Metabolic Engineering of a Toluene Degradation Pathway.

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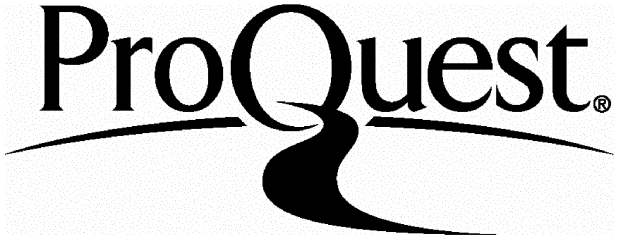

Abstract.

A mathematical model simulating metabolic flux through the TOL plasmid pWWO *meta*-cleavage pathway of *Pseudomonas putida* mt-2 (Regan *et al.*, 1991) has been experimentally investigated. Two of the predicted flux controlling steps in the pathway, the *xylXYZ* encoded benzoate dioxygenase (plasmid pQR189) and the *xylI* encoded oxalocrotonate decarboxylase (plasmid pQR194) have been individually co-expressed with the entire *meta*-cleavage pathway encoded on plasmid pQR150 in the same *E.coli* JM107 cell.

Co-expressing pQR189 and pQR150 in the same *E.coli* cell during benzoate metabolism has suggested a possible role of benzoate dioxygenase in the reactivation of catechol 2,3-dioxygenase.

Upon co-expression of pQR194 with pQR150, pathway metabolism was apparently shut-down in the presence of benzoate. It was predicted that this was due to an initial over-accumulation of the product of *xylI*, 2-hydroxypent-2,4-dienoate.

Kinetic analysis has revealed that 2-hydroxypent-2,4-dienoate acts as a non-competitive inhibitor of catechol 2,3-dioxygenase, with a K_i of 1.49×10^{-4} M. This, together with other findings, suggests that the physical coupling of oxalocrotonate decarboxylase and the *xylJ* encoded 2-oxopent-4-enoate hydratase exists to prevent the accumulation of this toxic intermediate.

Hybrid dioxygenases *xylX:bphA1A2A3A4* (pQR191) and *bphA1:xylYZ,* (pQR192) have been shown to be non-functional, thus indicating that enzyme subunits displaying an amino acid sequence identity of only 27% can not be interchanged.

The substrate range of the TOL plasmid *meta*-cleavage pathway has been broadened as a result of the co-expression of the *nahA* encoded naphthalene dioxygenase from *P.putida* G7 with the *meta*-cleavage pathway of the TOL plasmid. Such a dual-dioxygenase system is capable of the metabolism of naphthalene, biphenyl and benzoate as far as ring-cleavage intermediates.

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METABOLIC ENGINEERING OF A TOLUENE DEGRADATION PATHWAY

1.1 METABOLIC ENGINEERING.

1.1.1 Strategies for metabolic engineering.

Metabolic engineering can be defined as the use of recombinant DNA technology for manipulating the enzymatic, transport and regulatory functions of the cell (Bailey, 1991). It can be distinguished from the more traditional approaches of strain improvement by the ability to construct hybrid strains through the introduction of heterologous genes.

There are numerous applications of metabolic engineering that are described in the literature, such as the improved production of chemicals already produced by the host organism. Metabolic engineering of antibiotic production is a huge area in this respect, and is particularly attractive due to the clustered nature of antibiotic genes, making them easier to clone (Cameron and Teh-Tong, 1993). In addition, antibiotic genes are often positively regulated, thereby allowing improved production by over-expression of the regulator (Chater, 1990).

Another exciting area involves the production of chemicals new to the host organism. The production of novel polymers, in its own right described as biopolymer engineering (Peoples and Sinsky, 1990), and the genetic modification of polysaccharide biosynthesis (Hasler and Doherty, 1990), are two examples with great potential in this respect.

A very practical example of metabolic engineering is the extension of the substrate range of a host organism, a process that has obvious application in biodegradation and in the degradation of toxic chemicals. This particular aspect of metabolic engineering is discussed at length in a later section (section 1.1.1.4).

The construction of an optimised strain through metabolic engineering may be achieved by studying changes in cellular activities following each step in a cycle of genetic manipulations, a procedure already adopted in the development of protein engineering (Ulmer, 1983), and in the removal of impediments to the biosynthesis of aromatic acids from D-glucose (Dell and Frost, 1993) (see section 1.1.1.3). Such a step by step approach to metabolic engineering allows for the refinement of predictive design, hindered in many studies by the complex cellular responses that result from genetic manipulations of cellular processes.

In many cases, metabolic engineering by the introduction of a functional heterologous enzyme into an organism results in the appearance of new compounds that on further metabolism prove to be a central limitation in a metabolic engineering strategy (Bailey, 1991). Hence, following the introduction of heterologous genes into the constructs of genetically altered strains, it is imperative that the protein expressed is able to function with the desired activity, and for this reason a number of criteria must be met; the protein must resist proteolysis, must fold properly, and must form the correct assembly with any cofactors required.

In time the advance of metabolic engineering will establish the most effective methodologies for accomplishing a desired change in metabolic function.

The sections below review with the aid of specific examples the various methodologies and computational tools that are paramount in developing metabolic engineering strategies.

1.1.1.1 Transfer of an entire pathway.

The ability to transfer to a heterologous host a single continuous piece of DNA containing an entire metabolic pathway may provide more industrially robust and efficient strains (Bailey, 1991). Such metabolic engineering strategies are being employed in methods to improve the biosynthesis of biopolymers and antibiotics, the primary focus of the latter being the discovery of novel (hybrid) antibiotics (Chater 1990). Metabolic engineering of antibiotic biosynthetic pathways in this way may be facilitated by the clustering of the genes involved, and the cross-hybridization shown between genes of related pathways.

In 1984 a single continuous fragment of *Streptomyces coelicolor* DNA carrying the entire biosynthetic pathway for the antibiotic actinorhodin was isolated (Malpartida and Hopwood, 1984). The ability for this single fragment to 'complement' all seven phenotypic classes of actinorhodin non-producing *S.coelicolor* mutants provided evidence that the entire pathway for actinorhodin biosynthesis was encoded on this fragment.

In subsequent experiments using the isolated fragment cloned into a low copy-number vector, synthesis of the antibiotic was directed following the introduction of the fragment into another host, *S.parvulus*. The use of a low copy-number vector in such trials was believed to be advantageous in that the high-expression of physiologically active gene products is avoided, a situation that, as cited previously, may present difficulties in engineering strategies.

Since this first example of cloning an entire biosynthetic pathway and its subsequent expression in a heterologous host, several other examples have been

reported including that of the *S.coelicolor* red-pigmented antibiotic undecylprodigiosin (Malparpida *et al*, 1990). By cloning random fragments of the *S.coelicolor* DNA into the closely related *S.lividans*, 66 clones were isolated that over-expressed the antibiotic, presumably due to the presence of the positive regulating *redD* gene which activates the normally poorly expressed *red* genes of *S.lividans*. In further studies, two fragments from either end of the *red* cluster were cloned adjacent to one another on a low copy-number *Streptomyces* vector which was then used to screen *S.coelicolor* chromosomal DNA. Resulting homologous recombination lead to the isolation of the entire *red* cluster as a single cloned fragment in a low copy-number vector, thus enabling its introduction into a heterologous host. Such a procedure may be generalised for the use in sub-cloning of other gene clusters for metabolic pathways.

Further examples of subcloning and expression of entire biosynthetic pathways to a heterologous host include that of the clinically important *S.erythreus* produced antibiotic erythromycin (Stanzak *et al*, 1986) and penicillin V (Smith *et al*, 1990).

In the former, the biosynthetic pathway for erythromycin A, known to consist of about thirty enzymatic steps, was cloned into a cosmid vector bifunctional for *E.coli* and *Streptomyces*. Studies showed that transformation of *S.lividans* with a cosmid carrying a 35kb insert, believed to encompass the entire erythromycin synthetic pathway resulted in heterologous expression of the antibiotic, previously not expressed in such a host. Transformation of such a construct into *E.coli* failed to produce heterologous expression, indicative of a lack of key enzymes in *E.coli*, or poor expression of *Streptomyces* promoters in *E.coli*.

In the case of penicillin V, produced commercially from only one filamentous fungus, *Penicillium chrysogenum*, the penicillin biosynthetic pathway was cloned into a cosmid clone subsequently used to transform the related *Neurosporra crassa* and *Aspergilus niger*, neither of which produce β-lactam antibiotics (Smith *et al*, 1990).

The resulting heterologous expression confirmed that the penicillin biosynthetic genes are present as a gene cluster. The ability to isolate and subclone the entire biosynthetic pathway in this way has considerable commercial and clinical importance, particularly in the potential application of molecular genetics to strain improvement.

A more novel approach towards the introduction of an entire metabolic pathway into a heterologous host has recently been reported, and involves transferring and expressing the entire poly-β hydroxybutyrate (PHB) pathway of *Alcaligenes eutrophus* in plants, although results to date have only produced very small amounts of PHB (Poirier *et. al.*, 1992).

1.1.1.2 Extending existing pathways.

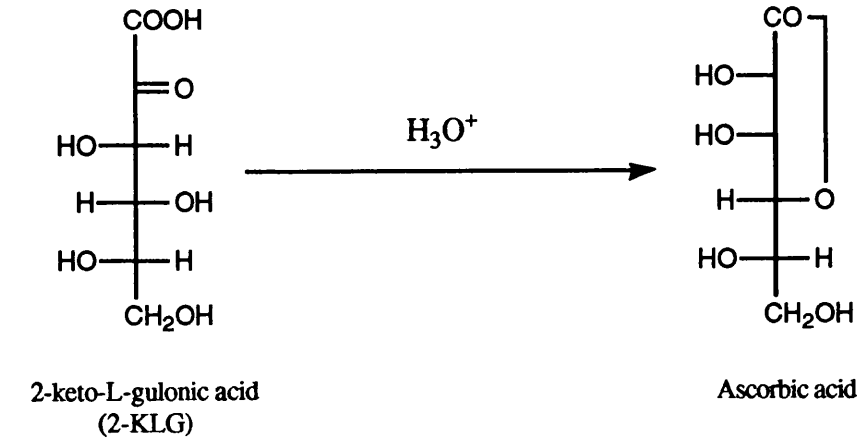
Despite the metabolic diversity that exists in nature, many organisms have metabolic characteristics in terms of substrate assimilation and product synthesis that are unattractive in a commercial sense. Metabolic engineering by means of extending naturally occurring microbial pathways is an obvious way to extend and enhance the capability of a particular organism. Indeed, by the introduction of carefully chosen heterologous genes, naturally occurring metabolites may be converted to more commercial products.

An example of such metabolic engineering of existing pathways involves the genetic modification of *Erwinia herbicola* to produce 2-keto-L-gulonic acid, an intermediate in the synthesis of L-ascorbate (Anderson *et al,*1985). The final step in the conventional synthesis involves a simple acid or base-catalysed cyclisation of 2-keto-L-gulonic acid (2-KLG) into L-ascorbic acid (Crawford, 1982) (fig 1.1a). This final intermediate 2-KLG may be synthesised by a simple stereoselective reduction of 2,5-diketo-D-gluconic acid (2,5-DKG) to 2-KLG, a reaction performed in a number a bacteria such as the *Corynebacterium*, as well as species of *Micrococcus, Pseudomonas* and several others (Sonoyama *et al*, 1982). Furthermore, the intermediate 2,5-DKG may be synthesised by *Erwinia* by a simple oxidation of D-glucose (Sonoyama *et al.*, 1982).

It is immediately obvious therefore that a dual fermentation of both appropriate organisms will produce 2-KLG, and thus simplify the manufacture of ascorbic acid (fig 1.1b). Further simplification has been accomplished however by combining the relevant traits of both the *Erwinia* and *Corynebacterium* in a single micro-organism (Anderson *et al,* 1985), thus enabling the conversion of D-glucose to 2-KLG in a single fermentation (fig 1.1c).

In the construction of the genetically modified *Erwinia*, the 2,5-diketo-D-gluconic acid reductase coding region from the *Corynebacterium sp.* that was responsible for the conversion of 2,5-DKG to 2-KLG was fused to an *E. coli trp* promoter, and the resulting construct introduced on a multi-copy plasmid into *E. herbicola*, itself naturally capable of producing 2,5-DKG via glucose oxidation.

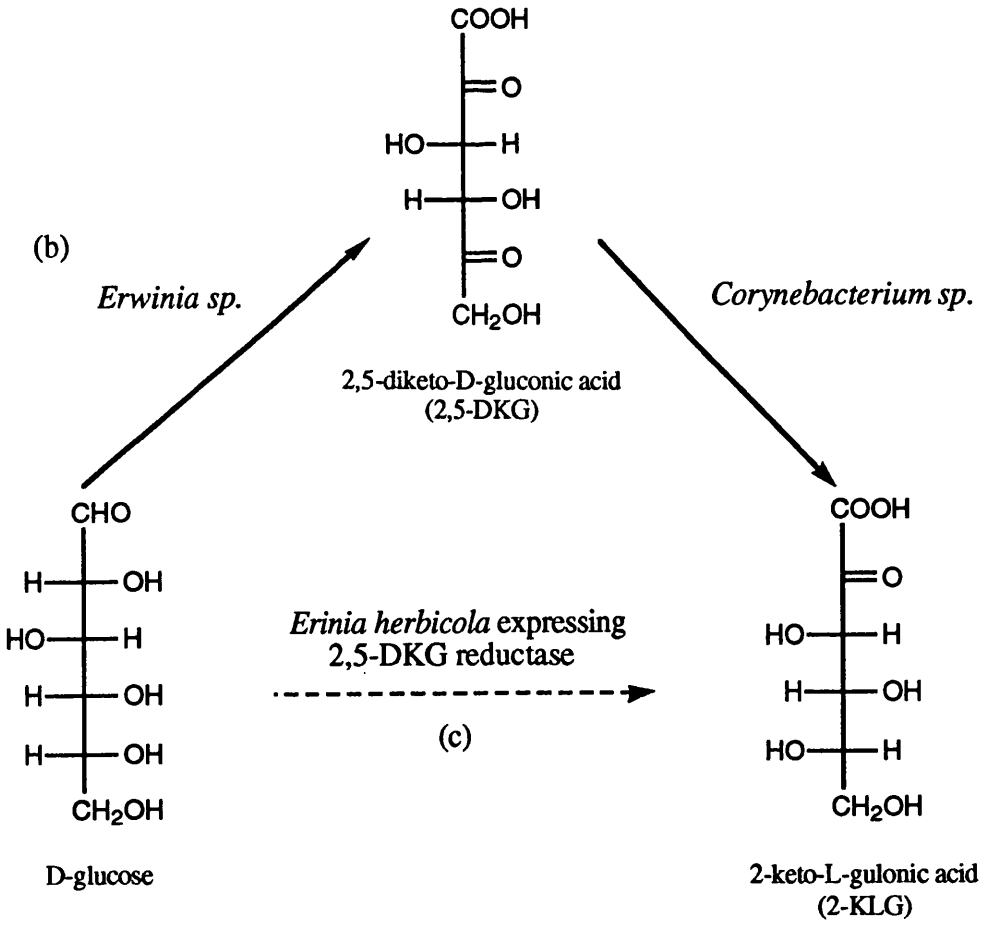
Such an example of metabolic engineering, although illustrating the feasibility of combining metabolic pathways for the production of a commercially important product, may also be used to highlight some inherent problems. The host dehydrogenases capable of oxidising D-glucose to 2,5-DKG are membrane-bound periplasmic enzymes linked to an electron transport chain (Ameyama *et al,* 1981), whereas the heterologous 2,5-DKG reductase is soluble, NADPH-linked and cytoplasmic. Hence an effective interface between the two separate pathways is required for the transport of 2,5-DKG. In addition, efficient bioconversion of glucose



2-keto-L-gulonic acid
(2-KLG)

Ascorbic acid

(a)



D-glucose

2-keto-L-gulonic acid
(2-KLG)

Fig 1.1 (a) The non aqueous acid-catalysed cyclisation of 2-keto-L-gulonic acid to L-ascorbic acid. (b) Synthesis of 2-keto-L-gulonic acid by dual fermentaion of *Erwinia* and *Corynebacterium*. (c) Synthesis of 2-keto-L-gulonic acid by recombinant *Erwinia herbicola*.

to 2,-KLG requires a NADPH regeneration system and also some means of 2-KLG efflux out of the cell. Thus it can be seen that the commercialisation of such a recombinant organism, by definition requiring a cost-effective production of L-ascorbic acid, relies upon several factors other than just the coexpression of both host and heterologous metabolic pathways.

Another example of metabolic engineering, extending a host pathway, involves the post-translational modification of a naturally expressed product by means of a heterologous protein processing enzyme. An example of such a modification involves the alteration of the N-linked oligosaccharide terminal sequences of Chinese Hamster Ovary cell glycoproteins by expression of a heterologous beta-galactoside alpha-2,6-sialyltransferase (Lee *et al,* 1989). Terminal glycoprotein glycosylation sequences are involved in mediating biological recognition, for example in the compaction of the mouse embryo at the 16-cell stage of development (Fenderson *et al,* 1984), and in modulation of cell adhesion during cell-migration in early development (Rutishauser *et al* 1988). Such terminal glycosylation sequences are normally absent from proteins produced from industrial cell-lines, including cloned erythropoietin. Galactoside α-2,6-sialyltransferase introduced into CHO cells competes with the endogenous sialyltransferase, leading to the production of protein that more closely resembles human erythropoietin (both possessing three asparagine linked sugar chains in one molecule, all being of the acidic complex type).

On the basis of the data obtained from this initial study it has been illustrated that metabolic engineering can be utilised successfully in the production of glycoproteins whose carbohydrate structures are common to the major sugar chains of the native one.

1.1.1.3 Maximising product formation by altering metabolic flow.

A characteristic of most metabolic pathways is the presence of points at which intermediates may enter alternative pathways, the choice often being dependent upon substrate, enzyme or transport systems (Bailey, 1991). The presence of branched pathways during metabolism is clearly disadvantageous in terms of maximising product formation, and as a result requires methods for minimising the flow through undesirable branches.

Amplification of an activity resulting in the desired metabolic flow is a common strategy used in metabolic engineering as a means of maximising product formation or of redirecting metabolic flow, and has, over the last decade, been largely achieved by the use of cloned genes. This technique allows the use of mutant enzymes desensitised to feedback repression.

An extension of this principle involves redirecting metabolic flow into newly acquired metabolic branches for the production of commercially viable end-products. A recent report (Draths and Frost, 1994) provides an excellent example of how such a metabolic engineering strategy has been developed, that involves the development of an environmentally compatible synthesis of adipic acid from D-glucose (fig 1.2).

The global demand for adipic acid, primarily used in the synthesis of nylon-6-6, exceeds $1.9x10^9$kg (Davis and Kemp, 1991). At present, all adipic acid manufacture is based upon the use of benzene as a starting material. Hydrogenation of benzene to cyclohexane is followed by air oxidation and subsequent nitric acid oxidation to yield adipic acid, together with nitrous oxide as a by-product. As adipic acid production in this way is thought to be responsible for as much as 10% of the annual increase in atmospheric nitrous oxide levels, it is likely that such a process will not meet environmental regulatory policies of future years.

A more acceptable synthetic process has now been established that utilises D-glucose as a starting material. The procedure is based upon the use of an initial mutant *E.coli* strain that lacks the aromatic amino acid biosynthetic enzyme shikimate dehydrogenase. Growth of such a strain on D-glucose consequently leads to an increased *in-vivo* synthesis of 3-dehydroshikimic acid (DHS).

By the co-expression of such a system with exogenous microbial metabolic enzymes, DHS can be converted to protocatechuic acid by the action of DHS dehydratase from *Klebsiella pneumoniae*. Other cloned enzymes enable the decarboxylation of protocatechuic acid to catechol by protocatechuate decarboxylase, and the conversion of catechol to *cis-cis* muconic acid by catechol 1,2 dioxygenase from *Acinetobacter calcoaceticus*. After the addition of 10% platinum on carbon to unpurified culture supernatants, subsequent hydrogenation has shown 90% yields of adipic acid. Although still subject to the considerations of optimisation and scale-up, such a biocatalytic conversion of D-glucose to *cis-cis* muconic acid warrants consideration as a viable microbial synthetic option for the manufacture of adipic acid.

Metabolic engineering of aromatic amino acid production in *Corynebacterium glutamium* (Ikeda and Katsumata, 1992), illustrates another strategy that has been utilised in redirecting metabolic flow to the desired product, in this case largely due to the development of host-vector systems for *C. glutamium* and related species (Yoshihama *et al*, 1985).

Essentially, the aromatic amino acids phenylalanine, tyrosine and tryptophan are synthesised via a common intermediate, namely chorismate . The first enzyme in the pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase is inhibited by phenylalanine and tyrosine. Similarly, chorismate mutase (CM) is itself inhibited by both phenylalanine and tyrosine. Prephenate dehydratase (PD) and anthranilate synthase (AS), which initiates the pathways to phenylalanine and

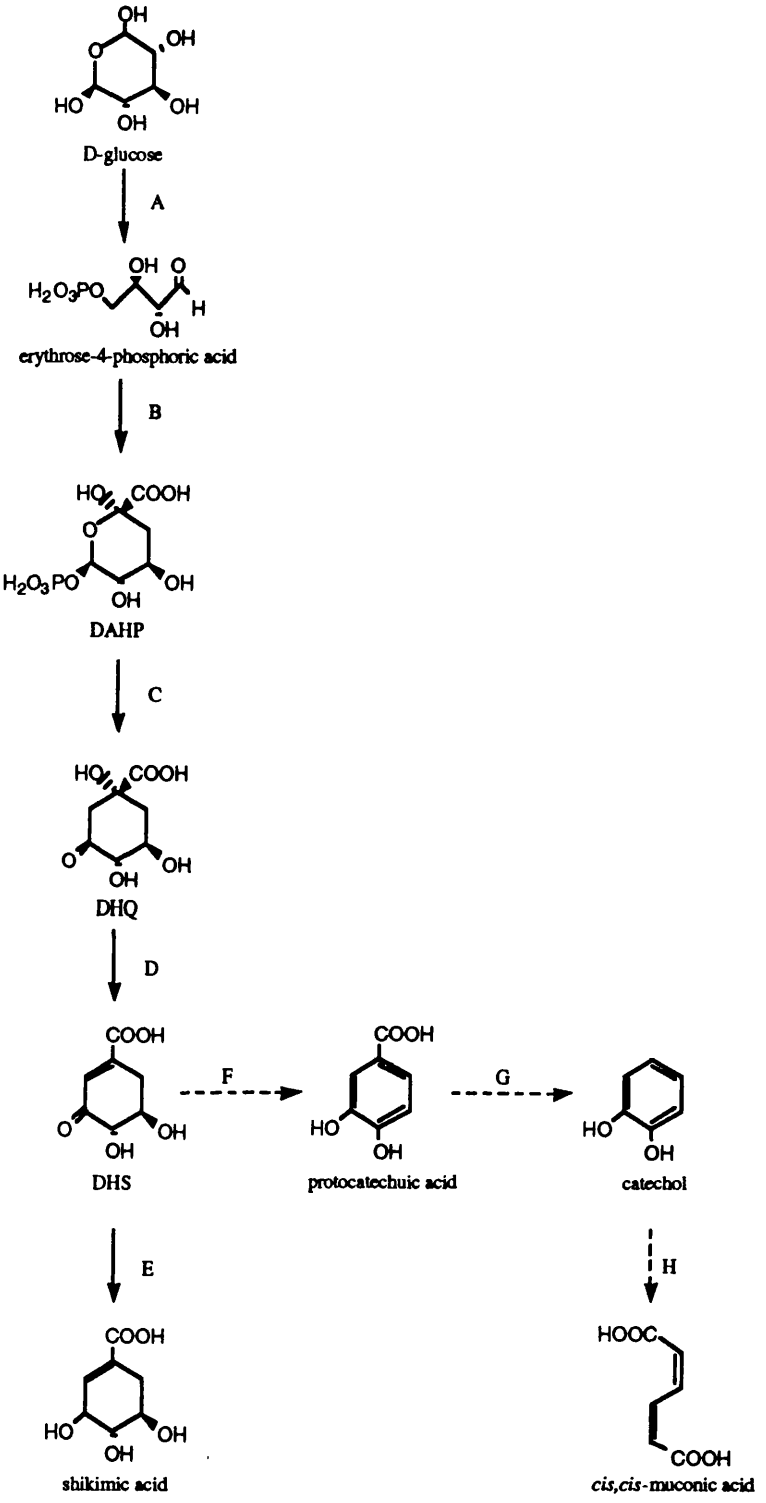


Fig 1.2 A biocatalytic pathway for the microbial conversion of D-glucose to *cis-cis*-muconate. Open boxes indicate enzymes recruited from *Klebsiella pseumoniae* (F and G), and from *Acinetobacter calcoaceticus* (H). Enzymes include (A) transketolase, (B) DAHP synthase, (C) DHQ synthase, (D) DHQ dehydratase, (E) shikimate dehydrogenase, (F) DHS dehydratase, (G) protocatechuate decarboxylase, (H) catechol 1,2-dioxygenase.

tryptophan respectively, are inhibited by end-product inhibition (Ikeda and Katsumata, 1992). Utilising the knowledge that aromatic amino acids are synthesised via a common pathway, amplification of a branch point enzyme in a certain amino acid producing strain would lead to an alteration of metabolic flow towards the amino acid corresponding to the amplified enzyme.

In this way, three biosynthetic genes coding DAHP synthase, CM and PD were individually cloned from regulatory mutants of *C. glutamium*, each of which possessed one of the enzymes desensitised to end-product inhibition, and assembled together on a *C. glutamium* multicopy vector. Following transfer into a tryptophan producing strain, enzymes specified on the plasmid were shown to be overexpressed seven-fold relative to chromosomally encoded enzymes, resulting in an acceleration of carbon through the common pathway as a result of amplified DAHP synthase, and a redirection of metabolic flow from tryptophan to phenylalanine or tyrosine.

Like the aromatic amino acids, the aspartate family share a common pathway and have shown to be influenced in the same way as above in terms of redirecting metabolic flow (Katsumata, 1986),, demonstrating that metabolic conversion by amplifying branch-point enzymes is a viable means of overproduction of a desired metabolite.

Another key aspect that must be considered in attempts to maximise product formation is in the removal of the rate-limiting steps of metabolism. In strategies designed to use microbes as catalysts in the synthesis of industrial or medicinal precursors, the highest possible percentage of pathway substrate must be directed into the pathway of interest in an effort to achieve maximum product formation. Amplification of enzymes that increase the channelling of a pathway substrate into such a desired route often leads to a situation where individual common pathway enzymes become rate-limiting. Subsequent build up of pathway intermediates can result in their rapid export into the culture supernatant. As a result, substrates of rate-limiting enzymes are effectively lost to metabolism, resulting in a reduction in percent conversion, together with a decreased purity of end-product.

The need to identify and remove such inefficiencies in metabolic processes is now widely recognised, and efforts to meet these requirements have been made by both the use of metabolic mutants, and in the development of mathematical models that predict the flux of substrates through a pathway (see section 1.1.1.5).

The channelling of substrates into a metabolic pathway, together with the removal of rate-limiting steps in order to maximise product formation have both been considered in methods to increase the flow of D-glucose into the aromatic biosynthetic pathways (fig 1.3). As mentioned above, it has been known for some time that DAHP synthase controls the flow of carbon into aromatic amino acid biosynthesis (Hermann, 1983). Only recently however has another enzyme, the transketolase, also been

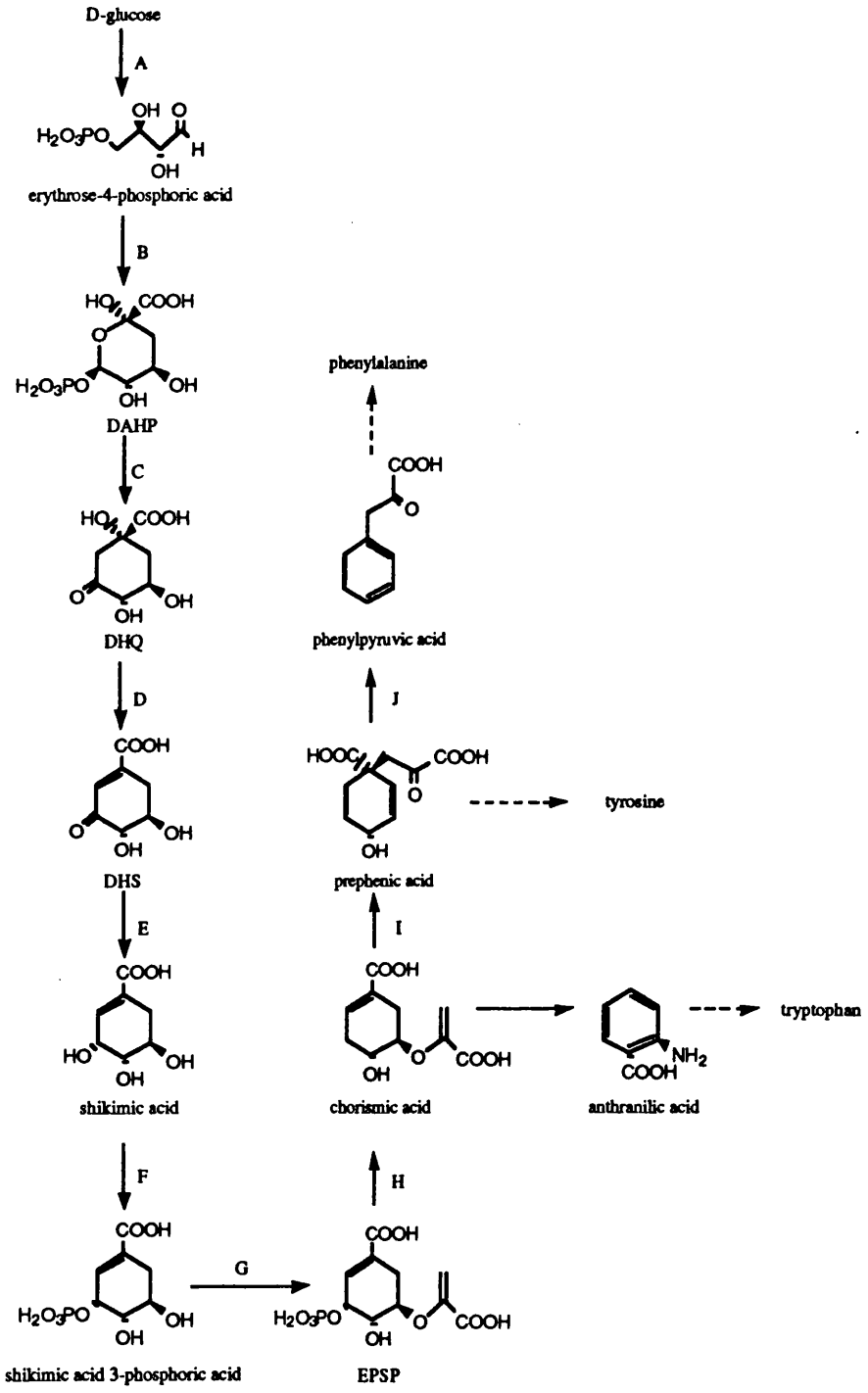


Fig 1.3 The aromatic amino acid biosynthetic pathway. Enzymes include (A) transketolase, (B) DAHP synthase, (C) DHQ synthase, (D) DHQ dehydratase, (E) shikimate dehydrogenase, (F) shikimate kinase, (G) EPSP synthase, (H) chorismate synthase, (I) chorismate mutase, (J) prephenate dehydratase.

considered a key component in such a biosynthetic pathway (Draths *et. al.*, 1992, Draths and Frost, 1990). Studies with manipulated aromatic acid biosynthetic pathways expressing amplified levels of DAHP synthase desensitized to feedback inhibition were used to reach a point where no further improvements in aromatic biosynthesis could be achieved. At this level of biosynthesis, by amplifying the levels of expression of the transketolase, an additional two-fold increase in the percentage of D-glucose channelled into aromatic acid biosynthesis was observed.

Having established methods to improve the *in vivo* catalytic activity of DAHP synthase, other rate-limiting steps of D-glucose biocatalysis were identified and removed in order to improve the channelling of intermediates to chorismic acid (Dell and Frost, 1993). The identification of such rate-limiting steps arose through the use of auxotrophic *E.coli* mutants lacking individual pathway enzymes. Initially, an *E.coli* strain carrying amplified levels of DAHP synthase and transketolase, but lacking 3-dehyroquinate (DHQ) synthase accumulated 20 mM 3-deoxy-D-*arabino*-heptulosnic acid (DAH), the substrate of DHQ synthase, in the culture medium when cultured in medium containing 56 mM glucose. Similar experiments, when performed with a strain lacking DHQ dehydratase instead of DHQ synthase, would also be expected to accumulate 20 mM of the end-product, namely DHQ. Instead however, only 14 mM of DHQ accumulated in the culture supernatant, together with 5 mM DAH. Such a reduction in the level of accumulated intermediate would suggest the presence of a rate-limiting enzyme. Indeed, when strains were supplemented with amplified levels of DHQ synthase, a disappearance of DAH in the culture supernatant resulted, together with the appearance of 19 mM DHQ. These results have been interpreted as indicative of DHQ synthase as a rate-limiting step in the biocatalysis of D-glucose for amino acid biosynthesis. Similar studies have been utilised to identify other rate-limiting enzymes in the same pathway.

As has been described, by determining the rate-limiting steps that need to be enhanced in order to improve metabolic flux through metabolic pathways, one of the key considerations that will determine the long-term utility of such biocatalytic syntheses can been met. In order to further improve the efficiency of engineered strains in this way, it is necessary in the development of metabolic engineering strategies to establish the minimum level of over-expression required to remove rate-limiting steps. Such considerations will be important in the optimisation of processes without the wasteful over-expression of unneeded enzyme.

1.1.1.4 Broadening the substrate range of metabolic pathways.

Another aspect of metabolic engineering that is attracting an increased amount of attention is broadening of the substrate range of a metabolic pathway. Much of this

work has centred around the engineering of organisms to use lactose, a by-product of the cheese-making industry. A successful example of this has involved the integration of the *lacYZ* genes from *E.coli* into the *Pseudomonas aeruginosa* chromosome, resulting in recombinant cells capable of producing rhamnolipid biosurfactants from lactose (Koch *et. al.*, 1988).

A primary example of such a metabolic engineering strategy involves the addition of new catabolic activities for the degradation of toxic chemicals. The first example of such a process was the construction of several *Pseudomonas* strains able to catabolize the degradation of a wide range of substrates such as camphor and naphthalene (Chakrabarty, 1974).

A more direct approach to engineering bacteria to degrade an increasing range of substrates has involved the construction of hybrid metabolic pathways. (Hirose *et al* 1994). Over the last few years it has become apparent that many aromatic compounds are biodegraded through evolutionarily related pathways (section 1.4). In 1992, Taira *et al,* reported the sequencing and analysis of the gene components from such a pathway that are responsible for the initial dioxygenation and ring *meta*-cleavage of biphenyl and polychlorinated biphenyls (PCB's) in *Pseudomonas pseudoalcaligenes* KF707. Of significance in the analysis was the nucleotide sequence similarity shown between genes encoding the biphenyl dioxygenase of KF707, and genes encoding the toluene dioxygenase of *P.putida* F1 (Zylstra and Gibson, 1989), despite their discrete substrate specificities.

Both biphenyl and toluene dioxygenases were found to be four component Class IIB dioxygenases encoded by *bphA1A2A3A4* and *todC1C2C3C4* respectively. On further analysis, the large subunit of the terminal dioxygenase of both enzymes, encoded by *bphA1* and *todC1* were found to be critical for their discrete substrate specificities (Furukawa *et al,* 1993). It was thus suggested that by altering the gene composition of aromatic dioxygenases, it may be possible to create novel degradative abilities.

In 1994, Hirose *et al,* reported the construction of hybrid biphenyl and toluene dioxygenases, by for example introducing the *bphA1* gene in the place of the *todC1* in the toluene dioxygenase gene cluster. As a result, a biphenyl degradative ability was introduced into a system that in the wild-type state has a substrate specificity restricted to benzene/toluene. It was concluded from these findings that in this way it may be possible to create novel dioxygenases that show a wider biodegradative range that would be advantageous in bioremediation applications. A more detailed discussion illustrating the use of metabolic engineering to facilitate the degradation of toxic chemicals can be found in section 1.4.

1.1.1.5 Metabolic Control Theory for the optimisation of metabolic pathways.

In terms of optimising a metabolic engineering strategy, the importance of optimising the flux through the pathway, and of minimising the number of side reactions and any possible production of toxic intermediates has already been stressed and illustrated (see section 1.1.1.3).

In order to maximise productivity in this way requires a thorough knowledge of the mechanisms involved, preferably through the formation of a model of the system, in which knowledge of the rate-limiting enzymes, and thus limiting fluxes, is established.

Given this need to quantitatively understand metabolic physiology, considerable attention has been devoted over the last few decades towards the mathematical description of metabolic flux analysis.

The simplest unstructured mathematical models box together details of the system in question without any regard for specific biochemical or kinetic data. Thus, although acceptable in certain situations, they are largely inadequate for maximising metabolism since more than one step within a box may affect metabolic flux.

A possible alternative is to consider the whole organism as an anonymous entity in which only the input and output of substrates, products and energy respectively are measured (Papoutsakis, 1984). This, as well as making qualification of potential product yields difficult, has the added problem of not providing information of individual steps in the pathway (Regan *et al,* 1991).

In comparison to unstructured models, highly structured models can provide a good insight into the regulatory mechanisms and limiting steps of the pathway, but in turn require a large number of input parameters, the result of which may result in data which is ambiguous and prone to errors.

In order to develop metabolic engineering strategies for maximising metabolic flux using computer generated mathematical models, DNA data bases that provide genetic information must be linked with information concerning metabolism. However, organisms and metabolic pathways are complex integrated systems, and as a result even very simple analyses such as the calculation of the maximum theoretical yield of a particular metabolite are difficult. A number of artificial intelligence programs have been developed (Seressiotis and Bailey, 1988; Mavrovouniotis *et al*, 1990) that generate all the potential routes from substrate to product, but are limited in their requirement for appropriate stoichiometric constraints.

In order to reduce the number of parameters that need to be analysed in a system, whilst still being able to maximise productivity, the regulatory points of the metabolic system need to be determined. The regulation may lie at the level of gene

expression, so that it is the level of particular enzymes in the system which control, for example, product yield. Alternatively, control may be achieved at the level of a specific enzyme activity, which may require interaction with a specific modulator to achieve maximal activity or down-regulation of the enzyme.

With the development of mathematical analyses of metabolic systems, it has been established that the control of flux is distributed over all of the enzymes of a given pathway to a varying degree. It is through the determination of such a flux control distribution that a rationale can be developed for the improvement of a pathway of interest. Furthermore, such a control systems approach will help determine the minimum levels of amplification required to maximise flux, a key consideration in the econonic and metabolic optimisation of industrial processes (Dell and Frost, 1993).

The development of flux control distributions over the last thirty years has largely been achieved through the use of Metabolic Control Theory (MCT) (Kacser and Burns, 1973; Heinrich *et al.*, 1977), a sensitivity analysis that is based upon a steady-state analysis of metabolic pathways. Such a MCT analysis results in the generation of control coefficients and elasticities that may be used to identify potential bottlenecks in a pathway.

In the analysis of any metabolic flux distribution by MCT, it is necessary to distinguish between parameters and variables of the system. Parameters are biological properties of the system that do not change during experimentation, such as the Km or k_{cat} of an enzyme, or physical properties such as temperature or pH. Variables, such as the concentration of a metabolite (X) , or the flux through a system (J), only achieve a constant value at steady state, this value being determined by the parameters.

As mentioned previously, MCT relies upon the calculation of control coefficients that describe how a variable of a system, such as flux, will respond to a parameter, such as enzyme concentration (E) or rate constant (V).

Flux control coefficients express in quantitative terms the extent to which an enzyme in a pathway controls the pathway flux (Y), and is expressed as:

$$C_E^Y = \frac{dY}{dE} \cdot \frac{E}{Y} \qquad (1)$$

In the case of Biochemical Systems Theory, the parameters are the rate constants (V) for synthesis and degradation of metabolic pools. In a similar fashion, flux control coefficients are thus defined as:

$$C_V^Y = \frac{dY}{dV} \cdot \frac{E}{Y} \qquad (2)$$

By virtue of the fact that flux control is distributed across the enzymes of the system as explained above, according to the flux-control summation theorem, the sum of the flux coefficients of 'n' enzymes in a pathway is equal to 1.

(3) $$\sum_{i=1}^{n} C_{V_i}^{J} = 1$$

MCT is not just limited to the control by flux coefficients. Indeed control coefficients may also be defined for pathway substrates, as well as for external inhibitors. In this case, the concentration control coefficient can be defined as the effect of a small change of a parameter (E or V) on a metabolite X.

(4) $$C_E^X = \frac{dX}{dE} \cdot \frac{E}{X}$$

There is a family of concentration control coefficients for each metabolite, one for each enzyme in the pathway, and in this way a summation theorem exists for concentration control coefficients that states that the sum of the concentration control coefficients for a set of enzymes on metabolite X is equal to zero (Heinrich *et al.* 1977).

(5) $$\sum_{i=1}^{n} C_E^X = 0$$

The turnover number of an enzyme is also a consideration in the determination of flux distribution within a pathway. This is reflected by an elasticity coefficient (ε), which determines the dependence of an enzyme reaction rate (V) on substrate concentration at constant levels of product concentration. Elasticity coefficients are traditionally defined as

(6) $$\varepsilon_X^V = \frac{X}{V} \cdot \frac{dV}{dX}$$

Elasticities can also be defined for changes in parameters (P), in which case the coefficients are defined as π-elasticities. Thus, as in eqn. 6

(7) $$\pi_P^V = \frac{P}{Y} \cdot \frac{dY}{dP}$$

Elasticities and flux coefficients are related by virtue of the connectivity theorem (Kacser and Burns, 1973), that states that the sum of the product of the flux coefficients of the enzymes in a pathway and their elasticities towards a given metabolite (X) is zero.

(8) $$\sum_{i=1}^{n} C_{V_i}^{Y} \varepsilon_X^V = 0$$

This is widely regarded as the most meaningful of the theorems, for it provides the route to understanding how the kinetics of the enzymes (represented by their elasticities) affect the values of the flux control coefficients. A large elasticity would suggest that an increase in an enzyme substrate results in a large substrate turnover. As a consequence, the enzyme in question would possess a low flux control coefficient, indicative of its small contribution to limiting flux.

The use of flux coefficients and elasticities with the summation and connectivity theorems provides the basis for the development of structured models, which use matrix based systems to generate theoretical predictions of flux coefficients using stoichiometric relationship of every element in the pathway.

Control coefficients are subsequently generated for a system in steady state that give a relative degree of importance to each enzyme for a desired flux. Consequently, the enzyme within a pathway with the highest control coefficient can be recognised as the rate limiting step within the pathway. The establishment of control coefficients as modelling parameters has lead to the development of sensitivity values, that are a function of time and are thus not restricted to steady state. The concept of sensitivity has been applied in several reported mathematical models (Malmberg *et al.*, 1991), (Regan *et al*, 1991).

Regan *et al,* have developed a mathematical model in such a way for the TOL plasmid encoded *meta*-cleavage pathway, making possible the determination of rate-controlling enzymes and metabolites for the system (Regan *et al* 1991). It has been shown that the concentrations of most of the metabolites in the pathway vary little on changing the input substrate concentration. Only the products of the *xylXYZ* encoded toluate dioxygenase, and the *xylH* encoded oxalocrotonate tautomerase have shown a significant change in this respect. The increase in both the concentration of the former, and the flux through it, coupled with unchanging concentrations of the following metabolites, has indicated that the first two enzymes play a large part in controlling the flux through the pathway. The increase in concentration of the latter, coupled with a lack of significant increase in its rate of formation, indicates this point in the pathway as another potential bottleneck. Such a mathematical model illustrates the points within the pathway which should be genetically manipulated in order to manipulate the flux at these stages.

Despite the development of such complex mathematical models that predict the flux of substrates through a biochemical pathway, doubt is still expressed as to the accuracy of sensitivity analysis that can be obtained. This is largely due to the complexity of biochemical systems, together with the difficulty of providing accurate kinetic data *in vitro*.

To overcome such potential limitations, the concept of the Characteristic Reaction Pathway (CRP) has been developed (Liao and Lightfoot, 1988), that attempts to estimate kinetic data *in vivo*. A second model that is independent of kinetic parameters has also been proposed (Delgardo and Liao, 1991), although both of these methods have yet to be tested on a model metabolic pathway system.

1.2 Metabolic engineering of the TOL plasmid.

Soil micro-organisms, particularly bacteria of the genus *Pseudomonas* possess metabolic pathways capable of degrading an enormous range of natural and synthetic organic materials, and thus play an important role in the carbon cycle and in maintaining a balanced ecosystem (Ramos *et al,* 1986). Biochemical pathways for the degradation of many compounds have now been elucidated and a number of the participating enzymes isolated and characterised. Indeed, many biodegradative pathways have been shown to share common ring fission and subsequent steps, and to employ isofunctional enzymes that exhibit varying substrate specificity.

Interest in the enzymatic mechanisms involved and in the genetics of the enzymes responsible has intensified over the past decade with the development of gene cloning techniques for soil bacteria (Harayama *et al.*, 1986a), and has stimulated interest in biotechnological routes for the production of useful chemicals (Mermod *et al*, 1986, ; Ensley, 1994; Errington, 1987; Davison *et al.*, 1990).

For example, the microbial catabolism of benzene, apparently by a method common to a number of different genera, involves the dioxygenation to benzene *cis*-glycol by a multicomponent enzyme system, followed by a dehydrogenation to catechol. Manipulation of these two conversions allows a commercially viable route to *cis*-glycols (Winstanley *et al*, 1987). Benzene *cis*-glycol formed by this method is of importance as an intermediate in the synthesis of polyphenylene (Ballard *et al.*, 1983).

One of the most common intermediates in the degradation of aromatic compounds such as benzoate, naphthalene, salicylate and toluene is catechol (1,2-dihydroxylbenzene). A diverse array of enzmyes may convert aromatics to catechol intermediates, although the subsequent reactions for oxygenative ring fission of catechol and its conversion to Kreb's cycle intermediates, are limited to one of two metabolic alternatives; that of the ortho or *meta*-cleavage pathways. Ortho-cleavage involves ring-cleavage between the two hydroxyl groups followed by a series of well defined reactions leading to β-ketoadipate (Dagley, 1986). The alternative, *meta*-cleavage pathway, involves ring cleavage adjacent to the two catechol hydroxyls, followed by degradation of the product to pyruvate and a short chain aldehyde. The use of one pathway or another is dependant upon the microbial species and/or the nature of the growth substrate (Shingler *et al*, 1992).

The *meta*-cleavage pathway was first studied in *Pseudomonas* strains capable of growing at the expense of phenols and cresols (Dagley and Gibson, 1965, Nishizuka *et al,* 1962), and since then has been identified in *Azotobacter, Alcaligenes* and a number of *Pseudomonas* species. More recently, enzymes common to *meta*-cleavage pathways have been identified in *Rhodococcus* species (Candidus *et al*, 1994).

The most comprehensively studied *meta*-cleavage pathway is that of the Inc-9 TOL plasmid pWWO, which encodes the toluene degradation pathway in *P.putida*. The presence of both the *meta*-pathway and enzymes such as benzoate dioxygenase, which exhibits a relaxed substrate specificity has made the TOL plasmid an attractive model system that is potentially very useful for both the extension of substrate range and for the creation of novel pathways by metabolic engineering routes. Such an example is the novel bacterial production of indigo, utilising a TOL plasmid specified hydroxylase (Mermod *et al*, 1986), and based upon the relaxed specificity of the TOL plasmid xylene oxidase enzyme (Morales *et al.*, 1990).

1.2.1 Characterisation of the TOL plasmid:

As has previously been mentioned, the TOL plasmid pWWO encodes a set of enzymes for the metabolism of toluene and its substituted derivatives to Kreb's cycle substrates. Degradation of such compounds is initiated by the progressive oxidation of methyl side chains of the aromatic ring.

The genetic organisation of the TOL plasmid was initially investigated by transposon mutagenesis with Tn5 and by gene cloning (Franklin *et al*, 1981). As a result, it was revealed that genes for toluene/xylene catabolic enzymes are organised into two clusters on the same plasmid, which are separated by a 14 kb stretch of DNA. The physical organisation of the genes into two clusters reflects a functional organisation into two blocks, one for the upper pathway operon, encoding enzymes for the metabolism of toluene/xylene to their corresponding carboxylic acids, and one for the lower '*meta*' pathway that encodes a series of enzymes which degrade benzoates/toluates to pyruvate, acetaldehyde/propionaldehyde and formate/acetate via extradiol (*meta*) ring cleavage of catechols (fig 1.4a and 1.4b).

1.2.1.1 The Upper Pathway.

By transposon mutagenesis and cloning analysis of the upper pathway genes of the TOL plasmid, expression of mutant and hybrid plasmids in both *E.coli* and *P.putida* allowed the determination of the order and position of upper pathway genes (Harayama *et al*, 1986a), which have subsequently been localised to a region of about 8kb (Nakazawa *et al.*, 1980) . However the three structural genes *xylC, A* and *B* that make up this region and encode benzaldehyde dehydrogenase (BZDH), xylene monooxygenase (XO) and benzyl alcohol dehydrogenase (BADH) respectively account for only about 50% of this region, suggesting the presence of other pathway genes, of which at least two have been identified, namely *xylM* and *xylN*. One of these, *xylM*, has been shown to encode one of the two polypeptide

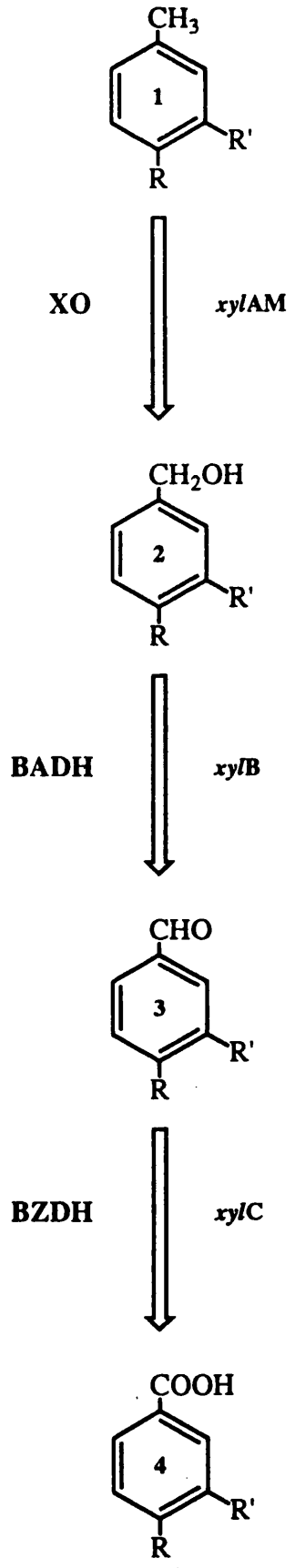


Fig 1.4 a The TOL plasmid upper pathway of *Pseudomonas putida* mt-2.
Enzyme abbreviations: XO, xylene monooxygenase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase. Compounds for R=H, R'=H: (1) toluene; (2) benzyl alcohol; (3) benzaldehyde; (4) benzoate.

components of xylene oxidase (XO). In subcloning analysis, (Harayama *et al,* 1989b), a hybrid plasmid encoding both *xylM* and *xylA* expressed XO activity, whereas its derivatives, expressing either *xylM* or *xylA*, gave no expression. To confirm that the XO is composed of two polypeptides, genetic complementation of the XO activity between *xylM* and *xylA* mutants was examined, showing that only a strain carrying both genes on separate plasmids was capable of oxidising toluene. Xylene monooxygenase catalyses the hydroxylation of the carbon side chain of toluene and benzyl alcohol, and seems to have a particularly relaxed substrate specificity (Harayama *et al,* 1986a).

Amino acid sequence analysis of the amino terminal of the XylA polypeptide has identified a similarity with chloroplast type ferredoxins, whilst the carboxyl-terminal domain has been assigned a feredoxin $NADP^+$ reductase function. As a result, the XylA protein is thought to act as an electron transport protein that transfers reducing equivalents of NADH to the XylM hydroxylase component (Shaw and Harayama, 1995).

The other TOL pathway specified upper pathway gene, *xylN* is thought to be the last gene in the operon that has an order *xylCMABN*. The XylN gene product is a 52-kDa protein which is processed to a 47 kDa polypeptide, the physiological role of which is as yet unknown.

The genetic characterisation and location of the upper pathway genes has revealed a 1.7 kb sequence between the promoter and the start of *xylC* from which no gene product has been identified (Harayama *et al,* 1986a). This region may be associated with a recently identified membrane protein of the toluene degradation pathway of *P. putida* F1. According to the findings of Wang *et al,* (1995), a 3 kb DNA region upstream of the toluene (tod) genes of *P. putida* F1encodes two open reading frames, one of which, the *todX* gene, has been found to be membrane bound. In addition, this previously unidentified gene has been found to share significant amino acid homology with the *E.coli* FadC protein, an outer membrane protein required for the uptake of long-chain fatty acids. It has been proposed as a result that the *todX* encoded protein, as well as a protein encoded by a homologous open reading frame upstream of *xylC* in the TOL plasmid, are both associated with the delivery of exogenous toluene inside the cell.

