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Investigation of global regulators influencing styrene metabolism and bioplastic synthesis in *Pseudomonas putida* CA-3

A Thesis Presented to the National University of Ireland for the Degree of

Doctor of Philosophy

by

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and

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January 2013

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For My Parents:

Their love gave me the strength to try, and their support gave me the means to succeed

ñAll truths are easy to understand once they are discovered; the point is to discover themò

Galileo Galilei

ñEarn gold and wear itò

Helen Ryan

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Declaration

I hereby certifythat this material, which I submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been submitted for another degree, either at UniversityCollegeCork or elsewhere.

Signed: _____

Student number: 104385527

Date: _____

Abstract

The anthropogenic accumulation of aromatic hydrocarbon contaminated wastes in the environment is of significant ecological concern given the potential toxicity of many such compounds and its recalcitrance to environmental degradation. Significant research into the microbial degradation of this alkenylbenzene has been conducted over the last 2 decades and has included the characterisation of styrene degradation in the bioreactor isolate, *Pseudomonas putida* CA-3, a unique feature of which is its ability to utilise the breakdown products of styrene catabolism to produce condensation polyesters collectively known as polyhydroxyalkanoates (PHAs). The genetic operons and biochemical pathways responsible for both these processes have been well characterised in previous studies by our group. However knowledge of the role played by regulators in controlling these pathways is required if their full biotechnological potential is to be realised. As a result our investigations have attempted to contribute to filling this knowledge gap in the model strain *P. putida* CA-3.

A mini-Tn*5* mutant library of *P. putida* CA-3 was generated and subjected to phenotypic screening to identify mutants exhibiting a reduced sensitivity to the effects of carbon catabolite repression of aromatic pathway activity. Our efforts identified a *clpX* disrupted mutant which was characterised as exhibiting wild-type levels of growth on styrene but significantly reduced growth on phenylacetic acid, which was restored upon *clpX* gene complementation. Investigations into the strains ability to utilise glucose demonstrated growth was unaffected in this strain suggesting core metabolism was not impaired. RT-PCR analysis of key PACoA catabolon genes, representative of discrete transcriptional units within the catabolon (i.e. *paaF;* encoding PaCoA ligase, *paaG;* encoding a member of the ring hydroxylation complex, and, *paaL;* encoding

phenylacetate permease), were conducted, but did not reveal altered transcriptional profiles between wild type CA-3 and the *clpX* mutant. Examination of cultures for distinct, post-transcriptional regulatory effects via SDS-PAGE protein profile analyses also failed to identify any alteration in PACoA pathway. Cloning and over-expression of the PaaL active transport protein, responsible for phenylacetic acid uptake in CA-3, further reduced the growth capacity of the strain suggesting that transport was not the limiting factor in growth on PAA. Thus while *clpX* disruption had a definitive impact on phenylacetic acid utilisation, correlation of this genotype with regulation of pathway specific elements could not be established and suggests the involvement of some intermediary, unknown component warranting further investigation.

The potential involvement of global regulators affecting the accumulation of PHAs in *P. putida* CA-3 was also studied. These biodegradable polyesters offer considerable commercial potential in a time of increasing costs associated with production of petrochemicals. Phenotypic screening of the mini-Tn*5* library revealed a *gacS* sensor kinase gene disruption which resulted in the loss of PHA accumulation capacity in *P. putida* CA-3. Recombinant expression of wild-type *gacS* from the pBBRgacS vector fully restored PHA accumulation capacity in the mutant strain. A combination of standard and gene-flanking based PCR screening strategies of the *P. putida* CA-3 genome identified gene homologues of the GacS/GacA - *rsm* small RNA regulatory cascade, with 96% similarity to homologues in published *P. putida* genomes. Subsequent SDS PAGE protein analyses of the wild type and GacS disrupted mutant strains identified post-transcriptional control of *phaC1* synthase as a key point of control of PHA synthesis in *P. putida* CA-3.

The potential for GacS mediated regulation of PHA accumulation to be was also investigated in *Pseudomonas* species other than *P. putida* CA-3. A second styrene degrading *P. putida* strain, S12, was therefore subjected to pKnockout disruption of the *gacS* homologue on its genome and PHA accumulation assessed. The *P. putida* S12 mutant demonstrated a similar reduction of PHA accumulation capacity confirming the importance of GacS regulation on bioplastic production, and ruling out concerns that our observations in *P. putida* CA-3 were strain specific. In addition, the regulatory function of *gacS* identified via screening under nitrogen limited growth was also assessed under phosphorus limitation to determine whether this regulation was solely associated with cellular responses to nitrogen deficiency. PHA accumulation was observed to be disrupted in the *gacS* mutant under phosphorus limited growth conditions, further suggesting a global regulatory role for GacS in the integration of PHA accumulation with general cell physiology. Finally, we investigated the role of the Gac/Rsm cascade associated sRNA, *rsmY,* in PHA accumulation via over-expression studies in both wild type CA-3 and *gacS* disrupted mutant. *rsmY* over-expression in *gacS* disrupted *P. putida* CA-3 did not restore PHA accumulation in the cell, suggesting atypical regulation of the PHA phenotype by the Gac/Rsm cascade. However, in wild type cells with an intact Gac/Rsm cascade, over-expression of *rsmY* results in an altered PHA monomer composition with a 21% increase in C_{10} monomers being observed in this strain. Interestingly, while the monomer composition was altered in this strain, the overall PHA production capacity was not affected, suggesting the *rsmY* overexpression effects may be mediated at the level of metabolic pathways feeding monomers to the PHA biosynthetic pathway.

Chapter 1

Regulation of aromatic hydrocarbon degradation and Polyhydroxyalkanoate production

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1. Introduction

The extensive use of aromatic hydrocarbons in industrial processes, coupled with inadequate waste management strategies, has led to a significant quantity of xenobiotic compounds building up in our environment. Aromatic hydrocarbons contain one or more benzene ring structures which confer these compounds with great thermostability. This has led to the widespread application of these compounds in many industrial processes and as an intermediate in the production of other compounds. However, the very properties which have led to the use of aromatic compounds in so many industrial processes have also contributed to their persistence in the environment. This poses a major environmental challenge in terms of remediation and waste management (Carmona *et al.,* 2009). A summary of some of some of the applications of aromatic hydrocarbons, quantities of waste released and their uses can be seen in Table 1 below (US EPA;b).

Compound	Total disposal (tonnes pa)	Uses
1,4-Dichlorobenzene	15.65	Pesticide, Disinfectant
Benzene	1,964.59	Production of pesticides, detergents, rubbers
Ethylbenzene	1,626.17	Intermediate in production of polystyrene
Styrene	11,153.59	Polymer production/processing
Hexachlorobenzene	15.54	Waste product in production of other chemicals

Table 1: Common aromatic hydrocarbons, quantities of the compounds disposed on and off site and their common uses. Figures obtained from US EPA TRI 2010 (US EPA;b).

Styrene, the simplest alkenylbenzene, is one such aromatic hydrocarbon; it is used extensively in the petrochemical industry and in processes such as synthetic polymer production and in polymer processing. The use of styrene in such processes results in the generation of styrene containing waste which is an undesirable outcome; as styrene is classified by the US EPA as a potential human carcinogen (US EPA;a) making remediation of such waste difficult. Approximately 29,122 tonnes of styrene contaminated waste was generated in 2010 of which 11,153 tonnes was released either on or off site. The remainder of this was recycled or used for other applications such as power generation (US EPA;b).

In response to the large quantities of styrene waste, considerable investigation of the microbial biodegradation of this compound has been undertaken with a view to developing biotechnology based remediation strategies offering environmental sensitivity and cost-effectiveness. Microbial degradation of styrene has been investigated for the last two decades most notably in *Pseudomonas* species. *Pseudomonas putida* CA-3 has previously been identified as one such styrene degrading strain (OôConnor *et al.*, 1993). *Pseudomonas putida* CA-3 also possesses the ability to accumulate medium chain length polyhydroxyalkanoates (mcl-PHA) under conditions of nitrogen limitation when grown on a variety of carbon sources including styrene (OôLeary *et al.,* 2005). These biodegradable polyesters have varied thermoplastic and elastomeric properties and have a number of industrial, medical and consumer applications.

Here we review research to date in the area of styrene degradation and the production of polyhydroxyalkanoate by microbes. We discuss the regulatory processes that are known to be involved in regulating these processes and highlight the shortcomings in our

knowledge in this area, specifically with respect to the impact of global regulatory features on these pathways.

2. Microbial Degradation of Styrene

The first genes classified as being required for catabolism of styrene were published by Marconi and co-workers (1996) in *P. fluorescens* ST. This initial investigation established the styrene monooxygenase and expoxystyrene isomerase encoding genes (Marconi *et al.,* 1996). Since then the full styrene catabolic operon has been identified in a number of species including; *Pseudomonas* sp. strain Y2 (Utkin *et al.,* 1991, Velasco, *et al.,* 1998), *Xanthobacter* strain 124X (Hartmans *et al.,* 1989), *Pseudomonas putida* CA-3 (OôConnor *et al.,* 1995, OôLeary *et al.,* 2002) and most recently Rhodococcus sp. ST-5 and ST-10 (Toda and Itoh, 2012) have been shown to be capable of styrene degradation. Interestingly the operonic organisation of the *sty* catabolic operon also appears to be highly conserved among a diverse range of species suggesting a similar evolutionary origin. A schematic diagram of the genetic organisation of the chromosomally encoded genes of the styrene degradative operons in a number of *Pseudomonas* species is presented in figure 1.

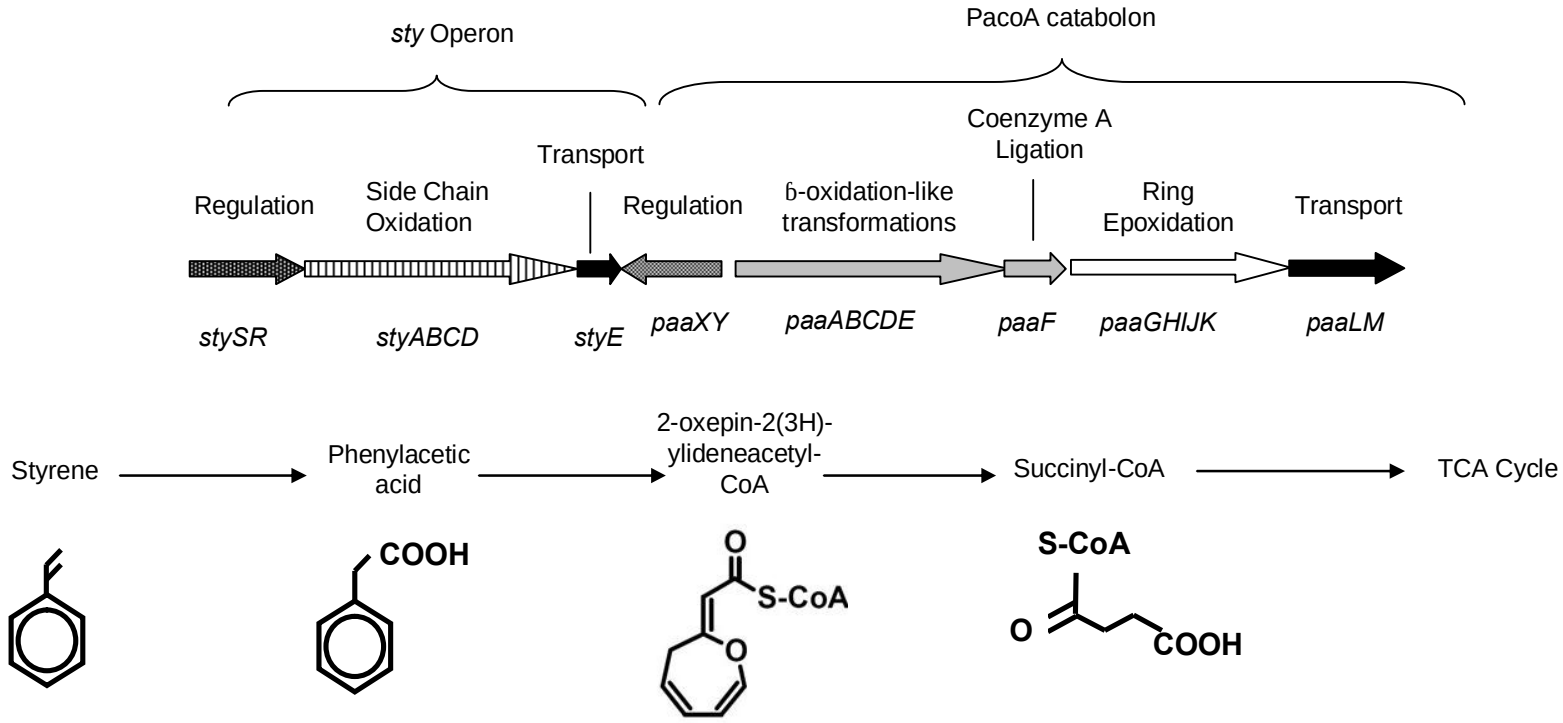


Figure 1: Graphical representation of key styrene metabolic and PACoA catabolic genes and major steps leading incorporation to TCA cycle intermediate succinyl-CoA (adapted from OõLeary *et al.,* 2011).

2.1 Side Chain Oxidation

Microbial degradation of styrene has been shown to proceed under both aerobic and anaerobic conditions. Two pathways have been identified for the aerobic degradation of styrene in bacteria; the first involves vinyl side chain oxidation which proceeds stepwise resulting in the formation of phenylacetic acid which is further degraded to produce TCA cycle intermediates for cell metabolism. The second pathway involves the direct oxidation of the benzene ring through the *cis*-glycol route to form acetylaldehyde and pyruvate (Warhurst *et al.,* 1994)

Side chain oxidation has been shown to be the predominant pathway for styrene metabolism in many of the bacterial strains studied to date (Mooney *et al.,* 2006). In side-chain oxidation of styrene, the initial degradation step is performed by a flavin adenine dinucleotide dependent styrene monooxygenase (SMO) encoded by *styA* and *styB* to form styrene oxide (Hartmans, 1995). Isomerisation of styrene oxide to phenylacetaldehyde (PAALDH) is subsequently achieved by a *styC* encoded styrene oxide isomerase (Hartmans *et al.,* 1990; Utkin *et al.,* 1991; Velasco *et al.,* 1998). Finally, PAALDH is oxidised to phenylacetic acid (PAA) through the action of an NAD^+ dependent, *styD* encoded phenylacetylaldehyde dehydrogenase. The *styABCD* genes form part of the *sty* operon with phenylacetic acid (PAA) the resulting end product of this catabolic route. A summary of the major steps and operonic organisation of genes involved can be seen in figure 1.

2.2 Direct Oxidation of Aromatic Ring/Anaerobic Degradation

Metabolism of styrene by direct oxidation of styrene has been reported in *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst *et al.,* 1994). Here styrene is degraded to styrene *cis*-glycol by the action of a dioxygenase and then further degraded to 3-vinylcathechol by cis-Glycol dehydrogenase. The pathway then branches in two; the first pathway involves the enzymes catechol 2,3-dioxygenase, semialdehyde hydrolase, hydratase and aldolase which convert the 2-vinylcathechol to acetylaldehyde and pyruvate (Warhurst *et al.*, 1994). The second branch accumulates 2-vinyl-*cis,cis*-muconate as a dead end product due to the action of catechol 1,2-dioxygenase (Warhurst *et al.,* 1994).

Anaerobic styrene metabolism has also been shown to proceed to phenylacetic acid via 2-phenylethanol and PAAL by Gbric-Galic in *Enterobacter* spp. and *Clostridium* spp. Anaerobic metabolism however diverges from the side chain oxidation method after formation of phenylacetic acid as it is converted to benzylalcohol and benzaldehyde in a manner similar to the meta-cleavage pathway of toluene degradation (Grbic-Galic *et al.,* 1990).

2.3 Phenylacetic Acid Metabolism

The degradation of phenylacetic acid has been widely reported among diverse microbial species, and is now recognised as one of the four major aromatic compound degradation routes in bacteria. The first step in the process of phenylacetic acid metabolism is uncharacteristic of aerobic catabolism and involves the Co-Enzyme A activation of PAA to phenylacetyl-CoA (PACoA). The PACoA generated then acts as the true inducer of the catabolic route (Olivera *et al.,* 1998; Ferrández *et al.,* 1998). NMR studies and mass

spectrometry have demonstrated that the activation of PAA to phenylacetyl-CoA in *Pseudomonas putida* and *E. coli* is catalysed by the *paaK* gene product, phenylacetyl-CoA ligase (Teufel *et al.,* 2010). The first stage in PACoA catabolism is the introduction of oxygen into the aromatic ring of PACoA by a multi component enzyme complex. The complex encoded by the *paaABCDE* genes functions as an oxygen dependent, NADPH consuming epoxidase and forms ring 1,2-epoxyphenylacetyl-CoA. The next step of the catabolic route was determined via purified, his-tagged PaaG. ^{13}C-NMR spectroscopy identified the product of this enzyme as an unsaturated seven-member heterocyclic enol ether, 2-oxepin-2(3OH)-ylidenacetyl-CoA. Thus PaaG acts as a ring 1,2-epoxyphenylacetyl-CoA isomerase. The *paaZ* encoded $NADP^+$ dependent hydratase converts this to 3-oxo-5,6-dehydrosuberyl-CoA. The PaaJ enzyme, 3-oxoadipyl-CoA thiolase, generates 2,3-dehydroadipyl-CoA and acetyl-CoA, before conversion by the PaaF CoA hydratase to 3-hydroxyadipyl-CoA. In the penultimate step, a NAD^+ dependent PaaH enzyme, 3-hydroxyadipyl-CoA dehydrogenase, forms 3-oxoadipyl-CoA, before a second CoA-thiolase (PaaJ) cleaves the 3-oxoadipyl-CoA into central metabolites acetyl-CoA and succinyl-CoA which enter central metabolism (Teufel *et al.,* 2010). An overview of the genes involved in the degradation of styrene and phenylacetic acid, respectively, with key catabolic intermediates are presented in figure 1.

A recent study by Martin and McInerney (2009) highlighted the dispersal of this pathway among 102 genomes and observed frequent clustering of the PAA degradation genes in several different lineages of species. The authorsô findings demonstrated that the *paaABCD* operon is highly conserved among the species tested and suggest that this is due to co-regulation of the genes, as these gene products must act in concert to be

effective. The authors suggest that of the 102 genomes tested that operon and cluster diversity is high and that the co-localisation of PAA degradation genes is quite common. They suggest that this can be explained by two main models; co-regulation model of genes, such as is seen in the *paaABCD* operon, and the protein immobility model which proposes a selective pressure to favour macromolecular crowding of proteins within the cell. This localises proteins of particular pathways together in order to speed up biochemical reactions. They further suggest it is probable that pressure to localise genes may also be due to the same transcription factor regulating more than one operon/ cluster thus localisation may encourage optimised expression of the all genes necessary for PAA degradation (Martin and McInerney, 2009).

The wide distribution of the genes required for PAA degradation in nature and the common themes of clustering and operonic structure in these distinct species suggest that many evolutionary pressures are at play influencing these common conformations. As previously discussed one such pressure could be the co-regulation of these genes. It is possible therefore that regulators common to many species control the activity of this pathway in many strains. Thus the identification of potential regulators in one strain may have relevance across a broad range of species.

Several regulators, both pathway specific and global, have to date been identified as impacting upon the styrene and PAA degradation pathways in bacteria. These regulators control the pathways in the context of the cell as a whole and are the subject of the next section of this introduction.

3. Pathway specific regulation

3.1 Regulation of the Side-Chain Oxidation Pathway in styrene catabolism

The genes responsible for the microbial degradation of styrene have been characterized extensively in a number of *Pseudomonas* strains as discussed above. In addition to the structural genes necessary for styrene metabolism (Figure 1) the *sty* operon harbours a two component regulatory system encoded by *stySR*, located upstream of, and transcribed separately from, the *styABCDE* genes.

The StySR proteins are responsible for detecting the presence of styrene and activating transcription of the upper pathway genes. StyS consists of 5 distinct domains (Figure 2); two histidine kinase domains (HK1 and HK2) both of which contain the conserved amino acid sequences H, N, G1, F (Parkinson and Kofoid, 1992), associated PAS input domains (Input 1 and Input 2) (Coschigano and Young, 1997; Lau *et al.,* 1997) as well as a receiver domain located between HK1 and Input 2 (Figure 2). The Input 2 domain contains the S1 (PAS) and S2 (PAS) sensory boxes and studies by Santos *et al*. (2000) have suggested that the heme group within the PAS domain may detect the presence of styrene as a result of an altered cell redox potential (Santos *et al.,* 2000; Taylor and Zhulin*.,* 1999). It has also been proposed that the second PAS domain functions in detecting the presence of extracellular styrene (Milani *et al.,* 2005). The role played by the individual domains of the StyS protein in regulating the activity of *styABC* encoded styrene monooxygenase, has previously been described by our group (Mooney, 2005). Results of these investigations indicated that the HK2 and Input 1 domains of the StyS sensor kinase are essential for activation of *styABC* transcription. StyS activity was also

assessed following exposure to extracellular and intracellular styrene to identify differences in sensor kinase activation. Results indicated that the Input 1 domain of StyS senses extracellular styrene and activates transcription of the genes accordingly while intracellular styrene, transported into the cell by the StyE transporter, is detected by the second input domain, input 2 resulting in StyS activation (Mooney, 2005).

StyR contains a receiver domain and a DNA-binding domain joined by a Q-linker region. The receiver domain belongs to the RA4 receiver subfamily and contains the conserved D, D, T and K amino acid residues found in many bacterial response regulators (Baikalov *et al.,* 1996; Robinson *et al.,* 2003). A consensus sequence of putative DNA-binding domains in the family 3 response regulators is also present in the carboxy-terminal end of StyR (Pao and Saier, 1995).

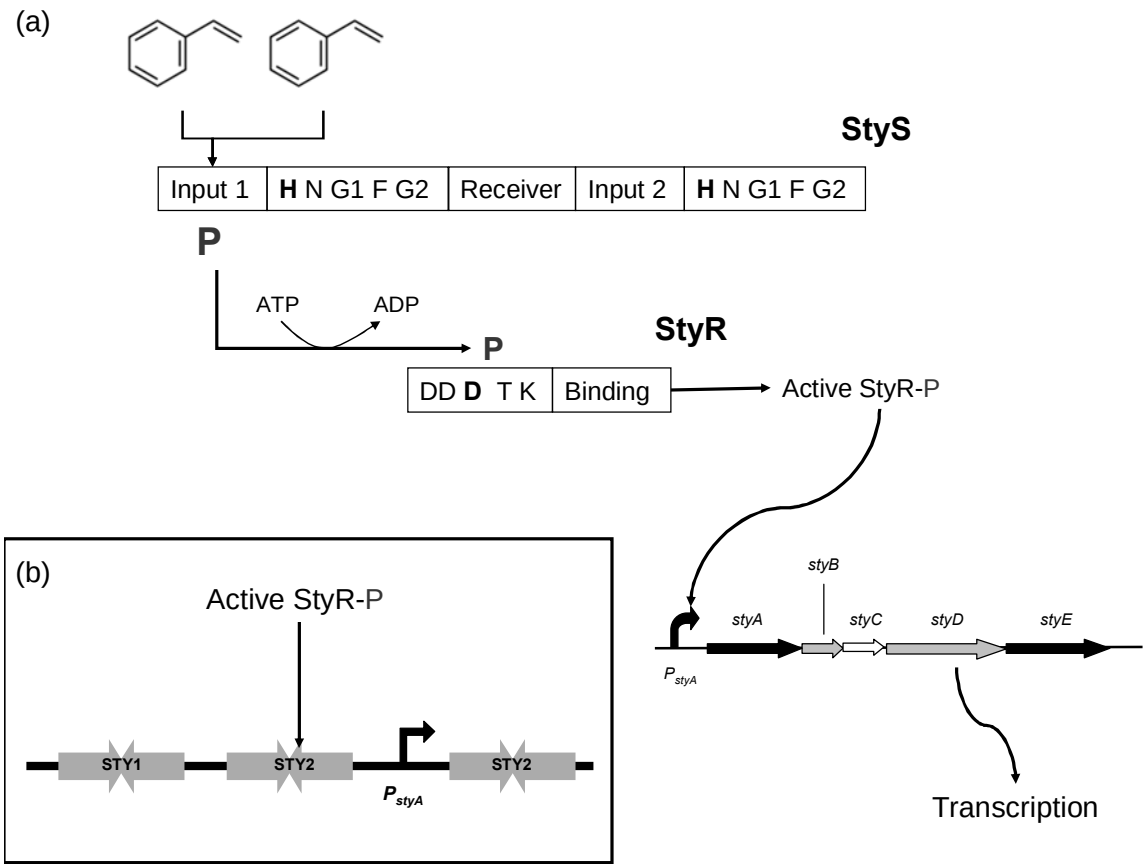


Figure 2: (a): Transcriptional activation of *styABCDE* genes. StyS detects presence of styrene which interacts with input 1, and becomes phosphorylated. The phosphoryl group is then transferred to the StyR receiver domain thus activating StyR. StyR-P dimer binds *styA* promoter region (P_{styA}) allowing transcription of the downstream genes to occur. **(b)**: StyR-P binding to STY2 high affinity binding site facilitating P_{styA} promoter activation and transcription initiation.

Once styrene enters the cell membrane the membrane associated StyS detects the presence of styrene through a sensory input domain, this results in kinase autophosphorylation of the His residue. This phosphoryl group is thought to be transferred to an Asp residue (**D**) of the receiver domain of StyR, activating the DNA-binding domain of the response regulator (Mooney, 2005). Phosphorylated StyR is then believed to dimerize and bind to the PstyA promoter at three distinct *cis*-acting elements (STY1, STY2 and STY3) within the promoter. StyR-P can bind to these with different affinities with different effects being observed from binding at each site (Leoni *et al.,* 2003).

The high-affinity site STY2 is essential for promoter activation while STY3 is responsible in part for catabolite repression. Upstream of the STY2 region is the URE (Upstream Regulatory Element), this contains the lower affinity StyR binding site STY1. The URE region is involved in both induction of the pathway in the presence of styrene and in carbon catabolite repression control of the pathway. The STY2 site is centred about a palindromic sequence -41 bp from the transcription start point within the *styA* promoter (P_{styA}). Under inducing conditions transcription is achieved when dimerized StyR-P binds the STY2 site (Rampioni *et al.,* 2008). As the P_{styA} promoter lacks a -35 consensus binding site StyR is believed to attract RNA polymerase (RNAP) to the transcription start site to initiate transcription of the *styABCDE* genes, (Figure: 2;b) (OõLeary *et al.,* 2002). Following activation and translation of the transcribed genes, increased levels of StyR, and thus phosphorylated StyR, are present in the cell. These can then bind the STY3 negative regulatory site and act as a repressor of transcription (Leoni *et al.,* 2005).

3.2 Pathway Specific Regulation of the Phenylacetyl Co-A Catabolon

The metabolism of phenylacetic acid (PAA), an intermediate of styrene metabolism, proceeds via the PACoA catabolon. Its metabolism is regulated independently of the *sty* genes which are responsible for encoding the upper pathway as previously described. Transcription of the PaCoA catabolic genes has been shown to be controlled by a GntR-type repressor, PaaX. Under repressive conditions PaaX binds to specific operator sites of the cognate promoter regions, controlling transcription of the genes responsible for PAA catabolism. Investigations into this regulation in *E. coli* using gel retardation assays identified a conserved PaaX binding site at the 5ô end of the noncoding strand necessary for PaaX binding in both the *Pa* promoter responsible for *paaABCDEFGHIJK* expression and the *Pz* promoter responsible for *paaZ* transcription. The expression of these genes is facilitated by the interaction of PaaX with PACoA generated from the atypical coenzyme activation of PAA to PACoA which releases the repressor from the operator region and allows transcription to take place (Ferrández *et al.,* 2000b).

Investigations into PaaX mediated regulation of PAA metabolism in *Pseudomonas sp.* strain Y2, which contains two functional copies of the *paa* gene cluster (*paa1* and *paa2*), by del Peso-Santos *et al.,* identified a more complex regulatory structure in this strain. Investigators noted that PaaX can also repress transcription from the P_{styA} promoter in the absence of PACoA in *Pseudomonas sp.* strain Y2 by binding a specific operator sequence located within the *styA* coding region. This binding site spans a region between +4 and +52 in respect to the transcriptional start site, and inhibits the binding of RNA polymerase. It is likely, given the location of the PaaX binding site, that the mode of

action involves inhibition of open transcription complex formation (del Peso-Santos *et al.,* 2006).

Investigations into the regulation of the *paa* catabolon in PAA and styrene degrading species have to date focused on species specific regulatory effects. As we have previously discussed the *paa* gene clustering and operonic structures are conserved among many PAA degrading organisms (Martin and McInerney, 2009) however little is known of the sequence conservation among promoter sequences. A recent bioinformatic study by OõLeary *et al.,* (2011) into *paaL* transcriptional regulation by the alternative ů-factor $ů^{54}$, identified two $ů^{54}$ binding sites within the *paaL* promoter, with one of these sites located next to the Shine-Delgarno (SD) sequence, typical of $ů^{54}$ binding sites.

The conservation of *paaL* promoter and gene sequences among styrene and PAA degrading *pseudomonads* were also investigated. The authors reported a much higher conservation of the *paaL* nucleotide sequence among styrene degrading strains, >70% identity, however promoter sequences were found to be much less conserved with many exhibiting <20% identity to other strains. Previous reports have identified $ů^{70}$ dependent regulation of the phenylacetic acid transport system in *Pseudomonas putida* U and *Pseudomonas* sp. strain Y2 (Schleissner *et al.,* 1994; Velasco *et al.,* 1998; Alonso *et al.,* 2003; Bartolome-Martin *et al.,* 2004). The identification of the involvement of other ů-factors may explain the low level of conservation among promoter sequences. Interestingly however OõLeary *et al.* identified 100% nucleotide identity in the *Pseudomonas* species Y2 *paaL* promoter sequence to that of *P. putida* CA-3. It is therefore possible that $ů^{54}$ dependent regulation of *paaL* expression in Y2 is also at play (OõLeary *et al.,* 2011).

The low *paaL* promoter sequence identity of *P. putida* CA-3 to other styrene and PAA degrading *Pseudomonas* species suggests that σ^{54} involvement in *paaL* expression may be a relatively recent evolutionary event. Despite this the significant difference that exists between *paaL* gene and *paaL* promoter nucleotide conservation suggests that much has yet to be elucidated in regard to regulation of the *paa* catabolon.

Although much work has been undertaken to improve our understanding of the pathway specific elements affecting expression of these operons in bacteria much remains to gain understanding of the impact global regulatory elements have on transcriptional activation of these pathways. The poor conservation in *paaL* promoter identity among PAA degrading organisms is a prime example of how different regulatory elements play differing roles in different strains. The next section of this review will expand the discussion of regulation of aromatic degradation by detailing the role played by global regulators in controlling the activity of these catabolic pathways.

4. Global Regulation of Aromatic Degradation

Both natural and synthetic aromatic compounds are highly abundant in the habitats of many microorganisms. This is likely to have resulted in evolutionary pressures leading these bacteria to evolve pathways to degrade and utilise these compounds. Previously we have discussed pathway specific controls of the styrene and phenylacetic acid degradative pathways, but knowledge of how global regulatory elements influence these pathways is also required in order to fully understand the integration of these pathways into overall cellular metabolism. Furthermore, as these bacteria offer the potential for development of bioremediation strategies for aromatic pollutants, insight into the impact of global

regulatory influences on these pathways is required to enable optimised biotechnological outputs from such whole-cell based systems.

