLT* genes 33
7. Til270 ultracentrifuge tube after a 48 hour spin at 43467 x g 50
8. Replica plate used in the screening of recombinant plasmids 67
9. Schematic representation of the assembly of a gel cassette 79
10. Schematic representation of a vertical agarose gel set for electrophoresis 81
11. Schematic representation of the ultraviolet radiation shadowing technique 84
12. Isolation of DNA from agarose gel slices by the electroelution technique 87
13. Overall strategy for the preparation of single stranded template 111
14. Agarose gel of single strand M13 template DNA 113
15. Summary of sequencing reaction protocol 118
16. Sample standard curve obtained using the Lowry method for the determination of protein concentration

17. Enzymatic reactions for the xylE and the xylLT gene products

18. Structural map of the pDKR1 cointegrate plasmid

19. Sequencing strategy utilized for the determination of the DNA sequence of the xylLT region

20. Autoradiograph of a 6% polyacrylamide sequencing gel

21. Nucleotide sequence and corresponding amino acid sequence for the xylL gene of the Pseudomonas putida HS1 pDK1 TOL plasmid

22. Nucleotide sequence and corresponding amino acid sequence for the xylT gene of the Pseudomonas putida HS1 pDK1 TOL plasmid

23. Restriction endonuclease map of the 'upper' and 'lower' operons together with the regulatory xylRS genes of the Pseudomonas putida pDK1 TOL plasmid

24. Comparison of the nucleotide sequences of the xylL genes

25. Comparison of the amino acid sequences encoded by the xylL genes

26. Comparison of the nucleotide sequences of the xylT genes

27. Amino acid sequence comparison of the xylT genes

28. DNA analysis of intergenic regions of the xylLT region from the pDK1 TOL plasmid

29. Comparison of the nucleotide sequence of stem and loop structures from the pDK1 and pWWO TOL plasmids

30. DNA analysis of operator/promoter regions
31. Comparison of nucleotide sequence of the upstream activator sequences

32. Construction of subclones

33. Construction of various subclones prepared for the characterization of the xylLTE region

34. Verification of the pBK291 (xylT) and pBK591 (xyle) subclones

35. Verification of the pBK191 (xylL) and pBK391 (xylLT) subclones

36. DHCDH activity towards benzoate/m-toluate dihydroxy diols (XylT CFE)

37. DHCDH activity towards p-toluate dihydroxy diol (XylTE CFE and XylL CFE)

38. DHCDH activity monitored towards m-toluate diol (XylLT CFE)

39. DHCDH activity towards benzoate dihydroxy diol (XylTE CFE and XylL CFE)

40. C230 activity monitored towards catechol (XylTE CFE)

41. C230 activity monitored towards catechol (XylE CFE)

42. C230 activity monitored towards catechol (XylLTE)

43. C230 activity monitored towards catechol (XylT CFE and XylE CFE)

44. Comparison of biodegradative pathways from the pDK1 TOL plasmid and the NAH7 plasmid

45. Phylogenetic relationships between various chloroplast-like ferredoxin genes and the xylT and the nahT genes from the pWWO, pDK1 TOL plasmids and the NAH7 plasmid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>Amp50</td>
<td>ampicillin (50 milligrams per milliliter)</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BAP</td>
<td>bacterial alkaline phosphatase</td>
</tr>
<tr>
<td>C</td>
<td>Centigrade or cytosine</td>
</tr>
<tr>
<td>Cbr</td>
<td>carbenicillin resistance</td>
</tr>
<tr>
<td>cc</td>
<td>cubic centimeter</td>
</tr>
<tr>
<td>CFE</td>
<td>cell-free extract</td>
</tr>
<tr>
<td>C23O</td>
<td>catechol-2,3-dioxygenase</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DHCDH</td>
<td>1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>kbp</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>Knr</td>
<td>kanamycin resistance</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>m-tol</td>
<td>meta-toluate</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PN</td>
<td>phosphate/nitrate</td>
</tr>
<tr>
<td>p-tol</td>
<td>para-toluate</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>Tcr</td>
<td>tetracycline resistance</td>
</tr>
<tr>
<td>TG</td>
<td>toluate-1,2-dioxygenase</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>U</td>
<td>uracil</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>vol/vol</td>
<td>volume to volume ratio</td>
</tr>
<tr>
<td>wt/vol</td>
<td>weight to volume ratio</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

The genus *Pseudomonas* includes a wide variety of organisms that commonly occur in the soil, water and other natural environments (Haas 1983). The Pseudomonads are Gram-negative, slightly curved rods with polar flagella that are present singly or in large tufts. These organisms for the most part do not pose any threat to healthy individuals. However, some species, such as *Pseudomonas aeruginosa*, may act as opportunistic pathogens. Although the pseudomonads are classified as aerobic organisms, under anaerobic conditions (for example, in water logged soils) some species are capable of anaerobic respiration, substituting nitrate for oxygen as the terminal electron acceptor.

Members of the genus *Pseudomonas* are metabolically diverse organisms, known for their ability to utilize a wide variety of compounds as carbon and energy sources (Chakrabarty 1976). An example of this versatility can be seen in *P. cepacia*, which can utilize any of well over 100 different compounds as a sole energy source (Haas 1983). The enzymes for the catabolism of these compounds may be encoded by genes located on either the bacterial chromosome or on
extrachromosomal genetic elements such as transmissible plasmids (Williams and Worsey 1976). The presence of such biodegradative plasmids was first observed in the pseudomonads in 1972 (Rheinwald et al. 1973). Since that time catabolic plasmids carried by the pseudomonads have been shown to degrade many substituted and unsubstituted aromatic hydrocarbons to metabolically useful products. In addition, these catabolic plasmids may bestow upon the host resistance to antibiotics and heavy metal compounds. Some non-transmissible biodegradative plasmids have also been described whereby the plasmids are transferred by the use of fertility factors (Mylorie et al. 1977). The versatility of these biodegradative plasmids may be due to their possession of mutator genes (Mylorie et al. 1977). This could help the organism adapt to foreign environments and utilize a wide spectrum of xenobiotic nutrient sources (Sakaguchi and Okanishi 1980). Transmissible plasmids involved in the catabolism of camphor (CAM) (Rheinwald et al. 1973), salicylate (SAL) (Chakrabarty 1972), octane (OCT) (Chakrabarty et al. 1973), naphthalene (NAH) (Dunn and Gunsalus 1973), benzoates, toluates (TOL) (Williams and Murray 1974; Wong and Dunn 1974) and toluenes and xylenes (TOL) (Worsey and Williams 1975) have been described in the literature. An overview of the substrate diversity of the
pathways encoded by these catabolic plasmids can be seen in Table 1. Many of these biodegradative plasmids are related to one another, having common biochemical activities as well as nucleotide sequence homology. Examples are the NAH, the TOL and the SAL plasmids. The catabolic genes of each of these three plasmids assigned to the Incp-9 (Heinaru et al. 1978) are arranged into two distinct operons (designated the upper and lower operons). These pathways share many similar enzymes and metabolic intermediates. For example, catechol is an intermediate common to all three biodegradative pathways and the three pathways are very similar beyond this point. Restriction endonuclease mapping and hybridization mapping studies have revealed a close relationship between these three biodegradative plasmids at the level of DNA sequence (Heinaru et al. 1978).

Although related catabolic pathways are often found encoded by genes on the bacterial chromosome, chromosomally specified pathways act on a narrower spectrum of metabolites as compared to plasmid-specified metabolic pathways. In some cases pathways are jointly encoded by a combination of plasmid and chromosomally located genes. An example of this is seen for the OCT plasmid, which encodes the enzymes responsible for the conversion of $n$-octane (C-6 to C-10 alkane) to octanealdehyde. However, the subsequent oxidation
Table 1. Plasmids that contribute to the metabolic diversity of the Pseudomonads (Adapted from Haas 1983).

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>Growth Substrate</th>
<th>Principal Metabolic Product(s)</th>
<th>Original Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOL (pWWG)</td>
<td>toluene, m-xylene, p-xylene</td>
<td>pyruvate+acetaldehyde, pyruvate+propional (meta cleavage)</td>
<td>P. putida</td>
</tr>
<tr>
<td>XYL-K</td>
<td>xylene, toluene.</td>
<td>same as above (meta cleavage)</td>
<td>P. putida</td>
</tr>
<tr>
<td>DWW50-1</td>
<td>naphthalene</td>
<td>salicylate\rightarrow succinate, acetyl CoA (ortho cleavage)</td>
<td>P. putida</td>
</tr>
<tr>
<td>NAH7</td>
<td>naphthalene</td>
<td>salicylate\rightarrow pyruvate, acetaldehyde (meta cleavage)</td>
<td>P. putida</td>
</tr>
<tr>
<td>SAL: pHWD1</td>
<td>salicylate</td>
<td>pyruvate+acetaldehyde (meta cleavage)</td>
<td>P. putida</td>
</tr>
<tr>
<td>CAM</td>
<td>camphor</td>
<td>isobutyrate</td>
<td>P. putida</td>
</tr>
<tr>
<td>OCT</td>
<td>(\pi)-octane (C6-C10 alkane)</td>
<td>alkane aldehyde</td>
<td>P. putida</td>
</tr>
</tbody>
</table>
steps are carried out by the gene products encoded by the *P. putida* chromosomal genes (Fennewald et al. 1979).

**TOL plasmids**

The work described in this dissertation deals with the TOL plasmid. *P. putida* was originally isolated from decaying materials in 1886 and is a natural host of the TOL and other degradative plasmids (Stanier et al. 1966). The majority of these TOL plasmids fall within a size range of approximately 50 to 300 kilobase pairs. The pseudomonads carrying these plasmids have been isolated from a variety of geographically remote locations (four continents) (Williams et al. 1988). Strains with the TOL plasmid were isolated from soil samples by selective enrichment on media containing toluene or meta-xylene. Many TOL plasmids have been isolated, but the locations and physical organizations of the catabolic genes have been extensively mapped for only three (Osborne et al. 1988). These include the archetype pWW0 plasmid (Worsey and Williams 1975; Nakazawa et al. 1978), pDK1 plasmid (Shaw and Williams 1988) and the pWW53 plasmid (Keil et al. 1987).

**Organization of TOL pathway genes**

The genes present on the TOL plasmids are organized into two operons. The 'upper pathway' operon consists of the
structural genes and is separated from the meta-cleavage operon by the xylRS regulatory genes. The meta-cleavage operon consists of the xylXYZLTEGKJQKI genes (Kunz and Chapman 1981; Williams et al. 1988; Shaw and Williams 1988). The physical map of the structural genes of the P. putida TOL pDK1 plasmid can be seen in fig. 1.

The 'upper pathway' operon encodes the enzymes responsible for the sequential oxidation of a methyl substituent of various aromatic substrates to a carboxylate group. This involves a three step process and the enzymes necessary are encoded by the genes xylCMABN. xylMA encodes the enzyme xylene oxidase, a two component monoxygenase. Xylene oxidase is responsible for the conversion of toluene to benzyl alcohol. Benzyl alcohol is oxidized to benzaldehyde by the action of benzylalcohol dehydrogenase, encoded by the xylB gene. The benzaldehyde is further metabolized to benzoic acid by the action of benzaldehyde dehydrogenase, encoded by the the xylC gene. The function of the xylN gene product has yet to be elucidated. An overview of the 'upper' and 'lower' pathways can be seen in Fig. 2.

The genes specifying the 'lower pathway' (meta-cleavage pathway), (Fig. 3) are physically separated from the genes of the first operon, and their expression is independently regulated. This operon encodes the enzymes for the
conversion of the carboxylic acids to central metabolites. The xylXYZ gene products form toluate-1,2-dioxygenase, which metabolizes benzoic acid to 1,2-dihydroxycyclohexa-3,5-diene carboxylic acid. The 1,2-dihydroxycyclohexa-3,5-diene carboxylic acid is converted to catechol by the xylL encoded enzyme 1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase (DHCDH). The catechols are then oxidatively cleaved to their ring fission products via one of two metabolic pathways. The plasmid-encoded meta-cleavage utilizes the xylE gene product (catechol-2,3-dioxygenase), while the ortho-cleavage pathway on the chromosome cleaves catechol by the action of a catechol-1,2-dioxygenase. The product of the catechol-2,3-dioxygenase on catechol is the ring fission product 2-hydroxymuconic semialdehyde. The ring fission products are in turn further metabolized via one of two branches of the pathway, the hydrolytic branch or the oxalocrotonate branch. The ring fission products of ortho- and para-substituted toluates and benzoates are metabolized via the more energetically favorable oxalocrotonate branch, whilst the meta-substituted toluates and xylenes are metabolized via the hydrolytic branch (Fig. 3). The oxalocrotonate and the hydrolytic branches converge to a common metabolite, 2-oxopent-4-enoate. The oxalocrotonate branch enzymes include the xylG-encoded enzyme,
Fig. 1. Structural genetic map of the *Pseudomonas putida* HS1 TOL plasmid. Two promoter and two operator regions are located at the start of the two upper and lower (meta) operons. The direction of transcription for the two operons is in opposite directions.
**PLASMID-ENCODED**

\[ \begin{array}{c}
\text{KULAM} \\
\text{Toluene} \\
\text{Hydroxylyase} \\
(TH)
\end{array} \]

\[ \begin{array}{c}
\text{KULB} \\
\text{Benzylic alcohol} \\
\text{Dehydrogenase} \\
(BADH)
\end{array} \]

\[ \begin{array}{c}
\text{KULC} \\
\text{Benzoaldehyde} \\
\text{Dehydrogenase} \\
(BZDH)
\end{array} \]

\[ \begin{array}{c}
\text{KULXYZ} \\
\text{Toluate-1,2-dioxynogenase} \\
(TO)
\end{array} \]

\[ \begin{array}{c}
\text{XUL} \\
1,2\text{-dihydroxycyclohexa-3,5-diene carboxylate} \\
\text{dehydrogenase} \\
(DHCDDH)
\end{array} \]

**CHROMOSOME-ENCODED**

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>toluene</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>m-xylene</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>p-xylene</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>3-ethyl toluene</td>
<td>C₂H₅</td>
<td>H</td>
</tr>
<tr>
<td>pseudocumene</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
</tbody>
</table>

\[ \begin{array}{c}
\text{KULD} \\
\text{Diot Dehydrogenase} \\
(DDH)
\end{array} \]

\[ \begin{array}{c}
\text{KUL} \\
\text{Toluene} \\
\text{Hydroxylyase} \\
(TH)
\end{array} \]
Fig. 2. Metabolic pathway of the *Pseudomonas putida* HS1 TOL plasmid pDK1. The 'upper' pathway genes consist of the *xylCMABN* and the 'lower' pathway genes consist of the *xylXYZLTEGFJQKIH*. At catechol the pathway bifurcates into the *ortho* and *meta* pathways. This metabolic pathway is identical to that found in the pWWO TOL plasmid (Adapted from Harayama et al. 1987).
Fig. 3. Bifurcation of the TOL pathway at catechol.
Utilization of catechol by catechol-2,3-dioxygenase (via the plasmid encoded meta pathway) to the hydroxymuconic semialdehyde with pyruvate and acetaldehyde being terminal end products. The chromosomally borne ortho pathway metabolizes catechol to a cis,cis-muconate with acetyl CoA and succinate as end products. This pathway is identical to that found in the pWWO TOL plasmid (Adapted from Harayama et al. 1987).
hydroxymuconic semialdehyde dehydrogenase, which converts the 2-hydroxymuconic semialdehyde ring fission product to its enol form, 4-oxalocrotonate. It is during this step that NADH is produced, making the oxalocrotonate branch more energetically favorable (Harayama et al. 1987a). This enol form is converted to its keto form by the action of the \textit{xylH}-encoded enzyme, 4-oxalocrotonate tautomerase. The keto compound is metabolized to 2-oxopent-4-enoate by the \textit{xylF}-encoded enzyme, 4-oxalocrotonate decarboxylase. The hydrolytic branch directly converts the 2-hydroxymuconic semialdehyde to 2-oxopent-4-enoate by the \textit{xylF} encoded enzyme 2-hydroxymuconic semialdehyde hydrolase. This reaction releases acetate as a by-product. The 2-oxopent-4-enoate is further metabolized to 4-hydroxy-2-oxovalerate by the \textit{xylJ} gene product 2-oxopent-4-enoate hydratase. The 4-hydroxy-2-oxovalerate is further metabolized to the TOL pathway end products (with toluene as the initial substrate) pyruvate and acetaldehyde by the action of the \textit{xylK} encoded enzyme 4-hydroxy-2-oxovalerate aldolase.

Recent reports in the literature have identified a total of 13 encoded polypeptides within the lower operon (Harayama and Rekik 1990). Two of these genes, \textit{xylT} and \textit{xylQ} have functions which remain to be elucidated. The present research provides some possible explanations for the
functions of the xyILT region in relation to both the DHCDH activity and the regulation of lower pathway.

The Ortho-cleavage Pathway

The ortho-cleavage pathway has been described for the P. putida mt-2 strain (Keil et al. 1985) and many other P. putida strains. The substrates for the ortho-cleavage pathway are benzoate and chlorobenzoate. Catechol is the shared intermediate for both the meta- and the ortho-cleavage pathways. In contrast to the action of catechol-2,3-dioxygenase in the meta-cleavage pathway, catechol is cleaved to the cis,cis-muconate product by catechol-1,2-dioxygenase by the chromosomally-encoded pathway. This is then further metabolized to beta-ketoadipate enol lactone by the catB-encoded product cis,cis-muconate lactonizing enzyme and subsequently converted to beta-ketoadipate by the action of the catC-encoded (muconolactone isomerase) and the catD-encoded (hydrolase I) products. The beta-ketoadipate is further metabolized to beta-ketoadipyl CoA and then finally metabolized into Krebs cycle intermediates which are succinyl CoA and acetyl CoA (Keil et al. 1985) Fig. 3.
Regulation of TOL pathway genes

Regulation of the expression of these two operons is controlled by two trans-acting effector proteins that are the products of the xylR and xylS genes. The complete nucleotide sequence of the xylR regulatory gene has been determined to be 1698 bp for pWW0, encoding a protein product of 63741 Da (Inouye et al. 1988). The nucleotide sequence of the pWW0 xylS regulatory gene has also been determined to be 963 bp with an encoded protein product of 36502 Da (Inouye et al. 1986). These proteins act at the level of transcriptional initiation by a positive control mechanism. In the presence of meta-xylene or meta-methylbenzylalcohol, the xylR gene product is responsible for the activation of both the xylCMABN operon and the xylS regulatory gene. The xylS gene product in turn activates the lower operon encoding the genes xylXYZLTEGRFQKIH (Inouye et al. 1987a). In the presence of meta-toluate, the xylS gene also possesses the ability to activate expression of xylXYZLTEGRFQKIH in the absence of upper operon expression (Inouye et al. 1987b). Recently, reports in the literature have shown that under certain conditions neither of the two regulatory gene products is required to induce some expression of the lower pathway, which suggests that a chromosomal regulator protein may also be involved (Cuskey and Sprankle 1988). A model for the
regulation of the TOL pathway genes has been proposed (Ramos et al. 1986). Figure 4 shows this proposed mechanism of regulation. In this model, the xylR gene product is produced constitutively and combines with toluene (the pathway's initial substrate) that enters the bacterial cell. As a result of this interaction the XylR protein is transformed to its active form which in turn binds to the xylCMABN operon. This promotes the binding of RNA polymerase to OPl with the assistance of the ntrA sigma factor. The ntrA protein is a sigma factor that allows certain promoter sequences, notably those involved in nitrogen regulation, to be recognized by the RNA polymerase (Burlage et al. 1989). The products of the xylCMABN-encoded pathway genes activate the lower pathway operon via the xylS regulatory gene product. Recent studies have shown that the xylR gene can produce a simultaneous activation of the upper and the lower pathways by utilization of the ntrA sigma factor by causing an overproduction of the xylS gene product. This overproduction of the xylS gene product causes a constitutive expression of the lower meta-cleavage pathway as has been shown by several studies (Inouye et al. 1987b; Mermod et al. 1987; Spooner et al. 1987). These studies involving the constitutive expression of the xylS regulatory gene were carried out using an heterologous inducible promoter for the meta-cleavage
pathway. The same promoter resulted in a similar effect when placed upstream of the xylR regulatory gene, producing a cascade effect that resulted in the transcription of the lower pathway genes in the absence of the usual inducers/substrates (e.g. methylbenzyl alcohol or meta-toluate) (Burlage et al. 1989). It has been shown that the xylS product is present in the cell at low concentrations and may bind to incoming inducer substrates, being converted from inactive to active forms (Ramos et al. 1987). The overproduction of the xylS gene product makes it more likely for the active molecule to bind the lower operon promoter sequence (Ramos et al. 1987). It has also been shown that the xylR gene is an autorepressing gene even in the presence of an inducing substrate (Inouye et al. 1987a).

**Structural and physical properties of TOL plasmids**

The best studied TOL plasmid is the archetype pWWO. This plasmid is 78 MDa (117 kb) in size. The pWWO plasmid was isolated from *P. putida* (arvilla) mt-2 (ATCC23973), by K. Hosokawa (Japan) in 1959 and has been given a Pawl designation (*Pseudomonas arvilla* Wales) in recognition of the site where it was first studied (Canovas et al. 1967). The region of the TOL plasmid pWWO encoding the toluene catabolic functions is 56 kb in size and is capable of being transposed
m-xylene, m-methylbenzyl alcohol

OP1  xyICMABN
    r  ntr A
    r  of f
      ntr A

OP2  xyICMABN

m-toluate

OP2  xyICMABN

xyIS  Ps

Pr  xyIR

Pr  xyIR
Fig. 4. Regulation of the *Pseudomonas putida* HS1 TOL plasmid. The *xylRS* gene products are positive regulators of both the upper and the lower operons. The *xylR* gene product binds *m*-xylene or *m*-methylbenzyl alcohol to achieve its active conformation. In its active conformation the R regulator activates the *Ps* promoter and the OP1 (*ntrA* required) simultaneously thus activating the upper pathway genes and the *xylS* gene. The *xylS* gene product activates the lower operon promoter OP2. The S regulator protein can activate the lower operon independently of the *xlyR* gene product in the presence of *m*-toluate.
from its plasmid location into the bacterial chromosome (Jeenes and Williams 1982a). There is an additional soil isolate, *Pseudomonas putida* MW100, that is capable of toluene/xylene catabolism, yet all the genes responsible are located on the chromosome and not on a plasmid (Sinclair et al. 1986). These data, taken together with the findings that the TOL genes of the pWW0 plasmid are located on a 56 kb transposon designated Tn4561 (Tsuda and Iino 1987) suggest that the wide variety of TOL plasmids may have arisen in part due to the transposition events of this 56 kb transposon into and out of several replicons. Since the discovery of the archetype pWW0 TOL plasmid, a number of additional TOL plasmids have been identified. These newly isolated plasmids exhibit marked differences from one another in size, transmissibility, ability to integrate into the chromosomal DNA of their hosts and the degree of antibiotic or any other compound. However the general organization of their catabolic genes in the two operons regulated by the *xylRS* is similar as these plasmids were more carefully studied differences were seen in their restriction endonuclease patterns and their capability to form deletion mutants (Kunz and Chapman 1981a). However, a comparison of all of these TOL plasmids reveals the biochemical pathway utilized to be for the most part quite similar. A comparision in the
physical mapping of TOL pathway genes within several of these plasmids can be seen in Fig. 5.

The TOL plasmid pDK1 was isolated from *P. putida* HS1 (also known as PpCl) strain. HS1 is a soil isolate from Minnesota, U.S.A (Kunz and Chapman 1981a). The plasmid used in this study is pDKR1, a cointegrate plasmid that contains the complete set of pDK1 TOL pathway genes integrated into the broad host range resistance plasmid RP1.

The TOL plasmid pDK1 is a non-conjugative plasmid, 82.5 MDa (125 kbp) in size which, like the archetype pWW0 TOL plasmid, contains the operons for the upper and the lower TOL pathways (Kunz and Chapman 1981a). The pDK1 plasmid is one of only a few TOL plasmids known at the present time that can undergo spontaneous deletions to create mutants that can utilize aromatic hydrocarbons by the concerted action of both plasmid and chromosomally-produced enzymes (Kunz and Chapman 1981b).

Three spontaneous deletion mutants, all smaller in size to the parent plasmid pDK1, were identified after growth on a medium containing benzoate. The first of the spontaneous deletion mutants is the PpCT1 strain, which carries the plasmid pDKT1. This plasmid possesses the genes that encode the enzymes for the upper pathway, but has lost those for the lower pathway. This was seen in the organisms' ability to grow on toluene and benzyl alcohol, but not on alkyl
Fig. 5. Comparasion of the physical structure of three TOL plasmids. The three TOL plasmids are pDK1, pWW53 and pWWO (Adapted from Williams et al. 1988).
substituted aromatics. However, the organism could oxidize the latter substrates to their respective benzoates (Kunz and Chapman 1981b). The second deletion mutant, PpCM1 strain contains a plasmid pDKR1 has lost the genes that encode the enzymes for the upper pathway and that regulation of the lower pathway has been altered. Alkyl substituted benzoates no longer serve as inducers for the meta-cleavage operon or lower pathway. This was seen in the organisms' ability to utilize meta-toluate and benzoate but no hydrocarbons (xylene or toluene) or alkyl substituted benzoates. However, this organism is able to utilize these same substrates after growth on media containing benzoate or meta-toluate (Kunz and Chapman 1981b). The third spontaneous deletion mutant strain is PpCC1 which harbors the plasmid pDKC1 posessses a very small plasmid and it is not clear if any of the TOL genes remain. A summary is given in Table 2.

