

STRIVE

Report Series No.65

Recombinant DNA Approaches to Enhance the Activity of the Pathway for the Degradation of the Toxic Pollutant Styrene in the Bioreactor Isolate *Pseudomonas putida* CA-3: a Biotechnologically Significant Metabolic Route

STRIVE

Environmental Protection
Agency Programme

2007-2013

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EPA STRIVE Programme 2007–2013

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Significant Metabolic Route**

(2007-FS-ET-9-M5)

STRIVE Report

Prepared for the Environmental Protection Agency

by

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ACKNOWLEDGEMENTS

This report is published as part of the Science, Technology, Research and Innovation for the Environment (STRIVE) Programme 2007–2013. The programme is financed by the Irish Government under the National Development Plan 2007–2013. It is administered on behalf of the Department of the Environment, Heritage and Local Government by the Environmental Protection Agency which has the statutory function of co-ordinating and promoting environmental research.

The authors would like to thank the members of the Environmental Microbial Genomics Group at the Environmental Research Institute, University College Cork (UCC), and the Department of Microbiology, UCC, for assistance throughout this project.

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The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

EPA STRIVE PROGRAMME 2007–2013

Published by the Environmental Protection Agency, Ireland

ISBN: 978-1-84095-385-5

Price: Free

Online version

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Executive Summary

Extensive use of styrene by the petrochemical and polymer processing industries generates over a hundred thousand tonnes of stored styrene waste annually, in addition to gaseous and effluent emissions to the environment. Styrene itself is a toxic alkenylbenzene, while its oxygenated derivative, styrene oxide, is carcinogenic, thus the anthropogenic release of styrene poses a threat to both the environment and to human health. As a result, there has been significant interest internationally in environmentally sensitive approaches to remediate styrene waste, which has largely focused on microbial degradation.

This project involved targeted genetic manipulation of the bacterial isolate *Pseudomonas putida* CA-3, in an attempt to enhance its ability to degrade the toxic pollutant styrene to carbon dioxide and water. The styrene degradation pathway has been well characterised in this strain at both the genetic and physiological levels. In addition, this strain has the novel ability to generate polyhydroxyalkanoate (PHA), a biodegradable polymer, from styrene under appropriate growth conditions. Polyhydroxyalkanoates have been recognised as a potential bio-plastic replacement for current petrochemical plastics. Thus, *P. putida* CA-3 not only offers a significant styrene remediation capability but also the opportunity to use a toxic waste compound as the starting material for the production of value-added environmentally friendly bio-plastic. This project has focused mainly on the styrene-degrading ability of *P. putida* CA-3, where styrene is degraded via phenylacetic acid (PAA), and seeks to develop the biotechnological potential of this strain by applying recombinant deoxyribonucleic acid strategies in an attempt to enhance the rate of styrene degradation. A considerable amount of data exists on the ability of bacteria to degrade styrene and a number of aspects of the styrene degradation pathway have been targeted in this study. Advanced molecular biology techniques, including binding site mutagenesis, tri-parental mating, gene over-expression and random mutagenesis, were used to

generate recombinant strains that were subsequently monitored for changes in the styrene degradation pathway.

When the presence of styrene is detected by the bacterial cell, the styrene degradation genes are activated and converted to proteins. These proteins facilitate the initial step-wise degradation of styrene to PAA. However, inhibitor compounds, particularly other carbon sources, can block transcription of the styrene pathway genes, thus disrupting degradation, and this could be a significant problem when trying to treat wastes since these are unlikely to consist of pure styrene. Manipulating the genetic information to block the interference from inhibitory compounds is a potential way of maintaining styrene degradation in mixed wastes. The recombinant strains were tested under a range of metabolic conditions and the genetic manipulations performed in this study did not appear to have a significant impact in *P. putida* CA-3; however, further analysis involving mixed substrates is required. The preliminary data suggest that these inhibitor sites may play a limited role in styrene degradation in *P. putida* CA-3.

For bacteria to successfully degrade many compounds, they must be transported across the cell membrane into the cell, where they are then degraded by cellular enzymes. Transport of styrene across the bacterial cell membrane presents a limiting factor in the rate of styrene degradation, and increasing the number of copies of the transport gene was seen as a potential way of increasing the rate of styrene degradation. Similarly, possessing additional copies of the styrene catabolic genes may lead to greater levels of enzyme activity and thus to increased styrene degradation. A number of recombinant strains were generated and analysis suggests that non-styrene-controlled expression and multiple copies of the genes are required to significantly increase styrene degradation.

In *P. putida* CA-3, PAA is a key intermediate in styrene degradation. Previous investigation of PAA breakdown

in this strain identified a critical gene involved in PAA metabolism which did not share significant similarity with reported PAA-associated genes. This suggested that novel single-copy genes are important for PAA degradation in this strain. Further investigation of *P. putida* CA-3 resulted in the identification of a novel mutant which had lost the ability to grow with PAA as a

sole carbon source. Additional analysis of the mutant strain revealed a link between the disrupted gene and transport of PAA into the cell. This further highlights the critical role that transport systems play in the effective operation of degradation pathways for aromatic compounds.

1 General Introduction

One of the consequences of living in a highly industrialised world is that a significant quantity of waste is generated in the manufacture, transport and disposal of many items that are now common in everyday use. Of particular concern is the fact that a substantial portion of this waste is of a hazardous nature, and thus poses a threat to the environment and to human health. The management of waste is controlled by legislation at both national and European Union (EU) levels. Waste management at EU level is regulated by the Waste Framework Directive (2006/12/EC), and includes, among others, the following provisions for Member States:

- To take steps to encourage the prevention of waste and the recovery of waste; and
- To ensure that waste recovery and disposal takes place without endangering human health or causing environmental pollution.

In addition, further provisions relating to hazardous waste are included in the Hazardous Waste Directive. In Ireland, EU directives are implemented by the Waste Management Acts 1996 to 2008. The Environmental Protection Agency (EPA) has recently

published a *National Hazardous Waste Management Plan 2008–2012*. The plan's objectives include efforts to reduce the generation of hazardous waste in Ireland and to manage the waste in a more self-sufficient manner, while protecting the environment and society. Further information on this plan is available at <http://www.epa.ie/downloads>. The focus of this project is the aromatic compound styrene, predominantly a man-made chemical, though also known to be formed in nature during the fungal decarboxylation of cinnamic acid in decaying plant material.

1.1 Styrene

Styrene is the simplest alkenylbenzene and originates from both natural and anthropogenic sources. It is used extensively by the petrochemical and polymer-processing industries to produce a wide range of industrially significant polymers, the most well known of which is polystyrene, as well as a range of other products such as styrene–acrylonitrile polymers, styrene–butadiene rubber and styrene–butadiene latex (Fig. 1.1). Styrene-derived products are used widely in the production of many common and well-known everyday items. Over twenty million tonnes are now used annually in industrial processes, resulting in

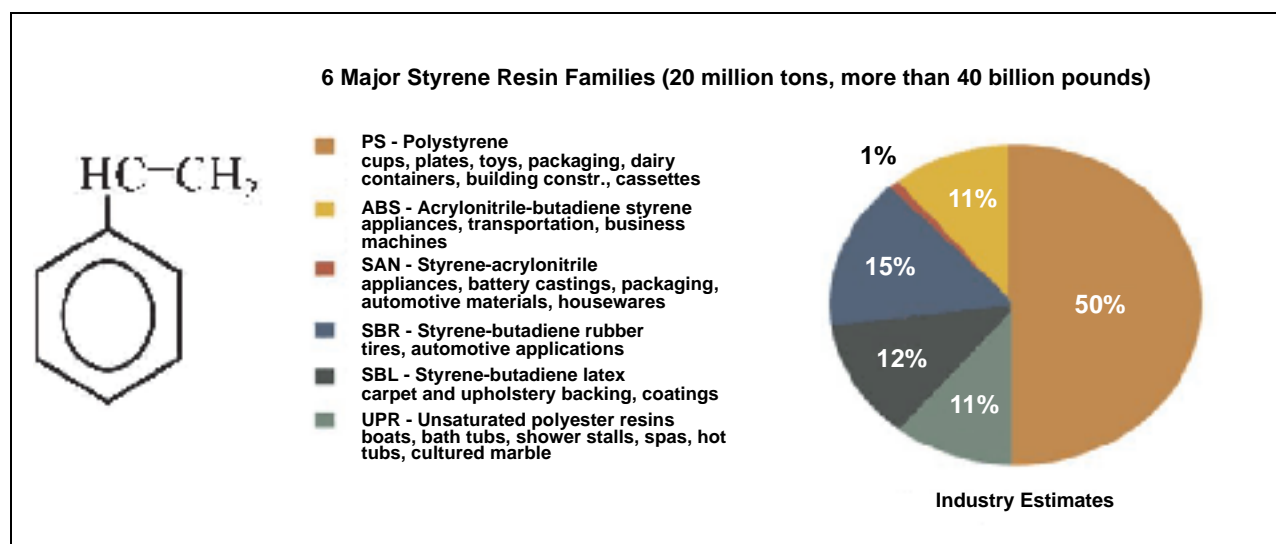


Figure 1.1. Chemical structure and industrial use of styrene (<http://www.styreneforum.org>).

the generation of styrene waste on a scale of over a hundred thousand tonnes per year. This wide-scale industrial use has led to a significant increase in the anthropogenic release of styrene into the environment, and the levels of anthropogenic release now greatly exceed the level of naturally occurring styrene (O'Leary et al., 2002; Mooney et al., 2006b). While a certain percentage of this waste is recycled by the petrochemical industry, a substantial portion has to be stored, which poses not only a threat to the environment, but also to human health. Human exposure to styrene through inhalation, ingestion or skin contact can cause disruption of the central nervous and respiratory systems, muscle weakness, fatigue and nausea. In addition, styrene is classified in the US EPA Toxic Release Inventory as a carcinogen, whereby it can be activated in mammalian cells to reactive epoxides which have been shown to have the ability to form deoxyribonucleic acid (DNA) adducts, leading to possible disruption of the genetic material within the cell. As a result of its widespread industrial use, potential release into the environment and potential destructive effects on human health, there has been increased interest in this molecule from environmental scientists and legislators in recent years. Several international research groups have focused extensively on various aspects of the microbial degradation of styrene in order to provide a biotechnological solution to styrene waste remediation. One such remediation strategy is the bacterial degradation of styrene and a number of bacterial strains have been well characterised for their ability to degrade this molecule, particularly members of the genus *Pseudomonas*. There is also now considerable interest in examining ways to convert some of the styrene-based products, such as polystyrene, back to styrene monomers which may subsequently be degraded biologically.

Styrene is used in large quantities in a number of EU countries, particularly France, Germany, Italy and Spain, as well as in the United States of America and some regions in Asia. Ireland is not a major producer of styrene-based products; however, it is heavily dependent on the import of large quantities of styrene polymers. In particular, considerable amounts of polystyrene are imported as packaging material, much of which is not widely recycled and therefore often

ends up in landfill. While efforts are being made to recycle expanded polystyrene by Rehab Recycle in the Greater Dublin area, there is still a significant quantity of waste that is not recycled countrywide. In the EU, the use of styrene is controlled by European Regulation ECC/793/93 – Existing Substances Regulation. Styrene is also controlled at national government levels, for example by the United Kingdom's Surface Water Regulations and Pollution Prevention and Control Regulations (<http://www.environment-agency.gov.uk>).

1.2 *Pseudomonas putida* CA-3

The focus of this reported project was the styrene-degrading strain *Pseudomonas putida* CA-3 (Fig. 1.2), which was initially isolated from a bioreactor treating styrene-contaminated waste. This strain has the ability to utilise styrene as a sole carbon source for growth, and completely degrades the styrene molecule to carbon dioxide and water, while also generating energy for the cell. However, it has recently also been reported that this strain has the ability to produce polyhydroxyalkanoates (PHAs), a potential source of biodegradable plastic, from styrene under appropriate growth conditions (O'Leary et al., 2005; Ward et al., 2005). Polyhydroxyalkanoates are polymers produced by bacteria in response to limited availability of essential nutrients, such as nitrogen, phosphorus or sulfur, in the presence of excess carbon (Kessler and Witholt, 2001). There is a wide range of potential uses



Figure 1.2. *Pseudomonas putida* CA-3 cells growing on Luria–Bertani agar containing indole.

and applications of PHA, such as biodegradable rubber, adhesives, binders in environmentally friendly paints and food packaging, and recently its potential use in the biomedical field has emerged, with suggested involvement in wound management and tissue engineering (Kessler and Witholt, 2001; van der Walle et al., 2001). In the case of *P. putida* CA-3, Ward et al. (2005) have reported maximal PHA accumulation from styrene of 21% biomass as cell dry weight under nitrogen-limiting growth conditions. Subsequently, O'Leary et al. (2005) identified a number of genes involved in the process, including a Class II medium chain length (MCL)-PHA synthase (*phaC1*), a PHA depolymerase (*phaZ*) and a second synthase (*phaC2*) organised in the typical Class II *pha* gene arrangement, which has previously been described in other species. The important role of a 3-hydroxyacyl-ACP-CoA¹ transferase (*phaG*) in sequestering *R*-3-hydroxyacyl-CoA PHA monomers from fatty acid synthesis for plastic production was also demonstrated in this strain. Additional studies have focused on the microbial process involved in PHA accumulation in *P. putida* CA-3. A novel two-step process has been developed in which polystyrene can be converted to styrene oil by pyrolysis and the styrene oil subsequently converted to PHAs by *P. putida* CA-3. The process was scaled up to a 7.5-l stirred tank reactor and led to further increases in PHA yield, with a single pyrolysis run followed by four fermentations, yielding a total of 6.4 g of PHA from 64 g of polystyrene (Ward et al., 2006). Further work using a nitrogen feeding strategy in the bioreactor led to a maximum amount of PHA accumulation from styrene at a feed rate of 1.5 mg/l/h for nitrogen. The nitrogen feeding strategy employed resulted in a 1.4-fold increase in the percentage of PHA within the *P. putida* CA-3 cell and improved the overall amount of PHA produced from styrene 3.1-fold per bioreactor run (Goff et al., 2007).

The ability of *P. putida* CA-3 to convert styrene to PHAs is of significant interest and promotes the idea of using a toxic waste product to produce a value-added product. This strain, therefore, not only has the ability to completely mineralise styrene to carbon dioxide and

water, but can also redirect styrene catabolism to produce PHAs, which are receiving considerable interest due to their potential use in a wide range of applications. As a result, this strain offers considerable biotechnological potential. One major drawback in the industrial production of PHAs is the elevated cost associated with the process when compared with the production of more widely available synthetic polymers, and the cost of the initial carbon sources has been cited as a major disadvantage. Therefore, the possibility of using a waste product, such as styrene, in the process could lead to more cost-effective production of PHAs, while also removing a pollutant from the environment.

The current project focused on the use of recombinant DNA approaches to investigate regulatory influences on pathway activation, with the overall aim of potentially enhancing the styrene-degradation capacity of this strain. While this study's particular focus has been on the styrene-degrading ability of this strain, improving styrene mineralisation may also potentially lead to increased accumulation of PHA. Tobin and co-workers recently reported that homologous over-expression of the *phaG* gene did not improve PHA accumulation from styrene in *P. putida* CA-3, suggesting that the availability of 3-hydroxyacyl-ACP substrate may be a limiting factor (Tobin et al., 2007). Therefore, efforts to improve the rate of styrene flux through the catabolic pathway is the focus of this current study and may enhance mineralisation of styrene, as well as produce maximal PHA accumulation from this toxic compound in *P. putida* CA-3. Thus, efforts to optimise the fermentation process, as described above, plus potential genetic manipulation of the strain may lead to the development of a cost-effective large-scale process in the future. A considerable amount of knowledge has been generated about bacterial degradation of styrene. Pathways and enzymes have been identified and characterised physiologically and at the genetic level. A summary of the current state of knowledge of the bacterial styrene degradation will be covered in the remainder of this section, with particular emphasis on *P. putida* CA-3.