1.2.1.2 The *Meta*-Cleavage Pathway.

In comparison to that of the upper pathway, the *meta*-cleavage pathway genes have been characterised in some detail. The TOL *meta*-cleavage operon comprises 13 structural genes (fig 1.4b) which encode a set of enzymes which transform

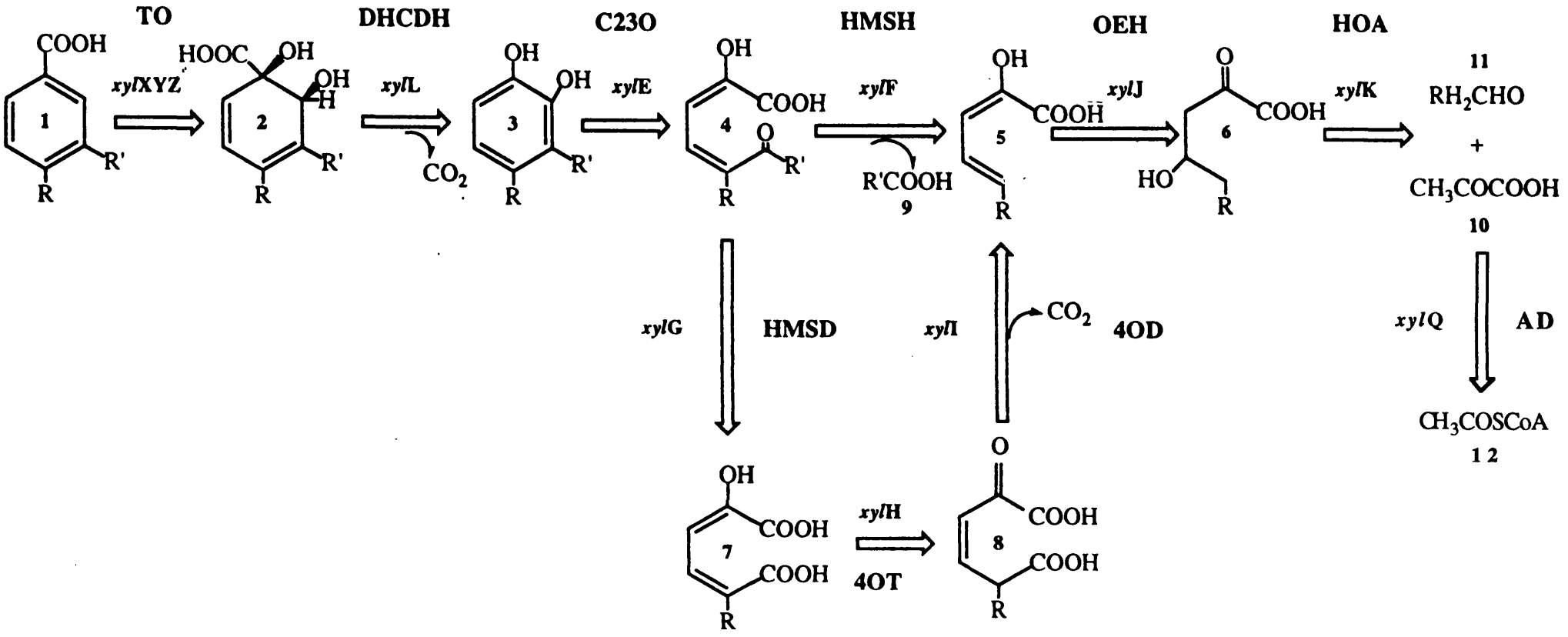


Fig 1.4 b. The TOL plasmid *meta*-cleavage pathway of *Pseudomonas putida* mt-2.
Enzyme abbreviations: TO, benzoate dioxygenase; DHCDH, *cis*-dihydrodiol dehydrogenase; C23O, catechol 2,3-dioxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; 4OT, 4-oxalocrotonate tautomerase; 4OD, 4-oxalocrotonate decarboxylase; (OEH), 2-oxopent-4-enoate; (HOA), 4-hydroxy-2-oxovalerate aldolase; (AD), acetaldehyde dehydrogenase.
Compounds for R=H, R'=H: (1) benzoate; (2) 1,2-dihydroxycyclohexa-2,5-diene-1-carboxylate; (3) catechol; (4) 2-hydroxymuconic semaldehyde; (5) 2-hydroxypent-2,4-dienoate; (6) 4-hydroxy-2-oxovalerate; (7) 4-oxalocrotonate (enol); (8) 4-oxalocrotonate (keto); (9) formate; (10) acetaldehyde; (11) pyruvate; (12) acetyl CoA.

benzoates/toluates to Kreb's cycle substrates via extradiol (*meta*-) cleavage of methylcatechol (Harayama and Shaw, 1985).

The first three genes in the operon, namely *xylX*, *Y* and *Z*, encode polypeptides of weight 57, 20 and 39kDa respectively, each known to be a subunit of the Class Ib benzoate1,2-dioxygenase (Harayama *et al,* 1986b).

Benzoate 1,2-dioxygenase is an example of a non-heme ring-hydroxylating dioxygenase, a group of enzymes first identified in *P. putida* by Gibson *et al,* (1970). These enzymes consist of two or three soluble proteins that interact to form an electron transport chain that transfers electrons from NADH via flavin and redox centres (FeS) to a terminal oxygenase. This interaction results in the incorporation of two atoms of oxygen into one molecule of substrate. In addition to molecular oxygen, such enzymes require cofactors and Fe(II) for the conversion of substrates to *cis*-dihydrodiols.

The *xylZ* gene of the benzoate 1,2-dioxygenase encodes the first component of the electron transport chain, the reductase system of the enzyme. The reductase comprises both the flavin and the iron-sulphur cluster in the same protein and catalyses the transfer of two electrons from NADH. It should be noted that in two component dioxygenases such as the benzoate dioxygenase, such an arrangement is common. In three-component systems however, the flavin and iron-sulphur ferredoxin are present in two individual enzyme subunits (Finnette *et al.*, 1984).

Amino acid homology studies between different reductase components has revealed two regions associated with an NAD-binding domain, characterised by a β–α–β-fold centred around a highly conserved Gly-X-Gly-X-X-Gly sequence. The conserved distance between these two motifs may be indicative of similar overall folding of such polypeptides.

The iron-sulphur flavoprotein of the XylZ protein transfers electrons from NADH to the terminal oxygenase component of the enzyme, via an electron acceptor such as cytochrome C.

The second, terminal oxygenase component is the catalytic centre of the enzyme, and is comprised of a large (50 kD) α-subunit, and a small (20 kDa) β-subunit, encoded by the *xylX* and *xylY* genes respectively. For hydroxylation of the substrate, both O_2 and Fe^{2+} must be present, and as a result, the terminal component contains an iron-binding site an a 2Fe-2S cluster.

The comparison of DNA and amino acid sequence homology data between different aromatic dioxygenase subunits has lead to the identification of conserved residues thought to be important in the function of such enzymes (Neidle *et al,* 1991). By comparing the *benABC* and *xylXYZ* encoded dioxygenases together with toluene, naphthalene and benzene 1,2-dioxygenases, amino acid sequence conservation has been found to be strongest in the N-terminal region in the case of the terminal oxygenase α-subunit. In particular, five conserved histidine and two conserved

tyrosine residues were detected in the alignment of five α-subunits. Considering the probable need for these amino acids in the coordination of a Rieske-type iron-sulphur cluster used to mediate the binding of oxygen, it is possible that the α-subunit plays such a role in dioxygenation. More recent studies investigating the function of the individual subunits of dioxygenase enzymes has illustrated that the α-subunit confers the substrate specificity of the enzyme (Hirose *et al*, 1994) (see section 1.1.1.4). In comparison to the large subunits however, the low degree of homology shared between β–subunits has suggested that these subunits are not directly involved in the common catalytic functions of dioxygenases. Of the nine conserved residues observed among β-subunits, five are charged residues. It is possible that the charged residues play a role in the association between α and β–subunits of the terminal oxygenases.

The enzyme encoded by *xylE*, namely catechol 2,3 dioxygenase (C23O) is responsible for the *meta*-ring cleavage of catechol, a key intermediate in the bacterial degradation of a number of aromatics as mentioned earlier. The C23O enzyme was first isolated by Kojima *et al*, (1967) who also demonstrated its sensitivity to oxygen.

Initial analysis of C23O showed the presence of at least four different polypeptides of weight 36, 35, 26 and 25-kDa (Harayama and Rekik, 1990). It is thought however that this observation of multiple polypeptides may be explained by firstly the presence of a ferrous iron prosthetic group in one or two active forms, giving rise to 35 and 36-kDa polypeptides, and secondly the presence of degradative products leading to 25 and 26-kDa polypeptides. It is now known that C23O consists of four identical subunits of 35kDa, and contains one catalytically essential Fe(II) ion per subunit.

From binding and kinetic studies, it has been proposed that the enzyme first combines with the substrate catechol, and is followed by the binding of oxygen. Both catechol and oxygen bind to the catalytic iron which is co-ordinated to two or more amino acid ligands on the polypeptide (Malbrouk *et al*, 1991). Results of X-ray absorption spectroscopy studies have confirmed such a proposal, and has revealed more detail concerning the mechanism of the reaction (Shu *et al*, 1995). It appears from the findings of this study that the redox state of the iron at the catalytic site of the enzyme strongly influences the binding mode of the catechol substrate, and the subsequent position of the attack by O_2. In Fe(II) enzymes such as the XylE catechol 2,3-dioxygenase, substrates appear to bind asymmetrically. This, together with the nature of the metal-bound oxygen, is thought to direct oxygen attack to a position where extradiol cleavage occurs.

In recent years, much work has centred upon the identification of the active site of catechol 2,3-dioxygenase (Nozaki *et al*., 1968;, Taira *et al*, 1992). According to the findings of Tatsumo *et al*, (1980) and Williams *et al*, (1990), histidine residues at the C-terminal end of the enzyme were predicted to be the most important in enzyme

activity. With these predictions in mind, amino acid sequence comparisons performed with ten isofunctional catechol 2,3-dioxygenases have revealed four conserved histidine residues, three of which are situated at the C-terminal end. Using a purified catechol 2,3-dioxygenase from *Rhodococcus rhodochrous* CTM, the conserved histidines were chemically modified, resulting in a loss of enzyme activity. As a result, it has been established that conserved histidines play a role in enzyme activity, although further analysis by site-directed mutagenesis is required to understand fully the role of the active site.

Although catechol 2,3-dioxygenase is capable of catalysing the ring-cleavage of substituted catechols as well as catechol, the enzyme shows only a low efficiency in the oxidation of 4-ethylcatechol, which serves as a suicide inhibitor. This is possibly due to the inefficient attack by iron-bound molecular oxygen on carbon-2 of 4-ethylcatechol (Cerdan *et al,* 1994). This would result in an increased probability of oxidation of the iron cofactor in the enzyme-oxygen-substrate complex.

3-methylcatechol and 4-methylcatechol, as well as their ring-cleavage products, have also been shown to be inhibitors of C23O (Polissi and Harayama, 1993). Attempts to understand the mechanism behind such inactivation have centred around the determination of k_{cat} and k_{inact} values for 3-methyl and 4-methylcatechol. From the work of Cerdan *et al* (1995), it appears that for 3-methylcatechol, the Km for suicide inhibition is much higher than that of catalysis, suggesting the presence of different binding sites for catalysis and inhibition. Such a proposal has been inferred before (Williams *et al,* 1990), which leads to the possibility that with this homotetrameric enzyme, negative cooperativity leads to a decrease in the binding affinity for 3-methylcatechol.

It was through the investigation into the mechanism of the inactivation of C23O that the otherwise unknown function of the *xylT* encoded gene product has been determined. The *xylT* gene is located between *xylL* and *xylE* in the TOL plasmid *meta*-cleavage operon, and is known to encode a polypeptide of 12kDa. Gene order and sequence analysis has shown that *xylT* shares features with genes from other degradative operons, namely *nahT* from the *sal* operon encoding genes for the degradation of salicylate, and *dmpQ* from the *dmp* operon encoding genes for the degradation of (dimethyl)phenol. Furthermore, the amino acid sequences conserved in the three gene products all possess a motif characteristic of chloroplast-type ferredoxins (Harayama *et al.*, 1991a, You *et al.*, 1991, Shingler *et al.*,1992). Such a characteristic would suggest a possible role for the *xylT* gene product in the reactivation of oxidised C23O subunits.

In order to investigate the potential functions of *xylT*, TOL plasmid pWWO mutants lacking a *xylT* expression showed an immediate drop in C23O activity upon metabolism of *m*- or *p*-toluate. Furthermore, C23O oxidised by H_2O_2 was not

reactivated in the absence of a functional *xylT*. This suggested that the XylT protein has a function in the reactivation of C23O by reducing the oxidised iron cofactor. The question that still needs to be addressed however is how does the oxidised *xylT* gene product become re-transformed to its reduced state. With a large proportion of the TOL plasmid pWWO having been elucidated, it is clear that there is no room for a gene that would encode an electron donor specific to *xylT*. Therefore it is possible that a non-specific electron doner may reduce the oxidised *xylT*. A possible candidate is the *xylZ* gene product that serves as an electron doner for benzoate dioxygenase. Alternatively, electrons may be provided from an electron transfer system independent of the TOL plasmid, for example the respiratory chain.

Following the aromatic ring cleavage of catechol or substituted catechols, the product is further metabolised by either a hydrolytic or dehydrogenative route, both of which result in the formation of 2-hydroxypent-2,4-dienoate, which isomerises rapidly to a form not recognised by downstream pathway enzymes. In the hydrolytic branch, the ring-cleavage intermediate is converted directly to 2-hydroxypent-2,4-dienoate by the action of 2-hydroxymuconic semialdehyde hydrolase (*xylF*), through one of the rarest known enzyme reactions, that of the hydrolysis of a C-C bond in a substrate. In the TOL plasmid *meta*-cleavage pathway, 3-methylcatechol is metabolised exclusively through this branch.

Recently, much work has centred upon the functional analysis of the XylF protein, and related hydrolases from different metabolic pathways. Amino acid sequence comparisons have revealed several conserved residues that form a 'lipase box' in a number of hydrolases (Ahmad *et al*, 1995). Such a consensus contains the triad Ser-Asp-His, the substitution of any of which by site directed mutagenesis results in a loss of enzyme activity. These observations suggest that enzymes such as XylF are serine hydrolases, a suggestion supported by the observation that enzyme activity is inhibited by PMSF, a classic inhibitor of serine hydrolase.

The identification of functionally active residues in XylF has lead to the analysis of residues flanking such lipase box sequences. This has in turn lead to the identification of a Phe to Met substitution at position 108 that results in a four-fold increase in the Km value for the ring-cleavage product of catechol (Diaz and Timmis, 1995). Such observations have provided the framework for the improvement of catalytic activity and altered substrate specificities of such enzymes.

Metabolism of catechol and 4-methylcatechol proceeds primarily through the dehydrogenative branch in the pathway, initiated by the *xylG* encoded $\alpha-$hydroxymuconic semialdehyde dehydrogenase. Metabolism via this route leads to the formation of the enol form of oxalocrotonate, the substrate of the *xylH* encoded oxalocrotonate tautomerase, cloned and sequenced by Chen *et al*, (1992). Characterisation of this enzyme has indicated it to be a pentamer of identical subunits

of 62 residues. Such a small subunit size would suggest that there is more likely to be one active site at the interface between subunits, rather than one active site per subunit. Such a possibility would explain the lack of sequence homology shared with other metabolic enzymes at the primary level.

Following the ketonization of 4-oxalocrotonate, the keto-form serves as a substrate for decarboxylation to 2-hydroxypent-2,4-dienoate by the *xylI* encoded oxalocrotonate decarboxylase (OD).

Characterisation of the gene product of *xylI*, together with the enzymes preceding and succeeding (Harayama *et al,* 1989a) has revealed that the OD and the XylJ gene product 2-oxopent-4-enoate hydratase (OEH) co-purify. This in turn has lead to the proposal that these two enzymes are physically associated as a complex *in vivo*.

It is thought that such a physical association between the OD and OEH would be required because the latter only metabolises the enol form of 2-hydroxypent-2,4-dienoate, which is spontaneously converted to the keto-form, 2-oxopent-4-enoate, which is not a substrate for OEH. Because of the unstable nature of the 2-hydroxypent-2,4-dienoate, the formation of a complex between OD and OEH may assure efficient transformation of this intermediate *in-vivo* (Harayama *et al,* 1989a) before side reactions can occur. Such a physical association between the enzymes OD and OEH initially suggests a possible interaction between OEH and HMSH due to the latter also producing 2-hydroxypent-2,4-dienoate by the hydrolytic branch. However these latter two enzymes have been successfully separated by DEAE chromatography, thus suggesting only a weak interaction between these two enzymes.

The presence of a second complex of physically associated enzymes within the *meta*-cleavage pathway has been proposed recently (Powlowski *et al,* 1993), following studies of the *dmp* operon-encoded pathway for phenol degradation in *Pseudomonas* sp. strain C600, which have revealed the presence of a previously unidentified enzyme activity, acetaldehyde dehydrogenase (*dmpF*), possibly conserved in the TOL *meta*-cleavage operon (Powlowski *et al.*, 1993).

By using a combination of deletion mapping, nucleotide sequence analysis and polypeptide analysis, the presence of the *dmpF* encoded acetaldehyde dehydrogenase was revealed (Shingler *et al,* 1992). By comparing the *dmp* genes with the TOL-encoded *xyl* genes, the gene order and sizes were found to be identical, except for between the *dmpF* and *xylQ* gene products, the latter having no previously known function. Despite this, given the similarity between all the other *meta*-cleavage genes, it would appear that *xylQ* encodes an isofunctional enzyme.

The possible presence of an acetaldehyde dehydrogenase in the TOL plasmid has presented some intriguing theories as to the fate of aldehydes following *meta*-cleavage. The action of the aldolase, the final step in *meta*-cleavage results in the

cleavage. The action of the aldolase, the final step in *meta*-cleavage results in the production of pyruvate, itself readily fed into the TCA cycle, and a toxic short-chain aldehyde, that requires further modification to produce a central intermediate. Metabolism of such aldehydes via *dmpF* in the case of the *dmp*- operon, or *xylQ* in the case of the TOL *meta*-operon, would be energetically favourable due the formation of an acyl coenzyme A that can be modified further.

Copurification of the 4-hydroxy-3-ketovalerate aldolase and the aldehyde dehydrogenase from *Pseudomonas* sp. strain C600 revealed that, like the *xylI* and *J* gene products these proteins are tightly associated. Despite being subjected to a wide range of separation procedures, the two activities were maintained at a similar ratio through each step. Furthermore it was shown that co-expression of the two appears to be required in order to observe aldolase activity.

As a result of such a physical association, the toxicity of aldehyde end-products of *meta*-cleavage would be minimised by keeping it enzyme bound, whilst efficiently metabolising it as it is generated. Secondly, physically associating the dehydrogenase with the aldolase would maximise the flux through the *dmpF/xylQ* dehydrogenase thus reducing the competition for the aldehyde by alcohol dehydrogenases, and therefore maximising the amount of aldehyde through the ATP-independent pathway.

Analysis of the coding capacities and the gene product sizes of *meta*-pathway genes has lead to the conclusion that most of the 10 kb DNA in the *meta*-pathway operon is composed of coding regions. Such a large coding region makes the *meta*-operon one of the biggest in bacteria, which thus raises questions as to whether any specific structure at the 3' end of the *meta* -operon mRNA exists to maintain its structure.

1.2.2 Regulation of the TOL plasmid degradative genes:

There are two regulatory genes, namely *xylR* and *xylS* located together, just downstream of the *meta*-operon genes (Franklin *et al,* 1983) that are involved in the regulation of the plasmid specified upper and *meta*- operons respectively. The XylS protein is activated by *meta*-pathway substrates and unlike the *xylR* activation of the upper pathway, is independent of nitrogen regulatory genes. Expression of the *meta* pathway is also induced by toluene/xylene activated XylR protein via a cascade regulatory system in which this protein, in combination with the NtrA protein, a sigma factor, stimulates transcription from the *xylS* promoter. Hyper-production of the XylS protein in turn provokes high-level expression of the *meta*-operon which is independent of *meta*-pathway substrates.

expression of the TOL plasmid degradative pathways, based on the presence of two regulatory genes, *xylR* and *xylS*.

As mentioned above, transcription from the upper operon promoter is mediated by the *xylR* product in conjunction with upper pathway substrates and a sigma factor, NtrA (Dixon, 1986). The involvement of a special sigma factor arose from the discovery of *xylABC* promoter sequences homologous to conserved regions found in nitrogen fixation (nif) promoters and in other promoters subject to nitrogen control (Reitzer and Magasanik, 1983). *P.putida xylA-lacZ* transcriptional and translational fusions were constructed (Dixon, 1986), and the expression from the *xylA* promoter was assayed in the presence of XylR and NtrA. In all cases, the expression was dependent upon the presence of the NtrA factor.

In comparison to the upper pathway promoter sequences, the regulatory region of the *meta*-pathway, although showing some homology with that of the upper operon, does not contain the unique features of an ntr consensus sequence. However, these promoters are regulated differently, since the *xylS* gene product is required for positive regulation of the *meta*-operon, in combination with benzoates or toluates (Worsey *et al,* 1978).

In order to analyse the function of the *xylS* encoded protein and to define essential structural features of *xylS* protein inducers, the effector activities of a wide range of benzoate derivatives have been analysed utilising constructs in which a *meta*-promoter sequence : *lacZ* fusion was present on a different plasmid from that carrying *xylS* in both *E.coli* and *P.putida* (Ramos *et al,* 1986). *Meta*-cleavage substrates benzoate and *m*-toluate were found to strongly induce β-galactosidase synthesis in both *E.coli* and *P.putida*, whereas upper pathway substrates, and intermediates of the *meta*-pathway did not, thus confirming the expression of the *meta*-cleavage pathway only in the presence of primary substrates of the pathway (Ramos *et al,* 1986). Further analysis of *xylS* effectors in terms of the influence of substituents on the aromatic ring of effector molecules has suggested that the presence of a carboxylic group at C-1 of the aromatic ring is essential for the establishment of productive contacts between effector and *xylS* product that in turn lead to activation of the latter.

In further attempts to understand the mechanisms of positive regulation of the TOL *meta*-cleavage pathway, two different groups of *xylS* mutants have been employed; namely XylS regulatory mutants with altered effector specificity (Ramos *et al,* 1990), and *xylS* mutants that encode regulators which constitutively mediate expression from the *meta*-pathway promoter. Of the former, six mutants were obtained which recognise as effectors benzoate analogues that are unable to activate the *xylS* wildtype protein. Those that were able to recognise effectors with unusual substitutions at position 2 or 3 showed a mutation in a putative β-pleated region at the N-terminal part of the protein, whereas mutants capable of recognising 2,5-

dimethylbenzoate and 3,5-dichlorobenzoate showed a change in the putative α-helix region at the central part and C-terminal part of the protein respectively. Conclusions drawn from these observations suggested that the effector binding pocket of XylS may be composed of two or more non-contiguous segments of its primary structure (Ramos *et al,* 1990).

As well as activating expression of the *meta*-cleavage pathway in the presence of benzoate, the *xylS* encoded protein also activates expression of the same operon in the presence of *m*-xylene or *m*-methylbenzyl alcohol, under the influence of XylR (Franklin *et al,* 1983) which is itself expressed constitutively. Activated XylR, together with NtrA, stimulates transcription of the *xylS* gene so that levels of XylS mRNA exceed those found in cells grown on *m*-toluate by at least 25-fold. Such a hyperproduction of XylS protein in turn leads to constitutive expression of the *meta*-operon in the absence of a *meta*- cleavage pathway substrate (Mermod *et al,* 1984).

More recent analysis of *meta*-operon regulation has centred upon investigating the mechanisms by which XylS interacts with upstream promoter sequences. In 1993, Kessler *et al* reported that the activation of promoter sequences by XylS is dependent upon a 36 bp *cis*-acting DNA sequence within the promoter that comprises two 15 bp direct repeats separated by 6 bp. XylS has subsequently been shown to bind to these two repeat sequences with very weak protein:DNA interactions.

Having established that it is possible for upper pathway substrates to stimulate *meta*-pathway expression in the presence of XylR, it is worth considering the physiological significance of such a system, when under normal circumstances the *meta*-pathway will be expressed anyway due to the metabolism of upper pathway substrates to benzoate or *m*-toluate. A possible explanation may concern the time taken to reach full induction of both upper and lower pathways. Unlike induction of the lac operon in *E.coli*, which takes several minutes, that of the TOL pathways takes several hours, resulting in a transient block in the flow of metabolites, should the expression of *meta*-pathway be dependent upon the metabolism of upper pathway substrates. Simultaneous co-induction of both upper and lower pathways would prevent such a transient block, and would as a result prevent a loss from the cell of carboxylic acid intermediates, themselves known to be chemoattractants for benzoate utilising bacteria. It is also feasible that, at low a concentration of upper pathway substrates, the resulting concentrations of carboxylic acid intermediates would be insufficient to effect the activation of *meta*-pathway expression.

1.3 Aromatic degradation.

Polycyclic hydrocarbons are widely distributed throughout the environment due to the incomplete combustion of organic materials (Blumer, 1976). Aromatic compounds are of special concern to man since, because of their hydrophobicity, the cell membrane does not provide an effective barrier and as a result compounds such as benzo-a-pyrene may become bioconcentrated in adipose tissue. In addition, such compounds may be genotoxic by a number of mechanisms including cross-linking intercalation and backbone breakage of DNA strands. The mechanisms of such toxicity have in many cases been associated with the physical shape of certain polyaromatics that have dimensions similar to that of a DNA base pair.

Like benz-a-pyrene, dioxins also accumulate in the adipose tissue, and primarily arise as by-products of the manufacture of herbicides, polychlorinated biphenyls (PCB's) and herbicides. PCB's are derivatives of biphenyl, which have found widespread use in industry as for example heat transfer fluids. Up to one third of the 10^8kg of PCB's produced since the 1930's is thought to have been released into the environment (Shehan 1994). These compounds are highly toxic, particularly to aquatic species, which compares with the relatively high tolerance shown by mammals. Thus there has been an increasing interest in the fate of aromatic hydrocarbons in the environment, particularly via microbial degradation. The first stage in microbial aromatic hydrocarbon degradation involves the oxidation of the unsubstituted aromatic compound by either a mono-oxygenase or a dioxygenase, which incorporates one or two oxygen atoms respectively into the organic substrate. In contrast to bacterial degradation, fungi oxidise aromatic hydrocarbons by a cytochrome P-450 mono-oxygenase catalysed reaction (Cerniglia and Gibson, 1978) in a reaction that appears to be similar to that reported for multi-enzyme systems.

1.3.1 Degradation of environmental pollutants.

As described above, increasing quantities of man-made organic chemicals are released into the biosphere each year, many of which are resistant to chemical, physical or biological degradation, thus constituting a potential environmental burden of considerable magnitude. As a result, there has developed a maze of governmental regulations to reduce the immediate toxic effects of such compounds on human beings and animals. The regulations are usually based on two major characteristics of the chemicals; namely their toxicological properties and their fate in the environment. If a compound is designated toxic, its release is highly regulated and seldom permitted. The issue is complicated however by the difficulty in determining the toxicity of a chemical, a problem that often allows the chemical industry to continue dumping

suspect chemicals into the environment. Other routes for environmental contamination by toxic pollutants include the accidental release of known hazardous chemicals, and the storage of toxic chemicals. The latter is of considerable concern in industrialised countries for which there are no particular technological or scientific answers for how to deal with such chemicals. Even the 'Superfund' program in the United States, designed to help decontaminate toxic dump sites, aims only at hauling the toxics away from the contamination site to a designated 'safe' site.

As has been mentioned previously, the genetic manipulation of microbial degradative pathways offers a powerful means by which to accelerate the evolution of biodegradative routes through which such compounds may be eliminated from the environment (Ramos *et al,* 1987a).

Restructuring of metabolic pathways has been utilised with the alkylbenzoate pathway of TOL plasmid pWWO, resulting in the creation of a pathway able to process 4-ethylbenzoate (4-EB). Initial studies investigating the ability of soil organisms to grow on 4-EB lead to the proposal that the recalcitrant nature of 4-EB could be due to one or more of four possible reasons: its toxicity, its failure to be taken up by bacteria, its failure to stimulate transcription, or its failure to be degraded by catalytic enzymes. The first two possibilities appear unlikely due to evidence that growth may be achieved on 3-EB. Furthermore, analysis using a TOL derivative pNM72 (Mermod *et al,* 1984) expressing the benzoate *meta*-cleavage pathway illustrated that growth could be achieved on 4-EB and glucose, at the expense of the latter, whilst accumulating 4-ethylcatechol (4-EC). It appears therefore, that the rate-limiting step involves the accumulation of 4-EC which inactivates the subsequent steps in the pathway (Ramos *et al,* 1987b). Another obstacle has been shown to lie in the inability of 4-EB to activate the *xylS* protein, resulting in a failure to transcribe the catabolic genes. These observations have lead to the isolation of mutants possessing a regulator capable of being activated by 4-EB. In this way, modification of the TOL *meta*-pathway by incorporating such mutant genes has lead to the ability of the host bacterium to degrade 4-EB in addition to the normal substrates of the pathway.

Such an example of engineering the *meta*-cleavage pathway has revealed the key targets that need to be addressed in order to achieve the degradation of various aromatics, namely the *xylS* gene product and the *xylE* encoded catechol 2,3-dioxygenase (C23O).

1.3.2 Degradation of halogenated compounds:

As described earlier, over the past few decades, chlorinated aromatic compounds have been released in massive amounts in the form of herbicides, pesticides or more simply, as hygienic household products. The presence of chlorine

atoms on such molecules renders them toxic for micro-organisms, as well as in some cases human beings. In the former this is largely due to the limited number of naturally occurring compounds having C-Cl bonds, rendering micro-organisms incapable of degradation of such compounds. This has resulted in the persistence of these compounds, creating an enormous problem in terms of toxic chemical production.

In spite of this, a number of micro-organisms have been identified that are able to slowly biodegrade either mono- or poly-chlorinated compounds, and with the development of metabolic engineering, more and more sophisticated biodegradation systems are being developed. Described below are some of the strategies that have been employed to enable such biodegradation.

There have been several reports in recent years of pure cultures capable of dissimilating simple chlorinated compounds. For example, 4-chlorobiphenyl (4CB is known to be metabolised by *Alcaligenes, Acinetobacter, Klebsiella* and others to 4-chlorobenzoic acid. The 4CB is initially converted through cyclic peroxide intermediates into dihydrodiols and then through a dioxygenase catalysed reaction into catechol derivatives (Ahmad *et al,* 1991), which are ultimately converted to chlorobenzoic acids. However, none of the pure cultures are capable of any further breakdown of the chlorobenzoic acids, which therefore accumulate in the medium. Cocultivation of the bacteria carrying the chlorobiphenyl degradative pathway (the *bph* gene cluster) with bacteria capable of degrading chlorobenzoic acids leads to the complete degradation of monochlorobiphenyls (Sylvestre *et al,* 1985). However, in situations in which chlorobenzoic acids accumulate in the medium, the *bph* genes are not the only ones operating, since chlorobenzoic acids are then converted into a number of phenolic and acidic metabolites.

In terms of the TOL plasmid encoded *meta*-cleavage operon, C23O, the key enzyme of the pathway, is inactivated by 3-chlorocatechol, a suicide substrate (Bartels *et al,* 1984). Extradiol cleavage of 3-halocatechols generates 5-haloformyl-2-hydroxypenta-2,4-dienoic acid, a highly reactive acyl halides which thus inactivate the C23O.

The inability of microorganisms to degrade halogenated catechols clearly suggests the requirement of a general strategy for the development of an engineered pathway for poorly biodegraded compounds such as chlorocatechol. One such method could include the restructuring of existing metabolic pathways, as described earlier in for the construction of a pathway able to metabolise 4-EB. A more versatile approach that has been described (Rojo *et al,* 1987) involves the patchwork assembly of new pathways by the combination of enzymes from different pathways. In this way a pathway constructed in a *Pseudomonas* species, strain B13, has enabled the engineered bacterium to grow well on mixtures of 3-chlorobenzoate (3-CB), as well as

4-chlorobenzoate (4-CB) and 4-methylbenzoate (4-MB) without the non-productive misrouting of intermediates.

This strain however could not mineralise *m*-toluate, a problem overcome following the isolation of a derivative harbouring a point mutation in *xylE*, that allows the degradation of *m*-toluate in the presence of 3-chlorobenzoate (Wasserfallen *et al*, 1991).

Trichlorinated and polychlorinated aromatics are long-lived environmental pollutants that persist due to the inability of a single indigenous soil micro-organism to utilise them as ground substrates. Nevertheless, following the recent advances that have been made concerning the molecular biology of bacterial aromatic degradation pathways, an increasing number of elegant engineered systems are being reported that provide improved degradation of tri and polychlorinated compounds. Highly halogenated compounds are most frequently metabolised by anaerobic bacteria as a result of reductive dehalogenation reactions, the products of which act as substrates for bacterial oxygenases.

Trichloroethylene has long been recognised as one of the most significant environmental pollutants in soil and ground water. However in 1989, a single bacterial species, *Pseudomonas mendocina* KR-1, that could efficiently catalyse the degradation of TCE by the action of toluene monooxygenase was reported (Winter *et al,* 1989). A similar observation was made (Wackett and Gibson, 1988) that reported that TCE degradation could also be achieved by the toluene dioxygenase of *P.putida* F1, implying that at least two diverse oxygenase systems involved in toluene metabolism can both oxidise TCE. Such new information has lead to the finding that the hybrid dioxygenase *todC1bphA2A3A4* (section 1.1.1.4) is capable of catalysing much faster rates of TCE degradation than observed in the wild-type dioxygenases. The reasons for such improved rates of degradation are unclear, although a tentative explanation involves the fact that unlike wild-type dioxygenases, the hybrid terminal oxygenase components form heterodimers as well as the more usual heterotetramers. Such dimeric structures may enhance enzyme:substrate affinity, or lead to a greater electron flow from NADH (Furukawa *et al*, 1994).

A different methodology (Wackett *et al*, 1994), utilises sequential reductive and oxidative reactions catalysed by cytochrome P450cam monooxygenase and toluene dioxygenase respectively in the same *Peudomonas* strain. Cytochrome P450cam monooxygenase reduces penta- and tetra-chloroethane to TCE, which, as described above, is then a substrate for toluene dioxygenase.

Despite the development of such systems for the degradation of highly chlorinated compounds, only recently has there been described a bacterium capable of degrading biphenyl compounds that are chlorinated on both aromatic rings. *Pseudomonas acidovorans* M3G7 (McCullar *et al*, 1994) was constructed with a

continuous amalgamated culture apparatus using two biphenyl and chloroacetate degraders. The resulting recombinant *Pseudomonas* has the capacity to utilise 3,4'-dichlorobiphenyl as sole carbon and energy source. However for reasons that are as yet unclear, the recombinant strain is not able to metabolise 3,3'-dichlorobiphenyl. This may however be due to the high substrate specificity of the bacterium, or the inhibitory effects of 3,3'-dichlorobiphenyl metabolite intermediates.

It is clear from the examples cited above that in time the directed laboratory evolution of metabolic pathways, draws on a knowledge of energy mechanisms and gene structure, will lead to the development of organisms that will be capable of metabolising the most toxic and environmentally persistent pollutants (Kunz, 1991).

1.4 Evolutionary relationships between TOL and other plasmids.

The biochemical pathways responsible for the aerobic catabolism of aromatic compounds share many common features (Horn *et al,* 1991), and involve the channelling of structurally diverse substrates to a central intermediate which is then cleaved by one of a few pathways.

The occurrence of common central routes in many catabolic pathways may be a consequence of divergent evolution in which a limited number of primordial central pathways have been selected among bacterial species, or convergent evolution in which different organisms have independently developed catabolic routes due to the limited number of useful reactions (Horn *et al,* 1991). In the case of the β-ketoadipate pathway, evidence for a common ancestor centres around a comparison of the amino acid sequences of isofunctional enzymes of several bacterial species (Ornston and Yeh, 1982).

A number of studies have investigated the evolutionary relationships between the TOL plasmid and the *Pseudomonas* degradative pathways for naphthalene and salicylate, encoded by the *nah* (Dunn and Gonsales, 1973), and *sal* (Chakrabarty, 1972) operons respectively; all of which are members of the *Pseudomonas* IncP-9 incompatibility group (Bayley *et al*, 1979).

Like the genes of the TOL plasmid, the catabolic genes for the oxidation of naphthalene are also encoded in two operons: the first, the *nah* operon, encodes six enzymes that specify the conversion of naphthalene to salicylate, and the second, the *sal* operon, encodes genes for the oxidation of salicylate to pyruvate (Harayama *et al,* 1987). Both sets of operons are induced by the *nahR* encoded regulatory protein which, on binding to the inducer (salicylate) activates transcription of the *nah* and *sal* operons (Schnell, 1986). Since expression of both these operons is co-ordinately controlled by *nahR*, sequences of both transcription start sites have been analysed by S1 nuclease mapping, in order to locate potential sites involved in common regulation. Furthermore, any possible homology between such sequences and analogous sequences of the TOL plasmid encoded upper and lower pathways would suggest origins of positive control systems of the environmentally important hydrocarbon degradative plasmids of *Pseudomonas* (Schnell, 1986).

Mapping techniques have revealed two 5' flanking regions of extensive homology between the *nah* and *sal* operons. One, a 12bp sequence at -35 represents the RNA polymerase binding site, whilst the other, a 21bp sequence at -70 suggests a region required for binding of the transcription activator, as this region between -60 to -80 has been found to be the site of action of other positive regulatory gene products; for example *araC* (Raibaud and Schwarz, 1984).

Comparison of *nah* and *sal* promoter sequences with the equivalent in the TOL specified pathways have shown very little homology, thus indicating an apparent independent evolution of regulatory systems for the NAH and TOL plasmids, despite their degradative genes being organised and regulated in a similar fashion. In comparison however, the extensive homology observed between the ***nah*** and ***sal*** promoters would suggest a common ancestor, or perhaps that the ***nah*** promoter evolved from the *sal* promoter.

In terms of the evolutionary relationship between different pathway enzymes, a number of chromosomally encoded *ortho* pathways for ring-cleavage of dihydric phenols have been shown to possess homologous enzymes derived from a common ancestor (Hartnett *et al*, 1990, Neidle *et al*, 1988). In contrast, the specific reactions following *meta*-cleavage are less clear in terms of their biochemical relatedness.

Early studies investigating the evolutionary relationships between the *meta*-cleavage pathways of TOL, SAL and NAH involved DNA hybridisation studies which illustrated the presence of a significant degree of homology, although the significance of these regions was at that time not defined (Heinaru *et al*, 1978).

Further analysis involved the theory that closely related plasmids yield similar cleavage fragment patterns upon restriction digests. A comparison of NAH and SAL pathways in this way however showed that each have a number of fragments that do not correspond in size to fragments from the other (Lehrbach *et al*, 1983), implying that the relationship between NAH and SAL cannot be explained simply by the loss of a single DNA segment from NAH to form SAL as had previously been suggested (Heinaru *et al*, 1978).

A number of other DNA hybridisation studies have been used to demonstrate the homology between the *meta*-cleavage pathways of NAH and TOL (Cane and Williams, 1986, Assinder and Williams, 1988), of which the latter used a sequential series of specific DNA hybridisation probes to elucidate the exact nature and extent of the structural similarities. A strong degree of homology was observed starting at the catechol 2,3-dioxygenase gene and extending downstream the full length of the NAH and TOL structural genes.

More recent studies (Horn *et al*, 1991) have centred around the post *meta*-ring fission genes, *xylGFJ* and their products in terms of their biochemical relatedness with other enzymes, and the evolutionary significance of any homology.

The *xylG* gene, encoding 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) was shown to share significant homology at the gene product sequence level with eukaryotic aldehyde dehydrogenases (ADH) and the *Pseudomonas* genes *xylC* and *xylH*, thus suggesting that the prokaryotic enzymes are members of the eukaryotic ADH superfamily. Incorporation of the *xylC,H* and *G* into the ADH superfamily in this way allows a broader analysis of the evolutionary divergence of the genes by

making use of an evolutionary dendrogram (Horn *et al,* 1991) generated by the method of Higgins and Sharp (1988). Using such a method, it was proposed that the *xylG* gene product is separated by less evolutionary distance from ADH's than that of *xylC*. This observation implies that after the divergence of *xylC* and *xylG*, significant gene exchange between kingdoms took place. It was also proposed from this that *xylC* and *xylG* evolved independently from a common ancestor, rather than by gene duplication and divergence, as is initially apparent for the two genes which are on the same plasmid and have related activities in the same catabolic pathway (Horn *et al,* 1991).

Such a suggestion has been reinforced by the findings of Roper *et al* (1995), who have reported sequence similarities between enzymes of the homoprotocatechuic acid (HPC) pathway of *E.coli* C. More specifically, sequence homology studies have revealed that 2-carboxymethyl-2-hydroxymuconic acid semialdehyde (CHMS) dehydrogenase of the HPC pathway is a member of the ADH superfamily, and shares 40% identity with the *xylG* encoded dehydrogenase of the TOL plasmid. This observation has lead to the suggestion as before, that the aldehyde dehydrogenases of different *meta*-cleavage pathways have evolved separately, but from the same ancestral source.

Sequence analysis revealed that both *xylF* and *xylJ* have analogues in the *tod* gene cluster, namely *todF* and *todJ*, which specifies a toluene/benzene degradative pathway. Despite this however, whilst the *xylFJ* genes are contiguously located within the TOL *meta*-operon, their analogues *todF* and *todJ* are separated by the *todC1C2BADE*, thus implying that the tod gene cluster may have been assembled by an insertion of the *todC1C2BADE* genes between *F* and *J*. The transfer of accumulated units in this way as a means of evolution provides, in terms of selective pressure, an increased advantage over the transfer of single genes.

In 1994, theories were proposed (Harayama, 1994) that suggested an independent evolution of the TOL plasmid upper and *meta*-cleavage pathways. Codon-usage patterns developed for both operons were similar for genes of the same operon, but different between genes of different operons. From this observation, it has been proposed that the two ancestral operons have evolved independently in different organisms.

By applying the same principle of codon-usage, from the knowledge that the *xylR* gene product influences the expression of the upper operon in the TOL plasmid, similar codon-usage patterns, indicating a co-evolution of the XylR gene product and the upper operon would be expected. This however has proven not to be the case, suggesting that the XylR ancestor may have been involved in the regulation of another gene, and was only recruited later by the TOL plasmid.

Over the past few years much attention has centred around the evolutionary relationships between different aromatic dioxygenases. With the increased availability of dioxygenase DNA sequences, DNA homology studies have been able to establish evolutionary links between such enzymes.

Comparison of the deduced amino acid sequences of the *benABC* encoded benzoate dioxygenase from *Acinetobactor calcoaceticus* (Neidle *et al*, 1991) with other multicomponent dioxygenases such as toluate. toluene, benzene and naphthalene dioxygenases has indicated that similarly sized terminal oxygenase subunits are derived from a common ancestor. Other studies (Harayama *et al*, 1991b) have supported this proposal, having reported aligned amino acid sequence identities between *benABC* and *xylXYZ* encoded subunits of up to 61%. A noticeable difference between the *benABC* and *xylXYZ* gene clusters however is the high frequency of arginine codons in XylX, a trait maintained to a lesser extent throughout the entire operon. Such an occurrence raises the possibility that the *xyl* structural genes were selected in an organism in which the tRNA corresponding to arginyl codons was particularly high.

A feature shared by a number of aromatic dioxygenase genes is the presence of repetitious DNA sequences (Neidle *et al*, 1991), (Harayama *et al*, 1991b). These are thought to have been acquired during the evolutionary divergence of such sequences from a common ancestral gene. In the case of the *benABC* and *xylXYZ* encoded dioxygenases, different patterns of repetition distinguish the coding regions. Such repetitions are thought to have evolved as components of three dimensional structures formed by slipped single stranded DNA. Such slippage structures occur when one DNA sequence hybridizes with a complementary sequence in another region, thus allowing the formation of an alternative slippage structure. Subsequent interactive slippage structures would generate genetic continuity, and when disrupted, generate cascades of mutation through mismatch repair. Such a model would account for the rapid genetic divergence of such bacterial systems, together with the maintenance of repeat sequences.

As the amino acid sequences of aromatic dioxygenases become known, the biochemical classification scheme for bacterial oxygenases is continually being established, and linked with the relationships between the genes encoding the enzymes. In this respect, Nakatsu *et al*, (1995) have identified regions in dioxygenase reductase components that are conserved in the Class IA enzymes. Furthermore, these identified regions have been shown to have occurred early in the evolution of the electron transport chain of bacterial oxygenases.

Evolutionary divergence in this manner is evident with the Class IB dioxygenases. Conserved amino acid sequences between the *benC* and *xylZ* encoded dioxygenase reductase components have been found to occur primarily in C-terminal

regions. In this respect it has been suggested that these portions of the proteins are responsible for the direct interaction with NADH In contrast, the BenC and XylZ proteins do not appear to be evolutionarily related to proteins of the benzene, toluene and naphthalene dioxygenases, which employ a two-protein electron transfer system. This in turn substantiates the knowledge that benzoate and toluate dioxygenases described here are members of a multicomponent aromatic ring-hydroxylating superfamily that is distinct from dioxygenases that are encoded by four coding regions.

The evolutionary relationship between ring-cleavage dioxygenases has also been investigated in recent years. In 1989 it was established that *ortho-* and *meta-* ring cleavage dioxygenases do not possess significant amino acid sequence homology, and are as a result classified into two different gene superfamilies. Within the *meta-*cleavage superfamily, substantial amino acid sequence identity has subsequently been reported between a number of such *Pseudomonas* enzymes (Harayama and Rekik, 1989, Carrington *et al*,1994). This analysis has revealed subfamilies within the *meta-* ring cleavage dioxygenases. The existence of these subfamilies is thought to reflect the nature of the substrate for the enzyme, either mono- or bicyclic. To confirm such a possibility, a C23O has been identified from the Gram-Positive *Rhodococcus rhodochrous* CTM that has substantial amino acid homology with a number of isofunctional *Pseudomonas* enzymes (Asturias *et al.*, 1995). This particular C23O has the ability to accept both mono- and bicyclic substrates, and, in accordance with the prior suggestion, shows homology with both monocyclic and bicyclic specific enzymes.

The overall similarity of genes from different pathways has lead to the development of a hypothesis for the evolution of *meta*-cleavage pathways (Williams and Sayers, 1994). The hypothesis is based upon evolution in a modular fashion by the juxta-positioning of pre-existing metabolic 'units' in sequence.

One unit would include the downstream genes of the *meta*-cleavage pathway, starting with the ring-cleavage enzyme. According to the hypothesis, such a unit would be incorporated into a pathway for degradation by recombination with the upstream genes in the *meta*-cleavage pathway. The result of this recombination would be the establishment of *meta*-cleavage pathways that bear little resemblance at the upper end, but share a high degree of identity from the C23O and downstream.

A further proposal of the hypothesis is that upper pathways (in the case of the TOL plasmid for the conversion of toluene to benzoate), would be acquired as a separate operon, and the development of an integrated regulation of the upper and meta-operons would occur at a later stage.

As has been discussed earlier, the understanding of the evolutionary relationships between isofunctional enzymes of *meta*-cleavage pathways will allow not

only the elucidation of the evolutionary process, but will also reveal functionally and structurally important amino acids. The identification of important residues in this way is exemplified by the work of Candidus *et al*, (1994), that illustrated the importance of conserved tyrosine residues in the activity of C23O.

Establishing the evolutionary and subsequently the functional relationship between such pathways will ultimately enable the design of mutagenesis studies to enhance or alter the substrate specificity of a particular enzyme or pathway.

Chapter 2 Materials and Methods.

All chemical reagents were of AnalaR grade or equivalent and were obtained from Sigma Chemical Company (Dorset, UK) or BDH Ltd. (Dorset, UK) unless stated otherwise. Bacteriological reagents were obtained from either Oxoid or Difco.

2.1 Strains.

All studies were performed with *E.coli* JM107 (Yanisch-Perron *et al* (1985).

2.2 Constructs.

The plasmid constructs used in this study are shown in table 2.1.

Table 2.1 Plasmid characteristics.

Plasmid	Characteristics	Reference
pUC18	*LacZ* Ap^r	Yanisch-Perron *et al.*, (1985)
pUC19	*LacZ* Ap^r	Yanisch-Perron *et al.*, (1985)
pUC119	LacZ Ap^r	Yanisch-Perron *et al.*, (1985)
pBGS18	*LacZ* Km^r	Spratt *et al.*, (1986)
pBGS19	*LacZ* Km^r	Spratt *et al.*, (1986)
pMMB66HE	*LacZ* Ap^r	Fürste *et. al.*, (1986)
pMMB66EH	*LacZ* Ap^r	Fürste *et. al.*, (1986)
pKTF11	*bphA1A2A3A4BC* in pHSG396 Cm^r	Taira *et al.*, (1992)
pJHF108	*bphA1A2A3A4* in pUC118 Ap^r	Hirose *et al.*, (1994)
pQR109	*xylTE* in pUC8 Ap^r	Ward (unpublished)
pQR149	*nahAaAbAcAd* in pUC18 Ap^r	Ward (unpublished)
pQR150	*xylXYZLTEGFJQKIH* in pBGS18 Km^r	Ward (unpublished)
pQR151	*xylJQKIH* in pBGS18 Km^r	Ward (unpublished)
pQR185	*xylXYZLTEGF* in pBGS19 Km^r	This study
pQR186	*xylXYZLTEGFJ* in pBGS19 Km^r	This study
pQR188	*xylXYZ* in pUC19 Ap^r	This study
pQR189	*xylXYZ* in pMMB66HE Ap^r	This study
pQR191	*xylXbphA2A3A4* in pBGS19 Km^r	This study
pQR192	*bphA1xylYZ* in pBGS18 Km^r	This study
pQR193	*xylXYZLTE* in pUC8 Ap^r	This study
pQR194	*xylI* in pMMB66EH Ap^r	This study
pQR225	*xylXYZLTE* in pUC8 Ap^r	This study
pQR226	*xylXYZLTE* in pBGS18 Km^r	This study

Plasmid	Characteristics	Reference
pQR220	*xylYZ* in pBGS18 Km^r	This study
pQR221	*bphA1* in pBGS18 Km^r	This study
pQR222	*bphA1A2A3A4* in pMMB66He Ap^r	This study
pQR223	*xylX:bphA2A3A4BC* in pHSG396 Cm^r	This study
pQR224	*xylI* in pBGS18 Km^r	This study
pQR227	*xylXYZL* + MCS in pBGS19 Km^r	This study
pSS2	*nahAaAbAcAd* in pMMB66EH Ap^r	Shrestha PhD Thesis 1994

Abbreviations: Ap^r, ampicillin; Cm^r, chloramphenicol; Km^r, kanamycin.
The plasmids pKTF11 and pJHF108 were obtained from K. Furukawa (University of Kyushu, Japan.) The vectors pMMB66HE and pMMB66EH were obtained from R. Drew (University College London).

2.3 Strain Maintenance.

E.coli strains were grown on selective nutrient agar plates, (25 gl^{-1} nutrient broth (Oxoid), 20 gl^{-1} bacteriological agar (Difco)). Glycerol stocks were prepared by resuspending colonies off selective plates in 2mls of 20% (w/v) glycerol using a sterile 10 ml plastic pipette (Sterilin). Resuspended cells were stored in Falcon tubes at -70°C.

Antibiotics and other supplements were used where necessary at the following concentrations: 100 μgl^{-1} ampicillin; 50 μgml^{-1} kanamycin; 30 μgml^{-1} chloramphenicol; 50 μgml^{-1} IPTG; 20 μgml^{-1} X-gal.

2.4 Strain selection.

In order to identify recombinant strains containing a recombinant *meta*-cleavage pathway including the *xylE* gene, colonies on selective nutrient agar plates were sprayed with a 100 mM catechol solution using an aerosol. Positive clones that turned yellow immediately due to the production of α-hydroxymuconic-semialdehyde were picked off and restreaked onto a fresh nutrient agar plate using a sterile loop.

To select positive clones containing the *nahA* gene, several crystals of indole were placed on the inside of the lid of selective nutrient agar plates following overnight growth of colonies. Plates were incubated in the presence of indole for 2-3 hrs at 30°C. Colonies exhibiting a blue colour due to the formation of indigo were picked off and restreaked onto a fresh nutrient agar plate using a sterile loop.

2.5 Growth conditions.

2.5.1 Shake flask experiments.

2.5.1.1 Wild-type and truncated *meta*-cleavage pathways.

Unless stated otherwise, shake flask experiments were performed using nutrient broth (25 gl^{-1} nutrient broth No. 2, Oxoid) as the growth medium.

5 ml volumes of sterile media were inoculated with 2 colonies from a fresh selective plate, and grown with reciprocal shaking at 37°C for 12 hours. 2 l Erlenmeyer flasks containing 200 ml sterile media were then inoculated with the overnight seed, and incubated with reciprocal shaking at 37°C. Antibiotics and IPTG were used where required at all stages at the concentrations indicated in section 2.3. Pathway substrates were used at 10 mM working concentrations for benzoate and *m*-toluate unless otherwise stated.

2.5.1.2 Wild-type *meta*-cleavage pathways supplemented with an additional dioxygenase.

20 ml volumes of sterile nutrient broth were inoculated with 2 colonies from a fresh selective nutrient agar plate, and grown with reciprocal shaking at 37°C overnight. The following morning 20 ml sterile nutrient broth in a 100 ml Erlenmeyer flask fitted with a moulded central well filled with sterile cotton wool, was inoculated with a 2 ml volume from the overnight seed culture, and incubated at 37°C with orbital shaking (200rpm). The cotton wool in the central wells were saturated with 10 mM concentrations of either toluene, biphenyl, naphthalene or benzene. Sodium benzoate was added directly to the media at a working concentration of 10 mM. Antibiotics and IPTG were used as described in section 2.3

2.5.2 Biotransformations.

2 l Erlenmeyer flasks containing 200 ml sterile nutrient broth supplemented with antibiotics and IPTG as in section 2.3 were inoculated with 2 colonies from a fresh nutrient agar plate. The cultures were grown with reciprocal shaking at 37°C for 12 hrs or until late-log phase. The cells were harvested at 9000rpm (Sorvall RC2-B, GSA rotor) , washed in 50 mM Tris-HCl buffer, pH 8.0, and reharvested before being

resuspended in the same buffer to an absorbance$_{600nm}$ of 1.0. 20 ml volumes of these resting cells were then transferred to sterile 100 ml Erlenmeyer flasks and incubated with orbital shaking (200 rpm) at 30°C in the presence of 0.5 mM concentrations of aromatic substrate.

