REGULATION OF *hyu* GENE EXPRESSION IN *Agrobacterium tumefaciens* STRAINS RU-AE01 AND RU-OR

THESIS

Submitted in fulfillment of the requirements for the Degree of

Doctor of Philosophy
(Microbiology)

in the

Department of Biochemistry, Microbiology and Biotechnology
Faculty of Science
Rhodes University

by

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June 2006

ABSTRACT

Several *Agrobacterium tumefaciens* strains have been isolated for their ability to produce D-amino acids from D, L-substituted hydantoins. The optically pure D-amino acids are used in the synthesis of pharmaceuticals, as food additives and as insecticides. This hydrolysis of D, L-substituted hydantoins is catalysed by two hydantoin-hydrolyzing enzymes, an hydantoinase and an *N*-carbamyl amino acid amidohydrolase. While the hydantoin-hydrolyzing enzymes have been studied in detail, the mechanisms that control expression of the *hyu* genes have not. The research reported in this work elucidates some of the mechanisms involved in the regulation of the *hyu* genes in *A. tumefaciens* strains.

The hydantoin-hydrolyzing enzyme activity from the environmental isolate *A. tumefaciens* RU-AE01 was characterized. A broad host range vector for the simultaneous analysis of divergent promoters was constructed. The promoter regions responsible for the activation of transcription of *hyuH* and *hyuC* were identified by deletion analysis. It was proposed that transcription of *hyuH* was activated by a putative σ^{54}-dependent promoter or a putative σ^{70}-dependent promoter identified upstream of the *hyuH* gene. The *hyuC* gene was activated by a putative σ^{70}-dependent promoter identified upstream of the *hyuC* gene.

The regulation of hydantoinase and *N*-carbamyl amino acid amidohydrolase enzyme activity was compared to the regulation of transcription from the RU-AE01 *hyuH-hyuC* region. Expression of the hydantoin-hydrolyzing enzymes was regulated by induction which correlated with reporter enzyme expression from the *hyuH* and *hyuC* promoter regions. However, the expression of the hydantoin-hydrolyzing enzymes was also regulated by nitrogen catabolite repression (NCR). This did not correlate to the reporter gene expression of the *hyuH* promoter region but did compare to the reporter gene expression of the *hyuC* promoter region. This suggested that NCR of *hyuH* was at the post-translational level whereas NCR of the *hyuC* promoter was at the transcriptional level.

Pathways involved in the regulation of the *hyu* genes were characterized. The production of the hydantoin-hydrolyzing enzymes in both *A. tumefaciens* strains

RU-AE01 and RU-OR were regulated by proteins involved in the global ntr pathway. The levels of the hydantoin-hydrolyzing enzymes in strain RU-AE01 were elevated in the presence of increased levels of NtrB and NtrC illustrating the importance of the ntr pathway in the regulation of the levels of the hydantoin-hydrolyzing enzymes. Similarly, in RU-OR the presence of exogenous NtrB and NtrC elevated levels of *N*-carbamyl amino acid amidohydrolase activity. However, the levels of hydantoinase enzyme activity in strain RU-OR were elevated in the presence of NtrC alone. In addition, the presence of a His_6-tagged NtrC molecule abolished the elevation in the levels of the hydantoinase but not the *N*-carbamyl amino acid amidohydrolase enzyme activity in strain RU-OR. This suggests that NtrC has a direct role in the regulation of the expression of *hyuH* in RU-OR. In addition, it indicates that the *hyu* genes in the two *A. tumefaciens* strains RU-AE01 and RU-OR are different.

The presence of the RU-AE01 *hyuH-hyuC* fragment caused a dramatic increase in the hydantoin-hydrolyzing enzyme activity in strain RU-OR but not strain RU-AE01. This implied the incidence of a possible repressor protein in RU-OR, which is titrated out by the presence of the RU-AE01 *hyuH-hyuC* fragment. Protein-DNA binding assays suggest that this putative repressor may be 38 kDa in RU-OR cells.

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LIST OF ABBREVIATIONS

AmmMM	Ammonium sulphate Minimal Medium
BSA	Bovine Serum Albumin
bup	Gene encoding a β-ureidopropionase in *Pseudomonas putida* $KM3_S$
CCR	Carbon Catabolite Repression
CRP	cAMP Receptor Protein
5-FU	5-fluorouracil
CAAMM	Casamino acids Minimal Medium
CHPG	*N*-carbamoyl-*p*-hydroxyphenylglycine
CTAB	Hexadecyltrimethyl-ammonium bromide
dhp	Gene encoding a dihydropyrimidinase in *Pseudomonas putida* $KM3_S$
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EMS	Ethylmethane sulphonate
GlyMM	Glycerol Minimal Medium
GMM	Glucose Minimal Medium
GDH	Glutamate Dehydrogenase
GOGAT	Glutamate Synthetase
GS	Glutamine Synthetase
HMM	Hydantoin Minimal Medium
HPG	Hydroxyphenylglycine
hyuA	Gene encoding an hydantoin racemase enzyme in *Agrobacterium tumefaciens* IP I-671*, Arthrobacter aurescens* DSM 3747
hyuA	Gene encoding a D-hydantoinase enzyme in *Pseudomonas* sp. NS 671
hyuB	Gene encoding an L- hydantoinase enzyme in *Pseudomonas* sp. NS 671
hyuC	Gene encoding an *N*-carbamyl amino acid amidohydrolase enzyme in *Agrobacterium tumefaciens* IP I-671, *Agrobacterium tumefaciens* NRRL B11291*, Arthrobacter aurescens* DSM 3747*, Pseudomonas* sp. NS 671
hyuD	Gene encoding a D-amino acid dehydrogenase enzyme in *Agrobacterium tumefaciens* IP I-671
hyuE	Gene encoding an hydantoin racemase enzyme in *Pseudomonas* sp. NS 671

hyuH	Gene encoding a hydantoinase enzyme in *Agrobacterium tumefaciens* IP I-671, *Agrobacterium tumefaciens* NRRL B11291*, Arthrobacter aurescens* DSM 3747
hyuN	Gene encoding an NAD(P)H flavin oxidoreductase enzyme in *Agrobacterium tumefaciens* IP I-671
hyuP	Gene encoding a permease enzyme in *Arthrobacter aurescens* DSM 3747
IPTG	Isopropyl-β-D-thiogalactosidase
LMM	Lactose Minimal Medium
NCAAH	*N*-carbamyl amino acid amidohydrolase
NCBI	National Centre for Biotechnology Information
NCG	*N*-carbamyl glycine
NCR	Nitrogen Catabolite Repression
nt	nucleotides
ONPG	*ortho*-nitrophenyl-β-D-galactopyranoside
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenyl methyl-sulphonyl fluoride
PNPG	*para*-nitrophenyl-β-D-glucuronide
RBS	Ribosome binding site
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
Tris	Tris-2-amino-2-(hydroxymethyl)-1, 3-propandiol
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronide

ACKNOWLEDGEMENTS

Prof Rosemary Dorrington - for her supervision and guidance, for her support and patience, and for her friendship. This project has been a long and hard road Doc, thank you for walking it with me.

Members of Lab 417, both past and present. Thank you for the friendship, camaraderie, humour and intellectual stimulation. I would like to mention in particular Gwynneth Matcher – for her patient support and her advice but most of all for her unconditional friendship; Carol Hartley – for helping me when I started out on the project, for sharing her work and her experience, for her friendship and her advice and of course, for her ever present offer of ‘just one drink’, Arno ‘If you don’t know what is what, then you don’t know what you’ve got’ Venter- for academic mentoring and friendship and last but definitely not least Val Hodgson – for keeping the lab running, at all times with efficiency and a smile.

My lovely family, Ami and Drew – thank you all for your love and support, for the patience and encouragement.

Beatrice – for all the hours you spend with me; the walks and talks were very helpful and much appreciated.

For proofreading this thesis I would like to thank Carol Hartley and Gwynneth Matcher.

For funding I gratefully acknowledge the Andrew Mellon Foundation.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The increased industrial focus on ‘green’ chemistry has meant that enzymes and microbial systems are rapidly replacing the classical chemical catalysts (Jaeger *et al*. 2002). Enzymes have the advantage over chemical synthesis methods as they ordinarily function under mild conditions (usually in aqueous medium at room temperature), at atmospheric pressure and near neutral pH (Jaeger *et al*. 2002, Koeller and Wong 2001). The generally low substrate specificity and high regio- (positional) and enantio-selectivity (chiral), make enzymes ideal candidates for the production of optically pure amino acids (Schulze and Wubbolts 1999, Syldatk *et al.* 1990a).

Amino acids are high value chemicals, approximated by Hols *et al.* in 1999 to have a market value of US $ 3 billion. A recent report states that the largest use of amino acids is in the synthesis market, for biotechnological and biopharmaceutical applications (http://www.bccresearch.com/biotech/B132R.html). As the worldwide demand for amino acids is expected to continue to rise at an average annual growth rate of 7% until 2009, no decrease in the production of amino acids is anticipated (http://www.bccresearch.com/biotech/B132R.html).

Industrial fermentation with microbes is not a new technology as it has been used for over thirty years to produce amino acids; for example the commercial production of monosodium glutamate by *Corynebacterium glutamicum* has been in use since 1957 (Fotheringham 2000, Kamphuis *et al.* 1990). More recently, companies including SmithKline Beecham (Great Britain), Ajinomoto (Japan), Kanegafuchi Chemical Industries (Japan), Bayer (Germany), Degussa (Germany), SNAM Progetti (Italy) and Recordati (Italy) have been awarded lucrative patents for the microbial production of optically pure amino acids (Bommarius *et al.* 1998, Brychem Business Consulting 1999, Schulze and Wubbolts 1999). In addition, most of the patents describe the use of the enzymes from members of the *Agrobacterium* sp. (US Patent 5869298, Galli *et al.* 1995), *Bacillus* sp. (US Patent 5962279, Nanba *et al.* 1996) and *Pseudomonas* sp. (US Patent 6087136, Gokhale *et al.* 1997).

Chiral amino acids have a wide range of applications (Table 1-1). Aromatic D- and L-amino acids are widely used in the pharmaceutical industry for the

production of drugs and bioactive peptides and as additives in the feedstock industry. For example, approximately 800 000 tonnes of monosodium glutamate with a market value of US $ 915 million are produced annually for use as a seasoning agent. A total of 300 000 tonnes of L-lysine are produced for use in the food and feed additive market and 13 000 tonnes of L-phenylalanine are produced for use in the synthesis of the antibiotic amoxicillin (Demain 2000). In addition there is a large market for unnatural amino acids, like D-citrullene and their derivatives, which are used in the pharmaceutical industry (Drauz *et al.* 1991).

Table 1-1: Examples of the application of optically pure amino acids in industry

Application	**Amino Acid**
PHARMACEUTICALS:	
Semi-synthetic penicillins	
amoxicillin	D-*p*-hydroxyphenylglycine[1], D-phenylglycine[5], D-cysteine[5], D-aspartic acid[5]
Semi-synthetic cephalosporins	
cephadroxil	D-*p*-hydroxyphenylglycine[6]
Angiotensin – converting enzyme (ACE) inhibitors	
Ramipril, Elanapril, Lisinopril, Quinapril, Trandolapril, Delapril, Benzapril, Siprapril, Cilazapril	D-serine[7], L-homophenylalanine[8], *trans*-4-hydroxy-L-proline[9]
Anti-virals	
Valaciclovir, Sandoz, Abbott, Biomega	L-valine[7], L-*tert*-leucine[1]
Anti-inflammatry	
RO-31-9790	L-*tert*-leucine[1]
Anti-tumour	
BB-2516, Ceterolix, Marimastat, Zeneca	L-*tert*-leucine[1], D-citrulline[7] , D-alanine[7]
Immuno-stimulants	
Libestatin, Bestatin	L-leucine[7]
Immuno-supressants	
Cyclosporin A	L-valine[10]
Cardiovascular	
Docarpamine, Tanadopa	L-methionine[7], L-proline[6]
Anti-arthiritics	
Ademetionine, Gumbaral	L-methionine[7]
FOOD AND FEED ADDITIVES	L-lysine[2], L-methionine[2], L-threonine[2], L-tryptophan[2]
NUTRACEUTICALS SWEETENERS	
Alitame, Aspartame	D-alanine[5], L-phenylalanine[4]
INSECTICIDES	
Fluvalinate	D-valine[3]
BIOACTIVE PEPTIDES:	
Optically active peptides	
Dextroamphetimine sulphate, Methamphetamine hydrochloride, *l*-Norephedrine hydrochloride	D-phenylalanine[4]
Neuropeptides	
Achatin, Dermophin	D-phenylalanine[4]

Bommarius *et al.* 1998 [1], Ottenheym and Jenneskens 1970 [2], Drauz *et al.* 1991 [3], Bae *et al.* 1999 [4], Yagasaki and Ozaki 1998 [5], Schulze and Wubbolts 1999 [6], Drauz 1997 [7], Kamphuis *et al.* 1992 [8], Ogawa and Shimizu 2002 [9], Kamphuis *et al.* 1990 [10].

The L-isomers of amino acids are predominant within all microbes as they are naturally synthesized by the cellular biosynthetic pathways (Hahn *et al.* 1954). However D-amino acids are not uncommon, being major constituents of bacterial cells walls and naturally occurring antibiotics (Tripathi *et al.* 2000, Yagasaki and Ozaki 1998). These D-amino acids are produced within the cell from the corresponding L-amino acid by the respective amino acid racemase or from an α-keto-acid by the combined actions of a D-specific transaminase and an amino acid racemase (Yagasaki and Ozaki 1998). Interestingly, under stressful conditions microbes tend to accumulate L-isomers of amino acids but not D-amino acids (Shahjee *et al.* 2002). It appears that while D-amino acids are generally more active and more stable than their L-analogs, they have an inhibitory effect on cell growth. Conversely, L-amino acids act as osmoprotectants within bacterial cells (Bommarius *et al.* 1998, Shahjee *et al.* 2002).

1.2 PRODUCTION OF CHIRAL AMINO ACIDS BY THE HYDANTOIN-HYDROLYZING ENZYMES

1.2.1 Hydantoin

In 1861, during the course of his study of uric acid, Baeyer discovered hydantoin (also known as 2, 4-imidazoledione and 2, 4-diketotetrahydroimidazole) (cited in Ware 1950). Hydantoins were first synthesized through the hydrogenation of allantoin however hydantoin has been found naturally in oriental plane tree buds and the shoots of the sugar beets (cited in Ware 1950). Hydantoin derivatives such as allantoin, carboxymethylenehydantoin and carboxyethylenehydantoin also occur naturally (Syldatk *et al.* 1990a).

Allantoin, an important metabolite in the degradation of purine nucleotides, is widespread in nature. The catabolism of guanosine and adenosine leads to the production of uric acid. In most organisms, uric acid is hydrolyzed by the enzyme 5-ureidohydantoinase (also known as allantoinase) (EC 3.5.2.5) to produce allantoin. Carboxymethylenehydantoin and carboxyethylenehydantoin are by-products of the L-selective degradation of dihydroorotic acid (a cyclic amide) and histidine respectively (Syldatk *et al.* 1990a).

There are three main techniques for the chemical synythesis of 5-monosubstituted hydantoins. Aldehydes or ketones (carbonyl compounds) can be condensed with hydantoins to produce an unsaturated C-5 substituted hydantoin derivative which is in turn reduced to a saturated C-5 substituted hydantoin (Figure 1-1A). Alternatively, Bucherer-Bergs synthesis of 5-monosubstituted hydantoins can be achieved from carbonyl compounds in the presence of potassium cyanide and ammonium carbonate (Figure 1-1B). Finally, α-amino acids, α-amino acid amides or α-amino acid nitriles can be converted to the respective hydantoic acid and in turn to the C-5 monosubstituted hydantoin by condensation reactions (Figure 1-1C). A number of criteria are used to select which of the methods above is used in the production of 5-monosubstituted hydantoins: the residue required at the C5 position, the availability of the reagents and the cost of the production of the final molecule (Syldatk *et al.* 1990a).

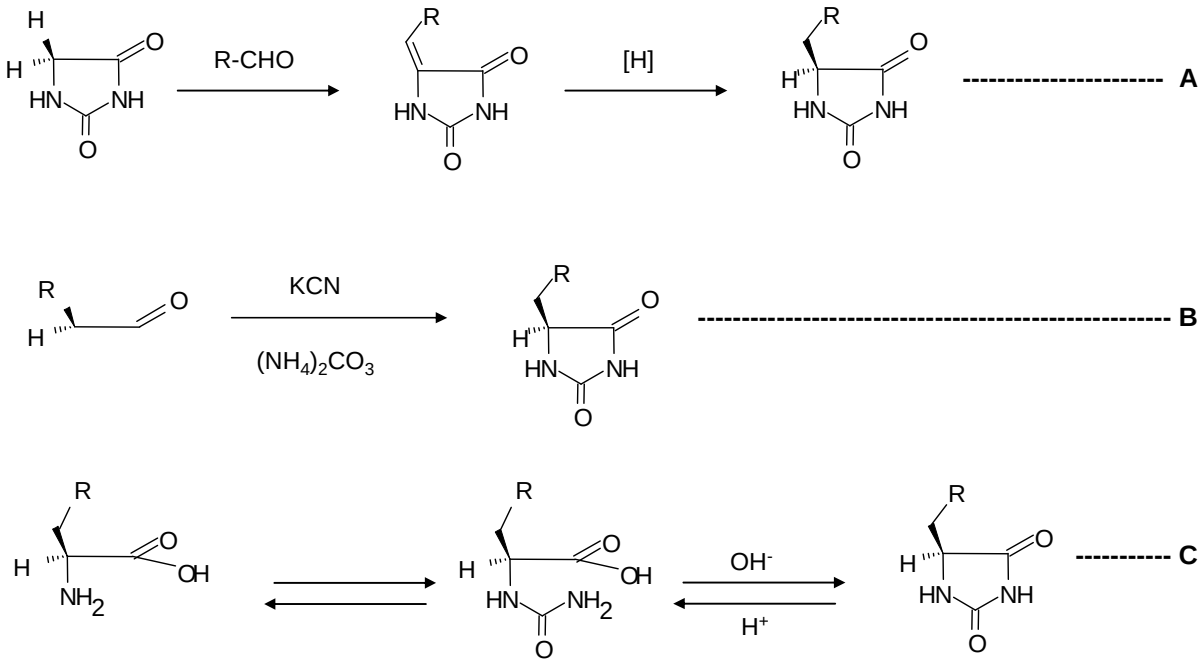


Figure 1-1: Chemical synthesis of 5-monosubstituted hydantoins by condensation of aldehydes (A) or Bucherer-Bergs synthesis (B) or by condensation of α-amino acids (C). Figure adapted from Syldatk *et al.* 1990a, Syldatk *et al.* 1992.

1.2.2 Hydantoin-hydrolyzing enzymes

While hydantoin-hydrolyzing activity has been found in plant sources (including legumes beans, peas, cucumbers, melon seeds, pine tissues, pea plants, wheat grains, tomato cells and rape seedlings) and animal sources (suspensions of livers and kidneys), hydantoin-hydrolyzing enzyme activity with incredible diversity and

significant industrial potential has been identified in microbes (Eadie *et al.* 1949, Morin 1993). Of the microbial hydantoin-hydrolyzing enzymes, extensive research and development has been done on the members of the *Agrobacterium*, *Arthrobacter*, *Bacillus* and *Pseudomonas* genera (Table 1-2).

Table 1-2: Strains of *Agrobacterium*, *Arthrobacter*, *Bacillus* and *Pseudomonas* in which the hydantoin-hydrolyzing enzymes have been studied in detail.

Genus	**Strain**	**Selectivity**		**Reference**
		HYD	NCAAH	
Agrobacterium	*Agrobacterium* sp.		D	Louwrier and Knowles 1996
	Agrobacterium sp. IP I-671	D	D	Runser and Ohleyer 1990
	Agrobacterium sp. KNK 712		D	Nanba *et al.* 1998
	A. radiobacter CCRC 14924		D	Hsu *et al.* 1999
	A. tumefaciens 47C	D		Durham and Weber 1995
	A. tumefaciens AM10		D	Sareen *et al.* 2001
	A. tumefaciens BQL9	D		Clemente-Jiminez *et al.* 2003
	A. tumefaciens NRRL B11291	D	D	Olivieri *et al.* 1979
	A. tumefaciens RU-OR	D	D	Hartley *et al.* 1998
Arthrobacter	*Arthrobacter* sp. BH20	Non	L	Syldatk *et al.* 1987
	Arthrobacter sp. DSM 3747	Non	L	Gross *et al.* 1990
	Arthrobacter sp. DSM 7330	Non		Volkel and Wagner 1995
	A. aurescens DSM 3745	SD		May *et al.* 1998a
	A. crystallopoietes AM2	D	D	Moller *et al.* 1988
	A. crystallopoietes DSM 20117	D		Siemann *et al.* 1999
Bacillus	*Bacillus* sp. AR9	D		Sharma and Vohra 1997
	B. brevis AJ-12299	L	L	Yamashiro *et al.* 1988
	B. circulans	D		Luksa *et al.* 1997
	B. stearothermophilus SD1	D		Lee *et al.* 1994a
	B. stearothermophilus NS1122A	D	L	Mukohara *et al.* 1993, 1994
	B. thermocatenulatus GH-2	D		Park *et al.* 1998
Pseudomonas	*Pseudomonas* sp. AJ-11220	D	D	Yokozeki *et al.* 1987a, b, c
	Pseudomonas sp. KBEL 101	D		Kim and Kim 1993
	Pseudomonas sp. KNK 003A		D	Ikenaka *et al.* 1998
	Pseudomonas sp. NCIM 5109	D		Sudge *et al.* 1998
	Pseudomonas sp. NS671	Non	L	Watabe *et al.* 1992a, b, c
	Pseudomonas sp. ON-4a		L	Ohmachi *et al.* 2002
	P. desmolyticum NCIM 2112	D		Gokhale *et al.* 1996
	P. putida CCRC 12857	D		Chien *et al.* 1998
	P. putida DSM 84	D		Morin *et al.* 1986
	P. putida RU-KM1	D	D	Burton *et al.* 1998
	P. putida RU- $KM3_S$	Non	L	Buchanan *et al.* 2001, Matcher *et al.* 2004
	P. putida RU-$KM3_L$	Non / L	L	Burton *et al.* 1998
	P. striata IFO-12996	D		Takahashi *et al.* 1978

Abbreviations: D- D-selective, HYD- hydantoinase, L- L-selective, NCAAH- *N*-carbamyl amino acid amidohydrolase, Non- Non-selective and SD- substrate dependent. Enzymes not specified have have either not been characterized or have not been isolated.

There are three enzymatic steps involved in the catabolism of hydantoin in these and other microbial strains (Figure 1-2). Racemization of 5-monosubstituted hydantoins between the D- and the L-enantiomers occurs via keto-enol tautomerism or as a result of the action of a racemase enzyme. Keto-enol tautomerism is also known as base catalysis as it occurs spontaneously between the pH values of 8 to 10 (Bommarius *et al.* 1998, Ogawa and Shimizu 1997). Hydantoin racemase enzyme activity has been detected in a number of strains but the enzyme has only been isolated from three genera- *Agrobacterium*, *Arthrobacter* and *Pseudomonas* (Hils *et al.* 2001, Martinez-Rodriguez *et al.* 2004, Wiese *et al.* 2001, Watabe *et al.* 1992a, b).

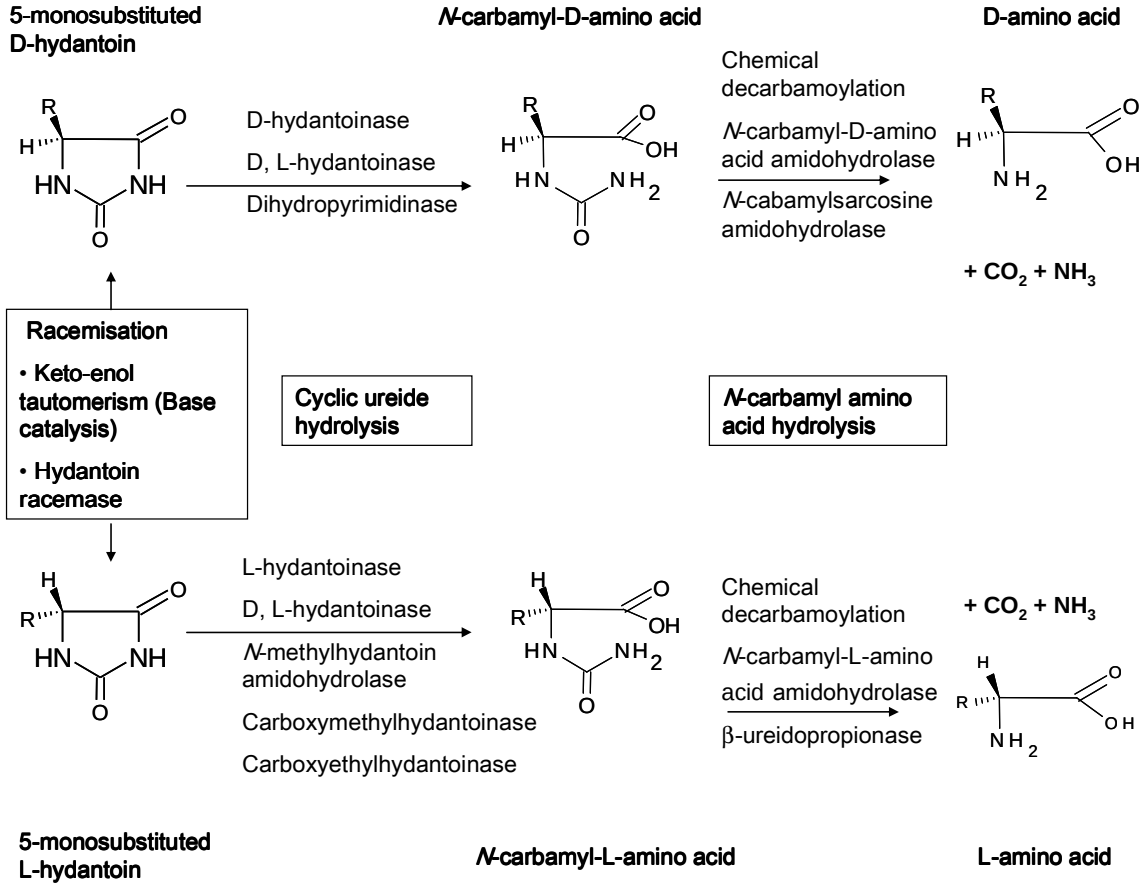


Figure 1-2: Mechanism for the enzymatic hydrolysis of hydantoins to chiral amino acids. Figure adapted from Altenbuchner *et al.* 2001, Ogawa and Shimizu 1997.

The 5-monosubstituted hydantoins are hydrolyzed to *N*-carbamyl amino acids by family of cyclic ureide hydrolyzing enzymes (Figure 1-2). Both D- and L-hydantoins can be hydrolyzed by a non-stereoselective D, L-hydantoinase (EC 3.5.2.X) resulting in the production of D- or L-*N*-carbamyl amino acids (Figure 1-2). D, L-hydantoinase

enzyme activity has been detected in members of the *Arthrobacter* and *Pseudomonas* genera (Syldatk *et al.* 1987, Watabe *et al.* 1992a). D-hydantoins can also be hydrolyzed to D-*N*-carbamyl amino acids by dihydropyrimidinases (EC 3.5.2.2) and D-hydantoinases (EC 3.5.2.2). In some bacterial genera like *Arthrobacter*, *Agrobacterium*, *Bacillus*, *Blastobacter* and *Pseudomonas*, the D-hydantoinase enzyme activity is identical to the dihydropyrimidinase activity (Moller *et al.* 1988, Durham and Weber 1995, Luksa *et al.* 1997, Ogawa *et al.* 1994a, Takahashi *et al.* 1978). In others, the two enzymes are distinct. For example in *Agrobacterium* sp. IP I-671, the D-hydantoinase enzyme activity is separate from the dihydropyrimidinase enzyme activity (Runser and Meyer 1993).

The hydrolysis of *N*-carbamyl amino acids to amino acids can occur by chemical decarbamoylation (Figure 1-2) which generally involves the use of nitrite under acidic conditions but enzymes that specifically hydrolyze *N*-carbamyl amino acids have also been identified (Ogawa and Shimizu 1997, Shimizu *et al*. 1980). *N*-carbamyl-D-amino acid amidohydrolases (D-NCAAH) (EC 3.5.1.77), which have been detected in *Blastobacter*, *Comamonas* and *Agrobacterium* specifically hydrolyze *N*-carbamyl-D-amino acids to D-amino acids (Ogawa *et al.* 1994a, Ogawa *et al.* 1993, Louwrier and Knowles 1996). Similarly, the *N*-carbamylsarcosine amidohydrolases (EC 3.5.1.60), which have been detected in *Pseudomonas*, catabolise *N*-carbamyl-α-amino acids to D-amino acids (Yamada *et al.* 1985).

L-hydantoins are hydrolyzed to *N*-carbamyl-L-amino acids by an L-hydantoinase (EC 3.5.2.X), which has been detected in *Arthrobacter* and *Bacillus*, or *N*-methylhydantoin amidohydrolases (EC 3.5.2.14), which have been detected in *Pseudomonas* (May *et al.* 1998a, Yamashiro *et al.* 1988, Ogawa *et al.* 1995a). The *N*-carbamyl-L-amino acids are in turn converted to L-amino acids. The enzyme *N*-carbamyl-L-amino acid amidohydrolase (L-NCAAH) (EC 3.5.1.87), present in *Alcaligenes*, *Arthrobacter*, *Flavobacterium* and *Pseudomonas* specifically hydrolyzes *N*-carbamyl-L-amino acids to L-amino acids (Ogawa *et al.* 1995b, Gross *et al.* 1990, Nishida *et al.* 1987, Ohmachi *et al.* 2002). Alternatively, the β-ureidopropionase (EC 3.5.1.6), which is L-selective is involved in the production of L-amino acids from *N*-carbamyl-β-amino acids, *N*-carbamyl-γ-amino acids and some *N*-carbamyl-α-amino acids (Ogawa and Shimizu 1997). The β-ureidopropionase

enzyme has been detected in *Blastobacter*, *Comamonas* and *Pseudomonas* (Ogawa *et al.* 1994b, Ogawa amd Shimizu 1994).

Two other L-selective enzymes, the carboxymethylhydantoinase (EC 3.5.2.4) and the carboxyethylhydantoinase (EC- 3.5.2.X) are present in bacteria. Carboxymethylhydantoinase hydrolyzes carboxymethylenehydantoin to L-aspartic acid and carboxyethylhydantoinases convert carboxyethylenehydantoin to L-glutamic acid (Ogawa and Shimizu 1997, Syldatk *et al.* 1990a).

1.2.3 Classification of the hydantoin-hydrolyzing enzyme sytems

At present, two different methods are used to classify the hydantoin-hydrolyzing enzymes. In the first, conventional enzyme nomenclature is based on enzyme biocatalytic function. In the case of the hydantoin-hydrolyzing enzymes, they are classified as hydrolases (EC 3) that act on carbon-nitrogen bonds (EC 3.5) in cyclic amides (EC 3.5.2) (http://www.chem.qmul.ac.uk/iubmb/enzyme/). While this classification specifies the type of bond the enzymes hydrolyze, it does not identify the differences between the enzymes. First, all dihydropyrimidinases (EC 3.5.2.2) are considered to be identical to D-hydantoinases thus are assigned one classification number. Second, other enzymes that are similar to dihydropyrimidinases but which are known to differ in their biocatalytic behaviour, for example the non-selective hydantoinases and L-hydantoinases, have not been allocated complete identifying numbers (http://www.chem.qmul.ac.uk/iubmb/enzyme/).

The second method of classifying the hydantoin-hydrolyzing enzymes is based on their selectivity thus the enzymes are separated into D-, L- and non-selective enzymes. However, in some cases, the selectivity of the hydantoin-hydrolyzing enzymes can be substrate dependent as is the case for the hydantoinase from *A. aurescens* DSM 3745 (May *et al.* 1998a). Also, the natural substrate selectivity of the enzyme is not always defined and can also be altered. May *et al.* (2000) used directed evolution to modify the natural preference of the non-selective hydantoinase of *A. aurescens* DSM 3747 from D-5-(2-methylthioethyl)hydantoin to the L-enantiomer while simultaneously increasing the activity five-fold.

Thus the present criteria used to classify the hydantoin-hydrolysing enzymes are not adequate. While it would be possible to use the natural functions of the enzymes to

group them, the true roles of all the enzymes that have been identified as able to hydrolyze hydantoin are not known. Only two hydantoinases have naturally occurring hydantoins as substrates: the carboxymethylhydantoinase and the carboxyethylhydantoinase (Syldatk *et al.* 1990a). The dihydropyrimidinase and β-ureidopropionase enzymes naturally catalyse the hydrolysis of pyrimidines to L-amino acids via an *N*-carbamyl-β-amino acid intermediate (Kao and Hsu 2003). The enzymes involved in pyrimidine catabolism are widely distributed in all organisms (Syldatk *et al.* 1990a). Yamada *et al.* (1978) identified D-specific hydantoin hydrolysis in all bacteria, actinomycetes, yeasts and moulds tested. Eadie *et al.* (1949) similarly identified D-hydantoinase activity in plant and mammalian extracts. With respect to the other hydantoin-hydrolyzing enzymes, Syldatk *et al.* (1999) postulated that they had an ancient function in the 'prebiotic conditions of the primitive earth' where hydantoins may have been in abundance.

1.2.4 Evolution of the hydantoin-hydrolyzing enzyme sytems

An alternative approach to the classification of the hydantoin-hydrolysing enzymes, to better represent the extraordinary diversity, may be in the evolutionary relationships of the hydantoin-hyrolysing enzymes to related enzyme groups. Holm and Sander (1997) used sequence similarity within the catalytic fold to hypothesize that the hydantoinases belong to a superfamily of cyclic amidohydrolases. The members of this superfamily form five different groups including L-hydantoinases (Group A), collapsing response mediator receptor proteins (CRMP) (Group B), dihydropyrimidinases (Group B), allantoinases (Group C), ureases (Group D) and dihydroorotases (Group E). May *et al.* (1998b) performed a phylogenetic analysis that showed that the L-hydantoinase from *A. aurescens* DSM 3745 formed its own cluster with an uncharacterized L-hydantoinase homolog from *Escherichia coli* with an amino acid sequence identity of 38% (Group A). Groups B, C, D and E showed 30%, 24%, 15% and 20% amino acid sequence identity with the L-hydantoinase respectively (May *et al.* 1998b). Unexpectedly, the *N*-methyhydantoinase enzyme and the non-selective hydantoinase from *Pseudomonas* spp. appeared to form a separate group of enzymes and did not belong to this superfamily of cyclic amidases (May *et al.* 1998b, Syldatk *et al.* 1999).

The low sequence similarity between the hydantoinases led May *et al.* (1998b) and Syldatk *et al.* (1999) to propose that the hydantoinase enzymes that belong to this

superfamily are all members of an ancient protein family and that they evolved from a common ancestor by divergent evolution. Taillades *et al.* (1998) reported that hydantoins and *N*-carbamyl-α-amino acids were more likely to be precursors for prebiotic peptides in the primordial soup than free α-amino acids. If this is the case, then the proposal that the hydantoinase enzymes may belong to a very old protein group is reasonable.

The hydantoin-hydrolyzing enzymes exhibit differences in the conditions under which they function and in their substrate selectivities, which reflects the diversity within the hydantoin-hydrolyzing enzyme groups (Ogawa and Shimizu 1997). However the secondary structures of the different hydantoin-hydrolyzing enzyme groups are similar (Holm and Sander 1997). Kim and Kim (1998) postulate that the minor insertions and deletions in the nucleotide sequence cause changes in the amino acid residues. These in turn lead to major changes in the biocatalytic behaviour of the enzyme (for example in substrate specificity) while maintaining the overall secondary structure of the proteins which are used to classify evolutionary relationships.

With respect to the enzymes involved in *N*-carbamyl amino acid hydrolysis, the D-NCAAH and β-ureidopropionase enzymes have been classified as members of the nitrilase superfamily which also includes nitrilases, aliphatic amidases and a β-alanine synthase (Pace and Brenner 2001, Nakai *et al.* 2000). All the members of the nitrilase superfamily have a conserved catalytic triad of glutamate, lysine and cysteine residues, including the D-NCAAH and β-ureidopropionase enzymes (Wang *et al.* 2001, Pace and Brenner 2001). The enzymes in this superfamily have diverse biological roles and only Branch 1, the nitrilases, actually hydrolyze nitriles. The other enzymes in this superfamily exhibit amidase activity with highly diverse substrate specificities (Brenner 2002). Unfortunately, nothing about the evolutionary relationships of the *N*-carbamylsarcosine amidohydrolases or the L-NCAAH has been published to date. This lack of information leaves a distinct gap in the knowledge about the enzymes involved in *N*-carbamyl amino acid hydrolysis.

1.3 GENETIC ORGANIZATION OF THE HYDANTOIN-HYDROLYZING ENZYME SYSTEMS

The genes encoding the enzymes required for hydantoin hydrolysis appear to be clustered on relatively large sections of DNA. The *hyu* clusters identified to date belong to the strains *Pseudomonas* sp. NS 671, *A. aurescens* DSM 3747, *A. tumefaciens* NRRL B11291 and *Agrobacterium* sp. IP I-671 (Watabe *et al.* 1992a, Wiese *et al.* 2001, Grifantini *et al.* 1998, Hils *et al.* 2001). In addition, Matcher *et al.* (2004) identified a gene cluster in *Pseudomonas putida* RU-KM3$_s$ encoding dihydropyrimidinase and β-ureidopropionase enzymes that were responsible for the hydantoin-hydrolyzing enzyme activity observed in the strain.

Most *hyu* gene clusters have been isolated by screening genomic DNA libraries. In the case of *A. aurescens* DSM 3747, *A. tumefaciens* NRRL B11291 and *Agrobacterium* sp. IP I-671 recombinant plasmids were screened with radioactive or DIG-labelled DNA probes derived from the nucleotide sequence of previously elucidated hydantoinase or NCAAH genes (Wiese *et al.* 2001, Grifantini *et al.* 1998, Hils *et al.* 2001). The native plasmid that encoded the *Pseudomonas* sp. NS 671 *hyu* gene cluster was identified by selecting *Escherichia coli* cells that contained recombinant plasmids able to hydrolyze 5-(2-methylthioethyl)hydantoin as the sole nitrogen source in a growth medium (Watabe *et al.* 1992a). The *hyu* gene cluster on the *Pseudomonas putida* RU-KM3$_s$ genome was identified by insertional inactivation of the *hyu* genes in *P. putida* RU-KM3$_s$ cells using a plasposon (Matcher *et al.* 2004)

Unfortunately, neither Grifantini *et al.* (1998) nor Watabe *et al.* (1992a) specified the exact number of *hyu* gene encoding plasmids isolated from genomic DNA libraries. However, for the *hyu* gene clusters isolated by screening genomic DNA libraries, only one of seven thousand *Agrobacterium* sp. IP I-671 genomic DNA-containing phages tested contained the *hyu* gene cluster (Hils *et al.* 2001). Similarly, only two plasmids encoding the *hyu* genes were isolated from a library of *A. aurescens* DSM 3747 genomic DNA-containing plasmids (Wiese *et al.* 2001). This suggests that the entire *hyu* operon was not simple to isolate. This may a due to the size of the *hyu* gene cluster or due to the toxicity of the *hyu* gene products when at a high copy number in cloning vectors.

1.3.1 L-specific hyu gene clusters

A. aurescens DSM 3747 was isolated by Gross *et al.* (1987) for its ability to produce L-amino acids from D, L-5-monosubstituted hydantoins. While the organization of the *hyu* genes was reported fourteen years later by Wiese *et al.* (2001), the location of the *hyu* cluster has not been specified. It is interesting to speculate whether the *A. aurescens* DSM 3747 L-selective *hyu* genes are present on a plasmid, as reported for the L-selective *hyu* system from *Pseudomonas* sp. NS 671 (Watabe *et al.* 1992a), or whether this is the first L-selective *hyu* cluster identified on a bacterial chromosome.

The *A. aurescens* DSM 3747 *hyu* gene cluster, present on an 8.7 kb DNA fragment, encodes 8 open reading frames (ORFs). The operon consists of a putative transport protein (*hyuP*), followed by an hydantoin racemase (*hyuA*), an hydantoinase (*hyuH*) and an NCAAH (*hyuC*) all in the same orientation (Figure 1-3). Upstream of *hyuP,* and in the opposite orientation, is a putative cytochrome P450 monooxygenase (ORF 1), a putative transport protein (ORF 2) and a putative ferrodoxin (ORF 3); all in the same direction. Downstream of *hyuC* and in the opposite orientation is ORF 8, which encodes a putative purine nucleotide synthesis repressor PurR (Wiese *et al.* 2001). The protein PurR was identified as a member of the LacI / GalR family of transcriptional regulators by Wiese *et al.* (2001). However, none of these ORFs appear to be linked to the pathways involved in the hydrolysis of hydantoins (Wiese *et al.* 2001). Using RT-PCR and Northern blot analysis, Wiese *et al.* (2001) confirmed that the *A. aurescens* DSM 3747 *hyuPAHC* genes were cotranscribed from a σ^{54}-dependent promoter upstream of the *hyu* operon. Since transcription of the *hyu* operon occurred at detectable levels only in the presence of the inducer, D, L-5-(3-indolylmethyl)-3-*N*-methylhydantoin, this suggested that induction may be an important mechanism of *hyu* regulation in strain DSM 3747 (Syldatk *et al.* 1990b, Wiese *et al.* 2001).

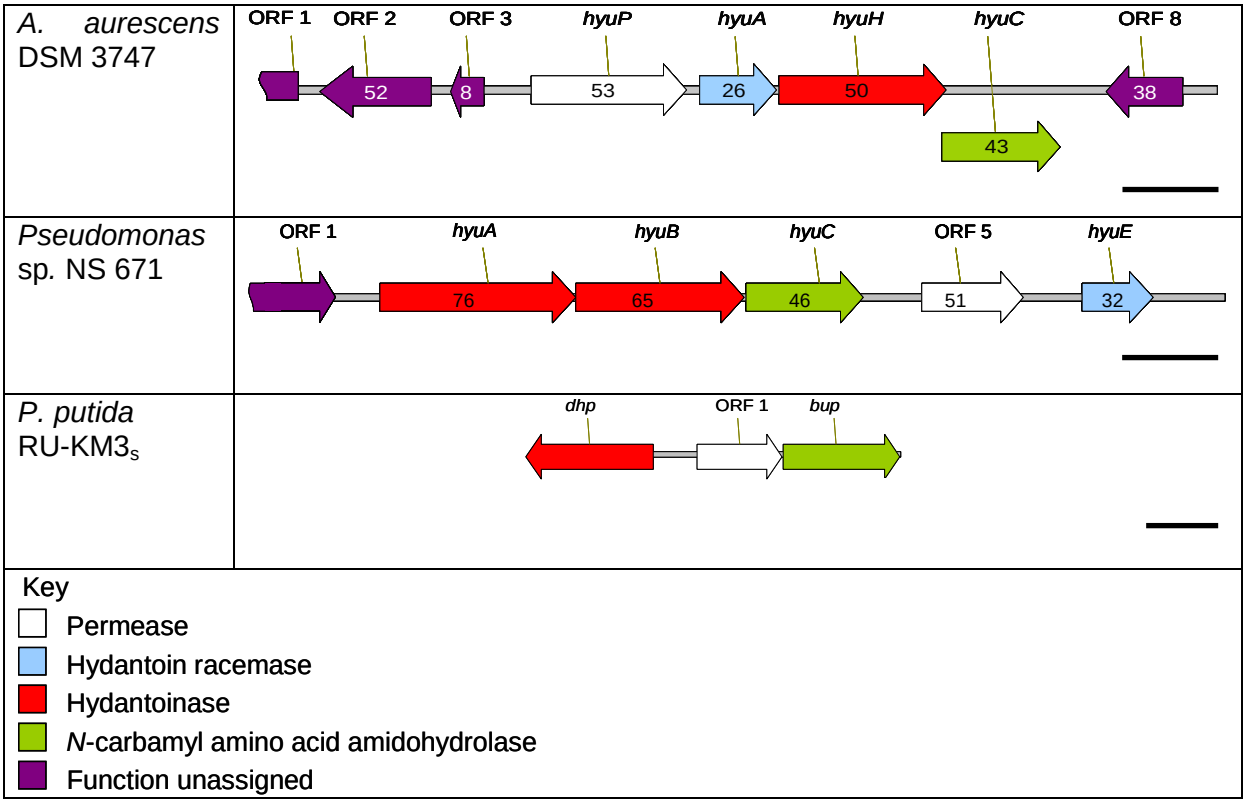


Figure 1-3: A schematic representation of the L-specific *hyu* gene clusters from *A. aurescens* DSM 3747, *Pseudomonas* sp. NS 671 and *P. putida* RU-KM3$_S$. The genes and the orientations are represented by arrows. The numbers within the arrows indicate molecular mass (in kDa) of the respective protein where available. Scale bars correspond to 1000 bp. Figure adapted from Wiese *et al*. 2001, Watabe *et al*. 1992a, Matcher 2004.

The *hyu* gene cluster from *Pseudomonas* sp. NS 671, another L-specific hydantoin-hydrolyzing bacterial strain, was isolated from a 172 kb plasmid pHN671 (Watabe *et al.* 1992a). The NS 671 *hyu* operon encodes two hydantoinases, a D-hydantoinase (*hyuA*) and an L-hydantoinase (*hyuB*) (Figure 1-3). The NCAAH gene, designated *hyuC* follows the *hyuA* and *hyuB* genes. Upstream of *hyuA* and ORF 5 are sequences similar to the -35 and -10 sequences for σ^{70}-dependent promoters in *E. coli.* In addition, overlapping the σ^{70}-dependent promoter sequences, are other sequences similar to the consensus sequence for *E. coli* σ^{54}-dependent promoters. Downstream of *hyuC* are two palindromic sequences followed by a stretch of Ts suggesting the presence of a ρ-independent transcriptional terminator (Watabe *et al.* 1992a). Interestingly, the HyuA and HyuB proteins of *Pseudomonas* sp. NS 671 bear significant identity at the amino acid level to the AgaF (41%) and AgaG (47%) proteins from *A. tumefaciens* plasmid pTi15955. The catabolic enzymes AgaF and AgaG are believed to be involved in the conversion of

agropinic acid to mannopinic acid, which are structurally similar to substituted hydantoins (Lyi *et al.* 1999). Hils *et al.* (2001) suggested a potential role for the Hyu enzymes in the hydrolysis of secondary plant metabolites. Downstream of the NS 671 *hyuC* is a putative permease (ORF 5) and an hydantoin racemase (*hyuA*) (Watabe *et al.* 1992a).

The clustering of the *hyu* genes in *A. aurescens* DSM 3747 and *Pseudomonas* sp. NS 671 suggests that they form *hyu* operons. Like the *hyu* gene cluster in *A. aurescens* DSM 3747, all the genes in the *Pseudomonas* sp. NS 671 are in the same orientation. The genes present in both *hyu* gene clusters are similar, both *A. aurescens* DSM 3747 and *Pseudomonas* sp. NS 671 *hyu* gene clusters encode an hydantoinase, an NCAAH, a permease and a racemase; however the order of the genes in the *hyu* gene clusters differs (Figure 1-3) (Wiese *et al.* 2001, Watabe *et al.* 1992a). While there appears to be some similarity between the amino acid sequences of the NCAAH, the permease and the hydantoin racemase enzymes of *A. aurescens* DSM 3747 and *Pseudomonas* sp. NS 671, the hydantoinase enzymes are significantly different. The L-selective hydantoinase enzyme of *A. aurescens* DSM 3747 is encoded by a single *hyuH* gene whereas the L-selective hydantoinase of *Pseudomonas* sp. NS 671 is encoded by two *hyuH* genes. The significant differences in the amino acid sequence of the two hydantoinase enzymes from *A. aurescens* DSM 3747 and *Pseudomonas* sp. NS 671 would explain the differences in substrate selectivity in the two strains (Watabe *et al.* 1992a, Wiese *et al.* 2001).

Matcher *et al.* (2004) identified the genes responsible for hydantoin hydrolysis in *Pseudomonas putida* RU-KM3$_s$ as *dhp* and *bup* genes (Figure 1-3), which encode a dihydropyrimidinase and a β-ureidopropionase respectively. The *dhp* and *bup* genes are separated by ORF 1, which encodes a putative transport protein. Insertional inactivation of ORF 1 led to the concomitant loss of β-ureidopropionase activity which indicates that ORF 1 and *bup* may share a common promoter and are transcribed on a polycistronic mRNA (Matcher *et al.* 2004). The clustering of ORF 1 with the *dhp* and *bup* suggests that the enzyme product of ORF 1 is directly linked to the hydrolysis of hydantoins (Matcher 2004, Matcher *et al.* 2004). The genes involved in hydantoin hydrolysis in *P. putida* RU-KM3$_s$ are significantly different to the *hyu* gene clusters from *A. aurescens* DSM 3747 and *Pseudomonas* sp. NS 671 (Matcher *et al.*

2004, Wiese *et al.* 2001, Watabe *et al.* 1992a). While the *P. putida* RU-KM3$_s$ dihydropyrimidinase and β-ureidopropionase are able to hydrolyze 5-monosubstituted hydantoins due to a low level of substrate selectivity, their main function resides in pyrimidine hydrolysis (Matcher 2004). As integral enzymes in pyrimidine hydrolysis, the dihydropyrimidinase and β-ureidopropionase enzymes are ubiquitous in bacteria. The different substrate selectivities of the RU-KM3$_s$ dihydropyrimidinase and β-ureidopropionase enzymes would also explain why these enzymes have not been isolated for hydantoin hydrolysis previously from other *P. putida* strains (Matcher 2004).

1.3.2 D-specific hyu gene clusters

Grifantini *et al*. (1998) first described the isolation of an *hyu* gene cluster from *A. tumefaciens* NRRL B11291. This *hyu* gene cluster is located on a plasmid 190 kb in size and is comprised of divergent *hyuH* and *hyuC* genes, which encode D-hydantoinase and D-NCAAH enzymes respectively (Figure 1-4) (Hils *et al*. 2001, Grifantini *et al*. 1998).

Like *A. tumefaciens* NRRL B11291, the *Agrobacterium* sp. IP I-671 *hyu* gene cluster is also located on an indigenous plasmid 160 kb in size. In this *hyu* gene cluster, the *hyuH* and *hyuC* genes, encoding D-hydantoinase and D-NCAAH enzymes respectively, are arranged divergently (Figure 1-4). Downstream of *hyuC* is *hyuD*, which encodes a D-amino acid dehydrogenase and *hyuA*, which encodes an hydantoin racemase (Hils *et al.* 2001). While the *hyuA* gene isolated from *Agrobacterium* sp. IP I-671 was the first to be reported for a D-specific hydantoin-hydrolyzing system, two further *hyuA* genes (*AtHyuA1* and *AtHyuA2*) from *A. tumefaciens* C58 have been reported (Hils *et al*. 2001, Las Heras-Vazquez *et al.* 2003, Martinez-Rodriguez *et al.* 2004). The two *A. tumefaciens* C58 hydantoin racemase enzymes, AtHyuA1 and AtHyuA2 share the highest amino acid sequence identity (50%). However the hydantoin racemases from *Agrobacterium* sp. IP I-671, *Pseudomonas* sp. NS 671 and *A. aurescens* DSM 3747 are 47%, 46% and 43% identical respectively to AtHyuA2 at the amino acid sequence level therefore indicating similar levels of sequence identity in the hydantoin racemase enzymes from the genera *Agrobacterium, Pseudomonas* and *Arthrobacter* (Martinez-Rodriguez *et al.* 2004).

Downstream and in the same orientation as the *Agrobacterium* sp. IP I-671 *hyuH* is *hyuN*, which encodes a putative NAD(P)H flavin oxidoreductase. No genes encoding putative permeases were identified on the *hyu* gene cluster from *Agrobacterium* sp. IP I-671 (Hils *et al.* 2001). The *hyu* gene cluster in *Agrobacterium* sp. IP I-671 is flanked by two partial ORFs that show similarity to genes involved in transposition (Figure 1-4); ORF 1 is 37% identical at the amino acid level to a transposase (*E. coli* Tn21) and ORF 7 is 72% identical at the amino acid level to a resolvase (*Pseudomonas pseudoalcaligenes* Tn5501) (Hils *et al.* 2001). While Hils *et al.* (2001) suggest that it is unlikely that ORFs 1 and 2 are part of the *hyu* gene cluster; they hypothesize that ORFs 1 and 2 may be part of a transposon that carries the *hyu* genes.

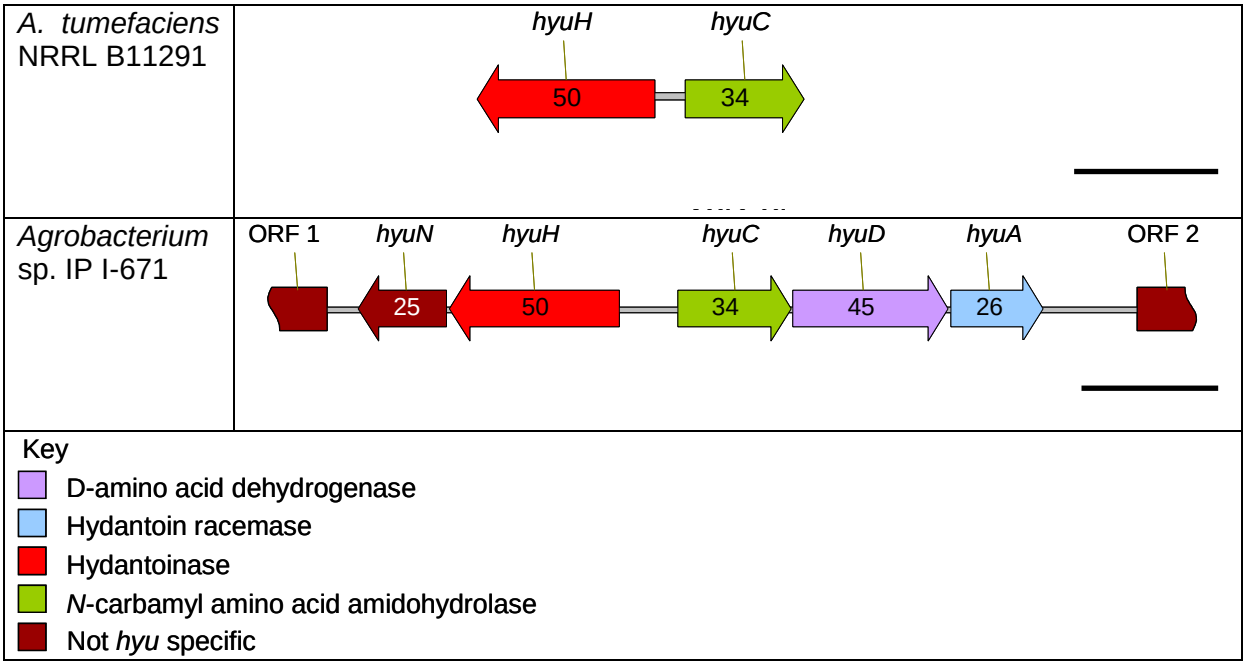


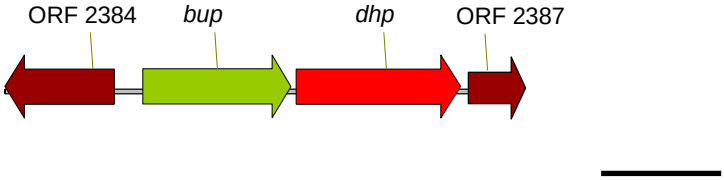
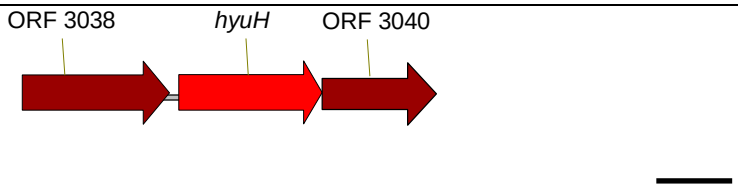
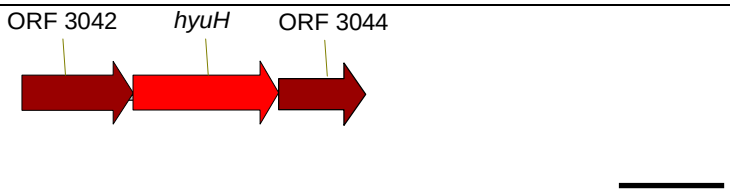
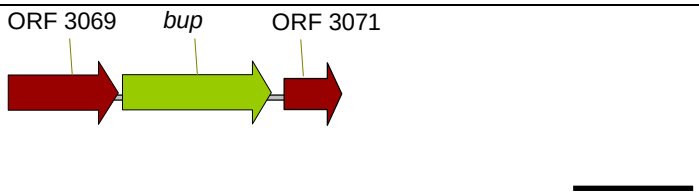
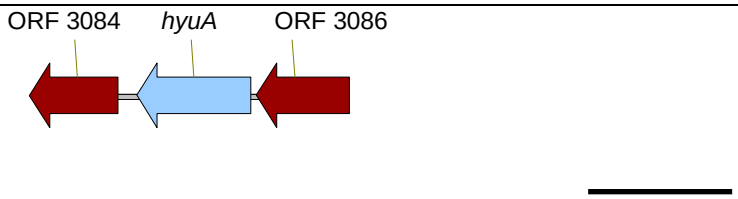
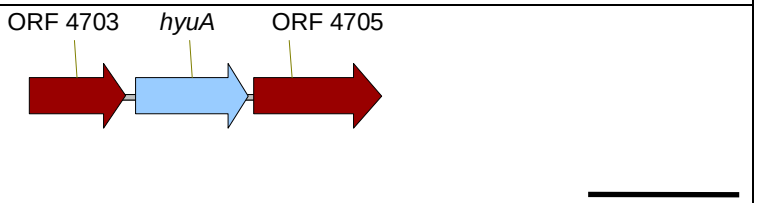
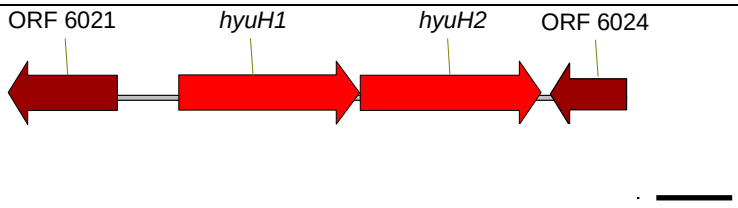
Figure 1-4: Organisation of the D-specific *hyu* gene clusters from *A. tumefaciens* NRRL B11291 and *Agrobacterium* sp. IP I-671. The genes and the orientations are represented by arrows. The numbers within the arrows indicate molecular mass (in kDa) of the respective protein where available. Scale bars correspond to 1000 bp. Figure adapted from Wiese *et al*. 2001, Hils *et al*. 2001.

While both the *Agrobacterium hyu* clusters have the *hyuH* and the *hyuC* genes arranged divergently, the length of the intergenic region between the *hyuH* and the *hyuC* genes varies from 470 nt in *Agrobacterium* sp. IP I-671 to 214 nt in *A. tumefaciens* NRRL B11291 (Hils *et al*. 2001). Grifantini *et al*. (1998) proposed that the *hyuH* and the *hyuC* genes in *A. tumefaciens* NRRL B11291 were

transcribed by overlapping promoters in opposite directions however no supporting data has been published as yet.

Insertional inactivation of the *hyuC* gene in *Agrobacterium* sp. IP I-671 led to the complete loss of NCAAH activity indicating that there was only one NCAAH-encoding *hyuC* gene in the strain. However insertional inactivation of the *hyuH* gene led to a decrease in hydantoinase enzyme activity but not a complete loss of activity. This suggested that a second hydantoinase-encoding *hyuH* gene was present elsewhere on the *Agrobacterium* sp. IP I-671 genome (Hils *et al*. 2001).

A bioinformatic analysis of the chromosomes and plasmids that form the *A. tumefaciens* C58 genome shows the presence of *bup* and *dhp* genes on the circular chromosome (Figure 1-5), which is not unexpected as the genes are essential for pyrimidine hydrolysis. Interestingly, the linear chromosome encodes genes for an *N*-methylhydantoinase, a hydantoinase, a second β-ureidopropionase and two hydantoin racemases (http://cancer.lbi.ic.unicamp.br/agroC58/). Isolation and biochemical characterization of the two hydantoin racemase enzymes identified on the *A. tumefaciens* C58 genome was reported by Las Heras-Vazquez *et al.* (2003) and Martinez-Rodriguez *et al.* (2004). However, none of the other genes identified in strain *A. tumefaciens* C58 have been studied. Also, all the genes identified existed alone on the chromosome and none of the genes form part of a cluster of genes involved in the hydrolysis of hydantoins as observed previously in *A. aurescens* DSM 3747, *Pseudomonas* sp. NS 671 and *Agrobacterium* sp. IP I-671 (Wiese *et al.* 2001, Watabe *et al.* 1992a, Hils *et al*. 2001). Interestingly, the pTi plasmid present in the strain *A. tumefaciens* C58 encodes two *hyuH* genes, *hyuH1* and *hyuH2,* representing two hydantoinase enzymes (http://cancer.lbi.ic.unicamp.br/agroC58/). The *hyuH1* and *hyuH2* genes in *A. tumefaciens* C58 are arranged in the same way as the *hyuA* and *hyuB* genes identified by Watabe *et al.* (1992a) on pHN671 in *Pseudomonas* sp. NS 671 (Figure 1-5). Additionally, the *hyuH1* and the *hyuH2* genes from *A. tumefaciens* C58 both share significant amino acid similarity to *hyuA* (30%) and *hyuB* (25%) from *Pseudomonas* sp. NS 671. Strangely, while a number of amidohydrolases genes were identified on the genome, no genes encoding NCAAH enzymes have been

Chromosome	Enzymes	Schematic map
Circular chromosome	β-ureidopropionase and Dihydropyrimidinase	
Linear chromosome	*N*-methylhydantoinase	
	Hydantoinase	
	β-ureidopropionase	
	Hydantoin racemase	
	Hydantoin racemase	
pTi plasmid	Hydantoinase A and Hydantoinase B	

Key

- Hydantoinase
- *N*-carbamyl amino acid amidohydrolase
- Hydantoin racemase
- Not *hyu* specific

Figure 1-5: A depiction of the *hyu* genes identified on the *A. tumefaciens* C58 genome and their location. The genes and the orientations are represented by arrows. Scale bars correspond to 1000 bp. Adapted from the information available at (http://cancer.lbi.ic.unicamp.br/agroC58/).

annotated (http://cancer.lbi.ic.unicamp.br/agroC58/). As the annotation of genomic sequence data was based primarily on the similarity of the sequence to known sequences in the database, the classification of genes and proteins may be inaccurate and inconsistent (Brenner 2002). Thus by inference the predicted biochemical and cellular function of the proteins may be erroneous. This implies that the putative *hyu* genes identified on the *A. tumefaciens* C58 genome require biochemical validation to be considered involved in hydantoin hydrolysis.

1.4 REGULATION OF THE HYDANTOIN-HYDROLYZING ENZYME SYSTEMS

To survive the rapid changes in their environment, microbes regulate the expression of genes coding for their enzymes and transport proteins required for growth in the new environment (Magasanik 2000). A number of regulation mechanisms exist to control hydantoin-hydrolyzing enzyme expression in bacterial strains including substrate induction, feedback regulation and nutritional regulation. In particular, the different carbon and nitrogen sources present in the growth medium of the bacterial cells have significant effects on the levels of enzyme activity produced (Sanchez and Demain 2002, Syldatk *et al.* 1992, Yamashiro *et al.* 1988, Nishida *et al.* 1987).

1.4.1 Induction

While the hydantoinase enzymes from *A. tumefaciens* NRRL B11291, *Bacillus* sp. AR9, *Pseudomonas* sp. KBEL 101, *Pseudomonas* sp. M-1, *Pseudomonas fluorescens* DSM 84 and *P. putida* are produced in the absence of inducers, most hydantoin-hydrolyzing enzymes have been reported to be subject to induction by hydantoin and hydantoin analogues (Table 1-3) (Deepa *et al*. 1993, Sharma and Vohra 1997, Kim and Kim 1993, Sudge *et al*. 1998, Morin *et al.* 1986, Morin *et al*. 1995, Syldatk *et al.* 1992). The diversity of the structures capable of inducing the hydantoinase and NCAAH enzymes demonstrates the variety in the hydantoin-hydrolyzing enzyme systems (Table 1-3) (Syldatk *et al.* 1992). The different inducers also have variable effects on the hydantoinase and NCAAH enzymes as observed in *A. crystallopoietes* AM2, which result in the production of different ratios of D-*N*-carbamyl amino acid: D-amino acid from various hydantoin substrates (Moller *et al.* 1988). Two main types of inducers are used for the induction of hydantoin-hydrolyzing enzymes: metabolizable and non-metabolizable inducers.

Metabolisable inducers such as hydantoin or D, L-5-(3-indolylmethyl)hydantoin are utilized by the cells and require replenishment throughout the bacterial fermentation whereas non-metabolizable inducers like 2-thiouracil and D, L-5-(3-indolylmethyl)-3-*N*-methylhydantoin can be added at low concentrations and last for the length of the fermentation (Syldatk *et al.* 1990b, Hartley *et al.* 1998).