Many diverse global regulatory factors can influence aromatic hydrocarbon metabolism. Metabolism of these compounds can be regulated in either a direct or indirect manner depending on the regulators involved. Direct control of pathway gene expression is mediated via pathway specific elements such as for example, a two-component system mediated activation system, responding to the presence of a pathway substrate. More complex forms of regulation take place in an indirect manner regulating expression of the pathways in response to non-pathway specific effects. Some of these indirect regulatory influences act as a result of carbon source availability such as carbon catabolite repression, shutting off aromatic metabolism in favour of a preferred carbon source; while others influence expression as a result of extracellular signals, carbon: nitrogen ratios, growth phase of the cell etc. Detailed below is an insight into some of the global regulatory factors which have been previously shown to impact on aromatic metabolism in various bacteria.

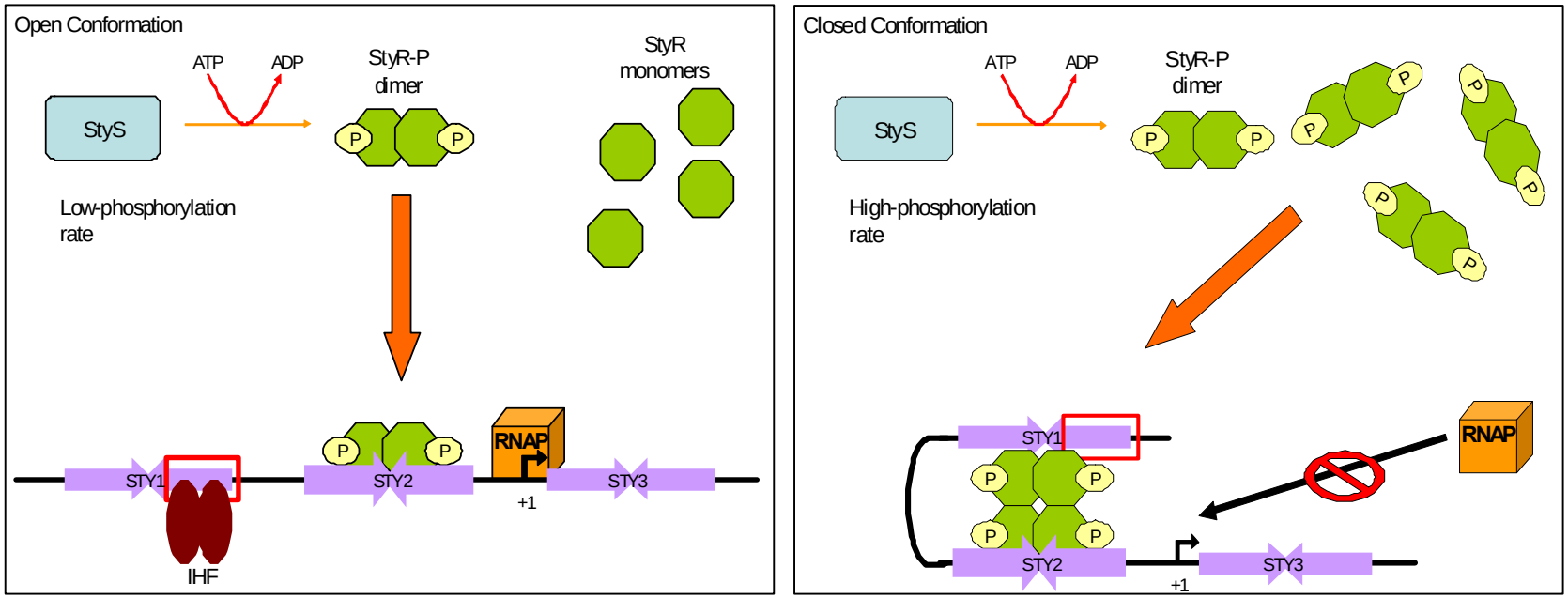
4.1 IHF mediated activator binding in aromatic metabolism

Integration host factor (IHF) is a global regulator which has been found to regulate over 100 genes in *E. coli* and has also been shown to be involved in regulating the degradation of several compounds in *Pseudomonas* as well as several other cellular processes (Rampioni *et al.,* 2008, Silva-Rocha *et al.,* 2012). IHF acts as a DNA-binding protein which recognises specific sites and bends DNA which is then free for interaction with other DNA associated elements. This regulator can act as either a transcriptional activator or repressor depending on the location of the IHF site in relation to the

transcriptional start site and the presence or absence of other regulatory factors (Martínez-Antonio and Collado-Vides, 2003). Studies into the effect that IHF has on aromatic metabolism have shown it to regulate expression of toluene metabolism genes in the *Pseudomonas* TOL plasmid by binding to the P*u* promoter of the plasmid, enabling σ^{54} dependent transcription of the toluene catabolism genes (Holtel *et al.,* 1992).

IHF mediated regulation has also been observed to regulate styrene metabolism in *P fluorescens* ST. As previously discussed three binding sites, each with different affinities, have been characterised for StyR-P binding within the P_{styA} promoter region known as STY1, STY2, and STY3 in *Pseudomonas fluorescens* ST. Using DNA protection assays Leoni *et al.* (2005) demonstrated the three distinct regions to which StyR-P binds at the locations detailed in figure 3; a. Mobility shift assays demonstrated that the STY2 site exhibits the highest affinity for binding by StyR-P of the three described. The authors demonstrated that under inducing conditions, StyR-P bind to the STY2 site while IHF binds to the URE. Under catabolite repression conditions, it was shown that levels of StyR-P increase in the cell, displacing IHF and resulting in a ñclosed promoterò conformation impairing the ability of RNA polymerase binding (Figure 3; b). The closed promoter conformation is formed when StyR-P binds both STY1 and STY2 sites forming a tetrameric structure, looping the DNA and preventing the binding of the RNA polymerase. It is hypothesised that the activity of the StyS sensor kinase, responsible for phosphorylation of StyR to StyR-P, is enhanced when cells are growing in conditions of high redox potential such as in the presence of glucose as well as styrene. The presence of two PAS and two kinase domains on the StyS sensor suggest that the sensing of two distinct signals may be possible by StyS (Rampioni *et al.,* 2008).

Although the above mechanism of control has been demonstrated in *P. fluorescens* ST not all styrene catabolic operons, such as that found in *P. putida* CA-3, are subject to carbon catabolite repression (CCR) control by glucose (OôConnor *et al,* 1995). It is possible however that alternative signal responses/mechanisms may trigger this type of control in *P. putida* CA-3.



(a) (b)

Figure 3: Open **(a)** and Closed conformation **(b)** of P_{styA} promoter region under repressive/ inducing conditions in *P. fluorescens* ST.

Adapted from Rampioni *et al.,* 2008

4.2 Sigma -factor regulation of aromatic degradation

All bacterial species have a housekeeping ů-factor responsible for transcription from the majority of promoters and most encode alternative ů-factor that redirect RNA polymerase (RNAP) to distinct sets of promoters (Gruber and Gross, 2003). These alternate ů-factors are used to globally alter and coordinate transcriptional responses to changing cellular demands. The variety of promoters targeted as well as the diversity of ů-factor present varies widely between bacterial species and reflects the environment and lifestyle of the bacteria itself (Kill *et al.,* 2005). With the exception of $ů^{54}$, all alternative ů-factor belong to the extensive $ů^{70}$ family, named after the housekeeping $ů^{70}$ in *E. coli.* A table of some common ů-factor, the genes which encode them and an example of known functions can be seen in table 2 below.

Sigma-Factor	Gene	Function	Reference
$ů^{S}$	*rpoS*	Stationary phase ů-factor	Fujita *et al.*, 1994; Hong *et al.,* 2005
$ů^{54}$	*rpoN*	Nitrogen metabolism, adhesion, QS, Aromatic metabolism	Ishimoto and Lory, 1989; Hoffmann and Rehm, 2005; OõLeary *et al.,* 2011
$ů^{70}$	*rpoD*	Housekeeping ů-factor	Tanaka and Takahashi, 1991; Aramaki and Fujita, 2000

Table 2: Common ů-factor found in *Pseudomonas* and functions. Adapted from Potvin *et al.,* 2008.

Our knowledge of ů-factor regulation and the pathways which they affect is growing constantly. Initially for example, the role of $ů^{54}$ was associated with nitrogen assimilation solely, however $ů^{54}$ proteins have since been found in many different organisms controlling a myriad of physiological processes including utilisation of alternative carbon sources, assembly of motility organs, control of detoxification systems and the production of extracellular alginate (reviewed in Valls *et al.,* 2004). In aromatic hydrocarbon degradation certain ů-factor, in particular $ů^{54}$, have been shown in to be involved in regulating toluene, dimethyl phenol and phenylacetic acid degradation in *Pseudomonas*.

The $ů^{54}$ class is solely represented by orthologues of *E. coli* $ů^{54}$ and is found in many phylogenetically diverse bacteria. These proteins recognise distinct promoter motifs usually located between -24 and -12 relative to the transcriptional start site (Barrios *et al.,* 1999). The method by which intracellular starvation and extracellular environmental signals which regulate ů-factor activity are detected in bacteria is poorly understood. It has been demonstrated however that the activity of the housekeeping ů-factor $ů^{S}$ and $ů^{54}$ in both *E. coli* and *Pseudomonas species* is regulated by the intracellular signalling molecule ppGpp potentially linking ů-factor activity to intra- and extra-cellular signals (van Delden *et al.,* 2001, Bernado *et al.,* 2009).

In toluene degrading *Pseudomonas putida* species both $ů^{70}$ and $ů^{54}$ ů-factor were identified as responsible for regulating expression of the *xyl* genes encoded on the *TOL* plasmid for toluene degradation in *Pseudomonas putida.* Transcription of the catabolic genes for toluene utilisation is activated by the pathway specific regulator XylR. Transcription of the XylR regulator is controlled by the housekeeping $ů^{70}$ however

transcription of the catabolic genes is dependent on the alternative ů-factor $ů^{54}$ (Ramos *et al,.* 1997). Similarly expression of the DmpR regulator responsible for activating dimethyl phenol degradation has been shown to be $ů^{54}$ dependent (OôNeill *et al.,* 1998). Investigations into the regulation of PAA metabolism in *P. putida* CA-3 by OôLeary *et al.,* (2011) identified a $ů^{54}$ deficient mutant capable of growth on styrene but not PAA. RT-PCR analysis of representative genes of the PA-CoA catabolon identified *paaG* and *paaF* expression in the *rpoN* negative mutant however expression of the PAA transporter, *paaL*, was absent. Complementation of the *paaL* in the *rpoN* disrupted mutant restored growth of the mutant on phenylacetic acid indicating a role for $ů^{54}$ in regulating expression of the PAA transporter *paaL* (OôLeary *et al.,* 2011). Comparison of the *paaL* promoter with nine other $ů^{54}$ regulated promoter sequences from *P. putida* KT2440 identified one highly conserved motif as a $ů^{54}$ binding site. The site identified contained the previously reported GG-N_{10}-GC,-24/-12 consensus sequence found in all $ů^{54}$ promoters (Barrios *et al,* 1999). Bioinformatic analysis however of the *paaL* promoter region failed to identify palindromic or inverted repeat regions typical of enhancer binding proteins (EBP) which are reported to be necessary for transcriptional activation of $ů^{54}$ dependent promoters (Cases *et al.,* 2003). This suggests therefore that the exact mechanism of $ů^{54}$ mediated expression of *paaL* is yet to be fully elucidated.

Interestingly the uptake mechanism of PAA has previously been shown in *P. putida* U to be expressed in conjunction with the catabolic pathway (Schleissner *et al.,* 1994). However recent proteomic studies of styrene grown *P. putida* CA-3 cells did not show the presence of the phenylacetic acid transport protein despite the presence of all other PACoA catabolon gene products (Nikodinovic-Runic *et al.,* 2009).

4.3 Catabolite Repression and transcriptional activation of aromatic metabolism

Expression of catabolic pathways in bacteria is tightly controlled with more easily accessible carbon sources being utilised preferentially over more complex ones. This phenomenon was first published by Jacques Monod in 1942 when he observed glucose-lactose diauxy in *E. coli* (Monod, 1942). Today carbon catabolite repression is defined as the phenomenon whereby the expression of genes required for the use of secondary carbon sources and/or the activities of the corresponding enzymes are reduced in the presence of a preferred carbon source.

In many organisms CCR of catabolic genes is achieved through a combination of both global and operon-specific regulatory mechanisms. The expression of styrene degrading genes has been reported on a number of occasions to be subject to carbon catabolite repression. In *P. putida* CA-3 this effect is mediated at the level of *stySR* expression (O'Connor *et al.,* 1995; O'Connor *et al.,* 1996; O'Leary *et al.,* 2001b). *stySR* transcripts were not detected when batch cultures were growth on styrene in the presence of phenylacetic acid, citrate, glutamate and thus the subsequent activation and transcription of the *styABCDE* genes did not occur (O'Leary *et al.,* 2001b). This system of control is not ubiquitous among *Pseudomonas* species however, with Santos *et al.* (2000) reporting the constitutive expression of *stySR* in *P. fluorescens* ST regardless of the carbon source present in the environment, although transcription of *styA* required the presence of styrene in this strain (Santos *et al.,* 2000).

4.4 The Crc global regulatory protein and aromatic metabolism

The Crc is a global regulatory protein which acts as a translational repressor to control carbon flow and metabolism of aromatic compounds in a number of *Pseudomonas* species and, more recently, in *Acinetobacter baylyi* (Morales *et al*., 2004; Zimmerman *et al*., 2009). Crc acts as a RNA-binding protein under catabolite repression conditions, recognizing specific sites located at the translation initiation regions of target mRNAs thus inhibiting translation and subsequent activity (Moreno and Rojo, 2008).

In the degradation of benzoate by *P. putida* KT2442 Crc has been shown to regulate the expression of the benzoate pathway by targeting and binding to mRNA encoding the *benR* response regulator responsible for the activation of the *benABCD* genes necessary for degradation of the compound. The first step in the degradation of benzoate in *P. putida* involves the oxidation of benzoate to catechol in a two-step process catalysed by benzoate dioxygenase, encoded by the *benABC* genes and a *cis*-diol dehydrogenase, encoded by *benD* to produce catechol. Activation of the catechol and subsequent operons is mediated by intermediates ensuring tight regulatory control of downstream processing. Investigations into the control of benzoate degradation by Crc identified the 5ô end of the *benR* mRNA as the target of Crc binding through the use of *lacZ* transcriptional and translational fusions (Moreno and Rojo, 2008).

Similarly in *P. putida* pWW0, a well-studied xylene and toluene degrading strain, Crc has been shown to modulate expression of XylS, the sensor kinase responsible for the transcriptional activation of genes required for xylene degradation. Interestingly however the role of Crc is not limited to the binding of transcriptional activators. Work investigating the role played by this protein in regulating the degradation of toluene and

xylene degradation in *P. putida* pWW0 confirmed the presence of recognition sites for Crc in the structural genes necessary for the degradation of these compounds using band-shift assays as well as the XylR transcriptional activator. This suggests that Crc can act to differentially modulate levels of certain genes with polycistronic mRNAs in order to optimize levels of individual components of particular pathways (Moreno *et al.,* 2010).

4.5 CRP regulation of aromatic metabolism in *E. coli*

Cyclic AMP receptor protein (CRP) is a regulatory molecule which controls a myriad of genes in prokaryotes which is activated by cAMP binding. The activated CRP protein affects gene expression by binding to target DNA and interacting with RNA polymerase to regulate expression.

Investigations into the global regulatory influences affecting CCR of the *paa* catabolic operon in *E. coli* identified that, in addition to the operon specific PaaX repressor, the *paa* catabolic cluster is subject to control by CRP and Integration Host Factor (IHF). Investigations with *lacZ*-translation fusions of the two (*Pa* and *Pz*) promoters of the *paa* catabolon demonstrated that CRP negative cells failed to activate expression of the fusions suggesting that CRP acts in the activation of *paa* gene expression. Gel retardation assays confirmed the binding of the cAMP-CRP complex to the *Pa* promoter further confirming that changing cAMP levels due to changes in the carbon source present result in the regulation of CRP activation and thus modulation of *paa* catabolon expression in *E. coli* (Ferrandez *et al.,* 2000a;b). This method of CCR control is unlikely to be seen in *Pseudomonas* species however as, unlike in *E. coli*, cAMP levels do not change depending on the carbon source supplied.

4.6 Repressive carbon source intermediates as signals for CCR

A major contributor in the regulation of carbohydrate metabolism within the cell is the phosphotransferase system (PTS). The term ñphosphotransferaseò is used to describe enzymes that transfer phosphate moieties, initially associated with phosphoenolpyruvate (PEP), from one member of the system to the next in a given order. Two types of PTSs are known; the phosphoenolpyruvate:carbohydrate (sugar PTS), responsible for transport and phosphorylation of carbohydrates within the cell, and the nitrogen PTS (PTS^{Ntr}) which exerts regulatory functions other than carbohydrate transport. Both the sugar PTS and the nitrogen PTS work in parallel and crosstalk with one another, although the nitrogen PTS can also receive independent signal inputs. Nitrogen PTSs are found only in Gram negative bacteria and in this system active phosphate is derived from PEP and transferred though the phosphotransferase proteins: enzyme I (EI^{Ntr}) and histidine protein (NPr). NPR then phosphorylates enzyme II ($EIIA^{Ntr}$) which then influences a variety of physiological responses both directly and indirectly (Aranda-Olmedo *et al.,* 2005). As no final acceptor has been found for $EIIA^{Ntr}$ to date this suggests that the PTS^{Ntr} system serves exclusively as a regulatory function within the cell.

PTS^{Ntr} was previously reported to play a role in the regulation of nitrogen metabolism because of the conserved localisation of two of its genes in the *rpoN* operon (Powell, 1995). However it has recently been found to affect the metabolism of other nutrients. In *Pseudomonas putida* mt-2 it has been identified as being involved in controlling toluene and xylene metabolism. The degradation of these aromatic compounds is mediated by a pathway encoded on two operons on the TOL plasmid pWW0. One of these operons is under the control of the σ^{54}-dependent *Pu* promoter which is subject to CCR in which

certain carbon sources down regulate the degradation of the aromatic compounds. The link between expression from this promoter and EII^{Ntr} was established when a loss of EII^{Ntr} was found to relieve CCR of the *Pu* promoter, while replacement of a histidine residue with an aspartate residue to mimic EII^{Ntr} phosphorylation resulted in permanent repression even in the absence of a repressive carbon source (Cases *et al.,* 1999).

A molecular mechanism for how the $EIIA^{Ntr}$ influences the activity of the *Pu* promoter still remains to be fully elucidated, however a hypothesis put forward by Aranda-Olmedo *et al.,* is that $EIIA^{Ntr}$ interferes with the binding of either σ^{54} or the effector-bound XylR to its upstream activator sequence, thereby preventing activation of the respective promoters (Aranda-Olmedo *et al.,* 2005). Investigations by del Castillo and Ramos to identify the signalling molecule responsible for this phenomenon identified high levels of the glucose intermediate 2-dehydro-3-deoxygluconate-6-phosphate as being responsible for reducing expression from the *Pu* promoter (del Castillo and Ramos, 2007).

The degradation of many structurally related aromatic hydrocarbons including styrene, ethylbenzene and 2-phenylethalanine occurs via PAA (O'Connor *et al.,* 1995; Utkin *et al.,* 1991) and many carbon sources have been demonstrated to repress the degradation of PAA within the cell. Efforts must be made therefore to understand CCR effects on PAA degradation in order to improve the bioremediation potential of the cell as a whole. Glucose and gluconate repression of PAA metabolism in *Pseudomonas putida* KT2440 is one such example of carbon source dependent repression. Investigations into the exact mechanisms by which glucose and gluconate repression occurs in *Pseudomonas putida* KT2440 by Kim *et al.,* identified the glucose metabolism intermediate 2-keto-3-deoxy-6-phosphogluconate (KDPG) as the signal required for CCR. The inhibitory effect of

KDPG on PAA metabolism in *P. putida* KT2440 was observed as repression of the PAA catabolic genes *paaA*, *paaG* and *paaL* in glucose + PAA grown cultures (Kim *et al.,* 2007). Later work by Kim and co-workers described how repression of PAA metabolism by glucose was found to be present in an *eda* mutant, encoding the KDPG aldolase, responsible for metabolism of KDPG to glyceraldehyde-3-phosphate and pyruvate while repression was relieved in an *edd* mutant. The mutant lacked 6-phosphogluconate dehydratase responsible for KDPG formation, thus identifying the compound between these two steps of glucose metabolism as the repressive intermediate (Kim *et al.,* 2009). Interestingly, in the same study Kim and colleagues investigated potential roles for the previously established PaaX repressor on the *paa* pathway in *P. putida* KT2440. In *paaA::gfp* reporter assays no activation of *paaA* transcription was found in a *paaX* negative mutant suggesting glucose mediated CCR in this strain is mediated by another repression mechanism. It is possible that an as yet to be elucidated link may exist between KDPG and the observed CCR repression effects.

In investigating the regulation of aromatic compounds many global regulatory elements have been identified as influential. Some elements identified, as detailed above, influence expression by binding directly to the promoter region of DNA to prevent or promote gene expression; such as IHF and ů-factors. Others regulate the catabolic operons indirectly through regulation of transcriptional activators such as the CCR of *stySR* expression in *P. putida* CA-3 and Crc protein regulation of *benR* transcription in *P. putida* KT2442. Finally the impact of repressive carbon source intermediates on aromatic degradation has been shown in species of *Pseudomonas.* As we have seen therefore a wide diversity of systems have been identified as playing a role in regulating

aromatic metabolism with many dependent on or working in concert with CCR. To date functional based approaches have yielded the best results in identifying specific regulatory elements, such as the identification of the repressive intermediate KDPG involvement in PAA metabolism and the Crc protein regulation of *benR* transcription, further investigations; specifically functional screen based approaches, are needed to identify more of global regulatory elements influencing aromatic metabolism. Furthermore work to elucidate the means by which these systems co-ordinate within the cell, based on intra- and extra-cellular signals and conditions to control expression of these systems to maximise the biotechnological potential of these cultures as whole cell based systems for bioremediation is also required.

5. Polyhydroxyalkanoate Production

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced by numerous bacterial species that have varied thermoplastic and elastomeric properties offering industrial, medical and consumer applications. The first PHA to be discovered was a homopolymer of polyhydroxybutyrate by Lemoigne in 1926. PHAs are typically synthesised by bacteria when an essential nutrient, e.g. nitrogen, sulphur, or phosphorous, becomes limiting in the presence of an excess of carbon (Lageveen *et al.,* 1988; Ramsay *et al.,* 1992; Huismann *et al.,* 1991; Garcia *et al.,* 1999). When the limiting nutrient is restored the PHA can undergo depolymerisation and be metabolised as a carbon and energy source. Several comprehensive reviews are available in the field of microbial PHA synthesis e.g. Grage *et al.,* 2009 and Kessler and Witholt, 2001.

PHAs are condensation polyesters of various hydroxyalkanoic acids found as inclusions within the cell cytoplasm. There are two main classes of PHA, based on the chain length of the monomer units which make up the polymer. Short chain length PHA, (scl-PHA), consisting of 3-5 carbon atom monomers and medium chain length-PHAs (mcl-PHA) consisting of 6-14 carbon atom monomers.

The two major classes of PHAs are synthesised by distinct polymerase enzymes, namely Class I synthases, (scl-PHA) or Class II, synthases (mcl-PHA), respectively (Witholt and Kessler, 1999). A third class has also been defined by Liu *et al.* (2003) as being composed of hybrid polymers with both scl- and mcl-hydroxyalkanoate monomers incorporated into the same polymer. The generation of monomer units can occur by several routes, but three principal pathways are commonly reported (Figure 4). scl-PHA 3-hydroxybutyrate monomers are derived from the condensation of acetyl-CoA moieties

from the TCA cycle, e.g. *Ralstonia eutropha* (Kessler and Witholt, 2001) (Figure 4; Pathway I). Pathway II proceeds with the metabolism of fatty acids and generates various hydroxyalkanoate monomers for PHA biosynthesis via substrate related conversions of keto-acyl-CoAs to corresponding 3-hydroxyacyl-CoAs during *β*-oxidation, e.g. *P. aeruginosa* (Tsuge *et al*., 2000) (Figure 4; Pathway II). PHA monomers can also be derived from unrelated carbon sources via acetyl-CoA diversion to *de novo* fatty acid synthesis, yielding 3-hydroxyacyl-ACP intermediates. These intermediates are converted to corresponding 3-hydroxyacyl-CoA monomers for PHA synthase by 3-hydroxy-ACP-CoA transacylases, e.g. *P. putida* (Rehm and Steinbüchel, 1999). This latter pathway is of specific biotechnological interest as it presents the potential for PHA production from organic wastes (Figure 4; Pathway III).

Medium chain length PHAs have a number of uses as biodegradable rubber, adhesive, environmentally friendly packaging etc., (van der Walle *et al.,* 1999; 2001) but one of the roadblocks to the commercialisation of mcl-PHA is the cost associated with its production when compared with standard petrochemical based plastics (Choi and Lee, 2000).

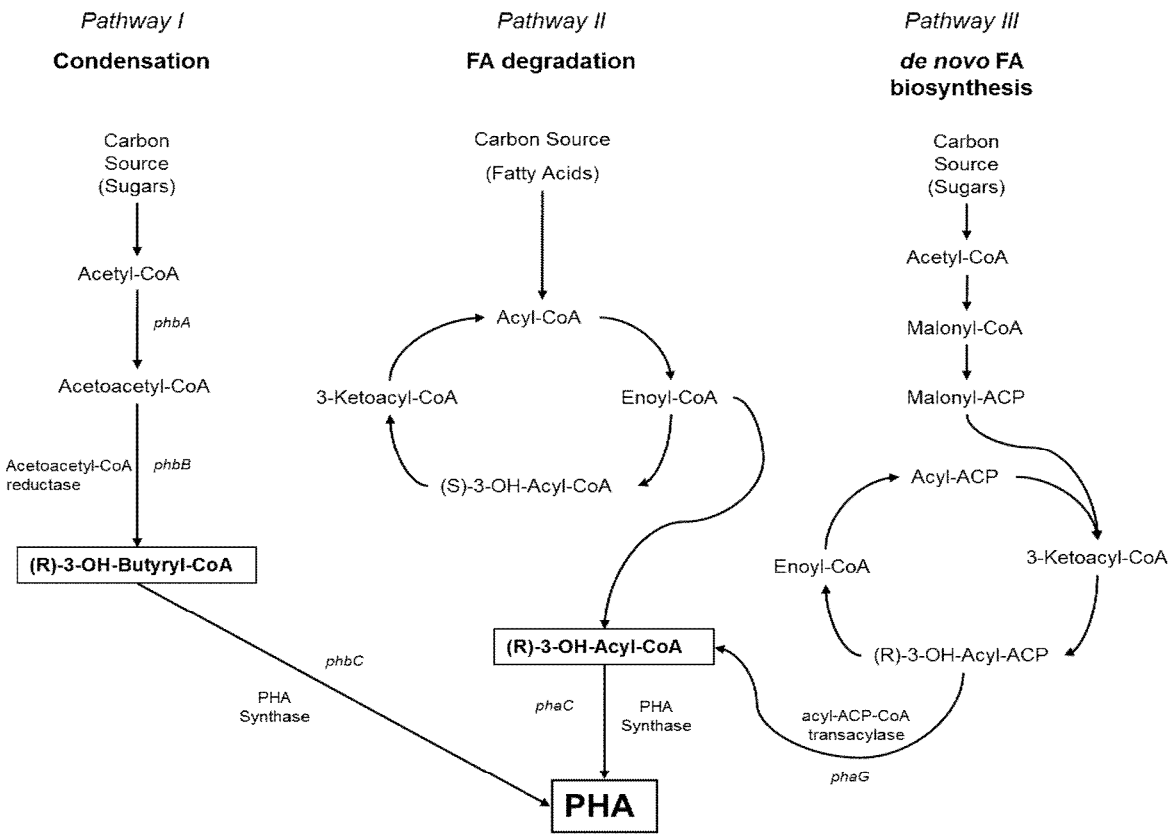


Figure 4: Major PHA accumulation pathways. Pathway I: Condensation of Acetyl-CoA moities; Pathway II: Fatty Acid (FA) Degradation (ɓ-oxidation pathway); Pathway III: *de novo* FA biosynthesis from unrelated carbon sources. (Adapted from Philip *et al.,* 2007).

5.1 Production and Genetics

Since the first discovery of *phb* genes was made in *Z. ramigera* by Peoples *et al.* in 1989 many bacterial species have been found to possess genes encoding PHA accumulation enzymes. For example, species of *Pseudomonas, Ralstonia, Bacillus, Rhodobacter* as well as certain *Archaea* have all been shown to accumulate forms of intracellular PHA (Philip *et al.,* 2007).

As discussed above, and described in figure 4, a number of different biochemical pathways exist for the production of PHA within the cell. It is not surprising therefore that a significant divergence exists in the organisation of genes required for PHA accumulation between genera. Many bacteria possess the scl-PHA accumulation genes *phbCAB* in their genome for example; in some species these genes are found together at the same loci, e.g. *Pseudomonas acidophila* (Umeda *et al.,* 1998) however, in others; the genes are often in different orders in for example the genome *R. eutropha* (Peoples *et al.,* 1989b;c). In other strains such as *Rhizobium meliloti* (Tombolini, *et al.,* 1995) and *Z. ramigera* (Peoples *et al,* 1989a) the *phbAB* and *phbC* loci are unlinked.

The properties of mcl-PHA which enable its use in a greater number of applications than scl-PHA have already been discussed. Furthermore the ability of strains to produce mcl-PHA via *de novo* FA biosynthesis presents the possibility of bioplastic production from waste streams such as PHA accumulation from styrene in *P. putida* CA-3 (OôLeary *et al.,* 2005). Interestingly the *pha* operonic structure has been found to be highly conserved among mcl-PHA accumulating *Pseudomonas* species with at least nine demonstrating similar operonic structures to that in figure 5 (de Euginio *et al.,* 2007).

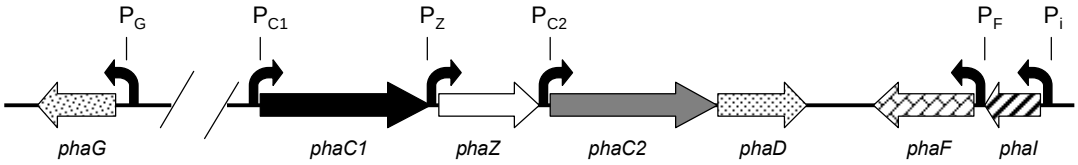


Figure 5: Putative organization of structural genes relevant for PHA biosynthesis in *P. putida* strains. Key: *phaC1 phaC2*, genes encoding PHA synthase; *phaZ,* encoding intracellular PHA depolymerase; *phaG*, encoding transacylase linking fatty acid biosynthesis with PHA biosynthesis; *phaD,* encoding TetR like transcriptional regulator; and phasing encoding *phaI* and *phaF*. Black arrows indicate the location of putative promoters.

Transcription of the *pha* structural genes has by and large been scarcely studied in many PHA accumulating strains. A recent study however by de Euginio *et al.,* (2010) investigated the promoter regions of the *pha* operon in *P. putida* KT2442 and identified functional promoters for *phaC1, phaZ* and *phaC2* genes (P_{C1}, P_Z and P_{C2} respectively) (Figure 5). The presence of independent promoters for *phaC1, phaZ* and *phaC2* genes is consistent with transcriptional data previously published by our group. O'Leary *et al.,* (2005) previously reported differential and independent expression of these genes in *P. putida* CA-3 cells. Furthermore they reported constitutive expression of *phaC1* while expression of *phaZ* and *phaG* was dependent on growth conditions further suggesting independent transcriptional activation of these genes (O'Leary *et al.,* 2005).