2.5.3 Fermentation growth studies.

2.5.3.1 Fermentation media.

The following mediums were used for fermentation studies with *E.coli* strains expressing wild-type or truncated *meta*-cleavage pathway constructs:

Per 1.5 l: 1.5 g Yeast extract
0.75 g NaCl
22.68 g $Na_2HPO_4.12H_2O$
4.5 g KH_2PO_4
11.1 g $(NH_4)_2SO_4$
15 ml Glycerol
15 ml Metal solution (stock: 100 mM $MgCl_2$,10 mM $CaCl_2$)
0.03 g $FeCl_3$

2.5.3.2 Fermenter Configuration.

Batch cultivations were carried out in a stirred tank 2 l (1.5 l working volume) LH 210 fermenter (Inceltech). The dissolved oxygen tension (DOT), pH and temperature were monitored by Ingold electrodes (Ingold, Switzerland), linked to Real Time-Data Acquisition System (RT-DAS) (Acquisitions Systems, Reading UK). Off gas concentrations of oxygen, nitrogen, carbon and argon were monitored by mass spectroscopy, (Fisons Scientific Instruments, Prima 600) and parameters such as oxygen uptake rate (OUR), carbon dioxide emission rate (CER) and respiratory quotient (RQ) were calculated and plotted by RT-DAS. The value of pH was held at pH 7.0 using 4 M NaOH, and the DOT maintained at 60 % using an air flow rate of 1 $vv^{-1}m^{-1}$ together with a variable stirrer speed of 300-1000 rpm. The bioreactor temperature was maintained throughout at 37°C. Mass spectrometry lines from the fermenter were protected by a home-made foam-trap consisting of a 500 ml flask containing 50 ml (polypropylene glycol) PPG connected to an off gas line. Off gas from the foam trap was filtered through a side-arm tube containing glass wool before entry to the mass spectrometer.

2.5.3.3 Fermenter growth conditions.

For *E.coli* fermenter studies, a 5 ml volume of fermentation media was inoculated with 2 colonies from a fresh selective nutrient agar plate and incubated with reciprocal shaking at 37°C for 2 hrs. A 500 ml Erlenmeyer flask containing 50 ml of the same media was inoculated with the 5 ml volume, and incubated with orbital shaking for 6 hrs at 37°C. This 50 ml volume was used to inoculate the fermenter. Antibiotics were used at the standard concentrations described in section 2.3

The cultures were induced with 50 $\mu g ml^{-1}$ IPTG after 12 hrs, followed by the addition of pathway substrate after an additional 2 hrs.

2.6 Sample analysis.

2.6.1 Optical density.

The optical density of a culture was measured at 600 nm against a blank of sterile culture medium.

2.6.2 Dry cell weights.

2 ml of an *E.coli* culture was vacuum filtered onto a Millipore filter AP25 (predried at 100°C overnight and weighed. The filter was washed with 2 ml phosphate buffered saline (PBS) and redried and weighed under the same conditions.

2.6.3 Synthesis of metabolic intermediates.

Commercially unavailable TOL plasmid *meta*-cleavage pathway intermediates were synthesized as follows.

2.6.3.1 Synthesis of 2-hydroxypent-2,4-dienoate.

2-hydroxypent-2,4-dienoate was prepared by the methods of Collinsworth *et. al.*, (1973). Briefly, 60 µmoles DL-allylglycine was added to a reaction mixture containing 200 µmoles KCl, 140 µmoles Tris buffer (pH7.2), 0.5 ml L-amino acid oxidase (5 units) and 0.1 ml catalase (250 units). The reaction mixture was incubated with shaking at 37°C for 1 hour, after which 1 ml of 6 % perchloric acid was added, and the precipitate centrifuged. The clear solution was subsequently extracted with 3 volumes of ether, and the solvent evaporated in a stream of air. The resultant oil was dissolved in water, and when analysed spectrophometrically, gave an absorbance peak of 265nm (Molar extinction coefficient 13, 000 M^{-1} cm^{-1}, Harayama *et al.*, 1989a), and showed the spectrum of 2-hydroxypent-2,4-dienoate.

2.6.3.2 Synthesis of benzoate and *m*-toluate *cis*-dihydrodiol.

Biotransformations were performed according to section 2.5.2 with *E. coli* cells expressing benzoate dioxygenase on plasmid pQR188. The resting cells were supplemented at half-hourly intervals with 1 mM working concentrations of benzoate or *m*-toluate and glucose. A total of 10 mM substrate was added, and the reaction followed to completion, with the concurrent accumulation of 10 mM benzoate or *m*-

toluate *cis*-dihydrodiol, as determined by the disappearance of substrate according to HPLC analysis (section 2.6.10).

2.6.4 Catechol 2,3-dioxygenase assay.

Catechol 2,3-dioxygenase activity was assayed by monitoring the absorbance increase at 375nm due to the formation of the product α-hydroxymuconic semialdehyde, based on the methods of Sala-Trepat and Evans (1971). 1ml of an *E.coli* culture was centrifuged at 13,000 rpm (Eppendorf microfuge) and resuspended in 1 ml 100 mM potassium phosphate buffer pH 7.5, 10% acetone. Resuspended bacterial pellets were then sonicated (5 x 10 secs., 8 microns), and centrifuged as previously. Supernatants were decanted, diluted 1:50 and assayed immediately.

Assays were performed in phosphate buffer pH 8.0 at 30°C in the presence of 2 mM catechol.

One unit of enzyme is defined as the amount that catalyses the formation of 1 μmole of product per minute at 30°C.

2.6.5 Batch purification of catechol 2,3-dioxygenase.

Catechol 2,3-dioxygenase, stable at 1:50 dilutions for a period of up to five hours, was purified by a variation of the methods of Cerdan *et. al*, (1994). A 2 ml volume of an overnight culture of *E.coli* JM107 grown in the presence of IPTG was centrifuged, and bacterial pellets resuspended in an equivalent volume of 10 mM ethylenediamine-hydrochloride buffer (pH 7.4) containing 10 % acetone. Cells were subsequently disrupted as in section 2.6.4. Active enzyme fractions from batch DEAE-anion exchange chromatography were eluted with the stepped addition of NaCl from 0-0.3 M, with most active fractions collected at a salt concentration of 0.3 M. Each step was performed in a 1.5 ml Eppendorf tube. Fractions were separated by centrifugation (5 mins, 13,000 rpm, Beckman centrifuge. Active fractions were adjusted to 25 % saturation of ammonium sulphate, 1 % BSA and 2 mM dithiothreitol (DTT).

2.6.6 Kinetic studies of Catechol 2,3-dioxygenase.

2.6.6.1 Determination of kinetic parameters.

The inhibition of catechol 2,3-dioxygenase by 2-hydroxypent-2,4-dienoate was determined by performing catechol 2,3-dioxygenase assays according to section 2.6.4, using enzyme purified according to the methods of section 2.6.5. For the inhibitory

studies, the lowest possible volume of C23O extract was in assays in order to minimise the inhibitory effect caused by acetone (Kobayashi *et al.*, 1995).

Indications of inhibition were provided by generating double-reciprocal (Lineweaver-Burk) and Eadie-Hofstee (V against V/[S]) plots. In the latter, parallel lines of slope -Km produced in different concentrations of 2-hydroxypent-2,4-dienoate were diagnostic of non-competitive inhibition.

In order to determine Ki values for the 2-hydroxypent-2,4-dienoate, a secondary plot of $1/V_{max}$ against [I] was performed, and the x-axis intersection taken as -Ki.

2.6.7 Protein assay.

1 ml *E.coli* cell extracts were prepared as in section 2.6.4 using 100 mM potassium phosphate buffer pH 7.5. Sonication, and preparations of supernatants from sonicated samples was performed as described in section 2.6.4.

The protein concentration of samples was determined using the Coomassie blue G-250 binding method of BioRad, with BSA as the reference protein at concentrations of 0-1.4 $mgml^{-1}$.

2.6.8 Spectrophometric determination of pathway intermediates.

A 1 ml broth was centrifuged (13,000 rpm 5 mins) and the supernatant retained.

For the analysis of benzoate metabolism, metabolism of benzoate was monitored by the disappearance of a peak at 225 nm. α-Hydroxymuconic semialdehyde was quantitated by the appearance of a peak at 375nm (molar extinction coefficient 33,000 M^{-1} cm^{-1}, Sala-Trepat and Evans, 1971). Oxalocrotonate was identified by the appearance of a peak of 350 nm in solutions adjusted to pH12 (Sala-Trepat and Evans, 1971). Quantitation of oxalocrotonate was performed against a standard curve of oxalocrotonate concentration (mM) produced using a synthesized standard (donated by L. Regan).

For the analysis of *m*-toluate metabolism, *cis*-dihydrodiol was identified at an absorbance maximum of 265nm. 2-hydroxy-6-oxohepta-2,4-dienoate was quantitated by the appearance of a peak at 388 nm (molar extinction coefficient 13,800 M^{-1} cm^{-1}, Sala-Trepat and Evans, 1971).

For the analysis of cells metabolising naphthalene, biphenyl or toluene, *cis*-dihydrodiols (264 nm, 303 nm, 265 nm respectively) and ring-cleavage intermediates (296 nm, 434 nm, 388 nm respectively) were monitored. The metabolism of biphenyl

and naphthalene was monitored by the disappearance of peaks at 225 nm and 220 nm respectively.

2.6.9 Capillary Zone Electrophoresis (CZE).

Meta-cleavage pathway substrates such as sodium benzoate and sodium *m*-toluate, together with the pathway intermediates catechol, *cis*-dihydrodiol, oxalocrotonate, and 4-hydroxy keto-valerate were quantitated using a Beckman P/ACE 2100 (CZE) system linked to System Gold software (Beckman).

By using the electrokinetic principles of electrophoresis and electro-osmosis, the CZE system separates the components of a sample within a fused silica capillary. Following the injection of a sample into the capillary by either pressure or voltage, a voltage applied to the capillary tube causes the migration of molecules by electrophoretic migration. In addition to this, silanol groups along the inner wall of the capillary ionize when in contact with electrolyte, thus causing a charge separation at the capillary wall/electrolyte interface. The voltage applied to the capillary leads to a net electroosmotic flow towards the cathode. As sample components pass a window in the capillary, a UV detector monitors them, and transmits a signal that is plotted as an electrophorogram.

For the quantitation of pathway substrates and intermediates, 1ml of fermentation broth was centrifuged at 13,000 rpm (Eppendorf microfuge) for 5 minutes, and the supernatants decanted. 20 µl of supernatant was injected into a 57 cm long, 50 µm internal diameter silicon fused capillary that had a 50cm length to the detector window. Electrophoresis was run at 20 kV (32 µA) for 25 minutes, using a 25 mM sodium tetraborate (pH9.3) running buffer. In addition, a separate injection of 1 % acetone as the electrophoretic flow marker together with iso-ketovaleric acid as the internal reference marker was made. These markers were included in the analysis so that the relative migration times of pathway intermediates would be consistent between different runs, thus allowing peak identification and quantitation using the Sytem Gold software. Intermediates were detected at either 254 nm or 300 nm.

2.6.10 HPLC analysis.

Acetate, pyruvate, formate and glycerol were quantitated by HPLC (LDC) using an Aminex HPX-87H anion exchange HPLC column (300mm x 7.8 mm) with a guard column (Biorad) held at 40^{o}C using a 4 mM sulphuric acid mobile phase, at a flow rate of 0.6 mlmin^{-1}. 1 ml samples were centrifuged for 5 minutes (13,000 rpm, Eppendorf microfuge), and the bacterial pellets resuspended in 0.5 % perchloric acid.

After incubating on ice for 10 minutes, the digested cells were recentrifuged under the same conditions, and the perchloric acid extract supernatants decanted for use immediately, or for storage at -20°C.

Benzoate, *m*-toluate, catechol, *m*-catechol, and *cis*-dihydrodiols were quantitated by HPLC (Perkin Elmer) using a Waters Bondapak C_{18} reverse phase hydrophobic HPLC column (300 mm), with a C_{18} Bondapak Guard-Pak precolumn at room temperature in order to confirm the findings of CZE analysis. Benzoate, *cis*-dihydrodiol and catechol were separated using a 20% acetonitrile: 80% water: 0.1% phosphoric acid mobile phase, at a flow rate of 0.8 $mlmin^{-1}$. Under these conditions, benzoate, *cis*-dihydrodiol and catechol possessed retention times of 16.1, 4.2 and 7.3 minutes respectively. *m*-Toluate, cis-dihydrodiol, and *m*-catechol were separated using the same mobile phase, at a flow-rate of 1 $mlmin^{-1}$. Under these conditions, *m*-Toluate, *cis*-dihydrodiol and *m*-catechol possessed retention times of 26.5, 3.9 and 10.4 minutes respectively.

2.7 DNA techniques.

2.7.1 DNA purification.

2.7.1.1 Large-scale plasmid DNA purification.

(i) Plasmid DNA was prepared using the method of Sambrook *et al*, (1989), which is based upon the unpublished procedure of Treisman and is a modification of the alkaline lysis method of DNA recovery developed by Birnbiom and Doly (1979) and Ish-Horowicz and Burke (1981). The method was used in conjunction with a further purification step involving equilibrium centrifugation of DNA samples in a CsCl-Ethidium bromide gradient (Sambrook *et al.*, 1989). Ethidium bromide was removed from the purified DNA by repeated isopropanol treatment. The plasmid DNA was ethanol precipitated at -20°C for 20 minutes and subsequently resuspended in 1 ml 10 mM TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA), pH 7.5.

(ii) Other large scale plasmid purifications were performed using the Wizard™ Maxiprep DNA purification system of Promega. DNA was eluted from the maxicolumn by centrifugation at 2,500 rpm for 5 minutes in a table-top MSE Minor 'S' centrifuge with a swinging bucket rotor.

2.7.1.2 Small-scale plasmid DNA purification.

(i) Plasmid DNA was prepared on a small-scale by the method of Sambrook *et al*, (1989) which is based upon those of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

(ii) Plasmid DNA was alternatively prepared using the Wizard™ Miniprep DNA purification system of Promega. 1.5 mls of a 5 ml overnight culture was applied to the purification column. Resulting DNA solutions were ethanol precipitated and resuspended in 1ml of 10 mM TE buffer, pH 7.5.

2.7.2 Restriction digests.

Restriction digests were performed according to the method of Sambrook *et al.* (1989). All reactions were carried out for 1-3 hours at 37°C unless otherwise stated, using a Universal 10 x restriction buffer (10 x RB) of 500 mM Tris-HCl pH 7.5, 50

mM $MgCl_2$. Restriction enzymes were obtained from New England Biolabs. Standard restriction digests were performed using 0.5-1.0 µg of plasmid DNA and 1 unit of restriction enzyme. For preparative restriction digests (see section 2.7.7), 2 µg of plasmid DNA were digested with 2-3 units of enzyme. Reactions for gel electrophoresis were stopped by the addition of a stop buffer, 0.1 M Na_2EDTA, 40 % (w/v) sucrose, 0.15 mgl^{-1} bromophenol blue. Reactions for further use in ligations were stopped by either heat inactivation at 70°C for 10 minutes, or by phenol-chloroform extraction of the digested DNA.

2.7.3 Ligation of plasmid DNA.

Ligation of linearised plasmid DNA was performed by the method of Sambrook *et al* (1989), performing reactions at 4°C overnight. The 10 x ligation buffer used was 0.66 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.1 M dithiothreitol and 1 mM ATP.

2.7.4 Preparation of *E.coli* competent cells.

A 5ml volume of nutrient broth supplemented with 20 mM $MgCl_2$ was inoculated with 2 colonies from a fresh selective nutrient agar plate of *E.coli* strain JM107, and incubated with reciprocal shaking at 37°C for 12 hours. A fresh 200 ml volume of the same media was then inoculated with the 5 ml overnight seed, and incubated with reciprocal shaking for 2-3 hours at 37°C. Cells were harvested at 9000 rpm (Sorvall RC2-B, GSA rotor) at 4°C for 10 minutes, then resuspended in 40 mls of ice-cold sterile 15 % (w/v) glycerol, 75 mM $CaCl_2$. The treated cells were reharvested under the same conditions and resuspended in 5 mls of the same solution. Individual 500 µl volumes of the competent cells were then either used immediately or stored at -70°C.

2.7.5 Transformation.

10-20 µl of plasmid DNA was added to 500 µl of ice-cold competent cells, mixed, and incubated on ice for 45 minutes. The cells were heat-shocked at 37°C for 10 minutes and then added to 5 ml nutrient broth and grown with reciprocal shaking for 1-2 hours. 100 µl volumes were then plates onto selective agar plates.

2.7.6 Agarose gel electrophoresis.

Agarose gel electrophoresis of DNA fragments was performed according to the methods of Sambrook *et al* (1989). The running buffer used was Tris-Borate-EDTA buffer (TBE) [90 mM Tris base, 90 mM boric acid, 1 mM Na_2EDTA, 0.05 $mgml^{-1}$ ethidium bromide]. 0.8 % (w/v) agarose (Sigma) gels were poured on 14.2 cm^2 glass plates and run in home-made plastic tanks at 150 V for 1.5-3 hours. The gels were photographed under ultraviolet light using a Polaroid MP-4 land camera.

2.7.7 Isolation of DNA restriction fragments.

2 μg of digested plasmid was loaded into three fused wells of an electrophoresis agarose gel, and the restriction fragments separated according to section 2.7.6

Segments of the agarose gel containing the desired DNA fragments were extracted from the gel using a sterile scalpel, and the DNA isolated using the Geneclean II kit of BIO 101 Inc. Alternatively, the DNA was extracted using a 0.2 mm cellulose acetate Spin-X tube (Costar UK Ltd). The gel segment was placed in the upper chamber of the Spin-X tube, and incubated at -20°C for 15 minutes. The Spin-X tube was then centrifuged at 10,000 rpm (Eppendorf microfuge) and the upper chamber discarded. DNA plasmid in the filtrate was ethanol precipitated and resuspended in 50 μl of TE buffer (pH7).

2.7.8 Oligonucleotides.

Oligonucleotides were synthesized and supplied by Pharmacia Biotech. For use in ligation, equimolar concentrations of complementary oligonucleotides were mixed in 10 mM TE buffer (pH7), placed in a 100°C water-bath and allowed to cool. Equivalent concentrations of isolated DNA fragments (see section 2.7.7) were added to the annealed oligonucleotides, and the combined DNA solution ethanol precipitated at -20°C. Ligations were performed as in section 2.7.3.

2.7.9 DNA sequencing.

Sequencing of plasmid DNA was performed by the Sanger dideoxy-mediated chain-termination method, according to the methods of Sambrook *et al*, (1989). All polyacrylamide gels were made and run according to Sambrook *et al*, (1989).

2.7.10 Phosphatase treatment of vector DNA.

5'-phosphate residues were removed from DNA, by using calf-intestinal alkaline phosphatase (CIP), according to the methods of Sambrook *et al*, (1989). Briefly, 1 μg of digested plasmid DNA was incubated at 37°C with 1 μl 10 x CIP buffer (10 mM $ZnCl_2$, 10mM $MgCl_2$, 100mM Tris.Cl (pH 8.3) and 1 unit of CIP in a total volume of 10 μl. After 15 minutes, 1 unit of CIP was added, and the mixture incubated for a further 45 minutes at 55°C. Following incubation, SDS and EDTA (pH 8.0) were added to final concentrations of 0.5% and 5 mM respectively. Proteinase K was added to a final concentration of 100 $\mu g ml^{-1}$, and the mixture incubated for 30 minutes at 56°C. The reaction mixture was subsequently cooled, and the DNA recovered by a phenol:chloroform extraction and ethanol precipitation.

Chapters 3 - 6 **Results.**

Chapter 3.

Experiments are described that investigate the effect of over-expression of either benzoate dioxygenase or oxalocrotonate decarboxylase on the metabolism of *meta*-cleavage pathway substrates.

Chapter 4.

Results from Chapter 3 suggest that over-expression of the *xylI* encoded oxalocrotonate decarboxylase causes a reduction in the metabolism of benzoate through the *meta*-cleavage pathway. It was predicted that this could be associated with the product of the XylI catalysed reaction, 2-hydroxypent-2,4-dienoate, exhibiting an inhibitory effect on C23O. This chapter discusses the partial purification of C23O that is subsequently used in inhibitory studies that reveal 2-hydroxypent-2,4-dienoate as a non-competitive inhibitor of C23O.

Chapter 5.

Given the inhibitory nature of 2-hydroxypent-2,4-dienoate, truncated *meta*-cleavage pathways were used to study the effect on *m*-toluate metabolism that resulted from terminating metabolism with the accumulation of 2-hydroxypent-2,4-dienoate or 4-hydroxy-2-oxovalerate. Results illustrate how co-expression of pQR194 with *meta*-cleavage pathways has a detrimental effect on substrate metabolism that can be associated with an accumulation of the substrate of XylJ.

Chapter 6.

Different strategies for expanding the substrate range of the *meta*-cleavage pathway of the TOL pathway are reported: (i) Constructing hybrid dioxygenases by exchanging enzyme subunits of biphenyl and benzoate dioxygenase. (ii) Co-expression of two wild-type dioxygenases in order to provide a route for substrates into the *meta*-cleavage pathway.

Chapter 3 Validation of the mathematical model of Regan *et al* (1991).

According to the mathmatical model simulating the flux of benzoate through the *meta*-cleavage pathway (Regan *et al*, 1991), two potentially rate-limiting steps that have been predicted are the *xylXYZ* encoded toluate dioxygenase, and the *xylI* encoded oxalocrotonate decarboxylase. In order to validate the predictions of this model, comparative studies were performed with *E.coli* strains carrying either (i) the wild-type *meta*-cleavage pathway alone encoded on plasmid pQR150, or (ii) the plasmid pQR150 together with plasmid pQR189 encoding toluate dioxygenase, or (iii) the plasmid pQR150 together with plasmid pQR194 encoding oxalocrotonate decarboxylase.

In the following sections, all experiments were performed in duplicate unless stated otherwise.

3.1 Analysis of a cell expressing pQR150.

3.1.1 Shake-flask analysis of *E.coli* cells carrying pQR150.

As an initial study into the metabolism of pathway substrates by the *meta*-cleavage pathway, *E.coli* cells carrying pQR150 were induced and grown in the presence of different pathway substrates according to the methods of section 2.5.1 During growth, 1 ml samples were removed for optical density and ring-cleavage intermediate analysis according to the methods of section 2.6.1 and 2.6.8. As expected, *E.coli* cells carrying pQR150 were capable of growing in the presence of initial 10 mM concentrations of either *m*-toluate, or benzoate. (fig 3.1 a), but with different rates of ring-cleavage intermediate accumulation (fig 3.1 b)

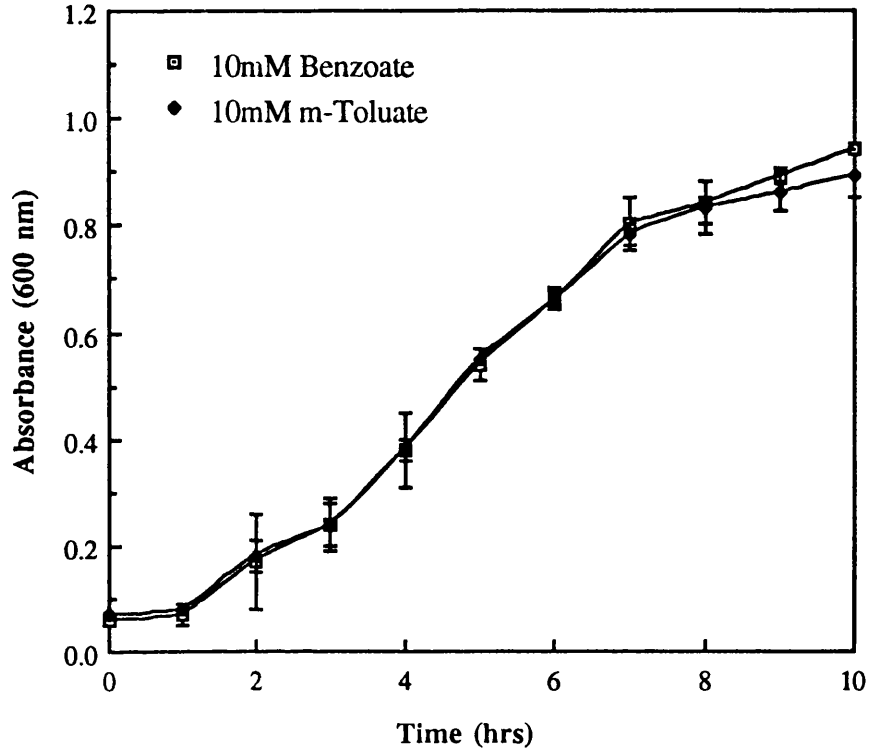


Fig 3.1 a. Growth of *E.coli* cells carrying pQR150 in the presence of different pathway substrates (10mM) added at T=0.

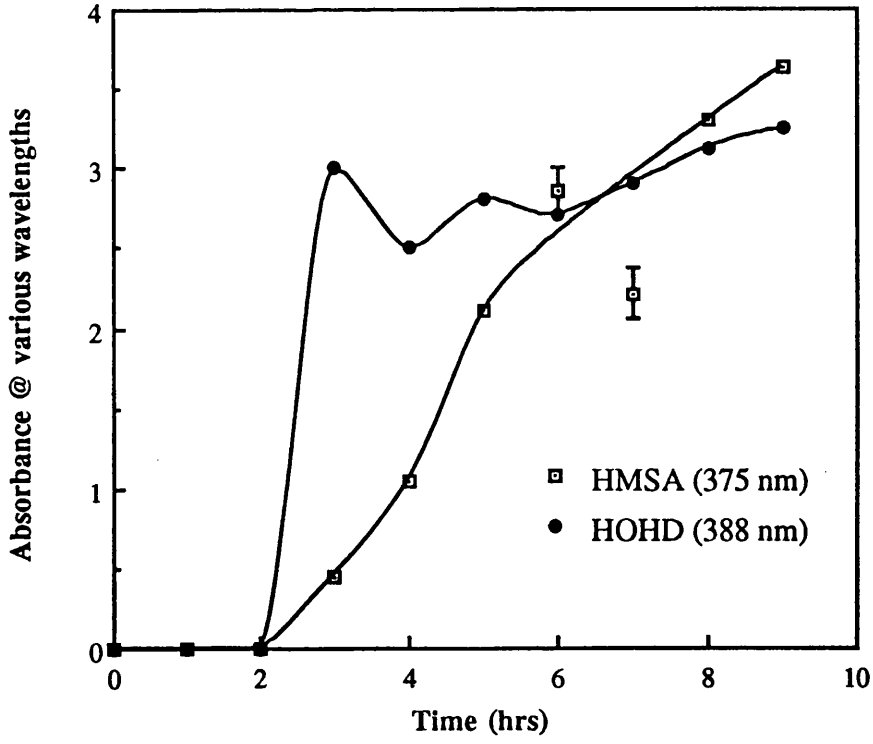


Fig 3.1 b. The production of ring-cleavage intermediates 2-hydroxymuconic semialdehyde (HMSA) and 2-hydroxy-6-oxohepta-2,4-dienoate (HOHD) during growth of *E.coli* cells carrying pQR150 in the presence of 10mM benzoate or *m*-toluate respectively. Substrate added at T=0.

3.1.2 Fermenter studies of pQR150.

In order to study the metabolism of pathway substrates by pQR150 in a more controlled environment, fermenter growth studies with *E.coli* cells carrying pQR150 were performed in fermenters on a 1.5 l scale as described in section 2.5.3. 1 ml sample volumes were removed as before, and, as indicated in fig 3.2, similar rates of growth were observed in the presence of either benzoate or *m*-toluate, which corresponds with the observations made during shake-flask analysis of this strain (fig 3.1 a). Furthermore, the concentration profiles of ring-cleavage intermediate observed during metabolism of either benzoate or *m*-toluate indicated reproducilibity between shake-flask and fermenter metabolism of *meta*-cleavage pathway substrates (fig 3.2 b).

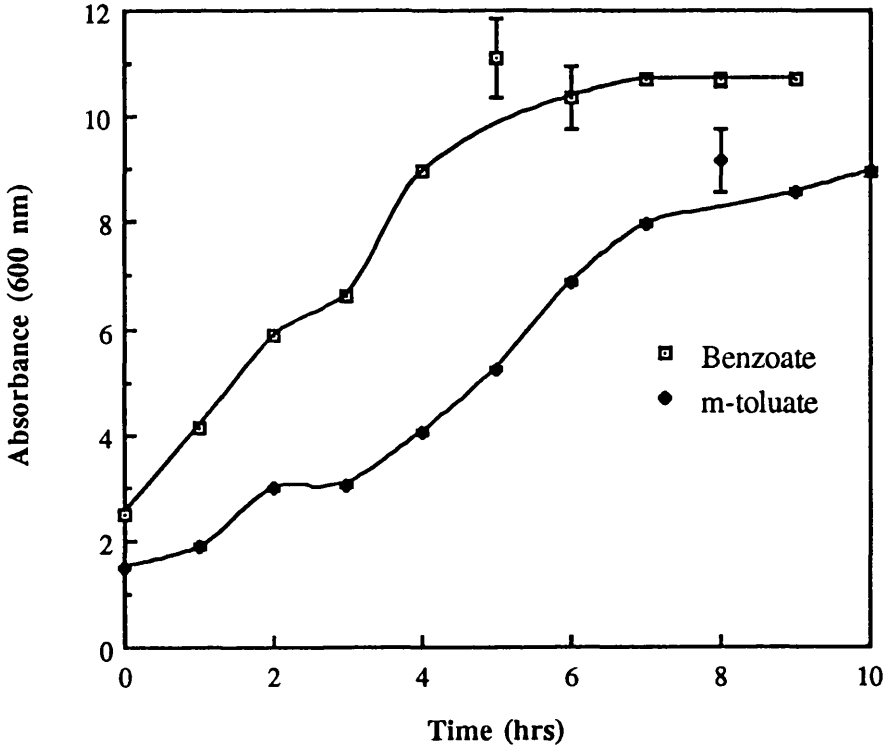


Fig 3.2 a. Growth in fermenters of *E.coli* cells carrying pQR150 in the presence of 10mM benzoate or *m*-toluate. Cells induced with IPTG at T=0. Substrate added at T=2.

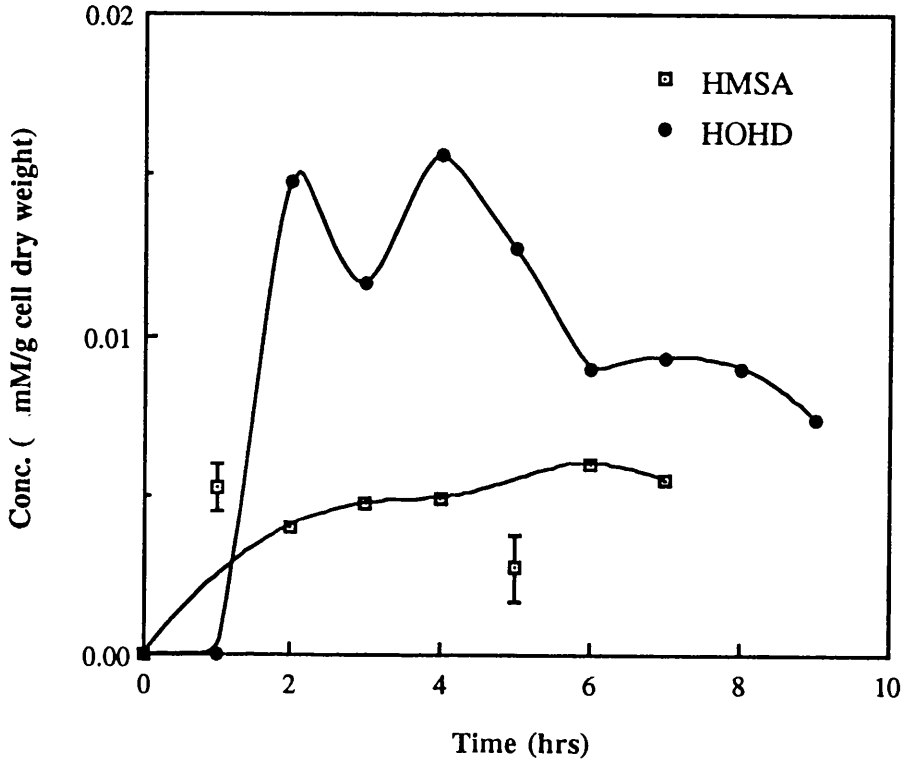


Fig 3.2 b. The production of either 2-hydroxymuconic semialdehyde (HMSA) or 2-hydroxy-6-oxohepta-2,4-dienoate (HOHD) during growth of *E.coli* cells carrying pQR150 in the presence of 10mM benzoate or *m*-toluate respectively. Induction with IPTG at T = 0. Substrate added at T = 2.

Culture supernatant concentrations of other *meta*-cleavage pathway intermediates and by-products were quantitated by the use of both HPLC and capillary zone electrophoresis according to sections 2.6.9 and 2.6.10, see figs 3.3 a and 3.3 b. As a result, rates of substrate degradation and levels of intermediate accumulation could be determined. These could then be compared with those observed during fermenter analyses of *E.coli* strains expressing amplified levels of the rate-limiting enzymes, in order to experimentally test the predictions of the mathematical simulation.

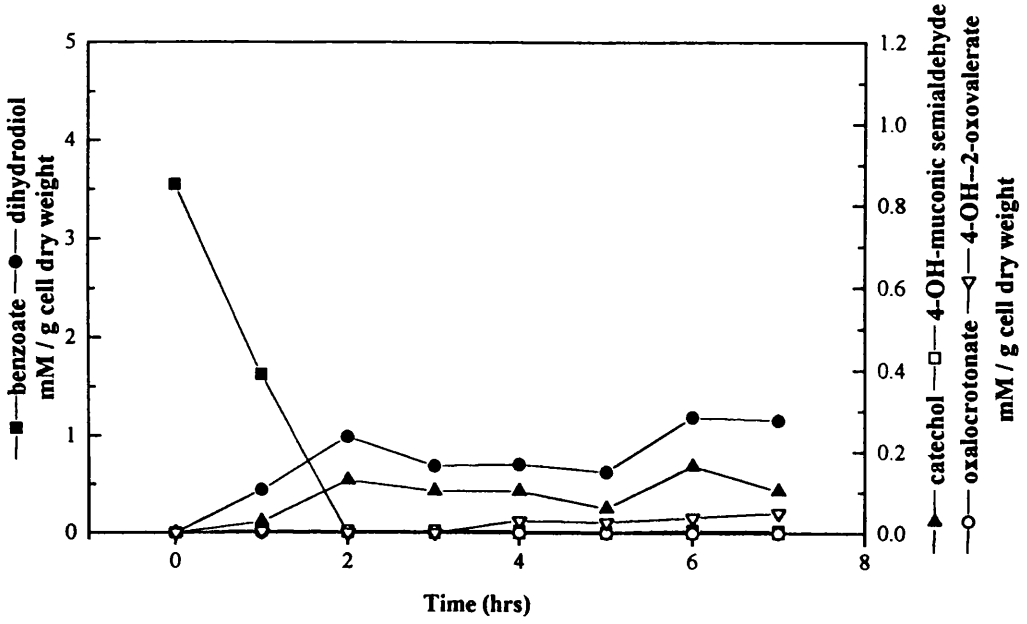


Fig 3.3 a. Supernatant concentrations of *meta*-cleavage pathway intermediates during growth in fermenters of *E.coli* cells expressing pQR150 in the presence of 10mM benzoate. Substrate added at T=0.

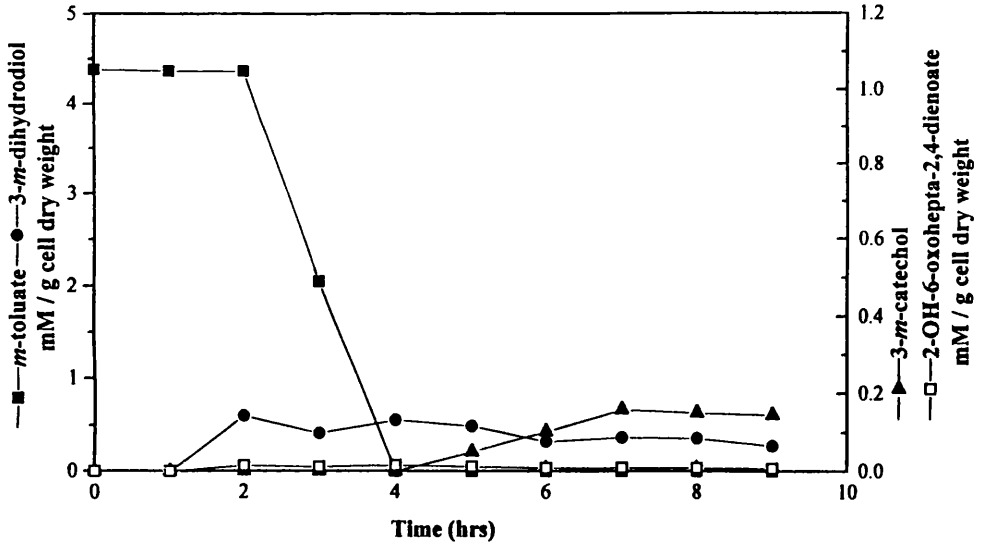


Fig 3.3 b. Supernatant concentrations of *meta*-cleavage pathway intermediates during growth in fermenters of *E.coli* cells expressing pQR150 in the presence of 10mM *m*-toluate. Substrate added at T=0.

Catechol 2,3-dioxygenase (C23O) levels were also monitored at hourly time-points during the period of induction and after the addition of pathway substrate, according to the methods of section 2.6.4. Such analysis served initially as an indication of the relative levels of plasmid expression during these stages. In addition, C23O lies at the branch-point of the *meta*-cleavage pathway, and is known to be subject to inhibition by both substrate, product, as well as by pathway substrates such as benzoate (Polissi and Harayama, *et.al*, 1993, Kobayashi, *et.al*, 1995). Changes in such enzyme levels during metabolism could be related to intermediate or substrate concentration, which could in turn be linked to the degree of flux through the pathway.

In the case of *E. coli* expressing pQR150, a more significant drop in C23O activity immediately following substrate addition is observed during benzoate metabolism (fig 3.4). This can possibly be related to the faster metabolism of benzoate (see fig 3.3) compared to *m*-toluate, that results in a higher concentrations of C23O inhibitors such as catechol.

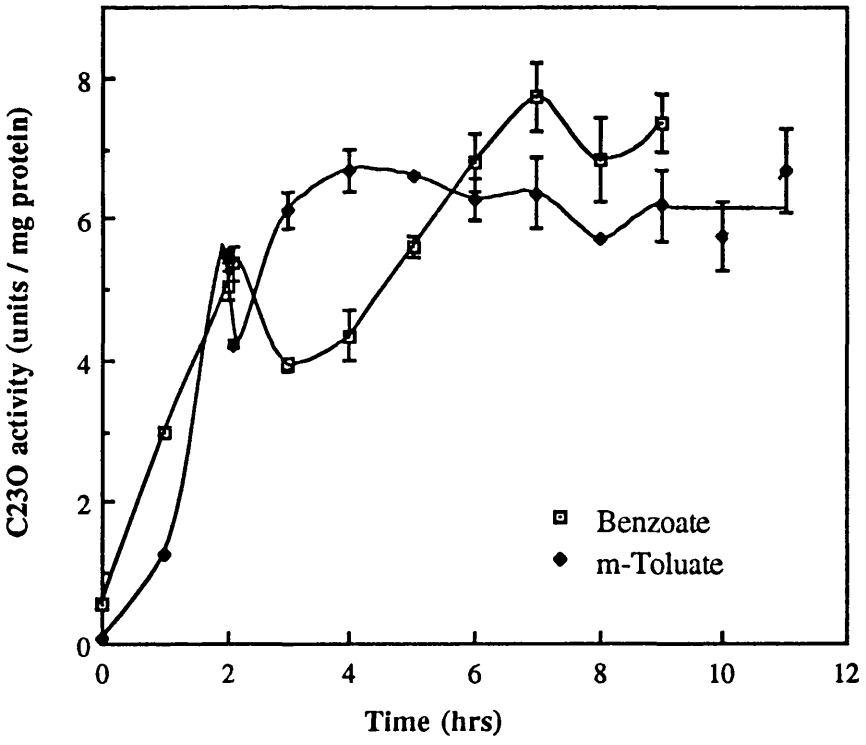


Fig 3.4. Catechol 2,3-dioxygenase specific activity during induction and metabolism of 10mM benzoate or *m*-toluate by *E.coli* cell carrying pQR150. Cells were induced at T=0 with IPTG, and substrate added at T=2.

These initial studies were necessary as a positive control for the proceeding studies, in which the potentially rate-limiting steps of the pathway were investigated during substrate metabolism.

3.2 Amplification of benzoate dioxygenase expression.

In order to determine the rate-limiting effect of benzoate dioxygenase on substrate metabolism, the *xylXYZ* gene cluster was effectively amplified by introducing into an *E. coli* cell carrying pQR150 a second, compatible plasmid, pQR189 (Table 2.1) that expresses *xylXYZ* under the control of the *tac* promoter. If the predictions of the mathematical model were correct, the expression of extra copies of the genes encoding toluate dioxygenase would remove the first bottleneck in the pathway, leading to a change in the concentrations of other pathway intermediates.

3.2.1 Construction of plasmid pQR189

The plasmid pQR189 was constructed as shown in fig 3.5 and used to transform competent *E. coli* harbouring pQR150 (section 2.7.5). All plasmids used in the construction are presented in table 2.1. Cells containing both pQR189 and pQR150 were selected on Amp/Km plates according to the methods of section 2.4.

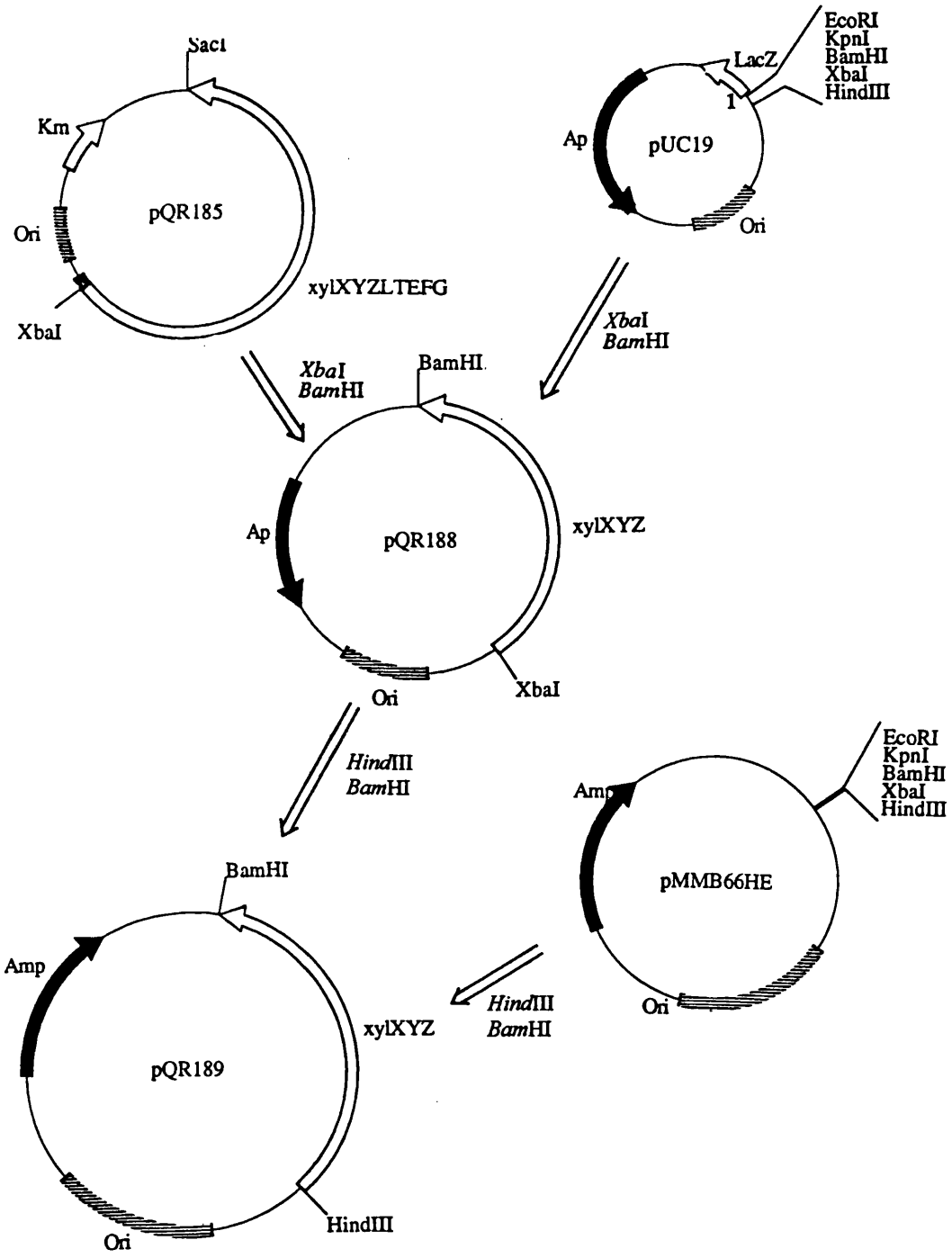


Fig 3.5. Construction of pQR189 (expressing *xylXYZ* under the control of the *tac* promoter in pMMB66HE). The presence of a unique *Bam*HI site within the *meta*-cleavage operon, at the 5' end of *xylL*, allowed the *xylXYZ* encoded toluate dioxygenase to be isolated on a 3.48 kb *Hind*III-*Bam*HI fragment. 1 µg of both pUC19 and pQR185 (Table2.1) were digested with *Hind*III and *Bam*HI according to the methods of section 2.7.2. The two digested plasmids were ligated and used to transform competent *E.coli* JM107 (section 2.7.5). Recombinant strains were selected in the presence of Amp/X-gal, and strains carrying plasmids expressing *xylXYZ* alone were further selected by picking white colonies from plates sprayed with catechol (section 2.4), as an indication of the absence of *xylE*. DNA was purified from these colonies (section 2.7.1.2) and the correct gene order of the recombinant plasmid pQR188 confirmed by *Hind*III/*Bam*HI double digests and *Eco*RI single digests. pQR188 was subsequently used as an intermediate plasmid for sub-cloning *xylXYZ* into pMMB66HE. 1 µg of pQR188 was digested with *Hind*III and *Bam*HI as above, and the 3.48kb fragment encoding *xylXYZ* isolated from an agarose gel by the methods of section 2.7.7. The gel purified fragment was subsequently ligated with 1 µg of *Hind*III/*Bam*HI digested pMMB66HE. Transformed *E.coli* were selected in the presence of Amp, and DNA purified from selected colonies was digested with *Hind*III and *Bam*HI or *Eco*RI, as an indication of the presence of *xylXYZ* in pMMB66HE, forming plasmid pQR189.

3.2.2 Shake-flask analysis of cells carrying pQR150 and pQR189.

In order to generate an initial indication into the effect of increasing the level of activity of benzoate dioxygenase, shakeflask growth experiments were performed as previously described in section 3.1.1 according to the methods of section 2.5.1. The accumulation of ring-cleavage intermediates was also monitored and compared with the results obtained during similar experiments with pQR150. The growth and simultaneous production of ring-cleavage intermediates by cells carrying either pQR189 and pQR150, or pQR150 alone as a control comparison are shown in fig. 3.6 a and fig. 3.6 b respectively.

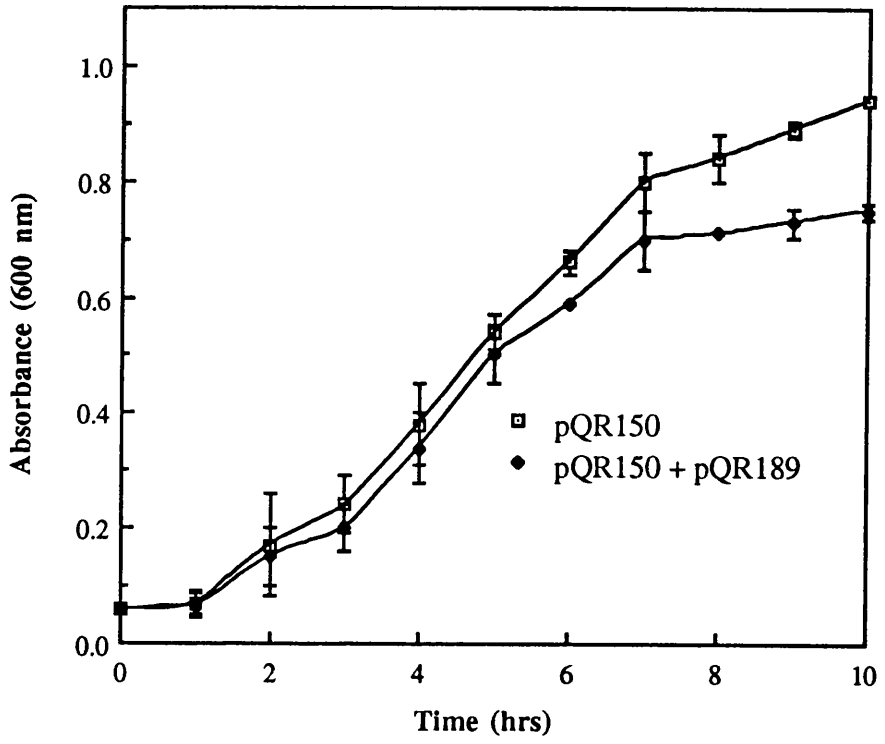


Fig 3.6 a. Shake-flask growth in the presence of 10mM benzoate of *E.coli* cells carrying either pQR150, or pQR150 together with pQR189. Benzoate added at T=0.

It would appear from fig 3.6 a that growth of cells carrying pQR150 is greater that of cells carrying both pQR150 and pQR189. However, because growth was conducted in the presence of rich media, different rates of benzoate metabolism would not necessarily correlate with differences rates of pathway substrate metabolism. Indeed, lower growth may simply occur due to the presence of an extra plasmid, a possibility supported by studying the growth of various strains in the absence of substrate (fig 3.7).

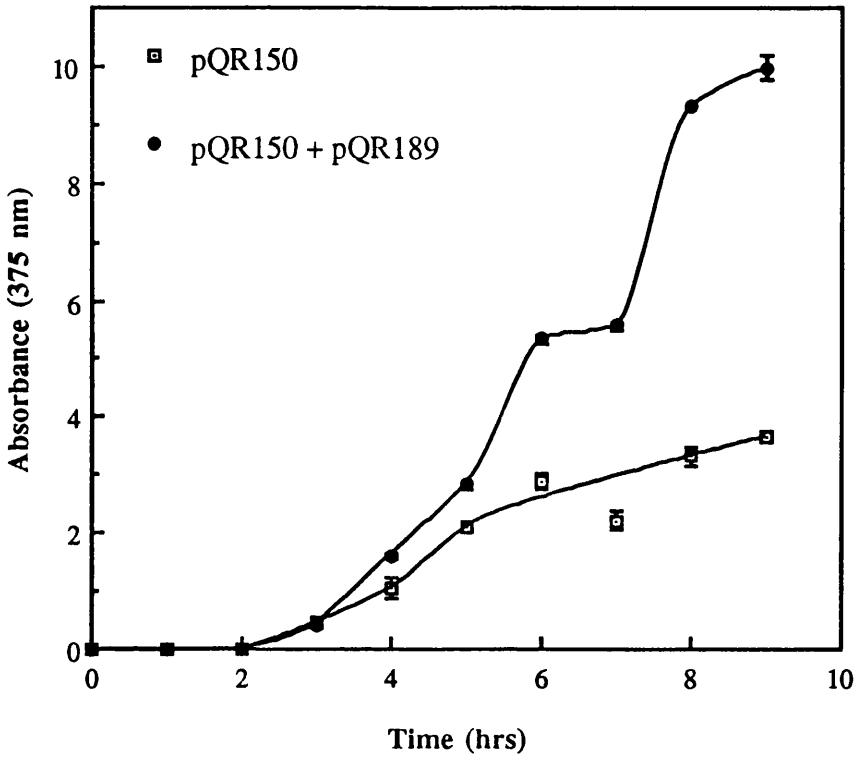


Fig 3.6 b. Production of α-hydroxymuconic semialdehyde from 10mM benzoate by *E.coli* cells carrying either pQR150 or pQR150 and pQR189. Benzoate added at T=0.

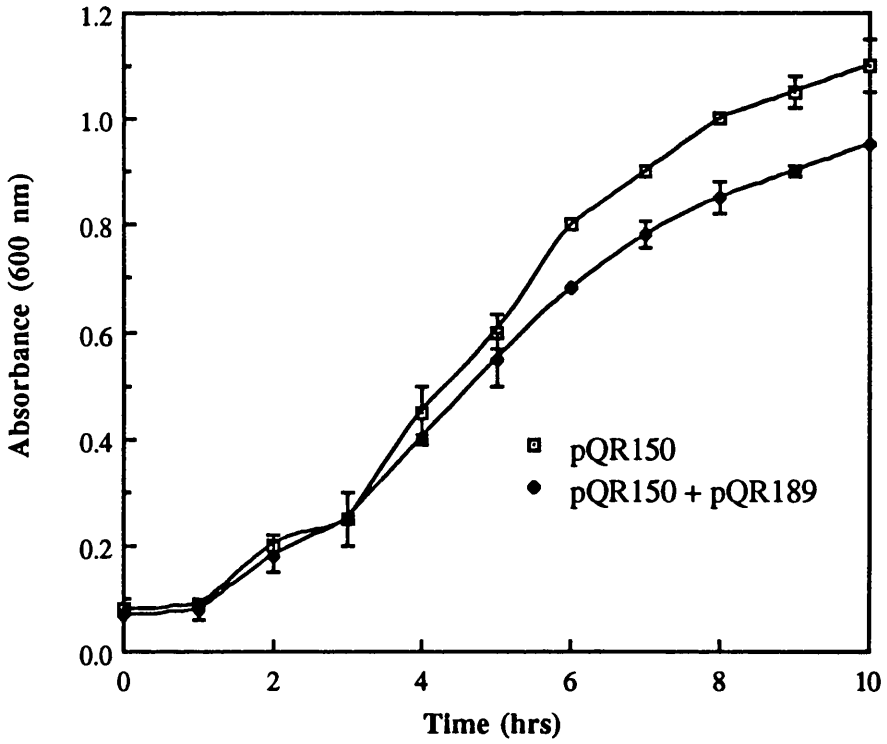


Fig 3.7. Growth in the absence of pathway substrate of *E.coli* cells carrying either pQR150 or pQR150 together with pQR189.

