Understanding the complexity of metabolic regulatory systems: An investigation into the regulation of hydantoin-hydrolysis in *Pseudomonas putida* RU-KM3$_s$

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ABSTRACT

It has been well-established that *Pseudomonas* species possess extremely versatile metabolic systems allowing them to utilise a wide range of nutrient sources and, furthermore, that the regulation of these enzyme systems involves highly evolved and sophisticated regulatory machinery. This study examined the complexity of metabolic regulation in *Pseudomonas* using the hydantoin-hydrolysing system of the environmental isolate, *Pseudomonas putida* RU-KM3$_s$. In this system, the genes encoding dihydropyrimidinase and β-ureidopropionase (*dhp* and *bup*) are arranged divergently on the chromosome, separated by a 616 bp intergenic region involved in the transcriptional regulation of these genes. The focus was on the transcriptional regulation of *dhp* expression. DHP activity was found to be sensitive to several environmental signals including growth phase, carbon catabolite repression (CCR), substrate induction and quorum sensing (QS). Bioinformatic analysis of the intergenic region upstream of *dhp* revealed a number of putative binding sites for transcriptional regulators, including recognition sequences for the alternate sigma factors σ^{54} and σ^{38}, as well as for the global regulators Anr (for anaerobic regulator) and Vfr (for virulence factor regulator). The targeted disruption of the genes encoding the transcriptional regulators, Vfr and the major CCR protein, Crc, resulted in a partial relief from repression for the *vfr*$^-$ mutant under quorum sensing conditions and a general decrease in activity in the *crc*$^-$ mutant. This data suggested that both Vfr and Crc were involved in regulating DHP activity. Mutational analysis of the *dhp* promoter revealed that at least two sites were involved in regulating transcriptional activity, one which mediated activation and the other repression. These sites were designated as a putative Anr box, situated 232 bp from the start codon of *dhp*, and a CRP-like binding site, at a position 213 bp upstream of *dhp*. Taken together, this data shows the involvement of several global regulatory factors in controlling the expression of *dhp*. A complex synergistic model was proposed for the transcriptional regulation of *dhp*, involving alternate sigma factors in addition to both global and specific regulators and responding to a number of environmental signals associated with growth phase, including nutrient availability, cell density and oxygen status.

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β-Gal	β-galactosidase, see also *lac*
β-gluc	β-glucuronidase or GUS
AHL	Acylated homoserine lactone
AMP	Adenosine monophosphate
Anr	Anaerobic regulator protein
BCKAD	Branched-chain keto acid dehydrogenase
bp	Base pair
bup	Gene encoding a β-ureidopropionase (BUP) in *Pseudomonas putida* RU-KM3$_s$
cAMP	Cyclic adenosine monophosphate
CCR	Carbon catabolite repression
Crc	Catabolite repression control protein
CRP	cAMP receptor protein
CTAB	Hexadecyltrimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
dhp	Gene encoding a dihydropyrimidinase (DHP) in *Pseudomonas putida* RU-KM3s
ECF	Extracytoplasmic (sigma) factor
EDTA	Ethylene diamine tetra-acetic acid
glnD	Gene encoding the uridyl transferase enzyme
gus	Gene encoding the β-Glucuronidase (GUS) enzyme
Hyd	Hydantoin
LasR	Global quorum sensing regulator mediating elastase production
LA	Luria-Bertani agar
lac	Gene encoding β-galactosidase
LB	Luria-Bertani broth
lux	As in *lux* box, binding site for quorum sensing regulators
NA	Nutrient agar
NB	Nutrient broth
***N*-carbamoylase**	*N*-carbamoylamino acid amidohydrolase
NCG	*N*-carbamylglycine
nt	nucleotides
ntrB	Gene encoding the nitrogen regulator protein B

ntrC	Gene encoding the nitrogen regulator protein C
$OD_{600\ nm}$	Optical density at 600 nm
ORF	Open reading frame
PCR	Polymerase chain reaction
PNPG	*para*-nitrophenyl-β-D-glucuronide
PpuR	LasR homologue in *P. putida* IsoF
QS	Quorum sensing
RhlR	Global quorum sensing regulator mediating rhamnolipid biosynthesis
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNAP	DNA-dependant RNA polymerase
rpm	Revolutions per minute
Succ	Succinate
TCA	Tricarboxylic acid
TE buffer	Tris-EDTA buffer
Tris	Tris-2-amino-2-(hydroxymethyl)-1,3-propandiol
Vfr	Virulence factor regulator
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

Within a single bacterial cell there are numerous enzymes that catalyse the reactions involved in driving cellular processes (Nelson *et al*., 2002; Perrenoud and Sauer, 2005). Some of these enzymes are required in equal amounts under all growth conditions and their genes are expressed constitutively. However, most are only required under certain conditions and their expression is tightly regulated by the metabolic state of the cell and stimuli from their constantly changing environments (Ma *et al*., 2004). This prevents the energy-expensive production of an enzyme when it is not needed by the cell (Perrenoud and Sauer, 2005). Many of these non-essential enzymes have found application in industry, either because the reactions they catalyse degrade harmful, recalcitrant pollutants or because the product of the reaction is economically valuable (Williams and Murray, 1974; Demain, 2000).

1.1 Microbial biocatalysis in industry

Certain bacteria possess specialised enzymes which are sought-after as the biocatalysts of industrially important chemical reactions. Pseudomonads, in particular, produce a wide range of industrial enzymes, which catalyse both biodegradative and biosynthetic reactions (Nakazawa *et al*., 1996). The former, biodegradation, refers to the process whereby organic material is broken down by enzymes produced by living organisms. Here again, microbes of the *Pseudomonas* species have proven useful as they are renowned for their ability to degrade a large variety of both natural and synthetic organics. Since many of these compounds are xenobiotics, the metabolically diverse pseudomonads are important in bioremediation (Nakazawa *et al*., 1996; Hester *et al*., 2000). For instance, it has been shown that diverse strains of *Pseudomonas citronellolis* have the ability to degrade “total petroleum hydrocarbons” (TPH) in environments polluted by “oily sludge” from oil refineries (Bhattacharya *et al*., 2003). Other examples of the biodegradative activities of pseudomonads include the ability to degrade toluene and xylene, encoded on the pWWO plasmid in *Pseudomonas putida* mt-2, with its

plasmid-free derivative, *P. putida* KT2440, being able to degrade a wide range of aromatics including *p*-hydroxybenzoate, vanillate, protocatechuate and a variety of alcohols, acids and aldehydes (Williams and Murray, 1974; Nelson *et al.*, 2002). Another strain, *P. putida* H, is able to degrade methylphenol using enzymes encoded on its pPGH1 plasmid (Hermann *et al.*, 1995). All of the biodegradative enzymes described above perform specialised, non-essential functions and are thus only produced when they are required by the cell; namely in the presence of a recalcitrant substrate in an environment where all the readily degradable nutrients have been depleted. Since such a substrate is not the preferred nutrient source, an understanding of the catabolic repression mechanisms operating under these conditions, reviewed in Section 3.1, could allow for the utilisation of more efficient organisms in bioremediation (Hester *et al.*, 2000).

1.1.1 Biosynthesis of optically pure amino acids

Besides their ability to degrade harmful pollutants in the environment, certain bacteria also produce a number of industrially important metabolites including amino acids, nucleotides, vitamins and organic acids in a process known as microbial biosynthesis (Demain, 2000; Leuchtenberger *et al.*, 2005). An important feature of these products is that they are enantiomerically pure, which is critical in pharmaceuticals due to the side effects and toxicity associated with racemic mixtures of stereoisomers (Maier *et al.*, 2001; Leuchtenberger *et al.*, 2005). In particular, amino acid production represents a multi-billion dollar industry where the products are used in the synthesis of antibiotics such as semi-synthetic penicillins and cephalosporins, antiviral and anti-tumour agents, as well as agrochemicals (Bommarius, 1998; Demain, 2000; Burton and Dorrington, 2004; Leuchtenberger *et al.*, 2005).

One source of these optically pure amino acids is the stereo-selective hydrolysis of 5-monosubstituted hydantoin by hydantoinase enzymes, as seen in a number of *Pseudomonas*, *Bacillus*, *Arthrobacter* and *Agrobacterium* species and even the hypothermophile, *Methanococcus jannaschii* (Ogawa and Shimizu, 1997; Syldatk *et al.*,

1999; Burton and Dorrington, 2004). Hydantoinases have been isolated from plants (Eadie, 1949; cited in Syldatk *et al*., 1999), animals (Wada, 1934; cited in Syldatk *et al*., 1999) and bacteria (Yokozeki *et al*., 1987; cited in Syldatk *et al*., 1999) and are classified as belonging to the EC 3.5.2 group, which includes the hydantoinase, dihydropyrimidinase, allantoinase, carboxymethylhydantoinase, carboxyethyl hydantoinase and *N*-methylhydantoinase enzymes (Syldatk *et al*., 1990). These biocatalysts are described as cyclic amidases and of the six listed above, only carboxymethylhydantoinase and allantoinase use hydantoin (imidazolidine-2,4-dione or 2,4-diketotetra-hydroimidazole) and its derivatives as their natural substrate. The remainder of the EC 3.5.2 enzymes are able to hydrolyse hydantoins, but these are not their preferred substrate (Syldatk and Prietzsch, 1995; Syldatk *et al*., 1999; Altenbuchner *et al*., 2001).

While a number of hydantoin derivatives exist in nature, the most industrially important of these molecules are the 5-monosubstituted hydantoins, which can be used as substrates for the production of D- or L-α-amino acids by hydantoinase-type enzymes (Burton and Dorrington, 2004; Leutenberger *et al*., 2005). The hydrolysis of 5-monosubstituted hydantoins to amino acids occurs via three different reactions (Figure 1.1). In the first reaction, the hydrolysis of the cyclic amide bond at position 2 opens the ring structure of the hydantoin to produce an *N*-carbamylamino acid. This intermediate can then be converted to the corresponding amino acid by either chemical or enzymatic means in the second reaction, with the latter involving an *N*-carbamoylase enzyme (Syldatk and Prietzsch, 1995; Burton and Dorrington, 2004). Finally, the unreacted hydantoin substrate undergoes spontaneous or enzymatic racemisation in the final reaction (Syldatk and Prietzsch, 1995; Altenbuchner *et al*., 2001). The most important compound produced in this way is D-*p*-hydroxy-phenylglycine, which is used as a precursor for semi-synthetic penicillins and cephalosporins (Leutenberger *et al*., 2005)

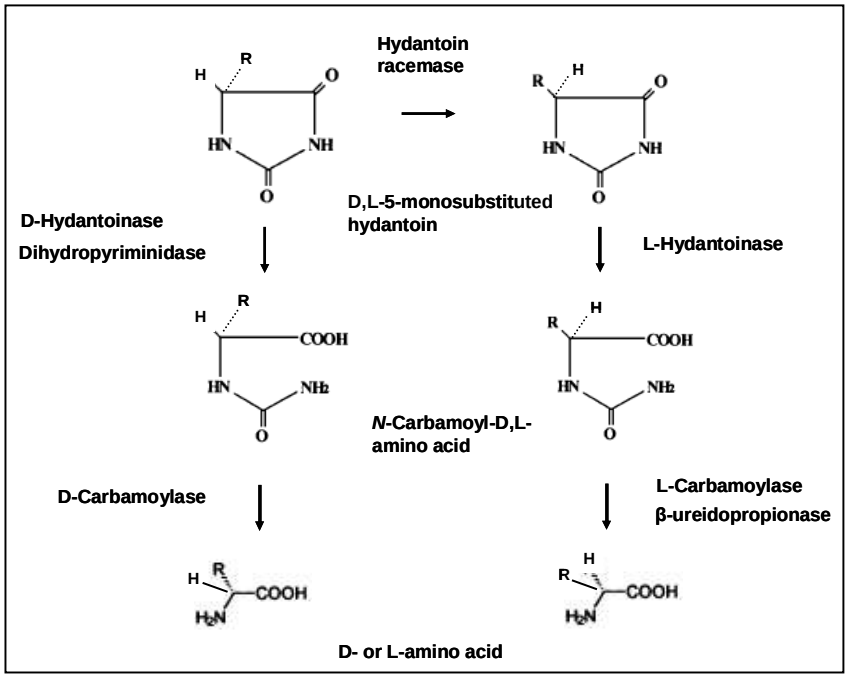

Figure 1.1. The stereoselective enzymatic hydrolysis of 5-monosubstituted hydantoins to form enantiomerically pure amino acids (adapted from Syldatk *et al*., 1999).

The hydrolysis of 5-monosubstituted hydantoins by hydantoinase enzymes is enantioselective and thus produces optically pure products (Syldatk *et al*., 1992; Burton and Dorrington, 2004). The process has a number of advantages for industry, most importantly the strictly stereo-selective conversion of an inexpensive racemic substrate to give yields of up to 100 % (Altenbuchner *et al*., 2001; Burton and Dorrington, 2004). The stereo-selectivity of the hydantoinase reaction may be provided by the hydantoinase, the *N*-carbamoylase, both of the latter two enzymes together, or a separate racemase; all of which ensure the production of optically pure products. D-selective systems are more common in nature, but those which are able to convert hydantoins with aromatic or non-natural aliphatic substitutions are less common (Burton and Dorrington, 2004).

1.1.2 The hydantoin-hydrolysing metabolic system of *P. putida* RU-KM3$_s$

Due to the high value of their optically pure amino acid products, the isolation of novel hydantoinase-producing strains has been the focus of a great deal of research in the recent decades (Burton and Dorrington, 2004). Such a strain, *P. putida* RU-KM3$_s$, was selected from the environment for its ability to utilise hydantoin as a sole nitrogen source in defined medium, a well-recognised method of isolating hydantoin hydrolysing strains (Burton *et al*., 1998). It was further discovered that this strain produced the less common L-amino acids, such as L-valine, L-*nor*-leucine and L–*tert*-leucine at a bioconversion rate of between 60 % and 100 % and that the stereo-selectivity was conferred by the *N*-carbamoylase enzyme (Buchanan *et al*., 2001). Thereafter, the production of amino acids by sonicated crude extract from *P. putida* RU-KM3$_s$ cells, immobilised on Eupergrit®C, was investigated. The immobilisation matrix bound 63 % of the soluble protein and retained 86 % of the original enzyme activity. It was further determined that the optimum pH and temperature for both immobilised and native enzyme were pH 9.0 – 10.0 and 40 ºC respectively. The enzyme activities remained 15 % of their original level after 4 weeks storage whereas the non-immobilised enzyme lost all activity after 2 weeks, indicating increased stability of the enzymes by immobilisation on Eupergrit®C (Bulawayo *et al*., 2007).

The genes responsible for hydantoin hydrolysis in strain RU-KM3$_s$ were identified by random transposon mutagenesis of the genome, using the plasposon, pTnMod-OKm (Matcher *et al*., 2004). In this way, the *bup* gene, encoding a β-ureidopropionase enzyme, was found to be responsible for *N*-carbamoylase activity. Further analysis revealed a *dhp* gene, encoding a dihydropyrimidinase enzyme, upstream of *bup*. Targeted insertional inactivation of *dhp* showed that this gene was responsible for hydantoinase activity. Furthermore, it was found that the *dhp* and *bup* genes were arranged divergently on the chromosome and were separated by an open reading frame (ORF), annotated as ORF1 (Figure 1.2). Bioinformatic analysis of ORF1 suggested that it might encode a putative permease, which may be involved in the transport of substrate into the cell (Matcher *et al*., 2004).

Previous studies concerning the regulation of hydantoin hydrolysis in RU-KM3$_s$ have shown that maximum activity of both dihydropyrimidinase and β-ureidopropionase occurs in early stationary phase and that the activity of both enzymes is strongly induced by the addition of the hydantoin substrate. Furthermore, activity is regulated by carbon catabolite repression (CCR), but is affected by neither the nitrogen status of the cell nor substrate and product inhibition (Buchanan *et al*., 2001; Matcher *et al*., 2004). Bioinformatic analysis of the *dhp*/*bup* intergenic region revealed the presence of a putative binding site for the cAMP receptor protein, CRP (Figure 1.2), which is responsible for mediating CCR in *Escherichia coli* (Collier *et al*., 1996; Matcher *et al*., 2004).

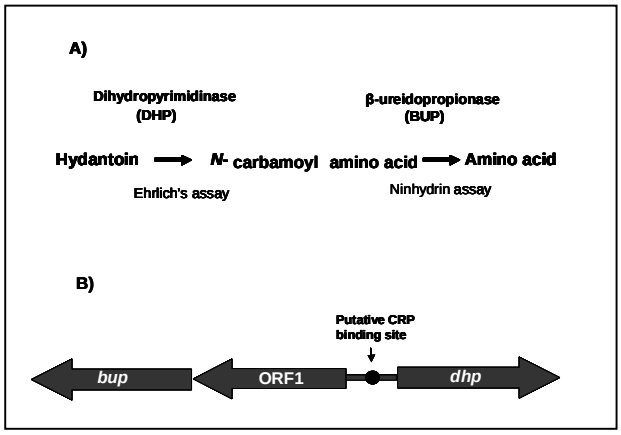

Figure 1.2. The hydantoin-hydrolysing system of *P. putida* RU-KM3$_s$. A) The enzymatic reaction responsible for hydantoin hydrolysis in RU-KM3$_s$ and the colorimetric assays used to detect enzyme activity. B) The organisation of the genes encoding the enzymes responsible for hydantoin hydrolysis in RU-KM3$_s$. The dihydropyrimidinase (*dhp*) and β-ureidopropionase (*bup*) genes are indicated as arrows pointing in the direction in which they are transcribed and the putative binding site for the cAMP receptor protein (CRP) in the intergenic region is indicated by a black oval (Adapted from Matcher *et al*., 2004).

A recent investigation into a dual phase fermentation process for producing the dihydropyrimidinase and β-ureidopropionase enzymes from *P. putida* RU-KM3$_s$ showed that in complex medium there was low enzyme activity, due to CCR of the enzymes; but

under low sugar conditions there was low biomass production. However, using a less favourable but still relatively efficient carbon source, in the form of fatty acids, yielded substantially improved biomass production of 7.3 g/L with increased enzyme activities of 187 nmol/min.mg and 22 nmol/min.mg for dihydropyrimidinase and β-ureidopropionase respectively (Kirchmann *et al*., 2007). It was immediately apparent, given these activity levels, that β-ureidopropionase activity was the rate-limiting factor in the conversion of hydantoins to amino acids under the conditions tested (Kirchmann *et al*., 2007).

In spite of extensive studies regarding the enzymatic degradation of hydantoin and its derivatives, considerably less is known about the genetic organisation in the native strains and the mechanisms that regulate expression of the genes involved (Wiese *et al*., 2001; Burton and Dorrington, 2004). The latter forms the focus of this study and therefore the various mechanisms of regulation of microbial biocatalysis enzyme systems, in particular transcription regulation, are reviewed.

1.2 Transcriptional regulation in prokaryotes

In bacteria, the regulation of cellular biochemical reactions in response to changes in environmental growth conditions occurs in one of two ways; either the amount or the activity of a particular enzyme is controlled. Regulation of the amount of enzyme occurs either at the level of transcription of the relevant genes or at the point of translation of the mRNA into protein, while regulation of the activity of the enzyme occurs post-transcriptionally (reviewed in Perrenoud and Sauer, 2005). In this study, the regulatory mechanisms of interest are those that occur at the first level of regulation, namely at the point of transcription of the genes.

Transcriptional regulation is regarded as the main method of controlling biocatalytic activity in bacteria (Perrenoud and Sauer, 2005). The pathways involved in regulating the transcription of non-essential genes are best understood in *E. coli* (Gralla, 1996). It

has been determined that, although small molecules often influence transcription levels, they do not do so directly. Instead they affect the binding of regulatory proteins to short stretches of conserved nucleotide sequences in a region of DNA close to the transcriptional start (Ishihama, 1993). Transcription factors bind DNA in a sequence-specific manner by the interaction of their amino acid side chains with nucleotide bases and this often occurs in the major groove of the DNA due to its larger size (Weber *et al*., 1982; Busby and Ebright, 1994). Inverted repeats are frequently the sites of binding of transcriptional regulators, which are often dimeric in structure, comprising two identical polypeptide chains (Busby and Ebright, 1994; Perrenoud and Sauer, 2005).

The helix-turn-helix motif of transcriptional regulator proteins is an important sub-structure required for binding DNA and consists of an α-helix joined to a three amino acid stretch containing a glycine, to allow the formation of a sharp "turn," followed by a second helix. The first helix is termed the recognition helix and interacts with the DNA (Busby and Ebright, 1994). Numerous transcriptional regulators in bacteria possess this structure including the *lac* and *trp* repressor proteins of *E. coli* (reviewed in Perrenoud and Sauer, 2005).

Once a transcription factor is bound to the DNA, it can affect gene expression in two general ways; either the protein blocks transcription by the DNA-dependant RNA polymerase, which is defined as negative regulation, or it activates transcription, termed positive regulation (Gralla, 1996). Induction and repression of transcription of a gene occurs in response to the substrate and product, respectively, of the reaction catalysed by the enzyme encoded by this gene (reviewed in Gralla, 1996). Often the enzyme will not be expressed if the product is present, resulting in catabolite repression of enzyme production; while a complementary process, called induction, ensures that an enzyme is only produced when its substrate is present in the growth medium (Gralla, 1996; Ma *et al*., 2004).

1.3 Global control systems and transcriptional regulation in bacteria

In their natural environment, bacterial cells need to regulate many different genes at the same time, in response to an external signal, and this requires global control mechanisms (Ma *et al*., 2004). As illustrated for *E. coli* in Table 1.1, the group of genes regulated in global systems may be very large and the regulatory pathways utilised very complex; in fact many genes form part of more than one global control system (Ma *et al*., 2004; Perrenoud and Sauer, 2005).

Table 1.1. Examples of global transcriptional control mechanisms in *E. coli*

Global control system	Environmental signal	Primary regulator	Number of genes regulated
Catabolite repression	Cyclic AMP concentration	Activator (cAMP receptor protein, CRP)	$\geq$100
Nitrogen utilisation	Ammonia limitation	Activator (NtrC) Alternative sigma factor (σ^{54})	$\geq$70
Aerobic respiration	Presence of oxygen	Repressor (ArcA)	$\geq$21
Anaerobic respiration	Absence of oxygen	Activator Fnr	$\geq$38
Heat shock	Temperature extremes	Alternative sigma factor (σ^{32})	$\geq$12
Oxidative stress	Oxidising agents	Activator (OxyR)	$\geq$30
SOS response	DNA damage	Repressor (LexA)	$\geq$20

(Adapted from Ma *et al*., 2004).

1.3.1. Carbon catabolite repression as a global regulatory system

In order to be competitive, microbial metabolism must be tightly regulated to allow for the utilisation of a wide variety of carbon and nitrogen sources, while ensuring the prioritisation of the most efficient such nutrient sources (Hutter and Niederburger, 1984; Collier *et al*., 1996). When presented with a range of potential energy sources, microorganisms display sequential utilisation of these substrates, with those allowing for

the fastest growth being metabolised first (Collier *et al*., 1996). In the case of carbon metabolism, this sequential utilisation is regulated by carbon catabolite repression (CCR), which is described as the repression of genes required for the utilisation of less efficient carbon sources in the presence of a preferred carbon source (Suh *et al*., 2002).

1.3.1.1. Carbon catabolite repression in *E. coli*

The mechanisms by which CCR occur in bacteria are best understood for the gram-negative enteric microbe, *E. coli*, where glucose is the preferred carbon source. Transport of glucose by the phosphoenolpyruvate-dependant transport system (PTS) drives a protein phosphorylation cascade (Figure 1.3) (Collier *et al*., 1996; Warner and Lolkema, 2003). This involves two phosphor-transfer proteins, Enzyme I (EI) and HPR, as well as a set of sugar-specific permeases, namely Enzymes II (EIIA and EIIB). The phosphoryl transfer proceeds from phosphoenolpyruvate (PEP) to glucose via EI, HPR, EIIA and EIIB respectively. In the absence of glucose, phosphorylated $EIIA^{glc}$ stimulates adenylate cyclase to produce cAMP, which binds to the cAMP receptor protein (CRP). CRP, in turn, binds to specific promoter regions to activate the transcription of certain genes, usually those encoding enzymes required for the utilisation of less favourable carbon sources (Figure 1.3A). In contrast, when glucose is available, the phosphate is transferred from $EIIA^{glc}$ to glucose instead, thus repressing adenylate cyclase activity and inhibiting the transcription of the above genes (Figure 1.3B) (Stulke and Hillen, 1999; Warner and Lolkema 2003).

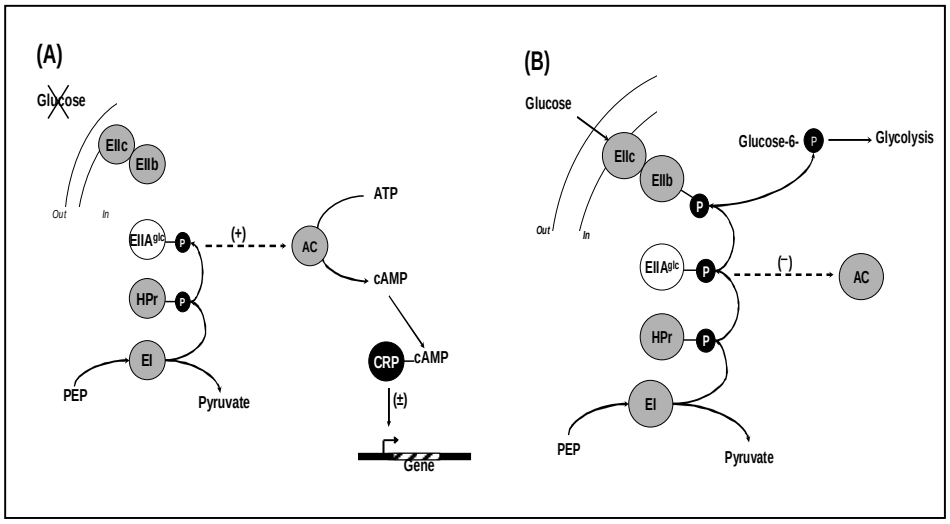

Figure 1.3. The phosphenolpyruvate-dependant transport system (PTS) as a mechanism of carbon catabolite repression in *E. coli*. (A) In the absence of glucose; (B) In the presence of glucose. Important components of the pathway are abbreviated as follows: Enzyme I (EI), Enzymes II (EIIA, EIIB and EIIC), Protein H (HPR), phosphoenol-pyruvate(PEP), adenyl cyclase (AC), phosphate (P) and cAMP receptor protein (CRP). (Adapted from Warner and Lolkema 2003).

The phosphoenolpyruvate-dependant transport system (PTS) described above is regarded as the master control circuit in carbon catabolite repression. CRP, as part of this system, controls the expression of more than a hundred genes in *E. coli* (Ma *et al*., 2004). Although most of the genes regulated by CRP are involved in the utilisation of carbon sources, genes involved in pH-regulated gene expression, the heat-shock response, flagellum synthesis and enterotoxin production have also been shown to be regulated in this manner in enteric bacteria (Saier and Ramseier, 1996; Suh *et al*., 2002; Ma *et al*., 2004).

1.3.1.2 Carbon catabolite repression in *Pseudomonas*

A CRP homologue, namely Vfr (virulence factor regulator), which exhibits 67 % amino acid identity to the *E. coli* CRP, has been identified in *P. aeruginosa* (West *et al*., 1994). Vfr is able to bind cAMP with a similar affinity to CRP, displaying dissociation constants for this binding of 0.4 and 1.6 μM for Vfr and CRP respectively (Suh *et al*.,

2002). Furthermore, Vfr can recognise the CRP binding site in the DNA of *E. coli* and interact with *E. coli* RNA polymerase to allow transcription of CRP-regulated genes (West *et al*., 1994; Suh *et al*., 2002). Indeed, it has been found that Vfr can substitute for CRP to regulate β-galactosidase and tryptophanase activity, thus restoring regulation by the phosphoenolpyruvate-dependant transport system in *E. coli* CRP^- mutants (West *et al*., 1994). However, unlike in *E. coli*, cAMP levels in *Pseudomonas* do not vary according to carbon source, and tricarboxylic acid (TCA) cycle intermediates such as succinate, rather than glucose, are the strongest repressors in *Pseudomonas* (Phillips and Mulfinger, 1981; Eschenlauer and Reznikoff, 1991; Diab *et al*., 2006). Thus, it has been suggested that CCR does not occur by Vfr-mediated transcriptional regulation in *Pseudomonas* (Collier *et al*., 1996; Suh *et al*., 2002).

Vfr as a global regulator of CCR in Pseudomonas?