In the production of mcl-PHA accumulation Rehm *et al.,* were the first to report on the *phaG* encoded 3-hydroxyacyl ACP-CoA transacylase being responsible for the transfer of 3-hydroxydecanoate moieties from the fatty acid biosynthesis acyl carrier protein to

coenzyme A in *E. coli* (Rehm *et al.,* 1998). This was confirmed by O'Leary *et al.,* to also be in the case in *P. putida* CA-3 by transcriptional analysis (O'Leary *et al.,* 2005).

PhaG transferred CoA moieties are packaged by the *phaC1* PHA synthase which catalyses the polymerization of hydroxyacyl-coenzyme A (CoA) to PHA (Figure 4; Pathway III). PHA synthases are bound to the surface of PHA granules in PHA accumulating cells (Ren *et al.,* 2009). Several PHA synthases from different bacterial sources have been identified and studied since the first example some 20 years ago. They are divided into classes based on their subunit composition and substrate sensitivity with the *P. putida* CA-3 PHA synthase representing an example of a class II enzyme producing medium chain length PHA.

5.2 Regulation of polyhydroxyalkanoate production

The production and genetics of PHA production have been extensively studied in a variety of bacterial species however little of the regulation of PHA production is understood. What is known is that there are four main levels of control influencing the production of PHA within the cell. These include transcriptional regulation of the *pha* genes themselves, regulation of the specific enzymes necessary for PHA accumulation within the cell, regulation of PHA enzymatic intermediate production and the regulation of PHA depolymerases controlling the biodegradation of PHA within the cell.

5.3 Transcriptional regulation of PHA production

The *pha* gene cluster is very well conserved among mcl-PHA producing strains and has been well studied in a variety of strains; however the regulation of PHA production has been poorly studied to date. Recent efforts have highlighted pathway specific regulation

influencing the production of PHA in *Pseudomonas* species in the form of the TetR like transcriptional regulator PhaD protein (de Euginio *et al.,* 2010). The gene encoding this regulator is found downstream of the *phaC1ZC2* operon (Figure 5) and RT-PCR investigations by de Eugenio *et al.,* into its activity in *P. putida* KT2442 identified it as being responsible for the carbon source dependent expression of the *pha* gene cluster (de Euginio *et al.,* 2010). The authors demonstrated that PhaD acts as an activator of *pha* gene expression by binding to both the P_{ci} and P_i promoters. However, the presence of *phaC1* mRNA transcripts in a *phaD* negative mutant suggested that it was not solely responsible for expression of the *pha* genes and that other factors were likely to be involved in *pha* gene expression. Furthermore, gel retardation assays demonstrated that PhaD activates transcription by binding upstream of the promoter regions of the P_{C1} and P_1 promoters responsible for *phaC1* synthase and *phaI* phasin gene expression. The authorôs findings suggest that although the binding of PhaD is required for expression of the *pha* genes it in itself is not sufficient to ensure gene expression (de Eugenio *et al.,* 2010).

5.4 Global regulatory influences affecting PHA accumulation

When grown in bioreactors microorganisms can be subjected to changes in various physiological conditions and stresses under controlled conditions. Previous studies have investigated the use of different carbon sources and fatty acid intermediates to achieve increased PHA accumulation by the cell (reviewed by Gomez *et al.,* 2012) however the use of pure carbon sources in the production of PHA significantly increases the associated costs. Some species as we have already discussed possess the ability to accumulate PHA from unrelated carbon sources enabling the use of waste streams in

plastic production. Knowledge of the role intracellular global regulatory elements play in regulating the accumulation of PHA is therefore of significant value if the production of these polyesters for commercial uses is to be fully exploited.

Some studies have identified and modified elements in the cell to promote conditions conducive to PHA accumulation. PHA accumulation requires aerobic conditions and thus production is highly energy intensive from a commercial perspective. Strategies therefore have been examined to improve the cells accumulation capacity under low-oxygen conditions through strain modification. Hong and co-workers reported recently on the expression of V*itreoscilla* haemoglobin in PHB producing *E. coli* cells and demonstrated improved growth and polymer yield in this strain (Horng *et al.,* 2010; 2011).

Another approached involved altering the redox potential of the cell to promote aerobic respiration. The ArcAB two component system controls expression of many operons according to the redox potential of the cell such as aerobic respiration and oxidative bioreactions. Lynch and Lin (1996) reported that *arc* negative *E. coli* mutants exhibited an elevated pool of reducing equivalents which could be funnelled into PHB production. Their approach demonstrated 35% PHB accumulation in an *arcA* mutant under conditions where the wild-type was seen to accumulate no PHB.

A further study focused on the CreBC system which regulates many cellular processes, including carbon metabolism (Avison *et al.,* 2001). Nikel *et al.* (2008a) reported an *arcA* and *creC* constitutive mutant demonstrated enhanced carbon source consumption as well as a reducing intracellular environment demonstrated increased PHB yield in *E. coli* cells harbouring the PHB biosynthetic genes from *Azotobacter* sp. strain FA8 (Nikel *et al.,*

2008b). A similar rationale employed anaerobic promoters to achieve PHB production under micro-aerobic conditions. Wei and co-workers reported the *E. coli* alcohol dehydrogenase promoter as most efficient at promoting micro-aerobic synthesis of PHB (Wei *et al.,* 2009).

To date few reports exist on the involvement of post transcriptional regulators on PHA accumulation however a report in 2000 by Castañeda *et al.* detailed the loss of poly-ɓ-hydroxybutyrate (PHB) accumulation and alginate production capacity in a *gacS* negative mutant of *Azotobacter vinelandii*. Their investigations utilised random mini-Tn5 mutagenesis to identify mutants exhibiting reduced capacity to accumulate PHB and alginate. A recent follow up study by Hernandez-Eligio *et al.,* (2012) further investigated the relationship between GacS/GacA and the accumulation of PHB by *A. vinelandii*. The authors reported that PHB accumulation in a GacA negative *A. vinelandii* mutant was significantly reduced. The GacS/GacA two component system has previously been reported to effect changes in the cell via the activation of transcription of small regulatory RNAs (sRNAs) as demonstrated in figure 6 below. The exact means by which activation of the GacS sensor kinase occurs has not yet been fully elucidated. Previous reports have indicated activation via *N*-acyl-homoserine lactone type molecules resulting in phosphorylation of the GacA response regulator (Dubuis and Haas, 2006). A more recent report however has demonstrated increased sRNA expression in *P. fluorescens* CHA0 in response to the presence of 2-oxoglutarate, succinate, and fumarate (Takeuchi *et al*., 2009). This suggests the possibility that TCA cycle intermediates act as GacS ligands potentially linking GacS signal activation to both the intracellular metabolic status of the cell and also potentially to the presence of preferred extracellular carbon sources.

Activated GacS then phosphorylates and activates GacA; this activated GacA in turn induces expression of the sRNA molecules. These sRNAs sequester the mRNA binding protein RsmA preventing translational repression of target genes. Hernandez-Eligio *et al.* reported a lack of sRNA expression in the GacA negative mutant and demonstrated RsmA binding to *phbB* and *phbR* mRNA. The *phbR* encoded regulator is required for expression of the *phbBAC* PHB biosynthesis operon. The authors therefore concluded that the lack of sRNA expression, owing to the GacA deficiency, resulted in an increase in RsmA binding to *phbR* transcripts which in turn reduced transcriptional activation of the *phb* biosynthetic operon (Hernandez-Eligio *et al.,* 2012).

Previous investigations into PHB production and regulation have, as we have already discussed, focused on intracellular factors such as redox potential and pathway intermediate production as well as extracellular conditions such as nutrient limitation and carbon availability. The recent reports of GacS/GacA two component system mediated regulation of PHB production introduces an element of population density based control of bioplastic production in the cell. It is likely that a variety of elements act in concert with one another to modulate PHA production in the cell however much has yet to be elucidated with respect to how they coordinate their responses to effect bioplastic production.

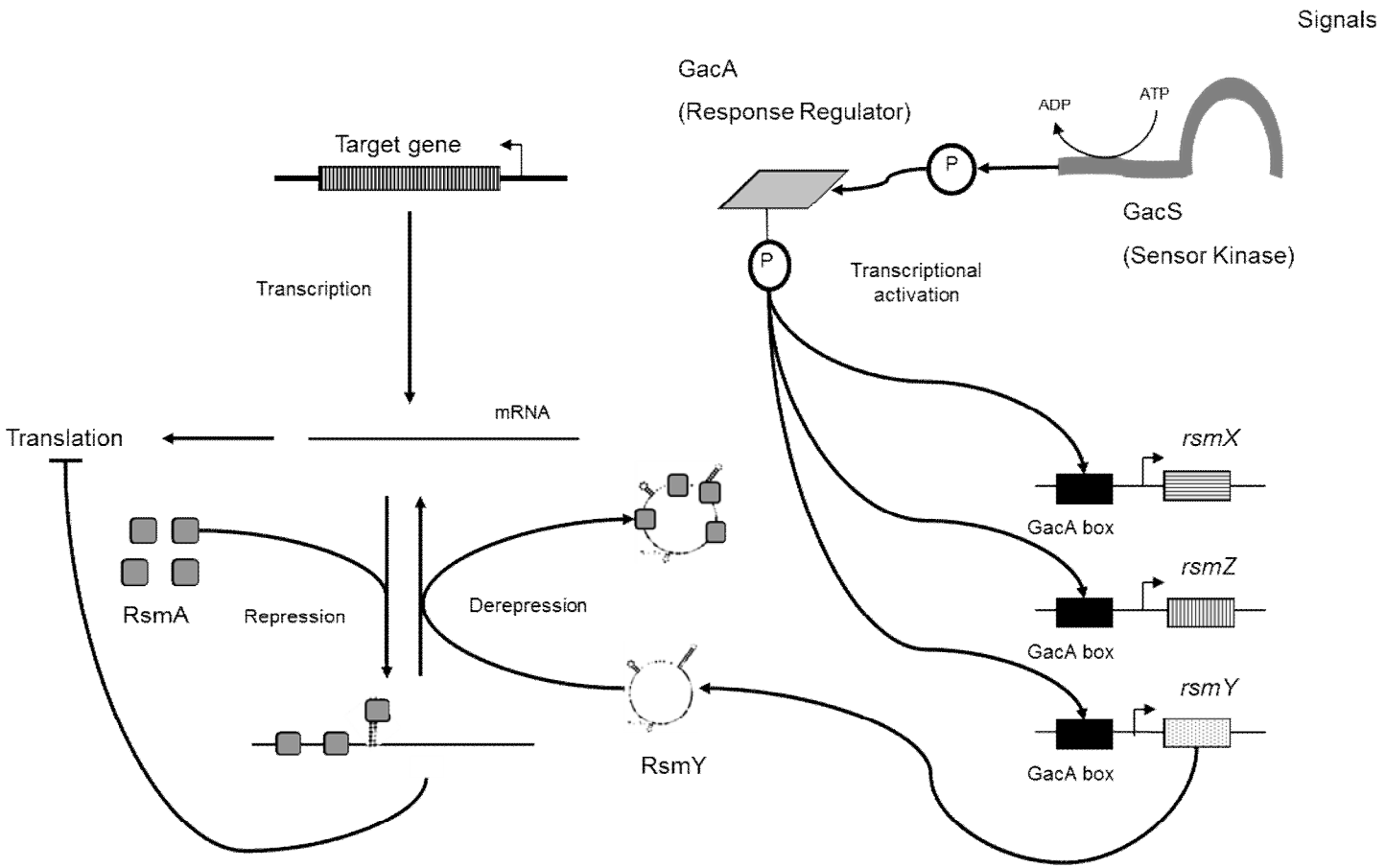


Figure 6: Current understanding of GacS/GacA cascade (based on *P. aeruginosa*). Under repressive conditions mRNA binding RsmA sequesters target mRNA preventing RNA polymerase binding and protein synthesis. Under non-repressive conditions RsmA is bound by *rsmY, rsmZ* and *rsmX* sRNAs allowing translation of mRNA to form protein

5.5 Sigma-factor and PHA accumulation

Sigma-factor control the expression of many genes and gene clusters in bacteria, as previously described in section 4.2 of this introduction, with a selection of the most common ů-factor and some known functions being previously described in table 2. Investigations into potential binding sites for these ů-factor upstream of *pha* genes, which are necessary for PHA accumulation, has identified ů-factor involvement in PHA production in a number of strains.

Sigma-factor involvement in PHA accumulation first identified a role for *rpoN* encoded $ů^{54}$ in *P. aeruginosa* when grown on gluconate (Timm and Steinbüchel, 1992). Further investigations into the relationship between $ů^{54}$ and PHA production in *P. putida* and *P. aeruginosa* strains when grown on different carbon sources by Hoffman and Rehm provided greater insight into the relationship. Their findings demonstrated a 40% increase in PHA accumulation by an *rpoN*-deficient mutant of *P. putida* compared with the wild-type when grown on octanoate under nitrogen limiting conditions but interestingly found no difference in PHA accumulation capacity when grown on gluconate under N-limiting conditions. Similar experiments on PHA accumulating *P. aeruginosa* strains PA01 and PAK showed that under nitrogen limited conditions *rpoN*-deficient PA01 and PAK mutants lose the capacity to accumulate PHA.

Subsequent RT-PCR analysis investigated the expression of the *pha* genes in both *P. putida* and *P. aeruginosa* wild type and *rpoN*⁻ mutant strains. The results demonstrated no difference in *phaC1* and *phaG* gene expression between *rpoN*⁻ and wild-type *P. putida* strains as expected. However in *P. aeruginosa* while *phaC1* gene expression was unaffected in the *rpoN*⁻ mutant RT-PCR analysis of *phaG* transacylase expression,

necessary for PHA production from non-related carbon sources via *de-novo* fatty acid biosynthesis, was repressed in the *rpoN*-deficient *P. aeruginosa* strain. This suggests that expression of *phaC1* and *phaG* genes are regulated independently in *P. aeruginosa* and that different regulatory controls may exist to control bioplastic production among the two different PHA accumulating *Pseudomonas* species.

The relationship between the *rpoS* encoded stationary ů-factor $ů^S$ and PHA production and stability has also been investigated in *Pseudomonas putida*. Studies into the effects of PHA degradation on the cell showed increased stress survivability and $ů^S$ levels in *P. putida* (Ruiz *et al.,* 2004). Subsequent studies into the relationship between $ů^S$, PHA and cell survivability by Raiger-lustman and Ruiz further implicated *rpoS* in stationary phase PHA production (Raiger-lustman and Ruiz, 2008). Their investigations on an *rpoS* negative *P. putida* KT2440 mutant demonstrated that an *rpoS* deficient mutant increased the rate at which PHA is degraded by the cell post stationary phase onset. The authors also demonstrated an increase in P_{C1} promoter expression in the *rpoS* negative mutant and speculated that the increased depolymerisation rate seen in the *rpoS* negative mutant could be attributed to co-transcription of the *phaZ* depolymerise gene along with the *phaC1* synthase gene from the P_{C1} promoter. However other promoters driving *phaZ* expression as seen in *P. putida* U could not be excluded. Lastly the authors demonstrated that *rpoS* negative mutants demonstrate decreased survivability and are less tolerant of oxidative stress than the wild-type under non-PHA accumulating conditions after onset of stationary phase. The alternative ů-factor *rpoS* has previously been shown to be responsible for the coordination of a number of cellular processes in response to stress. The relationship between PHA and *rpoS* further highlights the importance of PHA in

bacterial stress response and provides an insight into levels of control regulating PHA accumulation in the cell.

6 Aims and Objectives

The degradation of styrene and production of PHA by microorganisms has been well studied to date at the pathway specific level; with a breadth of knowledge reported in the biochemical pathways and organisation of genes involved in a variety of species, most notably *Pseudomonas* species. However, much remains to be elucidated with respect to the impact global regulatory factors play in affecting these biotechnologically significant pathways. Here we report on our attempts to identify global regulatory elements influencing styrene metabolism and PHA biosynthesis in *Pseudomonas putida* CA-3. Our efforts subjected the *P. putida* CA-3 genome to random Tn5 mutagenesis and screened the transformants which were generated for reduced sensitivity to carbon catabolite repression (CCR) of the *sty* pathway. Screening of mini-Tn5 mutant library identified two mutants with reduced sensitivity to citrate mediated CCR, CCR25 and CCR29, identified as *clpX::*Tn*5* and *luxR::*Tn*5* mutants respectively. Growth and RT-PCR analysis was employed to characterise the ability of these mutants to utilise styrene and phenylacetic acid as a sole carbon source as well as examining the growth of these under mixed carbon conditions.

Mutagenesis and subsequent functional screening was also employed to identify novel regulators of PHA biosynthesis via screening of the generated library for mutants with a reduced capacity to accumulate PHA. Solid media screening and subsequent fluorescence microscopy identified two *gacS*::Tn*5* disrupted mutants exhibiting this PHA negative phenotype, a regulator previously only associated with PHB accumulation in *A.*

vinelandii. Investigations were undertaken utilising RT-PCR and SDS-PAGE analyses to determine the means by while *gacS* regulates PHA production in the cell. The potential global impacts of this regulatory system were investigated under different PHA inducing stresses conditions and through disruption of *gacS* in other PHA accumulating strains. Lastly the biotechnological potential of GacS mediated regulation was examined by means of sRNA overexpression in a *gacS*::Tn*5 mutant* and in *gacS* positive *P. putida* CA-3 strains. We utilised GC analysis to monitor for any potential altered PHA production phenotypes including increased PHA production (wt/wt), altered PHA monomer composition and alteration in the quantities accumulated per cell.

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Chapter 2

Tn*5* mutagenic screening for, and characterisation of, a putative carbon catabolite repression regulator of phenylacetic acid metabolism in *Pseudomonas putida* CA-3

Abstract

The accumulation of the styrene contaminated wastes in the environment is of significant ecological concern given its toxicity and its recalcitrance to degradation. Significant research has thus been carried out into the microbial degradation of this compound and has included the characterisation of microbial styrene degradation. Little research however has been carried out to characterise impact of global regulators on this pathway. Here we report efforts to identify and characterise such regulatory elements in the styrene degrading organism, *Pseudomonas putida* CA-3. In our studies a mini-Tn*5* mutant library was generated and subjected to phenotypic screening to identify mutants exhibiting a reduced sensitivity to the effects of carbon catabolite repression on styrene degradation pathway activity. Our efforts identified a *clpX* disrupted mutant which was characterised as exhibiting wild-type levels of growth on styrene but significantly reduced growth on phenylacetic acid, which was restored upon *clpX* gene complementation. RT-PCR analysis of key PACoA catabolon genes, representative of discrete transcriptional units within the catabolon (i.e. *paaF*, *paaG* and *paaL*) were conducted, but did not reveal altered transcriptional profiles between wild type CA-3 and the *clpX::*Tn*5* mutant. Examination of cultures for distinct, post-transcriptional regulatory effects via SDS-PAGE protein profile analyses also failed to identify any alteration in PACoA pathway expression. Cloning and over-expression of the PaaL active transport protein, responsible for phenylacetic acid uptake in CA-3, further reduced the growth capacity of the strain suggesting that transport was not the limiting factor in growth on PAA. Thus while *clpX* disruption had a definitive impact on phenylacetic acid utilisation, correlation of this genotype with regulation of pathway

specific elements could not be established and suggests the involvement of some intermediary, unknown component warranting further investigation.

Introduction

Styrene is used extensively in the petrochemical industry as both a starting material and a solvent in synthetic polymer production and processing (Shingler, 2003). Styrene and its metabolites, such as styrene oxide, are classified by the US EPA and other bodies as potential carcinogens with toxic effects on the kidneys, gastrointestinal and respiratory tracts and the nervous system (US EPA). Microbial degradation of this compound has been the subject of much investigation, resulting in significant characterisation of the genetic organisation, transcriptional regulation and biochemistry of this pathway in a range of bacterial species (Luengo *et al*., 2001; O'Leary *et al*., 2002; Mooney *et al.*, 2006; Martin & McInerney, 2009).

In *Pseudomonas* species styrene is degraded in a stepwise fashion by one pathway comprised of two independently regulated catabolons. The *sty* catabolon, comprising the *styABCDE* genes, encodes the enzymes necessary for the first of these pathways in which styrene is degraded via oxidation of the vinyl side chain to form phenylacetic acid (PAA). Degradation of styrene by side chain oxidation is initiated by the *styAB* encoded two component styrene monooxygenase (SMO) which forms styrene oxide. The generated styrene oxide is then isomerised to phenylacetylaldehyde via a *styC* encoded isomerase from which the action of a *styD* encoded dehydrogenase generates PAA (Mooney *et al.,* 2006). Transcription of the *sty operon* is controlled by a two component regulatory system encoded by *stySR* located upstream of, and transcribed separately from, *styABCDE.* A *styE* encoded porin, previously shown to be involved in active transport of styrene across the cell membrane, is located downstream of the structural genes and is co-transcribed as part of the operon (Mooney *et al.,* 2005). The StySR system detects the

presence of styrene and activates transcription of the *styABCDE* genes. The PAA generated via this catabolic pathway is then further degraded by the PACoA catabolon to TCA cycle intermediates. The phenylacetic acid (PAA) generated is subsequently degraded by the second catabolic pathway. The first step is atypical of aerobic catabolism in that it involves Co-enzyme A activation of PAA to phenylacetyl-CoA (PACoA) by *paaK* encoded PACoA ligase (Martinez-Blanco *et al.,* 1990). PACoA acts as the true inducer of the pathway via interaction with a GntR-type transcriptional repressor, PaaX. This interaction releases PaaX from the operator region of the PACoA catabolon and permits transcription of the structural genes necessary for PACoA degradation (Ferrández *et al.,* 2000). The products of the PACoA catabolon then proceed to degrade PACoA to the TCA cycle intermediate succinyl Co-A in a stepwise fashion (Teufel *et al.,* 2010). In the first of these step the *paaABCDE* gene products performs a ϐ-oxidation like transformation of PACoA. Following these transformations *paaGHIJK* encoded proteins responsible for ring epoxidation take over to form succinyl-CoA (Teufel *et al.,* 2010). A detailed breakdown of the individual steps involved in PACoA degradation can be found in chapter 1 (section 2.1).

A number of carbon sources have been demonstrated to repress the expression of the pathways responsible for aromatic degradation in several bacterial strains. This method of regulatory control is known as carbon catabolite repression and has been identified as regulating a number of aromatic degradative pathways (reviewed in Görke and Stülke, 2008). This repressive phenomenon was first published by Jacques Monod in 1942 when glucose-lactose diauxy was observed in *E. coli* (Monod, 1942). It is defined as the phenomenon whereby the expression of genes required for the use of secondary carbon

sources and/or the activities of the corresponding enzymes are reduced in the presence of a preferred carbon source. Many aromatic degradation pathways including those necessary for PAA, styrene, xylene and benzoate degradation have been shown to be subject to CCR by various carbon sources (O'Connor *et al.,* 1995; O'Leary *et al.,* 2001; Aranda-Olmedo *et al.,* 2005; Moreno and Rojo, 2008).

The regulatory elements which effect this CCR in the cell to regulate pathways varies considerably between different aromatic pathways and strains. In the toluene and xylene degrading bacterium *Pseudomonas putida* mt-2 for example, CCR control of the TOL plasmid has been linked to the nitrogen PTS system. This system acts by controlling σ^{54} dependent expression of the *Pu* promoter responsible for catabolic gene expression (Cases *et al.,* 1999). However other global regulators have also been shown to respond to CCR conditions in the cell. These include integration host factor (IHF) which exerts CCR control in another xylene and toluene degrading strain *P. putida* pWW0 and in styrene degrading strain *P. fluorescens* ST. In these strains IHF has been found to control expression of the genes necessary for aromatic degradation through promoter binding which prevents RNA polymerase binding and transcriptional activation of the target genes (Holtel *et al.,* 1992; Leoni *et al.,* 2005).

Our group has previously reported that in *P. putida* CA-3 repression by citrate results in reduced transcription of *stySR* and *styABCDE* genes as long as the carbon source is present in the medium. Furthermore, transcription of *paaK*, a gene involved in the lower pathway and regulated independently of *stySR*, is also subject to catabolite repression (O'Leary *et al.*, 2001). These regulatory effects are also not ubiquitous among styrene degrading species with Santos *et al.* (2000) reporting constitutive expression of *stySR* in

P. fluorescens ST regardless of carbon source supplied, indicating a different CCR mechanism in this strain to that found in *P. putida* species. Many different regulatory controls have therefore been found to influence the expression and activity of these aromatic degradative pathways. This is perhaps not surprising given their relatively recent evolutionary development, especially examined in the context of the ñblack cat/white catò principle as proposed by Cases and de Lorenzo (2001). Thus it is possible that the regulatory control of these pathways has developed depending on the variety of signals found in their respective evolutionary environments and may therefore be poorly conserved between species.

In conclusion much research has taken place into pathway specific controls regulating expression of the catabolic pathways necessary for styrene metabolism, such as StySR activation of the *sty* operon in various strains. Current hypotheses suggest that a number of factors including StySR, IHF, feedback inhibition and CCR act in synergy to regulate these degradative pathways as described above. However the regulatory controls acting in these strains are often poorly conserved. Therefore the further elucidation global regulatory factors; including the identification of rate limiting steps, pathway bottlenecks and the involvement of global regulators, is required to fully harness the biotechnological potential of these cultures as whole cell based systems for bioremediation.

Our work seeks to address one aspect of this knowledge gap, the involvement of global regulators in the carbon catabolite repression of styrene/PAA metabolism. We detail here our attempts to utilise random Tn*5* mutagenesis together with functional screening to identify potential regulators of styrene/ phenylacetic acid metabolism in *P. putida* CA-3. Our efforts identified a putative role for the ClpX protease chaperone in regulating

phenylacetic acid and citrate metabolism. The ClpX chaperone is a member of a two component complex comprised of ClpX and protease core ClpP. Protein substrates degraded by this complex display a variety of recognition motifs and tags are identified by the ClpX chaperone. These are unfolded and translocated into the protease cylinder by ClpX before degradation by ClpP (Kress *et al.,* 2009) see figure 1. Almost all bacteria contain the ClpXP complex and previous studies have shown its involvement in many aspects of cell function including protein quality control, cell division, transposition and virulence. Specifically a study by Nikodinovic-Runic *et al.* (2009) identified ClpX as being upregulated under conditions of nitrogen limitation in *Pseudomonas putida* CA-3 when grown on styrene. Furthermore an investigation by Flynn *et al.* (2003) identified ClpXP as degrading the phenylacetyl-CoA monooxygenase component PaaA in *E. coli*. Our study attempted to characterise the role played by ClpX in carbon catabolite repression of styrene/ phenylacetic acid metabolism using growth profile, RT-PCR and SDS-PAGE analyses.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Pseudomonas putida CA-3, a styrene-degrading, bioreactor isolate has been described previously (O'Connor *et al.,* 1995). *E. coli* CC118λ*pir* hosted the mini-Tn*5* derivative pUT-Km1, (R6K origin of replication), which encodes resistance to kanamycin and ampicillin (de Lorenzo *et al.,* 1990). Plasmid pRK600 (Cm^r) was used as a helper in triparental mating experiments and encodes the *tra* functions facilitating pUT-Km1 mobilization. PCR2.1-TOPO vector (Invitrogen, California) was used in the cloning of polymerase chain reaction (PCR) amplification products. *Pseudomonas putida* CA-3 was routinely grown on E2-minimal medium (Vogel and Bonner, 1956) containing PAA, Citrate, Glucose, Styrene and combinations thereof, in 250ml flasks at 28°C, shaking at 180rpm. Carbon sources were added to the following concentrations pre-autoclaving; 20 mM phenylacetic acid, 23 mM citrate and 10mM glucose. Growth on styrene required substrate provision in the gaseous phase by the addition of 70 µl of liquid styrene to a tube within the flask. Cell growth was monitored by measuring the optical density of the culture at OD_{600nm}. *E. coli* strains were grown on Luria Bertani (LB) medium at 37°C. Host/plasmid associations were maintained during growth via the incorporation of appropriate antibiotics at the following concentrations; 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, 50 µg/ml kanamycin and 20 µg/ml gentamycin.

Nucleic acid manipulations

Genomic DNA isolation was performed according to Ausubel *et al* (1987). Plasmid DNA was isolated from *E. coli* using a plasmid Miniprep Kit (Qiagen), as per the manufacturerôs instructions. DNA visualisations were performed via 1% agarose gel electrophoresis in standard Tris-EDTA (TE) buffer followed by EtBr staining and photographic capture in a GeneWizard UV trans-illuminator/gel documentation system, (Syngene Bio Imaging). Oligonucleotide primers used in this study were synthesized by Sigma-Genosys, Ltd. (United Kingdom), and are listed in Table 1. Nucleic acid sequencing was performed by GATC Biotech AG, (Germany), using ABI 3730xl technology. Routine PCRs were carried out in a PTC-200 thermal cycler (MJ Research) using Taq DNA polymerase (Fermentas). High-fidelity amplification requirements were performed with proof-reading, VentR® DNA polymerase (NEB).

Random Tn*5* mutagenesis and selection

A triparental mating approach was used to introduce pUT-Km1 into *P. putida* CA-3. The mating mixture (1 ml), contained mid-log-phase, LB-grown recipient, donor and helper strains at a ratio of 7:2:1. The mixture was centrifuged for 2 min at 16,400 x g, resuspended in 50 µl fresh LB medium, spotted onto an LB agar plate, and incubated at 28°C for 24 h. *P. putida* CA-3 transconjugants expressing kanamycin resistance were subsequently isolated by plating serial dilutions of the mating mixture onto E2-citrate medium containing kanamycin (50 µg/ml). Screening for such transconjugants was performed on solid minimal media containing 23 mM citrate and 50 µg/ml kanamycin

supplemented with 0.03 g/l indole, a colourless, gratuitous inducer of the sty pathway which can be converted to a blue indigo derivative by styrene monooxygenase activity, see appendix 2 for steps in biotransformation. Blue colony screening was employed to identify sty pathway activation events in the presence of the repressive carbon source.

Identification of disrupted genes

Arbitrarily primed PCR was employed to map the Tn*5* insertion sites and identify disrupted genes. Identification of these sites was achieved via a 2 step PCR approach involving a Tn*5* specific and arbitrary primer. This was followed by a PCR reaction which utilised the resultant product as template and primers internal to those used in PCR 1. Oligonucleotide primer sequences and appropriate thermal cycling parameters were employed as described in Espinosa-Urgel *et al*. (2000). Products were visualised on 1% agarose gels, purified using a QIAGEN QIAquick Gel extraction kit. Sequencing of resultant PCR products to identify area of insertion employed the mini-Tn*5* internal primer, TNINT (Table 1). Sequencing of PCR products was performed on an ABI 3730xl Sanger sequencing system by GATC BioTech (UK). See appendix 3 for further details on mini-Tn*5* transposon mapping.