Fig 3.6 b suggests that greater rates of benzoate metabolism occur in the presence of the extra plasmid pQR189. There was a three-fold increase in the amount of 2-hydroxymuconic semialdehyde observed when an extra copy of the benzoate dioxygenase was expressed.

Although these experiments serve as an initial indication that the *xylXYZ* encoded benzoate dioxygenase is a rate-limiting enzyme within the *meta*-cleavage pathway, more detailed analysis was necessary in order to study the changes in pathway intermediate concentration and enzyme activity caused by amplification of the level of benzoate dioxygenase expression.

In order to achieve this, controlled growth studies in fermenters were performed with *E.coli* cells carrying pQR150 and pQR189.

3.2.3 Fermenter studies with *E.coli* carrying pQR150 and pQR189.

3.2.3.1 Benzoate metabolism:

Growth and analysis was performed as for cells carrying pQR150 alone (section 3.1.2), according to the methods of section 2.5.3 and 2.6.1 respectively. The comparative growth and production of 2-hydroxymuconic semialdehyde by cells carrying pQR150 and pQR189, and by cells carrying pQR150 alone are seen in fig 3.8 a and 3.8 b. Both growth and 2-hydroxymuconic semialdehyde production were comparable with shake-flask analysis (fig 3.6 a and b), as indicated by the 3-fold increase in the level of ring-cleavage product accumulated in the presence of amplified dioxygenase expression.

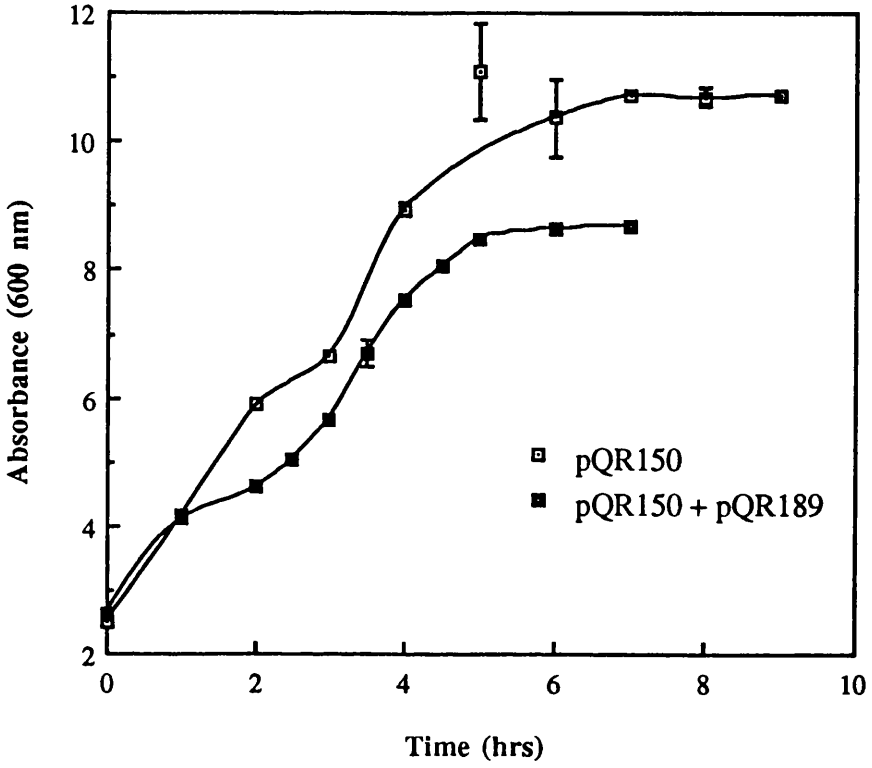


Fig 3.8 a. Growth in the presence of 10mM benzoate of *E.coli* cells carrying pQR150 or pQR150 and pQR189. Cells were induced with IPTG at T=0. Substrate added at T=2.

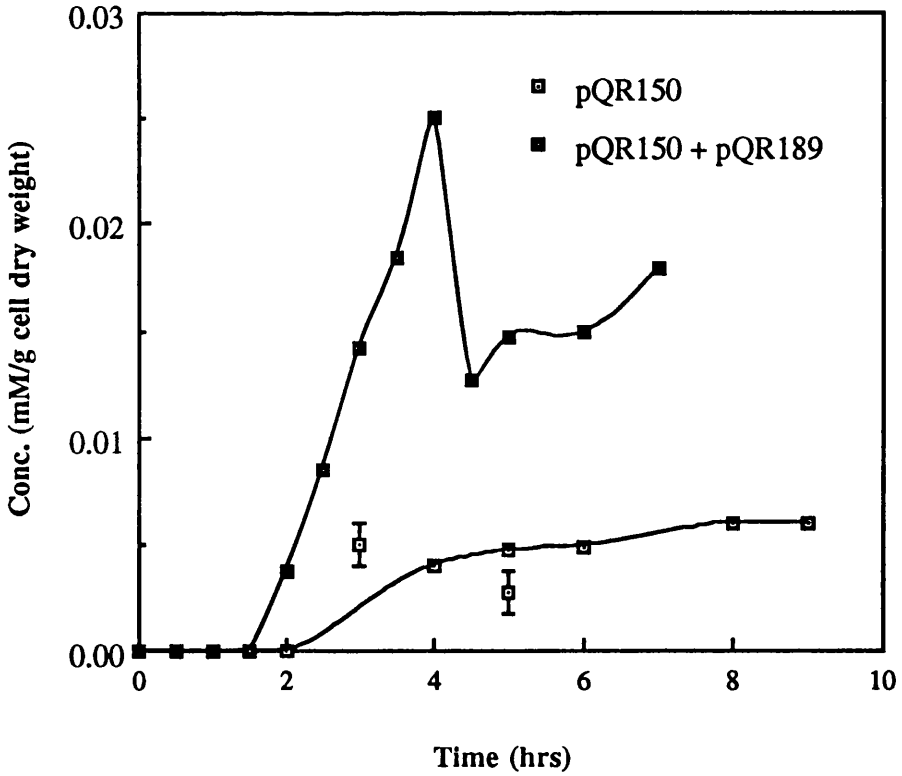


Fig 3.8 b. The production of 2-hydroxymuconic semialdehyde in fermenters during metabolism of 10mM benzoate by *E.coli* cells carrying either pQR150 or pQR150 and pQR189. Cells were induced with IPTG at T=0. Benzoate was added at T=2.

Meta-cleavage pathway intermediates were quantitated as before according to the methods of sections 2.6.9 and 2.6.10, as shown in fig 3.9. In comparison to the metabolism of benzoate by cells expressing pQR150 alone, metabolism by cells possessing an amplified level of benzoate dioxygenase lead to a three-fold increase in the concentrations of the early intermediates such as *cis*-dihydrodiol, catechol, and 2-hydroxy-muconic semialdehyde. In addition, a ten-fold increase in the concentration of 4-hydroxy-2-oxovalerate and 4-oxalocrotonate was also observed.

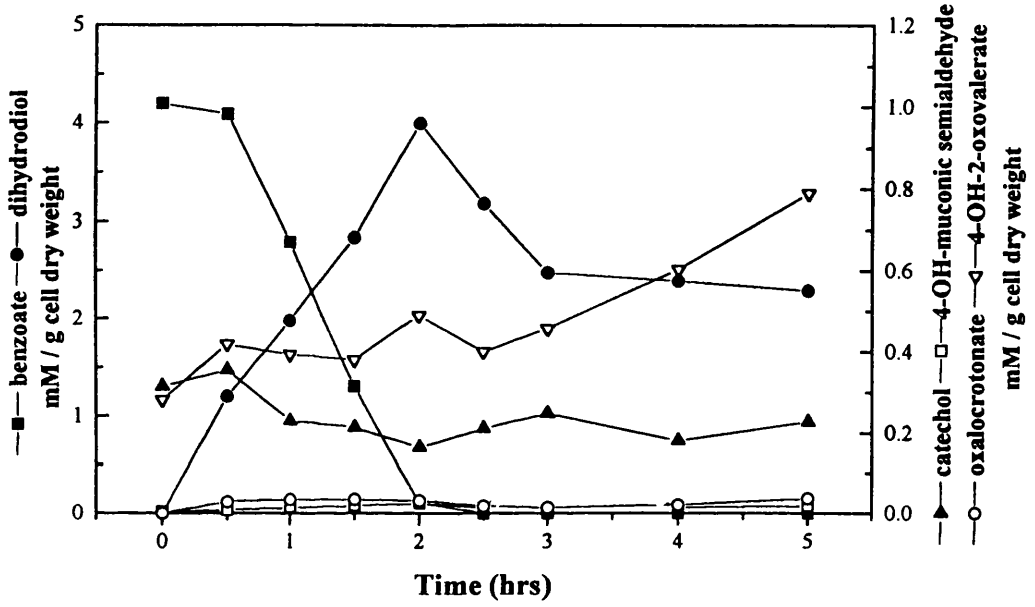


Fig 3.9. Supernatant concentrations of *meta*-cleavage pathway intermediates during the metabolism of 10 mM benzoate by *E.coli* cells expressing pQR150 and pQR189. Benzoate was added at T=0.

Levels of C23O per gram cell dry weight were also monitored (section 2.6.2 and 2.6.4) and compared with those observed during metabolism of benzoate by cells carrying pQR150 alone. Comparing C23O activity in this way was thought to be more representative than by comparing specific activities, as the latter would affected by the expression of an extra plasmid during studies of cells carrying pQR150 and pQR189.

According to fig 3.10, C23O activity per gram cell dry weight was found to be over four fold greater when an amplified level of toluate dioxygenase was expressed. Such an observation suggests that levels of plasmid expression are significantly different between experiments, or that amplification of toluate dioxygenase alters metabolic flux such that intermediates toxic to C23O are prevented from accumulating.

It is also possible that increased dioxygenase activity results in an increase in the basal level of dioxygenase activity.

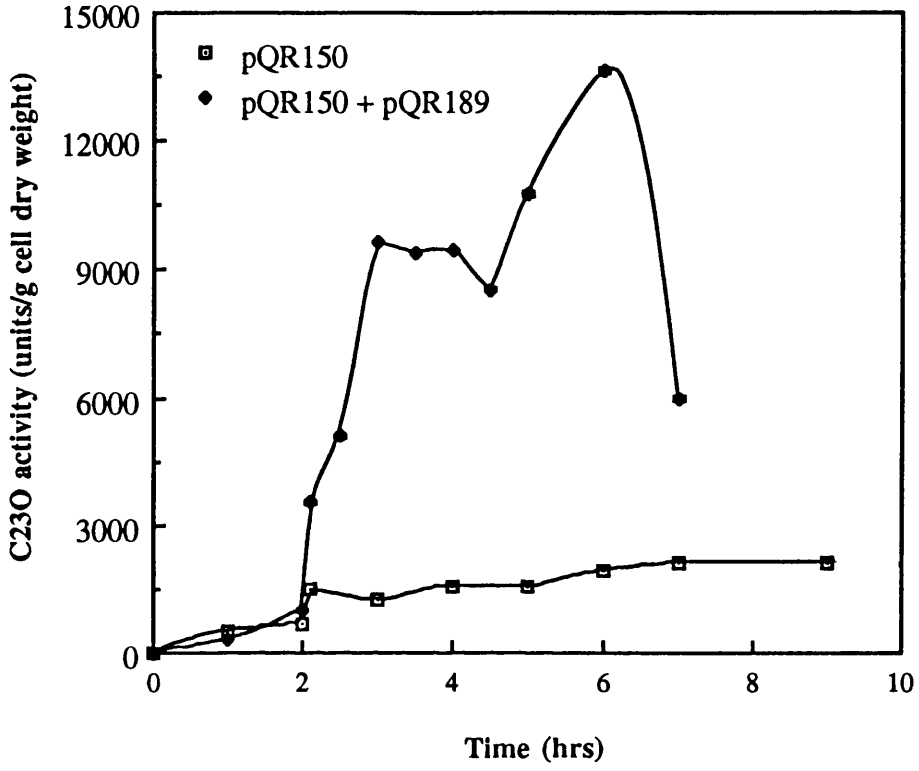


Fig 3.10. Catechol 2,3-dioxygenase activity during induction and metabolism of 10 mM benzoate by *E.coli* cells carrying either pQR150 and pQR189, or pQR150 alone. Cells were induced with IPTG at T=0, 10mM benzoate was added at T=2.

In order to investigate the first explanation, levels of protein per gram cell dry weight between control and amplified dioxygenase systems were compared according to the methods of section 2.6.7. As seen in fig 3.11, C23O activity was still seen to be four fold greater in systems expressing an amplified dioxygenase, in comparison with control studies.

Thus an increase in C23O activity during the metabolism of benzoate by cells expressing an amplified dioxygenase cannot be wholly attributed to differing levels of plasmid induction between different studies, and as a result must be an affect caused by the amplification of benzoate dioxygenase.

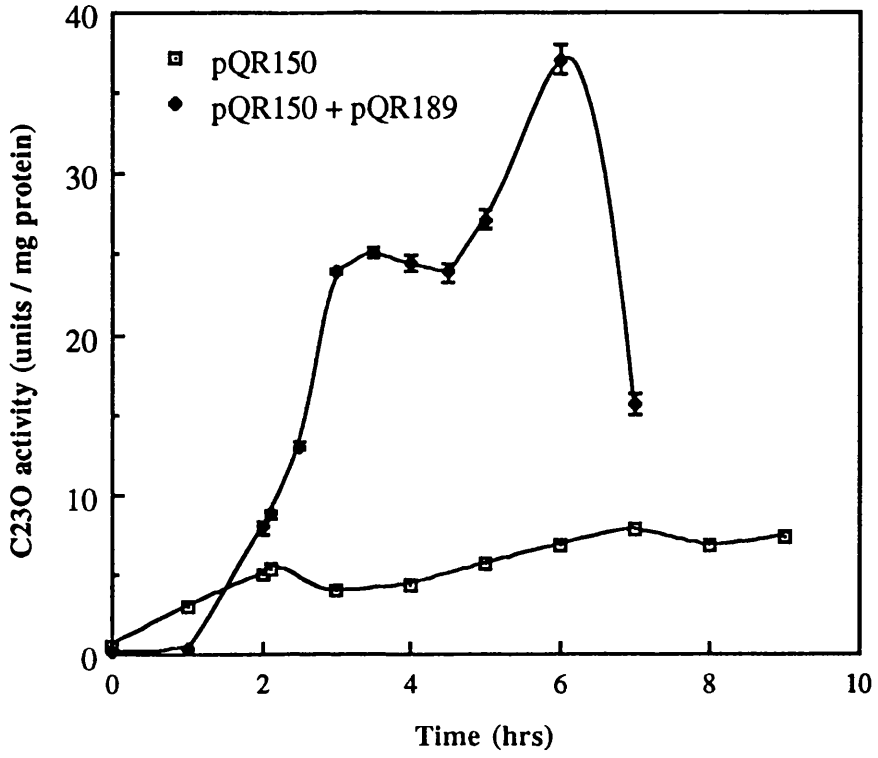


Fig 3.11. Catechol 2,3-dioxygenase specific activity during induction and metabolism of 10mM benzoate by cells carrying either pQR150 and pQR189, or pQR150 alone. Cells were induced with IPTG at T=0, and benzoate added at T=2.

3.2.3.2 *m*-Toluate metabolism:

Growth of cells carrying pQR150 and pQR189 was performed in fermenters as described previously (section 3.1.2), according to the methods of section 2.5.3. As seen in fig 3.12 a, growth of cells expressing the amplified dioxygenase was very similar to that of the control, but in contrast levels of the ring-cleavage intermediate 2-hydroxy-6-oxohepta-2,4-dienoate were 3-fold higher than seen during fermentations of cells carrying pQR150 alone (fig 3.12 b). It is interesting to note that despite such an increase in concentration, the same profile of fluctuation of the concentrations of this intermediate is apparent. Concentrations of other metabolic intermediates were determined as described previously, and as shown in fig 3.13. A comparison with the metabolism of *m*-toluate by *E.coli* expressing pQR150 alone (fig 3.3) reveals a 3-fold increase in the accumulation of *cis*-dihydrodiol in the presence of an amplified level of dioxygenase expression. In contrast to this however, it is interesting to note that the concentration of *m*-catechol does not increase upon amplification of benzoate dioxygenase.

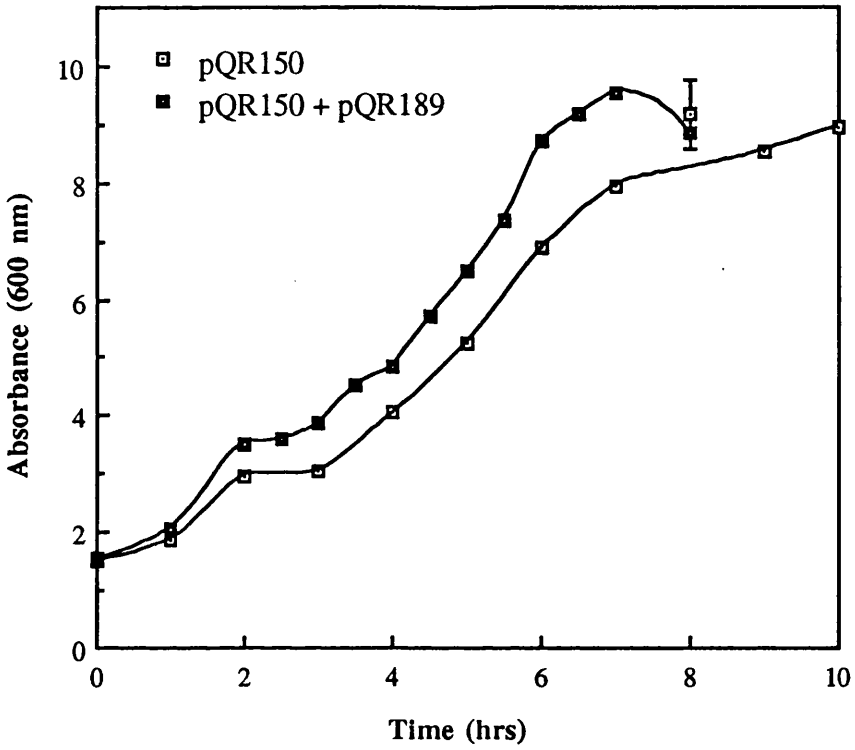


Fig 3.12 a. Growth in fermenters of *E.coli* cells carrying either pQR150 and pQR189 or pQR150 alone in the presence of 10mM *m*-toluate. Cells were induced with IPTG at T=0. *m*-toluate added at T=2.

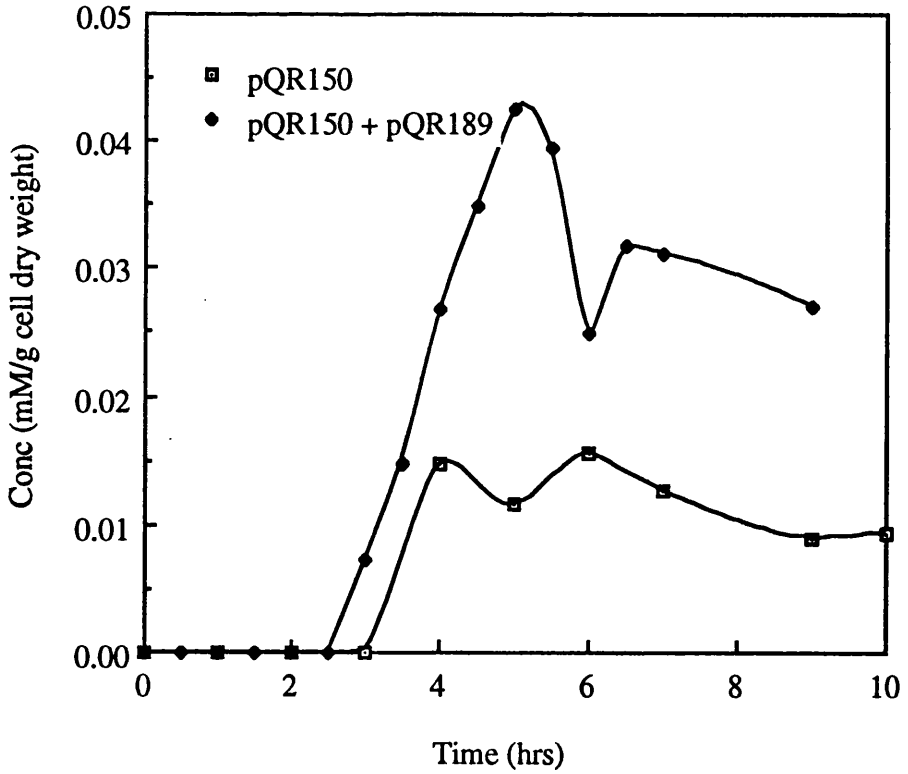


Fig 3.12 b. Production of 2-hydroxy-6-oxohepta-2,4-dienoate during the metabolism of 10mM *m*-toluate by *E.coli* expressing either pQR150 and pQR189 or pQR150 alone. Cells were induced with IPTG at T=0, and *m*-toluate added at T=2.

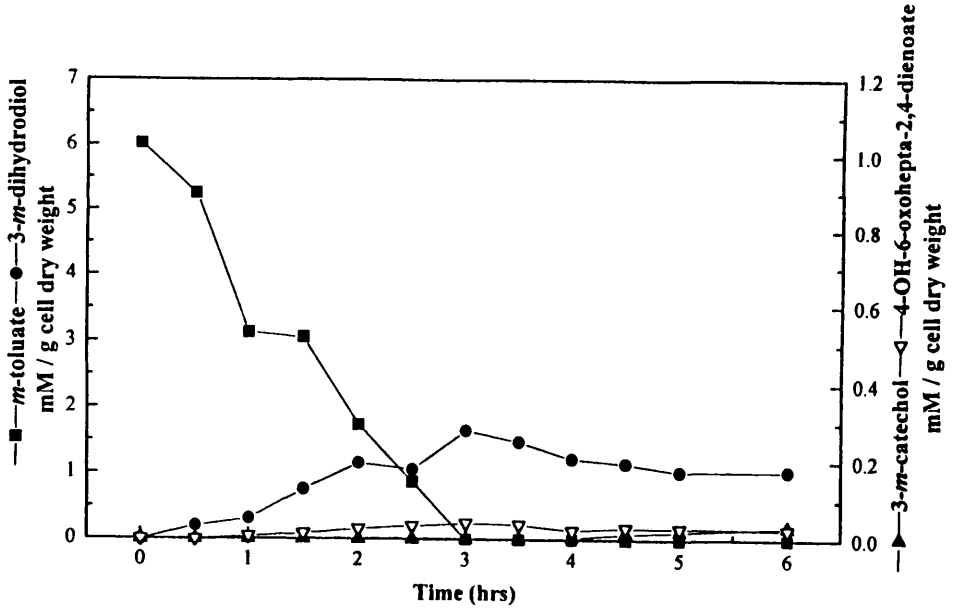


Fig 3.13 Supernatant concentration of intermediates during the metabolism of 10 mM *m*-toluate by *E.coli* cells expressing pQR150 and pQR189. *m*-toluate added at T=0.

Over-expression of benzoate dioxygenase during *m*-toluate metabolism leads to a reduction in C23O activity (fig 3.14), in direct contrast to the effect observed during benzoate metabolism (fig 3.10). It is possible that such a drop in activity seen during *m*-toluate metabolism is related to the higher concentrations of the toxic ring-cleavage product that accumulates (fig 3.12 b) as a result of dioxygenase over-expression. This can possibly be attributed to the faster initial rate of *m*-toluate metabolism due to the presence of amplified *xylXYZ* activity.

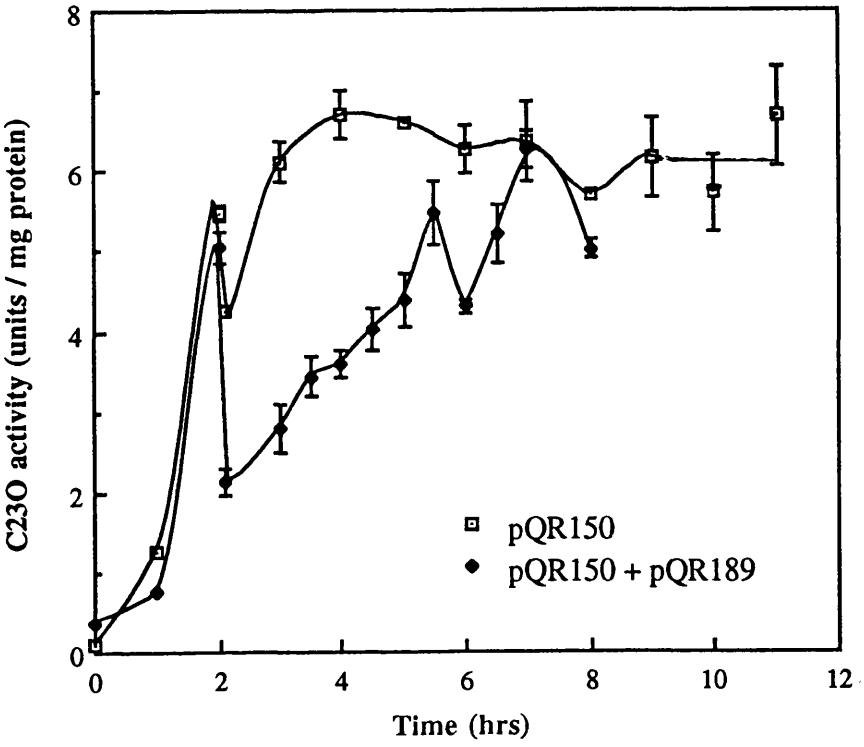


Fig 3.14. Catechol 2,3-dioxygenase specific activity during induction and metabolism of 10mM *m*-toluate by either pQR150 and pQR189 or pQR150 alone. Cells were induced with IPTG at T=0, and *m*-toluate added at T=2.

3.3 Amplification of oxalocrotonate decarboxylase expression.

3.3.1 Construction of plasmid pQR194.

In order to investigate the role of the *xylI* encoded oxalocrotonate decarboxylase in the control of the flux of substrates through the *meta*-cleavage pathway, the *xylI* gene was cloned into the broad host-range vector pMMB66EH, forming the plasmid pQR194. pQR194 was subsequently introduced into an *E. coli* strain already carrying the plasmid pQR150, according to the methods of section 2.7.5. The plasmid pQR194 was constructed according to fig 3.14. All plasmids used in the construction are presented in table 2.1.

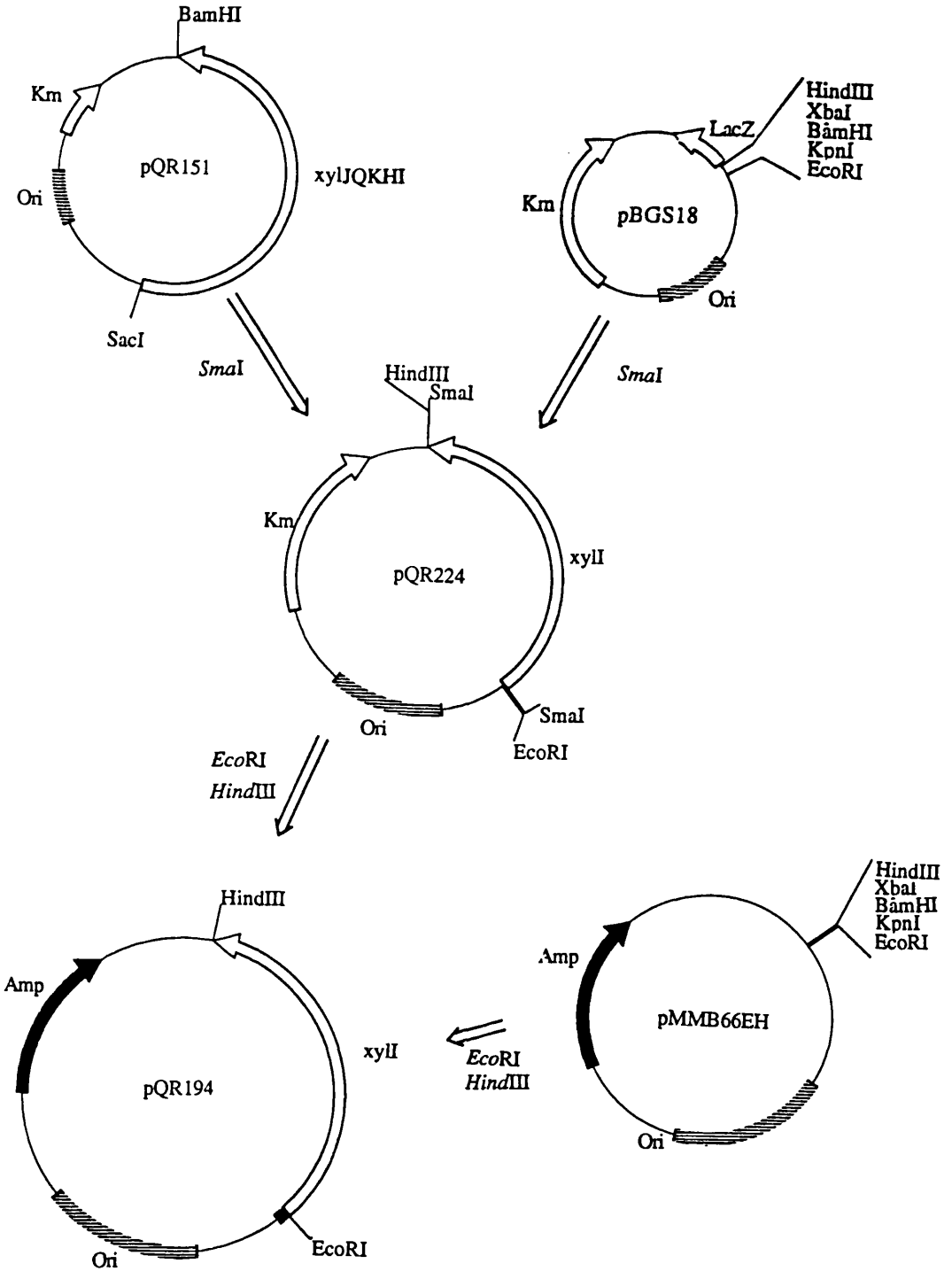


Fig 3.15. Construction of pQR194 (expressing *xylI* encoded oxalocrotonate decarboxylase). Due to the availability of *Sma*I restriction sites either side of the *xylI* gene in pQR151, the 1.5 kb fragment encoding *xylI* was subcloned into the broad host-range vector pMMB66EH. 1 µg of plasmid pQR151 was digested with *Sma*I, and the 1.5 kb fragment gel purified according to the methods of section 2.7.7. The isolated fragment was subsequently ligated with 1 µg of *Sma*I digested pBGS18, and the ligation mix used to transform *E.coli* according to section 2.7.5. Recombinant colonies were selected by blue/white screening in the presence of Km, X-gal and IPTG (section 2.4), and purified DNA screened for the presence of a 1.5 kb insert by performing *Sma*I restriction digests. The orientation of the *xylI* insert was determined by performing *Kpn*I restriction digests. This intermediate vector was named pQR224. The *xylI* gene was subsequently subcloned into pMMB66EH on a 1.5 kb *Eco*RI/*Hind*III fragment. 1 µg of both pQR224 and pMMB66EH were digested with *Eco*RI and *Hind*III, and the isolated 1.5 kb fragment from pQR224 ligated with the pMMB66EH. Following transformation of *E.coli*, purified DNA from isolated colonies was screened for the presence of the *xylI* insert by performing *Eco*RI and *Hind*III restriction digests. The resulting recombinant plasmid was named pQR194.

3.3.2 Shake-flask experiments with *E.coli* strains carrying plasmids pQR150 and pQR194:

As an initial study into the effect of over-expressing the expression of oxalocrotonate decarboxylase on *meta*-cleavage pathway metabolism, cells were grown and recombinant plasmids expressed in the presence of benzoate, according to the methods of section 2.5.1. Growth, and the production of 2-hydroxymuconic-semialdehyde as an indication of substrate metabolism was monitored according to the methods of 2.6.1 and 2.6.8. This study was performed alongside a control of cells carrying pQR150 alone. The comparative growth and production of ring-cleavage intermediate are shown in fig 3.16 a and 3.16 b.

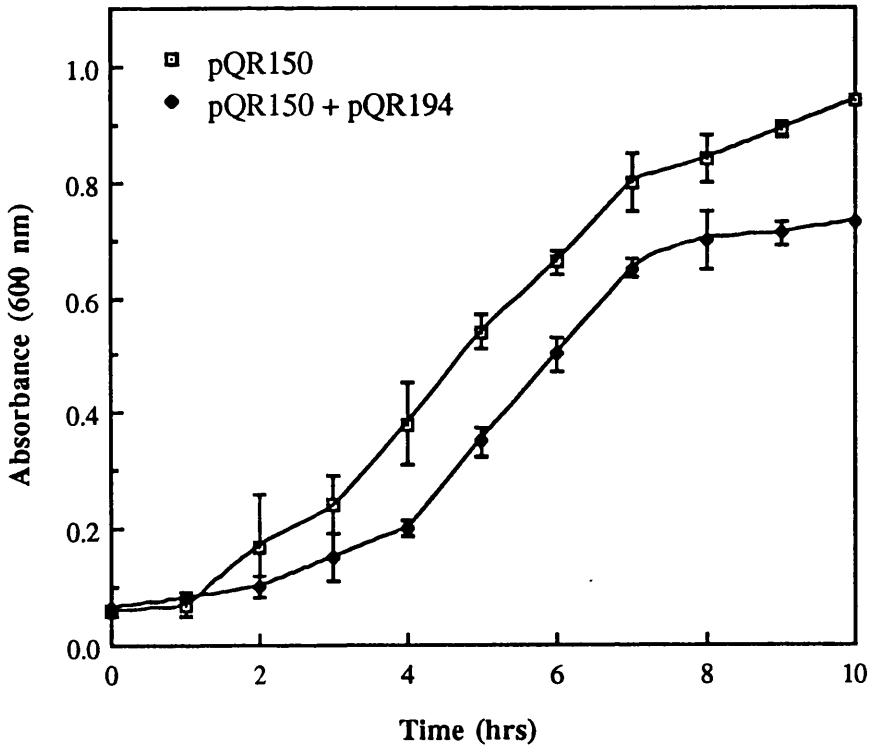


Fig 3.16 a. Shake-flask growth in the presence of 10 mM benzoate of *E.coli* cells carrying pQR150 or pQR150 and pQR194. Substrate added at T=0.

According to fig 3.16 a, the growth of cells carrying pQR150 alone is greater than that of cell carrying two plasmids. Such observation support the suggestion of section 3.2.2 that lower growth of certain strains in rich media is as a result of the metabolic load caused by an additional plasmid.

The results of fig 3.16 b suggest that only trace amounts of ring-cleavage intermediate accumulate during the metabolism of benzoate by cells expressing an

amplified level of oxalocrotonate decarboxlase expression. This serves as an indication that either (i) benzoate is metabolised very efficiently as a result of removing the rate-limiting effect at the level of oxalocrotonate decarboxylase; or (ii) the amplification of *xylI* significantly reduces the metabolism of benzoate by the engineered pathway.

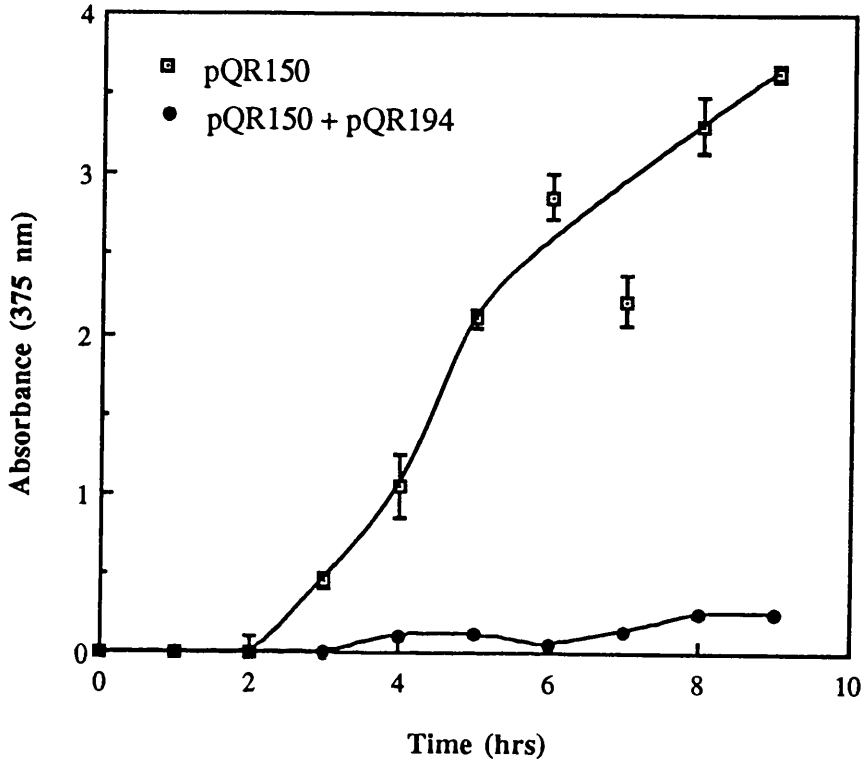


Fig 3.16 b. The production of 2-hydroxymuconic semialdehyde during growth of *E.coli* cells carrying pQR150 or pQR150 and pQR194 in the presence of 10mM benzoate.

3.3.3 Fermenter studies of cells carrying plasmids pQR150 and pQR194:

In order to investigate the proposals of section 3.3.2, cells co-expressing pQR150 and pQR194 were grown in fermenters in the presence of benzoate as previously described in section 3.1.2 according to the methods of section 2.5.3. Growth, *meta*-cleavage intermediate concentration and C23O activity were monitored as described in section 2.6.

As illustrated in fig 3.17 a and 3.17 b, relative growth and levels accumulation of 2-hydroxymuconic semialdehyde were as observed during shake-flask experiments described in section 3.3.2, the concentration of ring-cleavage intermediate being 2-fold lower in the presence of an amplified oxalocrotonate decarboxylase.

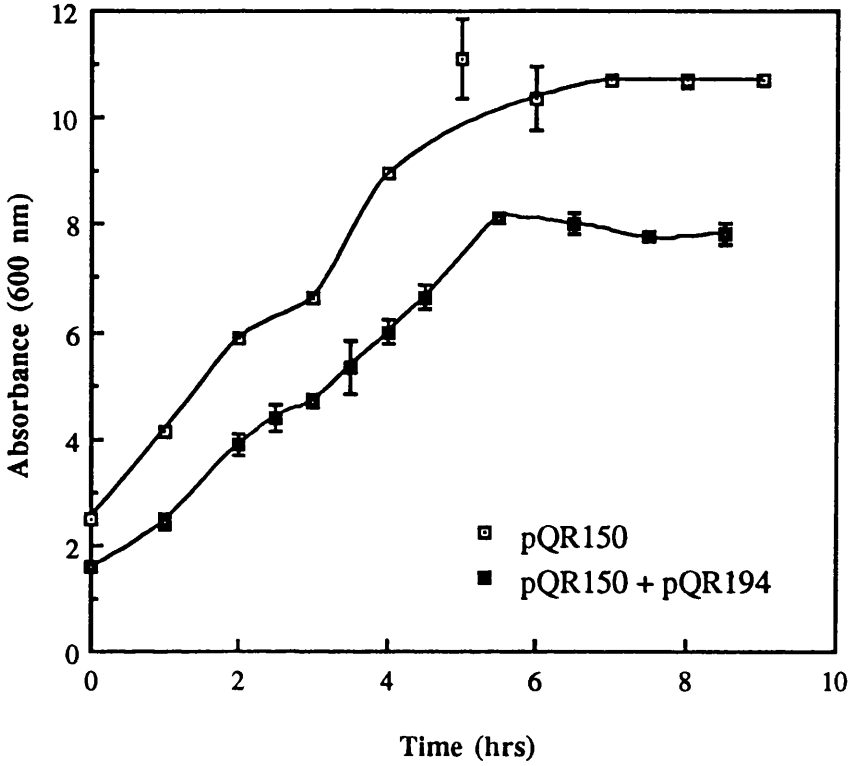


Fig 3.17 a. Fermenter growth in the presence of 10mM benzoate by *E.coli* cells carrying pQR150 and pQR194, or pQR150 alone. IPTG added at T=0. Substrate added at T=2.

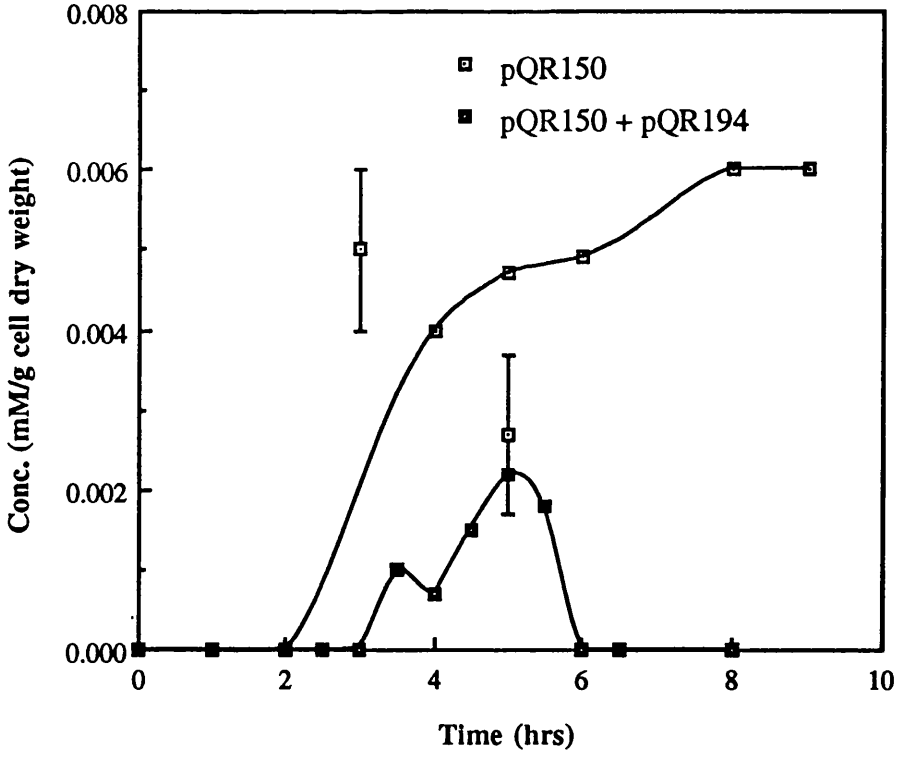


Fig 3.17 b. The production of 2-hydroxymuconic semialdehyde during growth in fermenters of cells expressing pQR150 and pQR194, or pQR150 alone in the presence of 10mM benzoate.

This is coupled with levels of oxalocrotonate and valerate 10-fold and 3-fold lower respectively (fig 3.18) than observed during metabolism of benzoate by a wild-type pathway (fig 3.3a).

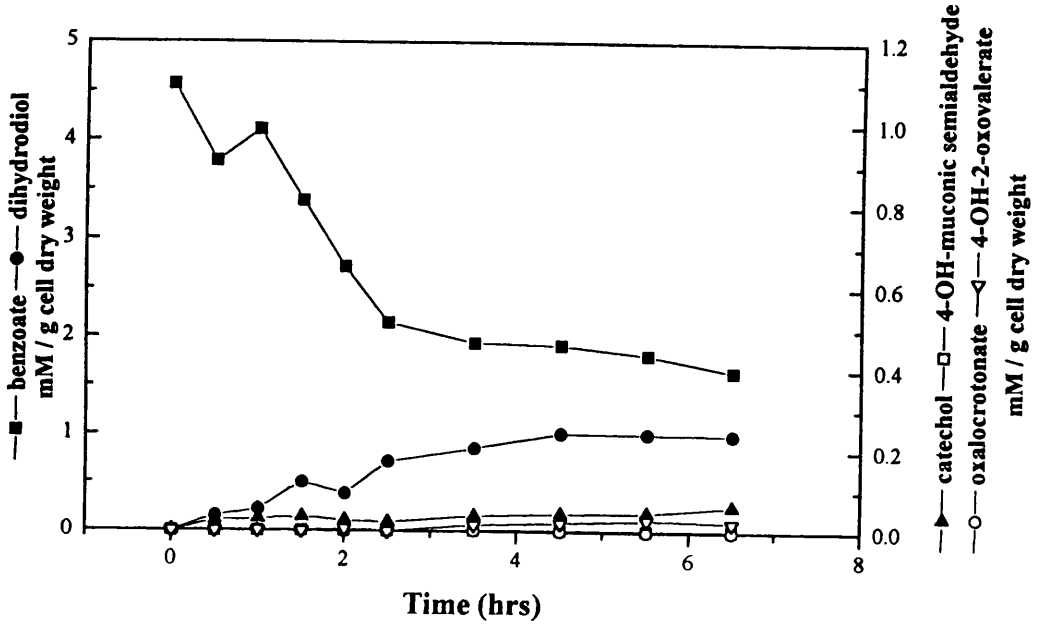


Fig 3.18. Supernatant concentration of *meta*-cleavage cleavage pathway intermediates during the metabolism of 10mM benzoate by *E.coli* cells carrying pQR150 and pQR194. Benzoate added at T=0.

In the light of these findings it is clear that amplification of oxalocrotonate decarboxylase expression results in a significant reduction in the overall metabolism of benzoate through the *meta*-cleavage pathway.

As a further indication of the effect on pathway metabolism caused by *xylI* amplification, levels of C23O activity in comparison with a wild-type pathway indicate that the amplification of *xylI* leads to an approximate three-fold drop in the level of activity when expressed per gram cell dry weight (fig 3.19).

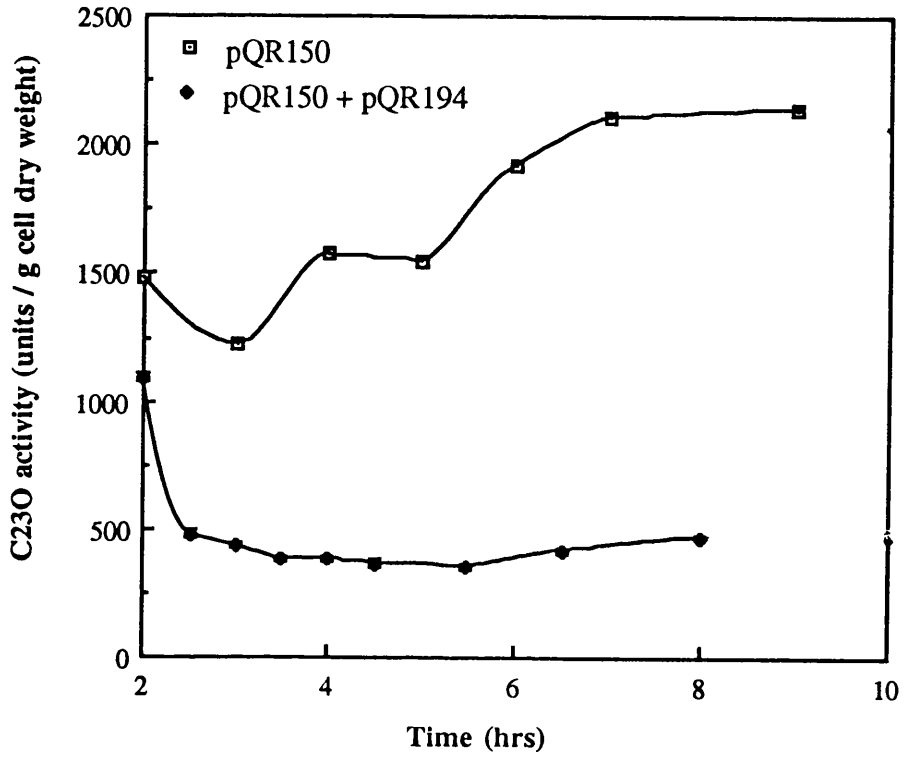


Fig 3.19. Catechol 2,3-dioxygenase activity during induction and metabolism of 10mM benzoate by *E.coli* cells carrying pQR150 and pQR194 or pQR150 alone. Cells were induced with IPTG at T=0, and benzoate added at T=2.

The determination and comparison of protein levels (section 2.6.7) during metabolism of benzoate by a wild-type pathway and one exhibiting an amplified dioxygenase suggests as previously that such differences in C23O activities between the two systems cannot be attributed to the effect of pQR194 on plasmid expression (fig 3.20). This is supported by the fact that levels of C23O specific activity are also in the region of 3-fold less in systems exhibiting an amplified *xylI* than in control systems expressing plasmid pQR150 alone fig 3.20. This indicates that as in section 3.2.3.1, such contrasting C23O activities cannot be attributed to differing levels of plasmid expression.

It is clear from the results of section 3.3 that co-expression of pQR194 with pQR150 results in a reduction on benzoate metabolism through the *meta*-cleavage pathway. It is possible that this is due to an alteration in metabolic flux caused by the presence of an amplified level of *xylI* activity. Alternatively, pQR194 could have a detrimental effect on the activity of *meta*-cleavage pathway enzymes that is independent of pathway substrates or intermediates. Both these possibilities are investigated in chapters 4 and 5.

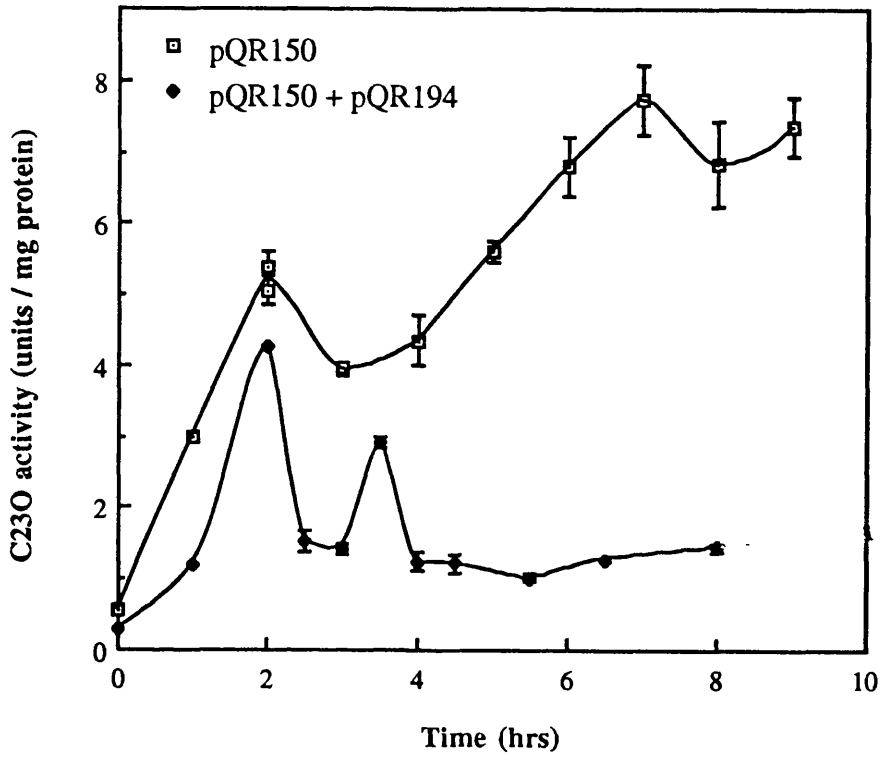


Fig 3.20. Catechol 2,3-dioxygenase specific activity during induction and metabolism of 10mM benzoate by *E.coli* cells carrying pQR150 and pQR194 or pQR150 alone. Cells were induced with IPTG at T=0, and benzoate added at T=2.

Chapter 4 The effect of overexpression of *xylI* on substrate metabolism.

In the following sections, all experiments were performed in duplicate unless stated otherwise.

4.1 Shake-flask analysis of benzoate metabolism by different engineered strains.

According to the results of section 3.3, amplified expression of the *xylI* encoded oxalocrotonate decarboxylase leads to a significant reduction in the metabolism of benzoate. In order to determine whether such a change in pathway activity is independent of substrate metabolism, comparative shake-flask experiments were performed with *E. coli* JM107, *E. coli* JM107 expressing pQR150 alone, and *E. coli* JM107 expressing pQR150 and pQR194. Shake-flask growth and induction was performed according to the methods of section 2.5.1.1, using the media described in section 2.5.3.1 as the growth media. Cells were induced with IPTG overnight.

Initial studies involved the comparative growth and metabolism of cells expressing pQR150 and pQR194 in the presence or absence of benzoate. Hourly samples were removed and analysed for optical density, protein content, dry weight and C23O as described in section 2.6.

Fig 4.1 a illustrates that the presence of benzoate reduces the growth of such engineered cells, in accordance with the observations of section 3.3.3. More significantly, the presence of benzoate leads to a three-fold drop in the level of C23O activity (fig 4.1 b).