Alignment of the amino acid sequences of Vfr and CRP reveals that the residues known to be important for CRP activity, including those involved in DNA binding, interaction with RNA polymerase and cAMP binding, are conserved in Vfr (Figure 1.4) (Ebright *et al*., 1987; Weber and Steitz, 1987; West and Iglewski 1988; Zhang and Ebright, 1990; Eschenlauer and Reznikoff, 1991; West *et al*., 1994). It is therefore plausible that Vfr may mediate CCR in *Pseudomonas* in a manner similar to that of CRP in *E. coli* (West *et al*., 1994; Suh *et al*., 2002). Six residues in CRP are predicted to be involved in the binding of cAMP, namely Gly71, Glu 72, Arg82, Ser83, Thr127 and Ser128 (Weber and Steitz, 1987; West *et al*., 1994). With the exception of Ser128, which has been replaced with Thr, all of these residues are identical in Vfr (Figure 1.4). Tyr99 and Arg123 of CRP have been linked to stabilisation of the cAMP binding site and are also conserved in Vfr (West *et al*., 1994).

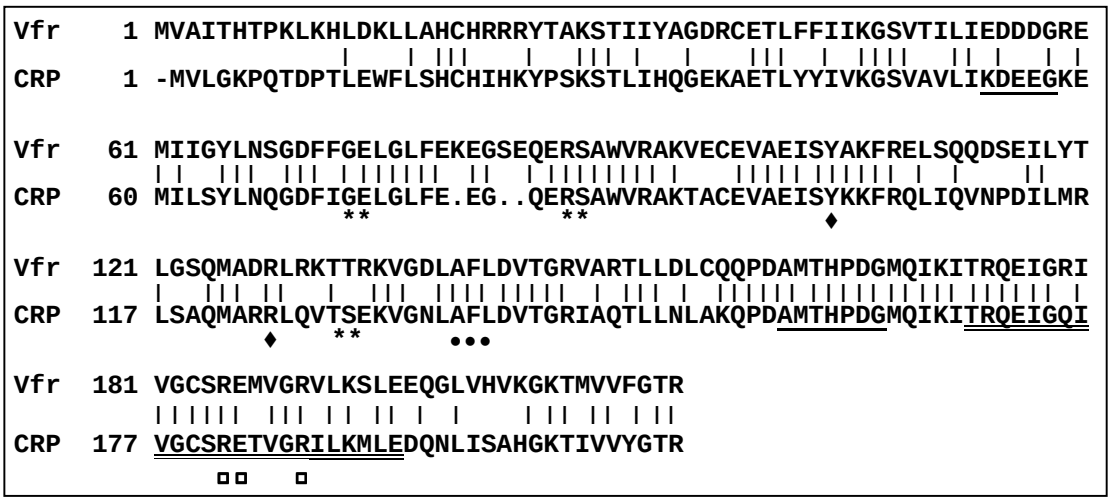

Figure 1.4. Alignment of the amino acid sequence of the Vfr protein of *P. aeruginosa* with that of *E. coli* CRP. Identical amino acids are indicated with vertical lines. Regions important for CRP activity are indicated below the sequence as follows: ***** - residues involved in cAMP binding, ♦ - residues which stabilise the cAMP binding pocket, ● - residues which form the hinge between the two domains of cAMP, single underline - residues which are involved in CRP-RNA polymerase interactions; double underline - residues which form the helix-turn-helix motif involved in DNA binding, and ▫ - residues of the HTH motif which make direct contact with the CRP binding site (adapted from West *et al*., 1994).

The C terminus helix-turn-helix (HTH) motif in CRP is responsible for DNA binding (Weber *et al*., 1982). With the exception of the Arg substitution of Gln174 in Vfr, the first helix of the HTH motif is identical in the two proteins (Hutter and Niederberger, 1984, West *et al*., 1994). The second helix, comprising amino acids 180 – 191, recognises and binds specific nucleotides and nine out of the twelve residues are identical in the two proteins (Ebright *et al*., 1987; Zhang and Ebright, 1990; West *et al*., 1994). Residues 52 to 56 and 156 to 162 of CRP form hydrophillic loops, which are though to be involved in interaction with RNA polymerase (West and Iglewski 1988; Eschenlauer and Reznikoff, 1991). In Vfr, residues 156 – 162 are identical to those of CRP, while only two out of five residues between amino acids 52 and 56 are conserved (West *et al*., 1994).

Despite these structural similarities and the fact that Vfr can complement an *E. coli crp*$^-$ mutant, CRP is unable to restore Vfr function in a *P. aeruginosa vfr*$^-$ mutant (West *et al*.,

1994; Kanack *et al*., 2006). A recent study of the DNA-binding specificity of Vfr revealed a consensus Vfr binding sequence that differs from the CRP consensus, suggesting that although Vfr shares many of the functions of CRP, it also performs many specialised functions that are unique to this global regulator (Kanack *et al*., 2006). The inability of CRP to complement a *vfr*$^-$ mutant in *P. aeruginosa* may be due to the fact that the levels of cAMP in this pseudomonad are ten-fold lower than those in *E. coli*, and thus may be too low to activate CRP (Petrushka *et al*., 2002).

Another possible explanation for the inability of CRP to substitute for Vfr in *P. aeruginosa,* is that Vfr binds a modulator other than cAMP in *Pseudomonas*. This hypothesis is supported by the fact that the cAMP binding domain of Vfr possesses three amino acids, namely Lys80, Ser83 and Glu84, which are not present in CRP, and could allow Vfr to bind another allosteric effector (Suh *et al*., 2002). Furthermore, the Thr substitution of Ser128 in Vfr, has been shown to allow Vfr to be activated by both cAMP and cGMP (Beatson *et al*., 2002). The modulator of Vfr in *Pseudomonas* has not yet been identified (Suh *et al*., 2002).

A further explanation for the failure of CRP to complement a *vfr*$^-$ mutant in *P. aeruginosa* may be an inability of CRP to interact in the same way as Vfr with the RNA polymerase of this organism. This may be due to differences in the spacing between the Vfr and RNA polymerase binding sites, differences in the RNA polymerase enzymes themselves or the inability of CRP to recognise the Vfr binding site in *P. aeruginosa* (West *et al*., 1994; Suh *et al*., 2002). Thus, whether Vfr plays a role in carbon catabolite repression in *Pseudomonas* remains to be determined (Diab *et al*., 2006; del Castillo and Ramos, 2007). On the other hand, it has been firmly established that Vfr acts as a global regulator of bacterial virulence in this species (Albus *et al*., 1997). The role of Vfr in mediating virulence is reviewed later in Section 1.3.2.2.

Crc as a global regulator of CCR in Pseudomonas

The first protein shown to participate in carbon catabolite repression in *Pseudomonas* was Crc (catabolite repression control protein) (MacGregor *et al*., 1991; Hester *et al*., 2000). This protein has been implicated in the repression of a number of genes involved in the metabolism of sugars and nitrogenated compounds in both *P. putida* and *P. aeruginosa* (Hester *et al*., 2000; Yuste and Rojo, 2001). One such system regulated by CCR is the *bkd* operon, encoding the branched-chain keto acid dehydrogenase (BCKAD) enzyme and its positive transcriptional regulator, BkdR (MacGregor *et al*., 1991). Repression of BCKAD by succinate, in the case of *P. aeruginosa* PA01, and glucose, in the case of *P. putida* KT2440, is lost in a *crc*$^{-}$ mutant and restored by the presence of extra-chromosomal (plasmid-borne) *crc*. Therefore this protein is proposed to play a central role in CCR in *Pseudomonas* (Hester *et al*., 2000).

The regulator, Crc, does not appear to mediate CCR of all genes linked to carbon metabolism. In the histidine utilisation pathway or *hut* system, inactivation of *crc* does not relieve succinate repression (Yuste and Rojo, 2001). It would therefore appear that, in *P. aeruginosa* PA01, there exist at least two succinate-responsive mechanisms of CCR; one that is dependant on Crc, for example the repression of amidase and mannitol dehydrogenase, and one that is Crc-independent, as in the case of the regulation of the *hut* pathway (Collier *et al*., 1996; Collier *et al*., 2001). Similar mechanisms of CCR, with respect to Crc, have been observed in *P. putida* KT2440, where Crc is 93 % similar and 86 % identical to that of *P. aeruginosa* PA01 (Hester *et al*., 2000; Nelson *et al*., 2002).

The Crc protein of *P. aeruginosa* shares between 25 % and 32 % amino acid sequence identity with a family of DNA repair enzymes, consisting of the apurinic and apyrimidinic endonucleases, yet appears to lack either DNA-binding or endonuclease activity (MacGregor *et al*., 1996). In addition, Crc in *P. aeruginosa* displays significant homology to the *E. coli* Exonuclease (ExoIII) enzyme, with respect to both amino acid sequence and predicted secondary structure. However, while Crc retains the structural

features required for the cleavage of phosphodiester bonds, it lacks either exo- or endonuclease activity (Gambello *et al*., 1993). These observations suggest that Crc may bind either to RNA, a small phospho-protein or a signalling molecule rather than to DNA (Collier *et al*., 1996).

A recent study has determined that both the amount and activity of Crc are modulated during regulation, suggesting that it forms part of a signal transduction pathway (Ruiz-Manzano *et al*., 2005). It has also been established that, in addition to its role as a repressor, Crc can also act as a transcriptional activator. An example of this positive regulation by Crc is the activation of expression of the cysteinyl-tRNA-synthetase and malate-quinone oxidoreductase genes in *P. putida* (Morales *et al*., 2004). Despite what has been documented about Crc, no specific biochemical activity has been assigned to this protein, and the mechanism by which it regulates gene expression remains unclear (del Castillo and Ramos; 2007).

1.3.2 Quorum sensing as a global control system

As outlined above, global control systems allow bacteria to co-ordinate the expression of genes in response to an environmental signal. One of these signals is the presence of other microbes of the same species and, consequently, certain bacteria have developed a regulatory mechanism known as quorum sensing (QS), which responds to the cell density of their own population (reviewed in Schuster and Greenberg, 2006). Quorum sensing is defined as “a population density-dependant intercellular signalling mechanism enabling bacteria to co-ordinate the expression of specific genes” and was first observed in the luminescent bacterium *Vibrio fischeri* (Nealson *et al*; 1970; Juhas *et al.*, 2005). The bacteria were shown to synchronise their behaviour by the secretion of signalling molecules in a manner that was dependant on cell density (Nealson *et al*; 1970). The name “quorum sensing” is derived from the critical or threshold concentration of bacteria known as the “quorum,” which is required for the induction or repression of

genes as a result of the secretion of signalling molecules (Fuqua *et al*., 1994; Schuster and Greenberg, 2006).

The diffusible molecules secreted during quorum sensing are either acylated homoserine lactones (AHL) in gram-negative bacteria, small peptides in the case of gram-positive bacteria or autoinducer-2 (AI-2) found in both bacterial groupings (Gambello and Iglewski, 1991; Passador *et al*., 1993, Pearson *et al*., 1994; Juhas *et al*., 2005). A model for the mechanisms of regulation by quorum sensing, based on studies involving marine vibrios, has been developed (Engebrecht and Silverman, 1984). In this model, AHL molecules are produced in a cell density-dependant manner by the LuxI family of proteins and then interact with the LuxR family of transcriptional activators, to regulate the expression of a number of quorum sensing-sensitive genes (reviewed in Schuster and Greenberg, 2006). LuxR is a homodimer possessing both an "amino-terminal membrane-bound regulatory domain," which binds AHLs; and a cytoplasmic carboxy-terminal domain, which binds DNA (Withers *et al*., 2001). The LuxR family of transcriptional regulators bind DNA at a palindromic recognition sequence termed the *lux* box (Fuqua *et al*., 1994). While there is a great deal of variability in confirmed *lux* boxes, the consensus sequence in *P. aeruginosa* is as follows: 5'-A**CCT**NCCANN**TCT**GGC**AG**NT -3', with highly conserved nucleotides indicated in bold (Pessi and Haas, 2000).

1.3.2.1 Quorum sensing in *Pseudomonas*

Quorum sensing has been observed in various *Pseudomonas* species and the human opportunistic pathogen *P. aeruginosa*, which causes infection in the lungs of cystic fibrosis sufferers, possesses the most extensively studied of these intercellular communication systems (Tummler *et al*., 1991; Juhas *et al*., 2005). According to microarray analysis, 5 % of all the genes in *P. aeruginosa* are regulated via quorum sensing (Wagner *et al*., 2003).

P. aeruginosa has two QS pathways, namely the *las* and *rhl* pathways (reviewed in Schuster and Greenberg, 2006). The *las* system is responsible for the regulation of elastase production and consists of the transcriptional activator protein, LasR, together with the autoinducer, PAI-1 [*N*-(3-oxododecanoyl)-*L*-homoserine lactone)] (Seed *et al*., 1995; Juhas *et al*., 2005). PAI-1 reaches a threshold level at high cell densities and complexes with the LuxR homologue, LasR, converting the protein into a transcriptional activator (Gambello and Iglewski, 1991; Latifi *et al*., 1996; Juhas *et al*., 2005). This complex activates the expression of *lasI, lasB,* and *lasA,* involved in the induction of elastase production; *apr*, encoding an alkaline protease and *toxA,* required for exotoxin A production (Toder *et al*., 1991; Seed *et al*., 1995; Pearson *et al*., 1997; Rumbaugh *et al*., 2000).

On the other hand, the *rhl* QS pathway involves the LuxR homologue, RhlR, and the autoinducer PAI-2 [*N*-butyryl-*L*-homoserine lactone) and controls transcription of *rhlA* and *rhlB,* which are involved in rhamnolipid biosynthesis (Ochsner *et al*., 1994; Juhas *et al*., 2005). A hierarchy governs the phenomenon of quorum sensing, where the *las* system is dominant and regulates the *rhl* system (Pesci *et al*., 1997; Schuster and Greenberg, 2006).

Not all pseudomonads, however, possess a QS system such as the one described for *P. aeruginosa*. The genome of *P. putida* KT2440 is 85 % similar to that of P. *aeruginosa*, but lacks the key QS genes *lasR* and *rhlR* and is therefore unable to produce elastases, lipases, exotoxins and other QS-dependant elements (Nelson *et al*., 2002). Another closely related pseudomonad, *P. fluorescens,* contains an *rhlR* gene on its chromosome which is similar to that found in *P. aeruginosa*, but lacks the coding sequence for the dominant transcriptional regulator, LasR (Kahn *et al*., 2005).

Besides the well-established role of QS in controlling pathogenesis in *P. aeruginosa*, it is now known that this cell density-dependant signalling pathway is also involved in mediating beneficial relationships between bacteria and eukaryotes (Gonzålez and Keshaven, 2006). An example of such an association is the bacteria-plant

communication displayed by certain strains of *P. putida* and *P. fluorescens* found in the rhizosphere (Gonzålez and Keshaven, 2006). Of 137 soil-borne and plant-associated *Pseudomonas* isolates, 39 % tested positive for AHL production (Steidle *et al*., 2002) Although AHL-dependant quorum sensing is not very common in *P. putida*, it has been found that some strains do produce AHL's (Bertani and Venturi, 2004). The regulons involved in QS in these strains of *Pseudomonas* are, to a large extent, unknown (Bertani and Venturi, 2004).

A pseudomonad whose QS machinery is highly similar to the LasR/RhlR system of *P. aeruginosa* has been isolated from the rhizosphere, namely *P. putida* IsoF. The AHL-dependant QS ability of IsoF is encoded by a four gene cluster containing: *ppuI*, whose gene product PpuI is a functional homologue of the LasI in *P. aeruginosa*; *ppuR*, encoding the LasR homologue, PpuR; *ppuA*, whose gene product is believed to be involved in biofilm formation; and *rsaL*, which is similar to the coding sequence of RsaL, a QS repressor in *P. aeruginosa* (Steidle *et al*., 2002). Furthermore, a palindromic sequence resembling a typical *lux* box was found upstream of the PpuR-regulated gene, *ppuI*, suggesting that PpuR is able to bind such a sequence in a manner similar to LasR (Steidle *et al*., 2002). The *ppu* system is absent in *P. putida* KT2440, but has been identified in the genome of another *P. putida* strain, designated as WCS358, and is identical to the system observed in IsoF (Nelson *et al*., 2002; Steidle *et al*., 2002; Bertani and Venturi, 2004).

1.3.2.2 Quorum sensing and virulence

In recent years, research has shown that QS is essential for the expression of numerous virulence factors (Hentzer *et al*., 2003). The dependence of virulence on QS in *P. aeruginosa* (summarised in Table 1.2) has provided a potential drug target to replace antibiotics, which have become problematic due to the frequent development of resistance to these compounds by the bacteria. Compounds which are able to override the cell-to-cell molecular signalling have been isolated from the marine environment. An

example of such an antagonist to QS is furanone, which has been shown to be successful in targeting and inhibiting virulence factor expression (Hentzer *et al*., 2003).

Table 1.2. Examples of virulence factors regulated through quorum sensing in *P. aeruginosa*.

LasR controlled virulence factors	**RhlR controlled virulence factors**
Alkaline protease	Alkaline protease
Elastase	Elastase
Lipase	Lipase
Hydrogen cyanide	Hydrogen cyanide
Catalase	
Aminopeptidase	
Superoxide dismutase	
	Chitinase
Exotoxin A	

(Adapted from Juhas *et al*., 2005)

Not only is QS required for virulence, it has further been shown that the transcriptional regulator Vfr, (for virulence factor regulator), regulates *lasR* expression. This places Vfr at the top of the regulatory QS cascade and firmly establishes the interdependence between QS and virulence (Albus *et al*., 1997). As a global regulator of virulence, Vfr has been implicated in the regulation of expression of at least 162 genes in *P. aeruginosa* and acts as either a transcriptional activator or repressor depending on the system (West *et al*., 1994; Suh *et al*., 2002).

1.3.3 Aerobic *vs*. anaerobic respiration as a global regulatory system

Microorganisms are ubiquitous and have been found to be capable of growth in a wide range of environmental oxygen levels (Pasteur, 1876; cited in Alexeeva *et al*., 2002). Aerobes are classified based on their ability to grow at a maximum oxygen tension which is equivalent to the 21 % oxygen level present in air (reviewed in Alexeeva *et al*.,

2002). These aerobic organisms are further classified as being either facultative or microaerophilic. Facultative aerobes, such as *E. coli*, are capable of growth under both aerobic and anaerobic conditions, while microaerophiles can only survive oxygen levels that are lower than that of air (Garrity, 1984; Alexeeva *et al*., 2002). Anaerobic microorganisms lack a respiratory system and cannot use oxygen as a terminal electron acceptor (reviewed in Imlay, 2002). Anaerobes are also divided into two sub-types; aerotolerant and obligate anaerobes. While aerotolerant microbes, such as *Streptococcus*, can tolerate oxygen in their environment and grow in its presence, they are unable to use it in respiration. Obligate anaerobes, such as *Methanobacterium*, are killed by oxygen due to their inability to remove the toxic by-products of oxygen metabolism (Garrity, 1984; Imlay, 2002).

1.3.3.1 Regulation of anaerobic respiration in *E. coli*

As outlined above, *E. coli* is a facultative aerobe capable of growth in aerobic as well as anaerobic conditions (Garrity, 1984). The switch from aerobic to anaerobic growth is regulated by two global transcriptional regulator systems. One is a two-component sensor-regulator system, consisting of ArcA and ArcB, which is able to repress certain aerobic genes under anaerobic conditions (Iuchi and Lin, 1988; Tseng *et al*., 1996). ArcB acts as an environmental oxygen sensor which activates ArcA under reduced oxygen conditions and ArcA, in turn, mediates both positive and negative regulation of gene expression. This occurs in environmental oxygen levels between 10 % and 20 % saturation, therefore ArcA has been designated as a microaerobic redox regulator (Tseng *et al*., 1996; Alexeeva *et al*., 2003).

The second global redox regulator system involves Fnr, which was named due to the discovery of mutants which were unable to use fumarate as an alternate electron acceptor in anaerobic conditions (Spiro and Guest, 1990; Becker *et al*., 1996). Fnr is a CRP homologue and possesses the characteristic helix-turn-helix DNA binding domain of transcriptional activators, while being able to function as both an anaerobic activator

and aerobic repressor (Spiro and Guest, 1990; Spiro, 1994). The dimeric protein has been isolated in monomeric form and has a molecular weight of 30 kDa (Spiro and Guest, 1990). Fnr is activated at low oxygen levels by acquiring $[4Fe\text{-}4S]^{2+}$ clusters, which function as oxygen sensors due to their instability in the presence of O_2. These clusters promote the formation of homodimers, by interacting with essential cysteine residues in the protein, resulting in increased sequence-specific DNA binding (Kiley and Beinert, 1998; Dibden and Green, 2005). DNA binding by these Fnr dimers occurs, under anaerobic conditions, at sites resembling the 22 base pair consensus sequence or Fnr box: 5' - TTGAT - N_4 – ATCAA - 3'. This Fnr recognition sequence differs only slightly from that of the CRP consensus sequence, although CRP is unable to bind Fnr box sequences (Eiglmeier *et al*., 1989; Dibden and Green, 2005). Fnr boxes are often present at a position approximately 30 nucleotides upstream of the transcriptional start site of genes regulated by Fnr (Eiglmeier *et al*., 1989; Dibden and Green, 2005).

1.3.3.2 Anaerobic growth of *Pseudomonas*

Although bacteria belonging to the genus *Pseudomonas* were originally classified as "organisms having a strictly respiratory metabolism," it has since been discovered that this term is not entirely accurate, especially in the case of *P. aeruginosa* (Palleroni, 1984; Sabra *et al*., 2002). The opportunistic pathogen, *P. aeruginosa,* was found to form macro-colonies giving rise to microaerobic to anaerobic environments (Worlitzsch *et al*., 2002). Furthermore, the deeper layers of biofilms, for example those found in the cystic fibrosis lung, were found to be anaerobic (Hassett *et al*., 2002). Chemostat experiments have shown that the microbe establishes "microaerobic milieus" for optimal growth (Sabra *et al*., 2002).

In the absence of oxygen, *P. aeruginosa* uses nitrate or nitrite as an alternate electron acceptor and generates energy by denitrification (Davies *et al*., 1989). When neither nitrate nor nitrate is available, *P. aeruginosa* may use arginine deimination to survive anaerobically (van der Wouven *et al*., 1984). Another means by which *P. aeruginosa*

can survive under anaerobic conditions is via pyruvate fermentation. Although pyruvate fermentation alone is not sufficient to sustain any considerable growth of the organism, the metabolic capacity provided allows survival in anaerobic conditions for up to 18 days (Eschbach *et al*., 2004). Thus *P. aeruginosa* may require reclassification as an aerotolerant anaerobe or even a facultative aerobe. There is, however, no evidence that *P. putida* strains are able to survive anaerobic conditions and the most frequently utilised laboratory strain, KT2440, is still regarded as a strict aerobe (Timmis, 2002).

Anr as a global regulator under anaerobic conditions in Pseudomonas

A diverse group of bacterial species possess Fnr homologues, which fulfil a number of different regulatory roles. *P. aeruginosa*, for example, possesses the Fnr homologue Anr, (named for its role as an anaerobic regulator) which controls the onset of anaerobic metabolism (Spiro, 1994). Genes that are activated by Anr display specific sequences in their promoters, termed "*anr* boxes" and it has been found that Anr binds these sequences with a recognition specificity which is similar, but not always identical, to that of Fnr (Wintler *et al*., 1996; Pessi and Haas, 2000). The Anr protein functions by means of a similar mechanism to that of Fnr, where it is converted to its active dimeric form under low oxygen levels, binds to the Anr box and then switches on the transcription of genes whose enzymes are required under anaerobic conditions (Spiro, 1994).

The anaerobic regulator, Anr, directly induces transcription of the genes required for arginine deimination, as well as a gene encoding a second Fnr-like protein called Dnr, which is responsible for the induction of denitrification pathways (Zimmerman *et al*., 1991; Arai *et al*, 1997; Arai *et al*., 2003; Schreiber *et al*., 2007). It was recently discovered that Anr plays an important role in stimulating the expression of the *cupA* fimbrial gene involved in biofilm formation by *P. aeruginosa* in the cystic fibrosis lung (Vallet-Gely *et al*., 2007). Although *P. putida* KT2440 is classified as an obligate aerobe, it does nonetheless possess an *anr* gene, which is intriguing and raises questions as to the role of Anr in this pseudomonad (Nelson *et al*., 2002)

1.4 Alternate sigma factors and regulation

Not all genes subject to global control systems make use of simple combinations of activators and repressors to regulate their expression. Another way in which cells are able to switch the pattern of transcription, in order to allow the expression of genes which are only required under certain conditions, is by the production of alternative sigma factors (reviewed in Venturi, 2003). In bacteria, transcription is carried out by RNA polymerase (RNAP), and specificity for the transcription of genes is provided by sigma factors. These sigma factors compete for a limited amount of RNAP core enzyme and direct the RNAP enzyme to the promoters of a certain subset of genes, depending on the specific sigma factor which is dominant at that time (Reviewed in Ishihama, 2000). Regulation is achieved by varying either the level or the activity of these different sigma factors, according to stimuli associated with various growth phases and growth conditions (Jishage and Ishihama, 1996; Ishihama, 2000).

1.4.1 Regulation of gene expression by sigma factors in *E. coli*

Eubacteria display two very different families of sigma factors, namely the σ^{70} family and the σ^{54} family (Lonetto *et al.*, 1992; Paget and Helmann, 2003). The σ^{70} family, in turn, is made up of two sub-families, the σ^{70} sub-family and the extracytoplasmic sigma factor (ECF) sub-family (Paget and Helmann, 2003). Most of the genes in *E. coli* require the 70 kDa vegetative or essential sigma factor, namely σ^{70}, for their transcription. Such genes possess specific -10 and -35 sequences, in relation to the transcriptional start of the genes, whose consensus sequences are TATAAT and TTGACA respectively (Wise *et al*., 1996; Paget and Helmann, 2003).

There are a variety of less common σ^{70} sub-family sigma factors, which are not essential for cell growth and are expressed at higher levels under certain environmental conditions (Paget and Helmann, 2003). An example of such an alternative σ^{70} family sigma factor is σ^{32} or σ^{H}, which allows for the expression of at least 127 genes required during high

temperatures in *E. coli* (Grossman *et al*., 1987; Paget and Helmann, 2003; Nonaka *et al*., 2006). This "heat shock" sigma factor has a consensus promoter recognition sequence that is different from that of σ^{70}, namely CCCCATWT (-10) and TTGAAA (-35), and the heat shock response is regulated directly by σ^{32}, as a result of an increase in the levels of this normally unstable sigma factor at high temperatures (Grossman *et al*., 1987; Nonaka *et al*., 2006).

Another alternative σ^{70} sub-family sigma factor is σ^{38}, also known as σ^{s}. This sigma factor was found to regulate the expression of more than 100 genes in *E. coli* in response to a variety of stresses associated with stationary phase; such as carbon starvation, limited oxygen availability, high osmolarity and the presence of toxic chemicals (Hengge-Aronis, 1993; Wise *et al*., 1996; Ishihama, 2000; Venturi, 2003). Although similar to σ^{70} in structure and molecular function, σ^{38} recognises a modified -10 sequence namely CTATACT and, instead of requiring a -35 sequence, makes use of a region upstream of the -10 sequence that is A+T rich and displays intrinsic DNA curvature (Lonetto *et al*., 1992; Espinos-Urgel *et al*., 1996; Lee and Gralla, 2001).

The extracytoplasmic function (ECF) sigma factors represent the second sub-family of the σ^{70} family of sigma factors (Lonetto *et al*., 1994; Paget and Helmann, 2003). Bacterial cells possess a number of ECF sigma factors which regulate transcription in response to extracytoplasmic stimuli, such as the presence of misfolded proteins in the periplasmic space, by means of post-translational interaction with an "anti-sigma factor" located in the cell membrane (Raivio and Silhavy, 2001; Paget and Helmann, 2003). In *E. coli*, examples of ECF sigma factor include the FecI sigma factor, which is involved in iron acquisition and regulates iron dicitrate uptake and σ^{E}, which ensures the correct assembly of outer-membrane proteins (Enz *et al*., 2000; Raivio and Sihavy, 2001; Paget and Helmann, 2003).