Mutant Complementation

Available nucleotide sequences of *clpX* genes from *P. putida* species were retrieved from the GenBank database and used to construct degenerate primers for the amplification of *clpX* from *P. putida* CA-3. Restriction sites were mis-primed into the oligonucleotides

clpXf-*Hin*d and clpXr-Xba, (Table 1), to facilitate directional cloning into the pBBR-1-MCS5 expression vector enabling *lac* promoter expression. PCR amplification of the full length *clpX* gene (2.4 kb) from genomic DNA involved 35 cycles of PCR amplification with *VENT* proofreading polymerase (NEB) followed by the addition of 2.5U *Taq* polymerase (Fermentas) and incubation at 72°C for 10 min to add a poly-A tail to facilitate cloning into the pCR2.1 cloning vector (Invitrogen). Amplification of the desired *clpX* target was confirmed by sequencing, prior to enzymatic restriction and ligation using standard conditions. The cloned fragments were then transformed into Top10 *E. coli* cells (Invitrogen) as per the manufacturerôs instructions. Transformations were carried out with Top 10Fô competent *E. coli* cells (Invitrogen, California) in accordance with the manufacturerôs instructions. Transformants generated were plated on LB agar plates containing 50 µg/ml kanamycin which were overlayed with 40 µl of 40 mg/ml X-gal to facilitate blue/white screening of colonies. Several of the white colonies generated were inoculated into liquid LB medium containing kanamycin and subjected to plasmid mini-preps (Fermentas) as per the manufacturerôs instructions. The resultant plasmid was cut with *Xba*I and *Hind*III restriction enzymes in a double digest at 37°C for 90 min with tango buffer (Fermentas). Sample of digested plasmid was run on a 1% agarose gel to confirm insert size prior to ligation into expression vector. The pBBR1MCS-5 expression vector was also digested in the same manner. Digestion of the expression vector was confirmed by running of a sample on 1% agarose gel as before. Conjugal transfer of this ClpX expression vector into *P. putida* CA-3 CCR25 (*clpX*::Tn*5*), was performed by tri-parental mating with the Top 10Fô *E. coli* host and the HB101 (pRK600) helper, as described above. Successful *P. putida* CA-3 CCR25clpX+

transconjugants were isolated from the mating mix by spread plating on E2 minimal salts media containing 23 mM citrate, 20 ε g/ml gentamycin and 50 µg/ml kanamycin.

Subsequent investigations into phenylacetic transport capacity of CCR25 mutant involved *paaL* overexpression in CCR25 mutant. Complementation of the full length *paaL* gene in *clpX::*Tn*5* mutant CCR25 to yield CCR25paaL+ was achieved in the same manner as detailed above for *clpX* complementation in CCR25. PCR amplification of the full length *paaL* gene for cloning utilised FpaaL-FW and FpaaL-RV primers (Table 1). Integrity and functional expression of cloned gene on plasmid was confirmed by sequencing of resultant plasmid and RT-PCR analysis as described below using gene specific primers (clpX-F/-R and L-F/-R).

RT-PCR analysis

RNA was isolated from *P. putida* CA-3 using a Fermentas GeneJET® RNA Purification Kit, as per the manufacturerôs instructions. The purified RNA was treated with TURBO DNA-freeÊ DNase kit, (Ambion), to ensure removal of DNA. Complete removal of genomic DNA from isolated RNA was confirmed by 16S PCR using 27F and 1492R primers (Table 1) prior to generation of cDNA. Reverse transcription (RT)-PCR was performed with 1µg of total RNA using random hexamer priming. Reactions also contained 1 mM dNTPs, 1U BioScript® (Bioline) reverse transcriptase with 5X reaction buffer (Bioline) in a 20 µl reaction volume. Reactions were incubated at 25°C for 10 min, followed by 30 min at 42°C and 10 min at 70°C. 2 µl of the respective RT-PCR reactions were employed as template in subsequent PCR reactions. Amplification of the 16S rRNA gene with primers 27F and 1492R (Table 1) acted as a positive control for RT-

PCR analyses and was also used for optimisation of RT-PCR product equivalence to ensure template cDNA concentrations were comparable between samples. Equivalence was achieved using the SynGene Genetools^{TM} v3.07 band comparison software to compare band intensities between samples. cDNA concentrations were optimised to within 10% of one another prior to analysis of individual gene expression. The following pathway operon specific targets were selected for transcriptional profiling; *paaF* encoding PaCoA ligase, *paaG* encoding a member of the ring hydroxylation complex, and, the *paaL* encoding phenylacetate permease. cDNA PCR cycle numbers were optimised by comparison of band intensities using SynGene Genetools^{TM} v3.07 band comparison software to ensure plateau phase of cDNA PCR reactions of target genes had not been reached. Oligonucleotide sequences for the respective gene targets are provided in Table 1.

SDS-PAGE Protein Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on soluble protein fractions from wild-type *P. putida* CA-3, mutant CCR25 (*clpX::*Tn*5*), CCR25clpX+ (*clpX* complemented mutant) cultures grown on PAA + glucose. Samples were incubated at 28°C with shaking at 180 rpm under conditions as described previously. After cultures had entered log-phase of growth they were sampled and 20 ml of actively growing culture was removed and centrifuged for 10 min at 5000 rpm (~5120 x g). Total protein was extracted from the resulting pellet using the B-PER Plus Halt Protease Inhibitor Cocktail Kit (Thermo Scientific). Pellets were resuspended in 4 ml of buffer containing 2 µl 20 mg/ml lysozyme (Sigma) and 2 µl DNase I

(Fermentas) and incubated for 15 min at room temperature. Separation of soluble and insoluble protein fraction was achieved by centrifugation of sample at 15000 g for 5 min. Resultant soluble protein fractions were resuspended in sample buffer and heated for 10 min at 100°C. The protein concentration of the crude extract was determined by the Bradford assay against a bovine serine albumin standard curve (Sigma-Aldrich). 5 εg of each protein sample was used for SDS-PAGE using an Atto AE-6450 system (Atto Corporation, Japan) according to the manufacturerôs instructions. Gels were run for 2 hrs. at 30 mA through a 5% acrylamide stacking gel and a 10% separating gel. Pre-stained protein marker weight standards (New England Biolabs) were used for molecular mass estimation. Gels were stained with PageBlue^{TM} Stain (Fermentas) overnight as per manufacturerôs instructions and destained with dH_2O for 10 min.

Results

Random Tn*5* mutagenesis, screening and insertion site mapping.

Random mini-Tn*5* mutagenesis of the *P. putida* CA-3 genome produced approximately 13,500 transconjugants resistant to kanamycin as described previously. As described above the solid media screening of generated mutants utilised concentrations of citrate sufficient to repress transcription of the *styAB* encoding styrene monooxygenase. This monooxygenase has previously been described as being capable of converting the colourless indole supplied in the medium to blue indigo. This conversion is performed by *styAB* encoded styrene monooxygenase and thus is indicative of *sty* operon expression. Mutants with reduced susceptibility to the repressive effects of the citrate on the *sty* pathway were selected based on their turning blue before the surrounding colonies. Three mutants, designated CCR25, CCR29 and CCR1, were identified using this screen as they exhibited the blue phenotype significantly before surrounding mutants. We therefore believed these mutants were likely to have a reduced susceptibility to the repressive effects of citrate (Figure 2).

The insertion sites of the mini-Tn*5* transposon in the three mutants of interest were mapped using two consecutive rounds of arbitrary PCR as described in the materials in methods, the resulting amplicons were sequenced and analysed using the GenBank, BLASTn algorithm. The downstream region of the mini-Tn*5* insertion in CCR25, CCR29 and CCR1 was found to share significant sequence similarity with *clpX* (94% sequence identity), *luxR* (92% sequence identity) and *tctB* (99% sequence identity) genes from other *P. putida* strains. The growth of CCR1, a putative *tctB* deficient mutant, on

various carbon sources was investigated and the mutant was found to be incapable of growth on citrate. This suggests its selection based on the blue phenotype was due to the cell lacking the ability to utilise citrate rather than a regulatory element being interrupted. Its inability to utilise the supplied repressive carbon source, citrate; however confirmed the efficacy of the screen at identifying mutants with reduced CCR sensitivity.

Growth profile analysis

Growth curves were conducted with wild-type *P. putida* CA-3, *clpX::*Tn*5* mutant CCR25, complemented *clpX::*Tn*5* mutant CCR25clpX+ and the *clpX* overexpression strain CA-3clpX+ Growth of these mutants over an 11 hour period which was sufficient to observe, lag, log and stationary phases under growth on the majority of carbon sources was assayed on E2-minimal medium supplemented with the Glucose (Figure S1), Citrate (Figure S2), Styrene (Figure S3), Phenylacetic acid (Figure S4), Citrate + Styrene (Figure S5). A tabulated breakdown of the major findings of the growth profile investigations can be seen in table 2.

Growth profiles demonstrated no change in the *clpX* disrupted mutantôs ability to utilise glucose when compared to wild-type CA-3 with average growth rates over the 11 hr period being 0.240 hr^{-1} and 0.254 hr^{-1} respectively (Table 2). Slower growth was seen in CCR25 when grown on citrate (0.139 hr^{-1}) compared with wild-type CA-3 (0.184hr^{-1}) and complementation of CCR25 with *clpX*, yielding CCR25clpX+, partially restored growth on citrate (0.153 hr^{-1}). A similar effect was seen in PAA grown cells with average CCR25 growth (0.152 hr^{-1}) lower than wild-type levels (0.193 hr^{-1}) (Table 2). Overexpression of the *clpX* gene in *P. putida* CA-3 yielding CA-3clpX+, improved cell

growth above wild-type levels when grown on citrate (Figure 3) (0.230 hr^{-1}) and PAA (Figure 5) (0.254 hr^{-1}). Growth on styrene was slightly reduced in CCR25 (0.168 hr^{-1}) compared to wild-type CA-3 (0.183 hr^{-1}) and restoration of growth did not occur in the complement CCR25clpX+ (0.168 hr^{-1}). A graphical representation of average growth rates of cultures grown on citrate, glucose, PAA and styrene can be seen in figure 3.

When media was supplemented with both citrate and styrene (Figure 6) growth was significantly more efficient in the wild-type (0.194 hr^{-1}) compared to CCR25 (0.070 hr^{-1}). Again complementation of *clpX* (CCR25clpX+) partially restored growth (0.140 hr^{-1}) while overexpression of *clpX* was found to have no effect on cell growth (0.195 hr^{-1}). Growth on PAA + glucose was also investigated in the wild-type CA-3, CCR25 and CCR25clpX+ (Figure S7), with results demonstrating growth on this carbon source was similar between the three cultures with average growth rates of 0.239 hr^{-1}, 0.251 hr^{-1} and 0.227 hr^{-1} being observed respectively (Table 2).

RT-PCR analysis of representative genes of *paa* catabolon

RT-PCR analysis was employed subsequent to the growth profiles to investigate the transcription of the PAA catabolic genes in the mutants compared to the wild-type when grown on E2 medium supplemented with PAA and either citrate or glucose. Three genes were selected as representatives of PAA catabolon expression which is necessary for both PAA and styrene metabolism. The genes selected for RT-PCR analysis, *paaL, paaG* and *paaF*, have previously been proposed by Alonso *et al.* (2003) to each be part of one of three discrete transcriptional units of the PACoA catabolon controlled by individual promoters. Expression of these genes has previously been proposed to be found to be

differentially regulated in different species thus making each of these a target for investigation in a mutant exhibiting altered growth on PAA.

RT-PCR investigations assessed expression of the phenylacetic acid degradation genes grown under a variety of conditions. Expression was first examined in cultures grown on PAA + glucose (Figure 4). Glucose, a non-repressive carbon source of the catabolon in *P. putida* CA-3, was supplied together with PAA to improve biomass levels and ensure sufficient RNA was present in the sample for extraction. As described above, equivalence of cDNA concentration was ensured prior to PCR reaction by band intensity analysis using SynGene GeneTools software to compare intensity of 16S PCR products prior to assessing *paaL* gene expression. RT-PCR profile analyses revealed equivalent expression of *paaL, paaG* and *paaF* between the mutant, complement or wild-type under these conditions (Figure 4).

The longer duration of the lag phase in the mutant CCR25 when grown on PAA and PAA + citrate (Table 2) led the author to investigate expression of the PAA transporter gene *paaL* in cultures during their early-exponential phase of growth. cDNA generated from these cells by RT-PCR as before, focused on the *paaL* transport gene, however, no apparent difference in expression of *paaL* was observed between the cultures at the time points sampled (Figure 5).

Transcription of the *paa* catabolon was also assessed in samples obtained from mid- and early-exponential phase cultures grown on phenylacetic acid + citrate (Figure 6). As can be seen in figure 6 (a) the quantity of cDNA supplied in the reactions was similar across all samples tested, again this was confirmed using SynGene band analysis software. Results of RT-PCR analysis of these genes suggests that *clpX* expression has little effect

on the transcription of *paaL* (Fig. 6: B), *paaG* (Fig 6: C) and *rpoN* (Fig 6: D) under the conditions tested. Results of the *paaF* (Fig 6: E). RT-PCR however did indicate increased band intensity in lanes 3 and 4 compared with both wild-type CA-3 and CCR25, lanes 1 and 2 respectively. SynGene Band intensity analysis of the resultant gel images indicated a difference of approximately 24% between expression in lanes 3 and 4 compared to 1 and 2. Analysis did not identify a comparable difference in expression between CA-3 wild-type and CCR25 however with only a 5% difference in band intensity evident (Table 3). This finding indicates an increased level of expression of the PACoA ligase in mutants overexpressing the *clpX* gene with both the *clpX* complemented mutant CCRclpX+ and *clpX* overexpressing CA-3clpX+.

SDS-PAGE Crude Protein Analysis of PAA grown cultures

SDS-PAGE analysis was employed to investigate if any alteration in the protein profiles in cultures grown on different carbon sources was evident. Samples were obtained from mid-log phase *P. putida* CA-3 WT, CCR25 and CCR25clpX+cultures grown on phenylacetic acid + glucose (Figure 7). Particular interest focused on the profiles of the gene products corresponding to *paaL, paaG* and *paaF* which were previously targeted in the RT-PCR investigation; especially given the apparent up-regulation in *paaF* expression in the *clpX* overexpression mutants. The proteins and their putative sizes, as predicted by the DNASTAR bioinformatics suite using EditSeq (standard genetic code translation) were identified as PaaL (55 kDa), PaaF (49 kDa) and PaaG (38kDa).

The profile obtained by SDS-PAGE analysis comparing phenylacetic acid + glucose grown cultures (Figure 7) showed distinct differences in protein profiles to cultures

grown on E2 + glucose (Figure 8) as one would expect however no conclusive differences in the band patterns were evident between the samples grown on phenylacetic acid + glucose which could explain the observed differences in growth.

Overexpression of *paaL* permease in the CCR25 mutant

Complementation of the *clpX* disrupted mutant CCR25 with the *paaL* permease gene was undertaken using the pBBR1MCS-5 vector harbouring the *paaL* permease gene as described in OôLeary *et al.* (2011) to assess whether transport of PAA was a limiting factor in the ability of the mutant to grow on PAA. Growth profiles revealed that the over-expression of *paaL* in CCR25 resulted in a reduction in the cellsô ability to utilise PAA as a sole carbon source for growth. The average growth rate for CCR25paaL+ was calculated to be 0.056hr^{-1} compared to 0.152 hr^{-1} and 0.193 hr^{-1} for CCR25 and CA-3 WT respectively (Figure 9).

Discussion

To date research into styrene and aromatic degradation by microbes has focused on genetic characterisation of the catabolic operon(s) required for degradation and pathway specific regulatory elements. Little is known however about the effect global regulators play in regulating these processes. The aims of this study were to (a) apply an indole to indigo based screen to identify mutants with a potentially altered catabolite repression genotype, associated styrene/phenylacetic metabolism in *Pseudomonas putida* CA-3 and, (b) characterise such mutants in an attempt to determine their site/mode of interaction with the *sty*/*paa* pathways. Mutants generated in the random Tn*5* mutagenesis reaction were plated on media containing citrate and indole, which was previously published as a gratuitous inducer of the *sty* pathway and, when active, converts the colourless indole to indigo (O'Connor *et al.,* 1997). Citrate is known to repress expression of the *sty* operon (O'Leary *et al*. 2001) therefore colonies exhibiting a strong blue phenotype compared with surrounding colonies were proposed to be less influenced by the repressive effects of citrate. The Tn*5* mutant CCR1, later identified as a *tctB* disrupted mutant, using this screen was found to be incapable of metabolising citrate. The isolation of this mutant confirmed the efficacy of the screen at distinguishing mutants with reduced sensitivity to the repressive effects of citrate on the *sty* pathway. The isolation of mutant CCR25; putative *clpX* protease chaperone disrupted mutant, had not to the author's knowledge, been previously associated with carbon catabolite repression. *clpX* had however has been previously reported to be upregulated in *P. putida* CA-3 when grown on styrene suggesting that it may have a role to play in aromatic hydrocarbon metabolism (Nikodinovic-Runic *et al.,* 2009). Furthermore a study by Goff *et al.* (2009) screening

for mutants with reduced capacity to accumulate PHA from styrene identified a ClpA negative mutant. This further indicates the potential involvement of this family of protease chaperones in hydrocarbon metabolism. The identification of the *clpX* disrupted mutant in this solid media screen on the basis of StyAB activity, as well as the previous identification of both ClpX and ClpA involvement in styrene metabolism and PHA accumulation, provided the basis for our continued focus on, and characterisation of the *clpX* mutant CCR25.

Growth profile analysis of the CCR25 mutant identified a 24.5 % reduction in the average growth rate compared with wild-type CA-3 (Table 2). This suggests that selection of the CCR25 mutant may have been due, similar to the isolation of *tctB* disrupted CCR1, to a reduced capacity of the culture to grow on citrate rather than a reduced sensitivity to carbon catabolite repression. However investigations of mutant growth on pathway specific carbon sources demonstrated a 44% reduction in the average growth of CCR25 on PAA which was not seen on styrene where a difference of only 15% was observed (Table 2). This is interesting as metabolism of PAA has previously been shown to proceed via phenylacetic acid in a stepwise fashion (O'Connor *et al.*, 1995). The growth of CCR25 on glucose (Figure 2) was also unaffected suggesting that *clpX* is not required for TCA cycle functionality, into which the *paa* pathway ultimately feeds (Ferrandez *et al.,* 1998). Complementation of the *clpX* gene in CCR25 on PAA and citrate was found to restore growth to wild-type levels (Table 2). This indicates that the observed differences in growth were unlikely to be due to any potential polar effects of the insertion of the mini-Tn*5* transposable element in the genome. Therefore the reduced growth rates observed in PAA and citrate grown mutant cultures are likely a direct result

of *clpX* disruption and further highlights a potential role for this protease in the metabolism of PAA and citrate.

Investigations by our group previously identified similar growth profiles in a σ^{54} disrupted mutant of *P. putida* CA-3 which was observed to be capable of growth on styrene but incapable of growth on PAA. Subsequent complementation of the *paaL* PAA permease in the σ^{54} mutant was found to fully restore growth on PAA thus identifying *paaL* as being dependent on σ^{54} for transcription (O'Leary *et al.*, 2011). Our investigations here therefore sought to identify if ClpXP was similarly required for expression of the *paaL* permease in *P. putida* CA-3. To determine whether *paaL* expression was indeed affected, the plasmid harbouring the full length *paaL* gene from a previous study by our group by O'Leary *et al.* (2011) expressed in the CCR25 mutant, generating CCR25paaL+. Interestingly growth analysis of this mutant on PAA demonstrated that the ability of CCR25 to grow on PAA was further inhibited by the introduction of the *paaL* encoding plasmid (Figure 9). This finding suggested that transport of PAA is not a growth limiting factor in the CCR25 mutant. Furthermore it suggested that metabolism of PAA is most likely to be affected in CCR25 following transport of the PAA into the cell. Interestingly O'Leary and co-workers noted in their investigations that *paaL* over-expression in *P. putida* CA-3 negatively affected growth, suggesting that increased concentrations of PAA in the cell may be damaging to cell growth (O'Leary *et al.,* 2011). Thus the growth profile investigations suggest a role for *clpX* in the metabolism of the pathway specific carbon source PAA, independent of an inability or reduced capacity of the cell to transport PAA into the cell.

RT-PCR investigations were then carried out to identify if disruption of the *clpX* gene in CA-3 led to altered expression from the *paa* catabolon. These investigations focused on three genes, chosen as representatives of individual operons within the catabolon, necessary for PAA metabolism. The expression of *paaL* permease expression was assessed at both early and mid-exponential phase to identify any delay between the CCR25 and the mutant in transporting the carbon source into the cell for utilisation; however none was evident (Figures 4 & 5). Expression of *paaG*, *paaF*, and the alternative sigma factor, $\mathring{u}^{54}$, was also investigated at mid-exponential phase (Figure 6) however no discernible difference was evident to suggest that altered transcriptional activity from the catabolon was linked to the reduced growth rate observed in the CCR25 mutant. Interestingly increased expression was observed in CCR25clpX+ and CA-3clpX+ mutants compared with CCR25 and *P. putida* CA-3 as described above. However expression levels were comparable between the mutant and wild-type (approximately 5% difference) suggesting *paaF* gene expression was unlikely to influence growth on PAA to the extent observed in growth analysis.

Finally, protein expression profiles were investigated using SDS-PAGE analysis of crude protein extracts obtained from CA-3 wild-type, CCR25 and CCR25clpX+ cultures grown under *paa* catabolon activating conditions (PAA + glucose). Results showed no difference between the SDS-PAGE band profiles of the three samples tested (Figure 7). SDS-PAGE analysis of these three cultures was also carried out when grown on glucose (Figure 8; lanes 2-4) for comparison to the PAA + glucose grown cultures. The resultant gels clearly showed an altered band profile to that of PAA + glucose grown cells (Figure 8; lanes 5-7) and suggest that proteins putatively involved in PAA metabolism are readily

identifiable by SDS-PAGE. However no discernible differences in band profile were attributable to *clpX* disruption by this method.

The results of the growth profile analysis demonstrating decreased growth of mutant CCR25 on PAA, coupled with expression of the *paa* catabolic genes tested and the similar protein profiles suggests the effect of *clpX* disruption on cell growth is not mediated at a pathway specific level. A previous report by Flynn *et al.* (2003) identified the *paaA* encoded PA-CoA oxygenease component as being a substrate of the ClpXP protease complex. Our finding however that the CA-3clpX+ over-expression strain grew at a higher rate than wild-type CA-3 suggests that this type of interaction is unlikely to affect PAA metabolism in *P. putida* CA-3. The previously reported role of the ClpXP protease complex in regulating stress responses within the cell highlights the potential that deletion of the *clpX* chaperone may inhibit the cellsô tolerance of intracellular PAA. This may explain the similar growth rates observed in styrene grown cultures, metabolism of which proceeds via PAA. It is possible that intracellular concentrations, limited by the rate of styrene degradation and PAA generation by the *sty* pathway are insufficient to induce a stress response requiring ClpXP intervention.

What is clear from this study is that the ClpX protease plays a role in regulating both PAA and citrate metabolism in *P. putida* CA-3 which is not seen at the level of transcription of the pathway specific genes nor readily identifiable in crude protein extracts using SDS-PAGE gel based analysis. The increased rate of PAA utilisation by the *clpX* overexpression strain also presents the potential that further research into this protease may enable improved aromatic hydrocarbon consumption by *P. putida* CA-3. A more in-depth study of the changes in the cells proteome in a *clpX* mutant, such as by 2D-

PAGE, would yield a more accurate picture of both up- and down-regulated proteins in *clpX* disrupted mutant; especially if these results could be compared to wild-type studies such as reported by Nikodinovic-Runic *et al.* (2009). These studies indicate *clpX* involvement in cell metabolism under growth on aromatic hydrocarbons while our finding further indicates a role for the protease chaperone aromatic hydrocarbon degradation. Our mutagenesis and screening approach set out to identify global regulatory influences which mediate CCR of styrene metabolism in *P. putida* CA-3 and the results reported here demonstrate a clear role for ClpX in this respect. While efforts were unable to identify the exact level at which the control is mediated within the cell it is clear however that ClpX has an effect. Previous studies have linked this family of protease chaperone to aromatic metabolism however none have proposed a mode of action to explain this control at the cellular level. Further work is therefore clearly required to fully elucidate the regulatory control mediated by ClpX.

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Name	Primer Sequences (5'-3')	Annealing temp (°C)	Source
rpoN-F	GCCTGCGCGTCAACCCACAA	65	This Study
rpoN-R	TGATTTTCCGCCGCAACCAGT	65	This Study
C1-F	ATGAGTAACAAGAACAACGAT	55	O'Leary et al., 2005
C1-R	TCAGCGCTCGTGAACGTAGGT	55	O'Leary et al., 2005
G-F	ATGAGGCCAGAAATCGCTGT	58	O'Leary et al., 2005
G-R	TCAGATGGCCAATGCATGCT	58	O'Leary et al., 2005
L-F	CGGCATGCTCGCGACCACCTG	60	O'Leary et al., 2010
L-R	AAAGCGATGTTCTGCGACTC	60	O'Leary et al., 2010
clpX -F	ATTGCTTTATTGCTCCTTCT	55	This Study
clpX-R	GCGGTTCTGGATGACCTT	55	This Study
FclpX-F	[1]*AAGCTT*ATGACTGACACCCGTAACGGCGA	65	This Study
FclpX-R	[2]*TCTAGA*TCAGGCGTCKGGYGCGGCYTT	65	This Study
27F	AGAGTTTGATCCTGGCTCAG	55	This Study
1492R	GGTTACCTTGTTACGACTT	55	This Study
TNINT	GACCTGCAGGCATGCAAGCTCGGC	59	Espinosa-Urgel et al., 2000

Table 1: Primers used in this study. Restriction sites italicised. 1: *HindIII* restriction site 2: *XcmI* restriction site

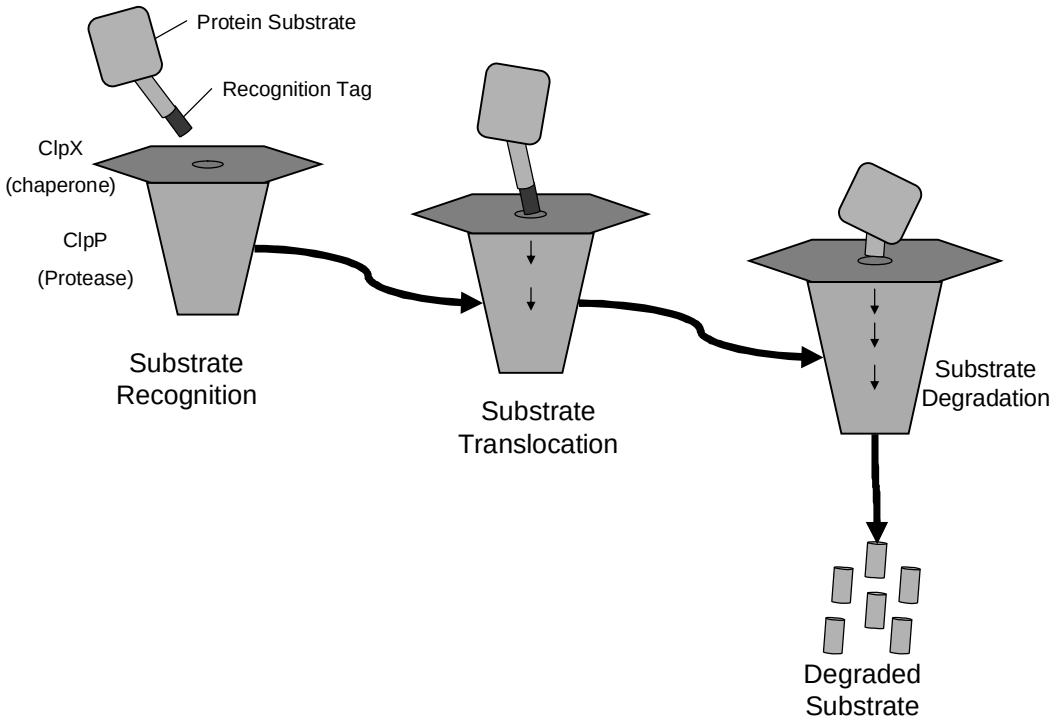


Figure 1: ClpXP Protease Chaperone Degradation of tagged substrate. I: Tag attached to protein destined for degradation by ClpXP complex is recognised by ClpX chaperone; II: ClpX chaperone translocates substrate protein into ClpP protease core for degradation; III: Protein substrate degraded by ClpP protease

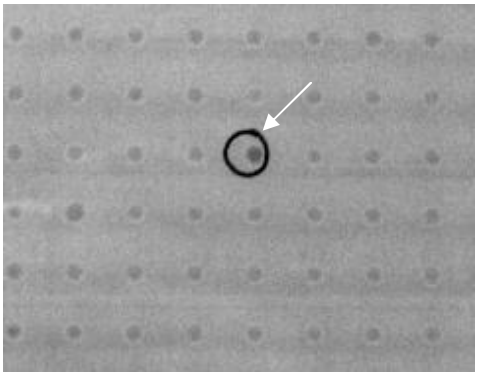


Figure 2: Solid media screening of mini-Tn*5* transconjugants on E2 minimal media supplemented with Citrate [40 mM]. 0.03g/L indole and Kanamycin [50 µg/ml]

	Glucose				***Citrate***			
	Avg. Growth Rate (hr^{-1})	*STDEV*	*T11 OD_{600nm}*	*Duration of lag phase (hrs)*	*Avg. Growth Rate (hr^{-1})*	*STDEV*	*T11 OD_{600nm}*	*Duration of lag phase (hrs)*
CA-3 WT	0.240	0.001	1.179	0	0.184	0.013	0.714	3
CCR25	0.254	0.006	1.149	1	0.139	0.011	0.378	5
CCR25clpX+	0.248	0.003	1.203	0	0.153	0.006	0.800	2
CA-3clpX+	0.247	0.000	1.208	0	0.230	0.001	1.077	3
	Phenylacetic Acid				***Styrene***			
	Avg. Growth Rate (hr^{-1})	*STDEV*	*T11 OD_{600nm}*	*Duration of lag phase (hrs)*	*Avg. Growth Rate (hr^{-1})*	*STDEV*	*T11 OD_{600nm}*	*Duration of lag phase (hrs)*
CA-3 WT	0.193	0.005	0.750	3	0.183	0.001	0.659	3
CCR25	0.152	0.008	0.417	6	0.168	0.005	0.560	5
CCR25clpX+	0.134	0.032	0.646	5	0.168	0.002	0.632	4
CA-3clpX+	0.254	0.003	0.777	4	0.173	0.010	0.612	4
	PAA + Citrate				***Citrate + Styrene***			
	Avg. Growth Rate (hr^{-1})	*STDEV*	*T11 OD_{600nm}*	*Duration of lag phase (hrs)*	*Avg. Growth Rate (hr^{-1})*	*STDEV*	*T11 OD_{600nm}*	*Duration of lag phase (hrs)*
CA-3 WT	0.258	0.016	0.263	7	0.194	0.017	0.553	5
CCR25	0.134	0.005	0.333	7	0.070	0.023	0.143	8
CCR25clpX+	0.059	0.010	0.140	8	0.140	0.013	0.451	5
CA-3clpX+	0.121	0.034	0.317	7	0.195	0.013	0.688	4
	PAA + Glucose							
	Avg. Growth Rate (hr^{-1})	*STDEV*	*T11 OD_{600nm}*	*Duration of lag phase (hrs)*				
CA-3 WT	0.239	0.015	1.163	0				
CCR25	0.251	0.015	1.07	0				
CCR25clpX+	0.227	0.002	0.877	1				
CA-3clpX+	N/A	N/A	N/A	N/A				

Table 2: Average growth rates, final OD_{600nm} reading and lag phase duration obtained from growth analysis of *P. putida* CA-3 WT; CCR25; CCR25clpX+ and CA-3clpX+ grown on Glucose; Citrate; Styrene; Phenylacetic Acid; Citrate + Styrene; Phenylacetic Acid + Glucose and Phenylacetic acid +Citrate. STDEV: Standard Deviation calculated from average growth rates of triplicate cultures.