Table 1-3: Inducers of hydantoin-hydrolyzing enzymes

Strain	Inducer	Enzymes affected	Reference
Agrobacterium sp. IP I-671	D, L-5-Methylthioethylhydantoin * 2-Thiouracil	D-Hyd D-NCAAH	Meyer and Runser 1993
A. tumefaciens 47C	Hydantoin D, L-5-Methylhydantoin	D-Hyd	Durham and Weber 1995
A. tumefaciens RU-OR	Hydantoin * 2-Thiouracil	D-Hyd D-NCAAH	Hartley *et al*. 1998
Arthrobacter sp. BH20	D-, L- and D, L-Indolylmethylhydantoin	Non-Hyd	Syldatk *et al*. 1987
A. crystallopoietes AM2	Hydantoin D, L-5-hydroxymethylhydantoin	D-Hyd D-NCAAH	Moller *et al*. 1988
A. aurescens DSM 3747	D, L-5-(3-indolylmethyl)hydantoin * D, L-5-(3-indolylmethyl)-3-*N*-methylhydantoin	Non-Hyd L-NCAAH	Syldatk *et al.* 1990b Wiese *et al*. 2001
B. brevis AJ-12299	5-Isopropylhydantoin	L-Hyd	Yamashiro *et al.* 1988
Pseudomonas sp. AJ-11220	D, L-5-Methylthioethylhydantoin D, L-5-Cyanoethylhydantoin	D-Hyd D-NCAAH	Yokozeki *et al*. 1987b
P. putida DSM 84	D, L-5-methylhydantoin * Thymine	D-Hyd	Morin *et al*. 1995
P. putida RU-KM1	Hydantoin	D-Hyd D-NCAAH	Burton *et al*. 1998
P. putida RU- $KM3_S$	Hydantoin	Non-Hyd L-NCAAH	Burton *et al*. 1998
P. putida RU-$KM3_L$	Hydantoin	L / Non-Hyd L-NCAAH	Burton *et al*. 1998

*- non-metabolizable inducer. Abbreviations: D- D-selective, Hyd- Hydantoinase, L- L-selective, NCAAH- *N*-carbamyl amino acid amidohydrolase and Non-non-selective.

Induction of the hydantoin-hydrolyzing enzymes in *A. tumefaciens* RU-OR by hydantoin led to a five-fold increase in both the hydantoinase and NCAAH activity (Hartley *et al.* 1998). Interestingly, when the non-metabolizable compound 2-thiouracil was used as an inducer of the hydantoin-hydrolyzing enzymes in *A. tumefaciens* RU-OR, the hydantoinase activity increased five-fold but the NCAAH activity showed a ten-fold increase in activity (Hartley *et al.* 2001). Similarly, the presence of the metabolizable inducer D, L-5-(3-indolylmethyl)hydantoin or the non-metabolizable inducer D, L-5-(3-indolylmethyl)-3-*N*-methylhydantoin in the growth medium of *A. aurescens* DSM 3747 cells led to a six-fold or a thirty-fold

increase in hydantoinase enzyme activity respectively (Syldatk *et al.* 1990b, Wiese *et al.* 2001). RT-PCR reactions and Northern blots on RNA extracted from *A. aurescens* DSM 3747 cells grown in the presence and the absence of D, L-5-(3-indolylmethyl)-3-*N*-methylhydantoin, demonstrated that induction of the *A. aurescens* DSM 3747 hydantoin-hydrolyzing enzymes is at the level of transcription (Wiese *et al.* 2001). Similarly, analysis of the hydantoin-hydrolyzing enzyme activity in *A. tumefaciens* RU-OR cells, grown in the presence and absence of the transcriptional inhibitor rifamipicin, showed that expression of the hydantoin-hydrolyzing enzymes was controlled at the transcriptional level (Hartley 2001). Unfortunately, the level at which induction is controlled in the other strains has not been elucidated. Also, the mechanisms by which induction occur have yet to be studied.

Generally, the non-metabolisable inducers like D, L-5-(3-indolylmethyl)-3-*N*-methylhydantoin and 2-thiouracil are toxic to bacterial cells at high concentrations (Syldatk *et al.* 1990b, Meyer and Runser 1993). Thus low concentrations of the compounds are added during bacterial fermentations. The lower concentrations of inducer used in the production of the hydantoin-hydrolyzing enzymes reduces the overall cost of the fermentation which has a positive effect on industrial processes (Morin *et al.* 1995, Meyer and Runser 1993, Syldatk *et al.* 1990b).

Inducer-independent mutant strains have been isolated in several genera. *Arthrobacter* sp. DSM 7330 was subjected to repeated cycles of growth on unique carbon and nitrogen sources in an attempt to isolate strains with higher levels of inducer-independent hydantoin-hydrolyzing enzyme activity (constitutive mutants). While most of the carbon-nitrogen combinations produced mutant strains, only the permutation of glucose and *N*-carbamoyl-L-methionine produced the desired mutant. The mutant strain selected for further analysis, DSM 9771, exhibited a three-fold increase in the hydantoinase levels when compared to the induced wild-type strain DSM 7330 (Wagner *et al.* 1996).

A. tumefaciens RU-OR was exposed to the chemical mutagen ethylmethane sulphonate (EMS) and constitutive mutant strains with elevated levels of hydantoinase activity were selected (Hartley *et al.* 1998). Mutants were selected for

the ability to grow in the absence of an inducer and in the presence of 5-fluorouracil (5-FU), a toxic hydantoin analogue. Inducer-independent expression of the hydantoinase would convert 5-FU to non-toxic 5-fluoroureidopropionic acid (Hartley *et al.* 1998). Two mutant strains were isolated: RU-ORLB3 which showed constitutive expression of the hydantoinase but the NCAAH enzyme was still inducible and RU-ORL5 which showed constitutive expression of both hydantoinase and NCAAH enzymes. The variation between the mutants RU-ORL5 and RU-ORLB3 led Hartley *et al.* (2001) to propose that while the induction of the hydantoinase and NCAAH may be controlled by the same factor, the induction of hydantoinase and NCAAH expression is independent of each other.

1.4.2 Feedback regulation

Feedback inhibition, a mechanism by which the cell controls the activity of enzymes already present within the cytosol, has been shown to play an integral role in the regulation of the hydantoin-hydrolyzing enzymes (Sanchez and Demain 2002, Olivieri *et al.* 1981). In both *Agrobacterium* sp. IP I-671 and in *A. tumefaciens* NRRL B11291 cells, the NCAAH enzyme activity was subject to feedback inhibition by ammonia however the hydantoinase enzyme activity was not affected (Runser *et al.* 1990, Olivieri *et al.* 1981). Similarly, the enzyme activity of the *Agrobacterium* sp. KNK 712 NCAAH enzyme expressed in *E. coli* cells was inhibited in the presence of ammonia (Nanba *et al.* 1998). The inhibitive effect observed was due to the presence of ammonia, the product of the NCAAH enzyme activity, which acted as a non-competitive inhibitor of the enzyme (Runser *et al.* 1990, Lee *et al.* 2001). The removal of the ammonia using specific adsorbents reversed the inhibitory effect, resulting in an increase in the NCAAH enzyme activity from 20% to 98%. Thus the mechanism of feedback inhibition on the NCAAH enzyme regulates the production of ammonia preventing its accumulation in the cell (Kim and Kim 1994).

1.4.3 Catabolite repression

Many studies have reported that bacteria produce the hydantoin-hydrolyzing enzymes after they have reached late logarithmic to early stationary growth phase. For example, *Agrobacterium* sp. IP I-672, *A. tumefaciens* NRRL B11291, *A. tumefaciens* RU-OR, *A. crystallopoietes* AM2, *B. circulans, Pseudomonas* sp. M1 all produce the highest levels of enzyme activity after exponential growth phase

(Runser *et al.* 1990, Deepa *et al.* 1993, Hartley *et al.* 1998, Moller *et al*. 1988, Luksa *et al.* 1997, Sudge *et al.* 1998), implying that catabolite repression plays a role in the regulation of expression of the hydantoin-hydrolyzing enzymes. This sequential utilization of nutrient sources during catabolite repression serves to catabolize the 'good' nutrient source; which most rapidly supplies carbon or nitrogen and energy for growth; before the 'poor' sources (Diaz and Prieto 2000, Sanchez and Demain 2002). The production of the hydantoin-hydrolyzing enzymes has been shown to be sensitive to carbon and nitrogen sources.

The L-tryptophan forming activity in *Flavobacterium* sp. I-3 was affected by both the carbon and the nitrogen source with the highest activity observed with 1% dextrin as a carbon source and 0.4% ammonium chloride as a nitrogen source (Nishida *et al.* 1987). In *B. brevis* AJ-12299, the hydantoin-hydrolyzing enzymes were affected by the carbon sources present in the medium, and cells grown in medium containing 2% xylose exhibited the highest levels enzyme activity (Yamashiro *et al.* 1988). Interestingly, some researchers report that excess levels of glucose or glycerol in the medium led to the detection of lower levels of hydantoin-hydrolyzing enzyme activities. This has been observed in *B. stearothermophilus* SD-1, *Pseudomonas* sp. NCIM 5109, *P. putida* DSM 84 and *P. putida* RU-KM3$_s$ (Lee *et al.* 1997, Sudge *et al*. 1998, Morin *et al.* 1995, Matcher *et al.* 2004). In the case of *P. putida* RU-KM3$_s$, the addition of glucose or succinate to the growth medium led to a four-fold and seven-fold decrease in the dihydropyrimidinase and β-ureidopropionase activities respectively (Matcher *et al.* 2004).

The hydantoin-hydrolyzing activity produced by *A. aurescens* DSM 3747 and *A. tumefaciens* RU-OR cells was affected by the nitrogen source used in the growth medium. The best activity was observed when 0.65% ammonium sulphate and 0.01% casamino acids were used as nitrogen sources in the growth medium of *A. aurescens* DSM 3747 and *A. tumefaciens* RU-OR cells respectively (Syldatk *et al.* 1990b, Hartley *et al.* 2001). Hartley *et al.* (2001) observed two levels of nitrogen catabolite repression (NCR) on the hydantoin-hydrolyzing enzymes of *A. tumefaciens* RU-OR: first at the level of transcription and second, at the post-translational level. In the case of post-translational regulation (termed the ammonia shock effect) induced cells with hydantoinase activity showed a dramatic

decrease in the hydantoinase, but not the NCAAH activity, when exposed to high concentrations of ammonia. However, upon removal of the ammonia the hydantoinase activity increased again to pre-ammonia shock levels (Hartley *et al.* 2001).

Hartley *et al.* (2001) went on to isolate 5-FU resistant *A. tumefaciens* RU-OR mutants with altered responses to NCR. One of the mutant strains, RU-ORPN1 showed constitutive hydantoinase and NCAAH enzyme activity under NCR growth conditions but the hydantoinase was still sensitive to ammonia shock. Further analysis of mutant strains RU-ORPN1F1 and RU-ORPN1F9 led Hartley *et al.* (2001) to propose that the ammonia shock effect was dependent upon glutamine synthetase activity in the cells. This will be discussed in more detail in Section 1.7.

Support for the role of catabolite respression in *hyu* gene expression is provided by sequence data showing the presence of putative σ^{54}-factor binding sites upstream of the *hyu* genes in *A. aurescens* DSM 3747 and *Pseudomonas* sp. NS 671 (Wiese *et al.* 2001, Watabe *et al.* 1992a). σ^{54} is involved in the activation of transcription of nitrogen-regulated promoters (Kustu *et al.* 1989). A putative σ^{54}-binding sequence was located upstream of *hyuP* and may be responsible for the activation of transcription of the *hyuPAHC* genes (Wiese *et al.* 2001). Two putative σ^{54}-binding sites were identified on the *hyu* cluster from *Pseudomonas* sp. NS 671; one upstream of *hyuA, hyuB* and *hyuC* and the second upstream of ORF 5 and *hyuE*. In addition, upstream of *hyuA* in *Pseudomonas* sp. NS 671 was ORF 1 which showed significant homology to the genes *nifA* (40 to 46% in 237 to 287 amino acids from different species), *ntrC* (42 to 44% in 248 to 264 amino acids from *Klebsiella pneumoniae* and *E. coli*), *dctD* (42% in 231 amino acids from *Rhizobium leguminosarum* and *Rhizobium meliloti*), *xylR* (40% in 229 amino acids from *Pseudomonas putida*, *tyrR* (40% in 229 amino acids from *E. coli*) and *hydG* (37% in 290 amino acids from *E. coli*). All of these proteins are transcriptional regulators (Watabe *et al.* 1992a). While the regulation of transcription in *Pseudomonas* sp. NS 671 has not been studied, the presence of the σ^{54}-binding sequences and ORF 1 suggests that ntr-dependent nitrogen catabolite repression may occur in these *hyu* clusters.

1.5 CARBON CATABOLITE REPRESSION IN GRAM-NEGATIVE BACTERIA

Dienert in 1900 first demonstrated that yeast grown in a mixture of glucose and galactose first metabolized glucose and then hydrolyzed galactose. This reseach was supplemented by Monod's 'diauxie' experiments, where the growth of bacterial cultures on two different carbon sources produced two separate growth cycles in the bacteria with a central lag period (reviewed in Ullmann 1996). This sequential utilization of carbon sources serves to catabolize the 'good' carbon source; which most rapidly supplies carbon and energy for growth; before the 'poor' carbon sources. This mechanism requires the enzymes involved in the catabolism of the 'poor' carbon sources to be repressed until the 'good' source is exhausted (Matin 1996). A regulatory mechanism termed carbon catabolite repression (CCR) describes the negative effect exerted by a preferential carbon source on the production of undesired enzymes. Thus CCR functions to organize the catabolism of the carbon sources when more than one is present in the environment (Sanchez and Demain 2002). While CCR is widely distributed in bacteria, the regulation and implementation of CCR in different bacteria varies significantly. Currently the best studied system, the *lac* system in *E. coli*, serves as the model sytem for CCR (Stulke and Hillen 1999).

1.5.1 CCR in E. coli

In *E. coli,* the transport and phosphorylation of carbohydrates by the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) requires two essential phosphotransfer proteins, Enzyme I (EI) and HPr and a series of sugar-specific permeases, known as Enzymes II (EII). EII is composed of three domains, EIIA and EIIB which are required for phosphate transfer and EIIC, which spans the membrane (Stulke and Hillen 1999). The transfer of the phosphoryl group from PEP therefore occurs via EI, HPr, $EIIA^{Glc}$ and EIIB to the respective sugar, in this case glucose (Figure 1-6). The resultant phosphorylated glucose (glucose-6-phosphate) subsequently enters the glycolysis pathway (Stulke and Hillen 1999).

In the absence of a 'good' carbon source, $EIIA^{Glc}$ remains phosphorylated, which stimulates the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) by adenylate cyclase (AC) (Warner and Lolkema 2003).

Sufficiently high levels of cAMP interact with cAMP receptor protein (CRP), generating complexes which interact directly with the promoters of catabolically repressed genes. This interaction serves to alleviate CCR and to activate transcription of genes that are subject to regulation by CCR (Stewart 1993). The central role in the mechanism of CCR in *E. coli* is played by $EIIA^{Glc}$. The level of phosphorylation of $EIIA^{Glc}$ is controlled by the amount of glucose being transported into the cell. Therefore the higher the levels of glucose available to the cell, the lower the levels of phosphorylated $EIIA^{Glc}$ in the cell (Warner and Lolkema 2003).

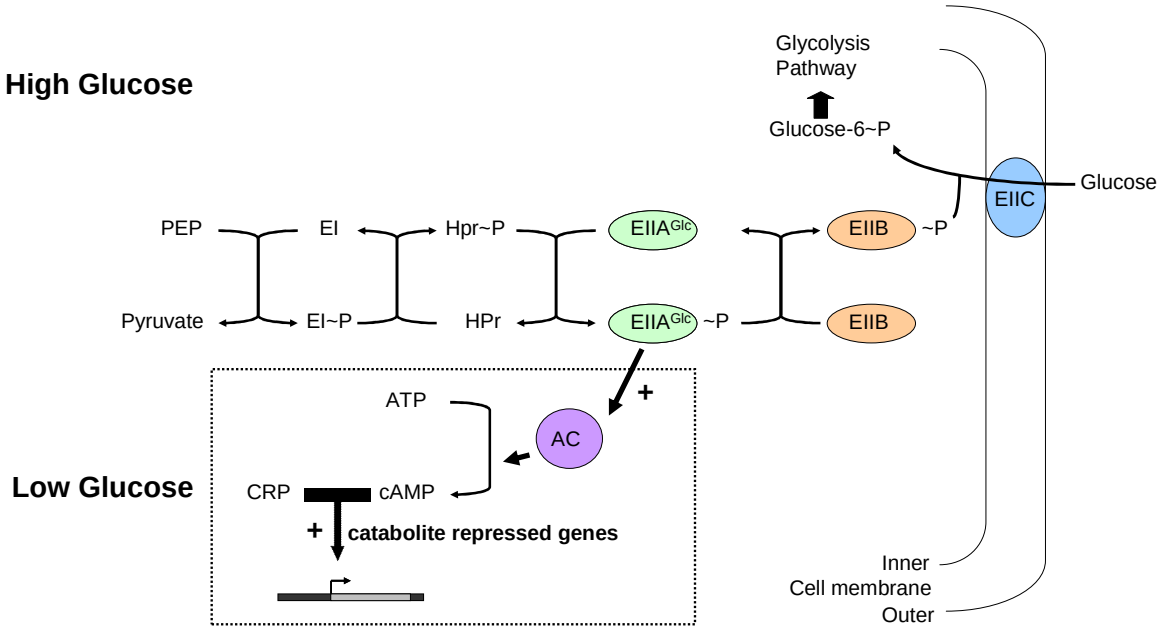


Figure 1-6: Proteins involved in the phophoenolpyruvate: phosphotranferase system which effects CCR in *E. coli*. The + indicates activation. Abbreviations: AC- adenylate cyclase, cAMP- cyclic adenosine monophosphate, ATP- adenosine triphosphate, CRP- cAMP receptor protein, E1- Enzyme I, EIIA- Enzyme II A, EIIB- Enzyme IIB, EIIC- Enzyme IIC, PEP- phosphoenol pyruvate. Figure adapted from Warner and Lolkema 2002.

In addition to the well-characterised PTS system, there are other proteins and pathways involved in the regulation of individual promoters that are sensitive to external carbon signals. In *E. coli*, the Cra (catabolite repressor / activator) protein mediates a second global catabolite control system. While initially identified as a fructose-specific repressor, its involvement in a number of the other carbohydrate catabolism pathways has been recognized (Cases and de Lorenzo 1998).

1.5.2 CCR in Pseudomonas

Catabolite repression, using CRP as an effector molecule, is not a universal occurrence. Despite the close phylogenetic relationship between *E. coli* and *Pseudomonas* spp., there are a startling number of differences in the CCR mechanisms between *E. coli* and the Pseudomonads (Cases and de Lorenzo 1998). While a CRP analog, Vfr, is present in *P. aeruginosa* it plays no role in catabolite repression. Vfr is involved in the quorum-sensing mechanisms in *P. aeruginosa* (Runyen-Janecky *et al.* 1997, Albus e*t al.* 1997). Instead, Crc, which shows no similarity to CRP, has a role in effecting catabolite repression in *P. aeruginosa.* The *crc* gene has been found in other Pseudomonads including *P. putida* and *P. fluorescens* but not in *E. coli* (Hester *et al.* 2000, MacGregor *et al.* 1996). Even the import of glucose, an almost universal inducer of catabolite repression, into the *Pseudomonas* cell is different. *P. putida* U does not hydrolyze glucose through the glycolytic pathway; instead the D-glucose is oxidized extracellularly to D-gluconic acid and then imported into the cell by an energy-dependent D-gluconic acid uptake system (Schleissner *et al.* 1997). This D-gluconic acid is phosphorylated internally and then enters the Entner-Doudoroff pathway (Sage *et al.* 1996). These modification mechanisms remove D-glucose from the environmental nutrient pool and give Pseudomonads an advantage over the other bacteria, which may be a reason for the development of these alternative pathways. The variation observed within genera and between the different genera suggests that the complete understanding of these systems requires further research. This variance between genera also cautions against extrapolating from one genus to another, despite phylogenetic relationships.

1.6 NITROGEN REGULATION IN GRAM-NEGATIVE BACTERIA

The research reported for *Agrobacterium* strains indicates that there are significant differences in the regulation of the *hyu* genes and in the expression of the Hyu enzymes between the strains. Of particular interest are the differences observed in the effect of nitrogen on the *hyu* genes and the activity of the Hyu enzymes in *Agrobacterium* sp. IP I-671, *A. tumefaciens* NRRL B11291 and *A. tumefaciens* RU-OR (Runser *et al.* 1990, Olivieri *et al.* 1981, Hartley *et al.* 2001). To better understand the mechanisms of *hyu* regulation, the different factors involved in the control of nitrogen will be reviewed in terms of their roles in the assimilation of

nitrogen and the regulation of the nitrogen levels within the cell. The interactions of these proteins will then be discussed.

1.6.1 Nitrogen assimilation in gram-negative bacteria

Bacteria can assimilate a diverse group of nitrogenous compounds as sources of nitrogen. The assimilatory pathways can be divided into two sets; those necessary for the utilization of nitrogen from extracellular compounds and those necessary for the biosynthesis of nitrogen compounds within the cell. The enzymes involved in these pathways are rigorously controlled by the intracellular nitrogen pool and by the availability of the required substrates (Tyler 1978). Organic nitrogen sources include amino acids and nucleosides, or compounds derived from them, and the profile of the nitrogenous compounds incorporated into the nitrogen pool is specific to the bacterium. For example, *E. coli* is able to hydrolyze adenine, adenosine, agmatine, L- and D-alanine, allantoin, γ-aminobutyrate, ammonia, arginine, asparagine, aspartate, cytidine, cytosine, glucosamine, glutamine, glutamate, glycine, ornithine, proline, putrescine, L- and D-serine and threonine as nitrogen sources. However, as ammonia enables the highest rate of grown for *E. coli*, it is the preferred nitrogen source (reviewed in Reitzer 2003).

The biochemical and genetic mechanisms that facilitate nitrogen control are well understood in enteric bacteria. *E. coli*, *Salmonella typhimurium* and *Klebsiella aerogenes* have attracted the most focus thus the pathways and mechanisms elucidated for them will be referred to in this review (Engleman and Francis 1978, Kustu and McKereghan 1975, Bender *et al.* 1977).

The transport of ammonia into the cell at high extracellular concentrations occurs by diffusion through the membrane (Burkovski 2003). In *E. coli,* when the diffusion across the membrane becomes limiting due to a decrease in the extracellular concentration of ammonia, a cytoplasmic transporter protein Ammonium / methylammonium transport B protein (AmtB) serves to transport ammonia against the concentration gradient into the cell (Soupene *et al.* 1998, Burkovski 2003). Interestingly, homologs of the *amtB* gene, which encodes AmtB, are found in all three domains of life, Archaea, Bacteria, and Eucarya (Soupene *et al.* 1998, Woese *et al*. 1990) suggesting that this mechanism is ubiquitous and essential.

Two major pathways in *E. coli* allow the assimilation of intracellular ammonia into the nitrogen pool of the cell, namely the GS / GOGAT pathway and the GDH pathway (Figure 1-7) (Tyler 1978). In the GS / GOGAT pathway, glutamine synthetase (GS) converts glutamate and ammonia to glutamine (NH_3 + Glutamate + ATP → Glutamine + ADP + P_i) (Ginsburg and Stadtman 1973) and glutamate synthetase (previously known as glutamine amide-2-oxoglutarate aminotransferase (GOGAT)) transfers the amide group from glutamine to α-ketoglutarate producing two glutamate molecules (Glutamine + α-ketoglutarate + NADPH → 2 Glutamate + $NADP^+$) (Miller 1973). Thus, in the overall reaction, GS and GOGAT serve to produce glutamate from ammonia and α-ketoglutarate (Stadtman 1973). In the GDH pathway, glutamate dehydrogenase (GDH) catalyses the reductive deamination of 2-ketoglutarate by ammonia in an NADPH dependent reaction to produce glutamate (NH_3 + α-ketoglutarate + NADPH → Glutamate + $NADP^+$) (Stadtman 1973). While the GDH pathway is energetically more efficient than the GS / GOGAT pathway, it has a lower affinity for ammonia which makes it ineffective in cells grown under nitrogen limiting conditions. The GS / GOGAT pathway allows the assimilation of ammonia at concentrations as low as 0.1 mM, which makes it the preferred pathway in the cell (Stadtman 1973, Merrick and Edwards 1995).

The GS / GOGAT pathway is ubiquitous in bacteria; however the GDH pathway is present in some but not all bacterial genera (Kumada *et al.* 1993, Magasanik 1982). In addition to the GS / GOGAT pathway and the GDH pathway, bacteria contain other pathways that enable the assimilation of ammonia. For example, Kenealy *et al.* (1982) demonstrated a role for alanine dehydrogenase in ammonia uptake.

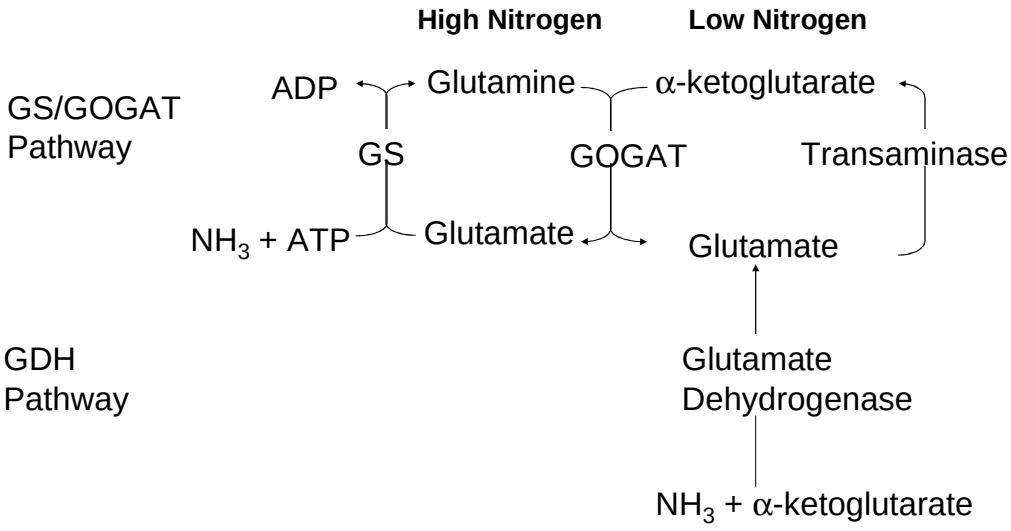


Figure 1-7: Assimilation of nitrogen in *E. coli*. Abbreviations: GS- glutamine synthetase, GOGAT- glutamate synthetase, GDH- glutamate dehydrogenase. Figure adapted from Tyler 1978.

In *E. coli,* GDH is a hexameric enzyme of identical subunits with a molecular mass of 48 kDa that is encoded by *gdhA* (McPherson and Wootton 1983). A second and different tetrameric glutamate dehydrogenase with a molecular weight of 180 kDa, encoded by *gdhB*, is present in *P. aeruginosa* (Lu and Abdelal 2001). The enzyme GOGAT, in *E. coli*, is comprised of two non-identical subunits, 53 kDa and 135 kDa in size. The two subunits are encoded by the *gltBD* genes which are transcribed contiguously in bacteria (Goss *et al.* 2001).

Most surprisingly, four different genes coding for GS enzymes have been identified; *glnA*, *glnII*, *glnN* and *glnT* (Table 1-4). Of these, the enzyme product of *glnA,* GSI, has been subjected to the most research and is the classic glutamine synthetase that is almost universally found in prokaryotes (Tiboni *et al.* 1993). GSI is a dodecameric enzyme where each subunit is approximately 50 kDa in size (Ginsburg and Stadtman 1973). GSI activity is controlled in four ways. First, it is regulated by feedback inhibition by eight nitrogonous compounds (histidine, tryptophan, AMP, CTP, carbamyl-phosphate, glucosamine-6-phosphate, alanine and glycine) (Stadtman 1990). GSI activity is also regulated by ADP-ribosylation and adenylation; both of which modify the activation level of the enzyme (Liu and Kahn 1995, Chung and Rhee 1984). Finally, the regulation of transcription of

the *glnA* gene regulates the biosynthesis of the GSI enzyme (Reitzer and Magasanik 1985).

ADP-ribosylation of GSI leads to the disruption of the dodecameric structure of GSI resulting in enzyme inactivation (Moss *et al.* 1990). It is interesting to note that a similar mechanism regulates the nitrogenase activity in *Rhodospirillum rubrum*; an enzyme which catalyzes the reduction of N_2 to ammonium (Pope *et al.* 1985, Lowery *et al.* 1986). The nitrogenase enzyme is regulated by ADP-ribosylation at Arg-101, a process that is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DraT). Removal of the moiety and consequent activation of the enzyme is catalyzed by dinitrogenase reductase activating glycohydrolase (DraG) (Ma and Ludden 2001, Fu *et al.* 1990). This enzyme system has also been identified in *Azospirillum* spp., *Azotobacter chroococcum, Rhodobacter capsulatus* and*, Rhodospirillum sphaeroides* (reviewed in Merrick and Edwards 1995).

Adenylation of the *E. coli* GSI is a function of the adenyltransferase enzyme (ATase) (Anderson *et al.* 1970). The *E. coli* ATase, encoded by the *glnE* gene, is a monomeric protein 115 kDa in size (Jaggi *et al.* 1990, Caban and Ginsburg 1976). ATase covalently adds an AMP group to a tyrosyl residue (Tyr-397) on each subunit progressively inactivating the GSI enzyme. As adenylation of a subunit inactivates only that subunit, GSI can exist in a number of different activity states (Anderson *et al.* 1970, Chung and Rhee 1984). Adenylation occurs in response to an increase in the intracellular nitrogen status such that as the cell becomes more nitrogen sufficient, GSI becomes more adenylated and the activity of GSI is progressively reduced (Engleman and Francis 1978, Foor *et al.* 1975).

Table 1-4: Distribution of the glutamine synthetase enzymes in prokaryotes

Enzyme	Structural Gene	Subunit MW (kDa)	Subunits	Distribution
GSI	*glnA*	~ 55	12	Almost ubiquitous
GSII	*glnII*	~ 36	8	*Rhizobium, Bradyrhizobium, Agrobacterium, Streptomyces, Frankia* spp.
GSIII	*glnN*	~ 75	6	*Bacteroides, Butyrivibrio, Synechocystis, Synechococcus, Gleocaspa, Pseudoanabana* spp.
GlnT	*glnT*	~ 47	8	*Rhizobium, Agrobacterium* spp.

Table adapted from Merrick and Edwards 1995.

GSI is the most predominant GS enzyme, but bacteria are now known to synthesize up to three other GS enzymes. In addition to GSI, GSII encoded by the *glnII* gene, has been identified in soil-dwelling microorganisms, and in eukaryotes (Fuchs and Keister 1980a, Pesole *et al.* 1995). The presence of GSII in eukaryotes and only the *Rhizobiaceae* led Carlson and Chelm (1986) to suggest that the *glnII* gene had been passed from plants to bacteria as a result of horizontal transfer. GSII differs from GSI in that it is an octomer, each subunit is approximately 36 kDa in size, and interestingly GSII is expressed only during nitrogen limitation conditions (de Bruijn *et al.* 1989, Fuchs and Keister 1980b). Immediately upstream of *glnII* in *R. leguminosarum* is *gstI*, which is expressed during nitrogen sufficiency and inhibits the translation of the *glnII* mRNA ensuring low levels of GSII in the cell during nitrogen sufficient conditions (Spinosa *et al.* 2000). A third and different GS, GSIII, was isolated from *Bacteroides fragilis* by Hill *et al.* (1989). Encoded by the *glnN* gene, GSIII is a hexameric enzyme and each subunit is 75 kDa in size. While GSIII has been identified in a number of strains since 1989, the enzyme has not been extensively characterized.

The GS enzyme product of *glnT*, isolated from *Rhizobium meliloti*, has been referred to as GlnT or GSIII (de Bruijn *et al.* 1989). Unfortunately, referring to the enzyme as GSIII leads to confusion with the GSIII enzyme isolated from *B. fragilis* thus for the purpose of this review it shall be called GlnT. A homologue of GlnT has been identified in *Agrobacterium* (Rossbach *et al.* 1988). The GlnT enzyme is an octomer and each subunit is 47 kDa in size (Shatters *et al.* 1993). GlnT, like GSI, is reported to be regulated by ADP-ribosylation; which also leads to the inactivation of GlnT. The purpose of ribosylating GlnT is unknown (Liu and Kahn 1995).

1.6.2 Nitrogen regulation

The *E. coli* GS / GOGAT pathway is linked to and regulated by the global nitrogen regulatory (ntr) system, which is composed of four main proteins – PII, UTase / UR, NtrB and NtrC. PII, also known as GlnB, is encoded by the *glnB* gene (Figure 1-8). The protein consists of trimers, each approximately 12 kDa in size; which are expressed constitutively in the cell (van Heeswijk *et al.* 2000, Forchhammer *et al.* 1999, van Heeswijk *et al.* 1993). The PII protein is modified in the cell by uridylyltransferase (UTase / UR; GlnD), an enzyme which has two separate activities. First, the UTase activity uridylylates PII by the addition of a UMP molecule and second, the UR activity of the enzyme deuridylylates PII (Figure 1-8) (Adler *et al.* 1975). This modification occurs in response to the nitrogen level in the cell; nitrogen insufficiency, indicated by low levels of glutamine, activates the UTase activity of the enzyme whereas in high nitrogen levels of nitrogen, the UR activity predominates (Jiang *et al.* 1998, Engleman and Francis 1978).

Deuridylylated PII (indicative of high nitrogen status) activates the enzyme ATase, which causes the adenylation of GSI. This results in the inactivation of the GSI enzyme and prevents the further production of glutamine (Brown *et al.* 1971). As the nitrogen levels in the cell drop, the UTase enzyme uridylylates PII by adding a UMP group to a Tyr-51 residue on each subunit of the protein (Jiang *et al.* 1998, Maheswaran and Forchhammer 2003). PII-UMP promotes the deadenylation of GS by ATase producing the active form of the enzyme which produces glutamine from ammonia (Brown *et al.* 1971). Thus UTase / UR and PII together provide a mechanism for sensing the intracellular nitrogen status. They also, as a result of their Jeckyl-Hyde forms, activate the enyme ATase whose enzyme activities essentially control the nitrogen status of the cell.

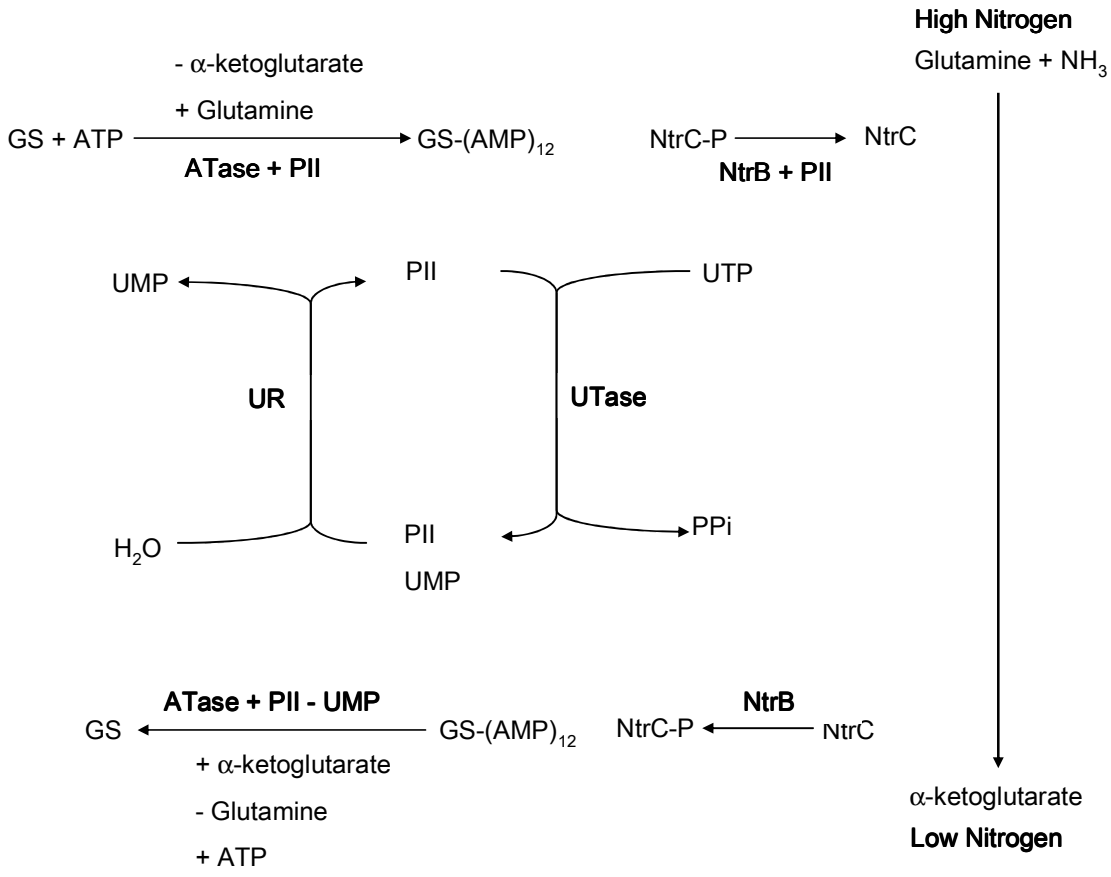


Figure 1-8 The proteins involved in the global nitrogen regulatory system of enteric bacteria. UTase / UR catalyses the uridylylation and deuridylylation of PII, ATase catalyses the adenylation and deadenylation of GS and NtrB catalyses the phosphorylation and dephosphorylation of NtrC. The reactions are stimulated (+) or inhibited (-) by levels of various metabolites as shown above. Figure adapted from Arcondeguy *et al.* 2001, Gottschalk 1986, Tyler 1978, Zubay 1988.

A PII homolog has been identified in *E. coli* cells. Like PII, GlnK is trimeric; however unlike PII which is expressed constitutively in the cell, the expression of GlnK is regulated by the nitrogen status of the cell. GlnK is detected only during nitrogen starvation conditions (van Heeswijk *et al.* 1995, 2000). Interestingly, GlnK can form heterotrimers with PII and is believed to work with PII in regulating the nitrogen regulatory cascade (van Heeswijk *et al.* 2000, Atkinson and Ninfa 1998). Atkinson and Ninfa (1999) demonstrated that though both PII and GlnK were uridylylated by the UTase activity of UTase / UR, only PII was effectively deuridylylated by the UR activity. In addition, the GlnK activity was regulated differently by α-ketoglutarate to PII. While GlnK was capable of fulfilling the role of PII in the *E. coli* cell, Atkinson and Ninfa (1999) proposed that GlnK was unlikely to have a significant role in ntr system but was more likely to have a role in the regulation of

nitrogen-status receptors in the cell. This has proved to be correct.

Javelle *et al.* (2004) demonstrated that the *E. coli* AmtB protein, which is involved in the transport of ammonia into the cell, was inactivated by the formation of a membrane bound complex with GlnK. The binding of GlnK to AmtB was moderated by its uridylylation state, such that in high nitrogen environments GlnK was deuridylylated and bound to AmtB resulting in the prevention of ammonia transport into the *E. coli* cell. This mechanism would be useful when transient and low levels of nitrogen became available to the bacterial cells. The AmtB protein therefore acts as a nitrogen sensor for the cell (Javelle *et al.* 2004). The *glnK* gene is found upstream of the *amtB* gene in a diverse group of eubacteria and archaea suggesting that the regulation of ammonia transport by GlnK and AmtB may exist in a wide variety of bacterial strains (Thomas *et al.* 2000).

1.6.3 ntr-regulated systems

In addition to regulating ATase activity, the PII protein regulates the activity of NtrB, a member of the sensory protein kinases. NtrB (NRII or GlnL), encoded by the *ntrB* (or *glnL*) gene, is a dimeric cytoplasmic signal protein and each subunit is 36 kDa in size (Keener and Kustu 1988, Kamberov *et al.* 1995). NtrB has two enzyme activities: it can behave as a phosphatase or a kinase depending on its conformation; which is regulated by PII (Pioszak and Ninfa 2003). NtrB autophosphorylates at the His-139 residue and phosphorylated NtrB acts as a kinase (Weiss and Magasanik 1988, Ninfa and Bennett 1991). The autophosphorylation and the transphosphorylation (transfer of a phosphate group from one subunit of NtrB to another) of NtrB is inhibited by PII (Jiang *et al*. 2000). Jiang *et al.* (2000) propose that binding of PII to NtrB (kinase) causes a conformational change which leads to the activation of the phosphatase activity of NtrB.

NtrB regulates the activity of a transcriptional activator NtrC (NRI, GlnG), which is encoded by the *ntrC* (*glnG*) gene. NtrC is also a dimeric protein with a subunit molecular mass of 55 kDa (Reitzer and Magasanik 1983). NtrC is phosphorylated at the Asp-54 residue by NtrB kinase (phosphorylated NtrB) (Weiss and Magasanik 1988, Sanders *et al.* 1992). Phosphorylated NtrC can then lose its phosphate group in two ways- it can autodephosphorylate, which occurs spontaneously, or it can be dephosphorylated as a result of the phosphatase activity

of NtrB phosphatase (Keener and Kustu 1988, Jiang *et al*. 2000). Thus, when the *E. coli* cell is in a condition of nitrogen insufficiency, NtrB kinase phosphorylates NtrC producing the active transcriptional factor, NtrC~P. Conversely, when high nitrogen levels exist within the cell, NtrC~P is dephosphorylated by NtrB phosphatase producing NtrC, the inactive transcription factor (Ninfa and Magasanik 1986, Ninfa and Atkinson 2000).

NtrB and NtrC are type members of a two-component regulatory system that has been extensively researched (Mettke *et al.* 1995). This is a mechanism of regulation based on a His-Asp phosphorelay circuit between a sensor kinase and a response regulator that work interactively. The sensor histidine kinase (NtrB) uses ATP to autophosphorylate a specific histidine residue in response to an environmental stimulus. The response regulator (NtrC) which has a conserved aspartate then recieves that phosphate and effects changes in gene expression (reviewed in Mizuno 1998).

NtrC dimers interact with enhancer DNA sequences which have a consensus sequence TGCACCANNNTGGTGCA (Weiss *et al.* 1992). While both phosphorylated and unphosphrylated NtrC can bind to the enhancer DNA sequences, only phosphorylated NtrC enables the covalent interactions between NtrC dimers which produce either tetramers or octomers (Weiss *et al.* 1992, Hwang *et al.* 1999). A model proposed by Rippe *et al.* (1998) requires the interaction of four dimers to form an oligomeric structure, which is feasible as each NtrC dimer can bind two further dimers. The oligomerization of NtrC results in activation of the ATPase activity of NtrC; which is essential for the activation of transcription from RNA polymerase-σ^{54} complexes (Mettke *et al.* 1995).

σ^{54} encoded by the *ntrA* gene (also known as *glnF* and *rpoN*) is required for the transcription of genes encoding amino acid transport components and catabolic enzymes. The expression of many ntr genes requires σ^{54} (Kustu *et al.* 1989). The interaction of NtrC and σ^{54}-containing RNA polymerase results in DNA bending which allows for the conformational changes required for the activation of transcription (Su *et al.* 1990, Carmona *et al.* 1997). Activation of transcription from all the known σ^{54}-dependent promoters requires the presence of an activator protein (like NtrC),

which makes σ^{54}–dependent transcription distinctive from σ^{70}-dependent transcription (Wosten 1998, Kustu *et al.* 1989). This means that the degree of σ^{54}-dependent transcription can be varied from none to very high levels depending on the presence of an activator.

The best studied ntr-regulated systems are the *glnAntrBC* operons of *E. coli, S. typhimurium* and *K. aerogenes* (Pahel *et al.* 1982, MacFarland *et al.* 1981, Rothman *et al.* 1982). The *E. coli* operon was originally isolated by Backman *et al.* (1981) and the genes subsequently identified by Pahel *et al.* (1982). In all three bacterial species expression of the *ntrB* and *ntrC* genes is adjacent to the *glnA* gene (Pahel *et al.* 1982, MacFarland *et al.* 1981, Rothman *et al.* 1982). *glnA* is expressed from one of two possible promoters, namely *glnA*p1 or *glnA*p2; the *ntrBC* genes are expressed either by readthrough from the *glnA* promoter or from a separate promoter p*ntrBC* (Figure 1-9) (Reitzer and Magasanik 1985, MacFarlane and Merrick 1985).

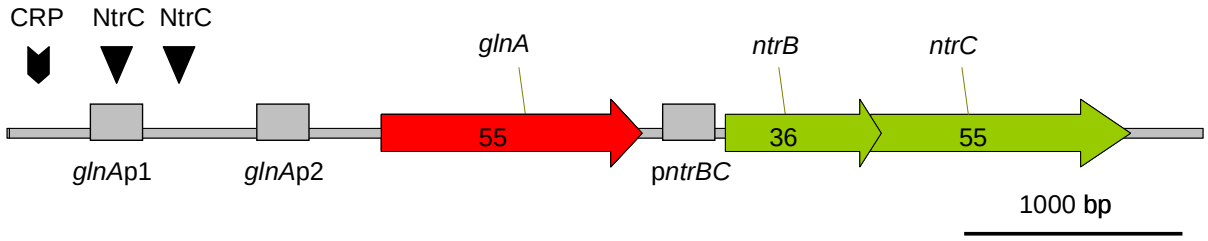


Figure 1-9 A schematic representation of the *glnAntrBC* operon from *E. coli.* The genes and the orientations are represented by arrows. The numbers within the arrows indicate molecular mass (in kDa) of the respective protein. The binding sites for CRP and NtrC are shown. Abbreviations: CRP- cAMP receptor protein, *glnA*p1- promoter 1 for *glnA, glnA*p2- promoter 2 for *glnA*, p*ntrBC*- promoter for *ntrB* and *ntrC*.

Under conditions of nitrogen sufficiency, *glnA* is transcribed from *glnA*p1 (Figure 1-9) by σ^{70}-dependent RNA polymerase (Reitzer and Magasanik 1985). Most of these transcripts terminate at the ρ-independent terminator downstream of *glnA* (Ueno-Nishio *et al.* 1984). At this time, low level of *ntrBC* expression occurs from p*ntrBC* and genes controlled by the ntr pathway are not expressed (Hanau *et al.* 1983, Pahel *et al.* 1982). However under nitrogen limiting conditions, phosphorylated NtrC binds to sites that overlap *glnA*p1 thus preventing

σ^{70}-dependent RNA polymerase mediated expression of *glnA*. Transcription of *glnA* by σ^{54}-dependent RNA polymerase from the *glnA*p2 promoter (Figure 1-9) occurs. A large proportion of these transcripts read through *ntrBC,* producing NtrB and NtrC (Reitzer and Magasanik 1985). This mechanism serves to provide low levels of GS, NtrB and NtrC (5 dimers per cell) under conditions of nitrogen sufficiency, which enables the cell to synthesize basal levels of glutamine required for nitrogen sufficient conditions. However during nitrogen limitation a marked increase in transcription causes a fourteen-fold increase in the levels of GS, NtrB and NtrC (Reitzer and Magasanik 1983).

There are two interesting interactions that occur between proteins in the regulation of the *E. coli glnAntrBC* operon. First, NtrC has the ablity to act as a transcriptional activator and a transcriptional repressor due to its DNA binding abilities. In the *glnAntrBC* operon, NtrC binding to sites that *glnA*p1 causes repression of *glnA* expression (Reitzer and Magasanik 1985). This is not the only report of NtrC acting as a repressor; the NtrC protein also assumes the role of a repressor in the regulation of the *aap* operon which is involved in the accumulation of a wide range of amino acids in *R. leguminosarum* (Walshaw *et al.* 1997). Secondly, binding sites for cAMP-CRP are present upstream of the *glnAntrBC* operon and the binding of cAMP-CRP interferes with expression from the *glnA*p2 promoter which in turn ensures low levels of *glnA* expression during nitrogen sufficent conditions (Reitzer and Magasanik 1985, Tian *et al.* 2001). CRP is also involved in the regulation of *dctA*, a gene which encodes a permease of the C_4-dicarboxylic acid transport system in *R. meliloti* (Wang *et al.* 1998). Wang *et al.* (1998) report that interaction of the cAMP-CRP complex with the *dctA* σ^{54} promoter results in the repression of *dctA* promoter activation.

Transcriptional regulators, like CRP, defined with respect to their roles in the carbon regulatory pathways have now been found to have roles in the nitrogen regulatory pathways. This suggests strong ties between carbon and nitrogen metabolism within enteric bacterial cells and inter-linking of the two global systems such that they cannot be separated into neat sets of pathways.

1.6.4 Genome organization in the Agrobacteria

A. tumefaciens C58, the type strain of the *Agrobacterium* genus, has a two-chromosome genome consisting of a 2.84 Mb circular chromosome and a 2.1 Mb linear chromosome. Circular chromosomes are ubiquitous in bacteria but linear chromosomes are found predominantly in eukaryotes (Wood *et al.* 2001). Both chromosomes encode DNA replication proteins and ribosomal proteins but most of the auxotrophic markers (86%) are localized to the circular chromosome (Goodner *et al.* 1999). This suggests that the circular chromosome was inherited directly from the α-proteobacterial ancestor and the essential genes present on the linear chromosome are a result of lateral transfer from the circular chromosome over a period of time (Goodner *et al.* 2001).

A total of 62.7% and 69.2% of the open reading frames on the circular and linear chromosomes respectively have been assigned function based on sequence identity to genes of known function. These figures are misleading as only 2.2% and 1.5% of the open reading frames on the circular and linear chromosomes respectively have known function. A further 4.6% and 4.3% of the open reading frames on the circular and linear chromosomes have possible function and 5.6% and 4.3% of the open reading frames on the circular and linear chromosomes have unknown function but have been identified in other bacterial strains (conserved unknown open reading frames). In addition, 24.9% and 20.7% of the genes on the circular and linear chromosomes respectively are novel providing a new pool of information to mine (Goodner *et al.* 2001, Wood *et al.* 2001).

In addition to the two chromosomes, *A. tumefaciens* C58 hosts a diverse group of plasmids varying in size from 45 kb (30 MDa) to 543 kb (358 MDa) (Currier and Nester 1976, White and Nester 1980, Wood *et al.* 2001). These plasmids are classified into two main groups, the tumor-inducing (Ti) and hairy-root inducing (Ri) plasmids (Yanofsky *et al.* 1985, White and Nester 1980). *A. tumefaciens* C58 hosts two plasmids, pTiC58 and pATC58 which are 543 kb and 214 kb in size respectively (Wood *et al.* 2001). The plasmid pTiC58, a typical nopaline-type plasmid has been the focus of most of the research as the plasmid encodes the genes required for virulence and hydrolysis of opines (Holsters *et al.* 1980). Suzuki *et al.* (2000) recently isolated a nopaline-type plasmid pTi-SAKURA. While 60% of pTi-SAKURA is very similar to pTiC58, 40% is significantly different

indicating that these mobile elements vary considerably in the genes they encode and in the order in which the genes are present (Suzuki *et al.* 2000).

1.6.5 Nitrogen regulation in the Agrobacteria

Agrobacterium spp. have been studied in great detail with respect to their ability to transform plants and their ability to catabolize opines; which can be used by the cells as both carbon and nitrogen sources (reviewed by Zhu *et al.* 2000, Cho *et al.* 1996). Unfortunately, very little has been reported about the fundamental mechanisms of nitrogen assimilation and regulation in *Agrobacterium* spp.

Bioinformatic analysis of the *A. tumefaciens* C58 genome (http://cancer.lbi.ic.unicamp.br/agroC58/) revealed that the C58 genome does encode all the genes required for the assimilation of nitrogen from the extracellular environment and all the genes required for the regulation of the intracellular nitrogen levels (Table 1-5). Strangely, strain C58 appears to encode six GS genes, GSI, GSII and GSIII (which should probably be named GlnT) and three other *glnA* genes whose enzymes have not been classified as yet. In addition, there appear to be three genes encoding the large subunit of the GOGAT enzyme and only one encoding the small subunit.

All but four of the genes involved in nitrogen assimilation were identified on the circular chromosome. One *glnA* gene encoding GSIII, one *gltD* gene encoding the small subunit of GOGAT and two *gltB* genes encoding the large subunit of GOGAT were identified on the linear chromosome. This emphasizes the paucity of auxotrophic markers on the linear chromosome (Goodner *et al.* 1999).

Table 1-5: Genes and proteins involved in the assimilation and regulation of nitrogen in *A. tumefaciens* C58 genome and their location. Adapted from the information available at (http://cancer.lbi.ic.unicamp.br/agroC58/).

Gene	Gene Number	Enzymes	Chromosome
amtB	2758	Ammonium transporter	Circular
gdh	2766	Glutamate dehydrogenase	Circular
glnA	0193	Unassigned	Circular
	0602	Unassigned	Circular
	1770	GSI	Circular
	2142	Unassigned	Circular
	2416	GSII	Circular
	4230	GSIII	Linear
glnB	1769	PII	Circular
glnD	0346	Uridylyltransferase	Circular
glnE	0981	ATase	Circular
glnK	2757	PII	Circular
gltD	3783	GOGAT (small subunit)	Linear
gltB	0145	GOGAT (large subunit)	Circular
	3784	GOGAT (large subunit)	Linear
	4229	GOGAT (large subunit)	Linear
ntrA (*glnF* / *rpoN*)	0332	σ^{54}	Circular
ntrB	1445	NtrB	Circular
ntrC	1446	NtrC	Circular

The *ntrC* gene was isolated from strain C58 by both Rossbach *et al.* (1987) and Wardhan *et al.* (1989). Rossbach *et al.* (1987) reported that the *ntrC* was not involved in the expression of GSI in strain C58 but was involved in the expression of GSII; which may explain why the *ntrBC* operon in strain C58 is not linked to the *glnA* gene (Wardhan *et al.* 1989). No further research has been reported. The lack of information available for *Agrobacterium* spp. leaves a large gap in the knowledge that requires further research.

1.7 RESEARCH PROPOSAL

A. tumefaciens strains have been found biologically important for two main reasons. Firstly, *A. tumefaciens* is a natural 'genetic engineer', able to transform fungi, plants and human cells, thus the species has incredible biotechnological potential (Gouke *et al.* 1999, de la Riva *et al.* 1998, Kunik *et al.* 2001). The mechanisms involved in the transformation of cells by *Agrobacteria* have been intensively studied by researchers and is well understood. Secondly, the hydantoin-hydrolyzing enzymes produced by *A. tumefaciens* are economically important. For example, the *Agrobacterium* D-NCAAH is presently being used in the manufacture of thousands of

tonnes of optically pure D-amino acids (Ogawa and Shimizu 2002). The application and the manipulation of the hydantoin-hydrolyzing enzymes to optimize the final concentrations of amino acids produced has also been the focus of much research (Kim and Kim 1995, Durham and Weber 1996, Oh *et al.* 2002, Chao *et al.* 2000). However, very little is known about the mechanisms that regulate the expression of the genes that encode hydantoin-hydrolyzing enzymes.

The complete genome sequencing of *A. tumefaciens* C58 has allowed the analysis of the circular and linear chromosomes and the two plasmids, pTiC58 and pATC58, within the strain (Wood *et al.* 2001). Genes have been annotated based primarily on sequence similarity to previously isolated genes. Only 4.3% of the *A. tumefaciens* C58 genes have been subjected to detailed analysis and have known function (Goodner *et al.* 2001). Analysis of the gene content of the C58 genome suggests that the mechanisms involved in the regulation of nitrogen in strain C58 are similar to those in *E. coli* and other enteric bacteria. However the large number of novel genes and the high degree of gene duplication present in strain C58 demands detailed analysis of the fundamental mechanisms of nitrogen regulation within *A. tumefaciens.*

Two *hyu* gene clusters have been isolated from *Agrobacterium* strains: from *A. tumefaciens* NRRL B11291 and *Agrobacterium* sp. IP I-671 (Grifantini *et al.* 1998, Hils *et al.* 2001). Both *hyu* clusters were located on large native plasmids within the *Agrobacterium* cells (Hils *et al.* 2001). The presence of different plasmids within the *Agrobacteria* provide a source of diversity within the various strains (Currier and Nester 1976). The *hyu* cluster from *Agrobacterium* sp. IP I-671 was flanked by sequences that bore similarity to transposon sequences (Hils *et al.* 2001). Wood *et al.* (2001) identified a large number of IS elements on the *A. tumefaciens* C58 genome, which suggests that a number of genes and gene clusters arrived in the strain C58 as transposons. While the transposon sequences can be classified based on sequence identity with known transposon sequences, the origin of the *hyu* genes is unknown and their regulation has not been studied.

The Rhodes University Hydantoinase Research Group has access to two *A. tumefaciens* strains, RU-AE01 and RU-OR, both of which are environmental isolates selected for their ability to hydrolyze hydantoin (Herrera unpublished,

Hartley *et al.* 1998). The regulation of the hydantoin-hydrolyzing enzymes in strain RU-OR was studied in detail by Hartley *et al.* (1998, 2001). Hartley *et al.* (1998) demonstrated that the hydantoin-hydrolyzing enzymes in strain RU-OR were induced in the presence of hydantoin and hydantoin analogues. Mutant strains of *A. tumefaciens* RU-OR with inducer-independent expression of the hydantoinase but not the NCAAH (RU-ORLB3) or inducer-independent expression of the hydantoinase and NCAAH (RU-ORL5) were isolated. A mutant (RU-ORPN1) which showed inducer-independent expression of the hydantoinase and NCAAH, like RU-ORL5, but also had elevated levels of hydantoinase activity was also isolated (Hartley *et al.* 2001).

NCR observed by Hartley *et al.* (2001) in strain RU-OR occurred at two levels: firstly at the level of transcription and secondly at the post-translational level (ammonia shock). In ammonia shock, induced RU-OR cells producing hydantoinase activity suffered a dramatic decrease in the detectable levels of the hydantoinase. However, upon removal of the ammonia the detectable levels of hydantoinase activity increased again to pre-ammonia shock levels (Hartley *et al.* 2001). The hydantoin-hydrolyzing enzymes in strain RU-ORPN1, which was isolated as an inducer-independent mutant, were no longer sensitive to NCR but were still susceptible to ammonia shock (Hartley *et al.* 2001). Subsequent isolation of ammonia shock-resistant mutant strains (RU-ORPN1F1 and RU-ORPN1F9) established that the the resistance to ammonia shock was a result of the irreversible adenylation of GS. As adenylated GS is inactive, it results in glutamine auxotrophy in the cells and as a result the inability to detect the nitrogen status of the cell. This in turn allows the expression of the hydantoin-hydrolyzing enzymes in the presence of ammonia in mutant strains RU-ORPN1F1 and RU-ORPN1F9 (Hartley *et al.* 2001).

Hartley (2001) isolated two NCAAH-encoding genes, *ncaR1* and *ncaR2.* Unfortunately, the gene encoding the hydantoinase enzyme in strain RU-OR has not been isolated as yet. A plasmid encoding the *hyuC* gene, with NCAAH activity, was isolated from strain RU-AE01 (Herrera unpublished) but the strain has not been further characterized.

The similarities between the *hyu* genes isolated from *Agrobacterium* sp. IP I-671 and *A. tumefaciens* NRRL B11291 allow speculation that the *hyu* genes in

A. tumefaciens strains RU-OR and RU-AE01 may be similar. The availability of strain RU-OR, in which the expression of the hydantoin-hydrolyzing enzymes has been well-studied, and strain RU-AE01, from which the genomic DNA encoding the *hyuC* gene has been isolated, allows us to address some of the gaps in the knowledge of the *hyu* gene clusters from *Agrobacterium* spp.

To this end two main objectives were identified.

1. To characterize the hydantoin-hydrolyzing enzyme production in strain RU-AE01 and to compare the levels and patterns of the hydantoin-hydrolyzing enzyme production of strain RU-AE01 with strain RU-OR.

2. To study the transcriptional regulation of the *hyu* genes in RU-AE01.

CHAPTER 2

DEVELOPMENT OF A BROAD HOST RANGE VECTOR FOR THE ANALYSIS OF *A. tumefaciens* RU-AE01 PROMOTER SEQUENCES

2.1 INTRODUCTION

Two methods have been used to isolate *hyu* genes from *Agrobacterium* strains: screening of genomic DNA libraries and PCR amplification. Firstly, the isolation of *hyu* genes from genomic DNA libraries includes selection for the ability of *E. coli* recombinants to utilize hydantoin and *N*-carbamyl amino acids as sole nitrogen sources (Ikenaka *et al.* 1998, Kim *et al.* 1997, Mukohara *et al.* 1993, Nanba *et al*. 1998, Watabe *et al.* 1992a). Secondly, the use of homologous DNA probes permits the screening for sequences similar to the published sequences (Chen and Tsai 1996, Griffantini *et al.* 1998). Though D-selective hydantoin-hydrolyzing enzyme activity has been detected in a number of *Agrobacterium* strains and some of the genes involved in the hydrolysis of hydantoins have been isolated, only two *hyu* operons have been isolated and characterized (Table 2-1). All the *Agrobacterium hyuH* genes isolated from genomic DNA libraries thus far have been linked to the *hyuC* gene (Grifantini *et al.* 1998, Nanba *et al.* 1998, Neal *et al*. 1999, Hils *et al.* 2001). In contrast, not all the *hyuC* genes isolated from genomic DNA libraries have been coupled to the *hyuH* gene (Hartley 2001).

Table 2-1: *hyu* genes isolated from different *Agrobacterium* strains**.**

Microbe	**Genes Isolated**	**Reference**
Agrobacterium sp. 80/44-2a	*hyuH, hyuC*	Neal *et al*. 1999
Agrobacterium sp. IP I-671	*hyuH, hyuC*, *hyuA, hyuD, hyuN*	Hils *et al.* 2001
A. radiobacter CCRC 14924	*hyuC* ∗	Hsu *et al*. 1999
A. radiobacter KNK712	*hyuH, hyuC*	Nanba *et al*. 1998
A. tumefaciens AM10	*hyuC* ∗	Sareen *et al.* 2001
A. tumefaciens BQL9	*hyuH* ∗	Clemente-Jimenez *et al.* 2003
A. tumefaciens C58	*hyuA* ∗	Las Heras-Vazquez *et al.* 2003
A. tumefaciens C58	*hyuA* ∗	Martinez-Rodriguez *et al.* 2004
A. tumefaciens NRRL B11291	*hyuH, hyuC*	Grifantini *et al.* 1998
A. tumefaciens RU-AE01	*hyuC*	Herrera unpublished
A. tumefaciens RU-OR	*hyuC1*∗, *hyuC2*	Hartley 2001

Abbreviations: *hyuH*- hydantoinase encoding gene, *hyuC*- NCAAH encoding gene, *hyuN*- NADPH encoding gene dependent flavin oxidoreductase, *hyuD*- D-amino acid dehydrogenase encoding gene and *hyuA*- racemase encoding gene. ∗ - genes isolated by PCR.

The strain *A. tumefaciens* RU-AE01 was isolated from hydantoin-rich strip gas liquor and selected for its ability to hydrolyse hydantoin (Herrerra unpublished results). Screening of a RU-AE01 genomic DNA library for NCAAH activity, using phenol red

as an indicator of ammonia production, led to the isolation of the plasmid pGH45 (Figure 2-1) which encodes *hyuC* and some of the activation sequences required for its expression in *E. coli* (Herrera unpublished results, Clark 2003).

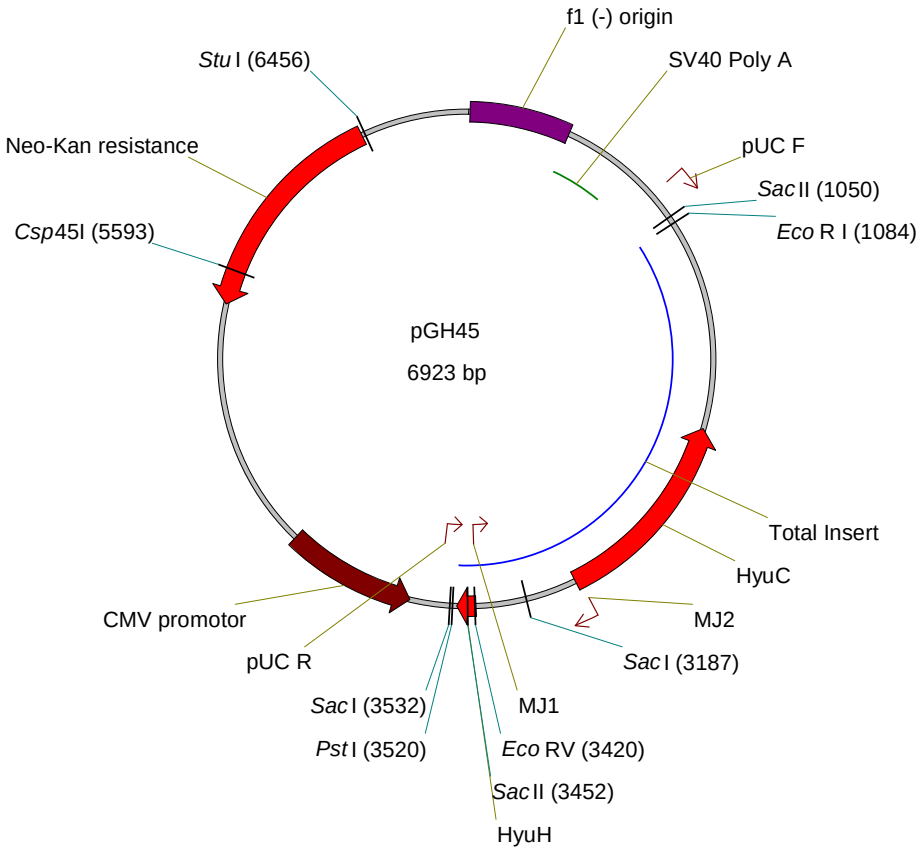


Figure 2-1: Schematic map of the construct pGH45. The 2.4 kb *A. tumefaciens* RU-AE01 genomic DNA insert in the vector pBK-CMV and the orientation of HyuC are shown by the blue line and the red arrow labeled HyuC respectively. Binding sites and orientation of the primers pUCF, pUCR, MJ1 and MJ2 are shown by the arrow heads.