The other major family of sigma factors is the σ^{54} family, which is classified separately due to the fact that σ^{54} is entirely different from other bacterial sigma factors in both its structure and mechanism of action (Lonetto *et al*., 1992; Buck *et al*., 2000; Paget and

Helmann, 2003). Unlike sigma factors of the σ^{70} family, σ^{54} works together with a transcriptional activator, which binds to an upstream activation sequence (UAS) at least 100 nucleotides from the transcriptional start site (Merrick, 1993; Buck *et al*., 2000; Reitzer and Schneider, 2001). Recognition of promoters by σ^{54} occurs via a palindromic binding sequence, namely: TGGCAC - N_{5-6} – TTGCT (Pearson *et al*., 1997). Binding of this transcriptional activator at the UAS causes the DNA to bend, forming a loop, which allows for direct contact between the σ^{54}-RNAP complex and the activator protein such that σ^{54}-bound RNAP is able to bind to the promoter and activate transcription (Merrick, 1993; Buck *et al*., 2000).

Another name for σ^{54} is σ^{N}, since the sigma factor was originally discovered to be nitrogen-specific (Hirschman *et al*., 1985; Buck *et al*., 2000). In recent years, however, σ^{54} has been implicated in the regulation of a number of very different genes, including those whose products are involved in the utilisation and transport of different carbon and nitrogen sources, in addition to genes involved in virulence as well as the phage-shock and zinc-tolerance responses (Reitzer and Schneider, 2001).

1.4.2 Regulation of gene expression by alternative sigma factors in *Pseudomonas*

The mechanisms of regulation of gene expression in *Pseudomonas* have long been thought to be among the most sophisticated of any bacterial genus. For example, in *P. aeruginosa*, 8 % of the total number of genes have been linked to regulation, which is the highest percentage observed for a bacterial genome (Stover *et al*., 2000). This suggests that these organisms possess regulatory machinery that is both complex and potentially novel. Furthermore, whereas *E. coli* displays 266 open reading frames coding for transcriptional regulators, *P. putida* and *P. aeruginosa* encode up to 450 such ORFs on their genome (Stover *et al*., 2000; Nelson *et al*., 2002). This apparent increase in the complexity of regulation in pseudomonads is also evident in the large range of sigma factors present in these bacteria. The genomes of *P. putida* KT2440 and *P. aeruginosa* PA01 possess 24 genes encoding sigma factors whereas *E. coli* has only

7 such genes (Stover *et al*., 2000; Nelson *et al*., 2002; Martinez-Beuno *et al*., 2002). Not surprisingly, this represents the highest number of sigma factors reported for any bacterial genome (Martinez-Beuno *et al*., 2002; Cases *et al*., 2003).

The first alternative sigma factor to be characterised in *P. putida* was σ^{38} (Ramos-Gonzalez and Molin, 1998). In this organism, bidimensional protein gel experiments have revealed that σ^{38} controls the expression of at least 50 genes under carbon starvation conditions (Ramos-Gonzalez and Molin, 1998; Venturi, 2003). In contrast to *E. coli*, where σ^{38} is a central regulator in the general stress response; this sigma factor has unique functions and has a less important role in the general stress response in *Pseudomonas* (Suh *et al*., 1999; Venturi, 2003; Paget and Helmann, 2003). For example, in *P. aeruginosa*, σ^{38} has been shown to affect the expression of a large number of quorum sensing genes, such as those involved in extracellular alginate and exotoxin A production, together with those responsible for the formation of type IV fimbriae, all of which are linked to the virulence of the organism (Latifi *et al*., 1996; Suh *et al*., 1999; Kazmierczak *et al*., 2005). Furthermore, the QS regulator, RhlR, was found to regulate σ^{38} expression, indicating an inter-dependence between this alternative sigma factor and the QS pathway (Latifi *et al*., 1996; Kazmierczak *et al*., 2005).

An additional σ^{70} sub-family sigma factor, which is not present in *E. coli*, was identified in *Pseudomonas* (Ditty *et al*., 1998). This alternative sigma factor, σ^{28}, also called FliA, due to its involvement in the regulation of flagellin biosynthesis, was first found in the strain *P. putida* PRS2000, but has since been identified in the genomes of both *P. aeruginosa* PAO1 and *P. putida* KT2440 (Ditty *et al*., 1998; Stover *et al*., 2000; Nelson *et al*., 2002). FliA is regulated by an "antisigma factor" called FlgM and the flagellin biosynthesis regulatory system falls under the control of σ^{54} and the transcriptional activator FleQ, which form the highest level of regulation in flagellum biosynthesis (Totten *et al*., 1990; Frisk *et al*., 2002; Kazmierczak *et al*., 2005).

In terms of ECF sigma factors in *Pseudomonas*, 19 new sigma factors have been identified in *P. putida* KT2440 (Martinez-Beuno *et al*., 2002). Interestingly, 13 of these

putative ECF sigma factors showed a high degree of similarity to the FecI sigma factor of *E. coli* and, since each is associated with a different activator protein, this allows for a number of different mechanisms of iron acquisition (Martinez-Beuno *et al*., 2002; Paget and Helmann, 2003). The ECF sigma factors of *P. putida* KT2440 and *P. aeruginosa* PA01 have been compared and five of these displayed a high degree of similarity between the two strains; whereas, for seven of the ECF factors in *P. aeruginosa* there were no homologous proteins in *P. putida* (Martinez-Beuno *et al*., 2002). These distinct sigma factors in the pathogen, *P. aeruginosa*, are most likely linked to virulence, which would explain their absence in the non-pathogenic strain, *P. putida* (Martinez-Beuno *et al*., 2002; Nelson *et al*., 2002). An example of such an ECF sigma factor involved in virulence in *P. aeruginosa* is AlgU, which regulates the biosynthesis of alginate in concert with the quorum sensing global transcriptional regulator, LasR (Kazmierczak *et al*., 2005).

In the case of the unique bacterial sigma factor, σ^{54}, which has previously been implicated in the regulation of a diverse set of genes in *E. coli*, a total of 55 promoters in *P. putida* have been predicted to fall under the control of this sigma factor (Reitzer and Schneider, 2001; Cases *et al*., 2003; Kazmierczak *et al*., 2005). A recent study of the σ^{54} regulon (sigmulon) of the complete genome of *P. putida* KT2440 revealed a range of new biological functions for σ^{54}, which were entirely different from those described for *E. coli* (Cases *et al*., 2003). Nine of the 55 predicted σ^{54}-regulated promoters were related to flagellum synthesis, required for motility of the organism and chemotaxis; 18 of the promoters were linked to genes involved in nitrogen metabolism, including the transport of amino acids, ammonia and polyamines; and a further nine of the σ^{54}-dependant promoters were found to drive the transcription of genes involved in carbon metabolism, including those linked to transport, particularly of dicarboxylates, as well as a number of dehydrogenase and oxidoreductase-type enzymes (Cases *et al*., 2003). In addition, σ^{54} has been implicated in the regulation of rhamnolipid biosynthesis by RhlA in the pathogenic strain, *P. aeruginosa*, since expression of *rhlA* decreased 15-fold in the absence of this sigma factor (Pearson *et al*., 1997; Kazmierczak *et al*., 2005).

What is clear from the literature regarding global control systems for the expression of non-essential genes in prokaryotes, is that these pathways are complex and are often integrated with other regulatory systems to give rise to hierarchies of interlinking control mechanisms (Ma *et al*., 2004). This is particularly true for the metabolically diverse genus *Pseudomonas*, where metabolic regulatory pathways overlap with QS systems and alternative sigma factor regulation, to allow for a co-ordinated and sophisticated response to a variety of related environmental signals (Stover *et al*., 2000; Withers *et al*., 2001; Nelson *et al*., 2002).

1.5 Research proposal

1.5.1 Knowledge gap and hypothesis

Although it is known that hydantoin-hydrolysing enzyme activity in *P. putida* RU-KM3$_s$ is subject to carbon catabolite repression (CCR) and is not affected by the nitrogen status of the cell, the mechanisms of regulation as well as the transcriptional factors involved, remain unknown (Matcher *et al*., 2004). To date, the only protein shown to play a global role in CCR in *Pseudomonas* is Crc (Hester *et al*., 2000; Yuste and Rojo, 2001). However, it has also been reported that inactivation of Crc does not relieve CCR in all enzyme systems linked to carbon metabolism (Yuste and Rojo, 2001). On the other hand, the presence of a putative CRP binding site in the intergenic region of the *dhp/bup* gene cluster in *P. putida* RU-KM3$_s$, together with the sequence similarity and conserved structural features between CRP and Vfr, suggest that Vfr may be able to mediate CCR of the hydantoin hydrolysis pathway in this organism (West, 1994; Suh *et al*., 2002; Matcher, 2004).

This study attempts to elucidate the mechanisms governing the complex metabolic regulatory systems in *Pseudomonas*, using the hydantoin-hydrolysing system of RU-KM3$_s$, in particular the expression of the *dhp* gene, as a model.

Hypothesis

The regulation of *dhp* expression in *P. putida* RU-KM3$_s$ requires two or more global transcription factors working in synergy, one of which is Vfr.

1.5.2 Overall aim of the project

Identification of the factors involved in regulating *dhp* expression in *P. putida* RU-KM3$_s$

1.5.3 Objectives

1) Bioinformatic analysis of the intergenic region upstream of *dhp* to identify potential transcription factor binding sites.
2) Characterisation of environmental factors affecting DHP activity, including confirmation of the observations reported by Matcher (2004).
3) Identification of potential global regulatory proteins affecting DHP activity.
4) Development of an experimental system to analyse *dhp* promoter activity.
5) Identification of putative transcription factor binding sites in the *dhp* promoter that affect gene expression.
6) Development of a model for the regulation of *dhp* expression in RU-KM3$_s$.

CHAPTER 2

METHODS AND MATERIALS

METHODS AND MATERIALS

2.1 Bacterial strains and culture conditions

The strains utilised and generated in this study are listed in Appendix 1, Table A1.1. *Pseudomonas putida* RU-KM3$_s$ and its plasposon-derived transconjugate strains were cultured with agitation at 200 rpm at 28 °C, while all *E. coli* strains were cultured at 37 °C. All strains were maintained on Luria-Bertani agar (LA) plates and liquid cultures cultured either in Luria-Bertani (LB) or nutrient broth (NB) (Sambrook *et al*., 1989). The following antibiotic concentrations were utilised for selective growth of desired strains: 25 μg/ml chloramphenicol, 10 μg/ml tetracycline, 100 μg/ml ampicillin and 50 μg/ml kanamycin.

Quorum sensing conditions were maintained by culturing *P. putida* RU-KM3$_s$ cells in double-strength medium i.e. 50 ml NB containing 0.1 g meat extract, 0.5 g peptone, 0.2 g yeast extract and 0.8 g sodium chloride. Carbon catabolite repression was achieved by the addition of 1 % succinic acid (succinate) to the growth medium. Anaerobic conditions were attained by culturing cells in tightly stoppered Schott bottles, in either NB alone or NB supplemented with 1 mM sodium nitrate as an alternate electron acceptor. The degree of aeration was varied by altering the volume of culture medium, and therefore headspace, in the growth vessel.

2.2 Introduction of plasmids into *E. coli* and *P. putida* cells

Recombinant plasmids were routinely maintained in *E. coli* DH5α cells. The preparation and transformation of competent *E. coli* cells was carried out according to Hanahan (1983) and is detailed in Appendix 2. Vectors were introduced into *P. putida* RU-KM3$_s$ cells by tri-parental mating, with *E. coli* DH5α hosting the desired vector and HB101 hosting the mobilising plasmid, pRK2013 (Santos *et al*., 2001), as described by Dennis and Zylstra (1998). This process was optimised for RU-KM3$_s$ by Matcher (2004) and accordingly a 1:1:1 ratio of RU-KM3$_s$:DH5α:HB101 was utilised. Transconjugate

strains were selected by plating onto LA plates containing chloramphenicol, to select against the *E. coli* strains, and the relevant antibiotic marker carried on the vector.

2.3 Construction of recombinant promoter probe vectors

The plasmids generated and utilised in this study are described in Appendix 1, Table A1.2/3. The specific details of all primers used in the cloning experiments below can be found in Appendix 1, Table A1.4/5, and the cycling parameters are described in Appendix 3.4, Table A3. The recombinant vectors were analysed at each cloning step by extraction from *E. coli* cells using the Easyprep method described by Berghammer and Auer (1993) and screening by restriction endonuclease digestion, followed by analysis by agarose gel electrophoresis (Sambrook *et al*, 1989). The desired plasmids were extracted to a high degree of purity using the High Pure Plasmid DNA isolation kit (Roche).

In order to analyse the regulation of *dhp* transcription by *cis*-acting elements in *P. putida* RU-KM3s, two different promoter probes were utilised, namely a chromosomal integration plasmid, pJD2, and a multi-copy broad host-range vector, pMJ449.

2.3.1 The plasposon-derived chromosomal integration vector, pJD2

To facilitate the insertion of a promoter probe into the chromosome of RU-KM3$_s$, a vector derived from a "modular self-cloning mini-transposon" or "plasposon," developed by Dennis and Zylstra (1998), was constructed. The *Eco* RI site of the plasposon, pTnMod-OTc (Figure 2.1) was removed by digesting with *Eco* RI, filling in the resulting nucleotide overhangs with Klenow DNA polymerase (Promega) and re-ligation with T4 DNA ligase (Promega). Thereafter, flanking *Eco* RI and *Bam* HI sites were introduced into the modified pTnMod-OTc vector adjacent to the translational stop codon of the pMB1 *oriV*, using Expand High Fidelity DNA polymerase (Roche) and the overlapping site-directed mutagenesis primers JAS11 (5'–CGAATTCGATGAGCTCGG GTTGGTTTG-3') and JAS13 (5'-CGAATTCGGATCCTTTCGTTCCACTGAGCGT

CAGAC-3') to generate pJAS26 (Figure 2.1, Step 1). The modification of pTnMod-OTc in terms of these restriction sites was necessary to allow for the cloning of the *gus* gene and subsequent fusion of the *dhp* promoter to this reporter in the plasposon.

The *gus* ORF, minus its native Shine-Delgarno sequence and encoding the β-glucuronidase enzyme, was PCR amplified from pMJ242 (Jiwaji, 2006), a plasmid derived from pCAMBIA (CAMBIA, Canberra, Australia). This was achieved using Expand High Fidelity DNA polymerase (Roche) and the primers JAS14 (5'-CCATGGATGTTACGTCCTGTAGAAACC-3') and JAS15 (5'-GGTACCTCGAGC TGCAGTCATTGTTTG-3'), which introduce a *Nco* I site on the 5´ end and a *Kpn* I site on the 3´ end of *gus* respectively. The PCR product was then ligated into the pGEM T-Easy vector (Promega) to generate pJAS21 (Figure 2.1 Step 2). The authenticity of the *gus* coding sequence was confirmed by DNA sequencing using the ABI Prism Big Dye Terminator protocol (Appendix 3.2) and the universal primers pUCF and pUCR. Thereafter, the *gus* gene was removed from pJAS21 as an 1824 bp *Eco* R1 restriction fragment and inserted into the single *Eco* RI site of pJAS26, yielding the construct pJAS29 (Figure 2.1, Step 3).

A sequence corresponding to the 255 bp intergenic region upstream of the *dhp* ORF, and including the ribosome binding site for the *dhp* transcript, was amplified from the genome of RU-KM3$_s$ using primers JAS6 (5'-GGATCCCATGGGGCCTTCTCCAGA TTT-3') and JAS10 (5'-CCATGGAGATCTGCCGTCTTCCTCG-3'), which introduced a flanking *Bam* HI and *Nco* I site at the 5' and 3' ends, respectively. The PCR products were first ligated into the pGEM-T-Easy vector, yielding pJD1 (Figure 2.1, Step 4) and the DNA sequence determined to confirm the integrity of the insert. Thereafter, this 255 bp fragment was inserted into pJAS29, using *Bam* HI and *Nco* I, to generate the final construct pJD2 (Figure 2.1; Step 5).

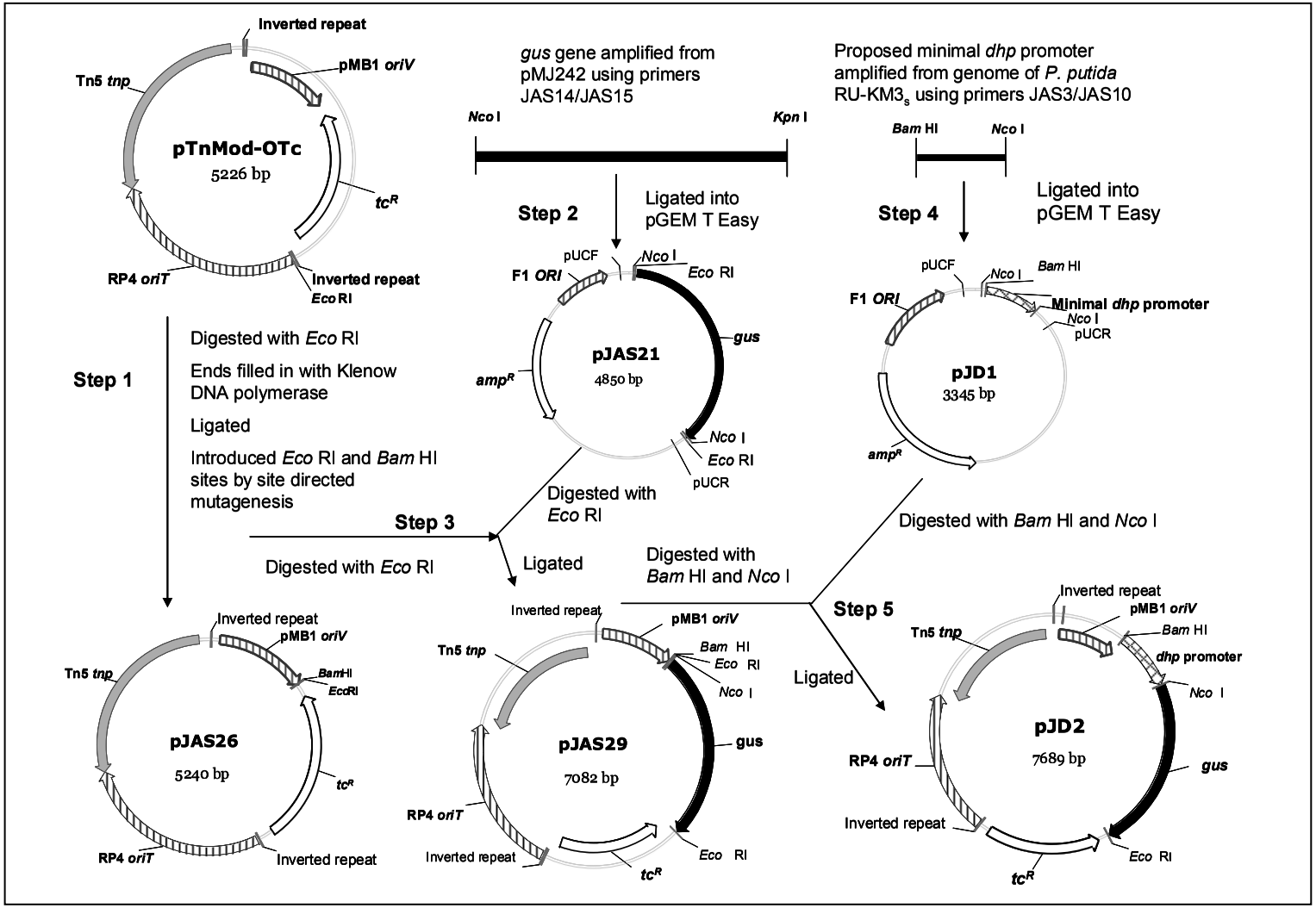

Figure 2.1. Cloning strategy for the construction of the integration-based promoter probe vector, pJD2.

The integration vector, pJD2, contained a conditional origin of replication (pMB1), preventing the plasposon from replicating in *P. putida*; an origin of transfer (*oriT*), to allow for introduction into RU-KM3$_s$ cells by tri-parental mating; a Tn5 transposase gene (*tnp*) together with inverted repeats, to allow for random integration into the chromosome; and the antibiotics resistance markers for Ampicillin (*amp*^{R}) and Tetracycline (*tc*^{R}).

To confirm the integration of pJD2 into the chromosome of RU-KM3$_s$, genomic DNA was extracted from the transconjugate strains using the detergent lysis/CTAB method (Ausubel *et al*., 1983, Appendix 3.3) and subjected to PCR analysis using Kapa*Taq* DNA polymerase (Kapa Biotech) and the *gus*-specific primers JAS14/JAS15, described above. The presence of an 1824 bp PCR product, corresponding to the *gus* gene, would indicate the successful insertion of pJD2 into the genome of a particular transconjugate strain.

2.3.2 The multi-copy broad host-range promoter probe vector, pMJ445.

A broad host-range vector, namely pMJ455, was constructed by Meesbah Jiwaji, a post-doctorate researcher in the laboratory. The vector contained an *oriT* gene, allowing the vector to be introduced into *P. putida* RU-KM3$_s$ by tri-parental mating, the broad host-range *rep* genes, to allow autonomous replication in RU-KM3$_s$, the *tc*^{R} gene to allow for antibiotic selection of transconjugate strains, as well as divergent *lacZ* and *gus* genes to allow for the analysis of a bi-directional promoter (Figure 2.2). The entire 616 bp intergenic region upstream of *dhp* was amplified from RU-KM3$_s$ genomic DNA using Expand High Fidelity DNA polymerase and the primers GFM38 (5'-AGATCTGGGG CCTTCTCCAGATTTTT-3') and GFM39 (5'-AGATCTGCCGTCTTCCTCGCAG-3'). These primers introduced *Bgl* II sites on either side of the sequence, allowing the promoter to be inserted into the *Bgl* II site of pMJ445. The PCR fragment was first ligated into the pGEM T-Easy and the sequence confirmed using the universal primers pUCF/R before inserting into pMJ445 using *Bgl* II to produce pMJ449. This construct contained the 616 *dhp* promoter region orientated in the direction of the *gus* reporter.

Recombinants were selected by their ability to degrade 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to produce blue colonies on agar plates containing 40 µl of a 40 µg/ml solution of the chromogenic substrate.

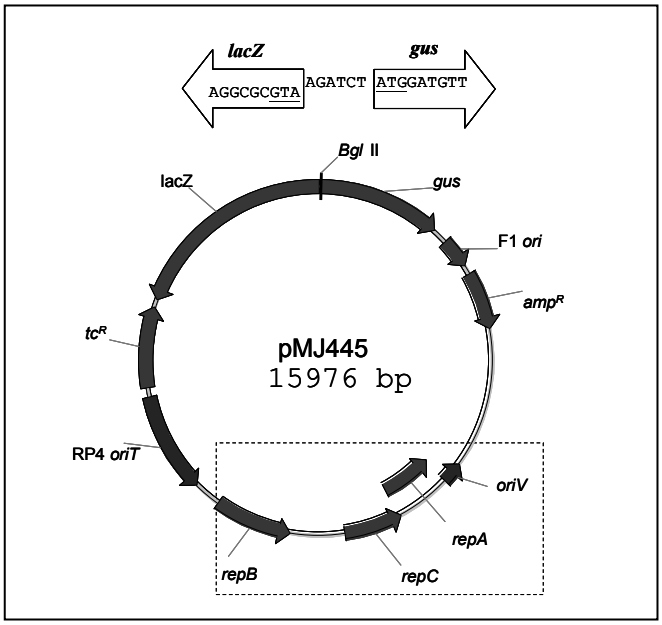

Figure 2.2. Schematic map of the broad host-range promoter probe vector, pMJ445, used in the analysis of the *dhp* promoter region. The vector contains a broad host-range origin of replication made up of the genes *repB*, *repC*, *repA* and *oriV* (boxed in dashed lines), an *E. coli* origin of replication (F1 *ori*), a plasposon-derived origin of transfer (*oriT*), the antibiotics resistance markers for Ampicillin (amp^R) and Tetracycline (tc^R), and divergent genes encoding the reporter enzymes β-galactosidase (*lacZ*) and β-glucuronidase (*gus*). The promoter is inserted between the *lacZ* and *gus* genes using the restriction enzyme *Bgl* II (Jiwaji *et al*., 2008).

2.4 Site-directed mutagenesis of the putative transcription factor binding sites in the *dhp* promoter

Mutagenesis was carried out on a pGEM T-Easy construct containing the 616 bp *dhp* promoter (pJAS20) using Kapa High Fidelity DNA polymerase (Kapa Biotech) and the amplification products were treated with *Dpn* I. The upstream half-site of the putative Anr box and the downstream half-site of the putative Vfr binding site were mutated to ensure that, in each case, the other site remained intact (Figure 2.3). For the Crp/Vfr

binding site mutagenesis, the half-site was mutated by the introduction of a *Sma* I site using the primers JD1 (5' - CAAAATGGTGAACAGCCCGGGCATTTTGGTGAACG CC - 3') and JD2 (5' - GGCGTTCACCAAAATGCCCGGGCTGTTCACCATTTTG - 3'). In the mutagenesis of the putative Anr box, the half-site was mutated by introducing a *Pst* I site using the primers JD3 (5' – CTTGACCAATTTGGCATCTGCAGAAGTGCG TCAAAATGGTG - 3') and JD4 (5' – CACCATTTTGACGCACTCTGCAGATGCC AAATTGGTCAAG - 3'). Once mutated, the promoters were sequenced using the universal primers pUCF/R, before sub-cloning into the broad host-range vector pMJ445, using *Bgl* II. This gave rise to pJD6 (mutated in the putative Vfr binding site) and pJD7 (mutated in the putative Anr box/σ^{54}) (Figure 2.3).

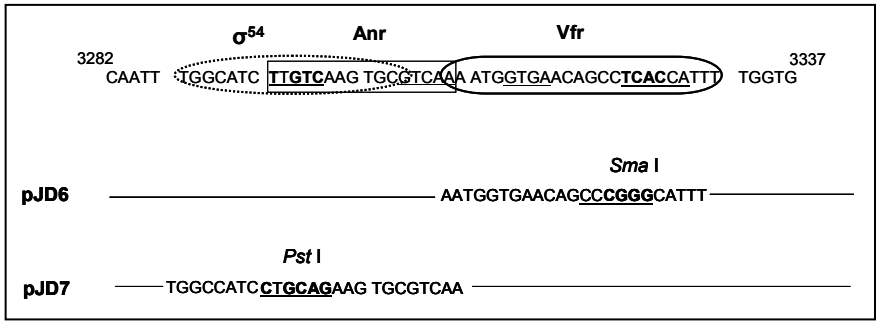

Figure 2.3. Strategy for site-directed mutagenesis of putative binding sites for Anr/σ^{54} and Vfr in the *dhp* promoter. The nucleotide sequences are annotated as follows: nucleotide co-ordinates relative to the start of the hydantoinase gene cluster flank the sequence; the putative σ^{54} binding site is circled in dashed lines, the Anr site is boxed and the putative Vfr site is circled in solid lines; nucleotides targeted for mutation are indicated in bold font, while both the half-sites and the restriction endonuclease recognition sequences are underlined. pJD6 and pJD7 refer to the final promoter probe constructs containing mutations in the Vfr and Anr/σ^{54} binding sites, respectively.

The vector constructs were screened using PCR followed by restriction digest with *Sma* I and *Pst* I. First, PCR amplification of the recombinant plasmids using primers specific to both the *dhp* promoter (GFM38) and the broad host range vector (MJ94: 5' - TTCGAAGATCGGCCCCCGCTCGACGCTC – 3') was carried out. Here, an 800 bp product would indicate that the *dhp* promoter was present in the vector, but not that the

putative binding sites were mutated. To determine if this was the case, the PCR products were digested with *Sma* I and *Pst* I, such that only the promoters containing a mutated Crp/Vfr or Anr binding site would be cut by either *Sma* I or *Pst* I respectively, to generate two distinct fragments of around 400 bp. The digests were analysed by agarose gel electrophoresis on 4 % agarose gels. This screening was carried out both before and after introducing pJD6/7 into RU-KM3$_s$ cells. It was particularly important to ensure that the cells used in reporter enzyme assays contained the correct vector construct. Therefore genomic extractions were performed on these cells to remove the vector and PCR was carried out on these genomic preparations, followed by restriction analysis, as before.

2.5 Targeted disruption of *vfr* and *crc*

In order to determine whether either of the global regulatory proteins Vfr or Crc were involved in regulating DHP activity in RU-KM3$_s$, the corresponding genes were disrupted by homologous recombination using plasposon-derived knockout vectors. The gene disruption vectors, pVfrKO and pCrcKO, were constructed by Dr Gwynneth Matcher (unpublished) and derived from the plasmids, pTnMod-OTc and pTnMod-OKm respectively (Dennis and Zylstra, 1998). The plasposons retained their *oriT* genes allowing them to be mated into RU-KM3$_s$, as well as the inverted repeats for insertion into the chromosome. However, in these plasmids, the transposase gene was removed and replaced by a 5' truncated fragment of the target gene, either *vfr* (550 bp) or *crc* (430 bp) (Figure 2.4). This allowed for the disruption of specific genes by homologous recombination on the chromosome of *P. putida* RU-KM3$_s$, resulting in the formation of two truncated non-functional copies of the target gene in the genome.