The TOL plasmid pWW53 is a nonconjugative plasmid that is carried by the P. putida strain MT53 (Osborne et al. 1988). The plasmid is 110 kbp in size and is unusual in its arrangement of TOL operons. The original wild-type plasmid carries two homologous, yet distinguishable repeats of the meta-cleavage pathway operon (xylXYZLTEGFJQKIH) which were designated xylXYZLTEGFJQKIH$_1$ and xylXYZLTEGFJQKIH$_2$. Located between these two homologous repeats are the upper pathway
Table 2. Growth characteristics of some *Pseudomonas putida* HS1 strains (Adapted from Kunz and Chapman 1981a).

<table>
<thead>
<tr>
<th>GROWTH SUBSTRATE</th>
<th>Growth of Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPC1</td>
</tr>
<tr>
<td>Toluene</td>
<td>•</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>•</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>•</td>
</tr>
<tr>
<td>Benzoate</td>
<td>•</td>
</tr>
<tr>
<td><em>m</em>-Xylene</td>
<td>•</td>
</tr>
<tr>
<td><em>m</em>-Toluene</td>
<td>•</td>
</tr>
<tr>
<td><em>m</em>-Methylbenzyl-</td>
<td>•</td>
</tr>
<tr>
<td>alcohol</td>
<td></td>
</tr>
<tr>
<td><em>p</em>-Xylene</td>
<td>•</td>
</tr>
<tr>
<td><em>p</em>-Toluene</td>
<td>•</td>
</tr>
<tr>
<td><em>p</em>-Methylbenzylalcohol</td>
<td>•</td>
</tr>
<tr>
<td>1,2,4-Trimethyl-</td>
<td>•</td>
</tr>
<tr>
<td>benzene</td>
<td></td>
</tr>
<tr>
<td>3,4-Dimethyl-</td>
<td>•</td>
</tr>
<tr>
<td>benzylalcohol</td>
<td></td>
</tr>
<tr>
<td>3,4-Dimethyl-</td>
<td>•</td>
</tr>
<tr>
<td>benzoate</td>
<td></td>
</tr>
<tr>
<td>3-Ethyltoluene</td>
<td>•</td>
</tr>
<tr>
<td>3-Ethylbenzoate</td>
<td>•</td>
</tr>
</tbody>
</table>
The *xyl* genes (Osborne et al. 1988). The *xyl* genes of this plasmid each possess their own internal promoters P3 and P4, respectively, which support the constitutive expression of these genes (Keil et al. 1987). The pWW53 plasmid is similar to the pDK1 plasmid in that it can also form spontaneous deletion mutants during growth on benzoates. One deletion involves the removal of the *xyl* operon (Osborne et al. 1988). Other deletion mutants have lost the regulatory control by the *xylR* and the *xylS* genes, suggesting ability of these deletion mutants to grow on benzoates by utilizing of the meta-cleavage pathway operon, while being unable to utilize the aromatic hydrocarbons (*MXY* phenotype, *xyl* genes required) (Osborne et al. 1988). It has been proposed that the structural organization of this particular plasmid may have some selective advantage in that the concerted action of the gene products of these two homologous operons may increase their catalytic activity and stability. In addition, a recombination event between the two homologous operons may allow the repair of mutational damage. Finally regulation may be such that under different growth conditions, the operons are expressed individually (Osborne et al. 1988).

There is a strong conservation of the catabolic genes between the three well characterized TOL plasmids (pWW0, pDK1
and pWW53), even though they were isolated from different geographical locations. The genes that encode the enzymes of the upper, lower and regulatory pathways are organized into three separate units xylCMABN, xylXYZLTEGFJQKIH and xylSR with the gene order being absolutely conserved for each unit (Keil et al. 1987). Within the genes encoding the meta-cleavage pathway found on pDK2 (therefore by inference on PDK1) 15 of 29 restriction endonuclease cleavage sites are in the same positions as found for pWW0 and 16 of these sites are shared with pWW53 (Keil et al. 1987). The xylCMABN operon of the pDK1 and the pWW53 plasmids are even more conserved in that 20 of restriction endonuclease cleavage sites present in this region of pDK1 are identical to in the corresponding region of pWW53, with the exception of one site found on pWW53 and not found on pDK1 (Keil et al. 1987). An example of a comparasion of the restriction sites found in these three TOL plasmids can be seen in Table 3.

Regulation of gene expression appears to be similar for these three TOL plasmids. The OP1 region, upstream of the xylCMABN operon, and the region identified to encode the xylRS genes are highly conserved and a corresponding homology of the region present in these three plasmids that encodes the OP2 and the xyJR products (Keil et al. 1987, Chang 1992; Hares et al. 1992). However, the position of the three
Table 3. Comparison of the restriction endonuclease sites of the three best studied TOL plasmids pWW0, pWW53 and pDK1 (Adapted from Williams et al. 1988).

### COMPARISON OF RESTRICTION SITES OF THE \textit{XyZCA} CODING REGIONS IN TOL PLASMIDS pWW0, pWW53 AND pDK1.

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>NUMBER OF SITES</th>
<th>No. OF SITES SHARED WITH pWW0</th>
<th>pWW53</th>
<th>pDK1</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWW0</td>
<td>17</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWW53</td>
<td>21</td>
<td>15</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pDK1</td>
<td>20</td>
<td>14</td>
<td>20</td>
<td>-</td>
</tr>
</tbody>
</table>

### COMPARISON OF THE RESTRICTION SITES OF THE \textit{mets} PATHWAY CODING REGIONS OF THE TOL PLASMIDS pWW0, pWW53(1), pWW53(2) and pDK1.

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>NUMBER OF SITES</th>
<th>No. OF SITES SHARED WITH pWW0</th>
<th>pWW53(1)</th>
<th>pWW53(2)</th>
<th>pDK1</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWW0</td>
<td>20</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWW53(1)</td>
<td>22</td>
<td>13</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWW53(2)</td>
<td>29</td>
<td>15</td>
<td>16</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pDK1</td>
<td>29</td>
<td>15</td>
<td>16</td>
<td>29</td>
<td>-</td>
</tr>
</tbody>
</table>
modules (\textit{xylRS}, upper and lower operons) in these plasmids is very much different as is the orientation of the units with respect to the direction of transcription. A comparison of these three TOL plasmids can be seen in Fig. 5 (Keil et al. 1987).

The \textit{xylL} region encoding 1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase (DHCDH)

The biochemical pathway for the conversion of aromatic hydrocarbons to Krebs cycle intermediates had been partially characterized by 1970 (Dagley 1971; Canovas et al. 1967; Gibson 1968). However, the intermediates of the pathway, then termed the benzoate oxidase system (Reiner and Hegeman 1971; Reiner 1971) were only elucidated in 1971. The enzyme DHCDH was first isolated from the bacterium \textit{Alcaligenes eutrophus} B9, a mutant strain that blocks the pathway at the conversion of 1,2-dihydroxycyclohexa-3,5-diene carboxylic acid (DHB) to catechol (Reiner and Hegeman 1971). This strain accumulates DHB, which is produced by the action of toluate oxidase on benzoates and related compounds. This conversion of benzoic acid to catechol was first thought to be carried out by an enzyme system referred to as the benzoate oxidase system. However, Reiner 1971 showed the presence of a specific enzyme in this system, DHCDH, which
was responsible for the dehydrogenation and decarboxylation of DHB to catechol. DHB was shown to be an intermediary metabolite of this system because it did not support the growth of any bacteria (four genera in the study carried out by Reiner). This was expected as the polar properties of this metabolite would not facilitate passive transport through the cell membrane and furthermore it is not found in large quantities in the environment so as to warrant the existence of an active transport system (Reiner 1971). This theory was put to the test by the production of mutants that were able to grow slowly at the expense of DHB (Reiner 1971). Several studies were carried out to show the actual presence of diol carboxylic acids as intermediates. First, the TOL plasmid pWW0 (the parent strain was *P. putida* mt-2) has a wide substrate specificity being able to utilize 3- and 4-substituted benzoates and 3,4-dimethyl benzoates) (Reineke and Knackmuss 1978). The recipient strain was the *P. putida* strain B13 which grows well on 3-chlorobenzoate but does not show significant oxidation of 4-substituted benzoates in which the substituents are larger than fluorine) (Reineke and Knackmuss 1978; Reineke et al. 1978). When the plasmid from the parent strain was transferred to the recipient strain, this resulted in the creation of an exconjugant that had the ability to utilize 3,5-dichloro-benzoate and 4-chlorobenzoate
(Reineke and Knackmuss 1980). Second, when the \textit{xylXYZ} and the \textit{xylL} genes from the TOL plasmid pWW0-161 were cloned into the \textit{P. putida} strain B13, growth occurred on 4-chlorobenzoate. However, both \textit{xylXYZ} and \textit{xylL} were required for growth on 3,5-dichlorobenzoate (Lehrbach et al. 1984). Finally, enzyme studies in \textit{Escherichia coli} strain K12 showed that the \textit{xylXYZ} gene products from the TOL plasmid pWW0 carried out the oxidation of benzoate to 1,2-dihydroxycyclohexa-3,5-diene carboxylic acid. However, both the \textit{xylL} and the \textit{xylXYZ} gene products were required for the NAD\(^+\) dependent oxidation of 3- and 5-chlorobenzoate-1,2-diols (Lehrbach et al. 1984). A diagrammatic view of the action of the \textit{xylXYZ} and \textit{xylL} genes and gene products can be seen in Fig. 6.

The 1,2-dihydroxycyclohexa-3,5-diene carboxylic acid requires NAD\(^+\) as a specific electron acceptor (NADP\(^+\) being unacceptable) the dihydrodiol is in the \textit{cis} configuration (Gibson 1968). However, an exception was noted with a NADP\(^+\) dependent DHCDH enzyme that was found to catalyze the dehydrogenation of the dihydrodiol to catechol (in rat liver) in the \textit{trans} configuration (Gibson et al. 1970). The benzoate oxidase system (\textit{xylL} and \textit{xylXYZ} genes) has a broad substrate specificity being capable of oxidizing 3- and 4-substituted benzoates, certain disubstituted benzoates.
**Key.**

- $R_1 = R_2 = H$ (toluene)
- $R_1 = CH_3; R_2 = H$ (m-xylene)
- $R_1 = H, R_2 = CH_3$ (o-xylene)
- $R_1 = R_2 = CH_3$ (1,2,4-trimethylbenzene)
- $R_1 = CH_2 CH_3; R_2 = H$ (3-ethyltoluene)
- DHB: 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid
- DHBDH: Dihydroxycyclo-3,5-hexadiene carboxylate dehydrogenase.

---

**Diagram:**

- **Toluene** $\rightarrow$ **Benzene** $\rightarrow$ **Catechol**

- **NADH, O_2** $\rightarrow$ TO encoded by *xyJ* gene
- **NAD** $\rightarrow$ DHBH, encoded by *xyL* gene

*Ring fission and production of TCA cycle intermediates*
Fig. 6. Mode of action of the \textit{xylD} and the \textit{xylLT} genes.
(Adapted from Reiner 1972). This study shows novel functions of the \textit{xylT} genes.
including 3,4-dimethylbenzoate, an intermediate in the biodegradation of pseudocumene) (Whited et al. 1986), a partial list of some of the dihydrodiols formed as intermediates to the formation of their respective catechols can be seen in Tables 4 and 5.

**Significance of research project**

Of the many synthetic chemicals that are added to the environment (herbicides, pesticides and other industrial wastes), many are derivatives of benzene. If the accumulation of these compounds in the soil and water should prove to recalcitrant to the action of the many microorganisms that are indigenous to these environments, then their accumulation could result in severe ecological changes. The approach by current research into the microbial degradation of these toxic aromatic compounds has taken any of three major directions. First, the study of metabolic intermediates of the various biodegradative pathways involved. Second the interpretation of the enzymatic mechanisms involved in hydroxylation and ring fission. Third, the regulatory control mechanisms of the catabolic pathway genes involved (Gibson 1968).

Although until very recently only a few of the TOL plasmid genes have been sequenced completely (Inouye et al.
Table 4. Dihydrocyclohexadiene substituted products from the oxidation of some aromatic compounds. (Adapted from Reiner and Hegeman 1971).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Diol</th>
<th>Diol Dehydrogenase Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>m-toluete</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>p-toluete</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>3-Ethylbenzoate</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>3,4-Dimethylbenzoate</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Table 5. Utilization of the dihydridiol products of the xylLT gene. Several genus of bacteria utilize the dihydridiols and convert them to various metabolically useable products (Adapted from Reiner and Hegeman 1971).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Biological Source</th>
<th>Suggested Intermediate in Formation</th>
<th>Evidence for Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Dihydridiol" /></td>
<td><em>Pseudomonas</em> Peroxide</td>
<td><em>18O incorporation</em></td>
<td>cis isomer</td>
</tr>
<tr>
<td><img src="image2.png" alt="Dihydridiol" /></td>
<td><em>Pseudomonas</em> Peroxides</td>
<td></td>
<td>cis isomers</td>
</tr>
<tr>
<td><img src="image3.png" alt="Dihydridiol" /></td>
<td><em>Bacillus</em> Oxide, <em>Pseudomonas</em> Oxide</td>
<td></td>
<td>trans isomer</td>
</tr>
<tr>
<td><img src="image4.png" alt="Dihydridiol" /></td>
<td><em>Pseudomonas</em> Oxide</td>
<td><em>18O incorporation</em></td>
<td></td>
</tr>
</tbody>
</table>
1988; Inouye et al. 1986; Nakai et al. 1983), recent advances in the study of the genetics of catabolic plasmids have produced organisms of a large phenotypic variety. First, the TOL plasmids are transmissable extrachromosomal pieces of genetic material that are not required in the normal life cycle of the bacterial cell. Hence, any recombination events or mutations in these plasmids (which can lead to an increase in the variety of toxic hydrocarbons utilized) may not directly affect the bacterium. Second, the catabolic pathway genes on the TOL plasmids are arranged in blocks (operons) that can be moved as such. This facilitates the production of new recombinant plasmids utilizing many host specific plasmid vector systems. Third, the Pseudomonads are easy to work with in that their growth temperature is 30°C, and they have the ability to grow on simple synthetically produced media. This facilitates the production of new transformants and conjugants via relatively easy processes.

The production of new transformants and conjugants is an important process in the production of new strains. Such new strains increase the substrate specificity in the biodegradation of toxic hydrocarbons. An example of recombinant DNA techniques and the resultant production of new transconjugants, the pWWO plasmid was transferred to the P. putida strain B13 (Reineke et al. 1982). This particular
strain of *Pseudomonas* contains chromosomally encoded enzymes that catabolize 3-chlorobenzoate. However, the newly created transconjugant could now utilize both toluene and 3-chlorobenzoate. When the *xylXYZ* and *xylL* genes of this transconjugant were activated by spontaneous mutations, substrate specificity was enhanced (Jeenes et al. 1982b).

The cloning of these genes and the manipulation of the pathways utilized by these genes can expand the number of substrates utilized by these catabolic plasmids, as well as allow the alteration of specific enzyme properties/specificities. Fine structural analysis also has the potential of revealing novel site by which the operons contained within these biodegradative plasmids are controlled. Regulation of these biodegradative pathways is necessary to increase the efficiency of utilization of substrates under varying conditions. A change in the kinetics of an enzyme within a biodegradative pathway may result in a productive change in the activity of the pathway or the production of a new end product which can be utilized in a more efficient biodegradative or commercial processes. The biodegradation of toxic aromatic hydrocarbons to safe metabolically useful products is important to alleviate the pressures placed on the environment and to waste management. Therefore, elucidation of the biochemistry and genetics of
these catabolic processes is of enormous importance.

The biodegradation of recalcitrant compounds by transmissible catabolic plasmids will only be further advanced through a better understanding of detailed physical and structural analysis of the genes, their regulatory elements and the structure/function of their enzyme products. To help facilitate such an understanding this research project provides detailed nucleotide sequence data of the TOL DHCDH region (\textit{P. putida} pDK1) and a characterization of its encoded gene products and regulatory elements.
CHAPTER II

MATERIALS AND METHODS

Bacterial strains and plasmids

*E. coli* DH5α (*supE44, hsdR17, recA1, endA1, gyrA96, thi-1 and relA1*) (Sambrook et al., 1989), was used as a recipient for transformation experiments in conjunction with the Col E1 plasmids pUC18 and pUC 19 (Bolivar 1978).

*E. coli* DH5α F' (*F', supE44, hsdR17, recA1, endA1, gyrA96, thi-1 and relA1*) (Sambrook et al., 1989), was used as the recipient strain for transformation experiments utilizing the M13 phage RF form and its recombinant derivatives. Such recombinant M13 phage were used as templates in the DNA sequencing protocols.

An *E. coli* JM101 strain (*SupE thi d(lac-proAB) F' [traD36 proAB+ lacIq lacZdelta M15]*) (Sambrook et al. 1989) containing the recombinant plasmid pBK190 (with the xyl1XYZ genes) was used for the production of the dihydroxy diols.

All subclones that were produced for this study were created from the original pDKR1 plasmid. The 97.6 kbp plasmid pDKR1 (66 kDa) is contained within the *P. putida* strain Paw630 (Shaw and Williams 1988). The pDKR1 is an in
vivo construct produced by the conjugation of strains carrying the broad host range R-plasmid, RP4 and pDK1, the TOL plasmid found in *P. putida* HS1. RP4 was transferred from *P. putida* AC34 (RP4) into HS1 by filter conjugation. The *P. putida* Paw630 strain carrying pDKR1 was obtained from Dr. D. A. Kunz, Department of Biological Sciences, University of North Texas. The *P. putida* Paw630 strain is a tryptophan auxotroph and is resistant to streptomycin (1 mg/ml). The R-plasmid genes confer kanamycin (100 μg/ml), tetracycline (50 μg/ml) and ampicillin (1 mg/ml) resistance.

**Media and culture conditions**

*E. coli* strains DH5α and DH5α F' were maintained on Luria-Bertani medium (LB) broth/agar. These were maintained at the optimal growth temperature of 37°C in the presence of the appropriate antibiotic(s) as necessary. All *E. coli* strains carrying recombinant plasmids in *E. coli* vector systems (pUC 18 or pUC19) were grown in the presence of 50 μg ampicillin per milliliter.

LB broth contained 10 grams Bacto Tryptone™, 5 grams Yeast Extract (Difco™) and 10 grams sodium chloride per liter of distilled deionized water. The pH was adjusted to 7.5 before sterilization (Difco Manual 1984). Luria-Bertani
agar contained 15 grams Bacto Agar™ per liter (1.5% w/v) (Difco Manual 1984) distilled deionized water.

Yeast Tryptone (YT) liquid medium contained 8 grams Bacto Peptone™, 5 grams yeast extract (Difco™) and 5 grams sodium chloride in a total volume of 1 liter distilled water. The pH was adjusted to 7.5 before sterilization (Difco Manual 1984). Yeast Tryptone agar contained 15 grams Bacto Agar™ per liter (1.5% w/v) distilled deionized water. The yeast tryptone soft top agar, used for the M13 transformation procedure, had the same composition as the regular agar except that 7.5 grams Bacto Agar™ per liter was used.

Media for enzyme expression in the production of dihydrodiols

The minimal medium used for the expression of enzymes or the accumulation of diols by recombinant E. coli strains was initially prepared as two separate solutions, PN (Phosphate/Nitrate) and R-salts (Kunz and Chapman 1981b). A stock solution of 10X PN was prepared by the addition of 91 grams of KH₂PO₄, 16.8 grams NaOH and 12 grams of (NH₄)₂SO₄ to 900 ml of distilled deionized water (Kunz et al. 1981b). The pH of the solution was then adjusted to 7.5 and the final volume was brought up to 1 liter with distilled deionized water. This stock solution was not sterilized.
A stock solution of 200X R-salts was prepared from two individual stock solutions. First, 40 grams of MgSO₄·7H₂O was dissolved in a final volume of 400 ml using distilled H₂O (this gave a 10% MgSO₄·7H₂O solution). A second solution was prepared by adding 1 gram of FeSO₄·7H₂O to 100 ml distilled water (this gave a 1% FeSO₄·7H₂O solution). The two solutions were mixed and 2 ml HCl were then added. The 200X R-salts solution was then autoclaved and stored at room temperature.

A 1X PN solution (total volume 50 ml) was prepared by adding 5 ml of 10X PN to 40 ml distilled water. This solution was then autoclaved. A 1X R-salts final concentration was attained by adding 250 μl from the 200X R-salts stock solution to the sterilized 1X PN solution. Stock solutions of 100 mM benzoic acid, meta-toluic acid, para-toluic acid and 3,4-dimethylbenzoic acid were prepared and used as substrates for toluate oxidase (Sigma™ molecular grade chemicals). Five milliliters was added to the 1X PN sterilized solution to give a final concentration of 10 mM of the desired hydrocarbon. A 1.25 ml volume of a stock solution of 2 molar glycerol solution was added to the 1X PN giving a final concentration of 5 mM (glycerol was used as
the growth carbon source). Thirty microliters from a stock solution of 100 mM isopropylthiogalactoside was added to the 1X PN solution giving a final concentration of 0.06 mM.

Fifty ml from a filter sterilized stock solution of ampicillin (50 mg/ml) was added to the 1X PN solution giving a final concentration of 50 µg/ml.

**Long-term storage of bacterial strains**

A single colony of a chosen bacterial strain was inoculated into 5 ml of culture medium (Both the plates and the liquid media possessed a final concentration of 50 µg ampicillin per ml, if appropriate). The 5 ml LB broth culture was then incubated overnight at 37°C and 250 rpm (to facilitate aerobic growth conditions) a New Brunswick™ G25 shaker/incubater was used for this purpose. Five hundred ml of the overnight culture were added to sterile air-tight microcentrifuge tubes. To this was added 500 µl of sterile 80% glycerol. The mixture of bacterial cells and glycerol was then vortexed well and placed at -70°C for indefinite storage (possibly several years).

For routine use of specific strains of bacterial cells, a LB-agar slant with a final concentration of 50 µg ampicillin per ml was streaked with cells and stored at 4°C.
Tanaka and Weisblum large scale plasmid isolation from E. coli strains (Johnston and Gunsalus 1977)

A 5 ml aliquot of LB liquid broth (50 μg ampicillin per ml) medium was inoculated with a single colony of the desired strain of E. coli. This was allowed to incubate overnight at 37°C at 250 rpm. This overnight culture was used to inoculate a 50 ml LB broth (50 mg ampicillin/ml) and allowed to incubate overnight on a shaker at 37°C and 250 rpm. One liter of LB broth (50 μg ampicillin/ml) in a 2800 ml Fernbach™ flask was inoculated from the overnight culture (10-50 ml was used). This culture was allowed to incubate at 37°C and 250 rpm. The absorbance of the suspension was measured at 550 nm at regular intervals until an absorbance of 0.8-1.0 was reached indicating log phase growth (a Beckman™ DU-40 spectrophotometer was used for this purpose with LB liquid broth as a blank). At this point, 2 ml of 85 mg chloramphenicol/ml dissolved in 95% ethanol was added (final concentration of 170 μg/ml.). The culture was then incubated overnight at 37°C and 250 rpm. The next day the cells were collected using a Sorvall™ GS3 rotor (approximately 500 ml per bottle) at 5213 x g for 6 minutes. The rotor and the centrifuge were precooled at 4°C. The supernatant was discarded and the cell pellet was resuspended
in cold 0.15 M NaCl (20 ml per liter of original culture)  
(All solutions except the 10% SDS should be stored on ice.  
The SDS should be stored at room temperature) and the  
suspension was transferred to a 45 ml Oak Ridge™ style  
centrifuge tube. Each tube should contain the cell pellet  
equivalent to 750-1000 ml of original culture. The Oak  
Ridge™ style tubes were centrifuged in a SA600 rotor at 5213  
x g for 5 minutes (4°C). Beyond this point all work was done  
in the cold room at 4°C. The supernatant from the SA600  
rotor tube was discarded and the cell pellet was resuspended  
in (all volumes per tube) 10 ml of 50 mM Tris, 25% Sucrose,  
pH 8.0 such that the suspension was complete with no clumps  
remaining. To this resuspension was added 2 ml of freshly  
prepared lysozyme solution (stock 5 mg lysozyme/ml). Tubes  
were capped and inverted two to three times to ensure good  
mixing. This solution was placed on ice for 5 minutes. Four  
ml of 0.25 M Na$_2$EDTA (pH 8.0) were added and mixed by  
inversion before being placed on ice for 5 minutes. Five ml  
of 5 M NaCl was added, followed by thorough mixing by  
inversion. The mixture was placed on ice for 5 minutes and 2  
ml of 10% SDS was then added. Again the mixture was mixed by  
inversion, yet unnecessary shaking was avoided which would  
have caused foam formation or shearing of DNA. The DNA
solution was placed on ice for two hours in the cold room.
The cells were centrifuged at 38712 x g for 60 minutes in an
SA600 rotor and the supernatant poured off into a graduated
cylinder to which one volume of isopropanol was added. This
mixture was transferred to a 250 ml Erlenmeyer™ flask. This
flask was placed into a dry ice/ethanol bath for 20 minutes
(or incubated overnight in a -20°C freezer). The contents of
the flask should freeze solid in the ethanol bath. This was
then melted by briefly placing in a water bath at room
temperature. The contents were then centrifuged in a
Sorvall™ GSA rotor for 20 minutes at 9268 x g. The
supernatant was discarded and the pellet resuspended in 7-8
ml 10 mM Tris, 1 mM EDTA, pH 8.0 (TE) per liter of original
culture. The pellet was resuspended in the TE solution by
placing in the cold room with a magnetic stirrer at moderate
speed so as to allow for adequate resuspension but without
excessive foaming or shearing of the DNA. Some DNase-free
RNase was then added (final concentration was 20 µg/ml) to
destroy any RNA. At this point the approximate plasmid yield
may be checked by loading 20 µl of the suspension onto a 1%
agarose gel (after a brief clarifying spin in a microfuge).
Any denatured proteins may be removed by centrifugation in an
SA600 rotor at 14481 x g for 10 minutes. The supernatant was
then poured into a graduated cylinder.