1. ACP, acyl carrier protein; CoA, coenzyme A.

1.3 Styrene-Degradation Pathways and Genes

1.3.1 Upper catabolic pathway

Two main pathways for styrene degradation have been proposed for bacteria growing under aerobic conditions, either direct attack on the aromatic ring or oxidation of the vinyl side chain. Direct ring cleavage has been proposed in a number of styrene-degrading strains (Bestetti et al., 1989; Hartmans et al., 1989), particularly *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst et al., 1994), where oxidation of the aromatic ring is initiated by a dioxygenase enzyme and results in the conversion of styrene to styrene *cis*-glycol. This molecule is converted to 3-vinyl-catechol, which is further degraded, via a *meta*-cleavage pathway, to intermediates that enter the tricarboxylic acid (TCA) cycle (Warhurst et al., 1994). However, the main microbial styrene-degradation pathway, and also the most characterised, proceeds via initial side-chain oxidation and can be divided into two interlinked pathways: an upper pathway involving styrene, styrene oxide, phenylacetaldehyde and phenylacetic acid (PAA), and a lower pathway beginning with the

conversion of PAA to phenylacetyl-CoA (O'Leary et al., 2002). Many of the genes and enzymes involved in these pathways have been identified. The initial step of the upper pathway is catalysed by a flavin adenine dinucleotide-dependent two-subunit monooxygenase and involves the epoxidation of the styrene vinyl side chain, leading to the formation of styrene oxide. Styrene oxide is isomerised to phenylacetaldehyde by styrene oxide isomerase and this compound is converted to PAA by the action of either an nicotinamide adenine dinucleotide (NAD⁺) or phenazine methosulfate-dependent dehydrogenase (Fig. 1.3a) (Hartmans, 1995).

This stepwise oxidation of the side chain has also been studied at the genetic level in a number of *Pseudomonas* strains, including *P. putida* CA-3 (O'Leary et al., 2001), but also in *P. fluorescens* ST (Marconi et al., 1996), *Pseudomonas* sp. strain VLB120 (Panke et al., 1998) and *Pseudomonas* sp. Y2 (Velasco et al., 1998). The chromosomally encoded catabolic genes of the upper styrene pathway are transcribed together as an operon and are arranged in the same order as the reactions they catalyse above.

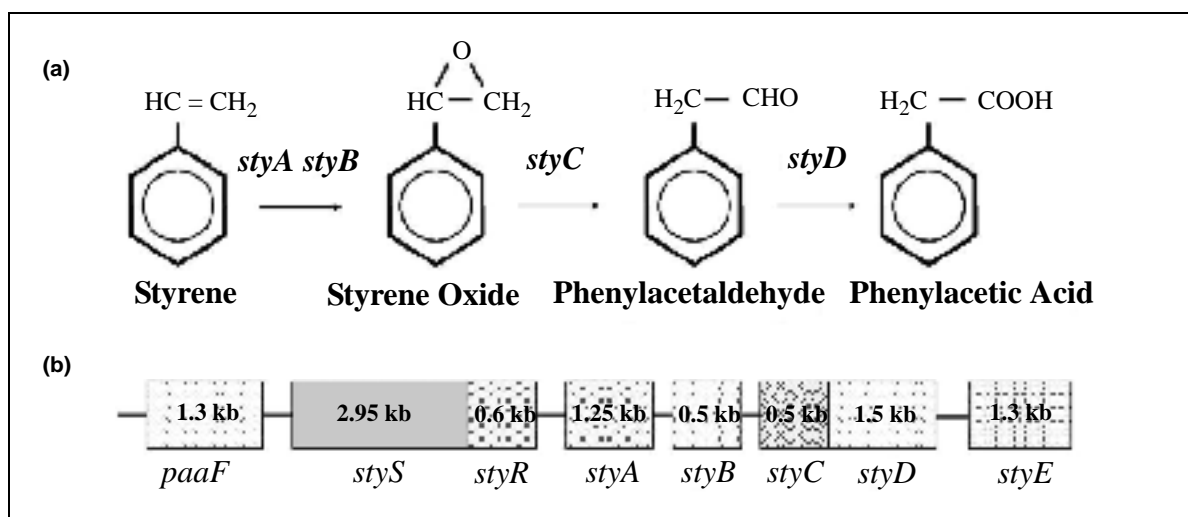


Figure 1.3. (a) Styrene upper pathway, vinyl side-chain oxidation of styrene, showing intermediates and styrene genes encoding enzymes responsible for degradation. (b) Organisation of the chromosomally encoded styrene genes in *Pseudomonas putida* CA-3. A similar arrangement occurs in a number of other *Pseudomonas* spp. The *styABCDE* operon encodes styrene monooxygenase subunits 1 and 2 (*styAB*), styrene oxide isomerase (*styC*), phenylacetaldehyde dehydrogenase (*styD*) and styrene active transport protein (*styE*); *styS* and *styR* are regulatory proteins and encode a sensor kinase protein and a response regulator, respectively; *paaF* is the gene for phenylacetyl-coenzyme A ligase, involved in the initial step of the lower catabolic pathway.

The first two genes of the operon are *styA* and *styB*, and encode the two subunits of the styrene monooxygenase. Adjacent to these genes are the *styC* and *styD* genes, which encode the styrene oxide isomerase and a phenylacetaldehyde dehydrogenase (Fig. 1.3b). Sequence analysis has revealed that these genes are remarkably similar in nucleotide sequence and organisation in a number of *Pseudomonas* strains. The high degree of similarity between these genes and the conserved arrangement of the operon suggest a conserved evolutionary origin. Furthermore, the conserved nature of the genes across a number of *Pseudomonas* species suggests that discrete horizontal transfer events resulted in the acquisition of the upper catabolic pathway by the different species. Also, another gene has recently been identified downstream from the *styABCD* genes in *P. putida* CA-3, which is co-transcribed with the rest of the operon. The product of this gene, StyE, appears to be involved in the active transport of styrene across the outer cell membrane (Mooney et al., 2006a).

1.3.2 Lower catabolic pathway

After styrene has been converted to PAA by the enzymes of the upper styrene pathway, the lower catabolic pathway begins with the conversion of PAA

to phenylacetyl-CoA by phenylacetyl-CoA ligase. The gene encoding the phenylacetyl-CoA ligase has been found adjacent to the styrene upper pathway genes in a number of *Pseudomonas* strains, but it also appears that another copy of this gene is generally also found on the genome. The conversion of PAA to phenylacetyl-CoA is followed by β -oxidation-like enzyme-catalysed reactions that eventually lead to intermediates that feed into the TCA cycle. Studies on the metabolic fate of phenylacetyl-CoA in *P. putida* CA-3, using random Tn5 mutagenesis, indicated that it is acetyl-CoA that ultimately enters the TCA cycle from phenylacetyl-CoA metabolism (Fig. 1.4). The final step in PAA metabolism, catalysed by the PaaE enzyme, has also been studied and it has been concluded that both succinyl-CoA and acetyl-CoA are the final intermediates that feed into the TCA cycle (O'Leary et al., 2005; Nogales et al., 2007). The PAA pathway has been characterised at the genetic level in a number of bacterial strains, including some *Pseudomonas* species. In addition, as styrene is degraded via PAA, it is not surprising that the pathway has been identified in styrene-degrading strains, such as *P. fluorescens* ST and *Pseudomonas* sp. Y2 (Alonso et al., 2003; Di Gennaro et al., 2007). A number of genes involved in PAA metabolism in *P. putida* CA-3 have also been

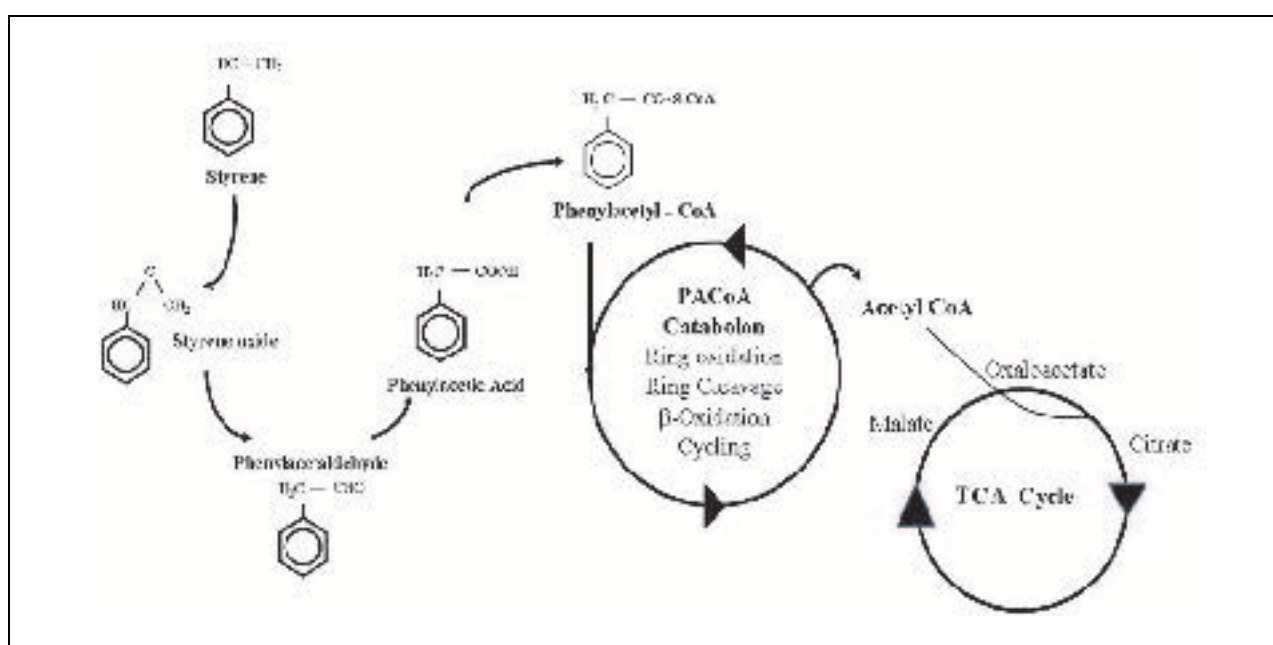


Figure 1.4. Overall scheme of styrene degradation showing upper and lower catabolic pathways, leading to the ultimate formation of acetyl-coenzyme A which feeds into the tricarboxylic acid (TCA) cycle.

identified, namely *paaX*, *paaY* and *paaN*, as well as the phenylacetyl-CoA ligase *paaF* (O'Leary et al., 2005), and the genes appear to be arranged in a similar manner to that which has been reported in *Pseudomonas* sp. Y2. Recently, it has been shown that the PAA catabolon is functionally duplicated on the *Pseudomonas* sp. Y2 genome (Bartolome-Martin et al., 2004). However, O'Leary and co-workers (2005) have also reported the identification of functional lower-pathway-type enzymes with only low levels of similarity to known phenylacetyl-CoA catabolon genes. The lower pathway involving the metabolism of phenylacetyl-CoA to acetyl-CoA is widely regarded as one of the four core metabolic routes known for aromatic hydrocarbon degradation in bacteria. However, the sequence and organisation of PAA genes in various bacterial species is much more diverse, in contrast to the highly conserved nature of the upper pathway styrene genes described above. It is possible that independent evolution of the upper and lower pathways may have occurred and that styrene-degrading species may have acquired both the upper and lower pathways separately.

1.4 Regulatory Control of Styrene-Degradation Genes

1.4.1 Transcriptional regulation

Transcription of the upper pathway *styABCDE* genes is activated when styrene is present and is mediated through a two-component signal transduction system encoded by the *styS* and *styR* genes. The involvement of a two-component regulatory system in aromatic hydrocarbon degradation is unusual and, apart from styrene metabolism, has only been described for toluene degradation in *P. putida* F1 and *Thauera* sp. strain T1 and for the degradation of biphenyls in *Rhodococcus* sp. strain M5 (Coschigano and Young, 1997; Labbe et al., 1997; Lau et al., 1997). The *styS* and *styR* genes are transcribed together, with the stop codon of *styS* overlapping the start codon of *styR*. The StyS sensor kinase protein contains five distinct domains: two histidine kinase domains, a receiver domain and two input domains (Lau et al., 1997; Velasco et al., 1998; O'Leary et al., 2002). Both histidine kinase domains contain the characteristic amino acid blocks H, N, G1, F and G2, while the receiver domain of StyS contains the D, D, S, K amino

acid residues typical of bacterial response regulators of the RA2 receiver subfamily (Grebe and Stock, 1999). Both sensory input regions also contain blocks of conserved amino acids typical of Per-ARNT-Sim (PAS) domains which have been identified in a number of prokaryotic, eukaryotic and archaeal proteins and are reportedly involved in sensing external signals, such as light and oxygen, sensing changes in the redox potential of the cell, as well as possessing the ability to bind small hydrophobic aromatic compounds (Taylor and Zhulin, 1999; Amezcua et al., 2002). The exact mechanism in which StyS senses the presence of styrene has not yet been fully elucidated, but it has been suggested that one input domain may sense changes in the redox potential of the cell when styrene is present, while the other input domain may detect the presence of extracellular styrene (Santos et al., 2000; Milani et al., 2005). The StyR response regulator contains two separate domains, the N-terminal regulatory domain and a C-terminal DNA-binding domain. The regulatory domain contains the essential conserved amino acids D, D, T and K found in a number of response regulators (Grebe and Stock, 1999). When the presence of styrene is detected by the StyS sensory input regions, this leads to phosphorylation of the sensor kinase and, in turn, phosphorylation of the StyR protein. It has been demonstrated that phosphorylation induces dimerisation of StyR and that it is a StyR dimer that binds to the *sty* promoter (Leoni et al., 2003). Studies have shown that a phosphorylated StyR dimer binds to a palindromic sequence centred at position -41 in the *sty* promoter and analysis has revealed that this 6, 4, 6 base-pair inverted repeat, designated STY2, is present in the *sty* promoter of *P. fluorescens* ST, *P. putida* CA-3 and *Pseudomonas* sp. Y2 (Beltrametti et al., 1997; Velasco et al., 1998; O'Leary et al., 2001). Binding of phosphorylated StyR to this region attracts ribonucleic acid (RNA) polymerase to the extended -10 box and initiates transcription. Furthermore, it has also been shown that the integration host factor binds to an adenine-thymine (AT)-rich region upstream from the STY2 sequence and is essential for optimal expression of the *sty* genes (Santos et al., 2002). Further analysis of the promoter region revealed the presence of two other StyR binding sites, STY1 and STY3, overlapping the integration host factor binding site and located

downstream of the *sty* operon transcription start site, respectively (Fig. 1.5) (Leoni et al., 2005). Phosphorylated StyR binds to the three sites with different affinities. While the STY2 site is essential for the transcription of the *sty* operon, the other two sites have been proposed as the target of negative regulatory influences, particularly when additional carbon sources are present. Thus, the overall regulation of the styrene catabolon appears quite complex, involving a number of specific regulators, as well as the undoubted interaction with other global regulators in the cell.

1.4.2 Catabolite repression

The expression of the styrene-degradative catabolon has been reported to be subject to catabolite repression whereby expression of the styrene catabolic genes can be switched off rapidly when certain other carbon sources become available. Therefore, the presence of additional carbon sources can have a significant impact on the ability of a strain to degrade styrene, as transcription of the genes and enzyme activity can be rapidly altered when more utilisable carbon sources are detected. This could be a significant problem for developing efforts to remediate styrene-contaminated waste as many waste streams

will not be pure and will likely contain additional compounds, including potential carbon sources. It has become clear that different carbon sources can have varying effects in different strains and while some carbon sources seem to have a similar repressive effect in both *P. putida* CA-3 and *P. fluorescens* ST, others can have very different effects. Glucose, succinate and acetate have no repressive effect in *P. putida* CA-3, while they have been shown to strongly repress induction of the *sty* operon in *P. fluorescens* ST (O'Connor et al., 1995; Santos et al., 2002). In *P. putida* CA-3, the effects of catabolite repression are exerted through expression of the *stySR* genes, with no *stySR* transcripts being detected in batch cultures grown on styrene in the presence of citrate, glutamate and PAA, and therefore no transcription of the *sty* catabolic genes occurs (O'Leary et al., 2001). In contrast, it appears that *stySR* is constitutively expressed in *P. fluorescens* ST and it has been suggested that the effects of catabolite repression are exerted through interference with the signal transduction process of the StySR system or by direct repression of the *sty* promoter (Santos et al., 2000). While it appears that many aspects of the regulation of the *sty* genes are similar in different strains, it has

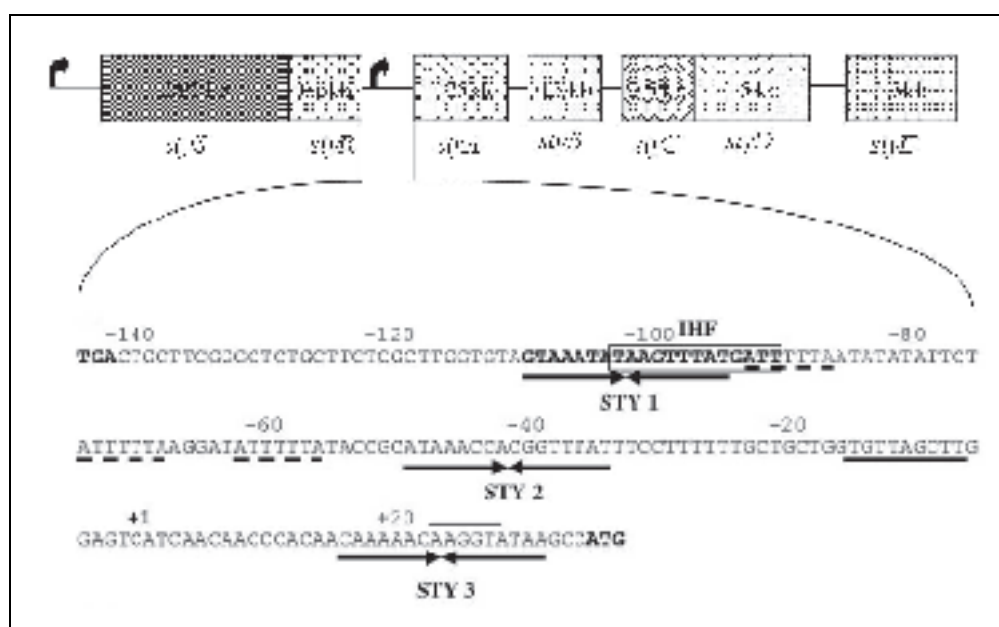


Figure 1.5. Sequence of the *sty* operon promoter region showing the phosphorylated StyR binding sites, STY1-3 and the integration host factor (IHF) binding site. The start codon of *styA* and stop codon of *styR* are indicated in bold. The extended -10 region is underlined. The transcription start site is indicated, +1.

become evident that this is a complex process and the fine-tuning of the regulatory process may differ slightly between the strains, through host-strain-specific interactions with global regulators in the cell.