Disruption of the genes on the chromosome of the transconjugate strains was confirmed by PCR analysis of the genomic DNA of these mutants using *Taq* polymerase (Bioline) and combinations of primers allowing for the wild-type chromosomal gene, knockout vector and disrupted chromosomal gene to be distinguished from one another. These primers corresponded to either the terminal end of the *vfr* gene (VfrR: 5'-GAATTCCTA GCGGGTACCGTGGACCACC-3'), internal *vfr* sequence [Vfr(int): 5'-CTGTGCTGGC

GATCGTGCCG-3'] or vector sequence (TnOTcF: 5'-CATTATGATTCTTCTCGGTTC CGGCGGC-3', TnOTcR: 5'-AAAGGCCAGGAACCGTAAAAAGGCCGCG-3' and TnOKmSF: 5'-TTTACGGTTCCTGGCCTT T-3').

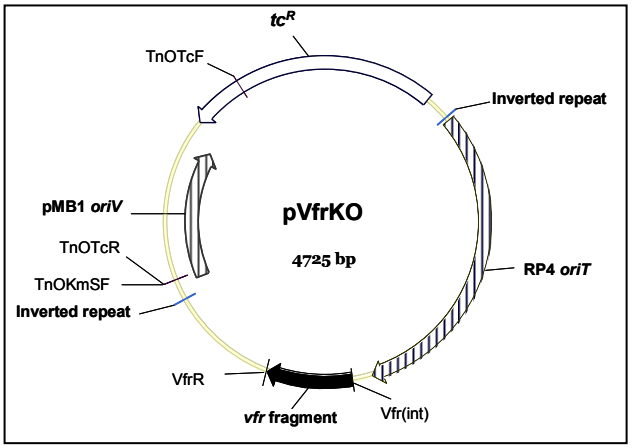

Figure 2.4. Schematic map of the gene disruption vector, pVfrKO. The plasmid contains a conditional origin of replication (pMB1 *oriV*), a plasposon-derived origin of transfer (*oriT*), the antibiotics resistance markers for Ampicillin (amp^R) and Tetracycline (tc^R), a set of inverted repeat sequences and a 515 bp internal fragment of *vfr*.

2.6 Biocatalytic colorimetric assays

2.6.1 Harvesting of cells

P. putida RU-KM3$_s$ cells were cultured in either nutrient broth (NB), NB containing 0.1 % hydantoin (Aldrich, ICN Biomedicals Inc) or NB containing 0.1 % hydantoin and 1 % succinate. These cultures were obtained by seeding 100 ml of medium with an overnight culture of RU-KM3$_s$ to an $OD_{600\ nm}$ of 0.02 and growing to stationary phase ($OD_{600\ nm}$ between 2.5 and 3.0) for 18 – 20 hours at 28 ^oC. Thereafter the cells were harvested by centrifugation in pre-weighed centrifuge bottles at 7000 rpm in a Beckman JA-14 rotor for 10 minutes. The supernatant was discarded and cells washed in half the original culture volume with cold 0.1 M potassium phosphate buffer, pH 8. After re-centrifugation as above, the wet cell mass of the resultant pellet was determined and the

pellet resuspended such that a final concentration of 40 mg wet cell mass/ml was achieved.

2.6.2 Dihydropyrimidinase (DHP) enzyme assays

Resting cell colorimetric assays of DHP activity, using Ehrlichs and Ninhydrin reagents, were carried out in triplicate according to Matcher *et al*. (2004). The 40 mg/ml cell suspension above was used to form reaction mixes as follows:

Table 2.1. Reaction components for biocatalytic assays

Components (replicates)	**Buffer blank (x1)**	**Substrate blank (x2)**	**Cell blank (x1)**	**Samples (x3)**
0.1 M Phosphate buffer, pH 8	2 ml	1 ml	1 ml	
100 mM Hydantoin substrate dissolved in 0.1 M phosphate buffer, pH 8		1 ml		1 ml
Cells	-	-	1 ml	1 ml

The reaction mixtures were then incubated at 40 °C for 3 hours with constant shaking after which the mixtures were transferred to 1.5 ml Eppendorf tubes and centrifuged at 13 000 rpm for 3 minutes in a Heraeus microfuge to pellet the cells. The resultant supernatant was analysed for *N*-carbamylamino acids and amino acids using Ehrlich's and Ninhydrin colorimetric assays respectively. Units of activity were measured as μmol/ml of product per 20 mg wet cell mass per ml, and expressed as an average of three replicates. DHP activity was calculated as the total μmol/ml of *N*-carbamylamino acid and amino acid produced from hydantoin substrate.

A. Ehrlichs assay

1 ml of the cell supernatant above was aliquoted into a test-tube and 0.5 ml 12 % trichloroacetic acid added to stop microbial conversion of *N*-carbamylamino acids to amino acids. The mixture was then shaken briefly and 0.5 ml Ehrlich's reagent (10 % ρ-dimethyl-aminobenzaldehyde in 6 M HCl) added, followed by 3 ml triple distilled

water. After incubation at room temperature for 20 minutes, the absorbance at 570 nm was determined and the concentration of product calculated using a standard curve of 0 – 50 mM *N*-carbamylglycine.

B. Ninhydrin assay

20 µl of the supernatant was aliquoted into test-tubes with 980 µl of 0.1 M potassium phosphate buffer, pH 8 and 1 ml of ninhydrin reagent (0.8 g ninhydrin and 0.12 g hydrindantin dissolved in 30 ml 2-methoxyethanol, before addition of 10 ml sodium acetate buffer of pH 5.5) was added. The samples were placed in a boiling water bath for 15 minutes. After cooling to room temperature, 3 ml 50 % ethanol was added and the samples incubated at room temperature for 15 minutes. The absorbance at 570 nm was determined and the concentration of product calculated using a standard curve of 0 – 50 mM glycine.
Note: Ninhydrin reagent was freshly prepared for each assay and stored in a dark glass container due to its sensitivity to light.

DHP activity was expressed as the total amount of both *N*-carbamylamino acid and amino acid produced (µmol/ml) during the biocatalytic reaction. All biocatalytic assays were independently repeated at least twice with freshly cultured cells.

2.6.3 β-Glucuronidase (GUS) reporter enzyme assays

GUS assays were carried out to monitor transcriptional activation supported by the *dhp* promoter in the multi-copy vector probe, pMJ449. *P. putida* cells were assayed for GUS activity using a protocol adapted from Jefferson *et al*. (1986). Cells were grown to stationary phase in the appropriate medium and harvested as described in Section 2.6.1 to give a final wet cell concentration of 40 mg/ml in 0.1 M phosphate buffer, pH 8.0. A total of 1 ml of these cells were then permeabilised by the addition of 1 ml permeabilisation buffer (32 mM $NaPO_4$, 2 mM EDTA, pH 8.0, 100 mM Tris HCl pH 8.0, 5 mM DTT, 4 % Triton X-100 and 0.4 mg/ml lysozyme) followed by incubation at 37 ºC for 1 hour. Thereafter 100 µl of the permeabilised cells was aliquoted into a microtitre plate and 50 µl substrate solution [20 mM PNPG dissolved in GUS buffer stock solution (50 mM

$NaPO_4$ buffer pH 7.0 and 1 mM EDTA, pH 8.0)] added to each sample. The microtitre plate was incubated at 37 ºC until a visible yellow colour was observed and the incubation time noted, before measuring the $OD_{600\ nm}$, $A_{405\ nm}$ and $A_{550\ nm}$ in a PowerwaveX Microtitre Plate Reader (Bio-Tek Instruments, Inc.). Activity was calculated in β-Gluc units, using the formula:

$$\frac{100 \text{ X } [A_{405nm}-(1.75 \text{ X } A_{550\ nm})]}{A_{600\ nm} \text{ X } (\Delta\text{Time}) \text{ X Volume (ml)}}$$

Once again, all reactions were carried out in triplicate and the standard deviation for each sample set calculated, while each assay was independently repeated at least twice with freshly harvested cells.

CHAPTER 3

RESULTS

RESULTS

3.1 Introduction

Matcher *et al*. (2004) showed that the hydantoin-hydrolysing activity of *P. putida* strain RU-KM3$_s$ was due to the activity of dihydropyrimidinase and β-ureidopropionase enzymes, encoded by chromosomal copies of *dhp* and *bup* respectively. Enzyme activity was tightly regulated by the growth phase as well as by the presence of hydantoin in the growth medium. Several mutant strains that were unable to grow in medium with hydantoin as the sole nitrogen source were isolated, a number of which retained wild-type hydantoinase (DHP) activity. The disrupted genes in these mutants were identified in each case as either *glnD*, *ntrC* or *ntrB,* which encode the enzyme uridyltransferase and the regulators NtrC and NtrB respectively. Since these proteins are involved in the nitrogen-sensitive Ntr regulatory pathway, the wild-type hydantoinase activity observed in these mutants suggested that DHP activity was not regulated in response to the nitrogen status of the cell.

Only one potential regulatory mutant displaying reduced hydantoinase activity was isolated, with the gene encoding a dihydrolipoamine succinyl transferase enzyme inactivated. This enzyme plays an important role in the TCA cycle in the conversion of α-ketoglutarate to succinyl co-A. Since disruption of the TCA cycle (and presumed subsequent changes in the concentration of cycle intermediates) decreased hydantoinase activity, this suggested a role for succinate and other readily utilisable carbon sources in regulating hydantoin-hydrolysing activity in RU-KM3$_s$. It was found that succinate, as well as glucose, substantially reduced activity, suggesting that the hydantoin-hydrolysing system of strain RU-KM3$_s$ is regulated by carbon catabolite repression (CCR). Finally, Matcher (2004) identified a putative CRP binding site in the intergenic region upstream of the *dhp* and *bup* coding sequences and showed that this site was functional in *E. coli*. It was hypothesised that this was the site through which CCR regulated the expression of *dhp* and *bup* (Matcher *et al*., 2004). The overall aim of this research project was to test this hypothesis and extend the understanding of the mechanisms which regulate the expression of these genes in *P. putida*.

3.2 Identification of potential *cis*-acting regulatory elements in the *dhp*-ORF1(*bup*) intergenic region of *P. putida* RU-KM3$_s$

First, the nucleotide sequence of the *dhp*-*bup* intergenic region was examined to determine whether there were other putative transcription factor binding sites present, in addition to the CRP site identified by Matcher (2004). The hydantoin-hydrolysing gene cluster consists of three open reading frames. The dihydropyrimidinase is encoded by an ORF of 1440 bp (479 amino acids), while the β-ureidopropionase ORF of 1284 bp encodes a predicted protein of 427 amino acids. A third ORF is present upstream of *bup*, which encodes a predicted transporter protein of 500 amino acids. The genes are arranged divergently to each other on the chromosome, separated by a 616 bp intergenic region (Figure 3.1).

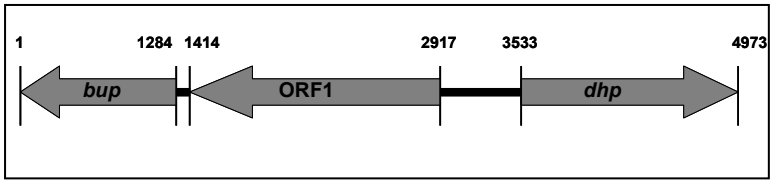

Figure 3.1. Organisation of the gene cluster responsible for hydantoin-hydrolysing enzyme activity in *P. putida* RU-KM3$_s$. Open reading frames are depicted as grey arrows and include the β-ureidopropionase (*bup*) and dihydropyrimidinase (*dhp*) genes as well as an open reading frame (ORF1) predicted to encode a permease. The nucleotide co-ordinates are given relative to the translational stop codon of *bup*.

Bioinformatic analysis of the *dhp*-ORF1(*bup*) intergenic region was carried out to identify potential binding sites for transcriptional regulators, including sigma factors and global regulatory proteins. These putative binding sites along with their degree of conservation, when compared to the consensus, are listed in Table 3.1.

Table 3.1. Identification of potential binding sites in the *dhp*/ORF1(*bup*) intergenic region of *P. putida* RU-KM3$_s$.

Site	Sequence		Percentage identity	Reference for consensus sequence
CRP	RU- KM3$_s$	3310-AAA**T-GTGA**TCTAGA**TCAC-A**TTT-3332	77 %	Gunasekera *et al*., 1992
		****** **** * **** ******		
	Consensus	AAA**TGGTGA**ACAGCC**TCACCA**TTT		
Vfr	RU- KM3$_s$	3310-AAAA**TGGTGA**ACAGCC**TCACCA**-3332	81 %	Kanack *et al*., 2006
		*** **** *** **** ***		
	Consensus	ANWW**TGN-GA**WNYAGW**TCAC-A**		
Anr box	RU- KM3$_s$	3294-**TTGTC**AAGTGC**GTCAA**-3310	70 %	Spiro, 1994
		***** ******		
	Consensus	**TTGAT**NNNN--**ATCAA**		
lux box	RU- KM3$_s$	3434-T**TCT**GTCAGG**TCT**GAC**AG**GAT-3455	75 %	Pessi and Haas, 2000
		**** ** **** *** ***		
	Consensus	A**CCT**NCCANN**TCT**GGC**AG-**NT		
σ^{70} -10	RU- KM3$_s$ (upstream of *bup*)	2943-**AATTAG**-2938 2963-**CATTAG**-2958	50 %	Wise *et al*., 1996
		**** *** **** ***		
	Consensus	**TATAAT** **TATAAT**		
		****** *** *********		
	RU- KM3$_s$ (upstream of *dhp*)	3345-**TATATT**-3351 3353-**TATAAA**-3358	83 %	
σ^{70} -35	RU- KM3$_s$ (upstream of *bup*)	2972-**CGGCCA**-2967 2982-**TGTAGA**-2977	50 %	Wise *et al*., 1996
		*** **** *** * ***		
	Consensus	**TTGACA** **TTGACA**		
		*** * *** ***** ****		
	RU- KM3$_s$ (upstream of *dhp*)	3332-**TGGTGA**-3337 3293-**TTGTCA**-3298	50 %, 83 %	
σ^{54}	RU- KM3$_s$	3286-**TGGCAT**CTTGTCAA**GTGCG**-3305	82 %	Pearson *et al*., 1997
		********** *******		
	Consensus	**TGGCAT**NNNNNN--**TTGCT**		
σ^{38}	RU- KM3$_s$	3423-**CTAG**AT**T**-3430	57 %	Espinos-Urgel *et al*., 1996
		***** ***		
	Consensus	**CTAT**AC**T**		

Key: For each binding site, the corresponding sequence in RU-KM3$_s$ and its nucleotide co-ordinates are shown above the consensus. The important consensus half-sites are indicated in bold and the areas of homology denoted by stars.

The putative CRP binding site (nts 3310 - 3332) in the *dhp*-ORF1(*bup*) intergenic region, although two nucleotides longer, displays significant homology to the *E. coli* CRP consensus sequence, particularly in the highly conserved half-sites, with 77 % identity overall (Table 3.1). This putative binding site also shows a high degree of conservation with the consensus binding site for Vfr (81 % identity) and all the nucleotides making up the important half-site are conserved (Table 3.1). Vfr is a functional CRP homologue in *E. coli*, but acts as a global virulence regulator in *Pseudomonas* and is required for the production of exotoxins, proteases and other virulence factors (Suh *et al*., 2002). Furthermore, it is known that Vfr activates the expression of the gene encoding the LuxR homologue, LasR, which is required for elastase and alkaline protease production in the virulent strain, *P. aeruginosa*. This highlights the interdependence of virulence and the cell-density dependant signalling mechanism known as quorum sensing (QS) in *Pseudomonas* (Aldus *et al*., 1997; Rumbaugh *et al*., 2000).

It is unknown whether *P. putida* RU-KM3$_s$ is a virulent strain, using its cell-to-cell communication in pathogenic interactions with other cells, or whether the strain uses QS to communicate with a plant host in a symbiotic relationship. Bearing in mind that RU-KM3$_s$ was isolated from soil (Burton *et al*., 1998), the latter seems more likely. Therefore it is plausible that RU-KM3$_s$ may possess a QS system involving the LasR homologue, PpuR, such as that found in the rhizosphere microbe, *P. putida* IsoF (Steidle *et al*., 2002). Considering this, it was interesting to discover a putative *lux* box (nts 3434 - 3455) at a position 88 bp upstream of the translational start of *dhp* (Table 3.1). The *lux* box has been identified as the binding site for both PpuR and the LasR co-regulator RhlR, which is involved in rhamnolipid biosynthesis (Juhas *et al*., 2005). When compared to the *lux* box consensus, the sequence in the *dhp*/ORF1(*bup*) intergenic region displayed 75 % identity and retained seven out of the eight nucleotides identified as being essential for recognition by LuxR-type QS regulators, including PpuR and RhlR (Table 1) (Pessi and Haas, 2000; Steidle *et al*., 2002).

Further analysis of the intergenic region revealed a potential Anr box (nts 3294 – 3310), centred 232 bp upstream of the translational start codon of *dhp* (Table 3.1). Anr is a global regulator which controls the switch from aerobic to anaerobic conditions in *Pseudomonas* (Spiro, 1994). The putative Anr box sequence was also two nucleotides longer than the consensus, but displayed 70 % identity in the conserved half-sites (Table 3.1). The downstream half-site (GTCAA), in particular, was almost identical to the consensus. Interestingly, this predicted Anr binding site overlaps almost completely with a putative σ^{54} binding site, while its downstream half-site overlaps to some extent with the putative Vfr binding site (Figure 3.2).

The *dhp*-ORF1(*bup*) intergenic region was also searched for the presence of sigma factor binding sites. In terms of the major sigma factor, σ^{70}, two potential -10 sequences or Pribnow boxes were identified at nucleotides 185 and 177 upstream of the *dhp* translation initiation codon (Figure 3.2), with the corresponding -35 sequence bearing little resemblance to the consensus (Table 3.1). In addition, two sets of overlapping poorly conserved -10 and -35 sequences were identified in the intergenic region upstream of the ORF1/*bup* coding sequences, with the putative Pribnow box sequences located 23 bp and 43 bp upstream of the ORF1 translational start codon and the putative -35 sequences positioned 43 bp and 63 bp upstream of the ATG of ORF1 (Figure 3.2). These σ^{70} recognition sequences in the putative ORF1/*bup* promoter, however, displayed only 50 % identity with the consensus (Table 3.1), suggesting that they are poor candidates for σ^{70}-mediated transcriptional regulation of ORF1-*bup*.

Further analysis of the intergenic region upstream of *dhp* revealed that the poorly conserved σ^{70} -35 sequence, which was identified based upon its position relative to the putative -10 sequences, was in fact located within a region designated as a CRP/Vfr binding site (Figure 3.2). Although a more highly conserved -35 sequence was found upstream of *dhp*, it was significantly further away from the putative Pribnow box sequences, at a position 237 nucleotides from the translation start of *dhp* (Table 3.1, Figure 3.2). Taken together, these factors suggested that it was highly unlikely that the

putative *dhp* promoter would be recognised by σ^{70} and, therefore, that the vegetative sigma factor probably plays a minor role in the hydantoinase regulon in RU-KM3$_s$.

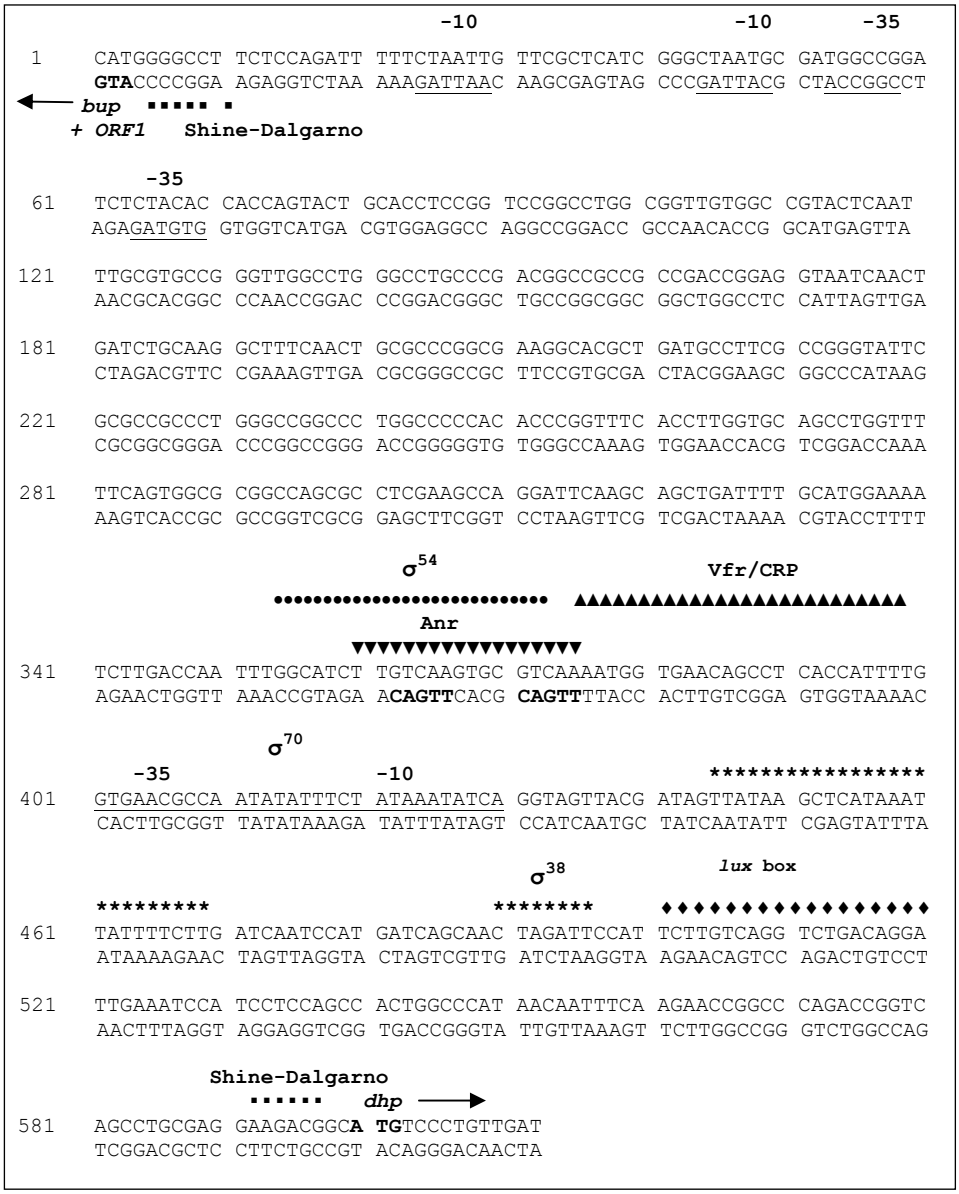

Figure 3.2. Intergenic region in the *dhp*-ORF1(*bup*) gene cluster of *P. putida* RU-KM3$_s$ showing putative binding sites for transcription factors. Symbols indicate binding sites as follows: • above the nucleotide sequence indicates a putative σ^{54} binding site, ▲ above the sequence indicates a putative Vfr binding site, ▼ is shown below the putative Anr binding site, , * above the sequence indicates a putative σ^{38} recognition sequence, ♦ above the sequence indicates a putative *lux* box and the putative σ^{70} recognition sequences are underlined (Gunasekera *et al*., 1992; Spiro, 1994; Wise *et al*., 1996; Espinos-Urgel *et al*., 1996; Pearson *et al*., 1997; Pessi and Haas, 2000).

On the other hand, a putative -10 recognition sequence for the stationary phase sigma factor, σ^{38}, was found at a position 106 nucleotides upstream of the translational start of *dhp* (Figure 3.2). This sigma factor regulates the expression of a wide variety of genes involved in various stress responses associated with stationary phase, such as carbon starvation and quorum sensing (Kazmierczak *et al*., 2005). The putative σ^{38} recognition sequence in the *dhp* promoter displays five out of seven nucleotides identical to the consensus and four out of the five nucleotides described as being essential for the functioning of the site (Table 3.1). In addition, the AT rich region upstream, required for DNA curvature, is present approximately 20 nt upstream of the binding site and is 25 nt in length (Figure 3.2). No such σ^{38} -10 recognition sequences were found upstream of ORF1/*bup*.

A sequence displaying 89 % identity to the palindromic recognition sequence of σ^{54} was identified approximately 238 bp from the translational start codon of *dhp* (Figure 3.2). This unique sigma factor is involved in the regulation of a diverse set of genes in *Pseudomonas*, including those whose products are involved in the utilisation and transport of different nitrogen and carbon sources, together with genes involved in virulence and the phage-shock response (Reitzer and Schneider, 2001). The first putative σ^{54} half-site in the *dhp* promoter is identical to the consensus, while the second half-site has only three out of the five nucleotides conserved (Table 3.1). The spacing between half-sites in the putative σ^{54} recognition sequence is eight nucleotides, two longer than that usually seen in such promoters (Table 3.1). This two nucleotide insertion is reminiscent of that seen in both the putative Vfr and Anr binding sites of the *dhp* promoter. Interestingly, the putative σ^{54} binding site overlaps almost completely with the Anr box identified in this promoter (Figure 3.2).

Thus, analysis of the *dhp*-ORF1(*bup*) intergenic region identified potential binding sites for seven transcriptional regulators upstream of the translational start of *dhp*, with only poorly conserved σ^{70} recognition sequences upstream of ORF1/*bup*. The putative transcription factor binding sites in the *dhp* promoter region included the recognition sequences for Vfr, Anr and PpuR/RhlR, as well for the alternate sigma factors, σ^{38} and

σ^{54} (Figure 3.2). It is interesting to note that all of these putative binding sites correspond to transcription factors that function under the various stress conditions associated with the onset of stationary phase, which is when hydantoin-hydrolysing activity is at its highest. In general, the stationary phase-specific expression of genes is controlled on a global level by the alternate sigma factors, σ^{38} and σ^{54} (Espinos-Urgel *et al*., 1996; Pearson *et al*., 1997). However, the response to specific environmental conditions occurs as follows: increased cell density stimulates the quorum sensing regulators PpuR and/or RhlR, along with the virulence regulator, Vfr (Pessi and Haas, 2000); decreased nutrient concentration, in particular poor carbon source, activates Crc and possibly Vfr (Suh *et al*., 2002), and decreased oxygen levels trigger the functioning of Anr (Spiro, 1994). Therefore, the presence of numerous overlapping potential binding sites for transcriptional regulators in the *dhp*-ORF1(*bup*) intergenic region suggests that the expression of this gene cluster may be sensitive to several global control mechanisms including: CCR, quorum sensing, and oxygen-sensitive regulation, all in addition to regulation by an alternate sigma factor.

It was decided to focus on the regulation of *dhp* expression to further characterise the mechanisms of transcriptional regulation in this system. This was because almost all of the potential transcription factor binding sites were clustered within 250 bp upstream of the translational start of the *dhp* open reading frame, with less significant recognition sequences located upstream of *bup*/ORF1. In addition the implications of the *bup* open reading frame being located downstream of ORF1, and thus 1500 bp away from its putative promoter region, are unknown and it is possible that the transcriptional regulation of *bup* is not mediated by this *dhp*/ORF1(*bup*) intergenic region.

3.3 Environmental factors that regulate DHP activity in *P. putida* RU-KM3$_s$

The results of the bioinformatic analysis of the intergenic region upstream of *dhp* suggested the involvement of several global regulatory systems; including carbon catabolite repression (CCR), quorum sensing (QS), virulence and oxygen limitation; in the regulation of *dhp* expression. Thus, the effect of the corresponding environmental conditions on DHP activity was examined. First, the findings of Matcher (2004), namely

that enzyme activity is induced in the presence of the substrate and repressed when a more efficient carbon source is added to the medium, were confirmed. Cells were grown to stationary phase ($OD_{600\ nm}$ 2.5 – 3.0) in complete medium (nutrient broth) without inducer or supplemented with either 0.1 % hydantoin or 0.1 % hydantoin and 1 % succinate (carbon repression) and assayed for DHP activity. For all assay results depicted, the experiments were repeated at least three times and showed the same trends in both the levels and regulation of enzyme activity.

There was an approximate 20–fold induction in enzyme activity in cells grown in complete medium supplemented with hydantoin (from 1.5 μmol/ml to 29.5 μmol/ml) (Figure 3.3). The addition of succinate resulted in a five-fold reduction in activity as compared to cells grown in the presence of inducer (6.4 μmol/ml *vs.* 29.5 μmol/ml respectively), which was still higher than that in uninduced cells (1.5 μmol/ml) (Figure 3.3). These results correlated with the observations of Matcher (2004).