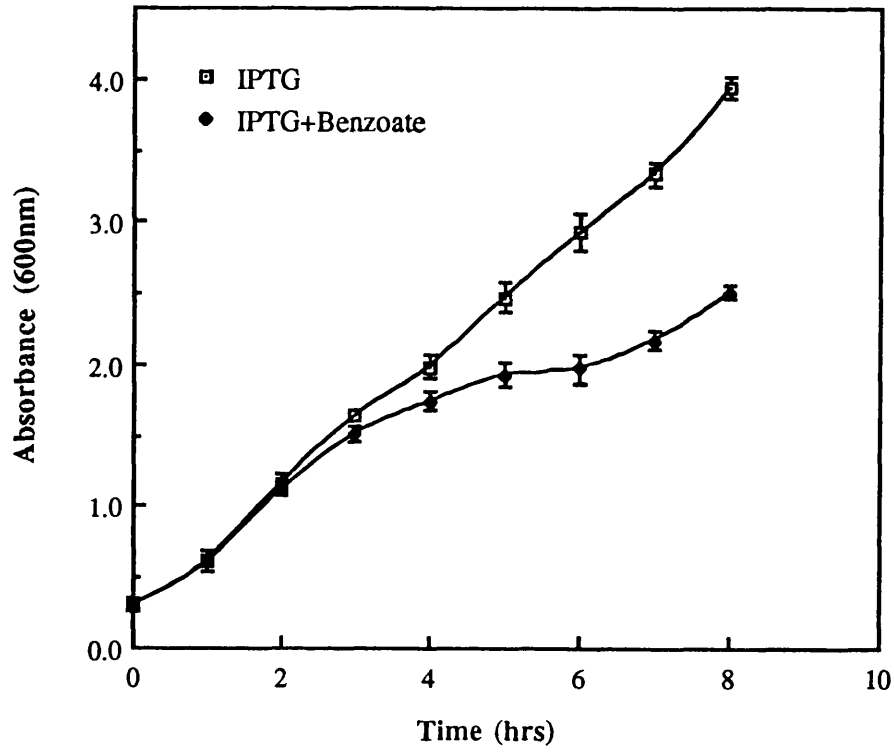


Fig 4.1 a. The comparative growth of *E.coli* cells expressing pQR150 and pQR194 in the presence or absence of 10mM benzoate. Benzoate added at T=0.

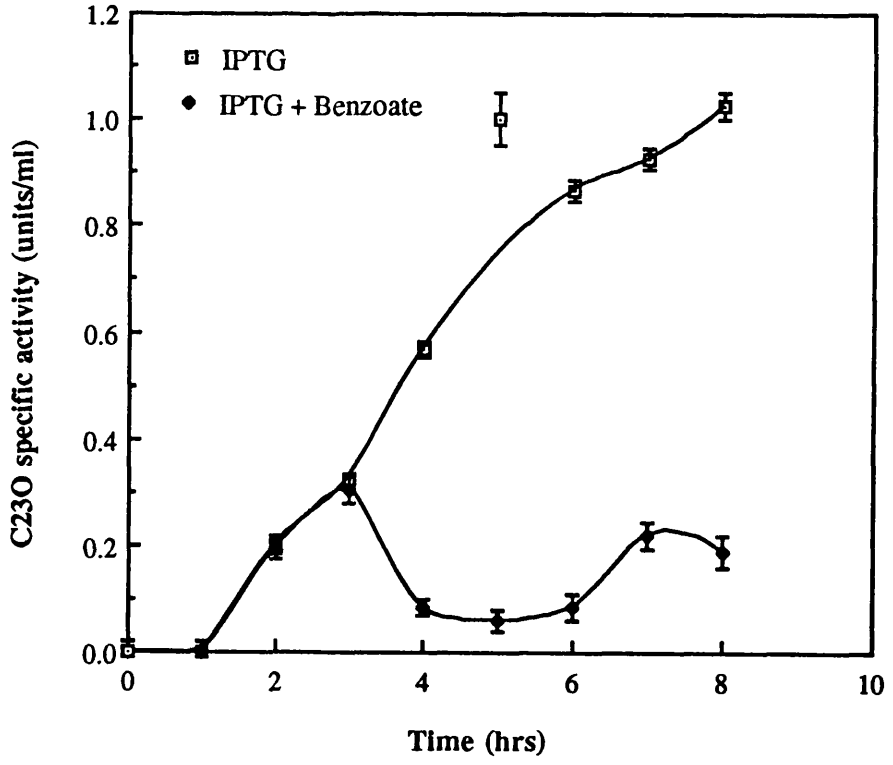


Fig 4.1 b. The effect of 10mM benzoate on C23O levels in cells carrying pQR150 and pQR194. Substrate added at T=0.

These initial observations suggest that low C23O levels are related to the presence of the pathway substrate benzoate, or an intermediate of benzoate metabolism, rather than by an inhibitory effect caused by the physical presence of an additional copy of the oxalocrotonate decarboxylase.

In order to confirm such suggestions, control experiments were performed with *E. coli* JM107 and cells expressing pQR150 alone.

As expected, the presence of benzoate was shown to have a detrimental effect on growth of both strains (fig 4.2 a and 4.2 b).

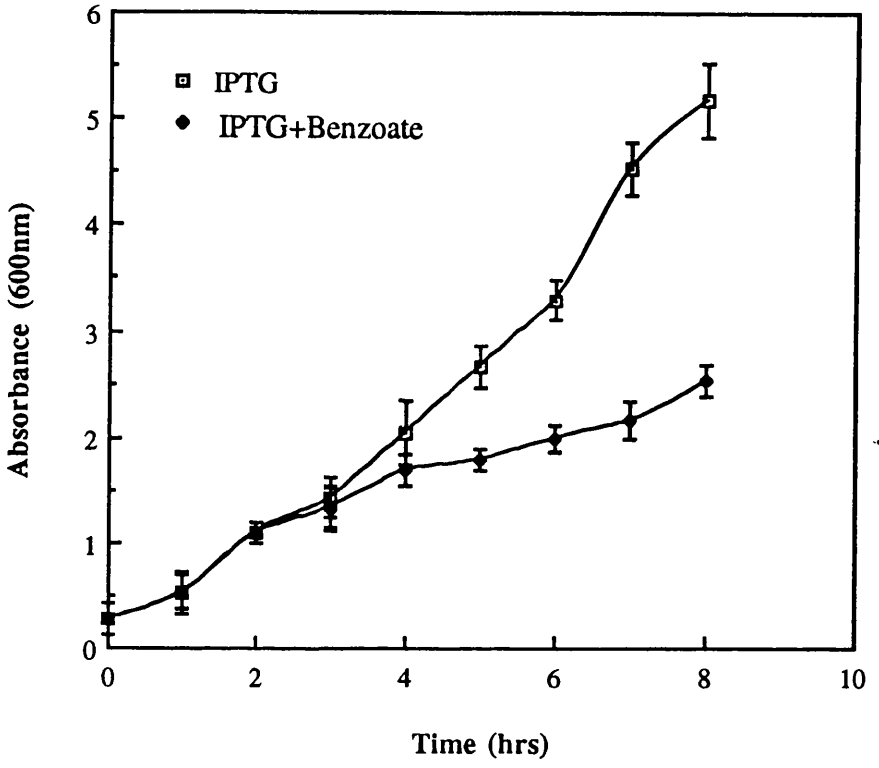


Fig 4.2 a. The growth of *E. coli* JM107 expressing pQR150 in the presence or absence of 10mM benzoate.

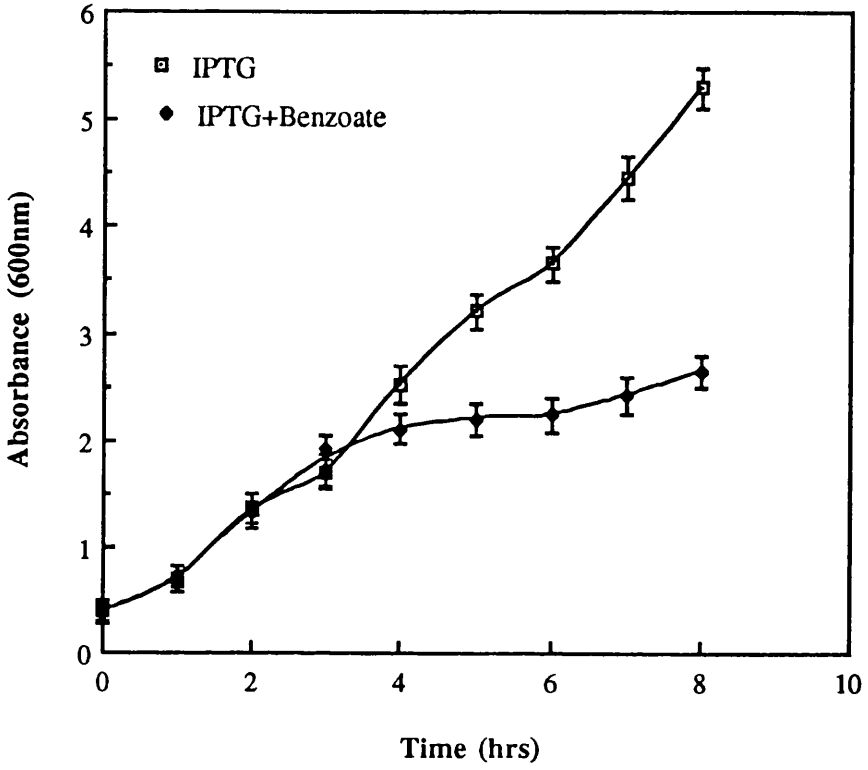


Fig 4.2 b. The growth of *E. coli* JM107 in the presence or absence of 10mM benzoate. Benzoate added at T=0.

By comparing the growth observed for all three strains in the absence of any pathway substrate, the presence of an additional plasmid, together with constraints caused due to the presence of an additional antibiotic result in a lower growth rate for cells expressing pQR150 and pQR194 (fig 4.3). This is in accordance with the observations of section 3.3.2.

Comparative levels of C23O activity between cells expressing either pQR150 or pQR150 and pQR194 indicate that even in the absence of substrates, expression of *xylI* has a detrimental effect on C23O activity (fig 4.4 a).

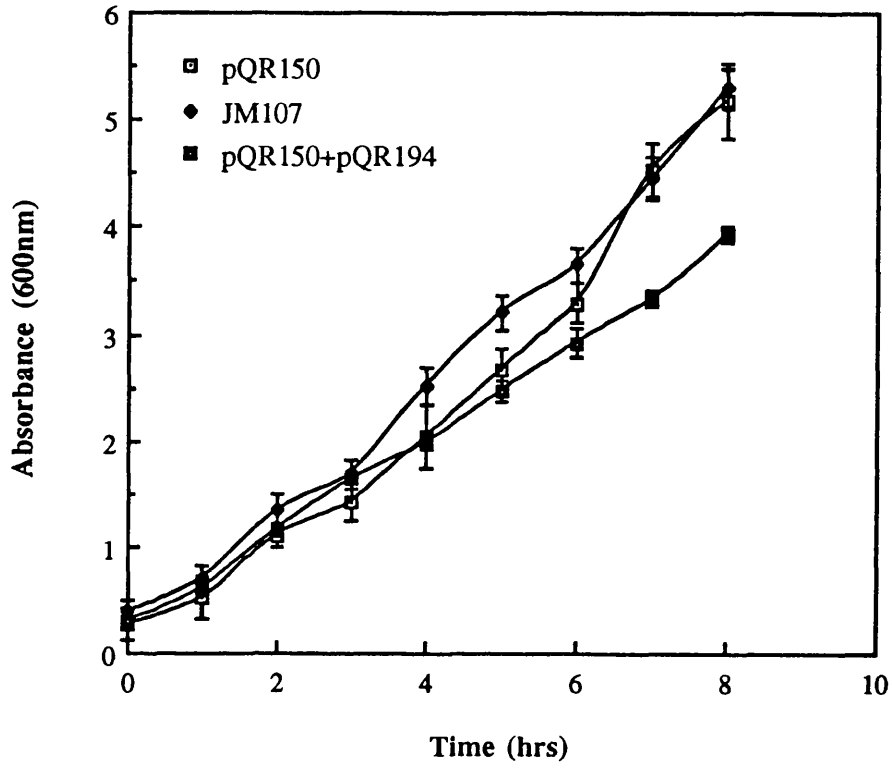


Fig 4.3. The comparative growth of different *E. coli* strains in the absence of benzoate.

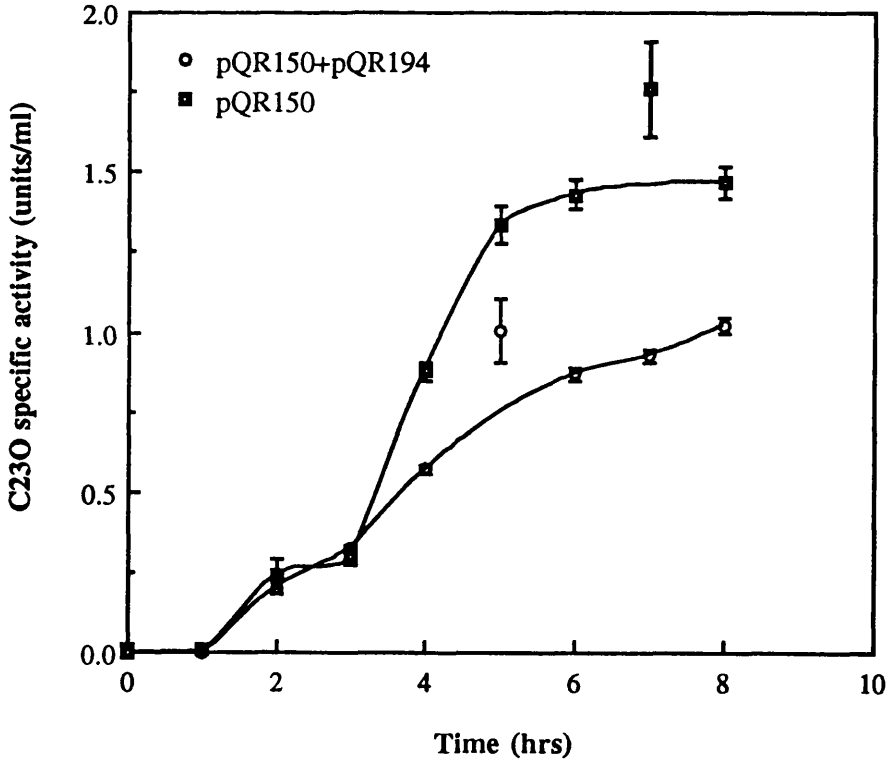


Fig 4.4 a. Comparative C23O activity during growth of cells expressing either pQR150 or pQR150 and pQR194 in the absence of 10mM benzoate. Benzoate was added at T=0.

As seen in fig 4.4 b, cells expressing pQR150 and pQR194 in the presence of benzoate show a C23O activity one-third that seen with cells expressing pQR150 alone. This is twice the difference in C23O activity shown between the two *E.coli* strains grown in the absence of benzoate (fig 4.4 a).

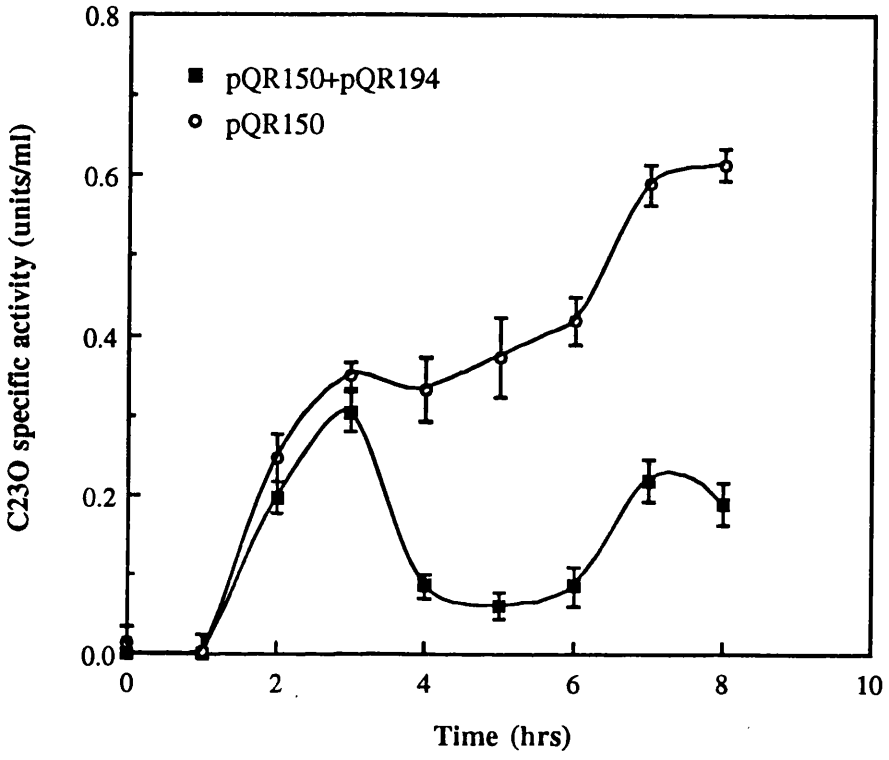


Fig 4.4 b. Comparative C23O activity during growth and metabolism of 10mM benzoate by *E.coli* cells expressing pQR150 or pQR150 and pQR194. Benzoate added at T=0.

Thus it appears that the reduction of benzoate metabolism in the presence of plasmid pQR194 is due to an alteration in metabolic flux resulting in the accumulation of a pathway intermediate to inhibitory levels, as well as an inherent detrimental physical effect. As this characteristic change in metabolic flux results from amplified expression of oxalocrotonate decarboxylase (OD), the product of OD was considered as a inhibitor of metabolic flux. It should be noted at this point that the product of OD, 2-hydroxypent-2,4-dienoate was not detected during studies performed in section 3.3.3. This in turn suggests that as an inhibitor 2-hydroxypent-2,4-dienoate would be inhibitory at low concentrations.

4.2 Development of a stable preparation of catechol 2,3-dioxygenase.

In order to perform a kinetic analysis of C23O, a preparation of the enzyme was required that maintained stability at 4°C over a period of 4-6 hours.

In all the following studies, C23O was prepared from an *E.coli* strain expressing a mutant *meta*-cleavage pathway pQR226 (see fig. 6.10 for construction), with metabolism terminated with the production of ring-cleavage product.
Initial investigations indicated that the methods described in section 2.6.4 were inadequate for this purpose, as a 60% drop in enzyme activity was observed within one hour of sonication of cell extracts (fig 4.5).

In order to improve the stability of the batch-prepared enzyme, various supplements were added to phosphate buffer prior to sonication of cell pellets (fig 4.5)

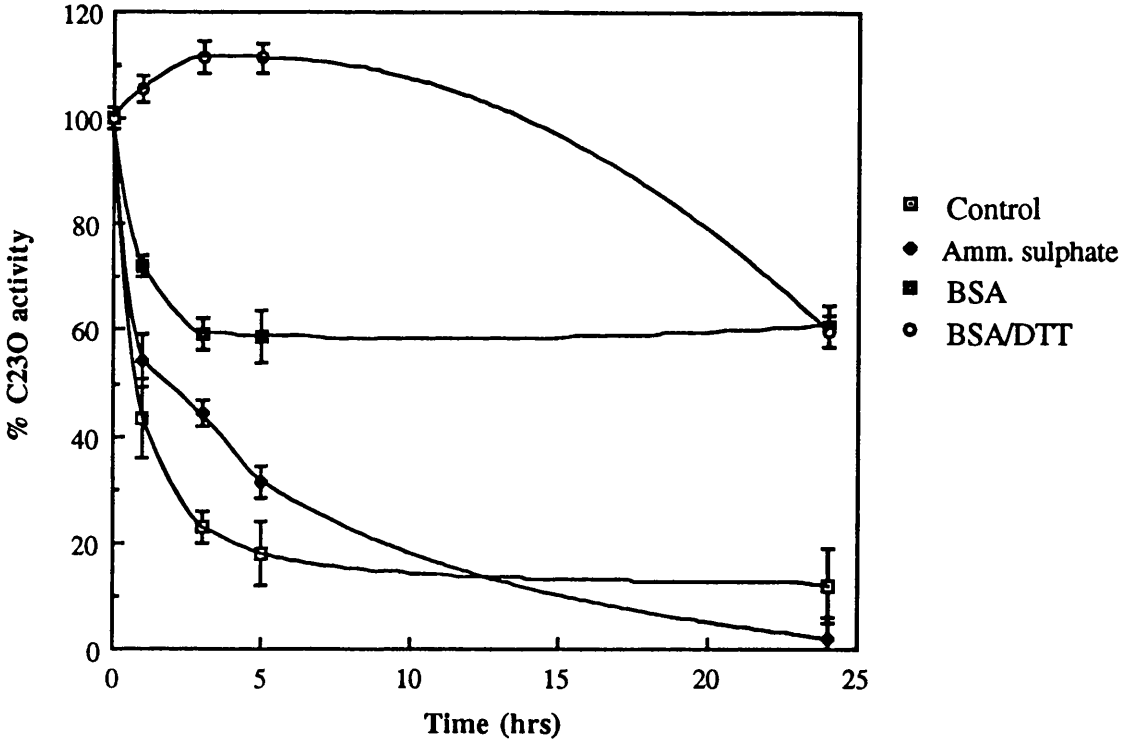


Fig 4.5. The stability of C23O in Potassium phosphate buffer (pH7.5 mM, 10% acetone) with various supplements.

According to the results of fig 4.5, the presence of 1% (w/v) BSA and 2mM DTT resulted in C23O stability being maintained for a period of five hours. Such observations however were made for concentrated enzyme preparations, and were not reproducible at the 1:50 (v/v) dilutions that would be required for accurate kinetic analysis (fig 4.6). Indeed, an 80% drop in activity over the desired period of 4 hours was observed in such cases.

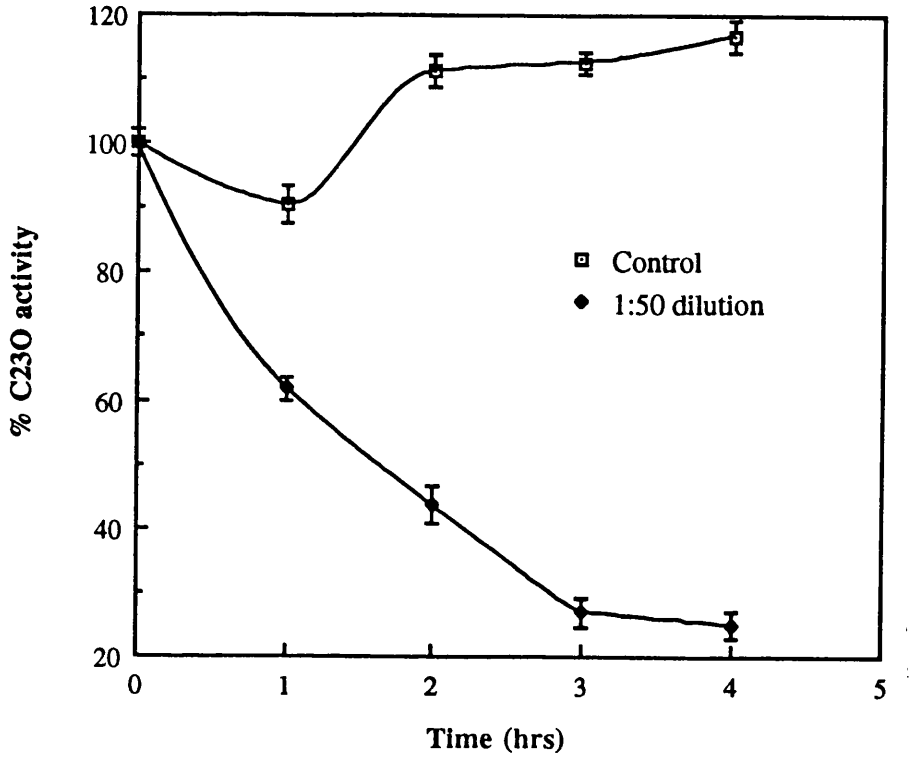


Fig 4.6. The effect of dilution on the stability of C23O prepared by an adaptation of the methods of section 2.6.5.

The use of divalent cations or protease inhibitors such as PMSF were also investigated, but were found to be detrimental to enzyme stability at both concentrated and diluted levels (fig 4.7).

From the results of figs. 4.6 and 4.7 it is clear that C23O extracts prepared by adaptations to the methods described in section 2.6.4 are not adequate enough to allow reproducible assays over a period of time sufficient to perform a series of kinetic studies.

As a result, the indications of fig. 4.5 were used to develop a batch purification procedure based upon the methods of Cerdan *et. al*, (1994) see section 2.6.5.

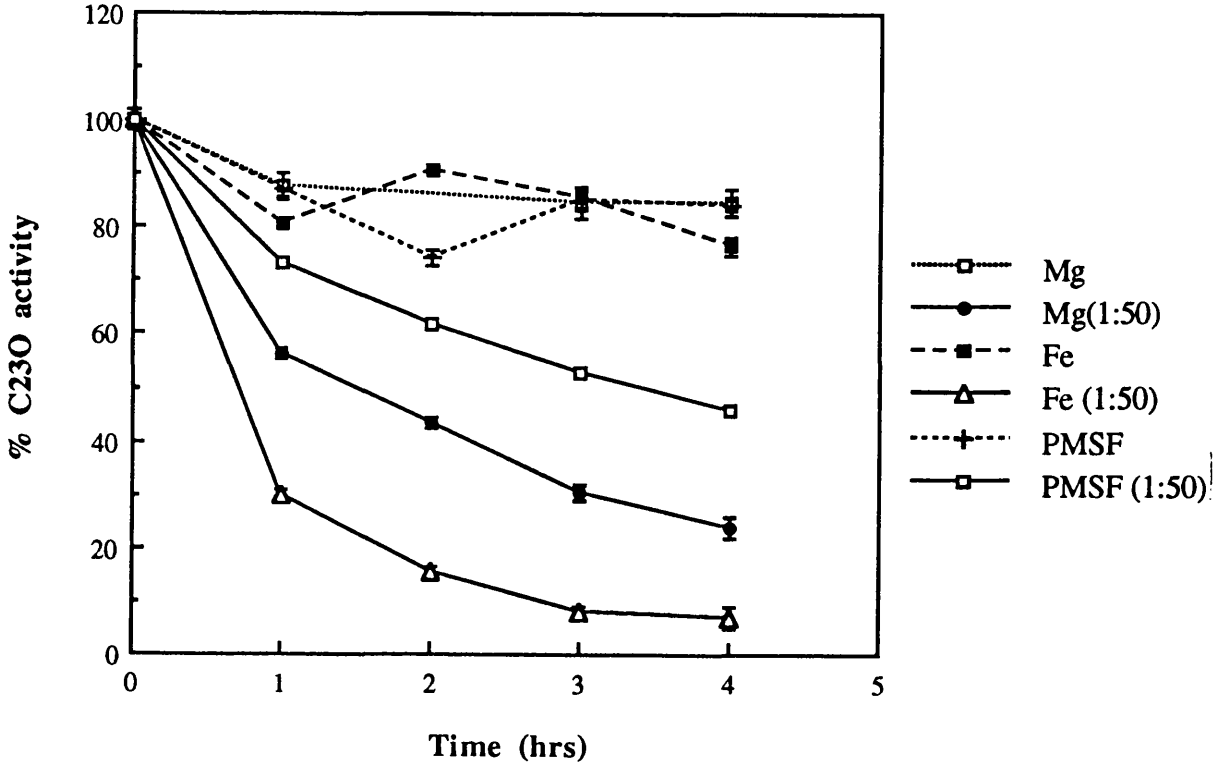


Fig 4.7. The effect of divalent cation or protease inhibitor on the stability of C23O prepared in potassium phosphate buffer supplemented with 10% (w/v) acetone, 1% (w/v) BSA, 2 mM DTT.

Using such a simplified DEAE based anion-exchange procedure, C23O was purified from an overnight culture of *E. coli* expressing pQR226 according to the purification table below (Table 4.1).

Table 4.1 Purification of catechol 2,3-dioxygenase from *E.coli* JM107 expressing pQR226

Sample	U_{TOT}	Prot. $(mg)_{TOT}$	Rel. Sp. Act.	Yield %
Crude	1.9	0.04	47.5	100
Unbound C23O	0.00855	0.00225	3.8	0.45
Wash 1	0	0.0024	-	0
Wash 2	0	ND(1)	-	0
0.1M NaCl	0.0018	ND	-	0.09
0.2M NaCl	0.0208	ND	-	1.09
0.3MNaCl (1)	0.5706	ND	-	30.03
0.3M NaCl (2)	0.306	ND	-	16.11
(1)+(2)	0.8586	0.0162	53	45.19

(1)ND: not determined

Samples of fractions containing active C23O were adjusted to 25% (w/v) $(NH4)2SO2$, with 1% (w/v) BSA, or 1% BSA and 2mM DTT. The adjusted fractions were then incubated at 4°C. Active fractions were assayed for C23O activity after 1 hour incubation.

Table 4.2 Stability of batch-purified catechol 2,3-dioxygenase

Sample		U_{TOT}	Prot. $(mg)_{TOT}$	Rel. Sp. Act.
(i)		0.752	0.0142	53
(ii)		0.05544	0.001	55.44
(iii)		0.0533	0.001	53.3
(i)	(1 hour)	0.640	0.016	40
(ii)	(1 hour)	0.0612	0.001	61.2
(iii)	(1 hour)	0.0695	0.001	69.5

1: Purified C23O. 2: C23O (25% (w/v) $(NH_4)_2SO_4$), 1% (w/v) BSA. 3: C23O (25% (w/v) $(NH4)2SO4$, 1% (w/v) BSA, 2mM DTT).

As illustrated in Table 4.2, the presence of 25% (w/v) ammonium sulphate with or without the presence of BSA and DTT lead to stability being maintained over a period of an hour. Over a period of 12 hours however, at 1:50 dilutions only fractions containing BSA and DTT maintained activity (fig 4.8).

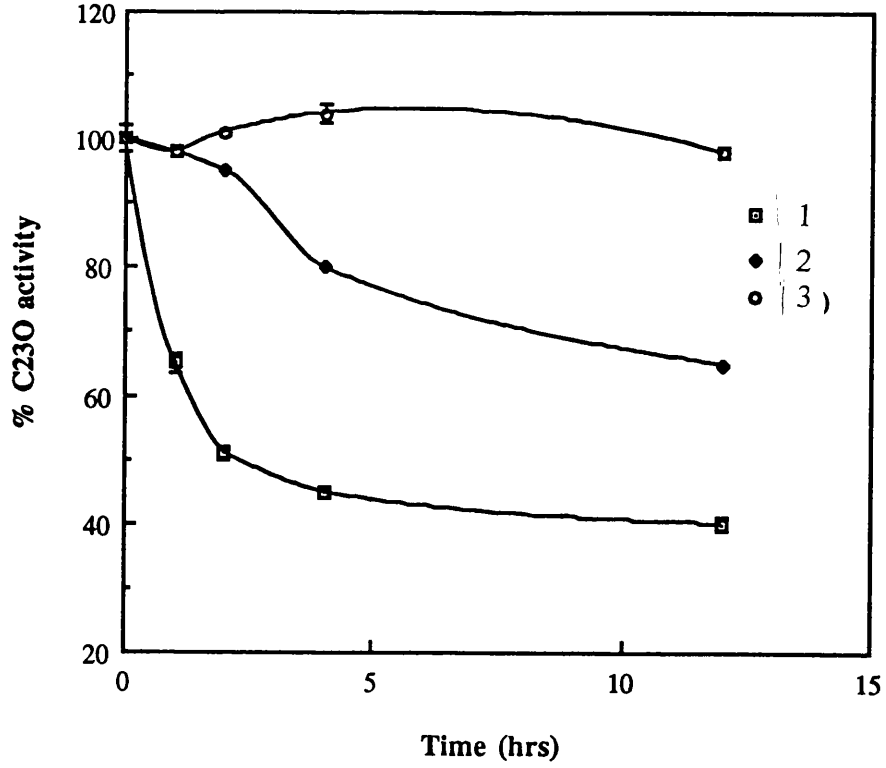


Fig 4.8. Stability of 1:50 diluted fractions of batch purified C23O.

As indicated in fig 4.8, the batch purification procedure described provides sufficient stability to allow reproducible assays to be performed over a period of time sufficient to carry out kinetic studies. Such a procedure removes the need for further preparation of the enzyme by gel filtration and hydrophobic interaction techniques.

4.3 2-hydroxypent-2,4-dienoate inhibition of catechol 2,3-dioxygenase.

The possibility that 2-hydroxypent-2,4-dienoate is responsible for the reduced levels of benzoate metabolism observed due to inhibition of enzymes in the pathway such as C23O was investigated. Inhibition studies were performed with C23O prepared according to the methods described in section 2.6.6, using 2-hydroxypent-2,4-dienoate at various concentrations ranging from 0-222 μM. 2-hydroxypent-2,4-dienoate was synthesized by the methods of Collinsworth *et. al.*, (1973) (see section 2.6.3.1)

The results shown in fig. 4.9 are not conclusive in determining the inhibitory effect of the 2-hydroxypent-2,4-dienoate on C23O. As a result, a second, Eaadie-Hofstee plot was generated (fig 4.10).

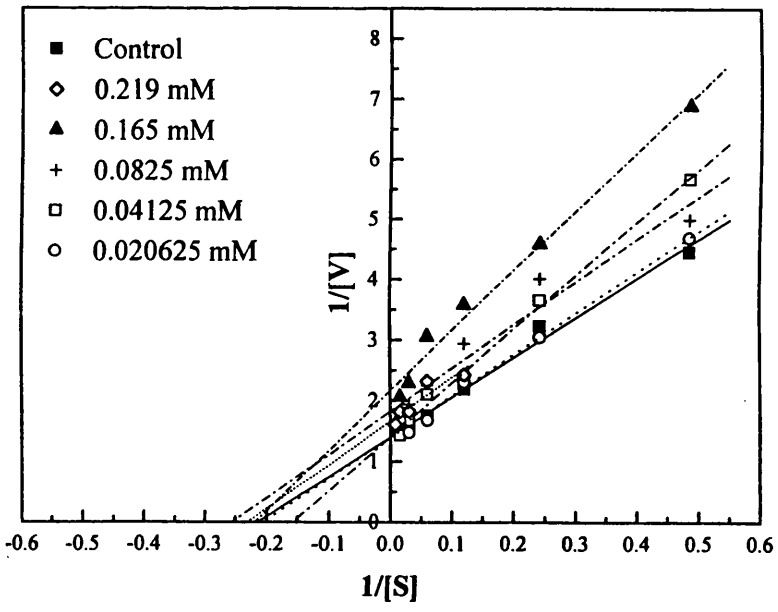


Fig 4.9. A Lineweaver-Burk plot showing the effect of different concentrations of 2-hydroxypent-2,4-dienoate on C23O activity.

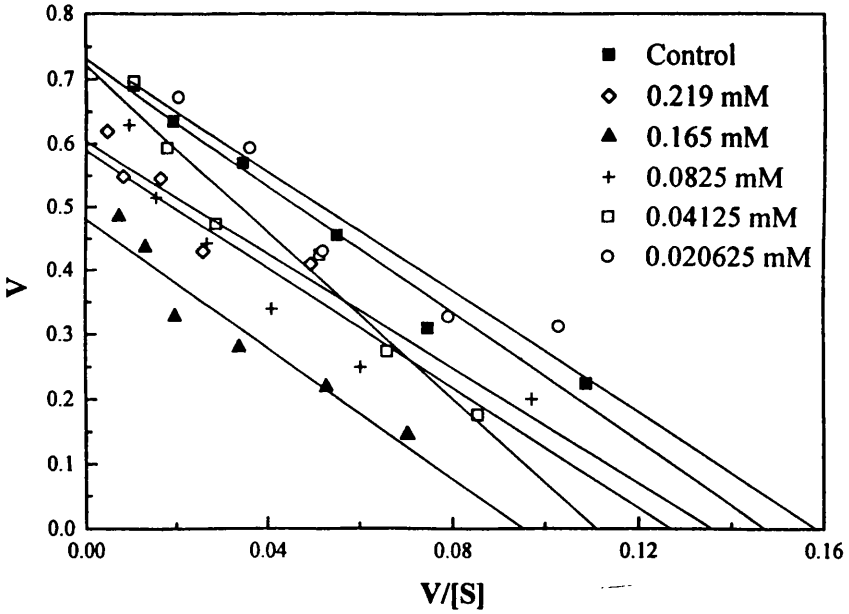


Fig 4.10. An Eaadie-Hofstee plot illustrating the apparent non-competitive inhibition of catechol 2,3-dioxygenase by 2-hydroxypent-2,4-dienoate.

The results of fig. 4.10 clearly show that the presence of 2-hydroxypent-2,4-dienoate upon C23O activity reduces the V_{max} of the reaction, but does not affect the Km. Such observations are indicative of non-competitive inhibition. In order to determine the inhibitor constant (Ki) for 2-hydroxypent-2,4-dienoate, a secondary plot was constructed from of $1/V_{max}$ against inhibitor concentration (fig 4.11).

The results of fig 4.11 indicate that the apparent Ki exhibited by 2-hydroxypent-2,4-dienoate is 149.5 μM. This value may be compared with the inhibition constants of the products of the C23O catalysed reactions. (Table 4.3).

Table 4.3 Comparison of different inhibitors of catechol 2,3-dioxygenase.

Substrate	Inhibitor	Ki (M)	Type of Inhibition
catechol	2-hydroxymuconic semialdehyde	0.4	Non-competitive[1]
3-methylcatechol	2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate	1.6×10^{-4}	Competitive[2]
4-methylcatechol	2-hydroxy-6-oxohepta-2,4-dienoate	1×10^{-4}	Competitive[2]
catechol	2-hydroxypent-2,4-dienoate	1.49×10^{-4}	Non-competitive

[1]Data collected from Hori *et al*, (1973). [2]Data obtained from Polissi and Harayama (1993).

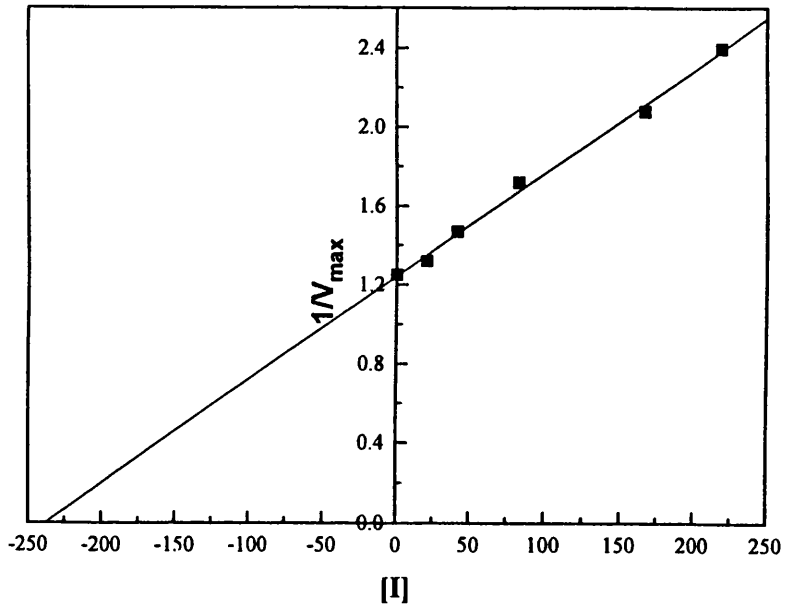


Fig 4.11. A secondary plot of $1/V_{max}$ against [I] for the non-competitive inhibition of catechol 2,3-dioxygenase by 2-hydroxypent-2,4-dienoate.

It is interesting to note from the data in table 4.3 that the inhibition constant of 2-hydroxypent-2,4-dienoate is similar to that of the ring-cleavage products of methyl catechols. It is therefore possible that, during *m*-toluate metabolism described in section 3.2.3.2, a drop in C23O activity following the amplification of benzoate dioxygenase may be due to inhibition by both the ring-cleavage product and the 2-hydroxypent-2,4-dienoate. During benzoate metabolism, it is apparent from table 4.3 that the ring-cleavage product of catechol is not as strong an inhibitor to C23O as 2-hydroxypent-2,4-dienoate. As a result, any inhibition to C23O observed during benzoate metabolism can be seen as a result of an accumulation of 2-hydroxypent-2,4-dienoate.

It is likely that the Ki values reported by Hori *et al*, (1973) and Polissi and Harayama, (1993) are higher than the actual values. This is due to the kinetic studies having been performed in the presence of inhibitory levels of acetone.

Chapter 5 The metabolism of *m*-toluate by truncated *meta*-cleavage pathways.

Having established the inhibitory effect of accumulated levels of 2-hydroxypent-2,4-dienoate on C32O activity, it was necessary to determine the mechanism behind such an accumulation during benzoate metabolism in the presence of amplified levels of OD expression.

It is possible that during benzoate metabolism, an amplified level of OD permits an increase in flux through the enzyme, resulting in an accumulation of 2-hydroxypent-2,4-dienoate. Alternatively, disruption of the equimolar concentrations of XylI and XylJ by an amplification of the former may reduce the activity of *xylJ* in the *xylI/xylJ* complex, resulting in a build-up of 2-hydroxypent-2,4-dienoate, the substrate of *xylJ*.

In order to distinguish between these two proposals, an initial analysis of *m*-toluate metabolism was performed using a truncated *meta*-cleavage pathway that lacked *xylI* and *xylJ*, and thus terminated metabolism with the accumulation of 2-hydroxypent-2,4-dienoate, reflected by a build up of 2-oxopent-4-enoate (section 1.2.1.2)

In the following sections, all experiments were performed in duplicate unless stated otherwise.

5.1 Metabolism of *m*-toluate by *E.coli* cells expressing pQR185.

The plasmid pQR185 encoding *xylXYZLTEGF* was constructed according to fig. 5.2. All plasmids used in the construction are presented in Table 2.1.

The metabolism of different pathway substrates through pQR185 is represented in fig. 5.1. Metabolism of oxalocrotonate branch substrates such as benzoate terminates with the production of 4-oxalocrotonate. In comparison, *m*-toluate is metabolised exclusively through the hydrolytic branch, ending in a potential accumulation of 2-oxopent-4-enoate.

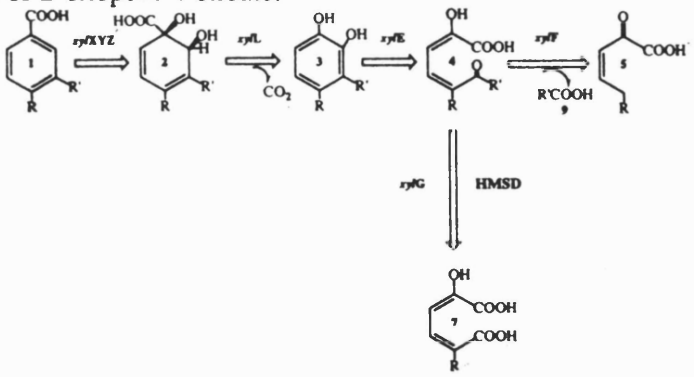


Fig 5.1. The metabolism of *meta*-cleavage pathway substrates through pQR185.

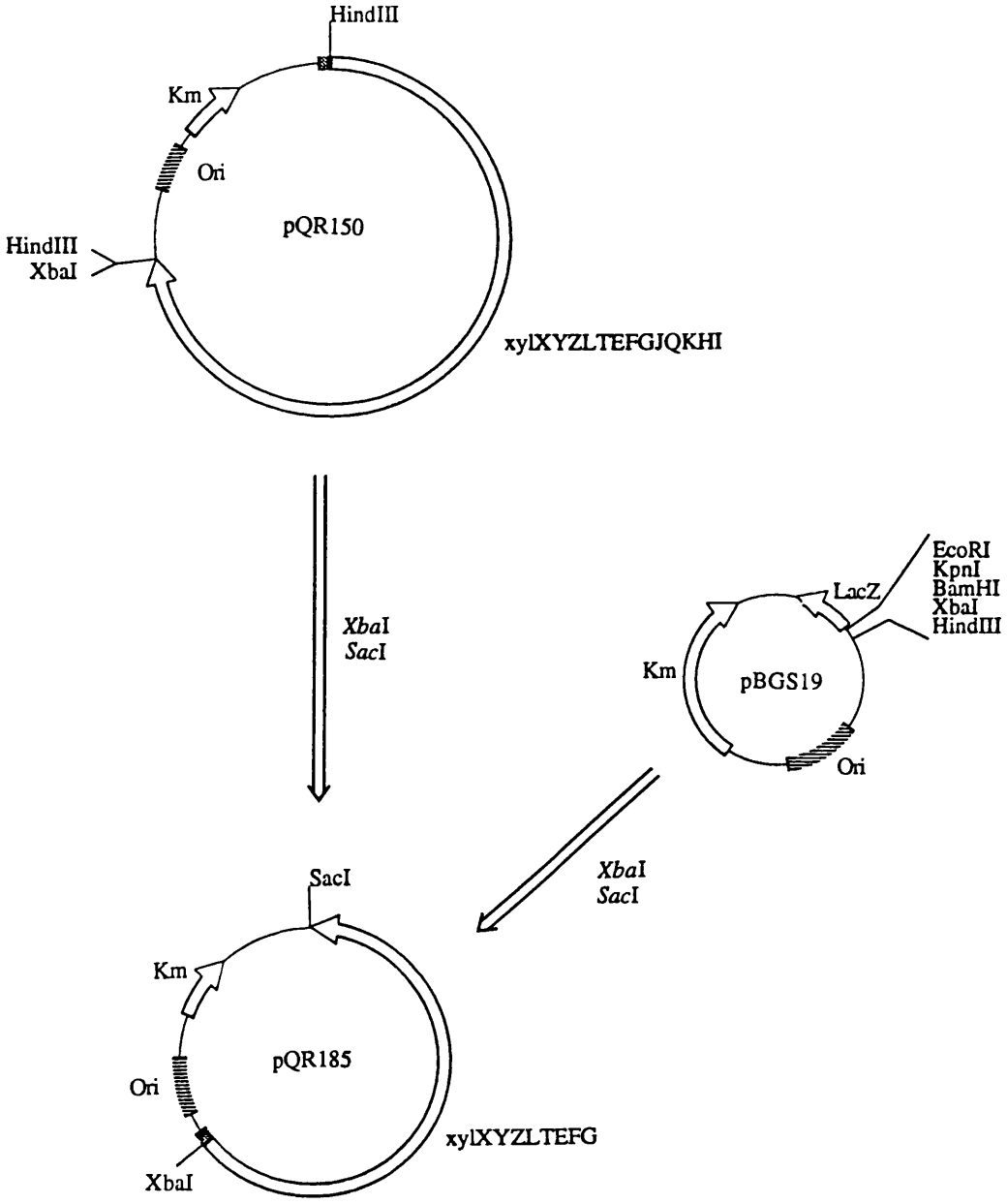


Fig 5.2. Construction of pQR185 (expressing the truncated *meta*-cleavage pathway *xylXYZLTEGF*). The presence of a unique *Sac*I restiriction site allowed the *xylXYZLTEGF* genes to be isolated from pQR150 on a 7.65 kb *Xba*I-*Sac*I fragament. 1 µg of both pQR150 and pBGS19 (Table 2.1) were digested with digested with *Xba*I and *Sac*I according to the methods of section 2.7.2. The two digested plasmids were ligated and used to transform *E.coli* JM107 (section 2.7.5). Recombinant strains were selected in the presence of Km by spraying plates with catechol. Colonies producing a characteristic yellow colour (section 2.4) were isolated and their DNA purified. Confirmation of the correct truncated *meta*-cleavage pathway gene order was determined by performing *XbaI*/*Sac*I and *Eco*RI/*Sac*I double digests.

5.1.1 Shake-flask experiments with *E.coli* cells carrying plasmid pQR185.

In accordance with previous studies performed with *E.coli* cells expressing *meta*-cleavage pathway constructs, shake-flask growth experiments were performed in order to give an initial indication of the effect on pathway activity caused by truncating the *meta*-cleavage pathway as in pQR185. Cells were grown and recombinant plasmids expressed in the presence of *m*-toluate according to the methods of section 2.5.1. Growth and concurrent accumulation of the ring-cleavage product, 2-hydroxy-6-oxohepta-2,4-dienoate during metabolism of *m*-toluate by cells expressing pQR185 were monitored according to the methods of sections 2.6, and compared with results obtained for *m*-toluate metabolism through the complete pathway, expressed by pQR150 (fig 5.3 a and 5.3 b).

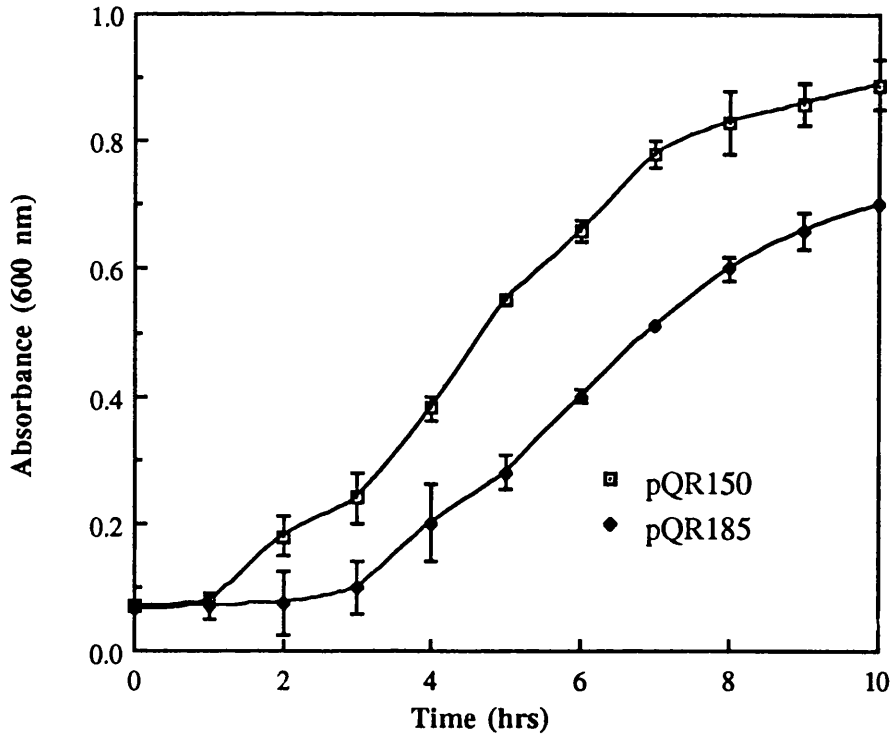


Fig 5.3 a. Shake-flask growth by *E.coli* cells expressing pQR150 or pQR185 in the presence of 10 mM *m*-toluate. *m*-toluate added at T=0.

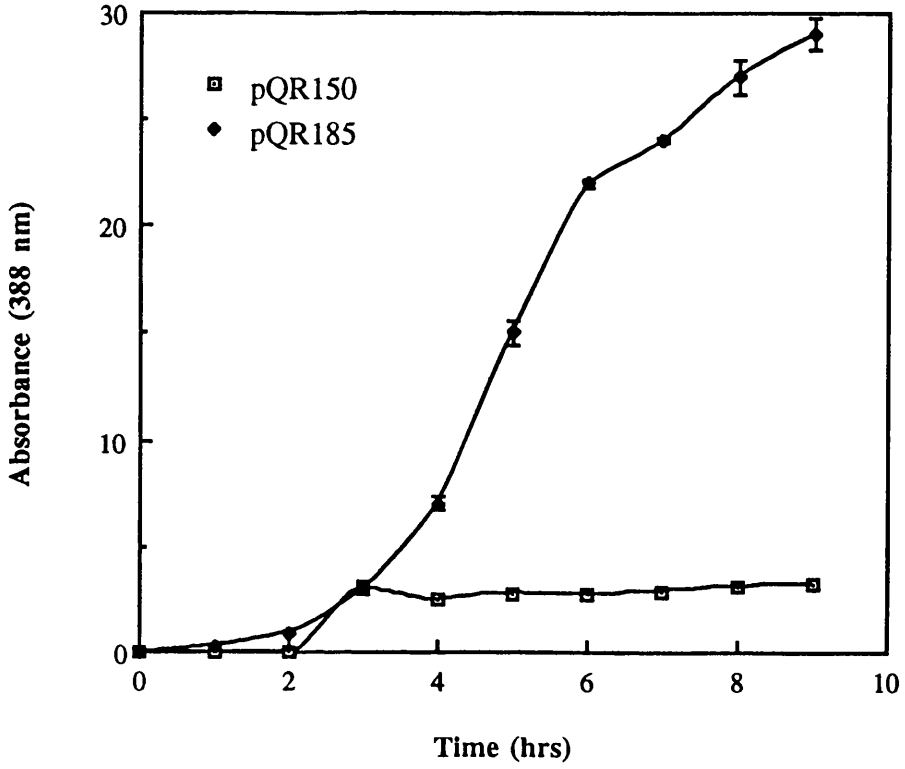


Fig 5.3 b. The production of 2-hydroxy-6-oxohepta-2,4-dienoate during 10mM *m*-toluate metabolism through pQR150 or pQR185. *m*-toluate added at T=0.

As can be seen in fig 5.3, by terminating *m*-toluate metabolism with the potential accumulation of 2-oxopent-4-enoate, growth of cells is reduced. Levels of ring-cleavage product also accumulate to levels ten-fold greater than achieved during metabolism through the entire pathway.

5.1.2 Fermenter studies of cells carrying plasmid pQR185.

In order to determine the effect of truncating *m*-toluate metabolism at 2-oxopent-4-enoate, growth of cells expressing pQR185 was performed in fermenters in the presence of *m*-toluate according to the methods of section 2.5.3. As seen in fig 5.4 a and 5.4 b the comparative growth and production of ring-cleavage intermediate by cells expressing either pQR150 or pQR185 was as observed in shake-flask experiments. As in section 3.2.3.2, the profile of 2-hydroxy-6-oxohepta-2,4-dienoate production is comparable between *m*-toluate metabolism through other manipulated *meta*-cleavage pathways.