1.5 Aims and Objective

The main focus of this project was to utilise the considerable knowledge base already generated about bacterial styrene degradation to develop the biotechnological potential of the styrene-degrading ability of *P. putida* CA-3. The overall aim of the project was to apply recombinant DNA strategies in an attempt to enhance the rate of flux of styrene through the catabolic route and ultimately lead to increased degradation of this toxic compound. In addition to having the potential to generate high degradation capability strains, it was anticipated that the enhanced catabolic flux might also, in turn, facilitate increased PHA accumulation from styrene, under appropriate growth conditions. Through focused recombinant DNA manipulations of the *P. putida* CA-3 genome, the project hoped to optimise the rate of styrene degradation to acetyl-CoA. Thus, it was hoped that physical outputs of this research might lead to recombinant strains capable of enhanced utilisation of styrene, which might offer biotechnological

applicability for both styrene degradation and biodegradable plastic production. However, the presence of other compounds, particularly more utilisable carbon sources, can occur in many styrene waste streams and additional carbon sources are known to impact negatively on styrene gene transcription and enzyme activity. The manipulation of these sites therefore may help to improve the styrene-degrading ability. Furthermore, the transport of many aromatic compounds, including styrene, into the cell may also be a limiting factor for degradation, and over-expression of styrene transport genes is a potential way to reduce such limitations. Previous identification of a novel gene in the PAA pathway indicated that critical single-copy genes may pose a limitation on the pathways, and efforts were made to identify additional PAA genes in this strain. This research project was divided into three core objectives as follows (Fig. 1.6):

1. Elimination of negative regulatory elements from the upper and lower catabolic operons;
2. Over-expression of key catabolic operon genes; and
3. Identification of functional genes contributing to lower pathway activity.

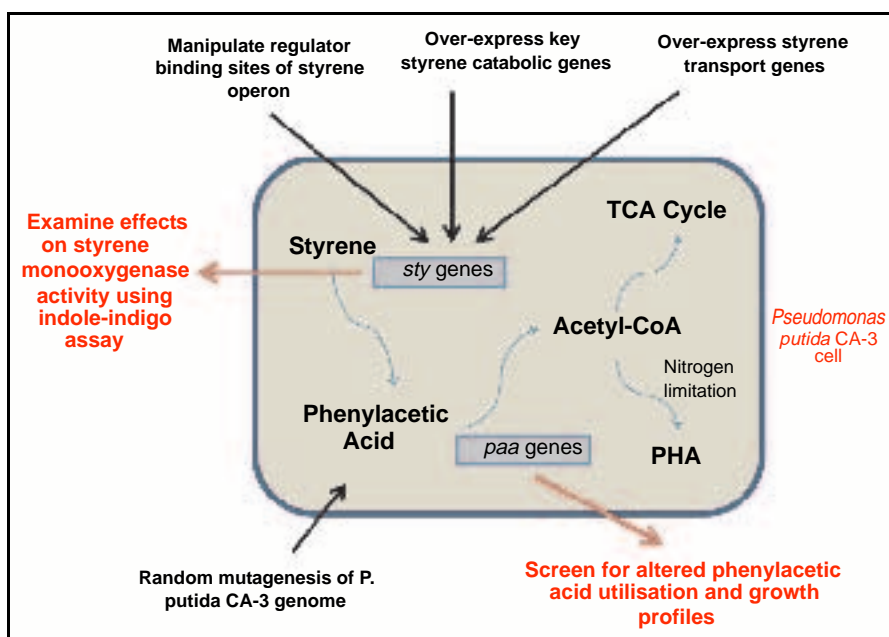


Figure 1.6. Summary of key objective and work plan. TCA, tricarboxylic acid; PHA, polyhydroxyalkanoate.

2 Genetic Manipulation of the Styrene-Degradative Gene to Improve Styrene Degradation

2.1 Introduction

Styrene is a toxic alkenylbenzene and its extensive industrial use has led to increased anthropogenic release of styrene, which poses a threat to both the environment and human health. While Ireland may not be a primary producer of styrene or styrene-based products, a considerable amount of these products are imported into the country every year, much of which are either not recycled or non-recyclable. Worldwide, the styrene industry produces vast quantities of styrene-based products and, as a consequence, large quantities of styrene waste. As a result, there has been significant interest in environmentally sensitive approaches to remediate and treat this styrene-contaminated waste. One such strategy is bacterial biodegradation and the styrene-degradative genes have been well characterised in a number of bacterial strains, particularly *Pseudomonas* spp., where the initial stages of the complete mineralisation of styrene are catalysed by enzymes encoded by the *sty* operon, and results in the stepwise conversion of styrene to PAA. Transcription of the *sty* genes is activated in the presence of styrene and controlled by the two-component system StyS/StyR. The presence of styrene is detected by the StyS sensory input regions, which leads to phosphorylation of the sensor kinase and subsequent phosphorylation of the StyR protein. Phosphorylated StyR then forms a dimer that binds to a palindromic sequence centred at position -41 in the *sty* promoter and analysis has revealed that this 6, 4, 6 base-pair inverted repeat, designated STY2, is present in the *sty* promoter of *P. fluorescens* ST, *P. putida* CA-3 and *Pseudomonas* sp. Y2 (Beltrametti et al., 1997; Velasco et al., 1998; O'Leary et al., 2001). Binding of phosphorylated StyR to the STY2 region is essential and attracts RNA polymerase to the extended -10 box and initiates transcription. In addition, it has also been shown that the integration host factor binds to an AT-rich region upstream from the STY2 sequence and is required for optimal expression of the *sty* genes (Santos et al., 2002). Further analysis of the promoter

region revealed the presence of two other StyR binding sites, STY1 and STY3, overlapping the integration host factor binding site and located downstream of the *sty* operon transcription start site, respectively, and it has been shown that phosphorylated StyR binds to these sites with different affinities (Leoni et al., 2005). As the STY1 site overlaps with the integration host factor binding site it has been proposed that there may be competition for binding between phosphorylated StyR and the integration host factor, with binding of StyR resulting in reduced levels of transcription. This site, therefore, plays a positive role under inducing conditions but also appears to be involved in glucose-mediated repression in *P. fluorescens* ST. Leoni et al (2005) have also reported that in *P. fluorescens* ST, under high redox potential conditions, a modulated StyS sensor kinase activity can alter levels of StyR phosphorylation, leading to it binding at the STY3 inhibitory site downstream from the *sty* operon transcriptional start site, thus blocking expression. In contrast, del Peso-Santos et al. (2006) have reported that, in *Pseudomonas* sp. Y2, the negative transcriptional regulator of the lower pathway, PaaX, binds to a site overlapping the inhibitory STY3 site and prevents high-level expression of the *sty* operon genes, unless the lower pathway substrate phenylacetyl-CoA is being generated, which, in turn, can alleviate PaaX binding (del Peso-Santos et al., 2006). The complex interplay of various proteins potentially binding to different sites clearly influences and modulates expression of the *sty* genes under different physiological conditions.

The current project focused on manipulating the STY3 site to try and reduce regulator binding and potentially improve overall levels of transcriptional activation. Attempts were also made to disrupt the negative transcriptional regulator of the PAA pathway, PaaX, by using a single crossover homologous recombination approach, and to determine the effect on activity of the styrene genes. Further work involved the stable integration of additional copies of the styrene catabolic

genes, *styABCD*, and the styrene transport gene, *styE*, into the chromosome of *P. putida* CA-3. Again, it was hoped that the introduction of additional copies of the genes would lead to increased levels of styrene degradation, particularly since previous work had indicated that the active intracellular transport of styrene across the cell membrane might be a limiting factor in the activation of transcription of the styrene genes (Mooney et al., 2006a).

2.2 Materials and Methods

2.2.1 Bacterial strains and culture conditions

Pseudomonas putida CA-3 was previously isolated from a bioreactor treating styrene-contaminated waste. Cultures of *P. putida* CA-3 were maintained on Luria–Bertani (LB) agar. *Pseudomonas putida* CA-3 cultures were grown in 100 ml of liquid minimal salts medium (MSM) at 30°C and 120 rev./min. The MSM contained 7.0 g K₂HPO₄, 3.0 g KH₂PO₄, 1.0 g (NH₄)₂SO₄ per litre, with 2 ml of 1 M MgSO₄ added post-autoclaving. Growth on styrene required the addition of 70 µl of liquid styrene to a test tube fixed centrally to the bottom of a baffled 1-l Erlenmeyer flask. Where required, *P. putida* CA-3 were grown in MSM with 0.2% glucose and induced with varying concentrations of indole. *Escherichia coli* strains were grown on LB medium at 37°C. *Escherichia coli* XL-1 Blue were used for routine maintenance and construction of plasmids. *Escherichia coli* TOP10 cells were used for TOPO[®] TA cloning of polymerase chain reaction (PCR) products. Where required, antibiotics were added to growth media at the following concentrations: ampicillin (100 µg/ml), kanamycin (50 µg/ml), gentamicin (20 µg/ml) and chloramphenicol (25 µg/ml).

2.2.2 Molecular techniques

Genomic DNA was purified from *P. putida* CA-3 using chloroform/isoamyl alcohol and phenol/chloroform/isoamyl alcohol and was precipitated with isopropanol (Ausubel et al., 1987). Plasmid DNA was isolated from *E. coli* using the Qiagen Plasmid Miniprep Kit. All restriction enzyme digestions were carried out according to the manufacturer's instructions (Roche Applied Science). The PCR was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research) and agarose gel electrophoresis of DNA was performed in 40 mM Tris–acetate, 1 mM EDTA. The PCR products

were cloned into pCR[®]2.1-TOPO (Invitrogen) using the A-overhang generated during amplification with *Taq* DNA polymerase and recombinant colonies selected on LB agar containing 100 µg/ml ampicillin and X-gal. Ligations were performed using T4 DNA ligase (NEB). High-fidelity amplification was carried out using Vent_R[®] DNA polymerase; reactions contained 100 ng each primer, 100 µM each dNTP, 1× ThermoPol reaction buffer, 4 mM MgSO₄, 4% DMSO and 1 unit Vent_R[®] DNA polymerase. Where required, the addition of A-overhangs to high-fidelity polymerase-generated PCR products was performed using *Taq* DNA polymerase. The PCR products were purified from agarose gel using the QIAEX II Gel Extraction Kit and the DNA was purified after enzymatic reactions using the QIAquick Purification Kit (Qiagen).

2.2.3 Tri-parental mating

Tri-parental mating was performed using the helper plasmid pRK600 which contains the *tra* function and facilitated the mobilisation of plasmids from *E. coli* donor strains to *P. putida* CA-3. Overnight cultures of the donor *E. coli* strains, *E. coli* HB101 containing pRK600 and *P. putida* CA-3, were inoculated in fresh LB containing the appropriate antibiotic. After approximately 6 h growth, 700 µl of *P. putida* CA-3 culture were incubated at 45°C for 10 min and 200 µl of the donor strain and 100 µl of the helper strain added. The cultures were mixed well and the pellet resuspended in 50 µl fresh LB and spotted onto the centre of an LB agar plate. After 24 h incubation at 30°C, the cells were scraped from the agar and washed five times in Ringer's solution to remove any residual media. To select for recombinant *Pseudomonas* strains the washed mating reaction was plated on E2 minimal media (Vogel and Bonner, 1956), containing 10 mM citrate as a sole carbon source, and supplemented with vitamin and mineral solutions. Using citrate as a sole carbon source prevents the growth of donor and helper *E. coli* strains.

2.2.4 Mutagenesis

Site-directed mutagenesis (SDM) was performed using splicing overlap extension (SOEing) PCR (Horton et al., 1990) and required a multi-step PCR-based approach to disrupt the required base pairs. The first two rounds of PCR used sets of primers designed

to disrupt four base pairs of the inhibitory binding site, with the mutated bases located at the 5' end of the primers. The primers bind around the mutagenesis site and allow mis-priming of the required base-pair changes, while keeping specificity for successful primer binding at the 3' end. The primers also contained an overlapping region of complementarity for the subsequent splicing reaction. Both PCR products were generated separately with Vent_R[®] DNA polymerase using the primer pairs pStyRF(411)/pstyAMutR1N and pstyMutF1N/pstyAR(321). The DNA fragments were purified and combined in a SOEing PCR reaction performed with the Expand High Fidelity PCR System and approximately 75 ng of each DNA fragment, with the addition of the outer primers after five cycles of amplification, to allow sufficient time for the polymerase to extend the annealed fragments. The resulting spliced product was subsequently cloned in the pCR2.1-TOPO vector after the addition of an A overhang by *Taq* DNA polymerase. This product, pCR2.1-Mut1, was used as the template in a second set of PCR reactions with another two sets of overlapping primers designed to disrupt another two base pairs and with the reverse primers designed to incorporate the already mutated base pairs of Mut1, pStyRF(411)/pstyAMutR2N and pstyMutF2N/pstyAR(321). The individual fragments were again spliced together by SOEing PCR and cloned into the pCR2.1-TOPO vector, generating a construct containing the 3' end of *styR*, the mutated *psty* promoter and the 5' end of the *styA* gene, pCR2.1-Mut2. Sequencing was performed to confirm that the base pairs had been successfully mutated. The pCR2.1-Mut2 vector was mobilised from the *E. coli* TOP10 cells into *P. putida* CA-3 by tri-parental mating.

The *psty-styABC* catabolic genes were amplified using primers pstyRF(411) and styCR, generating fragment RAC. The 3' end of *styA* plus *styBC* was amplified using styA(321)OvF and styCR. The styA(321)OvF primer contains a region of complementarity to the Mut2-mutated promoter fragment generated previously and SOEing PCR was used to splice both fragments together, generating a DNA product designated RACM. The resulting products were cloned in pCR2.1-TOPO. The RAC and RACM fragments were digested from pCR2.1-TOPO using the restriction enzymes *Xba*I and *Hind*III and ligated into the broad

host range vector pBBR1MCS-5 (Kovach et al., 1995). Where required, recombinant vectors were mobilised into *P. putida* CA-3 by tri-parental mating. The StySR two-component regulatory system, together with its promoter, was amplified from *P. putida* CA-3 genomic DNA using primers pStySRF(*Hind*III)/pstySRR(*Xba*I). The primers contained restriction sites for ligation into pBBR1MCS-5. All primers are listed in [Table 2.1](#).