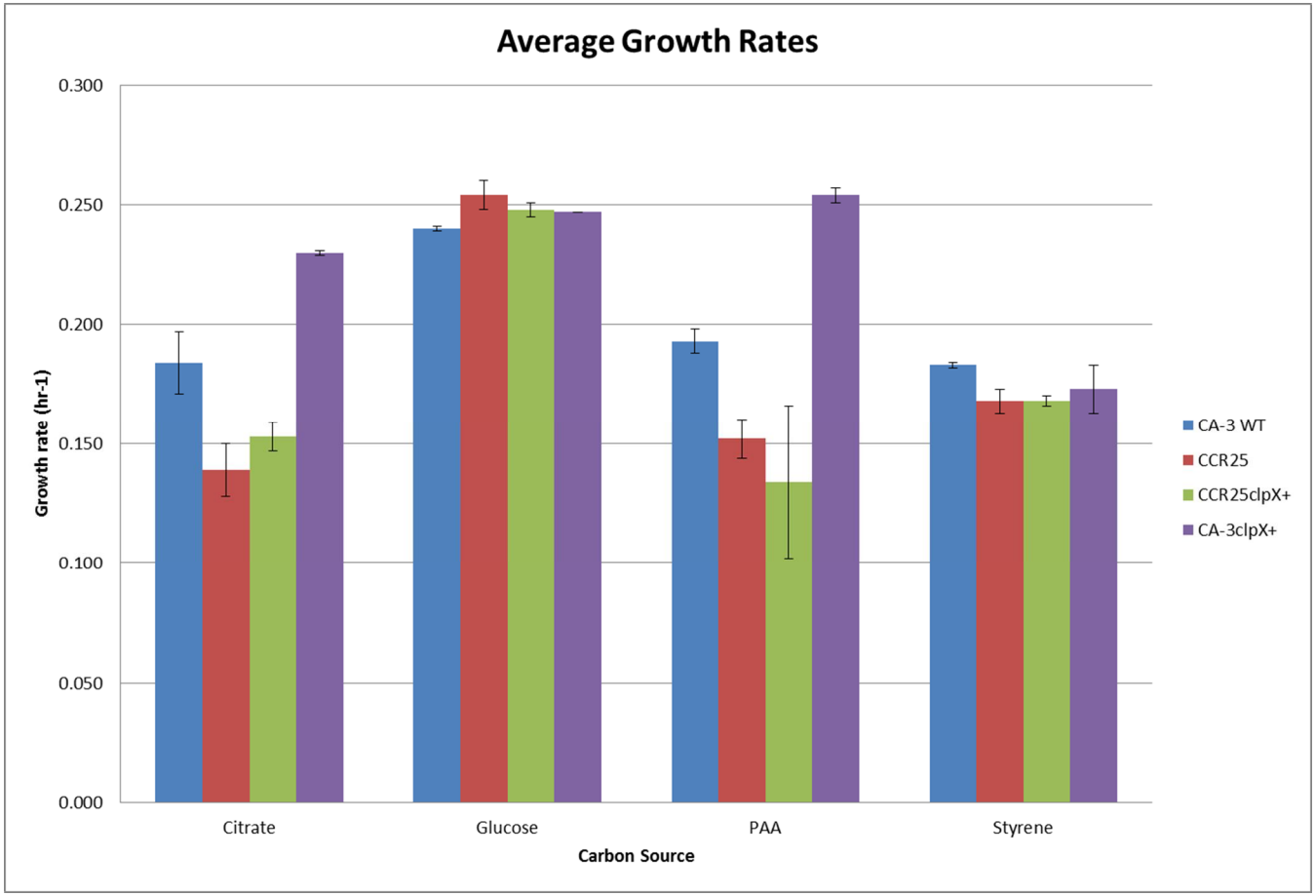


Figure 3: Average growth rates calculated from *P. putida* CA-3, CCR25, CCR25clpX+ and CA-3clpX+ growth curves. Cultures grown on E2 minimal media + carbon source described (supplemented with 50 µg/ml kanamycin and 20 µg/ml gentamycin where necessary). Error bars: Standard deviation calculated from average growth rates of triplicate cultures.

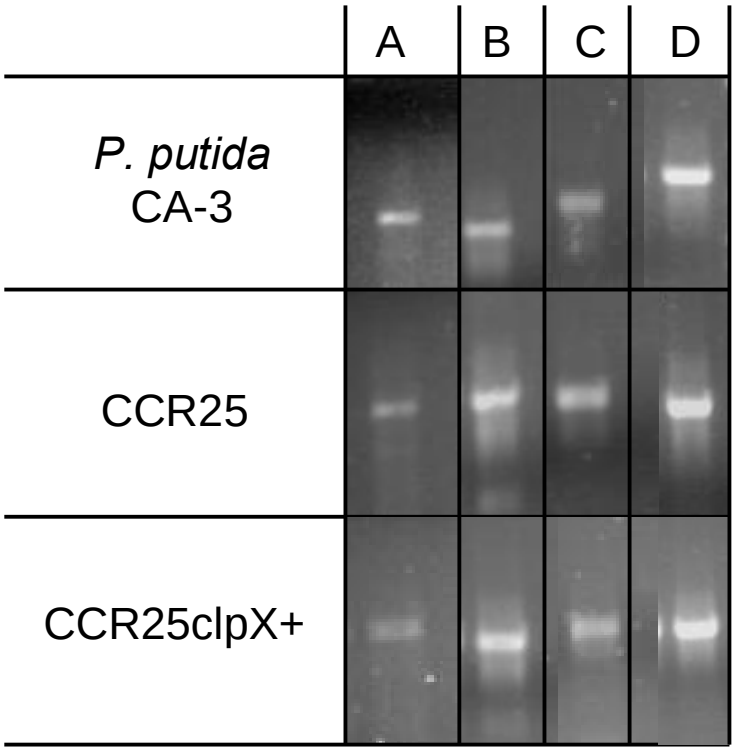
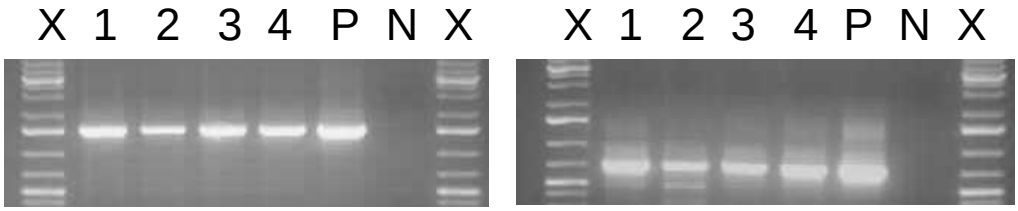


Figure 4: Mid exponential phase RT-PCR results from PAA + glucose grown cultures 16S (A), *paaL* (B), *paaF* (C), *paaG* (D).



(A) **(B)**

Figure 5: Early exponential phase RT-PCR results from PAA + citrate grown cultures 16S (A), *paaL* (B). Lanes; X= GeneRuler 1Kb+ DNA ladder, 2 = *P. putida* CA-3 WT, 3 = CCR25, 4 = CCR25clpX+, 5 = CA-3clpX+, P = Positive control ï *P. putida* CA-3 genomic DNA, N = Negative control ï DNA/RNA free dH_2O (Sigma-Aldrich)

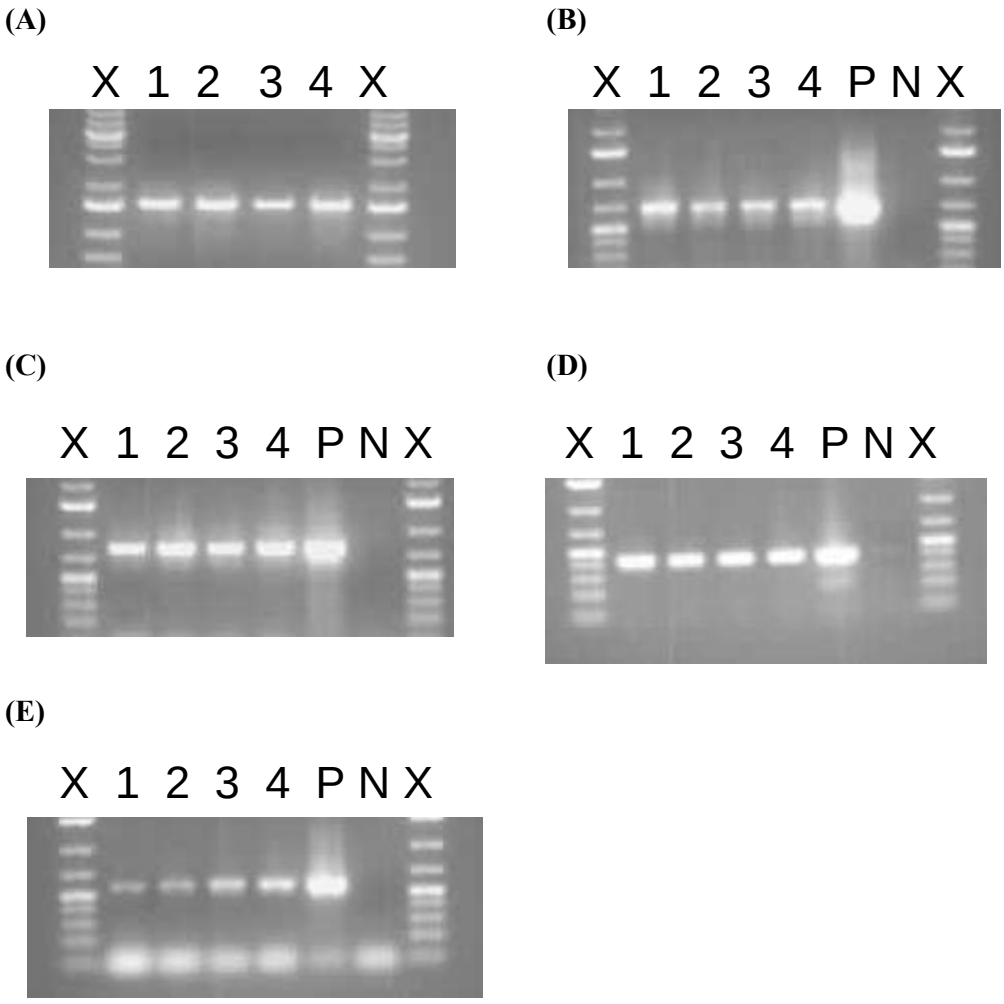


Figure 6: Mid exponential phase RT-PCR results from PAA + citrate grown cultures 16S (A), *paaL* (B), *paaG* (C), *rpoN* (D) and *paaF* (E) Lanes; X= GeneRuler 1Kb+ DNA ladder, 2 = *P. putida* CA-3 WT, 3 = CCR25, 4 = CCR25clpX+, 5 = CA-3clpX+, P = Positive control ï *P. putida* CA-3 genomic DNA, N = Negative control ï DNA/RNA free dH_2O (Sigma-Aldrich)

SynGene Band Analysis Results of Mid-log phase RT-PCR						
Gene	**Strain**	**Raw band intensity**	**Predicted DNA quantity (ng)**	**STDEV**	**MEAN**	**STDEV as % of MEAN**
16S	**CA-3 WT**	113525	254.177	8.4	266.6	3.1
	CCR25	121323	271.638			
	CCR25clpX+	120142	268.993			
	CA-3clpX+	121303	271.593			
paaF	**CA-3 WT**	28827.4	160.828	51.9	208.4	24.9
	CCR25	31259.1	174.395			
	CCR25clpX+	40070.1	223.551			
	CA-3clpX+	49291.2	274.996			

Table 3: SynGene Band Analysis results of 16S control and *paaF* RT-PCR analysis. Note: STDEV: Standard Deviation. Raw band intensity calculated by SynGene software, DNA quantity prediction calculated by SynGene software based on ng DNA per band in ladder.

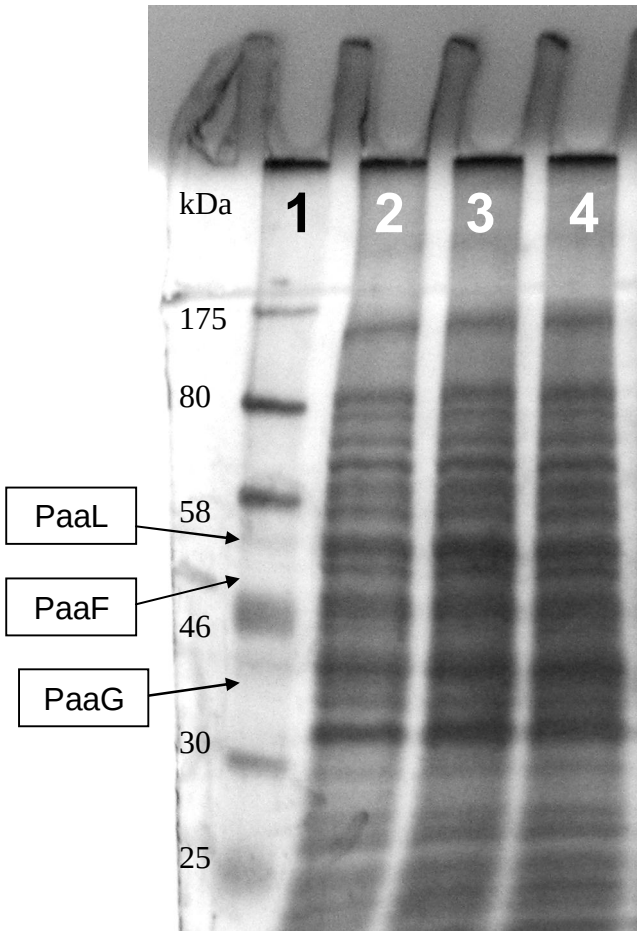


Figure 7: Mid exponential phase RT-PCR results from PAA + glucose grown cultures. Lanes; 1 = Prestained protein marker, Broad Range (NEB), 2 = *P. putida* CA-3 WT, 3 = CCR25, 4 = CCR25clpX+. Arrows indicate protein sizes of interest corresponding to predicted weights of PaaL, PaaF and PaaG proteins respectively from top to bottom.

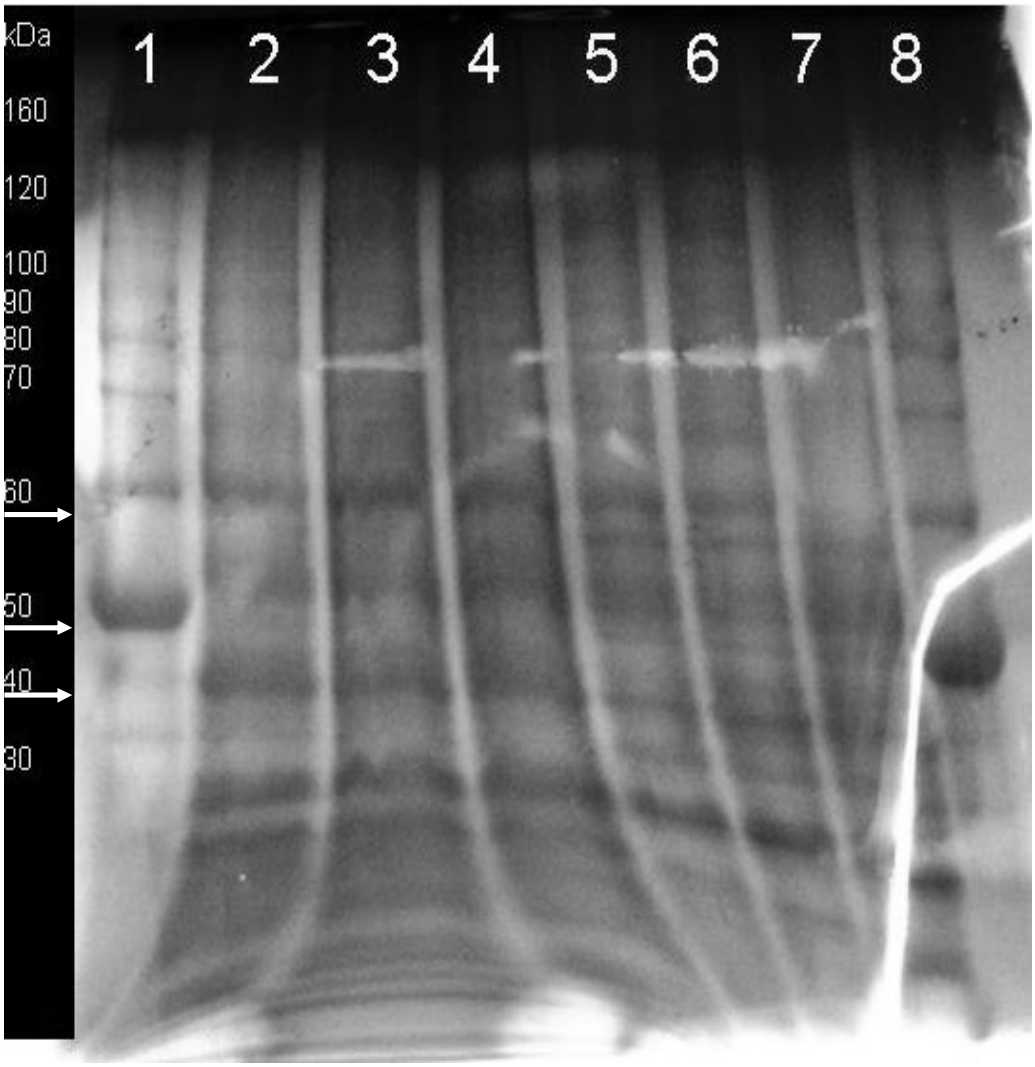


Figure 8: Mid exponential phase RT-PCR results from PAA + glucose and glucose grown cultures. Lanes; 1 = Prestained protein marker (Invitrogen), 2 = *P. putida* CA-3 WT ï Glucose; 3 = CCR25 ï Glucose; 4 = CCR25clpX+ ï Glucose; 5: *P. putida* CA-3 WT ï PAA + Glucose; 6 = CCR25 ï PAA+ Glucose; 7 = CCR25clpX+ ï PAA+ Glucose; 8: Prestained protein marker, Broad Range (NEB). Arrows indicate protein sizes of interest corresponding to predicted weights of PaaL, PaaF and PaaG proteins respectively from top to bottom.

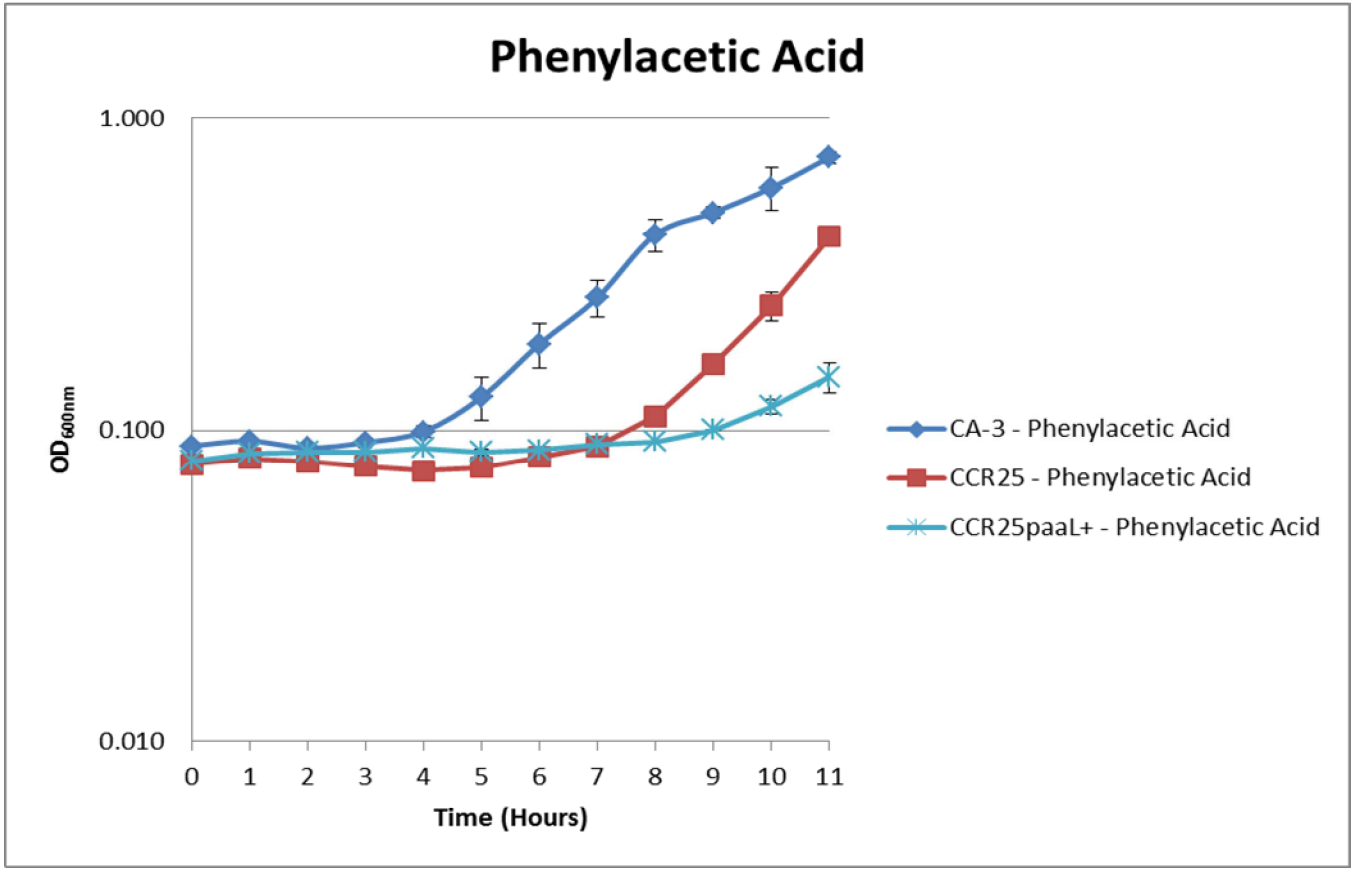


Figure 9: *P. putida* CA-3, CCR25, CCR25paaL+ growth curves. Cultures grown on E2 minimal media +Phenylacetic Acid (supplemented with 50 µg/ml kanamycin and 20 µg/ml gentamycin where required). Error bars calculated standard deviation of triplicate values.

Appendix 1

Supplementary Figures

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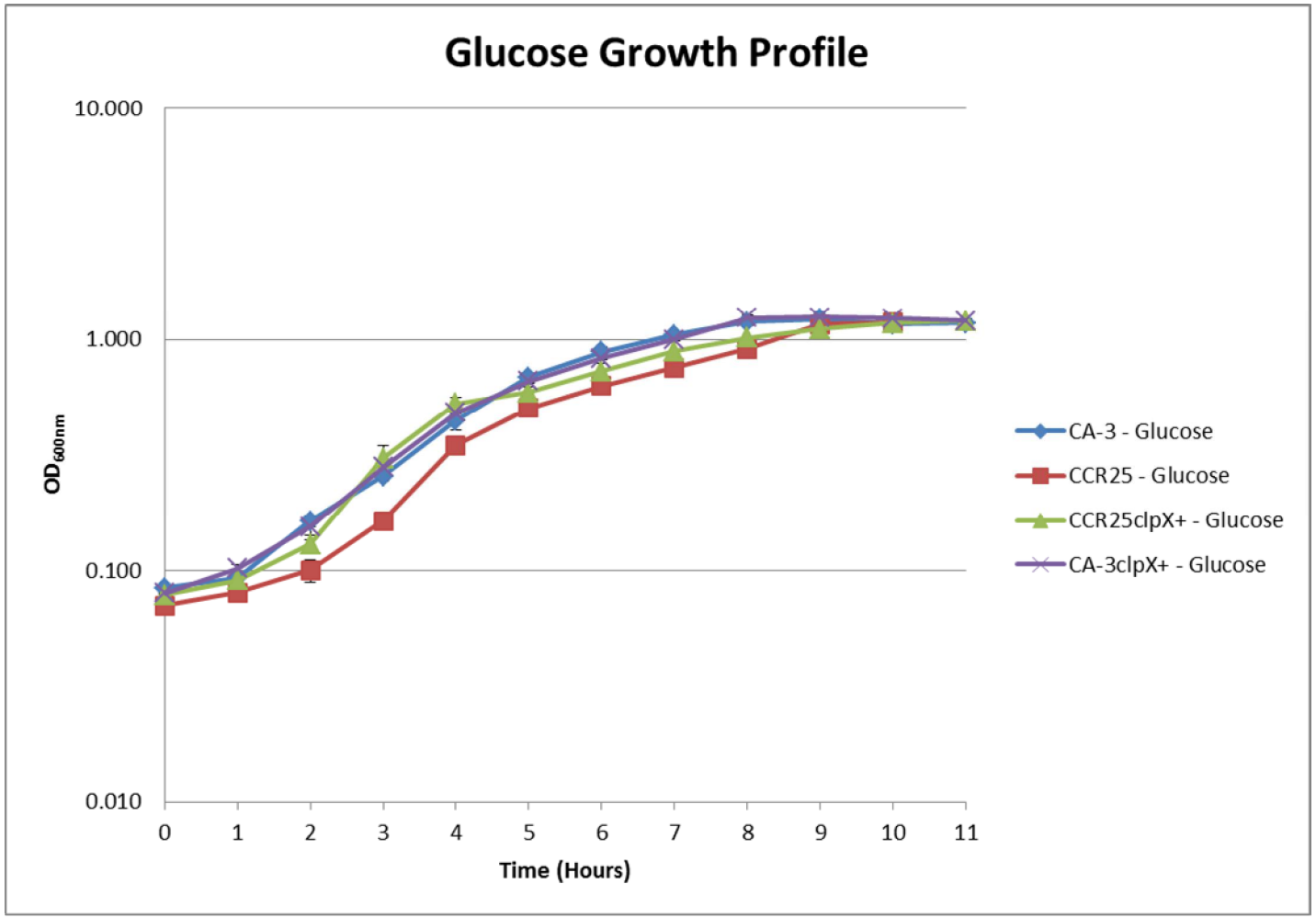


Figure S1: *P. putida* CA-3, CCR25, CCR25clpX+ and CA-3clpX+ growth curves. Cultures grown on E2 minimal media + Glucose (supplemented with 50 µg/ml kanamycin and 20 µg/ml gentamycin where required). Error bars standard deviation of triplicate data.

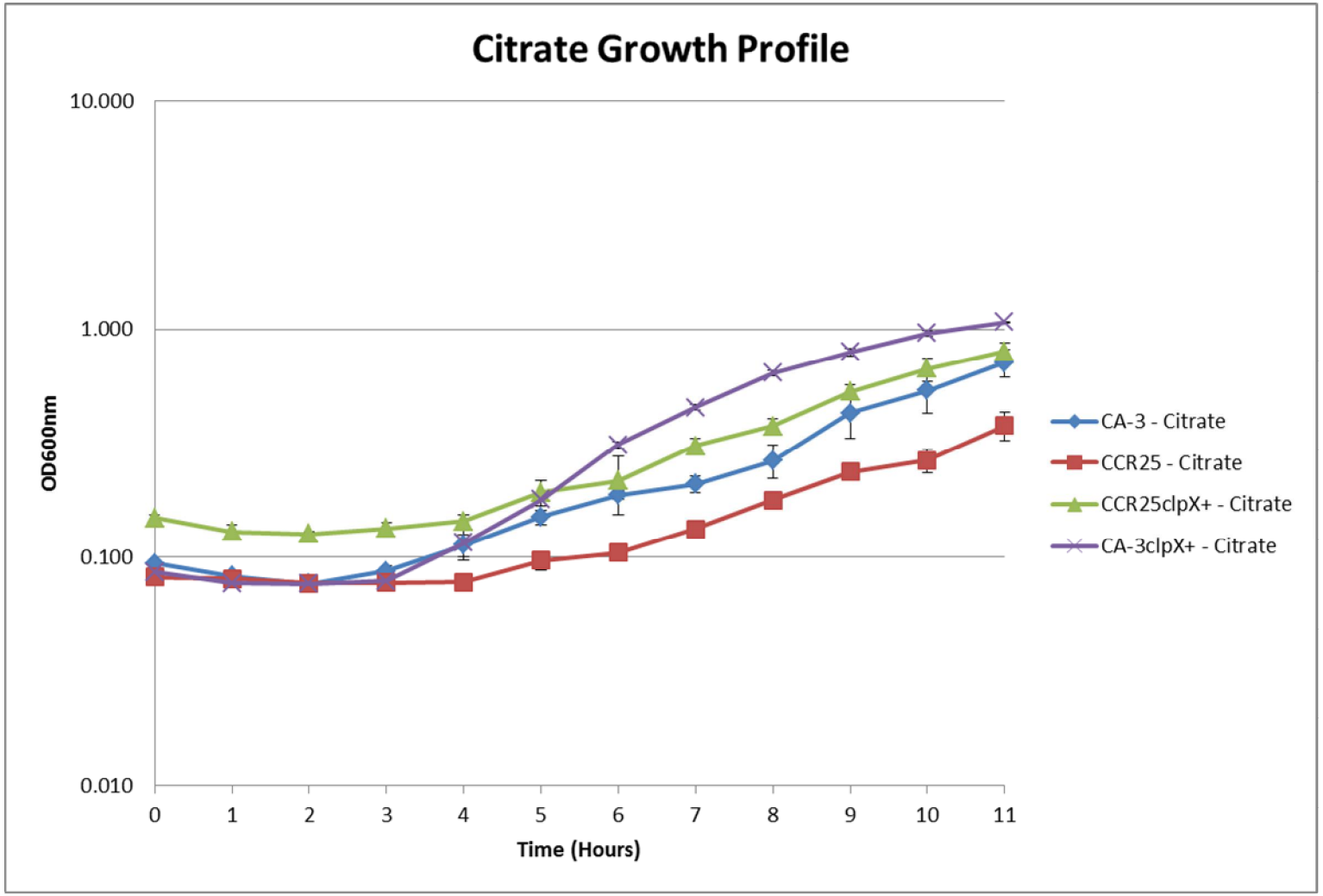


Figure S2: *P. putida* CA-3, CCR25, CCR25clpX+ and CA-3clpX+ growth curves. Cultures grown on E2 minimal media + Citrate (supplemented with 50 µg/ml kanamycin and 20µg/ml gentamycin where required). Error bars standard deviation of triplicate data.

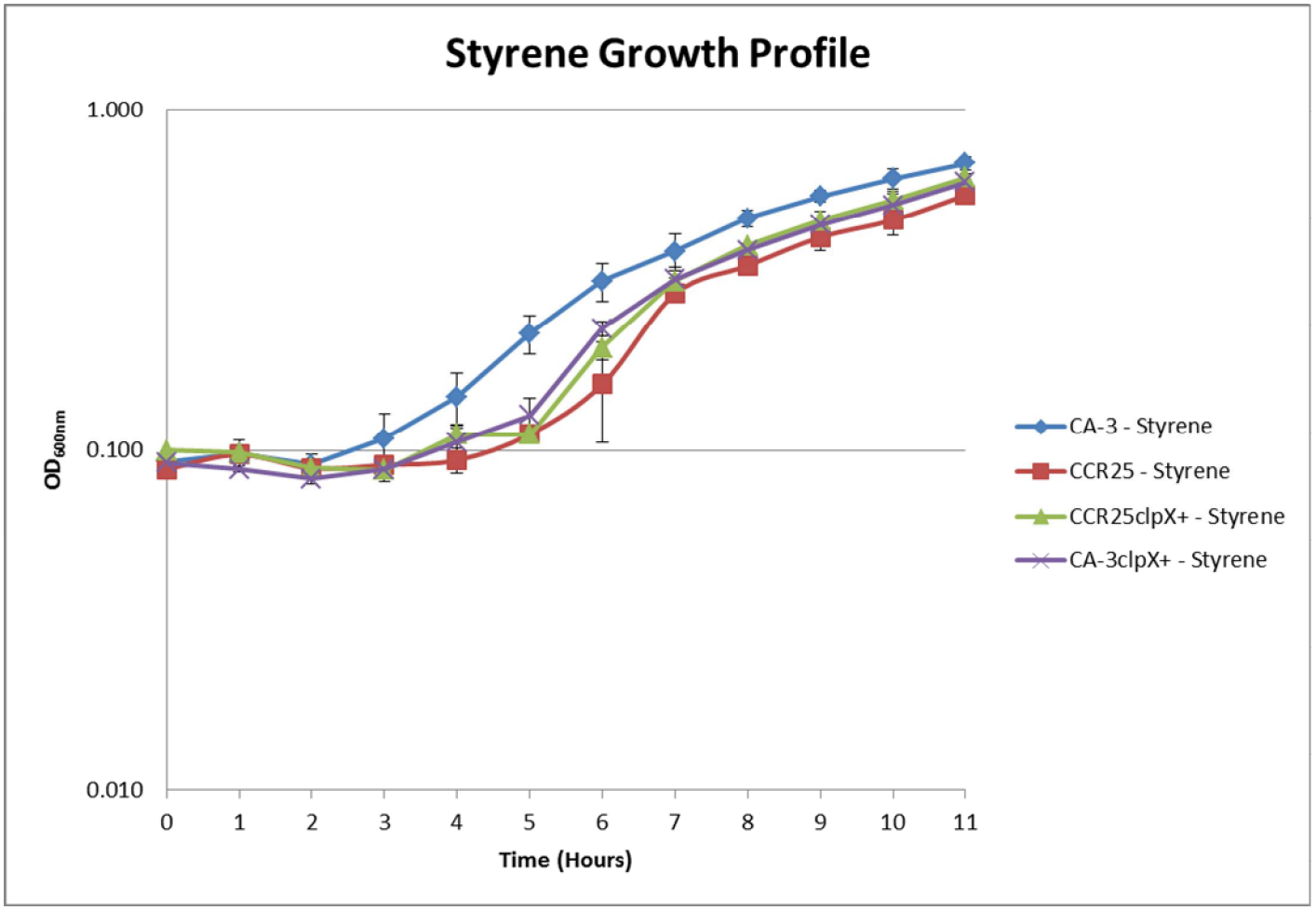


Figure S3: *P. putida* CA-3, CCR25, CCR25clpX+ and CA-3clpX+growth curves. Cultures grown on E2 minimal media + Phenylacetic Acid (supplemented with 50 µg/ml kanamycin and 20 µg/ml gentamycin where required). Error bars standard deviation of triplicate data.