Solid cesium chloride was added next to a final concentration of 5.3 grams per 5 ml TE. To this solution was added 400 μl of 10 mg/ml of ethidium bromide per 8 ml of solution (from this point on all work should be done in subdued light). One Ti1270 tube was used per liter of original culture. Each tube was filled to the top of the tube, leaving no air bubbles (mineral oil or cesium chloride balancing solution was used as necessary). A balancing solution of exactly 1.06 grams TE/ml (1.06 grams added to 1 ml TE) can be used to top off the tube. This ensures that the tube does not collapse during centrifugation. The tubes were then balanced to within 0.02 grams. This preparation was centrifuged in a Dupont™ OTD65 ultracentrifuge for 40 hours at 43467 x g. The bands of DNA can be visualized using a long wave ultraviolet lamp (310 nm). All work from this point had to be carried out under reduced light. The lower band was removed using a 20G hypodermic needle which was inserted through the side of the polyallomer tube. A diagrammatic view of the various layers formed in the polyallomer tube after an ultracentrifugation run can be seen in Fig. 7. The ethidium bromide was removed by repeated extraction with water-saturated butanol. An equal volume of
Fig. 7. Ti1270 ultracentrifuge tube after a 48 hour spin at 43467 x g. Two DNA containing bands were found along the cesium chloride gradient. The upper band corresponds to the chromosomal DNA of the bacterial cells. The lower band corresponds to the plasmid (recombinant) DNA of interest. Isopropanol and RNase treatments substantially reduced the protein and RNA components observed.
the water-saturated butanol was added for each extraction and
the solution mixed thoroughly by vortexing. The top pink
layer (butanol with ethidium bromide) was removed using a
Pasteur™ pipette. This process was repeated until there was
no indication of pink color in the upper butanol layer.
Beyond this point subsequent work was carried out under
normal light conditions. Two volumes of water and nine
volumes of ice-cold 100% ethanol were added to the solution.
This solution was mixed thoroughly and placed at -80°C for 15
minutes. The suspension was centrifuged at 14481 x g for 15
minutes and the supernatant discarded. The pellet was
treated with one-tenth volume of 3 M sodium acetate and three
volumes ice-cold 100% ethanol. The solution was mixed
thoroughly and centrifuged for 15 minutes at 14481 x g. The
supernatant was discarded and the pellet washed with 0.5 ml
ice-cold 70% ethanol and centrifuged for 5 minutes at 14481 x
g. The supernatant was discarded and the pellet washed with
0.5 ml of ice-cold 100% ethanol and centrifuged at 14481 x g
for 5 minutes. The supernatant was discarded and the final
pellet dried for 5-10 minutes in a Savant™ SpeedVac
centrifuge. The dried pellet was then resuspended in one
milliliter of TE. The concentration of the DNA was
calculated following spectrophotometric analysis using a
Beckman™ DU-40 spectrophotometer.

Digesting DNA with restriction endonucleases

Reaction conditions were according to the manufacturer and generally included two units of enzyme for every microgram of duplex DNA to be digested. A typical reaction mixture would be as follows:

- 2.0 µl Reaction Buffer (provided by the manufacturer)
- 7.0-16.0 µl Sterile distilled water (DNA + distilled water =17 µl).
- 1.0-10.0 µl DNA (1-10 µg in distilled water or TE buffer).
- 1.0 µl Restriction enzyme (10-20 units).
- 20 µl Total Volume.

The enzyme volume may be increased but under no circumstances was the enzyme volume more than 10-15% of the total reaction volume. This limitation was to minimize the effect of the the restriction endonuclease storage buffer which contains 50% w/v of glycerol. Specific enzymes required specific enzyme buffers, these were usually supplied by the manufacturer as 10X stock solutions. The buffer and the enzyme were stored at -20°C in a manually defrosted freezer.
The reaction mixture was routinely incubated at 37°C for 1-2 hours (for most enzymes). However, some enzymes required higher or lower temperatures (this information was provided by the manufacturer).

Once the DNA was cut, it could be utilized in a variety of ways. If a piece of DNA was to be used to insert into a cloning vector, the restriction digest needed to be cleaned up in order to remove any salts and enzymes that were present, which may hinder any ligation reactions that were to follow. If a cloning vector was to be used as a recipient of an inserted piece of DNA, the cut vector was treated enzymatically with bacterial alkaline phosphatase in order to prevent closing of its ends before incorporating the target DNA. If the samples were to be subjected to electrophoretic analysis immediately after the digestion, 5 μl of 5X agarose gel loading buffer was added (per 20 μl sample), followed by incubation at 70°C for 5 minutes.

Bacterial alkaline phosphatase treatment of vector DNA

After the cloning vector has been subjected to a restriction endonuclease digestion, it was enzymatically treated with bacterial alkaline phosphatase (BAP) in order to remove the 5’ phosphate group from the DNA and hence prevents
closing of the cut ends. Typically this enzymatic treatment was as follows. Into a sterile 1.5 ml microfuge tube was added:

5.0-40.0 µl Linear DNA (typically 5-10 µg of DNA) from restriction digest

9.0-44.0 µl TE buffer (DNA + TE = 49.0 µl)

1.0 µl bacterial alkaline phosphatase (100-200 units)

50.0 µl total volume

The reaction mixture was placed at 65°C for 3 hours. At 30 minute intervals the microfuge tube was centrifuged briefly at 14481 x g. This procedure prevented evaporation effects from changing the buffer concentration and hence the reaction conditions.

Phenol extraction of nucleic acids

Once the insert and the vector duplex DNA had been cut, it was necessary to remove the salts and enzymes and to precipitate the nucleic acids. The removal of proteins was achieved using an organic solvent such as phenol and/or a 1:1 mixture of phenol:chloroform. Phenol causes the denaturation of proteins and hence the precipitation of these proteins from the aqueous solution. However, the nucleic acids are
not denatured and hence remain in the aqueous solution. The phenol stock solution was saturated with a solution of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). To this TE-saturated phenol was added 8-hydroxyquinoline to a final concentration of 0.1%. The 8-hydroxyquinoline is an anti-oxidant. It also makes the phenol layer more visible by giving it a yellow/orange appearance.

The restriction endonuclease digests (or BAP treated vectors) were removed from their respective water baths and centrifuged briefly at 14481 x g to collect the contents to the bottom of the microfuge tube. To each of the microfuge tubes was added 50 μl of TE followed by 100 μl of TE-saturated phenol. The contents were mixed thoroughly and centrifuged for 3 minutes at 14481 x g. The lower colored layer was (phenol) removed and the upper layer transferred to a new microfuge tube. To this was added 100 μl of TE-saturated phenol. The contents were mixed thoroughly (vortexed) and then centrifuged for 3 minutes at 14481 x g. The lower colored phenol layer was again removed and to the remaining upper layer was added 500 μl of diethyl ether. The tube was sealed and the contents mixed thoroughly before being centrifuged briefly at 14481 x g. Using a 9" drawn out Pasteur™ pipette, the top diethyl ether layer was removed. The tube was left open at room temperature for a
few minutes in order to allow any remaining diethyl ether to volatilize. After a few minutes, 11 µl of 3.0 M sodium acetate was added to the tube, which was then mixed thoroughly. 350 µl of 100% ethanol was added next, followed again by mixing the solution well. The microfuge tube was then placed into a chilled Savant™ microfuge and centrifuged for 15 minutes at 14481 x g. The supernatant was removed by using a 9" Pasteur™ pipette (at this point a pellet of nucleic acid was visible). To the pellet was added 500 µl of 70% ethanol solution and the tube was inverted once or twice (rather than being mixed thoroughly). The contents were then centrifuged for 5 minutes at 14481 x g. The supernatant was then carefully removed using a 9" drawn out Pasteur™ pipette. To the pellet was added 500 µl of 100% ethanol and the tube inverted once or twice, being careful not to dislodge the pellet. Once again the contents were centrifuged for 5 minutes at 14481 x g. The supernatant was removed and discarded as before. The open microfuge tube was then placed in to a Savant™ Speed Vac centrifuge concentrator for 5 minutes. To the now dry pellet of nucleic acid was added 15 µl of distilled water and the contents mixed thoroughly so as to place the nucleic acid pellet into solution. If there was a difficulty in putting the nucleic
acid into solution by a single mixing then the microfuge tube was placed for 10 minutes at 65°C after which it was frozen at -20°C for 1-2 hours. The frozen mixture was then again placed at 65°C for 5 minutes and vortexed thoroughly. Storage of nucleic acids was at -20°C.

Qualitative and quantitative identification of DNA

Qualitative identification of DNA can be seen in the ability of ethidium bromide to intercalate between base pairs. When these fragments with intercalated ethidium bromide are exposed to ultraviolet light they emit a yellow/orange fluorescence. Unknown DNA samples were electrophoresed along side a marker DNA of known concentration and staining in a 50 µg ethidium bromide/ml solution. The two samples were then observed under illumination by long wave ultraviolet radiation. Comparison of the DNA of known concentration to the unknown samples provides an estimate of their relative concentrations, ethidium bromide also intercalates RNA. Therefore any RNA present was also observed.

Quantitative determination of the DNA concentration present was performed spectrophotometrically. DNA absorbs ultraviolet light at 260nm. A Beckman™ DU-40
spectrophotometer was used to determine the absorbance of DNA samples. A reading of 1.0 absorbance unit at 260 nm is equivalent to 50 μg of duplex DNA/ml. A scan of wavelengths between 220 nm and 320 nm reveals an absorbance maxima at 260nm. Any impurities if present, will frequently shift the absorbance maxima. Therefore, both a quantitative and qualitative assessment of the DNA solution could be obtained.

**Ligation of DNA for cloning into plasmids and phages**

Preparations of recombinant DNA served a two-fold purpose. First, recombinant DNA was required for the preparation of novel strains of bacteria in the expression of specific enzyme products. Further the recombinant DNA was employed as a substrate for DNA sequencing reactions. Protein expression studies required the preparation of recombinant DNA whereby the inserted genes in the appropriate cloning vector were readily expressed in a specific bacterial strain, for our purposes the bacterial strain was *E. coli* DH5α. The expression vector utilized for this purpose were the pUC plasmids pUC18 or pUC19. A typical ligation reaction using pUC cloning vectors was as described below.

A ratio of three moles of vector for every mole of target DNA was used (generally a total of no more than 1 μg
or no less than 0.1 µg). This ratio was varied from 10/1 to 1/10 vector/target depending upon the experimental demands and the availability of DNA's (Dugaiczyk et al. 1975). One microliter of T4 DNA ligase (approximately 1 unit of enzyme, as stated by the manufacturer), four microliters of 5X ligation buffer (250 mM tris-HCL pH 7.6; 25% (w/v) PEG 8000 (polyethylene glycol, av. Mol. Wt.= 8000); 50 mM MgCl₂; 5.0 mM ATP; 5.0 mM DTT) were added to a 500 µl microcentrifuge tube containing DNA and water. Sterile distilled deionized water was used to bring the final volume up to 20 µl.

The reaction mixture was incubated at room temperature (23°-26°C) for 4 hours. After the incubation period, the reaction mixture was diluted with an equal volume of TE buffer. This ligation mixture was stored for short periods at 4°C or placed at -20°C for long term storage.

When cloning into M13 vectors, a vector/insert ratio of 1:1 to 1:3 was employed with the M13 concentration about 2-20 µg/ml. A typical ligation mixture for the M13 phage vector system was as follows (BRL Instruction manual 1989):
1.0 µl restriction endonuclease-digested and alkaline phosphatase treated M13 vector (100 ng, about 20 fmol of DNA molecules) was used

1.0 µl restriction endonuclease-digested target DNA (100 ng, about 100-200 fmol of DNA molecules)

1.0 µl 5X DNA ligase reaction buffer (similar in composition to the buffer used in the pUC ligation reaction)

1.0 µl T4 DNA ligase (0.1 manufacturer's unit per µl for sticky end ligations, 1.0 manufacturer's unit per µl for blunt ends)

1.0 µl sterile distilled deionized water

5.0 µl total volume

The reaction mixture was incubated at room temperature (23°-26°C) for 4 hours. After the incubation was complete, the ligation reaction was stopped with 2.0 µl of 0.25 M EDTA and 18 µl of sterile distilled deionized water.

Preparation of competent cells (Huff et al. 1990)

*E. coli* DH5αF' bacterial cells were used for the transformation of M13 phages and *E. coli* DH5α bacterial cells were used for the transformation of pUC plasmids. Five milliliters of sterile liquid YT media was inoculated
with a single colony of the respective E. coli strain from a fresh YT agar plate. The inoculate was allowed to incubate at 37°C and 250 rpm (New Brunswick™ G25 incubator/shaker) overnight. Five hundred microliters of this overnight culture was used to inoculate 50 ml of sterile liquid YT medium in a 250 ml Erlenmeyer™ flask. This was in turn placed in the New Brunswick™ incubator/shaker under the same conditions as the previous inoculate. The culture was monitored spectrophotometrically by removing a 1 ml aliquot (aseptic technique was used) every 30 minutes, beginning 1 hour after inoculation. The sample aliquot was monitored at a wavelength of 550 nm using a Beckman™ DU-40 spectrophotometer. When an absorbance of 0.45 was reached, the flask was placed in an ice water bath for 10-20 minutes. 25 ml aliquots of the bacterial culture were placed into two sterile 30 ml Corex™ glass centrifuge tubes. The tubes were centrifuged for 15 minutes at 469 x g at 4°C. The supernatant was poured off and the bacterial cell pellet was resuspended gently in 12.5 ml/tube of ice-cold sterile 50 mM calcium chloride solution. Once the cells were suspended, the Corex™ tubes were placed on ice, for 30-40 minutes. After this incubation period, the tubes were centrifuged for 15 minutes at 579 x g at 4°C. The supernatant was removed
and the bacterial cell pellet was resuspended in 1.0 ml of ice-cold sterile 50 mM calcium chloride solution per tube. The Corex™ tubes containing the competent cells were then placed on ice, ready for use (for optimum results, the competent cells should be used within the next 8 hours) in the transformation procedure.

Transformation of *E. coli* strains DH5αF' and DH5α with recombinant pUC and M13 molecules

Recombinant M13 molecules (BRL Instruction manual 1989): A 300 μl volume of the freshly prepared competent cells was added to a 5 ml sterile glass culture tube. To this was added 0.5-1.0 μl (approximately 50 ng) of recombinant DNA (ligation mixture). The tubes were swirled a few times to mix the contents and then left on ice for 30 minutes. After this incubation time elapsed, the transformation mixture was heat shocked for 2 minutes at 42°C. This mixture was then added to an already prepared tube containing the following:
3.0 ml of YT soft agar (stood at 42°C.)

50.0 μl of 2% 5-bromo-4-chloro-3-indolyl-b-D-galactoside
(X-Gal; 25 mg 1.25 ml dimethylformamide, stored at 4°C)

10.0 μl of 100 mM isopropyl-b-D-thiogalactopyranoside
(IPTG; 23.8 mg/ml of water, stored at -20°C.)

200.0 μl lawn cells (DH5aF' lawn cells in log phase of growth (generally a 2-3 hour culture from an overnight inoculum))

Once the transformation mixture was added to the YT soft agar mixture, the entire contents were mixed gently by swirling and then poured onto a YT plate (prewarmed to 37°C). This was spread evenly by tilting the plate as necessary. The YT agar plate was left at room temperature for 10 minutes to allow the soft agar top to solidify before being placed overnight in a 37°C dry incubator. Plaques with recombinant phage were clear, while the nonrecombinant phage gave blue plaques.

pUC recombinant molecules (Huff et al. 1990): 10 ng to 1.0 μg of ligation mix was added to a total volume of 10-25 μl TE (a drop placed at the bottom of a sterile 12 mm x 75 mm glass tube). A 100 μl volume of freshly prepared competent cells was added to the drop of transforming DNA. The glass tubes were placed on ice for 10 minutes. After this incubation period, the
transforming mixture was heat shocked for 2 minutes at 42°C. 1.0 ml of sterile liquid YT medium was added to the glass tube containing the transforming mixture. This was allowed to incubate for 1 hour in a gyrorotatory shaker at 37°C and 250 rpm. Whilst the transforming mixture was incubating, 10 μl of 10 mM IPTG and 50 μl of 2% X-Gal was spread evenly on to an LB (50 μg ampicillin/ml) agar plate with a glass "hockey stick". The plates were placed at room temperature for 10 minutes to allow the X-Gal and the IPTG to soak into the agar. When the transforming mixture had completed its one hour of incubation, 10 μl and 100 μl aliquots were spread onto two separate LB/amp50/X-Gal/IPTG agar plates that were prepared previously. The transformed cells were spread as above and allowed 10 minutes at room temperature before being placed at 37°C in a dry incubator.

Colonies of ampicillin resistant *E. coli* DH5α grew on the plates. Recombinant plasmids were seen as white colonies. Nonrecombinant (vector with no insert) plasmids produced blue colonies.

*Screening of recombinant plasmids and phage*

Transformants carrying the pUC vector systems were screened on the basis of color formation and ampicillin (50 μg/ml)
resistance. The transformants carrying the M13 vector systems were screened on the basis of color formation. Both the vector systems produced white colonies for recombinant molecules and blue colonies for nonrecombinant vectors. An LB/Ampicillin (50 μg/ml)/ X-GAL/IPTG agar plate was prepared and placed on a 50 space screening grid or vinyl covered patching guide that could be seen in Fig. 8). A small piece of tape was placed at the bottom to prevent the plate from shifting position while work was in progress. A sterile toothpick was used to carefully touch a single colony on the transformation plate. A "short streak" was made over the #1 space of the grid. This procedure was repeated up to 50 times until all recombinant colonies were patch screened. The screening plates were transferred to a 37°C incubator and left overnight.

Rapid analytical scale isolation of plasmid DNA by the alkaline lysis technique (Birnboim and Doly 1979)

Once the screening plate colonies had grown overnight a few cells were inoculated from each streak into separate 5 ml LB liquid medium (ampicillin 50 μg/ml) culture tubes and allowed to incubate overnight at 37°C and 250 rpm. After the overnight incubation, 1.5 ml of the culture medium from each culture tube was placed into sterile 1.5 ml microfuge tubes and centrifuged
Fig. 8. Replica plate used in the screening of recombinant plasmids. The plates contained ampicillin with a final concentration of 50 µg/ml when recombinant plasmids with pUC18/19 vectors were used. In both cases of vectors that is with pUC18/19 or M13 the plates were spread with 60 µl of 2% X-Gal and 10 µl of 100 mM of IPTG.
for one minute at 14481 x g. The remainder of each of the overnight cultures was stored at 4°C. After the cells were centrifuged, the supernatant was removed by aspiration, leaving the bacterial pellet as dry as possible. The dry pellet was resuspended by mixing thoroughly in 100 µl of solution A (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl pH 8.0, 6.0 mg/ml lysozyme) and stored at room temperature for 5 minutes. After the incubation at room temperature, 200 µl of freshly prepared solution B (0.2 N NaOH, 1.0% SDS) was added. This was followed by one or two quick inversions of the tubes (vortexing at this point is not recommended). The microfuge tubes were allowed to incubate on ice for 5 minutes. After the incubation on ice, 150 µl of ice-cold potassium acetate, pH 4.8, was added and the tubes inverted one or two times (vortexing at this point was avoided). The tubes were then allowed to incubate on ice for 5 minutes. The microfuge tubes were centrifuged for 5 minutes in a chilled microfuge centrifuge at 14481 x g. The supernatant was transferred to a new sterile microfuge tube (the pellet was discarded) and 450 µl of a 1:1 solution of phenol:chloroform was added. The contents were then mixed by vortexing. The tubes were centrifuged for 2 minutes at 14481 x g. The supernatant was then removed and placed into another fresh microfuge tube. To this supernatant was added two volumes of 100% ethanol at
room temperature. The tubes were mixed thoroughly and then left at room temperature for 2 minutes. The microfuge tubes were then centrifuged for 2 minutes at 14481 x g at room temperature. The supernatant was removed and the tubes placed in an inverted position on a dry paper towel to allow all of the fluid to drain away. After this period 1 ml of 70% ethanol was added to the pellet and the mixture vortexed briefly before being centrifuged for 5 minutes at 14481 x g. Once again all of the supernatant was removed and the pellet dried in a vacuum dessicator. To the dried pipette was added 30 µl of TE (pH 8.0) with 25 µg/ml heat treated RNase A. The mixture was vortexed to put the DNA into solution and then stored at -20°C until needed for further analysis. The DNA at this point can be digested with restriction enzymes and analysed on an agarose or polyacrylamide gel.

**Agarose minigel gel electrophoresis**

An acrylic gel tray is sealed at its ends with masking tape and a slot former was placed at one end of the tray ("squared" to the tray). The agarose solution was placed in to the tray until it was 75% full (vertically). The solution was allowed to cool (gel) for about 45 minutes. Preparation of the agarose solution was as follows:
5X TBE Agarose gel electrophoresis running buffer.

54.0 g Tris base
27.5 g boric acid
20.0 ml 0.5 M EDTA, pH 8.0 (ethylenediaminetetraacetate, disodium form)
distilled water up to 1 liter total volume

Agarose Gel Solution (for preparation of 1% agarose gel).

1.0 g Electrophoretic grade agarose
20.0 ml 5X TBE agarose gel electrophoresis running buffer
80.0 ml distilled water

The contents of the agarose gel solution were placed into a 250 ml Erlenmeyer™ flask and weighed. A piece of Saran™ wrap was placed over the top of the flask and was then placed into a microwave oven for 1 minute. The flask was removed, the Saran™ wrap was removed and the flask weighed again. The lower difference in weight, due to evaporation of distilled water, was made up by adding distilled water until the original weight was reached. The agarose solution was allowed to cool to approximately 65° C before being poured into the gel tray.

The slot former was removed from the gel and the formed agarose gel, including the tray, was placed into the
electrophoresis tank. The tank was filled with 1X TBE agarose gel running buffer until the level of the buffer was 1-2 mm higher than the top of the gel.

5X agarose gel electrophoresis loading buffer was prepared as follows.

- 25.0% glycerol
- 0.5% SDS
- 0.1% bromophenol blue
- 0.1% xylene cyanol
- 50.0 mM EDTA

The 5X agarose gel loading buffer was added to the prepared restriction digests such that the final concentration of the loading buffer was 1X. The restriction digests in loading buffer were loaded in the gel slots using a hand held micropipettor. Electrophoresis was carried out at 80 volts for a 1 hour duration.

After the electrophoresis was complete the gel was stained in a gel staining solution (500 ml 1 µg ethidium bromide/ml and 500 ml distilled water) for approximately 30 minutes. Once the staining procedure was completed the gel was destained for 5-19 minutes in a gel destaining solution (1.0 mM MgSO₄).

The destained gel was examined under ultraviolet
illumination (approximately 300 nm wavelength). If it was necessary, the gel was photographed using an MP-4™ transilluminator system.

**Preparative scale isolation of cloned DNA fragments**

*(Restriction endonuclease digestion of recombinant plasmids)*

Restriction digestion on a preparative scale was carried out as follows:

- 100.0-155.0 μl plasmid DNA (approximately 150-250 μg)
- 20.0 μl 10X reaction buffer as provided by the manufacturer
- 25.0 μl (250 units) of restriction enzyme
- 0.0-100.0 μl distilled water
- 250.0 μl total volume

These volumes are approximate in the sense that they can be scaled up if the concentration of the DNA is especially dilute. However, the more concentrated the solution the better (optimal final DNA concentration is 0.5 mg/ml).