2.2.5 Over-expression of styrene catabolic and transport genes

The styrene promoter, *psty*, and the *styABCD* catabolic genes were amplified from the genome using the primer pairs, F1pstyA(*Not*I) and styDR(*Not*I) and Vent_R[®] DNA polymerase, generating a 4,052-bp product which was ligated into pCR2.1-TOPO. *Not*I restriction sites were included at the 5' end of each primer to allow subsequent cloning into *Not*I-digested pUT-Km1 (de Lorenzo et al., 1990). The pUT-Km1 vector allows the cloning of foreign DNA into the *Not*I site within the mini-transposon mobile element, and permits the stable integration of a cloned foreign gene into the chromosome after the recombinant vector is transferred into *P. putida* CA-3. Recombinant *P. putida* CA-3 strains containing an integrated extra copy of the *psty-styABCD* genes were selected by kanamycin resistance. The *sty* promoter, *psty*, was spliced to the *styE* gene by SOEing PCR (Horton et al., 1990). Briefly, the SOEing PCR consisted of the following steps:

1. Two separate PCR reactions generated the *psty* product (538 bp) using the primer pairs, pstyAFF2N(*Not*)/styARR2N, and the *styE* complete open reading frame (1,296 bp) with primers, styEF3N/styER4(*Not*). *Not*I sites were included to allow ligation into the pUT-Km1 vector;
2. Primers styARR2N and styEF3N were designed so that they contained an overlapping region of complementarity at their 5' end;
3. Both of the generated fragments were then used as the template in the next round of PCR using the outer primers pstyAFF2N(*Not*) and styER4(*Not*); and
4. After the initial denaturation step, the complementary regions of both DNA fragments

Table 2.1. Primers 1.

Primer	Sequence 5'–3' ¹	Annealing temperature (°C)
pStyRF(411)	acagctgttttcaccta	53
pStyAR(321)	gcatcggtgtagagacg	53
pstyAMutF1N	atgattaaaggtataagccatgaa	50
pstyAMutR1N	ttatacctttaatcattgtgggtgct	50
pstyAMutF2N	tatgagctatgaaaaagcgat	50
pstyAMutR2N	ttcatagctcatacctttaatcattgtgggt	50
styA(321)OvF	<u>tcttaccagccgatgctgatgcgtgc</u>	55
styCR	cgtcaattgcggaagag	55
pstySRF(HindIII)	tcaactaagcttctcgtcctgcacccctgctac	55
pstySRR(XbaI)	tactta tctagag atgactccaagctaacacca	55
F1pstyA(NotI)	<u>gcggccgcag</u> tgtgactgctcctggccctgttc	55
styDR(NotI)	<u>gcggccgctcag</u> taggcgataaccaacga	55
pstyAFF2N(Not)	<u>gcggccgccatgaaggccggagcgatagagtt</u>	50
pstyARR2N	<u>aggccgacgaacatggcttatacctttttgtt</u>	50
styEF3N	atgttctgcgcctcgag	50
styER4(Not)	<u>gcggccgctcagaaattatgggtatacgaa</u>	50
PaaX F	aaggcctcaagcgctctacag	60
PaaX R	tcgggcaaggaagcagtc	60

¹*gcggccga*, NotI; *aagctt*, HindIII; *tctaga*, XbaI. Complementary ends underlined. Mutated base pairs in bold.

anneal and the polymerase extends to fill the gaps and generates a spliced product as template for the successive rounds of amplification.

Pseudomonas putida CA-3 DNA was used as the template in each PCR reaction and the individual DNA products were amplified using Vent_R[®] DNA polymerase. The SOEing PCR reaction was performed with the Expand High Fidelity PCR System, with approximately 75 ng of each DNA fragment and the addition of the outer primers after five cycles of amplification, to allow sufficient time for the polymerase to extend the annealed fragments. Sequencing was performed to confirm that the DNA fragments had been successfully spliced together. The spliced *psty-styE* was cloned into pCR2.1-TOPO, digested with NotI and subsequently ligated into pUT-Km1. All primers are listed in Table 2.1. For multiple

copy expression, the *psty-styE* was digested from pCR2.1-TOPO with XbaI and HindIII and ligated into the broad host range vector, pBBR1MCS-5. Recombinant vectors were mobilised into *P. putida* CA-3 by tri-parental mating.

2.2.6 Gene knockout

Primers PaaX F and PaaX R (Table 2.1) were used to amplify a 408-bp section of the *paaX* gene from *P. putida* CA-3 genomic DNA. The resulting PCR products were cloned into pCR2.1-TOPO using the A-overhang generated during amplification with *Taq* DNA polymerase. Successful recombinant clones were identified by PCR and restriction digestion. The recombinant pCR2.1-paaX plasmid was mobilised from the host *E. coli* TOP10 cells into *P. putida* CA-3 by tri-parental mating, as described above.

2.2.7 Indole-indigo assay

The ability of styrene monooxygenase to convert indole to the blue dye indigo was used as an indication of pathway activity. Briefly, after growth and induction, cells are pelleted and re-suspended to a consistent cell dry weight of 1 mg/ml. Indole was dissolved in dimethylformamide (DMF) at a concentration of 100 mM, and this solution was further diluted in 50-mM sodium phosphate buffer (pH 7) to a final indole concentration of 0.25 mM. Next, 400 µl of the diluted indole solution were incubated at 30°C for 5 min and 100 µl of the cell suspension added. The formation of indigo by activity of the styrene enzymes is monitored over a 40- to 50-min period, with each time point requiring an individual sample. The indole/cell suspension was incubated at 30°C with vigorous shaking and at the appropriate time point the tubes were centrifuged at 13,000 rev./min. The supernatant was removed and 1 ml DMF added to the tubes, which were then vortexed for a further 10–15 min at 30°C. The tubes were centrifuged again at 13,000 rev./min and the supernatant transferred to a new tube. The optical density (OD) at 610 nm was recorded for each sample using a quartz cuvette (O'Connor et al., 1997).

2.3 Results and Discussion

The aim of the reported project was to utilise the extensive knowledge base about the degradation of styrene in bacteria to attempt to generate recombinant strains with improved styrene-degrading ability. A considerable amount of work has been carried out, particularly on members of the *Pseudomonas* genus, with pathways and genes characterised at the genetic and physiological levels. Further work has also examined transcriptional activation and positive and negative regulation events of the pathway. This project aimed to exploit this knowledge by applying recombinant DNA approaches to attempt to increase the rate of flux through the styrene-degradation pathway, thus potentially improving the rate of styrene degradation and possibly, in addition, the accumulation of PHA under appropriate growth conditions. However, the main focus was on the styrene pathway and the upper styrene catabolic operon, encoded by the *sty* genes.

2.3.1 Manipulation of the promoter region of the styrene operon

Sequencing and in silico analysis of the *P. putida* CA-3 *sty* promoter confirmed the presence of a number of potential regulator binding sites, and indeed the promoter region shared 100% identity with the *sty* promoter in *Pseudomonas* sp. Y2. While the binding of phosphorylated StyR to the STY2 site is essential to activate transcription, two inhibitory binding sites, STY1 and STY3, have been identified in the promoter region upstream and downstream of the STY2 site, respectively. Further analysis of the STY3 site, which is located downstream from the transcription start site, has shown that different regulators, namely phosphorylated StyR in *Pseudomonas fluorescens* ST and the negative regulator of the PAA pathway PaaX in *Pseudomonas* Y2, can bind to this site, possibly under different metabolic conditions, with binding of either regulator leading to the disruption of transcription of the styrene catabolic genes. Literature searches of consensus binding sites and in silico analysis of the STY3 site in *P. putida* CA-3 identified base pairs that may play a role in regulator binding. The STY3 site is located close to the translation start site of the *styA* gene, and overlaps the PaaX regulator binding site. The region also contains the putative ribosome binding site. Therefore, complete or partial removal of the STY3 site may adversely affect transcription or translation of the *sty* catabolic operon. A balance between disrupting enough to eliminate the binding site but not enough to eliminate expression of the *sty* genes was required. Analysis of the consensus sequences for both StyR and PaaX revealed a number of base pairs that may be important. The PaaX binding site is much longer than that for StyR and part of the consensus is located in the *styA* open reading frame. It was decided not to disrupt this part as it may interfere with the translation of the *styA* protein.

2.3.1.1 Site-directed mutagenesis of potential regulator binding sites

A site-directed mutagenesis approach was employed in an attempt to disrupt this inhibitor binding site and prevent or hamper regulator binding and the subsequent negative effect on *sty* gene transcription. In total, the primers were designed to change six bases pairs to disrupt part of the PaaX consensus and the

StyR inverted repeats (Fig. 2.1). Two of the mutations for StyR binding have previously been shown to relieve the promoter from glucose-mediated repression in *P. fluorescens* ST (Leoni et al., 2005). After initial mutagenesis attempts failed, a multi-step PCR-based approach was required to disrupt the required nucleotide bases. Subsequent sequencing analysis revealed that all base pairs were successfully altered as anticipated. The mutated promoter construct (a 717-bp fragment consisting of the 3' end of the *styR* gene, the mutated promoter region and the 5' end of the *styA* gene) was cloned into the pCR2.1-TOPO vector, generating pCR2.1-Mut2, and the vector transferred into the *P. putida* CA-3 by tri-parental mating. This vector is unable to replicate in *Pseudomonas* strains and it was anticipated that by including regions of the *styR* and *styA* genes a double crossover homologous recombinant event would occur and the native *sty* promoter would be replaced by the mutated promoter construct in the *P. putida* CA-3 genome. Numerous attempts were made to replace the native promoter using a double crossover. After the first mating, all the colonies (~1,000) were transferred to liquid media in 96-well plates and then replica-plated onto media containing the selection marker kanamycin. Resistant colonies suggest that a single crossover event occurred and that the kanamycin resistance cassette was integrated into the genome. The resistant colonies were grown again in liquid media and further screened for loss of kanamycin resistance, which completes the double crossover

event. However, from all the colonies screened the double crossover did not appear to have occurred. Control experiments were performed; however, a strain with the integrated mutated promoter was not created.

2.3.1.2 Construction of reporter system to analyse effects of mutated promoter on *sty* gene activity

While attempts were being made to produce a double crossover, other methods were used to try and analyse the effect of the mutated promoter. A reporter system was created in an attempt to quantify the effect of the mutated promoter base pairs on activity of the *sty* genes. The mutated promoter construct was spliced to the remainder of the *styA* gene, as well as *styB* and *styC*, by SOEing PCR and cloned in pCR2.1-TOPO, generating pCR2.1-RACM. The un-mutated native promoter plus *styABC* was also amplified directly from the *P. putida* CA-3 genome and cloned into the same vector to act as a control, pCR2.1-RAC. The *styABC* genes have previously been successfully used as a reporter system and when the genes are expressed they have the ability to convert indole to indigo, which can be quantitatively analysed and is an indication of styrene monooxygenase activity. For activation of the *sty* catabolic genes, the *stySR* genes are required, and these genes, along with the *stySR* promoter, were amplified and cloned into pBBR1MCS-5, generating pBB5-pstySR. In the initial experiments, the pBB5-pstySR vector was separately transformed into *E. coli* cells containing either pCR2.1-RAC or pCR2.1-RACM,

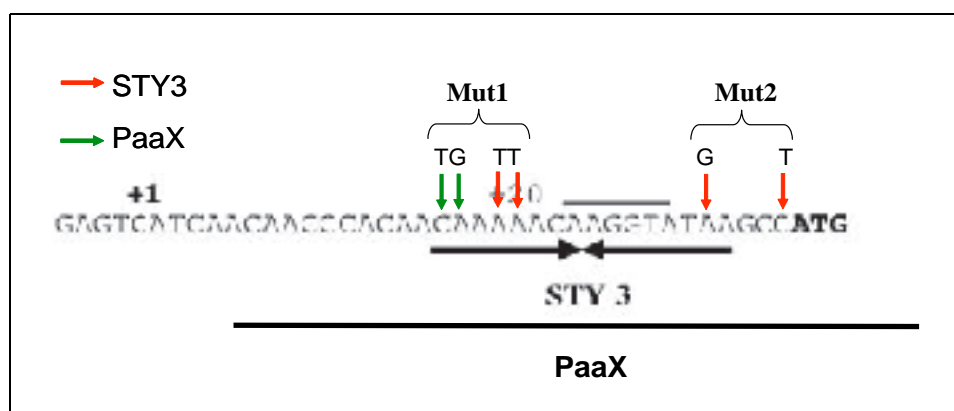


Figure 2.1. Styrene promoter region around the STY3 binding site. ATG indicates the start codon of the *styA* gene. Base pairs were mutated as indicated in two consecutive rounds of splicing overlap extension polymerase chain reaction in an attempt to disrupt the StyR and PaaX binding sites.

and both systems were analysed using the indole–indigo assay. In general, however, after a number of attempts to induce the system and determine the effects of the mutated base pairs on *sty* gene activity, the results were inconsistent. As the effects of the mutated promoter are likely mediated at the catabolite repression level, it is likely that other global host regulators are involved in the process that are absent from the *E. coli* cell and that are host specific to this study's *P. putida* CA-3 strain. Further to this the un-mutated promoter and mutated promoter constructs, RAC and RACM, were re-cloned into pBBR1MCS-5, generating pBB5-RAC and pBB5-RACM, respectively. These recombinant vectors have a broad host range and can replicate in the native *P. putida* CA-3 host. Both pBB5-RAC and pBB5-RACM vectors were successfully transferred into *P. putida* CA-3, where the entire regulatory apparatus is present and styrene

monooxygenase activity was analysed using the indole–indigo assay. Interestingly, under the range of metabolic profiles assessed to date, these disruptions did not appear to strongly impact on transcriptional repression events in *P. putida* CA-3. For example, when grown in the presence of styrene (Fig. 2.2), there was a slight increase in activity when either pBB5-RAC or pBB5-RACM was also present in the *P. putida* CA-3, compared with the wild-type cells. This is probably due to the fact that an extra copy of the *styABC* genes is now present in the cells. However, there were no differences in *sty* gene activity between the strains containing pBB5-RAC or pBB5-RACM. In a number of other different growth conditions a similar pattern was observed and the data tend to suggest that this binding site may only play a limited role in regulation of the styrene catabolic apparatus in *P. putida* CA-3, and indicates that while the styrene-degradation apparatus

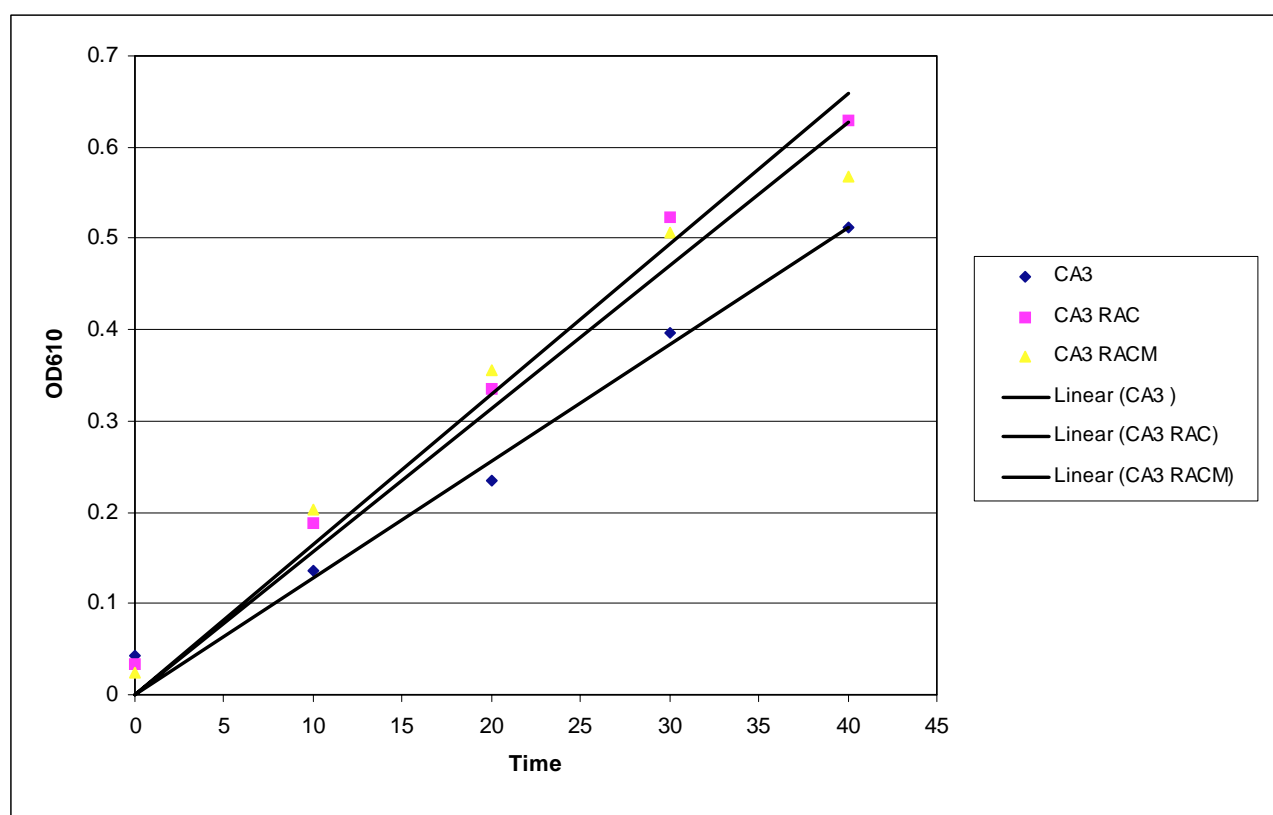


Figure 2.2. Formation of indigo by the action of the *sty* genes, as measured by monitoring optical density at 610 nm (OD610) over 40 min, for *Pseudomonas putida* CA-3 (CA-3), *P. putida* CA-3 containing pBBR1MCS-5-RAC (CA3 RAC) and *P. putida* CA-3 containing pBBR1MCS-5-RACM (CA3 RACM). RAC is the un-mutated promoter plus the *styABC* genes. RACM represents the mutated promoter spliced to the *styABC* genes.

appears conserved in a number of *Pseudomonas* strains subtle differences appear to exist in the fine-tuning of regulatory events. Recent work on catabolite repression in *P. fluorescens* ST also suggests that there are a number of slight differences in the way catabolite repression may be mediated compared with this study's *P. putida* CA-3 strain (Rampioni et al., 2008). Nevertheless, additional work is required to fully determine the significance of this regulator binding site in *P. putida* CA-3, especially in the presence of additional alternative carbon sources as the most evident effects are likely to be mediated under these metabolic conditions.