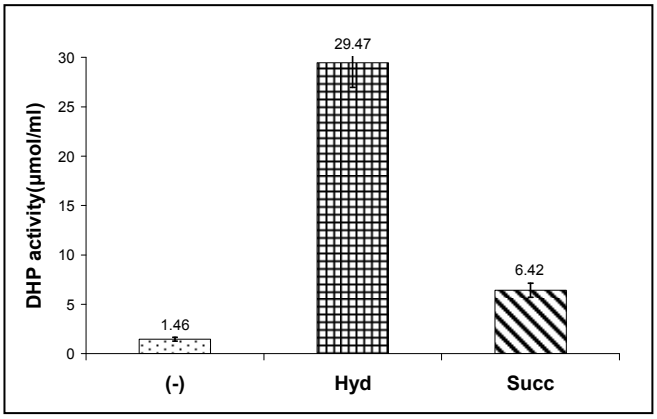

Figure 3.3. Regulation of DHP activity by the presence of inducer and a repressive carbon source. Cells were grown to stationary phase in complete medium. [(-): uninduced cells grown in nutrient broth; Hyd: induced with 0.1 % hydantoin; Succ: repressed by addition of 1 % succinate]. Error bars indicate the standard error of the mean where n = 3.

Next, it was important to determine whether other environmental conditions, which activate global regulatory pathways, also controlled DHP activity. The presence of a

potential *lux* box in the promoter region of *dhp* raised the possibility that enzyme activity would be sensitive to quorum sensing conditions. To investigate this, DHP activity was assayed in cells grown in half of the culture volume medium containing the same amount of nutrients as the control. Under these growth conditions, the cell density is increased ($OD_{600\ nm}$ of approximately 4.5) as opposed to standard growth conditions ($OD_{600\ nm}$ of around 2.8), inducing the quorum sensing response. DHP activity was repressed five-fold (35.8 µmol/ml *vs.* 6.5 µmol/ml) (Figure 3.4) under quorum sensing conditions, suggesting that *dhp* expression might be subject to regulation by PpuR and/or RhlR.

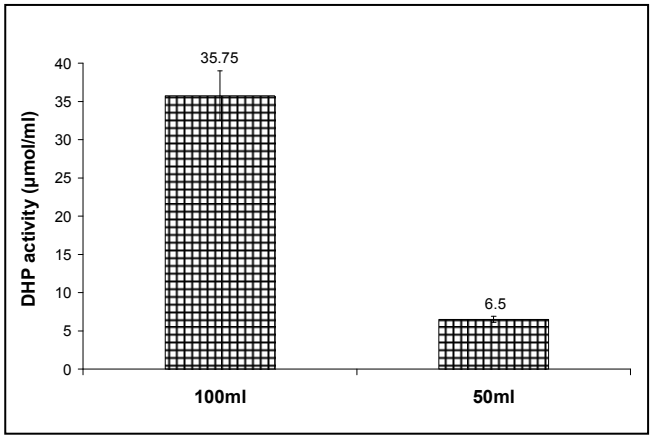

Figure 3.4. The effect of quorum sensing-specific environmental conditions on DHP activity. Cells were grown to stationary phase in either 100 ml or 50 ml nutrient broth, containing the same amount of nutrients, with 0.1 % hydantoin and harvested to the same cell density per ml used to assay enzyme activity. (Error bars indicate the standard error of the mean where n = 3).

The presence of a putative Anr box upstream of the *dhp* ORF, suggested that the oxygen status of the cell may also play a role in regulating DHP activity. Since it was unknown if strain RU-$KM3_s$ is capable of anaerobic growth, the first step was to culture the cells with varying levels of aeration in order to classify the organism as a facultative aerobe or aerotolerant anaerobe. Therefore, cells were grown in 100 ml nutrient broth with or without the alternate electron acceptor, sodium nitrate, in tightly stoppered Schott bottles of either 100 ml or 250 ml capacity. The optical densities were monitored over time. The

results suggested that *P. putida* RU-KM3$_s$ is an aerobic organism that displays only a mild tolerance towards anaerobic conditions. After four days of incubation at 28 ºC, the cells cultured under the most highly anaerobic conditions had not yet neared an $OD_{600\ nm}$ of 1.0, whereas cells grown under aerobic conditions had exceeded an $OD_{600\ nm}$ of 4.0 after 24 hours (Table 3.2). The presence of nitrate as an alternate electron acceptor did not affect the ability of cells to grow under anaerobic conditions, suggesting that RU-KM3$_s$ cells are unable to utilise this alternate electron acceptor and are simply making use of the small amount of residual oxygen in the vessel (Table 3.2). Whether or not DHP activity is affected by the oxygen level in the growth medium could not be determined due to insufficient biomass production under anaerobic conditions.

Table 3.2. Anaerobic growth studies for *P. putida* RU-KM3$_s$

Culture Vessel Volume	**OD_{600}**		**Incubation period (hr)**
	-	**1 mM $NaNO_3$**	
100 ml	0.572	0.464	96
250 ml	1.871	1.879	96
500 ml (control)	4.216	*nd	24

* not determined

Taken together, these results indicate the involvement of at least two distinct regulatory pathways in the control of DHP activity, namely the CCR system identified by Matcher (2004) and quorum sensing. The transcription factors mediating the cell's response to these specific environmental signals remain unknown, although the putative binding sites identified in the *dhp* promoter (Figure 3.2) hint at the involvement of the following regulatory proteins: Vfr in mediating CCR, PpuR/RhlR in response to quorum sensing and Anr in controlling the shift from aerobic to anaerobic conditions, as discussed in Section 3.1. Determining whether these transcription factors are, in fact, involved in regulating DHP activity in response to specific environmental signals required the targeted disruption of their genes to create transcription factor mutant strains. Since RU-KM3$_s$ appears to be a strictly aerobic strain, no experiments could be carried out under anaerobic conditions. Therefore it was decided to focus only on those factors regulating DHP activity under CCR and QS conditions.

3.4 Construction of *vfr*$^-$ and *crc*$^-$ mutant strains of RU-KM3$_s$

Previous studies have identified Crc as a global regulator of CCR in *Pseudomonas* (Hester *et al*., 2000), so this protein had a potential role to play in mediating the regulation of DHP expression by CCR. Since Crc does not bind DNA (MacGregor *et al*., 1991), it cannot be the protein that interacts with the putative CRP binding site upstream of *dhp* (Figure 3.2). In view of the fact that this site is also a putative Vfr recognition sequence, it was decided to investigate Vfr as a potential mediator of CCR in the regulation of DHP activity. In terms of the regulation of *dhp* expression under QS conditions, the proteins of interest were the global pseudomonad regulators, PpuR and RhlR. These transcriptional regulators are able to bind to conserved palindromic sequences known as *lux* boxes (Juhas *et al*., 2005; Steidle *et al*., 2002). Since such a sequence was identified upstream of *dhp* (Figure 3.2), it is feasible that PpuR and/or RhlR may be involved in the regulation of DHP activity in environmental conditions associated with QS. Confirmation of this hypothesis required mutational analysis of *ppuR* and *rhlR.*

First, it was important to determine whether the genes encoding Vfr, Crc, PpuR and RhlR were present on the RU-KM3$_s$ genome. Accordingly, PCR primers were designed, which corresponded to the 5' and 3' ends of the genes and whose sequences were derived from closely related pseudomonads. The primer sets VfrF/VfrR, derived from the *vfr* gene of *P. aeruginosa* PA01, and CrcF/CrcR, derived from the *crc* gene in *P. putida* KT2440 (primer sequences given in Methods and Materials) resulted in the successful amplification of a 645 bp *vfr* and a 780 bp *crc* gene respectively, from chromosomal DNA derived from RU-KM3$_s$ cells. Gene sequencing confirmed the presence of both genes in the strain. However, repeated attempts to amplify the quorum sensing genes, *ppuR* and *rhlR,* were unsuccessful. The primer sets PpuR-F/PpuR-R and RhlR-F/RhlR-R were designed based on the sequence of the *ppuR* gene in *P. putida* strain IsoF and the *rhlR* gene of *P. fluorescens*, respectively (see Methods and Materials). The experiments were repeated by Gwynneth Matcher (Department of Microbiology, Rhodes University) without success. This suggested that either RU-KM3$_s$ does not possess these genes; or that they are poorly conserved, when compared to the

rhlR of *P. putida* IsoF and *ppuR* of *P. fluorescens*, such that amplification of the genes using primers derived from these strains was not possible.

To determine whether Crc and/or Vfr are involved in modulating DHP activity, the corresponding genes were disrupted by homologous recombination. To generate the gene disruption, a vector (pVfrKO), derived from the plasmid pTnMod-OTc, was constructed for the targeted insertional inactivation of *vfr* in *P. putida* RU-KM3$_s$. This vector was created by inserting a 515 bp internal fragment of the target gene (*vfr*) into pTnMod-OTc, using the *Nde* I and *Eco* RI restriction sites, to allow for the specific disruption of *vfr* by homologous recombination on the chromosome. The construct, pVfrKO, was introduced into RU-KM3$_s$ cells by tri-parental mating, during which it was transferred from *E. coli* DH5α into the *Pseudomonas* cells, with the aid of *E. coli* HB101 cells containing the helper plasmid, pRK2013 (Dennis and Zylstra, 1998; Santos *et al*., 2001). Potential transconjugates were selected on agar plates containing chloramphenicol and tetracycline to select against both *E. coli* strains and RU-KM3$_s$ cells lacking pVfrKO. Thereafter, the disruption of *vfr* on the chromosome of the mutant, RU-KM3$_s$Δ*vfr*, was determined by PCR analysis using combinations of primers that would be able to distinguish between the wild-type chromosome and the disrupted locus (sequences given in Methods and Materials).

Primers Vfr(int) (A1) and VfrR (A2) were used to amplify a 550 bp fragment corresponding to the 3' end of the *vfr* gene in the wild-type and plasposon-generated mutant, which was not amplified from the knock-out vector, pVfrKO (Figure 3.5 B(i), Lanes 1 and 2 *vs.* Lane 3). Primers TnOTcF (B1) and TnOTcR (B2) amplified a 1060 bp fragment, corresponding to vector sequence, in the *vfr*$^-$ mutant and pVfrKO vector, but not in the wild-type chromosomal locus (Figure 3.5 B(ii), Lanes 1 and 3 *vs.* Lane 2). Finally, TnOKmSF (C1) corresponding to pVfrKO, and VfrR (A2) corresponding to the 3' end of the *vfr* gene, yielded a 1200 bp fragment in the *vfr*$^-$ strain only, confirming the disruption of *vfr* on the chromosome in the mutant (Figure 3.5 B(iii), Lane 1 *vs.* Lanes 2 and 3). These results confirmed that the mutant strain, RU-KM3$_s$Δ*vfr*, carried the disrupted *vfr* locus.

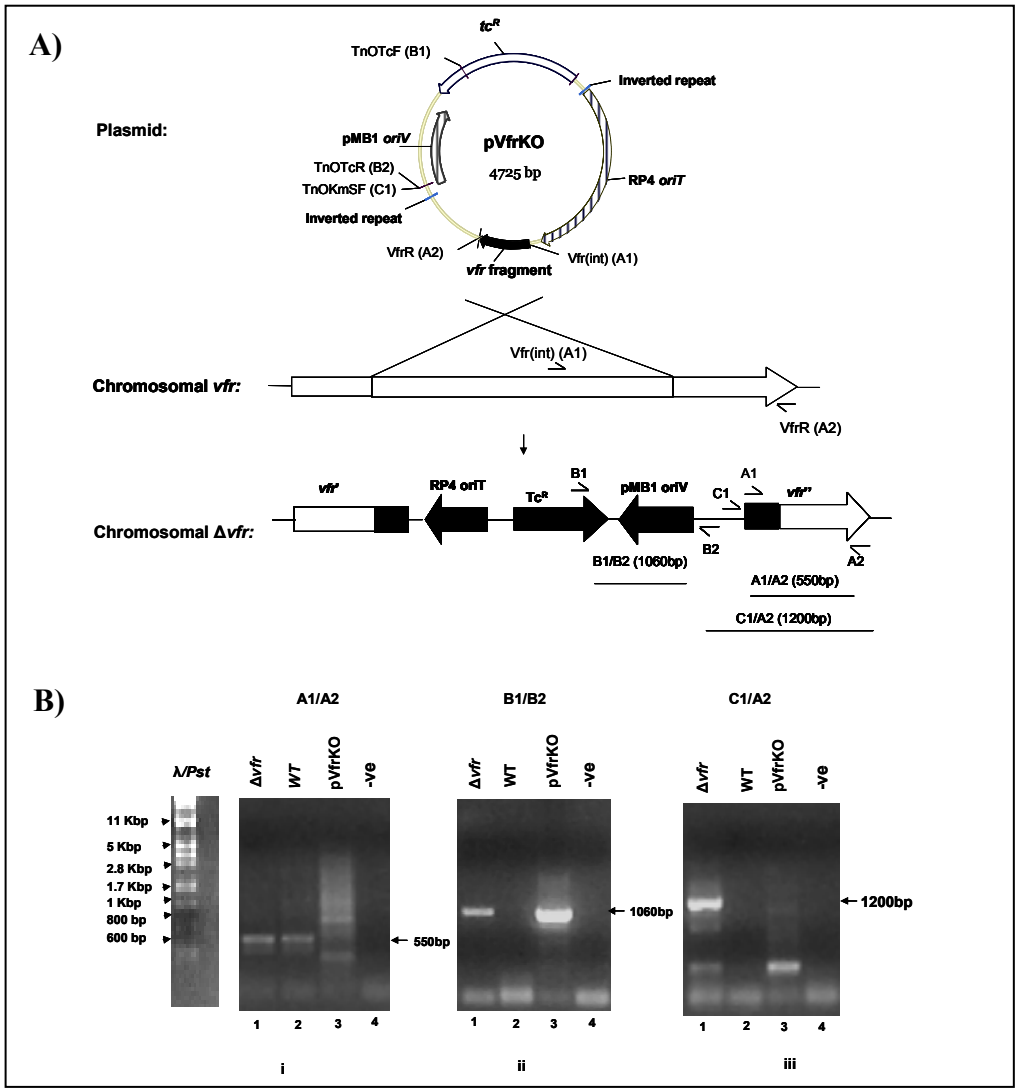

Figure 3.5. Construction of the *vfr*$^{-}$ mutant strain, RU-KM3$_S$Δ*vfr*, using pVfrKO to insertionally inactivate *vfr*. **(A)** Inactivation of chromosomal *vfr* by homologous recombination with pVfrKO to produce two truncated, non-functional *vfr* genes in the knock-out mutant. Primers are indicated by half arrows and anticipated PCR products as bold lines. **(B)** PCR verification of disruption of *vfr* in RU-KM3$_S$Δ*vfr*. PCR products were subject to agarose gel electrophoresis on ethidium bromide stained 1 % gels. **Bi, Bii and Biii** show amplification products using primers A1/A2, B1/B2 and C1/A2 respectively. DNA templates in each case: RU-KM3$_S$Δ*vfr* (Lane 1), wild-type (Lane 2), pVfrKO vector (Lane 3) and no DNA (Lane 4), respectively.

The results of the *vfr* disruption indicate the presence of single *vfr* gene on the chromosome of RU-KM3$_S$. This may be deduced from the absence of a wild-type *vfr* PCR product in the RU-KM3$_S$Δ*vfr* strain (Figure 3.5 Panel B(i), Lane 3). The presence

of only one *vfr* gene in this strain meant that no Vfr protein could be produced in the gene disruption mutant. This was an important observation, since no antibodies to Vfr were made in order to confirm the absence of this protein in RU-KM3$_s$Δ*vfr* cells.

In addition, a *crc*$^-$ strain (RU-KM3$_s$Δ*crc*) was obtained from Dr Gwynneth Matcher, who used a similar approach to generate this gene disruption mutant as was employed to create RU-KM3$_s$Δ*vfr*. The disruption of the *crc* locus was confirmed by PCR as for the *vfr*$^-$ strain and indicated the presence of a single *crc* gene in the *P. putida* RU-KM3$_s$ genome (data not shown).

3.4.1 DHP activity in *vfr*$^-$ and *crc*$^-$ mutant strains.

To determine whether DHP activity in RU-KM3$_s$ is regulated by Vfr and Crc , the wild-type cells, together with mutant strains, RU-KM3$_s$Δ*vfr* (*vfr*$^-$) and RU-KM3$_s$Δ*crc* (*crc*$^-$), were grown to stationary phase ($OD_{600\ nm}$ 2.5 – 3.0) in complete medium only, complete medium supplemented with hydantoin, or medium containing both hydantoin and succinate. The cells were assayed for DHP activity and a small, but noteworthy increase in hydantoin-induced DHP activity was observed in strain RU-KM3$_s$Δ*vfr* when compared to the wild-type (38.0 µmol/ml *vs.* 34.5 µmol/ml, respectively) (Figure 3.6 A). However, the inactivation of *vfr* did not appear to relieve carbon catabolite repression of DHP activity by succinate. The *vfr*$^-$ strain displayed a 9-fold reduction in enzyme activity under these conditions *vs.* a 5-fold decrease in wild-type cells (Figure 3.6 A). In addition, no major effect on induction by hydantoin, of DHP activity, was observed in this mutant (7-fold induction in the wild-type *vs.* 10-fold in RU-KM3$_s$Δ*vfr*). Thus, in RU-KM3$_s$, Vfr does not appear to function in the same way as *E. coli* CRP, which activates the transcription of genes whose products are required for the utilisation of less favourable carbon sources (Warner and Lolkema, 2003).

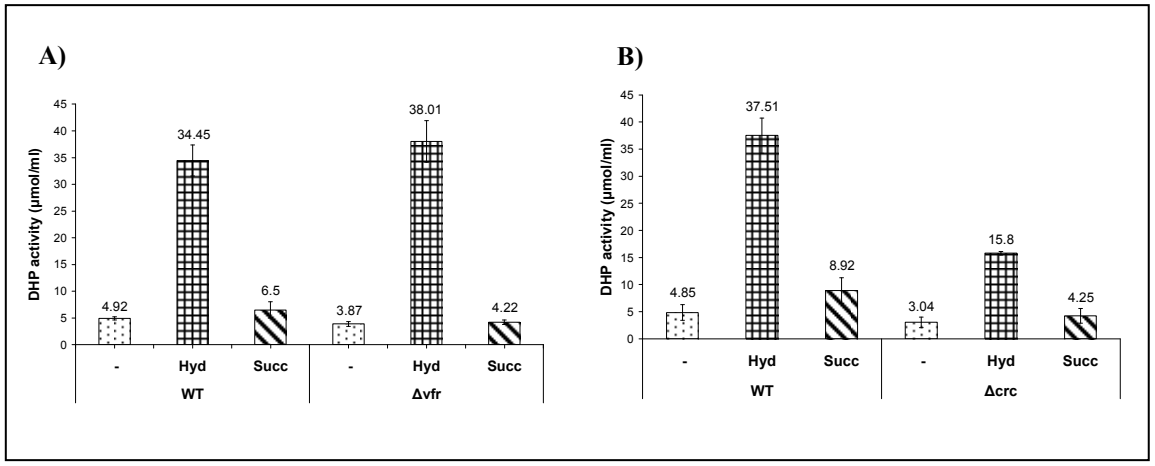

Figure 3.6. DHP activity in *vfr*$^-$ and *crc*$^-$ RU-KM3$_s$ strains. **A)** shows the effects of disruption of *vfr* on induction and CCR of DHP activity, while **B)** illustrates these effects on activity when *crc* is insertionally inactivated. (WT: Wild-type RU-KM3$_s$, Δ*vfr*: *vfr*$^-$ strain, Δ*crc*: *crc*$^-$ strain. (-): cells cultured in nutrient broth, Hyd: induced with 0.1 % hydantoin; Succ: repressed by 1 % succinate). Error bars indicate the standard error of the mean, where n = 3.

In the case of the *crc*$^-$ strain, RU-KM3$_s$Δ*crc*, the mutant displayed lower activity overall, with levels approximately half of those in the wild-type for all growth media, suggesting that Crc plays a general role in activation of DHP (Figure 3.6 B). Enzyme activity was not completely abolished in this mutant indicating that Crc is not functioning alone in the regulation of DHP activity. In terms of induction of DHP by hydantoin, the disruption of *crc* resulted in a noticeable decrease in this effect in RU-KM3$_s$Δ*crc* (8-fold induction in the wild-type *vs.* 5-fold in the mutant) (Figure 3.6 B), again pointing to a role in activation for Crc. However, CCR of DHP activity was identical in the wild-type and *crc*$^-$ strains showing a 4-fold reduction in both cases when cultured in medium containing succinate (Figure 3.6 B).

After studying the role of Vfr and Crc in regulating DHP activity under CCR conditions, the regulation of activity under quorum sensing conditions was examined. The identification of a putative *lux* box in the sequence upstream of the *dhp* ORF suggested that the global pseudomonad quorum sensing regulators, PpuR and/or RhlR, may be involved in mediating this cell density-dependant regulation of *dhp* expression in

RU-KM3$_s$. However, since attempts to amplify the PpuR- and RhlR-encoding sequences from the RU-KM3$_s$ genome were unsuccessful, it was not possible to construct knockout mutants for these transcription factors. Consequently, it was decided to investigate whether QS-responsive regulation of DHP activity was affected in the *vfr*$^-$ strain, as it has been shown that Vfr, the global regulator of virulence, also regulates QS (Albus *et al*., 1997). To this end, RU-KM3$_s$Δ*vfr*, together with wild-type cells, were inoculated into either 100 ml or 50 ml culture volume containing the same amount of nutrients and including hydantoin, and assayed for DHP activity as in Section 3.2. It was found that RU-KM3$_s$Δ*vfr* cells showed partial relief of repression under QS conditions, with approximately 3-fold reduction in DHP activity observed in the wild-type cells (from 39.5 μmol/ml to 14.4 μmol/ml) (Figure 3.7), compared to a two-fold decrease in activity in the *vfr*$^-$ mutant (from 28.9 μmol/ml to 19.6 μmol/ml) (Figure 3.7), suggesting that Vfr might play a role in the repression of DHP at high cell densities.

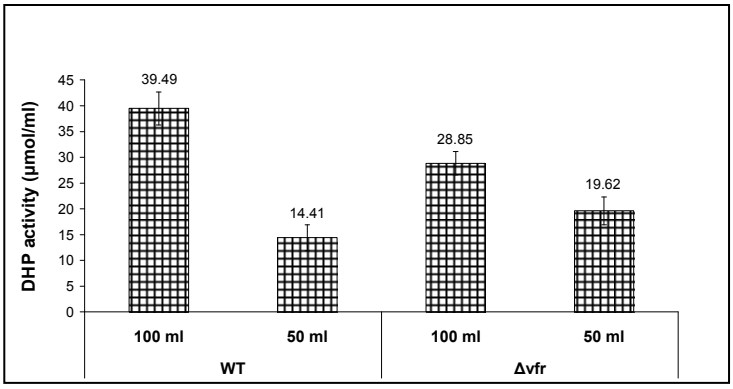

Figure 3.7. DHP activity in a *vfr*$^-$ mutant under quorum sensing conditions. Cells were grown to stationary phase in either 100 ml or 50 ml nutrient broth, containing the same amount of nutrients, with 0.1 % hydantoin as an inducer. (WT: Wild-type RU-KM3$_s$, Δ*vfr*: *vfr*$^-$ strain). The error bars indicate the standard error of the mean where n = 3.

The observation that this relief from repression was not complete suggests that an additional transcription factor may be operating under these conditions, perhaps through the *lux* box, and that the role of Vfr is auxiliary to this protein. The decrease in

repression of DHP, however, is specific to the quorum sensing conditions, since no relief from CCR was seen in the presence of succinate (Figure 3.6 A).

In summary, targeted gene disruption analysis of *vfr* and *crc,* in terms of their effect on the regulation of DHP activity, implicated both Vfr and Crc in the regulation of DHP activity. Crc appears to be a general activator of DHP, but does not play a role in CCR of this enzyme. It is unclear whether or not Vfr is involved in general activation of DHP, but this transcription factor appears to affect repression of DHP activity under quorum sensing conditions.

Although the global transcriptional regulatory proteins, Vfr and Crc, were identified as playing a role in the regulation of DHP in RU-KM3$_s$, the promoter sequences through which these factors act, remained to be determined. This required the development of a system that would enable the analysis of transcriptional activity supported by the *dhp* promoter.

3.5 Development of a promoter probe vector to analyse transcription regulation of *dhp* in RU-KM3$_s$ cells

To determine whether the putative transcription factor binding sites identified in the promoter region of *dhp* (Section 3.2) are involved in regulating *dhp* expression, a series of promoter probes were developed. Promoter probes are vectors into which DNA sequences suspected of having a regulatory function (such as the *dhp* promoter) may be cloned, upstream of a reporter gene, and then introduced into bacterial cells to facilitate the analysis of these sequences (Linn and St Pierre, 1990). Thus, the effect of mutations in the nucleotide sequence of a promoter of interest on its ability to regulate transcriptional activity may be measured by reporter enzyme assays, since the reporter is dependant on the upstream heterologous DNA for its transcription (Linn and St Pierre, 1990).

The first step in the development of a promoter probe is to select the type of vector to be used as the 'vehicle" for promoter studies. In this study, two types of promoter probe vectors were utilised, namely a multi-copy broad host-range (BHR) vector and a plasposon-based integration vector, which resulted in the introduction of a single copy of the promoter-reporter enzyme construct onto the chromosome of the RU-KM3$_s$ cells. The disadvantage of using a BHR vector as a promoter probe is the increase in copy number of the promoter-reporter gene fusion, which may result in transcriptional activities that do not necessarily reflect the expression of a single copy of the gene in the wild-type cells. This difference in copy number can affect the ratio of transcription factors to promoter binding sequences. Thus, any changes in reporter enzyme activity associated with alterations in the target promoter sequence are decreased by a factor relative to the copy number of the vector utilised. In this way, small variations in enzyme activity between cells containing a mutant or wild-type promoter could be overlooked, while large changes in activity are not as pronounced in a vector promoter probe system.

An alternative to using a multi-copy plasmid is to integrate a single copy of the promoter-reporter gene fusion into the chromosome (Marsch-Moreno *et al*., 1998). In this case, since the promoter probe is present as a single copy per cell, the problem of multiple copies of the promoter affecting reporter enzyme activities is overcome. The plasposon system described by Dennis and Zylstra (1998) and used for transposons mutagenesis of RU-KM3$_s$ cells in a previous study (Matcher *et al*., 2004), was selected to construct a promoter probe that could be integrated into the chromosome of RU-KM3$_s$ cells.

3.5.1 Construction of the integration vector, pJD2.

The *gus* gene was selected as a reporter for the plasposon-based vector, pJD2, since the encoded β-glucuronidase (GUS) enzyme has frequently been used as a reporter in promoter-probes and its suitability as such is well established (Jiwaji, 2006). The

cloning strategy for the construction of pJD2 is described in detail in Chapter 2, Section 2.3.1, but essentially involved two steps. First, the *gus* gene was amplified from pMJ242, a plasmid derived from pCAMBIA (Jiwaji, 2006). The gene was then introduced into an *Eco* RI site of pTnMod-OTc, yielding the construct pJAS29. The next step was to clone the entire intergenic region upstream of *dhp* (nts 2917 – 3533 relative to the translational stop codon of *bup*, Figure 3.1), including the Shine-Delgarno sequence, in frame with the *gus* ORF in pJAS29 using the *Bam* HI and *Nco* I restriction sites. Numerous attempts to insert the complete intergenic region into pJAS29 were unsuccessful. It was reasoned that this could be due to interference by the potentially bi-directional promoter with the pMB1 *oriV* of the plasposon, such that the resulting construct would be unable to replicate in *E. coli*. This explanation is based upon previous observations that transcription activation sequences orientated in the direction of another origin of replication can affect plasmid replication (Jiwaji, 2006).

To overcome this problem, a smaller intergenic DNA fragment was selected for cloning into pJAS29. The new promoter fragment consisted of the 255 bp upstream of the *dhp* ORF and contained all of the putative transcription factor binding sites identified earlier (Section 3.2), as well as the Shine Delgarno sequence for the *dhp* mRNA transcript. The rationale behind shortening the *dhp*/ORF1(*bup*) intergenic region to the half located immediately upstream of *dhp* was that the selected fragment was unlikely to contain the ORF1-*bup* promoter sequences. The 255 bp truncated promoter region was amplified from chromosomal DNA by PCR using primers which inserted flanking *Bam* HI and *Nco* I sites (primer sequences can be found in Methods and Materials). This fragment was successfully inserted into the *Bam* HI and *Nco* I sites of pJAS29, to generate the integration vector, pJD2 (Figure 3.8). Since this 255 bp fragment was cloned into pJAS29, it was likely that the ORF1-*bup* promoter had been deleted. However, it was possible that there were other sequences involved in regulating the *dhp* promoter that might also have been deleted.