The restriction digest was incubated at the specific temperature and time period as indicated by the manufacturer. Once the restriction digestion was complete, the buffer salts were removed by the following procedure.
Approximately one-tenth volume of 3 M sodium acetate was added to the restriction digest. After the sample was mixed thoroughly, approximately 3 volumes of ethanol was added and the solution mixed thoroughly again. The ethanol precipitated DNA was then centrifuged for 15 minutes at 14481 x g in a chilled microcentrifuge (4°C). The supernatant was removed using a drawn out 9" Pasteur™ pipette. Five hundred microliters of 70% ethanol was added to the pellet and the microfuge tube mixed by inversion two or three times times. The mixture was then centrifuged for 5 minutes at 14481 x g (4°C). The supernatant was again removed using a drawn out 9" Pasteur™ pipette. To the pellet was added 150 μl of 1x agarose gel electrophoresis loading buffer. The contents of the microfuge tube were mixed thoroughly and frozen. The sample was then allowed to thaw at 65°C to ensure that the DNA was well dissolved. Vortexing as necessary was done and the entire procedure would be repeated if required. The sample was stored at -20°C until ready for electrophoresis.

**Vertical agarose gel electrophoresis**

A. Glass plate cleaning and siliconization procedure.

The gel cassette consists of one 20 cm X 20 cm and one 20 cm X 22 cm glass plate. Each of the glass plates was
placed on four identical sized rubber stoppers, in order to prevent sliding of the plates during cleaning. The plates were thoroughly cleaned with glass cleaning solution (Windex™ in a spray bottle). The plates were then wiped with non-denatured 95% ethanol to remove any residual vacuum grease from previous electrophoretic runs. The plates were then wiped with a Kimwipe™ dampened with a solution of 1% dimethyldichlorosilane in heptane and allowed to dry at room temperature.

B. Cassette assembly.

Vacuum grease was applied to two Delrin™ spacers (20 cm X 1.0 cm X 1.5 cm) such that the grease covered both sides of the spacers but only on half of each side. The greased spacers were then carefully laid onto the sides of the 20 cm X 22 cm plate such that they were flush with the bottom and sides of the plate. A 18.5 cm X 1.0 cm X 5 mm piece of cellulose sponge was then soaked in a 1X agarose TBE buffer. All air bubbles from the sponge were removed by compressing the sponge while it was submerged in buffer. This sponge was then laid at the bottom of the 20 cm X 22 cm plate allowing it to overhang 2 mm from the bottom. The 20 cm X 20 cm plate was then placed over the sponge/spacer/plate assembly and the two plates were clamped together along the sides with three
medium clips. The Delrin™ spacers were then pressed in at the bottom to remove any space between them and the sponge spacer.

C. Clamping gel cassette into the vertical gel apparatus.

The assembled gel cassette was placed into the lower buffer chamber. A small amount of vacuum grease was applied to the bottom of two neoprene sponge spacers (1.0 cm X 2.0 cm X 6.0 mm) and these were placed onto the top of the Delrin™ spacers of the gel cassette. The three medium sized clips were moved to the center of the glass plates such that a large size binding clip could be used to clamp the top of the gel cassettes to the top buffer chamber. The neoprene spacers were then firmly pushed down to ensure that they were well seated. Adjustments were made to the large clips (by moving them up or down slightly) to ensure that the gel cassette was stood in a true vertical position.

D. Insertion of the well forming comb.

Vertical agarose gel combs were no more than 18 cm wide and had well slots at least 2.0-2.5 cm deep (a thickness of 1.5 mm was used). The comb was inserted about 1.5 cm into and between the gel cassette plates (leaving a gap between the top of the 20 cm X 22 cm back plate and the top of the slots of the slot former teeth) while it was on the gel
stand. The width of the preparative gel slots were determined by the amount of DNA to be applied (typically about 500 μg of purified plasmid digest was added to a 7-8 cm wide well).

E. Pouring of agarose into the gel cassette.

A 1% agarose gel solution in 1X agarose TBE running buffer solution was prepared as described previously. The bottom chamber of the electrophoresis apparatus was filled with the running buffer up to the top of the sponge gel cassette spacer (this level was not exceeded as this would have allowed buffer to run into the gel cassette). A 10 ml pipette was used to carefully pour 10-12 ml of agarose solution along the side of one spacer and into the bottom of the gel cassette. Care was taken not to introduce air bubbles into the agarose. The buffer level in the lower chamber was then increased to the top but not above the level of the agarose solution. The agarose solution was allowed to gel completely before the remainder of the cassette was filled using a 25 ml pipette. The agarose solution in the cassette was then allowed to cool. A cloudy coloration was noted as gelling was complete. The comb at the top was then removed carefully so that the well "teeth" were not disrupted.
F. Electrophoresis and buffer circulation system.

The upper and the lower buffer chambers were filled with 1X TBE agarose gel electrophoresis buffer. A circulation pump was connected such that buffer from the lower chamber was circulated into the upper chamber. The "overflow spillway" in the rear of the upper chamber returned excess fluid to the lower chamber, hence creating a completed circuit of buffer flow. A diagrammatic view of various aspects of the assembled electrophoresis unit can be seen in Fig. 9 and Fig. 10.

Once the circulating buffer circuit was set up, a voltage of no more than 80 volts was applied for a time period that varied according to the size of the DNA fragments to be separated. Five to six hours were required for the migration of the bromophenol blue dye to reach three-quarters of the distance down the gel.

Once the vertical agarose gel was ready to be stopped, the current was turned off along with the circulation pump. The gel cassette was removed from the electrophoresis chamber and the two glass plates were pried from each other, such that the gel adhered to one of the plates.
Fig. 9. Schematic representation of the assembly of a gel cassette. A cellulose sponge was used for vertical agarose gel cassette whereas a spacer was used in its place for the pouring of non-denaturing polyacrylamide gel cassettes.
Fig. 10. Schematic representation of a vertical agarose gel set for electrophoresis. The gel cassette was placed into the electrophoresis chamber and a pump connected such that electrophoresis running buffer was circulated from the lower to the upper chamber and returned by the overflow spillway.
Detection of DNA by ultraviolet shadowing

The gel was transferred from the glass plate to a piece of Saran™ wrap. The plastic wrap preparation was then laid across a thin layer chromatography plate impregnated with ultraviolet fluorescent indicator. A short wave (approximately 260 nm wavelength) hand held ultraviolet lamp was held over the agarose gel. The ultraviolet radiation caused the fluorescent indicator in the thin layer chromatography plate to fluoresce visible light, giving the plate a bright glow. However, DNA in the gel absorbs the ultraviolet radiation before it strikes the plate, which in turn leaves dark bands at those locations (A diagrammatic view of the procedure can be seen in Fig. 11). A sharp razor blade was used to cut out the dark bands of interest. The DNA contained in the gel slices was recovered from the agarose by electroelution (gel slices were placed at 4°C for storage).

Recovery of DNA from agarose gels by electroelution

A dialysis clip was placed at one end of a dialysis tubing (6 in. long and 10 mm in diameter) and approximately 500 µl of 0.25X agarose TBE was placed inside the dialysis tubing. A narrow spatula was used to insert the agarose gel
Ultraviolet light

Gel Containing DNA

Fluorescent Plate

Visible Light Produced (Bright Areas)
Fig. 11. Schematic representation of the ultraviolet radiation shadowing technique. This procedure was utilized for viewing DNA fragments that were to be cut from preparative gels for DNA fragment purification. The ultraviolet rays cause a specially prepared plate to fluoresce, while DNA fragments prevent this fluorescence resulting in a darkened area.
slice into the dialysis tubing. The open end of the dialysis tubing was secured with another clip. The dialysis tubing was then placed into a horizontal electrophoresis unit which had already been filled with 0.25X agarose gel running buffer. The level of buffer was adjusted to about 1-2 mm above the surface of the dialysis tubing. An electric field of 80 volts was applied across the tubing for two hours (A diagrammatic view of the electroelution procedure can be seen in Fig. 12. Upon completion of the electroelution, the current was reversed (the electrical leads were exchanged at their poles) for 15 seconds. The dialysis tubing was removed from the electrophoresis unit and the agarose gel in the tubing was moved around against the sides to further aid in the dislodgement of the DNA from the sides of the tubing. A siliconized 9" Pasteur™ pipette was used to remove the TBE buffer from the dialysis tubing. The buffer was transferred to a microfuge tube. The dialysis tubing was rinsed with 100 µl of 0.25X TBE buffer and the rinse was transferred to the microfuge tube with a Pasteur™ pipette. Phenol extraction and the ethanol precipitation was carried out on the electroeluate according to the procedures outlined previously.

The ethanol precipitated DNA pellet was resuspended in
Fig. 12 Isolation of DNA from agarose gel slices by the electroelution technique. The agarose strips containing the DNA fragments were placed in a dialysis bags and laid horizontally in an electrophoresis chamber. DNA migrated from the negative to the positive poles and remains in the bags but out of the agarose gel slices.
25 µl TE buffer. The DNA purity and yield was verified according to the qualitative and quantitative methods for determining DNA concentration outlined previously.

Nondenaturing polyacrylamide gel electrophoresis

A gel cassette of the same dimensions and preparation used for vertical agarose was used for non-denaturing polyacrylamide gel electrophoresis. The only difference was that no sponge was required at the bottom of the gel cassette and the electrophoresis unit did not require a spillway on the upper chamber, hence no circulation of buffer was required.

A stock solution of 40:1 acrylamide/bisacrylamide was made as follows:

- 40.0 grams of electrophoretic grade acrylamide
- 1.0 gram of electrophoretic grade bisacrylamide
- distilled water up to 100 ml

A stock solution of 10X non-denaturing polyacrylamide gel electrophoresis running buffer was prepared as noted below

- 500.0 mM tris pH 8.3 with boric acid (solid)
- 10.0 mM EDTA

A stock solution of 5X non-denaturing polyacrylamide gel
electrophoresis loading buffer was prepared as listed below.

50.0% glycerol
25.0 mM EDTA, pH 8.0
0.5% SDS (sodium dodecyl sulfate)
0.25% xylene cyanol
0.25% bromophenol blue

The nondenaturing 6% polyacrylamide gel solution was prepared as follows:

12.0 ml. 40:1 acrylamide:bisacrylamide
8.0 ml. 10X nondenaturing polyacrylamide running buffer
60.0 ml distilled water
0.1 gms. ammonium persulfate

The gel solution was poured into a 250 ml vacuum flask (filtration type) and the solution degased in vacuo for several minutes. After the solution was degased, 20 µl of TEMED was added and the solution was swirled rapidly (the solution was not mixed violently, as this would have reintroduced oxygen into the solution which would have inhibited polymerization of the gel). The gel solution was carefully poured into the assembled gel cassette and allowed to polymerize for approximately two hours. After this time the gel was preelectrophoresed for a one hour period at 200 volts (constant voltage). During this period 10 µl of DNA
sample was placed into a microfuge tube with 2.5 μl of 5X nondenaturing polyacrylamide gel electrophoresis loading buffer, giving a final concentration of loading buffer of 1X. The samples were mixed thoroughly and briefly centrifuged at 14481 x g. The samples were then loaded gently onto the gel using a 25 μl Hamilton™ microsyringe. Electrophoresis at 250 volts was carried out until the bromophenol blue dye had migrated to about 80% of the length of the gel. When the bromophenol blue dye had migrated its desired distance, the current was shut off, the gel cassette was removed from the electrophoresis unit and pried apart. The gel was transferred to a piece of nylon screen and placed in the ethidium bromide staining solution (preparation stated previously) for approximately 15 minutes. After the staining procedure the gel was destained for 5 minutes in a destaining solution (preparation previously described). At this point the DNA stained with ethidium bromide was clearly visualized by placing the gel slab onto an ultraviolet light table. If necessary the gel was photographed for later analysis and experimental record.
Preparation and electrophoresis of polyacrylamide sequencing gels

The polyacrylamide gels used were designed to detect and resolve radioactive DNA fragments differing in a size by a single nucleotide from the bands immediately above and below it. All the bands on the gel possess one end in common (the primer end) and one end that is different from any other band in the same fragment set (lane). There are four major factors affecting the resolution of band fragments on DNA sequencing gels (other than successful sequencing reactions). First, the diffusion of DNA samples prior, during and after electrophoresis. Second, the purity of the polyacrylamide gel, no contaminants must be present. Third, the intensity/strength of the radioactive emissions (beta particles for $^{35}$S-labelled samples) can affect resolution. Fourth, the actual vertical distance separating the bands (determined by the distance the gel is allowed to run) affects visual resolution. Diffusion is prevented most effectively by quickly loading the gel and applying voltage as soon as possible. Diffusion is also prevented during electrophoresis by high voltage (shorter running times) and after electrophoresis by drying or freezing. Gel purity was maintained by using molecular biology grade chemicals or
better. In addition, the solution was filtered to remove any solid materials which may retard or distort DNA migration. The intensity of the radioactive emissions was enhanced by using a thinner gel as this reduced the distance between the source of the emissions and the X-ray film. The thinness of the gel also allowed for an increased voltage to be applied across the gel (less wattage and heating) and hence the time for the actual run to be decreased.

Preparation of polyacrylamide sequencing gels (Sanger and Coulson 1978)

Sequencing gels were poured on the day prior to electrophoresis. This allowed for complete polymerization of the gel. Longer periods of polymerization can lead to the edges becoming dried out and are therefore avoided.

A. Assembly of the gel cassette.

Two glass plates (52 cm X 41 cm X 0.6 cm) were laid on four to six rubber stoppers. The glass plates were thoroughly cleaned with a commercial glass cleaner and then wiped with ethanol and left to completely dry. One side of each plate was labelled as the outside. The inside surfaces of both plates were then coated with a solution of 2% dichloromethylsilane dissolved in heptane. Two or three
coats were generally necessary to ensure that the plates were fully covered. The plates were then placed in a drying oven set at 100°C for 15 minutes. The plates were then placed on the rubber stoppers on the laboratory bench to cool. At this point the two glass plates were placed one on top of another such that they were offset by 13 mm (0.5 inches) lengthwise with the siliconized surfaces facing each other. One side of the top plate was lifted and a 51 cm X 13 mm X 0.25 mm Delrin™ spacer was placed between the edge lengthwise. The sides of the plates were then clamped together using 6-7 medium size binding clips. Another spacer was then inserted into the other lengthwise edge of the glass plates and secured in a similar manner. A third spacer (45 cm X 7 mm X 0.25 mm) was then inserted into the bottom of the cassette such that the bottom and the side spacers are flush with each other and secured into the place with four large binding clips. The bottom of the cassette would become the top of the gel when electrophoresis was carried out. One end of another piece of spacer material was then inserted into the top of the cassette (not more than 1 cm into the gel cassette) midway across the width of the cassette and secured into place with a large binding clip. A "funnel reservoir" was constructed across approximately one-third of the width.
of the gel cassette with warmed Plastocene™. The funnel was slightly higher at the outside edge and it tapered off as it approached the center of the cassette. This allowed a greater amount of polyacrylamide solution in the most desirable area. The polyacrylamide gel solution was poured along the side of the gel such that it ran down one side of the cassette. This prevented the trapping of air bubbles.

B. Pouring the sequencing gel.

125 ml of 6% polyacrylamide sequencing gel solution was prepared in a 250 ml beaker by adding 53 grams urea, 25 ml of 30% acrylamide/1% bis-acrylamide and 12.5 ml 10X TBE sequencing buffer. This was brought up to a total volume of 125 ml with distilled deionized water. The first three ingredients were first dissolved and then the volume brought up to a final volume of 125 ml (a small degree of heat was applied to dissolve the urea but the temperature was brought down to room temperature before the gel was poured).

Any undissolved particles were then removed from the gel solution by filtering the solution through a Buchner™ funnel which contained a piece of MFS™ or Whatman™ No. 1 qualitative filter paper. The filtrate was then collected in a 250 ml Erlenmeyer™ vacuum filtration flask. To this filtrate was added 0.15 grams ammonium persulfate. The flask
was then covered with a rubber stopper and allowed to degas
in vacuo until all dissolved gases were removed
(approximately 3-5 minutes of vacuum). The degased solution
was removed from the vacuum and placed into a 250 ml beaker.
25 μl of TEMED (N,N',N" tetramethylene diamine) was now added.
The solution was gently but thoroughly swirled manually
(being careful not to introduce air bubbles as this would
inhibit the polymerization of the gel) and poured into the
gel cassette. The acrylamide solution polymerized quickly
(approximately 20-30 minutes) and therefore was poured
immediately. During pouring the gel cassette was placed at
an incline of approximately 45° and was slightly tilted
towards oneself such that the solution was encouraged to flow
evenly down one side of the apparatus. This procedure helped
to prevent the trapping of air bubbles between the plates.
If air bubbles did form, then the gel cassette was tilted to
one side or the other to allow the level of the solution to
fall below the air pocket breaking the bubble. When the
cassette was approximately 80% full it was laid onto the
rubber stoppers and allowed to polymerize (this took
approximately 20-30 minutes). When the polymerization
procedure was complete, the exposed end of the gel cassette
was covered with Parafilm™ in order to prevent the gel from
drying out (Neither the clip nor the spacer at this end was removed).

C. Loading and electrophoresing the DNA sequencing gel.

The Parafilm™, binding clip and the Delrin™ spacer were removed from the top, together with the four binding clips and the spacer at the bottom of the gel. A surface temperature thermometer was attached to the front glass plate with vacuum grease and transparent tape. The gel cassette was placed into a sequencing electrophoresis stand such that the bottom of the gel during the pouring of the acrylamide solution was now at the top end of the stand. The upper and the lower chambers of the sequencing stand were both filled with 1X TBE sequencing buffer such that the buffer submerged both open ends of the gel cassette (care was taken to ensure that there were no air bubbles between or under the glass plates of the gel cassette). The 10X stock solution (1 liter) of gel sequencing buffer was prepared as follows: 100.0 mM tris base, 12.0 mM boric acid and 1.0 mM EDTA. Electrical leads connected to both the top and the bottom compartments and to a 3000 volts electrical supply unit. The gel was preelectrophoresed for one hour at 1700-2300 volts such that the temperature was not allowed to rise above 55°C. At the end of the preelectrophoresis the power supply
was turned off and any air bubbles accumulated during the preelectrophoresis were removed. Shark's teeth well forming comb units were inserted in between the plates until the teeth just touched the top surface of the gel. The electrical supply unit was turned on while the samples were prepared for loading. The samples were placed into a 90°C water bath for two minutes (this eliminated any intra-strand base pairing that might have influenced the migration rate. Samples were prepared in sets of 4-5 (each set containing 4 reaction tubes such that after the two minute incubation at 90°C the set of samples were transferred to a ice-water bath at 0°C. When the samples were ready to be loaded onto the gel the power supply was turned off a 10 μl Hamilton™ syringe with a 32G needle was used to transfer 0.5 μl of each sample into each of the wells. Samples were loaded in the order of GATC such that after each particular reaction the syringe was rinsed once or twice with running buffer before the next reaction mixture was loaded. One microliter of loading buffer (contained no DNA) was added to the left of the left most well followed by the loading of one microliter to the right of the right most well at the end of samples to be loaded. The time required for the electrophoresing of DNA sequencing gels depended upon the number of bases one desired
to read. As an indicator xylene cyanol was used as this dye migrated at about the same rate as a polynucleotide 70 bases long on a 6% polyacrylamide gel (if a longer sequence of nucleotides was desired to determined (i.e. farther from the primer hybridization site) then an aliquot of loading buffer was run for most of the length of the gel followed by another aliquot of loading buffer to monitor the migration distances.

 Autoradiography of sequencing gels

When the xylene cyanol had migrated the required distance down the length of the gel, then the power supply was turned off and the electrical leads disconnected from the electrophoresis unit and the power supply. The gel cassette was removed from the electrophoresis unit and placed onto the lab bench. All clips and spacers were removed and a thin spatula was inserted into one corner between the glass plates such that the top glass plate was lifted upwards. A piece of MPS™ filter paper was placed on the top of the gel such that it was lined up with the bottom of the gel and centered side to side (care was taken to ensure that all sample lanes were covered). Air bubbles between the gel and the filter paper were squeezed out and the excess acrylamide gel was trimmed away with a sharp razor blade. The gel adhered to the filter
paper surface and was removed from the surface of the glass plate. A piece of Saran™ wrap was stretched on the lab bench (transparent tape was used to stretch out the plastic wrap such that there were no wrinkles) and the filter paper with gel was positioned onto the plastic wrap with the gel in contact with the plastic wrap surface (excess plastic wrap was trimmed away with a sharp razor blade). The gel with the plastic wrap and the filter paper was then transferred to gel slab dryer and left under vacuum for one hour at a temperature of 80°C. At the end of this drying period the gel has been fixed onto the MPS™ filter paper such that the Saran™ wrap can be pulled clean away. The filter paper with the fixed gel was then placed into an autoradiography cassette followed by a piece of Kodak™ XAR-5 X-ray film (all work done in a dark room to prevent exposure of the X-ray film) and then closed with all the flaps of the autoradiography cassette in place. The cassette was then placed at room temperature under about 40-50 pounds weight for an exposure time of three days.

_Gel photography_

Once the gel (agarose or polyacrylamide) was stained and destained it was viewed under ultraviolet radiation
(protective goggles were worn). The gel was placed onto the plastic protective surface of the Fotodyne system™ (the gel tray was removed as the tray is ultraviolet opaque) and all air bubbles between the gel and the transilluminator surface were removed. The visible flood lights were turned on and the lens cap from the camera was removed. The red filter was swung out of place from between the lens and the gel. The camera aperture was opened to f/4.5 (this allowed the greatest amount of light to enter which allowed for easy focusing) which gave an exposure time of 1 minute. The camera height was adjusted so that it allowed the entire gel or region of interest to be within the field of view. Once the camera height was finalised, it was locked into position by a locking knob. The camera body was then swung to the right of the MP-4 system™ to bring the focusing screen over the lens, allowing the size of the area to be photographed to be adjusted. The red filter was returned to its original position between the gel and the lens. The lever on the right side of the camera was moved to the "L" or load position and the film was inserted into the camera until a click was heard. At this point the film was slid out (the film was out of the envelope and ready for exposure) and the camera body slid to the left of the MP-4 system™. This
placed the camera/film over the lens and in line of exposure. The film was exposed for one minute, at the end of which the ultra violet light was switched off and the camera body slid to the right of the MP-4 system™. The lever to the right side of the camera body was moved to the "P" or process position and the film was pulled from the camera body. The film was allowed to develop for 15-20 seconds and the film envelope was torn apart. The positive photograph was peeled back and coated with a fixer/hardener solution provided by the manufacturer. The negative (which gave a greater range of sensitivity for both over and under exposures) was conditioned for long term storage by soaking in 9% sodium sulphite.

_Dideoxy sequencing-Sanger method (Sanger et al.1977)_

The central concept of the Sanger method was that a complementary strand of DNA was enzymatically synthesised from a DNA template. The polymerase reaction is terminated by the incorporation of a dideoxynucleotide (ddNTP), which is an analog of a nucleotide (dNTP). Four enzymatic reactions are performed simultaneously with each of the reactions containing a separate dideoxynucleotide (ddGTP, ddATP, ddCTP, ddTTP) such that four distinct types of fragments are created.
each terminating in its own specific type of

dideoxynucleotide. These fragments can be electrophoresed on
a polyacrylamide gel and the different fragments can be
resolved by a difference of one nucleotide base pair.
Therefore the sequence of the DNA can be elucidated by
reading from the shortest fragment (bottom of the gel)
upwards to the largest fragment (top of the gel). The actual
enzymatic synthesis of the DNA fragments requires a
single-stranded DNA template, a primer that binds
specifically to a region of the single stranded template, a
polymerase that performs the elongation reactions, the
dideoxynucleotides triphosphates that are incorporated under
normal chain elongation and the dideoxynucleoside
triphosphates which terminate the elongation reaction when
incorporated.

The M13 is an E. coli male filamentous bacteriophage
(Messing et al. 1977) which attaches to the pilus of the
bacteria for infection. The virus contains single stranded
circular DNA (+) that serves as a template for the synthesis
of a complementary strand (−) in the host cell. This form of
the double-stranded DNA is called the replicative form. It
is this replicative form that undergoes amplification to
approximately 200 copies per cell. At the end of the
amplification stage of infection, the (-) strand is not synthesised but serves as a template for the synthesis of the (+) strand which is incorporated into a new assembled phage particle. The phage is expelled from the E. coli host via a non-lytic mechanism such that invivo observations show turbid "plaques" are observed rather than clear "plaques" indicating a reduced rate of growth of the infected E. coli cells.

DNA sequencing involves the insertion of the double strand target DNA into the M13 multiple cloning site (MCS). Screening of the newly created recombinant DNA involves colorimetric analysis that involves b-galactosidase which is encoded for by one of the enzymes of the lac operon. The protein encoded for by the lacZ gene of the lac operon is b-galactosidase which exists as a tetramer. The colorless substrate analog, 5-bromo-4-chloro-3-indoyl-b-D-galactoside (X-Gal), is converted to a blue chromophor and this gives visual indication of b-galactosidase activity. A mutation in the lacZ gene at the 5' end produces a non-functional enzyme which retains the ability to assemble as a tetramer. However, if this protein associates with the N-terminal portion of the enzyme that is missing the b-galactosidase activity is resumed. This association is termed as
a-complementation (Messing et al. 1977). a-complementation is facilitated by the particular \textit{E. coli} strain being used, in this work the \textit{E. coli} DH5\textalpha{}F' strain. The chromosomal DNA of the bacteria and the episome (F' plasmid) are both modified such that the genes encoding for enzymes involved in proline biosynthesis were deleted from the chromosomal DNA and inserted into the F' plasmid. The \textit{lacZ} gene from the chromosomal DNA is also deleted from the chromosome and a mutant form of the \textit{lacZ} gene (\textit{lacZ} DM15) that encodes for the N-terminal portion of an enzyme which interacts with the deleted \textit{lacZ} gene in the M13 genetically engineered phage (a-complementation) is inserted into the F' plasmid. Therefore the F' plasmid must be present for growth on minimal media. The F' plasmid also contains a \textit{lacI}\textgreek{G} mutation that encodes for a repressor, which allows the \textit{lac} operon to be regulated with an inducer analog isopropylthio-\textit{b-D-galactoside} (IPTG).