The availability of a recombinant plasmid potentially carrying the *hyuC* promoter sequences provided an opportunity for studying the regulation of *hyuC* expression using a reporter gene assay system. This would require a vector capable of replication in *E. coli* and *A. tumefaciens* with a suitable selectable marker that would preferably function in both strains and, most importantly, a robust reporter system. The advantage of using a reporter system is that the reporter enzyme retains its own enzyme characteristics, but is dependent on the heterologous DNA for transcription and translation. The fusion of the transcription activation sequence upstream of a reporter gene that encodes an easily assayed enzyme facilitates the analysis of expression of the affixed activation sequences (Linn and St Pierre 1990). A number of reporter genes have been used in transcriptional and translational fusions

including promoterless *amp*, *cat* or *tet* antibiotic resistance genes (Brosius 1984, Carbonelli *et al.* 1999), *lacZ* (Parales and Harwood 1993), *gus* (Bardonnet *et al.* 1988, Jefferson *et al.* 1986) and *gfp* (Dhandayuthapani *et al.* 1998).

This chapter describes the sequence analysis of the region upstream of the *hyuC* coding region in pGH45, the development of a broad host range promoter-probe plasmid for the analysis of the activation sequences and validation of the broad host range promoter-probe plasmid using the activation sequences present upstream of the *hyuC* coding region in pGH45.

2.2 METHODS AND MATERIALS

2.2.1 General culture conditions

Recombinant plasmids were maintained in *E. coli* DH5α cells (Appendix 8). *E. coli* DH5α cells were grown at 37 °C in medium containing the appropriate antibiotic to which the plasmid conferred resistance. Liquid cultures of *E. coli* DH5α were grown in Luria-Bertani (LB) broth (Appendix 2-1) or in *E. coli* minimal medium [including Glucose Minimal Medium (GMM), Lactose Minimal Medium (LMM), Glycerol Minimal Medium (GlyMM) or GlyMM containing IPTG] (Appendix 2-2) with shaking at 200 rpm. LB agar (Appendix 2-1) was used as a solid growth medium. Where required growth media were supplemented with 100 µg/mL ampicillin, 50 µg/mL kanamycin, 50 µg/mL chloramphenicol or 25 µg/mL tetracycline. Similarly, *A. tumefaciens* RU-AE01 and RU-OR cells were grown at 28 °C in LB broth with shaking at 200 rpm or on LB agar (Appendix 2-3). The growth medium was supplemented with the antibiotic (100 µg/mL ampicillin, 50 µg/mL kanamycin, 50 µg/mL chloramphenicol or 25 µg/mL tetracycline) to which the plasmid conferred resistance.

Recombinant plasmid DNA was extracted from *E. coli* DH5α cells for diagnostic purposes using the Easy Prep method (Berghammer and Auer 1993). The High Pure Plasmid Isolation Kit (Roche) was used to prepare DNA sequencing templates. Restriction digests of recombinant plasmids were performed as per the manufacturer's instructions and the resultant restriction fragments analysed by agarose gel electrophoresis (Sambrook *et al*. 1989). To confirm the presence of recombinant plasmids, total genomic DNA was extracted from

A. tumefaciens RU-AE01 and RU-OR cells (Ausubel *et al.* 1983) and transformed into *E. coli* DH5α cells with appropriate selection. Recombinant plasmids were isolated and analyzed as described above for *E. coli* DH5α cells.

2.2.2 Sources of strains and recombinant plasmids

A. tumefaciens RU-AE01 and the plasmid pGH45 were obtained from Gerardo Herrera (AECI Ltd) who isolated the plasmid as follows. Genomic DNA was extracted from RU-AE01 using the detergent lysis / CTAB method as described by Ausubel *et al.* (1983). The DNA was partially digested with *Sau* 3A1 and subjected to sucrose density ultracentrifugation fractionation as described by Maniatis *et al.* (1982). DNA from the sucrose fractions containing DNA fragments in the 3-7 kb range was concentrated by ethanol precipitation and cloned into the *Bam* HI site of the ZAP Express cloning vector pBK-CMV (Stratagene). The packaging of the DNA into λ phage, the infection of *E. coli* XL1-Blue MRF' cells (Appendix 8) and the *in vivo* excision of the recombinant phagemid vectors was done as per the manufacturer's instructions (Stratagene). *E. coli* XLOLR cells (Appendix 8) hosting recombinant pBK-CMV clones were subjected to a chromogenic assay to detect the production of NH_3 from *N*-carbamoyl-*p*-hydroxyphenylglycine (CHPG). The colorimetric assay was corroborated by the detection of D-hydroxyphenylglycine (HPG) by HPLC. Clone number 4 320 was isolated as one that was expressing NCAAH activity and the recombinant pBK-CMV construct was named pGH45 (Figure 2-1).

Other plasmids used in this chapter were obtained from Prof D Rawlings (pTV100) and Dr PA Venter (pAV14HPTrpGUS).

2.2.3 Isolation of the *hyu* intergenic region from pGH45

Partial DNA sequencing of the insert contained in pGH45 with M13 universal primers was used to design primers MJ1 and MJ2 (Table 2-2). MJ1 binds to the 24 nt corresponding to the 8 amino terminal amino acids of *hyuH* coding sequence and MJ2 binds to the 24 nt corresponding to the 8 amino terminal amino acids of *hyuC* coding sequence. The Expand High Fidelity PCR System (Roche) and the primers MJ1 and MJ2 were then used to amplify the region between the *hyuH* and *hyuC* open reading frames from pGH45 plasmid DNA. The PCR product was cloned into the pGEM-T Easy vector (Promega) generating the plasmid pMJ10 (Appendix 1-1).

The DNA sequence of the insert was determined with universal primers using the ABI Prism Big Dye Terminator (Amersham) protocol (Appendix 6-3).

Table 2-2: Sequences of PCR primers used in Chapter 2

Plasmid	Primer	Sequence (5' to 3') ([†] nts on the RU-AE01 intergenic sequence)	Reference
pMJ10	MJ1	[1]CGGGATCCTCCGTTCTTGATGATGATATCCAT[24]	This study
	MJ2	[515]GCCCTAGGCAGCAAGTATCATCTGACGTGTCAT[492]	This study
pMJ258 and pMJ260	MJ9	gaagatct [25]AAAGCAGCTCTCAGGGTTGATG[46]	This study
	MJ10	gaagatct [491]GAACCTTTGCTCCTTCGATAGTTAAT[466]	This study
	MJ46	gaagatct ATGGTCCGTCCTGTAGAAACCC	This study
	MJ47	aactgcag TCATTGTTTGCCTCCCTGCTGCG	This study
	Chao F1	cgggatcc [311]TTGACCCTGGTCCTTG[326]	Chao *et al.* 1999
	Chao R1	ccaagctt TCTCGATCGGATAGG	Chao *et al.* 1999
	Universal *lacZ* primer	GCCAGCTGCATTAATGAATCGGCCAACGCG	This study

[†] RU-AE01 *hyuH-hyuC* sequence detailed in Appendix 1-1
* Underlined sequence at the 5' terminus of the primers represents the sequence for a *Bgl* II (agatct), *Pst* I (ctgcag), *Bam* HI (ggatcc) or *Hind* III (aagctt) restriction enzyme sites.

2.2.4 Transformation of *E. coli* and *A. tumefaciens* strains

A protocol derived from Hanahan (1983) was used to transform competent *E. coli* resulting in an average transformation efficiency of 10^7 colonies / µg DNA. Similarly, a protocol adapted from Shaw (1995) was used to electroporate competent *Agrobacteria* cells with an average transformation efficiency of 10^4 colonies / µg DNA for *A. tumefaciens* strains RU-AE01 and RU-OR. Detailed protocols for the preparation and transformation of competent *E. coli* and *Agrobacterium* cells are reported in Appendix 7-1 and Appendix 7-2 respectively.

2.2.5 Reporter gene assays

Agar plate detection of β-glucuronidase and β-galactosidase activity

The presence of β-glucuronidase and β-galactosidase enzyme activity in *E. coli* DH5α cells, *A. tumefaciens* RU-AE01 cells and *A. tumefaciens* RU-OR cells was detected by streaking the cells on agar containing the chromogenic substrates 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). 50 µl of a 20 µg/mL X-Gluc solution was used per agar plate to detect β-glucuronidase enzyme activity and 40 µl of a 40 µg/mL X-Gal

solution was used per agar plate to detect β-galactosidase enzyme activity. Blue colonies were selected as cells that expressed β-glucuronidase or β-galactosidase enzyme activities in the presence of the chromogenic substrates.

Quantitative reporter enzyme assays

Starter cultures of *E. coli* K-12 (Appendix 8) and *E. coli* DH5α cells were grown to stationary phase in 50 mL of LB broth at 37 °C with shaking at 200 rpm. These starter cultures were then used to inoculate 100 mL of LB broth, *E. coli* GMM, LMM, GlyMM or GlyMM containing IPTG to an OD_{600nm} of 0.02. The cultures were incubated at 37 °C, shaking at 200 rpm; until they reached the desired phase of growth (stationary phase was generally reached after 24 hours). The wild-type strain *E. coli* K-12 was used as a positive control for the detection of β-glucuronidase and β-galactosidase reporter enzyme activity. *A. tumefaciens* RU-AE01 and *A. tumefaciens* RU-OR starter cultures were grown in 50 mL of HMM (Appendix 2-4) to stationary phase at 28 °C with shaking at 200 rpm, and then used to inoculate 100 mL of LB broth containing 1% hydantoin (Appendix 2-3), *A. tumefaciens* GMM, LMM, GlyMM, GlyMM containing IPTG (Appendix 2-4) or HMM to an OD_{600nm} of 0.02. The inoculated cultures were incubated at 28 °C, shaking at 200 rpm, until the desired phase of growth was reached (stationary phase was generally reached after 20 hours in GMM, after 36 hours in LMM, GlyMM and GlyMM containing IPTG and after 72 hours in LB broth containing 1% hydantoin and HMM). Absorbance readings for the *E. coli* and *A. tumefaciens* cultures were monitored using a Shimadzu UV-visible spectrophotometer with the uninoculated medium acting as a blank.

The *E. coli* and *A. tumefaciens* cells were collected at 3800 x g at 10 °C, washed in a half volume of 0.1 M potassium phosphate buffer (pH 9.0) and collected by centrifugation as above. The wet cell mass of the pellet was determined and used to resuspend the cells at 40 mg wet cell mass per mL in 0.1 M phosphate buffer (pH 9.0). This cell suspension was used for quantitative reporter enzyme assays.

The protocol for β-glucuronidase enzyme assays was adapted from Jefferson *et al.* (1986) and the protocol for β-galactosidase enzyme assays from

Miller (1992). Detailed protocols for quantitative assays for β-glucuronidase and β-galactosidase activities are described in Appendices 5-3 and 5-4 respectively. Each experiment was independently repeated at least three times with freshly transformed cells. The β-galactosidase activity was calculated in Miller Units using the following formula:

$$\frac{1000 \text{ X } (A_{420nm} - (1.75 \text{ X } A_{550nm}))}{A_{600nm} \text{ X } (\text{Time}_{final} - \text{Time}_{initial}) \text{ X Volume (mL)}}$$

and the β-glucuronidase activity was calculated in β-Gluc Units using the formula:

$$\frac{1000 \text{ X } (A_{405nm} - (1.75 \text{ X } A_{550nm}))}{A_{600nm} \text{ X } (\text{Time}_{final} - \text{Time}_{initial}) \text{ X Volume (mL)}}$$

The β-galactosidase and β-glucuronidase enzyme activities were both calculated as a function of the cell density as described by Miller (1992). The β-galactosidase and the β-glucuronidase activities varied between sets of experiments (< 30%) but the standard mean error between triplicates in each assay was very low and not significant (Valsa and Felzenszwalb 2001).

2.2.6 Construction of recombinant promoter-probe plasmids

Unless otherwise stated the Expand High Fidelity PCR System was used at an annealing temperature of 52 °C according to the manufacturer's instructions to amplify the desired region. The PCR amplification products were inserted into pGEM-T-Easy. The sequences of all PCR products were determined with the universal primers (Table 2-2) using the ABI Prism Big Dye Terminator (Amersham) protocol (Appendix 6-3).

Construction of pMJ26

The primers Chao F1 and Chao R1 (Table 2-2) were used to amplify a 1.2 kb fragment from the construct pG4 (Hartley 2001); which encoded the *A. tumefaciens* RU-OR *hyuC* coding sequence and 120 nt of the 5' preceeding sequence. The promoter of RU-OR *hyuC1* was identified by subjecting the PCR product to partial restriction digestion with the restriction enzyme *Sau* 3A1 and cloning the resulting fragments into the *Bgl* II site of pMJ17. Cells that were blue on

LA plates containing X-Gal were selected for further analysis. The recombinant plasmid pMJ26 was identified as the construct that encoded the RU-OR *hyuC1* promoter.

Construction of pMJ258 and pMJ260

The region between *hyuH* and *hyuC* in pMJ10 was amplified using the primers MJ9 and MJ10. The PCR product was digested with the restriction enzyme *Bgl* II and cloned into the *Bgl* II site of pMJ242 (Figure 2-7).

2.3 RESULTS AND DISCUSSION

2.3.1 DNA sequence analysis of pGH45

Initial sequencing of the *A. tumefaciens* RU-AE01 genomic DNA insert in pGH45 with the universal primer pUCR (Figure 2-1) revealed the presence of the 8 amino-terminal amino acids of *hyuH* coding sequence, 467 nt of sequence between the *hyuH* and *hyuC* coding sequence and 99 amino-terminal amino acids of the *hyuC* coding sequence. This demonstrated that the layout of *hyuH* and *hyuC* in *A. tumefaciens* RU-AE01 was similar to the layout of *hyuH* and *hyuC* in *Agrobacterium* sp. IP I-671 and *A. tumefaciens* NRRL B11291 (Figure 2-2).

The nucleotide sequence of the RU-AE01 genomic DNA insert obtained with the universal primer pUCF (Figure 2-1) showed that *hyuC* in RU-AE01 was not followed by any open reading frames (ORFs). Instead, 250 nt after the termination of the coding sequence for RU-AE01 *hyuC,* there was 200 nt of DNA sequence similar to sequence from IP I-671. These 200 nt corresponded to the intergenic sequence between *hyuA* and ORF 2 in IP I-671 (Figure 2-2).

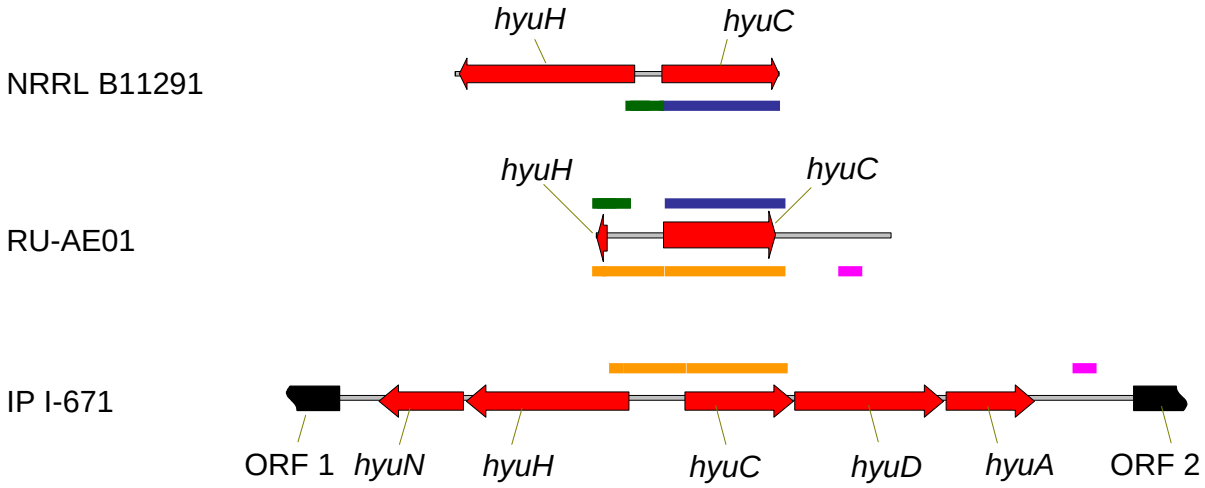


Figure 2-2: The nucleotide sequence identity between the *A. tumefaciens* RU-AE01 genomic DNA insert in pGH45 and the *hyu* operons from *A. tumefaciens* NRRL B11291 and *Agrobacterium* sp. IP I-671. The partial 5' sequence of *hyuH*, the region between *hyuH* and *hyuC* and the complete sequence of *hyuC* are depicted for RU-AE01. The complete *hyu* gene clusters are depicted for NRRL B11291 and IP I-671. Abbreviations: ORF 1- Open Reading Frame 1 which encodes a putative transposase, *hyuN*- gene encoding an NADPH dependent flavin oxidoreductase, *hyuH*- an hydantoinase encoding gene, *hyuC*- gene encoding an NCAAH, *hyuD*- D-amino acid dehydrogenase encoding gene, *hyuA*- gene encoding an racemase and ORF 2- Open Reading Frame 2 which encodes a putative resolvase.

Sequencing of the insert in pGH45 enabled the design of primers MJ1 and MJ2 to amplify and clone the RU-AE01 *hyuH-hyuC* sequence into pGEM-T Easy generating the construct pMJ10. This was done to determine the nucleotide sequence the RU-AE01 *hyuH-hyuC* region with universal primers in both directions to enable a detailed bioinformatics analysis of the region (Section 4.3.1). The sequence of the PCR product in pMJ10 was validated by comparing its sequence to pGH45.

2.3.2 Design and construction of a broad host range promoter-probe vector for *A. tumefaciens* strains RU-AE01 and RU-OR

The presence of the entire region between the *hyuH* and *hyuC* ORFs confirmed that this sequence contained a potentially divergent promoter and that this could be used in studies on the regulation of the expression of these two genes. The unavailability of promoter-probe vectors for use in *A. tumefaciens* necessitated the design and construction of a broad host range vector with a reporter gene system that was suitable for analyzing divergent promters. The requirements for such a vector were

suitable antibiotic markers, a stable broad host range origin of replication and two reporter enzymes.

Identification of suitable antibiotic markers

A. tumefaciens RU-AE01 and *A. tumefaciens* RU-OR cells were streaked onto LB agar plates and both strains were found to be resistant to ampicillin and kanamycin but sensitive to chloramphenicol and tetracycline. Luo and Farrand (1999) reported that genes conferring resistance to tetracycline were amongst the most abundant of the identified drug resistance elements. Furthermore, it was a well recognized observation that *Agrobacterium* C58 and its derivatives gave rise at a high frequency to spontaneous mutants resistant to tetracycline (Luo and Farrand 1999). For this reason, chloramphenicol was chosen as the selectable marker for the promoter-probe vector.

Construction of the basic host range vector

While a number of plasmids have been designed to analyze transcriptional and translational activation of reporter genes by promoters, few plasmids have been studied in *A. tumefaciens* strains. Plasmids that have been tested and shown to replicate in *A. tumefaciens* strains belong to the IncP (for example RK2), the IncQ (for example pSUP and pTF-FC2) and the IncW (for example pSa) incompatibility groups (Knauf and Nester 1982, Priefer *et al.* 1985, Dorrington and Rawlings 1990, Tait *et al.* 1983).

The plasmid pSa is not as well understood as the plasmids RK2 and pTF-FC2. While both pTF-FC2 and RK2 have been studied in detail, the plasmid pTF-FC2 has some advantages. First, the host range of the plasmid pTF-FC2 includes all the gram-negative bacteria tested including *E. coli, P. aeruginosa*, *T. novellas*, *R. melitoti*, and *A. tumefaciens* (Dorrington and Rawlings 1989, Dorrington *et al.* 1991, Rawlings *et al*. 1986, Dorrington and Rawlings 1990). Second, pTF-FC2 is relatively small (12.4 kb) compared to RK2 (56.4 kb) (Dorrington and Rawlings 1989, Figurski *et al.* 1979). Thirdly, pTF-FC2 has been fully sequenced (Rawlings and Kusano 1994) and the minimum replicon required for the autonomous replication of the plasmid has been identified (a 4.9 kb *Cla* I – *Eco* R1 fragment of pTF-FC2) (Dorrington *et al.* 1991). The minimum replicon was cloned into

pBluescript SK (Stratagene) by Dorrington *et al.* (1991) generating the plasmid pTV100. This construct was used as the starting point for a broad host range vector.

A *Csp* 451 DNA fragment encoding the *chloramphenicol acetyltransferase* (*cat*) gene in pBR325 was inserted into the *Cla* I site of pTV100 creating the plasmid pMJ11 (Figure 2-3, Step I). *E. coli* DH5α cells containing pMJ11 were blue in colour when streaked on LB agar containing X-Gal, suggesting that the activation of *cat* also activated the transcription of *lac alpha b* (Figure 2-3, Step I). This implied that the *cat* gene did not have a functional terminator sequence. All the recombinant plasmids analysed in this experiment contained the *cat gene* inserted in the opposite orientation to the broad host range origin of replication, suggesting that the transcription of *cat* interfered with the replication of the plasmid.

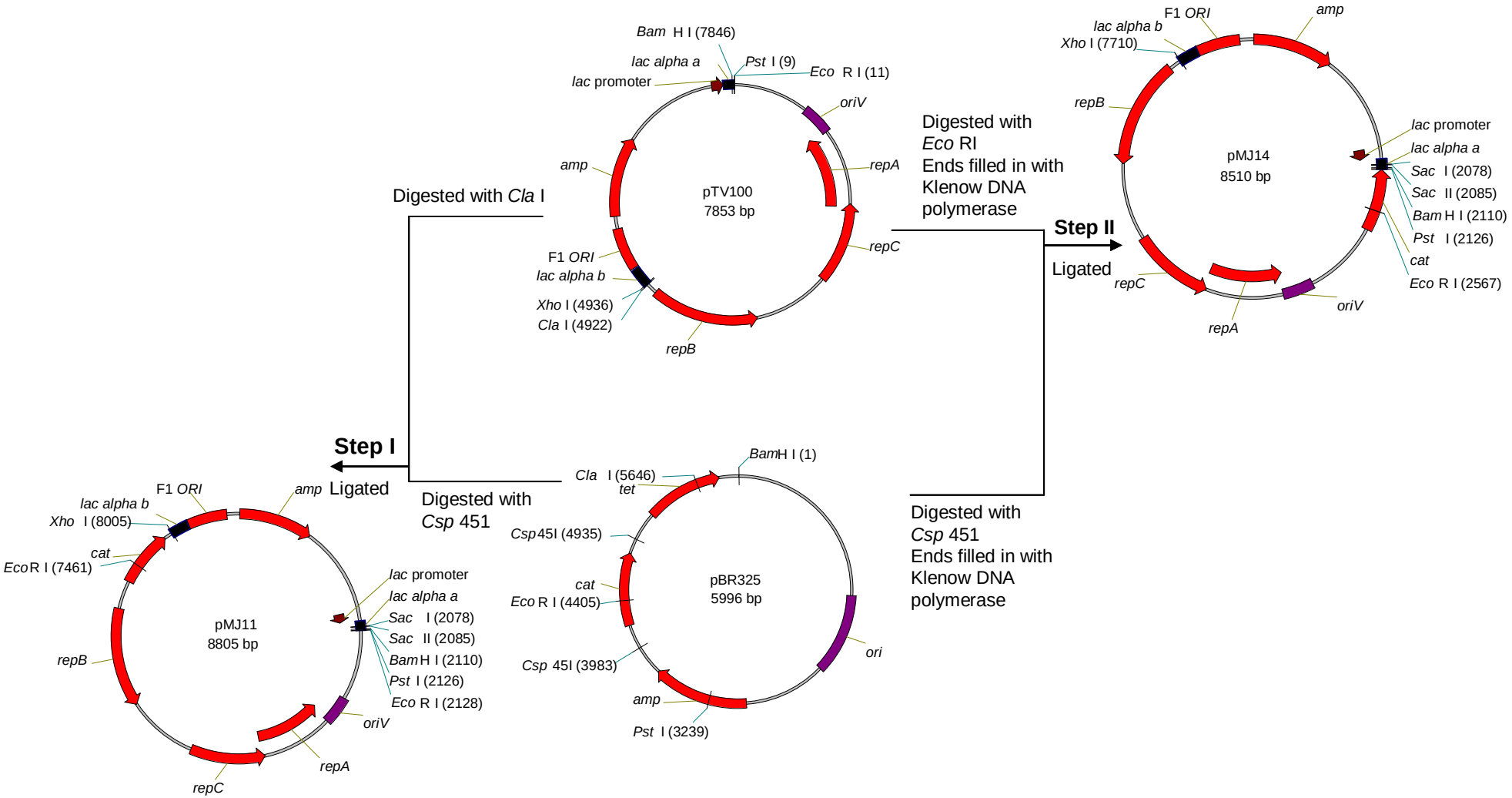


Figure 2-3: A flow diagram representing the construction of chloramphenicol resistant broad host range vectors pMJ11 and pMJ14.

To test the origin of replication and the antibiotic marker, *A. tumefaciens* RU-AE01 cells and *A. tumefaciens* RU-OR cells were electroporated with pMJ11. Transformants were selected on LB agar plates containing chloramphenicol. An average of 10^4 colonies per ug of plasmd DNA was obtained for *A. tumefaciens* RU-AE01 and *A. tumefaciens* RU-OR cells transformed with pMJ11, while no colonies were observed on plates with untransformed cells or cells transformed with pBR325, which does not replicate in *Agrobacterium* cells. Chloramphenicol resistant colonies were streaked onto successive LB agar plates contaning chloramphenicol and no decrease in cell viability was observed, demonstrating that the pTF-FC2 origin of replication was stably maintained within the *A. tumefaciens* strains RU-AE01 and RU-OR.

The activation of *lac alpha b* by *cat* in pMJ11 implied that any reporter gene introduced downstream of the *cat* is likely to be activated as well, which would obviously be a serious problem. The solution was to relocate the *cat* gene to the opposite end of the pTF-FC2 origin of replication in pTV100 such that it would be transcribed in the opposite orientation to the *lac alpha a* coding sequence. The ends of *Csp* 451 DNA fragment carrying *cat* were filled in with Klenow DNA polymerase. This fragment was inserted into the *Eco* RI site of pTV100, which had also been treated with the Klenow fragment, generating the plasmid pMJ14 (Figure 2-3, Step II). Again, all the recombinants isolated had *cat* facing away from the origin of replication supporting the theory that transcription of the *cat* gene in the direction of the origin was inhibitory to plasmid replication. *E. coli* DH5α cells hosting pMJ14 remained white in colour on LA plates containing X-Gal demonstrating that the *lac alpha* gene on pMJ14 was no longer being activated by *cat.* The appropriate reporter genes could therefore be selected for use in the broad host range vector.

Selection of β-galactosidase and β-glucuronidase as reporter enzymes

As previously discussed, the enzymes β-glucuronidase and β-galactosidase have been used frequently as reporters in promoter-probes. Initial plate assys for β-glucuronidase and β-galactosidase enzyme activities in the RU-AE01 and RU-OR cells showed that cells of both strains were white in colour when plated on LB agar containing X-Gluc and blue in colour when plated on LB agar containing X-Gal. This indicated that the *gus* gene was not present in the two strains but the *lacZ* gene was present in both RU-AE01 and RU-OR strains. As the *gus* gene is

found mainly in *E. coli* strains and in very few other strains (Rice *et al.* 1991), *gus* is an ideal reporter gene. This was verified by subjecting the RU-AE01 and RU-OR cells to quantitative β-glucuronidase assays (Figure 2-4B).

In contrast to *gus*, the *lacZ* gene is widely distributed in gram-negative bacteria in the environment (Erikson and Steers 1970). The regulation of *lacZ* transcription is sensitive to CCR (Tyler and Magasanik 1962). However the expression of the Hyu enzymes in RU-OR is subject to regulation by the nitrogen levels within the cells (Hartley *et al.* 2001). Thus *lacZ* appears to be an appropriate reporter gene for analysis of the *hyuH* and *hyuC* promoters in RU-AE01 and RU-OR. To ensure that the endogenous RU-AE01 and RU-OR β-galactosidase enzyme activity could be suppressed in the media chosen for the analysis of the *hyuH* and *hyuC* promoters, β-galactosidase assays were done on RU-AE01 and RU-OR cells in media with repressive and non-repressive carbon sources (Figure 2-4A).

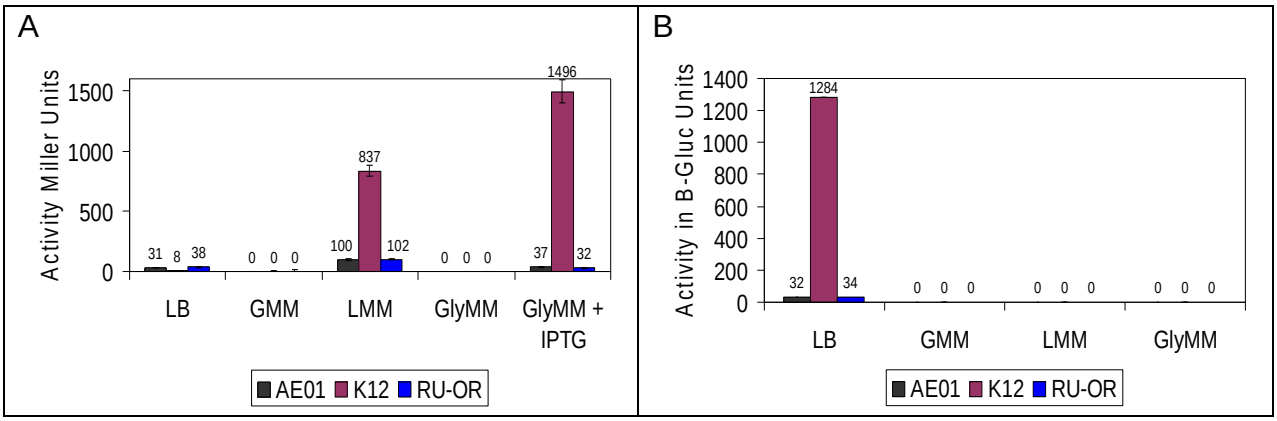


Figure 2-4: β-galactosidase and β-glucuronidase activities in native *E. coli* and *A. tumefaciens* strains. The β-galactosidase (A) and β-glucuronidase (B) activities in *A. tumefaciens* RU-AE01, *A. tumefaciens* RU-OR and *E. coli* K-12 cells. Abbreviations: LB- LB broth containing 1% hydantoin, GMM- glucose minimal medium, LMM- lactose minimal medium, GlyMM- glycerol minimal medium and GlyMM + IPTG- glycerol minimal medium supplemented with the inducer 1 mM IPTG. Results are the average of triplicate reactions. The standard mean error is shown by the bars.

E. coli K-12 cells showed high levels of β-galactosidase activity when induced in LMM and in GlyMM containing IPTG (837 and 1496 Miller units respectively). In constrast, low levels of β-galactosidase enzyme were detected in *A. tumefaciens* RU-AE01 (100 and 37 Miller units) and RU-OR cells (102 and 32 Miller units). The levels of β-galactosidase present in the media (LB broth containing 1% hydantoin and GMM) to be used for the growth and analysis of *A. tumefaciens* RU-AE01 and

A. tumefaciens RU-OR cells were negligible (Figure 2-4A). This data showed that while there was some β-galactosidase activity in both *Agrobacterium* strains, it could be repressed by the addition of glucose as a carbon source. The *lacZ* gene encoding β-galactosidase could therefore be used as a reporter gene if two criteria were met. First, that the levels of transcription from the test promters produced β-galactosidase activity that could be detected over the background levels. Second, that the promoters being analyzed were not subject to carbon catabolite repression.

With respect to β-glucuronidase activity, *E. coli* K-12 cells showed high levels of β-glucuronidase activity (1284 β-Gluc units) whereas there were very low levels of enzyme activity in *A. tumefaciens* RU-AE01 and *A. tumefaciens* RU-OR grown in LB broth containing 1% hydantoin (32 and 34 β-Gluc units respectively). No β-glucuronidase activity was detected for *E. coli* K-12, *A. tumefaciens* RU-AE01 or *A. tumefaciens* RU-OR cells in GMM, LMM or GlyMM (Figure 2-4B), which indicated that *gus* would be an appropriate reporter gene in the promoter-probe vector for *Agrobacterium* promoters.

The substrate in the quantitative assays for β-glucuronidase activity is PNPG; which is hydrolysed at low levels by β-galactosidase (Oakes *et al.* 2001). If PNPG was being hydrolyzed at low levels by β-galactosidase enzyme activity when RU-AE01 cells and RU-OR cells were cultured in LB, then PNPG hydrolysis would also have been observed in the β-glucuronidase reporter enzyme assays for RU-AE01 cells and RU-OR cells grown in LMM; under which conditions β-galactosidase enzyme activity was detected (Figure 2-4A). No β-glucuronidase activity was detected in *Agrobacterium* cells cultured in LMM (Figure 2-4B). This suggests that the low levels of β-glucuronidase enzyme activity detected for both RU-AE01 cells and RU-OR cells grown in LB broth are background levels of β-glucuronidase enzyme activity and are negligible. This is supported by the high level of β-glucuronidase enzyme activity detected for *E. coli* K-12 cultured in LB broth (1284 β-Gluc units, Figure 2-4B).

Construction of a single reporter enzyme promoter-probe vector

A 5.5 kb *Bam* HI - *Pst* I fragment containing all but the first three amino acids of the *lacZ* coding sequence from pHP41 (Park *et al.* 1992) was inserted into the *Bam* HI and *Pst* I sites of pMJ14 generating the plasmid pMJ16 (Figure 2-5 Step I). To generate a functional *lacZ* coding sequence, a double stranded oligonucleotide (MJ5/MJ6), which contained a single *Bgl* II restriction site, a ribosome binding site (RBS) and the missing amino terminal codons, was cloned into the *Bam* HI and *Xho* I sites of pMJ16 (Figure 2-5 Step II). The resulting construct, pMJ17, contained a promoterless-*lacZ* reporter gene with a functional ribosome binding site and a *Bgl* II restriction site that could be used for inserting promoter fragments upstream of *lacZ.*

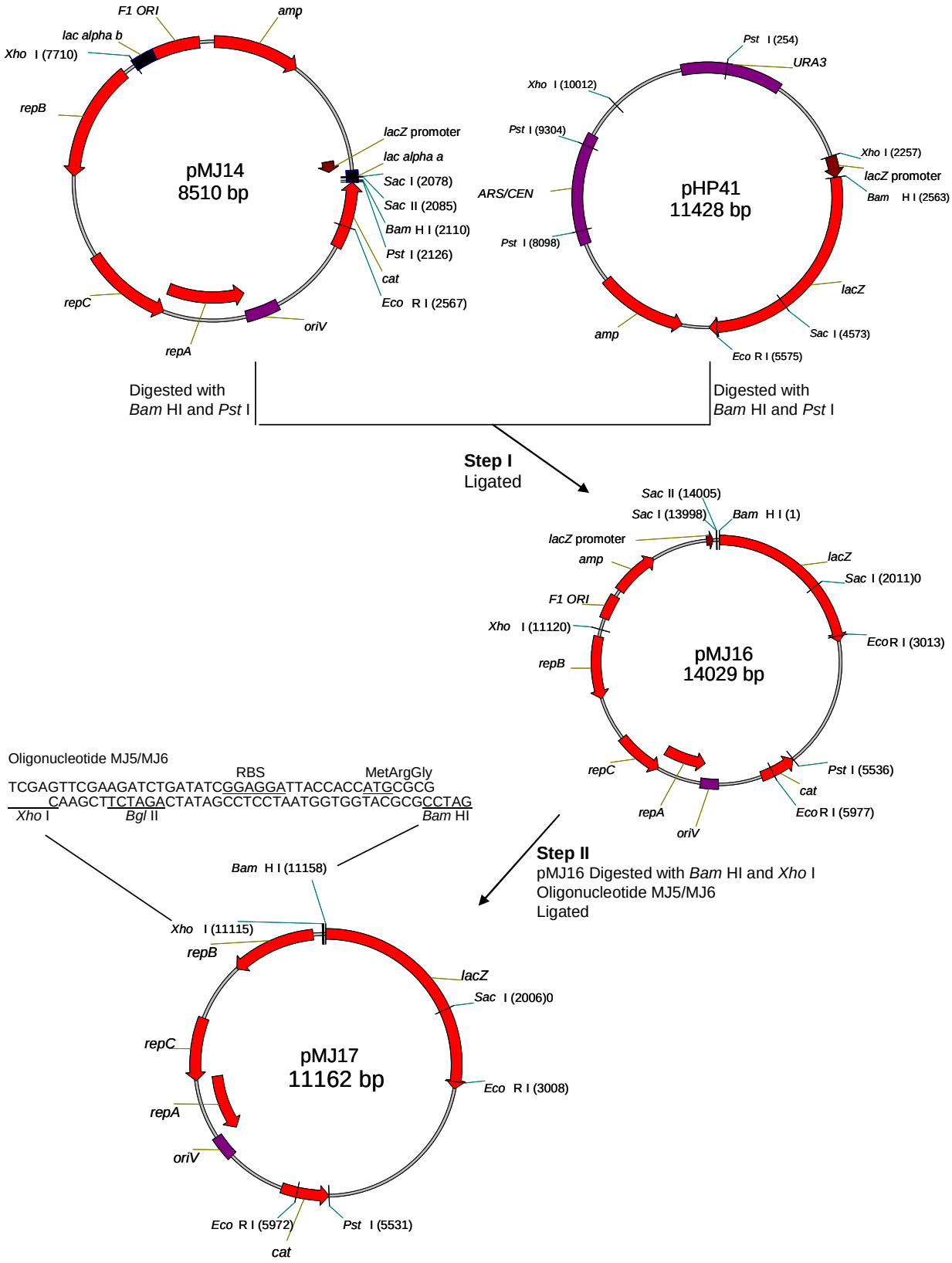


Figure 2-5: Steps involved in the contruction of the unidirectional promoter-probe pMJ17.

To validate pMJ17 as a promoter probe, a 432 nt *Sau* 3AI DNA fragment derived from the *A. tumefaciens* RU-OR *hyuC1* gene (pG4) was inserted into the *Bgl* II site of pMJ17. *E. coli* DH5α cells containing recombinant plasmids were then selected for their ability to activate transcription of *lacZ*. The insert in the recombinant, pMJ26, contained 270 nt of the RU-OR *hyuC1* coding sequence with 162 nt upstream corresponding to the promoter region that was active in *E. coli* (Hartley 2001) (Figure 2-6A). Next, pMJ26 was tested in *Agrobacterium* cells. As expected, the vector pMJ17 supported low levels of β-galactosidase activity compared to pMJ26 (10 versus 147 Miller units, respectively), showing a 14-fold increase in transcriptional activity when the RU-OR *hyuC1* promoter region was present (Figure 2-6B). This result confirmed the functionality of the *lacZ*-fusion construct and demonstrated that the activity resulting from activation by test promoters was easily detected above background enzyme levels.

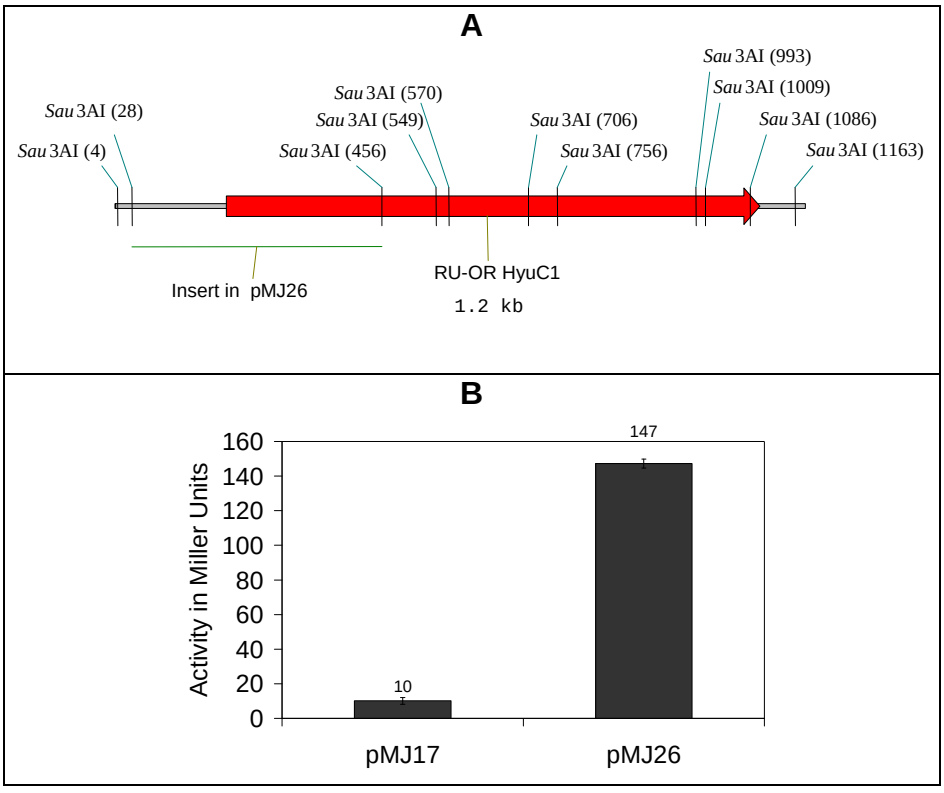


Figure 2-6: β-galactosidase activity in *A. tumefaciens* RU-OR cells transformed with pMJ26. (A) A diagrammatic representation of the *A. tumefaciens* RU-OR DNA insert in pMJ26, where *lacZ* is in the same orientation as *hyuC1.* (B) β-galactosidase assays of promoter-probe vector, pMJ17, and promoter-containing construct, pMJ26. Results are the average of triplicate reactions. The standard mean error is shown by the bars.

Of concern was the observation that, as with the *cat* gene, no recombinants were isolated in which the *hyuC1* promoter was oriented towards the broad host range origin of replication confirming that transcription activation sequences in the direction of the origin of replication affected plasmid replication. Since the RU-AE01 *hyuH-hyuC* region encodes divergent promoters for the *hyuH* and *hyuC* genes, cloning this fragment into pMJ17 could potentially be a problem.

Construction of a bidirectional promoter-probe vector

To allow the cloning of divergent promoters between the *repB* and *lacZ* genes, an oligonucleotide encoding a consensus *Agrobacterium* terminator sequence (MJ28/MJ29) was designed. This sequence would terminate the transcription initiated by divergent promoters inserted upstream of *lacZ* before they interfered with the regulation of *repB;* preventing the negative effect on plasmid replication observed in pMJ17. The consensus terminator sequence was derived from twenty *A. tumefaciens rho*-independent terminator sequences (Appendix 1-2) which were selected from a list available at The Institute of Genomic Research website (http://www.tigr.org/software/TransTermResults/ntat01.html). The double stranded oligonucleotide MJ28/MJ29 was inserted into the *Xho* I and *Bgl* II restriction sites of pMJ17, which are upstream of *lacZ,* generating pMJ185 (Figure 2-7 Step I).

To determine if the terminator was effective, attempts were made to insert the RU-OR *hyuC1* promoter and the divergent RU-AE01 *hyuH-hyuC* promoters upstream of *lacZ.* Plasmid constructs with the RU-OR *hyuC1* promoter in the opposite direction to *lacZ* were found but no plasmids containing the RU-AE01 *hyuH-hyuC* promoters were isolated. The inability to insert the RU-AE01 *hyuH-hyuC* promoters in the same orientation as *repB* in pMJ185 indicates that the terminator sequence was not effective against the activation sequences in the RU-AE01 *hyuH-hyuC* promoters. This implies that the RU-AE01 *hyuH-hyuC* promoters are stronger than the RU-OR *hyuC1* promoter which could be cloned in the same orientation as *repB.*

As the pTF-FC2 *repB* gene was sensitive to the presence of upstream activation sequences, a decision was made to invert the entire origin of replication to distance the *rep* genes from the promoters. The plamid pMJ185 was digested with *Xho* I and *Pst* I, the termini were filled in using the Klenow fragment and the fragments self-ligated generating pMJ203 (Figure 2-7 Step II). In pMJ203, the *cat* gene was

upstream of *lacZ* and in the same orientation but *E. coli* DH5α cells containing pMJ203 were white in colour when plated on LB agar containing X-Gal. This indicated that the activation of *cat* transcription in pMJ203 did not lead to activation of *lacZ* transcription as had occurred previously in pMJ11. This was attributed to the presence of the terminator sequence between the *cat* and *lacZ* genes. To verify that the inversion of the origin of replication now enabled the insertion of divergent promoters, the RU-AE01 *hyuH-hyuC* promoters were inserted into the *Bgl* II site of pMJ203. This proved that the presence of promoters in the same orientation as the origin of replication had a deleterious effect on plasmid replication and that in pMJ203, *cat* acted as 'stuffer' DNA preventing the promoter fragments from interfering with the origin of replication.

In pMJ203, only the RU-AE01 *hyuH* or *hyuC* promoter that was in the same orientation as *lacZ* could be analyzed. To enable the simultaneous analysis of both the RU-AE01 *hyuH* and the RU-AE01 *hyuC* promoters in pMJ203, a second reporter gene was inserted in the opposite orientation to *lacZ*. An oligonucleotide MJ44/MJ45 was designed with the sequence for *Bgl* II and *Pst* I restriction sites required for the introduction of *gus* into pMJ203. As the promoter-probe vector pMJ203 was designed to analyse transcriptional and translational activation of *gus* and *lacZ* by the RU-AE01 *hyuH-hyuC* promoters, the same oligonucleotide MJ44/MJ45 was also used to delete the ribosomal binding site (RBS) of *lacZ* that was present in pMJ17. The oligonucleotide MJ44/MJ45 was therefore inserted into the *Bam* HI and *Xho* I sites of pMJ203 generating pMJ224 (Figure 2-7 Step III).

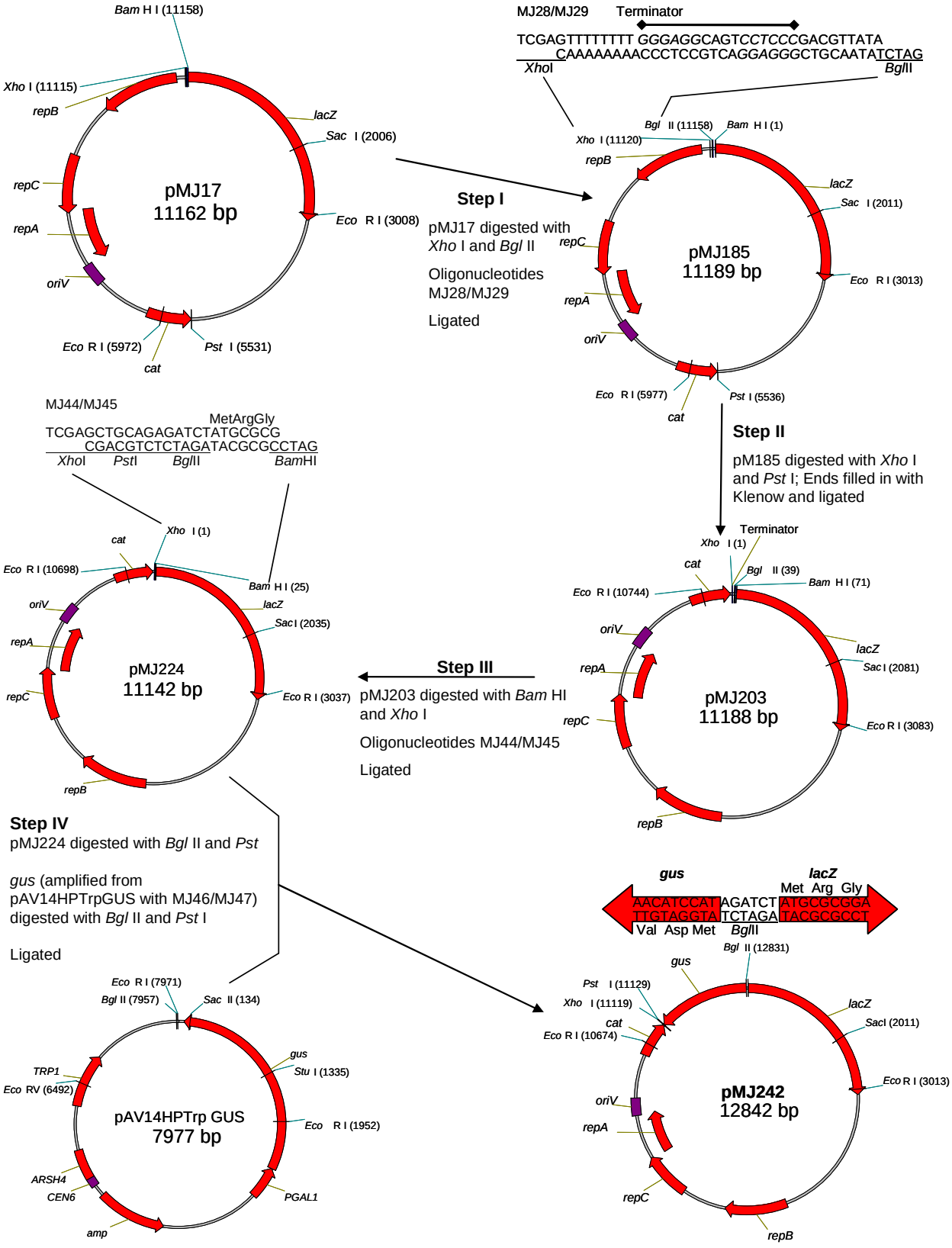


Figure 2-7: Construction of a bidirectional promoter-probe vector pMJ242

The *gus* gene, encoding the β-glucuronidase enzyme, was present on pAV14HPTrpGUS, a plasmid derived from pCAMBIA (CAMBIA, Canberra, Australia). The coding sequence for *gus* was amplified from pAV14HPTrpGUS using the PCR primers MJ46 and MJ47 (Table 2-2) and the resultant PCR product was inserted into the *BgI* ll and *Pst* I restriction sites of pMJ224 generating the plasmid pMJ242 (Figure 2-7 Step IV).

The final vector pMJ242 therefore has a broad host range origin of replication that is functional in *E. coli* and *A. tumefaciens* cells, a *cat* antibiotic resistance gene and promoterless reporter genes *gus* and *lacZ* to simultaneously analyze divergent transcriptional and translational activation by promoters of interest; in this case the RU-AE01 *hyuH* and *hyuC* promoters (Figure 2-7).

2.3.3 Insertion of the RU-AE01 *hyuH-hyuC* fragment into pMJ242

Primers MJ9 and MJ10 (Table 2-2) were used to specifically amplify the intergenic region between the *hyuH* and *hyuC* from pMJ10, excluding any *hyuH* or *hyuC* coding sequence (Figure 2-8A). The PCR product was flanked by *Bgl* II sites, which were used to insert the amplification product into the *Bgl* II site of pMJ242 (Figure 2-7). This resulted in two recombinant plasmids, pMJ258 and pMJ260, which had the promoter fragment oriented in opposite directions (Figure 2-8B). In pMJ258 the *hyuH* promoter and ribosome binding site was fused to the *gus* coding sequence and the *hyuC* promoter and ribosome binding site fused to the *lacZ* coding sequence. Contrarily, in pMJ260 the *hyuH* promoter and ribosome binding site was fused to the *lacZ* coding sequence and the *hyuC* promoter and ribosome binding site was fused to the *gus* coding sequence. Due to the presence of two reporter enzymes in opposing orientations, the promoter activity of both the *hyuH* and the *hyuC* promoters could be investigated simultaneously using β-glucuronidase and β-galactosidase enzyme assays. In addition, having constructs in which the same promoter sequence is placed upstream of both the *gus* and the *lacZ* coding sequence enables the analysis of a promoter using two reporter genes and direct comparison of the generated data.

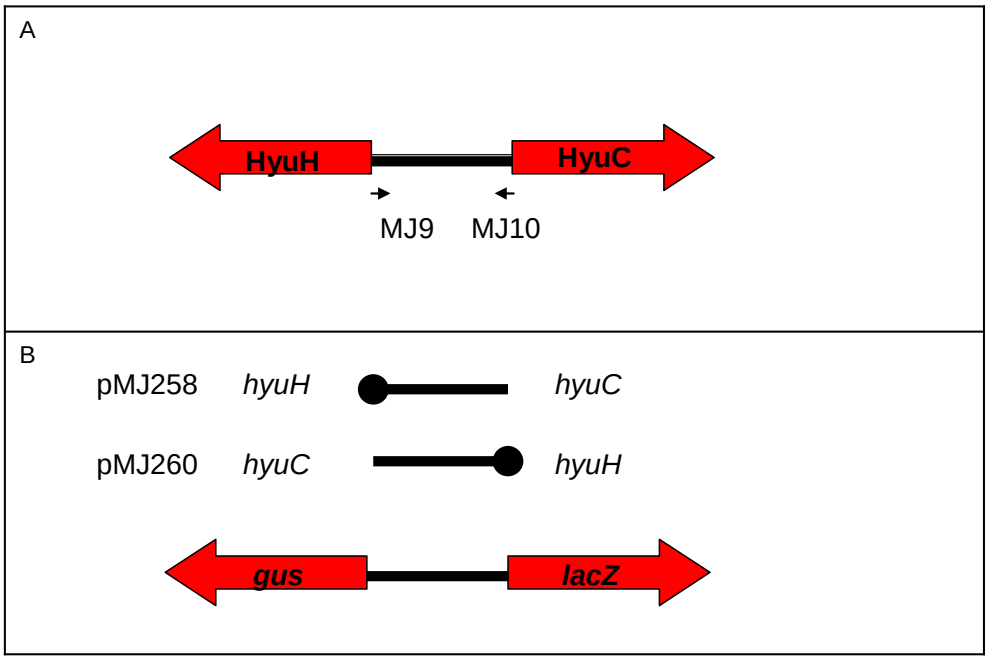


Figure 2-8: The *A. tumefaciens* RU-AE01 *hyuH-hyuC* promoters present in pMJ242. (A) A diagram depicting the binding sites for the primers MJ9 and MJ10 which were designed to amplify the 0.5 kb of sequence between the *hyuH* and *hyuC* coding sequence from the plasmid pMJ10. The *hyuH* and the *hyuC* coding sequences are shown. (B) The *A. tumefaciens* RU-AE01 promoter sequences present in the vector pMJ242; pMJ258 has the *gus* coding sequence on the *hyuH* side and the *lacZ* coding sequence on the *hyuC* side; pMJ260 has the *gus* coding sequence on the *hyuC* side and *lacZ* coding sequence on the *hyuH* side. The coding sequence for the two reporter enzymes, *gus* and *lacZ*, are depicted

2.3.4 Reporter gene assays in *E. coli*

There was evidence that at least the *hyuC* promoter and possibly the *hyuH* promoter were active in *E. coli* as NCAAH activity was detected in recombinant *E. coli* cells containing pGH45. To verify this, *E. coli* DH5α cells containing pMJ258 were grown in LB broth containing 1% hydantoin to establish the levels of of β-glucuronidase and β-galactosidase enzyme expression, the patterns of enzyme expression and the identification of the optimal stage of growth for enzyme assays in *E. coli* DH5α.

The growth of *E. coli* DH5α cells containing pMJ258 was monitored by measuring the OD_{600nm} (Figure 2-9A). The cells were observed to enter stationary growth phase at 13h. Assays of β-glucuronidase and β-galactosidase enzyme activity demonstrated transcriptional activity in both the *hyuH* and the *hyuC* directions. The *hyuH* promoter-derived transcription and translation of *gus* and Gus respectively enabled the detection of β-glucuronidase enzyme activity. Similarly the *hyuC*

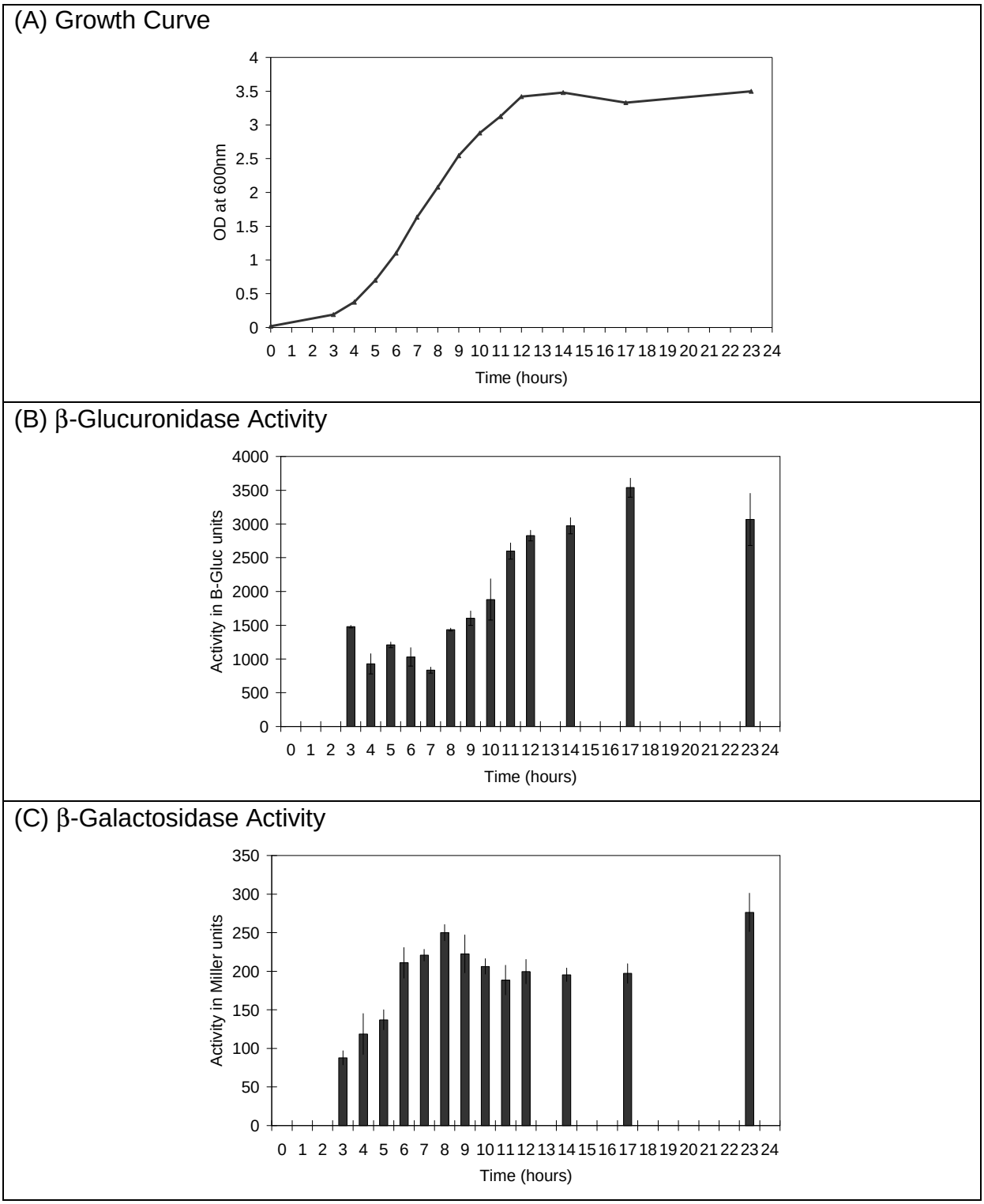


Figure 2-9: Reporter gene assays of *hyuH* and *hyuC* promoter activity in *E. coli* DH5α cells containing pMJ258 during culture in LB broth containing 1% hydantoin. (A) Growth of *E. coli* DH5α cells containing pMJ258 over 23h monitored at OD_{600nm}, (B) β-glucuronidase enzyme activity reported as β-Gluc units and (C) β-galactosidase enzyme activity reported as Miller units. Enzyme assays are an average of triplicate reactions. The standard mean error is shown for each reaction by the vertical bars.

promoter-derived transcription of *lacZ* and translation of LacZ led to the detection of β-galactosidase enzyme activity. This indicated that both the *hyuH* and *hyuC* promoters were functional in *E. coli* DH5α cells containing pMJ258 (Figure 2-9B and 2-9C). The β-glucuronidase activity reached a peak after 17h of growth (Figure 2-9B) and the β-galactosidase activity reached a peak after 8h of growth (Figure 2-9C) and these levels of β-glucuronidase and β-galactosidase were maintained throughout stationary growth phase (Figure 2-9A, 2-9B and 2-9C). The optimal time for assaying β-glucuronidase and β-galactosidase enzymes in *E. coli* DH5α cells containing pMJ258 was chosen as 20h.

E. coli DH5α cells containing the plasmids pMJ258 and pMJ260 (Figure 2-8) were analyzed to compare the β-glucuronidase enzyme activity derived from the *hyuH* promoter and ribosomal binding site to the β-galactosidase enzyme activity derived from the *hyuH* promoter and ribosomal binding site. Similarly, the levels of β-glucuronidase enzyme activity derived from the *hyuC* promoter and ribosomal binding site and the levels of β-galactosidase enzyme activity derived from the *hyuC* promoter and ribosomal binding site obtained from *E. coli* DH5α cells hosting the plasmids pMJ258 and pMJ260 were evaluated.

When fused to the *gus* reporter gene, the *hyuC* and *hyuH* promoters supported 1486 and 4291 units of β-Gluc enzyme activity respectively (Figure 2-10A). Similarly, when the promoters were fused with the *lacZ* gene, the *hyuC* promoter supported 244 Miller units of β-galactosidase activity while 531 Miller units of activity were detected from the *hyuH* promoter (Figure 2-10B). The results from both data sets indicated that the *hyuH* promoter supported higher levels of transcription than the *hyuC* promoter in *E. coli* cells.

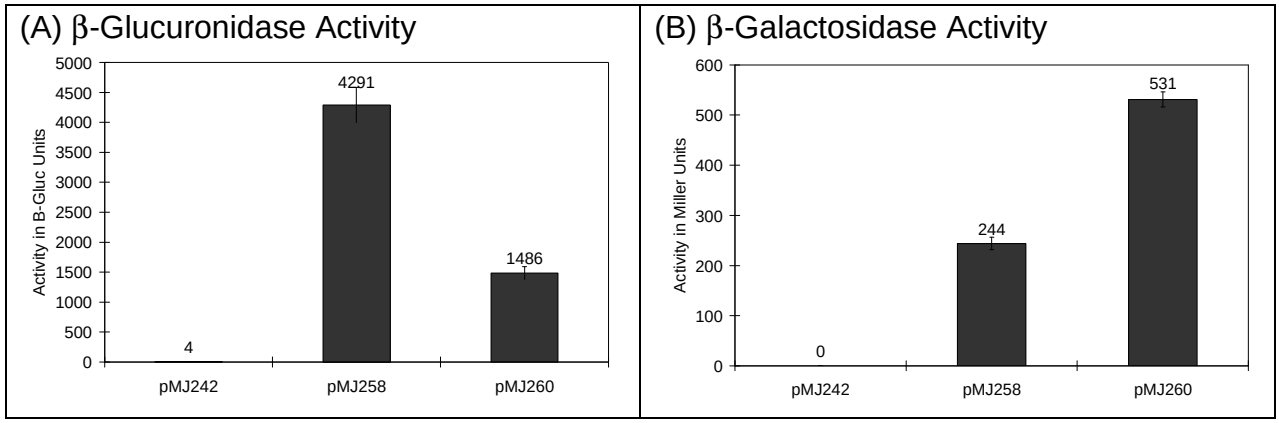


Figure 2-10: β-glucuronidase and β-galactosidase activities for pMJ242, pMJ258 and pMJ260 in *E. coli* DH5α cells grown in LB broth containing 1% hydantoin. (A) β-glucuronidase enzyme activity is reported as β-Gluc units. (B) β-galactosidase activity is reported in Miller units. Results are the average of triplicate reactions. The standard mean error is shown for each reaction by the vertical bars.

2.4 GENERAL CONCLUSIONS

The aims of the research described in this chapter were two-fold: (1) to analyse the nucleotide sequence of the region upstream of the *hyuC* coding sequence in pGH45 and (2) to construct a broad host range promoter-probe plasmid to analyse the transcriptional activity of the *hyuH-hyuC* promoter region.

Sequence analysis of the *A. tumefaciens* RU-AE01 genomic DNA encoding the NCAAH enzyme showed that the insert in pGH45 contained the entire *hyuC* together with the first eight amino acids of the *hyuH* coding sequence. The two genes were oriented divergently as was the case for other *Agrobacterium* strains such as IP I-671 and NRRL B11291 (Grifantini *et al.* 1998, Hils *et al.* 2001). In contrast to IP I-671, no ORFs were detected downstream of *hyuC* in RU-AE01. Instead, 200 nt of sequence similar to 200 nt of IP I-671 sequence between *hyuA* and ORF2 was identified downstream of *hyuC* in RU-AE01.

This indicates that while the arrangement of the *hyuH-hyuC* genes in RU-AE01 is similar to NRRL B11291 and IP I-671, there are some significant differences. The difference is not in the genes as the *hyuH* and *hyuC* genes from the *Agrobacterium* strains are closely related. The IP I-671 *hyuH* and NRRL B11291 *hyuH* share 87% identity at the DNA level and 90% identity at the amino acid level. The

IP I-671 *hyuC,* NRRL B11291 *hyuC,* RU-AE01 *hyuC* and RU-OR *hyuC1* all show greater than 90% identity at both the DNA and amino acid level. Variation is present in the sequence between the divergent *hyuH* and *hyuC* ORFs of RU-AE01, IP I-671 and NRRL B11291 and also in the sequence outside the *hyuH* and *hyuC* ORFs of RU-AE01, IP I-671 and NRRL B11291.

The divergent arrangement of the *hyuH* and *hyuC* genes implies that they are transcribed from divergent promoters. Thus, to enable simultaneous detection of transcriptional activation from the *hyuH-hyuC* promoter region a promoter-probe vector with two divergent reporter genes was required.

The plasmid pMJ17, an intermediate construct with *lacZ* as the single reporter gene, was used to test the functionality of the promoterless *lacZ* reporter gene. The region upstream of a *hyuC1* isolated from *A. tumefaciens* RU-OR (Hartley 2001) was inserted into pMJ17 and used to validate the β-galactosidase reporter system. The apparent inability to insert a DNA fragment into the cloning site of pMJ17 such that the promoter was facing the origin of replication suggested that the pTF-FC2 replicon was sensitive to transcription from active promoters in the direction of the origin of replication. The introduction of a ‘stuffer’ DNA fragment between the origin and the promoter fragments, in pMJ203, alleviated this problem highlighting the importance of efficient transcriptional terminators at the 3’ termini of genes and promoters when designing plasmid vectors based upon the pTF-FC2 origin.