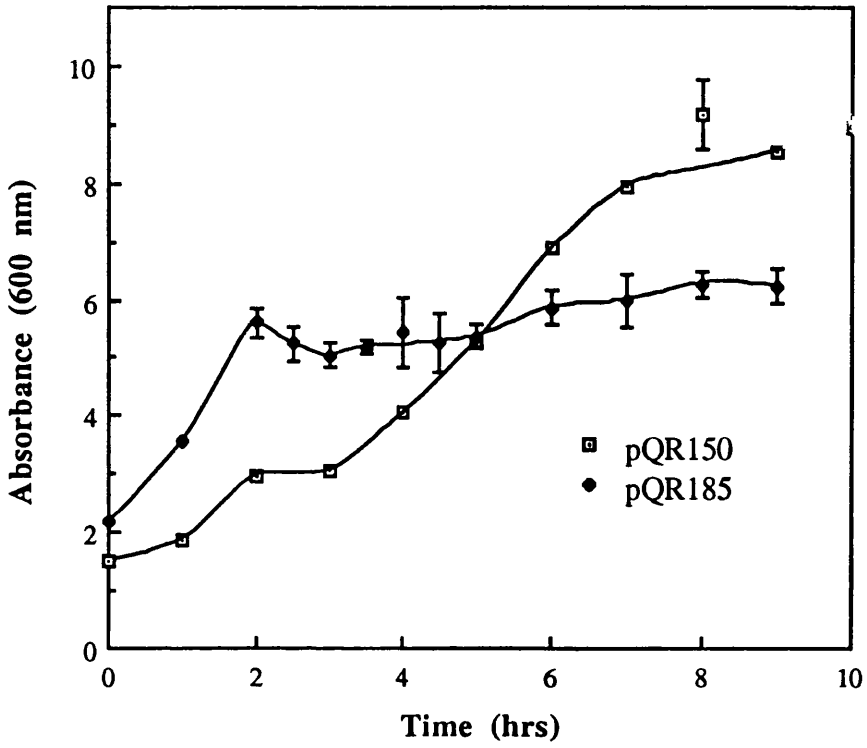


Fig 5.4 a. Growth in fermenters of *E.coli* cells expressing either pQR150 or pQR185 in the presence of 10 mM *m*-toluate. Cells induced with IPTG at T=0. *m*-toluate added at T=2.

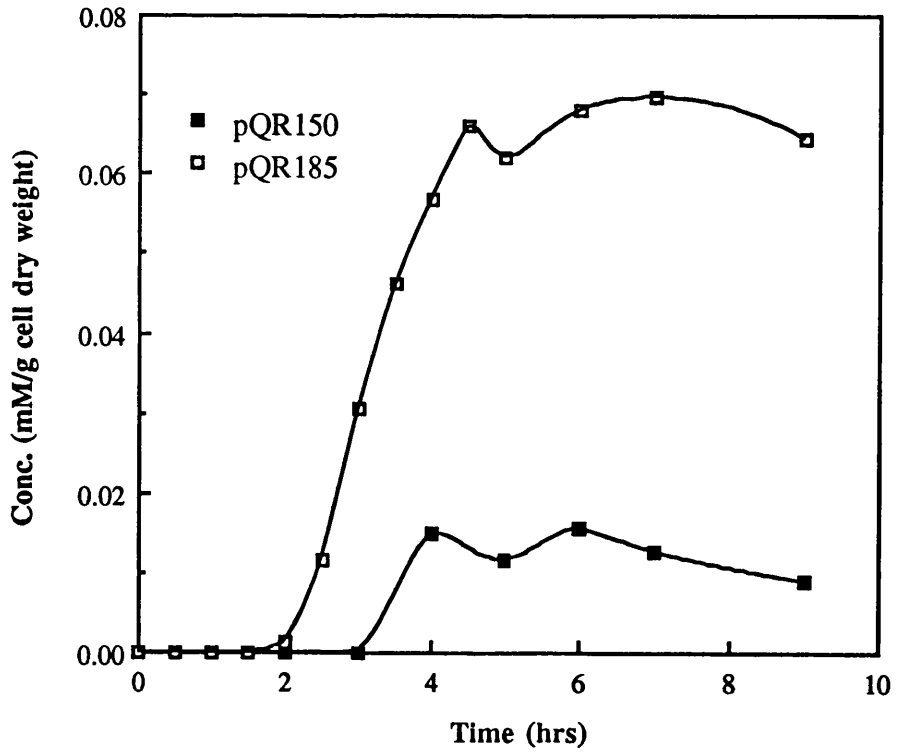


Fig 5.4 b. The production of 2-hydroxy-6-oxohepta-2,4-dienoate during metabolism of 10mM *m*-toluate through pQR150 or pQR185. Cells induced with IPTG at T=0. *m*-toluate added at T=2.

Concentrations of other pathway intermediates during metabolism were determined according to the methods of sections 2.6.9 and 2.6.10. Intermediate concentrations accumulated during metabolism through either pQR150 or pQR185 are shown in fig 5.5 a and 5.5 b respectively.

The most striking difference between the two concentration profiles shown in figs 5.5 a and 5.5 b concerns the three to four-fold increase in ring-cleavage product that results from terminating metabolism as in pQR185. Also significant is the increase in 2-oxopent-4-enoate from negligible concentrations observed during metabolism through wild-type pathways to that approaching 0.4 mM g cell dry weight^{-1}, as a result of truncating the pathway as in pQR185. It is interesting to note that such accumulations are coupled with a poor rate of *m*-toluate degradation.

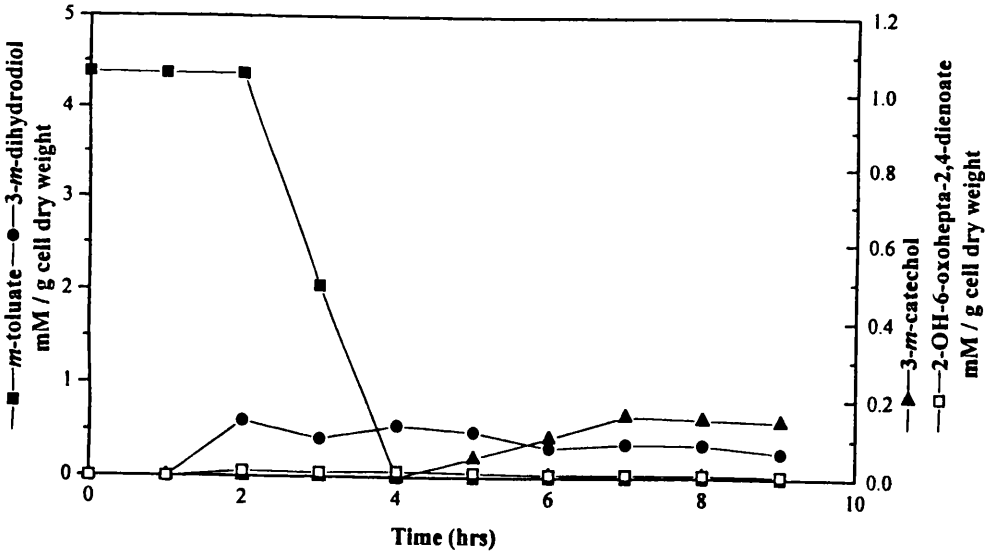


Fig 5.5 a. Supernatant concentrations of pathway intermediates during *m*-toluate metabolism by *E.coli* cells expressing pQR150. *m*-toluate added at T=0.

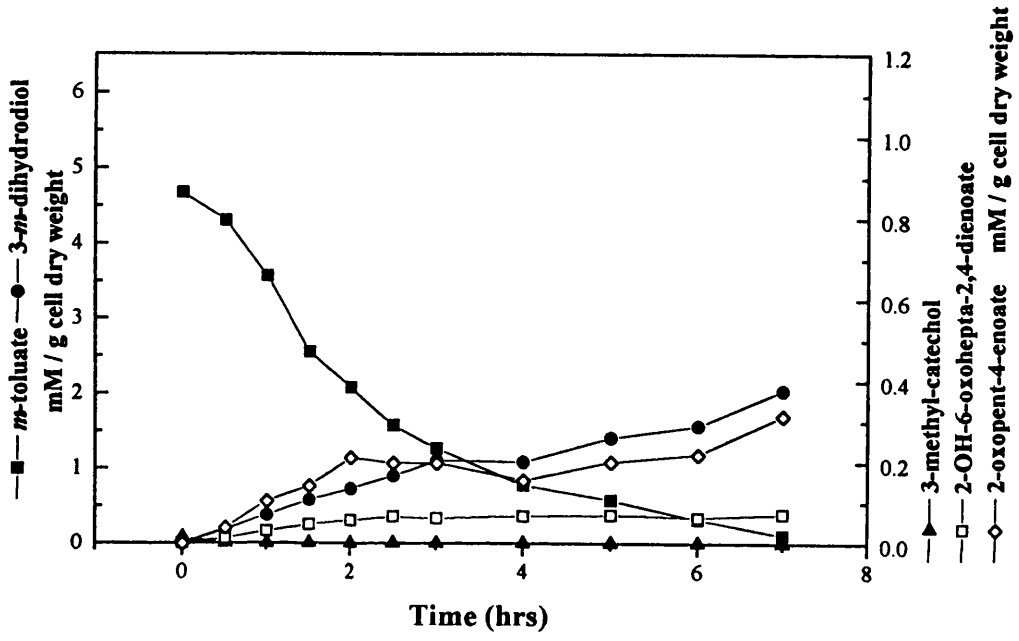


Fig 5.5 b. Supernatant concentrations of pathway intermediates during *m*-toluate metabolism by *E.coli* cells expressing pQR185. *m*-toluate added at T=0.

The effect on C23O of terminating the metabolism of *m*-toluate at 2-oxopent-4-enoate in comparison with C23O activity during metabolism in a wild-type pathway can be seen in fig 5.6.

Such a drop in C23O activity observed during metabolism through pQR185 can be accounted for by the high concentrations of both 2-oxopent-4-enoate and 2-hydroxy-6-oxohepta-2,4-dienoate.

The results of section 5.1.1.and 5.1.2 present the characteristic intermediate concentration and C23O activity profiles that can be expected during *m*-toluate metabolism in the absence of either *xylI* or *xylJ*.

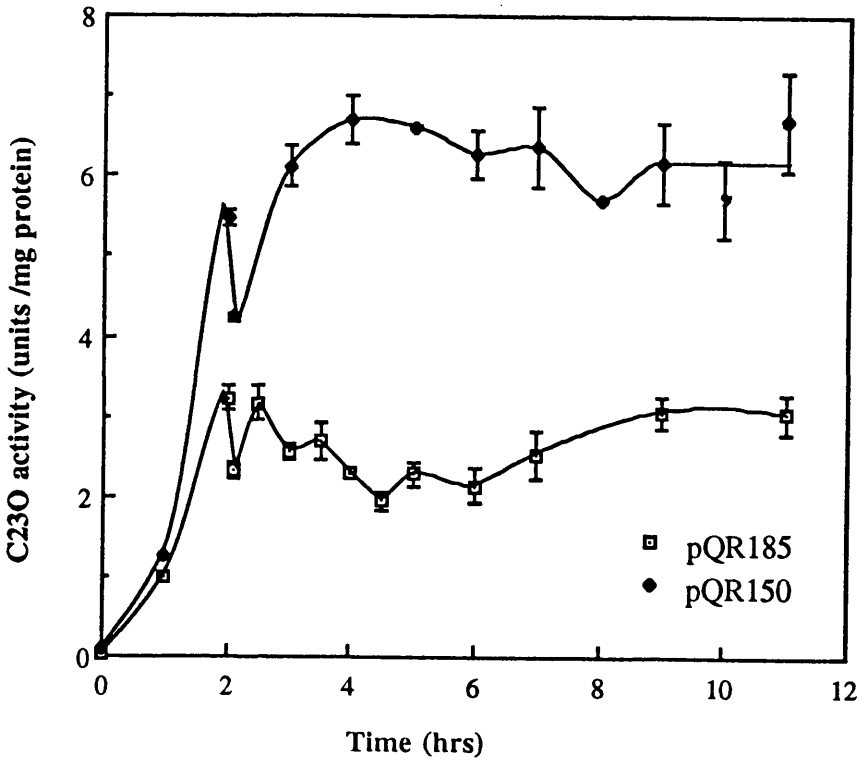


Fig 5.6. Catechol 2,3-dioxygenase specific activity during the metabolism of 10mM *m*-toluate by *E.coli* cells expressing either pQR150 or pQR185. Cells were induced with IPTG at T=0, and substrtae added at T=2.

5.2 Metabolism of *m*-toluate by *E.coli* carrying pQR186.

In order to establish the change in these profiles that would result from the presence of a fully active *xylJ* encoded OEH, the metabolism of *m*-toluate by *E.coli* expressing pQR186 was studied. The plasmid pQR186 (Table 2.1) encodes the truncated *meta*-cleavage operon *xylXYZLTEGFJ* that allows the metabolism of different *meta*-cleavage pathway substrates according to fig. 5.8. The construction of pQR186 was performed according to fig 5.7.

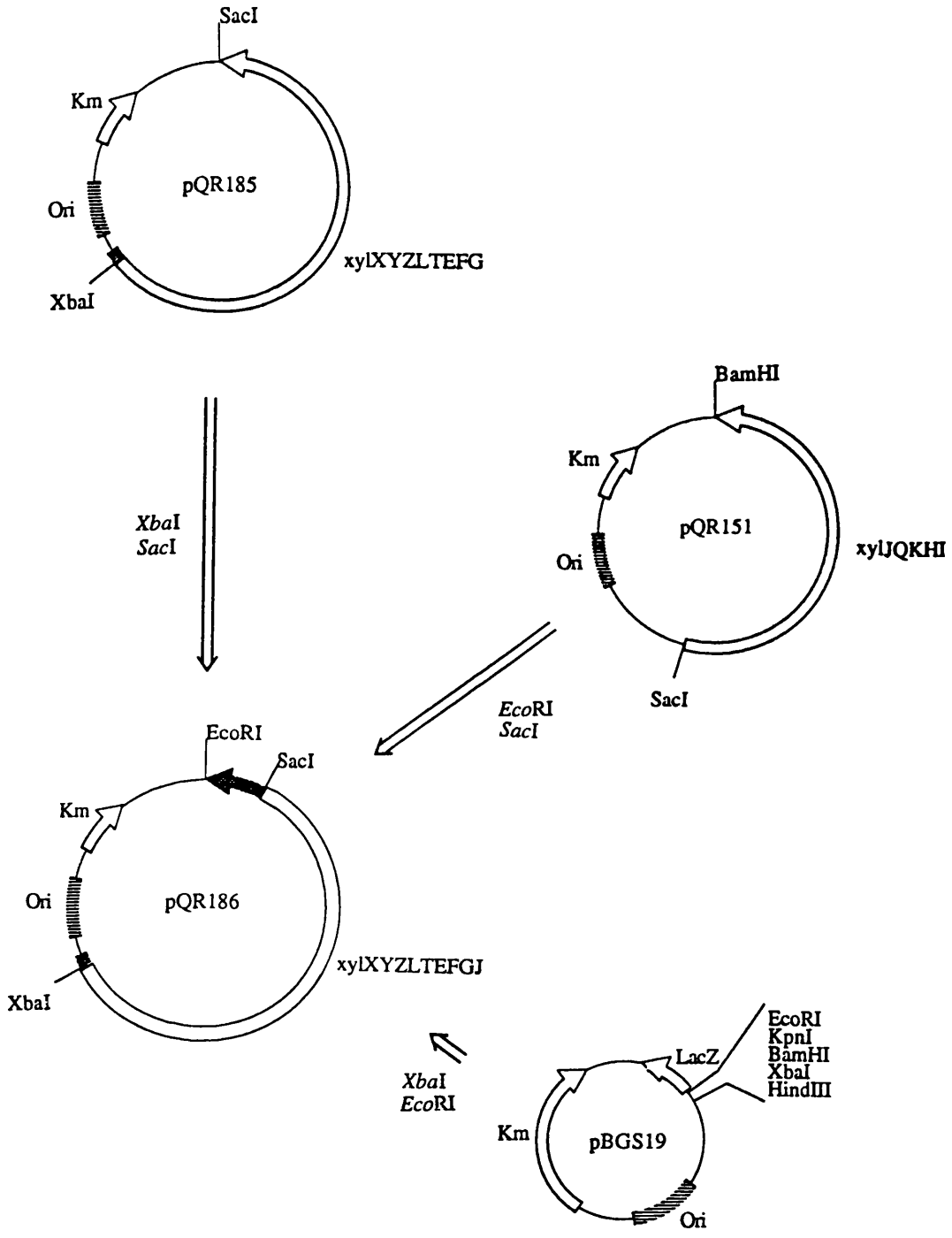


Fig 5.7. Construction of pQR186 (expressing the truncated *meta*-cleavage pathway *xylXYLTEGFJ*). The presence of an *Eco*RI site downstream of *xylJ* in *xylQ* enabled the *xylJ* to be introduced at the 3' end of pQR185 in the correct reading frame, on a 0.96 kb *Sac*I-*Eco*RI fragment. 1 µg of pQR185 was digested with *Xba*I and *Sac*I, and the 7.65 kb fragment encoding *xylX-J* was isolated from an agarose gel by the methods of section 2.7.7. 1 µg of pQR151 was digested with *Sac*I and *Eco*RI, and as above, the 0.96 kb *Sac*I-*Eco*RI fragment was isolated. The two gel purified fragments were subsequently ligated in equimolar amounts with pBGS19 that had been digested with *Xba*I and *Eco*RI. Following transformation of *E.coli* JM107, recombinant strains were selected as in fig 5.2, according to the methods of section 2.4. The correct gene order of recombinant plasmids was determined by performing *Eco*RI digests, together with *XbaI/SacI* and *XbaI/EcoRI* double digests on purified plasmid DNA.

The metabolism of *m*-toluate through pQR186 terminates with the production of 4-hydroxy-2-oxovalerate, one step further than achieved with pQR185.

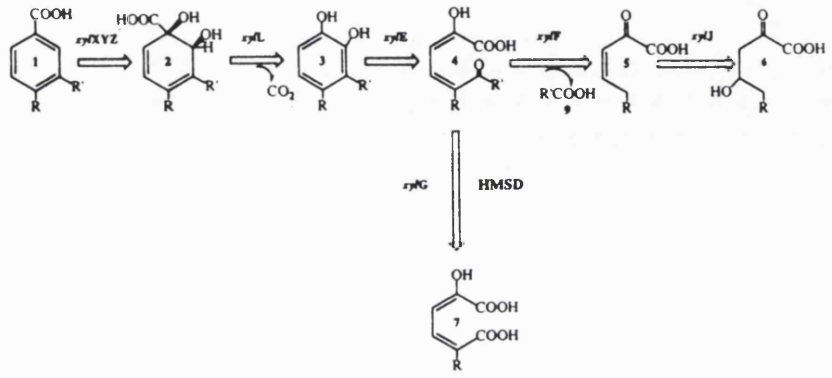


Fig 5.8. The metabolism of *meta*-cleavage pathway substrates through pQR186.

5.2.1 Shake-flask experiments with *E.coli* carrying plasmid pQR186.

As in studies with other pathway constructs shake-flask analysis of the growth and metabolism of *m*-toluate by cells carrying pQR186 was performed as described previously. Such studies served to provide an initial indication of the effects of expressing an extra step in the *meta*-cleavage pathway than pQR185. A comparison of the growth and production of ring-cleavage intermediate during *m*-toluate metabolism by *E.coli* cells expressing either pQR185 or pQR186 is shown is fig 5.9.

The significance of possessing XylJ activity in *m*-toluate metabolism is immediately obvious from fig 5.9. The effect of reducing 2-oxopent-4-enoate accumulation through the addition of the next step in the pathway results in growth and levels of ring-cleavage intermediate being restored to the levels observed during shake-flask metabolism of *m*-toluate by cells expressing pQR150 (fig 5.3).

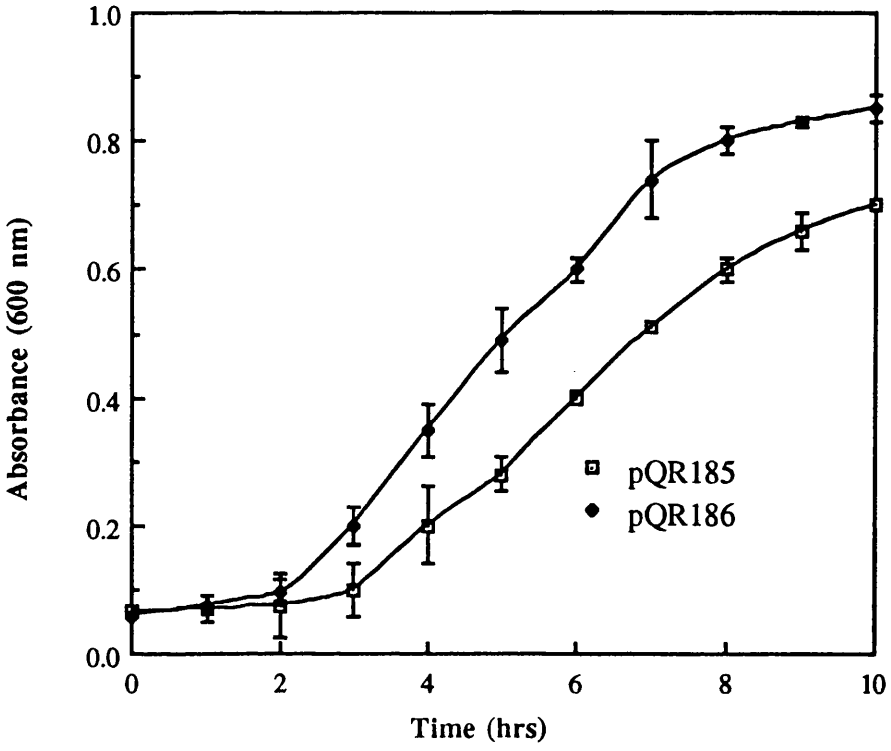


Fig 5.9 a. Shake-flask growth of *E.coli* cells expressing either pQR185 or pQR186 in the presence of 10 mM *m*-toluate. Substrate added at T=0.

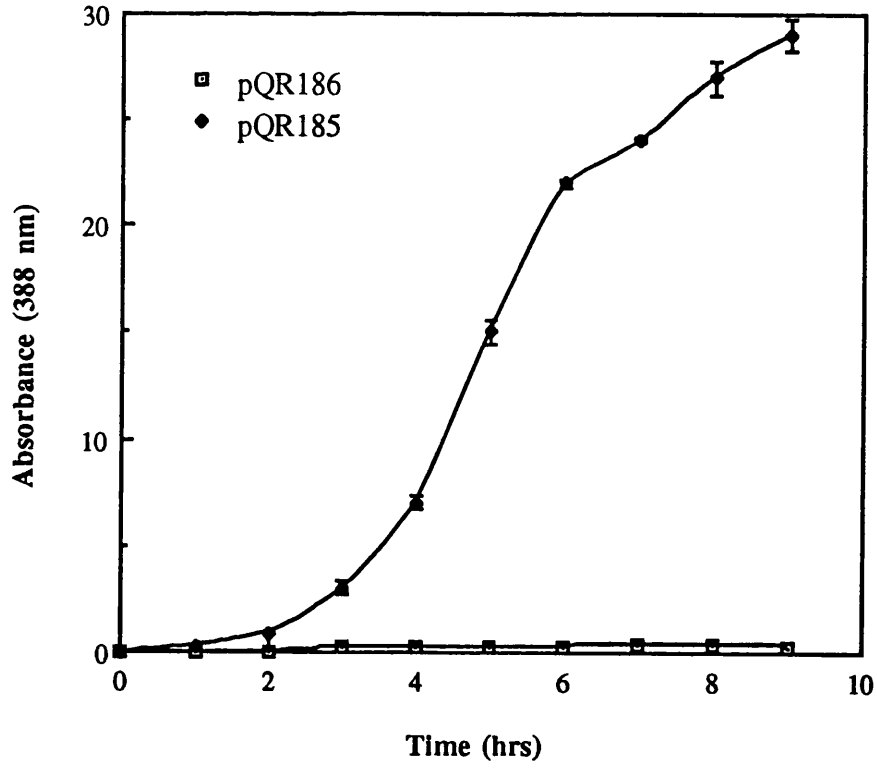
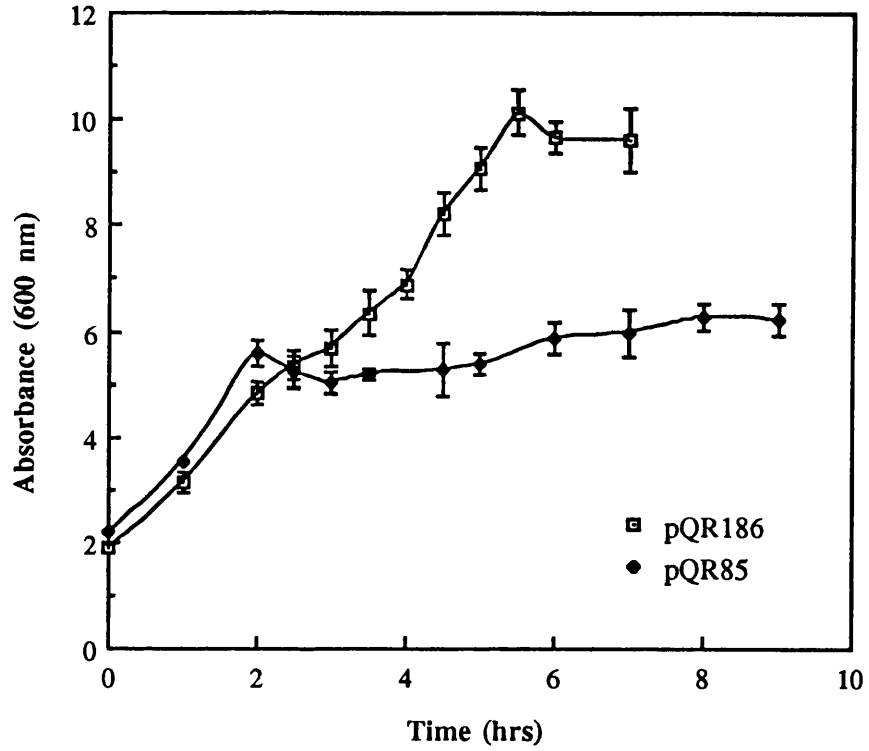


Fig 5.9 b. The production of 2-hydroxy-6-oxohepta-2,4-dienoate during the metabolism of 10 mM *m*-toluate by cells expressing pQR185 or pQR186. Substrate added at T=0.

5.2.2 Fermenter studies of cells carrying pQR186.

In order to demonstrate in more detail the effect of an active *xylJ* within a truncated *meta*-cleavage pathway, *E.coli* cells carrying pQR186 were grown under controlled conditions in the presence of an initial concentration of 10 mM *m*-toluate, as described previously (section 5.1.2). The growth of cells expressing either pQR185 or pQR186 (fig 5.10) clearly confirms the observation of section 5.1, that suggest that truncating the metabolism of *m*-toluate at 2-oxopent-4-enoate has a significant effect on growth that is removed by the addition of the next step in the pathway.



5.10. Fermenter growth of *E.coli* cells expressing pQR186 or pQR185 in the presence of *m*-toluate. Cells induced with IPTG at T=0. *m*-toluate added at T=2.

Fig 5.11 illustrates the fate of *m*-toluate and the concurrent accumulation of the other pathway intermediates during metabolism through pQR186. A comparison with fig 5.5 b indicates that twice as much end-product can accumulate during metabolism through pQR186 than through pQR185. In addition. the presence of XylJ/ activity results in the complete removal of *m*-toluate from the culture supernatant within two hours together with a reduction in the concentration of 2-oxopent-4-enoate to negligible amounts. Such metabolism has also been observed during *m*-toluate metabolism through the entire pathway (fig 5.5 a).

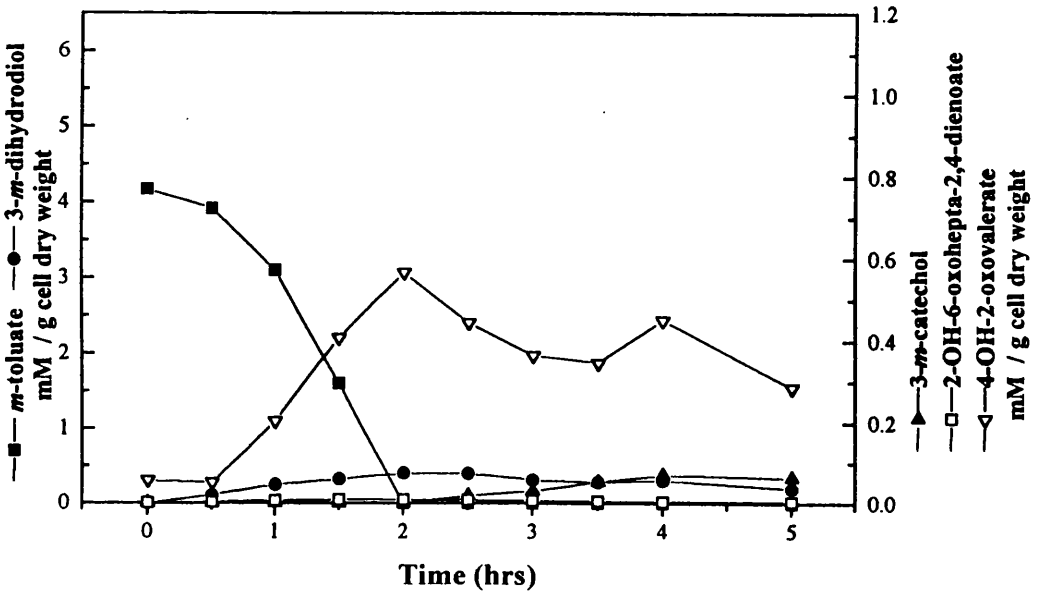


Fig 5.11. Supernatant concentrations of *meta*-cleavage pathway intermediates during the metabolism of 10mM *m*-toluate by *E.coli* cells expressing pQR186. Substrate added at T=0.

An analysis of C23O levels during *m*-toluate metabolism through pQR186 (fig 5.12) indicates a similar level of activity to that seen during metabolism of *m*-toluate through pQR150, and can be related to the negligible amounts of 2-oxopent-4-enoate and 2-hydroxy-6-oxohepta-2,4-dienoate that accumulates during pQR186 or pQR150 metabolism.

The results of section 5.1 and 5.2 demonstrate the effect on *meta*-cleavage pathway activity of the accumulation of 2-oxopent-4-enoate. In the case of metabolism of *m*-toluate through pQR185, such an accumulation occurs as a result of an absence of XylJ activity. By providing a XylJ activity as in pQR186, pathway activity as *m*-toluate metabolism resembles that seen during metabolism through a wild-type pathway.

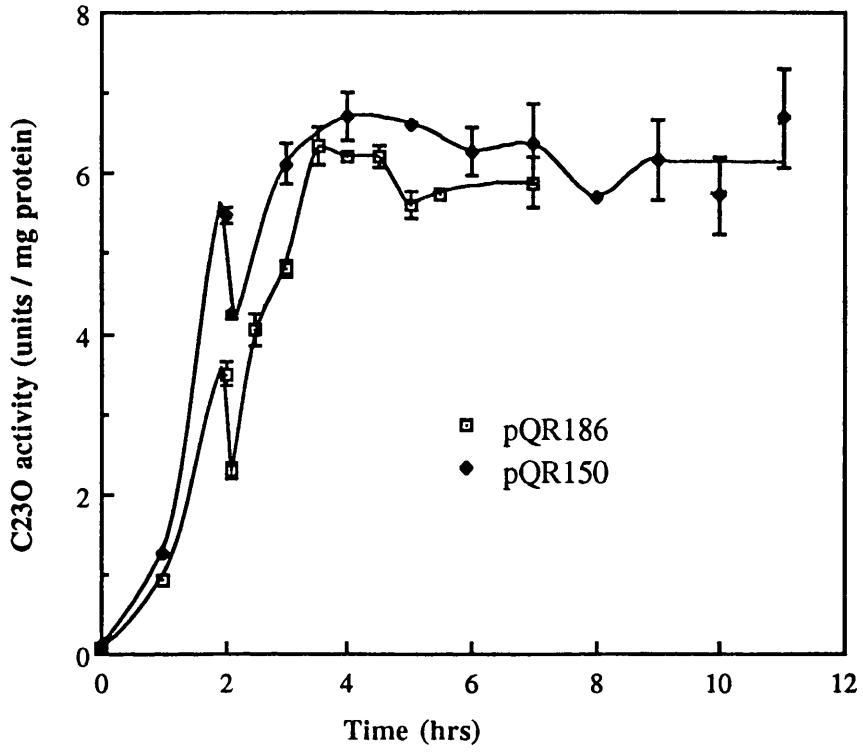


Fig 5.12. Catechol 2,3-dioxygenase activities during the metabolism of 10mM *m*-toluate by *E.coli* cells expressing either pQR150 or pQR186. Cells induced with IPTG at T=0. Substrate added at T=2.

5.3 Metabolism of *m*-toluate by *E.coli* cells expressing pQR186 and pQ194.

As a progression from these studies, pQR186 was co-expressed in *E.coli* with the plasmid pQR194 that expresses the *xylI* encoded oxalocrotonate decarboxylase. Metabolism of *m*-toluate through such cells was performed, and compared with the results of sections 5.1 and 5.2. Because *m*-toluate metabolism does not proceed through the *xylI* encoded step, any accumulation of 2-oxopent-4-enoate could not be attributed to an increase in flux through this step.

5.3.1 Shake-flask analysis of *m*-toluate metabolism by *E.coli* cells carrying pQR186 and pQR194.

Plasmids pQR186 and pQR194 are shown in Table 2.1, and were constructed according to the methods of fig 5.7 and fig 3.15 respectively. pQR194 was introduced

into an *E.coli* cell carrying pQR186 according to the methods of section 2.7.5, and the recombinant cells were grown, and the plasmids expressed in the presence of 10 mM *m*-toluate according to section 2.5.1.2.

The growth and production of the ring-cleavage intermediate were monitored as described previously, and the profiles shown in fig 5.13.

From fig 5.13, it appears that the presence of plasmid pQR194 reduces both the growth, and more significantly, results in a greater accumulation of 2-hydroxy-6-oxohepta-2,4-dienoate than seen during *m*-toluate metabolism by cells expressing pQR186 alone.

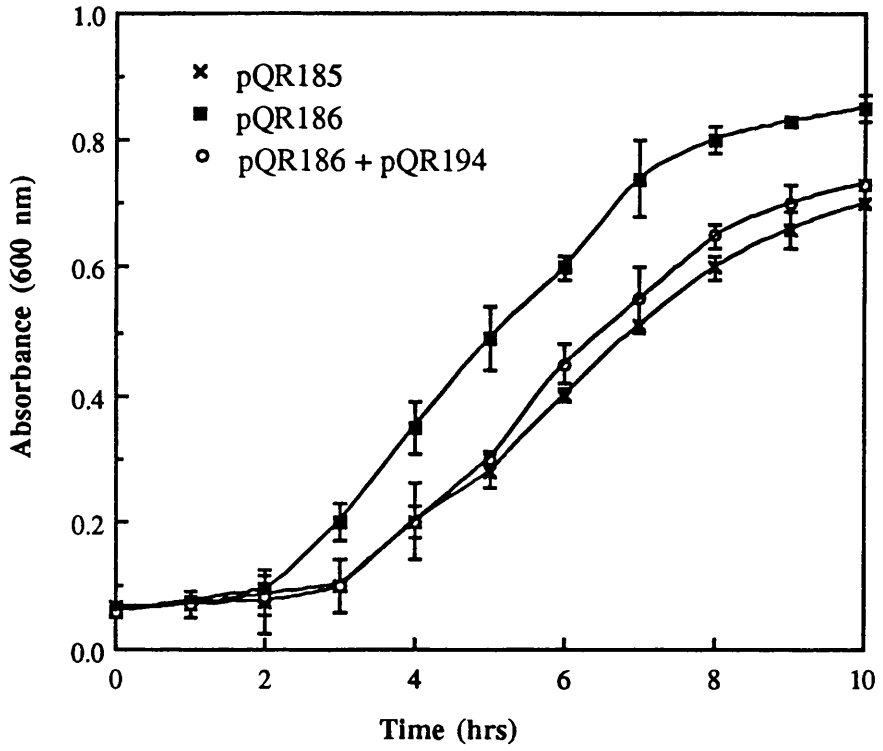


Fig 5.13 a. Growth of *E.coli* cells expressing either pQR185, pQR186 or both pQR186 and pQR194 in the presence of 10mM *m*-toluate. Substrate added at T=0.

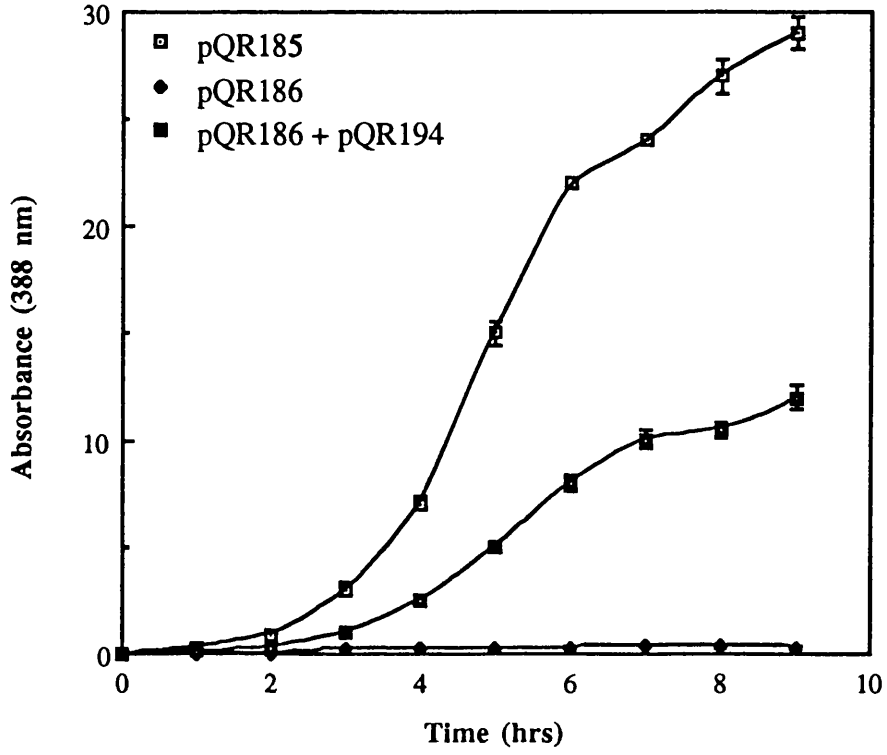


Fig 5.13 b. Production of 2-hydroxy-6-oxohepta-2,4-dienoate by *E.coli* cells expressing either pQR185, pQR186 or both pQR186 and pQR194 in the presence of 10mM *m*-toluate. Substrate added at T=0.

5.3.2 Fermenter studies of cells carrying plasmids pQR186 and pQR194.

In order to further investigate the effect on pQR186 activity by the co-expression of pQR194, controlled growth in fermenters was performed according to the methods of section 2.5.3. As in the shake-flask analysis of section 5.3.1, growth and more particularly ring-cleavage intermediate accumulation (fig 5.14 a, b) in the presence of plasmids pQR186 and pQR194 was significantly different to that observed during metabolism in the presence of pQR186 alone, suggesting that the presence of pQR194 induces an effect on metabolism that is seen more dramatically during metabolism by cells carrying pQR185.

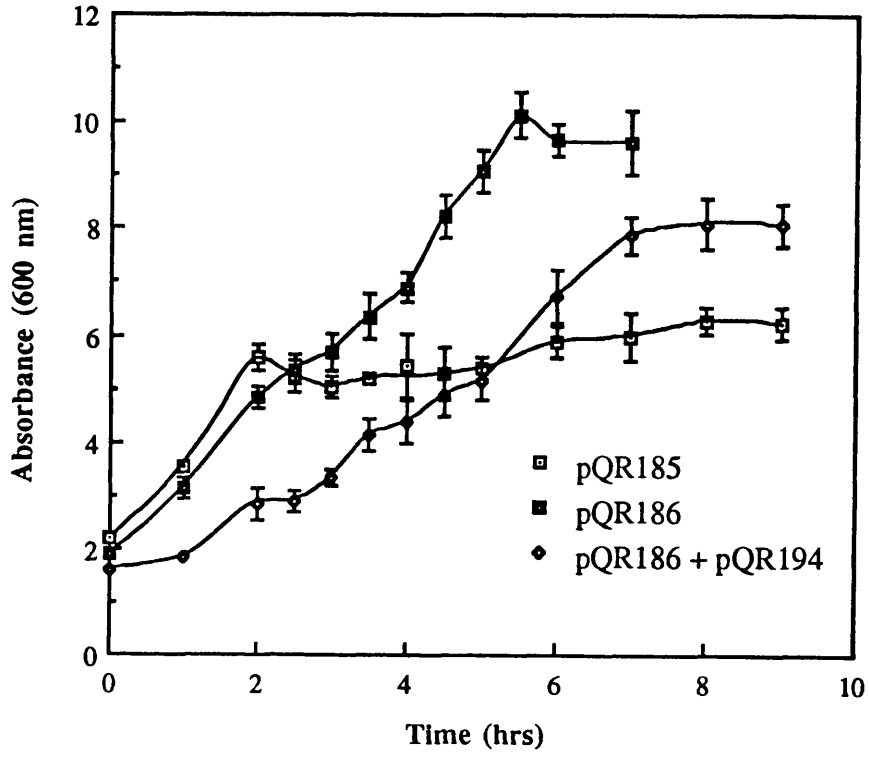


Fig 5.14 a. Growth of *E.coli* cells expressing either pQR185, pQR186, or both pQR186 and pQR194 in the presence of 10mM *m*-toluate. Substrate added at T=0.

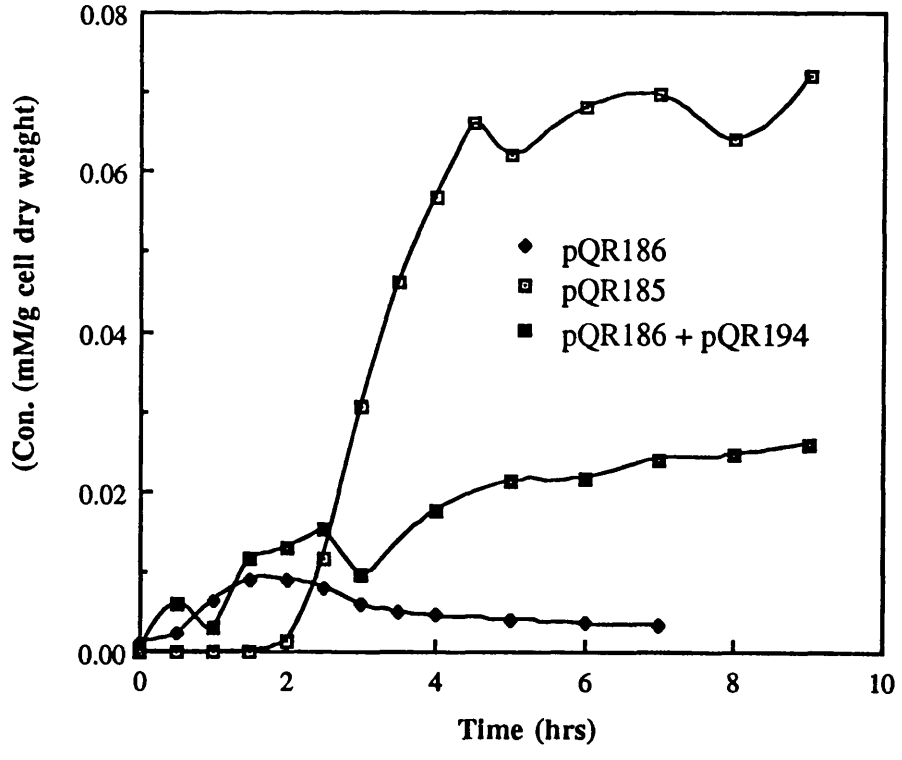


Fig 5.14 b. Production of 2-hydroxy-6-oxohepta-2,4-dienoate in fermenters by *E.coli* cells expressing either pQR185, pQR186, or both pQR186 and pQR194 in the presence of *m*-toluate. Substrate added at T=0.

Supernatant concentrations of other pathway intermediates were quantitated by HPLC and cze analysis (section 2.6.9 and 2.6.10) as shown in fig 5.15. As above, the profile of pathway intermediates resembled that seen during *m*-toluate metabolism by cells expressing pQR185 (fig 5.5b), characterised by low rates of *m*-toluate removal. In addition, levels of 4-hydroxy-2-oxovalerate accumulated during metabolism by cells expressing pQR186 and pQR194 were less than half that observed during metabolism by pQR186, suggesting a much lower XylJ activity in cells expressing pQR194 in addition to pQR186.

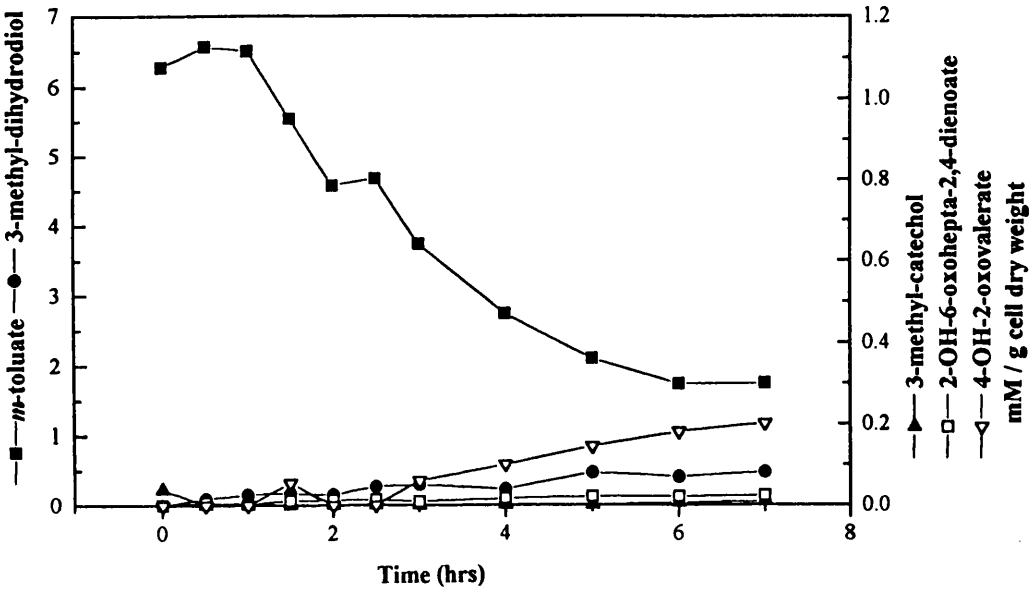


Fig 5.15. Supernatant concentrations of pathway intermediates during 10mM *m*-toluate metabolism by *E.coli* cells carrying plasmids pQR186 and pQR194. Substrate added at T=0.

This suggestion is reinforced by the comparative C23O activities observed during m-toluate metabolism by cells expressing pQR185, pQR186 or both pQR186 and pQR194 (fig 5.16).

In both pQR185 and pQR186 plus pQR194 metabolism, C23O activities drop significantly following the addition of pathway substrate, a characteristic associated with elevated levels of 2-oxopent-4-enoate. Thus, either 2-oxopent-4-enoate can be considered a C32O inhibitor as well as the 2-hydroxypent-2,4-dienoate, or high concentrations of 2-oxopent-4-enoate are indicative of inhibitory levels of the 2-hydroxypent-2,4-dienoate.

As stated previously, as *m*-toluate is not metabolised through the dehydrogenative branch of the *meta*-cleavage pathway, such a characteristic drop in C23O activity during *m*-toluate metabolism through pQR186 in the presence of

expressed plasmid pQR194 must be a result of an interaction between XylJ and the gene product of pQR194, resulting in reduced levels of XylJ activity, leading to increased pentenoate concentrations resulting in C23O inhibition. This in turn would account for the drop in 4-hydroxy-2-oxovalerate production by such cells. In his way the observations of section 3.3.3 can be explained in a similar fashion, indicating that in the case of benzoate metabolism, inhibition of C23O, together with the overall reduction in substrate removal is associated at least in part by a detrimental interaction between XylJ and pQR194 encoded XylI.

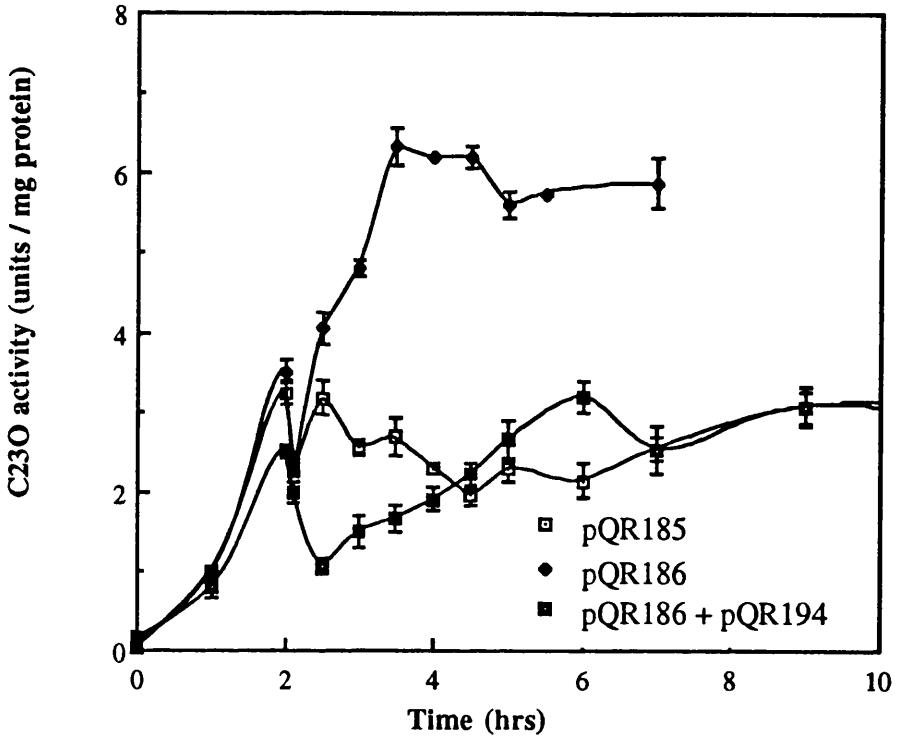


Fig 5.16. C23O specific activity during 10mM *m*-toluate metabolism by *E.coli* cells carrying either pQR185, pQR186 or both pQR186 and pQR194. Cells induced with IPTG at T=0. Substrate added at T=2.

5.4 The use of truncated *meta*-cleavage pathways to over-accumulate pathway intermediates.

The results of section 5.2 have already demonstrated how certain pathway intermediates can be accumulated as a result of constructing a truncated *meta*-cleavage pathway. In section 5.2, the pathway construct pQR186 was able to accumulate 2 mM levels of the chiral intermediate 4-hydroxy-2-oxovalerate from 10mM *m*-toluate.

As another example of this strategy of metabolic engineering, cells expressing the plasmid pQR185 were utilised to over-accumulate the product of the XylG catalysed step, 4-oxalocrotonate.

Fig 5.17 illustrates the growth and C23O activity observed during the metabolism of benzoate through the dehydrogenative branch of the *meta*-cleavage pathway (see fig 5.1 for a diagrammatic representation of pQR185 metabolism).

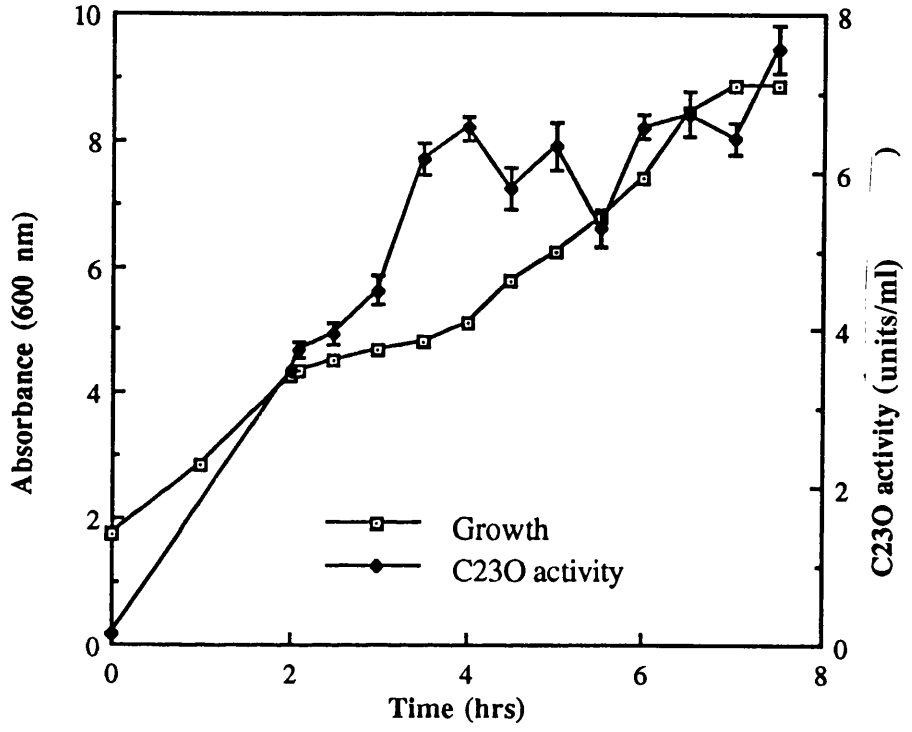


Fig 5.17. Growth and C23O activity during the metabolism in fermenters of 10mM benzoate by cells expressing pQR185. Benzoate added at T=0.

It is interesting to note that both growth and C23O activity were as observed during benzoate metabolism through the entire pathway (section 3.1.2).

Quantitation of pathway intermediates during pQR185 metabolism of benzoate (fig 5.18) illustrates the ability of such a truncated pathway to generate relatively large amounts of its end-product.

In comparison to section 3.1.2, a 100-fold increase in the concentration of 4-oxalocrotonate was generated through the use of a pathway that terminates metabolism at this step. The fact that rates of benzoate removal, together with levels of other pathway intermediates is comparable with that during metabolism through the entire pathway (fig 3.3) indicates that accumulating concentrations of 4-oxalocrotonate do

not affect the activity of the *meta*-cleavage pathway, unlike accumulating concentrations of 2-oxopent-4-enoate.