2.3.1.3 Disruption of the *paaX* negative regulator gene

A 408-bp section of the *paaX* gene, which is the negative regulator of the PAA pathway, was successfully amplified by PCR from *P. putida* CA-3 genomic DNA. The presence of this gene in *P. putida* CA-3 had previously been identified (O'Leary et al., 2005). The PCR product was subsequently cloned into pCR2.1-TOPO, with the intention of disrupting the genes in the *P. putida* CA-3 genome. This vector does not replicate in *P. putida* CA-3 and it was anticipated

that a single crossover event would occur to disrupt the *paaX* gene, and the effect of gene disruption of the activity of the *sty* gene and the phenylacetyl-CoA ligase could subsequently be examined. Numerous attempts were made to transfer this vector into *P. putida* CA-3 and select for a single crossover event; however, it could not be achieved using this technique.

2.3.2 Chromosomal duplication of the styrene catabolic genes

The effect of a chromosomal duplication of the styrene catabolic genes *styABCD* on the flux of styrene through the degradation pathway was investigated. The *styABCD* genes together with the *psty* promoter were amplified directly from the *P. putida* CA-3 genome and cloned into the pCR2.1-TOPO vector. The fragment was NotI digested and ligated into the mobile element of the pUT-Km1 mini Tn5 vector (Fig. 2.3). The pUT-Km1 vector allows the cloning of foreign genes at the NotI site, and after the recombinant pUT-Km1 was successfully introduced into *P. putida* CA-3 the mobile element of the pUT-Km1 containing the inserted genes should integrate into the genome. Stably integrating the genes into the genome,

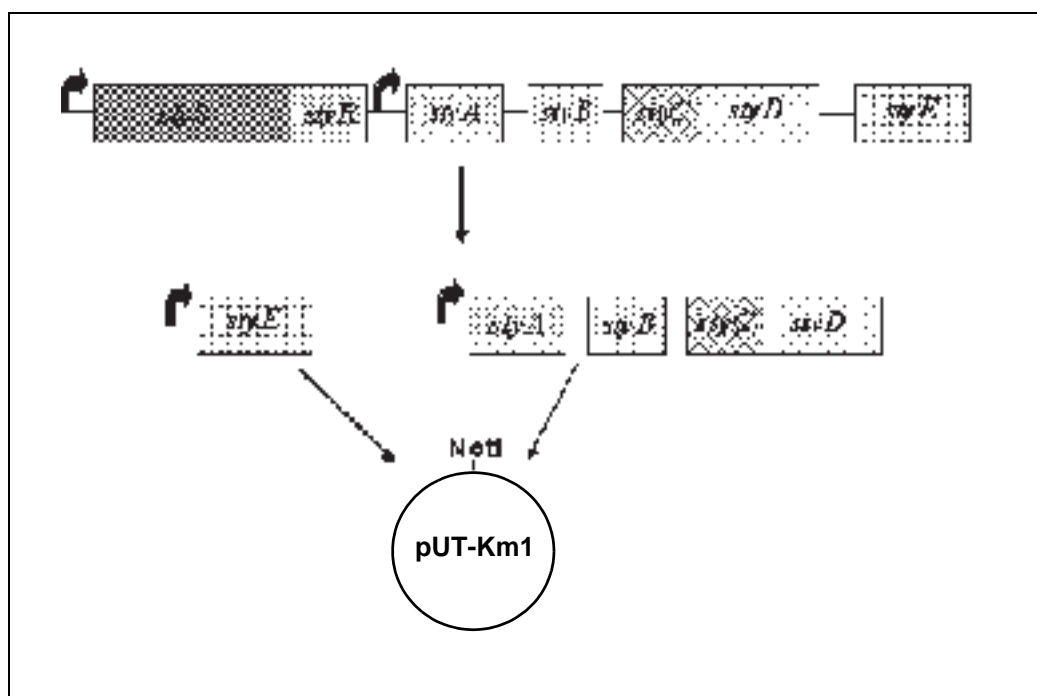


Figure 2.3. Splicing overlap extension polymerase chain reaction was used to splice the *sty* promoter to the *styE* gene. The *styABCD* genes plus the *sty* promoter were amplified from the *Pseudomonas putida* CA-3 genome. The DNA was cloned into the NotI site of the transposon delivery vector pUT-Km1.

rather than plasmid-borne homologous expression, has a number of advantages, especially for bioremediation applications, as it reduces the physiological burden on the cell, eliminates the requirement for costly antibiotic selection and also results in minimal chance of horizontal transfer. The recombinant vector was subsequently transferred to *P. putida* CA-3 by tri-parental mating and the resulting clones screened for stable integration of an additional copy of the catabolic genes into the genome. The activity of the styrene genes was then assessed by analysing the conversion of indole to indigo. While the additional copies of the catabolic genes appear to have integrated into the *P. putida* CA-3 genome, an increase in styrene monooxygenase activity was not observed. A number of independent indole–indigo assays were carried out but, again, a significant increase in enzyme activity was not detected. This suggests that a single additional copy of the genes may not be sufficient to significantly increase styrene monooxygenase activity and therefore improve styrene degradation.

2.3.3 Chromosomal and plasmid-borne duplication of the styrene transport gene

In addition, to examine whether active transport of styrene across the cell membrane presents a rate-limiting factor in the flux of styrene through the degradation pathway, an extra chromosomal copy of the *styE*-encoded membrane porin was constructed using the mini-transposon insertion strategy. To allow transcription of the *styE* gene and its concurrent transcription with the styrene catabolic genes, it was spliced to the *psty* promoter using SOEing PCR (Fig. 2.3). Again, it was hoped that introducing additional copies of the genes would lead to increased levels of styrene degradation by increasing transport of styrene into the cell and overcoming intracellular limitations. The spliced *psty-styE* fragment was misprimed with a *NotI* restriction site to permit ligation into the mobile element of the pUT-Km1 mini-Tn5 vector, and the recombinant vector transferred into *P. putida* CA-3 by tri-parental mating as before. The resulting colonies were screened by PCR and the successful integration of the spliced *psty-styE* construct into the *P. putida* CA-3 genome was observed, where the recombinant strain appeared to have both the full-length *psty-styABCDE* operon and the smaller *psty-*

styE construct amplified from genomic DNA (Fig. 2.4). The recombinant cultures were then examined for increases in styrene monooxygenase activity using the indole–indigo assay. However, after numerous attempts, an increase in styrene monooxygenase activity was not detected. The *styE* gene encodes a styrene active transport protein that transports styrene across the cell membrane, and previous work has indicated that this is essential for styrene degradation, as a knock-out mutant was no longer able to grow with this compound, and that transport of styrene across the cell membrane may be a limiting factor in styrene degradation (Mooney et al., 2006a). Therefore, with the incorporation of an additional copy of the gene, it was hoped that this limitation would be reduced. The mobile element of the pUT-Km1, into which the *psty-styE* constructs have been inserted, creates a recombinant strain with one additional genomic copy of the gene; however, it is possible that the presence of

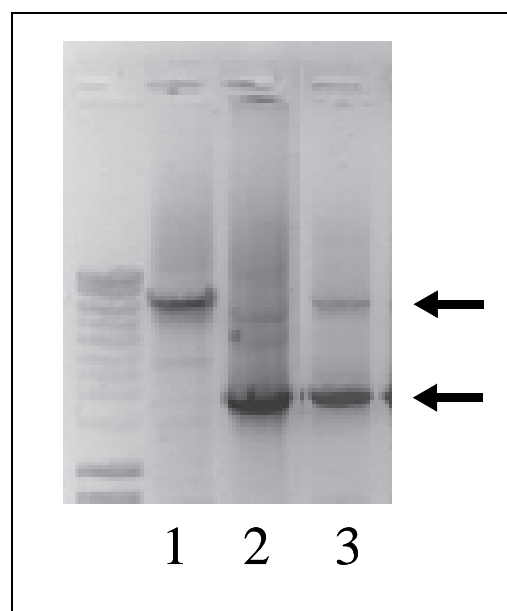


Figure 2.4. Polymerase chain reaction showing that the spliced *sty* promoter–*styE* gene construct appeared to integrate into the *Pseudomonas putida* CA-3 genome. Lane 1, *P. putida* CA-3 full-length *sty* promoter–*styABCDE*; Lane 2, *Escherichia coli* XL1 *sty* promoter–*styE* in pUT-Km1; Lane 3, *P. putida* CA-3 containing both the full-length *sty* promoter–*styABCDE* and the spliced *sty* promoter–*styE* construct.

only one extra chromosomal copy may not be sufficient to significantly overcome this limitation. Additional work was carried out by cloning the *psty-styE* into the broad host range vector pBBR1MCS-5, which can replicate in *P. putida* CA-3, and the recombinant vector was transferred to *P. putida* CA-3 by tri-parental mating, thereby introducing multiple copies of the *psty-StyE* construct into the cell. Assays were repeated to compare activity between *P. putida* CA-3, *P. putida* CA-3 containing the single integrated copy of *psty-styE*, and *P. putida* CA-3 with multiple plasmid-borne copies of *psty-styE* (Fig. 2.5). From the results, an increase in activity was not observed after numerous independent replications at various growth conditions. This may suggest that having extra copies of the promoter present may be a limiting factor and, in fact, the extra copies of the promoter may be subject to additional negative regulatory events. However, these observations have also suggested that single extra integrated copies of the genes do not appear to improve enzyme activity, and multiple copies of the

genes under the control of a constitutive promoter may be required in the cell to significantly increase styrene gene activity. Constitutive promoters are typically not influenced by the physiological conditions of the cell when styrene is present and, therefore, the styrene genes should be expressed regardless of the metabolic flux of the cell. Constitutive plasmid-borne expression of the *styE* gene had previously been shown to result in a 4.2-fold increase in styrene monooxygenase activity and an 8-fold increase in transcription of the *styA* gene (Mooney et al., 2006a). The aim of this project was to express stably integrated extra genomic copies of the styrene genes under the control of their own promoter, which is activated only in the presence of styrene, in a similar manner to what occurs in the wild-type cells grown in the presence of styrene. Styrene-activated expression would reduce the metabolic burden on the cell that may arise during constitutive expression of the genes. However, the results obtained tend to suggest that single integrated genomic copies are not enough to increase styrene

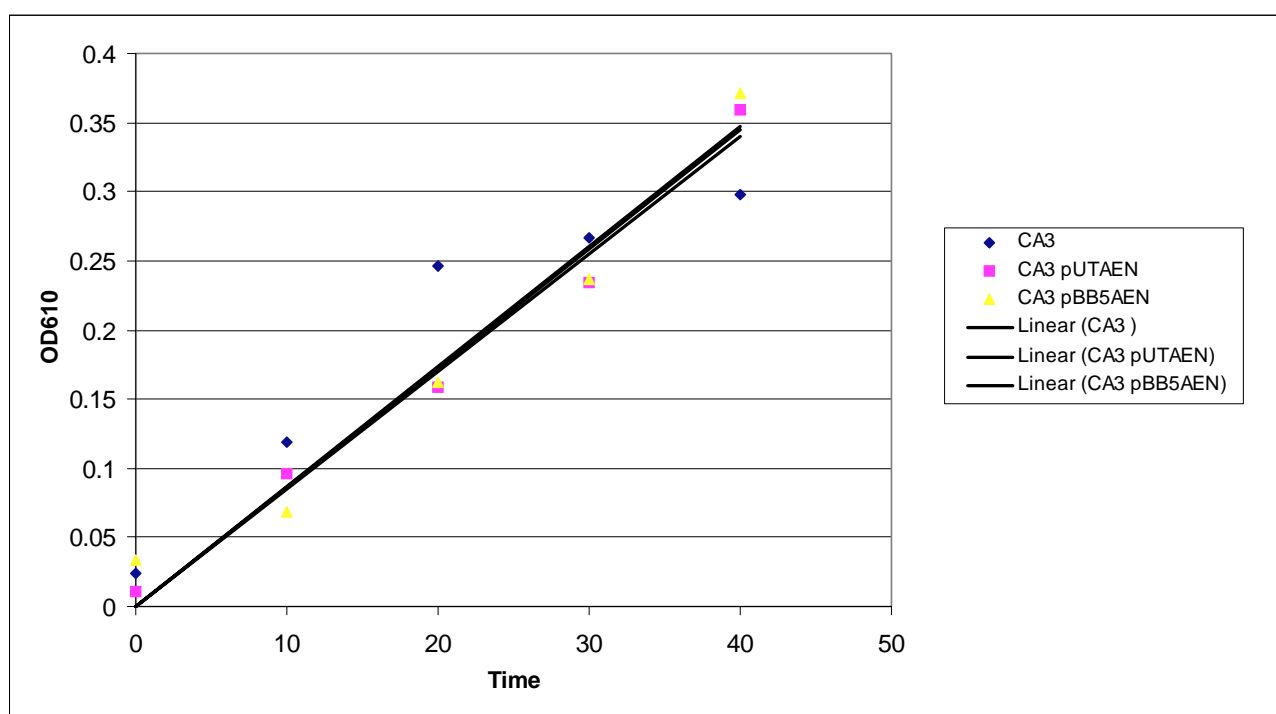


Figure 2.5. Formation of indigo by the action of the *sty* genes, as measured by monitoring optical density at 610 nm (OD610) over 40 min for *Pseudomonas putida* CA-3 (CA3), *P. putida* CA-3 containing pUT-Km1-AEN results in a single genomic extra copy of the gene (CA3 pUTAEN) and *P. putida* CA-3 containing pBBR1MCS-5- AEN multiple plasmid-borne copies of the gene (CA3 pBB5AEN). AEN represents the *sty* promoter spliced to the *styE* gene.

enzyme activity and that constitutive expression may be required to produce a significant increase in activity. It may be interesting to now examine whether constitutive expression of stably integrated genomic copies of the genes may be sufficient to improve activity.

2.4 Conclusion

The current project has enhanced knowledge of the styrene-degrading ability of *P. putida* CA-3. The results have suggested that the STY3 site may not be as important in this strain as a site of negative regulatory events and that additional elements may be involved in *P. putida* CA-3. Manipulation of sites involved in negative regulatory events is of particular interest for applications to bioremediation in the field, as many styrene waste sources may be contaminated with other pollutants and are unlikely to be pure. The presence of other compounds, particularly more utilisable carbon sources, is known to impact negatively on styrene gene transcription and enzyme activity. The manipulation of these sites, therefore, may help to improve the styrene-degrading ability. The transport of many aromatic compounds into the cell is another of the limiting factors for degradation, and transport of styrene across the cell membrane is no different. Over-expression of styrene transport genes

is a potential way to reduce such limitations; however, further work is required to exploit this to its full potential. Overall, the future goal is to make use of the remarkable ability of bacteria to completely mineralise a toxic pollutant and to create a styrene-degradation strain that is not limited by the transport of styrene across the cell membrane or indeed by negative regulatory events that disrupt aspects of the pathway in the presence of additional carbon sources. To summarise, the work presented here has indicated that:

- The STY3 binding site may not play a significant role in regulation of the styrene genes in *P. putida* CA-3, though it must be stressed that its role, as well as the role of other regulator binding sites, remains to be fully elucidated in this strain.
- A single additional chromosomal copy of the styrene catabolic genes, under the control of the styrene promoter, was not sufficient to improve styrene monooxygenase activity under these experimental conditions.
- Constitutive, rather than styrene-controlled expression, of additional copies of the styrene transport gene may be required to significantly improve styrene degradation.