Further modifications led to the development of pMJ242, a broad host range bidirectional promoter-probe vector. This plasmid is suitable for use in a number of gram-negative bacteria other than *E. coli* and *A. tumefaciens*. A limitation in the use of pMJ242 is that it has to be either chemically transformed or electroporated into cells of interest that must be sensitive to the antibiotic chloramphenicol. Another potential limitation of pMJ242 is its size. The largest natural IncQ-like plasmid isolated, pTF-FC14, was reported to be 14 kb (Rawlings and Tietze 2001). It is possible that large inserts could be maintained in pMJ242, a plasmid containing an origin of replication from pTF-FC2, an IncQ-like plasmid. However, it is also likely that the stability of the IncQ-like replicon may be dependent on the sequence of the insert and the size of the insert (Rawlings personal communication).

The RU-AE01 *hyuH-hyuC* promoter region was cloned into pMJ242 in both orientations generating the plasmids pMJ258, with the *hyuH* promoter fused to *gus* and the *hyuC* promoter fused to *lacZ*, and pMJ260, with the *hyuH* promoter fused to *lacZ* and the *hyuC* promoter fused to *gus*. The low levels of β-galactosidase enzyme activity compared to β-glucuronidase enzyme activity indicated that *gus* was more suitable for measuring the activity of the weaker promoter than *lacZ*. The plasmids pMJ258 and pMJ260 were studied in *E. coli* DH5α cells as they are easy to manipulate and thus provide a suitable environment for the isolation and testing of divergent promoter-containing plasmids. Transcription activation sequences from *A. tumefaciens* RU-AE01 were also expected to function in *E. coli* DH5α and this would allow preliminary analyses to be conducted in a strain similar to but less complicated than *A. tumefaciens* strains.

CHAPTER 3

COMPARISON OF THE TRANSCRIPTION FROM THE *hyuH-hyuC* PROMOTER TO THE EXPRESSION OF HyuH AND HyuC IN RU-AE01

3.1 INTRODUCTION

A number of HyuH, HyuC or HyuH and HyuC enzyme-expressing *Agrobacterium* strains have been reported in the literature including *Agrobacterium* sp. IP I-671, *A. radiobacter* CCRC 14924, *A. radiobacter* KNK712, *A. tumefaciens* AM10, *A. tumefaciens* BQL9, *A. tumefaciens* C58 and *A. tumefaciens* NRRL B11291 (reviewed in Section 1.2; Hils *et al.* 2001, Hsu *et al*. 1999, Nanba *et al*. 1998, Sareen *et al.* 2001, Clemente-Jimenez *et al.* 2003, Grifantini *et al.* 1998). While one *hyuC* gene has been isolated from most *Agrobacterium* strains, strain RU-OR contains two genes encoding the NCAAH enzyme, *hyuC1* and *hyuC2* (Hartley 2001).

The detailed study of the factors that affect Hyu enzyme activity have been reported only for *A. tumefaciens* RU-OR (Burton *et al.* 1998). The Hyu enzyme activity in RU-OR was regulated by three main mechanisms (1) catabolite repression, (2) ammonia shock and (3) induction (Hartley *et al.* 1998, Burton *et al.* 1998, Hartley *et al.* 2001). While the genomic DNA encoding a partial *hyuH,* the *hyuH-hyuC* promoter region and the *hyuC* have been isolated from RU-AE01, the Hyu enzymes in RU-AE01 have not been characterized. In this chapter, the regulation of the RU-AE01 Hyu enzyme activity is studied and compared to the patterns of hydantoin-hydrolyzing enzyme expression in strain RU-OR.

In the previous chapter, a broad host range promoter-probe vector was constructed and tested with the *A. tumefaciens* RU-AE01 *hyuH-hyuC* region in *E. coli*. As expected, the *A. tumefaciens* RU-AE01 *hyuH-hyuC* region encoded divergent promoters that were functional in *E. coli* DH5α. The localization of the *hyuH* and the *hyuC* promoters to the sequence between the *hyuH* and the *hyuC* ORFs makes possible the study of the regulation of the activation of transcription from the *hyuH-hyuC* sequence. In addition, the regulation of transcriptional activation by the *hyuH* and *hyuC* promoters can be compared to the expression of HyuH and HyuC in strain RU-AE01.

3.2 METHODS AND MATERIALS

3.2.1 General culture conditions

A. tumefaciens RU-AE01 cells were grown at 28 °C with shaking at 200 rpm in LB broth containing 1% hydantoin (Appendix 2-3) or in *A. tumefaciens* minimal medium [including 0.02% Casamino Acids Minimal Medium (CAAMM), 0.5% CAAMM and Hydantoin Minimal Medium (HMM)] (Appendix 2-4). Alternaltively, LB agar (Appendix 2-3) or HMM agar (Appendix 2-4) was used as a solid medium. Where required, the growth media was supplemented with the appropriate antibiotic to which the plasmid conferred resistance (50 µg/mL chloramphenicol or 25 µg/mL tetracycline).

Recombinant plasmids were transformed into competent RU-AE01 as described previously in Section 2.2.4. Plasmid isolation from RU-AE01 involved the extraction of total genomic DNA as described by Ausubel *et al.* (1983) and transformation of the the resulting supernatant into competent *E. coli* DH5α cells (Section 2.2.4). Recombinant plasmid DNA was subsequently extracted and analyzed from *E. coli* DH5α cells as described previously (Section 2.2.1).

3.2.2 Enzyme assays

A. tumefaciens RU-AE01 cells were harvested as described in Section 2.2.5 and resuspended at 40 mg wet cell mass per mL in 0.1 M phosphate buffer (pH 9.0). This cell suspension was used for quantitative β-glucuronidase and β-galactosidase enzyme assays (Section 2.2.5).

Enzyme assays for hydantoinase and NCAAH activity

The RU-AE01 cell suspension (40 mg wet cell mass per mL in 0.1 M phosphate buffer (pH 9.0)) was also used for resting cell biocatalytic reactions (Hartley *et al.* 1998) as described in Appendix 4. The reaction supernatant of the resting cell biocatalytic reactions were analysed for the presence of *N*-carbamylglycine and glycine using Ehrlich's reagent (Morin *et al.* 1986) and the Ninhydrin assay (Plummer 1987) respectively. The protocols are detailed in Appendices 5-1 and 5-2.

Hydantoinase activity is reported as the total (µmol/mL) *N*-carbamoyl glycine and glycine produced from hydantoin as a substrate (in 6h) by resting cells and NCAAH activity is reported as the amount of glycine (µmol/mL) produced from *N*-carbamoyl glycine as a substrate by resting cells over a 6h period. All experiments were independently repeated at least three times with freshly cultured cells. The enzyme activity varied between sets of experiments (up to two-fold) but the relative values were the same. The standard mean error between triplicates in each assay was low and not significant (Hartley *et al.* 1998).

3.3 RESULTS AND DISCUSSION

3.3.1 Optimisation of reporter gene assays in *A. tumefaciens* RU-AE01

The knowledge that the *hyuH-hyuC* region encoded divergent promoters that were active in *E. coli* cells enabled the study of the *hyuH* and *hyuC* promoters in the homologous host *A. tumefaciens* RU-AE01. RU-AE01 cells transformed with pMJ258 were grown in LB broth containing 1% hydantoin to determine the levels of β-glucuronidase and β-galactosidase enzyme expression supported by the *hyu* promoters and the patterns of Hyu enzyme expression relative to the growth phase of the culture. These results were then utilized to determine the optimal stage of growth for harvesting cells to conduct enzyme assays. Analysis of the increase in OD_{600nm} during growth in LB broth containing 1% hydantoin showed that the culture of RU-AE01 cells hosting pMJ258 remained in the logarithmic growth phase for approximately 20h following inoculation and entered stationary phase after approximately 25h (Figure 3-1A).

As in *E. coli*, both the *hyuC* and *hyuH* promters were transcriptionally active in RU-AE01 cells (Figure 3-1B and 3-1C). β-glucuronidase activity under control of the *hyuH* promoter, reached a peak after 22h of growth (Figure 3-1B) while the β-galactosidase activity, supported by the *hyuC* promoter reached a peak after 36h of growth (Figure 3-1C). While the β-galactosidase activity, representing the *hyuC* promoter-derived activity, was maintained throughout stationary growth phase (Figure 3-1C), the β-glucuronidase activity, representing the *hyuH* promoter-derived activity, declined during stationary phase (Figure 3-1B).

This suggested that transcription from the *hyuH* and *hyuC* promoters in *A. tumefaciens* cells, as in *E. coli* cells, was activated in late log and early stationary phase. In addition, the loss of β-glucuronidase activity suggested that the *hyuH* promoter was active for a relatively short period of time (8h) compared to the *hyuC* promoter which was active for a longer period of time (24h). For this reason, cultures were subsequently grown for 26h to ensure the optimal time for transcriptional activity from the *hyuC* and *hyuH* promoters in RU-AE01 cells.

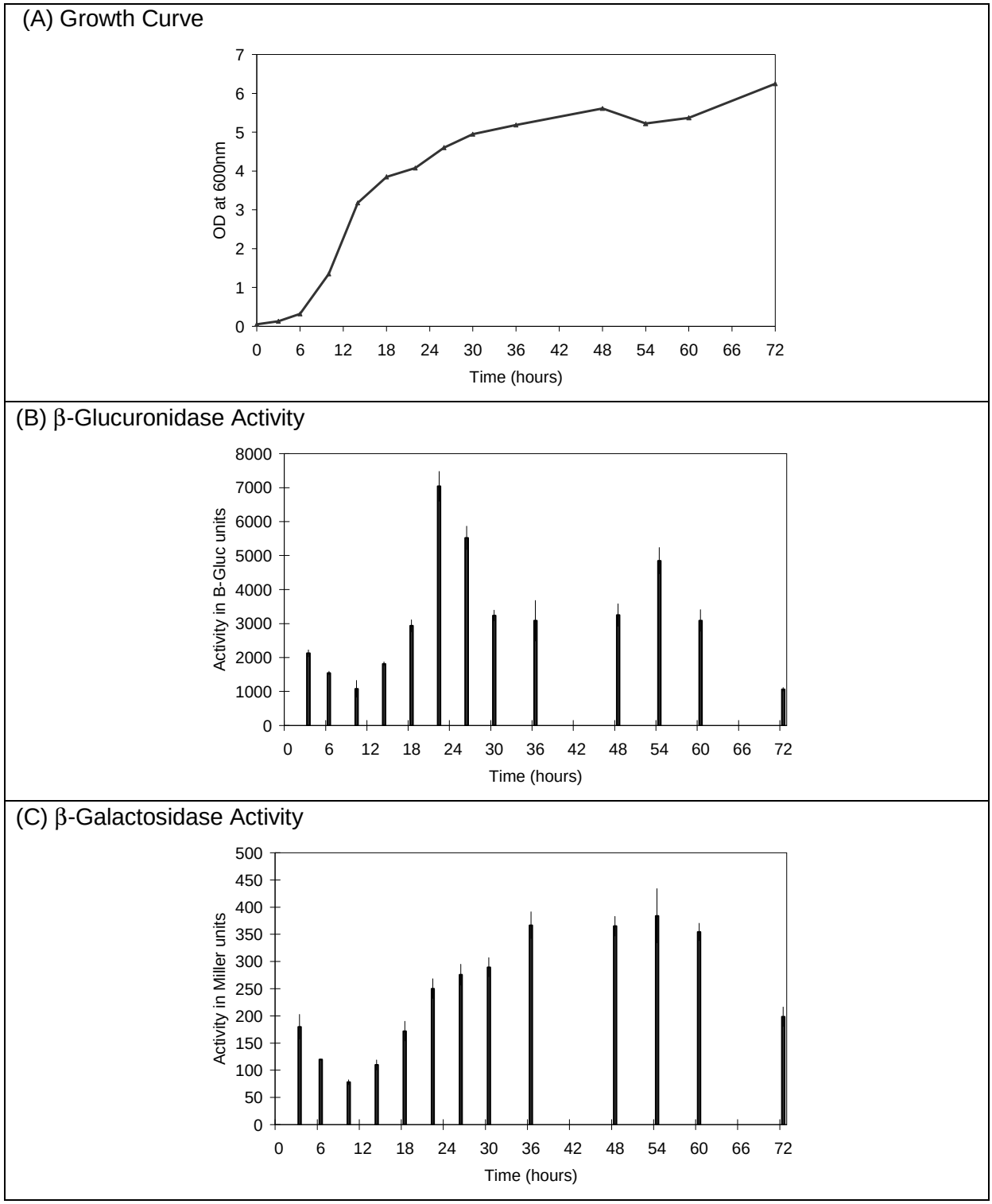


Figure 3-1: *hyuH* and *hyuC* promoter activity during growth of *A. tumefaciens* RU-AE01 cells hosting pMJ258 in LB medium containing 1% hydantoin. (A) Growth over 72h monitored at OD_{600nm}, (B) β-glucuronidase enzyme activity reported as β-Gluc units and (C) β-galactosidase enzyme activity reported as Miller units. Enzyme assays are an average of triplicate reactions. The standard mean error is shown for each reaction by the vertical bars.

The optimal time for reporter enzyme assays (26h) was determined from the levels of *hyuH*-promoter derived β-glucuronidase enzyme activity and the *hyuC*-promoter derived β-galactosidase enzyme activity obtained during the growth of RU-AE01 cells hosting pMJ258 in LB broth containing 1% hydantoin. It is important to note two limitations to determining the optimal time to assay reporter enzyme activity from this data. First, levels of mRNA are not always directly comparable to the levels of protein within the cell (Glanemann *et al.* 2003). Second, different enzymes have different half-lives within bacterial cells (More *et al.* 1995). This would make it difficult to infer promoter strength from the specific activity of a reporter enzyme.

While the levels of mRNA to protein may not always be comparable, Sperker *et al.* (2001) demonstrated that β-glucuronidase enzyme activity, protein concentration and mRNA amounts were all regulated in a similar manner when the enzyme β-glucuronidase was treated with xenobiotics. Similarly, Neidhardt *et al.* (1990) showed that specific β-galactosidase mRNA present in cells was proportional to the amount of enzyme synthesized. This suggests that the *hyuH*- or *hyuC*-derived promoter activity can be investigated using *gus* and *lac* as reporter genes and β-glucuronidase and β-galactosidase as reporter enzymes.

Girbal *et al.* (2003) report that the stability of β-galactosidase is often higher than the stability of β-glucuronidase in the cell. For this reason, plasmids pMJ258 and pMJ260 were constructed to compare *hyuH*-promoter derived β-glucuronidase enzyme activity to the *hyuH*-promoter derived β-galactosidase enzyme activity in RU-AE01 cells. in the same way, the plasmids pMJ258 and pMJ260 were used to compare the *hyuC*-promoter derived β-glucuronidase enzyme activity to the *hyuC*-promoter derived β-galactosidase enzyme activities in RU-AE01. If the *hyuH*-promoter derived β-glucuronidase enzyme activity and *hyuH*-promoter derived β-galactosidase enzyme activity exhibited the same trends and the *hyuC*-promoter derived β-glucuronidase enzyme activity and *hyuC*-promoter derived β-galactosidase enzyme activity exhibited the same trends, then the constructs pMJ258 and pMJ260 could be used to investigate transcription of *hyuH* and *hyuC* and to compare transcription of *hyuH* and *hyuC* to expression of HyuH and HyuC.

Reporter enzyme assays of the constructs pMJ258 and pMJ260 (Figure 3-2) in RU-AE01 cells showed similar results to those obtained in *E. coli.* The *hyuH* promoter appeared to support two to three-fold higher levels of transcription than the *hyuC* promoter (Figure 3-2A and 3-2B) in both pMJ258 and pMJ260.

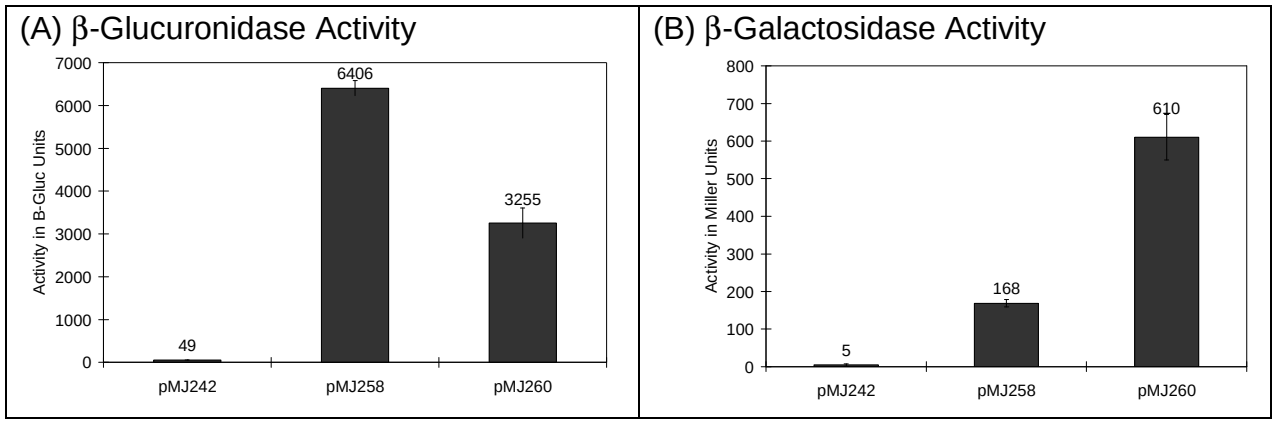


Figure 3-2: β-glucuronidase and β-galactosidase activities for pMJ242, pMJ258 and pMJ260 in *A. tumefaciens* RU-AE01 cells grown in LB broth containing 1% hydantoin. (A) β-glucuronidase activity is reported as β-Gluc units. (B) β-galactosidase activity is reported as Miller units. Results are the average of triplicate reactions. The standard mean error is shown for each reaction by the vertical bars.

3.3.2 Comparison of hydantoinase and NCAAH enzyme activities with the transcriptional activities of the *hyuH-hyuC* promoters

Little was known about the Hyu enzyme system in the strain RU-AE01 at the start of this research. Thus the first step was to establish the conditions for maximal enzyme activity and then to examine the response of the system to the presence of hydantoin in the growth medium (induction), CCR and NCR. The regulation of the hydantoin-hydrolyzing enzyme activities can then be compared to the regulation of the transcriptional activation of *gus* and *lacZ* under control of the RU-AE01 *hyuH-hyuC* promoter region.

Hydantoin-hydrolyzing enzyme activity during growth in batch culture

RU-AE01 cells containing pMJ258 (*hyuH-hyuC* promoters) were grown in LB broth supplemented with 1% hydantoin. As hydantoin and hydantoin analogs have been reported as inducers of the Hyu enzymes (Meyer and Runser 1993, Hartley *et al.* 1998), hydantoin was added to the growth medium as a metabolizable inducer. At predetermined time intervals, RU-AE01 cells hosting pMJ258 were

subjected to resting cell biocatalytic reactions followed by colorimetric assays to determine the stage of growth at which the hydantoinase and NCAAH activities were highest.

Low levels of hydantoinase and NCAAH activities were detected during the logarithmic growth phase (Figure 3-3A, 3-3B and 3-3C). As the cells entered stationary growth phase (after 30h), an increase was observed in both the hydantoinase and NCAAH activity until mid-stationary growth phase (after 60h). As the cells entered late-stationary growth phase (72h), the levels of both the hydantoinase and the NCAAH enzymes decreased.

The hydantoinase and NCAAH enzyme activities were highest during stationary growth phase indicating that the hydantoin-hydrolyzing enzymes are expressed under nutrient-limiting conditions after the ‘good’ carbon and nitrogen sources had been metabolized and the ‘poor’ carbon and nitrogen sources were being utilized. Hydantoin and hydantoin derivatives can be used as sole carbon (Moller *et al.* 1988) or as sole nitrogen sources (Yamashiro *et al.* 1988), or as combined carbon and nitrogen sources (Nishida *et al.* 1987). The decrease in hydantoin-hydrolyzing enzyme activity observed at 72h is likely to be a result of cell starvation due to the lack of carbon sources in the medium and therefore a lack of cell viability.

The pattern of hydantoinase and NCAAH enzyme activities during growth of cells in LB broth containing 1% hydantoin (Figure 3-3B and 3-3C) was similar to the pattern of β-glucuronidase and β-galactosidase enzyme activities expressed under control of the RU-AE01 *hyuH-hyuC* promoter region in pMJ258 (Figure 3-1B and 3-1C). The hydantoinase enzyme activity was maximal between 30-60h from early to mid-stationary growth phase; however it lagged 8h behind β-glucuronidase enzyme expression which peaked during early stationary phase at 22h and was detectable through stationary phase. The NCAAH activity was also at its maximum between 30-60h from early to mid-stationary growth phase and this correlated to the β-galactosidase activity which peaked at 36h but was detectable through stationary phase.

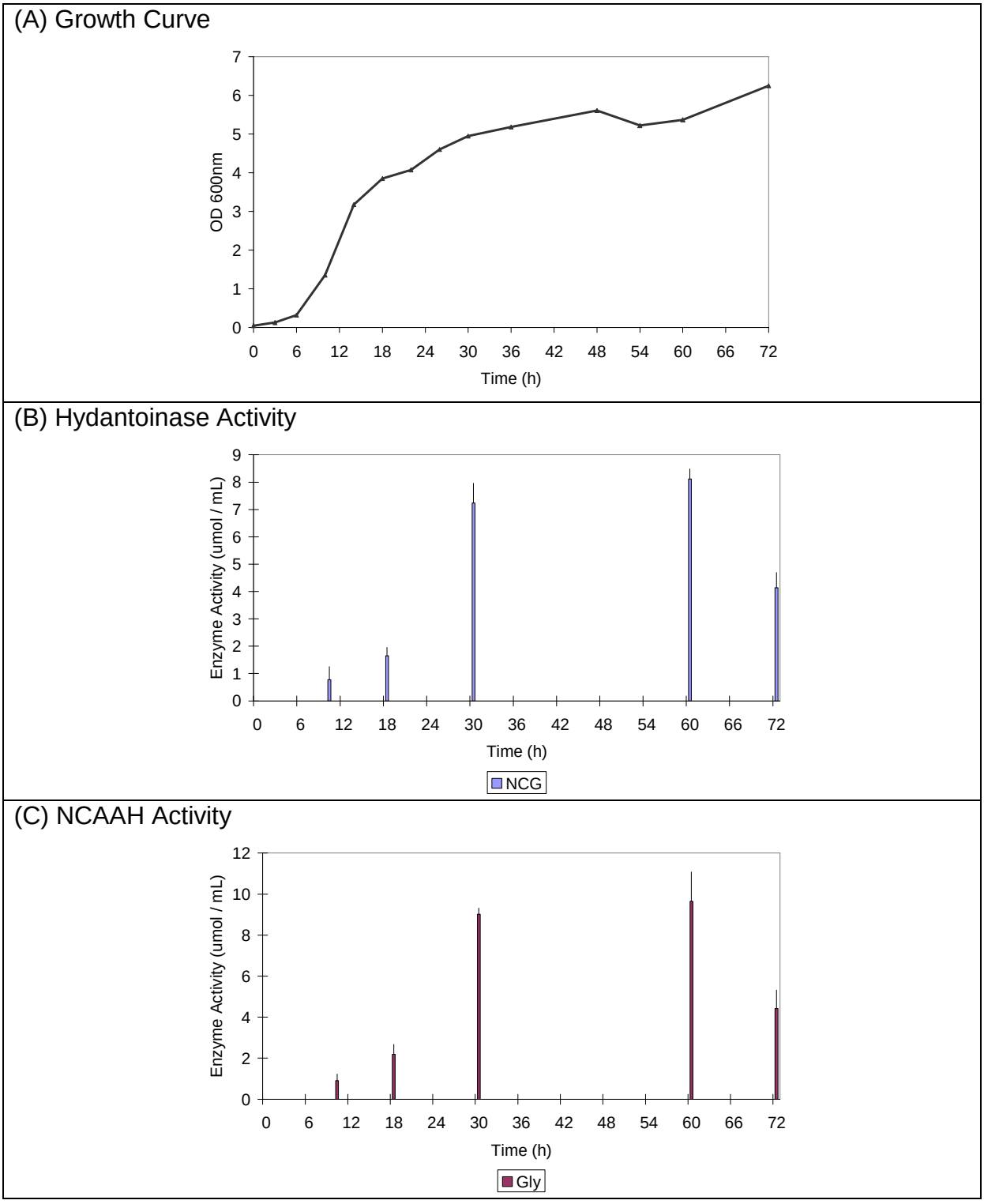


Figure 3-3: Hydantoinase and NCAAH enzyme expression by *A. tumefaciens* RU-AE01 cells containing pMJ258 in LB broth supplemented with 1% hydantoin. (A) Growth monitored over 72h at OD_{600nm}, (B) Hydantoinase activity and (C) NCAAH activity measured in µmol/mL. Results are the average of triplicate reactions. The standard mean error is shown by the vertical bars.

Slight differences in the composition of the reagents used to formulate LB broth often led to variable levels of Hyu enzyme activity. For reproducibility, a basic minimal medium recipe developed by Hartley *et al.* (2001) was used in which glucose was the sole source of carbon and hydantoin the sole source of nitrogen. Growth conditions and assay conditions for RU-AE01 cells in the minimal medium were subsequently optimized (data not shown). The optimized growth and assay conditions were then used to study induction and catabolite repression in RU-AE01 cells.

Induction of Hyu enzyme activity by growth in the presence of hydantoin

0.02% CAAMM is a minimal medium which contains a low concentration of nitrogen resulting in nitrogen-limiting conditions in which RU-OR cells show basal (low) levels of hydantoinase and NCAAH activity (Hartley *et al*. 2001). Growth of RU-AE01 cells in this medium resulted in 0.9 μmol/mL of hydantoinase and 1.4 μmol/mL of NCAAH enzyme activity, respectively (Table 3-1). RU-AE01 cells grown in medium with hydantoin as nitrogen source (HMM) showed elevated levels of enzyme activity, with 3.3 μmol/mL of hydantoinase and 3.8 μmol/mL of NCAAH enzyme activity, respectively (Table 3-1). There was therefore a 3.6 fold induction in the hydantoinase enzyme activity and a 2.7 fold increase in the NCAAH enzyme activity in medium containing hydantoin.

Table 3-1: Induction of the hydantoinase and NCAAH activity in *A. tumefaciens* RU-AE01

Medium	**Hydantoinase (μmol/mL)**	**NCAAH (μmol/mL)**
0.02% CAAMM	0.9 ± 0.10	1.4 ± 0.01
HMM	3.3 ± 0.10	3.8 ± 0.01

±- SEM (n=3)

To determine whether induction of enzyme activity occurred at the level of transcription, as had been observed by Hartley *et al*. (2001) in strain RU-OR, wild-type RU-AE01 cells grown to mid log growth phase in 0.02% CAAMM were transferred to 0.02% CAAMM, HMM or HMM containing 200 μg/mL rifampicin, which inhibits transcription. Basal levels of hydantoinase and NCAAH activity were detected in cells growing in 0.02% CAAMM (blue line Figure 3-4A and 3-4B) and elevated levels of enzyme activity in HMM (black line Figure 3-4A and 3-4B). However in HMM containing rifampicin, the levels of hydantoinase and NCAAH activity decreased to

below basal levels (green line Figure 3-4A and 3-4B), suggesting that induction of *hyu* expression in RU-AE01 was most likely at the level of transcription.

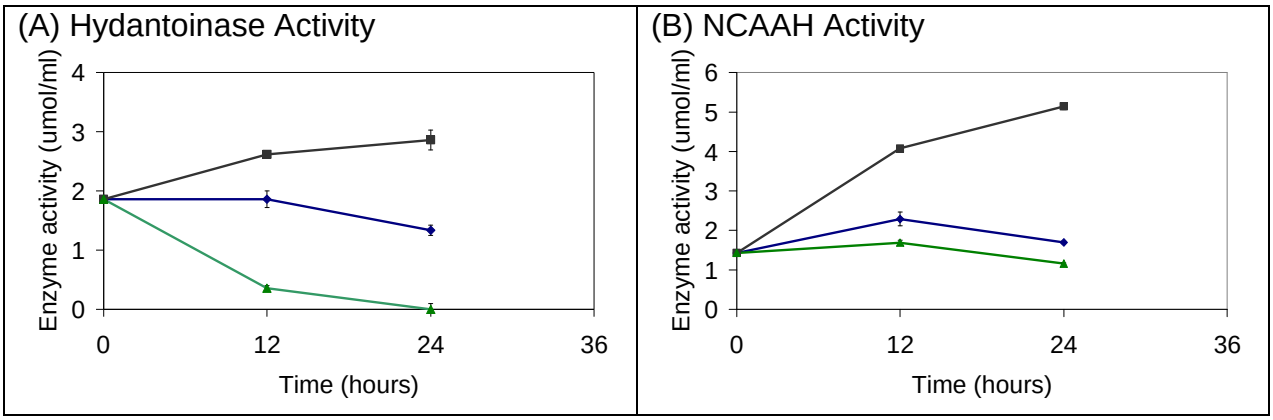


Figure 3-4: Transcriptional regulation of hydantoinase and NCAAH induction in strain RU-AE01. Cells were grown in 0.02% CAAMM, harvested and then divided equally between HMM (black line, ■), 0.02% CAAMM (blue line, ♦) and HMM + 200 µg/ml rifampicin (green line, ▲). Samples taken at 12 hour intervals were used to analyze the hydantoinase (A) and NCAAH (B) activities. Enzyme activity is represented in µmol/mL. Results are the average of triplicate reactions. The standard mean error is shown by the bars.

To confirm this, RU-AE01 cells containing the vector pMJ242 or pMJ258, which has the *hyuH* and *hyuC* promoter regions fused to *gus* and *lacZ* respectively, were grown in either 0.02% CAAMM or HMM. As expected, enzyme activity was induced in HMM compared with CAAMM (Table 3-2) with 5 fold and 2.5 fold increases in hydantoinase and NCAAH enzyme activities respectively. There was no detectable β-glucuronidase or β-galactosidase enzyme activity in cells containing the vector pMJ242, grown in either 0.02% CAAMM or HMM (Table 3-2). RU-AE01 cells hosting pMJ258, in contrast, produced 3693 units of activity in 0.02% CAAMM and 6761 units of β-glucuronidase activity in HMM (Table 3-2), indicating an approximately 2-fold induction of transcription by the *hyuH* promoter with a similar increase observed in β-galactosidase activity supported by the *hyuC* promoter (Table 3-2). This data confirmed that induction of the *hyuH* and *hyuC* promoters occurs at the level of transcription.

Table 3-2: Induction of the hydantoin-hydrolyzing enzymes in RU-AE01 cells hosting the broad host range plasmids pMJ242 or pMJ258

Plasmid	Medium	Hydantoinase (μmol/mL)	β-glucuronidase (β-Gluc units)	NCAAH (μmol/mL)	β-galactosidase (Miller units)
pMJ242	0.02% CAAMM	0.83 ± 0.11	0 ± 78	2.62 ± 0.42	0 ± 6
	HMM	4.64 ± 0.48	0 ± 89	5.43 ± 0.45	0 ± 6
pMJ258	0.02% CAAMM	0.69 ± 0.05	3693 ± 121	2.56 ± 0.51	963 ± 90
	HMM	3.75 ± 0.43	6761 ± 149	7.49 ± 0.17	1681 ± 164

±- SEM (n=3)

In the presence of the inducer, hydantoinase and NCAAH enzyme activity was induced 5 fold and 2.5 fold respectively. In constrast, *hyuH*-promoter derived β-glucuronidase activity and *hyuC*-promoter derived β-galactosidase activity only increased 1.8 and 1.7 fold. These differences could be attributed to the variation between the number of copies of the *hyu* genes and the number of copies of the reporter genes present in the RU-AE01 cells.

The effect of CCR on Hyu enzyme activity

To determine if the hydantoin-hydrolyzing enzyme system in strain RU-AE01 was subject to regulation by CCR, cells were grown in HMM with glucose, glycerol or mannitol as carbon sources. No significant variation was observed in the levels of hydantoinase and NCAAH activity in cells using glucose, glycerol or mannitol as carbon sources (Table 3-3). Thus, as with strain RU-OR (Hartley 2001), CCR did not play a role in the regulation of the expression of the Hyu enzymes from the RU-AE01 *hyu* genes.

Table 3-3: The lack of carbon catabolite repression on the Hyu enzymes in RU-AE01

Medium	Hydantoinase (μmol/mL)	NCAAH (μmol/mL)
HMM Glucose	3.3 ± 0.10	3.8 ± 0.00
HMM Glycerol	2.5 ± 0.05	4.9 ± 0.04
HMM Mannitol	2.1 ± 0.01	2.9 ± 0.03

±- SEM (n=3)

NCR of the Hyu enzymes

Hartley *et al*. (2001) reported that the hydantoin-hydrolyzing enzyme expression in strain RU-OR was regulated by NCR. To determine if NCR was similarly involved in strain RU-AE01, cells were grown under non-repressive (0.02% CAAMM) and repressive nitrogen conditions (0.5% CAAMM) followed by assays for hydantoinase and NCAAH activity. When RU-AE01 cells were grown in 0.02% CAAMM, basal levels of hydantoinase (1.2 μmol/mL) and NCAAH (3.6 μmol/mL) were produced (Table 3-4). In contrast, when the cells were grown in 0.5% CAAMM, little hydantoinase or NCAAH enzyme activity was detected (Table 3-3). Repressive nitrogen conditions resulted in a 6-fold decrease in hydantoinase activity and a corresponding 7-fold decrease in the level of NCAAH showing, as in strain RU-OR, that the expression of the RU-AE01 Hyu enzymes was subject to NCR.

Table 3-4: Nitrogen catabolite repression of the Hyu enzymes in RU-AE01

Medium	**Hydantoinase (μmol/mL)**	**NCAAH (μmol/mL)**
0.02% CAAMM	1.2 ± 0.07	3.58 ± 0.13
0.5% CAAMM	0.15 ± 0.03	0.54 ± 0.11

±- SEM (n=3)

To determine if NCR was exerted at the transcriptional level, RU-AE01 cells containing the plasmids pMJ242 and pMJ258 were grown in 0.02% CAAMM and 0.5% CAAMM and then subjected to hydantoin-hydrolyzing and reporter enzyme assays. As in the wild-type, cells containing the plasmids pMJ242 and pMJ258 showed a decrease in hydantoinase and in NCAAH activity (Table 3-5) under repressive nitrogen growth conditions. Low β-glucuronidase and β-galactosidase enzyme activities (Table 3-5), concomitant with promoterless *gus* and *lacZ* genes, were detected in cells hosting pMJ242 and pMJ258 grown in 0.02% CAAMM or in 0.5% CAAMM. In contrast, there were high levels of β-glucuronidase activity (*hyuH* promoter) in RU-AE01 cells hosting pMJ258 growing in either 0.02% CAAMM or 0.5% CAAMM (Table 3-5) with 3077 ± 159 β-Gluc units and 4170 ± 327 β-Gluc units respectively (Table 3-5). Transcriptional activity from the *hyuH* promoter thus appeared to be higher in 0.5% CAAMM than in 0.02% CAAMM which suggested that the NCR did not regulate this promoter. β-galactosidase activity (*hyuC* promoter) was detected in cells hosting pMJ258 growing in 0.02% CAAMM and in 0.5% CAAMM, but in contrast to the *hyuH* promoter activity, transcription levels were significantly

lower in cells grown in 0.5% CAAMM (784 ± 92 Miller units) versus 0.02% CAAMM (1005 ± 46 Miller units) (Table 3-5). This showed that NCR might be involved in the regulation of the *hyuC* promoter in strain RU-AE01.

Table 3-5: Repression of the Hyu enzymes by nitrogen in RU-AE01 cells containing the plasmids pMJ242 or pMJ258

Plasmid	Medium	Hydantoinase (μmol/mL)	β-glucuronidase (β-Gluc units)	NCAAH (μmol/mL)	β-galactosidase (Miller units)
pMJ242	0.02% CAAMM	1.56 ± 0.17	0 ± 47	3.87 ± 0.02	0 ± 1
	0.5% CAAMM	0 ± 0.03	58 ± 24	0.5 ± 0.06	0 ± 2
pMJ258	0.02% CAAMM	1.74 ± 0.07	3077 ± 159	3.64 ± 0.05	1005 ± 46
	0.5% CAAMM	0 ± 0.11	4170 ± 327	0.62 ± 0.17	784 ± 92

±- SEM (n=3)

3.4 GENERAL CONCLUSIONS

The strain RU-OR has been studied in detail by Hartley *et al.* (1998, 2001; Hartley 2001). However the hydantoin-hydrolyzing enzyme system in RU-AE01 has not previously been characterized. Before any analysis of the promoter region could begin, therefore, it was necessary first to establish the expression patterns and regulation of enzyme activity for strain RU-AE01. Thus the strain was analysed with respect to the stage of growth at which the hydantoin-hydrolyzing enzymes were produced and whether the enzymes were susceptible to induction or repression by nutrient sources.

When RU-AE01 cells were grown in complete medium (LB broth containing 1% hydantoin), the cells utilized the ‘good’ nutrient sources before expressing enzymes to metabolize the ‘poor’ nutrient sources such as hydantoin. Thus the cells produced the hydantoin-hydrolyzing enzymes to metabolise the hydantoin during stationary phase, after all the ‘good’ carbon and nitrogen sources had been utilized suggesting that the hydantoin-hydrolyzing enzymes are subject to catabolite repression. Hydantoinase and NCAAH activities were not affected by carbon catabolite repression, but rather by nitrogen catabolite repression as was reported by Hartley *et al.* (2001) for strain RU-OR. The RU-AE01 enzymes activities were

induced by growth in the presence of hydantoin, but induction occured only under conditions of nutrient limitation. Nitrogen catabolite repression appeared to prevent induction in nitrogen sufficient conditions. Thus the Hyu enzyme system of RU-AE01 appears be similar to that of strain RU-OR: (1) both are induced by hydantoin, (2) both systems are not subject to CCR and (3) both systems are regulated by NCR.

Reporter gene assays of the regulation of *hyuH* and *hyuC* promoter activity correlated with hydantoinase and NCAAH enzyme activity assays in that (1) transcriptional activation of both genes occurred during early stationary growth phase and (2) transcriptional activity was induced by the presence of hydantoin in the growth medium. The fold activation of the hydantoinase and NCAAH enzymes are not directly comparable to the fold activation β-glucuronidase and β-galactosidase enzyme activities. While there is evidence that the *hyu* operons elucidated thus far are present on plasmids, their copy number is unknown, though large plasmids often exist as single copies within the cell (Li and Farrand 2000). The pTF-FC2 replicon replicates at 12 to 15 copies per cell (Dorrington and Rawlings 1989), which is much higher than the native plasmids. This difference in copy number would unbalance the ratio of transcription factors relative to promoter binding sequences and could account for the differences between the levels of induction as measured by hydantoinase and NCAAH assays versus the reporter gene assays. To accurately compare the levels of activation between the hydantoin-hydrolyzing enzymes and the reporter enzymes, a plasmid that exists as a single copy in RU-AE01 cells needs to be constructed. Alternatively, the integration of a single copy of the *gus-hyuH-hyuC* promoter region-*lacZ* fusion reporter system on the chromosome would serve the same purpose.

The Hyu enzyme activity in RU-AE01 was subject to NCR in nitrogen sufficient conditions resulting in low levels of both hydantoinase and NCAAH enzyme activity. A parallel NCR effect was observed on the *hyuC*-promoter derived β-galactosidase activity where lower levels of β-galactosidase activity were detected in nitrogen sufficient conditions. The difference between the level of transcriptional repression of the *hyuC* promoter and the decrease in the NCAAH activity observed in nitrogen sufficient conditions can be attributed to the variation in the number of copies of the reporter genes present in the RU-AE01 cells and the number of copies of the *hyu*

genes. The *hyuH* promoter, however, did not appear to be affected by NCR - the level of β-glucuronidase activity was actually significantly higher in repressive medium than in non-repressive medium. This illustrates two important points. First, that the transcription of *hyuH* and *hyuC* is activated by distinct mechanisms which are regulated independently. Second, that the mechanisms of NCR with respect to the Hyu enzymes in the strains RU-AE01 and RU-OR differ.

In RU-OR, two nitrogen-dependent mechanisms exist- NCR and ammonia shock. The analysis of RU-OR mutants demonstrated that the two mechanisms are separate and distinct. Ammonia shock is a rapid but reversible effect observed on the hydantoinase activity as a result of high concentrations of NH_4^+ in the medium. NCR, on the other hand, is responsible for the low levels of hydantoinase and NCAAH enzyme activity during the exponential growth of RU-OR cells in nitrogen sufficient conditions as a result of low levels of enzyme expression. Thus in RU-OR, NCR is thought to prevent the transcription of the *hyuH* and *hyuC* genes when the cells are cultured in medium containing a repressive nitrogen source. However, in RU-AE01 NCR does not prevent the transcription of *hyuH* suggesting an alternative mechanism is responsible for the low levels of Hyu enzymes in nitrogen sufficient conditions. It is possible that in RU-AE01, ammonia shock alone exists as a mechanism to effect NCR.

Two important questions arise from the data reported in this chapter. First, what sequences on the RU-AE01 *hyuH-hyuC* fragment are responsible for the activation of transcription? This will be addressed in the following chapter, Chapter 4. Secondly, what mechanism does RU-AE01 employ to effect NCR? To undertand NCR in *A. tumefaciens* strains RU-AE01 and RU-OR, the effect of the global nitrogen regulator NtrC on the Hyu enzymes will be analysed in Chapter 5.

CHAPTER 4

ANALYSIS OF THE *A. tumefaciens* RU-AE01 *hyuH-hyuC* PROMOTER REGION

4.1 INTRODUCTION

In the previous chapter, the regulation of the activation of transcription by the RU-AE01 *hyuH-hyuC* promoters was studied. The data indicated that the *hyuH-hyuC* region encoded divergent promoters and that the *hyuH* and *hyuC* promoters were regulated at the transcriptional level by general catabolite repression and by induction. Additionally the *hyuC* promoter, but not the *hyuH* promoter, was subject to transcriptional regulation by NCR.

This chapter describes the bioinformatic analysis of the region between *hyuH* and *hyuC* in pMJ10 as well as those isolated from several other *Agrobacterium* strains leading to the identification of potential transcription activation or repression sequences. Subsequently, deletion analysis of the promoter region was used to determine which of the putative activation and repression sequences identified may be responsible for the transcription of *hyuH* and *hyuC* in RU-AE01.

4.2 METHODS AND MATERIALS

4.2.1 Source of *hyu* cluster sequences

Agrobacterium hyu cluster sequences (Table 4-1) were accessed from the NCBI database (http://www.ncbi.nlm.nih.gov/) unless stated otherwise.

Table 4-1: *Agrobacterium* DNA sequences

Strain	Source
IP I-671	NCBI Accession Number AF335479
NRRL B11291	NCBI Accession Number X91070
RU-AE01	This study
RU-OR *hyuC1*	Hartley (2001)

4.2.2 Construction of deletion plasmids

A series of deletions of the *hyuH-hyuC* promoter region were constructed by PCR amplification from pMJ10 using the Expand High Fidelity system and the primers listed in Table 4-2. The individual PCR products were digested with *Bgl* II or *Bgl* II and *Bam* HI and cloned into the *Bgl* II site of the vector pMJ242, generating the promoter-probe plasmids pMJ245, pMJ258, pMJ261, pMJ268 and pMJ361 (Table 4-2). The integrity of the DNA sequence of each insert was confirmed by sequencing.

Table 4-2: Primers used to construct the plasmids used in the deletion analysis of the *hyuH-hyuC* promoter region

Plasmid	Primer Name	Sequence	†RU-AE01 sequence (nts)
pMJ245	Chao F1	*cgggatcc ^{311}TTGACCCTGGTCCTTG^{326}	311-491
	MJ10	*gaagatct ^{491}GAACCTTTGCTCCTTCGATAGTTAAT^{466}	
pMJ258	MJ9	*gaagatct ^{25}AAAGCAGCTCTCAGGGTTGATG^{46}	25-491
	MJ10	*gaagatct ^{491}GAACCTTTGCTCCTTCGATAGTTAAT^{466}	
pMJ261	MJ9	*gaagatct ^{25}AAAGCAGCTCTCAGGGTTGATG^{46}	25-234
	MJ37	*gaagatct ^{234}TCCAGCTCGCCGAATAGGGTTTGG^{212}	
pMJ266	MJ9	*gaagatct ^{25}AAAGCAGCTCTCAGGGTTGATG^{46}	25-328
	MJ36	*gaagatct ^{328}GTCAAGGACCAGGGTCAAAATTTCC^{304}	
pMJ268	MJ35	*gaagatct ^{212}CCAACCCTATTCGGCGAGCTGGATT^{237}	212-491
	MJ10	*gaagatct ^{491}GAACCTTTGCTCCTTCGATAGTTAAT^{466}	
pMJ361	MJ9	*gaagatct ^{25}AAAGCAGCTCTCAGGGTTGATG^{46}	25-124
	MJ60	*gaagatct ^{124}CTTCCCTTCCCGCTTGACAGATAC^{91}	

* Underlined sequence at the 5' terminus of the primers represents the sequence for a *Bam* HI (ggatcc) or a *Bgl* II (agatct) restriction enzyme site.
† RU-AE01 *hyuH-hyuC* sequence detailed in Appendix 1-1

A. tumefaciens RU-AE01 cell cultures were grown as described in Section 3.2.1. The growth of RU-AE01 cells for β-glucuronidase and β-galactosidase enzyme assays was done as reported in Section 2.2.5.

4.3 RESULTS AND DISCUSSION

4.3.1 Multiple sequence alignment of *Agrobacterium hyuH* and *hyuC* promoter regions

The *hyuH-hyuC* regions of the *Agrobacterium* strains IP I-671, NRRL B11291 and RU-AE01 differ in length from 214 nt in NRRL B11291 (Grifantini *et al.* 1998) to 465 nt in IP I-671 (Hils *et al.* 2001) to 467 nt in RU-AE01 (this study). Two distinct *hyuC* genes were isolated from RU-OR (Hartley 2001) which do not appear to be linked to *hyuH* genes. The RU-OR *hyuC1* coding sequence (Figure 4-1D) showed over 90% identity at the nucleotide level with the *hyuC* sequences from RU-AE01, IP I-671 and NRRL B11291; which suggested that the 133 nt of sequence upstream of the HyuC1 ORF may be similar to the other *Agrobacterium* strains. In contrast, the *hyuC2* coding sequence (Figure 4-1D) showed only 60% identity at the nucleotide level with the *hyuC* sequences from RU-AE01, IP I-671 and NRRL B11291 suggesting that this second NCAAH which had been isolated from only RU-OR was different to the conventional *hyuC* genes. For this reason, the nucleotide sequence

upstream of the RU-OR *hyuC1* was included in the analyses conducted in this chapter but not the nucleotide sequence upstream of RU-OR *hyuC2*.

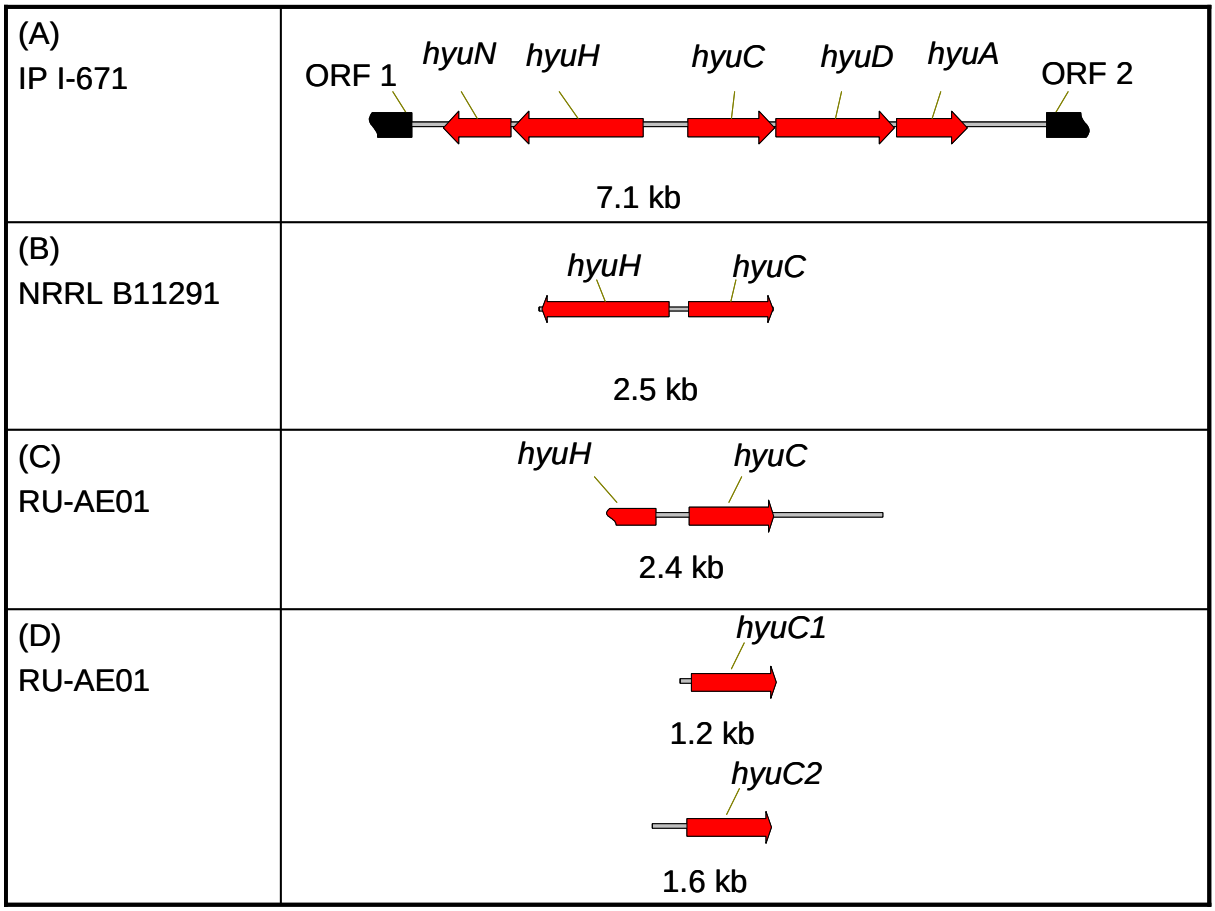
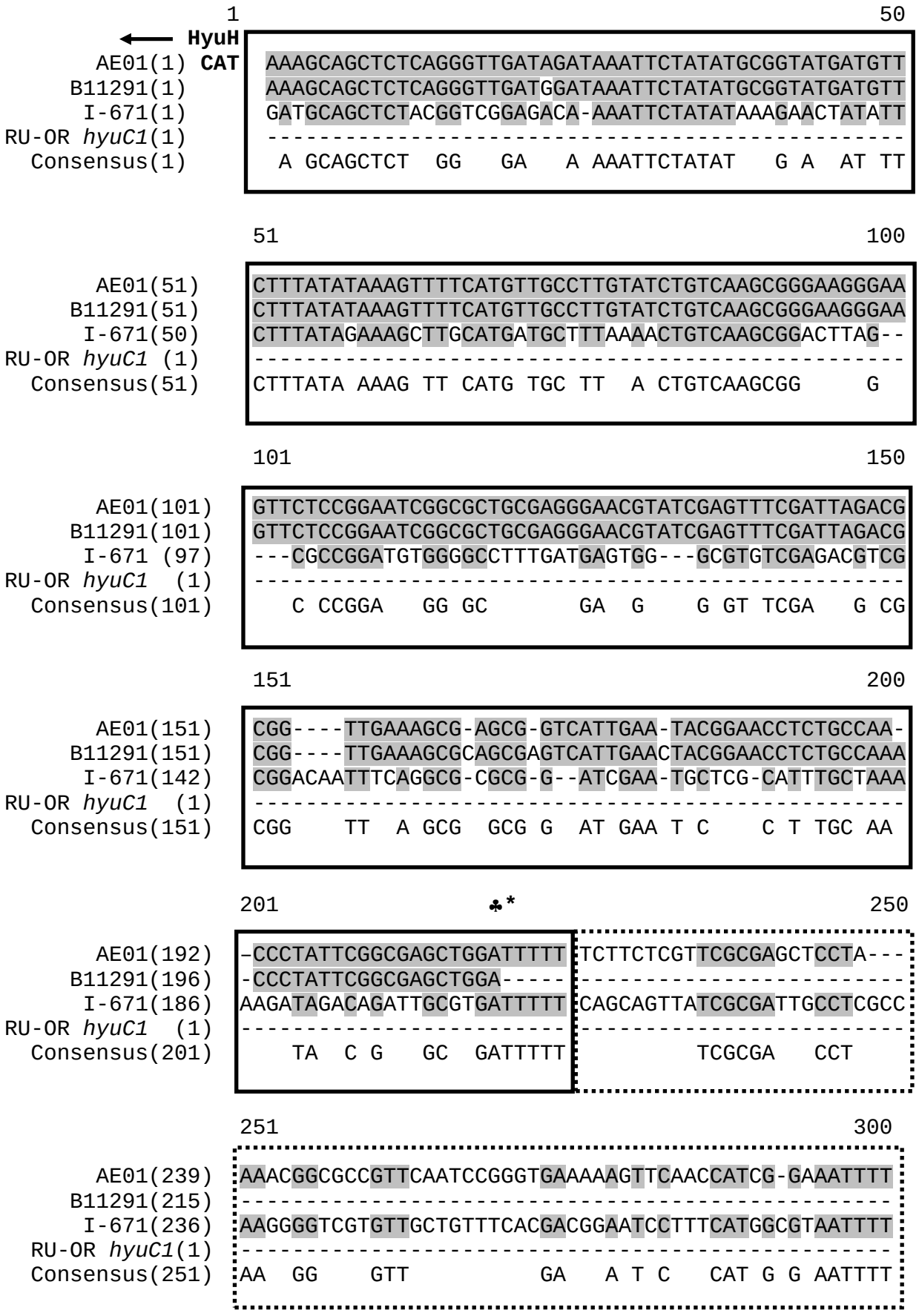


Figure 4-1: The genetic organization of the *hyu* gene clusters isolated from *Agrobacterium* strains (A) IP I-671, (B) NRRL B11291, (C) RU-AE01 and (D) RU-OR. The length of the *hyu* operon is shown below in base pairs (kb). The genes and the direction of transcription are shown by the arrows. Figure adapted from Grifantini *et al.* (1998), Hartley (2001) and Hils *et al.* (2001). Abbreviations: ORF 1- Open Reading Frame 1 which encodes a putative transposase, *hyuN*- gene encoding an NADPH dependent flavin oxidoreductase, *hyuH*- hydantoinase-encoding gene, *hyuC*- NCAAH-encoding gene, *hyuD*- gene encoding a D-amino acid dehydrogenase, *hyuA*- racemase-encoding gene and ORF 2- Open Reading Frame 2 which encodes a putative resolvase.

A multiple sequence alignment of available *Agrobacterium hyuH-hyuC* promoter regions was used to determine whether there was any sequence conservation between the promoter sequences. Alignment of the sequences from IP I-671 and NRRL B11291, and the nucleotide sequence upstream of RU-OR *hyuC1*, with that of RU-AE01 revealed extensive regions of homology between the promoter regions of all four strains (Figure 4-2).


1
50
HyuH
CAT
AE01(1)
B11291(1)
I-671(1)
RU-OR hyuC1(1)
Consensus(1)
AAAGCAGCTCTCAGGGTTGATAGATAAATTCTATATGCGGTATGATGTT
AAAGCAGCTCTCAGGGTTGATGGATAAATTCTATATGCGGTATGATGTT
GATGCAGCTCTACGGTCGGAGACA-AAATTCTATATAAAGAACTATATT
A GCAGCTCT GG GA A AAATTCTATAT G A AT TT
51
100
AE01(51)
B11291(51)
I-671(50)
RU-OR hyuC1 (1)
Consensus(51)
CTTTATATAAAGTTTTCATGTTGCCTTGTATCTGTCAAGCGGGAAGGGAA
CTTTATATAAAGTTTTCATGTTGCCTTGTATCTGTCAAGCGGGAAGGGAA
CTTTATAGAAAGCTTGCATGATGCTTTAAAACTGTCAAGCGGACTTAG--
CTTTATA AAAG TT CATG TGC TT A CTGTCAAGCGG G
101
150
AE01(101)
B11291(101)
I-671 (97)
RU-OR hyuC1 (1)
Consensus(101)
GTTCTCCGGAATCGGCGCTGCGAGGGAACGTATCGAGTTTCGATTAGACG
GTTCTCCGGAATCGGCGCTGCGAGGGAACGTATCGAGTTTCGATTAGACG
---CGCCGGATGTGGGGCCTTTGATGAGTGG---GCGTGTCGAGACGTCG
C CCGGA GG GC GA G G GT TCGA G CG
151
200
AE01(151)
B11291(151)
I-671(142)
RU-OR hyuC1 (1)
Consensus(151)
CGG----TTGAAAGCG-AGCG-GTCATTGAA-TACGGAACCTCTGCCAA-
CGG----TTGAAAGCGCAGCGAGTCATTGAACTACGGAACCTCTGCCAAA
CGGACAATTTCAGGCG-CGCG-G--ATCGAA-TGCTCG-CATTTGCTAAA
CGG TT A GCG GCG G AT GAA T C C T TGC AA
201
♣*
250
AE01(192)
B11291(196)
I-671(186)
RU-OR hyuC1 (1)
Consensus(201)
–CCCTATTCGGCGAGCTGGATTTTT
-CCCTATTCGGCGAGCTGGA-----
AAGATAGACAGATTGCGTGATTTTT
TA C G GC GATTTTT
TCTTCTCGTTCGCGAGCTCCTA---
CAGCAGTTATCGCGATTGCCTCGCC
TCGCGA CCT
251
300
AE01(239)
B11291(215)
I-671(236)
RU-OR hyuC1(1)
Consensus(251)
AAACGGCGCCGTTCAATCCGGGTGAAAAAGTTCAACCATCG-GAAATTTT
AAGGGGTCGTGTTGCTGTTTCACGACGGAATCCTTTCATGGCGTAATTTT
AA GG GTT GA A T C CAT G G AATTTT

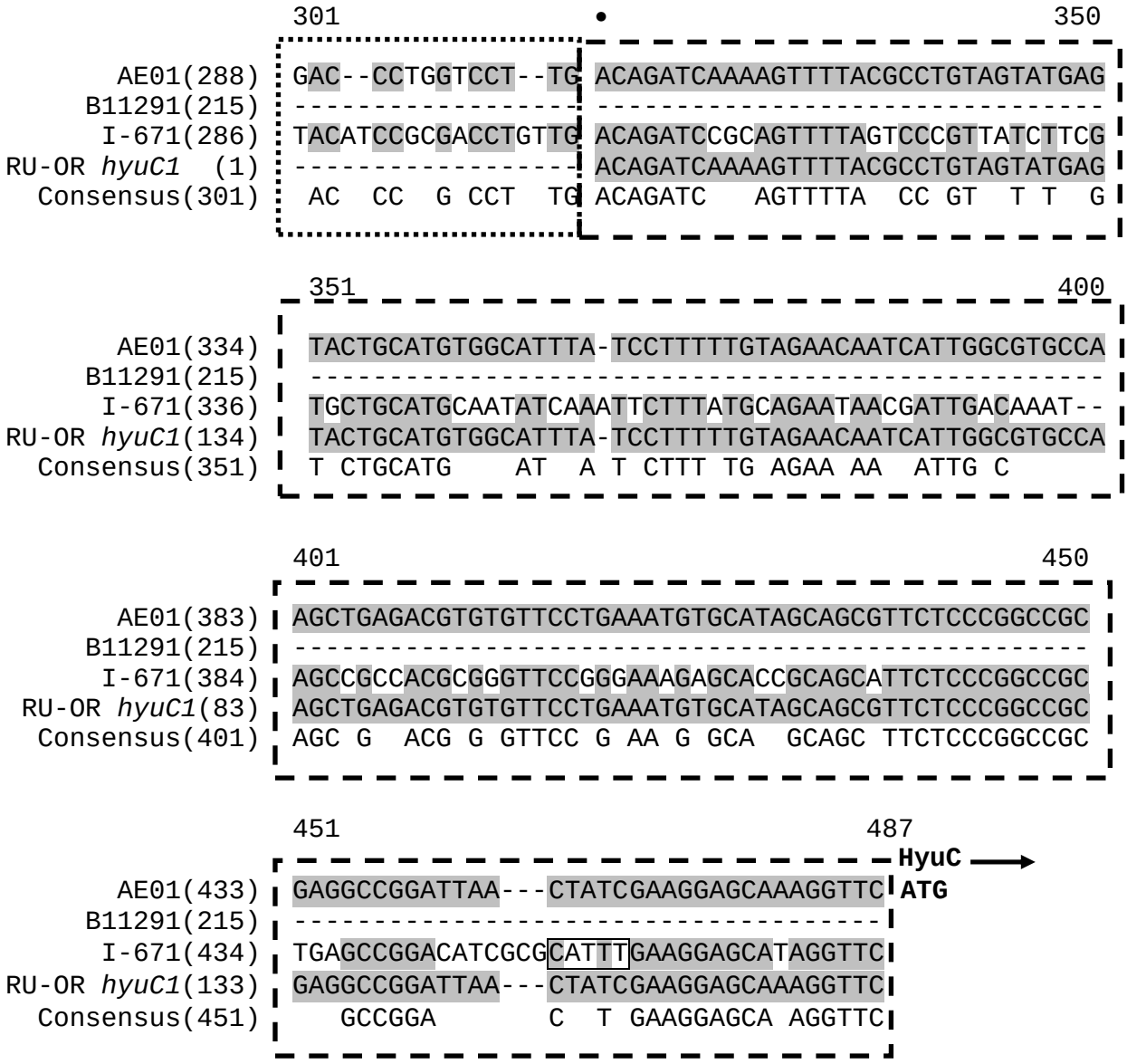


Figure 4-2: Alignment of the promoter regions of the *hyu* genes from *Agrobacterium* strains. Sequences start at the nucleotide directly upstream the A of the ATG start codon for both the *hyuH* and the *hyuC* genes. The A of the ATG start codon for 5' terminus of the *hyuH* coding sequence is at nucleotide position 1 for RU-AE01 and the A of the ATG start codon for the 5' terminus for the *hyuC* coding sequence is at nucleotide position 487 for RU-AE01. The consensus sequence is shown below the alignment. ♣: denotes the last nucleotide of the intergenic sequence in NRRL B11291 (nt 220 on the RU-AE01 sequence); ***:** denotes the position at which the A of the ATG start codon for the HyuC in NRRL B11291 is positioned (nt 221 on the RU-AE01 sequence); **•:** denotes the position at which the nucleotide sequence for RU-OR *hyuC1* starts (302 nt on the RU-AE01 sequence). The sequence boxed by a bold line represents Region I; the sequence boxed by the dotted line represents Region II; the sequence boxed by the dashed line represents Region III. The boxed sequence (nt 449-453) on the I-671 sequence is a possible Pribnow box which is discussed in Section 4.3.2.

The IP I-671 sequence was 58% identical to the RU-AE01 nucleotide sequence, while the 214 nt of NRRL B11291 promoter sequence was 99% identical to the integenic sequence of RU-AE01. The NRRL B11291 promoter region ended at position 220 (marked by the symbol ♣ above the sequence in Figure 4-2) and the A of the ATG start codon for *hyuC* followed at position 221 (marked by the symbol ***** above the sequence in Figure 4-2). This suggests a deletion of the sequence between nts 221 and 486 in NRRL B11291 or an insertion of DNA sequence at nt 221 in IP I-671 and RU-AE01. The nucleotide sequence for the region upstream of RU-OR *hyuC1* started at position 319 on the RU-AE01 sequence (marked by the symbol • above the sequence in Figure 4-2) and was 100% identical to the sequence for RU-AE01.

When aligning the promoter regions of the *hyu* genes from *Agrobacterium* strains, there appeared to be three distinct regions (I, II and III) based on their levels of sequence conservation. Region I, a region of high conservation immediately 5' to the *hyuH* coding sequence, showed 99% nucleotide sequence identity between NRRL B11291 and RU-AE01 and 63% nucleotide sequence identity between IP I-671 and RU-AE01, respectively. In contrast, Region II, a region of low conservation adjacent to Region I, had 48% nucleotide sequence identity between IP I-671 and RU-AE01 and was not present in the other promoter regions. Region III, is a region of high conservation adjacent to Region II and immediately 5' to the coding sequence of the *hyuC* gene, which shows 100% nucleotide sequence identity between RU-OR *hyuC1* and RU-AE01 sequences and 67% nucleotide sequence identity between IP I-671 and RU-AE01, respectively. Region III was not present in the *hyuH-hyuC* sequence from NRRL B11291. The presence of regions of high sequence conservation flanking a region of low conservation suggests that the regulatory sequences required for the transcription of the RU-AE01 *hyuH* and *hyuC* genes might be present in Regions I and III, respectively. This assumption presumes that the *hyu* genes in the *Agrobacterium* strains IP I-671, NRRL B11291, RU-OR and RU-AE01 are regulated in the same manner. This hypothesis is supported by the nucleotide sequence identity between the *Agrobacterium* strains.

4.3.2 Analysis of the RU-AE01 *hyuH-hyuC* region

Transcription activation requires recognition of promoter sequences by the σ subunit of the RNA polymerase enzyme. Most prokaryotic genes are recognized by σ^{70}-subunit of RNA polymerase, the binding at which is regulated at the -35 / -10 promoter sequences (Sivaraman *et al*. 2005). The consensus sequences for σ^{70}-dependent promoters at the -10 region is TATAAT (Pribnow 1975) and -35 region TTGACA (Seeburg *et al*. 1977). The genes for nitrogen metabolism are often regulated by an alternative σ subunit, σ^{54} which has -24 / -12 promoter recognition sites. The consensus for σ^{54}-dependent promoters at the -12 site is TTGCA and at the -24 site is CTGGNA (Barrios *et al*. 1999) where the GC and GG are most highly conserved nucleotides. While these consensus sequences have been derived from *E. coli*, they are well conserved in the gram negative bacteria (Barrios *et al*. 1999, Helmann and Chamberlain 1988). The *hyuH* and the *hyuC* genes in RU-AE01 are both subject to NCR thus it is possible that σ^{54}-dependent promoters may be responsible for the activation of transcription. In addition, σ^{54}-binding sites have been identified upstream of *hyu* genes in other bacterial strains including *Pseudomonas* sp. NS 671 and *A. tumefaciens* NRRL B11291 (Watabe *et al*. 1992a, Grifantini *et al*. 1998). For these reasons, the RU-AE01 *hyuH-hyuC* sequence was analyzed for σ^{70}-dependent and σ^{54}-dependent promoters.