The multiple cloning site (MCS) of the M13 phage is a clustering of restriction endonuclease sites. A "universal" oligonucleotide primer is hybridized to a region adjacent to the multiple cloning site such that sequences of the DNA inserted into the multiple cloning site can be deduced (Heidecker et al. 1980; and Anderson et al. 1980).
polymerase initiates synthesis at the 3' end of the "universal" primer and continues elongation in the 5'--> 3' direction. The primer, insert or even the site into which the insert was placed are different and the M13 phage are constructed in pairs such that the multiple cloning sites of the each pair are in different orientations with respect to the primer hybridization region. This last feature permits cloning and hence sequencing of both strands of the target region/fragment.

The polymerase employed is the Klenow fragment of T4 DNA polymerase. This fragment of the native polymerase does not possess the 5'--> 3' exonuclease activity and hence cannot correct for the incorporation/termination of the dideoxyribonucleoside triphosphates.

The M13 phage system has several advantageous features including
1) The presence of the F' plasmid, necessary for infection, is ensured by the modifications in the bacterial chromosome and the F plasmid itself.
2) Recombinant forms of the M13 phage, carrying DNA inserted into the MCS, can be visually monitored by the β-galactosidase α-complementation activity.
3) Due to the M13 phage being filamentous it can accommodate
reasonably large fragments (1000-3000 bp) into the MCS, hence the cloning of recombinants are not constrained by insert size.

4) The replicative form of the M13 phage has a high copy number, hence large quantities can be readily acquired for use in ligation reactions.

**Preparation of single strand recombinant M13 phage (Schreier et al. 1979)**

Five milliliters of liquid YT medium was inoculated with *E. coli* strain DH5αF'. The inoculate was allowed to incubate overnight at 37°C with shaking at 250 rpm. A 1:50 dilution of an overnight culture was obtained by adding 100 μl of the overnight culture to 5 ml of YT liquid medium (the number of 5 ml YT tubes that were inoculated depended upon the number of clones (plaques) being processed. Each of the 1:50 diluted YT tubes were inoculated with an agar plug that was picked from a YT agar plate and contained a single isolated clear recombinant M13 plaque. A sterile 9" Pasteur™ pipette was used to transfer the plaque to the media. Care was taken to utilize only isolated plaques since contamination from other recombinant or non-recombinant plaques would give uninterpretable DNA-sequence data (more
than one sequence overlapping in the same lanes). The YT tubes with the 1:50 diluted overnight culture and the agar plugs were allowed to incubate at 37°C with shaking at 250 rpm for 5 hours. After this incubation period, 3.5 ml from each YT tube was transferred to 5 ml polypropylene tubes. These polypropylene tubes were centrifuged at 14481 x g for 8 minutes in a table top microcentrifuge. The supernatant was transferred to a new polypropylene tube, being careful not to disturb the pellet. The pellet contained cells with the RF DNA and was stored at 4°C for later small scale preparations of specific clones. The supernatant contained the single-stranded phage template. This can also be stored at 4°C if it is not to be used immediately. The supernatant was centrifuged again at 14481 x g for 8 minutes to ensure complete removal of the RF DNA/bacterial cells. The supernatant was once again transferred to a new polypropylene tube and to this was added 900 μl of a solution of 20% PEG (polyethylene glycol) and 2.5 M NaCl. This mixture was mixed thoroughly and left at room temperature for 15 minutes. After this period, the polypropylene tube was centrifuged at 14481 x g for 10 minutes in order to pellet the phage particles. The supernatant was removed by using a sterile 9" Pasteur™ pipette, being careful not to disturb the visible
pellet. The tube was then inverted and the lip of the tube was wiped with a paper towel. The phage pellet was resuspended by vortexing in 100 μl TES buffer (20 mM Tris-HCl pH 7.5; 10 mM NaCl; 0.1 mM Na₂EDTA). The phage proteins were extracted by adding 50 μl of phenol-saturated with TE buffer and mixing thoroughly (the yellow phenol layer is at the bottom). The tube was then centrifuged for 2 minutes at 14481 x g to separate the aqueous and phenol phases, the latter phase being removed by a mechanical pipettor set at 80 μl. The top aqueous layer was thoroughly extracted with 50 μl of chloroform. The mixture was centrifuged for 2 minutes at 14481 to separate the aqueous and the organic phases. The aqueous phase was removed with a mechanical pipettor set at 80 μl and placed into a 1.5 ml microfuge tube. Care was taken not to include the interphase and the lower phase, as these contain organic solvents which would inhibit sequencing reactions. Nine microliters of 3 M sodium acetate was added (300 mM final concentration) and mixed thoroughly. Two hundred microliters of 100% ethanol was then added and mixed well. The microfuge tube was placed at -70°C for 15 minutes in order to precipitate the DNA. After this incubation, the DNA was collected by centrifugation for 10 minutes at 14481 x g. The resulting pellet was visible to the eye. The
supernatant was removed and the pellet was washed with 1 ml of 80% ethanol. Care was again taken not to disturb the pellet. The 80% ethanol wash was placed at -70°C for 15 minutes before the supernatant was carefully poured off. The open microfuge tube was then placed into a Savant™ Speed Vac vacuum concentrator for 5-10 minutes to remove any residual liquid. The final pellet was resuspended in 30 µl of TE buffer. The resuspended single-stranded template DNA to be used for sequencing reactions was stored at -20°C. An overview of the subcloning and preparation of single-stranded template can be seen in Fig. 13 (BRL Instruction Manual). The presence of any recombinant single-stranded template was verified by loading 3 µl (of the original 30 µl single-stranded preparation) onto a 1% agarose minigel. Nonrecombinant M13 DNA was used as a control. The recombinant M13 molecules carrying cloned fragments of 300 bp or greater had a reduced mobility and could readily be identified when the gel was stained with ethidium bromide and visualized using ultraviolet irradiation. A sample gel of recombinant and non-recombinant molecules can be seen in Fig. 14.
M13mp white plaque (recombinant)

infect *E. coli* host

grow 6-8 hrs. at 37°C

centrifuge

**cell pellet**

M13mp RF DNA with insert

small-scale plasmid preparation

**culture supernatant M13mp virus**

precipitate with PEG/NaCl 15 min., R.T.

centrifuge

supernatant (discard)

**viral pellet**

resuspend in Tris/EDTA

phenol/CHCl₃ extract

EtOH precipitate

centrifuge

pellet

single-stranded M13mp DNA with insert

supernatant (discard)
Fig. 13. Overall strategy for the preparation of single-stranded template. Single-strand recombinant DNA consisted of the M13 vector and the cloned fragment of *Pseudomonas putida* HS1 TOL plasmid pDK1. The RF form was also prepared and can be saved for later use (storage was at 4°C) (Taken from BRL Instruction Manual).
Fig. 14. Agarose gel analysis of single-stranded M13 template DNA. The single-strand M13 DNA that contained target DNA electrophoresed at a slower rate than single strand M13 DNA that did not contain target DNA.

Five microliters of the single-stranded template was added to a 0.5 ml microcentrifuge tube. Care was taken to label the tubes appropriately for each specific clone. Recombinant clones were processed in sets of twelve (a smaller number of clones required a proportionate adjustment of total volumes given below). To a 0.5 ml microcentrifuge tube was added 13.0 μl of "universal" primer, 26 μl of reaction buffer and 26 μl of sterile distilled water, giving a total volume of 65 μl. Five microliters of this solution was added to each of the twelve tubes containing 5 μl of the respective single-stranded recombinant M13 clones. This gave a total volume of 10 μl in each microfuge tube. The template/primer mixture was placed at 65°C for 2 minutes. The microfuge tubes were then removed from the water bath and placed on the bench in a Pyrex™ tray that contained water at 65°C. This tray was left at room temperature to cool to about 37°C, (requires approximately 30-40 minutes). During the cooling process, sets of "termination tubes" were prepared for each of the recombinant M13 DNA's. Each set consists of four 0.5 μl microfuge tubes labelled appropriately with the specific clone number and the
dideoxyribonucleotide base to be incorporated (G, A, T, or C) for termination to occur. It was most convenient to use a different colored tube for each termination reaction. The sample clone number was placed on the top lid (green = G, red/pink = A, blue = T and yellow = C). This procedure reduced the chance for accidental misidentification of the correct tube while the various manipulations of the DNA sequencing reactions or sequencing gel loading were carried out. Two and a half microliters of ddGTP termination analog was added to the G tubes, 2.5 μl of ddTTP was added to the T tubes, 2.5 μl of ddATP was added to the A tubes and 2.5 μl of ddCTP was added to the C tubes. At this point the labelling solution was prepared by adding to a 0.5 ml microfuge tube 13.0 μl of 0.1 M DTT (dithiothrietol), 22.8 μl distilled water, 5.2 μl labelling mix (provided for by the manufacturer) and 6.5 μl of $^{35}$S-ATP (65 μCi), giving a total volume of 45.5 μl. After the primer/template annealing mixture had cooled to less than 35°C, 3.5 μl of the labelling mixture solution was added to each of the annealed primer/template tubes. Also at this point, an 8-fold dilution of the Sequenase™ version 2.0 was made in a 0.5 ml microfuge tube using sequenase dilution buffer (provided by the manufacturer). For twelve samples, 3.3 μl of Sequenase™
was added to 23.1 μl of sequenase dilution buffer giving a total volume of 26.4 μl. Two microliters of this diluted Sequenase™ was added to each of the tubes containing the primer/template/labelling mixtures. These tubes were allowed to incubate at room temperature for 4 minutes. While this incubation was being carried out, the termination mixes were placed at 37°C. Upon completion of the 4 minute incubation, 3.5 μl of the incubated mixture was added to each of the G/A/T/C microfuge tubes. The two solutions were mixed as they were added by pipetting the contents up and down in the tip several times. The G/A/T/C termination tubes were placed back at 37°C as each was complete and allowed to incubate for a further 20 minutes. At the end of this incubation period, 4 μl of formamide stop solution was added to each tube (An overview of the timed sequencing reactions (for a set of twelve reactions can be seen in Fig. 15). The tubes were mixed manually and given a brief centrifugation. The samples at this point were stored at -20°C until they were ready to be loaded onto a sequencing gel. Prior to loading the samples onto a 6% polyacrylamide sequencing gel, the samples were placed at 80°-85°C for 2 minutes.
SEQUENCING REACTIONS:

TIME/MIN.  REACTION
41 Place all termination tubes for templates 1-4 at 37 deg. C.
40 Add 2ul diluted Sequenase to template #1. Mix, place at R.T.
39 Add 2ul diluted Sequenase to template #2. Mix, place at R.T.
38 Add 2ul diluted Sequenase to template #3. Mix, place at R.T.
37 Add 2ul diluted Sequenase to template #4. Mix, place at R.T.
36 Dispense 3.5ul of template #1 to each GATC tube (@37 deg.)
35 Dispense 3.5ul of template #2 to each GATC tube (@37 deg.)
34 Dispense 3.5ul of template #3 to each GATC tube (@37 deg.)
33 Dispense 3.5ul of template #4 to each GATC tube (@37 deg.)
32 TAKE A BREAK! (You may use this time to catch up)
31 Place all termination tubes for templates 5-8 at 37 deg. C.
30 Add 2ul diluted Sequenase to template #5. Mix, place @ R.T.
29 Add 2ul diluted Sequenase to template #6. Mix, place @ R.T.
28 Add 2ul diluted Sequenase to template #7. Mix, place @ R.T.
27 Add 2ul diluted Sequenase to template #8. Mix, place @ R.T.
26 Dispense 3.5ul of template #5 to each GATC tube (@37 deg.)
25 Dispense 3.5ul of template #6 to each GATC tube (@37 deg.)
24 Dispense 3.5ul of template #7 to each GATC tube (@37 deg.)
23 Dispense 3.5ul of template #8 to each GATC tube (@37 deg.)
22 TAKE A BREAK! (You may use this time to catch up)
21 Place all termination tubes for templates 9-12 @ 37 deg. C.
20 Add 2ul diluted Sequenase to template #9. Mix, place @ R.T.
19 Add 2ul diluted Sequenase to template #10. Mix, place @ R.T.
18 Add 2ul diluted Sequenase to template #11. Mix, place @ R.T.
17 Add 2ul diluted Sequenase to template #12. Mix, place @ R.T.
16 Dispense 3.5ul of template #9 to each GATC tube (@ 37 deg.)
15 Dispense 3.5ul of template #10 to each GATC tube (@ 37deg.)
14 Dispense 3.5ul of template #11 to each GATC tube (@ 37deg.)
13 Dispense 3.5ul of template #12 to each GATC tube (@ 37deg.)
12 Add 4ul of Stop solution to all template 1-4 tubes.
11 Add 4ul of Stop solution to all template 5-8 tubes.
10 Add 4ul of Stop solution to all template 9-12 tubes.
Fig. 15. Summary of sequencing reaction protocol. The subclones (single-stranded recombinant M13 phage DNA) were subjected to the sequencing protocol in sets of four, with a total of three sets (twelve subclones) being sequenced per occasion.
Sequencing double-stranded DNA templates by the dideoxy-ribonucleotide method (USB step-by-step protocols 1989)

Approximately 4 μg of clean (no RNA, etc.) recombinant pUC18/19 plasmid DNA was placed in a 0.5 ml microcentrifuge tube. Sterile water was then added up to a total volume of 30 μl. To this solution was added 3 μl of 2 N NaOH. The mixture was then allowed to incubate at room temperature for five minutes. After the incubation 120 μl of 100% ethanol (stored at -20°C) was added and the contents mixed well. This was followed by the addition of 5 μl of 3 M sodium acetate (pH 5.0) which again was mixed well. The tube was then incubated at room temperature for five minutes. At the end of the incubation period the mixture was centrifuged at 14481 x g for 20 minutes in a microfuge at 4°C (excessive warmth may cause DNA to redissolve and hence be lost). The supernatant was removed and the open microfuge tube was placed in a Savant™ Speed Vac concentrator to remove any residual liquid. The dry pellet (denatured DNA) was resuspended in 8 μl of the sequencing primer and water solution (which was prepared exactly as described previously for single-stranded templates). To this solution was added 2 μl of the Sequenase™ sequencing reaction buffer (provided by the manufacturer), giving a total volume of 10 μl. The
primer was annealed to the template at 37°C for 45 minutes. From this point the procedure was performed exactly as described above for single-stranded sequencing reactions.

Rapid preparation of double-stranded DNA sequencing (Zhou et al. 1990; Crouse and Amorese 1989; Kraft et al. 1988)

Single colonies of bacterial cells carrying recombinant plasmids (white colonies on X-Gal/IPTG plates) were inoculated into tubes containing 5 ml of LB liquid medium. If the double-stranded recombinant DNA was pUC 18/19 ampicillin at 50 µg/ml final concentration was included. These tubes were then incubated overnight at 37°C with shaking at 250 rpm. Three milliliters of the overnight cultures were centrifuged for 2 minutes at 14481 x g in 1.5 ml microcentrifuge tubes this involved two centrifugations of 1.5 ml each. The supernatants were poured off and the tubes refilled for the second centrifugation. The final supernatant was gently decanted leaving approximately 50-100 µl of medium with the cell pellets. These were resuspended in this residual medium by vortexing thoroughly. To the resuspended cells was added 300 µl of TENS (3.875 ml TE buffer, 1.0 ml 0.5 M NaOH, 0.125 ml 20% SDS, giving a total volume for the solution of 5 ml) by vortexing for 2-5
seconds. This produces a viscous solution. The solution was then centrifuged for 2 minutes in a table top microfuge at 14481 x g in order to pellet the chromosomal DNA and the large bacterial cell debris. The supernatant was transferred to a new microfuge tube and 900 µl of ice-cold 100% ethanol was added and mixed thoroughly. This mixture was then centrifuged for 2 minutes at 14481 x g to collect the recombinant plasmid DNA and RNA. The supernatant was discarded and the pellet was washed twice with 1 ml ice-cold 70% ethanol. After the pellet was dried in a Savant™ Speed Vac concentrator for 5 minutes, it was resuspended in 40 µl of TE buffer. Since this solution contained RNA, 1 µl of 10 mg/ml heat-treated RNase was added to each tube and the tubes incubated for one-half hour at 37°C. After the incubation period 10 µl of 7.5 M ammonium acetate was added. This was followed by incubation at room temperature for thirty minutes. The solution was centrifuged at 14481 x g for 15 minutes at 4°C to remove precipitated proteins. The supernatant was placed into a new 1.5 ml microfuge tube and 160 µl of ice-cold 100% ethanol was added and mixing well. Samples were then incubated on wet ice (0°C) for thirty minutes. The tubes were then centrifuged for 20 minutes at 14481 x g to pellet the DNA. The supernatants were
discarded. One milliliter of ice-cold 70% ethanol was added to each pellet, mixed thoroughly, and the tubes centrifuged at 14481 x g for 1 minute at 4°C. The supernatants were discarded and the pellets dried in a Savant™ Speed Vac concentrator for 5 minutes. The pellet were then resuspended in 16.8 µl of distilled water. To these were added 3.2 µl of 5 M NaCl and 20 µl of 13% PEG 8000 (130 mg of PEG 8000 in a total volume of 1 ml). After the tubes were mixed well and incubated on ice for 1 hour in the cold room, they were centrifuged for ten minutes at 14481 x g and 4°C. The supernatants were discarded and the pellets washed with 1 ml of ice cold 70% ethanol. The samples were then centrifuged for 1 minute at 14481 x g, the supernatants discarded and the pellets dried in a Savant™ Speed Vac concentrator. The pellets were resuspended in 30 µl of TE buffer and to this solution was added 3 µl of 2 N NaOH (prepared fresh each time immediately before use). The tubes were incubated at room temperature for five minutes. At the end of the incubation, 120 µl of ice-cold 100% ethanol was added and the tubes mixed well. To this was added 5 µl of 3 M sodium acetate (pH 5.0) and the tubes were incubated at room temperature for five minutes. The microfuge tubes were then centrifuged for 20 minutes at 14481 x g and 4°C. The supernatants were removed
and the pellets dried in a Savant™ Speed Vac concentrator for 5 minutes. The pellets were resuspended in 5 µl of primer/distilled water solution (as described previously), followed by the addition of sequencing reaction buffer (provided by the manufacturer) to give a total volume of 10 µl per tube. The annealing reaction was carried out at 37°C for 45 minutes. Beyond this point the procedure was performed exactly as to the single-stranded template sequencing reactions as described previously.

Preparation of dihydrodiols (Kunz and Chapman 1981b)

A single colony of *E. coli* strain DH5α (carrying the pBK190 plasmid (xylXYZ) was inoculated from an agar plate into 5 ml LB (ampicillin 50 µg/ml) liquid medium. This culture was allowed to incubate overnight at 37°C with a shaking speed of 250 rpm. The next day this first overnight culture was inoculated into a 50 ml LB (ampicillin 50 µg/ml) liquid medium and allowed to again incubate overnight at 37°C and 250 rpm shaking. Twenty five milliliters of the second overnight culture was then inoculated into 250 ml LB (ampicillin 50 µg/ml) liquid medium. This culture was allowed to incubate overnight at 37°C and 250 rpm in a New Brunswick™ G25 incubator/shaker. The next day the entire
overnight culture was placed into a sterile 500 ml centrifuge bottle using aseptic technique. The cells were centrifuged at 9268 x g for 15 minutes. The supernatant was then removed and the cells resuspended (by vortexing) in a sterile solution of 250 ml 0.85% NaCl. The cells were then centrifuged once again at 9268 x g for 15 minutes and the supernatant removed. The final bacterial cell pellet was resuspended in 4 ml of 0.85% NaCl. This suspension of cells was kept on ice until ready for use. Fifty microliters of 50 μg/ml filter sterilized ampicillin and 30 μl of 100 mM IPTG were added to a 250 ml Erlenmeyer™ flask which contained 45 ml of sterile distilled water and 5 ml of sterile 10X PN liquid medium. Seven hundred and fifty microliters of 2 M glycerol was added as a carbon source. Five milliliters of 100 mM benzoic acid (m-toluate, p-toluate as desired) was added as a substrate for the xylXYZ gene product, toluate-1,2-dioxygenase. Two hundred and fifty microliters of 200X R-salts were also added to the 250 ml Erlenmeyer™ flask (all of the above solutions were sterile). One milliliter of the washed E. coli cells were now inoculated into the prepared Erlenmeyer™ flask. The flask was allowed to incubate at 37°C with 250 rpm shaking for a two day period. At the end of this time, 50 ml of the culture from
the flask was poured into a 50 ml centrifuge bottle and centrifuged at 14481 x g for 20 minutes. The supernatant contained the required dihydrodiol and was separated into 10 ml aliquots for storage at -70°C until ready for use in enzymatic assays.

Preparation of cell-free extracts (Kunz and Chapman 1981b)

A single colony of *E. coli* strain DH5a carrying the appropriate recombinant pUC18/19 plasmid (expressing *xylL*, *xylT*, *xylLT*, *xylTE*, *xylE* or *xylLTE*) was inoculated into 5 ml of sterile LB (ampicillin 50 μg/ml) liquid medium and allowed to incubate overnight at 37°C with shaking at 250 rpm. The entire 5 ml of overnight culture was then placed into 500 ml of LB liquid medium contained within a 2 liter Erlenmeyer™ flask. To this flask was also added 500 μl of 50 mg/ml filter sterilized ampicillin (final concentration was 50 μg/ml) and 300 μl 100 mM IPTG. The 2 liter culture flask was then allowed to incubate at 37°C with shaking at 250 rpm for a period of 2 days. At the end of this incubation period the entire 500 ml culture was transferred to a preweighed 500 ml centrifuge bottle and centrifuged at 9268 x g for 30 minutes. The supernatant was discarded and the centrifuge bottle containing the cell pellet was weighed. The cells were then
resuspended in a volume of SP-50 buffer equal to two times the weight of the cells. The resuspension of the cells was carried out using a glass rod. The cell suspension was then transferred to a French™ press cell and pressed at a pressure of 1000 lbs/in². The extract that was removed from the French™ press and a few crystals of pancreatic DNaseI was added and left at room temperature for 10 minutes. The ruptured bacterial cell suspension was then centrifuged for 30 minutes at 30230 x g. The cell free supernatant containing the expressed enzyme product(s) encoded by the cloned TOL segment was divided into 500 µl aliquots and stored at -70°C until ready for use.

*Modified Lowry procedure for determination of protein concentration (Lowry et al. 1951)*

The Lowry method for the determination of protein concentration is sensitive to as little as 0.2 µg of protein. Overall protein determination utilizing a copper and Folin™ reagent had several advantages. First, the enzyme for which the concentration was to be measured was not required to be digested as with other reagents for protein determination (Nessler's reagent). Second, the Lowry reagents were much more sensitive (0.2 µg of protein could have been detected)
than UV spectrophotometric analysis at 280 nm and are also less susceptible to disturbance by turbidity. Third, the Lowry protein concentration determination method was shown to be several-fold more sensitive than ninhydrin and a hundred times more sensitive than a Biuret reaction.

There is however a disadvantage to the Lowry method for protein concentration determination in that the amount of color formation at the end of the reaction incubation time varied with different proteins being assayed.

The Lowry procedure involved the use of several reagents that were prepared as described below.

Reagent A

2% Na₂CO₃ in 0.1 N NaOH (can be stored indefinitely)

Reagent B

0.5% CuSO₄.5H₂O in 1% NaK Tartarate (can be stored indefinitely)

Reagent C

49 ml of A + 1.0 ml of B (prepared fresh)

Reagent D

1N Phenol (Folin™ reagent)

Standard tubes were set up as follows:

1 mg/ml bovine serum albumin

0 μl 5 μl 10 μl 15 μl 20 μl 25 μl
Distilled water
200 µl  195 µl  190 µl  185 µl  180 µl  175 µl

The experimental tubes were set up by making dilutions of the cell free extracts. Dilutions were made in the order of 1:5  1:10  1:20  1:50

Five microliters of the dilutions were added to the tubes containing 195 µl of distilled deionized water.