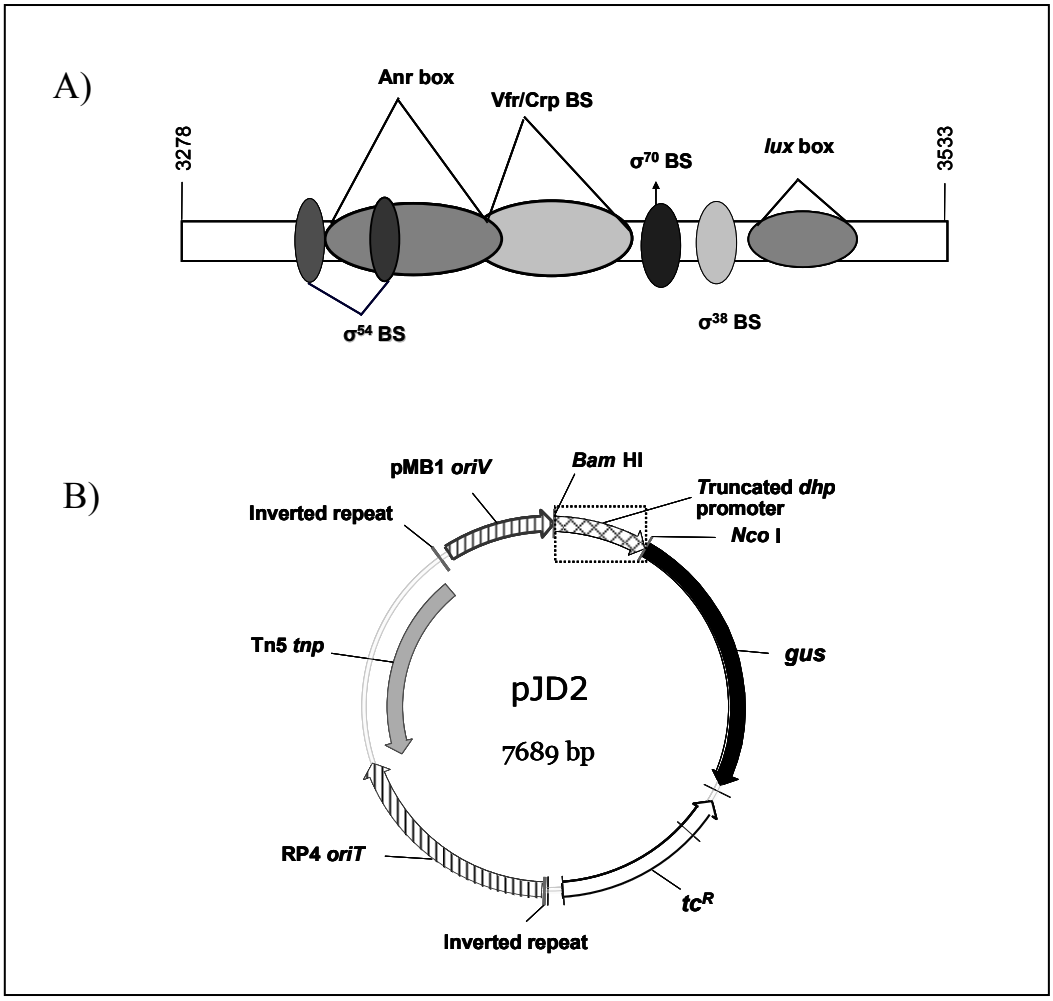

Figure 3.8. The plasposon-derived promoter probe, pJD2, used in the analysis of putative *cis*-acting regulatory elements in the *dhp* promoter. **A)**: The 255 bp truncated promoter with putative transcription factor binding sites are indicated as ovals. The nucleotide co-ordinates above the fragment are given in relation to the translation stop codon of *bup* in terms of the hydantoinase gene cluster in RU-KM3$_s$. The *Bam* HI and *Nco* I recognition sequences used to clone the promoter fragment into the plasposon are shown below the insert. **B)**: Schematic diagram of the promoter probe, pJD2. The boxed area indicates the promoter region which is expanded in **(A)**. pJD2 was constructed by introducing this 255 bp fragment, fused to the *gus* gene of pCAMBIA, into pTnMod-OTc.

Plasmid pJD2, carrying the P_{dhp}-*gus* fusion (Figure 3.9 A) was introduced into RU-KM3$_s$ cells by tri-parental mating, and transconjugates selected on agar plates containing chloramphenicol and tetracycline To confirm the integration of pJD2 into the genome, PCR analysis of the chromosomal DNA of the transconjugates was carried out using primers corresponding to the 5' and 3' ends of the *gus* ORF (JAS14 and JAS15, Methods and Materials). Since RU-KM3$_s$ does not encode an endogenous *gus* gene (Jiwaji *et al.*, 2008), the presence of a PCR product would indicate the successful insertion of the plasposon into the chromosome. The chromosomal integration of pJD2 can be assumed due to the fact that the plasmid does not contain a broad host-range origin of replication enabling it to replicate autonomously in RU-KM3$_s$. Thus, the only way that the *gus* gene would be found in successive generations would be if the plasmid were integrated into the genome of the host cells.

For five strains generated by the introduction of plasposons both with and without the truncated *dhp* promoter (pJAS29 and pJD2, respectively), all displayed the 1824 bp PCR product corresponding to the *gus* gene of the vector, while the wild-type control did not generate such a product (Figure 3.9 B). The five transconjugate strains carrying pJAS29 were named ΔRU-KM3$_s$1 - ΔRU-KM3$_s$5, while the strains containing pJD2 were named ΔRU-KM3$_s$6 - ΔRU-KM3$_s$10. Strains ΔRU-KM3$_s$3 and ΔRU-KM3$_s$7 were selected for further studies.

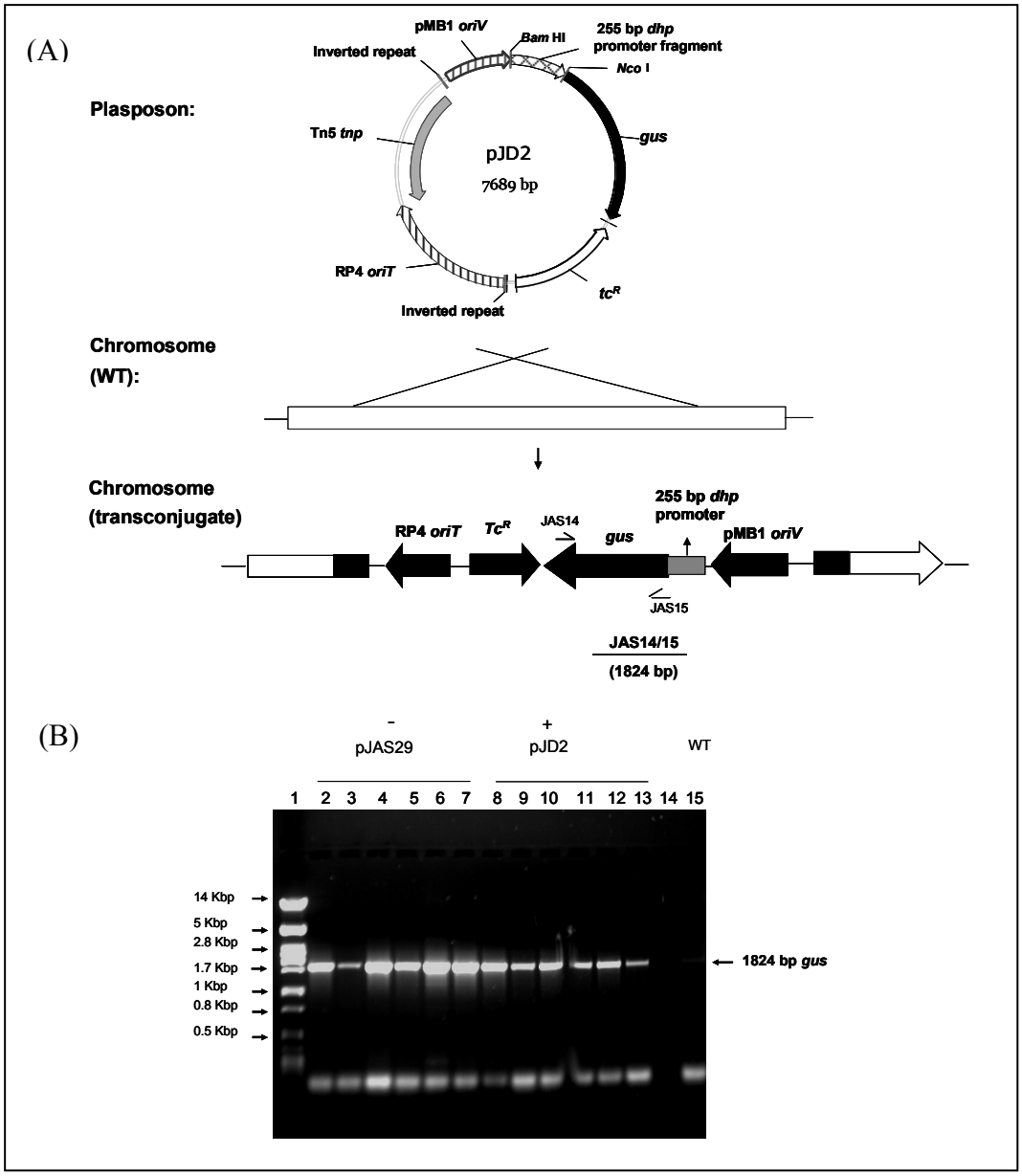

Figure 3.9. Confirmation of the integration of the promoter probe, pJD2, into the chromosome of RU-KM3$_s$. **A)**: Schematic diagram showing the random integration of pJD2 into the chromosome. Primers are indicated as half arrows and the predicted PCR product as a bold line **B)**: PCR verification of the integration of pJD2 and pJAS29 into the chromosome using the *gus*-specific primers, JAS14 and JAS15. PCR products were subject to agarose gel electrophoresis on ethidium bromide stained 1 % gels. Chromosomal DNA templates: transconjugate strains ΔRU-KM3$_s$1-5 containing the control plasmid, pJAS29, with no promoter insert (-) (Lanes 2 - 7); strains ΔRU-KM3$_s$6-10 containing the plasposon, pJD2, with the 255 bp "minimal *dhp* promoter" insert (+) (Lanes 8 - 13); wild-type RU-KM3$_s$ as negative control (Lane 15). Lane 1 contains the λ/*Pst* I molecular marker.

After confirming the presence of the *gus* gene in the transconjugate strains, GUS reporter enzyme assays were carried out for one such strain. To determine whether transcription of the P_{dhp}-*gus* gene fusion was induced by hydantoin and sensitive to CCR, ΔRU-KM3$_s$7 cells were grown to stationary phase in complete medium supplemented with hydantoin and/or succinate and assayed for GUS activity. No product could be detected after an hour of incubation, suggesting very low levels of GUS activity in whole cells. The experiment was repeated on cell-free extracts derived from sonicated cells. The yellow breakdown product of the chromogenic substrate, PNPG, was only visible after a number of hours leading to the conclusion that GUS activity was very low (44.9 β-Gluc units, Figure 3.10). The reporter enzyme activity appeared to display the general trends of induction by hydantoin and repression by succinate (Figure 3.10), suggesting that the deletion *dhp* promoter fragment was able to mediate transcriptional activation, with evidence of induction and CCR. However, the low levels of activity and consequent lack of reproducibility, as well as the high standard deviations in values (+/- 14 β-Gluc units, Figure 3.10) indicated that this system would not be suitable for further studies of transcriptional activation.

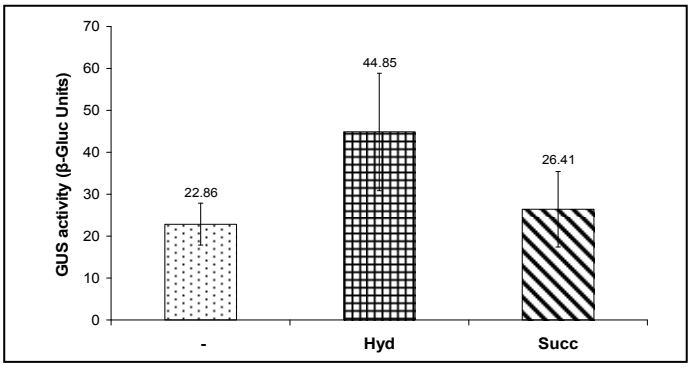

Figure 3.10. Transcriptional activation of GUS activity mediated by the 255 bp truncated *dhp* promoter in the transconjugate strain, RU-ΔKM3$_s$7. This mutant strain was generated by random integration of the plasposon, pJD2, into the chromosome of RU-KM3$_s$, and the ability of the *dhp* promoter region to mediate induction by hydantoin and repression by succinate of GUS activity was examined. Cells were grown to stationary phase in complete medium. (-: uninduced cells, Hyd: cells induced by addition of 0.1 % hydantoin, Succ: cells repressed by addition of 1 % succinate). Error bars indicate the standard error of the mean where n = 3. GUS activities in β-Gluc Units are calculated by:
[1000 x ($OD_{405\ nm}$ − $OD_{550\ nm}$)]/OD_{600} x ΔTime x Volume (ml) (Jefferson *et al*., 1986).

It was important to ensure that no genes involved in the transcriptional regulation of *dhp*, under the environmental conditions of interest, had been disrupted by the random integration of pJD2. If this were the case, it would interfere with the native *dhp* regulon, and may affect the ability of key regulatory factors to bind to the promoter probe and regulate reporter enzyme activity. Therefore, the wild-type and transconjugate strains were grown to stationary phase in complete medium substituted with either 0.1 % hydantoin alone or hydantoin and 1 % succinate and assayed for DHP activity.

DHP activity was induced in both strains ΔRU-KM3$_s$3 (containing the promoterless *gus* insert, derived from pJAS29) and ΔRU-KM3$_s$7 (containing the P$_{dhp}$-*gus* fusion derived from pJD2) when cells were grown in the presence of hydantoin. In the case of the ΔRU-KM3$_s$3 cells, there was a 6.5-fold increase in DHP activity, while a 20-fold increase was observed in ΔRU-KM3$_s$7 cells (Figure 3.11). Growth in medium containing succinate resulted in a 9-fold and 4.5-fold repression of DHP activity in ΔRU-KM3$_s$3 and ΔRU-KM3$_s$7 cells, respectively (Figure 3.11). In previous assays on the wild-type strain (RU-KM3$_s$), DHP activity was increased 20-fold in medium containing hydantoin, with a 5-fold repression of activity in the presence of succinate (Figure 3.3).

Thus, the levels of induction and repression of endogenous DHP activity in ΔRU-KM3$_s$7 were almost identical to that of the wild-type, suggesting that this transconjugate strain did not contain a disruption in an important gene associated with the *dhp* regulon. On the other hand, the level of induction of activity was substantially reduced in ΔRU-KM3$_s$3, in relation to the wild-type (Figure 3.11). This suggested that ΔRU-KM3$_s$3 harboured a disruption in a gene involved in mediating induction of DHP.

Interestingly, there was a considerable reduction in the levels of DHP activity, in the ΔRU-KM3$_s$3 mutant strain as compared with those of the wild-type RU-KM3$_s$ and ΔRU-KM3$_s$7 cells (12. 8 μmol/ml *vs.* 29. 5 μmol/ml *vs.* 27.5 μmol/ml, respectively) (Figure 3.11). This result suggested that the insertion event in ΔRU-KM3$_s$3 might have inactivated or up-regulated a factor involved in the regulation of DHP expression. The

disruption may have occurred either within a gene encoding a transcription regulator protein or within a previously unknown activation sequence.

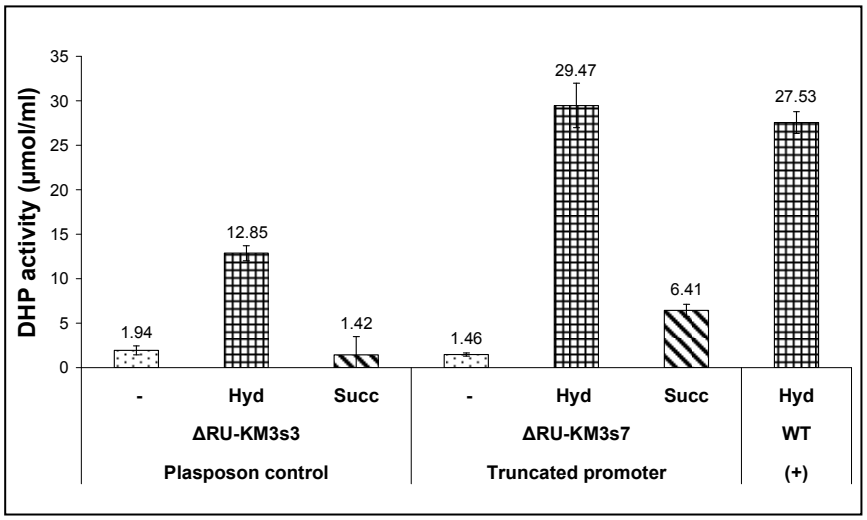

Figure 3.11. Effect of chromosomal integration of a plasposon promoter probe on the regulation of wild-type DHP activity. The consequence of transposition was determined for transconjugates ΔRU-KM3$_s$3, containing the control plasmid pJAS29 with no promoter insert (plasposon control) and ΔRU-KM3s7, containing the plasposon pJD2 with the 255 bp "minimal" *dhp* promoter (truncated promoter) fused to *gus*; in comparison with DHP activity in the wild-type (+). Cells were grown to stationary phase in complete medium (-: cells grown in nutrient broth alone, Hyd: cells grown in NB + 0.1 % hydantoin, Succ: cells grown in NB + 0.1 % hydantoin + 1 % succinate, WT: wild-type). Error bars indicate the standard error of the mean where n = 3.

Sequencing of the surrounding genomic DNA at the insertion site of pJAS29 would form an interesting future study and could potentially lead to the identification of a component of the *dhp* regulon. However, the inability to obtain meaningful assay data for the transcriptional activity of the *dhp* promoter, together with the fact that the entire *dhp*/ORF1(*bup*) intergenic region could not be analysed using this system, prompted the use of a different probe to study the *dhp* promoter.

3.5.2 Promoter studies using the multi-copy vector, pMJ445

The availability of a broad host-range vector (pMJ455), constructed by Meesbah Jiwaji (Rhodes University Dept of Biochemistry, Microbiology and Biotechnology), offered an alternative system for studying the transcriptional regulation of *dhp*. The vector contains an *oriT*, allowing it to be introduced into *P. putida* by conjugation; an IncQ broad host-range origin of replication, which is functional in RU-KM3$_s$ cells, and a tetracycline resistance selectable marker to allow for selection of transconjugate strains. In addition, the vector, lacking ribosome binding sequences, carries divergent promoterless *lacZ* and *gus* genes separated by a *Bgl* II site, to allow for the analysis of a bi-directional promoter (Jiwaji *et al*., 2008) (See Methods and Materials for construct map).

The full-length intergenic region upstream of *dhp* was amplified by PCR with primers GFM38 and GFM39 (Methods and Materials), which introduced flanking *Bgl* II sites, and successfully cloned into the *Bgl* II site of pMJ445, such that the *dhp* promoter was inserted upstream of the *gus* ORF. The ability to insert the full promoter region into pMJ445, which was not possible for the plasposon promoter probe (pJD2), may have been due to the presence of a second reporter gene in the BHR vector. This *lacZ* gene in pMJ445 may have acted as 'stuffer' DNA between the promoter and the *oriV*, thereby preventing the potentially bi-directional promoter from interfering with the origin of replication of the vector.

A recombinant plasmid with the insert orientated such that the *dhp* promoter was fused to the *gus* ORF was selected (pMJ449) and introduced into RU-KM3$_s$ cells by tri-parental mating. The *gus* fusion was selected over that of *lacZ*, due to the previous observation that 100-fold lower β-Galactosidase (β-Gal) activity levels were obtained for pMJ445, when compared to the β-Glucuronidase (GUS) enzyme of the vector (Jiwaji *et al*., 2008), suggesting that β-Gal may not be as accurate in detecting small changes in activity between strains.

GUS activity levels supported by the multi-copy vector were determined and compared to those generated by the integrated promoter probe (pJD2, Figure 3.10). RU-KM3$_s$ cells containing the vector (pMJ449, full-length *dhp* promoter) were cultured to stationary phase in complete medium substituted with hydantoin and assayed for GUS activity after 3 hours. Enzyme activities in the cells containing the broad host-range vector, with the full *dhp* promoter fused to *gus*, were routinely found to be in excess of 5000 β-Gluc units (data not shown), which was approximately 100-fold higher than that observed in cells where the truncated promoter-*gus* construct was integrated into the chromosome (Figure 3.10).

The substantial increase in GUS activity in the BHR promoter probe (pMJ449), over the integrated vector (pJD2), prompted a comparative assay of GUS activity in the full-length and truncated *dhp* promoters using this system. This was important in order to determine whether the increase in promoter activity observed in the vector system was due to an increase in copy number, as compared with the single copy integration system, or due to the presence of additional regulatory sequences in the full-length promoter fragment. Therefore, the 255 bp truncated *dhp* promoter fragment used to construct the integration vector (pJD2, Figure 3.8) was inserted into pMJ445, with the resulting construct (pMJ480) carrying the *dhp* promoter fragment, including the ribosome binding site, fused to the *gus* ORF. The two promoter constructs, pMJ449 (full-length *dhp* promoter) and pMJ480 (truncated promoter), together with the vector control (pMJ445) were introduced into RU-KM3$_s$ by conjugation and the cells assayed for promoter activity. All strains were grown to stationary phase in complete medium supplemented with succinate and/or hydantoin and assayed for GUS activity.

Overall, the activity of cells containing the full-length *dhp* promoter was approximately 2-fold higher than cells where GUS was expressed via the truncated *dhp* promoter (7957 *vs*. 3301 β-Gluc units, respectively), suggesting that the truncated promoter fragment lacked important regulatory elements, such as activation sequences, required for optimal enzyme activity (Figure 3.12). However, a comparison of GUS activity between wild-type cells containing the truncated promoter construct (pMJ480) and those

in the transconjugate strain with the same promoter fragment (ΔRU-KM3$_s$7) revealed a 67-fold increase in reporter enzyme activity (Compare Figure 3.12 with Figure 3.10). This indicated that the higher GUS activity obtained for the full-length promoter construct (pMJ449) (Figure 3.12), in comparison with that of the transconjugate (ΔRU-KM3$_s$7) in the previous assay (Figure 3.10), was not solely due to the presence of additional regulatory sequences in the complete promoter, but that the presence of multiple copies of the vector probe also contributed to the substantial increase in GUS activity.

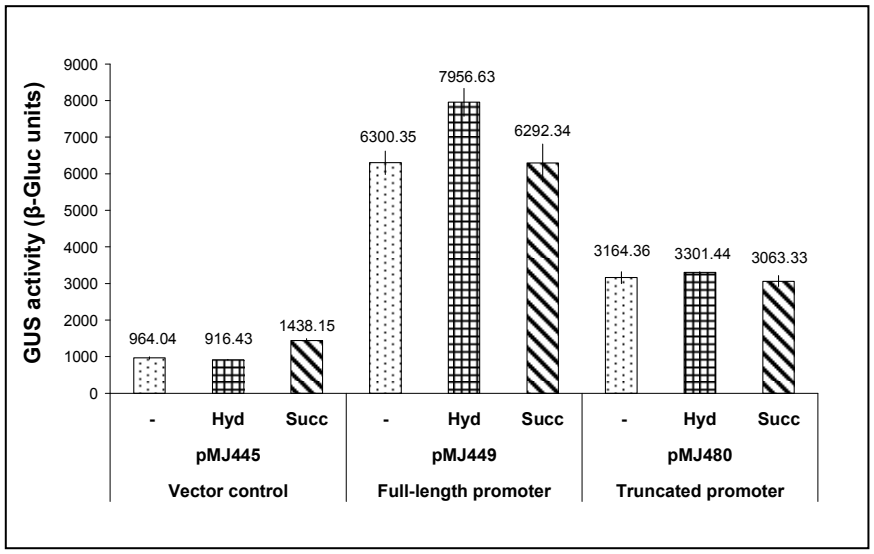

Figure 3.12. Comparison of the regulation of GUS activity by the truncated and full-length *dhp* promoter, in terms of induction by hydantoin and repression by succinate. The vector control represents cells containing the promoter probe vector (pMJ445) only, the full-length promoter indicates cells containing the promoter probe with the full *dhp* promoter fused to *gus* (pMJ449), and the truncated promoter indicates cells containing the vector probe with a 255 bp truncated *dhp* promoter fused to *gus* (pMJ480). (-: uninduced, Hyd: induced with 0.1 % hydantoin, Succ: repressed with 1 % succinate). Error bars indicate the standard error of the mean where n = 3. GUS activities in β-Gluc units are calculated using the formula developed by Jefferson (1986).

In terms of the regulation of reporter enzyme activity supported by the full-length promoter fragment (pMJ449), there was a 21 % induction of GUS activity in cells grown with hydantoin (6300 β-Gluc units in uninduced cells *vs.* 7957 β-Gluc units in induced

cells) as well as a 21 % repression of GUS activity in cells grown in succinate (7957 β-Gluc units *vs.* 6292 β-Gluc units, respectively) (Figure 3.12). On the other hand, the regulation of GUS activity supported by the truncated *dhp* promoter in pMJ480, in terms of induction by hydantoin and repression by succinate, suggested that the truncated promoter had lost, to a large extent, the ability to mediate induction and repression of enzyme activity [3164 β-Gluc units (uninduced) compared with 3301 β-Gluc units (induced) and 3063 β-Gluc units (repressed)] (Figure 3.12). This inability of the 255 bp promoter fragment to modulate reporter activity, in terms of induction and repression, once again suggested the loss of important regulatory elements in the truncated promoter.

The extent of induction and repression of GUS activity supported by the full-length promoter construct (pMJ449) was substantially reduced when compared to the DHP activity trends routinely observed in the wild-type *dhp* system (1.3-fold induction in the promoter probe *vs.* 20.2-fold in the native *dhp* of RU-KM3$_s$ and 1.3-fold repression in the probe compared to 4.6-fold in the *dhp* system) (Compare data in Figure 3.12 to Figure 3.3). These "muted" regulatory effects in reporter enzyme activity are likely due to the presence of multiple copies of the promoter in the cell, which titrate out transcription factors and skew enzyme assay results.

It was decided to utilise the multi-copy promoter probe system to further analyse the regulatory sequences responsible for *dhp* transcription, since it allowed an investigation of the full promoter sequence.

3.6 Mutational analysis of the putative Vfr and Anr binding sites in the *dhp* promoter

Bioinformatic analysis of the intergenic region upstream of the *dhp* ORF identified putative binding sites for the transcription factors Vfr and Anr (Section 3.2; Figure 3.2). It was also shown that inactivation of the *vfr* gene had an effect on DHP activity under quorum sensing conditions, suggesting that the putative Vfr binding site in the *dhp*

promoter might be functional in mediating transcriptional regulation of *dhp* expression. It was therefore decided to use site-directed mutagenesis to inactivate the putative Vfr binding site and determine the effect on transcriptional activation by the *dhp* promoter. Since the putative Anr box overlaps with the Vfr binding site it was decided to also mutate this putative binding site to determine whether Anr plays a role in the transcriptional regulation of *dhp*.

For each binding site, it was decided to mutate one of the two half-sites that are proposed to be important for recognition and binding by the relevant regulatory proteins (Kanack *et al*., 2006; Pessi and Haas, 2000). The upstream half-site of the putative Anr box and the downstream half-site of the putative Vfr binding site were mutated to ensure that, in each case, the other site remained intact despite the overlap of the binding sequences. In the case of the Vfr binding site, the 3' half-site was mutated by the introduction of a *Sma* I site; while the 5' half-site of the Anr box was mutated by introducing a *Pst* I site (see Methods and Materials for a schematic representation of the mutagenesis strategy). The introduction of the *Pst* I site into the Anr box would also result in mutation of a putative overlapping σ^{54} binding site (Figure 3.2). The mutated promoter sequences were then removed from their pGEM T-Easy constructs and inserted into the *Bgl* II site of the promoter probe vector, pMJ445, upstream of the *gus* reporter. Plasmids, pJD6 and pJD7, thus contained the full-length *dhp* promoter region, with a mutated Vfr or Anr box/ σ^{54} binding sequence, respectively.