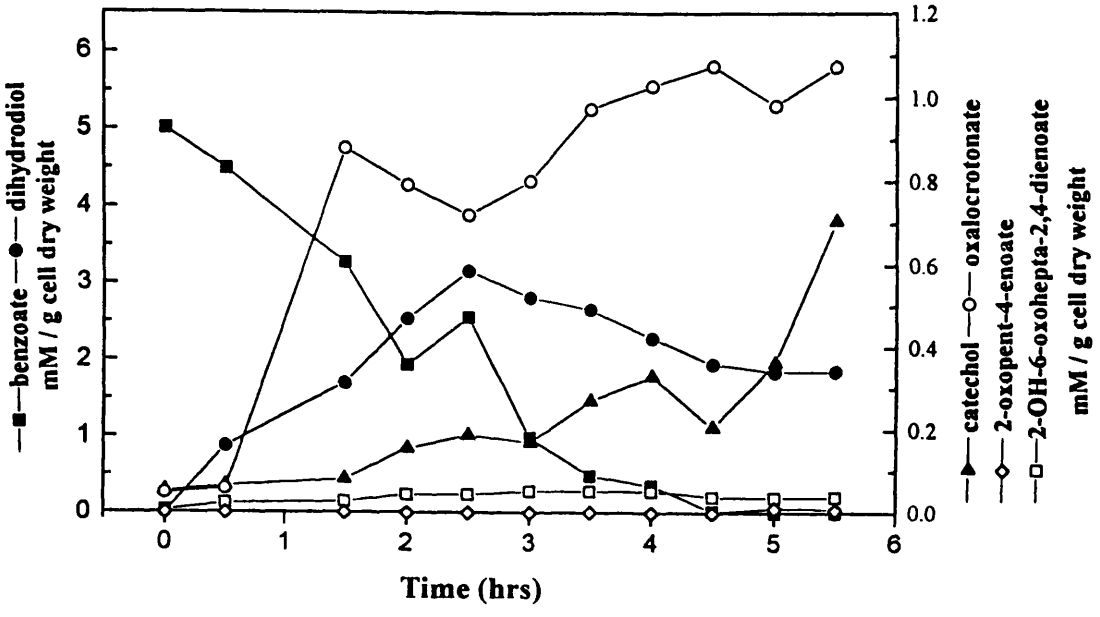


Fig 5.18. Supernatant concentrations of pathway intermediates during the metabolism of 10mM benzoate by *E.coli* cells expressing pQR185. Benzoate added at T=0.

Chapter 6: Broadening the substrate-range of the TOL plasmid *meta*-cleavage pathway.

In the following sections, all experiments were performed in duplicate unless stated otherwise.

6.1 Construction of hybrid benzoate (*xyl*) and biphenyl (*bph*) dioxygenases and their functional analysis.

Hirose *et al* (1994) have previously shown that active hybrid bacterial aromatic dioxygenase enzymes can be constructed by interchanging terminal oxygenase components. (section 1.1.1.4). In this study, hybrid gene clusters were constructed between genes of the TOL plasmid encoded benzoate dioxygenase (*xylXYZ*), and those of the *P.pseudoalcaligenes* KF707 biphenyl dioxygenase (*bphA1A2A3A4*) (Taira *et al*, 1992) as shown in figs 6.2 and 6.3.

The plasmids containing *xyl* and/or *bph* genes are shown in Table 2.1, and are schematically represented in fig 6.1. pQR191 and pQR192 contain various combinations of the hybrid *xyl-bph* gene clusters used in this analysis.

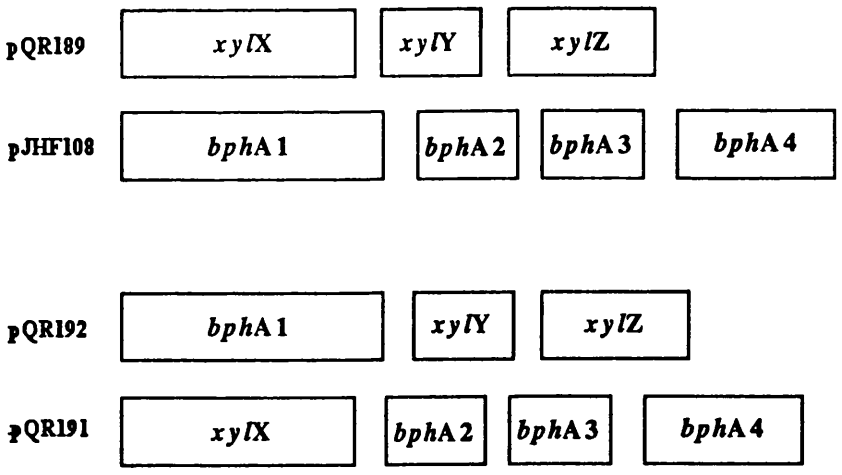


Fig 6.1. Schematic representations of plasmids carrying various hybrid and wild-type aromatic dioxygenase gene clusters

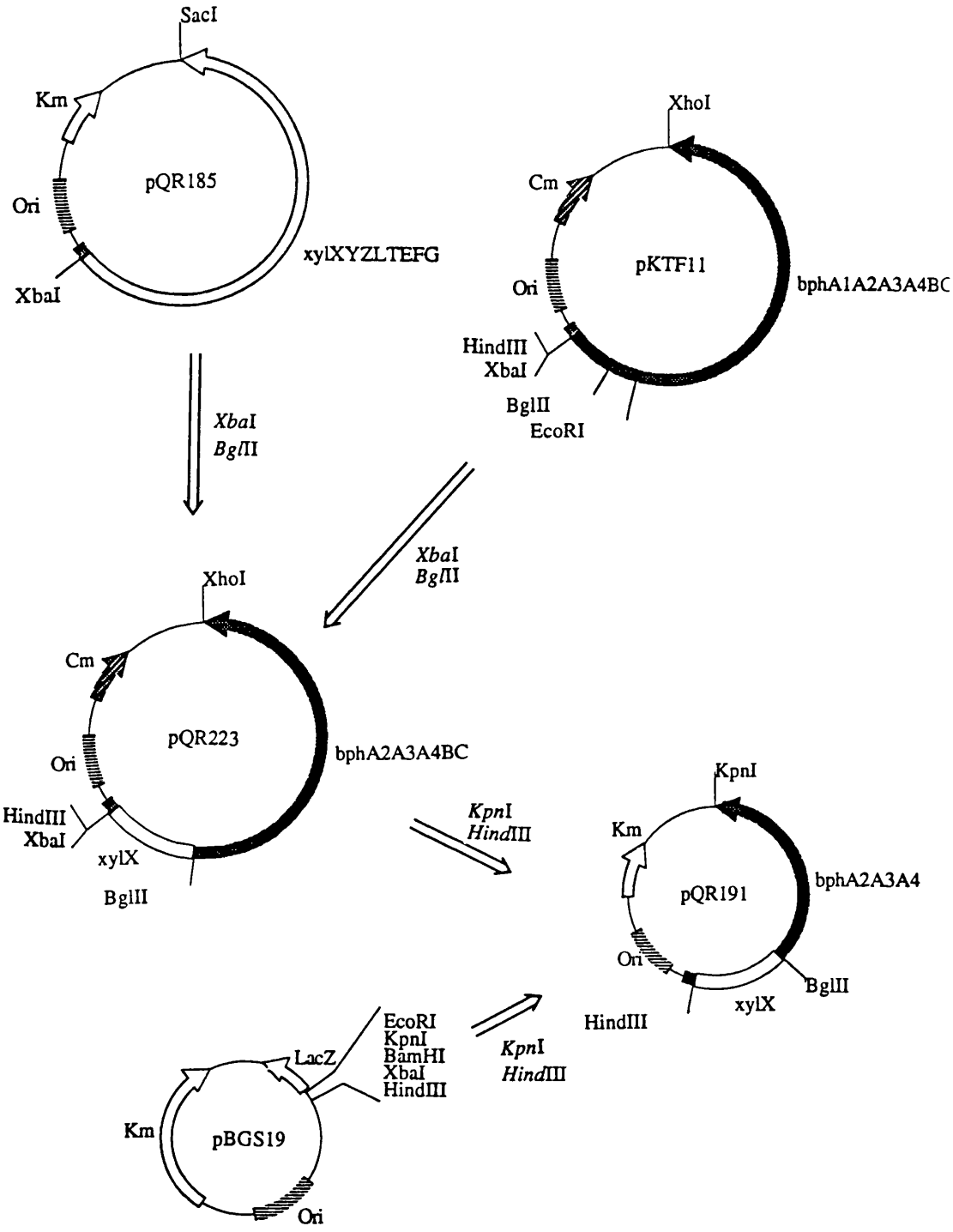


Fig 6.2. Construction of plasmid pQR191 (encoding the hybrid gene cluster *xylX:bphA2A3A4*). The presence of a *Bgl*II site downstream of *xylX* in pQR185 (this study), and a unique *Bgl*II site in the flanking region between *bphA1* and *bphA2* in pKTF11 (Taira *et al*, 1992) enabled the replacement of *bphA1* with *xylX*. 1 µg of both pQR185 and pKTF11 were double digested with *Xba*I and *Bgl*II (section 2.7.2) and the digested DNA combined in a ligation (section 2.7.3). Following transformation of competent *E.coli* JM107 (section 2.7.5), recombinants were sprayed with biphenyl and white colonies (indicating the absence of *bphA1* subcloned. DNA was purified from subclones (section 2.7.1.2) and the presence of the desired *xylX:bphA2A3A4BC* sequence (pQR223) confirmed by *Eco*R1 restriction digests. In order to isolate the hybrid dioxygenase sequence, (pQR223) and pBGS19 were double digested with *Hind*III and *Kpn*I, and ligated. Following transformation, recombinants were sprayed with catechol and yellow colonies subcloned. The presence of the 4.3kb *xylX:bphA2A3A4* insert in pBGS19 (pQR191) was confirmed by *Hind*III/*Xho*I digestion of purified DNA.

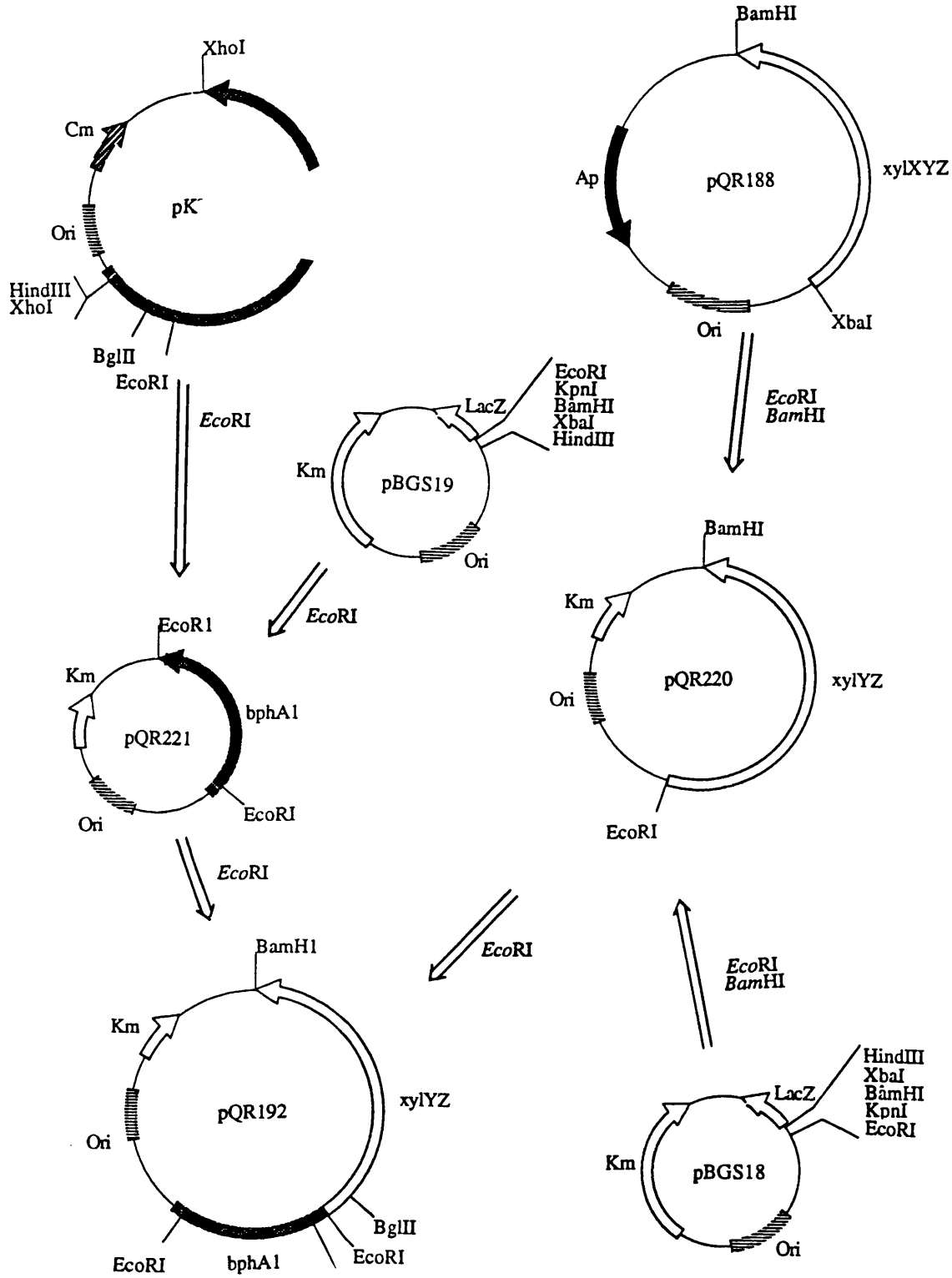


Fig 6.3. Construction of pQR192 (encoding the hybrid gene cluster *bphA1:xylYZ*). *BphA1* was isolated from pKTF11 by virtue of flanking *Eco*R1 sites either side of the gene. The *bphA1* gene was then linked to an *Eco*RI-*Bam*HI fragment encoding *xylYZ* in pBGS19. 1 µg of pKTF11 was digested with *Eco*RI, and the 1.5kb *bphA1* fragment gel purified (section 2.7.7). The gel purified fragment was subsequently ligated into 1 µg *Eco*RI digested pBGS19, forming the recombinant plasmid (pQR221), confirmed from transformed *E.coli* by *Eco*RI digestion. 1 µg of plasmid pQR188 was digested with *Eco*RI and *Bam*HI and the 2.3kb fragment encoding *xylYZ* was gel purified (section 2.7.7). The fragment was then ligated with *Eco*RI/*Bam*HI digested pBGS18 forming the resulting plasmid (pQR220). 1 µg of both (pQR221) and (pQR220) were subsequently digested with *Eco*RI and ligated together. Following transformation of competent *E.coli*, recombinant plasmids possessing the desired *bphA1:xylYZ* gene complex, together with the correct orientation of *bphA1* were selected by *Eco*RI digestion of purified DNA.

The substrate ranges of the wild-type and hybrid dioxygenases onstructed were examined for their ability to degrade the host substrates *m*-toluate and biphenyl. *E.coli* resting cells carrying various plasmids were incubated with the substrates according to the methods of section 2.5.2. The formation of dihydrodiols was monitored by measuring the absorption maxima of the respective dihydrodiol compounds (fig 6.4).

It has previously been shown that *E.coli* cells carrying plasmid pJHF108 (expressing wild-type *bphA1A2A3A4*) have the ability to readily convert biphenyl, 4-chlorobiphenyl and 4-methylbiphenyl to the respective dihydrodiols (Hirose *et al*, 1994). Similar rates of biphenyl conversion were obtained in this study, together with the observation that wild-type biphenyl dioxgenase is unable to convert *m*-toluate to the didydrodiol. In a similar fashion, *E.coli* cells carrying pQR188 (expressing wild-type *xylXYZ*) were able to convert *m*-toluate to the *cis*-dihydrodiols, but were as expected unable to catalyse the biotransformation of biphenyl, in accordance with the established narrow substrate specificity of benzoate dioxygenase.

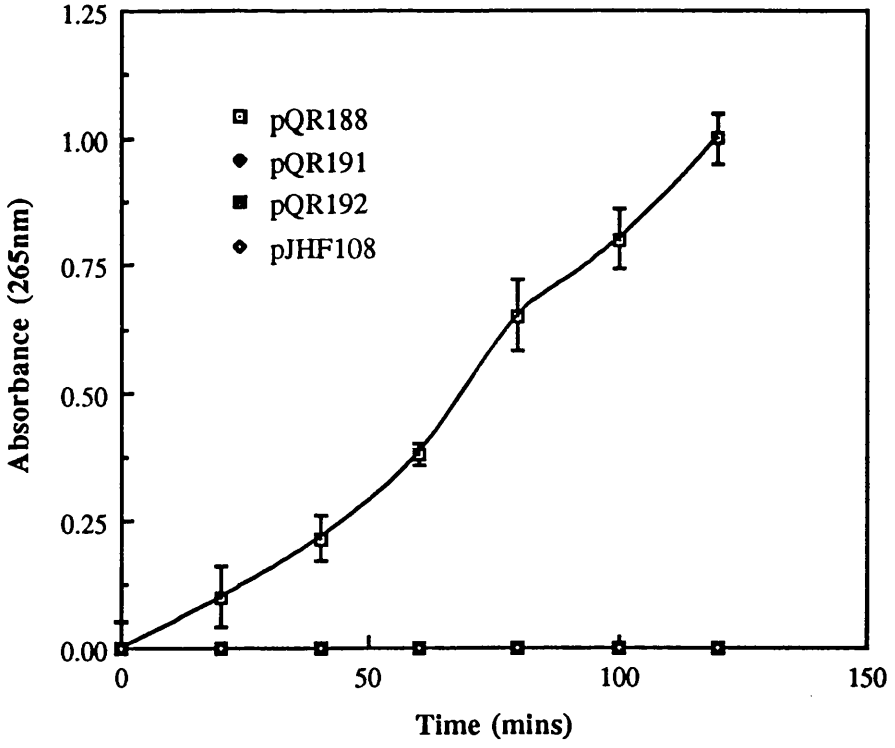


Fig 6.4 a. The production of *cis*-dihydrodiol from *m*-toluate by *E.coli* stains expressing wild-type or hybrid aromatic dioxygenases. (pQR188: *xylXYX*; pQR191: *xylXbphA2A3A4*; pQR192: *bphA1xylYZ*; pJHF108; *bphA1A2A3A4*).

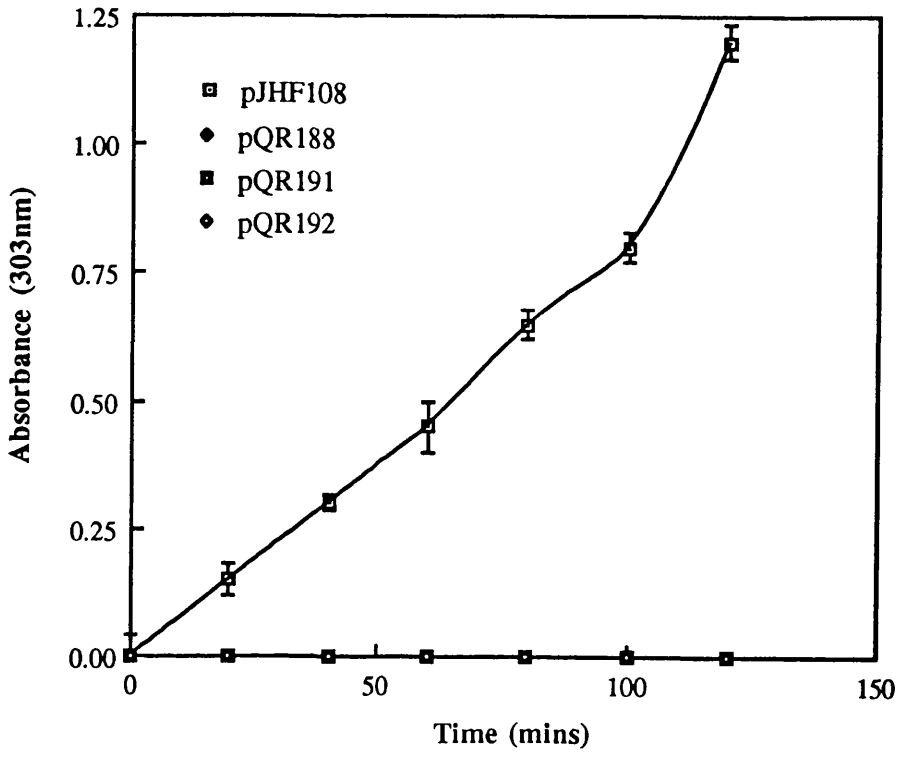


Fig 6.4 b. The production of *cis*-dihydrodiol from biphenyl by *E.coli* stains expressing wild-type or hybrid aromatic dioxygenases. (pQR188: *xylXYX*; pQR191: *xylXbphA2A3A4*; pQR192: *bphA1xylYZ*; pJHF108; *bphA1A2A3A4*).

As a result of interchanging the large terminal oxygenase subunits between the *xyl* and *bph* gene clusters, (forming the hybrid dioxygenases encoded by pQR191 [expressing *xylX:bphA2A3A4*] and pQR192 [expressing *bphA1:xylYZ*]) all enzymatic activity was lost. These results further validate the finding that the genes encoding the large subunits of aromatic dioxygenase terminal oxygenases (in this case *xylX* and *bphA1*) are critical for the enzymatic activity, and furthermore the substrate specificity of the wild-type enzymes. It appears however that such substrate specificities are not exchangeable with one another between the benzoate and biphenyl dioxygenenase complexes.

In order to investigate the inactivity of hybrid dioxygenases encoded by pQR191 and pQR192, amino acid sequence alignments of terminal oxygenase components were performed. The amino acid sequence identity between the *xylX* and *bphA1* gene components is 26% (fig 6.5), and that between *xylX* and the *todC1* gene component of the toluene dioxygenase of *P.putida* F1 27% (fig 6.6). These values are less than half the observed sequence identity shared between the *bphA1* and *todC1* (quoted as 65%, Furukawa *et al*, 1993), that are readily exchangeable in the active hybrid dioxygenases constructed by Hirose *et al* (1993).

```
bphA1.pep       MSSSIKEVQGAPVKWVTNWTPEAIRGLVDQEK--GLLDPR--IYADQSLYELELERVFGR
xylX.pep        MTMTM-----------HLGLDYIDSLVEEDENEGIYRCKREMFTDPRLFDLEMKHIFEG
                *. ..            .   . * .**...   *.   .  ...*  *..**. ..*

bphA1.pep       SWLLLGHESHVPETGDFLATYMGEDPVVMVRQKDKSIKVFLNQCRHRGMRICRSDAGNAK
xylX.pep        NWIYLAHESQIPEKNDYYTTQMGRQPIFITRNKDGELNAFVNACSHRGATLCRFRSGNKA
                .*. *.***..** *. .* ** .*. . *.**  .. *.* * ***  .**  .**

bphA1.pep       AFTCSYHGWAYDIAGKLVNVPFEKEA-FCDKKEGDCGFDKAEWGPLQ--ARVATYKGLVF
xylX.pep        THTCSFHGWTFSNSGKLLKVKDPKGAGYPD------SFDCDGSHDLKKVARFASYRGFLF
                . ***.***..  .***..*  * * . *      .**      *.  ** *.*.*..*

bphA1.pep       ANWDVQAPDLETYLGDARPYMDVMLDRTPAGTVAIGGMQKWVIPCNWKFAAEQFCSDMYH
xylX.pep        GSLREDVAPLEEFLGESRKVIDMVVDQSPEGLEVLRGSSTYVYEGNWKVQVEN-GADGYH
                ..   . . ** .**..*  .*...*..* *   . *    *   ***   *.  .* **

bphA1.pep       AGTMSHLSGILAGMPPEMDLSHAQVPTKGNQFRAGWGGHGSGWFVDEPGM----------
xylX.pep        VSTV-HWN--YAATQQQRKLRDAGDDIRAMT-ASSWGGDGGGFYSFENGHQMVWARWGDP
                 .*. * .   *.   .  * .*    ..    ..***.*.* .  * *

bphA1.pep       ----LMAVMGPKVTQY------WTEGPAADLA-------EQRLGHTMPVRRMFGQHMSVF
xylX.pep        KNRPLFAERDRLASEFGEARADWMIGVSRNLCLYPNLYLMDQFGSQLRITRPLSVDRTEI
                    * *  .   ...      * * . .*          ...*  . . * .. . . .

bphA1.pep       PTCSFLPaINTIRTWHPRGPNEIEVWAFTLVDADAPAEIKEEYRRHNIRTFSAGGVFEQD
xylX.pep        TIYCIAPKGETPR--RARRVRQYEDF-FNVSGMATPDDL-EEFRACQ-EGFAGGGMNDMS
                    . *  .* *  ..*   . *   * . .  .* .. **.*  .   *..**. .

bphA1.pep       DGE-NWVEIQKGLRGYKAKSQPLNAQMGLGRSQTGHPDFPGNVGYVYAEEAARGMYHHWM
xylX.pep        RGAKHWIEGPDE----GAKEIDLHPKLSGVRSE-DEGLFVMQHKY-WQQQMIKAVKREQD
                 *  .*.*         **   *.....  **. ..  *  .  *   ..  ... ..

bphA1.pep       RMMSEPSWATLKP
xylX.pep        RLIHAEG-----V
                *..   .
```

Fig 6.5. Computer generated amino acid sequence homology between *xylX* and *bphA1*. (* indicates identical amino acids.)

```
xylX.pep        MTMT----MHLGLDYIDSLVEEDENEGIYRCKREMFTDPRLFDLEMKHIFEGNWIYLAHE
tod.pep         MNQTDTSPIRLRRSWNCSEIEALFDEHAGRIDPRIYTDEDLYQLELERVFARSWLLLGHE
                *  *    ..*      * .*   .*   *    ..** *..**. ..*  .*. *.**

xylX.pep        SQIPEKNDYYTTQMGRQPIFITRNKDGELNAFVNACSHRGATLCRFRSGNKATHTCSFHG
tod.pep         TQIRKPGDYITTYMGEDPVVVVRQKDASIAVFLNQCRHRGMRICRADAGNAKAFTCSYHG
                .**    ** ** ** .*. . *.**. .  *.* * ***  .**  .**  . ***.**

xylX.pep        WTFSNSGKLLKVKDPKGAGY-PDSFDCDGSHDLKKV-ARFASYRGFLFGSLREDVAPLEE
tod.pep         WAYDTAGNLVNV------PYEAESFACLNKKEWSPLKARVETYKGLIFANWDENAVDLDT
                *..  .*.*..*       * ..** *    .   . **  .*.*..*..  *.   *.

xylX.pep        FLGESRKVIDMVVDQSPEGLEVLRGSSTYVYEGNWKVQVEN-GADGYHVSTV-HWN--YA
tod.pep         YLGEAKFYMDHMLDRTEAGTEAIPGVQKWVIPCNWKFAAEQFCSDMYHAGTTSHLSGILA
                .***..  .* ..*..  * * . *    *   ***   *.  .* ** .*  * .   *

xylX.pep        ATQQQRKLRD-AGDDIRAMTASSWGGDGGGFYSFENGHQMVWARWGDPKNRPLFAERDRL
tod.pep         GLPEDLEMADLAPPTVGKQYRASWGGHGSGFYV-----------GDPNL--MLAIMGPK
                .  ..  . * *   .     .****.*.***             ***.   ..*  .

xylX.pep        ASEFGEARADWMIGVSRNLCLYPNLYLMDQFGSQLRITRPLSVDRTEITIYCIAPKGETP
tod.pep         VTSY------WTEGPASEKA-------AERLGSVERGSK-LMVEHMTVFPTCSFLPGINT
                 . .      *  * . .          ...**  * .. * *..  .   *    *

xylX.pep        RRARRVRQYEDF----FNVSGMATPDDL-EEFR-ACQEGFAGGGMNDMSRGAKHWIEGPD
tod.pep         VRTWHPRGPNEVEVWAFTVVDADAPDDIKEEFRRQTLRTFSAGGVFEQDDGE-NWVEIQH
                *... *  ..     * * .  .***. ****      *..**. .   *  .*.*  .

xylX.pep        ----EGAKEIDLHPKLSGVRS-EDEGLF---VMQHKY----------WQQQMIKAVKRE
tod.pep         ILRGHKARSRPFNAEMSMDQTVDNDPVYPGRISNNVYSEEAARGLYAHWLR-MMTSPDWD
                    . *.   ... .*  .. ... ..   . .. *          * . *..  ..

xylX.pep        QDRLIHAEGV
tod.pep         ALKATR----
                  . .
```

Fig 6.6 Computer generated amino acid sequence homology between *xylX* and *todC1*. (* indicates identical amino acids.)

6.2 Broadening of the substrate range of the TOL plasmid by the *in-vivo* co-expression of *nahA* with pQR150.

As a result of the inability to alter the substrate range of the TOL plasmid *meta*-cleavage pathway by the *in-vitro* construction of hybrid aromatic dioxygenases, using a combination of *xyl* and *bph* genes, a different strategy was developed that involved the introduction into an *E. coli* cell carrying the plasmid pQR150 of a second plasmid expressing a different and compatible wild-type aromatic dioxygenase.

In developing this strategy it was necessary to identify an aromatic dioxygenase that itself possesses a suitably broad substrate range. The biphenyl dioxygenase of *P.putida* KF707 is able to metabolise a range of chlorinated biphenyls such as 4-chloro- and 4-methylbiphenyl, but is unable to oxidise benzene, naphthalene, toluene (Hirose *et al,* 1994), *m*-toluate and benzoate (this study). As a result biphenyl dioxygenase was not initially considered in this strategy.

The naphthalene dioxygenase of *P.putida* G7 had previously been cloned into the broad host range vector pMMB66EH (Shresthra, 1994) forming the plasmid pSS2. The substrate range of this bacterial dioxygenase was examined for benzene, toluene, biphenyl and naphthalene. *E.coli* resting cells carrying pSS2 were incubated with these substrates according to the methods of section 2.5.2. The formation of dihydrodiols was monitored by measuring the absorption maxima of the respective dihydrodiol compounds (fig 6.7). As a control experiment, JM107 *E.coli* resting cells harbouring no plasmid were incubated under the same conditions. In this case no dihydrodiol accumulation was observed.

Having confirmed the ability of pSS2 to oxidise toluene, biphenyl, benzene as well as the host substrate naphthalene to the respective dihydrodiols, the plasmid pSS2 was transformed into competent *E. coli* cells carrying pQR150 (see Table 2.1) using the methods of sections 2.7.4 and 2.7.5 Recombinant *E. coli* cells carrying both pSS2 and pQR150 were selected on Km/Amp agar plates, and the presence of *nahA* activity confirmed by ability of recombinant strains to form dark-blue colourations due to the formation of indigo in the presence of indole (see section 2.4). *E. coli* cells carrying pQR150 alone were unable to produce indigo, indicating that this activity is as a result of the *nahA* as aposed to endogenous pQR150 activity. The presence of pQR150 activity in recombinant cells was confirmed by the production of α-hydroxymuconic-semialdehyde in the presence of catechol (section 2.4).

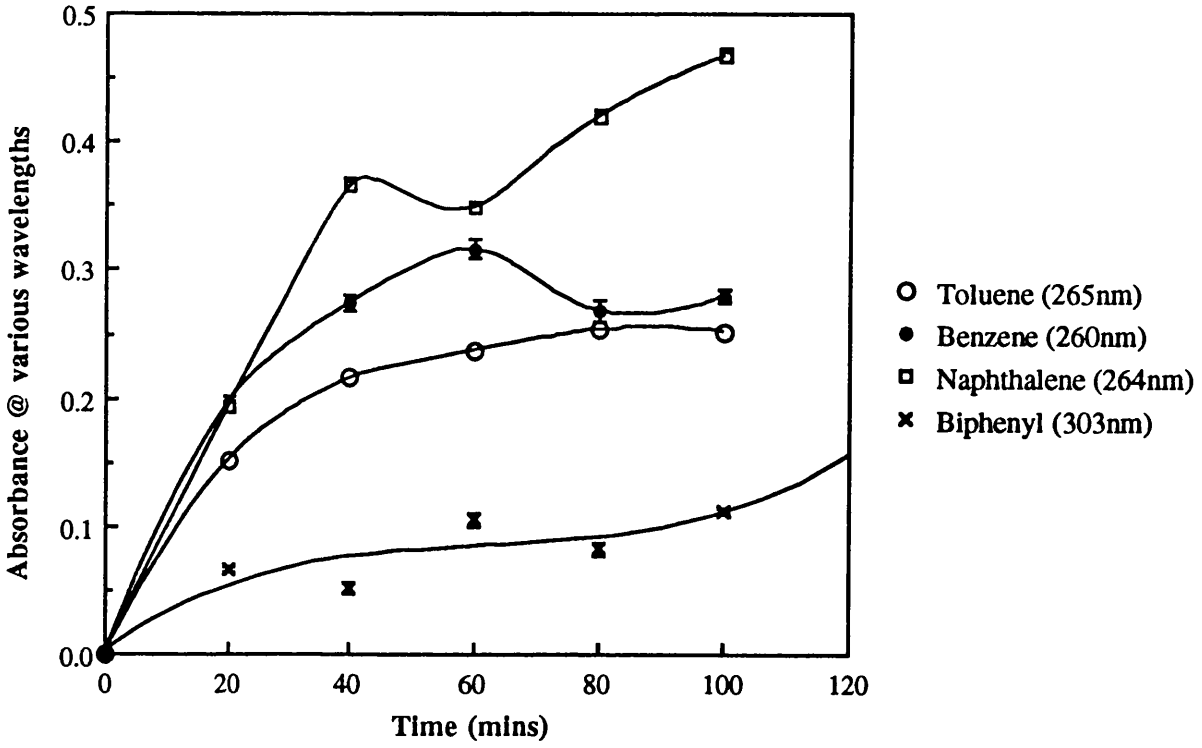


Fig 6.7. The production of dihydrodiol compounds from different aromatic substrates by *E.coli* cells carrying pSS2.

E.coli cells carrying both pSS2 and pQR150, or pQR150 alone as a negative control were grown in the presence of different aromatic substrates according to the methods of section 2.5.1.2, and the growth monitored spectrophometrically (fig 6.8).

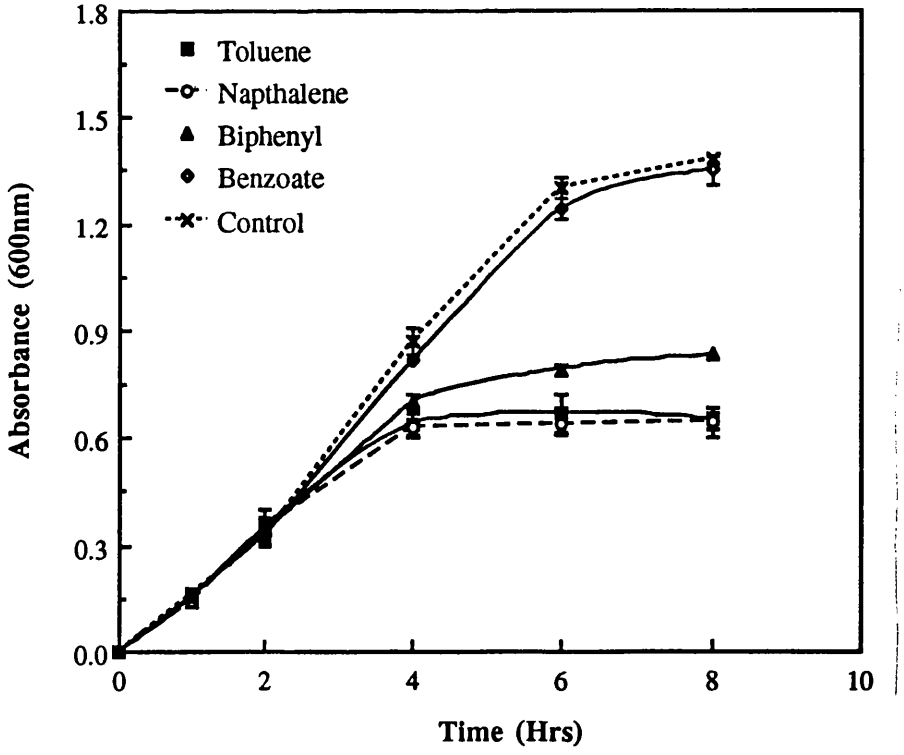


Fig 6.8 a. Growth of *E.coli* cells expressing pSS2 and pQR150 in the presence of biphenyl, benzoate, naphthalene or toluene.

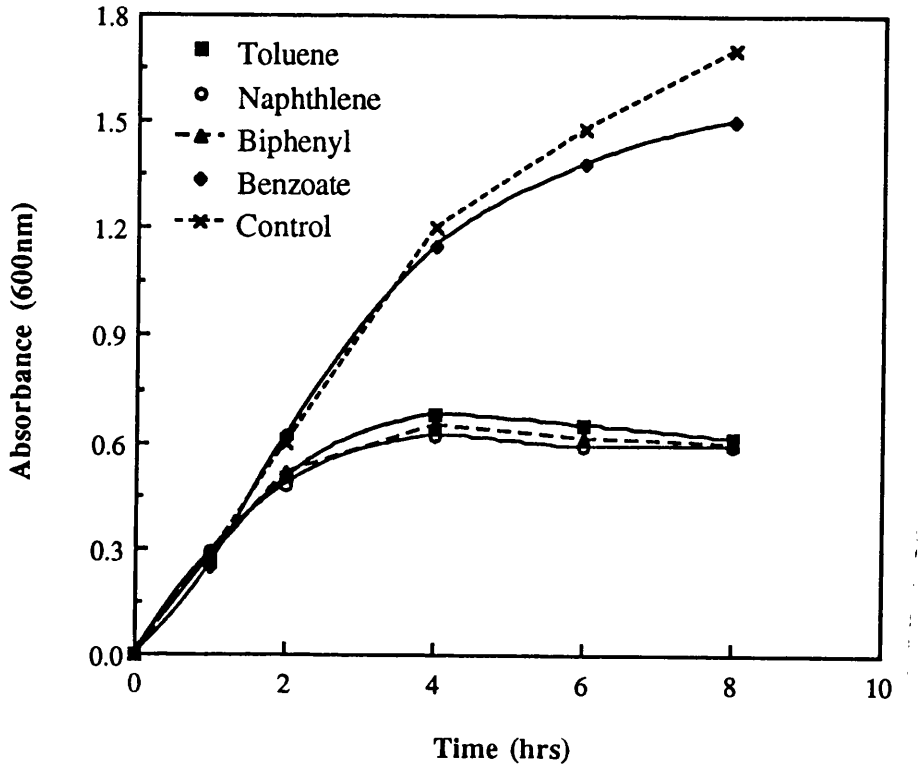


Fig 6.8 b. Growth of *E.coli* cells expressing pQR150 in the presence of biphenyl, benzoate, naphthalene or toluene.

As an indication of the metabolism of the aromatic substrates during the period of growth, sample were monitored by scanning spectrophotometrically ection 2.6.8) for the production of ring-cleavage intermediates (fig 6.9).

In the case of cells harbouring pSS2 together with pQR150, the spectrophometric data shown in fig 6.9 indicates that benzoate, biphenyl, naphthalene and toluene are all metabolised as far as ring-cleavage intermediates. In the case of the metabolism of toluene, biphenyl and benzoate, the appearance of yellow bacterial pellets upon centrifugation was also a further indication of the presence of ring-cleavage intermediates.

For cells harbouring pQR150 alone, such characteristic results are only apparent during the metabolism of the host-substrate benzoate.

According to the observations shown in fig.6.8, the similarities in growth shown between cells in the presence or absence of aromatic compounds indicates that the use of flasks with central wells in this way does not provide significant levels of aqueous solvent. In addition, the transient levels of ring-cleavage intermediate observed may be related to the presence of enzymes downstream of C23O

metabolising the small amounts of ring-cleavage intermediate generated from toluene, biphenyl and naphthalene. This in itself is an interesting speculative finding.

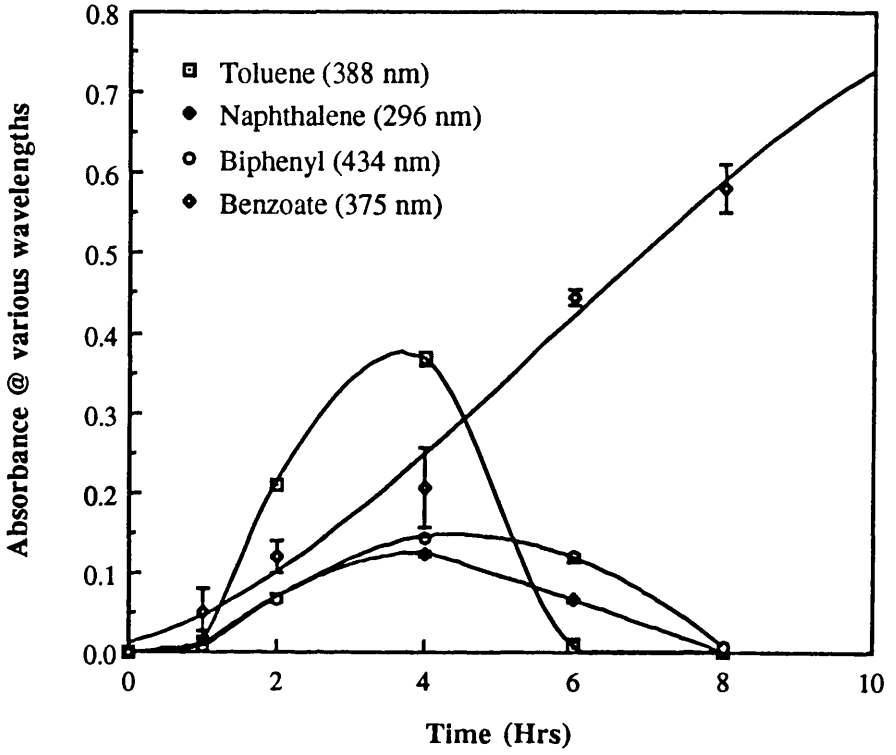


Fig 6.9 a. The production of *meta*-ring cleavage intermediates as a result of metabolism of benzoate, biphenyl, naphthalene and toluene by *E.coli* cells expressing pSS2 and pQR150.

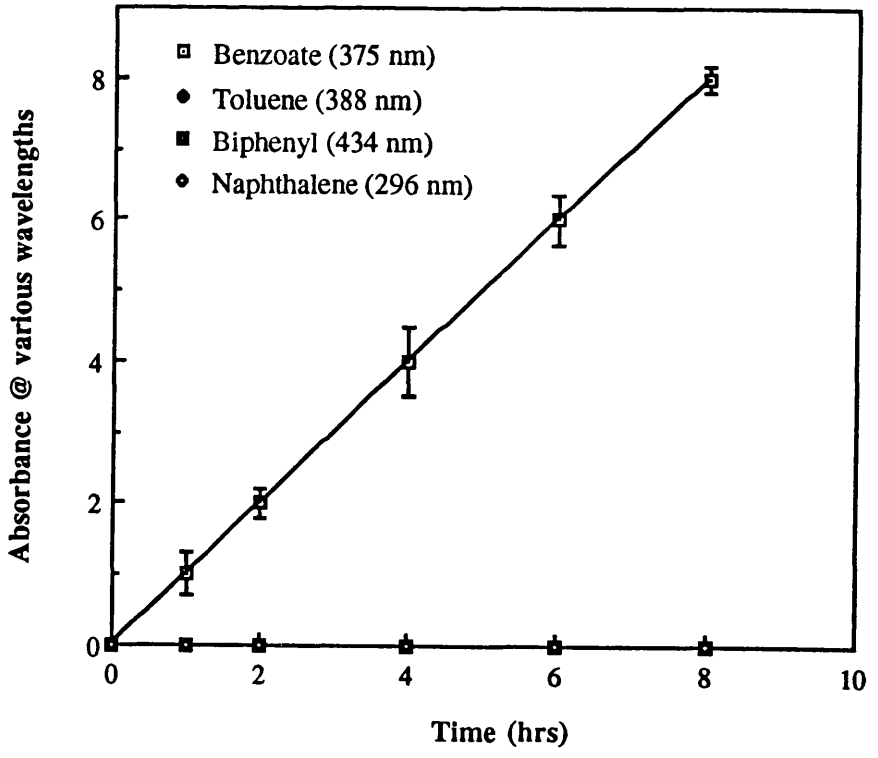


Fig 6.9 b. The production of *meta*-ring cleavage intermediates as a result of metabolism of benzoate, biphenyl, naphthalene and toluene by *E.coli* cells expressing pQR150.

6.3 The co-expression of *nahA* with pQR226.

In order to improve the design of the above experiments, a truncated mutant of the *meta*-cleavage pathway was constructed in which metabolism of benzoate or *m*-toluate is terminated with the production of ring-cleavage intermediates. The construction of this plasmid, pQR226 is shown in fig.6.10.

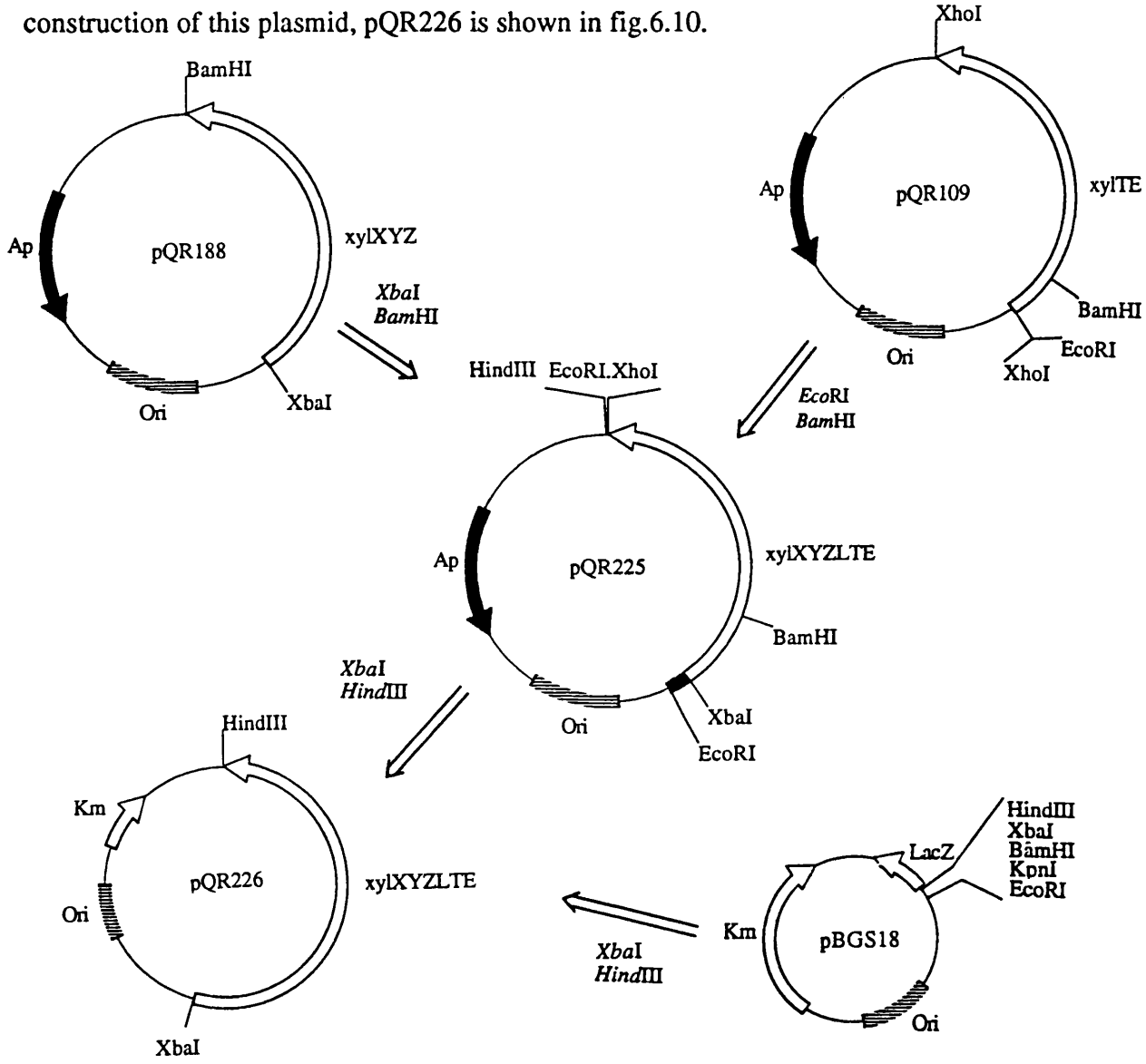


Fig 6.10 Construction of pQR226 (expressing *xylXYZLTE*). 1 µg of pQR188 was digested with *Xba*I and *Bam*HI, and the 3.48 kb fragment encoding *xylXYZ* separated and gel purified according to the methods of section 2.7.7. In addition, 1 µg of pQR109 was digested with *Eco*RI and *Bam*HI, and the 4.74 kb fragment encoding the *xylLTE* gene cluster, together with other pUC8 sequences was gel purified in a similar manner. The two fragments were subsequently ligated with an equimolar concentration of synthesized oligonucleotide linkers (Pharmacia Biotech.), designed to possess *Eco*RI and *Xba*I restriction sites in order to the two isolated fragments. The ligation mix was used to transform *E.coli* JM107 according to the methods of section 2.7.5. Recombinant cells containing a *meta*-cleavage pathway truncated at *xylE* were selected as yellow colonies on agar plates containing IPTG, Amp, and 10 mM sodium benzoate. DNA was purified from isolated colonies and digested with *Xba*I and *Bam*HI in order to confirm the structure of the truncated pathway. This construct was naned pQR225 The *xylXYZLTE* gene cluster was subsequently sub-cloned into pBGS18 on a *Xba*I-*Hind*III fragment according to the methods of section 2.7, forming pQR226.

E.coli resting cells carrying the plasmid pQR226 and pSS2 were subsequently used in biotransformation studies according to methods based on those described in section 2.5.2. Substrates were injected directly through air-tight bungs into the resuspended cells by the use of a Hamilton syringe to a working concentration of 0.5 mM. In this way, any loss of substrate through evaporation was limited. The degradation of the aromatic substrates benzoate, naphthalene, biphenyl and toluene, together with the concurrent production of dihydrodiol and ring-cleavage intermediate were measured as described previously and are shown in figs. 6.11, 6.12 and 6.13.

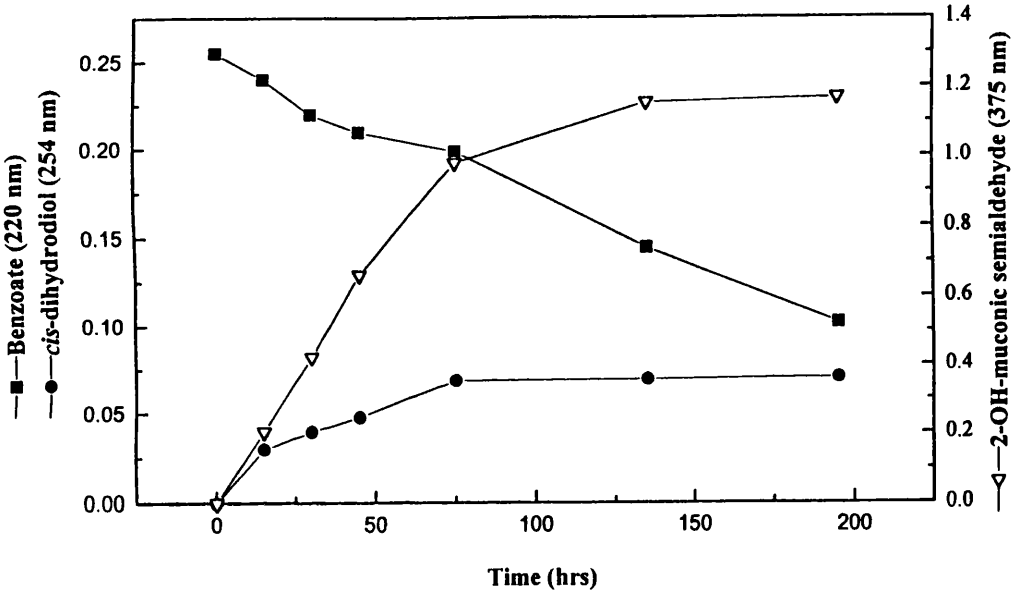


Fig.6.11. The metabolism of benzoate by *E.coli* cells expressing pQR226 and pSS2.

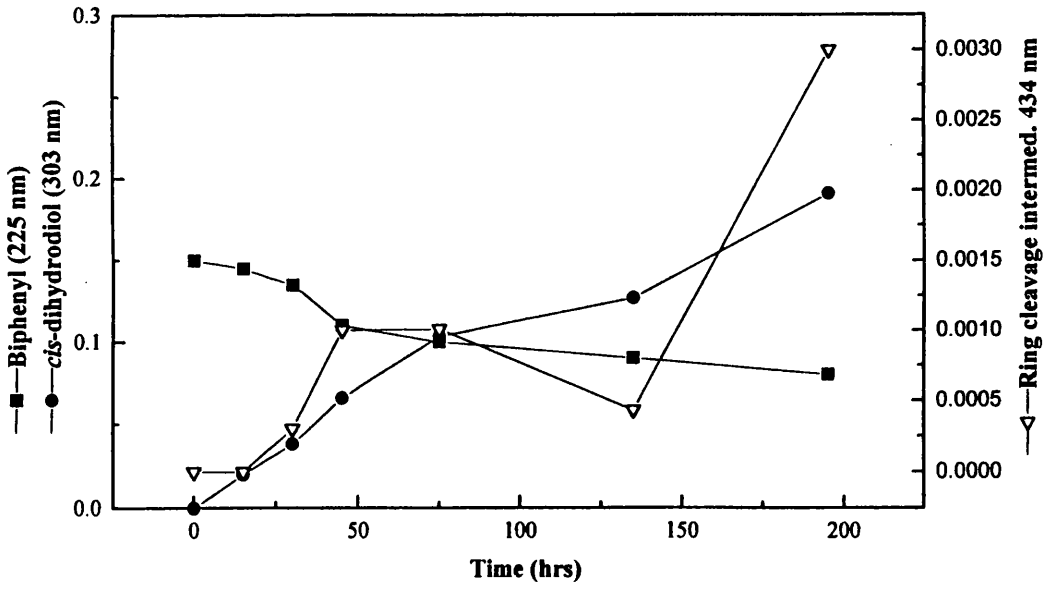


Fig.6.12. The metabolism of biphenyl by *E.coli* cells expressing pQR226 and pSS2.