3 Identification of Genes Involved in Phenylacetic Acid Degradation in *Pseudomonas putida* CA-3

3.1 Introduction

A number of *Pseudomonas* strains have to date been characterised for their ability to degrade the toxic aromatic pollutant styrene to carbon dioxide and water, with the concomitant generation of energy for the cell. The most common route of styrene degradation proceeds via oxidation of the vinyl side chain of the molecule, with the stepwise conversion of styrene to PAA. Phenylacetic acid is subsequently activated to phenylacetyl-CoA, and further β -oxidation reactions lead to the ultimate generation of intermediates that enter the TCA cycle. Therefore, in a number of styrene-degrading strains the mineralisation of styrene proceeds through PAA, and indeed the PAA catabolon has been suggested to be one of the four main routes of aromatic compound degradation in bacteria, thus playing a central role in cellular metabolism. Further analysis of the genome of styrene-degrading strains has identified the presence of genes required for the breakdown of this compound, and in some case the genes have been found adjacent to the styrene catabolic genes. The entire PAA catabolon has been identified in *Pseudomonas* sp. Y2 and in *P. fluorescens* ST (Alonso et al., 2003; Di Gennaro et al., 2007), and it has been reported that there is a second functional gene cluster for PAA degradation in *Pseudomonas* sp. Y2 (Bartolome-Martin et al., 2004). Three copies of the *paaF* gene, which encodes phenylacetyl-CoA ligase that catalyses the critical conversion of PAA to phenylacetyl-CoA, have now been found in *Pseudomonas* sp. Y2 and further analysis has revealed that expression of one copy of this gene, located adjacent to the styrene catabolic genes, is in fact controlled by the styrene regulatory system StyS/StyR (del Peso-Santos et al., 2008), while the negative regulator of the PAA pathway, PaaX, has also been shown to have the ability to bind to the promoter of the styrene catabolic genes and decrease levels of transcription (del Peso-Santos et al., 2006). These findings have revealed an integrated mechanism of regulation of the entire catabolon. A

number of genes involved in PAA metabolism in *P. putida* CA-3 have also been identified, namely *paaX*, *paaY* and *paaN*, as well as the phenylacetyl-CoA ligase *paaF*, and they appear to be arranged in a similar manner to those found in *Pseudomonas* sp. Y2. However, during previous investigation of PHA accumulation in *P. putida* CA-3, the unanticipated Tn5 disruption of a 3-hydroxyacyl-CoA dehydrogenase involved in PAA metabolism occurred, and this gene did not share significant identity (<30%) with other reported PAA 3-hydroxyacyl-CoA dehydrogenases, suggesting that novel genes may be involved in this strain. Despite reported duplication of the PAA catabolon in *Pseudomonas* sp. Y2, disruption of individual genes can apparently disrupt the ability of *P. putida* CA-3 to degrade styrene and PAA. Therefore, *P. putida* CA-3 potentially has genes with only low levels of homology to other reported genes, as well as apparently single-copy functional genes, which may therefore introduce a rate-limiting element into the lower PAA pathway. In this project efforts focused on subjecting the *P. putida* CA-3 genome to further rounds of random Tn5 mutagenesis in an attempt to disrupt and subsequently identify other potential genes involved in PAA metabolism in this strain.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

Pseudomonas putida CA-3 was isolated from a bioreactor treating styrene-contaminated waste and cultures were maintained on LB agar for routine use. *Pseudomonas putida* was grown in 100 ml of liquid MSM in 1-l flasks at 30°C, shaken at 120 rev./min. The MSM contained 7.0 g K₂HPO₄, 3.0 g KH₂PO₄, 1.0 g (NH₄)₂SO₄ per litre distilled water, and 2 ml of 1 M MgSO₄ added post-autoclaving. Carbon sources were added as follows: 0.2% glucose, 15 mM PAA, 10 mM citrate. Growth on styrene required the addition of 70 μ l of liquid styrene to a test tube fixed centrally to the bottom of a baffled 1-l Erlenmeyer flask. Cell growth was monitored by measuring optical density at 540 nm

and 600 nm. *Escherichia coli* XL1-Blue was used for plasmid maintenance and general cloning. The *E. coli* strains were grown on LB medium at 37°C. Where required, antibiotics were used at the following concentrations: 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, 50 µg/ml kanamycin and 20 µg/ml gentamicin.

3.2.2 Molecular techniques

Genomic DNA was purified from *P. putida* CA-3 using chloroform/isoamyl alcohol and phenol/chloroform/isoamyl alcohol and precipitated with isopropanol (Ausubel et al., 1987). Plasmid DNA was isolated from *E. coli* using the Qiagen Plasmid Miniprep Kit. All restriction enzyme digestions were carried out according to the manufacturer's instructions (Roche Applied Science). The PCR was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research) and agarose gel electrophoresis of DNA was performed in

40 mM Tris–acetate, 1 mM EDTA. Ligations were performed using T4 DNA ligase (NEB). Routine PCR was carried out using *Taq* DNA polymerase (Fermentas). High-fidelity amplification was carried out using Vent_R[®] DNA polymerase; reactions contained 100 ng each primer, 100 µM each dNTP, 1× ThermoPol reaction buffer, 4 mM MgSO₄, 4% DMSO and 1 unit Vent_R[®] DNA polymerase. The PCR products were purified from agarose gel using the QIAEX II Gel Extraction Kit and the DNA was purified after enzymatic reactions using the QIAquick Purification Kit (Qiagen). Primers for the PCR reactions are listed in [Table 3.1](#).

3.2.3 Random Tn5 mutagenesis

Random mutagenesis was performed using the transposon delivery vector pUT-Km1, which contains a mini-Tn5 transposon encoding kanamycin resistance (de Lorenzo et al., 1990), and can insert randomly into

Table 3.1. Primers 2.

Primer	Sequence 5'–3'	Annealing temperature (°C)
ARB1A	ccacgcgtcgactagtagtactnnnnnnnnngatat	55
TNEXT2	cttattgattccatttacact	55
ARB2A	ccacgcgtcgactagtagt	65
TNINT2	cctgcaggcatgcaagcttcggc	65
16S 27F	agagttgatcatggctcag	55
16S 1492R	ggttacctgttacgactt	55
PaaF F	ggttgagcatgtaggacggt	55
PaaF R	gccataaccgccttgctga	55
PaaL F	cggcatgctcgaccacctg	60
PaaL R	aaagcgatgttctgcgactc	60
Sig54 F Hind	tattacaagccttatgaaccatcgctgtcctaaaaatg ^a	60
Sig54 R Xba	atcattctagactacatcagtcgcttcgctcga ^b	60
paaLproF	gccgcgcaacagccagagc	63
paaLproR	cgccgagatgccaggaagg	63
paaL FIF Hind	tattacaagccttatgacagccctgcgctcctcacctt ^a	60
paaL RIF Xba	atcattctagactagtggttactggccttgct ^b	60
paaL-N F	cgcgctgtcccttactcca	63
paaL-N R	ggcgcaactctccagcatc	63

^aHindIII restriction site.

^bXbaI restriction site.

the *P. putida* CA-3 genome. The pUT-Km1 vector was mobilised from *E. coli* into *P. putida* CA-3 by tri-parental mating. Log-phase LB-grown cultures of *P. putida* CA-3, the pUT-Km1 donor strain and the pRK600 helper strain were mixed in the ratio 7:2:1, as described in [Chapter 2](#). The mating reaction was plated on E2 minimal media containing 10 mM citrate and 50 µg/ml kanamycin, to select for the recombinant *P. putida* CA-3 strains where the mini-Tn5 transposon was inserted into the genome. Approximately 12,500 individual colonies were transferred by hand to 100 µl of liquid citrate media in 96-well plates after each round of mutagenesis. The mutant libraries were replica-plated onto liquid citrate media containing 8% glycerol for long-term storage at -80°C. The libraries were subsequently screened for transposition events that disrupted PAA metabolism by replica plating the mutants onto agar plates containing either 10 mM citrate or 15 mM PAA as the sole carbon sources. The plates were screened for clones that retained the ability to grow with citrate but had reduced or no ability to utilise PAA. Potential mutants were selected and further analysed. The insertion site of the mini-Tn5 transposon was mapped using arbitrary PCR, consisting of two consecutive PCR reactions. The first round of PCR was performed using a mutant colony as the template, with an arbitrary primer, ARB1A and an internal primer of the mini-Tn5, TNEXT2. A further PCR reaction was performed using 5 µl of the first reaction as the template and primers that corresponded to the conserved region of the ARB1A primer, ARB2A, and a second internal primer of the mini-Tn5, TINT2 ([Table 3.1](#)). The resulting products were visualised on agarose gels and the required DNA bands purified from the gel using a QIAEX II Gel Extraction Kit. The DNA was sequenced using the mini-Tn5 internal primer, TINT2.

3.2.4 Reverse transcription (RT)-PCR analysis

Ribonucleic acid was isolated from *P. putida* CA-3 using the Qiagen RNeasy® Mini Kit. The purified RNA was treated with the TURBO DNA-free™ DNase Kit to ensure complete removal of DNA (Ambion). To examine the integrity of the RNA, agarose gel electrophoresis was performed in 45 mM Tris-borate, 1 mM EDTA. The concentration of RNA was determined by spectrophotometry. Reverse

transcription was carried out on 1 µg total RNA with 0.08 A₂₆₀ units random primers p(dN)₆ (Roche), 20 units SUPERaseIn™ RNase Inhibitor (Ambion), 1 mM each dNTP, 1× Transcriptor RT Reaction Buffer and 10 units Transcriptor Reverse Transcriptase (Roche). The reaction was incubated at 25°C for 10 min, followed by 30 min at 55°C, and 2 µl were used in subsequent PCR reactions. The PCR reactions contained 100 ng forward primer, 100 ng reverse primer, 200 µM each dNTP, 1× NH₄ buffer, 1.5 mM MgCl₂, 4% DMSO and 2.5 units *Taq* DNA Polymerase (Fermentas) in a total volume of 50 µl. Primers to monitor expression of the 16S ribosomal RNA gene (16S 27F/1492R), *paaF* gene (PaaF F/R) and *paaL* gene (PaaL F/R) are listed in [Table 3.1](#).

3.2.5 Complementation

The nucleotide sequences of six different *P. putida* *rpoN* genes, which encode the σ₅₄ sigma factor, were aligned. Primers were designed to amplify the full coding sequence of the gene. Restriction sites were mis-primed into the primers to allow subsequent ligation in the pBBR1MCS-5 vector (Kovach et al., 1995). The forward primer contains the HindIII restriction site (Sig54 F Hind) and the reverse primer contains the XbaI site (Sig 54 R Xba) to allow correct orientation in the vector to permit constitutive expression of the gene from the *lac* promoter in *P. putida* CA-3. Both the vector and the amplified DNA were digested with both restriction enzymes and ligated overnight at 16°C with T4 DNA ligase. The ligation reaction was transformed into competent *E. coli* XL-1 cells and confirmation of a successful ligation was performed by colony PCR and restriction digestion of the plasmid. The recombinant plasmid was transferred into *P. putida* CA-3 by tri-parental mating, with selection on 10 mM citrate and 20 µg/ml gentamicin.

3.2.6 Amplification of permease gene promoter and gene expression

Primers were designed to amplify the promoter region of the *paaL* gene based on the sequence data of the PAA pathway of *Pseudomonas* sp. Y2 obtained from the National Center for Biotechnology Information (NCBI) – *paaLproF* and *paaLproR* ([Table 3.1](#)). The primer set was designed to amplify a 964-bp region spanning the 3' end of the *paaK* genes, the intergenic

region and the 5' end of the *paaL* genes. The PCR product was amplified using high-fidelity Vent_R® DNA polymerase and subsequently sequenced. In an attempt to identify the gene downstream of *paaL*, primers were also designed to amplify a product based on the sequence of the *Pseudomonas* sp. Y2 sequence data – *paaL*-N F and *paaL*-N R (Table 3.1). The nucleotide sequences of four PAA permease genes were aligned. The nucleotide sequences of the *paaL* genes of *P. fluorescens* ST and the *Pseudomonas* sp. Y2 were more closely related and, as these strains are also styrene degraders, the primers were designed to amplify the full-length *paaL* gene based on homology between these genes. Restriction sites HindIII and XbaI were again mis-primed into the primers for subsequent cloning in the pBBR1MCS-5 vector. The full-length *paaL* gene was amplified using the primer pair, *paaL* FIF Hind and *paaL* RIF Xba, from *P. putida* CA-3 genomic DNA, digested with restriction enzymes and ligated into the digested pBBR1MCS-5 vector. Recombinant *E. coli* XL1 cells were identified by PCR and restriction digestion of the plasmid. The recombinant plasmid was then mobilised into *P. putida* CA-3 by tri-parental mating, and recombinant *P. putida* CA-3 selected on 10 mM citrate and 20 µg/ml gentamicin.

3.2.7 Indole–indigo assay

The indole–indigo assay was used to monitor activity of the styrene monooxygenase enzyme. The reaction was carried out as described in Chapter 2.

3.2.8 Nucleotide sequencing, assembly and analysis

Sequencing was carried out by GATC-Biotech (Germany), using ABI 3730xl technology. Sequences were assembled and processed using DNASTAR. The Basic Local Alignment Search Tool (BLAST) at NCBI was used to search DNA and protein databases for homologous DNA and protein sequences from other bacterial genomes (Altschul et al., 1997). Conserved functional or structural protein domains were identified using the Conserved Domain Database at the NCBI (Marchler-Bauer et al., 2005). Sequences were aligned using CLC Free Workbench Version 4 (<http://www.clcbio.com>). In silico restriction digestion of DNA sequences was performed using the NEBcutter V2.0, at the New England Biolabs UK website

(<http://www.neb.uk.com>). Potential σ 54 sigma factor RNA polymerase binding sites were identified using the PromScan programme (<http://molbiol-tools.ca/promscan>).

3.3 Results and Discussion

In a previous study, the *P. putida* CA-3 genome was subjected to random mutagenesis to identify genes involved in PHA accumulation and PAA catabolism (O'Leary et al., 2005). The investigation identified a critical 3-hydroxyacyl-CoA dehydrogenase involved in PAA metabolism in *P. putida* CA-3 which did not share significant identity with reported PAA-associated 3-hydroxyacyl-CoA dehydrogenases. This finding revealed that, despite published works suggesting genomic duplications of the PAA catabolism genes, novel single-copy genes that are essential for PAA degradation operate in *P. putida* CA-3, which should be fully investigated to assess rate-limiting implications in the overall catabolism of styrene, via PAA, to carbon dioxide and water.

3.3.1 Random Tn5 mutagenesis of *P. putida* CA-3 genome

Further rounds of mini-Tn5 random mutagenesis of the *P. putida* CA-3 genome were performed in this study using the transposon delivery pUT-Km1 mini-Tn5 vector to try to identify additional genes involved in PAA degradation in this strain. After initial optimisation of the tri-parental mating reaction, the pUT-Km1 vector was transferred to *P. putida* CA-3 by tri-parental mating with subsequent selection of recombinant colonies on media containing citrate as a carbon source. Initial rounds of mutagenesis were performed on a small scale, generating approximately 2,000–3,000 recombinant colonies. Once the process had been optimised the reaction was scaled up to generate mini-Tn5 mutant libraries with approximately 12,000 recombinant colonies per mating reaction. The colonies were transferred by hand to 96-well plates containing citrate broth with kanamycin and 8% glycerol and grown at 30°C for 24 h. The recombinant *P. putida* CA-3 strains were subsequently replica-plated from the liquid citrate culture onto solid media containing either citrate or PAA as sole carbon sources to identify colonies that have lost the ability or have reduced ability to utilise PAA. Three separate

independent rounds of mutagenesis were performed, generating approximately 35,000 recombinant strains in total, which were hand-picked and replica-plated. After screening on both citrate and PAA media, approximately 20 strains that had altered PAA utilisation ability were identified. However, after further analysis of the growth profile of a number of these strains, it appears that the ability to utilise PAA had not been disrupted and the lack of initial growth was probably due to transfer issues or possibly other physiological stress at that point. Nevertheless, a novel mutant was identified from the screening which was unable to grow with PAA as the sole carbon source and this was chosen for further analysis (Fig. 3.1).