The hyuH promoter sequences

Analysis of the sequence upstream of *hyuH* revealed three possible promoter regions (Figure 4-3). The first two were σ^{70}-dependent promoters ‘A’ and ‘B’ with -35 and -10 regions that were similar to the consensus -35 and -10 sequences for prokaryotes. The σ^{70}-dependent promoter ‘A’ had a -35 sequence between nts 87-82 and a -10 sequence between nts 56-52. The second σ^{70}-dependent promoter ‘B’ had a -35 region between nts 170-165 and a -10 region between nts 147-142. The third putative promoter was a σ^{54}-dependent promoter with sequences similar to the consensus prokaryotic -24 and -12 sequences identified between nts 191-186 and nts 179-175 respectively. All the regulatory sequences identified were in Region I between nts 1-225 on the RU-AE01 *hyuH-hyuC* sequence.

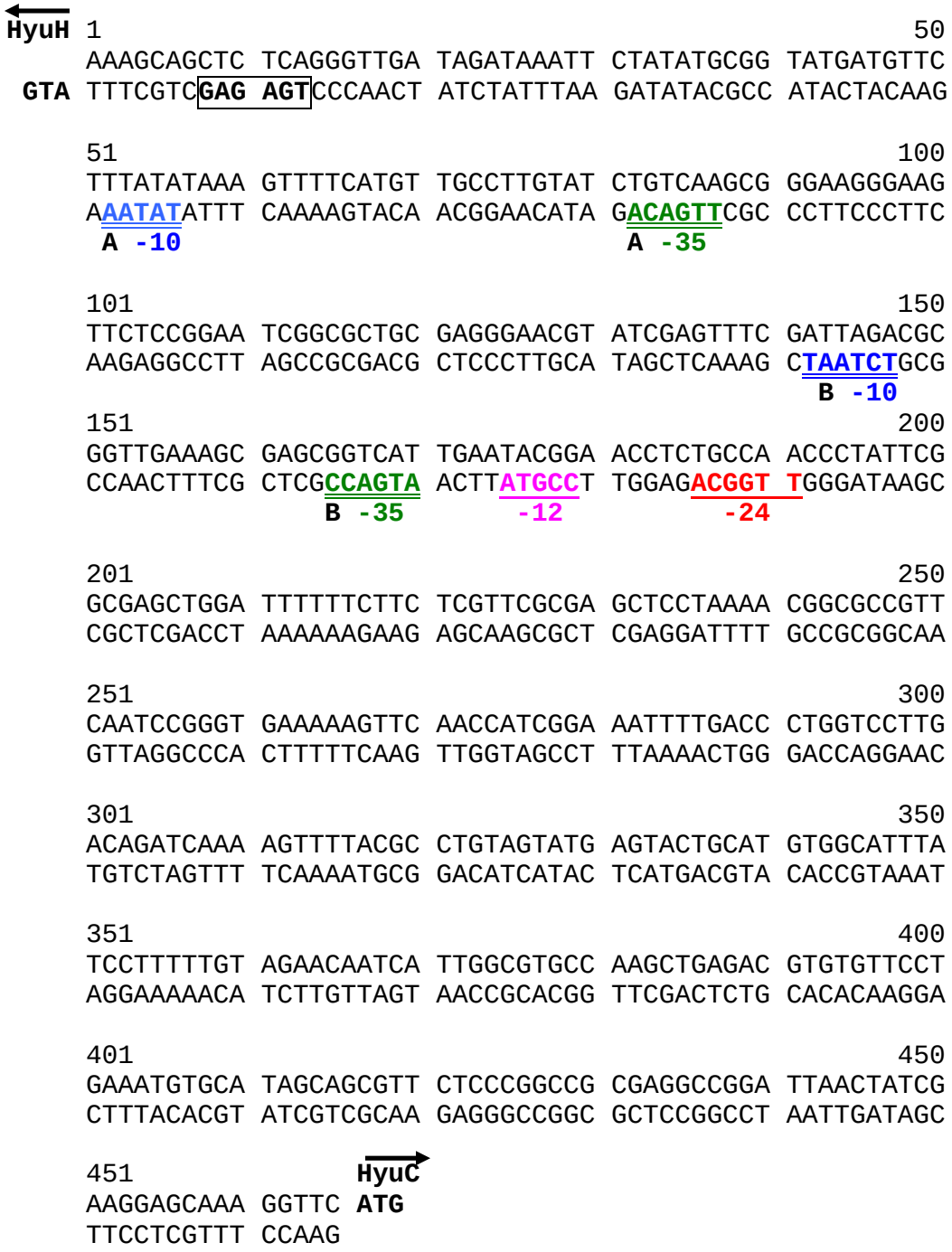


Figure 4-3: Identification of putative transcription and translation signals for *hyuH*. The RU-AE01 nucleotide sequences starts at the nucleotide directly upstream the A of the ATG start codon for both the *hyuH* and the *hyuC* genes. Putative σ^{70}-dependent -35 regions are shown in a **green font** (consensus sequence - TTGACA) and -10 regions are shown in a **blue font** (consensus sequence - TATAAT). Putative σ^{54}-dependent -24 regions are shown in an **orange font** (consensus sequence - CTGGNA) and -12 regions shown in a **pink font** (consensus sequence - TTGCA). Putative Shine-Dalgarno sequences are indicated by the boxed hexanucleotide sequences (consensus sequence - AGGAGC).

The hyuC promoter regions

Four putative σ^{70}-dependent promoters labeled ‘A’, ‘B’, ‘C’ and ‘D’ each consisting of a -35 region and a -10 region were identified upstream of *hyuC* (Figure 4-4). The first σ^{70}-dependent promoter ‘A’ encodes a -35 sequence between nts 17-22 and a -10 sequence between nts 41-46. The next promoter ‘B’ encodes a -35 sequence between nts 153-158 and a -10 sequence between nts 171-176. The third promoter ‘C’ encodes a -35 sequence between nts 298-303 and a -10 sequence between nts 322-327. Finally, the fourth promoter ‘D’ encodes a -35 sequence between nts 419-424 and a -10 sequence between nts 439-444. While the σ^{70}-dependent promoter sequences are named -35 and -10 regions and the optimal distance between the two sequences is 17 $\pm$ 1 nts, the distances between the -35 and -10 regions can vary from 15 to 21 nts (Harley and Reynolds 1987, Huerta and Collado-Vides 2003). In addition, variation in the weakly conserved nucleotides in the consensus recognition sequences for the -35 (TTGACA) and -10 (TATAAT) regions do occur in the promoter sequences providing a possible explanation for the deviation from the consensus sequences observed in the σ^{70}-dependent promoters identified on the RU-AE01 *hyuH-hyuC* fragment (Harley and Reynolds 1987).

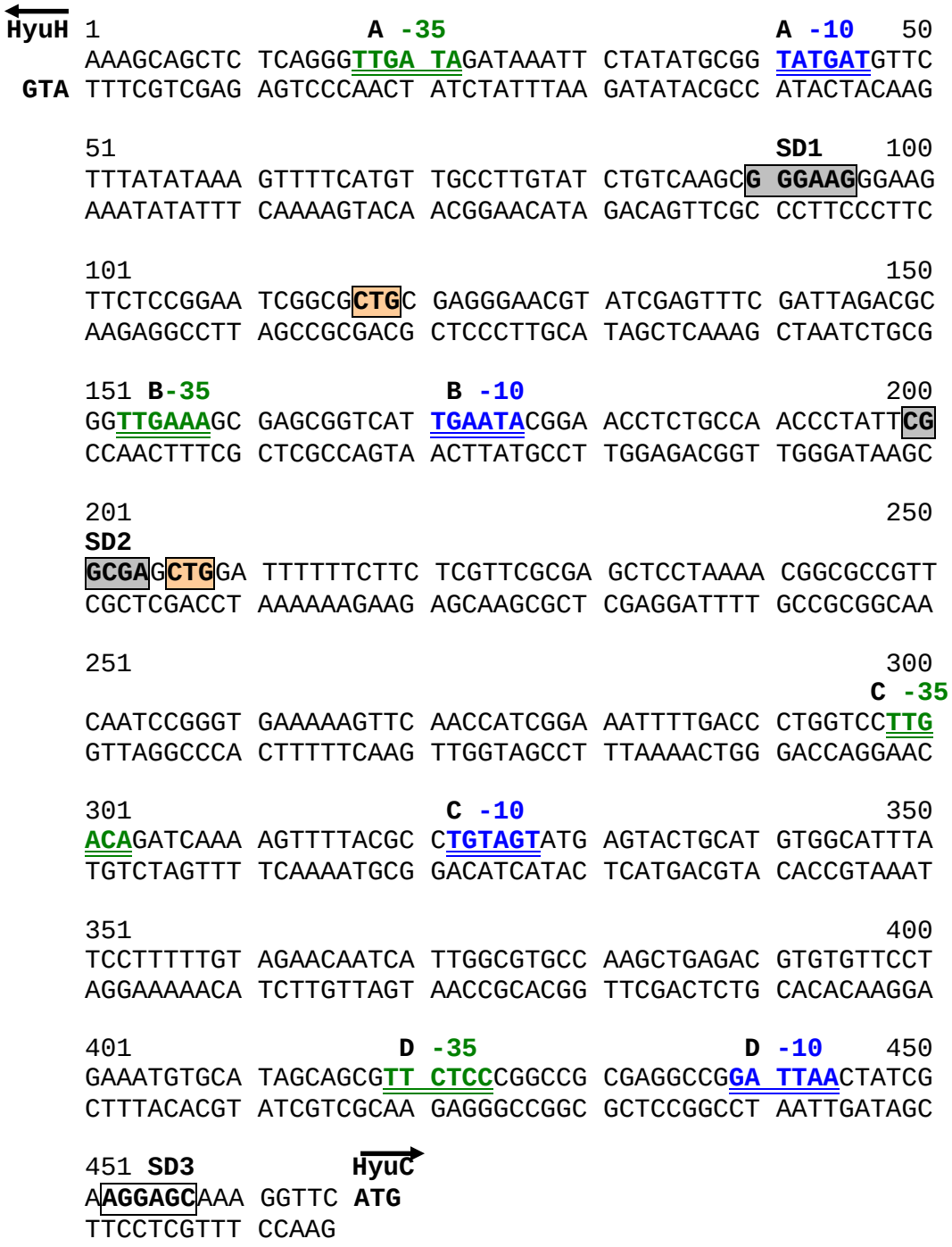


Figure 4-4: Identification of putative transcription and translation signals for *hyuC*. The RU-AE01 nucleotide sequences starts at the nucleotide directly upstream the A of the ATG start codon for both the *hyuH* and the *hyuC* genes. Putative σ^{70}-dependent -35 regions are shown in a **green font** (consensus sequence - TTGACA) and -10 regions are shown in a **blue font** (consensus sequence - TATAAT). Four sets of putative σ^{70}-dependent -35 and putative -10 sequences identified are marked A, B, C and D. A putative Shine-Dalgarno sequence (SD3) is indicated by the boxed hexanucleotide sequences (consensus sequence - AGGAGC). Sequences that bear similarity to Shine-Dalgarno sequences but that do not have the start codons (AUG) downstream are indicated by the boxed hexanucleotide sequences (SD1 and SD2) (consensus

sequence - AGGAGC). Possible alternative start codons (GUG, UUG and CUG) are indicated by the boxed tan-shaded sequences.

hyuH and hyuC regulatory regions

Translation of mRNA is mediated by ribosome binding to the Shine-Dalgarno sequence whose consensus sequence has been identified as AGGAGC (Shine and Dalgarno 1975). Though the spacing between the Shine-Dalgarno and the translational start (AUG) has been shown to vary between 2 to 34 nts, the optimal spacing is 5 nts (Chen *et al.* 1994). While the most frequently used start codon in *E. coli* is AUG (Met), the codons GUG (Val), UUG (Leu) and CUG (Leu) can also serve as initiation codons (O'Donnell and Janssen 2001).

In addition to the promoter sequences identified on the RU-AE01 *hyuH-hyuC* region, one putative Shine-Dalgarno sequence was identified between nts 13-8 upstream of *hyuH* (Figure 4-3). Three possible Shine-Dalgarno sequences were identified upstream of *hyuC* between nts 90-95 (SD1), nts 199-204 (SD2) and nts 452-457 (SD3) (Figure 4-4). Of the three putative Shine-Dalgarno sequences identified upstream of *hyuC*, only SD3 is followed by an AUG start codon. Putative Shine-Dalgarno sequences SD1 and SD2 are both followed by a CUG codon.

These sequences could serve as sites for ribosome binding and subsequent translation of the mRNA provided a suitable translational start was present downstream of the Shine-Dalgarno sequence.

Identification of repeat sequences on the RU-AE01 hyuH-hyuC fragment

The responsiveness of the *hyuH* and *hyuC* promoters to induction (Section 3.3.2) suggests that transcription factors may be involved in the regulation of *hyuH-hyuC* transcriptional activation. Transcription factors bind specific target DNA sequences and these binding sites are often symmetrical sequences consisting of direct, inverted or everted repeats (Yanofsky 1992, Pedersen and Valentin-Hansen 1997, Jensen *et al*. 1999). Two GCGAG sequences were identified between nts 159-163 and nts 430-434 respectively (highlighted by blue shading, Figure 4-5). These direct repeats are between the -35 and -10 sequences of two putative σ^{70}-dependent promoters which were identified upstream of *hyuC*; 'B' and 'D' respectively. Three inverted repeat (palindromic) sequences were identified in the *hyuH-hyuC* promoter regions of RU-AE01. Two perfect hexanucleotide palindromic sequences were

observed in the RU-AE01 sequence between nts 50-61 (P1) and between nts 239-250 (P2) (Figure 4-5). Another imperfect palindromic sequence was present between nts 255-294 (P3). An extensive search of the published literature and of transcription factor databases did not yield any information suggesting that sequences similar to these sites have not been reported as binding sites for transcription factors nor have they been reported as being involved in regulation of transcription.

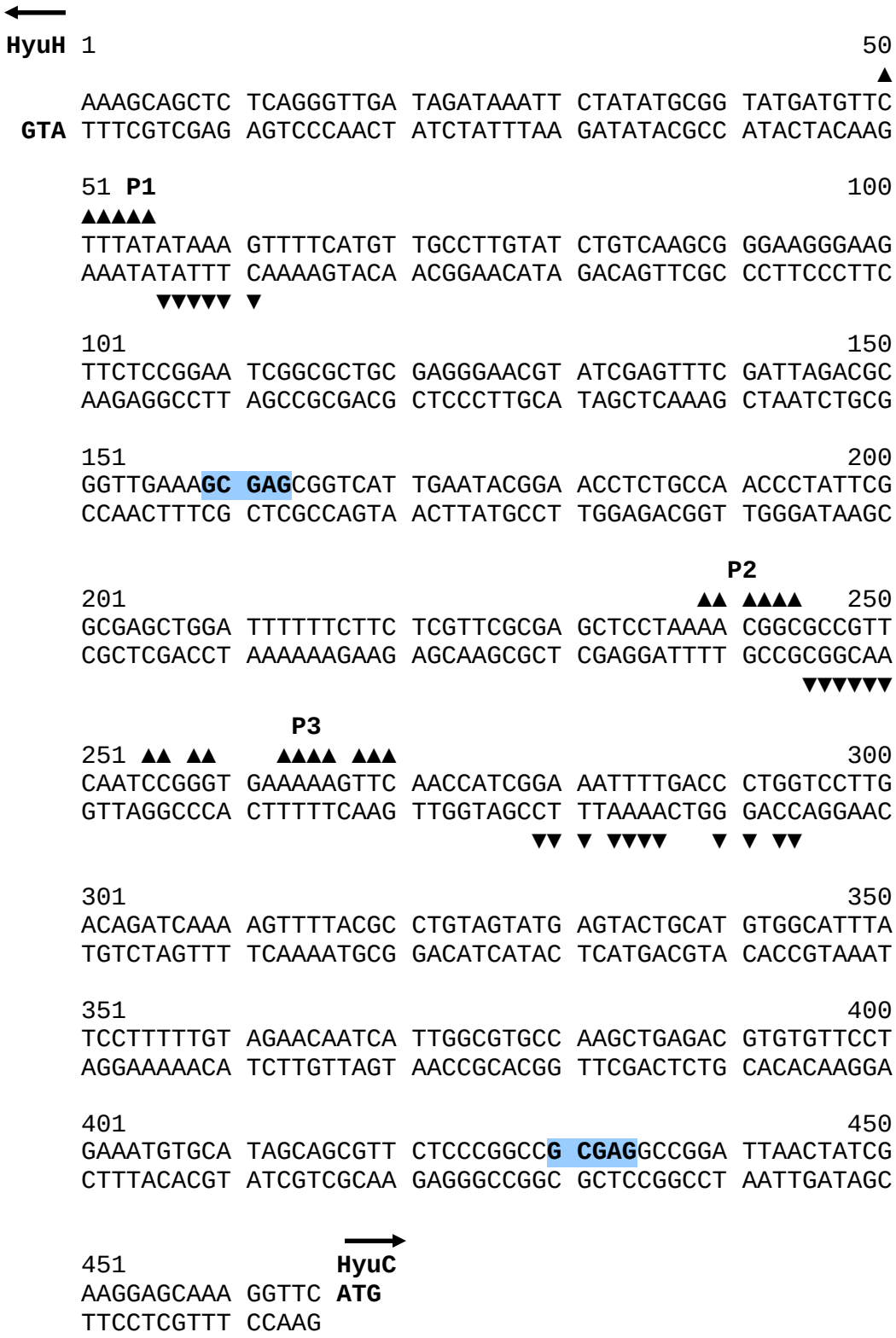


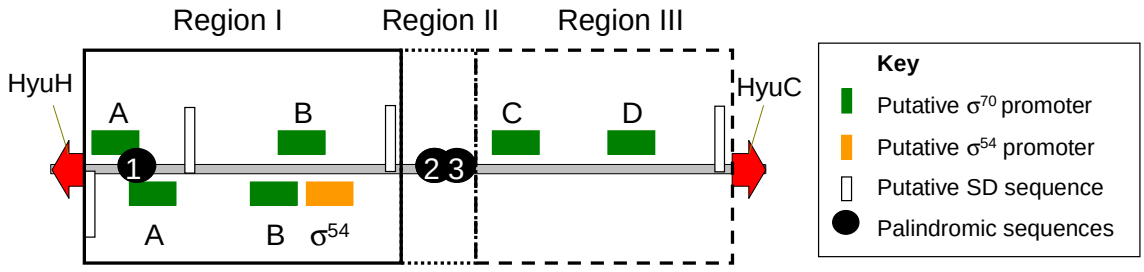
Figure 4-5: Putative palindromic sequences on the RU-AE01 *hyuH-hyuC* intergenic region. The RU-AE01 nucleotide sequences starts at the nucleotide directly upstream the A of the ATG start codon for both the *hyuH* and the *hyuC* genes. Direct repeats identified on the RU-AE01 sequence are highlighted in blue and palindromic sequences identified on the RU-AE01 sequence are labeled by a (▲) above or a (▼) below the sequence.

4.3.3 Deletion analysis of the *hyuH-hyuC* region

A deletion analysis of the *hyuH-hyuC* region was used to investigate which of the putative promoter sequences and potential transcription factor binding sites (identified in Figures 4-3, 4-4 and 4-5) were functional. Six deletion plasmids were constructed (Figure 4-6) in pMJ242 (Section 2.3.2). These plasmids were transformed into RU-AE01 cells, grown to stationary phase in LB containing 1% hydantoin and reporter enzyme assays were done as described in Section 2.2.5.

Plasmid pMJ258, which contains the entire promoter region between *hyuH* and *hyuC* from RU-AE01, was used as the parental plasmid, with the *hyuH* promoter fused to *gus* and *hyuC* promoter fused to the *lacZ* gene. Deletion plasmids pMJ266, pMJ261 and pMJ361 have 189, 253 and 367 nts, respectively, deleted from the *hyuC* terminus of the promoter fragment (Figure 4-6). Thus in pMJ266, the putative σ^{70}-dependent promoter sequences designated ‘C’ and ‘D’, upstream of the *hyuC* gene, corresponding to Region III was deleted. In pMJ261 Regions II and III, encoding two of the three inverted repeats was also deleted, leaving only Region I. Lastly, deletion plasmid pMJ361 contained only the 100 nt immediately upstream of *hyuH* encoding the putative σ^{70}-dependent promoter ‘A’ sequence but not the putative σ^{70}-dependent promoter ‘B’ or the putative σ^{54}-dependent promoter sequence. In the *hyuC* direction, this plasmid carried a putative σ^{70}-dependent promoter sequence denoted ‘A’ (Figure 4-6). Plasmid pMJ268 carried 279 nt immediately upstream of the *hyuC* gene corresponding to Regions II and III, with Region I deleted and plasmid pMJ245 carried 182 nt directly upstream of the *hyuC* gene corresponding to the sequence for the putative σ^{70}-dependent promoter ‘D’ and the putative Shine-Dalgarno sequence SD3 (Figure 4-6).

A



B

Plasmid	Area of the *A. tumefaciens* RU-AE01 *hyuH-hyuC* intergenic fragment present in the plasmid	Nts	*hyuH* β-Gus ± SD	*hyuC* β-Gal ± SD
pMJ242	---	---	-1 ± 3	2 ± 1
pMJ258		1-467	5084 ± 300	689 ± 69
pMJ266		1-278	4729 ± 66	nd
pMJ261		1-214	4858 ± 34	80 ± 1
pMJ361		1-100	201 ± 22	398 ± 11
pMJ268		188-467	288 ± 53	788 ± 35
pMJ245		285-467	nd	311 ± 16

Figure 4-6: Reporter enzyme activities for the deletion constructs of the RU-AE01 *hyuH-hyuC* fragment. (A) Summary of the *hyuH* and *hyuC* sequences identified on the *hyuH-hyuC* region of RU-AE01. The 8 amino terminal amino acids of HyuH, the *hyuH-hyuC* region and the 8 amino terminal amino acids of HyuC are depicted. The Regions I, II and III identified in Figure 4-2 are depicted by the bold, dotted and dashed boxes respectively. The transcription activation signals upstream of *hyuH* are shown on the bottom strand. The transcription activation signals upstream of *hyuC* are shown on the top strand. Diagram is not drawn to scale. (B) β-glucuronidase and β-galactosidase enzyme assays for the deletion constructs of pMJ258. Results are the average of triplicate reactions. The standard mean error is shown by the SD values. Abbreviation: nd- not determined.

The levels of β-glucuronidase enzyme activity (*hyuH* fusion) detected in RU-AE01 cells hosting pMJ258 (full length *hyuH-hyuC* promoter fragment), pMJ266 (Region III deletion) and pMJ261 (Regions II and III deleted) were comparable (Figure 4-6). This indicated that the *hyuH* promoter and Shine-Dalgarno sequence was located in the first 214 nts immediately upstream of the *hyuH* coding sequence. The presence of palindromic sequences P2 and P3 had no effect on the levels of β-glucuronidase enzyme activity suggesting these sequences were not involved in the regulation of *hyuH* transcription or translation. There was an approximately 20 fold drop in β-glucuronidase enzyme activity in cells hosting pMJ361 (201 ± 22) suggesting that the putative σ^{70}-dependent promoter sequence 'A' between nts 87-52 does not play a

significant role in activating *hyuH.* By elimination, this implies that the area of importance for the activation of *hyuH* might lie between nts 101 and 214. This is the region where two putative promoters, a σ^{70}-dependent promoter sequence 'B' between nts 170-142 and a putative σ^{54}-dependent promoter between nts 191-175, and a putative Shine-Dalgarno sequence were identified. Thus it is possible that these sequences might be largely responsible for the activation of *hyuH* transcription and translation. Deletion plasmid pMJ268, in which Region I had been deleted, supported low levels of β-glucuronidase activity consistent with the observation that the major activation sequences for *hyuH* were located in Region I. While levels of β-glucuronidase activity supported by promoter fragments in pMJ361 (Regions II and III deleted) and pMJ268 (Region I deleted) were low, they were still significantly higher than that supported by the promoterless vector plasmid pMJ242 (201 and 288 vs 0 β-Gluc units respectively) (Figure 4-6). This β-glucuronidase enzyme activity can be attributed to low levels of activation due to weak transcription and translation signals present on the inserts in pMJ268 and pMJ361, but not in the vector pMJ242.

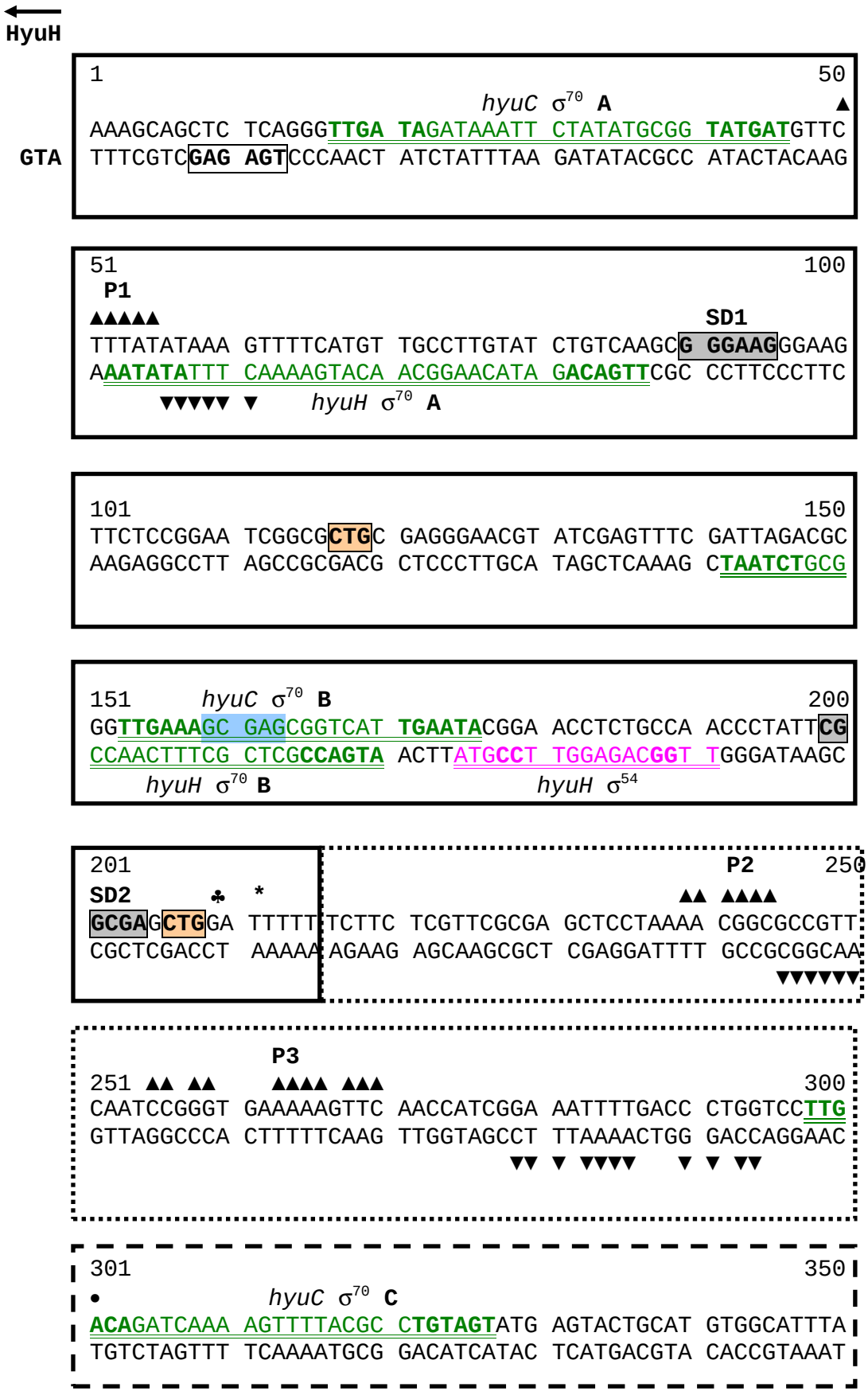
Approximately equivalent levels of transcriptional and translational activity were supported by the promoter fragment in pMJ268 (containing Regions II and III) compared with the full length promoter fragment in pMJ258 (788 vs 689 Miller units of β-galactosidase enzyme activity). This result suggested that the sequences required for the activation of transcription and translation of *hyuC* were between nts 188-467, a region which contains two putative σ^{70}-dependent promoters (labelled 'C' and 'D' in Figure 4-6) and a putative Shine-Dalgarno sequence. Plasmid pMJ245 which contained the nucleotide sequence for the putative σ^{70}-dependent promoter 'D' and the Shine-Dalgarno sequence SD3 (Figure 4-6) supported half the β-galactosidase enzyme activity (311 Miller units) as compared to pMJ258 and pMJ268. This implies while the region between nts 285-467 encodes some transcription and translation activation sequences, other sequences important for the activation of transcription and translation are located outside this region. The results of the β-galactosidase enzyme assays imply that these other important sequences are between nts 188-285. Two palindromic sequences (denoted P2 and P3, Figure 4-5) were identified in this region.

RU-AE01 cells containing the plasmid pMJ361 and RU-AE01 cells hosting pMJ261 both supported β-galactosidase enzyme activity (398 and 80 Miller units respectively). The levels of β-galactosidase produced by pMJ361 and pMJ261 suggested that further transcription and translation activation sequences were present between nts 1-100 in pMJ361 and pMJ261. Interestingly, the presence of additional sequence (between nts 100–214) resulted in a ten-fold drop in transcriptional and translational activation (pMJ261) (Figure 4-6). This demonstrated that transcriptional and translational activation by the sequence between nts 1-100 in pMJ261 was repressed by sequences located between nts 100 and 278.

4.4 GENERAL CONCLUSIONS

The aims of the research presented here were to (1) conduct a detailed bioinformatic analysis of the *hyuH-hyuC* regions from *Agrobacterium* strains including IP I-671, NRRL B11291, RU-OR *hyuC1* and RU-AE01, (2) to identify putative regulatory sequences on the RU-AE01 *hyuH-hyuC* fragment and (3) to conduct a deletion analysis of the *hyuH-hyuC* region to analyse the regulatory sequences identified in section 4.3.2.

The sequence alignment of the *hyuH-hyuC* promoter regions demonstrated that the *hyuH-hyuC* fragments from *Agrobacterium* strains had two regions of high sequence identity (named Regions I and III) flanking a region of low sequence identity that was 84 nt in length (Region II). When all the regulatory sequences identified in this chapter were overlayed (Figure 4-7), it was observed that all the sequences identified as putative promoter sequences or as putative Shine-Dalgarno sequences were located in the highly conserved Regions I and III (depicted in Figure 4-7). The NRRL B11291 sequences, the IP I-671 sequences and the RU-OR *hyuC1* sequences were 98%, 74% and 100% identical to the RU-AE01 sequence over the putative regulatory regions; which suggests a high level of sequence conservation between the strains. The σ^{70}-dependent -10 region (marked ‘D’ in Figure 4-4) was poorly conserved in IP I-671, showing only 33% identity to the RU-AE01 sequence. An alternative sequence (boxed sequence CATTT between nts 467-471 on the RU-AE01 sequence in Figure 4-2) was identified as a putative -10 sequence for strain IP I-671.


HyuH
1
50
hyuC σ70 A
AAAGCAGCTC TCAGGGTTGA TAGATAAATT CTATATGCGG TATGATGTTC
GTA TTTCGTCGAG AGTCCCAACT ATCTATTTAA GATATACGCC ATACTACAAG
51
100
P1
SD1
TTTATATAAA GTTTTCATGT TGCCTTGTAT CTGTCAAGCG GGAAGGGAAG
AAATATATTT CAAAAGTACA ACGGAACATA GACAGTTCGC CCTTCCCTTC
hyuH σ70 A
101
150
TTCTCCGGAA TCGGCGCTGC GAGGGAACGT ATCGAGTTTC GATTAGACGC
AAGAGGCCTT AGCCGCGACG CTCCCTTGCA TAGCTCAAAG CTAATCTGCG
151
hyuC σ70 B
200
GGTTGAAAGC GAGCGGTCAT TGAATACGGA ACCTCTGCCA ACCCTATTCG
CCAACTTTCG CTCGCCAGTA ACTTATGCCT TGGAGACGGT TGGGATAAGC
hyuH σ70 B
hyuH σ54
201
SD2
250
P2
GCGAGCTGGA TTTTTTCTTC TCGTTCGCGA GCTCCTAAAA CGGCGCCGTT
CGCTCGACCT AAAAAAGAAG AGCAAGCGCT CGAGGATTTT GCCGCGGCAA
P3
251
300
CAATCCGGGT GAAAAAGTTC AACCATCGGA AATTTTGACC CTGGTCCTTG
GTTAGGCCCA CTTTTTCAAG TTGGTAGCCT TTAAAACTGG GACCAGGAAC
301
350
hyuC σ70 C
ACAGATCAAA AGTTTTACGC CTGTAGTATG AGTACTGCAT GTGGCATTTA
TGTCTAGTTT TCAAAATGCG GACATCATAC TCATGACGTA CACCGTAAAT

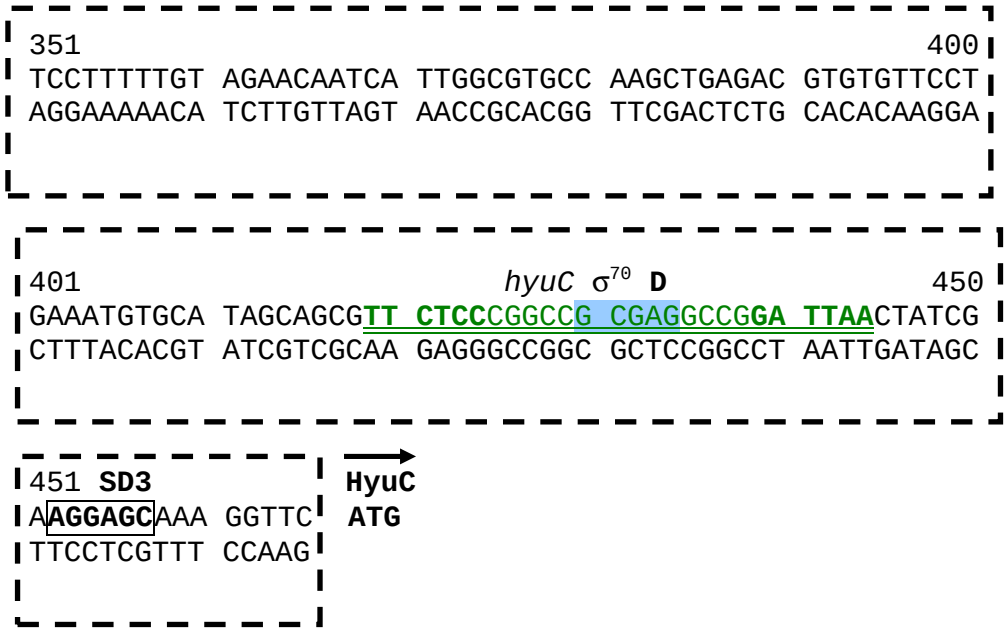


Figure 4-7: Summary of the results of the bioinformatics analyses of the region between *hyuH* and *hyuC* from RU-AE01. The sequences used in the alignment start at the nucleotide directly upstream the A of the ATG start codon for both the *hyuH* and the *hyuC* genes. ♣: denotes the last nucleotide of the intergenic sequence in NRRL B11291; *: denotes the position at which the A of the ATG start codon for the *hyuC* in NRRL B11291 is positioned; •: denotes the position at which the nucleotide sequence for RU-OR *hyuC1* starts (nt 301 on the RU-AE01 sequence above). The sequence boxed by a bold line represents Region I; the sequence boxed by the dotted line represents Region II; the sequence boxed by the dashed line represents Region III. Putative σ^{70}-dependent -35 regions are shown in a **green font** and putative σ^{54}-dependent regions are shown in a **pink font**. Two putative σ^{70}-dependent promoters and one putative σ^{54}-dependent promoter are shown upstream of *hyuH.* Four putative σ^{70}-dependent promoters are shown upstream of *hyuC.* Putative Shine-Dalgarno sequences are indicated by the boxed hexanucleotide sequences. One putative Shine-Dalgarno sequence is indicated upstream of the HyuH AUG and one upstream of the HyuC AUG. Sequences that bear similarity to Shine-Dalgarno sequences but that do not have the start codon (AUG) downstream are indicated by the boxed hexanucleotide sequences. Alternative start codons (GUG, UUG and CUG) are indicated by the boxed tan-shaded sequences. Two potential Shine-Dalgarno sequences with CUG start codons are shown upstream of HyuC. Direct repeats identified on the RU-AE01 sequence are highlighted in blue and palindromic sequences identified on the RU-AE01 sequence are labeled by a (▲) above or a (▼) below the sequence.

Deletion analysis of the RU-AE01 *hyuH* promoter region implied that either the σ^{70}-dependent promoter 'B' or the putative σ^{54}-dependent promoter identified upstream of *hyuH* may be responsible for the activation of *hyuH* transcription. From the high nucleotide sequence identity observed between RU-AE01, IP I-671 and NRRL B11291, it is possible that this putative promoter may also be responsible for the activation of *hyuH* in strains IP I-671 and NRRL B11291 as well as RU-AE01.

Similarly, deletion analysis of the RU-AE01 *hyuC* promoter suggested that the putative σ^{70}-dependent promoter 'C' or 'D' might be the activator of *hyuC* transcription in RU-AE01. A high degree of sequence similarity between RU-AE01, IP I-671 and RU-OR *hyuC1* indicates that the same promoter may be responsible for the activation of *hyuC* transcription in IP I-671 and RU-OR *hyuC1.* Consensus sequences for two other putative σ^{70}-dependent promoters were identified upstream of RU-AE01. Preliminary reporter enzyme assays on *hyuC* promoter deletions implies that the putative σ^{70}-dependent promoter 'A' is functional but that 'B' is not.

To produce *hyuH* promoter-derived β-glucuronidase enzyme activity and *hyuC* promoter-derived β-galactosidase activity in pMJ242, transcription and translation activation sequences are required on the promoter fragment. Thus the deletion analysis of the *hyuH-hyuC* promoter fragment in Section 4.3.3 enabled the identification of regions of DNA capable of activating both transcription and translation of the *hyuH* or the *hyuC* genes.

The regulatory signals were identified based on sequence similarity to consensus prokaryotic sequences. It has been observed that high densities of promoter-like signals are found on the bacterial chromosomal DNA that match the consensus sequences for prokaryotic promoters but these sequences do not encode active promoters (Huerta and Collado-Vides 2003). The authors speculate that this may be a result of the high evolutionary rate present in prokaryotes or that the promoter-like signals may play a regulatory role. If the promoter-like signals serve as competitors for the RNA polymerase then they would function as repressors. However if the promoter-like signals channeled the RNA polymerase into the promoter, they would function as activators. This may propose a role for the putative σ^{70}-dependent promoter downstream of the putative σ^{54}-dependent *hyuH* promoter. It is possible that *hyuH* expression from the putative σ^{70}-dependent promoter occurs under different conditions to expression of the *hyuH* from the putative σ^{54}-dependent promoter. This would mirror the expression of *glnA* from one of two possible promoters depending on the nitrogen levels within *E. coli* cells (Reitzer and Magasanik 1985). The palindromic sequences identified on the RU-AE01 *hyuH-hyuC* region may also be involved in the regulation of the activation of transcription of *hyuH* and *hyuC.*

To validate the reporter enzyme data from the deletion analysis, the putative promoters would need to be inactivated by site-directed mutagenesis and these mutants analyzed for their inability to activate transcription. In addition, a primer extension protocol in conjunction with DNA sequence analysis can be used to identify the transcriptional start site for both the *hyuH* and the *hyuC* promoters. This would allow for the conclusive identification of the -10 / -35 or -12 / -24 sequences responsible for the activation of transcription of *hyuH* and *hyuC.*

CHAPTER 5

IDENTIFICATION OF TRANSCRIPTION FACTORS INVOLVED IN THE REGULATION OF HYDANTOIN HYDROLYSIS IN *A. tumefaciens* STRAINS RU-AE01 AND RU-OR

5.1 INTRODUCTION

Hartley *et al.* (2001) reported that Hyu enzyme activity in *A. tumefaciens* RU-OR was NCR sensitive and that NCR occurred via two mechanisms; first there was the repression of *hyu* expression in 'good' nitrogen sources which is classic NCR and second there was immediate inhibition of the Hyu enzymes in the presence of ammonia termed 'ammonia shock'. Like RU-OR, both the hydantoinase and NCAAH activities were NCR sensitive in *A. tumefaciens* RU-AE01 cells (section 3.3.2). While the transcription of the *hyuC* promoter was subject to NCR, the activation of transcription of the *hyuH* promoter was independent of the nitrogen levels in the cells. Thus the repression of the Hyu enzymes in RU-AE01 was not due to the lack of *hyuH* transcription but a result of ammonia shock (section 3.3.2).

The mechanism of ammonia detection in the cell is entwined with the global nitrogen regulatory mechanisms, in which NtrB and NtrC are central. The role of the NtrB and NtrC in regulating the expression of genes involved in nitrogen metabolism has been discussed in detail in Chapter 1 (section 1.6). Essentially, the phosphorylation status of NtrC, as determined by the nitrogen status of the cell, enables NtrC to act as an activator of transcription from σ^{54}–dependent promoters during conditions of low nitrogen and as a repressor of transcription from σ^{54}–dependent promoters during conditions of high nitrogen (Atkinson *et al.* 1994, summarized in Figure 5-1).

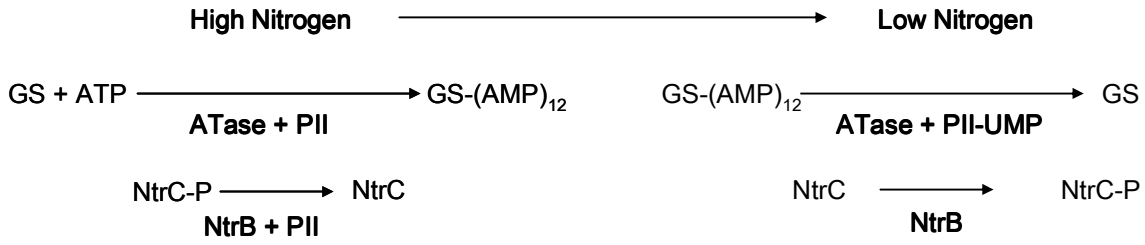


Figure 5-1: The adenylation state of GS and the phosphorylation state of NtrC as determined by the nitrogen status of the cell. ATase catalyses the adenylation and deadenylation of GS and NtrB catalyses the phosphorylation and dephosphorylation of NtrC. Figure adapted from Arcondeguy *et al.* 2001.

The experiments described in this chapter attempt to unravel the mechanisms by which NCR affects the Hyu enzymes in *A. tumefaciens* strains RU-AE01 and RU-OR. To this end, the role of the NtrB and NtrC proteins in the regulation of the

Hyu enzymes will be studied. It must be noted that the over-expression of proteins on plasmids, especially cellular signaling molecules and transcription factors, may upset the natural balance within the cell. Thus the effects generated by the over-expressed proteins need to be interpreted with caution. However, by artificially increasing the number of molecules of essential proteins, gross phenotypes may be observed which will enable the analysis of the role of the transcription factors within the cells.

5.2 METHODS AND MATERIALS

5.2.1 Bacterial plasmids, strains and culture conditions

Recombinant plasmids were hosted in *E. coli* DH5α cells and heterologous expression of NtrC was done in *E. coli* BL12 (DE3) cells (Appendix 8). Unless otherwise stated, *E. coli* strains and *A. tumefaciens* strains were cultured as described in sections 2.2.1 and 3.2.1 respectively. The plasmid pSR146, which carries *ntrBC* cloned from *A. tumefaciens* strain C58, was obtained from Dr Sylvia Rossbach (Rossbach *et al.* 1987). *A. tumefaciens* strains RU-AE01 and RU-OR transformants were selected on LB agar supplemented with the appropriate antibiotic to which the plasmid conferred resistance (50 µg/mL chloramphenicol or 25 µg/mL tetracycline).

β-glucuronidase and β-galactosidase enzyme assays and hydantoinase and NCAAH enzyme assays were done as described in sections 2.2.5 and 3.2.2 respectively. The levels of hydantoinase and NCAAH activity varied between sets of experiments (< 30%). To prevent the introduction of inter-experimental variation, the results from one complete experiment are presented in this chapter. The data set is attached in Appendix 12. The level of hydantoin-hydrolyzing activity was normalized to 100% for the control sample (wild-type cells or wild-type cells containing the vector pMJ14) which allowed the effect of the broad host range NtrBC plasmids to be represented as a fold-increase or fold-decrease on the wild-type activity.

5.2.2 Recombinant DNA contructs

The Expand High Fidelity System (Roche) was used according to the manufacturer's instructions at an annealing temperature of 52 °C. The sequence of all PCR amplification products was confirmed by DNA sequencing (Appendix 6-3).

Table 5-1: PCR and sequencing primers used in this chapter

Plasmid	Primer Name	Primer-P / S	Sequence	Coding sequence
pMJ191	MJ21	S + P	^{1}GCCGATATTCCCACTATCGCCGTTGAAC^{28}	NtrBC
	MJ23	S + P	^{2946}CTGAAGTCAGGCAAGACTCCCCCATCAGG^{2918}	
pMJ211	MJ40	P	catatg^{1385}ACAGCTACGATCCTCGTCGCCG^{1421}	NtrC
	MJ41	P	cccggg^{2833}GCTGGGGCGGGAACTACGATAGACG^{2808}	
	MJ22	S	^{1352}CAGCTCTTTGCCGAACACGACAGGAAATG^{1380}	
	MJ42	S	^{1759}CGCCAAGCTCGATGACGACATGC^{1781}	
	MJ43	S	^{2418}ATGACTTCCAGCGCCTCCGTCTCG^{2395}	

- Primer binding sites are depicted in Figure 5-2A
- Underlined sequence at the 5' terminus of the primers represent the sequence for a *Nde* I (catatg) or a *Sma* I (cccggg) restriction enzyme site.
Abbreviations: P- PCR, S- sequencing

Table 5-2: Plasmids generated in this chapter

Plasmid Name	Plasmid Vector	Insert Description	His_6-tag
* pMJ191	pTF-FC2	†NtrBC	None
* pMJ193	pTF-FC2	NtrB	None
* pMJ198	pTF-FC2	NtrC	None
pMJ211	pGEM-T-Easy	†NtrC	None
pRD109	pCW21 (pT7-7 derivative)	His_6-NtrC	Amino-terminal
pMJ219	pIVEX 2.3	His_6-NtrC	Carboxy-terminal
pMJ234	pT7-7	His_6-NtrC	Carboxy-terminal
* pMJ364	pTF-FC2	NtrB His_6-NtrC	Carboxy-terminal
* pMJ366	pTF-FC2	His_6-NtrC	Carboxy-terminal

†- PCR products
*- Broad host range origin of replication

Construction of broad host range NtrBC expression vectors

The *A. tumefaciens* C58 genome sequence (http://cancer.lbi.ic.unicamp.br/agroC58/) was used to design primers MJ21 (forward) and MJ23 (reverse), which correspond to 239 nts upstream of the AUG start codon of *ntrB* and 110 nts downstream of the UGA stop codon of *ntrC* respectively (Table 5-1). The *ntrBC* coding sequences were amplified from pSR146 using the primers MJ21 and MJ23. This *ntrBC* PCR fragment was used to construct the broad host range *ntrBC-, ntrB-* and *ntrC*-encoding plasmids.

The 5' and 3' termini of the *ntrBC* PCR product were filled in (Klenow) and the DNA fragment cloned into the filled in *Kpn* I site of the broad host range vector pMJ14 (Figure 2-3, Chapter 2) generating the *ntrBC*-encoding plasmid pMJ191 (Figure 5-2B). The *ntrBC* PCR fragment was also subjected to digestion with *Kpn* I and the termini filled in using Klenow. The resultant *ntrB*-encoding DNA fragment was cloned into the filled in *Kpn* I site of pMJ14 to generate pMJ193 (Figure 5-2C).

Finally, the *ntrBC* PCR fragment was subjected to digestion with *Eco* RI and the termini filled in with Klenow. The resultant *ntrC*-encoding DNA fragment was cloned into the filled in *Kpn* I site of pMJ14 generating pMJ198 (Figure 5-2D). The sequence of the inserts in pMJ191, pMJ193 and pMJ198 were validated using primers MJ21, MJ22, MJ23, MJ42 and MJ43 (Table 5-1).

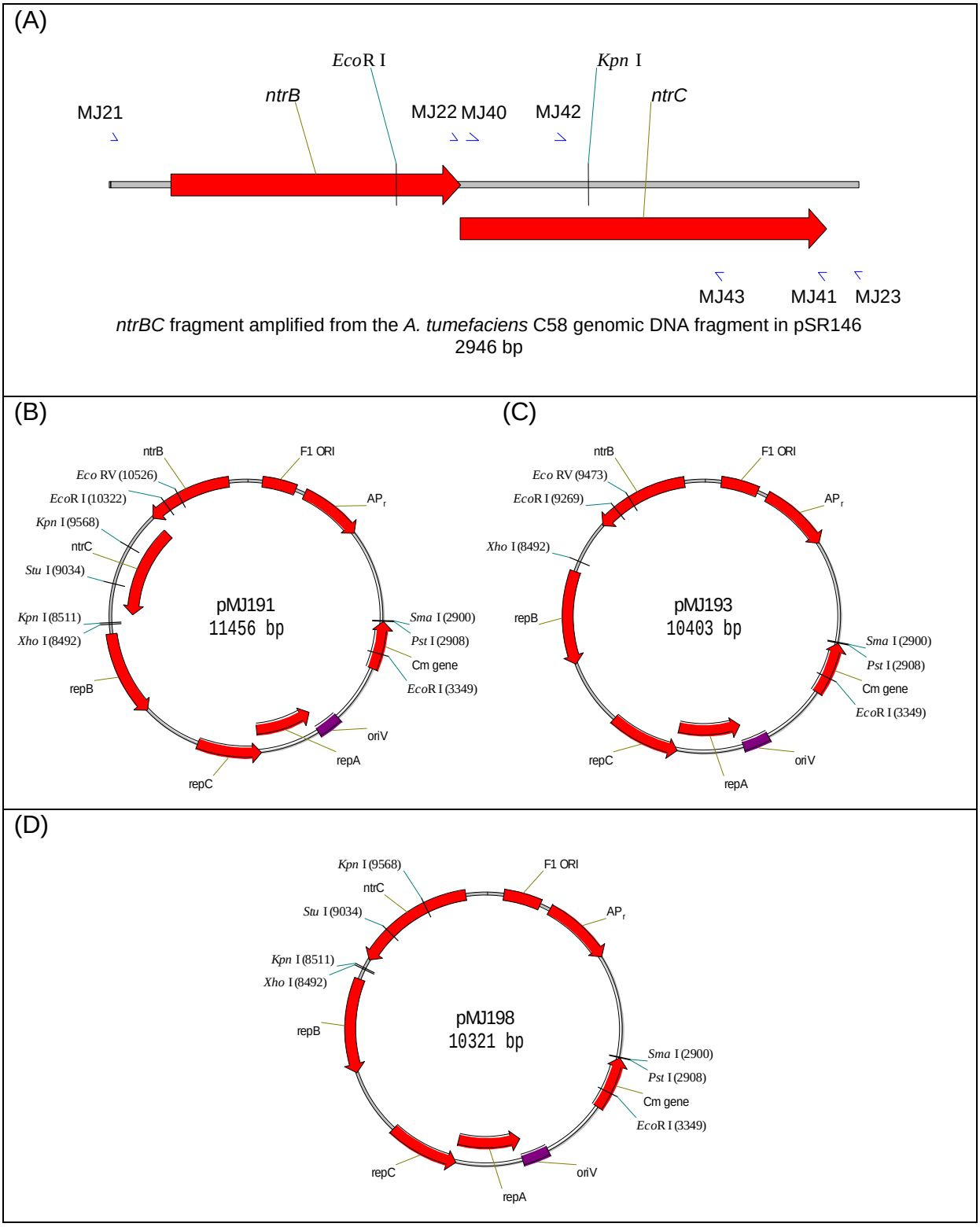


Figure 5-2: The broad host range *ntrBC*-, *ntrB*- and *ntrC*-encoding plasmids. (A) A linear map of the *A. tumefaciens* C58 *ntrBC* region amplified by PCR with MJ21 and MJ23, (B) *ntrBC*-encoding plasmid pMJ191, (C) *ntrB*-encoding plasmid pMJ193 and (D) *ntrC*-encoding plasmid pMJ198. The location of the primers MJ21, MJ22, MJ40, MJ42, MJ23, MJ41 and MJ43 are shown on the *ntrBC* fragment.

Construction of E. coli His_6-NtrC expression vectors

The *ntrC* coding sequence was amplified from pSR146 using primers MJ40 and MJ41 (Table 5-1) and cloned into pGEM-T-Easy (Promega) generating the plasmid pMJ211 (Table 5-2). An *Nde* I fragment encoding the *ntrC* gene was excised from pMJ211 and cloned into the *Nde* I site of pCW21 (Walter unpublished), a derivative of pT7-7 with an amino-terminal His_6-tag, generating plasmid pRD109 (Dorrington unpublished). The *ntrC* gene was excised from pMJ211 with *Nde* I and *Sma* I and cloned into the *Nde* I and *Sma* I sites of the expression vector pIVEX 2.3 (Roche) generating pMJ219 (Figure 5-3A) which resulted in the NtrC amino acid sequence fused to a carboxy-terminal His_6-tag. The DNA sequence encoding NtrC with a carboxy-terminal His_6-tag was excised from pMJ219 using *Nde* I and *Eco* RI and inserted into the *Nde* I and *Eco* RI sites of pT7-7 (Tabor 1987), generating pMJ234 (Figure 5-3B), which carries the His_6-tagged NtrC-coding sequence under control of the T7 promoter. The sequence of the inserts in pGEM-T-Easy (Promega), pT7-7 (Tabor 1987) and pIVEX 2.3 (Roche) was verified by DNA sequencing.

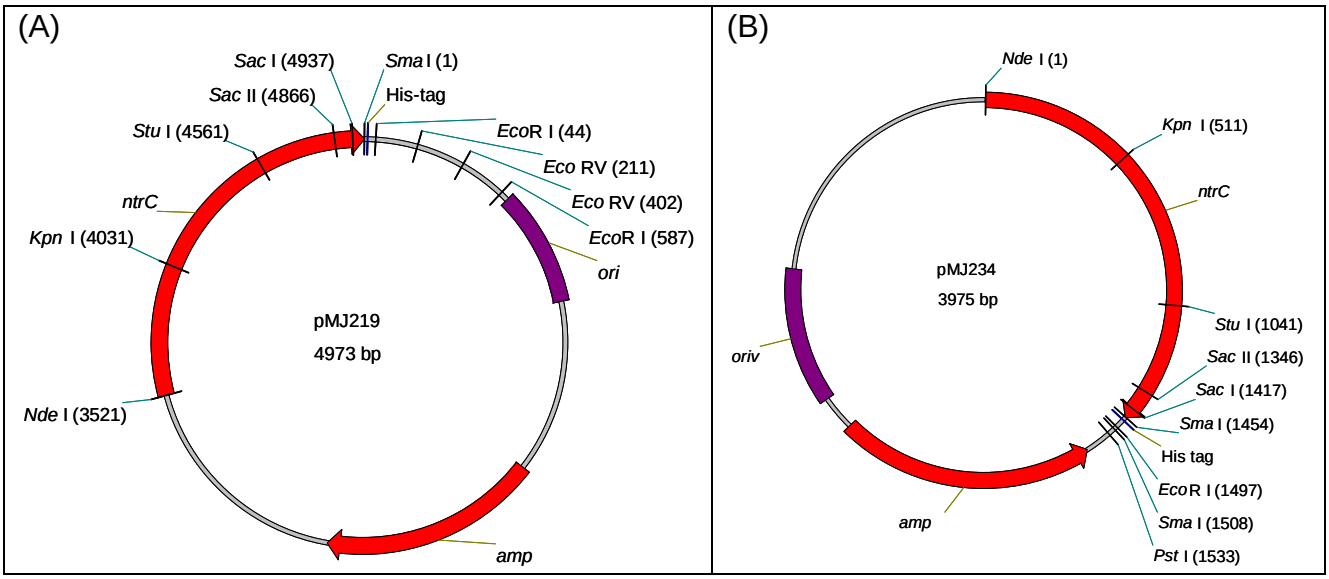


Figure 5-3: His_6-NtrC expression plasmids. NtrC His_6-tagged at the carboxy-terminal inserted into pIVEX 2.3 (A) and pT7-7 (B).

Construction of broad host range His_6-NtrC expression vectors

The broad host range plasmids pMJ191 and pMJ198 were digested with the restriction enzymes *Stu* I and *Xho* I. The 10.9 kb and 9.8 kb DNA backbones of pMJ191 and pMJ198 were gel purified using the GFX gel purification kit (Amersham),

thus removing the DNA sequence encoding 142 carboxy-terminal amino acids of NtrC and a stop codon (UGA). A DNA fragment encoding the corresponding 142 carboxy-terminal amino acids and a His_6-tag were excised from pMJ234 using the restriction enzymes *Stu* I and *Sal* I, gel purified and ligated into the *Stu* I and *Xho* I sites of pMJ191 and pMJ198 generating plasmids pMJ364 and pMJ366 respectively.

Construction of hyuH-hyuC deletion constructs

The construction of plasmids pMJ242 and pMJ258 was reported in section 2.3.2 and 2.2.6 respectively. A description of the plasmids pMJ245, pMJ261, pMJ266 and pMJ268 was reported in section 4.2.2.

5.2.3 Expression of His_6-NtrC encoding constructs

Recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells. A single colony was inoculated into Overnight Express Medium (Invitrogen) and incubated at 37 °C, with shaking for 16 hours. Cells were harvested by centrifugation at 3800 x g for 10 minutes. The supernatant was discarded and the pellet was frozen at -80 °C until further analysis.

For expression in *A. tumefaciens* RU-AE01 cells, a colony containing the desired broad host range plasmid was inoculated into 200 mL LB broth containing 1% hydantoin and grown at 28 °C, shaking at 200 rpm, for 72 hours. Cells were harvested by centrifugation at 3800 x g for 10 minutes and the pellets were frozen at -80 °C until further analysis.

5.2.4 SDS-PAGE and Western analysis

His_6-tagged NtrC was purified under native conditions using the Ni-NTA Spin Kit and Columns (Qiagen) as outlined in the manufacturer's instructions. Protein samples were separated by SDS-PAGE using 12.5% SDS-polyacrylamide gels. Electrophoresis was carried out using the Mini-PROTEAN 3 Electrophoresis System (Biorad) with 1 X Q buffer at 150 V (constant voltage) for 1 hour. Protein bands were visualized on the SDS-polyacrylamide gels with Coomassie Brilliant Blue staining solution (Appendix 3).

For Western analysis, protein samples were transferred from SDS-polyacrylamide gels to Hybond-C+ nylon membranes (Amersham) using the Mini-PROTEAN 3 Mini Trans-Blot module (Biorad) according to the manufacturer's instructions. Membranes were stained with Ponceau-S (Salinovich and Montelaro 1986) for 1 minute to confirm the transfer of proteins to the nylon membrane and to mark the positions of the protein molecular weight markers.

The membrane was incubated overnight in Blotto (5% non-fat milk powder in TBS-Tween), washed twice with 50 mL TBS-Tween and then incubated in a 1:2000 dilution of primary anti-His_6 antibody (Sigma) in Blocking Reagent (1% BSA in TBS-Tween) for 2 hours. The membrane was washed twice for 15 minutes and 4 times for 5 minutes in TBS-Tween and then incubated in a 1:10 000 dilution of secondary antibody (anti-rabbit anti-mouse conjugated to horseradish peroxidase provided in the Roche Chemiluminescent Western Blotting Kit) in Blocking Reagent for 1 hour. The membrane was then washed twice for 15 minutes and 4 times for 5 minutes in TBS-Tween before the membrane was placed in the luminol solution (provided by the Roche Chemiluminescent Western Blotting Kit). Chemiluminesence was detected using X-ray film (Kodak).

5.2.5 DNA-Protein interaction assays

Biotinylated MJ59 and MJ10 (Table 5-3) were used with the Expand High Fidelity system to amplify the RU-AE01 *hyuH-hyuC* promoter region from pMJ10 generating the DNA probe for protein-binding assays. The PCR product was purified using the DNA Clean and Concentrator Kit (Zymo) and then incubated with Tetralink Avidin (Promega) with rocking at 4 °C for 16h. This avidin-bound biotinylated DNA fragment was used for subsequent DNA-protein binding assays.

Table 5-3: Sequences of the primers used in the construction of *hyuH-hyuC* promoter construct pMJ245

Primer Name	**Sequence**	**†RU-AE01 sequence (nts)**
MJ10	*gaagatct ^{466}GAACCTTTGCTCCTTCGATAGTTAAT^{491}	25-491
MJ59	Biotin-gaagatct ^{25}AAAGCAGCTCTCAGGGTTGATG^{46}	

* Underlined sequence at the 5' terminus of the primers represents the sequence for a *Bgl* II (agatct) restriction enzyme site.
† RU-AE01 *hyuH-hyuC* sequence detailed in Appendix 1-1

Protein extracts were prepared from 2 L of *A. tumefaciens* RU-AE01 cells and RU-OR cells grown in 0.02% CAAMM or HMM as specified in section 3.2.1. Cells were harvested by centrifugation at 3800 x g for 10 minutes at 10 °C. The supernatant was discarded and the cells were frozen at -80 °C. The frozen cells were resuspended at 2 g *Agrobacterium* cells per 5 mL ice cold Sonication Buffer (200 mM Tris-HCl pH 7.4, 100 mM KCl, 1 mM DTT, 10 mM $MgCl_2$; 1 mM PMSF) and sonicated using a microtip (Vibra Cell, Sonics and Materials Inc, USA) for 20 cycles of 10 second bursts at 35% power, punctuated by 10 seconds on ice. Cell debris was removed by centrifugation at 8000 x g for 30 minutes at 4 °C. Glycerol was added to the supernatant at a final concentration of 20% and the aliquots were stored at -80 °C. Protein concentration was estimated using the Bradford's Method (Bradford 1976). The average concentration of the preparations was approximately 100 μg/mL.

A total of 500 μg of protein extract was thawed and incubated with 1 mL of RU-AE01 *hyuH-hyuC* DNA-linked avidin for 1 hour at room temperature with rocking. The mixture was transferred to column and centrifuged at 1000 x g for 2 minutes to separate DNA-linked avidin from unbound protein extract (flow through). The DNA-linked avidin was washed 5 times in 1 mL Incubation Buffer (4% glycerol, 4mM Tris-HCl pH 8, 100 mM NaCl, 4 mM $MgCl_2$, 0.2 mg/mL sheared single stranded salmon sperm DNA, 1 mM DTT). Protein was eluted in 750 uL of Elution Buffer (4% glycerol, 4mM Tris-HCl pH 8, 1 M NaCl, 4 mM $MgCl_2$, 0.2 mg/mL sheared single stranded salmon sperm DNA, 1 mM DTT).

Elutions were concentrated and desalted by trichloroacetic acid (TCA) precipitation as follows: Deoxycholate (2%) was added to a final concentration of 0.02% and the samples incubated at room temperature for 15 minutes. TCA (24%) was added to a final concentration of 6% and the samples incubated on ice for 1 hour. Samples were spun at 1700 x g for 15 minutes at 4 °C, the pellet washed in 500 uL ice-cold acetone and samples incubated on ice for 15 minutes. Samples were then spun at 1700 x g for 15 minutes at 4 °C. The supernatant was discarded and the pellet air dried. The pellet was then resuspended in 40 μL Elution Buffer. The analysis and detection of the protein was done by SDS-PAGE as described in section 5.2.4.

5.2.6 RU-AE01 *hyuH-hyuC* fragment-NtrC protein interaction assays

A total of 2 L RU-AE01 cells containing pMJ364 or pMJ366 were grown to confluence in LB containing 1% hydantoin as described in section 5.2.3. His_6-NtrC protein was purified from RU-AE01 cells containing pMJ364 or pMJ366 under native conditions using the Ni-NTA Spin Kit and Columns (Qiagen) as specified in the manufacturer's instructions. Protein samples were incubated with the avidin-bound biotinylated RU-AE01 *hyuH-hyuC* fragment for 1 hr at room temperature with rocking. The mixture was treated as described in section 5.2.5. Proteins bound to the RU-AE01 *hyuH-hyuC* DNA were eluted in high salt buffer, subjected to TCA precipitation and the pellets resuspended in 15 μL Elution Buffer. The analysis and detection of the protein was done by SDS-PAGE and Western analysis using anti-His_6 antibodies as described in section 5.2.4.

5.3 RESULTS AND DISCUSSION

5.3.1 Exogenous NtrBC and hydantoin-hydrolyzing activity

Data presented in Chapter 3 indicated that the RU-AEO1 *hyuC* gene expression is sensitive to NCR. NtrC has been shown to act as both an activator and repressor of nitrogen-sensitive genes. Thus one would expect that if *hyu* gene expression is regulated by NtrC, the presence of exogenous NtrB and NtrC would result in either an increase or decrease in Hyu enzyme activity. The cosmid pSR146 contains a 20 kb *Eco* RI fragment of *A. tumefaciens* C58 genomic DNA including 2.9 kb of DNA encoding *ntrBC* (Rossbach *et al.* 1987). pSR146 was transformed into RU-AE01 cells and transformants selected for their ability to grow on LB agar supplemented with tetracycline. The cells were grown to early stationary phase in 0.02% CAAMM, in which basal levels of Hyu enzyme activity was previously observed (section 3.3.2).