Plasmids, pJD6 (δVfr) and pJD7 (δAnr/σ^{54}) together with pMJ449 (wild-type *dhp* promoter) were introduced into RU-KM3$_s$ cells and the effect of these mutations on the ability of the promoter to activate transcription was determined by measuring GUS activity. Cells containing the wild-type promoter construct (pMJ449), as well as those containing the mutated promoters (either pJD6 or pJD7), were grown to stationary phase in complete medium or medium supplemented with either hydantoin or hydantoin and succinate before assaying for GUS activity. In addition, cells containing only the promoter probe vector with no insert (pMJ445), grown to stationary phase in complete medium and supplemented with hydantoin, were included as a negative control.

The full-length *dhp* promoter generated GUS activities that were only half as high overall as those previously obtained for this construct (3110 *vs.* 7957 β-Gluc units, respectively) and similar to the levels observed for the truncated promoter (3301 β-Gluc units) (Figure 3.12). Repeating the assay several times yielded similar GUS activities that were consistently lower in cells containing pMJ449, than those obtained in the previous assays (Figure 3.12 *vs.* Figure 3.13). The degree of induction and repression was also lower in this assay when compared to the results displayed in Figure 3.12, perhaps due to the overall decrease in activity levels.

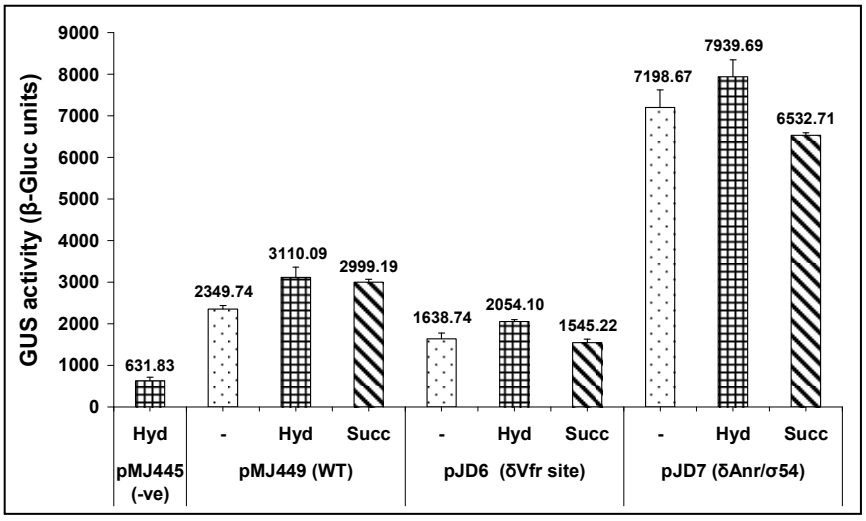

Figure 3.13. Effect of mutation of the putative Vfr and Anr binding sites on the ability of the *dhp* promoter to regulate GUS reporter enzyme activity. Strains and plasmids are indicated as follows: the negative control (-ve) contains only the promoter probe vector (pMJ445), (WT) represents a strain containing the promoter probe with wild-type *dhp* promoter (pMJ449), (δVfr site) and (δAnr/σ^{54} site) represent strains containing the promoter probe plasmid with *dhp* promoter sequences mutated in the putative Vfr binding site (pJD6) and Anr and σ^{54} sites (pJD7), respectively. Cells were grown to stationary phase in complete medium (-: uninduced, Hyd: induced with hydantoin, Succ: repressed with succinate). Error bars indicate the standard error of the mean where n = 3. GUS activities in β-Gluc units are calculated according to Jefferson (1986).

Mutation of the putative Vfr binding site in the *dhp* promoter region resulted in GUS activities that were approximately 25 % lower overall than those corresponding to the wild-type promoter (Figure 3.13). On the other hand, mutation of the Anr box resulted in

a two-fold increase in GUS activity overall (Figure 3.13). In addition, it appears that neither the sequence designated as a putative Vfr binding site, nor that predicted to be an Anr/σ^{54} recognition sequence, are involved in mediating the induction and repression observed for DHP in the wild-type RU-KM3$_s$.

It is possible that the sequence designated as a putative Anr box might play a role in repression of the system that is not linked to carbon source, but rather to another environmental condition associated with stationary phase. This deduction was based on the observation that although GUS activity for this promoter mutant was 2-fold higher, in general, than for the wild-type promoter, it nonetheless displayed the low levels of carbon catabolite repression (6532 *vs.* 7939 β-Gluc units, respectively) (Figure 3.13) routinely observed for the wild-type *dhp* promoter in pMJ449 (Figure 3.12). Inducibility, albeit at low levels (7199 *vs.* 7939 β-Gluc units, respectively), was also detected for the Anr box mutation, suggesting that Anr does not mediate this effect under these specific assay conditions (Figure 3.13).

The putative Vfr site, on the other hand, may play a minor role in activation of the system, which is not related to the presence of the substrate. This was inferred from the result that the Vfr binding site mutation displayed a 25 % decrease in activity overall, yet retained the wild-type trend of substrate induction (1639 *vs.* 2054 β-Gluc units, respectively) (Figure 3.13). Carbon catabolite repression was also retained in this mutant promoter (1545 *vs.* 2054 β-Gluc units, respectively) (Figure 3.13).

In order to ensure that the correct constructs were present in the RU-KM3$_s$ cells employed in the experiment, plasmid DNA was extracted from the cells used for the GUS assays and the *dhp* promoter region amplified by PCR, using primers specific to the *dhp* promoter and the vector probe. To confirm the presence of the correct promoter insert in the vector, the PCR products were digested with *Sma* I and *Pst* I, to confirm the presence of the wild-type and mutant promoter sequences. The 800 bp wild-type promoter fragment was not digested by either *Sma* I or *Pst* I (Figure 3.14, Lanes 2 and 6), while the promoter in the δVfr mutant was digested by *Sma* I only (Figure 3.14,

Lanes 3 and 7) and the δAnr box/σ^{54} binding sequence mutant was digested by *Pst* I only (Figure 3.14, Lanes 4 and 8), confirming the presence of the correct plasmid in each case.

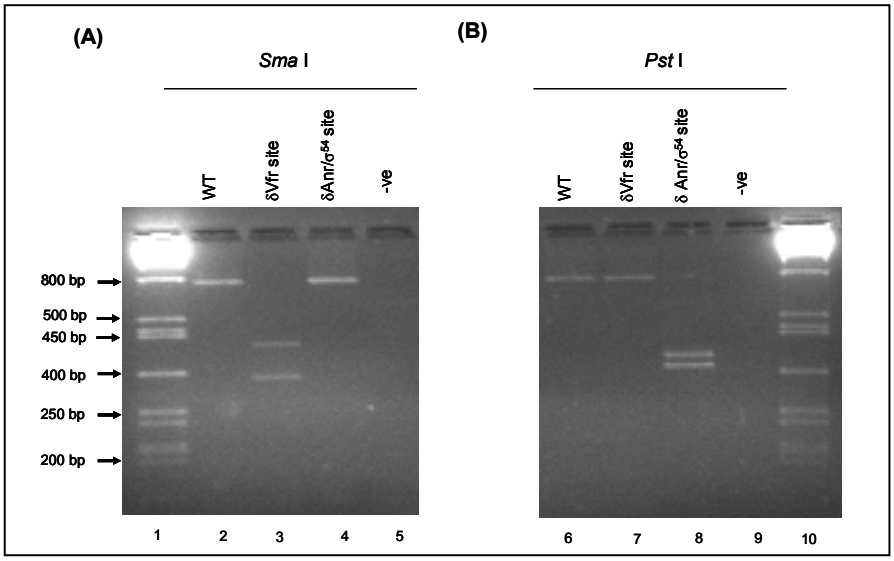

Figure 3.14. Restriction analysis of *dhp* promoter constructs used in GUS reporter assays. **A)**: 4 % Ethidium bromide stained agarose gel containing PCR products cut with *Sma* I to verify the mutation of the Vfr binding site. **B)**: 4 % agarose gel containing PCR products digested with *Pst* I to verify the mutation of the putative Anr box. Lanes 1 and 10 contain the λ/*P*st I molecular weight marker. WT indicates a strain containing the promoter probe vector with wild-type *dhp* promoter (pMJ449), while "δVfr site" and "δAnr/σ^{54} site" represent strains containing the promoter probe with *dhp* promoter fragments mutated in the putative Vfr (pJD6) and Anr/σ^{54} (pJD7) sites, respectively.

Thus, the mutations in the putative Vfr and Anr binding sites were confirmed and indicated that the altered reporter activities in cells containing these vectors were due to specific changes in the DNA sequence of the *dhp* promoter.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

4.1 Introduction

It has been well-established that *Pseudomonas* species possess extremely versatile metabolic systems allowing them to utilise a wide range of nutrient sources and, furthermore, that the regulation of these enzyme systems involves highly evolved and sophisticated regulatory machinery (Nakazawa *et al*., 1996; Stover *et al*., 2000; Nelson *et al.*, 2002). The aim of this research was to study these complex metabolic regulatory mechanisms using the hydantoin-hydrolysing system of the environmental isolate *P. putida* RU-KM3$_s$ (Burton *et al*., 1998). In particular, the focus was on the transcriptional regulation of the *dhp* gene. Up to date, very little is known about the regulation of expression of the genes responsible for hydantoinase activity (Burton and Dorrington, 2004). It was hypothesised that there were several factors regulating *dhp* expression based on the fact that, in general, pseudomonads possess a large number of transcriptional regulators in relation to their genome sizes, such that up to 8 % of their genes encode regulatory elements (Stover *et al*., 2000). This hypothesis was supported by the observation that DHP activity in RU-KM3$_s$ was tightly regulated by growth phase in addition to induction by hydantoin and carbon catabolite repression (CCR) (Matcher *et al*., 2004). In order to test the hypothesis, the significance of putative transcription factor binding sites in the intergenic region upstream of *dhp,* as well as the role of certain corresponding transcriptional regulatory proteins in controlling DHP activity, were examined.

4.2 Identification of putative transcription factor binding sites in the *dhp* promoter region

Bioinformatic analysis revealed the presence of putative binding sites for the global regulators Vfr, Anr, PpuR and RhlR, in addition to the sigma factors, σ^{38} and σ^{54} (Section 3.2). It was interesting to note that all of the putative binding sites identified are recognised by transcription factors which are known to function during stationary phase

and that they respond to specific stresses associated with this growth phase (reviewed in Yuste *et al*., 2006). This is also when hydantoinase activity is at its maximum (Matcher *et al*., 2004). At this point, the original hypothesis was expanded by suggesting that the transcription of *dhp* might be regulated in response to a variety of different environmental signals specific to stationary phase, by means of four global regulatory pathways, as follows:

(1) The general stress response, mediated by the alternate sigma factors, σ^{38} or σ^{54}, which direct RNAP to the *dhp* promoter via their respective recognition sequences and represent the highest level of regulation in a complex hierarchy.
(2) Carbon catabolite repression (CCR), mediated by a CRP homologue such as Vfr and acting through the putative CRP/Vfr binding site;
(3) Quorum sensing (QS), by means of RhlR and/or PpuR operating via the putative *lux* box at high cell densities;
(4) Anaerobiosis, by way of Anr binding to the putative Anr box at low oxygen levels.

The identification of a putative QS-responsive element (the *lux* box) in the *dhp* promoter region was unexpected, since current literature regarding genes sensitive to QS in *Pseudomonas* has reported mainly virulence factors and there is very little information concerning transcriptional regulation of metabolic activity by QS. However, it has been suggested that cell density information detected by the QS system forms part of a complex network of sensory input, which reflects both the chemical and physical status of the environment, including nutrient availability, oxygen levels, temperature, osmolarity and pH (Withers *et al*., 2001; Bertani and Venturi, 2004). In fact, a new QS model, called the *Pseudomonas* quinolone signal (PQS), has been described in *P. aeruginosa*. The PQS has been implicated in the regulation of both fatty acid metabolism and, significant to this research, amino acid biosynthesis, in particular tryptophan (Withers *et al*., 2001). This information may explain why the promoter region of *dhp* might contain such a binding site and added weight to the hypothesis regarding the role of QS in regulating a non-virulent, metabolic system such as the hydantoinase (*dhp*) of strain RU-KM3$_s$.

The presence of a putative Anr box upstream of *dhp* was also unexpected, since very little has been reported about the role of Anr in regulating carbon metabolism. Instead it has been established that Anr stimulates denitrification pathways in order to enable the cells to grow under anaerobic conditions (Arai *et al*., 2003). It has, however, been reported that *P. aeruginosa* can survive under anaerobic conditions for long periods of time via pyruvate or amino acid fermentation (Eschbach *et al*., 2004). Thus, it is plausible that stimulation of the production of amino acids by Anr, through the hydantoinase reaction, might aid in the survival of RU-KM3$_s$ cells under the limited oxygen levels associated with stationary phase.

Upon closer examination of the putative binding sites in the intergenic region upstream of *dhp*, it was noted that a number of these sites were two nucleotides longer than their respective consensus sequences and, furthermore, that several binding sites overlapped with one another. In terms of the former, the two nucleotide insertion in the region between the important half-sites was seen in the putative Vfr, Anr and σ^{54} recognition sequences. It is possible that the elongated binding sites might reflect specific modifications in the DNA binding domains of the transcriptional regulatory proteins, or that the additional nucleotides may aid in looping of the DNA upon binding of transcription factors.

The putative Anr box overlapped significantly on its 5' end with the σ^{54} recognition site and, to a lesser degree, with the CRP-like binding site on its 3' end. A similar merging of binding sites was described in a study regarding the transcriptional control of the hydrogen cyanide biosynthetic genes (*hcn*ABC), where it was found that regulation was mediated by the QS global regulators, LasR and RhlR, in addition to the oxygen-sensitive Anr protein and a RNAP sigma factor (Pessi and Haas, 2000). In terms of the regulation of *dhp* expression, binding to either of the overlapping Anr and CCR-responsive recognition sequences in the *dhp* promoter may be competitive and dependant on specific environmental stimuli, such that Vfr (or a similar CRP homologue) would dominate under CCR conditions and Anr would prevail at low oxygen levels.

4.3 Development of an experimental system to study transcriptional regulation of *dhp*

Although bioinformatic analysis revealed a number of putative binding sites for transcriptional regulators, it was unknown whether any of these sites were functional in mediating the regulation of *dhp* transcription. In order to study the role of the potential regulatory sequences in the intergenic region upstream of *dhp*, a suitable promoter probe was required, the construction of which was an important objective of this study (Section 3.5). It was initially decided to develop a promoter probe that would integrate into the chromosome of RU-KM3$_s$. This could allow for promoter studies without having to take into account the effect of vector copy number, since the probe would be present as a single copy in the genome. However, this approach encountered a number of experimental problems. First, repeated attempts to clone the full 616 bp intergenic region into the vector were unsuccessful; and second, the final construct generated reporter enzyme activity that was too low to allow for accurate comparisons to be made between wild-type and mutant promoters.

The low reporter enzyme activities achieved using this probe may have been due to the loss of activation sequences in the truncated promoter used in initial studies with this system or, alternatively, as a result of the single copy of the probe in mutant cells. The results of these experiments did, however, provide the opportunity to critique the suitability of a pTnMod-derived probe in the analysis of potentially bi-directional promoters. It was proposed that the sensitive *oriV* of the plasposon prevented the cloning of the full-length *dhp* promoter region, which implied that this system may not be suitable for the study of a bi-directional promoter.

The alternate promoter probe system based upon a broad host-range vector, designed specifically for the analysis of bi-directional promoters, proved to be more successful. Despite the limitations of a multi-copy system, in particular, the risk that numerous copies of the vector may dilute out transcription factors, this approach does have certain advantages over the integration probe. The benefits of using a multi-copy probe lie in the fact that the construct is not integrated into the chromosome of the host cell. This means

that the shortcomings associated with integration, namely the effect that the surrounding genomic DNA sequence may have on the promoter of interest, as well as non-reproducibility in terms of the insertion site of the probe, are avoided. Furthermore, the ability to characterise the full-length *dhp* promoter in the multi-copy system and the relatively high reporter enzyme activities achieved in the recombinants, implies that this promoter probe would be more suitable for the analysis of the putative transcription factor binding sites identified in the *dhp* promoter region.

The vector, pMJ445, also afforded the opportunity of comparing the regulation of reporter enzyme activity via the truncated *dhp* promoter, containing all of the potential binding sites for transcriptional regulators, with that of the full *dhp*-ORF1 intergenic region. It was found that transcriptional activity mediated by the truncated promoter was only half that produced by the full promoter, and that the inducibility and CCR of enzyme activity, by hydantoin and succinate respectively, was lost in the truncated promoter (Section 3.4.2). This implies that important regulatory sequences upstream of *dhp*, which were not identified in the bioinformatic analysis, may be deleted in the truncated promoter fragment.

With respect to the full-length *dhp* promoter, the overall extent (fold) of induction and repression of activity was considerably lower than those observed in the wild-type system (1.3-fold induction in the promoter probe *vs.* 20.2-fold in the native DHP system). This was an important observation, since it implies that multiple copies of the promoter sequence are "muting" important regulatory trends. This would reduce the sensitivity of this assay system with respect to detecting subtle changes in transcriptional activation.

4.4 Mutational analysis of putative transcription factor binding sites in the *dhp* promoter

Two of the potential binding sites for transcription factors in the *dhp* promoter were selected for mutational analysis. The sequence designated as a putative CRP/Vfr site was

of particular interest since this site was originally considered to be the chief candidate in the regulation of DHP by CCR (Matcher *et al*., 2004). In addition, the site designated as a putative Anr box was chosen since it overlapped with the Vfr binding site and also contained a σ^{54} recognition sequence. It was considered that mutation of this site could offer insight regarding the hypothesis that binding to overlapping regulatory sequences was competitive and dependent on specific environmental conditions.

Mutation of the putative Vfr binding site resulted in a 25 % decrease in activity, while retaining the wild-type trends of induction by hydantoin and repression by succinate. On the other hand, mutation of the putative Anr box resulted in a doubling of enzyme activity when compared to the wild-type promoter, but the pattern of induction and repression was also retained (Section 3.6). The Vfr and Anr binding sites were mutated in only one of their two half-sites, such that in each case the other binding site remained intact. Nonetheless, the extensive overlap of the Anr box with the putative σ^{54} recognition sequence meant that it was possible that mutation of the 5' half-site of the Anr box might affect the ability of σ^{54} to bind the *dhp* promoter. However, the region of the σ^{54} recognition sequence that does not overlap with the Anr box (*i.e.* the sequence not affected by site-directed mutagenesis) contains the half-site that has been reported to make the greatest contribution to DNA binding by σ^{54}. Furthermore, it has been shown that even if the 3' half-site were absent, the sigma factor would retain its ability to bind the promoter (Buck *et al*., 2000). Therefore, although it is possible that the mutation of the Anr box would affect the functioning of the σ^{54} site, it is more likely that it is the mutation of the former that is responsible for the increase in DHP activity.

Based on the mutational analysis of the putative Vfr site and Anr box, it was suggested that the Vfr site may play a role in mediating the generalised activation of DHP, while the Anr site might play a role in repression of DHP. Whether or not Vfr and/or Anr actually bind to these sites and regulate transcriptional activity, is unknown. To determine if this were the case, *in vitro* DNA binding studies between the promoter fragment and purified Vfr or Anr would be required. It is also possible that other

transcriptional CRP-like regulator proteins may interact with these sites and modulate activity.

4.5 Environmental factors affecting DHP activity

Up until the start of this study, the following was known about the regulation of DHP activity: that it was (1) growth phase-dependant, (2) inducible by the hydantoin substrate, (3) repressed by more favourable carbon sources such as glucose and succinate and (4) independent of the nitrogen source in the growth medium (Matcher *et al*., 2004). The effect of QS conditions and oxygen levels on enzyme activity was unknown. First, the above findings by Matcher (2004) were confirmed, in terms of the induction and repression of DHP during stationary phase, before assaying for DHP activity at a cell density that was twice as high as that routinely used in enzyme assays, in an attempt to simulate QS conditions (Section 3.3). It was observed that activity was significantly reduced under these conditions. To the best of our knowledge, this is the first report implicating QS in the regulation of hydantoinase activity.

The potential involvement of Anr in regulating *dhp* expression raised the question of whether RU-KM3$_s$ cells were capable of growth under anaerobic conditions. The only report of a *Pseudomonas* species being able to establish itself in such an environment is in the cystic fibrosis lung, where *P. aeruginosa* forms macro-colonies in microaerobic to fully anaerobic conditions (Hasset *et al*., 2002; Worlitzch *et al*., 2002). Before being able to study the effect of oxygen levels on DHP activity in strain RU-KM3$_s$, it was necessary to determine the tolerance of the cells to various levels of anaerobiosis. Preliminary results showed that the strain favoured aerobic conditions, such that growth decreased significantly as oxygen levels were decreased and that the addition of the alternate electron acceptor, sodium nitrate, did not have an effect on the strain's ability to grow at low oxygen levels (Section 3.3). This suggests that strain RU-KM3$_s$ is an obligate aerobe, which would agree with the classification of the species. The results also meant that it was not possible to determine the effect of low oxygen levels on DHP

activity, since these conditions generated insufficient biomass for enzyme assays. A more in-depth study of the growth of RU-KM3$_s$ under varying oxygen levels could be conducted using a sealed growth vessel, to which a measured amount of oxygen could be introduced, and correlating this to the $OD_{600\ nm}$ of the culture; such that a survival curve could be plotted for this strain.

4.6 Transcriptional regulatory proteins that influence DHP activity

While it was found that the putative Vfr and Anr/ σ^{54} recognition sequences in the intergenic region upstream of *dhp* appear to play a role in regulation, the proteins that bind these sites to activate or repress transcription remain unknown. In this study, two proteins were selected for mutational analysis: the CRP homologue, Vfr, and the chief pseudomonad CCR regulatory protein, Crc. Disruption of the *vfr* gene did not appear to have an effect on either induction or CCR of DHP activity. This result suggested, that although Vfr is a CRP homologue, it does not mediate CCR in *P. putida* RU-KM3$_s$ and thus does not function in the same manner as CRP in *E. coli*. Another possibility is that Vfr may still be expressed from an alternative, duplicate locus in the mutant. The only way to confirm that this is not the case would be to generate antibodies specific to Vfr and perform Western analysis of cell-free extracts of this strain, cultured under DHP assay conditions. However, the latter possibility is unlikely in view of the fact that the Δ*vfr* mutant cells exhibited a reduction in DHP activity, suggesting that the disruption of the *vfr* gene resulted in altered expression of *dhp*.

It has been reported that Vfr forms part of the QS regulatory cascade (Albus *et al*., 1997). This prompted an assay for DHP activity in the *vfr*$^-$ strain under QS conditions, which revealed a partial relief of repression in the mutant. Thus, Vfr was implicated in the QS-responsive regulation of *dhp* expression. There are two mechanisms by which Vfr may regulate *dhp* expression under QS conditions: (1) via the *lux* box upstream of the *dhp* coding sequence or (2) by direct binding of Vfr to the putative CRP-like binding site further upstream. It is more likely that it is the *lux* box that is responsible for

regulation via QS, since transcriptional activity supported by a *dhp* promoter containing a mutated CRP-like binding site remains subject to repression under QS conditions (Meesbah Jiwaji, personal communication). Confirmation of this model of regulation would require site-directed mutagenesis of the *lux* box. Since repression was incomplete it is possible that Vfr functions in conjunction with another QS regulator, such as PpuR or RhlR.

A comparison of the results generated by the site-directed mutagenesis of the putative Vfr binding site in the *dhp* promoter (Section 3.6) with the targeted *vfr* gene disruption (Section 3.4) revealed that, while the Vfr site was implicated in general activation of *dhp* expression, Vfr itself may play a role in repression under QS conditions. Whilst the two sets of experiments appear to contradict one another, they do highlight several other possibilities regarding the regulation of *dhp* expression. First, in the absence of direct evidence that Vfr binds the putative Vfr binding site, it is possible that this protein may function indirectly via the activity of other transcription factors such as PpuR or RhlR. It is also possible that the putative Vfr binding site might be the recognition sequence of another transcription factor, perhaps a different CRP homologue, which mediates transcriptional activation of *dhp*.

Since Vfr is not the factor responsible for mediating CCR of *dhp* expression, another candidate, Crc, was considered for this role. The specific disruption of *crc* resulted in an overall decrease in DHP activity, without affecting either induction or CCR, suggesting that Crc plays a general role in activation of DHP. Again, as with *vfr*, although the *P. putida* KT2440 genome sequence indicates the presence of only a single *crc* gene on the chromosome, there might be a Crc homologue that mediates CCR in RU-KM3$_s$. While this is unlikely, antibodies to Crc would need to be generated and the disruption strain subjected to Western analysis to confirm the absence of Crc. Thus far, the role of Crc in the repression of several catabolic pathways has been well-documented. However, there have also been reports of this protein mediating activation of such genes, for example the malate-quinone oxidoreductase gene in *P. putida* KT2440 (Morales *et*

al., 2004). This is the first time that Crc has been shown to be involved in the regulation of a hydantoin-hydrolysing enzyme system.

Disruption of two global transcription factors and two putative binding sites in the *dhp* promoter did not identify the elements involved in mediating either induction by hydantoin (now referred to as HYDp) or succinate-responsive CCR (referred to as CCRp) of DHP, suggesting the involvement of factors other than Vfr, Crc or Anr. Bearing in mind that induction and repression of reporter activity were largely lost in the 255 bp truncated promoter, it is plausible that the sequences responsible for mediating these effects are located further upstream (Figure 4.1).

4.7 A model for the regulation of *dhp* expression

The final objective of this study was to develop a model for the regulation of *dhp* expression in RU-KM3$_{s.}$ This model will attempt to describe the mechanisms of transcriptional regulation of *dhp*, focusing on putative binding sites identified in the intergenic region upstream of the *dhp* coding sequence. Since it was not determined whether Anr or Vfr bind to putative binding sites in the *dhp* promoter, the sites that were mutated will be referred to as binding site "X" and "Y" respectively. This study showed that site X is involved in repression of DHP, while site Y appears to play a role in activation of DHP. The model therefore proposes a competitive binding system where, under sub-optimal conditions for *dhp* expression, transcription factor X (perhaps Anr or σ^{54}) binds to this site and represses activity. This repression is not complete, therefore it is likely that transcription factor X forms part of a signal transduction pathway involving other repressors. Repression via binding site X does not respond to the presence of a more efficient carbon source *i.e.* CCR.

In terms of the putative σ^{54} recognition sequence, it is assumed that mutagenesis did not affect the ability of the sigma factor to bind to this site, based on previous observations that the 3' end of this sequence was not essential to the functioning of the binding site.

Even if this were not the case, the σ^{54} site does not overlap with site Y (previously known as the Vfr binding site), and thus the sigma factor binding site is excluded from the competitive binding model

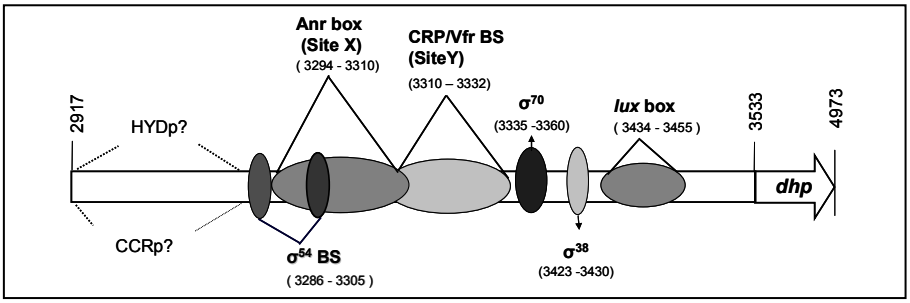

Figure 4.1. Schematic representation of the intergenic region upstream of the *dhp* ORF showing putative transcription factor binding sites. Putative binding sites are indicated as ovals and nucleotide co-ordinates are given in relation to the translation stop codon of *bup* in terms of the hydantoinase gene cluster in RU-KM3$_s$. NOTE: not drawn to scale.

Under the conditions where DHP activity is required for survival (in response to a stimuli other than the presence of substrate *i.e.* induction), a transcriptional activator protein Y (not Vfr) is able to displace transcription factor X and bind to site Y to activate *dhp* expression. This activator may be a regulatory protein that is specific to the *dhp* gene (DhpR), in the same way that XylR regulates the xylene catabolic genes and BkdR regulates the expression of enzymes responsible for the degradation of branched-chain keto acids (Hester *et al*., 2000; Sze *et al*., 2002). Since activation is incomplete, it is probable that transcription factor Y is co-operating with other activators as part of a regulatory cascade. A potential component of such a cascade is Crc, which appeared to play a role in activation of DHP. Crc does not bind DNA, but has been proposed to form part of a signal transduction pathway that modulates carbon metabolism and includes components activated by phosphorylation or dephosphorylation (Morales *et al*., 2004; Ruiz-Manzano *et al*., 2005).