3.3.2 Identification of transposon disrupted gene

The insertion site of the mini-Tn5 transposon was subsequently mapped using two consecutive rounds of arbitrary PCR. The PCR and subsequent sequencing identified the transposon disrupted gene as a gene encoding a putative $\sigma 54$ sigma factor, which is associated with RNA polymerase and is often involved in regulation of complex carbon utilisation profiles in *Pseudomonas* species. The core RNA polymerase enzyme in bacteria can associate with a number of interchangeable sigma factors, which recognise promoter regions of specific genes. The primary $\sigma 70$ sigma factor is present in all bacterial genomes and is involved in transcription of housekeeping genes required for normal cellular activity. In addition, bacteria contain a number of alternative sigma factors, including $\sigma 54$ sigma factor, which control transcription of genes involved in a range of other processes that

may only need to be switched on under certain physiological conditions (Wigneshweraraj et al., 2008). The $\sigma 54$ sigma-factor gene in *P. putida* CA-3 therefore appeared to play a role in transcription of the PAA metabolic genes.

3.3.3 Characterisation of the transposon mutant

Further analysis of the growth characteristics of this $\sigma 54$ mutant revealed that it could grow in the presence of both glucose and citrate, but interestingly also retained the ability to grow with styrene as a sole carbon source, despite the fact that styrene is degraded via PAA. The growth rate of the $\sigma 54$ mutant with styrene was, however, slightly less than that of *P. putida* CA-3, and styrene monooxygenase activity was subsequently analysed in both strains. The indole–indigo assay revealed that styrene monooxygenase activity was also slightly lower in the $\sigma 54$ mutant, approximately 1.5 times lower than *P. putida* CA-3, but also revealed that transcription of the *sty* genes was largely unaffected in the $\sigma 54$ mutant, so was therefore likely to be under the control of another sigma factor. This suggested that the catabolic genes of both the styrene and PAA pathways were unaffected and that styrene could still be degraded via PAA in the mutant strain. Therefore, if PAA was generated internally in the cell, the catabolic genes were active for further degradation. These observations tended to point towards the possible disruption of PAA transport genes. Searches of the literature indicated that some PAA metabolic pathways contain a permease, encoded by the *paaL* gene, which is transcribed separately from the PAA catabolic genes. The PAA

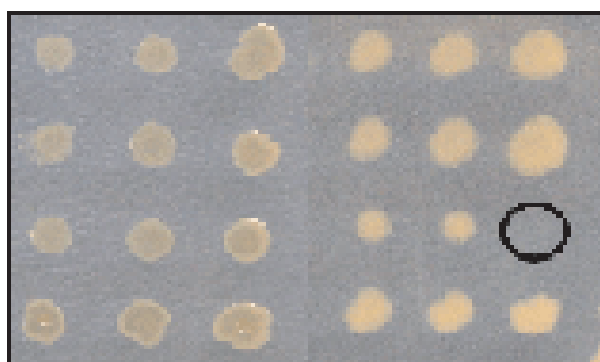


Figure 3.1. Replica plating of recombinant colonies after random Tn5 mutagenesis on citrate (left) and phenylacetic acid media. Circle indicates a strain no longer able to utilise phenylacetic acid as a sole carbon source.

catabolon appears to be arranged in a number of operons, and one such operon contains the permease and a ring-opening enzyme, encoded by the *paaN* gene, in both *Pseudomonas* sp. Y2 and *P. fluorescens* ST (Alonso et al., 2003; Di Gennaro et al., 2007). The *paaABCDEF* and the *paaGHIJK* operons encode the catabolic genes necessary for degradation, and analysis of the promoter regions of both operons suggests the involvement of a $\sigma 70$ sigma-factor RNA polymerase complex. Furthermore, a second copy of

the *paaN* gene is located adjacent to the *paaXY* regulator genes in *Pseudomonas* sp. Y2. Expression of a second copy of the gene may overcome the lack of $\sigma 54$ sigma-factor-mediated transcription of the *paaN* adjacent to the *paaL* gene in the $\sigma 54$ mutant strain. A number of genomic sequences of the *paaL* permease genes were retrieved from the NCBI genomic databases and a nucleotide and amino acid alignment performed to identify conserved regions (Fig. 3.2). From the conserved regions, a set of PCR primers was

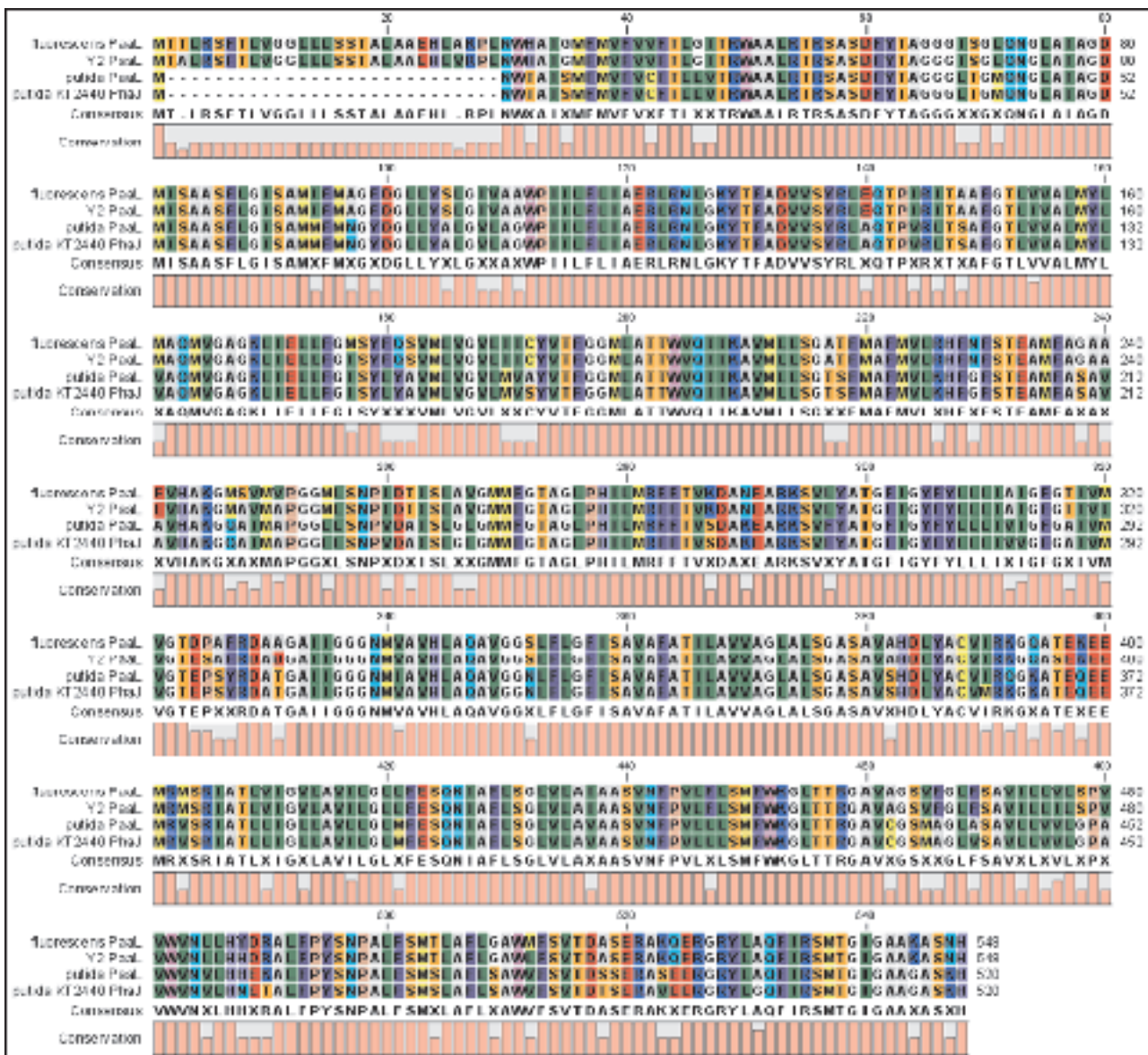


Figure 3.2. Alignment of the amino acid sequence of four phenylacetic acid permease genes, *paaL/phaJ* from the NCBI. The *paaL* genes from both the styrene-degrading strain *Pseudomonas fluorescens* ST and *Pseudomonas* sp. Y2 appear more closely related and contain a number of additional amino acids. Primers were designed from a conserved region of the gene.

designed to amplify a 696-bp internal region of the gene, and these were used to successfully identify the presence of the *paaL* gene in *P. putida* CA-3.

3.3.4 Analysis of gene expression

Both *P. putida* CA-3 and the $\sigma 54$ mutant were grown in the presence of different carbon sources and RNA was subsequently isolated and DNase treated. Gene expression patterns were examined using RT-PCR analysis, focusing on the 16S ribosomal RNA gene as a control constitutively expressed housekeeping gene, the *paaF* genes, encoding the phenylacetyl CoA ligase, which catalyses a critical step in the overall pathway by activating PAA to phenylacetyl-CoA, and the *paaL* gene (Fig. 3.3). The 16S ribosomal RNA gene was expressed under all conditions, as expected. The results indicated that the $\sigma 54$ sigma factor does not control the expression of the *paaF* gene, but does

indeed appear to play a role in expression of the *paaL* gene. While there appears to be low levels of transcription of the *paaL* gene in *P. putida* CA-3 grown on styrene, there appears to be no transcription of the gene in the $\sigma 54$ mutant. The PAA transporter is not required for styrene degradation and the low levels of expression of the *paaL* gene are possibly due to the presence of intracellular PAA produced during the catabolism of styrene. Thus, the results obtained strongly suggest that the $\sigma 54$ sigma factor disrupted in the mutant is involved in transcription of the transport gene. The transcription of the catabolic PAA genes appears to be controlled by $\sigma 70$ -type RNA polymerase in other *Pseudomonas* species and the involvement of a $\sigma 54$ sigma factor appears to be a novel finding and indicates a different regulatory control from the other catabolic genes.

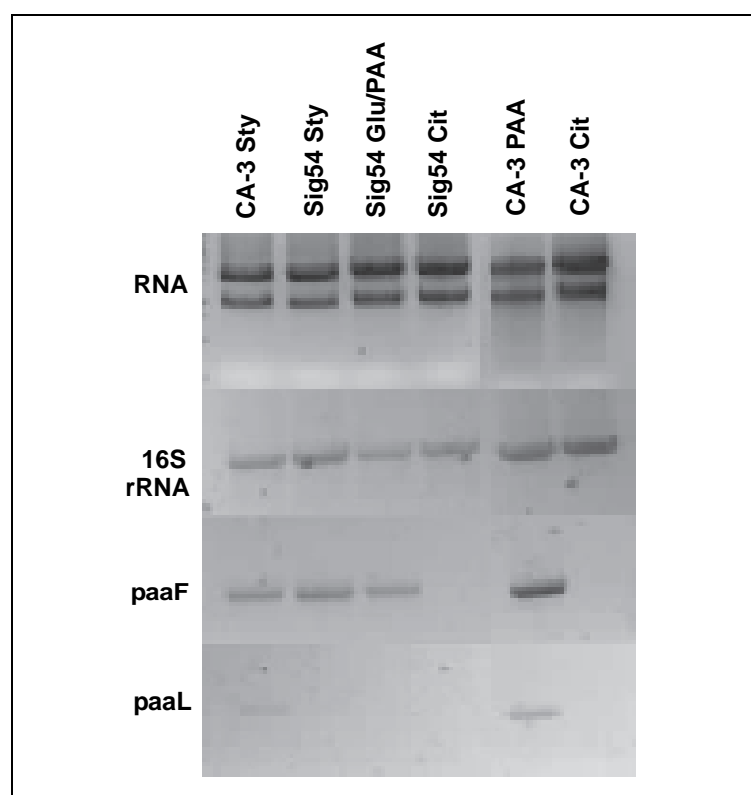


Figure 3.3. Reverse transcription polymerase chain reaction showing gene expression in the presence of different carbon sources. The top line illustrates the RNA isolated from both *Pseudomonas putida* CA-3 (CA-3) and *P. putida* CA-3 $\sigma 54$ mutant (Sig54) after growth with styrene (Sty), phenylacetic acid (PAA), glucose (Glu) or citrate (Cit) as indicated. Expression patterns of the 16S ribosomal RNA (rRNA) gene, the *paaF* gene (phenylacetyl-CoA ligase) and the *paaL* gene (phenylacetic acid permease) were analysed.

3.3.5 Gene complementation studies

Additional work has been carried out to further characterise this PAA $\sigma 54$ mutant. The nucleotide sequences of six *Pseudomonas* spp. *rpoN* genes, which encode the $\sigma 54$ sigma-factor subunit, were retrieved from the NCBI database and an alignment of the sequences was performed (Fig. 3.4). The nucleotide sequence of the *rpoN* genes is highly

conserved across the *Pseudomonas* strains, particularly at the 5' and 3' ends, and primers were designed to amplify the full open reading frame from *P. putida* CA-3 (Fig. 3.5). The *rpoN* gene was then successfully ligated in the pBBR1MCS-5 vector, where the gene can potentially be expressed when the recombinant vector is mobilised into the *P. putida* CA-3 $\sigma 54$ mutant. Plasmid-borne expression of the *rpoN*

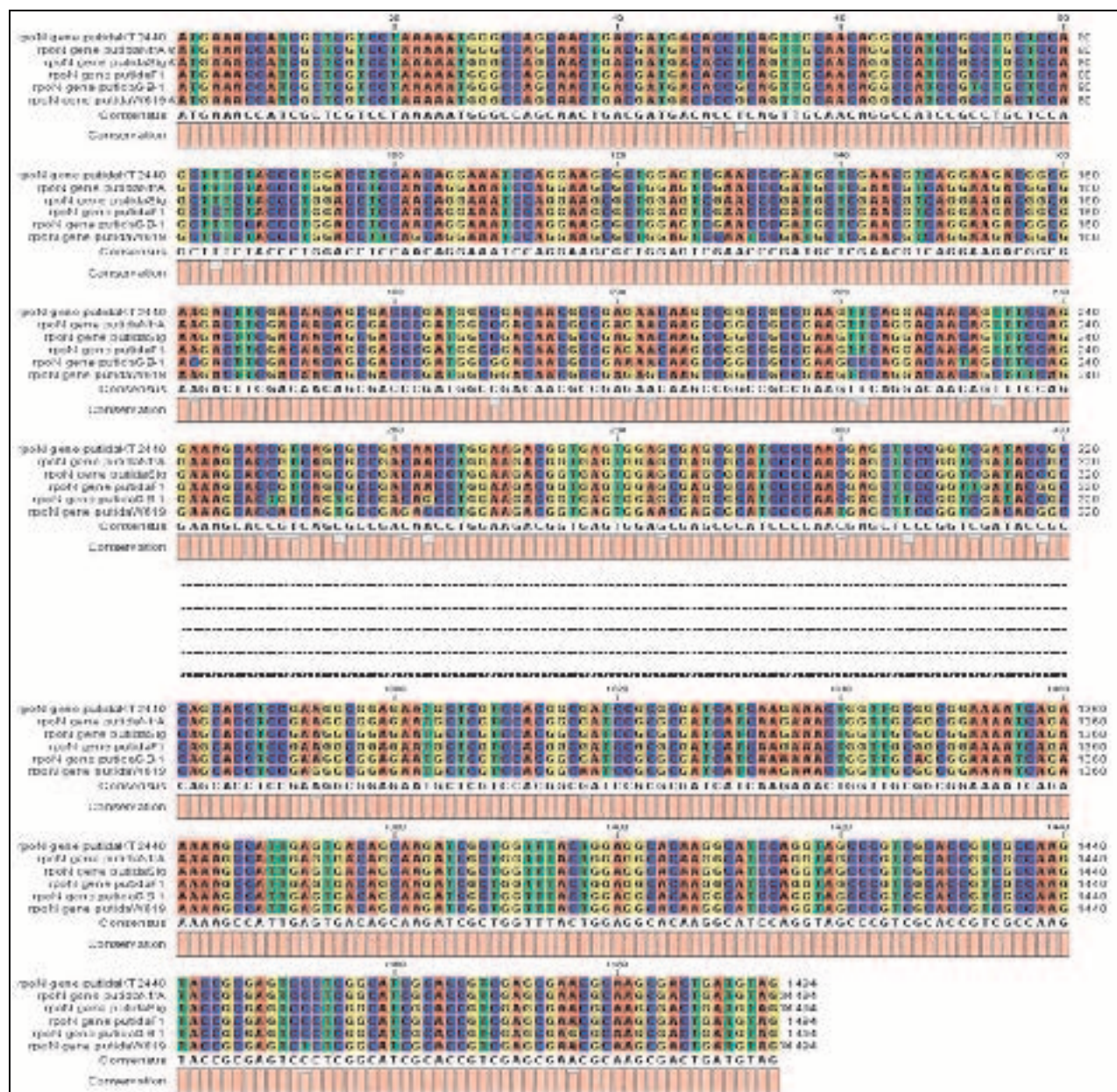


Figure 3.4. Alignment of the nucleotide sequence of six *rpoN* genes, which encode the $\sigma 54$ sigma factor subunit of RNA polymerase, of different *Pseudomonas* spp. The gene is 1,494 bp and the above displays both the 5' and 3' ends of the alignment and illustrates that the gene is highly conserved in these species. Primers were designed from this alignment to amplify the full-length *rpoN* gene from *P. putida* CA-3.