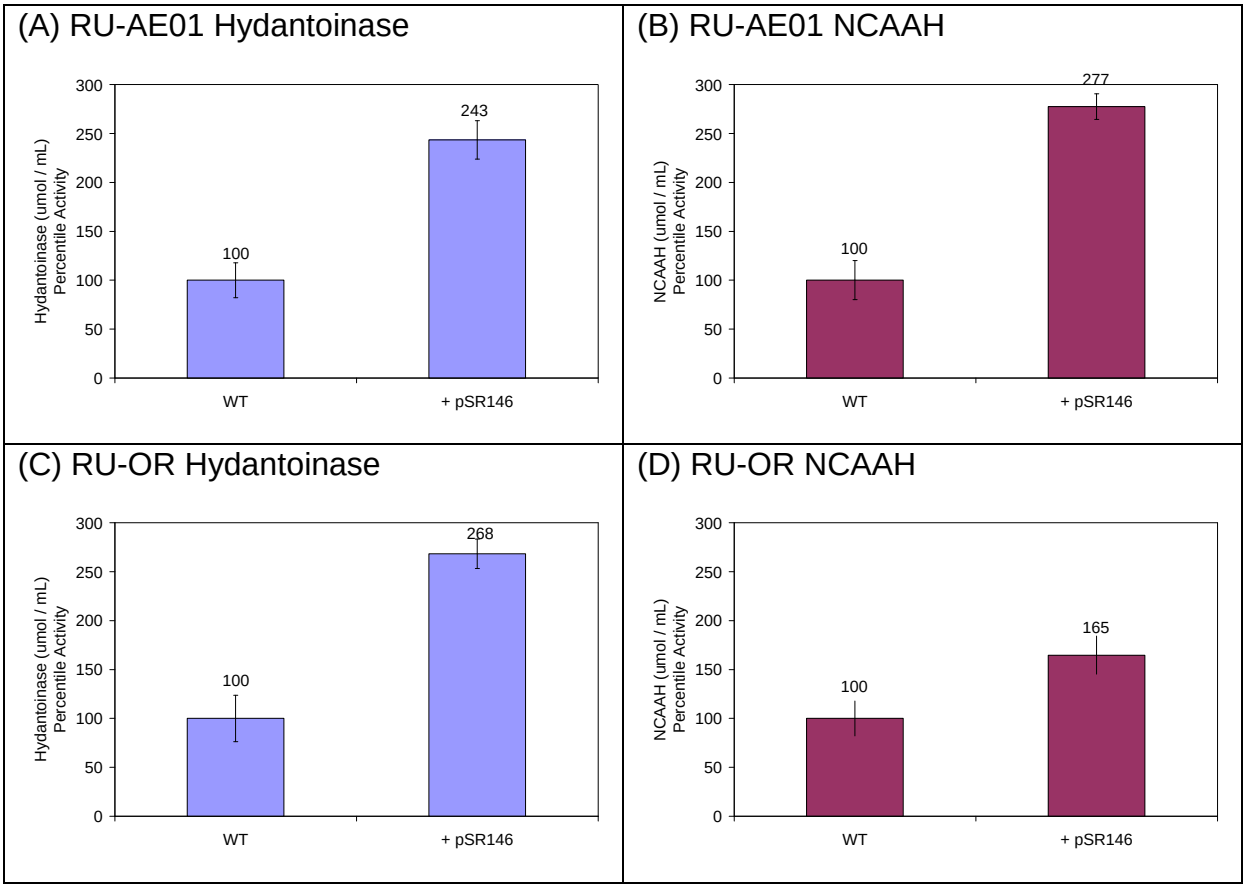


Figure 5-4: The effect of multiple copies of *ntrBC* on the Hyu enzyme activity. Resting cell biocatalytic activity for RU-AE01 (A and B) and RU-OR (C and D) in 0.02% CAAMM. Results are the average of triplicate reactions. The standard mean error is shown by the bars. The activity in the wild type strain (WT) was normalized at 100%. Abbreviations: WT- wild-type, +pSR146- wild-type containing pSR146.

The presence of pSR146 in RU-AE01 cells increased both the hydantoinase and NCAAH enzyme activities 2.4 and 2.7 fold respectively (Figure 5-4A and 5-4B). Similarly, when pSR146 was transformed into RU-OR cells, both the hydantoinase and the NCAAH enzyme activity increased. The hydantoinase activity increased 2.7 fold (Figure 5-4C) but the NCAAH activity increased only 1.6 fold in RU-OR cells (Figure 5-4D). This result suggested that an increased level of expression of one of the genes encoded on pSR146 was resulting in the up-regulation of the *hyu* genes in both *A. tumefaciens* strains RU-AE01 and RU-OR.

To determine whether this increase in hydantoinase and NCAAH activity was due to the presence of *ntrBC,* RU-AE01 and RU-OR cells were transformed with plasmids expressing NtrB and NtrC (pMJ191), NtrB (pMJ193) or NtrC (pMJ198) and assayed for hydantoinase and NCAAH activity.

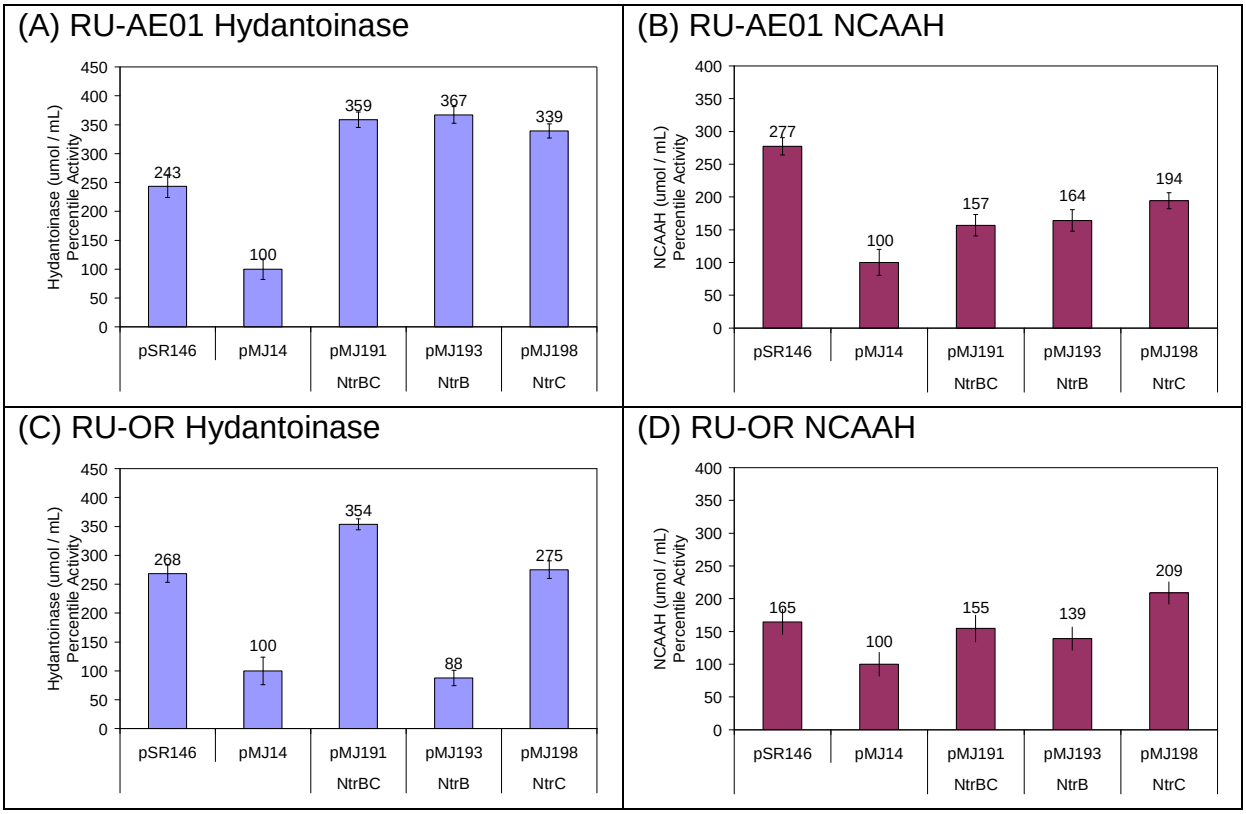


Figure 5-5: The effect of broad host range *ntrBC*-expression plasmids on Hyu enzyme activity. Resting cell biocatalytic activity for RU-AE01 (A and B) and RU-OR (C and D) in 0.02% CAAMM. Results are the average of triplicate reactions. The standard mean error is shown by the bars. The activity in the wild type strain containing the vector pMJ14 was normalized at 100%. Plasmids: pMJ14- vector, pMJ191- *ntrBC*, pMJ193- *ntrB* and pMJ198- *ntrC*-encoding broad host range plasmids.

The presence of exogenous NtrBC, NtrB or NtrC in RU-AE01 cells resulted in a similar 3.5-fold increase in hydantoinase activity (Figure 5-5A), which was higher than the approximately 2.4-fold increase in enzyme activity observed in the presence of the cosmid, pSR146. A similar effect was observed on the NCAAH enzyme, where the presence of exogeneous NtrBC, NtrB or NtrC increased NCAAH activity 1.5 to 2.0 fold (Figure 5-5B). However, the increase in NCAAH activity was lower than that observed in cells hosting the cosmid (2.7 fold versus 1.5 to 2 fold). The reduction in response in the cells containing pSR146 relative to the pMJ14-based plasmids could be attributed to the copy number of the different plasmids; the RK2 origin of pSR146 exists at 4 to 7 copies per chromosome (Blasina *et al.* 1996) whereas the pTF-FC2 origin of pMJ14 exists at 12 to 15 copies per chromosome (Dorrington and Rawlings 1989).

The response of RU-OR cells, in terms of hydantoinase and NCAAH enzyme activity, to the presence of increased levels of either NtrBC, NtrB or NtrC was unexpectedly different to that observed in RU-AE01 cells. Increased levels of both NtrBC and NtrC alone resulted in an increase in hydantoinase activity of 3.5 and 2.7 fold respectively, which was equivalent to the increase observed in the presence of the cosmid pSR146. However, increased levels of NtrB had no effect on the hydantoinase activity (Figure 5-5C). With respect to NCAAH activity, the effect of NtrB and NtrC was similar to that observed for RU-AE01 cells, where the presence of the plasmids increased the NCAAH enzyme activity 1.5 to 2.0 fold (Figure 5-5D). These results supported the hypothesis that the increase in hydantoinase and NCAAH activity observed in both *Agrobacterium* strains was due to elevated levels of NtrB and / or NtrC.

5.3.2 Expression of His_6-NtrC in *E. coli* and *A. tumefaciens*

The results reported in section 5.3.1 indicate that *Agrobacterium* strains RU-AE01 and RU-OR may respond differently to the elevated levels of NtrC with respect to hydantoinase activity. However, it would need to be confirmed that the presence of pMJ191 and pMJ198 did result in elevated levels of NtrC in the cells. Since NtrC antibodies were not available, the NtrC protein was tagged with a hexa-histidine (His_6) tag which would allow detection of the fusion protein using anti-His_6 antibodies.

First, pMJ234, which contains the NtrC coding sequence fused at its carboxy-terminus to a His_6 tag, was transformed into *E. coli* BL21 (DE3) cells. Low levels of His_6-NtrC expression were observed in *E. coli* BL21 (DE3) cells but no obvious 55 kDa band was detected following SDS-PAGE analysis and Coomassie staining of crude proten extracts derived from cells containing pMJ234 compared with the vector pT7-7 (Figure 5-6A, lane 3 versus lane 2). However Western analysis with anti-His_6 antibodies detected the His_6-NtrC protein in cells containing pMJ234 but not pT7-7 (Figure 5-6B, lane 3 versus lane 2). The His_6-NtrC protein was subsequently purified by native nickel affinity chromatography. SDS-PAGE and Western analysis showed the presence of a band that corresponded in size to NtrC (55 kDa) (Figure 5-6A and 5-6B, lane 5). Several high molecular weight proteins that co-purified with His_6-NtrC (Figure 5-6A, lane 5), were not detected by the anti-His_6 antibodies (Figure 5-6B, lane 5).

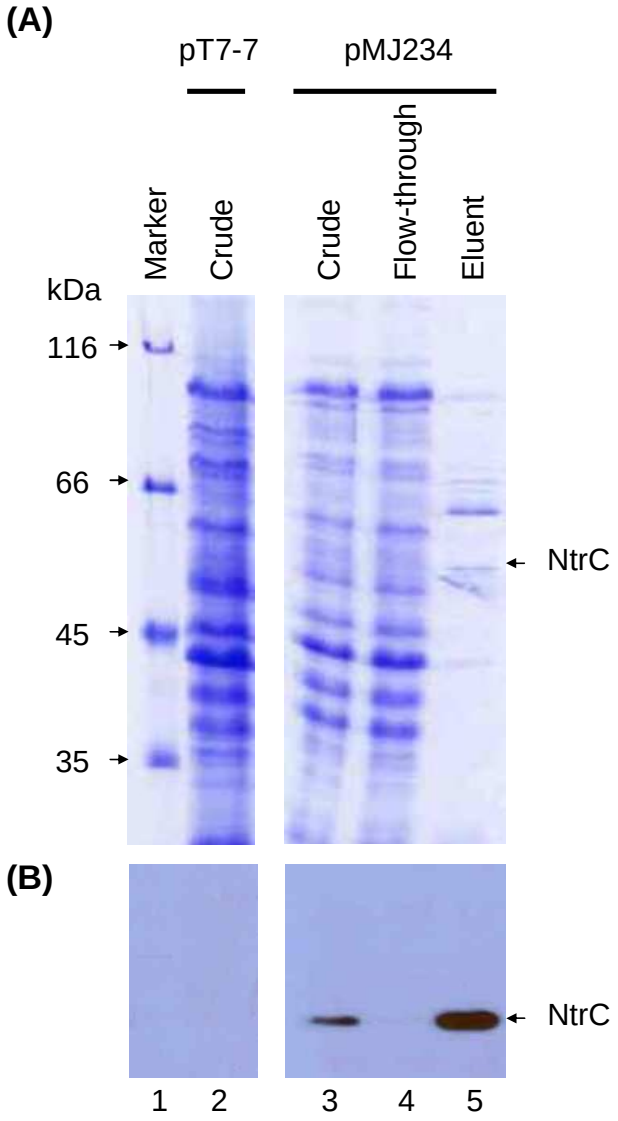


Figure 5-6: Affinity purification of His_6-NtrC from *E. coli* BL21 (DE3) cells. (A) Coomassie stained SDS-polyacylamide gel and (B) Western analysis of protein extracts. Lane 1: Molecular Weight Markers with their weights listed along the left hand side, Lane 2: *E. coli* BL21 (DE3) containing pT7-7 and Lanes 3-5: *E. coli* BL21 (DE3) containing pMJ234. NtrC- 55kDa protein marked by the arrow.

E. coli BL21 (DE3) cells containing pRD109, which encodes NtrC with an amino-terminal His_6-tag, produced no detectable levels of NtrC protein after SDS-PAGE and Western analysis of crude protein extracts (data not shown). The amino-terminal of NtrC, which was crystallized by Volkman *et al.* (1995), encodes the receiver domain of the protein including Asp-54, which forms the phosphorylation site. It is possible that the addition of the His_6-tag at the amino-terminal was masked by the secondary structure of the amino-terminal domain of NtrC.

The ability to purify the NtrC protein from *E. coli* BL21 (DE3) cells containing pMJ234 showed that, at least in *E. coli,* His_6-NtrC tagged at the carboxy-terminus appeared to be soluble and that the fusion protein could be detected using anti-His_6 antibodies. To determine whether the anti-His_6 antibodies could be used to detect His_6-tagged NtrC in the two *A. tumefaciens* strains, pMJ364 and pMJ366 were constructed in which the wild-type carboxy-terminus of NtrC was replaced with the His_6-tagged carboxy-terminus from pMJ234.

The plasmids pMJ14 (vector), pMJ191 (NtrB and NtrC), pMJ198 (NtrC), pMJ364 (NtrB, His_6-NtrC) and pMJ366 (His_6-NtrC) were transformed into RU-AE01 cells and grown to stationary phase in LB broth with 1% hydantoin. His_6-tagged NtrC protein was purified from all the cultures and subjected to SDS-PAGE and Western analysis.

As with *E. coli,* several high and low molecular weight proteins co-purified with the His_6-NtrC expressed in RU-AE01 cells containing pMJ364 and RU-AE01 cells containing pMJ366 (Figure 5-7A, lanes 5 and 6). These bands were also visible in the controls where no His_6-tagged NtrC had been expressed (Figure 5-7A, lanes 2, 3 and 4). However, Western analysis using anti-His_6 antibodies detected a faint 55 kDa band in the lane corresponding to RU-AE01 cells containing plasmid pMJ364 (Figure 5-7B, lane 5), indicating that these cells were expressing the NtrC protein, but at low levels.

A total of 80 optical density units (ODU) of RU-AE01 cells were used for SDS-PAGE and Western analysis (Figure 5-7A and 5-7B, lanes 2-6) compared to 2.4 ODU of *E. coli* BL21 (DE3) cells. (Figure 5-7A and 5-7B, lanes 8-9). Thus the expression of NtrC from the native promoter in the plasmids pMJ364 and pMJ366 was much lower than the level of expression of NtrC by pT7 promoter from the plasmid pMJ234 in *E. coli* BL21 (DE3) cells.

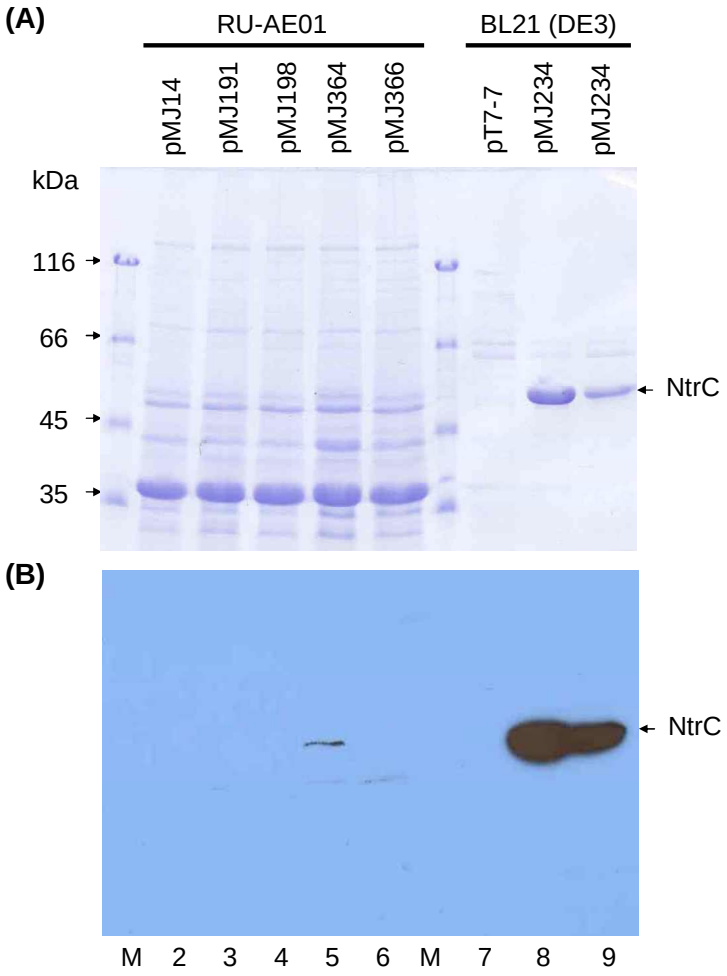


Figure 5-7: Affinity purification of His_6-NtrC protein from *A. tumefaciens* RU-AE01 cells and *E. coli* BL21 (DE3) cells. (A) Coomassie stained SDS-polyacrylamide gel and (B) Western analysis of His_6-NtrC protein extracts. Lanes: M- Molecular Weight Markers with their weights listed along the left hand side, Lanes 2 - 6: elutions from affinity chromatography protein purifications from *A. tumefaciens* RU-AE01 cells; Lane 2- pMJ14, Lane 3- pMJ191, Lane 4- pMJ198, Lane 5- pMJ364 and Lane 6- pMJ366. Lanes 7 - 9: elutions from affinity chromatography protein purifications from *E. coli* BL21 (DE3) cells; Lane 7- pT7-7, Lane 8- pMJ234 (500 ng) and Lane 9- pMJ234 (100 ng). NtrC- 55 kDa protein marked the arrow.

Since His_6-NtrC was being expressed in RU-AE01 cells containing the broad host range construct pMJ364, it follows that pMJ366 was also producing NtrC. Thus both constructs would be suitable to continue studying the effect of NtrC on the Hyu enzyme activity, provided the His_6-tagged NtrC protein was functional. To this end, the response of RU-AE01 and RU-OR cells to the presence of elevated levels of His_6-tagged NtrC as opposed to the native protein, was investigated. *A. tumefaciens* RU-AE01 and RU-OR cells were electroporated with pMJ14 (vector), the *ntrBC*-encoding plasmids pMJ191 (wild-type NtrB and NtrC) and pMJ364 (wild-type

NtrB and His_6-NtrC), and the NtrC-encoding plasmids pMJ198 (wild-type NtrC) and pMJ366 (His_6-NtrC) and assayed for hydantoinase and NCAAH activity.

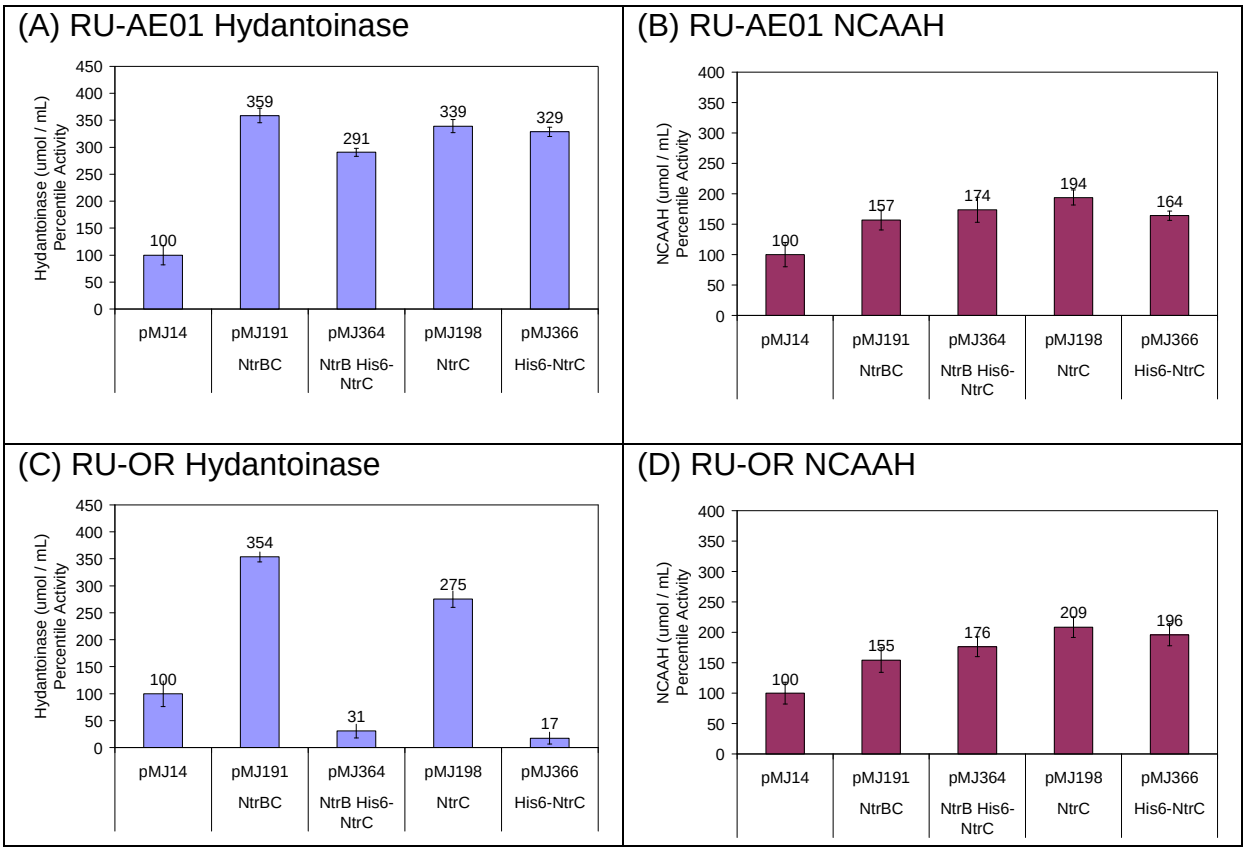


Figure 5-8: The effect of His_6-NtrC-expression on Hyu enzyme activity. Resting cell biocatalytic activity for *A. tumefaciens* RU-AE01 (A and B) and *A. tumefaciens* RU-OR (C and D) in 0.02% CAAMM. Hydantoinase (A and C) and NCAAH (B and D) enzyme activities for RU-AE01 cells and RU-OR cells in 0.02% CAAMM. Enzyme activity is reported in µmol/mL. Results are the average of triplicate reactions. The standard mean error is shown by the bars. The activity in the wild-type strain containing the vector pMJ14 was normalized at 100%. Plasmids: pMJ191- NtrBC, pMJ364- NtrBHis_6- NtrC, pMJ198- NtrC and pMJ366- His_6- NtrC-encoding broad host range plasmids.

The addition of the carboxy-terminal His_6-tag to NtrC did not alter the effect of exogenous NtrC on hydantoinase and NCAAH enzyme activities in RU-AE01 cells. The hyantoinase and NCAAH enzyme activities were up-regulated in cells hosting pMJ364 and pMJ366 as they were in cells containing pMJ191 and pMJ198 (Figure 5-8A and 5-8B). This was not the case in RU-OR cells. The NCAAH enzyme activity appeared to respond in the same way to the presence of the His_6-tagged NtrC as compared with the untagged NtrC protein (Figure 5-8D). In contrast, the hydantoinase activity, which increased in the presence of wild-type NtrC, was severely repressed in the presence of His_6-tagged NtrC protein (Figure 5-8C). These

different responses of RU-AE01 and RU-OR cells to the presence of His_6-tagged NtrC supported the results in section 5.3.1 that suggested that there were differences in the regulation of the *hyuH* gene expression in the two strains, even though the transcription factors involved may be the same (i.e. NtrB and NtrC).

5.3.3 Transcriptional regulation of the *A. tumefaciens* RU-AE01 *hyu* genes by NtrC

Previously it was demonstrated that the RU-AE01 Hyu enzyme system was regulated by NCR and that transcription of the *hyuC* gene was repressed under nitrogen sufficient conditions (section 3.3.2). Experimental data presented in this chapter provided evidence that the presence of increased levels of NtrBC resulted in elevated levels of both hydantoinase and NCAAH activities in RU-AE01 cells. This raised the question of whether or not NCR of Hyu enzyme activity is mediated through transcriptional regulation via the NtrBC system. NtrC is known to be a major transcriptional regulator in gram-negative bacteria, which binds specific sequences of dyad symmetry and activates transcription from σ^{54}-dependent promoters (Thony and Hennecke 1989). To answer the question of whether or not NtrC is involved in the regulation of the *hyu* gene expression, the first step would be to examine the promoter region for the presence of potential NtrC binding sites.

Identification of possible NtrC binding sites on the A. tumefaciens RU-AE01 hyuH-hyuC promoter fragment

While consensus NtrC binding sites are available for some enteric bacterial species (Table 5-4), they vary between strains and between the different reports (Robison *et al.* 1998, Contreras and Drummond 1988, Ames and Nikaido 1985, de Zanaroczy *et al.* 1990, Spinosa *et al.* 2000). There are no reported consensus sequences for NtrC binding sites in *Agrobacterium* species. Thus the available data (including NtrC sites in *Rhizobium leguminosarum* and *Azospirillum brasilense*, which are more closely related to *Agrobacteria* than the enteric bacteria *E. coli*, *K. pneumoniae* and *S. typhimurium*) were used to derive a consensus NtrC binding sequence with which to search the *hyu* promoter regions (Table 5-4).

Table 5-4: Generation of an NtrC consensus sequence for NtrC binding sites in gram-negative bacteria

NtrC Binding Sequence	Gene	Bacterial Species
AGCACCATGTTGGTGCA	*glnA*	*E. coli* [1]
AGCACTATATTGGTGCA	*glnA*	*E. coli* [1]
CGCACCAGATTGGTGCC	*glnHp2*-1	*E. coli* [1]
TGCCCCAGAATGGTGCA	*glnHp2*-2	*E. coli* [1]
TGCACTAAAATGGTGCA	*glnL*	*E. coli* [1]
CTGGNNNNTTGCA	Consensus	*E. coli* , *K. pneumoniae*, *S. typhimurium* [2]
TGCACCAAAATGGTGCA	Consensus	*S. typhimurium* [3]
TGCACCANNNTGGTGCA	Consensus	*Azospir*illum *brasilense* [4]
TGCTCTAATTTTGTGCA	*glnII*	*Rhizobium leguminosarum* [5]
TGCATAAACTATGTGCA	*glnII*	*Rhizobium leguminosarum* [5]
TGCACNANNNTGGTGCA	**CONSENSUS**	

Genes- *gln*- glutamine synthetase genes. Where genes have not been specified, sequences were reported as consensus sequences in the gram-negative bacterial strain. References: Robison *et al*. 1998 [1], Contreras and Drummond 1988 [2], Ames and Nikaido 1985 [3], de Zanaroczy *et al.* 1990 [4] and Spinosa *et al*. 2000 [5].

The binding sites from *E. coli*, *K. pneumoniae*, *S. typhimurium* as well as *R. leguminosarum* and *A. brasilense* were used to derive the consensus: TGCACNANNNTGGTGCA (Table 5-4). While a high degree of conservation was observed in the sequence of the NtrC binding sites within the *gln* genes of *E. coli*, the binding sites were not as well conserved in *R. leguminosarum*; which is more closely related to *Agrobacterium.* The consensus sequence reported for *E. coli*, *K. pneumoniae* and *S. typhimurium* by Contreras and Drummond (1988) suggested the sequence **TGCA** was important for NtrC interaction. Alignment of the sequences reported in Table 5-4 revealed that a dyad repeat **TGCA**N_9**TGCA** was important.

Based on these findings, sequences with similarity to the NtrC consensus binding sequence or sharing dyad symmetry, were identified in the RU-AE01 *hyuH-hyuC* region (Figure 5-9). While the consensus NtrC binding site had TGCA dyad repeats, the sequence was not a perfect palindrome. This suggested that putative NtrC binding sites could be identified on both strands of the DNA. Five putative NtrC binding sites were identified (Figure 5-9, highlighted in purple).

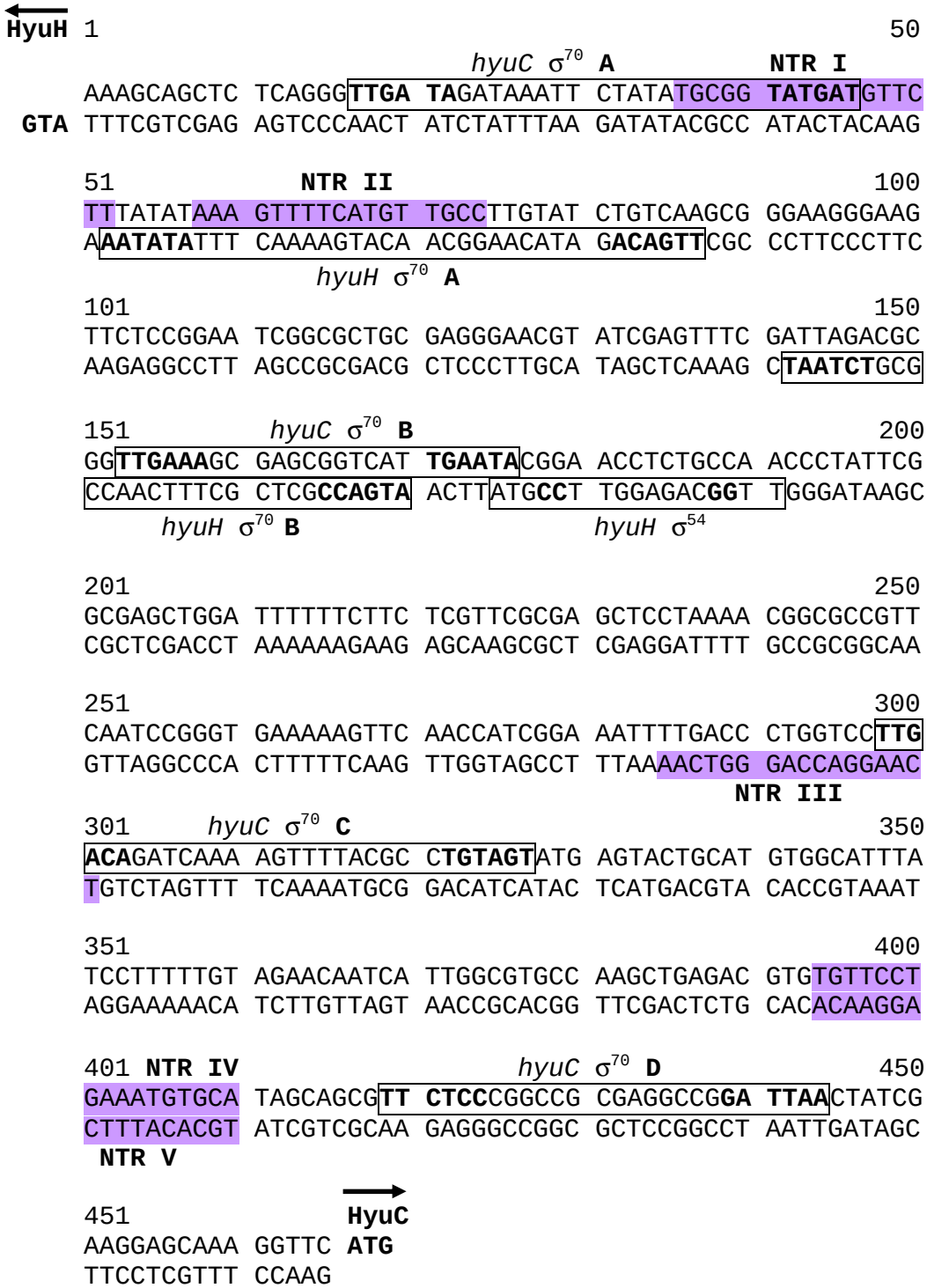


Figure 5-9: Putative NtrC binding sites within the *hyuH-hyuC* region from *A. tumefaciens* RU-AE01. The RU-AE01 nucleotide sequences starts at the nucleotide directly upstream the A of the ATG start codon for both the *hyuH* and the *hyuC* genes. The putative NtrC binding sites shaded in purple were identified using the consensus sequence TGCACNAN$_3$TGGTGCA as a reference. Putative σ^{70}-dependent and σ^{54}-dependent promoters identified in section 4.3.2 are boxed and labelled.

NTR I was only 54% identical to the consensus NtrC binding sequence, but it was selected as the TG repeats (nts 36-37 and nts 46-47) and a central A (nt 42) which were previously identified as important, (Table 5-4) were conserved. *NTR II* was selected because the sequence at the 3' terminal TGTTGCC (nts 68-73) was 71% identical to the consensus NtrC binding sequence TGGTGCA. *NTR I* and *NTR II* were arranged in a similar way in *A. tumefaciens* RU-AE01 (Figure 5-9) as they were in the *glnII* genes in *R. leguminosarum* (Spinosa *et al*. 2000) which suggested that the DNA sequences *NTR I* and *NTR II* may be potential DNA binding sites for the NtrC protein. *NTR I* and *NTR II* are upstream of *hyuC.* The *NTR I* sequence partially overlaps nucleotide sequences identified as the -10 sequence of a putative σ^{70}-dependent promoter 'A' upstream of *hyuC* (section 4.3.2) (Figure 5-9). *NTR I* and *NTR II* are also on the opposite DNA strand to the putative σ^{70}-dependent promoter 'A' identified upstream of *hyuH* (Figure 5-9), with the *NTR II* binding site lying between the -10 and -35 sequences respectively.

NTR III was chosen due to the TCAA dyad repeats at nts 285-288 and at nts 298-301 (Table 5-5, underlined sequence) and the conserved A at nt 295 (Table 5-5, marked by a ▪). In addition, the number of nucleotides between the dyad repeats was identical to that in the consensus NtrC binding sequence. *NTR IV* was selected as it was very similar to the consensus sequence reported by Contreras and Drummond (1988) for *E. coli*, *K. pneumoniae* and *S. typhimurium* (Table 5-4). *NTR IV* is upstream of the *hyuC* σ^{70}-dependent promoter 'D' (Figure 5-9). The sites *NTR III* and *NTR V* are upstream of the *hyuH* σ^{70}-dependent promoter 'B' and the *hyuH* σ^{54}-dependent promoter (Figure 5-9). *NTR V* was selected as the sequence at the 5' terminal (nts 406-410) was identical to the consensus NtrC binding sequence TGCAC and the nucleotides GG at nts 398-399 were conserved (Table 5-5).

Table 5-5: Comparison of the putative NtrC binding sites with the consensus sequence derived for gram-negative bacteria

	Possible binding sites for NtrC and position on the RU-AE01 nucleotide sequence nt ▪ nt	**Similarity to the consensus sequence (%)**
Consensus	**TGCACNANNNTGGTGCA**	
NTR I	36 TGCGGTATGATGTTCTT 52	54
NTR II	58 AAAGTTTTCATGTTGCC 74	38
NTR III	301 TCAAGGACCAGGGTCAA 285	54
NTR IV	394 TGTTCCTGAAATGTGCA 410	62
NTR V	410 TGCACATTTCAGGAACA 394	69

The putative *NTR* sites identified in the *hyuH-hyuC* promoter region have low levels of conservation with the consensus NtrC binding sequence varying from 38% to 69% (Table 5-5). There is also no discernable consensus sequence between the five *NTR* sites identified in the *hyuH-hyuC* region of strain RU-AE01. Experimental evidence from section 3.3.2 showed that transcription of *hyuH* was not regulated by NCR, which implies that *NTR I* and *NTR II* probably do not function as NtrC binding sites for *hyuH.* However, NCR decreased the level of transcription of *hyuC* in RU-AE01 cells (section 3.3.2). The sequence between nts 1-100 and nts 188-467 were shown to be capable of activating the transcription and translation of *lacZ* and β-galactosidase respectively (section 4.3.3). This suggests that *NTR I* and *NTR II* may be involved in the regulation of the putative σ^{70}-dependent promoter 'A' and that *NTR IV* may be involved in the regulation of putative putative σ^{70}-dependent promoter 'C'. Taken together, this suggests that direct binding of NtrC to specific sequences on the RU-AE01 *hyuH-hyuC* promoter region may be responsible for the up-regulation of the Hyu enzymes observed in *A. tumefaciens* RU-AE01 when plasmids encoding NtrC were present.

5.3.4 Transcriptional activity of the RU-AE01 *hyuH-hyuC* promoters in RU-OR cells

Experimental evidence presented thus far has indicated that the mechanisms that regulated Hyu enzyme activity are distinct in strains RU-AE01 and RU-OR. Firstly, exogenous NtrB resulted in an increase in hydantoinase activity in strain RU-AE01

but not in strain RU-OR. Secondly, the presence of increased levels of endogenous NtrC and His_6-tagged NtrC both resulted in an increase in Hyu enzyme activity in strain RU-AE01. However, in strain RU-OR, the addition of a His_6-tag to NtrC negated the increase in the hydantoinase activity observed with untagged NtrC. The addition of the His_6-tag to NtrC had no effect on the NCAAH enzyme activity in strain RU-OR, both His_6-tagged NtrC and untagged NtrC resulted in an increase in enzyme activity.

There are two explanations for these observations. Either the global regulatory machinery in the two strains is different, or alternatively, there are differences in the regulatory sequences on the promoters and consequently regulation of transcriptional activation of the *hyuH* genes in the two strains. This question would have been easier to answer if the nucleotide sequence of the *hyuH* gene from strain RU-OR was available. In the absence of this information, the only other way to address this question was to examine the regulation of transcription by the RU-AE01 *hyuH-hyuC* promoter in strain RU-OR and to compare the transcriptional regulation in strain RU-OR with strain RU-AE01.

Plasmid pMJ258, which carries the entire *hyuH-hyuC* promoter sequence fused to *gus* and *lacZ* respectively, (Figure 2-8) was electroporated into both RU-AE01 and RU-OR cells to compare reporter enzyme activity in the two strains. The sensitivity to NCR and induction by hydantoin were used as the two criteria to compare transcriptional regulation of the *hyuH-hyuC* fragment in the two *Agrobacterium* strains.

As expected, hydantoinase activity was repressed in RU-AE01 cells transformed with pMJ242 (vector) grown in 0.5% CAAMM as compared with 0.02% CAAMM (0 μmol/mL versus 1.6 μmol/mL respectively). The same trend was observed in RU-AE01 cells hosting the promoter construct pMJ258 (Figure 5-10A). In contrast, *hyuH* transcription, as a function of β-glucuronidase activity was not repressed during growth in 0.5% CAAMM. In fact, there was a significant increase in *hyuH* activation in cells grown in this medium (Figure 5-10B), as had been observed before (section 3.3.2). In RU-OR cells, the RU-AE01 *hyuH* promoter activity also appeared to be unaffected by NCR (Figure 5-10D). However, analysis of the hydantoinase activity in RU-OR cells transformed with the promoter construct pMJ258, revealed an

unexpected result. As was the case for RU-AE01 cells, hydantoinase activity in RU-OR cells transformed with pMJ242 (vector) was repressed during growth in 0.5% CAAMM. Unexpectedly, the presence of pMJ258 in cells grown in 0.02% CAAMM resulted in a ten-fold increase in hydantoinase activity, which was completely repressed in 0.5% CAAMM (12.1 µmol/mL versus 0 µmol/mL, Figure 5-10C).

Thus the hydantoinase enzyme activity in both RU-AE01 and RU-OR is subject to NCR, with no significant levels of hydantoinase enzyme activity being detected in repressive nitrogen conditions. Similarly, the expression of the *hyuH* promoter was comparable in both strains. The *hyuH* promoter derived β-glucuronidase activity increased when RU-AE01 cells were grown in repressive medium as opposed to non-repressive medium. In RU-OR, the expression of the *hyuH* promoter was comparable in both 0.02% CAAMM and in 0.5% CAAMM. Thus transcription of the *hyuH* promoter was not sensitive to NCR in both strains.

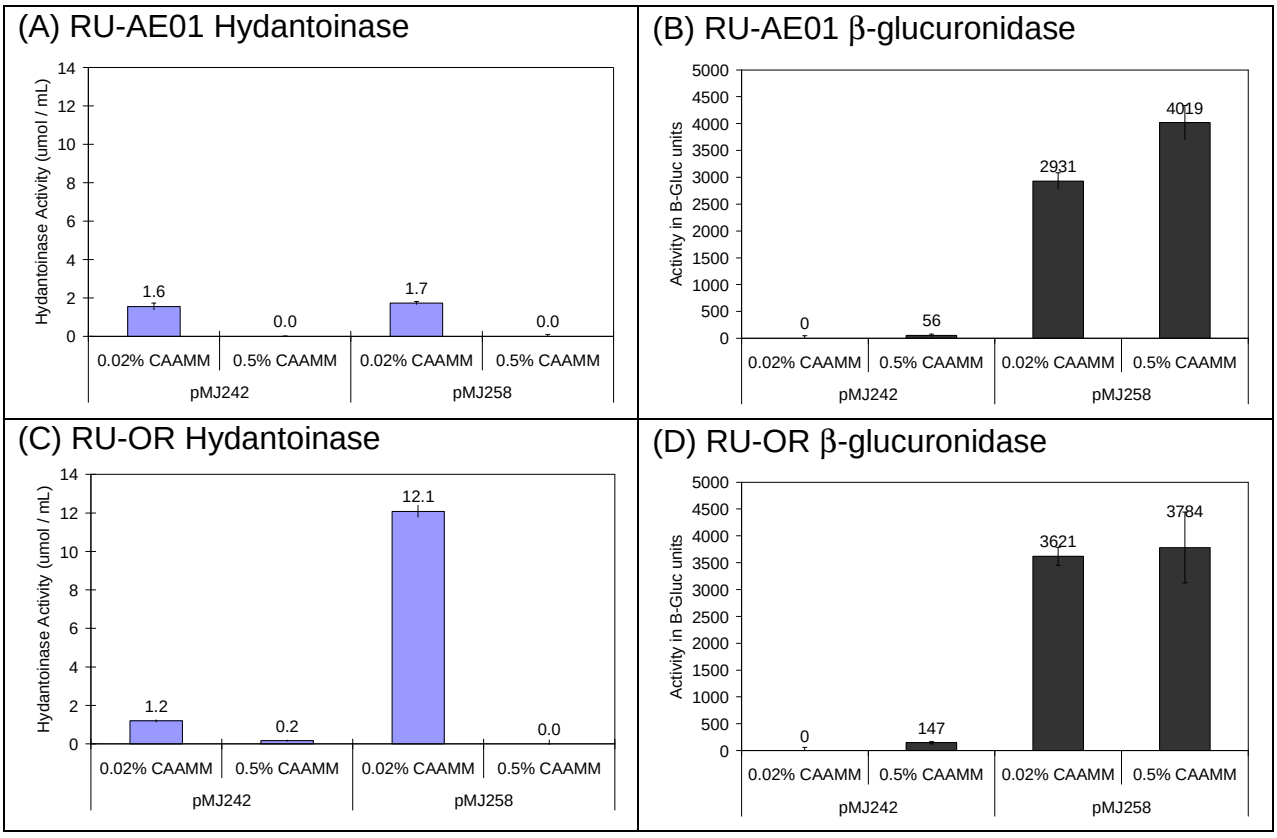


Figure 5-10: Comparison of the effect of NCR on *hyuH* expression and hydantoinase activity in RU-AE01 and RU-OR cells. Hydantoinase enzyme activity is reported in µmol/mL of product produced and β-glucuronidase enzyme activity is reported in β-Gluc units. Results are the average of triplicate reactions. The standard mean error is shown by the bars.

In RU-AE01 cells transformed with pMJ242, NCAAH activity decreased from 3.9 μmol/mL to 0.5 μmol/mL in 0.02% and 0.5% CAAMM respectively (Figure 5-11A). A similar decrease was observed with cells hosting pMJ258. As observed previously, there was a corresponding decrease in *hyuC* transcriptional activation in cells grown in 0.5% CAAMM as compared with 0.02% CAAMM (735 versus 943 Miller units of β-galactosidase activity respectively, Figure 5-11B). NCAAH enzyme activity in RU-OR cells decreased from 3.2 to 0.4 μmol/mL respectively for cells containing pMJ242 and from 3.3 to 0.5 μmol/mL in 0.02% and 0.5% CAAMM for cells containing pMJ258 (Figure 5-11C). A concurrent decrease was observed in the activation of *hyuC* transcription for cells grown in 0.5% CAAMM compared to 0.02% CAAMM (1055 versus 1970 Miller units of β-galactosidase activity, Figure 5-11D).

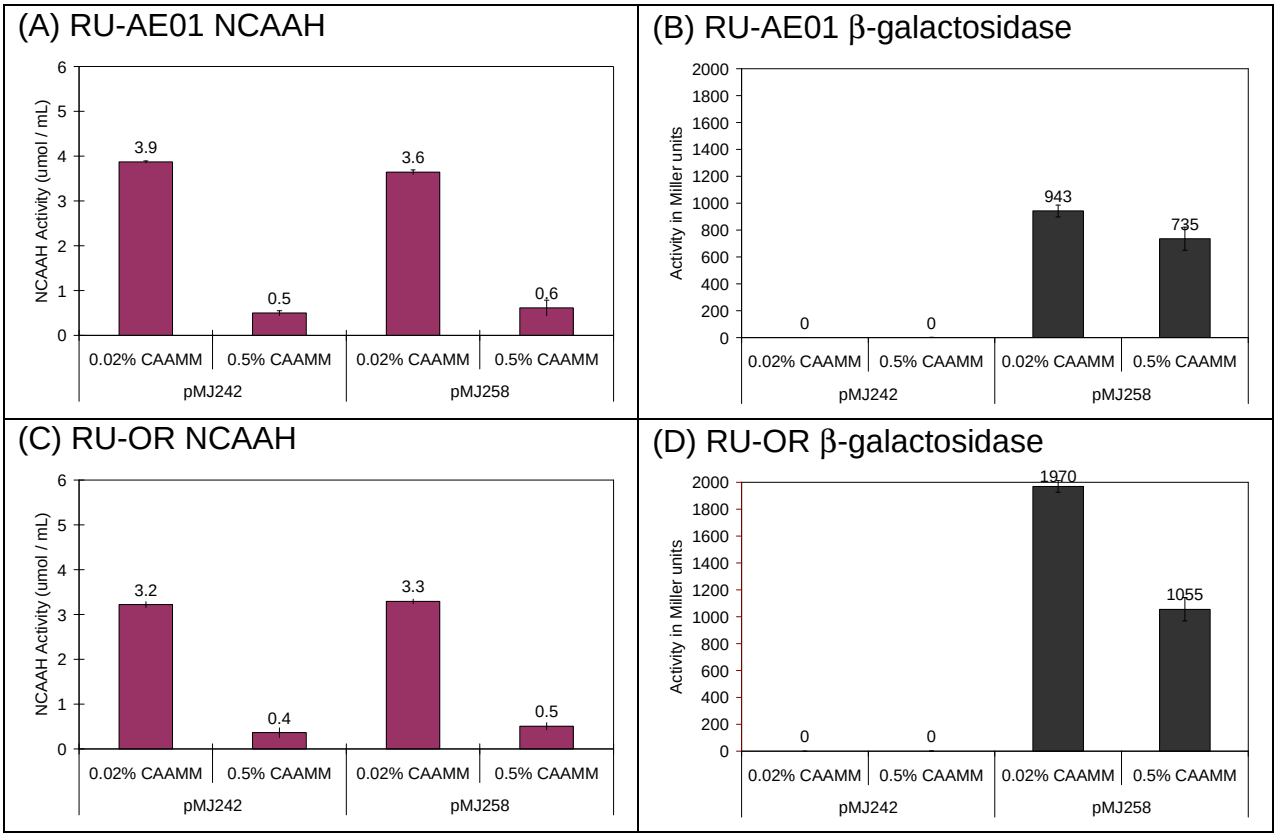


Figure 5-11: The NCR effect on *hyuC* expression and NCAAH activity in RU-AE01 and RU-OR cells. NCAAH enzyme activity is reported in μmol/mL of product produced and β-galactosidase enzyme activity is reporter in Miller units. Results are the average of triplicate reactions. The standard mean error is shown by the bars.

Like the hydantoinase activity, the NCAAH activity in both RU-AE01 and RU-OR is subject to NCR. In both strains, transcription of the *hyuC* promoter is sensitive to NCR. The regulation of RU-AE01 *hyuH-hyuC* transcription in response to NCR consequently appears to be the same in both RU-AE01 and in RU-OR.

The second criterion selected to analyse the transcriptional regulation of the RU-AE01 *hyuH-hyuC* genes was induction by hydantoin (characterized previously in section 3.3.2). Both RU-AE01 and RU-OR were again transformed with the vector (pMJ242) and promoter-probe (pMJ258) and grown in either 0.02% CAAMM (non-repressive nitrogen source) or HMM (inductive medium).

In both strains RU-AE01 and RU-OR, the cells containing the control vector, pMJ242, showed an approximately six-fold increase in the levels of hydantoinase enzyme activity in HMM when compared to 0.02% CAAMM (Figure 5-12A and 5-12C). The response of RU-AE01 and RU-OR to growth in HMM, as compared with 0.02% CAAMM, was again unexpectedly different when they were transformed with the promoter construct, pMJ258. Hydantoinase activity in RU-AE01 cells increased from 0.70 μmol/mL in 0.02% CAAMM to 3.75 μmol/mL in HMM, respectively. However, a 2.5-fold decrease was observed in RU-OR cells grown in 0.02% CAAMM as compared with HMM (12.0 μmol/mL to 4.8 μmol/mL). In both RU-AE01 and RU-OR, the *hyuH* promoter activity represented by the β-glucuronidase activity doubled when cells were grown in medium containing the inducer hydantoin (from 4345 to 8451 β-Gluc units in RU-AE01 and from 8224 to 17440 β-Gluc units in RU-OR respectively, Figures 5-12B and 5-12D).

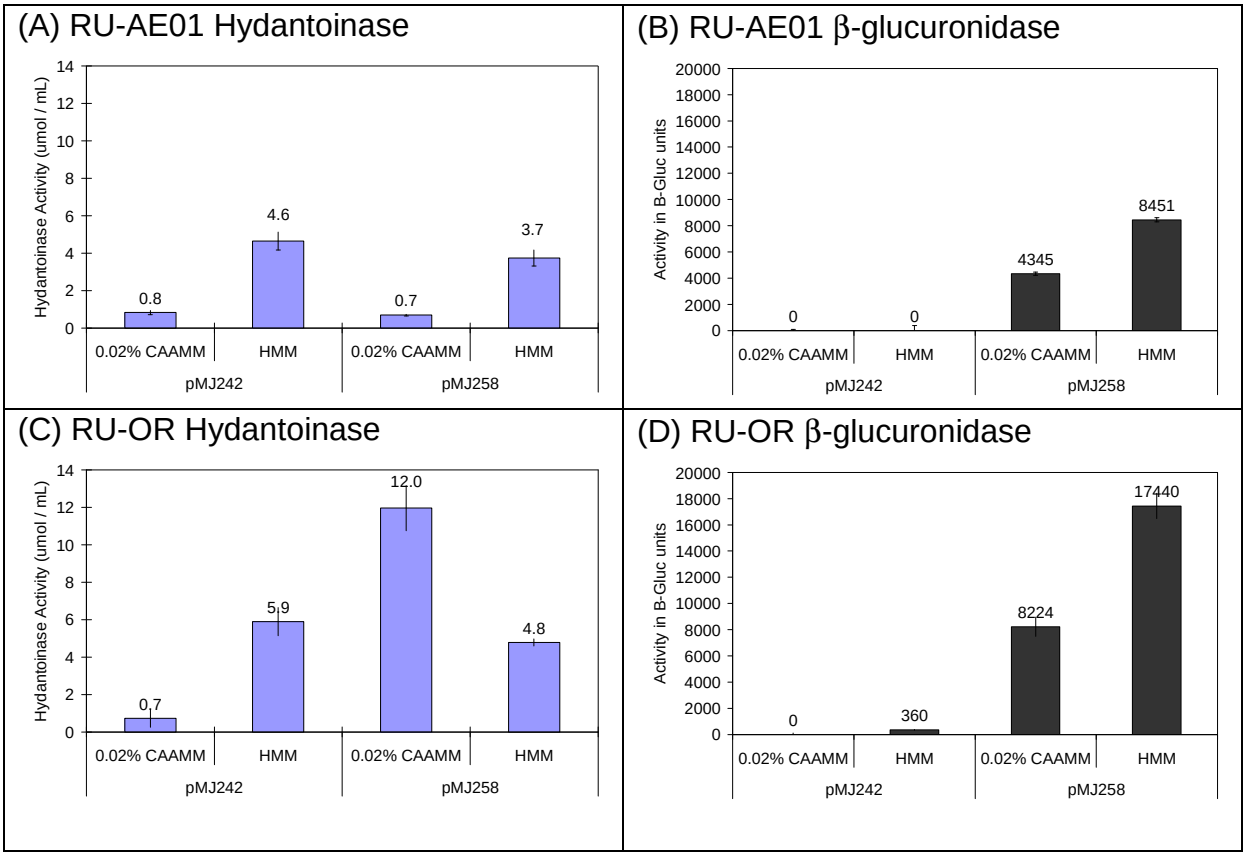


Figure 5-12: Comparison of the effect of induction on the expression of *hyuH* and on hydantoinase activity in RU-AE01 and RU-OR. Hydantoinase activity is reported in µmol/mL and β-glucuronidase enzyme activity is reported in β-Gluc units. Results are the average of triplicate reactions. The standard mean error is shown by the bars.

RU-AE01 cells containing pMJ242 showed a two-fold increase in the NCAAH activity as did RU-AE01 cells containing pMJ258 (Figure 5-13A). There was a concomitant two-fold increase in the transcriptional activity of *hyuC* (1133 to 2102 Miller Units of β-galactosidase activity, Figure 5-13B) in RU-AE01 cells containing pMJ258. Similarly, the NCAAH activity of RU-OR cells containing pMJ242 and of RU-OR cells containing pMJ258 doubled when grown in HMM (Figure 5-13C). RU-OR cells containing pMJ258 showed an equivilant increase in *hyuC* transcriptional activation when grown in medium containing inducer (from 1974 to 4235 Miller Units of β-galactosidase activity, Figure 5-13D).

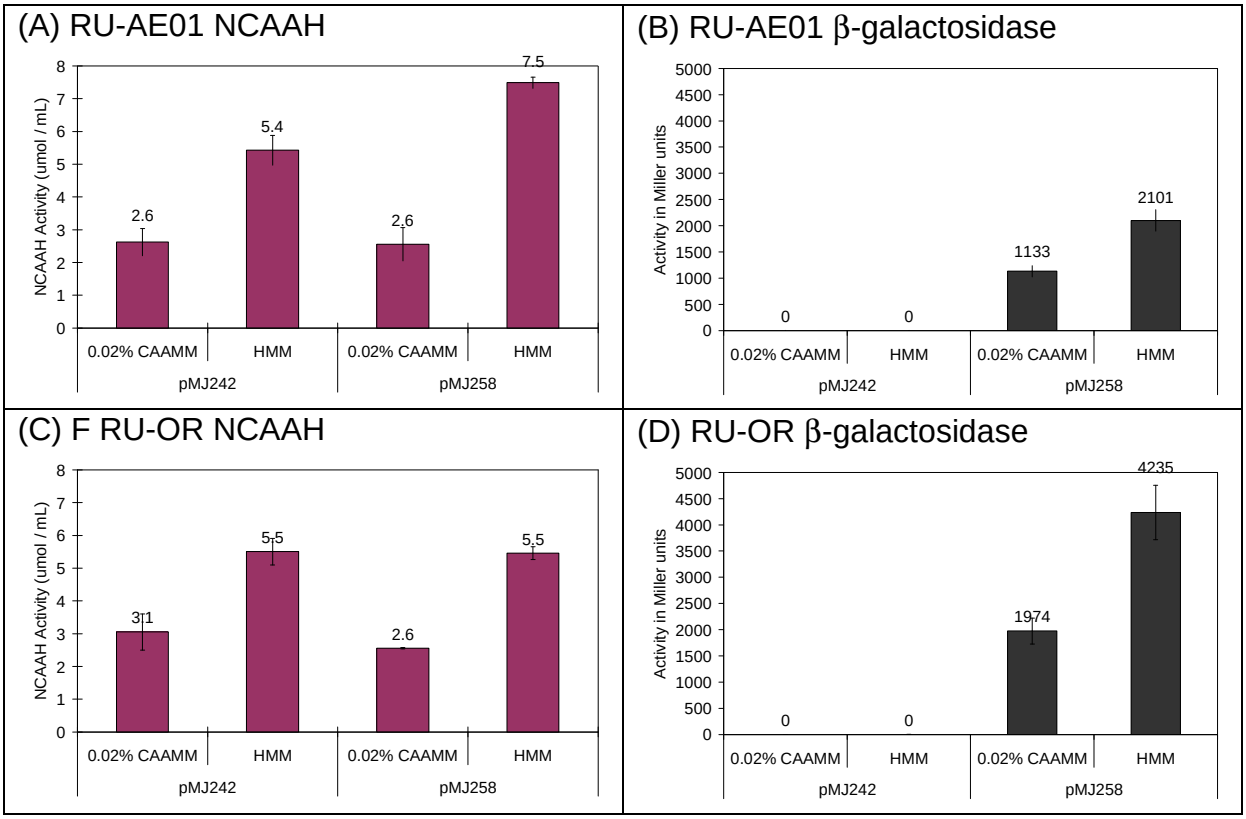


Figure 5-13: The effect of induction on the expression of *hyuC* and on NCAAH activity in RU-AE01 and RU-OR. NCAAH enzyme activity is reported in μmol/mL and β-galactosidase enzyme activity is reporter in Miller units. Results are the average of triplicate reactions. The standard mean error is shown by the bars.

The hydantoinase and the NCAAH enzymes in both RU-AE01 and RU-OR were induced in the presence of hydantoin. Similarly, transcription of the RU-AE01 *hyuH* and *hyuC* promoters was sensitive to induction in both RU-AE01 and RU-OR. The RU-AE01 *hyuH-hyuC* fragment was therefore regulated in the same manner in both RU-AE01 and RU-OR.

The similarities in the regulation of the *hyuH-hyuC* region in strains RU-AE01 and RU-OR indicates that the global mechanisms of regulation in the *Agrobacterium* strains RU-AE01 and RU-OR are comparable. This suggests that NtrBC have different effects on the Hyu enzyme activities in RU-AE01 and RU-OR because the *hyu* genes in the two strains are different. The levels of β-glucuronidase and β-galactosidase activities in RU-OR cells were approximately twice as high when compared with RU-AE01 cells (Figure 5-11D versus 5-11B, 5-12D versus 5-12B and 5-13D versus 5-13B). This effect can be attributed to the variation in the levels of

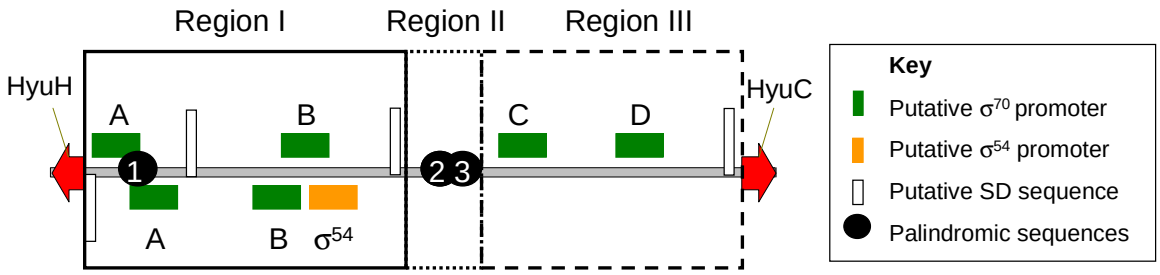
cellular proteins involved in the regulation of the *hyu* genes in the two different *Agrobacterium* strains.

5.3.5 The effect of the RU-AE01 *hyuH-hyuC* promoter fragment on hydantoinase activity in RU-OR cells

A dramatic increase in hydantoinase enzyme activity was observed when RU-OR cells containing pMJ258 were grown in 0.02% CAAMM (non-repressive) but not when the RU-OR cells containing pMJ258 were grown in 0.5% CAAMM (repressive) or HMM (inductive) (Figures 5-10C and 5-12C). This increase in hydantoinase activity was not observed when RU-OR cells containing the vector (pMJ242) (Figures 5-10C and 5-12C) or when RU-AE01 cells containing either pMJ242 or pMJ258 were grown in 0.02% CAAMM (Figures 5-10A and 5-12A).

The increase in hydantoinase activity when RU-OR cells containing pMJ258 are grown in basal medium (0.02% CAAMM) suggests that the RU-AE01 *hyuH-hyuC* fragment encodes binding sequences for a putative transcription factor that is involved in the regulation of *hyuH* transcription in strain RU-OR. The elevation of *hyuH* transcription could be a result of the titration of a potential repressor protein by the multiple copies of the RU-AE01 *hyuH-hyuC* fragment in non-repressive conditions. Thus it was hypothesised that the repressor is active during conditions of nitrogen sufficiency and prevents the transcription of the *hyu* genes. However, during nitrogen poor conditions, the repressor is inactivated enabling transcription and translation of the *hyu* genes. This repressor would also be inactivated in the presence of an inducer, especially if the mechanisms of NCR and induction are distinct in RU-OR cells.

In this model, the RU-AE01 *hyuH-hyuC* fragment would need to encode potential DNA binding sequences for a transcription factor that represses the hydantoinase activity in RU-OR cells in the absence of hydantoin. To determine where such a binding sequence might lie within the RU-AE01 *hyuH-hyuC* promoter region, plasmids containing a deletion series of the RU-AE01 *hyuH-hyuC* promoter fragment were transformed into RU-OR cells and the levels of the hydantoinase enzyme activity determined. The selection of deletion plasmids was informed by the presence of putative promoter sequences identified in Chapter 4 (Figure 4-7).



Plasmid	Area of the *A. tumefaciens* RU-AE01 *hyuH-hyuC* intergenic fragment present in the plasmid	Nts	Hydantoinsae Activity (µmol/ml)
pMJ242	---	---	1.3 ± 0.25
pMJ258		1-467	9.8 ± 0.78
pMJ266		1-278	1.9 ± 0.4
pMJ261		1-214	1.2 ± 0.4
pMJ268		188-467	0.43 ± 0.5
pMJ245		285-467	0.83 ± 0.29

Figure 5-14: The minimum RU-AE01 *hyuH-hyuC* promoter region required for the increase in hydantoinase activity observed in the presence of the plasmid pMJ258 but not pMJ242 in RU-OR cells. The 8 amino-terminal amino acids of HyuH, the *hyuH-hyuC* region and the 8 amino-terminal amino acids of HyuC are depicted. The area boxed by a bold line represents Region I; the area boxed by the dotted line represents Region II and the area boxed by the dashed line represents Region III. The signals upstream of *hyuH* are shown on the bottom strand and the signals upstream of *hyuC* are shown on the top strand. Diagram is not drawn to scale.

Hydantoinase assays (Figure 5-14) demonstrated that only the presence of the full-length RU-AE01 *hyuH-hyuC* promoter fragment resulted in increased hydantoinase enzyme activity in RU-OR cells. Surprisingly, the fragment containing the sequence for *hyuH* activity alone had no effect. The requirement for the presence of the entire RU-AE01 *hyuH-hyuC* promoter fragment implies that there is more than one binding site for the repressor and that binding is co-operative.