One milliliter of reagent C (49:1 of A:B) was added to each of the tubes in ordered succession and allowed to incubate at room temperature for precisely 10 minutes. At the end of the incubation period 0.1 ml of Folin™ reagent (1 N) was added to each of the tubes in the same ordered succession as before. The tubes were then incubated at room temperature for 30 minutes or 1 hour. At the end of the incubation period the optical density of the standard and experimental tubes were read spectrophotometrically at a wavelength of 750nm. A graph of the absorbance of the standard tubes was plotted and from this standard curve the concentration of the total protein in the cell-free extracts were then determined. An example of the standard curve and a sample calculation can be seen in Fig. 16.
Sample calculation (\textit{lytF} cell-free extract protein concentration determination).

\[ 30 \, \mu g \times 1.5 \text{ dilution factor} = 30 \, \mu g/ml = 30 \, mg/ml \text{ protein}. \]

5 \mu l extract in cuvette
Fig. 16. Sample standard curve obtained using the Lowry method (Lowry et al. 1951) for the determination of protein concentration.
Enzyme assays (Kunz and Chapman 1981b)

All the enzymatic assays were performed at room temperature utilizing masked quartz cuvettes and a Perkin-Elmer™ dual beam spectrophotometer (0.5 interval; 2.0 slit; 480 nm/min). 1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase activity was determined from the formation of NADH from NAD$^+$ (monitored spectrophotometrically at a wavelength of 342 nm) (Fig. 17). The enzyme reaction mixture contained the following (Kunz and Chapman 1981b)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reference Cuvette</th>
<th>Experimental Cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diol (6-8 mM)</td>
<td>0.00 µl</td>
<td>100.00 µl</td>
</tr>
<tr>
<td>Tris (50 mM, pH 8.0)</td>
<td>500.00 µl</td>
<td>500.00 µl</td>
</tr>
<tr>
<td>Distilled H$_2$O</td>
<td>300.00 µl</td>
<td>200.00 µl</td>
</tr>
<tr>
<td>NAD (10 mM)</td>
<td>200.00 µl</td>
<td>200.00 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>1000.00 µl</td>
<td>1000.00 µl</td>
</tr>
</tbody>
</table>

To the above prepared solutions was added no more than 10 µl of either undiluted or diluted cell-free extract.

The catechol-2,3-oxygenase (C230) activity was measured by monitoring the production of the hydroxymuconic semialdehyde ring fission product (Fig. 17). The formation
1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase

\[
\text{NAD}^+ \rightarrow \text{NADH} + H^+
\]

3,5-cyclohexadiene-1,2-diol-1-carboxylic acid

Catechol-2,3-dioxygenase

\[
\text{Catechol} \rightarrow 2\text{-hydroxy}
\]

2-hydroxymuconic semialdehyde
**Fig. 17.** Enzymatic reactions for the *xyle* and the *xylLT*
gene products. The *xylLT* region encoded
1,2-dihydroxycyclohexa- 3,5-diene carboxylate dehydrogenase,
the activity of which was monitored spectrophotometrically at
a wavelength of 342 nm or by utilizing the *xyle* subclone
gene products as a reporter function. The *xyle* gene coded
for catechol-2,3- dioxygenase was monitored
spectrophotometrically at a wavelength of 375 nm and visually
by the formation of a yellow ring fission product.
of the ring fission product was monitored
spectrophotometrically at a wavelength of 375nm.

The assay conditions were as follows (Kunz and Chapman 1981b)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reference Cuvette</th>
<th>Experimental Cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol (10 mM)</td>
<td>0.00 μl</td>
<td>33.00 μl</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>33.00 μl</td>
<td>0.00 μl</td>
</tr>
<tr>
<td>KH₂PO₄ (pH 7.5)</td>
<td>970.00 μl</td>
<td>970.00 μl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1003.00 μl</td>
<td>1003.00 μl</td>
</tr>
</tbody>
</table>

As before no more than 10 μl of either diluted or
undiluted cell-free extract was added to the above solutions.

A sample calculation of enzymatic specific activity was
performed as follows (Fig. 16)

\[
\text{Slope of assay (Absorbance/sec.)} \times 60 \text{ sec.} = \frac{\text{Extinction coefficient (mmol}^{-1} \times \text{mg/ml protein (CFE))}}{10^6}
\]

\[
= \text{nmol/min/mg}
\]

The protein concentration of the cell-free extract was
determined using the Lowry procedure as described previously.
The extinction coefficients for 1,2-dihydroxy-cyclohexa-
3,5-diene carboxylate dehydrogenase at pH 8.0 was 3000 mm⁻¹
and catechol-2,3- dioxygenase at pH 7.0 was 33000 mm⁻¹ (Kunz
CHAPTER III

RESULTS

Nucleotide sequence determination

The determination of the DNA sequence of the xylLT region of the Pseudomonas putida HS1 pDK1 TOL plasmid was facilitated by the subcloning of the pDKR1 lower operon (Fig. 18) into the E. coli vector pBR322. A HindIII fragment carrying all the structural genes of the lower pathway of the P. putida HS1 TOL plasmid pDK1 was cloned by Azadpour into the pBR322 vector and the recombinant plasmid was designated pBK489 (Azadpour 1991). A HindIII/KpnI/KpnI fragment from pBK489 was subcloned into the E. coli expression vector pUC19, producing a recombinant plasmid containing the xylXYZ genes capable of producing the dihydroxy diols from appropriate (benzoate, m-toluate, p-toluate or 3,4-dimethylbenzoate) substrates (Azadpour 1991). A 5.4 kbp EcoRI fragment from the pDKR1 cointegrate plasmid was also inserted into an EcoRI site of the E. coli plasmid pBR325 by Voss (Voss 1989). This EcoRI fragment contained the xylZLTE genes and portions of the xylY and xylG genes and was designated pBK188 (Benjamin et al. 1991 and Benjamin et al. 1990).
Fig. 18. Genetic map of the pDKR1 cointegrate plasmid. The pDKR1 plasmid (100 kbp) carries both the 'upper' and 'lower' pathways of the pDK1 TOL plasmid isolated from the *Pseudomonas putida* HS1 strain.
Subcloning of portions of the \textit{xylLTE} region required a large scale preparation of the pBK188 plasmid followed by digestion with \textit{EcoRI}. At least 500 \(\mu\)g of the pBK188 plasmid was digested and the completed digest applied to a preparative vertical agarose gel in order to purify the cloned TOL fragment. The fragments were visualized by UV shadowing and the desired TOL segment cut from the gel. The DNA was electroeluted from the agarose slice and purified. The \textit{EcoRI} fragment contained the \textit{xylLTE} structural genes, together with portions of the \textit{xylY} and \textit{xylG} genes. Restriction enzymes (compatible with sites in the multiple cloning site of the M13 phage) were used to digest this \textit{EcoRI} fragment and the fragments created were inserted into the M13 phage. These single stranded recombinant DNA molecules were then subjected to Sanger dideoxy sequence analysis. Sequencing reactions were performed on the single stranded templates inserted into M13 phage such that both strands of the \textit{xylLT} region were determined. The sequencing strategy used in this study to achieve this result can be seen in Fig. 19. A representative polyacrylamide sequencing gel can be seen in Fig. 20. The complete nucleotide sequence of the \textit{xylL} and the \textit{xylT} region (the first nucleotide sequence data for the region of any TOL plasmid previously assigned to \textit{xylLT}) can be seen in Fig. 21 and in Fig. 22 respectively.
Fig. 19. Sequencing strategy utilized for the determination of the DNA sequence of the xyILT region. Several subclones were generated that included stretches of DNA in the xyILT region. Subclones were generated for both strands of DNA such that sequencing reactions were carried out with both strands of the xyILT region.
Fig. 20. Autoradiograph of a 6% polyacrylamide sequencing gel. This is an example of a ladder gel which enables one to read the nucleotide sequence of DNA (Nucleotide sequence was read from the 5' to 3' ends beginning at the bottom of the gel). Samples were loaded into wells in the following order: dideoxyguanosine triphosphate, dideoxyadenosine triphosphate, dideoxythymidine triphosphate and dideoxycytosine triphosphate (GATC).
144

xylL Region
1210

123?

1264

12*3

ACGCTCCTTTGGTCCGCTTACTTGGTGACCCGGCCTGTTTTATTCCTGCAGTAGAGCCCATCTTTCC7CCCAACT7TCCCAAA
1310

1330

1354

1374

ATTGCAT CTGGGCTACA CAACCGAGC TCGTTC &LQ AAC AAA CGT TTC CAG GAC AAG ACT GCC GTT A7C ACC
(ORF-1J
1377

met asn lys arg phe gin a s p lys thr ala val

1398

lie thr

141*

1440

CGC GCT GCC CAG GGC ATC CGT CGC CGC GTG GCC GAA CGG ATG GCG GCC CAA GGA CGT CGG CTG CTG
g l y ala ala g i n gly lie gly arg arg val ala glu arg met ala ala glu gly gly arg leu leu
1443

1464

1485

1506

CTG GTC GAC CGT TCC GAG CTA ATA CAT CAG CTG GCC GAC GAA CTG GTC GGA GTC GCT GAC CTG CTG
leu val a s p arg ser glu leu lie his glu leu ala asp glu leu val gly val ala glu val leu
1509

1530

1551

Xho

T 1572

ACC CTG ACC GCC GAC CTT GAG CAG TTC CCC GAT TGC CAA CGG CTG ATC GCG GCG CGG CTC GAG CGC
t h r leu t h r a l a asp leu glu gin p h e ala a s p cys gin arg val met ala ala arg leu glu arg
1575

1596

1617

1638

TTC GGT CGT CTG GAC ATT CTG ATC AAC AAC CTT GGC CGC ACC ATT TCG GCT AAG CCA TTC GAG CAT
p h e g l y arg leu a s p l i e leu lie asn asn v a l g l y g l y thr lie t r p ala lys pro phe glu his
1641

1662

1683

1704

TAC CAG GAA CAC GAG ATC GAG GCC GAA GTG CGT CGT TCG CTG TTC CCC ACC CTG TGG TGC TGC CAC
t y r gin glu h i s glu l i e glu ala glu v a l arg arg aer leu p h e p r o thr leu trp cys cys his
1707

Jfho T 1731

1749

1770

GCC GCC CTG CCG CCA ATG CTC GAG CAG GGC AGT GGA GCC ATC CTC AAC GTT TCC TCC CTC GCC ACG
a l a ala v a l p r o p r o m e t leu glu g i n g l y ser g l y a l a lie v a l asn val ser ser val ala t h r
1773

1794

1815

1836

CGC GGG GTC AAT CGC GTC CCC TAC GGC GCG GCA AAG GCT GGC GTT AAC CCC CTG ACC CCC TGC CTA
a r g gly v a l asn arg v a l p r o t y r g l y ala ala ly« gly gly v a l asn ala leu thr ala cys leu
1839

1860

1881

1902

GCC TTT CAA ACC GCC GAG CGC GGT ATC CGG GTC AAC GCC ACC GCG CCG GGT GGC ACC GAG GCG CCT
a l a p h e glu thr ala glu arg gly l i e arg v a l asn ala thr ala p r o gly g l y thr glu ala p r o
1905

1926

1947

1968

CCA CGG CGG ATT CCG CGC AAC AGC GCC CAG CCG AGC GAG CAG GAG AAG GTC TGC TAC CAG CAG ATC
p r o arg arg lie pro arg asn ser ala glu pro ser glu gin gly lys val t r p tyr gin gin
1971

1992

2013

lie
2034

GTC GAC CAG ACC CTC GAC AGC AGC CTG ATG AAA CGC TAC GGC AAC ATC GAC CAA CAG CCC GGG GCG
v a l a s p gin t h r leu a s p ser ser leu met
2037

lys arg tyr gly asn lie asp glu gin ala gly ala

2058

2079

2100

ATT CTG TTC CTG GCC TCC GAC GAC GCT GCC TAC ATC ACC CGT GTA ACT CTT CCG CTG GCA GGG GGA
lie leu p h e leu ala ser a s p a s p ala ala tyr ile thr gly val thr
2103

2120

leu p r o val ala gly gly

2150

a s p leu gly O P A
2180

Fsd

I

2210

P2A0

CCCTGAGACT CATTTTCGGG GTTGCGCAGG CATCACCCCA GAGCTCTTGG GGGGATCCTT CCGTCATCTT TAAGGGCATC


Fig. 21. Nucleotide sequence and corresponding amino acid sequence for the xylL gene of the *Pseudomonas putida* HS1 pDK1 TOL plasmid.
xyLT Region

2260 2276 2297 2315
TGAGGDAAC C ATG GAC AAC ACT TAT GAC GTG CAG CGG ATG AAC GCC CAG GTG TTC CCG TGC
(ORF-2) met asp ser ser tyr glu val arg glu arg ile ser gly gin val phe arg trp

2318 2339 2360 2381
CTG CCG GAC CAG TCG TCC CTC CGG GCC ATC CAG CAG GGC AAG GCT TGC CCG GTC GGT TGC
leu pro glu gin ser val leu arg ala met glu glu gin gly lys arg cys val pro val gly cys

2384 2405 2426 2447
CTG GCT GGT GCC TGG TCC TGC AAG GTC GCC CTC GGC ATC AAC GCC TAC CAG TGC GCC AAC ATG
arg gly gly gly cys gly leu cys lys val arg val leu ser gly asp tyr gin cys gly arg met

2450 2471 2492 2513
ACC TGC ACT CAG CTT CCA CCG GAC GCC GGC AAC CAG GGC CGG GTG GCC TGG CTG CAA CTT TAT CCA
ser cys ser gin val pro pro glu ala ala lys gin gly leu ala leu ala cys gin leu tyr pro

2516 2537 2558 2580
CGC GCT GAT CTG TAC ATC GAA TGC CTT CGG CCA GTG CGA ACC AAT GCC TGA CAACAA GAAACATTAA
arg ala asp leu tyr ile glu cys leu arg gin val arg thr asn pro OPA
Fig. 22. Nucleotide sequence and corresponding amino acid sequence for the xylT gene of the Pseudomonas putida HSl pDKI TOL plasmid.
DNA sequence analysis

DNA sequence analysis of the xylLT region indicates the presence of an additional XhoI fragment not previously observed in the original restriction mapping of the lower operon region (Williams and Shaw 1988; Williams and Osborne 1988). The M13-derived nucleotide sequence of a SalI fragment, which included the 0.15 kilobase XhoI (a detailed restriction endonuclease map can be seen in Fig. 23) was used for final verification of the order and orientation of the three primary XhoI fragments. The xylLT region was found to contain two open reading frames each with an identical putative ribosome binding site sequence of 5'-GAGGTG-3' located at -10 with respect to the AUG initiation codons. This had not been previously reported. Previous reports described a single open reading frame encoding the xylL gene. The xylL gene consisted of an open reading frame of 777 base pairs (337 codons) and with a predicted encoded polypeptide of 27,853 Da. The xylT gene consisted of an open reading frame of 303 base pairs (101 codons) and with a predicted encoded polypeptide of 11,133 Da. This data can be seen in Table 6. Also seen in Table 7 is a comparison of the putative ribosome binding sites of some of the structural genes (both regulatory proteins and pathway enzymes).

Further analysis of the DNA sequence of the xylLT region
Fig. 23. Detailed restriction endonuclease map of the upper and lower operons together with the regulatory xylRS genes of the *Pseudomonas putida* pDK1 TOL plasmid.
Table 6. Comparasion of the \textit{xylL} and the \textit{xylT} structural genes. Specific reference to putative ribosome binding sites, size of open reading frames (nucleotide base pairs and amino acid codons) and predicted polypeptide size is shown.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Putative Ribosome Binding Site</th>
<th>ORF Length</th>
<th>Predicted Polypeptide (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{xylL}</td>
<td>GAGGTG</td>
<td>777 (259)</td>
<td>27,853</td>
</tr>
<tr>
<td>\textit{xylT}</td>
<td>GAGGTG</td>
<td>303 (101)</td>
<td>11,133</td>
</tr>
</tbody>
</table>
Table 7. Comparasion of the putative ribosome binding sites of the thirteen structural genes of the meta pathway.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ribosome Binding Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylX</td>
<td>AATAATGGAGGCACGAAAATGAACCTGGGT</td>
</tr>
<tr>
<td>xylY</td>
<td>CATGCGGAGGGCGCGTAAATGACTATCTCC</td>
</tr>
<tr>
<td>xylZ</td>
<td>CACGTCTGAGGTCCGCCATGACACACAAG</td>
</tr>
<tr>
<td>xylL</td>
<td>ACACAACCGAGGTGGTTCATGAACAACGTT</td>
</tr>
<tr>
<td>xylT</td>
<td>GGGCATCTGAGGTGAACCATGGACAGCATG</td>
</tr>
<tr>
<td>xylE</td>
<td>ACATTAAGAGGTTGCTGATGAAACAAGGA</td>
</tr>
<tr>
<td>xylG</td>
<td>ATTGAAGAGATTGGCAGATGAAAGGAATC</td>
</tr>
<tr>
<td>xylF</td>
<td>AACTCTGAGGACCTGGTCATGAACGCCGG</td>
</tr>
<tr>
<td>xylJ</td>
<td>TTCCTGAGAGAGCAATATGGACAGACA</td>
</tr>
<tr>
<td>xylR</td>
<td>AAAACAAGAGGAAATACAAATGTCCCTTATA</td>
</tr>
<tr>
<td>xylS</td>
<td>ATAAGAGAAACGTAGCTATGGATTTTTCG</td>
</tr>
</tbody>
</table>
included nucleotide and amino acid sequence comparisons to the \textit{xylLT} region of the pDK1 TOL plasmid and the known sequence of the same region from the pWWO TOL plasmid. The homology in nucleotide sequences between the \textit{xylL} genes from the TOL plasmids pDK1 and pWWO was calculated to be 90\% with 70\% of the non-homologous base pairs being third base differences, 16\% being second base differences and 13\% being first base pair differences. These data can be seen in Fig. 24 and Fig. 25. Comparisons of the nucleotide and amino acid sequences of the \textit{xylT} gene of the pDK1 TOL plasmid were also made to the \textit{nahT} gene of the NAH7 plasmid (these data can be seen in Fig. 26 and in Fig. 27).

The nucleotide sequence analysis also demonstrated the presence of two relatively large gaps between the \textit{xylZ} and the \textit{xylL} genes (115 bp) and between the \textit{xylL} and the \textit{xylT} genes (139 bp). These were the largest noncoding regions observed within the 'lower' operon of the pDK1 TOL plasmid. The relative sizes of the intergenic spaces can be seen in Table 8. Analysis of these gaps between the structural genes revealed the presence of palindromic sequences potentially able to form stem and loop structures. Fig. 28 shows the possible stem and loop structures in these intergenic regions. On comparison with intergenic regions at the same locations from the TOL plasmid pWWO it was found that the
Fig. 24. Comparison of the nucleotide sequences of the \textit{xyLL} genes. Nucleotide sequence from the \textit{P. putida} TOL plasmid pDK1 and the related \textit{P. putida} TOL plasmid pWWO are shown. Non-homologous nucleotides are shown by lower case letters.
MNKRFQDKTA VITGAAQGIG RRVAERMAAE GGRLLLVDRS ELIHELADEL
MNKRFQGKVA VITGAAQGIG RRVAERMAAE GGRLLLVDRS ELIHELADEL

60 70 80 90 100
VGVAEVLTLT ADLEQFADCQ RVMAAALERF GRDLILINNV GGTIWAKPFE
VGVAEVLTLT ADLEQFAECQ RVMAAALERF GRDLILINNV GGTIWAKPFE

110 120 130 140 150
HYQEHEIEAE VRRSLFPTLW CCHAAVPPML EQGSGAIVNV SSVATRGVNR
HYQERIEIEAE VRRSLFPTLW CCHAA1pPMI EQGSGAIVNV SSVATRG1hR

160 170 180 190 200
VPYGAAAKGGV NALTACLAFE TAERGIRVNA TAPGGTEAPP RRIPRNAEAP
VPYGAAAKGGV NALTACLAFE TAERGIRVNA TAPGGTEArh RggfRNSAEP

210 220 230 240 250
SEQEKVWYQQ IVDQTLDSKL MKRYGNIDEG AGAILFLASD DAAAYITGVTLP
SEQEKVWYQQ IVDQSLDSSL MKRYGIDEQ veAILFLASD aAsYITGtLP

260
VAGGDLGZ  pDK1
VAGGDLGcQS  pM20
Fig. 25. Comparison of the amino acid sequences encoded by the xylL genes. Amino acid sequence from the P. putida TOL plasmid pDK1 and the related P. putida TOL plasmid pWWO are shown.
pwWO  gGGatgAatATGaACAGtg cccgctacGA GGTGttcGAA GTGCTAAGCC
pDK1  TCTCAGTGGAACCATGGACAGCA GTTAT---GA GGTGCGTtGAG CCGATTtAGCC
nah7   GGAAGGAGATATGTCAGAGG  TCTTT GA  AATCACTGTG CAGCCTtGGtG

110 120 130 140 150 160
GCCAGTCATT CCGCTGT--- GCCGAGGCCCC AGTCCG-TACtG GCGCCGCAATG GAAGCtCCAGG
GCCAGGTTtG CCGTtGGCCTG CCGGAG C  AGTCGGTCCT  GtCGGGCCATG GAGGACAGG
GAGAGCGGTT TGTtGGtCAG CAGCAGCAAT CAGCGT T  GCAGtCCATG GAGACCCAGG

170 180 190 200 210 220
GCAAGGCCCTG CATACCCGttG GGCtGtCCCG GTGGCGGttTG CCGCttTGTtGt AGAGTCCGCG
GCCAGGtGtG CTTGGCCGttGC GGGGtGGtGTG TGGtGtTtGtGC AAGGtGCCCG
GCAAGGCCCTG CTTACCTGTG CCGCTGTCGCG  GGGGCGGttTG TGGCTTGTGC  AAGGtGAAAGG

230 240 250 260 270 280
TGtGACCGGG AGCCtACCCGtG AGCGGCCGCA TGAGCGGCGG TGACtCGCGG GCGCttCCGCG
TGtGACCGGG CGACTACCGG TGCGGCCGGC AAGGCGCAGG TGAGtCCGCA GCGGtCCCGC

290 300 310 320 330 340
CtCCGCCGACC GCTGGCCCTG GCC T  GTCAAGTGttG TCCGtAAACC GACtTGACCA
---CtGAAG- -AGGGGtCCtG GCtCTGCGCC GTCAACTGtG TCCAGGCGCT GACTGTCtCA
---GtGAA- -CAAGGtCtAT GCtCTGCGCC GtGGCCCGAC GATCTTTGtA

350 360 370 380 390 400
TCGAGTACtT TCGCCtACGtT tGGCGGtAAC AAACtGGA-- CAACATtGA ACTATGAGA
TGtGATAcTt TCGGtCCAGtG CAGAGCtCtC CCtGA------ CAACAGA AACA
TCGAGCtGTt ACTtAAAGGtG STGGAGtGGA AGTACtGGtG ACCAAACA A
Fig. 26. Comparison of the nucleotide sequences of the xylT genes. Nucleotide sequence from the *P. putida* TOL plasmids, pDK1 and the related *P. putida* TOL plasmid pWWO are shown. Also compared with these TOL plasmid nucleotide sequences is the *nahT* gene from the nah7 plasmid. The homology in nucleotide sequence between the three plasmids is not significant, however the nucleotide sequence coding for the six cysteine residues typically found in this chloroplastic-type ferredoxin are homologous to a significant degree (these sites are noted with asterisks).
<table>
<thead>
<tr>
<th></th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
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<tbody>
<tr>
<td>pWWO</td>
<td>MmsaqYEVfE</td>
<td>v1SGQsFRca</td>
<td>egOSVLRAME</td>
<td>aQGKRCiPVG</td>
<td>CRGGGCGLCr</td>
</tr>
<tr>
<td>pDK1</td>
<td>-MDSSYEVRRE</td>
<td>RISGQVFRWl</td>
<td>PEQSVLRAME</td>
<td>EQGKRCVPVG</td>
<td>CRGGGCGLCK</td>
</tr>
<tr>
<td>NAH7</td>
<td>-MsevfEitv</td>
<td>qpqGerFvcq</td>
<td>PqQSalhAME</td>
<td>tQGKRCiPVG</td>
<td>CRGGGCGLCK</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWWO</td>
<td>VRVLSGaYrs</td>
<td>GRMSrghVFa</td>
<td>kAAaeaLALA</td>
<td>COvfPqtDlt</td>
<td>IEyfRhVggN</td>
</tr>
<tr>
<td>pDK1</td>
<td>VRVLSGDYQC</td>
<td>GRMSCSQQVPP</td>
<td>EAAKQGLALA</td>
<td>CQLYPRADLY</td>
<td>IECLRQVRTN</td>
</tr>
<tr>
<td>NAH7</td>
<td>VRVLaGDYes</td>
<td>GRvSCKh1Pv</td>
<td>EAreQGyALA</td>
<td>CrLfaRsDLc</td>
<td>IEryskpcse</td>
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</table>

<table>
<thead>
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<th></th>
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</thead>
<tbody>
<tr>
<td>pWWO</td>
<td>kPDNMNYEEV TS</td>
</tr>
<tr>
<td>pDK1</td>
<td>P</td>
</tr>
<tr>
<td>NAH7</td>
<td>sTVDQQQR</td>
</tr>
</tbody>
</table>
Fig. 27. Amino acid sequence comparison of the xylT genes. Amino acid sequence from the *Pseudomonas putida* TOL plasmids pWWO and pDK1 and the nahT gene from the NAH7 plasmid is shown. Non-homologous amino acids are shown by lower case letters.
Table 8. Comparasion of the nucleotide spacing between the structural genes of the meta-cleavage pathway. The nucleotide space between the \textit{xylZ} and the \textit{xylL} genes (115 bp) and the \textit{xylL} and the \textit{xylT} genes (139 bp) are the largest in the meta-cleavage pathway.