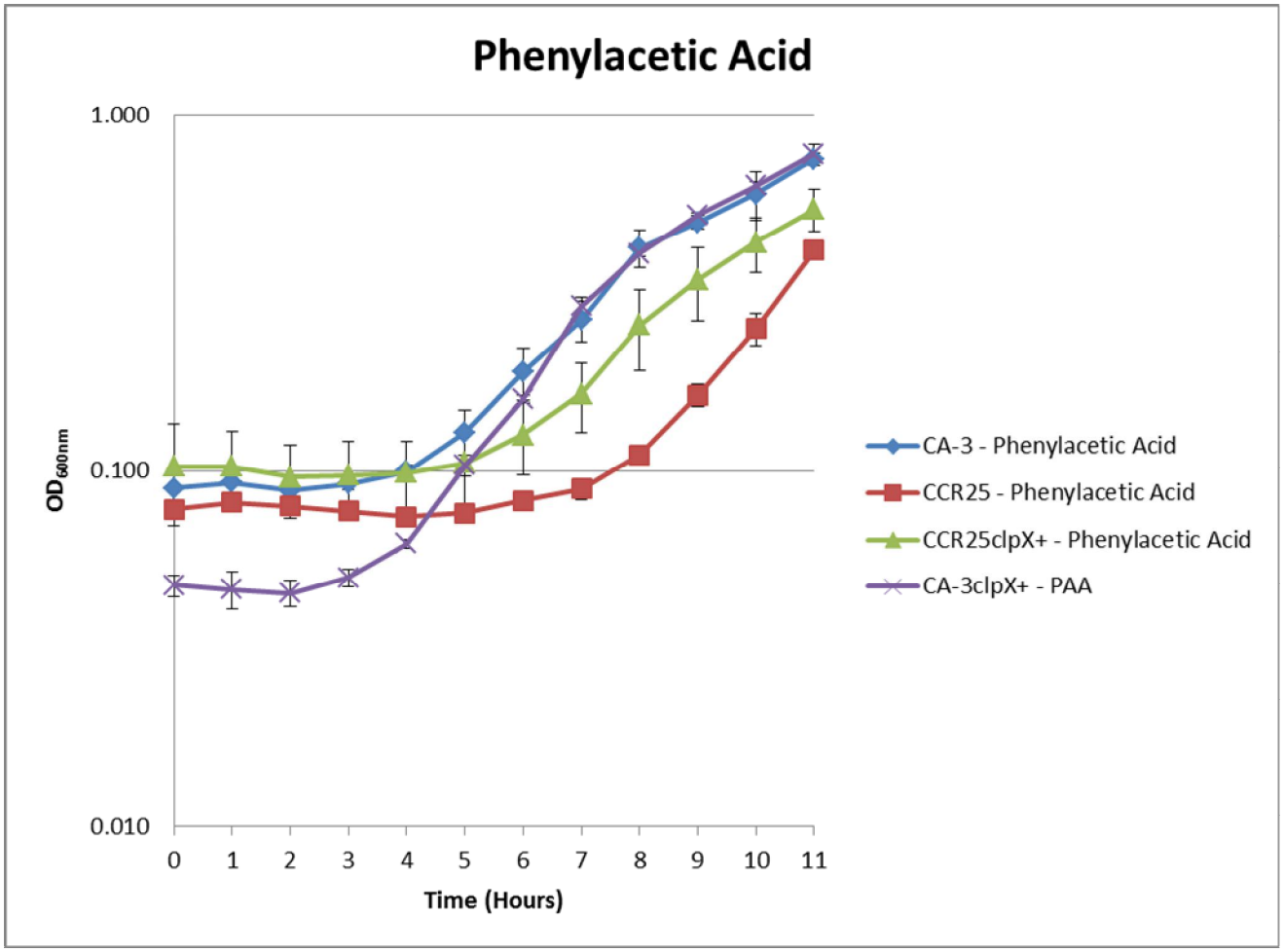


Figure S4: *P. putida* CA-3, CCR25, CCR25clpX+ and CA-3clpX+ growth curves. Cultures grown on E2 minimal media + Phenylacetic Acid (supplemented with 50 µg/ml kanamycin and 20 µg/ml gentamycin where required). Error bars standard deviation of triplicate data.

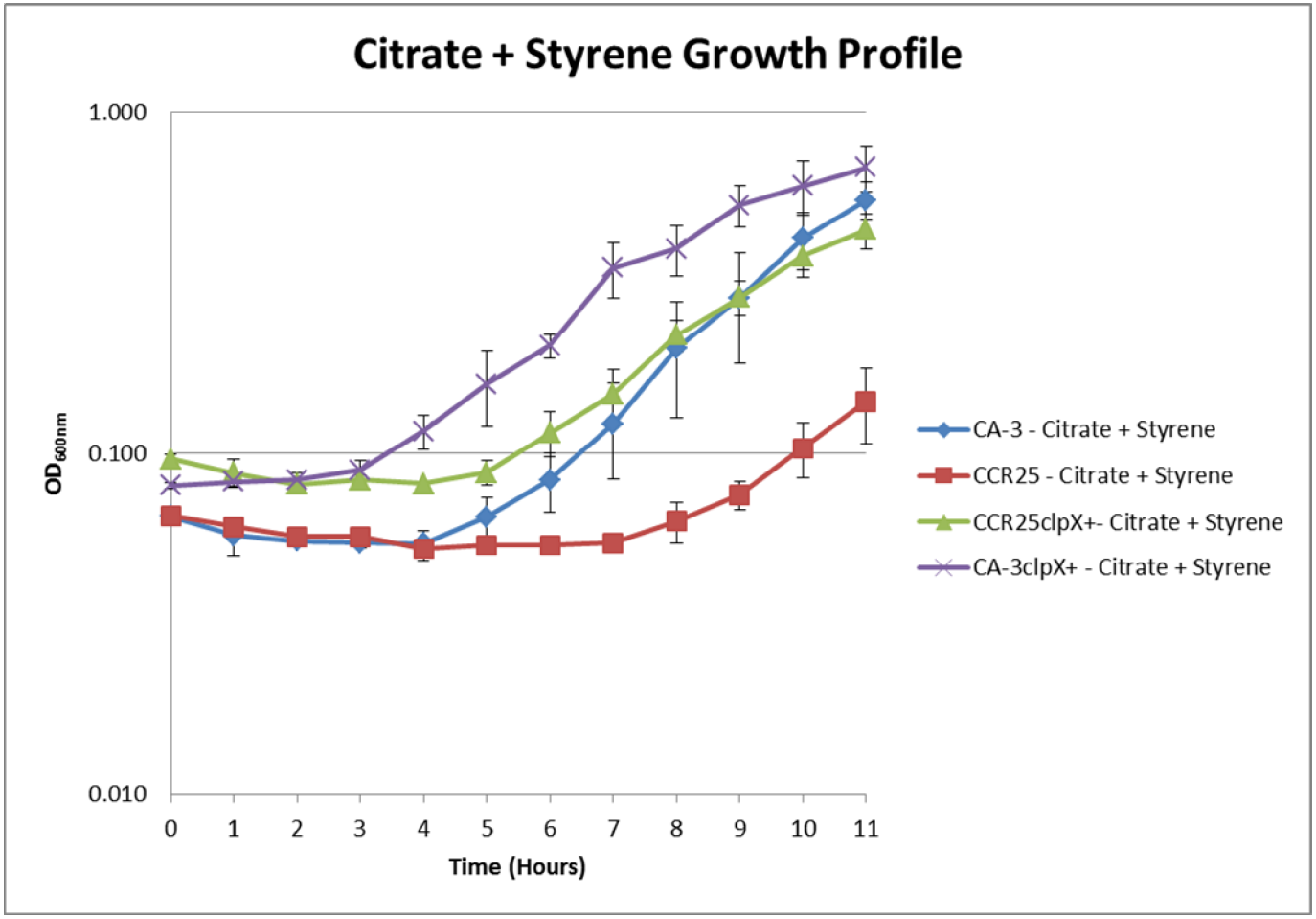


Figure S5: *P. putida* CA-3, CCR25, CCR25clpX+ and CA-3clpX+ growth curves. Cultures grown on E2 minimal media + Citrate + Styrene (supplemented with 50µg/ml kanamycin and 20µg/ml gentamycin where required). Error bars standard deviation of triplicate data.

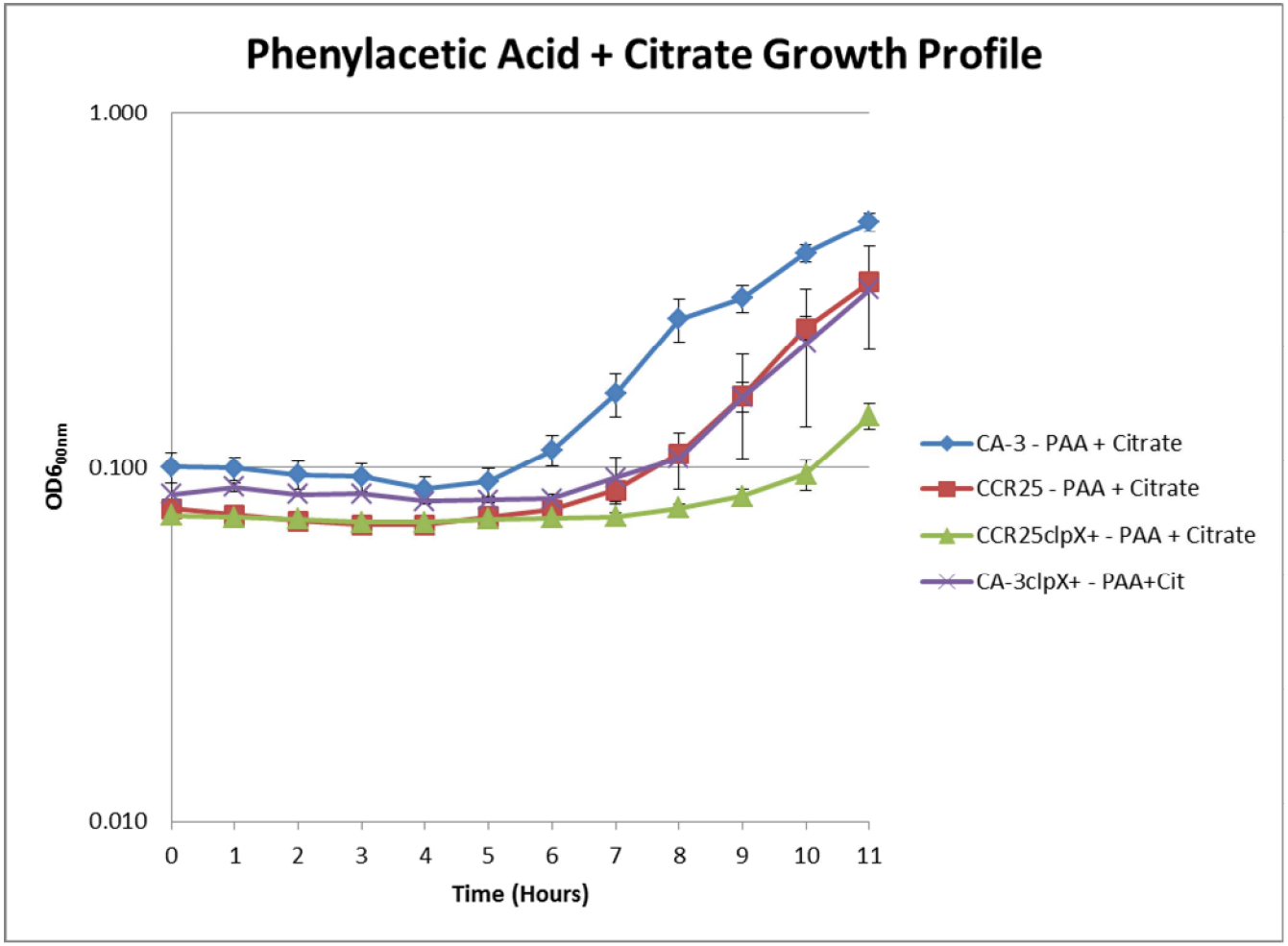


Figure S6: *P. putida* CA-3, CCR25, CCR25clpX+ and CA-3clpX+ growth curves. Cultures grown on E2 minimal media + Phenylacetic Acid + Citrate (supplemented with 50µg/ml kanamycin and 20µg/ml gentamycin where required). Error bars standard deviation of triplicate data.

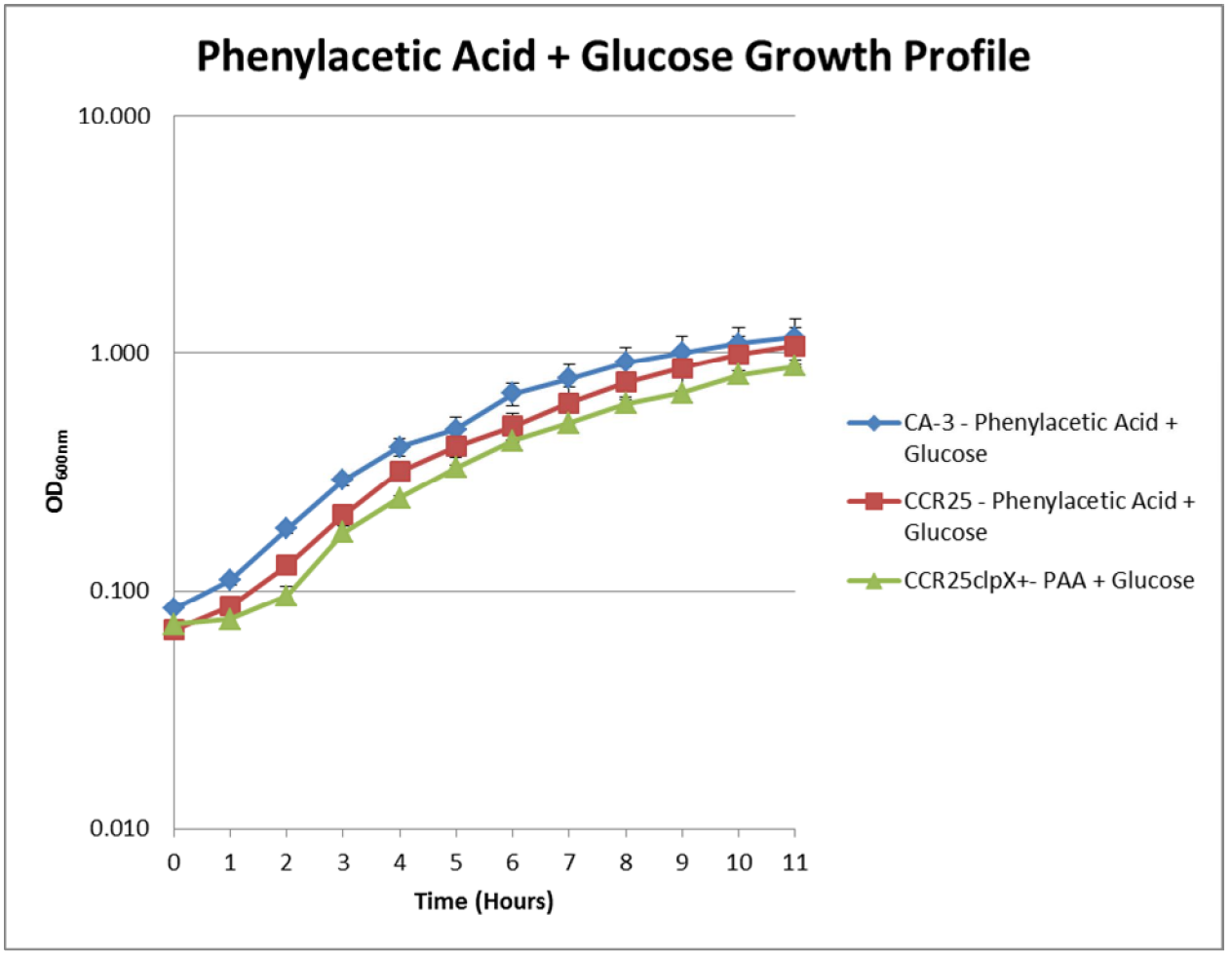


Figure S7: *P. putida* CA-3, CCR25, CCR25clpX+ growth curves. Cultures grown on E2 minimal media + Phenylacetic Acid + Glucose (supplemented with 50µg/ml kanamycin and 20µg/ml gentamycin where required). Error bars standard deviation of triplicate data.

Appendix 2

Indole to Indigo conversion

Styrene transformation to styrene oxide and phenylacetaldehyde via the action of styrene monooxygenase (SMO) and styrene oxide isomerase (SOI) as described in introduction. Indene is converted to indene oxide and 2-indanone via the action of these same enzymes. Indole conversion to indigo proceeds via indole oxide and indoxyl followed by dimerization to form indigo. Studies did not identify the formation of 2-oxidole in this strain suggesting styrene oxide isomerase does not act on indole oxide in the same manner as indene oxide.

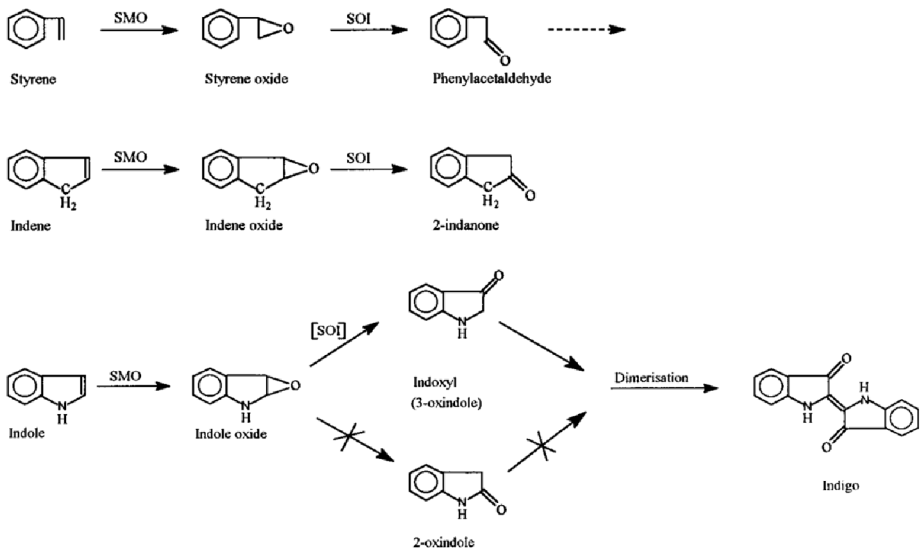


Figure 1: Biotransformation of styrene, indene and indole by *P. putida* S12.

(Adapted from O'Connor *et al.,* 1997)

Appendix 2

Mapping of mini-Tn5 transposon inserts

DNA sequences from mini-Tn*5* transposon mutants are determined by two rounds of arbitrarily primed PCR. The first round of PCR uses chromosomal DNA as template with an arbitrary primer (ARB1; 5ô-GGCACGCGTCGACTAGTACNNNNNNNNNNGATAT-3ô) and an internal primer of mini-Tn*5*, unique for the right end (TNEXT; 5ô-TGATGAATGTTCCGTTGCGCTGCC-3ô). The ýrst round was as follows: 3 min at 95°C; 6 cycles of 30 min at 95°C, 30 min at 30°C, and 1 min at 72°C; 30 cycles of 30 min at 95°C, 30 min at 50°C, and 1 min 72°C; and an extension period of 7 min at 72°C. A second round of ampliýcation was done with 5 µl of the ýrst-round reaction as the template as follows: 3 min at 95°C; 30 cycles of 30 min at 95°C, 30 min at 57°C, and 1 min at 72°C; and 7 min at 72°C. Primers used for the second round were those corresponding to the conserved region of ARB1 (ARB2; 5ô-GGCACGCGTCGACTAGTAC-3ô) and a second internal primer of mini-Tn5, closer to the end (TNINT; 5ô-GACCTGCAGGCATGCAAGCTCGGC-3ô) (Figure 1).

Reaction mixtures were electrophoresed, and the most intense bands were isolated with a Qiagen gel extraction kit and sequenced. The ~40-bp distance between this primer and the end of the mini-Tn5 provides an internal control to ensure that the obtained sequence corresponds to the junction between the transposon and the chromosome. Sequences were analyzed and compared with the GenBank database by using BLAST programs.

(Adapted from Espinosa-Urgel *et al.,* 2000)

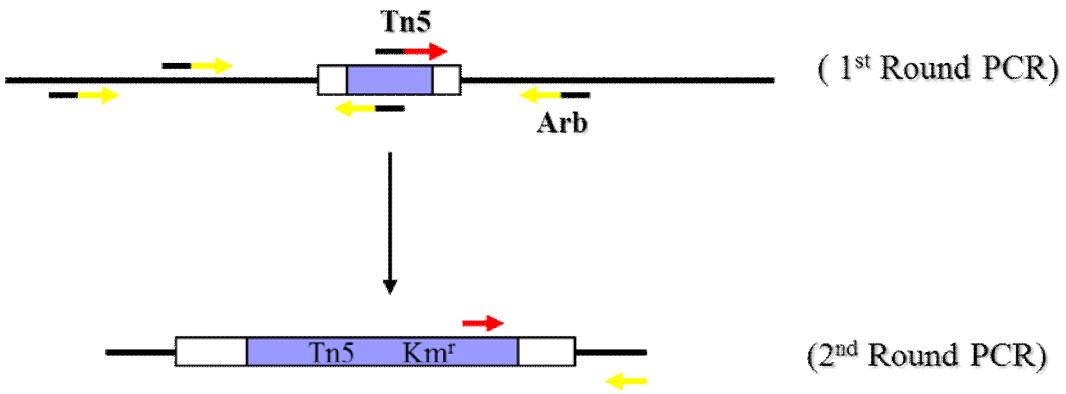


Figure 1: Mapping of mini-Tn*5* transposon insert (blue box). 1st round PCR uses mutant genomic DNA as template and involves arbitrary primer ARB1 (yellow & black arrow) and mini-Tn*5* transposon specific primer TNEXT (red & black arrow). 2nd round PCR uses 1st round PCR product as template and primers corresponding to conserved regions of 1st round primers ARB1; ARB2 (yellow arrow) and TNEXT; TNINT (red arrow).

Chapter 3

GacS dependent regulation of polyhydroxyalkanoate synthesis in *Pseudomonas putida* CA-3

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Abstract

Polyhydroxyalkanoates, (PHAs), are microbially derived, biodegradable polyesters which offer considerable commercial potential as they possess material properties facilitating their use in a range of industrial, medical and consumer applications. Medium chain length PHAs from *Pseudomonas* species are of particular interest due to their compositional diversity and many have been extensively characterised at the chemical, biochemical and genetic levels. However, to date there have been limited reports on the regulatory systems specifically exerting control over PHA synthesis. Here we identify gacS dependent regulation of medium chain length PHA production in *Pseudomonas putida* CA-3 following random mini-Tn*5* disruption of the sensor kinase gene. Recombinant expression of wild-type *gacS* from the pBBRgacS vector fully restored PHA accumulation capacity in the mutant strain. PCR based screening of the *P. putida* CA-3 genome identified gene homologues of the GacS/GacA - *rsm* small RNA regulatory cascade with 96% similarity to published *P. putida* genomes. However, RT-PCR analyses revealed transcription of the *rsmY* and *rsmZ* sRNAs in *gacS::*Tn*5* disrupted *P. putida* CA-3, which is atypical of the commonly reported Gac/Rsm regulatory cascade. Subsequent SDS PAGE protein analyses of the wild type and *gacS*::Tn*5* mutant strains identified post-transcriptional control of *phaC1* synthase as a key point of control of PHA synthesis in *P. putida* CA-3.

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced by numerous bacterial species that have varied thermoplastic and elastomeric properties offering a number of industrial, medical and consumer applications. PHAs are typically synthesised by bacteria when an essential nutrient such as nitrogen, sulphur, or phosphorous becomes limiting in the presence of an excess of carbon (Legeveen *et al*., 1988; Ramsay *et al*., 1992; Huismann *et al*., 1991; Garcia *et al.,* 1999). When the limiting nutrient is restored, PHA undergoes depolymerisation and is utilised by the host cell as a carbon and energy source. Several comprehensive, recent reviews are available in the field of microbial PHA synthesis (e.g. Philip *et al*., 2007; Grage *et al*., 2009; Gomez *et al.,* 2012).

PHAs are condensation polyesters of various hydroxyalkanoic acids, forming inclusions within the cell cytoplasm. There are 2 main classes of PHA which are divided based on the chain length of the monomer units which make up the polymer. Short chain length PHA, (scl-PHA), consisting of 3-5 carbon atom monomers and medium chain length-PHAs (mcl-PHA) consisting of 6-14 carbon atom monomers. The pathways responsible for the production of scl-PHAs have been extensively studied and the polyesters produced are typically highly crystalline. The resulting plastics therefore demonstrate brittle/stiff characteristic properties, which limit their commercial applications to disposable packaging and bulk materials. In contrast, mcl-PHAs offer greater strength and flexibility, yielding more diverse applications beyond packaging, such as biomedical implants, gauzes, osteosynthetic materials and slow release drug matrices (van der Walle *et al*., 1999; 2001; Chen and Wu, 2005).

The two major classes of PHAs are synthesised by distinct polymerase enzymes, namely Class I synthases, (scl-PHA) or Class II, synthases (mcl-PHA), respectively (Witholt and Kessler, 1999) which can demonstrate amino acid sequence identities as low as ~21% (Rehm and Steinbüchel, 1999). Generation of the various scl- and mcl- monomers can occur by several routes, but three principal pathways are commonly reported. In the first scl-PHA 3-hydroxybutyrate monomers are derived from the condensation of acetyl-CoA moieties from the TCA cycle, (e.g. *Ralstonia eutropha*) (SenioŘ and Dawes, 1971). Alternatively fatty acid metabolism generates various hydroxyalkanoate monomers for PHA biosynthesis via substrate related conversions of keto-acyl-CoAs to corresponding 3-hydroxyacyl-CoAs during *β*-oxidation, (e.g. *P. aeruginosa*) (Tsuge *et al*., 2000). In the third route PHA monomers can be derived from unrelated carbon sources via acetyl-CoA diversion to *de novo* fatty acid synthesis, yielding 3-hydroxyacyl-ACP intermediates. These intermediates are converted to corresponding 3-hydroxyacyl-CoA monomers for PHA synthase by 3-hydroxy-ACP-CoA transacylases (e.g. *P. putida*) (Rehm and Steinbüchel, 1999). This latter pathway is of specific biotechnological interest as it presents the potential for PHA production from organic wastes (Philip *et al*., 2007). One example of this is the ability of *Pseudomonas putida* CA-3 to accumulate mcl-PHA from the alkenylbenzene, styrene (Ward *et al.*, 2005).

Given the multiple pathways capable of contributing to PHA synthesis it is no surprise that several studies have observed that metabolic networks and carbon flux play a central role in PHA production. Indeed, a number of core cellular metabolic enzymes have been manipulated in attempts to improve PHA yields in various bacterial species. Research by Lim *et al*. (2002) demonstrated that overexpression of glucose-6-phosphate

dehydrogenase and 6-phosphogluconate dehydrogenase redirected catabolic flux through the pentose phosphate pathway. The result was an elevated NADPH/$NADP^+$ ratio, which favoured PHA accumulation to levels approximately 41% higher than recombinant *E. coli* harbouring only the PHB synthesis operon from *Ralstonia eutropha* (Lim *et al*., 2002). Research by Hong *et al.* (2003) also investigated the role played by the Entner-Doudoroff pathway in PHB synthesis and demonstrated reduced PHB accumulation in an *eda* deficient mutant lacking 2-keto-3-deoxy-6-phosphogluconate aldolase. In addition to specific metabolic enzymes, signal transduction systems have also been shown to play a role in the detection of extracellular conditions which induce PHA accumulation and the co-ordination of central metabolic pathways involved. The *arc* system in *E. coli,* which regulates the expression of several operons, relative to the redox state of the environment, has previously been manipulated to improve *E. coli* as a host strain for PHB biosynthesis (Nikel *et al.,* 2006). In addition, *arc* gene mutants demonstrate the expression of TCA cycle components under micro-aerobic conditions, elevating levels of reducing equivalents, which can be channelled into PHB production. The authors demonstrated a 35% (wt/wt) increase in PHB production in such *arc* negative *E. coli* mutants over wild type strain levels. The *cre* regulon has also been investigated as a target for improving PHA production. The CreBC two component system controls several genes involved in carbon flux in *E. coli* (Avison *et al.,* 2001). Nikel *et al.* (2008) achieved increased PHB yields in an *arcA, creC* double mutant of *E. coli*, owing to the induction of a reduced redox state coupled with increased carbon utilisation, respectively. Key cellular metabolic networks, their effect on PHA production and manipulations of same for

enhanced PHA production in the cell have been the subject of recent reviews (e.g. Gomez *et al.*, 2012).

Despite an increased understanding of how metabolic intermediates, carbon flux and redox potential affect PHA synthesis, relatively little remains known in relation to global cellular regulatory systems directly controlling PHA synthesis processes. In this study we sought to determine whether functional links could be established between global regulatory/signalling mechanisms and the PHA synthetic pathway within the well characterised mcl-PHA producer *P. putida* CA-3. Random mini-Tn*5* mutagenesis of the *P. putida* CA-3 genome was combined with a solid media screen to identify deficient/altered PHA accumulation phenotypes. 13,500 mutants were generated by this process of which approximately 35 demonstrated altered PHA accumulation profiles. Here we present our findings on the characterisation of one of these mutants, PHA45A, which hosted a *gacS* gene disruption and establish a direct regulatory influence, at the post-transcriptional level, on PHA synthesis in *P. putida* CA-3.

Materials and Methods

Bacterial strains, plasmids, culture media, and growth conditions.