5.3.6 Characterization of proteins binding to the RU-AE01 *hyuH-hyuC* promoter region

Having hypothesised that the increase in hydantoinase enzyme activity was as a result of the titration of a repressor in RU-OR cells, it was decided to attempt to isolate the protein. The approach involved the use of a biotin-tagged PCR product

containing the RU-AE01 *hyuH-hyuC* region to “pull-down” potential DNA binding proteins using avidin-affinity chromatography. Two main assumptions were made in the design of this series of experiments. First, that the binding of the protein to sites on the AE01 *hyuH-hyuC* promoter fragment was based on ionic interactions. Second, that the binding of the repressor was competitive. Both these assumptions have been made by other researchers (Kadonaga and Tjian 1986, LeBlond-Francillard *et al.* 1987) who successfully purified repressor proteins.

Proteins were extracted from RU-OR cells grown in HMM as well as RU-OR and RU-AE01 cells grown in basal medium (0.02% CAAMM). The proteins were then passed through a column containing the biotinylated RU-AE01 *hyuH-hyuC* DNA fragment bound to avidin. The column was washed to remove the unbound proteins. The proteins that remained bound to the RU-AE01 *hyuH-hyuC* intergenic DNA were eluted using high salt concentrations that should disrupt ionic interactions between the putative repressor and the DNA fragment. The eluents were then analysed by SDS-PAGE (Figure 5-15).

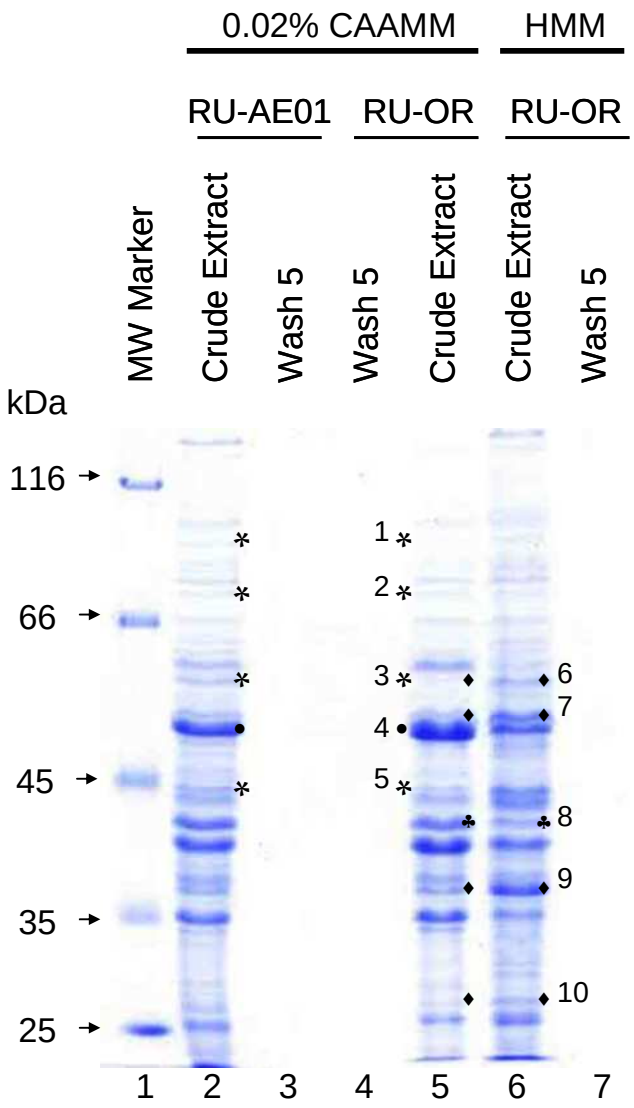


Figure 5-15: SDS-PAGE analysis of column flow-through. Coomassie Brilliant Blue stained SDS-PAGE gel of protein extracts from RU-AE01 and RU-OR cells. Lane 1: Molecular Weight Markers with their weights listed along the left hand side, Lanes 2, 5 and 6 contain protein extracts obtained after sonication of cells. Lanes 3, 4 and 7 contain the elutions after the fifth wash of the DNA-avidin columns. Symbols: * and • - depict differences between RU-AE01 and RU-OR protein extracts from cells grown in 0.02% CAAMM, ♦ and ♣ - show differences between protein extracts from RU-OR cells grown in 0.02% CAAMM and HMM.

The profiles of the protein extracts from RU-AE01 cells and RU-OR cells grown in 0.02% CAAMM showed that while most of the proteins appeared to be present in protein extracts from both RU-AE01 and RU-OR, there were some differences (Figure 5-15, lanes 2 and 5). For example protein bands labeled 1, 2, 3 and 5 (Figure 5-15, lanes 2 and 5, indicated by the symbol *) were clearer in protein extracts from RU-AE01 cells than from proteins extracted from RU-OR cells. However the protein band labeled 4 (Figure 5-15, lanes 2 and 5, shown by the

symbol •) is brighter in protein extracts from RU-OR cells grown in 0.02% CAAMM. The protein extract from RU-OR cells grown in HMM had a very different protein profile to that of RU-OR cells grown in 0.02% CAAMM (Figure 5-15, lanes 5 and 6). For example, protein bands labeled 6, 7, 9 and 10 (Figure 5-15, lanes 5 and 6, indicated by the symbol ♦) appear to be present in lower concentrations or absent in protein extracts from RU-OR cells grown in 0.02% CAAMM but are clear in proteins extracted from RU-OR cells grown in HMM. Conversely, the protein band labeled 8 (Figure 5-15, lanes 5 and 6, shown by the symbol ♣) is brighter in proteins extracted from RU-OR cells grown in 0.02% CAAMM than in protein extracts from RU-OR cells grown in HMM. The protein profiles indicate that the genes expressed, and consequently the proteins produced, are determined by the nutrients available to the cells. Thus the variation in the protein profiles of RU-OR cells grown in 0.02% CAAMM and HMM are a result of the differences in the nitrogen sources provided to the strain. However, the differences observed between RU-AE01 cells and RU-OR cells grown in 0.02% CAAMM are a result of the different genes expressed in the different strains. The variation in the protein profiles implies that despite the similarity between the strains RU-AE01 and RU-OR, there are some differences. These different proteins may be responsible for the different effects observed with respect to the regulation of the *hyu* genes in the two strains.

The flow-though after the fifth wash (Figure 5-15; lanes 3, 4 and 7) showed no visible protein bands. This indicated that all the proteins from the crude extracts (Figure 5-15, lanes 2, 5 and 6) that did not bind the RU-AE01 *hyuH-hyuC* DNA fragment were washed off the column after five washes. This implied that the proteins in the elution would represent the proteins that had bound the RU-AE01 *hyuH-hyuC* intergenic DNA fragment.

The high salt elutions were analysed by SDS-PAGE and visualized using Coomassie Brilliant Blue stain (Figure 5-16). A number of bands were visible suggesting that several proteins were binding to the DNA fragment or that there was a degree of non-specific interaction between the cellular proteins and the DNA fragment or the avidin. To eliminate avidin as the source of non-specific interaction, protein extracted from RU-OR cells was passed through a column that contained avidin beads but no RU-AE01 *hyuH-hyuC* DNA fragment (Figure 5-16, lane 6). No bands were visible

indicating that proteins present in the eluent from columns containing the RU-AE01 *hyuH-hyuC* DNA fragment were interacting with the RU-AE01 *hyuH-hyuC* DNA.

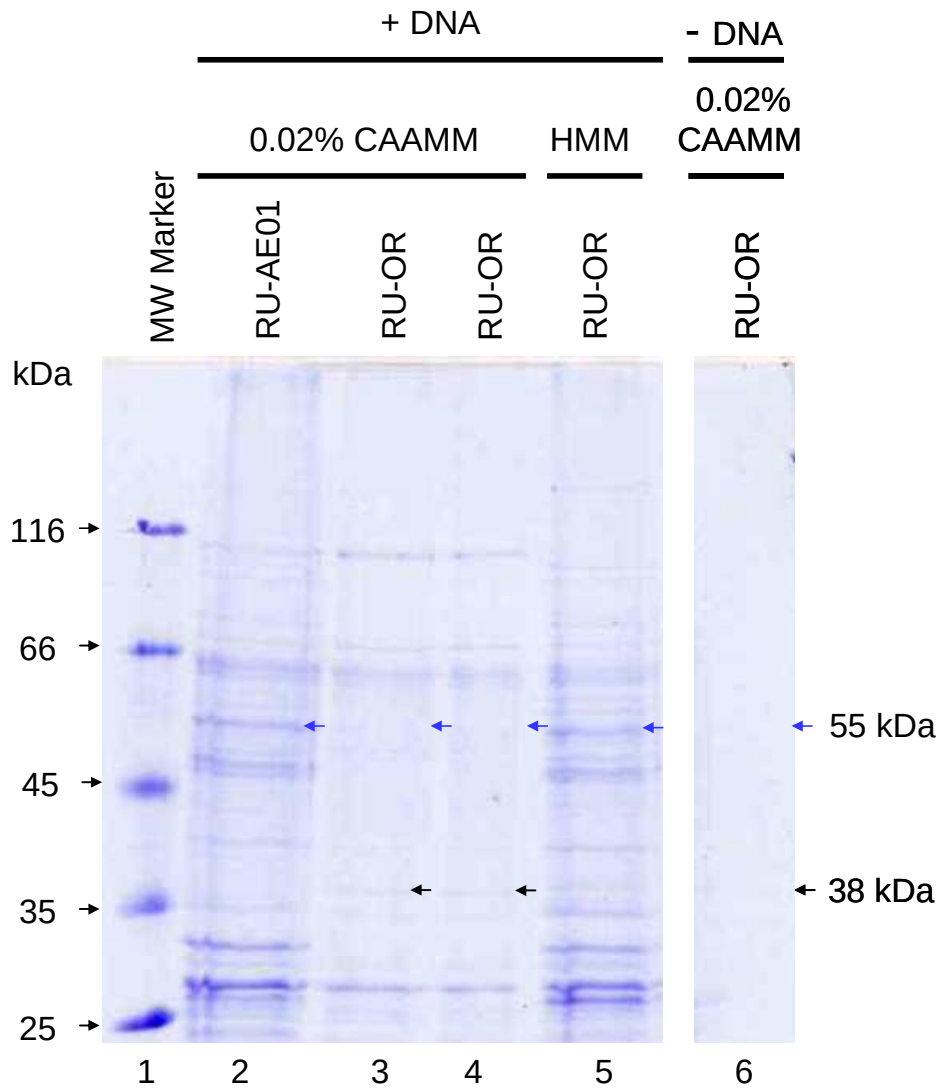


Figure 5-16: RU-AE01 and RU-OR proteins that bound to the RU-AE01 *hyuH-hyuC* promoter region. Coomassie stained SDS-PAGE gel of protein extracts from RU-AE01 cells and RU-OR cells. Lane 1: Molecular Weight Markers with their weights listed along the left hand side, Lanes 2 – 5 contains proteins that were passed through avidin bound biotin-linked RU-AE01 *hyuH-hyuC* DNA and Lane 6 contains proteins that were passed through avidin that was not bound to any biotin-linked DNA. Black arrowheads show the position of the putative repressor at 38 kDa and blue arrowheads show the position of a protein at 55 kDa.

If the hypothesis that a repressor was present in strain RU-OR was correct, it would be expected that the transcriptional factor titrated out by the RU-AE01 *hyuH-hyuC* promoter fragment was likely to be present in RU-OR cells but not in RU-AE01 cells grown in 0.02% CAAMM. This is because the increase in hydantoinase enzyme

activity was observed in RU-OR cells containing pMJ258 but not in RU-AE01 cells containing pMJ258 grown in 0.02% CAAMM (section 5.3.4). A faint band of approximately 38kDa in size (p38) was present in RU-OR cells grown in 0.02% CAAMM but not in RU-AE01 cells (Figure 5-16, lanes 3 and 4 versus lane 2). There was also a faint band present in RU-OR cells grown in HMM but it appeared to migrate slightly higher that the 38 kDa band identified in RU-OR cells grown in 0.02% CAAMM (Figure 5-16, lanes 3 and 4 versus lane 5). This suggests that the putative repressor protein, p38, which is present in RU-OR cells, but not in RU-AE01 cells, is 38 kDa in size.

A number of limitations existed for the experiments reported in this section. The low cell densities obtained for *Agrobacterium* in minimal medium required large volumes of cells to be grown for protein extraction, especially regulatory proteins that are present in low concentrations in the cell. The extraction and purification of proteins that are present in low concentrations in the cells required a large amount of biotin-linked RU-AE01 *hyuH-hyuC* DNA and avidin. This limited the number of controls that could be done in this set of experiments. Also, the elution and concentration of DNA binding protein in high salt buffers reduced the reproducibility of the SDS-PAGE analyses that were conducted. In addition, the quantity of protein detectable by Coomassie Brilliant Blue staining was limited suggesting the optimization of a more sensitive method like silver staining would be required to detect lower quantities of proteins.

Furthermore, while SDS-PAGE was a simple technique to perform and useful for preliminary analyses, it yielded very little information. Only the presence of the protein and its approximate size could be determined by using SDS-PAGE. For more information other techniques need to be explored, including 2D-DIGE (DIfferential Gel Electrophoresis) which would allow the direct comparison of two protein samples from different sources. Proteins isolated from 2D-PAGE or 2D-DIGE gels could also be subjected to amino-terminal protein sequencing and subsequently be identified. This information would allow the detailed study of the mechanism of repression of the *hyu* genes in RU-OR cells.

5.3.7 Is NtrC the repressor protein in strain RU-OR?

Experimental evidence presented in this chapter has demonstrated that the proteins NtrBC have different and distinct effects on the Hyu enzyme activities in strains RU-AE01 and RU-OR. Analysis of the RU-AE01 *hyuH-hyuC* promoter region led to the identification of five putative NtrC binding sites (section 5.3.3). As the NCR effect observed on the RU-AE01 *hyuH* was not transcriptional (section 3.3.2), the *NTR* binding sites are unlikely to be involved in the regulation of *hyuH* in strain RU-AE01. However, the NCR effect observed on the RU-AE01 *hyuC* was at the transcriptional level (section 3.3.2). Additionally, putative *NTR* binding sites were identified in the vicinity of *hyuC* promoter sequences (section 5.3.3). This implies that NtrC may bind the putative *NTR* binding sites upstream of *hyuC* and may be involved in the moderation of NCR on *hyuC.* In addition, the DNA-protein binding assays (section 5.3.6) showed that a number of proteins, other than p38, were isolated. One of these protein bands was approximately 55 kDa in size, the same as size as NtrC, and was identified in protein extracts of RU-AE01 cells grown in 0.02% CAAMM and in protein extracts of RU-OR cells grown in 0.02% CAAMM and HMM (Figure 5-16, blue arrows). Is it therefore possible that NtrC binds the RU-AE01 *hyuH-hyuC* DNA fragment? Does NtrC behave as a repressor of the *hyu* genes in 0.02% CAAMM in RU-OR cells?

To determine if NtrC was capable of binding the RU-AE01 *hyuH-hyuC* DNA fragment, His_6-NtrC was purified from RU-AE01 cells and passed through a column containing the DNA fragment bound to avidin. The column was washed five times to remove unbound proteins. The proteins that remained bound to the DNA were eluted in a high salt buffer. Due to the low concentrations of DNA binding proteins obtained by this method, the proteins in the eluent were concentrated by precipitation. The concentrated proteins were resuspended in a small volume of Elution Buffer and then subjected to SDS-PAGE and Western analysis (Figure 5-17).

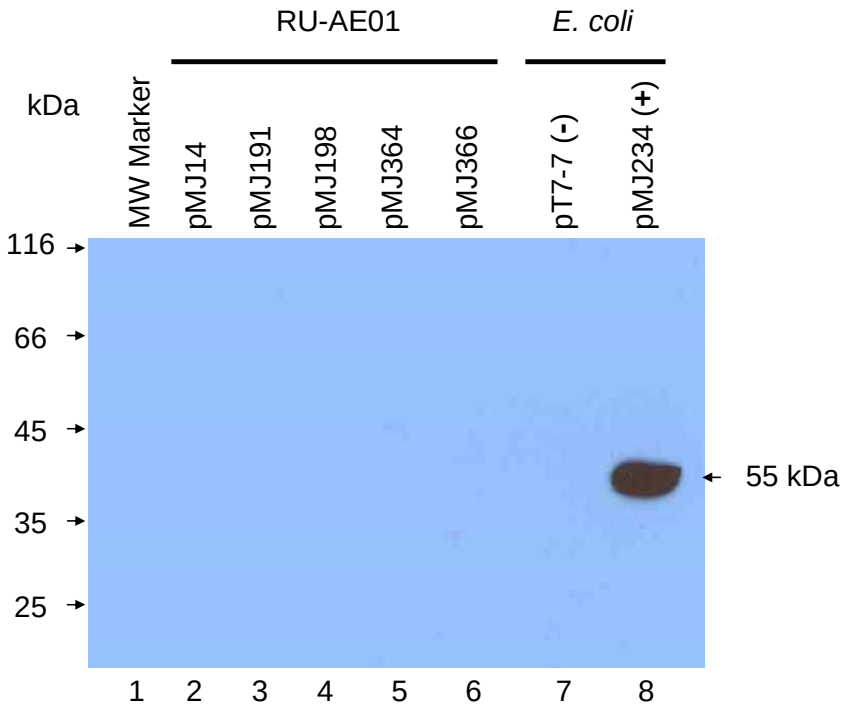


Figure 5-17: Western analysis of the His_6-NtrC protein, eluted from the RU-AE01 *hyuH-hyuC* DNA-avidin column, with anti-His_6 antibodies. Lane 1: Molecular Weight Markers with their weights listed along the left hand side, Lanes 2-6: Eluents of the His_6-NtrC protein (purified by affinity chromatography from RU-AE01 cells containing pMJ14, pMJ191, pMJ198, pMJ364 or pMJ366) from a column containing the RU-AE01 *hyuH-hyuC* DNA fragment. Lane 2: pMJ14, Lane 3: pMJ191, Lane 4: pMJ198, Lane 5: pMJ364 and Lane 6: pMJ366. Lanes 7-8: His_6-tagged NtrC purified by affinity chromatography from *E. coli* BL21 (DE3) cells. Lane 7: pT7-7 (negative control) and Lane 8: pMJ234 (positive control). Black arrowhead shows the position of His_6-NtrC at 55 kDa.

No His_6-NtrC protein produced by RU-AE01 cells containing pMJ364 or pMJ366 was detected in the elutions that were subjected to SDS-PAGE and Western analysis (Figure 5-17, lanes 5 and 6). His_6-NtrC purified from *E. coli* BL21 (DE3) cells containing pMJ234 was readily detected by the anti-His_6 antibodies (Figure 5-17, lane 8). This indicates that the His_6-NtrC protein bound to the RU-AE01 *hyuH-hyuC* DNA fragment was at very low concentrations thus was not detected by the Western analysis or that His_6-NtrC did not bind to the RU-AE01 *hyuH-hyuC* DNA fragment.

Weiss *et al.* (1992) reported that both phosphorylated and unphosphorylated NtrC could bind enhancer DNA sequences. While the levels of His_6-NtrC produced by RU-AE01 cells were very low (section 5.3.2), significant levels of His_6-NtrC protein were produced by *E. coli* BL21 (DE3) cells (section 5.3.2). Thus His_6-NtrC protein

produced by *E. coli* BL21 (DE3) cells was passed through a column containing the RU-AE01 *hyuH-hyuC* DNA fragment, the column was washed five times and the proteins bound to the DNA fragment eluted. The eluents were subsequently analyzed by SDS-PAGE and Western blotting.

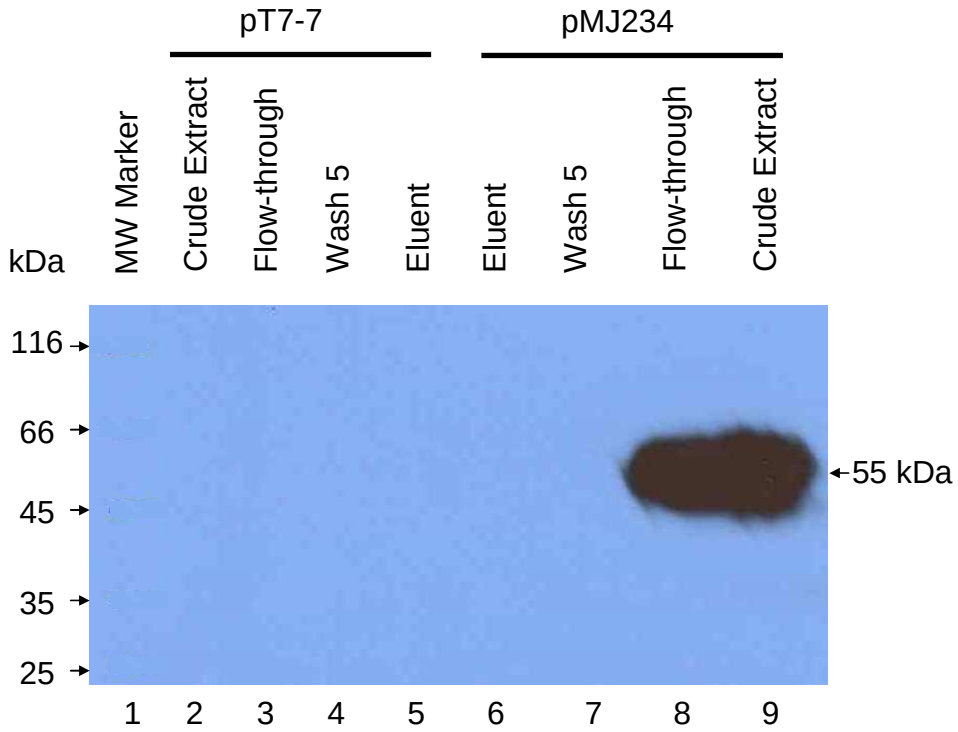


Figure 5-18: Western analysis of *E. coli* BL21 (DE3) His_6-NtrC protein eluted from the RU-AE01 *hyuH-hyuC* DNA-avidin column with anti-His_6 antibodies. Lane 1: Molecular Weight Markers with their weights listed along the left hand side, Lanes 2-5: Protein extracted from *E. coli* BL21 (DE3) containing pT7-7. Lane 2: Crude Extract, Lane 3: Flow-through, Lane 4: Wash 5, Lane 5: Eluent. Lanes 6-9: Protein extracted from *E. coli* BL21 (DE3) containing pMJ234. Lane 6: Eluent, Lane 7: Wash 5, Lane 8: Flow-through, Lane 9: Crude Extract. Black arrowhead shows the position of His_6-NtrC at 55 kDa.

A large amount of His_6-NtrC protein was present in the crude protein extract from *E. coli* BL21 (DE3) cells (Figure 5-18, lane 9). However, when passed through the column, most of the His_6-NtrC was in the flow-through (Figure 5-18, lane 8). Subsequent washes removed unbound protein and no His_6-NtrC protein was detected by the anti His_6 antibodies (Figure 5-18, lane 7). No His_6-NtrC protein was detected in the eluent from the RU-AE01 *hyuH-hyuC* column (Figure 5-18, lane 6). This indicates that His_6-NtrC did not bind the RU-AE01 *hyuH-hyuC* promoter region directly.

This data demonstrates that the binding of NtrC to the *hyuH-hyuC* DNA fragment alone is not enough to produce the increase in hydantoinase enzyme activity which is observed in RU-OR cells. However, it is possible that co-operative binding between NtrC and other transcription factors produces a transcription-activating complex. This protein complex may be responsible for the increase observed in the hydantoinase enzyme activity in strain RU-OR. The data presented in this section eliminates the possibility that NtrC binds the RU-AE01 *hyuH-hyuC* DNA fragment. However, it does not remove the possibility that NtrC, in conjunction with other transcription factors, behaves as a repressor of the *hyu* genes in RU-OR cells in 0.02% CAAMM.

5.4 CONCLUSIONS

Analysis of the Hyu enzyme expression in strain RU-AE01 (section 3.3.2) indicated that the hydantoinase and NCAAH enzymes were both sensitive to induction by hydantoin and to NCR. These two mechanisms were also involved in the regulation of the Hyu enzymes in strain RU-OR (Hartley *et al.* 1998, 2001). Regulation of the RU-AE01 *hyuH-hyuC* DNA fragment in RU-AE01 cells demonstrated that the induction of both *hyuH* and *hyuC* was regulated at the transcriptional level. In addition, NCR of *hyuC,* but not *hyuH,* occurred at the transcriptional level. The regulation of the *hyu* genes at the level of transcription suggests the involvement of transcription factors that are present in the RU-AE01 cells. The aim of the research described in this chapter was to examine the role of transcription factors in the regulation of the *hyu* genes and in the expression of the Hyu enzymes in *A. tumefaciens* strains RU-AE01 and RU-OR. The susceptibility of the Hyu enzymes in both strains to NCR suggested a role for the global nitrogen pathway in the regulation of the *hyu* genes. To this end, the function of the effectors of the global nitrogen regulatory pathway, NtrB and NtrC, in the regulation of the *hyu* genes was studied.

The data reported in chapter 5 demonstrates:
(1) That the NtrBC proteins play a role in the regulation of the Hyu enzymes in strains RU-AE01 and RU-OR.
(2) The roles of the NtrB and NtrC proteins in the regulation of the Hyu enzymes are different in the two strains. This is supported by two pieces of evidence. First, in strain RU-AE01, NtrBC caused the up-regulation of the *hyuH* gene but only NtrC

caused the up-regulation of the *hyuH* genes in RU-OR. Second, the addition of a His_6-tag to NtrC did not affect the ability of NtrC to increase the hydantoinase activity in RU-AE01 whereas it did in RU-OR. This led to the hypothesis that while the same factors may be involved in the regulation of the *hyu* genes in RU-AE01 and RU-OR, the mechanisms of regulation in the two strains were different.
(3) Analysis of the RU-AE01 *hyuH-hyuC* fragment in strain RU-OR demonstrated that the global mechanisms of regulation in the two strains are similar. This disproved the hypothesis that the regulation of the *hyu* genes in the two strains was different. It also indicated that the *hyu* genes in strain RU-OR are different to those in strain RU-AE01.
(4) The identification of p38, a putative repressor protein which is present in strain RU-OR but absent in strain RU-AE01 supports the theory that the *hyu* genes in the two strains are different. In addition, it adds a third mechanism of regulation of the *hyu* genes in strain RU-OR.

The regulation of the *hyu* genes by the global nitrogen pathway is not unexpected. The proteins NtrBC regulate a large number of genes involved in the regulation of the nitrogen levels within the cell (Kustu *et al.* 1989). In *E. coli,* approximately 2% of the genes are regulated by NtrC (Zimmer *et al.* 2000). During low nitrogen conditions, NtrC is phosphorylated by NtrB, producing the active transcription factor NtrC~P. NtrC~P directly or indirectly causes the up-regulation of genes required for the production of glutamine. Conversely, during nitrogen sufficiency, NtrC~P is dephosphorylated by NtrB, producing the inactive molecule NtrC (Ninfa and Atkinson 2000).

In RU-AE01, the presence of NtrB and NtrC causes an increase in the Hyu enzyme activity. This demonstrates that NtrB, the protein kinase, and NtrC, the response regulator, are both involved in the up-regulation of the *hyu* genes. It is interesting to speculate whether this indicates that NtrC~P is responsible for the effect observed in RU-AE01. As NtrC~P is a signal of poor nitrogen levels within the cell, this would be a reasonable supposition. This theory would also explain the NCR effect observed on the Hyu enzymes during high nitrogen conditions, where NtrC would signal nitrogen sufficiency to the cells and prevent the transcription of the genes required for the metabolism of poor nitrogen sources, like hydantoin. Data presented in section 3.3.2 demonstrated that the NCR effect on the *hyuH* was not at the level of transcription.

This indicates that the effect observed in RU-AE01 cells is likely to be a direct result of the nitrogen status of the cell. This effect was termed ammonia shock by Hartley *et al.* (2001). In contrast to the regulation of *hyuH*, the NCR effect observed on *hyuC* was at the transcriptional level (section 3.3.2). While putative DNA binding sequences for NtrC were identified on the RU-AE01 *hyuH-hyuC* fragment, NtrC did not bind to the DNA. This suggests that direct interaction of NtrC with the fragment does not occur. However, it does not exclude the interaction of NtrC with other transcription factors and interaction between the transcription factor complex and the RU-AE01 *hyuH-hyuC* DNA fragment.

NtrC, but not NtrB, played a role in increasing the levels of the hydantoinase enzyme in strain RU-OR. This is significantly different to the effect of NtrBC in strain RU-AE01 as it suggests that the unphosphorylated NtrC protein is involved in the regulation of the *hyuH* gene in RU-OR. Unphosphorylated NtrC is a cellular signal for nitrogen sufficiency thus it is unlikely to be an activator of the *hyu* genes, which are involved in the hydrolysis of the poor nitrogen source hydantoin. However, the interaction of NtrC, whether phosphorylated or unphosphorylated, with other transcriptional proteins may in turn activate the transcription of the *hyu* genes. This suggests that the regulation of the *hyuH* genes in RU-OR is controlled not by the nitrogen status of the cell, as hypothesized for RU-AE01, but by the interaction of transcription factors.

One transcription factor of particular interest is the Nac (nitrogen assimilation control) protein, which is 32 kDa in size and belongs to the LysR family of regulatory proteins (Schwacha and Bender 1993). The *nac* gene was first described in *Klebsiella aerogenes* and is also present in *E. coli* and *A. tumefaciens* C58 (Bender *et al.* 1983, Muse and Bender 1998, http://cancer.lbi.ic.unicamp.br/agroC58/) but not in *Salmonella typhimurium* (Muse and Bender 1998). Generally, under conditions of nitrogen limitation when the concentration of NtrC~P increases, *nac* expression occurs from a σ^{54}-dependent promoter (Atkinson *et al*. 2002, Feng *et al.* 1995a). Thus *nac* expression is directly controlled by the NtrBC proteins (Macaluso *et al.* 1990). Interestingly, Nac acts directly to repress further *nac* expression by preventing interaction of NtrC~P and σ^{54}-RNA polymerase at the *nac* promoter (Feng *et al.* 1995b). Thus the Nac protein itself regulates the cellular levels of Nac. Nac is involved in the activation of the *hut* (histidase), *put* (proline oxidase) and *ure* (urease) genes and the repression of the *gdh* (GDH) and *gltBD* (GOGAT) gene expression

(Macaluso *et al.* 1990). A number of the systems regulated by Nac are also controlled by other regulatory mechanisms. For example cAMP-CRP is involved in the regulation of the *hutUH* operon (histidine utilization) (Macaluso *et al.* 1990, Osuna *et al.* 1991). Macaluso *et al.* (1990) suggest that the Nac system may be a mechanism by which the normally σ^{54}-dependent ntr system regulates σ^{70}–dependent operons. It is interesting to speculate whether Nac or a Nac-like protein may be involved in the regulation of the *hyuH* gene in strain RU-OR.

The experimental evidence proposes that the *hyuH* gene in RU-OR is different to the *hyuH* gene in strain RU-AE01 and the other *Agrobacterium* strains. This theory is supported by the observation that RU-OR cells, but not RU-AE01 cells, encode a putative repressor protein (p38) which is approximately 38 kDa in size. Thus this means that the *hyuH* genes in strain RU-OR have three mechanisms of regulation: (1) induction by hydantoin and hydantoin analogues, (2) NCR and potentially (3) repression by p38.

The over-expression of NtrB and NtrC had a clear effect on the hydantoin-hydrolyzing activity in *Agrobacterium* strains RU-AE01 and RU-OR. It would be interesting to inactivate the NtrB and NtrC genes in RU-AE01 and RU-OR using a suicide plasmid (Hils *et al.* 2001) or transposon mutagenesis (Dennis and Zylstra 1998) and then determine the effect of no NtrB/NtrC and compare it to the effect of exogenous NtrB/NtrC expressed at varying levels.

Hartley *et al.* (1998, 2001) isolated three classes of *A. tumefaciens* RU-OR mutants. Class I includes mutants that showed inducer-independent hydantoinase enzyme activity (RU-ORLB3) or hydantoinase and NCAAH enzyme activity (RU-ORL5). Class II represented mutants that exhibited inducer-independent hydantoin-hydrolyzing enzyme activity that was also insensitive to NCR (RU-ORPN1). Class III contains mutants that exhibited inducer-independent hydantoin-hydrolyzing enzyme activity, insensitivity to NCR and resistance to ammonia shock (RU-ORPN1F1 and RU-ORPN1F9). Hartley *et al.* (2001) proposed that mutations in the signalling pathways resulted in the phenotypes observed for the RU-OR mutants. NtrC is an important signalling molecule in the global nitrogen regulatory pathway. What is the role of the NtrC protein in these mutant strains? These studies would permit the unravelling of the complex mechanisms that interact

to regulate the *hyu* genes in strain RU-OR. By extension, these studies would also allow the study of nitrogen regulation in the *Agrobacterium* strains.

The RU-OR *hyuC1* enzyme, like the RU-AE01 *hyuC,* was up-regulated in the presence of NtrB and NtrC proteins. In addition, the modification of NtrC, by addition of a His_6-tag, did not affect the ability of the NtrC protein to increase the levels of the NCAAH enzymes in RU-OR or in RU-AE01. The *hyuC* and *hyuC1* genes in strains RU-AE01 and RU-OR respectively share over 90% identity at both the DNA and amino acid level. Thus the similar behaviour of the *hyuC*(*1*) genes and the HyuC(1) enzymes in strains RU-AE01 and RU-OR is not surprising. The presence of a second *hyuC* gene in strain RU-OR, which shares only 60% identity at the nucleotide level with RU-AE01 *hyuC* and RU-OR *hyuC1*, and the presence of a number of other potential *hyu* genes on the C58 genome suggests that strain RU-OR encodes different *hyu* genes that may be controlled by alternative regulatory mechanisms.

It is therefore important to bear in mind that the hydantoinase and NCAAH activities obtained for RU-AE01 cells and RU-OR cells represent the sum of the hydantoinase and the NCAAH enzyme activities from all the functional *hyuH* and *hyuC* genes in the strains, respectively. In contrast, the levels of β-glucuronidase and β-galactosidase enzyme activities obtained from pMJ258 represent the transcriptional and translational activation of one *hyuH* gene and one *hyuC* gene. In the case of strain RU-OR, the *hyu* genes first need to be isolated. However, in the case of RU-AE01, this can be addressed by cloning the *hyuH*-RU-AE01 *hyuH-hyuC* promoter-*hyuC* fragment and the *gus*-RU-AE01 *hyuH-hyuC* promoter-*lac* fragment into the same broad host range vector backbone and analyzing the enzyme activities in a bacterial strain that does not encode the *hyuH* or the *hyuC* genes. The *hyuH* transcription and translation can be compared to the hydantoinase enzyme activity and the *hyuC* transcription and translation can be compared to the NCAAH enzyme activity, without interference from other *hyuH* and *hyuC* genes or from other hydantoinase and NCAAH enzyme activities, thus providing data specific for the one *hyuH* gene and one *hyuC* gene being analyzed.

CHAPTER 6

GENERAL CONCLUSIONS

6.1 INTRODUCTION

Agrobacterium strains are economically important for their use in plant boiotechnology and industrially important for the production of hydantoin-hydrolyzing enzymes (Gelvin 2000, Ogawa and Shimizu 2002). Research has focused on the molecular characterization of the Hyu enzymes from *Agrobacterium* strains. However, very little is known about how their expression is regulated. In this study, the pathways involved in the regulation of the *hyu* genes from *A. tumefaciens* strains RU-AE01 and RU-OR were studied.

6.2 REGULATION OF *hyu* GENE EXPRESSION

Analysis of the Hyu enzyme activities in strain RU-AE01, under different growth conditions, revealed that the hydantoinase and NCAAH enzymes were produced under the same conditions as in strain RU-OR (Hartley *et al.* 1988, 2001). The Hyu enzyme activities of strain RU-AE01 were induced in the presence of hydantoin and subject to NCR.

A novel promoter-probe vector was used to study *hyu* gene expression. Initial data showed that the Hyu enzyme activity followed the same patterns as the reporter enzyme activity demonstrating that the *hyuH* and *hyuC* promoters were regulating the expression of the *hyu* genes in the same manner as the reporter genes. Thus the promoter-probe vector was used to study the transcriptional and translational regulation of the RU-AE01 *hyu* genes.

The *hyuH* and *hyuC* promoter activity, like the Hyu enzyme activity, was subject to induction in the presence of hydantoin indicating that induction was regulated at the level of transcription in RU-AE01. Both the HyuH and the HyuC enzyme activities were sensitive to NCR but only the *hyuC* promoter was subject to repression by nitrogen at the transcriptional level. The degree of repression exerted by nitrogen on HyuC and the level of repression observed on the *hyuC* promoter activity was not equivalent (Section 3.3.2) but the difference between the two ratios was attributed to the difference in the copy number of the the *hyuC* gene and the copy number of the *hyuC* promoter-reporter gene fusion. It is also possible that the difference could be the result of a second level of NCR exerted on the HyuC enzyme, at the

post-translational level. The *hyuH* promoter was unaffected by NCR indicating that NCR observed on *hyuH* was exerted at the post-transational level, as described for the same enzyme in RU-OR cells by Hartley *et al.* (2001). This leads to the conclusion that the regulation of *hyuC* expression was comparable between the strains RU-AE01 and RU-OR. As the available promoter and coding sequence of RU-OR *hyuC1* was over 90% identical to the RU-AE01 *hyuC* promoter and coding sequence, this is not unexpected. The second *hyuC* gene (*hyuC2*), which is present in RU-OR, may involve alternative regulatory mechanisms and pathways which have not been elucidated. The regulation of expression of *hyuH* however was different in the strains RU-AE01 and RU-OR. While NCR of *hyuH* was exerted at the transcriptional and post-translational levels in strain RU-OR (Hartley *et al.* 2001), only the post-translational regulatory pathway was active in strain RU-AE01.

Deletion analysis of the RU-AE01 *hyuH-hyuC* promoters conducted in the promoter-probe vector was used to detect regions capable of activating transcription and translation of the *hyuH* and *hyuC* genes. Overlapping putative σ^{54}-dependent and putative σ^{70}-dependent promoter sequences were identified upstream of *hyuH.* A region of DNA encoding a putative σ^{54}-dependent promoter sequence and a putative σ^{70}-dependent promoter sequence upstream of the *hyuH* gene was able to activate transcription of a reporter gene.

σ^{54}-dependent promoters are responsible for the transcription of a wide variety of genes (Kustu *et al.* 1989). Activation of the σ^{54}-dependent promoter relies on the presence of an activator protein belonging to the NtrC family of activator proteins that includes NtrC, NifA, DctD and XylR. NtrC acts as an activator of genes involved in the assimilation of nitrogen, NifA activates genes expressed during low oxygen conditions, DctD activates genes required for the uptake of dicarboxylic acids while XylR activates the expression of genes required for the uptake of toluene and xylene (Kustu *et al.* 1989). The transcriptional activity of NtrC (in *S. typhimurium*) and DctD (in *Rhizobium meliloti)* is regulated by phosphorylation of the response regulator by a kinase via the two-component system (Nohaile *et al.* 1997, Kern *et al.* 1999, Lee *et al.* 1994b). NifA (in *R. meliloti*) is produced in an active state and its activity is modulated in response to oxygen (Beynon *et al.* 1988). XylR (in *P. putida*) is

activated by binding the substrates of the xylene or toluene catabolic pathways (Inouye *et al.* 1987).

The presence of a σ^{54}-dependent promoter suggests that the transcription of RU-AE01 *hyuH* is activated by an activator protein of the NtrC family. This protein is not NtrC as protein-DNA binding studies showed that His_6-NtrC did not bind the RU-AE01 *hyuH-hyuC* DNA fragment. Thus the activator of *hyuH* gene transcription has yet to be isolated. The mechanism by which the transcriptional activator is triggered varies significantly between the different activators. However the requirement for hydantoin suggests that the transcriptional activator may be induced by substrate binding as is the case for XylR.

The presence of overlapping σ^{54}-dependent and σ^{70}-dependent promoters has been best studied in the *glnAntrBC* operon of the enteric bacteria *E. coli, S. typhimurium* and *K. aerogenes* (Pahel *et al.* 1982, MacFarland *et al.* 1981, Rothman *et al.* 1982). It was determined that the *glnA* gene was expressed from the promoter *glnA*p1 or the promoter *glnA*p2 depending on the nutritional status of the cell (Reitzer and Magasanik 1985). The presence of overlapping σ^{54}-dependent and σ^{70}-dependent promoters upstream of *hyuH* in the plasmid-borne *hyu* cluster of *Pseudomonas* sp. NS 671 *hyu* gene cluster is noteworthy (Watabe *et al.* 1992a). This makes it interesting to speculate about the conditions under which the σ^{54}-dependent and σ^{70}-dependent promoters of *hyuH* are active and whether the overlapping promoter sequences allow different levels of expression to occur at different stages in the growth of the cells. It is possible that these overlapping promoters may be part of a regulatory mechanism that has yet to be elucidated. The DNA sequence for the σ^{54}-dependent and putative σ^{70}-dependent promoters of RU-AE01 showed high identity with the same region in *Agrobacterium* strains NRRL B11291 and IP I-671. This implies that similar mechanisms of *hyuH* transcription may occur in these *Agrobacterium* strains.

Four putative σ^{70}-dependent promoters were identified upstream of *hyuC*, two of which (‘A’ and ‘D’) were able to activate low levels of reporter gene expression and one of which (‘C’) was able to activate high levels of reporter gene expression. The putative σ^{70}-dependent promoters ‘C’ and ‘D’ are present immediately upstream of

hyuC in RU-AE01 and also upstream of *hyuC1* in strain RU-OR. This suggests that these putative σ^{70}-dependent promoters might be responsible for the activation of *hyuC* and *hyuC1* in strains RU-AE01 and RU-OR respectively. Interestingly, the sequence for putative σ^{70}-dependent promoter 'A' is present in the promoter of strain NRRL B11291, and may therefore provide transcriptional activation for the expression of *hyuC* in this strain.

The deletion analysis of the RU-AE01 *hyuH-hyuC* region indicated which transcription activation sequences were most likely to be responsible for the activation of transcription of the *hyuH* and *hyuC* genes. To confirm the functions of these sequences, one would need to conduct mutational analysis of each putative promoter sequence.

6.3 NITROGEN REGULATION OF HYDANTOIN HYDROLYSIS

The NtrB and NtrC proteins are integral members of the global ntr pathway, which functions to regulate the levels of cellular nitrogen. NtrC, the response regulator, is phosphorylated by the protein kinase, NtrB, under low nitrogen conditions (Ninfa and Magasanik 1986, Ninfa and Atkinson 2000). The phosphorylated NtrC acts as a transcriptional activator for a number of genes involved in the assimilation of nitrogen (Kustu *et al.* 1989). Conversely, under nitrogen sufficient conditions, NtrC is usually dephosphorylated and inactivated (Ninfa and Magasanik 1986, Ninfa and Atkinson 2000).

A. tumefaciens strains RU-AE01 and RU-OR were both isolated for their ability to hydrolyze hydantoin as the sole source of nitrogen (Hartley 1995, Herrera unpublished). The HyuH and HyuC enzymes in strains RU-AE01 and RU-OR were both sensitive to NCR, which suggests the involvement of the ntr pathways. In addition, analysis of RU-OR mutant strains indicated that the resistance to ammonia shock, which was observed on the hydantoinase enzyme, was a result of the irreversible adenylation of glutamine synthetase (Hartley *et al.* 2001). Glutamine synthetase is an effector enzyme of the global nitrogen regulatory pathway. Thus it feasible to hypothesize that the global nitrogen regulator NtrC plays a role in the regulation of the *hyu* genes.

The research reported in this thesis allows four main conclusions to be drawn with respect to the regulation of the Hyu enzymes by nitrogen in *A. tumefaciens* strains RU-AE01 and RU-OR (summarized in Figure 6-1).

(1) The regulation of the *hyuH* gene in RU-AE01 occurs via the global ntr pathway, specifically through the action of the phosphorylated NtrC protein. This is supported by two pieces of evidence. Firstly, the presence of exogenous NtrB and exogenous NtrC in RU-AE01 cells leads to the detection of dramatically high levels of HyuH enzyme activity. This implies that phosphorylated NtrC is required to exert the effect in RU-AE01 cells. Secondly, the observation that the *hyuH* promoter was not subject to NCR at the transcriptional level, implies that the phosphorylated NtrC protein acts as a signaling molecule rather than as a transcriptional activator (Figure 6-1A). Thus phosphorylated NtrC signals a nitrogen poor status to the cellular nitrogen sensors leading to the activation of transcription of the genes required for the hydrolysis of poor nitrogen sources including hydantoin.

(2) The regulation of the RU-AE01 *hyuC* gene is subject to control by the ntr pathway. Firstly, the levels of the HyuC enzyme activity in RU-AE01 were higher in the presence of exogenous NtrB and NtrC. Secondly, transcription of the *hyuC* promoter was observed to be sensitive to NCR (Figure 6-1A). While three putative NtrC binding sites were identified upstream of *hyuC,* His_6-NtrC protein-RU-AE01 *hyuH-hyuC* DNA binding assays demonstrated that the NtrC protein did not bind the RU-AE01 *hyuH-hyuC* DNA fragment. This indicates that the NtrC protein may bind weakly to the RU-AE01 *hyuH-hyuC* DNA fragment thus was not detected in the His_6-NtrC protein-RU-AE01 *hyuH-hyuC* DNA binding assays. Alternatively, the NtrC protein may not bind the promoter region upstream of *hyuC.* Instead it may stimulate other transcription factors that activate the transcription of *hyuC.*

(3) Like RU-AE01, the RU-OR *hyuC* gene(s) are sensitive to regulation by the global ntr pathway. An increase in the levels of NtrB and NtrC protein in the RU-OR cells led to an increase in the HyuC enzyme activity detected (Figure 6-1B). Two *hyuC* genes are present in RU-OR (Hartley 2001). As a result of the sequence identity between RU-AE01 *hyuC* and RU-OR *hyuC1,* it is most likely that these two genes are closely related. Thus the mechanisms that regulate the *hyuC* and *hyuC1* genes are expected to be the same or similar. A plasmid construct encoding the RU-OR *hyuC1* gene

(pG4) hosted in *E. coli* DH5α cells produces glycine from *N*-carbamylglycine as a substrate in Ninhydrin assays indicating that the promoter sequence of *hyuC1* is encoded in pG4. However, it is unknown whether the full promoter sequence is present. All attempts to isolate the sequence upstream of *hyuC1* to date have met with failure. Until the full promoter sequence upstream of *hyuC1* is isolated and studied, the proposed relationship between RU-AE01 *hyuC* and RU-OR *hyuC1* remains speculative. This avenue is an objective for future research. While a plasmid construct encoding the *hyuC2* gene was shown to produce an active HyuC2 enzyme (Hartley 2001), this plasmid construct has since been lost. All attempts to re-isolate the *hyuC2* gene have also been unsuccessful. Again, the re-isolation of *hyuC2* from strain RU-OR is a task for future research. Considering the differences between *hyuC2* and the conventional *hyuC1* genes, the study of *hyuC2* should be a priority.

(4) The regulation of *hyuH* in RU-OR involves unphosphorylated NtrC. This is supported by two lines of evidence. Firstly, the HyuH enzyme activity in strain RU-OR increased dramatically in the presence of exogenous NtrC (Figure 6-1B). Secondly, the addition of a His_6-tag to NtrC dramatically reduced the levels of HyuH enzyme activity. It is possible that the structural integrity of the NtrC protein was altered by the addition of a His_6-tag to the carboxy-terminus and that NtrC, with its tertiary structure intact, was required to increase the levels of the hydantoinase enzyme activity. It is also possible that NtrC binds to alternative transcription factors which in turn leads to the activation of transcription of *hyuH.* Weiss *et al.* (1992) reported that both phosphorylated and unphosphorylated NtrC bound enhancer DNA sequences thus NtrC binding to other transcription factors and subsequent activation of *hyuH* is a possibility. However, this does not explain the unexpected observation that the presence of NtrC alone led to the increased levels of HyuH enzyme activity as NtrC is a signal for high nitrogen levels within the cell and hydantoin is a poor nitrogen source.

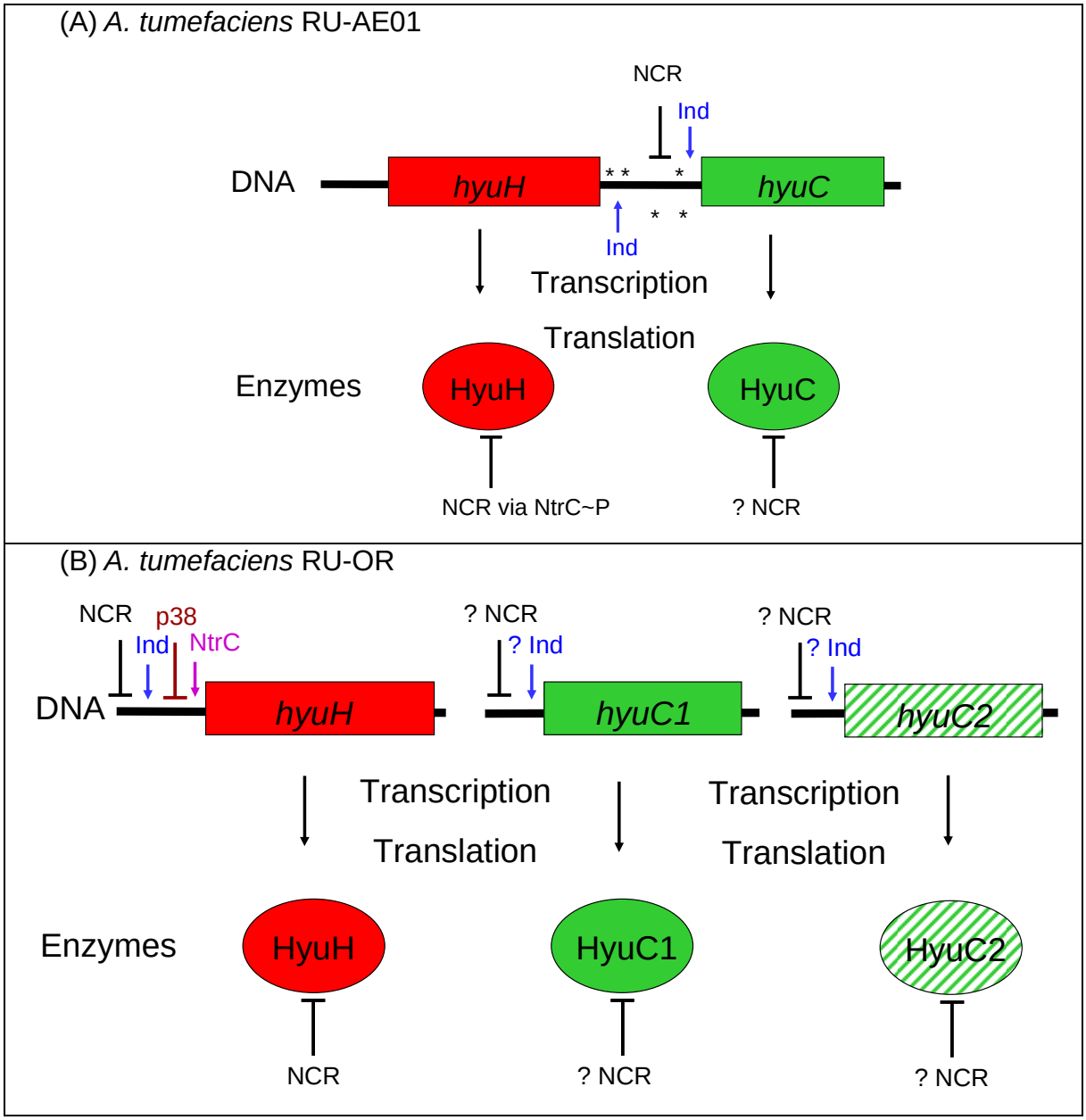


Figure 6-1: Factors involved in the regulation of the *hyu* genes in *A. tumefaciens* strains RU-AE01 and RU-OR. Arrows represent induction and terminated lines indicate repression. Abbreviations: NCR- nitrogen catabolite repression, Ind- Induction and *- putative NtrC binding sites.

The differences observed between the conditions under which elevated levels of HyuH are observed in strains RU-AE01 and RU-OR indicate that the regulation of the *hyuH* genes in the two strains is different. The differences in the *hyu* genes in the two *A. tumefaciens* strains RU-AE01 and RU-OR are highlighted by the presence of a possible repressor protein, p38, in RU-OR (Figure 6-1B). Attempts to identify the factor that interacts with the RU-AE01 *hyuH-hyuC* DNA fragment in RU-OR led to the identification of a 38 kDa protein that was present in strain RU-OR but absent in

strain RU-AE01. A 38 kDa protein was the expected product of ORF 8 from *A. aurescens* DSM 3747 (Wiese *et al.* 2001). This protein was identified as a putative repressor protein from the LacI / GalR family of transcriptional regulators by Wiese *et al.* (2001). However, no research has been done to study whether this protein regulates the expression of the *hyu* genes in *A. aurescens* DSM 3747.

6.4 THE *HYU* SYSTEMS IN *AGROBACTERIA*

Initial bioinformatic analyses showed that the *Agrobacterium hyu* gene clusters were all similar; the *hyuH* and *hyuC* genes were arranged divergently in all the operons. However, the *hyu* gene clusters all also showed some variation. The sequence between *hyuH* and *hyuC* in NRRL B11291 was ~250 nt shorter than IP I-671 and RU-AE01 suggesting either a deletion event in strain NRRL B11291 or an insertion of DNA in the other strains. There was also 200 nt of intergenic sequence downstream from the *hyuC* gene in RU-AE01 that was similar to sequences downstream from the *hyuD* and *hyuA* genes in IP I-671 implying that *hyuD* and *hyuA* had been deleted in strain RU-AE01. This variation between the *hyu* gene clusters can be attributed to two main factors: the diversity of the plasmids present in the *Agrobacterium* strains and transposition events.

The *A. tumefaciens* C58 genome is 5.7 Mb in size. It includes a 2.8 Mb circular chromosome, a 2.1 Mb linear chromosome and two plasmids, pATC58 and pTiC58 which are 0.5 Mb and 0.2 Mb in size respectively (Goodner *et al.* 2001). The octopine-type and nopaline-type Ti plasmids present in *Agrobacterium* cells show tracts of sequence that are identical between plasmids yet they also show areas of significant differences (Suzuki *et al.* 2000). The *hyu* gene clusters that have been isolated from strains IP I-671 and NRRL B11291 were located on two native plasmids, 190 kb and 160 kb in size respectively (Hils *et al.* 2001). This indicates that *Agrobacteria* also host other plasmids giving this genus incredible variety in the genetic information it contains. The diverse nature of plasmids in *Agrobacterium* strains provides a large potential pool of information to mine and study and also explains the differences between closely related *Agrobacterium* strains.

Plasmids pATC58 and pTiC58 present in strain C58 encode the genes required for plasmid replication and transfer (Wood *et al.* 2001) and are thus independently

mobile. In addition, sequence analysis of bacterial genomes indicates that a large proportion of the genome encodes transposon-based sequences, which provides a high degree of plasticity within the bacterial genomes. The IP I-671 *hyu* gene cluster is flanked by transposon-derived sequences (Hils *et al*. 2001) which suggests that the *hyu* operons are mobile elements in *Agrobacterium* that are capable of jumping from one position to another. In addition, the mechanisms of bacterial transposon excision and recombination are error-prone. Furthermore, transposons tend to rearrange generating variation between different isolates of the same species (Hauptmann and Schmitt 2006, Nagy and Chandler 2004). Thus transposition events would also explain the differences in the structure of the *hyu* gene clusters present in the different *Agrobacterium* strains namely IP I-671 and RU-AE01. Hils *et al.* (2001) reported that the strain IP I-671 encoded two *hyuH* genes but only one *hyuC* gene. Hartley (2001) isolated two *hyuC* genes from the strain RU-OR. Analysis of the C58 genome showed the presence of two *bup* genes, two *hyuH* genes and two *hyuA* genes on the circular and linear chromosomes. Two additional *hyuH* genes were identified on the pTi plasmid. The presence of the *hyu* cluster on a transposon would also explain the *hyu* gene duplication observed in the different *Agrobacteria.*

The function of the *dhp* and *bup* genes in pyrimidine hydrolysis has been well characterized (Kao and Hsu 2003). However, the natural function of the *hyuH* and *hyuC* genes remains to be elucidated. The proposal that hydantoins and *N*-carbamyl-α-amino acids may have been the precursors for prebiotic peptides rather than free α-amino acids suggests a historical purpose for the HyuH and HyuC enzymes in primitive bacterial cells (Taillades *et al.* 1998). These ancient genes may have been retained by the *Agrobacterium* strains, and evolved through time to hydrolyze the hydantoins that form their present substrate range. Further evolutionary studies are required to fully understand the presence of the *hyu* genes in *Agrobacterium* cells.

Cases *et al.* (2003) suggest that there is a correlation between the number of transcriptional regulators in a strain and its genome size. For *Agrobacterium* with a genome size of 5.7 Mb, it suggests that there are a lot of transcriptional factors present that are not present in bacteria with smaller genomes. Bacterial species that exist in highly competitive environments are under pressure to regulate and coordinate gene expression (Cases *et al.* 2003). The *Agrobacterium* species are

under environmental pressure to develop and maintain regulators for catabolic degradation pathways and these pathways may vary within the same species, as observed for RU-AE01 and RU-OR.

Based on the conditions under which the Hyu enzymes were expressed and the Hyu enzyme characteristics, the Hyu enzyme systems in the strains RU-AE01 and RU-OR would have been considered identical. This is now known to be incorrect. The data reported indicates that the techniques that are presently used to classify the hydantoin-hydrolyzing enzymes in bacteria are imperfect. Grouping of the Hyu enzyme systems based on the conditions under which the Hyu enzymes are expressed and the characteristics of the enzymes that are expressed does not do justice to the diversity of the Hyu enzymes present in microorganisms. A more detailed system is required to classify Hyu enzymes, possibly one based on the evolutionary relationships between the enzymes.

6.5 RESEARCH OPPORTUNITIES

There are three main areas of interest that require further investigation to enable a more complete understanding of the *hyu* gene systems in *Agrobacterium* species.
(1) The isolation of the RU-OR *hyuH* gene and identification of the location of the *hyu* gene cluster on the RU-OR genome.
(2) p38 characterization.
(3) Role of NtrC in *hyu* regulation in strain RU-OR.

Numerous attempts have been made to isolate the *hyuH* gene from RU-OR. However all of them have been unsuccessful. The isolation of the *hyuH* genes from RU-OR remains an important task for the future. The availability of the *hyuH* gene will allow the isolation of the *hyu* gene cluster. This will allow the comparison of *hyu* genes from strain RU-OR to the existing sequence data from the other *Agrobacterium* strains. In addition, research on the transcriptional regulation of the RU-OR *hyu* genes would add significantly to the information presented in this thesis. The elucidation of the *hyu* sequence will also enable the study of the effect and possibly the interaction of the unphosphoylated NtrC protein with the *hyu* promoter sequence or with alternative transcription factors.

The role of the putative repressor protein, p38, from RU-OR needs to be investigated further. The inability to purify enough p38 protein from RU-OR cells hampered attempts to study the putative repressor protein. Large volume fermentations would enable the production of increased cell biomass which would allow the purification of higher concentrations of p38 protein. Another option would be to inactivate the gene encoding the putative repressor protein in RU-OR cells using transposon mutagenesis (Dennis and Zylstra 1998) and to then select for cells that producing high levels of hydantoinase enzyme in basal minimal medium (0.02% CAAMM). Identification of the site of transposon insertion would allow the isolation of the coding sequence of the putative repressor protein. This would increase the possibility of isolating enough p38 protein for analysis. With enough p38, the protein could be subjected to 2D-electrophoresis, purified from a polyacrylamide gel and the amino-terminal sequenced. Once identified, p38 could be used in DNA-binding studies and the binding sites of p38 identified by DNA footprinting studies.

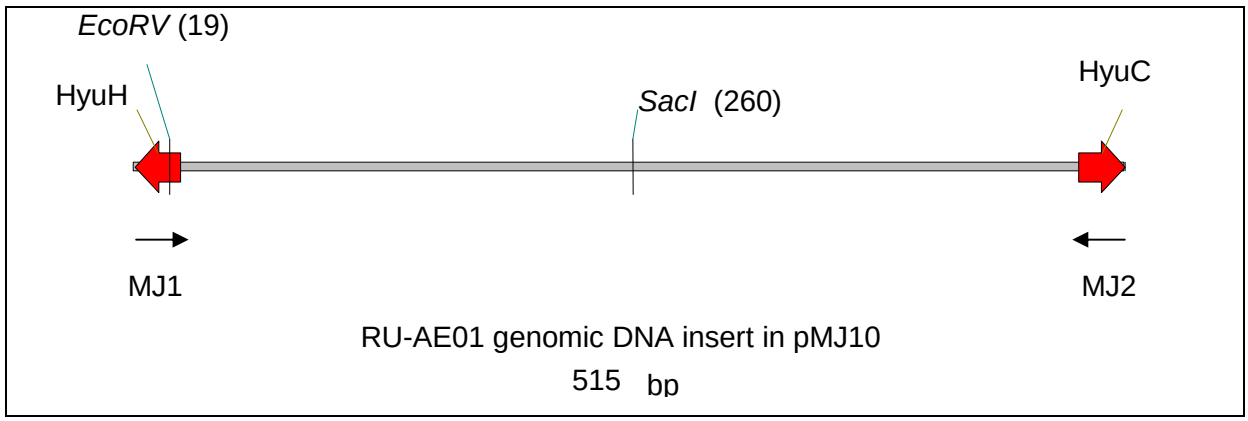
The availability of three classes of *A. tumefaciens* RU-OR mutants with altered HyuH and HyuC enzyme activity (Hartley *et al.* 1998, 2001) provide a resource to study the role of NtrC in the regulation of *hyu* gene expression. As the addition of a His_6-tag to NtrC affected the activity of the protein, anti-NtrC antibodies need to be produced. These anti-NtrC antibodies would allow the study of the role of wild-type NtrC in strain RU-OR and its mutants.

This project has developed the tools to study the regulation of *hyu* gene expression in a number of gram-negative bacterial strains. Understanding the regulation of the *hyu* expression at the gene level is a valuable tool for the use of *A. tumefaciens* strains RU-AE01 and RU-OR as industrial organisms for the production of optically pure amino acids. The knowledge about *hyu* gene regulation also explains some of the limitations experienced with applying *Agrobacteria* to large-scale fermentations. However it allows for the modification of the present methods, optimizing them for improved biocatalytic conversion. Finally, the analysis of the *hyu* gene regulation in *A. tumefaciens* strains RU-AE01 and RU-OR has resulted in the study of nitrogen regulation within the *Agrobacteria*, elucidating some of the mechanisms of nitrogen regulation within the genus and highlighting the diversity that exists between species of the same genus.

APPENDICES

Appendix 1: DNA sequences used or generated in this study

1-1: DNA sequence of the *A. tumefaciens* RU-AE01 insert in pMJ10 (Chapter 2)




```
                        EcoRV
            HyuH        ~~~~~~~
 1    TCCGTTCTTG ATGATGATAT CCATAAAGCA GCTCTCAGGG TTGATGGATA
      AGGCAAGAAC TACTACTATA GGTATTTCGT CGAGAGTCCC AACTACCTAT

51    AATTCTATAT GCGGTATGAT GTTCTTTATA TAAAGTTTTC ATGTTGCCTT
      TTAAGATATA CGCCATACTA CAAGAAATAT ATTTCAAAAG TACAACGGAA

101   GTATCTGTCA AGCGGGAAGG GAAGTTCTCC GGAATCGGCG CTGCGAGGGA
      CATAGACAGT TCGCCCTTCC CTTCAAGAGG CCTTAGCCGC GACGCTCCCT

151   ACGTATCGAG TTTCGATTAG ACGCGGTTGA AAGCGAGCGG TCATTGAATA
      TGCATAGCTC AAAGCTAATC TGCGCCAACT TTCGCTCGCC AGTAACTTAT

201   CGGAACCTCT GCCAACCCTA TTCGGCGAGC TGGGATTTTT TTCTTCTCGT
      GCCTTGGAGA CGGTTGGGAT AAGCCGCTCG ACCCTAAAAA AAGAAGAGCA

           SacI
          ~~~~~~
251   TCGCGAGCTC CTAAAACGGC GCCGTTCAAT CCGGGTGAAA AAGTTCAACC
      AGCGCTCGAG GATTTTGCCG CGGCAAGTTA GGCCCACTTT TTCAAGTTGG

301   ATCGGAAATT TTGACCCTGG TCCTTGACAG ATCAAAAGTT TTACGCCTGT
      TAGCCTTTAA AACTGGGACC AGGAACTGTC TAGTTTTCAA AATGCGGACA

351   AGTATGAGTA CTGCATGTGG CATTTATCCT TTTTGTAGAA CAATCATTGG
      TCATACTCAT GACGTACACC GTAAATAGGA AAAACATCTT GTTAGTAACC

401   CGTGCCAAGC TGAGACGTGT GTTCCTGAAA TGTGCATAGC AGCGTTCTCC
      GCACGGTTCG ACTCTGCACA CAAGGACTTT ACACGTATCG TCGCAAGAGG

451   CGGCCGCGAG GCCGGATTAA CTATCGAAGG AGCAAAGGTT CATGACACGT
      GCCGGCGCTC CGGCCTAATT GATAGCTTCC TCGTTTCCAA GTACTGTGCA

501   CAGATGATAC TTGCT
      GTCTACTATG AACGA
       HyuC
```

1-2: *A. tumefaciens* C58 *rho*-independent transcriptional terminator sequences used to generate the consensus sequence for the terminator oligonucleotide (MJ28/MJ29) (Chapter 2)

	Sequence	Gene No	Gene Name
1	aaaaaaaa ccggcgtcacgcgccgg tcttaatt	Atu 2277	Conserved hypothetical protein
2	acgaaaaa ggcggccgattggccgcc tttttata	Atu 2303	Conserved hypothetical protein
3	taacaaaa gggcggcctgagccgccc ttaaacca	Atu 2445	RNA polymerase sigma-32 factor
4	taaaaaaa gcaccggtcgcccggtgc tttgtgct	Atu 2552	nodulation protein T precursor
5	aataaaaa ggcgcccaaaggcgcc tttttgca	Atu 2669	RhtB family transporter
6	acaaaaaa ccccggagcgatccgggg tttttctt	Atu 2709	3-isopropylmalate dehydratase, large subunit
7	acgaaaaa ggcggccttggccgcc ttttttat	Atu 2303	Conserved hypothetical protein
8	acgaaaaa ggggcctttcggcccc tttctgtt	Atu 0171	ABC transporter, membrane spanning protein [phosphonate]
9	taaaaaaa gcccggttttaccgggc ttttttgt	Atu 0826	conserved hypothetical protein
10	gaaaaaaa gcggccttcgggccgc ttttttat	Atu 0324	chromosomal replication initiator protein DnaA
11	agaaaaaa gcgggttaccgc gcaactgt	Atu 0253	conserved hypothetical protein
12	aaaaaaaa ccctccgtcaggaggg ctgcaata	Atu 0051	two component sensor kinase
13	aataaaaa cccgggtgaagaacccggg ttttgttt	Atu 0005	DNA polymerase III, epsilon chain
14	ataaaaaa gcccggcacatggccgggc ttttgtcg	Atu 0148	conserved hypothetical protein
15	taaaaaaa gcgggatgtggccccgc ttttgatt	Atu 0294	bacitracin resistance protein
16	aatgtcca gccggtccatcgaccggc ttttttat	Atu 0916	transcriptional regulator
17	atgaaaaa gggggctgaaagccccc tttttcat	Atu 2174	conserved hypothetical protein
18	agtaaaaa gaaagggccggtaaaaaccggcccctcc aaaatcat	Atu 2043	serine protease DO-like precursor
19	attgggaa cgggccttcgggcccg tttttgtt	Atu 1616	fumarate hydratase
20	acgagaaa ggcggcgaaagccgcc ttttttgc	Atu 1550	polypeptide deformylase

Transcriptional terminator sequences were acquired from The Institute for Genomic Research transcriptional terminator database

(http://www.tigr.org/software/TransTermResults/ntat01.html). The genes to which the transcriptional terminators belonged were identified by comparing the terminator sequence against the *Agrobacterium tumefaciens* C58 genomic DNA sequence at (http://cancer.lbi.ic.unicamp.br/agroC58/).