### Organization of the Meta-Cleavage Operon of the \textit{P. putida} TOL Plasmid pDK1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene Spacing</th>
</tr>
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<tbody>
<tr>
<td>\textit{xylX-xylY}</td>
<td>-12</td>
</tr>
<tr>
<td>\textit{xylY-xylZ}</td>
<td>-2</td>
</tr>
<tr>
<td>\textit{xylZ-xylL}</td>
<td>115 (38/60 A+T)</td>
</tr>
<tr>
<td>\textit{xylL-xylT}</td>
<td>139 (39/71 A+T)</td>
</tr>
<tr>
<td>\textit{xylT-xylE}</td>
<td>16</td>
</tr>
<tr>
<td>\textit{xylE-xylG}</td>
<td>21</td>
</tr>
<tr>
<td>\textit{xylG-xylF}</td>
<td>-2</td>
</tr>
<tr>
<td>\textit{xylF-xylJ}</td>
<td>-2</td>
</tr>
<tr>
<td>\textit{xylJ-xylQ}</td>
<td>2</td>
</tr>
<tr>
<td>\textit{xylQ-xylK}</td>
<td>-2</td>
</tr>
<tr>
<td>\textit{xylK-xyll}</td>
<td>-18</td>
</tr>
<tr>
<td>\textit{xyll-xylH}</td>
<td>37</td>
</tr>
</tbody>
</table>
(-) C=G (-)
A  G
C=G
T=A
A=T
C=G (A)
G=C
G=T
A=T
(G) C C
G=C

[xyll] --> 5'CGGGTTGCG=GTCACTTTAAGGGCATCTGAGGTGAACCATG (T)

(A)
T
T  G
C=G
A=T
T-G
T=A
C=G
G=C
C=G
C=G
C=G
T-G
G=C

[xylj] ----> 5'CTCCTTTG=CTGTTTTATT-3' ----> [xyll]
**xylL-xylT intergenic regions**

Intergenic region between the *xylL* and the *xylT* genes.

```
pDK1   GTCCCCCTGAGACTCATTTTCGGGTTTGGGAGGCCAGGCATCAACCCCAAGAGCTGTT
pWWO   GACCCCCTGAGGCATTTTCGGGTTATGGCGGCATCA-CCCAGAGCTGTT
        * * *
```

```
pDK1   GGGGGGATGCTTCCGTCATGTTTA
pWWO   GGGGGATACCTTCCGTCATGTTTA
        * *
```

Intergenic region between the *xylZ* and the *xylL* genes.

```
pDK1   AGGCTCCTTTGGTCCGCTTACTTTGAGGGGGGCTGTTTTATTCGGCAGTAG
pWWO   AGGCTCCTTTGGTCCGCTTACTTGGGAGGGGCTGTTTTATTCGGCAGTAG
        *
```
Fig. 29. Comparison of the nucleotide sequence of stem and loop structures from the pDK1 and pWWO TOL plasmids. The nucleotides that actually comprise the stem and loop structure are underlined. Non-homologous nucleotides are indicated by asterisks and their position in the stem and loop structure can be seen in Fig. 28 as indicated by parentheses.
nucleotide sequence was very near homologous (Fig. 29). Discrepencies in nucleotide sequence (shown in parentheses in Fig. 28) at these intergenic locations tend to cancel each other out (in reference to the overall stability of the stem and loop structure).

Analysis of the nucleotide sequence (possible operator/promoter regions) upstream of the $xylLT$ region (located at 2132-2182 base pairs on Fig. 21) from the pDK1 TOL plasmid included comaparisions in nucleotide sequence from the OP1 (promoter/operator region of the 'upper' pathway structural genes) of the PWWO TOL plasmid, and the OPs (operator/promoter region of the $xylS$ gene). A strong homology exists between the three sets of nucleotide sequences so as to suggest the possibility of an operator/promoter region upstream of the $xylLT$ genes. These comparasions can be seen on Fig. 30. A comparison nucleotide sequences of a suggested activator sequence (located at 2100-2128 base pairs in Fig. 21) upstream of the $xylL$ gene (of the TOL pDK1 plasmid) to those of upstream activator sequences of the $xylS$ gene (TOL pWWO plasmid) and the $xylCMABN$ operon (TOL pWWO plasmid) can also be seen in Fig. 31. This could be of potential significance as this sequence may be responsible for the binding of regulatory gene products such as the NtrA sigma factor, which in turn may
<table>
<thead>
<tr>
<th>Consensus</th>
<th>AA-AAG</th>
<th>TC</th>
<th>TGG-G-T</th>
<th>TTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPI (pWW0)</td>
<td>GATGAAATAAGGG-GATCCTTATAAGCAATGCCATGGGCTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xylLT (pDX1)</td>
<td>GG-TTAAAGAGAGAAGATCGAGAGATGCTGGAGAAAGGACGTCACG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPs (PWW0)</td>
<td>TTCTTAAAGAAGATCGTTCTGGGTGTGGCTGGGCTTATTTTG</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Fig. 30. DNA analysis of operator/promoter regions. A suggested operator/promoter region for the xyI\text{LT} region from the \emph{P. putida} pDK1 TOL plasmid shows a significant homology in nucleotide sequence to that of the OP1 and OPs regions from the \emph{P. putida} pWWO TOL plasmid. Nucleotides that are between these regions are underlined.
UAS xylS  TAGCATTTCCTAAGGCTTCCTGAAAGATTAACCAATTGATGATT-TG  (pWWO)
xylL  GTGTAACTCTTCCGCTGCCAGGGGAGAC---CTCGTGTGATCTTTATG  (pDK1)
UAS xylCAB  GATCAAAAATCGACAGGTGTTATG-------CGCGATTGATGATT-TG  (pWWO)
**Fig. 31.** Comparison of nucleotide sequence of the upstream activator sequences between the *xylS* gene from the *Pseudomonas putida* pWWO TOL plasmid, the *xylL* gene from the *Pseudomonas putida* pDK1 TOL plasmid and the *xylCAB* gene from the *Pseudomonas putida* pWWO TOL plasmid.
induce the appropriate response in the expression of downstream genes.

Subcloning of the xylLT region

In order to determine the function of the individual genes and other sequences encoded by the xylLT region a series of subclones of the pBK188 DNA were constructed. Subcloning involved the insertion of the xylLTE genes into suitable E. coli expression vector systems. The expression vectors utilized in this study were pUC18 and pUC19. The pBK188 clone which carried the 5.4 kbp EcoRI fragment of the pDKR1 cointegrate plasmid in the EcoRI site of PBR325 was digested using suitable restriction enzymes and fragments and portions subcloned in the proper orientation into suitable restriction sites of the expression vectors. The recombinant DNA was then transformed into E. coli DH5α. Utilizing data from our DNA analysis of the determined nucleotide sequence, we were able to construct a detailed restriction endonuclease map of the entire xylZLTE region of the pDK1 TOL plasmid. Complete knowledge of the restriction endonuclease sites facilitated the creation of several important subclones from pBK188. A PvuII/FspI fragment (this FspI restriction endonuclease site proved to be extremely helpful as it was located in between the xylL and the xylT genes) was inserted
into the SmaI site of the multiple cloning site of the pUC19 expression vector. This novel plasmid was designated pBK191, was 4.3 kbp in size, and encoded the \textit{xylL} gene. A XhoI fragment from pBK188 was inserted into the SalI site of the multiple cloning site of the pUC18 expression vector. This novel plasmid was designated pBK189, was 4.1 kbp in size and encoded the \textit{xylTE} genes. A \textit{PvuII/EcoRI} fragment of pBK188 was inserted into the \textit{SalI/EcoRI} site of the multiple cloning site of the pUC19 expression vector. This novel plasmid was designated pBK692, was 6.4 kbp in size and encoded the \textit{xylLTE} genes. A \textit{PvuII/SmaI} fragment of pBK188 was inserted into the \textit{SmaI site} of the multiple cloning site of the pUC19 expression vector. This novel plasmid was designated pBK391, was 4.8 kbp in size and encoded the \textit{xylLT} genes. A \textit{FspI/PstI} fragment of pBK188 was inserted into the \textit{SmaI site} of the multiple cloning site of the expression vector pUC19. This novel plasmid was designated pBK591, was 3.8 kbp in size and encoded the \textit{xyle} gene. A \textit{FspI/SmaI} fragment of pBK188 was inserted into the \textit{SmaI site} of the multiple cloning site of pUC19. This novel plasmid was designated pBK291, was 3.1 kbp in size and encoded the \textit{xylT} gene. An overview of the subcloning strategy and the subclones created in this study can be seen in Fig. 32. The pUC expression vectors utilized in the creation of the various subclones involved in this
Fig. 32. Construction of subclones. The pDKR1 cointegrate plasmid HindIII fragment was first cloned into an E. coli transmissible plasmid pBR322 (this fragment contained all the genes of the lower pathway and done by Elahe Azadpour) designated pBK489. A HindIII/KpnI fragment from the pBK489 was subcloned into an E. coli expression vector pUC19 which resulted in recombinant DNA containing the xylXYZ genes capable of producing the dihydrodiols from benzoate substrate. An EcoRI fragment from the pDKR1 cointegrate plasmid was inserted into an EcoRI site of the E. coli transmissible plasmid pBR325 (done by John Voss). This EcoRI fragment contained the xylZLTE genes together with portions of the xylXY and the xylG genes. The EcoRI fragment was inserted into the EcoRI site of an E. coli expression vector pUC19 and the recombinant DNA designated pBK188. Various fragments from the pBK188 plasmid were cloned into the E. coli expression vectors pUC18/19. A FspI/SmaI fragment was inserted into the SmaI site of pUC18 (xylT) and designated pBK291. A FspI/PstI fragment was inserted into the SmaI/PstI site of pUC19 (xylE) and designated pBK591. A PvuII/SmaI fragment was inserted into the SmaI site of pUC19 (xylLT) and designated pBK391. A PvuII/FspI fragment was inserted into a SmaI site of pUC19 (xylL) and designated pBK191. A PvuII/FspI fragment was inserted into the SmaI site of pUC19 (xylLTE) and designated pBK692. A XhoI fragment was inserted into the SalI site of pUC19 (xylTE) and designated pBK189.
Fig. 33. Construction of various subclones prepared for the characterization of the xylLTE region. The *E. coli* expression vectors pUC18 and pUC19 were used as cloning vectors. The respective genes were placed into the multiple cloning site of the genetically constructed vectors such that the open reading frame of the inserted genes were in the correct orientation just downstream of the *lac* promoter.
Verification of subclones

Verification of the identity of subclones was routinely carried out by restriction endonuclease mapping of the various recombinant plasmids, verifying the presence and location of known restriction sites within the expression vector and the cloned structural genes. Verification of the subclones required the selection of restriction endonuclease sites that were off-center in the target DNA (structural gene(s)) hence distinguishing between two similarly sized digestion fragments. Also required was the presence of at least two identical restriction endonuclease sites either both present in the target or vector DNA, or one restriction site present in the target DNA and the other in the vector DNA. The size of the restriction fragments generated from the digestions verified the presence of both the presence of the required structural gene and the orientation of the start codon (ATG) of the inserted gene to be correctly positioned in front of the α-peptide start region of the pUC vector lac promoter. The only difference of the genetically engineered pUC expression vectors (pUC18 and pUC19) was that their multiple cloning sites in front of the lac promoter are reversed. This helped in the proper identification of the
Fig. 34. Verification of the pBK291 (xylT) and pBK591 (xylE) subclones. *Pst*I restriction endonuclease digestion of the pBK291 (xylT) subclone (lane 1) resulted in 200 bp and 2936 bp DNA fragments. *Pst*I restriction endonuclease digestion of the pBK591 (xylE) subclone (lane 13) resulted in 640 bp and 3136 bp fragments.
Fig. 35. Verification of the pBK191 (xylL) and pBK391 (xylLT) subclones. SalI restriction endonuclease digestion of the pBK191 (xylL) subclone resulted in 510 bp, 230 bp and 3596 bp fragments. KpnI restriction endonuclease digestion of the pBK191 (xylL) subclone resulted in 1070 bp, 270 bp and 2996 bp fragments. SalI restriction endonuclease digestion of the pBK391 (xylLT) resulted in 900 bp, 570 bp and 3316 bp DNA fragments. KpnI restriction endonuclease digestion of the pBK391 (xylLT) subclone resulted in 1080 bp, 700 bp and 3006 bp fragments.
generated subclones. Fig. 34 shows agarose gel analysis of the restriction endonuclease digestions of subclones pBK291 and pBK591 (carrying the *xylT* and the *xylE* genes, respectively) with restriction endonuclease *PstI*. The *PstI* digestion of the pBK291 (*xylT*) subclone yielded 200 bp and 2936 bp DNA fragments (lane 1). The *PstI* digestion of the pBK591 (*xylE*) subclone yielded 640 bp and 3136 bp DNA fragments (lane 13). Fig. 35 shows the restriction endonuclease digestion of subclones pBK191 and pBK391 (carrying the *xylL* and the *xylLT* genes) with a variety of restriction endonucleases. The pBK191 (*xylL*) subclone was digested with *SalI* to yield 510 bp, 230 bp and 3596 bp DNA fragments (lane 11). The pBK191 (*xylL*) subclone was also digested with *KpnI* to yield 1070 bp, 270 bp and 2996 bp DNA fragments (lane 6). The pBK391 (*xylLT*) subclone was digested with *SalI* to yield 900 bp, 570 bp and 3316 DNA fragments (lane 9). The pBK391 (*xylLT*) subclone was also digested with *KpnI* to yield 1080 bp, 700 bp and 3006 bp DNA fragments (lane 4). The pBK291 subclone was digested with *SalI* to yield 230 bp, 500 bp, 1200 bp and 2856 bp DNA fragments (Data not shown). In addition to restriction endonuclease verification of the various subclones, the recombinant DNA were also subjected to double-stranded nucleotide sequencing to determine both the presence of the required structural gene
and the correct orientation of the gene with respect to the lac promoter of the pUC expression vector.

Enzyme assays

E. coli DH5α strains carrying various subclones were cultured and their cell-free extracts prepared. Using the Lowry method (Lowry et al. 1951) for protein determination, the protein concentration of the cell-free extracts were calculated. Assays were performed for DHCDH and C230 enzymatic activity as described in the Methods and Materials.

The results from Fig. 36 shows a DHCDH assay using the XylT cell-free extract produced from a DH5α carrying pBK291. Biologically produced benzoate/m-toluate dihydroxy diols were used as substrates for the DHCDH assay. Enzymatic activity was monitored as a function of time by following the rate of NADH formation. This was measured spectrophotometrically at a wavelength of 340 nm. The assay showed no observable DHCDH activity.

The results from Fig. 37 shows a DHCDH assay using the XylTE cell-free extract (produced from a DH5α carrying pBK189) alone at first followed by the addition of XylL cell-free extract (produced from DH5α carrying pBK191) after 60 seconds. The substrate for the DHCDH was biologically
produced $p$-toluate dihydroxy diol. Enzymatic activity was monitored as a function of time for the production of ring fission product (hydroxymuconic semialdehyde), measured spectrophotometrically at a wavelength of 382 nm. The assay showed no DHCDH activity with the XylTE cell-free extract, however on addition of the XylL cell-free extract a significant increase in DHCDH activity was observed, however this activity was 3-fold less than the DHCDH activity of the xylLT (pBK391) cell-free extract.

The results from Fig. 38 shows a DHCDH assay using the XylLT cell-free extract produced from DH5α carrying pBK391 and using biologically produced $m$-toluate dihydroxy diol as a substrate. The assay showed the highest level of DHCDH activity observed using the various subclones with a 3-fold increase in DHCDH activity than the XylL (pBK191) cell-free extract.

The results from Fig. 39 shows a DHCDH assay using the XylTE cell-free extract (produced from DH5α carrying pBK189) at first followed by the XylL cell-free extract (produced from DH5α carrying pBK191) after 60 seconds. Benzoate dihydroxy diol was used as the substrate for DHCDH. Enzymatic activity was monitored as a function of time for NADH formation, measured spectrophotometrically at a
wavelength of 340 nm. The assay showed no DHCHD activity with the XylTE cell-free extract. The addition of XylL cell-free extract did not give DHCDH activity but this activity was 3-fold less than the DHCDH activity of the XylLT (pBK391) cell-free extract. Thus the addition of the XylL and the XylT extracts showed no increase in DHCDH activity from that of the XylL extracts alone.

The results from Fig. 40 shows a C23O assay using XylTE cell-free extract produced from DH50α carrying pBK189 only. Catechol (Sigma™ Chemical Company) was used as a substrate for C23O. Enzymatic activity was monitored as a function of time for ring-fission product (hydroxymuconic semialdehyde), measured spectrophotometrically at a wavelength of 375 nm. This was the highest level of C23O activity of all the subclones assayed showing a 8-fold increase in C23O activity as compared to the XylE (pBK591) cell-free extract.

The results from Fig. 41 shows a C23O assay using XylE cell-free extract produced from DH50α carrying pBK591 only. Catechol (Sigma™ Chemical Company) was used as a substrate for C23O. Enzymatic activity was monitored as a function of time for ring-fission product (hydroxymuconic semialdehyde), measured spectrophotometrically at a wavelength of 375 nm. The assay showed a C23O activity that was 8-fold lower than
Extract (0.02 mg protein)
**Fig. 36.** DHCDH activity towards benzoate/m-toluate dihydroxy diols. Enzymatic activity was monitored spectrophotometrically at a wavelength of 340 nm. Reaction conditions were as follows:

- 50.0 mM tris-HCl pH 8.0
- 0.4 μmol biologically prepared diol
- 1.0 mM NAD⁺
- 1.0 ml total volume

0.02 mg of protein was added as cell-free extract prepared from E. coli DH5α cells containing pBK291 (xylT).
Accumulation of Ring-fission Product

- **L Extract** (0.02 mg protein)
- **TE Extract** (0.9 mg protein)

Chemical structure:

\[
\text{H}_3\text{C} - \text{C} - \text{COOH}
\]

**Absorbance (382 nm)**

**Time (sec)**

0 60 120 180
Fig. 37. DHCDH activity towards p-toluate dihydroxy diol.
Enzymatic activity was monitored spectrophotometrically at a wavelength of 382 nm (hydroxymuconic semialdehyde formation). Reactions conditions were as follows 50.0 mM tris-HCl pH 8.0, 0.4 µmol biologically prepared dihydroxy diol, 2.0 mM NAD⁺, 1.0 ml total volume. 0.9 mg of protein as cell-free extract prepared from E. coli DH5α cells containing pBK189 (xylTE) and this followed by the addition of 0.02 mg protein as cell-free extract prepared from E. coli DH5α cells containing pBK191 (xylL).
LT Extract (0.04 mg protein)
Fig. 38. DHCDH activity monitored towards m-toluate diol.

Enzymatic activity was monitored spectrophotometrically at a wavelength of 340 nm (NADH formation). Reactions conditions were as follows 50.0 mM tris-HCl pH 8.00, 4 μmol biologically prepared dihydroxy diol, 2.0 mM NAD$^+$, 1.0 ml total volume. 0.04 mg protein as cell-free extract prepared from E. coli DH5α cells containing pBK391 (xylLT).
Graph showing absorbance (340 nm) over time (seconds) for two extracts:

- L Extract (0.02 mg protein)
- TE Extract (0.02 mg protein)
**Fig. 39.** DHCDH activity monitored towards benzoate dihydroxy diol. Enzymatic activity was monitored spectrophotometrically at a wavelength of 340 nm (NADH formation). Reactions conditions were as follows 50.0 mM tris-HCl pH 8.00, 4 μmol biologically prepared dihydroxy diol, 1.0 mM NAD⁺, 1.0 ml total volume. 0.02 mg protein as cell-free extract prepared from *E. coli* DH5α cells containing pBK189 (xylTE) and this followed by the addition of 0.02 mg protein as cell-free extract prepared from *E. coli* DH5α cells containing pBK191 (xylL).
the XylTE (pBK189) cell-free extract C230 activity.

The results from Fig. 42 shows a C230 assay using XylLTE cell-free extract produced from DH5α carrying pBK692 only. Catechol (Sigma™ Chemical Company) was used as a substrate for C230. Enzymatic activity was monitored as a function of time for the ring-fission product (hydroxymuconic semialdehyde), measured spectrophotometrically at a wavelength of 375 nm. The assay showed the lowest C230 activity of almost an 80-fold drop in activity as compared to the XylTE (pBK189) cell-free extract activity.

The results from Fig. 43 shows a C230 assay using XylE and XylT cell-free extracts produced from separate cultures of DH5α carrying pBK591 and pBK291. Catechol (Sigma™ Chemical Company) was used as a substrate for C230. Enzymatic activity was monitored as a function of time for ring-fission product (hydroxymuconic semialdehyde), measured spectrophotometrically at a wavelength of 375 nm. The assay showed an activity that was comparable to the C230 activity of the XylE cell-free extract. A comparison of specific enzymatic activities of DHCDH (with various substrates) and C230 using catechol as a substrate can be seen in Table 9 and in Table 10 respectively.

Analysis of the data showed that the xylLT gene product
showed the highest levels of DHCDH activity. The *xylL* gene product also expressed DHCDH enzymatic activity but at only 30% the level for strains carrying the *xylLT* genes. The relative activities towards benzoate, meta and para-toluate dihydroxydiols were the same. Also observed was that no DHCDH activity was detected in cell-free extracts of the *xylT* (that is *xylTE* and *xylT*) structural genes nor did their addition to those of either the *xylL* or the *xylLT* improve enzymatic activities above that detected alone. The C230 activities of the various subclones showed that the *xylTE* gene product showed the highest C230 enzymatic activity (three to five times the specific activity of the *xylE* gene product). The *xylLTE* gene product however showed the lowest C230 enzymatic activity. The *xylT* and the *xylE* gene products, when added separately showed no increase in C230 enzymatic activity over the *xylE* gene product when added alone. This last enzymatic assay helped suggest a location for the regulatory control of the *xylT* gene which is at the transcriptional (DNA) level rather than at the protein level.
Fig. 40. C23O activity monitored towards catechol.

Enzymatic activity was monitored spectrophotometrically at a wavelength of 375 nm (hydroxymuconic semialdehyde formation). Reactions conditions were as follows 48.50 mM KH$_2$PO$_4$, 0.33 mM catechol, 1.00 ml total volume. 3.5 µg protein as cell-free extract prepared from E. coli DH5α cells containing pBK189 (xylTE).
**Fig. 41.** C230 activity monitored towards catechol.

Enzymatic activity was monitored spectrophotometrically at a wavelength of 375 nm (hydroxymuconic semialdehyde formation). Reactions conditions were as follows 48.50 mM KH$_2$PO$_4$, 0.33 mM catechol, 1.00 ml total volume. 3.4 µg protein as cell-free extract prepared from *E. coli* DH5α cells containing pBK591 (xylE).
Fig. 42. C230 activity monitored towards catechol. Enzymatic activity was monitored spectrophotometrically at a wavelength of 375 nm (hydroxymuconic semialdehyde formation). Reactions conditions were as follows 48.50 mM KH$_2$PO$_4$, 0.33 mM catechol, 1.00 ml total volume. 37.5 µg protein as cell-free extract prepared from *E. coli* DH5α cells containing pBK692 (xylLTE).
Fig. 43. C230 activity monitored towards catechol. Enzymatic activity was monitored spectrophotometrically at a wavelength of 375 nm (hydroxymuconic semialdehyde formation). Reactions conditions were as follows 48.50 mM KH₂PO₄, 0.33 mM catechol, 1.00 ml total volume. 0.66 µg protein as cell-free extract prepared from *E. coli* DH5α cells containing pBK291 (xylT) and 0.62 µg protein as cell-free extract prepared from *E. coli* DH5α cells containing pBK591 (xylE).
Table 9. Activity table showing the rates of DHCDH specific activities (nmol/min·mg⁻¹ protein). *E. coli* DH5α cells carrying plasmids pBK391 (*xylLT*), pBK191 (*xylL*), pBK189/pBK291 (*xylTE/xylT*) and pBK291 (*xylT*) individually were grown for 48 hours in Luria-Bertani broth containing 5 mM IPTG before harvesting and preparation of cell-free extracts.

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>xylLT</em></th>
<th><em>xylT</em></th>
<th><em>xylTE/xylT</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoyl diol</td>
<td>3250</td>
<td>975</td>
<td>&lt;1</td>
</tr>
<tr>
<td><em>m</em>-Toluate diol</td>
<td>2975</td>
<td>925</td>
<td>&lt;1</td>
</tr>
<tr>
<td><em>p</em>-Toluate diol</td>
<td>215</td>
<td>55</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
Table 10. Activity table showing the rates of C23O specific activities (nmol/min·mg⁻¹ protein). *E. coli* DH5α cells carrying plasmids pBK692 (xylLTE), pBK191/pBK291 (xylL/xylT), pBK591 (xylE) and pBK189 (xylTE) individually were grown for 48 hours in Luria-Bertani broth containing 5 mM IPTG before harvesting and preparation of cell-free extracts. All assays for C23O activity utilized catechol (0.33 mM final concentration) as a substrate.