Pseudomonas putida CA-3, a styrene-degrading, bioreactor isolate has been described previously (O'Connor *et al.,* 1995). *E. coli* CC118λ*pir* hosted the mini-Tn*5* derivative pUT-Km1, (R6K origin of replication), which encodes resistance to kanamycin and ampicillin (de Lorenzo *et al.,* 1990). Plasmid pRK600 (Cm^r) was used as a helper in triparental mating experiments and encodes the *tra* functions facilitating pUT-Km1 mobilization. PCR2.1-TOPO vector (Invitrogen, California) was used in the cloning of PCR amplification products. The expression vector pBBR1MCS-5 was used for expression of cloned genes in complementation studies as described below (Kovach *et al*., 1995). *Pseudomonas putida* CA-3 was routinely grown on E2-minimal medium (Vogel, 1956) containing 23 mM citrate as the sole carbon source. PHA accumulation was achieved by reducing the nitrogen content of the E2 medium in the form of NH_4SO_4 from 8mM to 1.5mM and allowing cultures to grow for ~8h. All *E. coli* vector hosts were maintained on standard LB agar plates containing the appropriate antibiotic(s) and were inoculated into 10ml LB overnight broths prior to desired applications.

Cultures were routinely tested for PHA accumulation by microscopic fluorescent visualisation of Nile Red stained samples exposed to UV excitation. 1ml samples of respective cultures actively growing under nitrogen limiting (1.5 mM) conditions were centrifuged, (16,400 x g), the supernatants removed and pellets washed in phosphate buffered saline, (PBS), before staining with 10µl of 10 mg/ml Nile Red (ex 485 nm/em 525 nm) in DMSO. The stained pellets were incubated at room temperature for 5 min, protected from ambient light exposure, before being made up to 1ml with PBS. Cells

were visualised under fluorescence (Filter I3: ex450 nm/490 nm) at 1000X magnification for the presence of PHA granules using Leica DM3000 microscope fitted with Leica DFC490 camera and analysed with Leica Application suite v3.1.0.

Random mini-Tn5 mutagenesis.

A triparental mating approach was used to introduce pUT-Km1 into *P. putida* CA-3. The mating mixture (1 ml), containing mid-log-phase LB-grown recipient, donor, and helper strains at a ratio of 7:2:1, which was centrifuged for 2 min at 16,400 rcf, resuspended in 50µl fresh LB medium, spotted onto an LB agar plate, and incubated at 28°C for 24h. *P. putida* CA-3 transconjugants expressing kanamycin resistance were subsequently isolated by plating serial dilutions of the mating mixture onto E2-citrate medium containing kanamycin (50 µg/ml). Approximately 13,500 isolated colonies were individually transferred to 96-well microtiter plates containing 200µl of E2-citrate broth in each well. These master plates were used to identify transposition events resulting in a qualitative loss/reduction of the parent strainôs ability to accumulate PHAs from the unrelated carbon source citrate. PHA-negative phenotypes were identified by first growing colonies in E2-citrate broth containing 1.5 mM nitrogen, (28°C for 24 h), followed by transfer to E2-citrate agar plates lacking any nitrogen, incubation for 48 h at 30°C, and monitoring thereafter for an absence of colony opacity associated with PHA accumulating microorganisms (OôLeary *et al.,* 2005).

Mapping of transposon insertion sites.

Arbitrarily primed PCR was employed to map the gene disruption sites utilising previously published oligonucleotide primers and appropriate thermal cycling parameters (Espinosa-Urgel *et al*., 2000). Products were visualised on 1% agarose gels, purified using a QIAGEN QIAquick Gel extraction kit and sequenced using the mini-Tn*5* internal primer, TNINT (Table 1).

Mutant complementation.

Genomic DNA was isolated from *P. putida* CA-3 according to Ausubel *et al.* (1987). The primer pair FgacS-FW and FgacS-RV (Table 1) were designed based on available *Pseudomonas putida gacS* gene sequences in GenBank. PCR amplification of the full length *gacS* gene (GenBank Accession Number: JX826483) from genomic DNA involved 35 cycles of PCR amplification with *Pfu* proofreading polymerase followed by the addition of 2.5U *Taq* polymerase and incubation at 72°C for 10 minutes to add a poly-A tail to facilitate cloning into the pCR2.1 cloning vector (Invitrogen). The cloned fragments were then transformed into Top10 *E. coli* cells (Invitrogen) as per the manufacturerôs instructions. Transformants were plated on LB agar plates containing 50 µg/ml kanamycin which were overlaid with 40 µl of 40 mg/ml X-gal to facilitate blue/white screening of colonies. Several of the white colonies generated were inoculated into liquid LB medium containing kanamycin and subjected to plasmid mini-preps (Fermentas) as per the manufacturerôs instructions. The resultant plasmid was cut with *Xba*I and *Hind*III enzymes in a double digest at 37°C for 90min with tango buffer (Fermentas). The pBBR1MCS-5 expression vector was also digested in the same

manner. The *gacS* fragment obtained from the double digest was ligated into the pBBR1MCS-5 vector using T4 DNA ligase (Fermentas) as per manufacturerôs instructions to yield pBBRgacS. Transformation of pBBRgacS into chemically competent Top10 *E. coli* cells (Invitrogen) was achieved as previously described and the resultant transformants were screened on LB agar plates containing 20 µg/ml gentamycin. Transconjugation of pBBRgacS expression vector into *P. putida* CA-3 *gacS::*Tn*5* PHA45A mutant was performed via triparental mating as before. Successful transconjugants were isolated on solid E2 minimal media containing 50 µg/ml kanamycin and 20 µg/ml gentamycin. Successful mating reactions yielded the complemented *gacS::Tn5* mutant named PHA45AgacS+. Complementation of full length *phaC1* gene in the *phaC1::Tn5* mutant B11 to yield B11phaC1+ was achieved in the same manner as detailed above for *gacS* complementation. PCR amplification of the full length *phaC1* gene from *P. putida* CA-3 utilised the primers FphaC1-FW and FphaC1-RV (Table 1), with thermal cycling conditions as described above for amplification of full-length *gacS*. Integrity and functional expression of cloned gene on plasmid was confirmed by sequencing of resultant plasmid and RT-PCR analysis as described below using gene specific primers (gacs-FW/-RV and C1-F/-R).

RT-PCR analyses.

Total RNA was isolated from *P. putida* CA-3 wild type and Tn*5* insertion mutants using a Fermentas GeneJET^{TM} RNA Purification Kit, as per the manufacturerôs instructions. The purified RNA was treated with TURBO DNA-freeÊ DNase, (Ambion), to ensure complete removal of DNA. Complete removal of genomic DNA from isolated RNA was

confirmed by 16S PCR using 27F and 1492R primers (Table 1) prior to generation of cDNA. Reverse transcription was performed with 1 µg of total RNA using random hexamer priming, 1 mM dNTPs, 10U BioScript reverse transcriptase with 1X reaction buffer, (Bioline) in a 20 µl reaction volume. Reactions were incubated at 25°C for 10 min, followed by 30 min at 42°C and finally at 70°C for 10 min. 1 µl of the respective RT reactions were employed as template in subsequent PCR reactions to confirm generation of cDNA. Amplification of the 16S rRNA gene from CA-3 genomic DNA acted as a positive control for RT analyses and was performed with the universal primers 27f and 1429r (Table 1) at an annealing temperature of 55°C and standard thermocycling conditions. 16S rRNA gene RT-PCR amplification was used for optimisation of RT-PCR product equivalence to ensure template cDNA concentrations were comparable between samples (Figure 4). Equivalence was achieved using the SynGene Genetools^{TM} v3.07 band comparison software to compare band intensity between samples. PCR cycle numbers were also optimised in assessing Gac/Rsm homologue expression, to ensure amplifications did not reach a plateau phase.

Oligonucleotide primers, (Table 1), were also designed for transcriptional profiling of pathway related genes; *gacS* encoding the GacS sensor kinase and *gacA,* encoding the response regulator of the GacS/GacA two component system; *rsmA* encoding the secondary metabolism regulator RsmA, and both *rsmY* and *rsmZ* non-coding sRNAs.

Bioinformatic analysis of the published *rsmZ* and *rsmY* gene sequences identified poor sequence conservation among various *Pseudomonas* species (data not shown). However, the genome positions of *rsmZ* and *rsmY,* demonstrated high degrees of conservation within the genes flanking the respective sRNA sites. A flanking PCR and sequencing

approach was therefore employed to obtain the sequence of both *rsmY* and *rsmZ* in *P. putida* CA-3. PCR utilising an *rpoS* gene based forward primer, (rpoS-817), coupled with an *feS* gene specific reverse primer (feS-45), (Table 1), was employed to amplify the intergenic region harbouring the *rsmZ* gene. The PCR product was sequenced and found to contain a full length *rsmZ* sRNA (GenBank Accession Number: JX826485), which was subsequently used to design *P. putida* CA-3 specific *rsmZ* primers. A similar flanking PCR approach utilising primers ACDH-1520 and lysR-524 was used to amplify the intergenic region harbouring the *rsmY* gene located between the *lysR* and *ACDH* genes. The amplicon generated included the full length *rsmY* gene sequence (GenBank Accession Number: JX826484) which was used to design the *rsmY* specific primers *rsmY*-FW and *rsmY*-RV. Transcriptional profiling of the genes required for PHA synthesis, i.e. *phaC1* (encoding the mcl-PHA synthase) and *phaG* (encoding an ACP-CoA transferase), was also performed using previously published primer pairs C1-F, C1-R and G-F, G-R respectively (O'Leary *et al.,* 2005).

SDS-PAGE Protein Analysis.

SDS-PAGE analysis was performed on soluble protein fractions from wild-type *P. putida* CA-3, mutant PHA45A (*gacS::*Tn*5*), PHA45AgacS+ (*gacS* complement), B11 (*phaC1::*Tn*5*) and B11 pBBRphaC1 (*phaC1* complement) under PHA accumulation conditions. Samples were incubated at 28°C with shaking at 180rpm under nitrogen limiting conditions as described. After approximately 6 hours cultures were sampled and analysed for PHA accumulation as previously described. Upon confirmation of PHA accumulation, 20 ml of actively growing culture was removed and centrifuged for 10min

at 5000 x g. Total protein was extracted from the resulting pellet using the B-PER Plus Halt Protease Inhibitor Cocktail Kit (Thermo Scientific). Pellets were resuspended in 4 ml of buffer containing 2 µl of 20 mg/ml lysozyme (Sigma) and 2 µl DNase I (Fermentas), and incubated for 15 min at room temperature. Separation of soluble and insoluble protein fractions was achieved by centrifugation of samples at 15000 x g for 5 min. Resultant soluble protein fractions were resuspended in sample buffer and heated for 10 min at 100°C. The protein concentration of the crude extract was determined by the Bradford assay against a bovine serine albumin standard curve (Sigma-Aldrich). Five micrograms of each protein sample was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Protean II xi system (Bio-Rad) according to the manufacturerôs instructions. Gels were run for 2 h at 30 mA through a 4% acrylamide stacking gel and a 12% separating gel. Pre-stained protein marker weight standards (New England Biolabs) were used for molecular mass estimation. Gels were stained with PageBlue^{TM} Stain (Fermentas) overnight as per manufacturerôs instructions and destained with dH_2O for 10 min.

Results

Cloning and characterization of PHA negative mutants.

Random mini-Tn*5* mutagenesis of the *P. putida* CA-3 genome produced 13,500 kanamycin resistant transconjugants. Solid media screening on E2 Nitrogen limited media identified 44 transconjugants exhibiting reduced opacity, indicative of reduced PHA accumulation capacity (Figure 1A). Fluorescent microscopic analyses further confirmed PHA accumulation deficiencies within these isolates, Figure 1B. The mini-Tn*5* insertion site was mapped in each isolate using two consecutive rounds of arbitrary PCR as previously described (Espinosa-Urgel *et al.,* 2000) and the resulting amplicons sequenced. Comparative analyses of the sequence data against the GenBank, non-redundant nucleotide database was performed using the NCBI BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/). Two of the isolates were found to harbour disruptions within the *phaC1* synthase gene, which has previously been shown to be crucial for PHA accumulation in *P. putida* CA-3 (OõLeary *et al.,* 2005). This finding confirmed the validity/efficacy of this screening strategy for identifying functionally significant genes/enzymes involved in PHA accumulation. The region downstream of the mini-Tn*5* insertion in two of the other transconjugants, PHA39B and PHA45A, were found to share over 96% sequence similarity with *gacS* gene sequences from other *P. putida* strains. In order to rule out any possible polar effects caused by the insertion of mini-Tn*5*, the *gacS* gene disruption in PHA45A was complemented. Expression of *gacS* from the pBBR1MCS-5 derived *gacS* expression vector pBBRgacS in PHA45AgacS+ was found to completely restore the strainõs ability to accumulate intracellular PHA (Figure 2B).

Identification and transcriptional analyses of Gac/Rsm cascade gene homologues in *P. putida* CA-3.

The GacS/GacA two-component system which positively controls the expression of regulatory sRNAs has been reported in a variety of different bacterial species (reviewed in Lapouge *et al.,* 2008). A mechanistic model of the cascade is presented in Figure 3.

A PCR approach was employed to screen the *P. putida* CA-3 genome for homologues of genes potentially involved in the Gac/Rsm regulatory cascade in the strain; namely *gac*S, *gacA, rsmA, rsmY* and *rsmZ* Amplicons corresponding to the appropriate sizes of *gacS* and *gacA* genes were obtained in each case from *P. putida* CA-3 and following sequence analyses were found to possess high levels of nucleotide identity (85-99%) with respective genes from previously published *P. putida* genomes (Table 2). PCR primers specific to conserved regions of previously published *rsmA* sequences were also employed to identify an *rsmA* homologue in CA-3, the resultant amplicon when sequenced was found to possess a percentage nucleotide identity of between 88 to 100% to other known PHA accumulating *Pseudomonas* species. Due to poor sequence conservation in published genomes for the *rsmY* and *rsmZ* sRNA genes, flanking PCR approaches were employed to facilitate the design of CA-3 specific primers as previously described. Subsequent identification of *rsmY* and *rsmZ* gene homologues in *P. putida* CA-3 revealed 89-99% and 91-97% identities, respectively, when compared to putative gene sequences obtained from published *P. putida* genomes, (Table 2). An *rsmX* homologue could not be identified in *P. putida* CA-3, possibly die to the low conservation in the genes flanking *rsmX* in species in which this sRNA has previously been reported.

In *Pseudomonas* species the GacS/GacA two component system regulates the expression of sRNAs by sequestering the RsmA post-transcriptional regulator (Figure 3). RT-PCR analysis was employed to assess expression of these genes in both *P. putida* CA-3 wild-type and the *gacS::Tn5* PHA45A mutant. As described in materials and methods the SynGene GeneTools band analysis software was employed to analyse the relative yields of control 16S PCR amplicons, with a <10% difference in band intensity between CA-3 WT and PHA45A cDNA samples being observed. The results of these analyses were used to ensure equivalent cDNA quantities were employed in the subsequent PCR analysis of Gac/Rsm gene homologue expression. It was observed that *gacA, rsmA, rsmY* and *rsmZ,* are all expressed in both the CA-3 wild type and PHA45A mutant strains under PHA accumulation conditions. The expression of *gacS* was also observed in wild-type CA-3 but could not be detected in the PHA45A mutant, suggesting a single functional/transcribed copy exists in the *P. putida* CA-3 genome (Figure 4).

Expression of PHA biosynthetic genes in wild-type *P. putida* CA-3 and *gacS* mutant.

Our group has previously reported transcriptional regulation of the key *pha* genes, (*phaC1* polymerase and *phaG* encoded ACP-CoA transacylase), in *P. putida* CA-3 under nitrogen-limiting and non-limiting conditions (O'Leary *et al*., 2005). Here *pha* gene expression profiles were examined in both the *gacS::Tn5* mutant PHA45A and *P. putida* CA-3 wild type with similar levels of transcription for both the synthase and transacylase being observed in both strains (Figure 5). We therefore sought to determine whether GacS induced control of PHA biosynthesis in *P. putida* CA-3 was mediated at the level

of translation of the biosynthetic apparatus. SDS-PAGE analyses were performed on the soluble protein fractions from wild-type *P. putida* CA-3 and the *gacS::Tn5* mutant PHA45A (Figure 6; lanes 1 and 2, respectively). Samples were obtained from cultures grown under PHA accumulation conditions as described by O'Leary *et al*. (2005). A protein of ~ 60 kDa in size which is present in wild-type CA-3 (Figure 6; lane 1) was found to be absent in the *gacS::Tn5* negative mutant PHA45A (lane 2). Previously our group reported that the *P. putida* PHA synthase (*phaC1::Tn5)* disrupted mutant B11 was incapable of PHA accumulation (O'Leary *et al.*, 2005). Bioinformatic analysis of the *P. putida* CA-3 *phaC1* gene sequence detailed in that study predicted a putative size for the phaC1 protein of 62.2 kDa.

Here we used samples of the soluble protein fraction obtained from this B11 mutant and compared them with the protein profile of *gacS::Tn5* negative mutant PHA45A. Results from the SDS-PAGE analysis of mutant B11 (Figure 6, lane 3) identified the absence of a similar ~60 kDa protein to that identified in PHA45A (Figure 6, lane 2). Complementation of *phaC1::Tn5* mutant B11 with the full length *phaC1* gene to form B11phaC1+ was found to restore this ~60 kDa protein (Figure 6; lane 5) as well as to restore PHA granule production (Figure S1; b). Similarly complementation of the full length *gacS* gene in the PHA45A mutant to generate PHA45AgacS+ resulted in restoration of this ~60 kDa protein (Figure 6, Lane 4) and the PHA accumulation phenotype (Figure 2; b).

Discussion

The two component GacS/GacA system forms the sensory apparatus of the Gac/Rsm cascade, which involves interactions with small RNA regulatory molecules affecting a range of microbial metabolic pathways (Lapouge *et al.,* 2008). A mechanistic model of the cascade is presented in figure 3 and demonstrates the sensing of appropriate extracellular signals via the GacS sensor kinase, resulting in autophosphorylation. A phosphor-transfer event is then believed to activate the associated response regulator GacA, which is found distally encoded in *P. putida* genomes published to date. Phosphorylated GacA acts as a transcriptional activator of several, non-operonic sRNA genes, *rsmX, Y* and *Z*. These sRNAs act to sequester a negative regulator of mRNA translation, RsmA, enabling affected protein synthesis to proceed.

While GacS dependent regulation of mcl-PHA production in *Pseudomonas* species has not been previously reported, an early study by CastaÑeda and co-workers observed the loss of scl-PHA production in a *gacS* negative mutant of *Azotobacter vinelandii* (CastaÑeda *et al.,* 2000). A recent, follow up study by Hernandez-Eligio *et al.* (2012) investigated GacA regulation of PHB accumulation in the same strain. The authors reported that regulation of PHB biosynthesis proceeds in *Azotobacter vinelandii* via post-transcriptional regulation of PhbR, via mRNA binding by RsmA. PhbR has previously been shown to be required for transcriptional activation of the *phbBAC* biosynthetic operon for PHB. Regulation of *phbR* mRNA transcripts thus reduces expression from the *phbBAC* operon, resulting in reduced PHB accumulation by the cell. The correlation between the PHA deficient phenotypes in the *gacS* mutants of *A. vinelandii* and *P. putida* CA-3, despite the dissimilarities between scl- and mcl- PHA biosynthetic routes, raises

the possibility of a common or convergent regulatory mechanism controlling PHA production across many species of bacteria. However, the mechanism of Gac/Rsm mediated regulation of PHB production proposed by Hernandez-Eligio is unlikely to affect PHA accumulation in *P. putida* CA-3, as PHA regulation in this strain has not been demonstrated to be dependent on a dedicated transcriptional regulator such as, for example, PhbB in *A. vinelandii*. Prieto *et al.* have indicated that PhaF, a PHA granule associated protein, can exert carbon source dependent, (i.e. octanoate vôs citrate), regulatory influences on the expression of *phaC1* in *P. oleovorans* GPo1 (Prieto *et al.,* 1999). It would not appear that such an effect is relevant in this study however, as we report that transcription of the *phaC1* and *phaG* genes, central to PHA biosynthesis, are unaffected by *gacS* disruption in citrate grown *P. putida* CA-3. Thus the GacS/GacA system does not appear to be involved in controlling expression of any intermediate transcriptional regulator affecting PHA operon gene expression in this strain.

The presence and expression of typical Gac/Rsm cascade genes (*gacA, rsmA, rsmY* and *rsmZ*) were demonstrated in both wild type and PHA45A mutant strains of *P. putida* CA-3; however an *rsmX* homologue could not be identified. It should be noted that while analyses of sequenced genomes of *P. aeruginosa* and *P. entomophila* have identified *rsmY* and *rsmZ* homologues, *rsmX* equivalents could not be identified in either strain (reviewed in Lapogue *et al*., 2008). It is possible therefore the inability to clone an *rsmX* homologue in *P. putida* CA-3 in this study reflects a similar absence in our strain. Pursuant to the generation of a genome sequence for this strain it is not possible to be definitive on this matter. Bioinformatic analyses of *rsmY* and *rsmZ* sRNA sequences revealed archetypal RsmA binding sites in both. In *Pseudomonas fluorescens* RsmA has

previously been shown to bind the sequence 5ô-$^{A}/_{U}$CANGGANG$^{U}/_{A}$-3ô (Lapouge *et al.,* 2007; Schubert *et al.,* 2007). In our study, a sequence 5ô-TCAAGGATGA-3ô, matching this binding site was identified in *rsmY* at the +35 site with respect of the putative transcriptional start site. A similar sequence with a single base pair deletion was also identified at the +20 site in the *rsmZ* sequence; 5ô-TCA_GGATGA-3ô. However, the observed expression of both *rsmY* and *rsmZ* in the PHA45A mutant is not consistent with the widely held model of transcriptional regulation within the cascade, for which GacS is believed to be essential. Therefore, despite the presence in *P. putida* CA-3 of the key Gac/Rsm regulatory elements, (exhibiting conserved structural characteristics, e.g. putative RsmA binding sites within the sRNAs), our findings suggest that GacS may not be the sole factor regulating *rsmY* and *rsmZ* sRNAs under PHA accumulating conditions. Similarly, a recent report by Lalouna and co-workers reported expression of both *rsmY* and *rsmZ* sRNAs in a GacA negative mutant of *Pseudomonas brassicacearum* (Lalouna *et al.,* 2012). The authors suggested that the expression of the *rsmY* and *rsmZ* sRNAs is not completely controlled by the GacS/GacA system and proposed that other regulators may be involved in regulating transcription of these sRNAs. Our identification of *rsmY* and *rsmZ* sRNA expression in *P. putida* CA-3 also suggests that an alternative mediation of sRNA expression by additional, unknown elements may be possible. Despite the expression of these sRNAs in the *gacS*::Tn*5* mutant, *gacS* disruption in *P. putida* CA-3 definitively inhibits PHA accumulation. We therefore sought to establish whether the loss of *gacS* expression in mutant PHA45A affected post-transcriptional expression of the PHA synthetic apparatus in *P. putida* CA-3.

SDS-PAGE analyses of N-lim cultures indicated a ~60 kDa protein absent in PHA45A, when compared with wild type protein profiles. The size as indicated on the gel is consistent with the predicted size of PhaC1 in *P. putida* CA-3, of ~62 kDa. A comparative analysis of the protein profile from a previously generated *phaC1* disrupted mutant, B11, revealed a similar protein absence from the gel. Complementation of *gacS* and *phaC1* in these mutants, respectively, restored this protein and the PHA accumulation phenotype in both strains (Figure 6). It appears therefore that a key interaction between the Gac/Rsm regulatory cascade and PHA synthesis in *P. putida* CA-3 occurs via the post-transcriptional regulation of *phaC1*, a protein which we have previously shown to be required for PHA synthesis in this strain (O'Leary *et al.,* 2005). It should be noted that efforts undertaken to identify a consensus RsmA binding site 5'-$^{A}/_{U}$CANGGANG$^{U}/_{A}$-3' within the promoter region of published *phaC1* sequences did not reveal any significant sequence similarity within the promoter sequences. The latter contributes further to the atypical nature of GacS dependent regulation of PHA synthesis observed in this strain however it does not rule out the potential involvement of an as yet unidentified RsmA recognition motif associated with *phaC1* mRNA.

Regulation of PHA accumulation in bacteria is known to be dependent on many physiological and regulatory factors, including transcriptional regulation, redox potential, nutrient limitation and availability of metabolic intermediates. The physiological status of the cell is therefore vital to the accumulation of these biopolymers. The findings reported here further develop this understanding as we demonstrate, for the first time, the regulation of mcl-PHA production via GacS. The GacS/GacA system has previously been described in many bacterial genera as regulating a variety of cellular elements

including biocontrol characteristics, stress resistance, motility, virulence, central cellular metabolism and PHB biosynthesis, (reviewed in Lapouge *et al.,* 2008). The observance of GacS mediated regulation of PHA production is perhaps not surprising given its role in dealing with stress resistance and central metabolism, which often coincide with known PHA accumulation conditions (e.g. N-, S- and P-limitation). It is possible that the GacS/GacA sensor kinase acts in concert with other environmental sensors involved in carbon availability and redox sensing to coordinate the cellôs response, e.g. by switching the metabolic focus from carbon utilisation and growth to storage. Such processes must clearly involve global/common regulatory mechanisms, affecting various cellular processes including the production of PHA in different species. Indeed, this may potentially explain why both scl- and mcl- PHA synthesis are regulated via mechanisms involving GacS, despite being distinct both genetically and biochemically. Future work in this area is required to further explore the role of GacS and the specific operation of the associated sRNA regulatory cascade in mcl-PHA production and to determine whether any biotechnological advances in PHA synthesis can be achieved via recombinant manipulations of the regulatory cascade in PHA producing strains.

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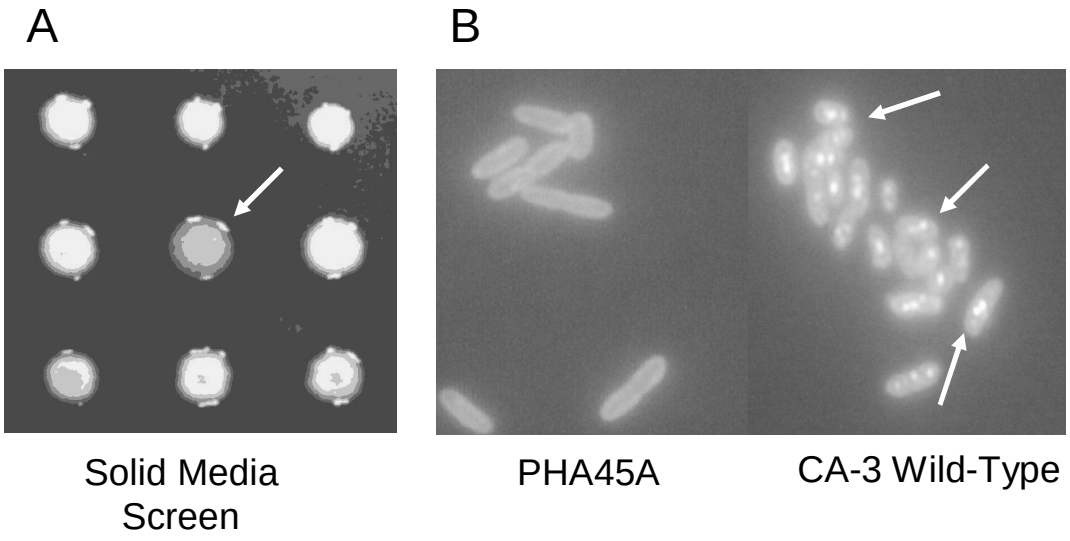


Figure 1: (A) Solid media screen of Tn5 mutants on Nitrogen Limiting E2- Minimal media supplemented with 60 mM Citrate. Arrow indicates mutant exhibiting reduced opacity phenotype (B) Fluorescence microscopy of Tn5 mutant PHA45A, and wild-type *P. putida* CA-3 grown on Nitrogen Limiting E2- Minimal media supplemented with 60 mM Citrate stained with Nile Red at 1000X magnification using Filter I3. Intracellular PHA granules indicated with arrow.

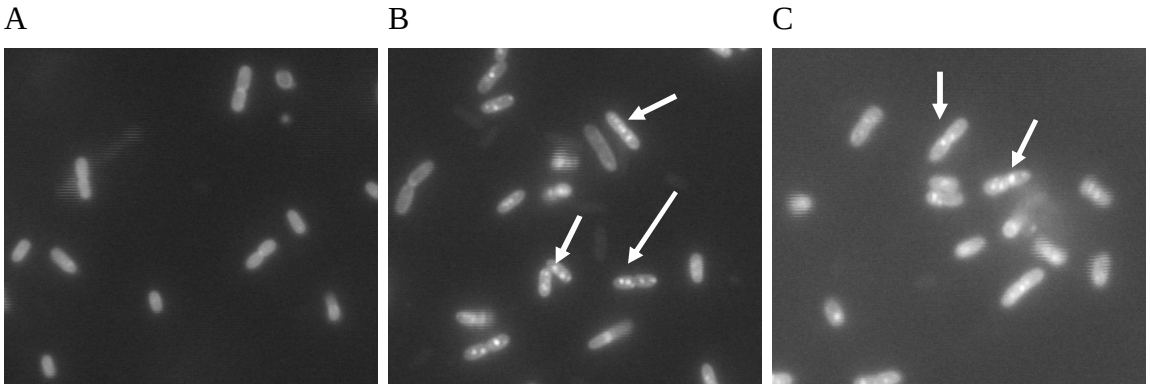


Figure 2: Fluorescent microscopy of Tn5 mutant PHA45A (A), PHA45AgacS+ (B), and *P. putida* CA-3 wild-type (C) grown on Nitrogen Limiting E2- Minimal media supplemented with 60 mM Citrate stained with Nile Red at 1000X magnification using Filter I3. PHA granules indicated with arrow

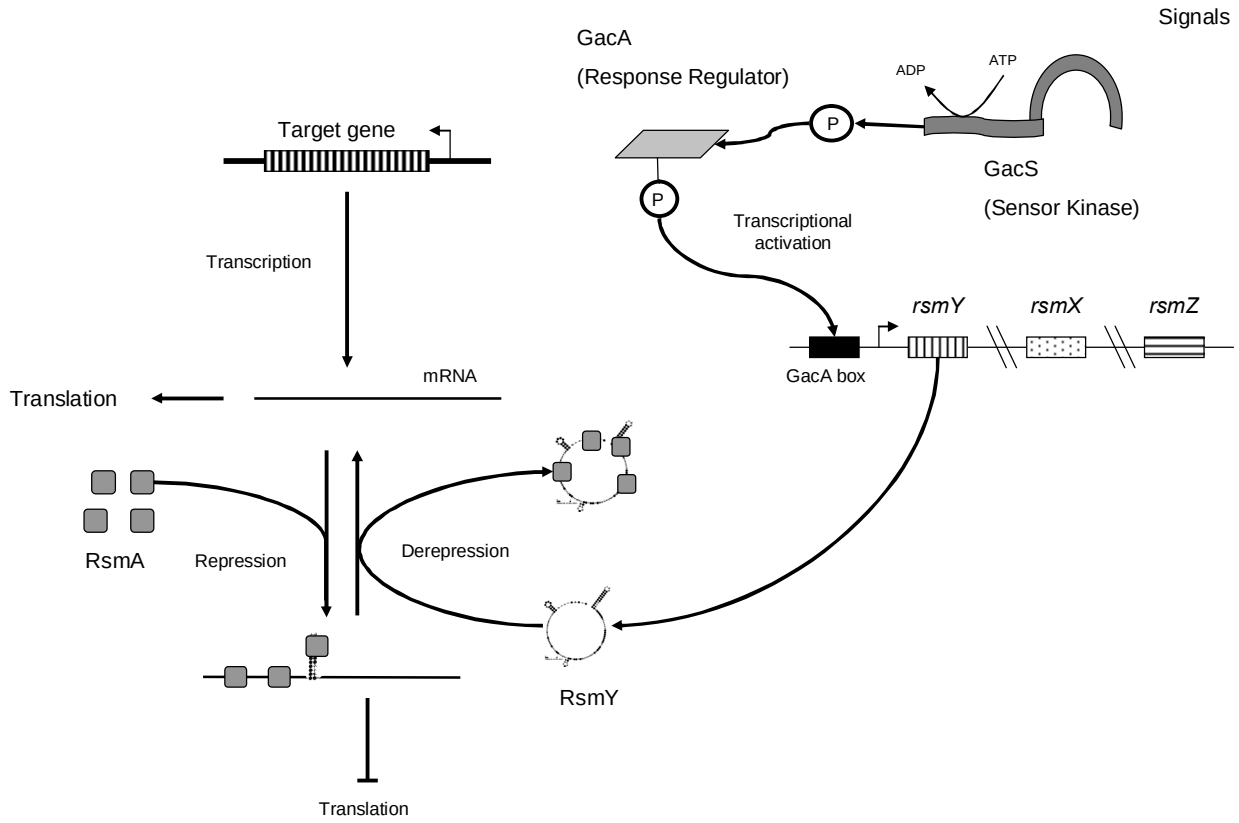


Figure 3: Current understanding of GacS/GacA cascade. Under repressive conditions mRNA binding RsmA sequesters target mRNA preventing RNA polymerase binding and protein synthesis. Under non-repressive conditions RsmA is bound by *rsmY, rsmZ* and *rsmX* sRNAs allowing translation of mRNA to form protein.