Demonstration of the involvement of Vfr, Crc, Repressor X and Activator Y in regulating the *dhp* system substantiated the hypothesis, which stated that: “the regulation

of *dhp* expression in *P. putida* RU-KM3$_s$ requires two or more global transcription factors working in synergy, one of which is Vfr." The relatively large number of potential transcription factor binding sites identified in the intergenic region upstream of *dhp* and the degree of overlapping of these binding sites point to a complex and potentially novel mechanism of transcriptional regulation of *dhp* expression. Expanding on the above model of competitive binding and taking into consideration the arrangement of the putative binding sites for transcriptional regulators in the promoter, a synergistic or co-operative system of transcriptional regulation of *dhp* is proposed. Belyaeva *et al.* (1998) developed a model for transcriptional activation at promoters carrying tandem CRP binding sites in *E. coli*, whereby two CRP homodimers, one bound to recognition sequences centered 42 bp upstream of the transcriptional start site, and the other located further upstream, interact with the C- and N-terminal domains of the alpha sub-unit of RNA polymerase by means of DNA looping and a flexible RNAP linker domain (Figure 4.2)

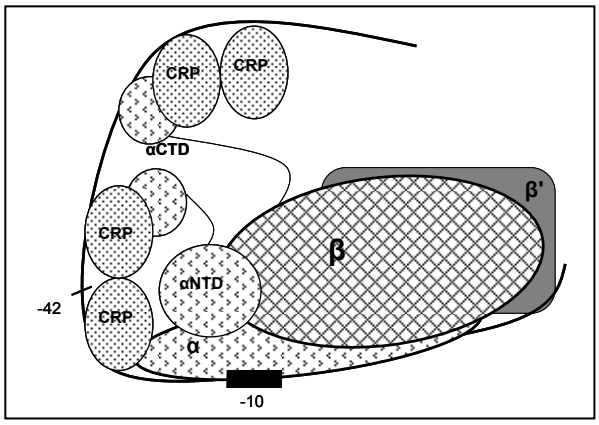

Figure 4.2. Model for synergistic regulation at *E. coli* promoters carrying tandem CRP recognition sequences. The nucleotide co-ordinates of the promoter are given below the DNA strand and transcription factors are indicated as follows: β and β' refer to the two β sub-units of RNA polymerase, while α refers to the α sub-unit; αCTD and αNTD refer to the C- and N–terminal domains of this RNAP sub-unit (Modified from Belyaeva *et al*., 1998).

Expanding from this model, the second CRP molecule may be replaced by another transcriptional regulator that is a CRP homologue, such as Fnr in *E coli*., thereby

allowing a single promoter to respond to a number of environmental signals (Belyaeva *et al*., 1998). A characteristic feature of such ambidextrous promoters is a 42.5 bp spacing between the centre of a transcription factor binding site and the transcriptional start. Therefore it would be interesting to perform primer extension analysis of the promoter region of *dhp* in order to determine the position of the transcriptional start of the promoter and whether any of the putative binding sites occupy such a position (Guest, 1992; Belyaeva *et al*., 1998).

A similar mechanism of synergistic activation has been observed in the promoter of the hydrogen cyanide producing genes, *hcnABC*, in *P. aeruginosa*. Here, transcriptional activity is co-regulated by Anr, which binds at an Anr box centered at 42.5 bp upstream of the transcriptional start, and a hetero-dimer comprising of LasR and RhlR, which binds to a *lux* box centered at 71 bp from the +1 site (Pessi and Haas, 2000). Activation of the *hcn* genes, achieved by the binding of individual transcription factors and favouring the Anr protein, is optimal when all four proteins Anr, LasR, RhlR and RNAP are bound to the DNA. Pessi and Haas (2000) proposed that binding of the proteins cause the DNA to bend into a loop conformation, thus allowing the transcriptional regulators to interact and co-operate with one another (Figure 4.3).

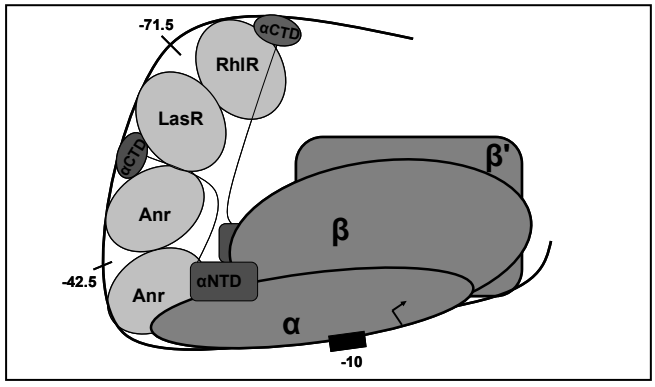

Figure 4.3. Model for the regulation of the hydrogen cyanide biosynthetic genes in *P. aeruginosa*. Transcriptional regulation occurs via the *hcn* promoter by the regulatory proteins Anr, LasR, and RhlR, that interact with the RNA polymerase αCTD (C-terminal domain of the α sub-unit) and αNTD (N-terminal domain of the α sub-unit). Nucleotide positions relative to the transcription start site are indicated below the sequence (modified from Pessi and Haas, 2000).

The *dhp* promoter region displays a tandem cluster of transcription factor binding sites separated by a poorly conserved σ^{70} promoter (Section 3.2). Considering the models put forward by Belyaeva *et al*. (1998), as well as Pessi and Haas (2000), a complex synergistic mechanism of transcription regulation is proposed for *dhp* (Figure 4.4). The upstream cluster is made up of a recognition sequence for σ^{54} as well as overlapping binding sites for Anr (or Repressor X) and Vfr (Activator Y/DhpR), for which a competitive binding model has been proposed above. The downstream cluster, the analysis of which was beyond the scope of this study, comprises the σ^{38} recognition sequence together with a putative *lux* box.

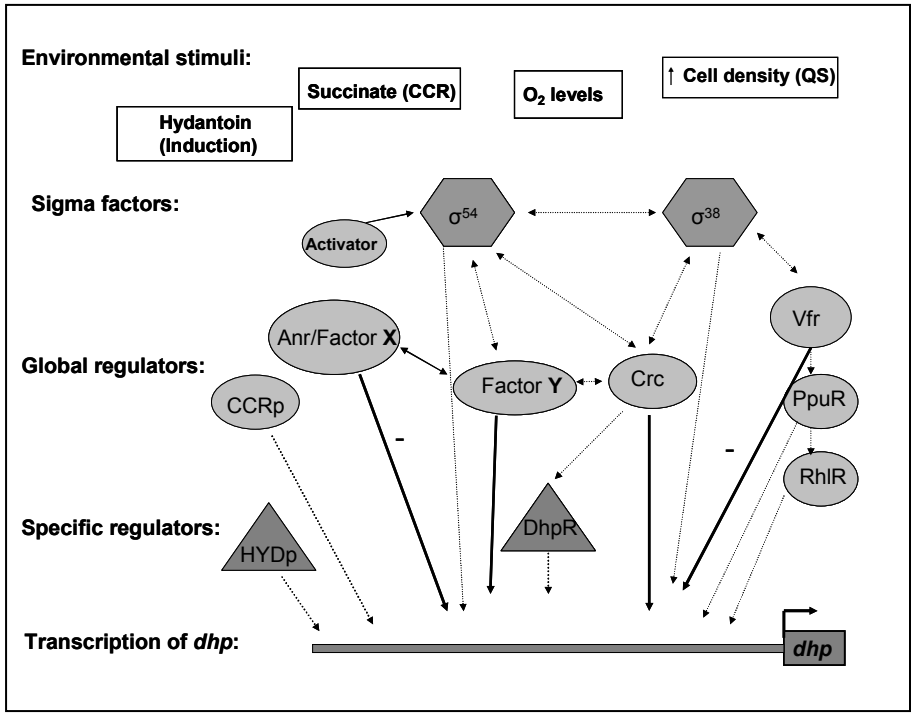

Figure 4.4. Synergistic model for the transcriptional regulation of *dhp*. Regulation is achieved by a complex hierarchy of sigma factors, as well as global regulators and specific regulators. Sigma factors are indicated by hexagons, global regulators by ovals and specific regulators by triangles. Arrows indicate an activation effect, except where accompanied by (-), which indicates a repression effect. Solid arrows represent relationships examined and confirmed in this study, while dotted arrows indicate hypothesised interactions.

In this system, when cells are growing exponentially in complete medium under ambient conditions, RNAP is unable to bind the promoter via the poorly conserved σ^{70} recognition sequence and thus transcription of *dhp* is prevented. However, should binding by RNAP inadvertently occur, repression via binding site X would prevent *dhp* expression. On the other hand, in the specific environmental conditions under which σ^{54} dominates, it is proposed that RNAP binds via this sigma factor to the *dhp* promoter, along with the transcriptional activator Y (possibly DhpR) and that these proteins interact and co-operate with one another by means of looping of the DNA to activate *dhp* expression. The stationary phase sigma factor, σ^{54}, has been shown to function in a manner that is entirely different to that of the σ^{70} family of sigma factors. More specifically, that it requires the binding of an activator protein which is located significantly further upstream of its recognition sequence. This corroborates with the finding that removal of 360 bp on the 5' terminus of the intergenic region upstream of *dhp* results in a 50 % decrease in DHP activity (Section 3.5). Thus, it is proposed that this 360 bp nucleotide stretch in the *dhp* intergenic region harbours a σ^{54}-specific upstream activation sequence. Furthermore, it is put forward that when environmental conditions favour σ^{38}, RNAP will bind to the *dhp* promoter via this sigma factor recognition site to activate *dhp* transcription. In terms of the adjacent putative *lux* box, it is suggested that quorum specific regulators such as Vfr, PpuR and RhlR may bind to this site to activate or repress expression of *dhp*. It is also possible that σ^{38} interacts and co-operates with the transcriptional regulatory protein(s) bound to the *lux* box.

Previous reports have shown that σ^{54} may activate the expression of other sigma factors and function independently of the RNAP holo-enzyme, such that it acts as a global transcriptional activator. It is therefore possible that σ^{54} may activate production of σ^{38}, which in turn would drive transcription of *dhp*. This would mean that *dhp* is subject to a complex hierarchy of regulatory processes that are comprised of various layers of activation or repression, which have a cumulative effect on *dhp* expression. Thus DHP activity would be maximal when both sigma factors σ^{54} and σ^{38}, the specific activator DhpR, Crc and other upstream activators are present (Figure 4.4). This co-operation of transcription factors is similar to that proposed by Pessi and Haas (2000).

4.8 Concluding remarks and future research opportunities

This study has demonstrated that transcriptional regulation of *dhp*, the gene responsible for hydantoinase activity in *P. putida* RU-KM3$_s$, occurs via at least two binding sites upstream of the *dhp* ORF, one mediating activation and the other repression. The involvement of two global transcriptional regulatory proteins, namely Vfr and Crc, was also demonstrated, with Vfr playing a role in repression and Crc in activation of DHP activity. The regulatory machinery involved in modulating *dhp* expression is sensitive to growth phase, substrate induction, CCR and QS. As a future research opportunity, these effects on the transcriptional regulation of *dhp* may also be studied by quantitative reverse transcriptase PCR to determine the levels of *dhp* expression, which could prove to be more sensitive than a reporter study.

The information regarding the regulation of the DHP activity provided in this study could assist in the development of bioprocesses using RU-KM3$_s$. It has been reported that this strain has industrial potential as a whole-cell biocatalyst for the production of chiral amino acids, finding applications in the pharmaceutical, food and cosmetic industries (Buchanan *et al*., 2001; Bulawayo *et al*., 2007). It is now known that at very high cell densities, the expression of *dhp* is repressed via QS. However, genetically modified RU-KM3$_s$ strains, in which the *vfr* gene has been inactivated would allow a certain degree of relief from repression, thus improving DHP production. In addition, disruption of either the putative Anr binding site (site Y) or the protein that binds this site, may further enhance expression of *dhp*. To date, very little information is available regarding the use of such transcriptional regulatory mutants to optimise amino acid production by hydantoinase activity (Hartley *et al*., 2001; Leuchtenberger *et al*., 2005). Another avenue for improving enzyme production in strain RU-KM3$_s$ is the over-expression of transcriptional activators, such as Crc.

It is already well established that supplementation of the growth medium with hydantoin improves DHP activity (Matcher *et al*., 2004). However, further studies regarding the identification of the factors responsible for mediating induction by hydantoin would

allow for the development of an inducer-independent strain that would negate the need for an expensive substrate in the growth medium. In terms of CCR of DHP activity, a study has been conducted to find a carbon source that has a reduced repressive effect, while supporting reasonable biomass production (Kirchmann *et al*., 2007). An alternative approach to overcoming the adverse effects of CCR is to study the molecular mechanisms governing CCR and develop a genetically modified strain resistant to CCR. Such a strain would enable production the DHP enzyme in the presence of a more efficient carbon source, allowing for greater biomass generation in a fermentation reactor.

Finally, besides the potential benefits to industry afforded by an understanding of the regulation of hydantoinase activity in *P. putida* RU-KM3$_s$, the novelty of such studies must also be considered. Elucidation of the mechanisms governing the sophisticated regulatory systems in *Pseudomonas* promises to contribute greatly to the current fundamental knowledge about transcriptional regulation in bacteria.

APPENDICES

APPENDIX 1

Strains, plasmids and primers utilised and generated in this study

Table A1.1. Strains utilised and generated in this study

Strains	**Description**	**Phenotype[a]**	**Source or reference**
P. putida			
RU-KM3$_s$	Wild-type isolate	Cm^R, Amp^R	Burton *et al*., 1998
RU-KM3$_s$Δ*vfr*	Mutant of RU-KM3$_s$ with *vfr* disrupted	Cm^R, Amp^R, Tet^R	This study
RU-KM3$_s$Δ*crc*	Mutant of RU-KM3$_s$ with *crc* disrupted	Cm^R, Amp^R, Kan^R	Matcher, unpublished
ΔRU-KM3$_s$3	Mutant of RU-KM3$_s$, with pJAS29 randomly integrated into the chromosome	Cm^R, Amp^R, Tet^R	This study
ΔRU-KM3$_s$7	Mutant of RU-KM3$_s$, with pJD2 randomly integrated into the chromosome	Cm^R, Amp^R, Tet^R	This study
E. coli			
DH5α	*supE44*, *lac*U169, (φ*80lacZ*ΔM15), *hsdR1*, *recA1, endA1, gyrA96, thi-1, relA1*		Hanahan, 1983
HB101	*supE44, hsdS20,* (Γ_B^-, m $_B^-$), *recA13, ara-14, proA2,lacY1, galK2, rpsL20, xyl-5, mtl-1*	Kan^R	Sambrook *et al*., 1989

Table A1.2. Plasmids utilised in this study

Plasmid	**Description**	**Reference**
pGEM T-Easy	General cloning vector used to insert PCR products and sequence inserts	Promega
pTnMod-OTc	Plasposon conferring resistance to tetracycline	Dennis and Zylstra, 1998
pTnMod-OKm	Plasposon conferring resistance to kanamycin	Dennis and Zylstra, 1998
pVfrKO	pTnMod-OTc derived gene disruption vector for targeted insertional inactivation of *vfr*	Matcher, unpublished
pCrcKO	pTnMod-OKm derived gene disruption vector for targeted insertional inactivation of *crc*	Matcher, unpublished
pMJ242	pCAMBIA-derived promoter probe vector containing *gus* reporter gene	Jiwaji, *et al*., 2008
pMJ445	Broad host-range promoter probe vector	Jiwaji, *et al*., 2008
pMJ449	pMJ445 containing the entire intergenic region upstream of the *dhp* of RU-KM3$_s$ fused to *gus*	Jiwaji, *et al*., 2008
pMJ480	pMJ445 containing the "minimal" *dhp* promoter region of RU-KM3s fused to *gus*	Jiwaji, unpublished

Table A1.3. Plasmids generated in this study

Plasmid	Description
pJAS20	pGEM T-Easy construct containing the 616 bp intergenic region upstream of *dhp* in RU-KM3$_{s,}$ with flanking *Bam* HI and *Nco* I sites
pJAS21	pGEM T-Easy construct containing the *gus* gene amplified from pMJ242 with flanking *Nco* I and *Kpn* I sites
pJAS26	Modified pTnMod-OTc plasposon with original *Eco* RI site removed and *Eco* RI and *Bam* HI sites introduced
pJAS29	pJAS26 containing the *gus* gene from pJAS21, cloned with *Eco* RI
pJD1	pGEM T-Easy construct containing the 255 bp truncated *dhp* promoter of RU-KM3$_s$ with flanking *Bam* HI and *Nco* I sites
pJD2	pJAS29 containing the 255 bp truncated *dhp* promoter of RU-KM3$_s$ from pJD1, cloned using *Bam* HI and *Nco* I
pJD4	pJD1 mutated in the putative CRP/Vfr binding site of the "minimal" *dhp* promoter to introduce a *Sma* I site
pJD5	pJD1 mutated in the putative Anr binding site of the "minimal" *dhp* promoter to introduce a *Pst* I site
pJD6	pMJ445 containing the mutated "minimal" *dhp* promoter of pJD4
pJD7	pMJ445 containing the mutated "minimal" *dhp* promoter of pJD5

Table A1.4. Primers utilised in this study

Primer	Description	Sequence	**
VfrF	Primer for *vfr* in RU-KM3$_s$ (with *Nde* I site) and corresponding to the start codon of the ORF. Diagnostic for *vfr* gene disruption mutant.	*CATATGGTTGCCTCCGCCCTACCCGCC	+
VfrR	Primer for *vfr* in RU-KM3$_s$ (with *Eco* RI site) and corresponding to the stop codon of the ORF. Diagnostic for *vfr* gene disruption mutant.	GAATTC**CTA**GCGGGTACCGTGGACCAC C	-
Vfr(int)	Primer for *vfr* and corresponding to nt 128 in the ORF. Diagnostic for gene disruption.	CTGTGCTGGCGATCGTGCCG	+
TnOTcF	Primer corresponding to the *Tc^R* gene in pTnMod-OTc. Diagnostic for plasposon.	CATTATGATTCTTCTCGGTTCCGGCGG C	+
TnOTcR	Primer corresponding to 5' end of the *oriV* gene in the pTnMod-OTc. Diagnostic for plasposon .	AAAGGCCAGGAACCGTAAAAAGGCCGC G	-
TnOKmSF	Primer corresponding to 5' end of the *oriV* gene in the pTnMod-OTc. Diagnostic for plasposon	TTTACGGTTCCTGGCCTTT	+
CrcF	Primer for *crc* in RU-KM3$_s$ (with *Nde* I site) and corresponding to the start codon of the ORF. Diagnostic for *crc* gene disruption mutant	CAT**ATG**CGGATCATCAGTGTGAACGTA AAT	+
CrcR	Primer for *crc* in RU-KM3$_s$ (with *Eco* RI site) and corresponding to the stop codon of the ORF. Diagnostic for *crc* disruption	GAATTCT**TAG**ATGGTCAGCGTCCAGTC ATA GTCG	-
Crc(int)	Internal primer for *crc* in RU-KM3$_s$ corresponding to nt 347 in the ORF. Diagnostic for gene disruption	CGGCCTGCTCGTTGTCCGGC	+
GFM38	Primer for the 616 bp intergenic region upstream of the *dhp* translational start codon in RU-KM3s (with *Bgl* II site).	AGATCTGGGGCCTTCTCCAGATTTTT	+
GFM39	Primer for the 616 bp intergenic region upstream of the *dhp* translational start in RU-KM3$_s$ (with *Bgl* II site)	AGATCTGCCGTCTTCCTCGCAG	-
MJ94	Primer corresponding to *gus* in pMJ449, diagnostic for integration	TTCGAAGATCGGCCCCCGCTCGACGCTC	-
pUCF	Universal sequencing primer for pGEM T-Easy and pUC18 plasmids	CGCCAGGGTTTTCCCAGTCACGAC	+
pUCR	Universal sequencing primer for pGEM T-Easy and pUC18 plasmids	TCACACAGGAAACAGCTATGAC	-

* Underlined sequences correspond to restriction endonuclease sites. ** + and – indicate sense (5'-3') and anti-sense (3'-5') primer direction.

Table A1.5. Primers generated in this study

Primer	Description	Sequence	**
JAS6	Primer for the 255 bp *dhp* promoter fragment in RU-KM3$_s$ (with *Bam* HI site).	*GGATCCCATGGGGCCTTCTCCAGATTT	+
JAS10	Primer for the 255 bp *dhp* promoter fragment in RU-KM3$_s$ (with *Nco* I site).	CCATGGAGATCTGCCGTCTTCCTCG	-
JAS11	Site-directed mutagenesis primer for the introduction of an *Eco* RI site into pTnMod-OTc	CGAATTCGATGAGCTCGGGTTGGTTTG	+
JAS13	Site-directed mutagenesis primer for the introduction of an *Eco* RI and a *Bam* HI site into pTnMod-OTc	CGAATTCGGATCCTTTCGTTCCACTGA GCG TCAGAC	-
JAS14	Primer for the *gus* gene of pMJ242 and corresponding to the translation start of the ORF (with *Nco* I site)	CCATGG**ATG**TTACGTCCTGTAGAAACC	+
JAS15	Primer for the *gus* gene of pMJ242 and corresponding to the stop codon of the ORF (with *Kpn* I site)	GGTACCTCGAGCTGCAG**TCA**TTGTTTG	-
JD1	Site-directed mutagenesis primer for the disruption of the putative CRP/Vfr binding site in the *dhp* promoter of RU-KM3$_s$ by the introduction of a *Sma* I site	CAAAATGGTGAACAGCCC GGGCATTT TGGTGAACGCC	+
JD2	Site-directed mutagenesis primer for the disruption of the putative CRP/Vfr binding site in the *dhp* promoter of RU-KM3$_s$ by the introduction of a *Sma* I site	GGCGTTCACCAAAATGCCCGGGCTGTT CAC CATTTTG	-
JD3	Site-directed mutagenesis primer for the disruption of the putative Anr binding site in the *dhp* promoter of RU-KM3$_s$ by the introduction of a *Pst* I site	CTTGACCAATTTGGCATCTGCAGAAGT GCG TCAAAATGG TG	+
JD4	Site-directed mutagenesis primer for the disruption of the putative Anr binding site in the *dhp* promoter of RU-KM3$_s$ by the introduction of a *Pst* I site	CACCATTTTGACGCACTTCTGCAGATG CCA AATTGGTCAAG	-

* Underlined sequences correspond to restriction endonuclease sites. ** + and – indicate sense (5'-3') and anti-sense (3'-5') primer direction.

APPENDIX 2

Preparation and transformation of competent *E. coli* cells

2.1. Preparation of competent *E. coli* cells

The protocol for the preparation of competent *E. coli* cells was developed from Hanahan (1983). A test-tube containing 5 ml Luria-Bertani broth was inoculated with *E. coli* DH5α and grown to confluence overnight at 37 °C with shaking at 200 rpm. Four Ehrlenmeyer flasks, each containing 100 ml Luria-Bertani broth were then inoculated with 1.5, 1.0, 0.7 and 0.3 ml of the overnight culture respectively and incubated at 37 °C for approximately 2 hours until an $OD_{600\ nm}$ of between 0.6 and 0.8 was obtained. The flasks were then cooled for 5 to 10 minutes on ice, after which the flask contents were processed separately until the final step. The cultures were centrifuged in a Beckman JA-14 rotor at 5000 rpm for 10 minutes, at 4 °C. The supernatant was discarded and the pellet resuspended in 50 ml RF1 (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15 % glycerol, pH 5.8), followed by a further 20 minutes incubation on ice. The cells were once again pelleted by centrifugation as above and the supernatant discarded. All four pellets were then resuspended together in a final volume of 4 ml of RF2 (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15 % glycerol, pH 6.8). The resuspended cells were frozen and stored at -80°C in 500 µl aliquots until required.

2.2. Transformation of competent *E. coli* cells

After thawing on ice, 100 µl competent *E. coli* cells were mixed with the plasmid DNA in a sterile 1.5 ml Eppendorf tube and incubated on ice for 20 minutes. The cells were then subjected to a heat-shock step by incubation at 42 °C for exactly 45 seconds, followed immediately by incubation on ice for 5 minutes. 1 ml cold Luria broth was then added and the cells incubated at 37 °C for 1 hour after which the cells were spread-plated onto Luria agar plates containing the appropriate selective antibiotic.

APPENDIX 3

General protocols used in DNA manipulation

3.1 Plasmid DNA extraction from *E. coli*

E. coli DH5α cells containing the recombinant plasmid were grown to confluence overnight at 37 ºC, with agitation at 200 rpm, in 5 ml Luria broth supplemented with the relevant antibiotic. The plasmid constructs were then removed from the *E. coli* cells using the 'Easyprep' method described by Berghammer and Auer (1993). The constructs were screened by means of restriction endonuclease digestion according to manufacturer's specifications. Thereafter the resulting DNA fragments were analysed by electrophoretic separation on 1 % agarose gels (unless stated otherwise) containing 10 μg/ml ethidium bromide. The buffer used in the preparation and electrophoresis of the agarose gels was 1 x TAE [50x TAE (1L): 242 g Tris HCl, 57.1 ml glacial acetic acid and 100 ml 0.5 M Na_2EDTA, pH8.0]. The DNA in the agarose gels was visualised under UV light and images captured using the "UviPro Chemiluminescence and Fluorescence Documentation System".

The desired plasmids were then extracted from *E. coli* to a high degree of purity using the High Pure Plasmid DNA isolation kit (Roche), according to manufacturer's specifications. This high quality DNA was used in further manipulation and in-depth analysis using DNA sequencing.

3.2. Sequencing of DNA

Where constructs required DNA sequencing, the "Di-deoxy Chain Termination Method" (Sanger *et al*., 1997) using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (PE Applied Biosystems) was carried out. The thermal cycling reaction parameters were performed according to the manufacturer's instructions for the GeneAmp PCR System 9700 thermal cycler. The amplified products were then purified using the DNA Clean and Concentrate kit (Zymo) and dried by vacuum centrifugation (Speedvac concentrator, Savant). Thereafter the samples were sequenced using an

ABI Prism 3100 Genetic Analyser (Hitachi Applied Biosystems) at the Rhodes University Sequencing facility.

3.3. Genomic DNA extraction

Genomic DNA was extracted from *P. putida* RU-KM3$_s$ and its transconjugate mutants using the detergent lysis/CTAB and organic solvent extraction method, developed by Ausubel (1983). The resultant DNA was treated with 5 µl of 10 µg/ml RNAse A to remove the RNA, and the DNA was then precipitated by adding a tenth of the volume of 3 M sodium acetate buffer (pH 5.2) together with two times the volume of ice-cold 96 % rectified ethanol and incubated at -20 ºC overnight. The DNA was then pelleted by centrifugation at 13 000 rpm for 10 minutes in a Heraeus microfuge, washed twice with ice-cold 70 % rectified ethanol and dried by vacuum centrifugation (Speedvac concentrator, Savant), before resuspending in 50 µl TE buffer (pH 7) for 3 hours at room temperature. The genomic DNA was then analyzed by agarose gel electrophoresis as detailed in Section 3.1 above.

3.4. PCR amplification of DNA

In this study, four different DNA-dependant DNA polymerases were utilised, namely *Taq* DNA polymerase (Bioline), Kapa*Taq* DNA polymerase (Kapa Biosystems), Kapa High Fidelity DNA polymerase (Kapa Biosystems) and Expand High Fidelity DNA polymerase (Roche). All enzymes were used according to manufacturer's specifications. The general cycling parameters are detailed in Table A2.

Table A3. General reaction conditions for PCR carried out in this study

Step	**Cycling parameters for each DNA polymerase**				**No. of cycles**
	Bioline Taq	Kapa Taq	Expand High Fidelity	Kapa High Fidelity	
Initial denaturation	92 ºC, 2 min	94 ºC, 2 min	94 ºC, 2 min	95 ºC, 2 min	1
Denaturation	92 ºC, 45 s	94 ºC, 30 s	94 ºC, 30 s	98 ºC, 15 s	30
Annealing	X ºC, 45 s	X ºC, 45 s	X ºC, 45 s	X ºC, 15 s	
Extension	72 ºC, 1min/Kb	72 ºC, 1min/Kb	72 ºC, 1min/Kb	72 ºC, 30 s/Kb	
Final Extension	72 ºC, 3 min	72 ºC, 2 min	72 ºC, 3 min	72 ºC, 1 min	1

Where the annealing temperature, X ºC, varies according to the melting temperatures of the primer pair used in the reaction.

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