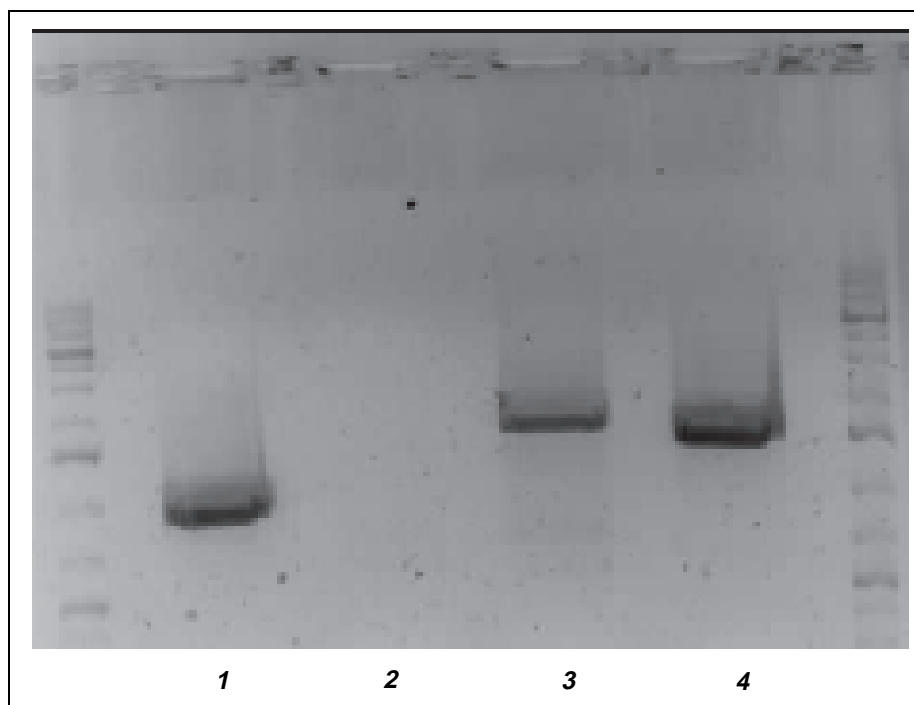


Figure 3.5. High-fidelity polymerase chain reaction to generate the following: 1, *paaL* promoter region of *Pseudomonas putida* CA-3 (964 bp); 2, downstream region of *paaL*; 3, full-length *paaL* open reading frame (1,647 bp); 4, full-length *rpoN* σ 54 open reading frame (1,494 bp).

gene should complement the disrupted *rpoN* gene in the *P. putida* CA-3 σ 54 mutant genome and restore wild-type expression patterns. The recombinant vector was successfully transferred to the *P. putida* CA-3 σ 54 mutant, but initially did not appear to fully complement the mutant, though preliminary data suggested that some degree of complementation was occurring. This may be due to an issue with optimal expression of the gene from the plasmid, or it is also possible that the mini-Tn5 insertion in the *P. putida* CA-3 genome is having a polar effect and may also be disrupting expression of genes adjacent to *rpoN* which may be important for optimal activity. Further optimisation of the procedure however resulted in complementation of the disrupted *rpoN* gene and restoration of the ability of the *P. putida* CA-3 σ 54 mutant to utilise PAA. This indicates that the *rpoN* gene plays a strong role in PAA utilisation in this strain.

The full-length open reading frame of the *paaL* gene was also amplified from *P. putida* CA-3 (Fig. 3.5) and cloned into the pBBR1MCS-5 vector and the recombinant vector mobilised successfully into the *P.*

putida CA-3 σ 54 mutant. This was performed to determine whether plasmid-borne expression of the *paaL* permease gene on its own was sufficient to restore the ability of the mutant strain to transport PAA into the cell. So far, the data obtained suggest that additional transport genes may be required. However, further studies are needed to obtain a definitive answer. A second functional PAA degradation gene cluster has been identified in *Pseudomonas* sp. Y2 (Bartolome-Martin et al., 2004), and sequence annotation and analysis of gene arrangement have identified additional transport genes adjacent to the *paaL* gene in this second gene cluster, namely *paaM*, encoding a porin, and a small membrane protein, encoded by *paaP*, located downstream and upstream of *paaL*, respectively. In the other PAA gene cluster in *Pseudomonas* sp. Y2, the *paaM* gene does not appear to be present and the *paaN* gene is located directly downstream of the *paaL* gene. The sequence and gene arrangement of the styrene gene cluster and a number of *paa* genes in *P. putida* CA-3 appear to share high levels of similarity to those in *Pseudomonas* sp. Y2. In silico sequence analysis of the *paaL*–*paaN*

region of *Pseudomonas* sp. Y2 allowed primers to be designed to try to determine if these genes are indeed located adjacent to each other in *P. putida* CA-3. However, a DNA product could not be amplified from *P. putida* CA-3 genomic DNA. The amplification conditions were varied but no product was obtained (Fig. 3.5). This may suggest that in *P. putida* CA-3 the *paaL* and *paaN* genes are not adjacent to each other and may be separated by the *paaM* gene, similar to the second PAA gene cluster in *Pseudomonas* sp. Y2. These three genes may form an operon and these observations further suggest that expression of the entire operon is disrupted in the $\sigma 54$ mutant and that the *paaM* gene may also be required to efficiently transport PAA across the cell membrane.

3.3.6 Analysis of the *paaL* promoter region

Primers were also designed to amplify the promoter region of the *paaL* gene from *P. putida* CA-3, again based on in silico analysis of the *Pseudomonas* sp. Y2 PAA gene cluster. Primers were designed to amplify a 964-bp region spanning the 3' end of the *paaK* gene,

the promoter region and the 5' end of the *paaL* gene, and a PCR product was successfully generated from *P. putida* CA-3 genomic DNA (Fig. 3.5). The DNA was subsequently sequenced and putatively annotated as shown in Fig. 3.6. Both the 3' end of the *paaK* gene open reading frame and the 5' end of the *paaL* gene were identified. At the nucleotide level, the *P. putida* CA-3 region is 99% similar to gene cluster 1 in *Pseudomonas* sp. Y2 PAA and 98% similar to *P. fluorescens* ST. Analysis of the intergenic region identified a putative open reading frame, with the stop codon overlapping the start codon of the *paaL* gene. Homology searches have revealed similarity to a putative membrane protein, with 99% identity at the amino acid level to a similar protein in the *P. fluorescens* ST PAA gene cluster (*paaW*). This gene may also play a role in PAA transport. A potential $\sigma 54$ sigma factor RNA polymerase binding site was identified upstream from the putative *paaW* gene using the PromScan programme. Further analysis will be required to identify a binding site for enhancer proteins,

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AGGCCGGGGTGTGCTCGACCTGCAAGTGCAAGGTGGTCGAGGGCGAAGTGGAAATGGACAGCAACTTCGCCC
TGGAGGACTACGAAGTCGCCGCCGGCTACGTGCTGTCTGTCTCAGACCTTCCCGATCAGCGACAAGGTGGTAC
TCGACTTCGACCAGATTTGATTGAGCACCCGACATGGCCCAGGAAACGCATGTGGGCATGGCCAGCATGCGC
GCGGGCAAATACTGCAGTAGTACCCCGTTTCGGCTGAATAGCCTTGCCGCTCTGCAGCCGACCGAATGCCTCAC
AACAATCACAAAAGAGAGAGCGACCATGACTCCGCAAGACCTGGAACAGCTACAGCGAAACCCAGATTTCA
TCCAATTGGTTCGCCGCAAGCAGCGCCTCACCTGGTCGCTGACCGCGAGCATGCTGGTGATCTACTACGGTT
TCGTCCTGCTGATGGCCTTTGCCCCACAGGTGCTCGGGCGACCGATCGACGGGGGTGCCACCAACGTGCGCA
TCCTCCTGACGGTGGCGGTAATCCTGCTTTCTTCGCCCTCACCGCGCTCTACGTGTGGCAGACCAACAAGG
TTCTCGATCCGCTCACAGAGAAGCTGCTCCGGGAGGATCGCCCATGACAGCCCTGCGCTCCTTCACCTTGG
TCGGCGGCCTGCTGCTGAGCAGCACTGCGCTGGCTGCCGAGCACTTGGTGCGCCCCCTTGAAGTGGCACGCCA
TCGGCATGTTTCATGGTGTTTCGTCGTGTTTACCCTCGGCATCACCCGGTGGGCCGCGCTGCGCACGCCTCGGC
CAGCGATTTCTATACAGCCGGTGGCGGCATCAGCGGCCTGCAGAACGGC

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Figure 3.6. Sequence and putative annotation of the *paaL* promoter region of *Pseudomonas putida* CA-3. The sequences in red represent the 3' end of the *paaK* gene (top) and the 5' end of the *paaL* gene, including the ATG start codon. Analysis of the intergenic region identified a putative open reading frame (highlighted), with the stop codon overlapping with the *paaL* start codon. Homology searches have revealed similarity to a putative membrane protein. A potential $\sigma 54$ sigma factor RNA polymerase binding site was identified using PromScan (underlined).

which are required for σ_{54} sigma factor RNA polymerase transcription, but are often located a considerable distance from the transcription initiation site.

3.4 Conclusion

To the authors' knowledge, this is the first report of a σ_{54} sigma factor involved in controlling expression of

the PAA genes and it further highlights the critical role of transport systems in the effective operation of catabolic pathways for styrene and PAA. It is also interesting that transcription of the catabolic genes and transport genes appears to be controlled differently in the *P. putida* CA-3 strain. Further work is, however, required to fully elucidate the role of the σ_{54} sigma factor in transcription of the PAA gene cluster.

4 Overall Conclusions

The reported project has focused on a number of strategies, including:

- Site-directed mutagenesis of the promoter of the styrene catabolic genes to alleviate transcription inhibitor binding events;
- Homologous expression of catabolic genes to enhance flux through the pathway;
- Chromosomal insertion and homologous expression of the styrene transport gene to overcome intracellular substrate limitations; and
- Random mutagenesis of the *P. putida* CA-3 genome to identify additional genes of the PAA pathway, a key intermediate of styrene degradation.

The overall aim of the project was the optimisation of the rate of styrene degradation, via PAA, to acetyl-CoA through focused recombinant DNA manipulations of the *P. putida* CA-3 genome. Site-directed mutagenesis was performed in an attempt to eliminate negative regulator binding sites from the promoter region. While the mutagenesis was successful, attempts to replace the native promoter on the genome with the mutated promoter were not. However, a reporter system was instead employed to determine the effect of the mutated promoter on activity of the styrene genes. The results have suggested that the mutated binding site may not play a significant regulatory role in *P. putida* CA-3, though further investigation of the promoter region in this strain is required. It is becoming clear that subtle differences appear to exist in the fine-tuning of regulatory events in different *Pseudomonas* strains. Over-expression of key styrene catabolic and transport genes was attempted by stably integrating single extra copies of the genes into the chromosome. Extra copies of the genes may enhance pathway activity, particularly in relation to the transport gene, as transport of aromatic compounds across the cell membrane is often a limiting factor for subsequent degradation. However, a single extra copy did not seem to affect the activity of the pathway. Further tests

with multiple plasmid-borne copies of the transport gene suggest that single integrated genomic copies are not enough to increase styrene enzyme activity and that constitutive expression may be required to produce a significant increase in activity. Additional work focused on the identification of functional genes contributing to lower pathway activity. Random mutagenesis was employed to disrupt potential genes involved in PAA degradation. While PAA genes were not directly detected during mutagenesis, a gene was identified that clearly plays a role in PAA catabolism in this strain. The gene was determined to encode a $\sigma 54$ sigma factor and subsequent analysis appears to reveal an involvement in transcription of PAA transport genes. This again highlights the critical role that transporters play in the degradation of aromatic compounds in bacterial cells and suggests targets for future recombinant DNA strategies to enhance pathway activity. While the creation of recombinant strains with increased ability to degrade styrene was not achieved in this project, the work undertaken has contributed considerably to the existing knowledge base.

To summarise, several key issues have been targeted in this investigation and as a result the authors have:

- Identified the likely involvement of $\sigma 54$ sigma factor in the regulation of PAA transport, which may have a link to global regulation in the cell;
- Conducted mutagenesis of a binding site on the styrene promoter, which may have revealed a limited regulatory role for this site in *P. putida* CA-3 and the suspected involvement of other novel functional elements; and
- Determined that additional single extra copies of the styrene transport and catabolic genes may not be sufficient to increase the activity of the styrene pathway.

The research outputs of this project have greatly enhanced understanding of the styrene-degradation pathway in *P. putida* CA-3 in general and may point to

the involvement of novel functional elements within the pathway. These results are likely to be of interest to a number of international research groups, in particular those involved in studying aromatic hydrocarbon degradation. While the styrene-degrading ability has been characterised in a number of other *Pseudomonas* strains, *P. putida* CA-3 remains the only strain reported to be capable of styrene conversion to PHA, and this promotes the idea of using a toxic waste to produce a value-added product. The research described above may in future lead to the generation of additional strains with enhanced styrene-degrading ability, but may also facilitate an enhanced research strategy towards the generation of recombinant strains with improved styrene flux to PHA. Such outputs would offer strong intellectual property potential as they would be of significant interest to industrial groups involved in the management of styrene waste. Recent years have seen significant advances in our understanding of the molecular biology of microbial

styrene degradation, resulting in the identification of the structural genes encoding the degradative enzymes and the elucidation of the regulatory apparatus of the upper and lower pathways, involving complex positive/negative transcriptional regulation events. But, despite the substantial amount of information already generated, further work is required to fully elucidate a number of critical events, for example the exact mechanism of activation of the sensor kinase. There also remains the task of establishing in more detail how catabolite repression exerts control over the styrene-degradation pathway under different physiological conditions, particularly those that exert negative regulatory influences. Undoubtedly these issues and others will be the focus of research over the next few years and will further enhance our knowledge of the remarkable ability of bacteria to degrade the toxic pollutant styrene to carbon dioxide and water.

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Acronyms and Annotations

ACP	Acyl carrier protein
AT	Adenine–thymine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CoA	Co-enzyme A
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EU	European Union
LB	Luria–Bertani medium
MCL	Medium chain length
MSM	Minimal salts medium
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
OD	Optical density
PAA	Phenylacetic acid
PAS	Per-ARNT-Sim
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
RNA	Ribonucleic acid
RT	Reverse transcription
σ	Sigma factor
SDM	Site-directed mutagenesis
SOEing	Splicing overlap extension
STY	Styrene
TCA	Tricarboxylic acid
Tn5	Tn5 transposon
Tris	Tris(hydroxymethyl)aminomethane

An Gníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil agus Rialtais Áitiúil a dhéanann urraíocht uirthi.

ÁR bhFREAGRACHTAÍ

CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreal.
- Scardadh dramhuisce

FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain.
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce.
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí chomhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOIL

- Monatóireacht ar chaighdeán aer agus caighdeáin aibhneacha, locha, uisce taoide agus uisce talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntiú a dhéanamh.

RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdeán aer agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Gníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstíúrthóir agus ceithre Stíúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.