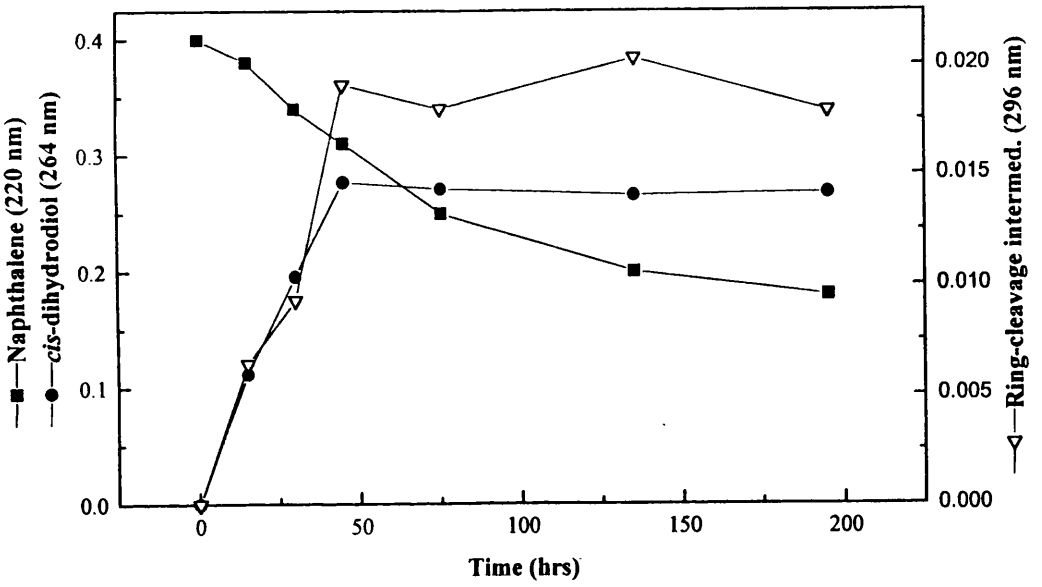


Fig.6.13. The metabolism of naphthalene by *E.coli* cells expressing pQR226 and pSS2.

In accordance with the observation shown in fig.6.9 b, cells carrying pQR226 in the absence of pSS2 were only capable of benzoate metabolism.

As can be seen in fig. 6.13, the use of a truncated *meta*-cleavage pathway results in a accumulation of the ring-cleavage intermediate upon the metabolism of naphthalene. In the case of biphenyl metabolism, significant levels of dihydrodiol were obtained, but with only low levels of ring-cleavage intermediate. The metabolism of toluene failed to yield reproducible results, possibly due to the toxic nature of residual toluene.

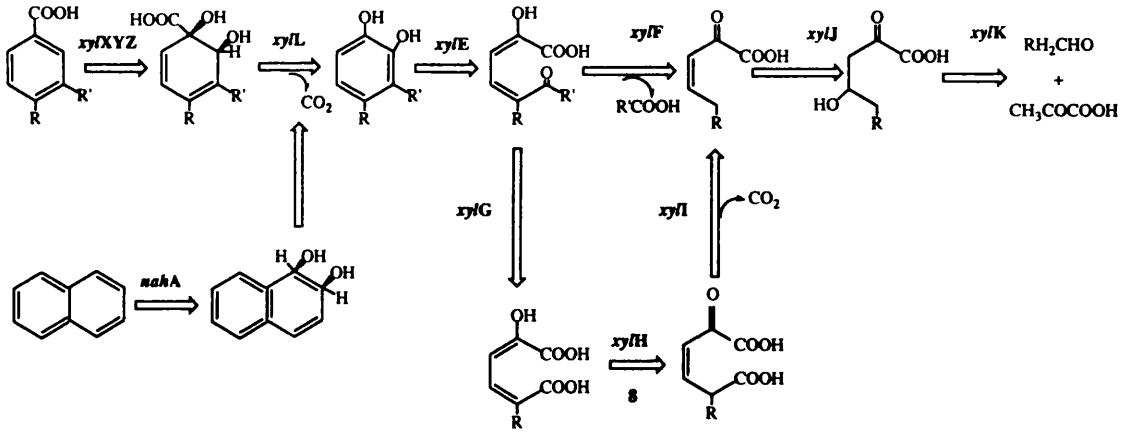


Fig 6.14. The channelling of aromatic substrates into the TOL plasmid *meta*-cleavage pathway as a result of the co-expression of *nahA* with pQR150.

These findings suggest that the co-expression of *nahA* with pQR150 enables the channelling of intermediates from different metabolic pathways into the TOL plasmid *meta*-cleavage pathway. Such a 'throats and stomachs' arrangement for metabolism is illustrated in fig.6.14

6.4 Development of an expression cassette for the production of novel metabolic intermediates.

Having established that it is possible to channel naphthalene into the TOL plasmid *meta*-cleavage pathway, such a metabolic arrangement may be further exploited to produce novel metabolic intermediates. Such a system may involve the incorporation of the *catA* encoded catechol 1,2-dioxygenase, and the *catB* encoded *cis,cis*-muconate lactonizing enzyme from the β-ketoadipate pathway of *Acinetobacter calcoaceticus*. In this way, substrates such as naphthalene may be channelled through to the production of *cis,cis*-muconate and muconolactone versions of these compounds.

In order to facilitate the introduction of such exogenous enzymes into a pre-existing metabolic pathway, a multiple cloning site has been incorporated downstream of the *xylL* encoded *cis*-dihydodiol dehydrogenase, forming pQR227 The construction of plasmid pQR227 is shown in fig.6.15. The multiple cloning site of pQR227 contains restriction sites not present in either the *xylXYZL* or *catABC* gene clusters. As a result, the *catA* or *catB* genes may be amplified from genomic DNA of *A.calcoaceticus* using primers incorporating restriction sites that will allow the introduction of such genes into pQR227 in the correct reading frame and orientation.

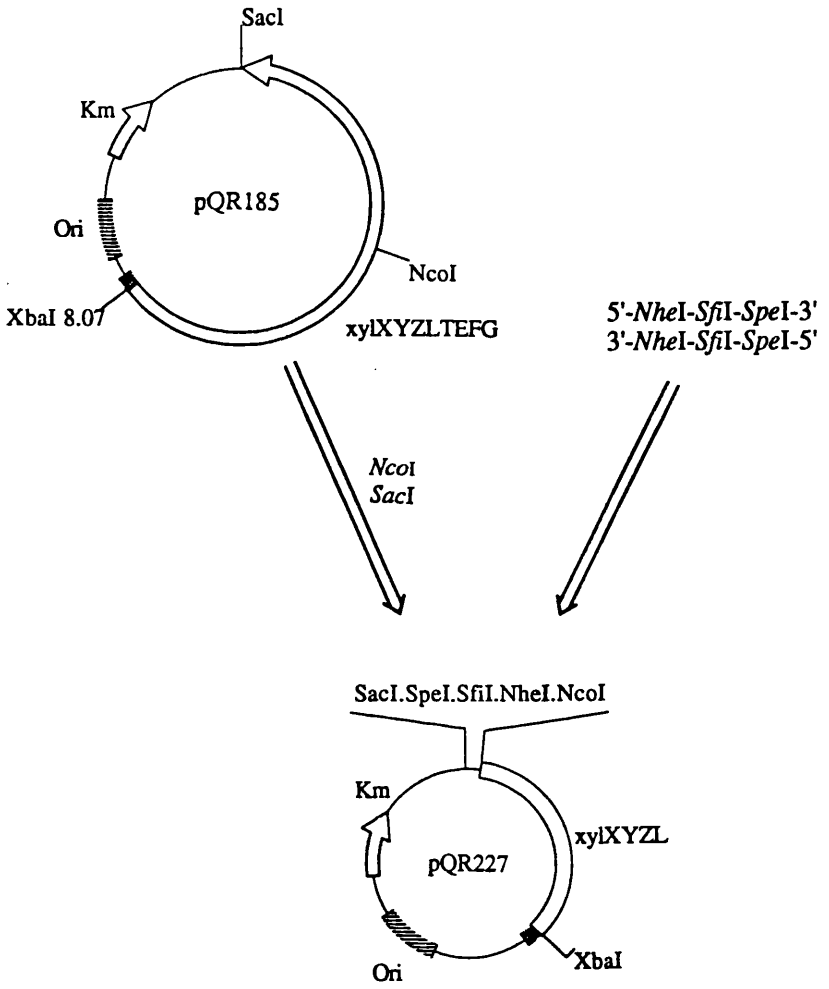


Fig 6.15. Construction of plasmid pQR227 1 µg of plasmid pQR185 was digested with *Nco*I and *Sac*I. The large 8.5 kb fragment was separated and gel purified according to the methods of section 2.7.7. This fragment was then ligated with an equimolar concentration of two self-complimenting oligonucleotides possessing the sequence 5'CATGGCCTAATAGGCCATGCTAGCATCGACTAGTTCAGGAGCT3', and 5'CCTGAACTAGTCGATGCTAGCATGGCCTATTAGGC3' that encode a 5'-*Nhe*I-*Sfi*I-*Spe*I 3' multiple cloning site. The ligation mix was used to transform *E.coli* JM107 by the methods of section 2.7.5. Colonies were isolated that remained white in the presence of catechol (due to the absence of *xylE*). DNA was purified from such colonies, and the presence of the multiple cloning site confirmed by performing digestion *Nhe*I, *Sfi*I and *Spe*I digestions, as well as *Xba*I/*Sac*I double digestions. The correct sequence of the inserted oligonucleotide was confirmed by dideoxy sequencing using M13 reverse primers (see section 2.7.9).

Chapter 7 Discussion.

The use of metabolic engineering as a means of achieving higher efficiencies in either the production of pathway metabolites, or in the rate of degradation of a pathway substrate is now widely recognised. In the latter case, this has been achieved largely by the identification and subsequent removal of rate-limiting steps within a metabolic pathway.

An example of how such a strategy has been adopted is described in section 1.1.1.3. This example illustrates how auxotrophic *E. coli* mutants lacking different aromatic acid biosynthetic pathway enzymes were generated and used to indicate how control of metabolic flux is distributed through such a pathway (Dell and Frost, 1993).

Although effective in pin-pointing the main flux controlling enzymes within the aromatic amino acid biosynthetic pathway, the methodology described above required the use of extensive and time-consuming genetic manipulation that could be avoided through the application of a more rational approach such as Metabolic Control Analysis (MCA) described in section 1.1.1.5.

The use of MCA to identify flux-limiting steps in metabolic pathways is receiving increased attention as a tool for metabolic engineering, and is thought by many to have much potential in terms of the optimisation of both bacterial strains, and related bioprocesses (Varma and Palsson, 1994). Indeed, as an indication of the recent increase in the application of MCA, the production of β-lactams through the penicillin biosynthetic pathway, itself traditionally improved by repeated rounds of mutation and selection, has recently been a focus of MCA in an effort to remove flux-limiting steps (Jørgensen *et al*, 1995).

In this study, a mathematical model that simulates the flux of benzoate through the TOL plasmid encoded *meta*-cleavage pathway of *Pseudomonas putida* mt2, and in addition predicts the flux-limiting steps in the pathway has been experimentally tested.

According to the mathematical model (Regan *et al*, 1991), the first enzyme in the pathway, benzoate dioxygenase, is the greatest single flux-controlling enzyme in the pathway. This, together with the prediction that the product of benzoate dioxygenase, benzoate *cis*-dihydrodiol, is one of only two pathway intermediates that varies in concentration with varying input substrate concentrations suggested that the benzoate dioxygenase enzyme was a suitable target for over-expression. If the predictions of the mathematical model were correct, it was thought that over-expressing the benzoate dioxygenase would result in a significant change in pathway flux.

In order to investigate the effect of enzyme over-expression in this way, control studies were initially performed in order to determine the profile of *meta*-cleavage

pathway intermediates that would result from metabolism of benzoate through a wild-type pathway. *m*-Toluate metabolism was also studied through a wild-type pathway, as a basis for determining whether the predicted flux-controlling steps in benzoate metabolism could be extended to *m*-toluate metabolism.

Studies (section 3.1) illustrated that growth of *E. coli* expressing pQR150 in the presence of either pathway substrate was comparable, as was the level of C23O activity during induction and towards the end of metabolism. Despite this, C23O activity during *m*-toluate metabolism was shown to plateau very quickly upon the addition of substrate (fig 3.4, section 3.1.2), in step with the characteristic profile of ring-cleavage intermediate accumulation during metabolism (fig 3.2 b, section 3.1.2). Such distinctions from benzoate metabolism can be attributed to the inhibitory nature of 2-hydroxy-6-oxohepta-2,4-dienoate (Polissi and Harayama, 1993).

Another interesting difference between benzoate and *m*-toluate metabolism through the wild-type *meta*-cleavage pathway was in the rate of substrate utilisation which was almost two-fold greater when benzoate was the pathway substrate.

In order to investigate the prediction that benzoate dioxygenase is the main flux-controlling enzyme during benzoate metabolism, the *xylXYZ* genes encoding benzoate dioxygenase were cloned into the expression vector pMMB66HE, forming plasmid pQR189 (fig 3.5, section 3.2.1). pQR189 was subsequently co-expressed with pQR150 in the same *E. coli* cell, thereby amplifying the level of dioxygenase expression

Metabolism of benzoate by *E. coli* cells expressing extra copies of toluate dioxygenase (section 3.2) was coupled with a poorer growth rate than seen with cells expressing the plasmid pQR150 alone. This however was shown to be a result of the presence of an additional plasmid rather than due to a change in metabolic activity. Indeed, co-expression of pQR150 and pQR189 resulted in an increased rate of benzoate utilisation in comparison with the case of the wild-type pathway in the presence of pQR189. Such a difference can be related to an increase in the activity of benzoate dioxygenase as a result of co-expression of pQR189 with pQR150. By increasing the activity in this way, the flux through benzoate dioxygenase was increased thus reducing the flux control coefficient of this step.

In future studies it would be of interest to obtain quantitative values of for example V_{max} for benzoate dioxygenase with or without the co-expression of pQR189 and pQR150. These values were not calculated in this study due to the inherent instability of benzoate dioxygenase in cell extracts preventing reproducible assays.

The removal of benzoate dioxygenase as the principle flux controlling enzyme in the pathway was illustrated by the three-fold increase in 2-hydroxymuconic semialdehyde concentration, and the ten-fold increase in 2-hydroxy-4-oxovalerate

concentration observed during the metabolism of benzoate by cells expressing an amplified dioxygenase, in comparison to metabolism by cells expressing pQR150 alone. (fig 3.9, section 3.2.3.1).

Despite such apparent increases in pathway flux as described above, it appears from comparative *cis*-dihydrodiol and catechol concentrations between control and amplified benzoate dioxygenase catalysed metabolism of benzoate (fig 3.3a, section 3.1.2 and fig 3.9, section 3.2.3.1 respectively), that the removal of benzoate dioxygenase as a flux-limiting enzyme results in the *xylL* encoded *cis*-dihydrodiol dehydrogenase becoming a significant flux-limiting enzyme.

According to fig 3.9, despite the aforementioned increase in the utilisation of an initial concentration of 10mM benzoate under conditions of amplified dioxygenase activities, *cis*-dihydrodiol concentrations were found to reach concentrations of 4.0 mM g cell dry weight^{-1} by the end of benzoate utilisation. This was almost four-fold the concentration of *cis*-dihydrodiol observed during metabolism through the wild-type pathway (fig 3.3 a). Concentrations of catechol however were found to remain constant during metabolism through both systems. These observations suggest that the *cis*-dihydrodiol dehydrogenase possesses a low elasticity, and subsequently a high flux control coefficient, as defined in section 1.1.1.5.

Such a property of the *cis*-dihydrodiol dehydrogenase would explain why, during metabolism through either wild-type or amplified dioxygenase systems levels of cis-dihydrodiol accumulate to concentrations approximately ten-fold that of other pathway intermediates.

From a physiological point of view, the flux-limiting role of the *cis*-dihydrodiol dehydrogenase apparently serves to control and therefore limit the flux of a varying concentration of cis-dihydrodiol to catechol, a known inhibitor of pathway and cellular metabolism.

In future studies it would be interesting to over-express the *cis*-dihydrodiol dehydrogenase with or without the over-expression of benzoate dioxygenase. Such a system would serve to demonstrate whether an amplification of this enzyme results in an accumulation of catechol to concentrations that become toxic to cellular metabolism.

Another noticeable difference between benzoate metabolism through either a wild-type or amplified benzoate dioxygenase system was seen in the relative levels of C23O activity (fig 3.10, section 3.2.3.1). Following induction with IPTG and addition of benzoate, C23O activities in cells expressing an amplified activity of benzoate dioxygenase rose rapidly to levels five-fold greater than during control activities. It is interesting to note that such a rise in activity reaches a plateau is in line with the disappearance of benzoate, and the maximum production of *cis*-dihydrodiol (fig 3.9). It is also noticeable that such a plateau accompanied by a change in the rate of 2-hydroxymuconic semialdehyde accumulation that is apparent in both shake-flask

and fermenter analysis (figs 3.6, section 3.2.2 and 3.8b, section 3.3.2.3.1 respectively).

According to Kobayashi *et al*, (1995), benzoate acts as a competitive inhibitor of C23O, with an inhibition constant of 4.9 mM. Thus in one respect, in the early stages of substrate metabolism, amplified levels of benzoate dioxygenase activity may serve to remove benzoate at a rate sufficient to prevent C23O inhibition. Such an explanation would result in the higher levels of C23O activity that were observed under such conditions.

Another explanation for the high C23O activities observed during the metabolism of benzoate through an amplified benzoate dioxygenase system lies in the mechanism behind the reactivation of inhibited C23O by the *xylT* encoded ferredoxin. The studies of Polissi and Harayama, (1993) have suggested that reduced XylT reactivates C23O by reducing the oxidised iron cofactor (section 1.2.1.2). For this system of reactivation to operate, continual regeneration of reduced XylT is required. In the light of these suggestions, Polissi and Harayama (1993) have speculated that the reduced electron donor of benzoate dioxygenase, XylZ, may serve this purpose by donating electrons to oxidised XylT.

In systems expressing amplified levels of benzoate dioxygenase activity, a high turnover of benzoate, coupled with a relatively low turnover of *cis*-dihydrodiol may result in a high concentration of reduced XylZ, that may not be reoxidised. If the proposals of Polissi and Harayama are correct, an excess of reduced XylZ may provide the capability for electron donation that is required for the regeneration of XylT. Such a scenario would explain the significant differences in C23O activity during metabolism of benzoate by wild-type or amplified benzoate dioxygenase systems.

Upon the complete utilisation of benzoate by amplified dioxygenase systems, C23O activities (section 3.2.3) were seen to plateau at the end of benzoate utilisation, as described above. In line with the proposal that amplified benzoate dioxygenase facilitates the rapid regeneration of C23O, such a plateau could have been associated with C23O activity becoming independent of benzoate dioxygenase activity.

More work is required to develop a full understanding of the effect of benzoate dioxygenase amplification on C23O activity. In particular, an explanation is required for the dramatic spike in C23O activity that was observed during the end of pathway metabolism in such systems (fig 3.10, section 3.2.3.1).

It would be interesting to study C23O activity during a pulsed supply of benzoate to cells expressing an amplified dioxygenase. The observation of a fluctuating C23O activity in line with benzoate utilisation, together with an absence of

such a pattern during metabolism through a wild-type pathway would strongly suggest a link between benzoate dioxygenase and the regeneration of reduced XylT.

During the metabolism of *m*-toluate by cells expressing an amplified benzoate dioxygenase activity, as in the metabolism of benzoate, the flux through the first step was significantly increased, resulting in a significant increase in the accumulated concentration of the *cis*-dihydrodiol (fig 3.13, section 3.2.3.2). In accordance with benzoate metabolism, levels of *cis*-dihydrodiol metabolism were found to be significantly greater than other pathway metabolites with or without the additional dioxygenase activity. As a result the *cis*-dihydrodiol dehydrogenase can be implicated as an important flux-controlling enzyme during *m*-toluate metabolism as well as during benzoate metabolism.

An interesting distinction between benzoate and *m*-toluate metabolism by cells exhibiting an amplified benzoate dioxygenase activity was found to be in the levels of C23O activity found in relation to the concentrations of ring-cleavage intermediate. Like during benzoate metabolism, *m*-toluate metabolism resulted in levels of ring-cleavage intermediate three-fold greater than seen during control metabolism by cells expressing QR150 alone. However, this was coupled with C23O activities significantly less that observed during control studies. It is reasonable to assume that such activities can be associated with the high concentrations of the ring-cleavage product 2-hydroxy-6-oxohepta-2,4-dienoate, a known competitive inhibitor of C23O (see Table 4.3).

The question that remains is how is such a flux to 2-hydroxy-6-oxohepta-2,4-dienoate maintained despite such apparent low levels of C23O activity. In the light of the proposals concerning the effect of amplified benzoate dioxygenase on XylT regeneration, it is possible that the continual reactivation of C23O as a result of such amplified dioxygenase levels results in high concentrations of ring-cleavage products, in itself leading to inactivation of C23O, thus leading to a cycle of inactivation, regeneration and inactivation that prevents the C23O activities reaching those observed during benzoate metabolism.

It is clear from the results of chapter 3 that an amplification of benzoate dioxygenase activity facilitates a shift in the distribution in flux control to other steps within the pathway. Having established this ability, it would be interesting to determine whether such a shift could be achieved by the amplification of either the reductase or the terminal oxygenase components alone. In the case of the three-component benzene dioxygenase of *Pseudomonas putida* ML2, activity has been found to be stimulated *in vitro*, but not *in vivo*, by expression of additional copies of the gene encoding the ferredoxin (Tan *et al*, 1994). More significantly from the point

of view of this study was the discovery that the *in vivo* activity could be stimulated by the addition of the purified reductase, suggesting that the reductase component is in part a limiting factor in the activity of the benzene dioxygenase.

If the reductase component of benzoate dioxygenase is the critical component limiting dioxygenase activity, this would support the proposal that increased C23O activity in the presence of amplified benzoate dioxygenase is associated with improved regeneration of XylT by amplified levels of *xylX* encoded reductase.

According to the mathematical model simulating flux through the pathway, the substrate of the *xylI* encoded oxalocrotonate decarboxylase was predicted to accumulate on increasing the concentration of input substrate. Furthermore, the build-up of this intermediate was predicted to cause the greatest single negative effect on the other pathway intermediates. As a result, attempts were made to prevent any such accumulation through the introduction into a cells carrying pQR150 a second plasmid, pQR194, that expresses *xylI* under the control of the *tac* promoter of pMMB66EH (fig 3.15, section 3.3.2).

A comparison of the metabolism of benzoate by cells carrying pQR150 alone, with cells carrying pQR150 and pQR194 (sections 3.3.2 and 3.3.3) illustrated the detrimental effect on pathway metabolism that was caused by the co-expression of pQR150 and pQR194. As can be seen in figs 3.17 and fig 3.18, section 3.3.3, such an effect manifested itself in a significant drop in the rate of benzoate utilisation when pQR150 was expressed together with pQR194. In addition, intermediates such as 2-hydroxymuconic semialdehyde accumulated to levels less than half that observed during with a wild-type pathway. Such low concentrations were attributed to poor substrate utilisation rather than to very efficient pathway metabolism as was initially expected.

Comparative C23O activities supported the suggestion that co-expression of pQR150 and pQR194 resulted in an apparent shutdown of pathway activity in the presence of a pathway substrate. According to fig 3.20, section 3.3.3, upon the addition of benzoate to cells expressing pQR194 and pQR150, C23O activities dropped to levels three-fold less than observed during control metabolism. It is interesting to note that despite such poor C23O activities in such systems, this did not result in a gradual accumulation of the substrate of C23O, namely catechol, as would perhaps be expected. On the contrary, and in accordance with the observations concerning benzoate degradation through amplified dioxygenase systems, utilisation of benzoate in systems expressing pQR150 and pQR194 resulted in a concurrent accumulation of cis-dihydrodiol, with catechol levels remaining stable. This provides further evidence to suggest that the *xylL* encoded dihydrodiol dehydrogenase plays a significant role in controlling pathway flux.

Such detrimental effects on metabolism were reasoned to be as a result of either (i) the expression of pQR194 facilitating a change in flux leading to the accumulation of an intermediate to levels that effects both C23O and pathway activity, or (ii) an effect caused by the plasmid pQR194 that was entirely independent of substrate metabolism, and was thus not a reflection of a change in metabolic flux.

The second proposal outlined above was subsequently dismissed by the results of section 4.1, in which an analysis of the growth and C23O activity was monitored for cells expressing pQR150 and pQR194 in the presence or absence of benzoate, against a control analysis of cells expressing pQR150 alone and cells lacking any *meta*-cleavage pathway construct.

As result, *meta*-cleavage pathway 'shut-down' was reasoned to be associated mainly with an inhibition of C23O and subsequently a change in flux caused by the over-expression of *xylI*.

In order to verify such a proposal, the product of the XylI catalysed reaction was considered a candidate inhibitor of C23O activity at high concentrations.

Kinetic studies were performed in order to determine the effect of purified 2-hydroxypent-2,4-dienoate on C23O activity (section 4.3), using a DEAE batch-purified preparation of C23O (section 4.2). Results indicated that 2-hydroxypent-2,4-dienoate acts as a non-competitive inhibitor of C23O with a Ki of 1.49×10^{-4} M (fig 4.10), comparable with the Ki values of ring-cleavage products of methyl-substituted benzoates (see Table 4.3).

Having established 2-hydroxypent-2,4-dienoate as an inhibitor of C23O, it is interesting to consider this in view of the known physical association between the XylI and XylJ gene products, first reported by Harayama *et al*, (1989a).According to these initial reports, physical association in this way was thought to occur in order to prevent rapid conversion of 2-hydroxy-2,4-dienoate to the inactive 2-oxopent-4-enoate. This reasoning has also been used to explain a similar physical association between the oxaolcrotonate decarboxylase and 2-oxopent-4-enoate hydratase (encoded by *dmpH* and *dmpE* respectively) of the phenol catabolic pathway of *Pseudomonas* sp. strain CF600 (Shingler *et al*, 1992).

In the light of the findings of this study it is reasonable to suggest that such a physical association between such enzymes of the benzoate and phenol degradation pathways exists not only to prevent conversion of 2-hydroxy-2,4-dienoate to 2-oxopent-4-enoate, but also to prevent the resulting build-up of the toxic intermediate. Such an explanation can also be used to explain the flux-controlling nature of the *xylI* gene product that is predicted by the mathematical model of Regan *et al*, 1991). By possessing such a control on overall pathway flux, it is clear that the elasticity and subsequent flux control coefficient associated with XylI provides an internal regulation

within the pathway in order to prevent excess build-up of 2-hydroxypent-2,4-dienoate.

With this in mind, in order to generate a more accurate model of flux control through the *meta*-cleavage pathway, in future studies the Ki of 2-hydroxypent-2,4-dienoate will need to be introduced as a new parameter into the mathematical model, in order to determine a new profile of flux control that takes into account the significance of this intermediate.

Enzyme inhibition in relation to the development of models predicting flux through metabolic pathways has already been recognised as an important parameter, and has been incorporated into a co-metabolic kinetics model of TCE degradation through an ammonia-utilising *Nitrosomonas* strain (Ely *et al*, 1995a, 1995b). In the development of this particular model, a pseudo-steady state was assumed, despite the fact that enzyme inhibition and reinactivation all create an inherent unsteady state condition. The error associated with such an assumption however was found to be negligible, which is reassuring for the future modelling of the TOL plasmid *meta*-cleavage pathway. The studies of Ely and co-workers also emphasises the significance of enzyme recovery in modelling studies, and how such a process must be incorporated into kinetic models. In the context of this study, regeneration of C23O activity appears to be, as already discussed, associated with the activity of one of the flux-controlling enzymes. A full understanding of the relationship between these two enzymes is clearly required for incorporation into the model.

As a progression from these findings, in future studies it would be interesting to determine whether mathematical models predicting the distribution of flux control during *m*-toluate metabolism predicts the *xylF* encoded 2-hydroxy-6-oxohepta-2.4-dienoate hydrolase as a flux controlling enzyme.

The studies of Harayama *et al*, (1989a) and Shingler *et al*, (1993) have both reported a lack of physical association between the hydrolase and hydratase enzymes of both the *xyl* and *dmp* operons, suggesting that 2-hydroxypent-2,4-dienoate accumulation is prevented by some other mechanism other than by physical coupling of the enzymes required for its production and removal. This in turn suggests a flux control distribution different to that predicted by the model for benzoate degradation.

Following the determination of 2-hydroxypent-2,4-dienoate as an inhibitor of C23O, and therefore an inhibitor of pathway metabolism as a whole, studies were performed in order to investigate the mechanism behind inactivation of C23O by the co-expression of pQR150 and pQR194 (chapter 5). The findings of Harayama and Shingler have concluded that activity of oxalocrotonate decarboxylase is dependent upon the activity of the 2-oxopent-4-enoate hydratase. Indeed, the decarboxylase has been found to be unstable in the absence of the hydratase.

With this in mind, it would be unlikely that the co-expression of pQR194 and pQR150 leads to an increase in 2-hydroxypent-2,4-dienoate accumulation through an increased metabolic flux through *xylI* caused by an amplified level of decarboxylase activity. On the contrary, build-up of the 2-hydroxypent-2,4-dienoate would appear to be a consequence of inactivation of 2-oxopent-4-enoate hydratase by co-expression of pQR194 with pQR150.

The results of section 5.1 indicate the consequences of a loss of XylJ activity during the metabolism of *m*-toluate by cells expressing pQR185. As would be expected, an accumulation of the end-product of metabolism through pQR185, namely 2-oxopent-4-enoate, resulted in a significant drop in C23O activity (fig 5.6, section 5.1.2) in comparison with metabolism by cells expressing pQR150. It is interesting to note that despite such a significant drop, the same profile of activity is maintained, suggesting that the nature of reactivation is maintained between the two systems.

It is reasonable to suggest that the observed levels of 2-oxopent-4-enoate are indicative of high concentrations of the 2-hydroxypent-2,4-dienoate, in a dynamic equilibrium between the enol and keto forms. Alternativly, the 2-oxopent-4-enoate may itself be an inhibitor of C23O. As has been stated previously, 2-hydroxypent-2,4-dienoate is clearly an inhibitor of C23O. However it is known to spontaneously convert to the keto form, and so in future studies it would be interesting to determine whether inhibition of C23O is due to 2-hydroxypent-2,4-dienoate alone, or in combination with 2-oxopent-4-enoate.

The addition of a XylJ activity to the truncated pathway expressed by pQR185 (forming plasmid pQR186, fig 5.7, section 5.2) resulted in metabolism of *m*-toluate similar to that seen through the wild-type pathway. C23O activities were restored as a result of minimising the accumulation of 2-oxopent-4-enoate (fig 5.12, section 5.2.2), and as a result the pathway flux as a whole was improved in comparison with pQR185-linked metabolism, as can be seen by the increase in both substrate utilisation and end-product accumulation (figs 5.5b, section 5.1.2, and fig 5.11, section 5.2.2).

The co-expression of plasmids pQR186 and pQR194, as described in section 5.3, lead to a metabolism of *m*-toluate very similar to that observed in section 5.1 for the metabolism through cells expressing pQR185, both in terms of reduced C23O activities and in the reduced rate of *m*-toluate utilisation. In addition, the accumulation of 2-hydroxy-4-oxovalerate by cells expressing pQR150 and pQR194 was found to be significantly less than in pQR186 studies. Such observations cannot be related to an increase in flux through to 2-oxopent-4-enoate as a result of *xylI* over-expression, because *m*-toluate metabolism does not proceed through the dehydrogenative branch of the pathway, and so would be unaffected by changes in flux through the oxalocrotonate decarboxylase. Thus it appears that, as predicted, the expression of *xylI* by pQR194 appears to reduce the activity of the XylJ gene product, possibly

through a detrimental physical association caused by the expression of *xylI* and *xylJ* from different transcripts. In support of this proposal, it has been suggested that the co-expression of the *dmpH* and *dmpE* within the phenol catabolic pathway is required from the same transcript is required for a successful physical association between the two enzymes (V. Shingler, personal communication).

It should be noted that in studies with cells expressing pQR186 and pQR194, expression of *xylI* occurred solely from pQR194 due to the absence of the *xylI* gene in the truncated operon encoded by pQR186. In the case of co-expression of pQR150 and pQR194, *xylI* was expressed in both plasmids. In future studies it would be interesting to assay the activity of both XylI and XylJ during metabolism of *m*-toluate by cells expressing firstly pQR150 with or without the presence of pQR194, and secondly cells expressing pQR186 and pQR194, in order to determine whether *xylI* expression from a different transcript competes for XylJ with XylI expressed from the same transcript.

Another aspect of metabolic engineering that has been approached in this study involves the over-accumulation of pathway intermediates through the use of truncated *meta*-cleavage pathways. Metabolic engineering strategies of this type have been established for some time, for example in the production of benzene *cis*-glycol (see section 1.2), and more reports are continually appearing in the literature. A recent example involves the use of a mutant *P.putida* that is capable of producing high quantities of *cis,cis*-muconic acid (32g l^{-1}) from benzoic acid over a continual feeding period of 40 hours (Bang and Choi, 1995).

In this study, as has been mentioned previously, by truncating the *meta*-cleavage pathway at the last step in the pathway (as in pQR186), the chiral intermediate 2-hydroxy-4-oxovalerate can be forced to accumulate to levels of over 0.4 mM g cell dry $weight^{-1}$, which represents a conversion yield of 10% (fig 5.11). This is in itself approximately a 10-fold improvement in the conversion yield obtained by substrate metabolism through the wild-type pathway (fig 3.3 a and 3.3 b).

Other studies (section 5.4) have shown the capability of a strain expressing the truncated plasmid pQR185 in accumulating over 1 mM g cell dry $weight^{-1}$ concentrations of oxalocrotonate (fig 5.18, section 5.4). Such an accumulation represents a 20% conversion yield of substrate to oxalocrotonate over a six hour period, a 100-fold increase in yield than observed during metabolism through the wild-type pathway.

It is clear that through the optimisation of pathway metabolism, achieved by an understanding of flux-control within the pathway, together with the development of an effective fed-batch system for the continual production and removal of desired end-

product, bioprocesses can be established in which the conversion yields quoted above can be significantly increased.

Studies are underway with these intentions in mind, to maximise the potential of *E.coli* cells expressing pQR226 to over-accumulate the ring-cleavage product of benzoate, 2-hydroxymuconic semialdehyde, which may be used as a precursor in the synthesis of picolinic acid (F. Vanier, personal communication).

The increasing number of methods available that enable protein and pathway engineering provide an opportunity not only to increase the rate of degradation of a pathway substrate, but also to broaden the substrate range of a pathway enzyme. Enzyme recruitment has been reported over a number of years as a method for generating hybrid metabolic pathways that display degradative activities towards new substrates such as alkylbenzoates (Ramos *et al*, 1987a). In the last two years even more subtle methods of genetic manipulation have been employed to improve or broaden the activity of degradative enzymes such as the aromatic dioxygenases (Hirose *et al*, 1994).

As discussed in section 1.1.1.4, the construction of various biphenyl and toluene degrading mutant strains revealed that the large α-subunits of the terminal dioxygenases, in this example encoded by *bphA1* and *todC1*, were critical for the discrete substrate specificities exhibited by the biphenyl and toluene dioxygenases respectively (Furukawa *et al*, 1993). Furukawa and co-workers further revealed that it was possible to establish a toluene degradative activity in the biphenyl degrading strain KF707 by introducing either the *todC1* or both *todC1* and *todC2* on a separate plasmid. This work was superseded by the more interesting discovery that hybrid multicomponent dioxygenases could be constructed by exchanging the terminal oxygenase components between different systems (Hirose *et al*, 1994).

As an extension to this work, the hybrid dioxygenase gene clusters *xylX:bphA2A3A4* (pQR191) and *bphA1:xylYZ* (pQR192) were constructed by combining subunits from biphenyl and benzoate dioxygenase (see section 6.1). The hybrid enzymes were analysed for their ability to degrade a range of aromatic substrates. Results indicated that the hybrid dioxygenases were unable to metabolise even the host substrate of the α-subunits in either hybrid, despite the fact that substrate recognition is governed by this subunit. These observations suggest that combinations of *xyl* and *bph* terminal dioxygenase genes do not form functionally active units.

The degree of amino acid sequence homology between the *xylX* and *bphA1* reveals only a 27% homology, over two-fold less than the identity shared between the *bphA1* and *todC1* encoded subunits (65%), that were successfully exchanged in the construction of active hybrid dioxygenases. The low degree of homology observed

would explain the non-overlapping substrate specificity of the biphenyl and benzoate dioxygenases.

Such a lack of amino acid homology can also be associated with the apparent inability of XylX and BphA2, and also of BphA1 and XylY to interact correctly.

Nucleotide sequence homology studies (Neidle *et al*, 1991), have suggested that such an interaction between the α– and β-subunits of terminal oxygenase components is governed by the β–subunits. Furthermore, it has been suggested that the catalytic centre of the oxygenase component lies at the interface of the two subunits. If such proposals are correct, it would suggest that sequence homology between β-subunits is as equally important as between α–subunit for the construction of functionally active gene clusters. In the light of this it would be interesting to determine the level of homology between *bphA2* and *xylY*, in order to ascertain whether such a low homology could be partly responsible for the inactivity of hybrid enzymes. According to the observations of Furukawa *et al.*, 1993, the *bphA2* and *todC2* subunits involved in the functionally active Bph:Tod hybrids exhibit a 60% amino acid identity. In comparison, although identities have not been reported for the *bphA2/xylY* homology, sequence data suggests that the *todC2* and *xylY* components share only a 20% homology. As a result it can be reasoned that the homology between the *bphA2* and *xylY* would be equally as low.

Clearly, if conserved β-subunit sequences are responsible for catalytic activity of αβ terminal oxygenases, the predicted low amino acid identity between BphA2 and XylY would explain the inability of for example a *bphA:xylYZ* gene cluster to recognize biphenyl as a pathway substrate.

In future studies, it would be interesting to construct hybrid dioxygenases by exchanging entire terminal oxygenase between enzymes. By introducing *xylXY* in the place of *bphA1A2* in the *bph* operon, the ability of the resulting hybrid enzyme to metabolise benzoate would be dependent upon the interaction of the exogenous terminal oxygenase with the ferredoxin and reductase components of the *bph* pathway.

Ultimately, site-directed mutagenesis may also be utilised as an alternative method to alter the substrate range of benzoate dioxygenase. This approach has already been successful in broadening the substrate range of biphenyl dioxygenase LB400 (Erickson and Mondello, 1993). By introducing four amino acid changes in the region 335-341, the resulting hybrid enzyme maintained its wild-type specificity, but in addition gained the ability to metabolise *para*-substituted substrates.

In order to overcome the difficulties imposed by the low degree of amino acid homology shared between the *xyl* and *bph* systems, a different strategy involving enzyme recruitment was employed in order to broaden the substrate range of the TOL plasmid *meta*-cleavage pathway.

As already mentioned, enzyme recruitment is a process that has been established over a number of years and, like the use of hybrid gene clusters has allowed the development of improved genetically engineered organisms for the degradation of organic compounds. In order to broaden the substrate range of the TOL plasmid *meta*-cleavage pathway, the *nahA* encoded naphthalene dioxygenase from *Pseudomonas putida* G7 plasmid NAH7 was co-expressed with the *meta*-cleavage pathway in the same cell. A similar arrangement, in which two multicomponent oxygenases are coexpressed in a single strain was employed by Wackett *et al.* (1994) in the development of an engineered *Pseudomonas* that has the ability to completely degrade polyhalogenated compounds. In this particular system, as described in section 1.3.2, cytochrome $P450_{CAM}$ monooxygenase was co-expressed with toluene dioxygenase.

In this study, the substrate specificity of NahA was studied in order to determine whether this dioxygenase was a suitable candidate for enzyme recruitment together with pQR150 into an *E.coli* cell. According to results obtained from biotransformation experiments performed with strains expressing *nahA*, the enzyme was found to be capable of catalysing the conversion of benzene, toluene and biphenyl, as well as the host substrate naphthalene to *cis*-dihydrodiols.

The plasmid pSS2 expressing *nahA* was subsequently introduced into an *E.coli* strain carrying pQR150. Recombinant cells grown in welled flasks with substrate saturated cotton wool were found to be capable of metabolism of biphenyl, naphthalene, benzoate and toluene, indicated by a transient accumulation of ring-cleavage intermediate.

By co-expressing a truncated *meta*-cleavage pathway, (pQR226, fig 6.10, section 6.3) together with pSS2, biotransformations were performed by injecting aromatic substrate directly into the resuspended cells. In this way it was hoped firstly that accumulating rather than transient levels of ring-cleavage intermediate would occur. Secondly, the direct addition of substrate was thought to increase the aqueous concentration of substrate in comparison with systems using welled flasks.

Biphenyl metabolism in such a system resulted in significant amounts of biphenyl dihydrodiol accumulation, coupled with only a very low level or ring-cleavage intermediate. The most successful metabolism was observed with naphthalene as a pathway substrate, which resulted in the accumulation of 2-hydroxychromene-2-carboxylate (HCCA), the first detectable ring-cleavage intermediate according to the proposed pathway for the bacterial metabolism of naphthalene (Eaton and Chapman, 1992). Furthermore, levels of HCCA produced were comparable with levels produced through the use of a truncated NAH7 pathway (Eaton and Chapman, 1992). These observations suggest that the TOL plasmid

catechol 2,3-dioxygenase is able to metabolise the substrate 1,2-dihydroxynaphthalene at a rate sufficient to minimise its rapid oxidation in water to 1,2-naphthoquinone.

The addition of toluene directly to resuspended cells resulted in a total absence of substrate metabolism. This is in contrast with the results obtained when toluene was supplied in the vapour phase using welled flasks, and can be attributed the highly toxic effect of toluene. The supply of toluene by diffusion through a silicon tubing at the bottom of a bioreactor would provide more control over the transfer rate of toluene. Such a system has already been employed (Jang-Young, *et al.*, 1994) in studies illustrating the ability of a dual dioxygenase system involving the TOL plasmid *meta*-cleavage pathway to metabolise toluene. These reports confirm the observations made in this study concerning the metabolism of toluene by growing cells carrying pSS2 and pQR150.

In the light of the results of this study it appears that successful metabolism of a range of aromatic substrates occurs by one of two mechanisms. Either the combination of benzoate and naphthalene dioxygenase subunits *in vivo* form an active hybrid dioxygenase capable of catalysing the oxygenation of a range of substrates, or the two aromatic dioxygenases function as separate wild-type enzymes. In either case, substrate metabolism would result in the conversion of for example naphthalene to naphthalene *cis*-dihydrodiol, which would serve as a substrate for XylL. For successful metabolism to ring-cleavage intermediates, both XylL and XylE would require a substrate specificity more relaxed than that of XylXYZ.

The work of Jang-Young *et al.*, 1994 has provided evidence that XylL and XylE do indeed possess such a relatively broad substrate range. In their study, the wild-type TOL plasmid pWWO was co-expressed in *Pseudomonas* with toluene dioxygenase from *Pseudomonas putida* F1. Studies indicated that under anaerobic conditions, the *xylL* encoded dihydrodiol dehydrogenase was able to metabolise toluene or benzene cis-glycols, with a concurrent production of equimolar amounts of NADH and reaction product, namely a catechol derivative. In aerobic conditions, no catechol derivatives were seen to accumulate, indicating that metabolism was able to proceed through to ring-cleavage as a result of the oxygen dependent catechol 2,3-dioxygenase.

More recent analysis involving a crude extract of XylL have confirmed its ability to metabolise a number of dihydrodiol compounds (Jang-Young, *et al.*, 1995a).

From the results of this study, and from the findings of Jang-Young and co-workers, it would appear that the substrate range of the wild-type TOL plasmid *meta*-cleavage pathway is significantly restricted by benzoate dioxygenase, and that in the presence of a second aromatic dioxygenase, the substrate range can be enhanced to include benzene, toluene naphthalene and biphenyl.

It is interesting to note that the dual dioxygenase system employed in this study is capable of the metabolism of biphenyl through to the production of dihydrodiol and, to a lesser extent, ring-cleavage intermediate, whilst the hybrid dioxygenases constructed show no activity towards biphenyl. Such differences could be used as the basis of determining whether the dual dioxygenase system operates as two wild-type enzymes, or as *in vivo* constructed hybrids. Firstly, it would be necessary to establish whether a system co-expressing the *bphA* encoded biphenyl dioxygenase with the TOL plasmid *meta*-cleavage pathway is capable of degrading biphenyl in a similar fashion to that of the pSS2/pQR150 system. If such metabolism is achieved, hybrid systems could be developed in which the *meta*-cleavage pathway is co-expressed with either *bphA1A2* or *bphA1:xylY*. The absence of any metabolism in such systems would point to the need for a complete biphenyl dioxygenase for the first metabolic step in the pathway. From this it could be concluded whether dual dioxygenase systems as described here operate by the action of to independent aromatic dioxygenases.

More recent work concerning dual dioxygenase systems (Jang-Young, *et al.*, 1995b) has provided an interesting link between the two main metabolic engineering strategies employed in this study, namely the broadening of substrate range and the maximisation of metabolic flux. Following their initial studies concerning the co-expression of toluene dioxygenase with the TOL plasmid *meta*-cleavage pathway, Jang-Young and co-workers have reported an improvement in the rate of degradation of various aromatic substrates by as much as 9-fold. These improvements have been achieved by amplifying the activity of the toluene dioxygenase, which they report to be the rate-limiting step in the metabolism of the various aromatic aromatic substrates.

Having established the ability of the *meta*-cleavage pathway to accept an increased range of aromatic substrates, a multiple cloning site (MCS) has been designed and inserted into a truncated *meta*-cleavage pathway downstream of *xylL*, forming plasmid pQR227, (fig 6.15, section 6.4). Using such an expression cassette, the MCS has been constructed such as to allow the easy insertion of the *catA* encoded catechol 1,2-dioxygenase from the chromosomally encoded ortho-cleavage pathway for benzoate degradation of *Acinetobacter calcoaceticus* (Neidle and Ornston, 1986). The catechol 1,2-dioxygenase catalyses the ortho-cleavage of catechol to form *cis,cis*-muconate, a metabolic intermediate already recognised industrially as a precursor of adipic acid manufacture (see section 1.1.1.3).

By employing a hybrid metabolic pathway that incorporates *catA* in pQR227, together with a second, over-expressed aromatic dioxygenase, it is possible that alternative routes for the bacterial synthesis of *cis,cis*-muconic acid could be established.

More significantly, it is accepted that the chromosomally encoded ortho-pathway for benzoate degradation possesses a narrow substrate specificity (Neidle, *et al.*, 1987). Using the hybrid system discussed above, it is conceivable that metabolism of substrates such as naphthalene or biphenyl could be degraded, with the concurrent production of novel *cis,cis*-muconates.

As an extension to this strategy, the MCS of pQR227 is designed to enable the *catB* encoded *cis.cis*-muconate lactonizing enzyme to be inserted downstream of *catA*, allowing the potential production of novel lactones as a result of channelling a range of substrates into the hybrid *meta*-cleavage pathway.

Conclusions.

Through investigating the predictions generated by the mathematical model (Regan *et al*, 1991) simulating flux through the TOL plasmid *meta*-cleavage pathway, some interesting characteristics of the pathway have been revealed. Identification of 2-hydroxy-2,4-dienoate as a non-competitive inhibitor of C23O has suggested that the physical association of XylI and XylJ occurs to prevent accumulation of this toxic intermediate.

Furthermore, amplification of benzoate dioxygenase activity with the *meta*-cleavage pathway has suggested a link between C23O regeneration and the level of expression of the *xylZ* encoded benzoate dioxygenase reductase component.

Such results serve as an indication that mathematical modelling of metabolic pathways, although sound in principle, cannot identify up front the inevitable enzyme-enzyme interactions that occur within such systems. A more general, physiological engineering approach is more likely to deal with these complexities.

Given that there is a high degree of homology shared between the TOL plasmid *meta*-cleavage pathway and other aromatic degradative pathways, it is reasonable to suggest that these new insights into internal pathway regulation can be found throughout other such pathways. Clearly as one of the long-term goals of metabolic pathway engineering is to develop bacterial strains capable of degrading environmental pollutants in the field, details of this study will help in optimising such systems through a detailed understanding of the internal regulations involved.

It is important to appreciate however that all studies described here involve the use of recombinant plasmid systems expressed and maintained through a carefully controlled fermentation system. For use in a natural environment for degradation of environmental pollutants, recombinant pathways will need to be integrated into the host chromosome.

Hydrid aromatic dioxygenases of biphenyl and benzoate dioxygenases constructed by substituting enzyme subunits has illustrated that not all aromatic dioxygenases can be manipulated in this way to expand their substrate range. A more general approach involving the co-expression of two wild-type dioxygenases however facilitates channelling of an expanded range of substrates into the TOL *meta*-cleavage pathway. Further studies will be able to reveal the mechanism behind such a change in pathway substrate specificity, which in turn will reveal further information concerning the mechanism behind aromatic substrate dioxygenation.

Chapter 8 References.

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Chapter 9: Appendix

List of Abbreviations

2,5-DKG	2,5-diketo-D-gluconic acid
2-KLG	2-keto-L-gulonic acid
3CB	3-chlorobenzoate
4CB	4-chlorobenzoic acid
4EB	4-ethylbenzoate
4OD	4-oxalocrotonate decarboxylase
4OT	4-oxalocrotonate tautomerase
AD	Acetaldehyde dehydrogenase
ADH	Alcohol dehydrogenase
Amp	Ampicillin
AS	Anthranilate synthase
BADH	Benzyl alcohol dehydrogenase
BenABC	Benzoate 1,2-dioxygenase
BphA1A2A3A4	Biphenyl dioxygenase
BSA	Bovine serum albumin
BZDH	Benzaldehyde dehydrogenase
C23O	Catechol 2,3-dioxygenase
CatA	Catechol 1,2-dioxygenase
CatB	*cis-cis*-muconate lactonizing enzyme
CIP	Calf intestinal phosphatase
Cm	Chloamphenicol
CM	Chorismate mutase
CZE	Capillary zone electrophoresis
DAH	3-deoxy-D-*arabino*-heptulosnic acid
DAHP	3-deoxy-D-*arabino*-heptulosonate 7-phosphate
DHCDH	*cis*-dihydrodiol dehydrogenase
DHQ	3-dehydroquinate
DmpE	Phenol pathway 2-oxopent-4-enate hydratase
DmpH	Phenol pathway oxalocrotonate decarboxylase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HCCA	2-hydroxychromene-2-carboxylate
HMSA	Hydroxymuconic semialdehyde
HMSD	2-hydroxymuconic semialdehyde dehydrogenase
HMSH	2-hydroxymuconic semialdehyde hydrolase
HOA	4-hydroxy-2-oxovalerate aldolase
HOHD	2-hydroxy-6-oxohepta-2,4-dienoate
HPC	Homoprotocatechuic acid
HPLC	High performance liquid chromatography
IPTG	Isopropylthiogalactoside
Km	Kanamycin
LacZ	β-galactoside

MCA	Metabolic control analysis
MCS	Multiple cloning site
MCT	Metabolic Control Theory
NADH	Nicotinamide adenine dinucleotide
NahAaAbAcAd	Naphthalene dioxygenase
NahR	NAH operon regulator
OEH	2-oxopent-4-enoate hydratase
PCB	Polychorinated biphenyl
PD	Prephenate dehydratase
TCE	Trichloroethylene
TO	Benzoate dioxygenase
TodA1A2A3A4	Toluene dioxygenase
XO	Xylene monooxygenase
XylAM	xylene monooxygenase
XylB	Benzyl alcohol dehydrogenase
XylC	Benzaldehyde dehydrogenase
XylE	Catechol 2,3-dioxygenase
XylF	Hydroxymuconic semialdehyde hydrolase
XylG	Hydroxymuconic semialdehyde dehydrogenase
XylH	4-oxalocrotonate tautomerase
XylI	4-oxalocrotonate decarboxylase
XylJ	2-oxopent-4-enoate hydratase
XylK	4-hydroxy-2-oxovalerate aldolase
XylL	cis-dihydrodiol dehydrogenase
XylQ	Acetaldehyde dehydrogenase
XylR	TOL upper pathway regulator
XylS	TOL *meta*-pathway regulator
XylT	Ferredoxin
XylXYZ	Toluate dioxygenasae

PhD Thesis

Metabolic Engineering of a Toluene Degradation Pathway

By Graham Jackson

Minor Corrections

- ✓ There is a need for a list of abbreviations.
- ✓ Title of chapters should be included at the beginning of each chapter e.g. Introduction.
- ✓ It is useful to include a section on Conclusions/ suggestions for future work.

Page	Line	Comment
14	19	Chater *et al* is only Chater in the list
15	26	change fungi to fungus
16	19	Sonoyama. 1981 is 1982 in the list
21	16	Katsumata *et al*, is not *et al* in the list
23	1, 2	Draths...90 and 92 check with the list
69	fig 3.2a	y-axis should be checked and altered
70	fig 3.2b	y-axis correct mmM to mM
78	fig 3.8a	y-axis should be checked and altered
89	3	fig 3.3 should change to fig 3.3a
101	fig 4.8	need legend for i, ii, iii
102	12	change Collongworth to Collinsworth
116	5 from bottom	correct "activity"
122	7 from bottom	correct 320 to 230
124	2	correct sentence
124	4 from bottom	correct spelling
134	4	correct spelling
141	27	make "et al" italics
147	24	Harayama 1989 is it "a" or "b" ?
147	30	Shingler 1993 is not in the list,it is 1992
152	14	add the date for the reference
155	8 from bottom	1987 ref. is 1986 in the list

✓Some references in the list do not seem to be referred to in the text. These are identified by X and need to be added.

CORRECTIONS

p 1 – line 16 (with)
13 – making them easier to clone
21 – 2nd para bottom – been made
30 bottom (of which at least of which)
30 Skims up from bottom 'ferredoxins'.

Top 107 Ammonium Sulphate SO_4

p 58 – Lamio(acid) oxidase.

p 103 1st para – recording plot etc.

112 under figure – substrate!

122/123 begin repeated line/phrase.

124 – were added/were employed

124 unit/mg / units/ml p 97 (carefully).

129 15 line deoxygenase.

147 3rd para 'a'

151 – Superseded! – extenuation

ABBREVIATIONS (back of).