<table>
<thead>
<tr>
<th>Cloned genes/vector</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylLTE/pUC19</td>
<td>223</td>
</tr>
<tr>
<td>xylL/pUC19 + xylT/pUC19</td>
<td>3028</td>
</tr>
<tr>
<td>xylE/pUC19</td>
<td>3044</td>
</tr>
<tr>
<td>xylTE/pUC19</td>
<td>25435</td>
</tr>
</tbody>
</table>
CHAPTER IV

DISCUSSION

Concern about an environmentally unsafe world has prompted the United States Congress to pass the Toxic Substances Control Act (TSCA) in 1976, which required all new chemical substances that may prove to be hazardous, to be approved by and registered with the Environmental Protection Agency. Other Acts of Congress passed were the Federal Insecticide, Fungicidal and Rodenticide Act (FIFRA) and The Clean Water Act. These laws call for an in depth study of these toxic substances and their impact on the environment and the general population together with an assessment of the means of biodegradation of these toxic substances of which the understanding of the roles of these microorganisms might play and of the catabolic processes involved.

Many synthetic chemicals that are added to the environment are derivatives of benzene, toluene and xylenes. If these 'man-made' chemicals in the form of pesticides, herbicides and industrial effluents should prove to be recalcitrant in nature to microbial biodegradation then accumulation in soils and water levels would be environmentally disastrous. Serious environmental damage
together with accumulation in animal tissues may be the eventual result. Occurrences such as this pose a threat to the environment and to populations in general make it imperative that we study microorganisms that possess the ability to biodegrade both synthetic and natural pollutants.

Due to the high negative resonance stabilization energies possessed by aromatic hydrocarbons (benzene and its derivatives) these compounds are extremely stable in our environment. However, as discussed in this Dissertation, microorganisms possess a variety of metabolic pathways which under normal growth conditions can degrade a variety of toxic aromatic compounds to metabolically safe intermediates that can be utilized by the microorganism in normal biochemical processes.

This interest in elucidating the fine structure of genes and operons encoding the bacterial enzymes for aromatic degradation prompted us to subclone regions of the P. putida HS1 pDK1 TOL plasmid DNA for further study. The 'lower' operon of TOL plasmids encodes enzymes for the conversion of aromatic acids (benzoates, m-toluate and p-toluate, 3,4-dimethylbenzoate and 3-ethylbenzoate) to catechols and subsequently to Krebs cycle intermediates via the meta-cleavage pathway. My research has dealt primarily with the region of the TOL 'lower' pathway which had been
previously determined to encode the enzyme
1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase
(DHCDH).

A 5.4 kbp EcoRI restriction fragment from the P. putida
HS1 TOL plasmid pDK1, encoding the xylZLTE region, was cloned
into the E. coli plasmid pBR325 by Voss to create pBK188
(Voss 1989, Benjamin et al. 1991). The enzymes encoded by
this fragment are 1,2-dihydroxycyclohexa-3,5-diene
carboxylate dehydrogenase, catechol-2,3-dioxygenase and a
subunit of the toluate-1,2-dioxygenase complex. The findings
of this investigation of the pDK1 TOL region located between
the xylZ and xylE genes and encoding DHCDH are now discussed.

Previous studies by other laboratories noted that the
xylL gene contained one internal XhoI restriction
endonuclease site (Shaw and Williams 1988). However,
nucleotide sequence data obtained in this study identified an
additional XhoI restriction endonuclease site not previously
reported in the literature (creating a 0.15 kbp fragment
within the xylL gene) (Fig.1). We were able to verify the
presence and orientation of this new XhoI site/fragment by
first subcloning a SalI fragment of the xylL region, which
overlapped these sites, into an M13 phage. The nucleotide
sequence of this fragment verified the presence of the
additional XhoI site. In order to avoid problems of this
type, the DNA sequence described here was always completed for both complementary strands and no two sequences were 'connected' without at least 30 bp of overlap. In this manner the entire nucleotide sequence of the DHCDH region was determined (Fig. 21 and Fig. 22). It is of significant interest to note that this study provided the first nucleotide sequence data reported for the 1,2-dihydroxycyclohexa-3,5-diene carboxylate dehydrogenase region (DHCDH) from any TOL plasmid.

Computer analysis of the derived nucleotide sequence revealed the presence of two open reading frames within the DHCDH region (Table 6). The first open reading frame has been assigned a putative ribosome binding site of 'GAGGTG' and is 777 bp in length (259 codons). It encodes a predicted polypeptide of 27,853 Da. It has kept the original xylL notation. On comparison with the xylL gene from the pDK1 TOL plasmid to that of the pWWO TOL plasmid we found a 93% homology in nucleotide sequence. Of the non-homologous nucleotides 70% were third position differences, 16% were second position differences and 13% were third position differences. The second open reading frame has been assigned a putative ribosome binding site of 'GAGGTG' and is 303 bp in length (101 codons). This open reading frame encodes a predicted polypeptide of 11,233 Da. This gene has been
assigned the designation \textit{xyl}T. With this information we proceeded to attempt to elucidate the function(s) of these two gene products. Several possibilities existed. First, the DHCDH enzyme might consist of two different subunits, heterodimers for example. Second, there was the possibility that one gene product might have novel functions not previously noted in TOL studies. Both could encode separate DHCDH activities with different substrate preferences or one could have a function unrelated to DHCDH.

We therefore proceeded to investigate these and other possibilities. To aid us in this investigation, several subclones were prepared from the original pBK188 (\textit{xylZLTE}) recombinant plasmid (Fig. 32). Subclones constructed included ones encoding \textit{xylLT} (pBK391), \textit{xylL} (pBK181), \textit{xylT} (pBK291), \textit{xylTE} (pBK188), \textit{xylLTE} (pBK692) and \textit{xylE} (pBK591). Each of these utilized a pUC \textit{E. coli} expression vector (Fig. 33). The genes were placed into suitable restriction endonuclease sites within the multiple cloning site and in the proper orientation to allow gene products to be produced under the control of the plasmid's genetically engineered \textit{lac} promoter. Cell-free extracts were prepared from \textit{E. coli} DH5\(\alpha\) strains carrying each of the subclones after a two day growth period (the \textit{lac} promoter is under the control of the CAP
protein and hence not expressed well under log phase high growth conditions). A lac inducer isopropylthiogalactopyranoside (IPTG), was added to all cultures but, due to the high copy number of the pUC plasmids, this inducer proved to be unnecessary (the high operator copy number diluted out the few copies of the lac repressor found per cell).

Expression of DHCDH by recombinant strains was ascertained either by (i) coupled assays using XylTE extracts expressing C230 as a reporter function or (ii) direct spectrophotometric measurement of the rate of NADH formation.

My work showed that, of the four xyl subclones of the xylLT region (xylLT-pBK391, xylL-pBK191, xylTE-pBK189 and xylT-pBK291), the XylLT (pBK391) extracts exhibited the highest levels of DHCDH activity (Table 9). However, xylL (pBK191) subclones also expressed DHCDH activity, but at only about 1/3 of the activity levels for E. coli strains carrying the xylLT (pBK391) subclone (Fig. 37 and Fig. 39). The relative DHCDH activities against the benzoate, meta-toluate and the para-toluate diols were very similar for both extracts. It was further observed that no DHCDH enzymatic activity was detected in cell-free extracts of the XylT subclones (pBK291) or the XylTE (pBK189) (Fig. 36, Fig. 37 and Fig. 39). Surprisingly, the addition of these extracts (XylT
or XylTE) to XylL preparations (pBK191) failed to increase the observed DHCDH activity towards any of the diol substrates (A summary of DHCDH activities can be seen in Table 9). Taken together this is most consistent with the possibility that the DHCDH enzymatic activity is associated with only the xylL gene product in the P. putida HS1 pDK1 TOL plasmid. The increased activity when xylT is included downstream of the xylL in subclones appears to be due to an effect on gene expression at the level of transcription or translation.

It was suggested in previous reports in the literature that this region may be an additional site of regulatory control for the 'lower' operon of the TOL pDK1 plasmid. The xylLTE region provides an interesting area of the 'lower' operon to be investigated due to it being the set of genes that encode the enzymes responsible for the metabolism of catechol. Catechol is an important metabolite in several other pathways, for example the nah operon (Patel and Barnsley 1980; Patel and Gibson 1974; Ensley and Gibson 1983; Yen and Gunsalus 1982) and the sal operon (Catterall et al. 1971; Yen and Gunsalus 1982) and it is also the substrate that is acted upon which leads to the bifurcation of the pathway into the meta- and ortho-cleavage pathways. It would seem plausible to suggest that this region could be under
some type of regulatory control, either at the DNA or protein level.

The possibility of functional regulatory elements within the DHCDH region has been previously suggested by Keil et al. (1987) who observed a weak promoter activity located upstream of xylE. The meta-cleavage operon from the *P. putida* HS1 pDK1 TOL plasmid is one of the largest operons found in bacteria. The collective work carried out by our laboratory has yielded the entire nucleotide sequence of the meta-cleavage operon from pDK1 (consisting of thirteen genes and extending over 10 kbp of DNA). Of the twelve intergenic regions, six pairs of genes overlap (negative distance of separation) and four are 21 bp or less (see Table 8). The remaining three are significantly larger and are found between xylI and xylH (37 bp) and upstream (115 bp) and downstream (139 bp) of xylL. Computer analysis of these three intergenic regions has revealed each of them to possess palindromic nucleotide sequences theoretically capable of forming stem and loop structures. Those for the xylZL and xylLT intergenic regions are shown in Fig. 28 and in Fig. 29. The xylZL intergenic region palindromic structure shows strong homology to previously characterized *E. coli* rho-independent terminators. Both these palindromic regions are highly conserved when the two TOL plasmid sequences are
compared. On examination of the comparison in nucleotide sequence between the intergenic regions (xylZ/xylL and xylL/xylT) between the pDK1 and pWWO TOL plasmids (Fig. 29) we see a significant homology and the differences can be explained. The single difference in the stem and loop structure between the two TOL plasmids xylZ/xylL intergenic region is located in the loop (Fig. 28) and does not affect the stability of the stem and loop overall structure. There are a few nucleotide sequence differences in the xylL/xylT intergenic region, yet these differences can be explained by their location. The two negative signs in Fig. 28 show base pairs not found in pWWO but present in pDK1. The positions of these base pairs is exact in that their effect is cancelled. The dATP found in pWWO as opposed to the dGTP found in the same site of pDK1 destabilizes the stem and loop structure. However, there is a dGTP found in pWWO (as opposed to the dCTP in the same position in pDK1) that stabilizes the stem and loop structure. The dATP at the base of the stem is not accounted for but its net effect may not be of any significance.

As mentioned above, questions have been previously posed in the literature as to whether of there would be a secondary promoter inside the operon (Harayama and Rekik 1990). According to Harayama and Rekik this suggested that the
meta-cleavage operon resulted from a fusion of two DNA modules (xyl\textsubscript{XYZL}(T) and xyl\textsubscript{T}EGFQKH) each originally possessing their own promoter regions (Harayama and Rekik 1990).

These suggestions are supported by my data. The xyl\textsubscript{TE} intergenic region may have its own promoter as shown by the significant DNA sequence homology between the intergenic region of the xyl\textsubscript{TE} (pDK1) and the OP1 region of the 'upper' pathway genes in the pWWO TOL plasmid and the OPs region of the xyl\textsubscript{S} gene of the pWWO TOL plasmid (Fig. 30). Also seen is a certain degree of homology between the upstream activator sequences for xyl\textsubscript{CMABN} (pWWO) and xyl\textsubscript{S} (pWWO) and the xyl\textsubscript{LT} intergenic region (Fig. 31).

The similarity of these intergenic sequences to known transcriptional regulatory elements lead us to construct a further series of subclones for additional expression studies. Each of the following subclones created expressed C230 (XylE) activity (Fig. 32). The pBK591 subclone encoded only xyl\textsubscript{E}, pBK189 encoded xyl\textsubscript{TE} plus the xyl\textsubscript{LT} intergenic region, pBK692 encoded xyl\textsubscript{LTE} with both the xyl\textsubscript{ZL} and the xyl\textsubscript{LT} intergenic regions and pBK792 encoded xyl\textsubscript{YZL}TE with both the xyl\textsubscript{ZL} and the xyl\textsubscript{LT} intergenic regions.

Enzymatic analyses showed that \textit{E. coli} strains carrying the xyl\textsubscript{TE} (pBK189) subclone expressed catechol-2,3-
dioxygenase activity (Fig. 40) at levels five to eight times the C230 activity of *E. coli* strains carrying the *xylE* (pBK591) subclone (Fig. 41), using catechol as a substrate. This means that the addition of the *xylLT* intergenic region and *xylT* in some way is able to increase the enzymatic expression of both DHCDH (the next gene upstream as described earlier) and C230 activity (the next gene downstream).

The further inclusion of the *xylZL* intergenic region, in contrast, reduced the expressed C230 activity substantially. This is seen by comparing enzymatic activities in cell-free extracts from *E. coli* DH5α strains carrying the *xylLTE* (pBK692) subclone (Fig. 42) with those carrying *xylTE* (pBK189) (Fig. 40). In this case the XylLTE cell-free extract was shown to possess 10% of the C230 activity of the *xylE* subclone (pBK591) subclone and only 1% the C230 activity expressed by the *xylTE* (pBK189) subclone. It was still considered a possibility that this stimulatory and/or inhibitory effects could be at the post-translational level. Two or more protein products could interact to increase or decrease C230 activity. We therefore performed additional coupled enzymatic assays using cell-free extracts. Complementation assays using cell-free extracts of XylLT (pBK291) and the XylE (pBK591) were performed, with catechol
used as the C320 substrate for the reaction. The C230 activities obtained were the same whether only the XylE was present (pBK591) or when XylL or XylT cell-free extracts were included. This data suggests that basis for the stimulatory effect at the protein level is not very likely, but rather more plausible to be at the level of transcription. A summary of C230 activities expressed can be seen in Table 10.

These results are consistent with there being both positive and negative transcriptional control elements within the DHCDH region. To summarize, a transcriptional terminator appears to be located upstream of \textit{xylL} and a positive acting element appears to be found immediately downstream of \textit{xylL}. The possible requirement for the \textit{xylT} gene product for the action of either of these elements cannot be ruled out at this time.

Previous reports in the literature indicated a high level of nucleotide sequence homology between the \textit{xylT} gene (of the TOL plasmids pDK1 and pWWO) and the \textit{nahT} gene of the NAH7 plasmid carried by \textit{P. putida} strain \textit{PpG7} to chloroplast-type plant ferredoxins. A similarity to the \textit{nahT} gene is at the level of a shared amino acid motif, that is both appear to be members of a family of ferredoxin type proteins. \textit{PpG7} was the original strain which carried the NAH7 plasmid responsible for naphthalene biodegradation to
catechol (Worsey and Williams 1975; Yen and Gunsalus 1982; Dunn and Gunsalus 1973). Catechol undergoes further oxidation to citric acid cycle intermediates. Also found on the NAH7 plasmid is the sal operon which encodes nahGHINLJK. The nahG is the structural gene encoding salicylate hydroxylase. The nahH, nahI, nahN, nahL, nahJ and nahK are isofunctional structural genes corresponding to the xylE, xylG, xylF, xylJ, xylH and xylI genes, respectively, of the TOL pDK1 plasmid (an overview of the two pathways can be seen in Fig 44. Nucleotide sequence analysis of both the NAH and TOL structural genes have shown extensive homologies between the xylE and the nahH and between the xylG and nahI genes between the TOL and NAH plasmids, respectively (Harayama et al. 1987; Ghosal et al. 1987). However, the reaction catalyzed by the nahG gene product is completely different from that catalyzed by the xylL gene product (Harayama et al. 1991) even though both are the respective genes upstream of nahH (NAH7) and its counterpart xylE (TOL pDK1). There is also no nucleotide sequence homology between the xylL and the nahG structural genes. However Harayama and Rekik showed that homologous recombination occurred between the xylT region of pWWO and the region upstream of nahH of the NAH7 plasmid. Based on this observation, they proposed that the NAH7 plasmid encodes a gene that is analagous to the xylT.
Fig. 44. Comparison of biodegradative pathways from the pDK1 TOL plasmid and the NAH7 NAH plasmid. Both pathways converge onto a common product, catechol. The nahH, nahI, nahN, nahL, nahJ and nahK are isofunctional structural genes of the nah7 plasmid corresponding to the xylE, xylG, xylF, xylJ, xylH and xylI structural genes of the TOL pDK1 plasmid (Adapted from Harayama et al. 1987b).
structural gene, and that this gene is located in the region between the *nahG* and the *nahH* structural genes on the NAH7 plasmid. This homology in the nucleotide sequence between the *xylT* gene on the pWWO TOL plasmid and the now designated *nahT* gene of the NAH7 plasmid may possibly be due to the physiological roles that these structural genes play in the metabolism of catechol (Harayama and Rekik 1990).

Harayama and Rekik (1990) also noted that portions of the amino acid sequence of the XylT and NahT proteins were similar to those found in chloroplast-type ferredoxins, specifically the amino acid sequence Cys-XXXX-Cys-XX-Cys is present in each case. These ferredoxins belong to a class of electron transfer proteins that possess a characteristic 2Fe-2S cluster coupled with four non-variant cysteine residues. This class of ferredoxins were originally found in photosynthetic organisms, such as plants, algae and cyanobacteria (Higgins and Sharp 1988). However, this same class of ferredoxins has also been shown to exist in non-photosynthetic archaebacteria, with the *Halobacterium* species being an example, and most recently in a photosynthetic bacterium *Rhodobacter capsulatus*. Harayama et al. (1991) proposed that subunits of oxygenase type proteins have arisen due to the fusion of this class of chloroplast-type ferredoxins and the carboxyl-terminal
sequences (responsible for electron transfer). Some examples include a monoxygenase (monoxygenase encoded for by the MmoC gene), xylene monoxygenase (xylA component) (Suzuki et al. 1991), benzoate dioxygenase (benC component) (Neidle et al. 1987) and toluate dioxygenase (xylZ component) Harayama et al. 1986). Harayama et al. (1991) have also proposed an evolutionary relationship of these ferredoxin type proteins and in fact classified these ferredoxins into five subfamilies, some of which can be seen in Fig 45.

Ferredoxin genes of the xylT from pWWO (and pDKl in this study) and the nahT were shown to be similar not so much in nucleotide and amino acid sequence but more significantly in the position of nucleotide sequences that encode the cysteine residues that are consistent with this class of chloroplast-type ferredoxins. In this study I have shown a significant homology in the nucleotide sequence that encodes the specific cysteine (six residues) codons of functional interest between the xylT gene of the pDKl TOL plasmid to the xylT of the pWWO TOL plasmid and the nahT gene of the NAH7 plasmid. A comparison of the nucleotide sequence between the nahT gene from the NAH7 plasmid and the xylT gene from the pDKl and pWWO TOL plasmids can be seen in Fig. 26. However, these two structural genes show little similarity with the other subclasses of the chloroplast-type ferredoxins. In
Fig. 45. Phylogenetic relationships between various chloroplast-like ferredoxin genes and the xylT and the nahT genes from the pWWO, pDK1 TOL plasmids and the NAH7 plasmid. MmmoC-amino terminal sequence of the electron transfer component of methane monooxygenase, benCN-amino terminal sequence of benC, an electron transfer component of benzoate-1,2-dioxygenase, xylZN-amino terminal sequence of the xylZ, an electron transfer component toluate-1,2-dioxygenase, PHP5N-amino terminal sequence of Polypeptide 5 of phenol hydroxylase, vanBC-carboxyl terminal sequence of vanB, an electron transfer component of vanillate decarboxylase, xylA-the amino terminal sequence of xylA, an electron transfer component of xylene monooxygenase (Adapted from Harayama et al. 1991).
fact Harayama et al. showed that even though the \textit{xylA}, \textit{xylT} and \textit{xylZ} are encode by both the TOL plasmids (pWWO and pDK1) these genes do not show substantially greater similarities for each other than they do with the other chloroplast-type ferredoxin genes. This observation prompted Harayama et al. 1991 to suggest that the ferredoxin genes on the pWWO (pDK1 in this study) TOL plasmid did not have a common ancestral gene but rather were each derived from different ancestral genes. Also suggested was that even though the \textit{xylT} gene and the \textit{nahT} gene were located on separate biodegradative plasmids, they both possessed strong nucleotide sequence and amino acid homologies for each other, and hence must be responsible for a common step in the metabolism of catechols to their respective intermediates which can be utilized by the citric acid cycle.

In summary the \textit{xylLT} region consists of two open reading frames. The open reading frame for the \textit{xylL} structural gene is 777 base pairs in length and encodes a polypeptide of 27,853 Da, which is responsible for DHCDH activity. The open reading frame for the \textit{xylT} structural gene is 303 base pairs in length and encodes a polypeptide of 11,133 Da. The \textit{xylL} gene from the TOL pDK1 plasmid shows a 93\% homology in nucleotide sequence with the corresponding region in the related TOL pWWO plasmid. The \textit{xLT} gene from the TOL pDK1
plasmid also shows a significant homology in nucleotide sequence to the TOL pWWO plasmid and the NAH7 plasmid (xylT and nahT genes respectively) in the regions of the respective genes that encode the cysteine residues of this particular class of ferredoxins.

The intergenic regions between the xylZ/xylL and xylL/xylT may represent significant transcriptional control elements within the TOL meta-cleavage operon. However, as mentioned previously by Harayama and Rekik, this region may simply be the result of a physical joining of two sub-operons which may have formed the meta-cleavage operon. The intergenic region upstream of the xylL (downstream of xylZ) contains a palindromic nucleotide sequence that suggest the presence of a possible rho-independent terminator. If this transcriptional terminator element were 100% effective, then the xylL gene may never be expressed. Obviously this is not the case. However, a possibility that the xylT gene product may act as trans effector and negate the rho-independent terminator effect exists. The intergenic region between the xylL and the xylT genes may possess an operator/promoter region, which would not directly effect the transcription of the xylL gene, it being located in a downstream position. However, this very same operator/promoter region could be regulated by an unknown product (possibly the xylT gene
product) which would cause the enhanced expression of the structural genes downstream of the intergenic region. As an example, in this study C230 activity as encoded by the \textit{xylE} gene did seem to be enhanced. Further work centered around site-directed mutagenesis of the \textit{xylT} region would no doubt help answer some of these questions asked about the \textit{xylT} gene product function.
REFERENCES


Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7:1513-1515


Canovas J, Ornston LN, Stanier RY (1967) Science 156:1695


Ghosal D, You I-S, Gunsalus IC (1987) Nucleotide sequence and
expression of gene nahH of plasmid NAH7 and homology
with gene xylE of TOL plasmid pWWO. Gene 55:19-28

Gibson DT (1968) Microbial degradation of aromatic compounds.
Science 161:1093-1097

Gibson DT, Hensley M, Yoshioka H Mabry TJ (1970) Formation of
(+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from
toluene by Pseudomonas putida. Biochemistry 9:1626-1630

Haas D (1983) Genetic aspects of biodegradation by
Psedomonads. Experimentia 39:1199-1213

Harayama S, Rekik M, Timmis KN (1986) Genetic analysis of a
relaxed substrate specificity aromatic ring dioxygenase,
toluate-1,2-dioxygenase, encoded by TOL plasmid pWWO of

Harayama S, Rekik M, Wasserfallen A, Bairoch A (1987b)
Evolutionary relationships between catabolic pathways
for aromatics: Conservation of gene order and nucleotide
sequences of catechol oxidation genes of pWWO and NAH7
plasmids. Mol Gen Genet 210:241-247


Hares D, Azadpour E, Benjamin RC (1992) Nucleotide sequence of the xylXYZ region of the Pseudomonas putida TOL plasmid pDK1 and expression of the the encoded toluate-1,2-dioxygenase in Escherichia coli. ASM General Meeting, New Orleans. #K41


for DNA sequencing in the M13 mp2 cloning system. Gene 10:68


Reineke W, Knackmuss HJ (1978) Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. Biochim Biophys Acta 542:424-429


Sanger F (1981) Determination of nucleotide sequences in DNA.
Science 214:1205

Schreirer PH, Cortese R (1979) A fast and simple method for sequencing DNA cloned into the single stranded bacteriophage as an aid to rapid DNA sequencing. J Mol Biol 143:161


Stanier RY, Palleroni NJ, Suzuki M, Tanaka T, Weisblum B


Identification of cis-dioxols 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, Murray K (1974) Metabolism of benzoate and the
methylbenzoates by *Pseudomonas putida* (arvilla) mt-2:
evidence for the existence of a TOL plasmid. J
Bacteriol 120:416-423

Williams PA, Worsey MJ (1976) Ubiquity of plasmids in
coding for toluene and xylene metabolism in soil
bacteria: evidence for the existence of new TOL

of and relationships between catabolic genes of TOL
plasmids, pp 339-358. In Hagedorn R, Hanson RS, Kunz DA
(ed) Plasmids in Bacteria. Plenum Publishing Corp New
York

Wong CL, Dunn NW (1974) Transmissible plasmid coding for the
degradation of benzoate and *m*-toluate in *Pseudomonas*