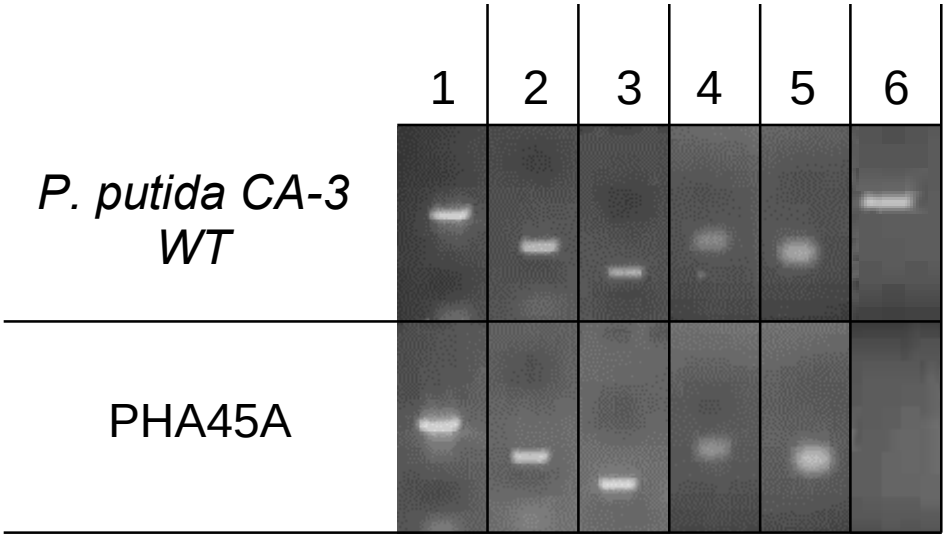


Figure 4: RT-PCR analysis of elements involved in GacS signalling cascade. 1; 16S control, 2; *gacA* response regulator, 3; *rsmA* mRNA binding protein, 4; *rsmY* sRNA molecule, 5; *rsmZ* sRNA molecule, 6; *gacS* sensor kinase

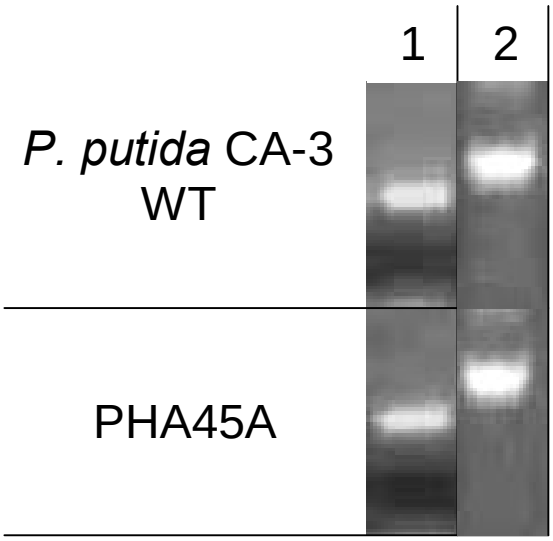


Figure 5: RT-PCR analysis of expression of genes required for PHA accumulation. 1; *phaC1* class II poly-(3)-hydroxyalkanoate synthase, 2; *phaG* 3-hydroxyacyl-CoA:ACP acyl transferase.

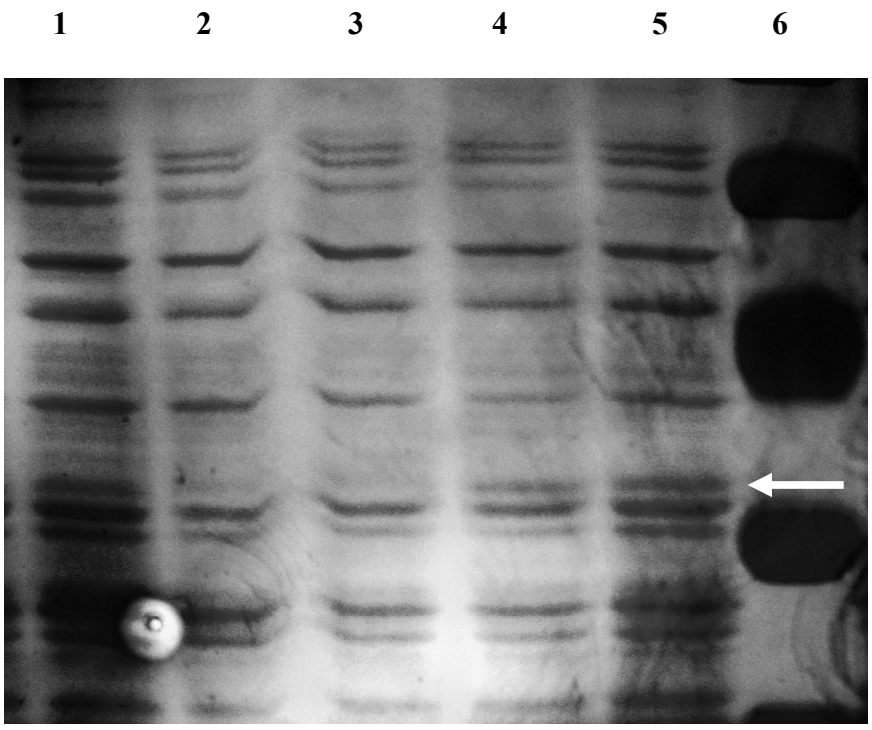


Figure 6: SDS-PAGE of crude soluble protein extracts. Lanes: 1; *P. putida* CA-3 WT, 2; PHA45A, 3; B11, 4;PHA45AgacS+, 5; B11phaC1+, 6; Molecular mass standards. PhaC1 protein band denoted with arrow.

Name	Primer Sequences	Annealing temp (°C)	Source
rpoS-817	ACCCGACAAGCAGCAGCGTGAAGTG	65	This Study
feS-45	CGACTGCGTGGAAGTCTG	65	This Study
ACDH-1520	CAACCCGAATGAAATTGGCG	65	This Study
lysR-524	TGAAGGACTTCAAGAAGGTGTC	65	This Study
gacS-FW	GTACCGGCACCCAACTCCTG	65	This Study
gacS-RV	GGATCAGGTCCCGCAGGTTG	65	This Study
gacA-FW	GATCTGGTACGAACCGGTATTACA	60	This Study
gacA-RV	GACGATCATCAGGGCAATCTGGAT	60	This Study
rsmA-FW	GATCTGGTACGAACCGGTATTACA	62	This Study
rsmA-RV	GACACGCTCCAGCAGCAGGT	62	This Study
rsmY-FW	ACGTTGCGCAGGAAGCGCATA	65	This Study
rsmY-RV	CGCCGAAGCGGGGTTTTCCA	65	This Study
rsmZ-FW	CGCTTGCTCGCCTGGCTGAA	65	This Study
rsmZ-RV	GCCTTCATCCTGAAAGCCTGTGC	65	This Study
C1-F	ATGAGTAACAAGAACAACGAT	55	O'Leary *et al.*, 2005
C1-R	TCAGCGCTCGTGAACGTAGGT	55	O'Leary *et al.*, 2005
G-F	ATGAGGCCAGAAATCGCTGT	58	O'Leary *et al.*, 2005
G-R	TCAGATGGCCAATGCATGCT	58	O'Leary *et al.*, 2005
FrsmY-FW	AAGCTTACATGGACGTTGCGCAG	65	This Study
FrsmY-RV	*TCTAGA*AAAACCCCGCCGAAGCG[a]	65	This Study
FgacS-FW	*AAGCTT*GTGCTCGATCGCTTGGGAATCC[b]	65	This Study
FgacS-RV	*TCTAGA*TCAAGCGCTCAATCTGGCCTGT[a]	65	This Study
FphaC1-FW	*AAGCTT*ATGAGTAACAAGAACAACGAT[b]	60	This Study
FphaC1-RV	*TCTAGA*TCAACGCTCGTGAACGTA[a]	60	This Study
27F	AGAGTTTGATCCTGGCTCAG	55	This Study
1492R	GGTTACCTTGTTACGACTT	55	This Study
TNINT	GACCTGCAGGCATGCAAGCTCGGC	59	Espinosa-Urgel *et al.,* 2000

Table 1: Oligonucleotide sequences and annealing temperatures of primers utilised in polymerase chain reaction amplification of gene targets from *P. putida* CA-3 in this study. a: XbaI restriction site; b: HindIII restriction site.

		P. putida **F1**	***P. putida*** **GB1**	***P. putida*** **KT2440**	***P. putida*** **BIRD1**	***P. putida*** **W619**	***P. fluorescens*** **Pf-5**
P. putida CA-3	*phaC1/ phaA*	98.1	95	*98.5*	97.6	89.3	83.6
	phaG	99	93.7	*98.8*	98.3	86.8	75.8
	gacS	98.3	92.6	98.6	98.4	83.3	77.7
	gacA	99.3	91.5	98	98.5	84.5	79.3
	rsmA/csrA	100	98.4	100	87.8	92.9	87.4
	rsmY	98.4	98.4	98.4	99.2	88.9	83.1
	rsmZ	97.3	94.5	97.3	95.5	90.8	73

Table 2: BLAST n % nucleotide identity. Comparison of the *pha* and Gac/Rsm cascade genes cloned from *P. putida* CA-3 to other PHA accumulating *Pseudomonads*.

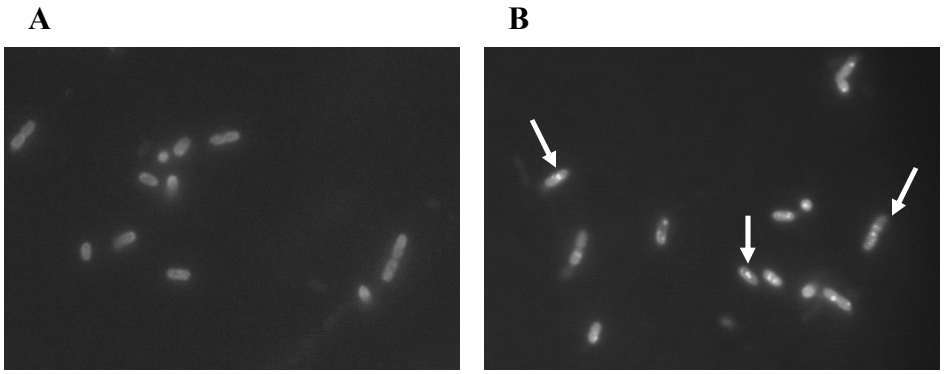


Figure S1: Fluorescent microscopy of Tn5 mutant B11 (A) and B11phaC1+ (B) grown on Nitrogen Limiting E2- Minimal media supplemented with 60 mM Citrate stained with Nile Red at 1000X magnification using Filter I3. PHA granules indicated with arrow.

Appendix 1

Supplementary Table

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

CORA
Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Ryan, W.J. 2013. *Investigation of global regulators influencing styrene metabolism and bioplastic synthesis in Pseudomonas putida CA-3*. PhD Thesis, University College Cork.

Access to pages 166 – 204 is restricted for 12 months by request of the author. Full text will be available from 22 January 2013

CORA Cork Open Research Archive http://cora.ucc.ie

Chapter 5

General Discussion

The potential to develop microbial based strategies for bioremediation of aromatic compounds has been the focus of multiple research groups over the last number of decades. Similarly, microbial bioplastic production has also been the subject of intense investigation since PHA accumulation was first observed in 1926 by Lemoigne. To date however our understanding of the nature by which these processes function intracellularly have largely been limited to characterisations of the specific pathways involved at the genetic and biochemical levels. These studies, although necessary as a basis for understanding the mechanisms involved provide a poor insight into how these biotechnologically significant pathways are incorporated into general cellular metabolism. Knowledge of this integration is required to enable thorough optimisation of the use of such microorganisms as whole cell based systems for biotechnological applications. The present study sought to further our understanding in this field by screening and characterising putative global regulatory molecules influencing the styrene catabolic pathway and PHA accumulation capacity in the well characterised bioreactor isolate *P. putida* CA-3.

Carbon catabolite repression is a common regulatory control influencing the expression and functioning of pathways responsible for aromatic hydrocarbon degradation, particularly in the mixed carbon environments commonly associated with aromatic contamination. CCR mediates its effect by selectively repressing aromatic metabolism in favour of an energetically preferential carbon source present in the environment, integrating global and pathway specific impacts. Previous work into the effects of CCR on the metabolism of styrene, the simplest alkylbenzene, identified CCR regulation of the pathway in the presence of citrate in *P. putida* CA-3 (O'Leary *et al.,*

2001). The current study focused on identification of putative regulators responding to carbon catabolite repression (CCR). O'Connor *et al.* (1997) previously reported the use of a colorimetric assay to detect activity of the *styAB* encoded monooxygenase necessary for styrene metabolism. The authors reported transformation of the colourless compound, indole, to dark blue indigo by styrene monooxygenase activity enzyme under a variety of conditions. In the present study we employed these previous observations to develop a solid media screen combining indole and citrate to identify colonies with reduced sensitivity to the effects of CCR. A Tn*5* library of *P. putida* CA-3 mutants generated in this study was screened on the citrate/indole media and identified a *clpX* mutant displaying the desired phenotype. A literature review of previously described roles for *clpX* in this area highlighted a proteomic study by Nikodinovic-Runic *et al.* (2009) which described an increase in ClpX protein expression in *P. putida* CA-3 under growth on styrene. ClpX forms part of the ClpXP two component system and degrades both damaged and tagged proteins in the cell. ClpX recognises and unfolds target proteins and feeds them into the associated ClpP protease for degradation (Kress *et al.,* 2009).

Analysis of the growth of the *clpX* disrupted mutant on styrene and glucose sources suggested comparatively similar growth between wild type and mutant cultures. However, phenylacetic acid (PAA) and citrate metabolism were significantly affected with reductions in growth rates of 56% and 53%, respectively, in ClpX deficient mutant cultures when compared with CA-3 wild type equivalents. This presented somewhat of a quandary however, as styrene metabolism is known to proceed via PAA (reviewed in Mooney *et al.,* 2006). Subsequent RT-PCR analysis of key gene representatives of the

PACoA catabolic operons demonstrated expression of the all pathway genes under the conditions tested, suggesting that the effect on PAA metabolism was mediated at another level. O'Leary *et al.* (2011) described a similar phenotype of growth on styrene and no apparent growth on PAA in a σ^{54} encoding *rpoN* mutant of *P. putida* CA-3. Their efforts identified σ^{54} dependent expression of the PAA transport gene *paaL* and expression of a vector harbouring *paaL* restored growth in this mutant on PAA to wild-type levels. We therefore sought to determine whether disruption of *clpX* in our mutant was having a similar effect on PAA transport into the cell, and thus on growth, via introduction of the *paaL* harbouring plasmid into the *clpX* mutant CCR25. Similarly the constitutive expression of *paaL* in the mutant would overcome any potential delay in initiation of *paaL* transcription which could potentially explain the increased lag phase observed in this mutant when grown on PAA (Chapter 2; Supplemental Figure S6; pg. 115). However analysis of this complemented mutant failed to show restoration of growth on PAA to wild type levels. It would therefore appear that transport of PAA is not a limiting factor to growth in a *clpX* disrupted mutant, a finding supported by the identification of *paaL* and other examined PACoA catabolon gene transcripts in both mid and early exponential phase CCR25 cultures by RT-PCR.

In evolutionary terms the large-scale production and environmental dissemination of aromatic hydrocarbons is a relatively recent event. Similarly the development of pathways responsible for degradation of styrene and phenylacetic appears to reflect a more recent evolutionary event. ClpX has previously been reported to be involved in regulating proteins involved in stress by mediating protein turnover (Flynn *et al.,* 2003; Stephani *et al.,* 2003) and the presence of aromatic hydrocarbons are often detected as

stresses by the cell (Shingler, 2003). Thus the disruption of *clpX* may be reducing the cells overall fitness in dealing with the presence of aromatic compounds. This theory could be supported by the low solubility of styrene in water, levels of which may not be sufficiently toxic to the cell to warrant a full stress response. However the increased solubility of PAA in water increases the potential for a stress response to be imposed. It is also possible that ClpX regulation of PAA metabolism developed in parallel with the evolution of the PACoA catabolon. Investigations into styrene and PAA metabolism in *Pseudomonas putida* Y2 by Bartolomé-Mártin and co-workers (2004) previously identified the presence of two functional, yet evolutionarily distinct, *paa* catabolic operons with quite distinct features and regulatory influences. Their work demonstrated that disruption of the *paaL* gene did not prevent metabolism and growth on styrene in their strain. Genetic characterisation subsequently identified one *paa* catabolon associated with the *sty* operon and a second involved solely with the metabolism of phenylacetic acid. Thus regulatory control by *clpX* may not be fully incorporated in the *sty* operon associated PACoA catabolon. Our growth curve analyses also show some support for this theory given the similar growth profiles on styrene and the fact that log phase growth rates on PAA of both CA-3 WT and CCR25 mutants are comparable. In the absence of a genome sequence for *P. putida* CA-3 it is not possible to be definitive on this possibility yet the presence of a second *paa* cluster cannot be discounted. However the similar growth characteristics of our *clpX* mutant, exhibiting similar growth on styrene while significantly reduced growth on PAA, could indeed suggest that potential. Therefore while ClpX appears not to be a terminal regulator of PAA metabolism it perhaps acts to mediate a switch to PAA metabolism which is not required when PAA is

fed from an upstream pathway such as the *sty* catabolon. Further work remains to be carried out in this area, perhaps including a systems biology approach, to elucidate the flux rates through various steps in the PAA catabolism route, to identify the role of ClpX in regulating aromatic metabolism and confirm its biotechnological potential for improving aromatic metabolism in bacteria.

As discussed in chapter 1, PHAs are biodegradable polyesters with a variety of commercial, industrial and medical applications. PHA production has been described in a number of bacterial species and genera including *Bacilli, Ralstonia,* and *Pseudomonas* among others. As such the genes associated with PHA accumulation have been identified in a wide and diverse range of bacterial genera suggesting an extended evolutionary history for this pathway (reviewed in Philip *et al.,* 2007). Two major types of PHA are found in nature; short chain length, scl-, and medium chain length, mcl-, PHA as described in chapter 1. However the properties exhibited by mcl-PHAs, such as those produced by *P. putida* CA-3, enable their use in a far greater number of commercial and industrial applications. Furthermore as mankind has clearly passed the peak in global oil production, alternatives to petrochemical plastic production are not only appealing but also essential to our future (Murray and King, 2012).

Interestingly, while many strains have been found to possess the genes necessary for PHA accumulation, and indeed have been demonstrated to accumulate the bioplastic, the organisation of the genes responsible for their accumulation can differ markedly in both operonic structure and regulatory control. Many strains for example possessing the genes required for scl-PHA (*phbABC*) accumulation have been found to exhibit different organisation of the *phb* operon with different genes in different orders such as

Pseudomonas acidophila (Umeda *et al.,* 1998) and *R. eutropha* (Peoples *et al.,* 1989a;b). Others, such as *Rhizobium meliloti* (Tombolini, *et al.,* 1995) have demonstrated two separate, unlinked loci for the three genes. Interestingly however the organisation of the genes necessary for mcl-PHA accumulation in *Pseudomonas* species has been demonstrated to be highly conserved suggesting the potential for common regulatory controls to also be in effect across these strains (de Euginio *et al.,* 2007).

Production of PHAs is dependent on a cells possession of the appropriate genetic tools and the provision of essential monomers via central carbon metabolism. In scl-PHA producing strains polyhydroxybutyrate formation proceeds strictly via the condensation of two acetyl-CoA moieties, followed by reduction to the hydroxyacyl-CoA monomer required for PHB synthesis. However in some mcl-PHA producers such as *P. putida* CA-3 monomers can be produced from unrelated carbon sources such as industrial waste streams. This therefore highlights the potential for employing this strain to produce a high quality bioplastic in the form of mcl-PHA from an unrelated waste product such as styrene. To date investigations into the effect of general cellular metabolism and the effect of the supplied carbon source on PHA production have yielded interesting results. Studies have previously shown that the supply of substrates such as fatty acids and glycerol results in the majority of PHA formed comprising 3-hydroxoctanoate monomers while the supply of sugars such as glucose results in a majority of 3-hydroxydecanoate monomers (Haywood *et al.,* 1990; Huijberts *et al.,* 1992). Investigations into the biochemical pathway responsible for PHA accumulation have also demonstrated that the same gene locus is responsible for PHA accumulation regardless of carbon source supplied (Huijberts *et al.,* 1992). Thus it would appear that effects mediated outside of

the pathway encoded by the *pha* gene locus play a role in regulating PHA accumulation and that a knowledge of PHA production regulation outside pathway specifics is required to fully exploit the biotechnological potential of PHA production. Studies have been undertaken investigating the effect of altered intracellular redox potential and the perception of extracellular stress to increase PHA accumulation capacity of strains. Investigations such as those by Lim *et al.* (2002) demonstrated that a high intracellular $NADPH/NADP^+$ ratio resulting from disruption of genes encoding glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase favoured PHA accumulation, improving production by 41% wt/wt. Other studies have investigated the role of regulators of redox potential and carbon consumption on PHA synthesis. Lynch and Lin (1996) reported that increasing the pool of reducing equivalents in the cell through *arcA* disruption can result in PHA production up to 35% (wt/wt) under conditions previously not permissive of PHA accumulation. Furthermore Nikel *et al.* (2008) have combined this with disruption of *creC*, a regulator of carbon utilisation, to increase PHA accumulation compared to wild-type. These studies demonstrate therefore the importance of knowledge of metabolic networks feeding into/influencing PHA biosynthetic pathways in bacteria. They also demonstrate that an overall understanding of PHA accumulation will only be developed with the elucidation of as comprehensive a picture as possible of the network of global regulatory influences acting on same. Here we utilised a solid media screen previously published by our group to identify mutants with reduced ability to accumulate PHA based on a reduction in colony opacity (OõLeary *et al.,* 2005). Mapping of the gene disruption sites of the mutants selected based on this

screen identified two mutants displaying the desired phenotype in which the *gacS* gene was disrupted.

Characterisation of the *gacS* disrupted mutant confirmed the lack of PHA accumulation via fluorescence microscopy. GacS is part of the Gac/Rsm cascade and regulation by this cascade has previously been reported to be mediated at the post transcriptional level, a review of Gac/Rsm cascade regulation can be seen in chapter 1; section 5.4. Our investigation in *P. putida* CA-3 concluded that the loss of PHA accumulation was attributable to a loss of PhaC1 protein expression in the *gacS* disrupted mutant despite RT-PCR analysis identified expression of the *phaC1* gene. This finding is consistent with previous reports of post transcriptional regulation by the Gac/Rsm cascade (Lapouge *et al.,* 2008). GacS involvement in regulation of mcl-PHA had not previously been characterised, although a report by Castaneda *et al.* (2000) did identify a similar loss of scl-PHA production in *A. vinelandii* following *gacS* disruption. The means by which this control was effected was not identified in that study. Our analysis of transcription of other Gac/Rsm cascade genes revealed an atypical expression profile in our strain with *rsmY* and *rsmZ* both transcribed in the *gacS* disrupted mutant. Expression of these sRNAs has previously been reported to be dependent on activation by the GacS/GacA two component system. In order to understand the cascade in more detail we first considered the merits of utilising a knockout of the post-transcriptional regulator RsmA to look for restoration of PHA production. This protein has previously been reported to bind recognition sites in the promoter regions of target mRNA molecules and prevent protein translation and are sequestered by the GacS/GacA regulated sRNAs (Lapouge *et al.,* 2008). However previous reports have suggested that disruption of

central regulatory control such as RsmA has the potential to impose detrimental disruptions of core metabolic networks, resulting in poor cell growth and limiting the capacity for accurate interpretation of any findings (reviewed in Lapouge *et al.,* 2008). An alternative strategy to over-express the *rsmY* sRNA was subsequently devised, as it exhibited a strong consensus RsmA binding site, and minimised the risks of physiological imbalances likely to have occurred following an *rsmA* gene deletion. Thus *rsmY* was cloned and expressed from a suitable vector in the *gacS* disrupted mutant in an attempt to facilitate restoration of the PHA accumulation phenotype in this strain. However, this proved unsuccessful as PHA production was not observed in the *rsmY* over-expressing *gacS* mutant. It is possible that the overexpression of *rsmY* in the cell had unintended consequences for cellular metabolism and therefore prevented accumulation of the bioplastic. Follow up studies into overexpression of this sRNA in wild-type CA-3 identified an altered PHA monomer composition in the overexpression strain to that of the wild-type CA-3 with a 16% reduction of C_8 and a corresponding increase in C_{10} monomers detected in the *rsmY* overexpression strain. The observed altered monomer composition supports our previous suggestion of metabolic networks effects beyond the level of PHA synthase activity as the supply of monomer units is controlled by the core carbon metabolism and fatty acid biosynthesis pathways rather than solely by PhaC1 synthase or PhaG transacylase activity.

What was clear from out findings was that GacS disruption results in disruption of PhaC1 translation as demonstrated in chapter 3 however the exact means by which it this occurs has yet to be identified. The regulation of mRNA translation is mediated by a promoter recognition site by mRNA being bound by RsmA. Bioinformatic investigations

performed in this study failed to identify an archetypal RsmA binding site in the promoter region upstream of *phaC1*. This in itself is interesting given the evidence which exists for conserved Gac/Rsm cascade systems operating in other bacteria, commonly characterised by RsmA repression of target gene translation being relieved via GacS activated expression of sRNAs which sequester RsmA out of solution and relieve said repression events. Our studies did identify an archetypal binding site as being present in both *rsmY* and *rsmZ* sRNA sequences, suggesting this cascade is present in *P. putida* CA-3. However the relationship between GacS and PhaC1 expression raises the interesting prospect of the involvement of another RsmA like protein in post transcriptional regulation. Further work is required to fully characterise the regulation of PhaC1 expression in *P. putida* CA-3, potentially via modification of the *phaC1* promoter region by directed evolution and mRNA binding assays with RsmA to identify altered PhaC1 expression phenotypes. Furthermore characterisation of the means by which PhaC1 expression is controlled may prove to be of significant biotechnological potential in improving PHA production.

Finally, we sought to determine whether GacS regulation of PHA production was restricted to PHA synthesis in *P. putida* CA-3 alone, and whether the reliance on N-lim in the screen created the bias for the detection of regulators which responded solely to extracellular nitrogen stress. In relation to the latter query, previous work by OôLeary *et al.* (2011) reported the novel regulatory associations operating in this strain such as ů^{54} regulation of PaaL, a phenylacetic acid transporter. Furthermore, in a follow-up study to that of CastaÑeda *et al.* (2000), Hernandez-Eligio *et al.* (2012) described the Gac/Rsm mediated regulation of scl-PHA accumulation in *A. vinelandii* as being a result of RsmA

binding to a pathway specific transcriptional activator of *phb* gene expression. This method of control appears quite distinct from that observed in *P. putida* CA-3 in which regulation is mediated at the post-transcriptional level of PhaC1 production. This therefore further raised the question of whether the involvement of GacS in controlling PHA accumulation was unique to our strain. The disruption of *gacS* in second PHA producing *P. putida* strain, *P. putida* S12, was thus carried out and investigated to determine whether this regulation was more widespread among *Pseudomonas putida* species. Results demonstrated a similar loss of PHA accumulation in *gacS* disrupted *P. putida* S12 to that observed in CA-3. Thus while the finding is in no way interpreted as a comprehensive assessment of the *Pseudomonas* genus, it is clear that GacS regulation of PHA production is not a unique feature of our strain and this finding may have broader impacts in relation to PHA accumulators within this genus.

In order to establish PHA accumulation by the cell under alternative stress conditions, phosphorus limitations were imposed on wild type and *gacS* disrupted mutant cultures. P-lim stress also produced a PHA negative phenotype in the *gacS* disrupted mutant. This finding suggests the response mediated by the GacS sensor kinase is not dependent on one particular stress, N-lim, but rather responds to intracellular triggers, rather than particular components in the cellôs environment. Interestingly, P-lim induced PHA accumulation approximately 3 hours earlier than was observed under N-lim during growth curves. This is likely due to a greater cellular demand for phosphate for elements such as the DNA backbone, intracellular signalling (e.g. ppGpp) and energy transfer (e.g. ATP, GTP etc.) compared with cellular nitrogen demands which stem mostly from amino acid production (NH_2). The common PHA deficient phenotype observed in the *gacS*

disrupted mutant under distinct stress conditions is perhaps not surprising as, although the exact means by which GacS activation occurs is unknown, a previous report by Takeuchi *et al.* (2009) identified increased sRNA expression in the presence of 2-oxoglutarate, succinate and fumarate in *P. fluorescens* CHA0. This finding highlights the potential for TCA cycle intermediates to act as GacS ligands; potentially linking Gac/Rsm system activation to both the intracellular status of the cell and also the potentially the presence of preferred extracellular carbon sources. This provides an interesting platform for further study as production of PHA for commercial use will depend a great deal on the optimisation of batch culturing of cells, wherein intracellular conditions as well as nutrient limitation may play a central role in ensuring an efficient process.

In summary the case for the provision of a bioplastic alternative to replace the increasingly expensive petrochemical materials in use today necessitates the need for research in this area to continue if commercial production is to be realised. While research has taken place into diverse PHA producing strains and respective classes of bioplastic, mcl-PHA still provides the greatest opportunity for real world application given mcl-PHA plastics are more flexible and have a wider variety of potential uses than scl-PHA. The capacity of strains to produce mcl-PHA from unrelated carbon sources further adds to their appeal as substrates for production are widely available, many at low cost. The mcl-PHA producing strain studied in this thesis, *P. putida* CA-3, possesses the ability to produce this plastic from such unrelated carbon sources. Furthermore this strain also harbours the ability to metabolise environmentally damaging industrial pollutants such as styrene from which it can also produce PHA (OôConnor *et al.,* 1993). In this strain therefore we have the potential to utilise an industrial waste product in the

production of an environmentally friendly value added product which can easily substitute the plastics that have become vital to our way of living. Our efforts detailed in this thesis have attempted to investigate and overcome some of the hurdles currently hampering the commercial application of this technology. It is clear that many factors are at play in the regulation and integration of these processes with overall cellular physiology and metabolism. Indeed, as time goes on it is likely that further elucidation of these complex regulatory networks will come to light, enabling recombinant strategies to explore new platforms for optimisation of bioremediation and bioproduction strategies.

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