1-3: *Agrobacterium* spp. *hyuH-hyuC* sequences used for the bioinformatic analysis (Chapter 4)

The AUG (start codon) for both the *hyuH* and the *hyuC* are highlighed, except in the case of RU-OR *hyuC1*, which was a PCR amplification product (Hartley 2001).

***Agrobacterium* sp. IP I-671**

CATGATGCAG CTCTACGGTC GGAGACAAAA TTCTATATAA AGAACTATAT TCTTTATAGA
AAGCTTGCAT GATGCTTTAA AACTGTCAAG CGGACTTAGC GCCGGATGTG GGGCCTTTGA
TGAGTGGGCG TGTCGAGACG TCGCGGACAA TTTCAGGCGC GCGGATCGAA TGCTCGCATT
TGCTAAAAAG ATAGACAGAT TGCGTGATTT TTCAGCAGTT ATCGCGATTG CCTCGCCAAG
GGGTCGTGTT GCTGTTTCAC GACGGAATCC TTTCATGGCG TAATTTTTAC ATCCGCGACC
TGTTGACAGA TCCGCAGTTT TAGTCCCGTT ATCTTCGTGC TGCATGCAAT ATCAAATTCT
TTATGCAGAA TAACGATTGA CAAATAGCCG CCACGCGGGT TCCGGGAAAG AGCACCGCAG
CATTCTCCCG GCCGCTGAGC CGGACATCGC GCATTTGAAG GAGCATAGGT TC**ATG**

***A. tumefaciens* NRRL B11291**

CATAAAGCAG CTCTCAGGGT TGATGGATAA ATTCTATATG CGGTATGATG TTCTTTATAT
AAAGTTTTCA TGTTGCCTTG TATCTGTCAA GCGGGAAGGG AAGTTCTCCG GAATCGGCGC
TGCGAGGGAA CGTATCGAGT TTCGATTAGA CGCGGTTGAA AGCGCAGCGA GTCATTGAAC
TACGGAACCT CTGCCAAACC CTATTCGGCG AGCTGGA**ATG**

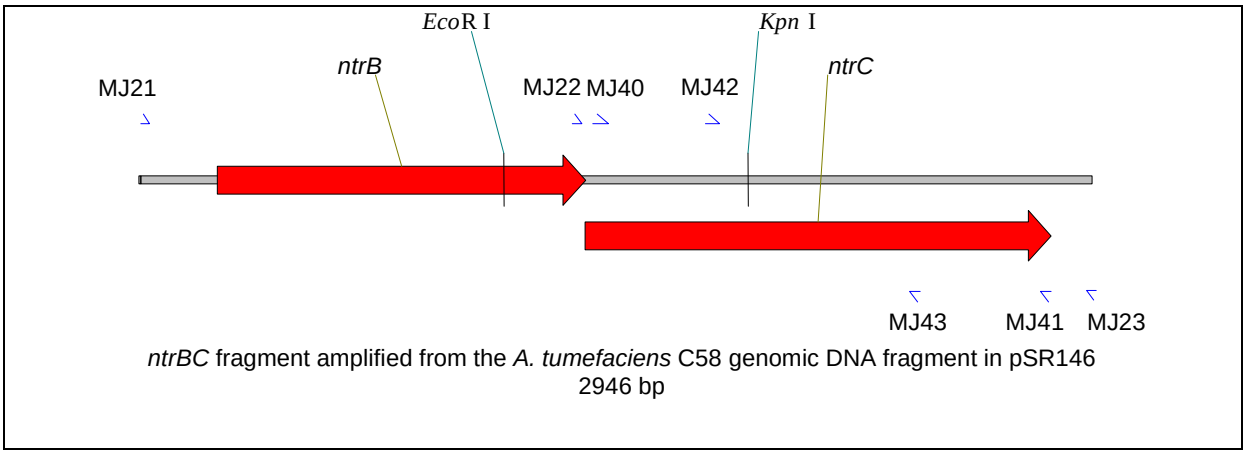
***A. tumefaciens* RU-AE01**

CATAAAGCAG CTCTCAGGGT TGATAGATAA ATTCTATATG CGGTATGATG TTCTTTATAT
AAAGTTTTCA TGTTGCCTTG TATCTGTCAA GCGGGAAGGG AAGTTCTCCG GAATCGGCGC
TGCGAGGGAA CGTATCGAGT TTCGATTAGA CGCGGTTGAA AGCGAGCGGT CATTGAATAC
GGAACCTCTG CCAACCCTAT TCGGCGAGCT GGATTTTTTC TTCTCGTTCG CGAGCTCCTA
AAACGGCGCC GTTCAATCCG GGTGAAAAAG TTCAACCATC GGAAATTTTG ACCCTGGTCC
TTGACAGATC AAAAGTTTTA CGCCTGTAGT ATGAGTACTG CATGTGGCAT TTATCCTTTT
TGTAGAACAA TCATTGGCGT GCCAAGCTGA GACGTGTGTT CCTGAAATGT GCATAGCAGC
GTTCTCCCGG CCGCGAGGCC GGATTAACTA TCGAAGGAGC AAAGGTTC**AT G**

A. tumefaciens* RU-OR *hyuC1

ACAGATCAAA AGTTTTACGC CTGTAGTATG AGTACTGCAT GTGGCATTTA TCCTTTTTGT
AGAACAATCA TTGGCGTGCC AAGCTGAGAC GTGTGTTCCT GAAATGTGCA TAGCAGCGTT
CTCCCGGCCG CGAGGCCGGA TTAACTATCG AAGGAGCAAA GGTTC**ATG**

1-4: *ntrBC* region amplified from pSR146 using the primers MJ21 and MJ23 (Chapter 5)



ntrBC fragment amplified from the *A. tumefaciens* C58 genomic DNA fragment in pSR146
2946 bp

DNA sequence of the primers used for the amplification and sequencing of the *ntrBC* region are shown in red, with the names of the primers above the highlighted sequence.

```
                    MJ21
  1  GCCGATATTC CCACTATCGC CGTTGAACAT TACGAGATGA TGCTGGAATT
     CGGCTATAAG GGTGATAGCG GCAACTTGTA ATGCTCTACT ACGACCTTAA

 51  TTATGGAAGG GAAGCCGGTC TGCGGCATGC CCGTAAACAT CTGGGCTGGT
     AATACCTTCC CTTCGGCCAG ACGCCGTACG GGCATTTGTA GACCCGACCA

101  ATCTCGACCG TTCCGCTCCC GACATCGCCA CGCCGGAAAA GGCTCAAATC
     TAGAGCTGGC AAGGCGAGGG CTGTAGCGGT GCGGCCTTTT CCGAGTTTAG

151  ATGACATCGC GTGACACGGG GGAGGTGGCG GATCTGCTTC GCTCCGCCCT
     TACTGTAGCG CACTGTGCCC CCTCCACCGC CTAGACGAAG CGAGGCGGGA

201  GAGCGAAAAT GCGGGCGAAG AAGCCGCAAG GAAGGCCGCA TGAGCGCCGA
     CTCGCTTTTA CGCCCGCTTC TTCGGCGTTC CTTCCGGCGT ACTCGCGGCT

251  TAAACACAGC CCGGCAGATG CGAACCAGCT CGCCATGGCG GTTCTGAACG
     ATTTGTGTCG GGCCGTCTAC GCTTGGTCGA GCGGTACCGC CAAGACTTGC

301  CAGTGCAGAA CCCGGTCATT CTGGTGGATG GCGAAGGTTT CATTTCATTC
     GTCACGTCTT GGGCCAGTAA GACCACCTAC CGCTTCCAAA GTAAAGTAAG

351  GCCAATTGGG AAGCCGAAGC CTTTTTCGGG GCGAGCGCCT CCCATCTTGC
     CGGTTAACCC TTCGGCTTCG GAAAAAGCCC CGCTCGCGGA GGGTAGAACG

401  CCGTTATCGC GTCTCCACTT TCATTCCCTT CGGCAGCCCG CTTCTGGCGC
     GGCAATAGCG CAGAGGTGAA AGTAAGGGAA GCCGTCGGGC GAAGACCGCG

451  TGATCGATCA GGTGCGGGAG CGCCGTGCGC CGGTCAACGA ATATCGCGTC
     ACTAGCTAGT CCACGCCCTC GCGGCACGCG GCCAGTTGCT TATAGCGCAG
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 501  GATCTGAGTT CGCCACGCCT CGGTCAGGAC AAGCTGGTTG ATCTCTACGT
      CTAGACTCAA GCGGTGCGGA GCCAGTCCTG TTCGACCAAC TAGAGATGCA

 551  TGCGCCCGTC GTCAGTGAAC CGGGGTCGGT TGTCGTCGTC TTCCAGGAAC
      ACGCGGGCAG CAGTCACTTG GCCCCAGCCA ACAGCAGCAG AAGGTCCTTG

 601  GGTCGATGGC CGACAAGATC GACCGTCAAC TGACCCATCG CGCCGCCGCC
      CCAGCTACCG GCTGTTCTAG CTGGCAGTTG ACTGGGTAGC GCGGCGGCGG

 651  CGCTCGGTTA CCGGCCTTGC CTCGATGCTG GCGCATGAGA TCAAGAATCC
      GCGAGCCAAT GGCCGGAACG GAGCTACGAC CGCGTACTCT AGTTCTTAGG

 701  GCTGTCCGGT ATTCGTGGCG CGGCCCAGTT GCTGGAAATG TCGGTGCCTG
      CGACAGGCCA TAAGCACCGC GCCGGGTCAA CGACCTTTAC AGCCACGGAC

 751  ATGAGGACCG CGCCCTTACC AGGCTTATCT GCGACGAGAC TGACCGCATC
      TACTCCTGGC GCGGGAATGG TCCGAATAGA CGCTGCTCTG ACTGGCGTAG

 801  GTGTCATTGG TCGATCGTAT GGAAATCTTT TCCGACGAGC GGCCGGTCGA
      CACAGTAACC AGCTAGCATA CCTTTAGAAA AGGCTGCTCG CCGGCCAGCT

 851  CCGCGTGCCG GTCAATATCC ACTCCGTGCT TGATCACGTG AAGGCGATTG
      GGCGCACGGC CAGTTATAGG TGAGGCACGA ACTAGTGCAC TTCCGCTAAC

 901  CCAAGGCCGG TTTTGCCCGC AACATCAAGA TATCGGAGAA TTATGATCCC
      GGTTCCGGCC AAAACGGGCG TTGTAGTTCT ATAGCCTCTT AATACTAGGG

 951  TCGCTGCCGG CGGTCTATGC CAATCGCGAT CAACTGGTGC AGGTCTTCCT
      AGCGACGGCC GCCAGATACG GTTAGCGCTA GTTGACCACG TCCAGAAGGA

1001  CAATCTGGTG AAGAATGCAG CCGAGGCAGT CGCCAATCAG CCGGATGGTG
      GTTAGACCAC TTCTTACGTC GGCTCCGTCA GCGGTTAGTC GGCCTACCAC

1051  AGGTTGTGCT GACGACCGCC TATCGCCCCG GTATTCGGCT TTCGGTTGCC
      TCCAACACGA CTGCTGGCGG ATAGCGGGGC CATAAGCCGA AAGCCAACGG

                                        EcoRI
                                        ~~~~~~
1101  GGTAGCCGCG AGCGCATCTC GCTGCCGCTT GAATTCTGCG TGCATGATAA
      CCATCGGCGC TCGCGTAGAG CGACGGCGAA CTTAAGACGC ACGTACTATT

1151  TGGACCGGGG GTCCCTTCCG ATCTTCTGCC GCATCTCTTC GATCCCTTCA
      ACCTGGCCCC CAGGGAAGGC TAGAAGACGG CGTAGAGAAG CTAGGGAAGT

1201  TCACCACCAA GACCAATGGT TCGGGCCTCG GCCTGGCGCT TGTCGCCAAG
      AGTGGTGGTT CTGGTTACCA AGCCCGGAGC CGGACCGCGA ACAGCGGTTC

1251  CTGATTGGCG CCCATGGCGG CATTGTCGAA TGCGACAGCC AGAACCACCG
      GACTAACCGC GGGTACCGCC GTAACAGCTT ACGCTGTCGG TCTTGGTGGC

1301  CACGACTTTC CGCGTATTGA TGCCCGTCTC GCCGGAAGTG GCGCTTGACG
      GTGCTGAAAG GCGCATAACT ACGGGCAGAG CGGCCTTCAC CGCGAACTGC

                    MJ22                              MJ40
1351  ACAGCTCTTT GCCGAACACG ACAGGAAATG ACAGATGACA GCTACGATCC
      TGTCGAGAAA CGGCTTGTGC TGTCCTTTAC TGTCTACTGT CGATGCTAGG
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1401  TCGTCGCCGA TGATGATGCC GCAATCCGCA CGGTGCTGAA CCAGGCGCTC
      AGCAGCGGCT ACTACTACGG CGTTAGGCGT GCCACGACTT GGTCCGCGAG

1451  AGCCGTGCCG GTTATGACGT GCGCATCACC TCCAATGCCG CAACGCTTTG
      TCGGCACGGC CAATACTGCA CGCGTAGTGG AGGTTACGGC GTTGCGAAAC

1501  GCGCTGGGTG TCGGCGGGTG AGGGCGATCT CGTCGTGACC GATGTTGTGA
      CGCGACCCAC AGCCGCCCAC TCCCGCTAGA GCAGCACTGG CTACAACACT

1551  TGCCGGATGA AAACGCCTTC GACCTTCTGC CGCGCATCAA GAAGGCCCGC
      ACGGCCTACT TTTGCGGAAG CTGGAAGACG GCGCGTAGTT CTTCCGGGCG

1601  CCGGACCTGC CGGTTCTCGT CATGAGCGCG CAGAACACCT TCATGACGGC
      GGCCTGGACG GCCAAGAGCA GTACTCGCGC GTCTTGTGGA AGTACTGCCG

1651  CATCAAGGCT TCGGAAAAGG GCGCTTATGA TTATCTGCCC AAGCCCTTCG
      GTAGTTCCGA AGCCTTTTCC CGCGAATACT AATAGACGGG TTCGGGAAGC

1701  ACCTGACGGA ACTGATCGCC ATCATCGGCC GCGCGCTCTC CGAGCCGAAG
      TGGACTGCCT TGACTAGCGG TAGTAGCCGG CGCGCGAGAG GCTCGGCTTC

                          MJ42
1751  CGCAAGCCCG CCAAGCTCGA TGACGACATG CAGGACGGCA TGCCGCTCGT
      GCGTTCGGGC GGTTCGAGCT ACTGCTGTAC GTCCTGCCGT ACGGCGAGCA

1801  CGGCCGCTCC GCAGCGATGC AGGAAATCTA CCGCGTTCTC GCGCGCCTGA
      GCCGGCGAGG CGTCGCTACG TCCTTTAGAT GGCGCAAGAG CGCGCGGACT

                                                   KpnI
                                                  ~~~~~~~
1851  TGCAGACCGA TCTGACGCTG ATGATCACGG GTGAATCCGG TACCGGCAAG
      ACGTCTGGCT AGACTGCGAC TACTAGTGCC CACTTAGGCC ATGGCCGTTC

1901  GAGCTGGTGG CGCGGGCGCT GCATGATTAC GGCAAGCGCC GCAACGGTCC
      CTCGACCACC GCGCCCGCGA CGTACTAATG CCGTTCGCGG CGTTGCCAGG

1951  CTTTGTCGCC ATCAATATGG CCGCCATCCC GCGCGACCTG ATCGAATCGG
      GAAACAGCGG TAGTTATACC GGCGGTAGGG CGCGCTGGAC TAGCTTAGCC

2001  AACTGTTCGG CCATGAGAAG GGCGCCTTCA CCGGCGCACA GAACCGTTCC
      TTGACAAGCC GGTACTCTTC CCGCGGAAGT GGCCGCGTGT CTTGGCAAGG

2051  ACCGGCCGTT TCGAGCAGGC CGAGGGTGGC ACACTGTTCC TAGATGAAAT
      TGGCCGGCAA AGCTCGTCCG GCTCCCACCG TGTGACAAGG ATCTACTTTA

2101  CGGCGACATG CCGATGGATG CCCAGACGCG TCTGCTGCGT GTGTTGCAGC
      GCCGCTGTAC GGCTACCTAC GGGTCTGCGC AGACGACGCA CACAACGTCG

2151  AGGGCGAATA TACGACCGTG GGCGGGCGCA CGCCGATCCG CACCGATGTC
      TCCCGCTTAT ATGCTGGCAC CCGCCCGCGT GCGGCTAGGC GTGGCTACAG

2201  CGCATCGTTG CCGCCACCAA CAAGGACCTG AAACAGTCGA TCAATCAGGG
      GCGTAGCAAC GGCGGTGGTT GTTCCTGGAC TTTGTCAGCT AGTTAGTCCC

2251  CCTCTTCCGC GAGGACCTTT ATTATCGCCT CAACGTCGTG CCGTTGCGCC
      GGAGAAGGCG CTCCTGGAAA TAATAGCGGA GTTGCAGCAC GGCAACGCGG
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2301  TGCCGCCGCT GCGCGATCGT GCCGAGGATA TTCCTGATCT CGTCCGCCAT
      ACGGCGGCGA CGCGCTAGCA CGGCTCCTAT AAGGACTAGA GCAGGCGGTA

2351  TTCATCCAGA CGGGTGAGAA AGAAGGGCTG GAGGGCAAGC GTTTCGAGAC
      AAGTAGGTCT GCCCACTCTT TCTTCCCGAC CTCCCGTTCG CAAAGCTCTG

2401  GGAGGCGCTG GAAGTCATGA AGGCCTATGC CTGGCCGGGC AACGTCCGCG
      CCTCCGCGAC CTTCAGTACT TCCGGATACG GACCGGCCCG TTGCAGGCGC
            MJ43

2451  AGTTGGAAAA CCTGATCCGC CGCCTGATGG CGCTTTATCC GCAGGAAGTC
      TCAACCTTTT GGACTAGGCG GCGGACTACC GCGAAATAGG CGTCCTTCAG

2501  ATCACCCGCG AAATCATCGA GCAGGAATTA CAGTCGGATG TTCCCGATAG
      TAGTGGGCGC TTTAGTAGCT CGTCCTTAAT GTCAGCCTAC AAGGGCTATC

2551  CCCGTTGGAC AAGATGGCGG TTCGCACCGG TTCGCTCACC ATTTCGCAGG
      GGGCAACCTG TTCTACCGCC AAGCGTGGCC AAGCGAGTGG TAAAGCGTCC

2601  CTGTCGAGGA GAACATGCGG GATTATTTCG CCAGCTTCGG CGATGGCCTG
      GACAGCTCCT CTTGTACGCC CTAATAAAGC GGTCGAAGCC GCTACCGGAC

2651  CCGCCGCCCG GCCTTTACGA CCGCGTGCTG CGCGAACTCG AATATCCGCT
      GGCGGCGGGC CGGAAATGCT GGCGCACGAC GCGCTTGAGC TTATAGGCGA

2701  GATTCTCGCG GCTCTGACGG CAACCCGCGG CAACCAGATC AAGGCCGCCG
      CTAAGAGCGC CGAGACTGCC GTTGGGCGCC GTTGGTCTAG TTCCGGCGGC

2751  ATCTTCTTGG CCTCAACCGC AATACGTTGC GCAAGAAAAT CCGCGAGCTC
      TAGAAGAACC GGAGTTGGCG TTATGCAACG CGTTCTTTTA GGCGCTCGAG

2801  GGCGTTTCCG TCTATCGTAG TTCCCGCCCC AGCTGACAAT GTTGACAAGG
      CCGCAAAGGC AGATAGCATC AAGGGCGGGG TCGACTGTTA CAACTGTTCC
                        MJ41

2851  CCACAGTTGT CGTTGCATTT TCGCCACATT GTGTTGCTTG GAAAACACTA
      GGTGTCAACA GCAACGTAAA AGCGGTGTAA CACAACGAAC CTTTTGTGAT

2901  GGAAGGATAT AGATTCGCCT GATGGGGGAG TCTTGCCTGA CTTCAG
      CCTTCCTATA TCTAAGCGGA CTACCCCCTC AGAACGGACT GAAGTC
                                  MJ23
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Appendix 2: Media for growth of *E. coli* and *A. tumefaciens* strains

2-1 Complete growth medium for *E. coli*

Luria-Bertani broth (1L) - 10 g tryptone, 5 g yeast extract and 5 g NaCl. For Luria-Bertani agar add 20 g bacteriological agar.

Luria-Bertani broth + 1% hydantoin (1L) - 10 g tryptone, 5 g yeast extract, 5 g NaCl and 10 g hydantoin. For Luria-Bertani agar add 20 g bacteriological agar.

2-2 Minimal growth medium for *E. coli*

A basic minimal medium recipe for *E. coli* Glucose Minimal Medium (GMM) was adapted from Sambrook *et al.* (1989) as follows-

Stock solutions	Volume used in 1 L medium	Final concentration
Na_2HPO_4	6 g	0.6%
KH_2PO_4	3 g	0.3%
NaCl	0.5 g	0.05%
40% Glucose ♣	25 mL	1%
1 M $MgSO_4$	1 mL	1 mM
1 M $CaCl_2$	130 µL	130 µM
1% Thiamine HCl	1 mL	0.001%
Agar (for plates)	20 g	2%
H_2O	972 mL	---

♣ 40% glucose can be replaced by lactose, glycerol or mannitol as required producing *E. coli* Lactose Minimal Medium (LMM), *E. coli* Glycerol Minimal Medium (GlyMM) or *E. coli* Mannitol Minimal Medium (MMM).

All the components are autoclaved separately (20 minutes at 15 psi pressure at 121 °C).

Appropriate amounts of nitrogen sources were added to the medium as required. Some of the commonly used nitrogen sources are shown below-

Medium	Nitrogen source	Stock solution	Volume used in 1 L medium	Final concentration
0.02% CAAMM	Casamino acids	5%	4 mL	0.02%
0.5% CAAMM	Casamino acids	5%	100 mL	0.5%
AmmMM	$(NH_4)_2SO_4$	20%	5 mL	0.1%

An appropriate amount of inducer was added to the medium as required. The commonly used induction conditions are shown below-

Inducer	Stock solution	Volume used in 1 L medium	Final concentration
Hydantoin	1%	100 mL	0.1%
IPTG	0.84 M	1.2 mL	1 mM

For solid medium, add 20 g bacteriological agar to the desired minimal medium resulting in a final concentration of 2% agar.

2- 3 Complete growth medium for *A. tumefaciens*

Luria-Bertani broth + 1% hydantoin (1L) - 10 g tryptone, 5 g yeast extract, 5 g NaCl and 10 g hydantoin. For Luria-Bertani agar add 20 g bacteriological agar.

2-4 Minimal growth medium for *A. tumefaciens*

A basic minimal medium recipe for *A. tumefaciens* Glucose Minimal Medium (GMM) was adapted from Sambrook *et al.* (1989) as follows-

Stock solutions	Volume used in 1 L medium	Final concentration
10 X M9 salts [a]	100 mL	1 X
40% Glucose ♣	25 mL	1%
Trace Elements [b]	10 mL	---
1 M $MgSO_4$	500 µL	500 µM
1 M $CaCl_2$	500 µL	500 µM
Agar (for plates)	20 g	2%
H_2O	615 mL	---

[a] 10 X M9 salts (1L) - 60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl.

♣ 40% glucose can be replaced by lactose, glycerol or mannitol as required generating *A. tumefaciens* Lactose Minimal Medium (LMM), *A. tumefaciens* Glycerol Minimal Medium (GlyMM) or *A. tumefaciens* Mannitol Minimal Medium (MMM).

[b] Trace elements (1L) - 50 mg boric acid, 40 mg $MnSO_4.2H_2O$, 40 mg $ZnSO_4$, 20 mg $(NH_4)_6Mo_2O_{24}.4H_20$), 10 mg KI, 4 mg $CuSO_4$ and 20 mg $FeCl_3$ (autoclave $FeCl_3$ separately from the rest of the trace elements and add when the solution is cool).

All the components are autoclaved separately (20 minutes at 15 psi pressure at 121 °C).

Appropriate amounts of nitrogen sources were added to the medium as required. Some of the commonly used nitrogen sources are shown below-

Medium	Nitrogen source	Stock solution	Volume used in 1 L medium	Final concentration
0.02% CAAMM	Casamino acids	5%	4 mL	0.02%
0.5% CAAMM	Casamino acids	5%	100 mL	0.5%
HMM	Hydantoin	4%	250 mL	1%
AmmMM	$(NH_4)_2SO_4$	20%	5 mL	0.1%

An appropriate amount of inducer was added to the medium as required. The commonly used inducer conditions are shown below-

Inducer	Stock solution	Volume used in 1 L medium	Final concentration
IPTG	0.84 M	1.2 mL	1 mM

For solid medium, add 20 g bacteriological agar to the desired minimal medium resulting in a final concentration of 2% agar.

Appendix 3: SDS-PAGE and Western analysis buffers

Reagent	Recipe
12.5% Resolving Gel (10 mL)	3.13 mL 40% acrylamide, 0.5 mL 2% bisacrylamide, 3.75 mL 1M Tris HCl pH 8.8, 0.1 mL 10% SDS, 0.04 mL 10% APS, 2.47 mL H_20, 0.01 mL TEMED
4% Stacking Gel (5 mL)	0.5 mL 40% acrylamide, 0.35 mL 2% bisacrylamide, 1.25 mL 1M Tris HCl pH 6.8, 0.05 mL 10% SDS, 0.025 mL 10% APS, 2.8 mL H_20, 0.05 mL TEMED
1 X Q buffer (1L)	28.8 g glycine, 6 g Tris-HCl pH 8.3, 2 g SDS
5 X Sample Buffer (10 mL)	0.6 ml 1M Tris HCl pH 6.8, 5 mL 50% glycerol, 2 mL 10% SDS, 0.5 mL β-mercaptoethanol, 1 mL 1% bromophenol blue, 0.9 mL H_20
Transfer Buffer (1L)	3.03 g Tris HCl, 14.4 g glycine, 200 mL methanol
TBS-Tween (1L)	100 mL 1M Tris HCl pH 7.5, 30 mL 5 M NaCl, 1 mL Tween-20
Coomassie Brilliant Blue Stain (200 mL)	0.5 g R250 Coomassie Brillliant Blue in 200 mL Destain I. Mix overnight and then filter.
Destain I (1 L)	45% methanol, 10% glacial acetic acid
Destain II (1 L)	5% methanol, 7% glacial acetic acid
Destain III (1 L)	5% methanol, 7% acetic acid, 3% glycerol
10 X Ponceau Stain (100 mL)	2 g Ponceau S, 30 g Tricarboxylic acid, 30 g sulphosalicylic acid

Appendix 4: Standard procedure for culturing, harvesting and conducting resting cell biocatalytic reactions for the analysis of hydantoinase and NCAAH enzyme activities in *Agrobacterium* strains

4-1 Culture conditions

A single colony of *A. tumefaciens* was inoculated from a HMM agar plate into 50 mL of HMM. The cells were grown with shaking at 200 rpm at 30 °C to stationary phase (OD_{600nm} of 3.0).

Once grown to confluence, the starter culture was used to inoculate the required medium to a starting OD_{600nm} of 0.2. These flasks were grown with shaking at 200 rpm at 28 °C to the desired OD_{600nm}. CAAMM grew to early stationary phase (OD_{600nm} of 0.8 – 1.0) in approximately 36 hours and HMM grew to early stationary phase (OD_{600nm} of 2.5 – 3.0) in approximately 72 hours.

4-2 Harvesting conditions

Cells were collected by centrifugation at 5 000 rpm for 10 minutes at 10 °C in a Beckman centrifuge in a JA14 rotor. The supernatant was discarded and the cells were washed in a half volume of 0.1 M potassium phosphate buffer (pH 9.0). The cells were then collected by centrifugation as above.

After centrifugation, the wet cell mass of the pellet was determined and used to resuspend the cells to 40 mg wet cell mass per mL in 0.1 M phosphate buffer (pH 9.0). This cell suspension was used for the resting cell biocatalytic reactions.

4-3 Resting cell biocatalytic reactions

The cell suspension from above was used to set up resting cell biocatalytic reactions as described below.

Reaction	Contents
Buffer blank	2 ml 0.1 M phosphate buffer (pH 9.0)
Substrate blank	1 ml 0.1M phosphate buffer (pH 9.0) + 1 ml substrate (100 mM hydantoin / 50 mM *N*-carbamyl glycine dissolved in 0.1 M phosphate buffer (pH 9.0))
Cell blank	1 ml 0.1M phosphate buffer (pH 9.0) + 1 ml cells (40 mg wet cell mass per mL of 0.1 M phosphate buffer (pH 9.0))
Samples in triplicate	1 ml substrate (100 mM hydantoin / 50 mM *N*-carbamyl glycine dissolved in 0.1 M phosphate buffer (pH 9.0)) + 1 ml cells (40 mg wet cell mass per mL of 0.1 M phosphate buffer (pH 9.0))

The reaction mixes were incubated with constant shaking at 40 °C for 6 hours. A 1.5 mL volume of the reaction mix was then centrifuged at 13 000 rpm for 3 minutes to pellet the cells. The supernatant was analysed for *N*-carbamyl amino acids and amino acids using the Ehrlich's and Ninhydrin colorimetric assays respectively.

Activity was measured as µmol/mL of product for 20 mg wet cell mass per mL. Activity was expressed as an average of 3 replicas. Hydantoinase activity was represented as the total µmol/mL of *N*-carbamyl amino acid and the total µmol/mL of amino acid produced from hydantoin as a substrate. *N*-carbamyl amino acid was represented as the total µmol/mL of amino acid produced from *N*-carbamyl amino acid as a substrate.

Appendix 5: Analytical Methods

5-1 Ehrlich's assay for the detection of *N*-carbamyl amino acids

Protocol was derived from Morin *et al.* (1986). The presence of *N*-carbamyl amino acids was quantified using the Ehrlich's assay as described below.

Each sample was prepared as follows-

0.5 mL 12% Trichloroacetic acid

1 mL reaction supernatant

0.5 mL Ehrlich's reagent (10% dimethylaminobenzaldehyde in 6 M HCl)

3 mL H_2O

The tubes were vortexed, allowed to react for 20 minutes and then read at 420 nm and compared against a standard curve of 0 – 50 mM *N*-carbamylglycine. Fresh standard curves were done for each assay. A sample of typical standards, their absorbances and a standard curve is shown in Table A1 and Figure A1. All reactions were done in triplicate and the standard deviation calculated.

Table A1: Typical values obtained for a 0 – 50 mM *N*-carbamylglycine standard curve after the Ehrlich's reaction.

Standard (mM)	0.1 M phosphate buffer (mL)	100 mM *N*-carbamyl glycine in 0.1 M phosphate buffer	A_{420nm} after the Ehrlich's reaction
0	1.0	0	0
10	0.9	0.1	0.18
20	0.8	0.2	0.326
30	0.7	0.3	0.514
40	0.6	0.4	0.629
50	0.5	0.5	0.8

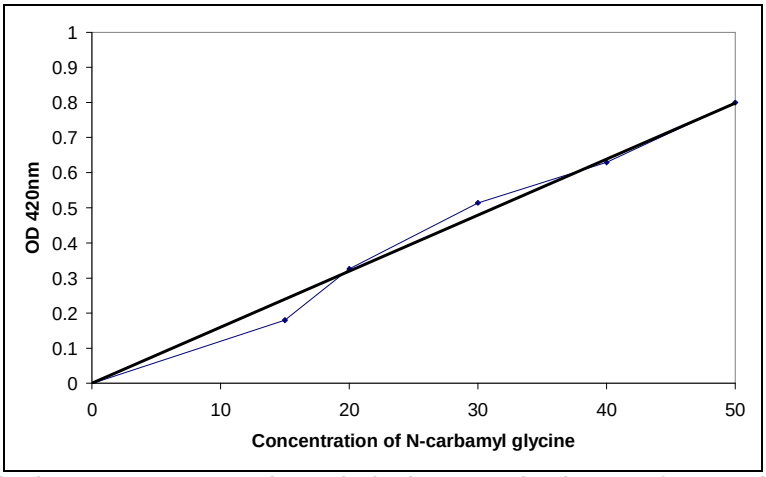

Figure A1: A typical 0 – 50 mM *N*-carbamyl glycine standard curve for an Ehrlich's reaction.

5-2 Ninhydrin assay for the detection of amino acids

Protocol was derived from Plummer (1987). The presence of amino acids was quantified using the Ninhydrin colorimetric assay as described below.

Each sample was prepared as follows:

0.020 mL reaction supernatant

0.998 mL 0.1 M phosphate buffer

1 mL Ninhydrin reagent (0.8 g ninhydrin, 0.12 g hydrindantin, 30 mL methoxyethanol, 10 mL 4 M sodium acetate buffer (pH 5.5))

The tubes were capped and boiled for 15 minutes. When cool, 3 mL 50% ethanol was added and the tubes were read at 595 nm and compared against a standard curve of 0 – 0.5 mM glycine. Fresh standard curves were done for each assay. A sample of typical standards, their absorbances and a standard curve is shown in Table A2 and Figure A2. All reactions were done in triplicate and the standard deviation calculated.

Table A2: Typical values obtained for a 0 – 0.5 mM glycine standard curve after the Ninhydrin reaction.

Standard (mM)	0.1 M phosphate buffer (mL)	1 mM glycine in 0.1 M phosphate buffer	$A_{595\ nm}$ after the Ninhydrin reaction
0	1.0	0	0
0.1	0.9	0.1	0.254
0.2	0.8	0.2	0.455
0.3	0.7	0.3	0.653
0.4	0.6	0.4	0.835
0.5	0.5	0.5	1.061

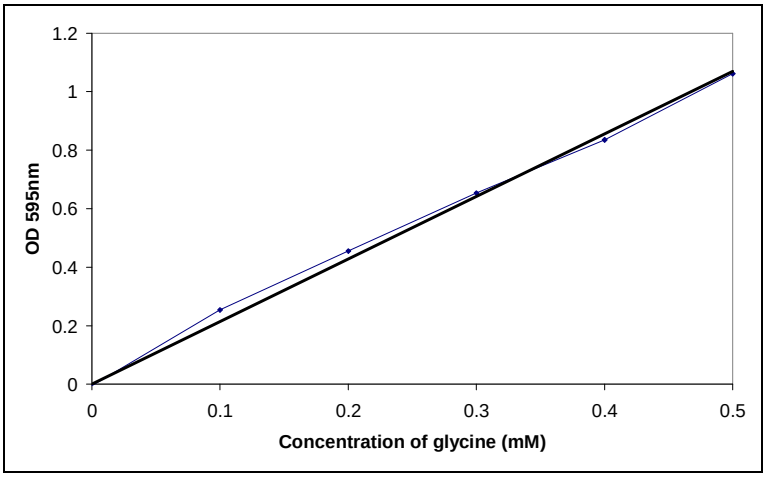

Figure A2: A typical 0 – 0.5 mM glycine standard curve for a Ninhydrin reaction.

5-3 β-glucuronidase (GUS) assay

Protocol was developed from Jefferson *et al.* (1986). Cells were grown to the desired $OD_{600\ nm}$ in the required medium. A total of 20 μL of cells was aliquoted into a microtitre plate. A total of 50 μL of permeabilisation buffer (32 mM $NaPO_4$, 2 mM EDTA (pH 8), 100 mM Tris HCl (pH 8), 5 mM DTT, 4% Triton X-100 and 0.4 mg/mL lysozyme) was added to the cells and the microtitre plate was incubated at 37 °C for 30 minutes. A 50 μL volume of substrate solution (20mM PNPG dissolved in GUS buffer stock solution (50 mM $NaPO_4$ buffer (pH 7), 1 mM EDTA (pH 8))) was added to each well. The microtitre plate was incubated at 37 °C until adequate colour development occurred. The $OD_{600\ nm}$, $A_{405\ nm}$ and $A_{550\ nm}$ were measured in a microtitre plate reader and the lapsed time for the colour development noted.

Activity was calculated as –

$$\frac{1000 \times (A_{405nm} - (1.75 \times A_{550nm}))}{A_{600nm} \times (Time_{final} - Time_{initial}) \times Volume\ (mL)}$$

Triplicate samples were averaged and the standard deviation calculated.

5-4 β-galactosidase assay

Protocol was developed from Miller (1992). Cells were grown to the desired $OD_{600\ nm}$ in the required medium. A total of 200 μL of cells was aliquoted into a test tube and 800 μL Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.03 M β-mercaptoethanol (add β-mercaptoethanol just before use)) was added to the cells. One drop of 0.1% SDS and two drops of chloroform were added to each test tube and the test tube vortexed vigorously for 10 seconds. The reaction mixes were pre-warmed in a water bath to 30 °C and 200 μL of substrate solution (4 mg/mL ONPG dissolved in Z buffer) was added. When sufficient yellow colour had developed, the reaction was stopped with 0.5 mL 1 M Na_2CO_3 and the lapsed time for the colour development noted. The $OD_{600\ nm}$, $A_{420\ nm}$ and $A_{550\ nm}$ were measured in a microtitre plate reader.

Activity was calculated as –

$$\frac{1000 \text{ X } (A_{420nm} - (1.75 \text{ X } A_{550nm}))}{A_{600nm} \text{ X } (Time_{final} - Time_{initial}) \text{ X Volume (mL)}}$$

Triplicate samples were averaged and the standard deviation calculated.

Appendix 6: General protocols for manipulation of DNA

6-1 Preparation and analysis of plasmid DNA from *E. coli* cells

E. coli cells containing plasmids were grown overnight at 200 rpm at 37 °C in 5 mL LB broth supplemented with the appropriate antibiotic. Plasmid DNA was extracted from these confluent cultures using the 'Easyprep' method described by Berghammer and Auer (1993).

The constructs were screened using restriction endonuclease digestion and electrophoretic separation on an agarose gel (0.7 – 2%) containing 10 μg/mL of ethidium bromide. The gel was made with 1 X TAE and run in 1 X TAE (50 X TAE (1L) – 242 g Tris HCl, 57.1 mL glacial acetic acid, 100 mL 0.5 M Na_2EDTA (pH 8.0)). The DNA was visualised under UV light and images were captured on a Kodak DC 120 Gel Imaging System.

High quality DNA was isolated from cells containing the desired constructs using the Roche High Pure Plasmid DNA Isolation Kit or the Bio-Rad Quantum Prep Plasmid Midiprep Kit according to the manufacturer's instructions. This DNA was used for further restriction analysis, manipulation and DNA sequencing.

6-2 Genomic DNA and plasmid DNA extraction from *A. tumefaciens* cells

Genomic and large plasmid DNA was extracted using the detergent lysis / CTAB method as described by Ausubel *et al*. (1983). The DNA was treated with RNaseA to degrade the contaminating RNA and the DNA was precipitated as described by Ausubel *et al*. (1983). The precipitated DNA was dried by vacuum centrifugation (Speedvac concentrator, Savant) and resuspended in 100 μL $dddH_20$. The DNA was quantified by comparison to known concentrations of Lambda DNA on a 0.8% agarose gel containing 10 μg/mL of ethidium bromide. The DNA was visualised under UV light and images were captured on a Kodak DC 120 Gel Imaging System.

6-3 DNA sequencing

The nucleotide sequence of double stranded DNA was determined by the dideoxy-chain termination method (Sanger *et al.* 1977) using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (PE Applied

Biosystems). The thermal cycling reactions were performed according to the manufacturer's instructions for the GeneAmp PCR System 9700 Thermal Cycler.

The PCR products were purified using the DNA Clean and Concentrator Columns (Zymo) according to the manufacturer's instructions. The samples were dried by vacuum centrifugation (Speedvac concentrator, Savant) and submitted for sequencing on an ABI Prism 3100 Genetic Analyser (Hitachi Applied Biosystems) at the Stellenbosch University or Rhodes University Sequencing Facilities.

6-4 PCR amplification

Three different polymerases were used during the course of this dissertation- Taq DNA polymerase (Bioline), Expand Hi-Fidelity PCR System (Roche) and Vent^{TM} DNA polymerase (New England Biolabs). All the enzymes were used according to the manufacturer's instructions unless otherwise stated. The general reaction conditions are shown below.

Step	Reaction step	Temperature	Time	Number of cycles
1	Initial denaturation	95 °C	2 minutes	1 cycle
2	Denaturation	95 °C	1 minute	30 cycles
	Annealing	X °C	1 minute	
	Extension	72 °C	3 minutes	
3	Final Extension	72 °C	2 minutes	1 cycle

The annealing temperature (X °C) varied depending on the primers and the reaction requirements and is stated where necessary.

Appendix 7: Preparation and transformation of *E. coli* and *A. tumefaciens* competent cells.

7-1 Preparation and transformation of competent *E. coli* cells

This protocol was developed from Hanahan (1983).

A 5 mL volume of LB broth was inoculated with the required *E. coli* strain and grown with shaking to confluence at 200 rpm at 37 °C overnight. Four Erlenmeyer flasks, each containing 100 mL of LB broth, were inoculated with 1.5, 1.0, 0.7 and 0.3 mL of the overnight culture respectively. These four Erlenmeyer flasks were incubated with shaking at 200 rpm at 37 °C until an OD_{600nm} of 0.6-0.8 was obtained for the flask inoculated with 1.5 mL of the overnight culture (approximately 2 hours). The flasks were cooled on ice for 10 minutes. All the flasks were processed separately until the final step. The cells were collected by centrifuging in a Beckman centrifuge with a JA14 rotor at 5000 rpm for 10 minutes at 4 °C. The supernatant was discarded and each pellet was resuspended in 50 mL of RF1 (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15% glycerol (pH 5.8)) followed by a 20 minute incubation on ice. The cells were collected by centrifugation as above. The supernatant was discarded and each pellet was suspended in 1 mL of RF2 (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15% glycerol (pH 6.8)). The resuspended cells were pooled and frozen in 200 µL aliquots at -80 °C until needed.

When required, the 200 µL aliquots of *E. coli* cells were thawed on ice. A 150 µL volume of *E. coli* cells was mixed with plasmid DNA (1 to 20 ng) and incubated on ice for 20 minutes. The cells were then subjected to a heat shock at 42 °C for 45 seconds and then immediately placed on ice for a further 10 minute incubation. A volume of 1 mL LB broth was added to the transformation reaction and the cells were incubated at 37 °C for 1 hour after which the cells were plated onto the desired selective medium and incubated at 37 °C until colonies developed.

7-2 Preparation and transformation of competent *A. tumefaciens* cells

This protocol was developed from Shaw (1995).

Agrobacterium tumefaciens cells were grown with shaking in LB broth at 200 rpm at 28 °C overnight. A total of 3.0 mL of the overnight culture was collected by centrifugation at 13 000 rpm at room temperature. The pellet was resuspended in 0.5 mL ice cold 1 mM HEPES / KOH (pH 7.0) and recentrifuged at 13 000 rpm at room temperature. This was repeated twice.

The pellet was resuspended in 0.5 mL ice cold 10% glycerol (filter-sterilized) and spun as above. Each pellet was resuspended in 20 μL ice cold 10% glycerol and two resuspended pellets were combined to make a total volume of 40 μL which was used for each electroporation. Plasmid DNA (0.5 to 1.0 μg) was added to the competent cells and the cells were incubated on ice for 2 minutes.

The DNA-bacteria mixture was pulsed at 2500 V in a warm cuvette (0.2 cm or 1 cm with an average time constant of 4 - 9 ms). A volume of 1 mL of LB broth was added to the electroporation reaction immediately. The electroporated cells were incubated at 28 °C for 4 to 6 hours, then plated on selective medium and incubated at 28 °C until colonies developed.

Appendix 8: Bacterial strains used in this study

8-1: *Escherichia coli* strains used in this study

Strain	Description	Reference
BL21 (DE3)	*F dcm ompT hsdS*($r_B^- m_B^-$) *gal*λ[DE3]	Studier and Moffat 1986
DH5α	*F*$^-$*Φ80dlacZΔM15*Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* ($r_k^- m_k^-$) *deoR thi-1 supE44* λ$^-$*gyrA96 relA1*	Hanahan 1983
K-12 MG1655	F- lambda- *ilvG- rfb*-50 *rph*-1	Blattner *et al*. 1997
XLOLR	Δ(*mcrA*)183Δ(*mcrCB-hsdSMR-mmr*)173 *endA1 thi*-1 *recA1 gyrA96 relA1 lac*[F'*proAB lacI*^{q}*Z*ΔM15 Tn10(Tet^r)] Su$^-$ (non-supressing) λ^r (lambda resistant)	ZAP Express Predigested Gigapack Cloning Kit – Instruction manual
XL1 Blue MRF'	Δ(*mcrA*)183Δ(*mcrCB-hsdSMR-mmr*)173 *endA1 supE44 thi*-1 *recA1 gyrA96 relA1 lac*[F'*proAB lacI*^{q}*Z*ΔM15 Tn10(Tet^r)]	ZAP Express Predigested Gigapack Cloning Kit – Instruction manual

8-2: *Agrobacterium tumefaciens* strains used in this study

Strain	Description	Reference
RU-AE01	Environmental isolate from hydantoin-rich strip gas liquor, South Africa	AECI Ltd, This study
RU-OR	Soil isolate from Hogsback, South Africa	Hartley 1995

Appendix 9: Plasmids used or generated in the study

9-1: Plasmids used in this study

Plasmid	Description	Reference
pAV14 HP Trp GUS	*gus* containing plasmid	Venter unpublished
pBK-CMV	ZAP Express vector	Stratagene
pBR325	Cloning vector	Bolivar 1978
pG4	1.2 kb *hyuC1* from RU-OR in pGEM-T-Easy (Chao F1 and Chao R1)	Hartley 2001
pGH45	2.4 kb genomic DNA from RU-AE01 in pBK-CMV	Herrera unpublished
pGEM-T-Easy	Cloning vector	Promega
pHP41	Reporter expression vector	Park *et al.* 1992
pIVEX 2.3	Expression vector	Roche
pRD109	NtrC-expression (with an amino-terminal His_6-tag) from pCW21 (a pT7-7 derivative)	Dorrington unpublished
pSR146	~ 20 kb of C58 genomic DNA in pLAFRI	Rossbach *et al.* 1987
pTnmodOCm	Plasposon	Dennis and Zylsta 1998
pTnmodOTc	Plasposon	Dennis and Zylsta 1998
pT7-7	Expression vector	Tabor and Richardson 1987
pTV100	BHR origin of pTF-FC2 in pBluescript SK	Dorrington *et al.* 1991
pUC18	Cloning vector	Yanisch-Perron *et al.* 1985

Abbreviation: BHR- broad host range.

9-2: Plasmids generated in this study

Plasmid	Description
pMJ10	8 amino acids of HyuH, *hyuH-hyuC* intergenic fragment and 8 amino acids of HyuC from RU-AE01 in pGEM-T-Easy
pMJ11	Chloramphenicol resistant pTV100
pMJ14	Chloramphenicol resistant pTV100
pMJ16	pMJ14 *lac* (*lac* non-functional as missing a Shine-Dalgarno sequence and missing a start codon (ATG))
pMJ17	pMJ16 with a functional *lac*
pMJ26	*hyuC1* promoter in pMJ17
pMJ185	pMJ17 with a terminator sequence inserted upstream of *lac*
pMJ191	*ntrBC* in pMJ14
pMJ193	*ntrB* in pMJ14
pMJ198	*ntrC* in pMJ14
pMJ203	pMJ185 with the BHR origin inverted
pMJ211	*ntrC* in pGEM-T-Easy
pMJ219	NtrC-expression (with a carboxy-terminal His_6-tag) from pIVEX 2.3
pMJ224	pMJ203 with a multiple cloning cassette
pMJ234	NtrC-expression (with a carboxy-terminal His_6-tag) from pT7-7
pMJ242	pMJ224 with *gus* – BHR bidirectional promoter-probe vector
pMJ245	nts 311-491* of the RU-AE01 *hyuH-hyuC* fragment in pMJ242 with with *hyuH* on the *gus* side and *hyuC* on the *lac* side
pMJ258	nts 25-491* of the RU-AE01 *hyuH-hyuC* fragment in pMJ242 with *hyuH* on the *gus* side and *hyuC* on the *lac* side
pMJ260	nts 25-491* of the RU-AE01 *hyuH-hyuC* fragment in pMJ242 with *hyuH* on the *lac* side and *hyuC* on the *gus* side
pMJ261	nts 25-234* of the RU-AE01 *hyuH-hyuC* fragment in pMJ242 with *hyuH* on the *gus* side and *hyuC* on the *lac* side
pMJ266	nts 25-238* of the RU-AE01 *hyuH-hyuC* fragment in pMJ242 with *hyuH* on the *gus* side and *hyuC* on the *lac* side
pMJ268	nts 212-491* of the RU-AE01 *hyuH-hyuC* fragment in pMJ242 with *hyuH* on the *gus* side and *hyuC* on the *lac* side
pMJ361	nts 25-124* of the RU-AE01 *hyuH-hyuC* fragment in pMJ242 with *hyuH* on the *gus* side and *hyuC* on the *lac* side
pMJ364	pMJ191 with the sequence encoding a His_6-tag at the carboxy-terminal of NtrC
pMJ366	pMJ198 with the sequence encoding a His_6-tag at the carboxy-terminal of NtrC

* - nts on the RU-AE01 *hyuH-hyuC* sequence as detailed in Appendix 1-1
Abbreviation: BHR- broad host range.

Appendix 10: Primers used in the study

10-1: Universal sequencing primers

Primer	Description	Sequence (5' to 3')
pUCF	Universal primer F	CGCCAGGGTTTTCCCAGTCACGAC
pUCR	Universal primer R	TCACACAGGAAACAGCTATGAC
T7-F	Sequencing primer for pT7-7 and pIVEX 2.3 F	GGGAGCTGCATGTGTCAGAGG
T7-R	Sequencing primer for pT7-7 R	CGCTGAGATAGGTGCCTCAC
T7-T	Sequencing primer for pIVEX 2.3 R	GCTAGTTATTGCTCAGCGG
Universal *lacZ* primer	*lacZ* R	GCCAGCTGCATTAATGAATCGGCCAACGCG

10-2: Primers used in this study

Primer	Description	Sequence (5' to 3')
CH1	16S rRNA F	GCGGATCCGCCTAACACATGCAAGTCGAACGG
CH3	16S rDNA R	CGGGATCCCGACGATTACTAGCGATTCCAGCT
Chao F1	NRRL B11291 *hyuC* F	cgggatcc TTGACCCTGGTCCTTG
Chao R1	NRRL B11291 *hyuC* R	ccaagctt TCTCGATCGGATAGG
MJ1	†Nts 1-24	CGGGATCCTCCGTTCTTGATGATGATATCCAT
MJ2	†Nts 515-492	GCCCTAGGCAGCAAGTATCATCTGACGTGTCAT
MJ5	Oligo for pMJ16 top	TCGAGTTCGAAGATCTGATATCGGAGGATTACCACCATGCGCG
MJ6	Oligo for pMJ16 bottom	GATCCGCGCATGGTGGTAATCCTCCGATATCAGATCTTCGAAC
MJ9	†Nts 25-46	gaagatct AAAGCAGCTCTCAGGGTTGATG
MJ10	†Nts 491-466	gaagatct GAACCTTTGCTCCTTCGATAGTTAAT
MJ21	*ntrB* F	GCCGATATTCCCACTATCGCCGTTGAAC
MJ22	*ntrC* F	CAGCTCTTTGCCGAACACGACAGGAAATG
MJ23	*ntrC* R	CTGAAGTCAGGCAAGACTCCCCCATCAGG
MJ28	Terminator top	TCGAGTTTTTTTTGGGAGGCAGTCCTCCCGACGTTATA
MJ29	Terminator bottom	GATCTATAACGTCGGGAGGACTGCCTCCCAAAAAAAAC
MJ35	†Nts 212-237	gaagatct CCAACCCTATTCGGCGAGCTGGATT
MJ36	†Nts 328-304	gaagatct GTCAAGGACCAGGGTCAAAATTTCC
MJ37	†Nts 234-212	gaagatct TCCAGCTCGCCGAATAGGGTTTGG
MJ40	*ntrC Nde* I F	catatg ACAGCTACGATCCTCGTCGCCG
MJ41	*ntrC Sma* I R	cccggg GCTGGGGCGGGAACTACGATAGACG
MJ42	*ntrC* F2	CGCCAAGCTCGATGACGACATGC
MJ43	*ntrC* R3	ATGACTTCCAGCGCCTCCGTCTCG
MJ44	BHR oligo top	TCGAGCTGCAGAGATCTATGCGCG
MJ45	BHR oligo bottom	GATCCGCGCATAGATCTCTGCAGC
MJ46	*gus* F *Bgl* II	gaagatct ATGGTCCGTCCTGTAGAAACCC
MJ47	*gus* R *Pst* I	aactgcag TCATTGTTTGCCTCCCTGCTGCG
MJ59	Biotinylated MJ9	Biotin- gaagatct AAAGCAGCTCTCAGGGTTGATG
MJ60	†Nts 124-91	gaagatct CTTCCCTTCCCGCTTGACAGATAC

† - nts on the RU-AE01 *hyuH-hyuC* sequence as detailed in Appendix 1-1

Abbreviation: BHR- broad host range.

Appendix 11: Comparison of the 16S rDNA sequence of *A. tumefaciens* strains RU-AE01 and RU-OR.

Genomic DNA was extracted from *A. tumefaciens* RU-AE01 and *A. tumefaciens* RU-OR using the detergent/CTAB method described in Appendix 6-2. The 16S rDNA PCR primers CH1 and CH3 (Appendix 10-2) were used to amplify the 16S rDNA sequence using the Expand High Fidelity PCR system (Roche) under standard amplification parameters (Appendix 6-4) at an annealing temperature of 60 °C. The resultant PCR product was cloned into the pGEM-T-Easy (Promega) and the subsequent plasmid construct used for sequence analysis. The nucleotide sequence of the 16S rDNA product was determined using the ABI Prism Big Dye Terminator (Amersham) protocol with universal primers (Appendix 10-1). Sequence data was compared to the sequences on the NCBI database (http://www.ncbi.nlm.nih.gov/blast/). The sequences for the two strains, RU-AE01 and RU-OR, were aligned using the Vector NTI DeLuxe software v5.0 (Informax Inc.). General recombinant techniques were performed as described by Sambrook *et al.* (1989) or according to the manufacturer's instructions.

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RUOR     (1) GCGGATCCGCCTAACACATGCAAGTCGAACGGCCCGCAAGGGGAGTGGCA
AEO1     (1) GCGGATCCGCCTAACACATGCAAGTCGAACGGCCCGCAAGGGGAGTGGCA

RUOR    (51) GACGGGTGAGTAACGCGTGGGAACATACCCTTCCTGCGGAATAGCTCCG
AEO1    (51) GACGGGTGAGTAACGCGTGGGAACATACCCTTTCCTGCGGAATAGCTCCG

RUOR   (101) GGAAACTGGAATTAATACCGCATACGCCCTACGGGGGAAAGATTTATCGG
AEO1   (101) GGAAACTGGAATTAATACCGCATACGCCCTACGGGGGAAAGATTTATCGG

RUOR   (151) GGAAGGATTGGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTAC
AEO1   (151) GGAAGGATTGGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTAC

RUOR   (201) CAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGA
AEO1   (201) CAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGA

RUOR   (251) CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
AEO1   (251) CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA

RUOR   (301) CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTT
AEO1   (301) CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTT

RUOR   (351) AGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAA
AEO1   (351) AGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAA

RUOR   (401) GAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCT
AEO1   (401) GAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCT

RUOR   (451) AGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAA
AEO1   (451) AGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAA
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RUOR    (501) GTCAGGGGTGAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATACTGG
AEO1    (501) GTCAGGGGTGAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATACTGG

RUOR    (551) GTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAA
AEO1    (551) GTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAA

RUOR    (601) TTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCA
AEO1    (601) TTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCA

RUOR    (651) TTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
AEO1    (651) TTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC

RUOR    (701) CTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTATACT
AEO1    (701) CTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTATACT

RUOR    (751) GTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTC
AEO1    (751) GTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTC

RUOR    (801) GCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
AEO1    (801) GCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG

RUOR    (851) CATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCTCTTGACAT
AEO1    (851) CATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCTCTTGACAT

RUOR    (901) TCGGGGTATGGGCATTGGAGACGATGTCCTTCAGTTAGGCTGGCCCCAGA
AEO1    (901) TCGGGGTATGGGCATTGGAGACGATGTCCTTCAGTTAGGCTGGCCCCAGA

RUOR    (951) ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
AEO1    (951) ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA

RUOR   (1001) GTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTTAGTTGGG
AE01   (1001) GTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTTAGTTGGG

RUOR   (1051) CACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACG
AEO1   (1051) CACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACG

RUOR   (1101) TCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGT
AEO1   (1101) TCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGT

RUOR   (1151) GGTGACAGTGGGCAGCGAGACAGCGATGTCGAGCTAATCTCCAAAAGCCA
AEO1   (1151) GGTGACAGTGGGCAGCGAGACAGCGATGTCGAGCTAATCTCCAAAAGCCA

RUOR   (1201) TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGCTGGAATCGC
AEO1   (1201) TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGCTGGAATCGC

RUOR   (1251) TAGTAATCGTCGGGATCCCG
AEO1   (1251) TAGTAATCGTCGGGATCCCG
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Figure A3: Alignment of the 16S rDNA sequence from the strains *A. tumefaciens* RU-AE01 and *A. tumefaciens* RU-OR (Informax Inc.).

Alignment of the 16S rDNA sequence from the two *Agrobacterium* strains RU-AE01 and RU-OR (Appendix 11 Figure A3) indicated 99.92% identity over the 1251 bp sequenced.

Appendix 12: A complete data set showing the effect of the proteins NtrB and NtrC on the Hyu enzyme activities in *A. tumefaciens* strains RU-AE01 and RU-OR (as presented in Chapter 5)

			Hydantoinase			
Strain	Plasmid		Hyd	SD	% of At pMJ14	% SD
RU-AE01	pSR146		5.25	1.02	243.35	19.52
	pMJ14	Vector	2.16	0.38	100.00	17.79
	pMJ193	NtrB	7.91	1.16	367.07	14.61
	pMJ198	NtrC	7.31	0.89	339.18	12.12
	pMJ366	His6-NtrC	7.09	0.61	328.65	8.54
	pMJ191	NtrBC	7.73	1.03	358.71	13.28
	pMJ364	NtrB His6-NtrC	6.27	0.48	290.67	7.63
RU-OR	pSR146		5.12	1.00	268.30	15.08
	pMJ14	Vector	1.91	0.45	100.00	23.72
	pMJ193	NtrB	1.67	0.24	87.69	13.44
	pMJ198	NtrC	5.25	0.64	275.31	15.12
	pMJ366	His6-NtrC	0.33	0.03	17.44	10.86
	pMJ191	NtrBC	6.74	0.90	353.67	9.35
	pMJ364	NtrB His6-NtrC	0.58	0.04	30.63	12.68

			NCAAH			
Strain	Plasmid		NCAAH	SD	% of At pMJ14	% SD
RU-AE01	pSR146		8.98	1.18	277.42	13.15
	pMJ14	Vector	3.24	0.64	100.00	19.85
	pMJ193	NtrB	5.32	0.86	164.18	16.25
	pMJ198	NtrC	6.28	0.77	194.05	12.23
	pMJ366	His6-NtrC	5.32	0.41	164.34	7.64
	pMJ191	NtrBC	5.08	0.82	156.85	16.21
	pMJ364	NtrB His6-NtrC	5.63	1.16	173.81	20.60
RU-OR	pSR146		4.93	0.96	164.63	19.44
	pMJ14	Vector	3.00	0.54	100.00	17.85
	pMJ193	NtrB	4.17	0.73	139.07	17.62
	pMJ198	NtrC	6.25	1.05	208.68	16.79
	pMJ366	His6-NtrC	5.87	1.07	195.98	18.22
	pMJ191	NtrBC	4.63	0.93	154.63	20.15
	pMJ364	NtrB His6-NtrC	5.28	0.86	176.30	16.28

Each result is an average of triplicate reactions. This experiment was independently repeated four times.

REFERENCES

Adler, A., Purich, D. and Stadtman, E. (1975) Cascade control of *Escherichia coli* glutamine synthetase, *Journal of Biological Chemistry 250,* 6264-6272.

Albus, A., Pesci, E., Runyen-Janecky, L., West, S. and Iglewski, B. (1997) Vfr controls quorum sensing in *Pseudomonas aeruginosa*, *Journal of Bacteriology 179,* 3928-3935.

Altenbuchner, J., Siemann-Herzberg, M. and Syldatk, C. (2001) Hydantoinases and related enzymes as biocatalysts for the synthesis of unnatural chiral amino acids, *Current opinion in Biotechnology 12*, 559-563.

Ames, G. F.-L. and Nikaido, K. (1985) Nitrogen regulation in *Salmonella typhimurium*: identification of an ntrC protein-binding site and definition of a consensus binding sequence, *The EMBO Journal 4*, 539-547.

Anderson, W., Hennig, S.B., Ginsburg, A. and Stadtman, E. (1970) Association of ATP:glutamine synthetase adenyltransferase activity with the PI component of the glutamine synthetase deadenylation system, *Proceedings of the National Academy of Science USA 67,* 1417-1424.

Arcondeguy, T., Jack, R., and Merrick, M. (2001) PII signal transduction proteins, pivotal players in microbial nitrogen world, *Microbiology and Molecular Biology Reviews 65*, 80-105.

Atkinson, M., Kamberov, E., Weiss, R., and Ninfa, A. (1994) Reversible uridylylation of the *Escherichia coli* PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC), *Journal of Biological Chemistry 269*, 28288-28293.

Atkinson, M. and Ninfa, A. (1998) Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli, Molecular Microbiology 29,* 431-447.

Atkinson, M. and Ninfa, A. (1999) Characterization of the GlnK protein of *Escherichia coli, Molecular Microbiology 32,* 301-313.

Atkinson, M. R., Blauwkamp, T. A., Bondarenko, V., Studitsky, V. and Ninfa, A. J. (2002) Activation of *glnA, glnK* and *nac* promoters as *Escherichia coli* undergoes the transition from nitrogen excess growth to nitrogen starvation, *Journal of Bacteriology 184,* 5358-5363.

Ausubel, F.M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. and Struhl, K. (1983) Current protocols in molecular biology (3rd Edition), Greene Publishing Associates and Wiley-Interscience, New York.

Backman, K., Chen, Y.-M., and Magasanik, B. (1981) Physical and genetic characterization of the *glnA-glnG* region of the *Escherichia coli c*hromosome, *Proceedings of the National Academy of Science USA 78*, 3743-3747.

Bae, H.-S., Lee, S.-G., Hong, S.-P., Kwak, M.-S., Esaki, N., Soda, K., and Sung, M.-H. (1999) Production of aromatic D-amino acids from α-keto acids and ammonia by coupling of four enzyme reactions, *Journal of Molecular Catalysis B: Enzymatic 6*, 241-247.

Bardonnet, N., Trautwetter, A., Couchoux-Luthaud, G., and Blanco, C. (1988) Plasmids with the *uidA* reporter gene for the detection of promoters and transcription signals, *Molecular Genetics and Genomics 212*, 390-392.

Barrios, H., Valderrama, B., and Morett, E. (1999) Compilation and analysis of σ^{54}-dependent promoter sequences, *Nucleic Acids Research 27*, 4305-4313.

Bender, R., Janssen, K., Resnick, A., Blumenberg, M., Foor, F., and Magasanik, B. (1977) Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*, *Journal of Bacteriology 129*, 1001-1009.

Bender, R. A., Snyder, P. M., Bueno, R., Quinto, M. and Magasanik, B. (1983) Nitrogen regulation system of *Klebsiella aerogenes:* the *nac* gene, *Journal of Bacteriology 156,* 444-446.

Berghammer, H. and Auer, B. (1993) 'Easy preps': Fast and easy plasmid minipreparation for analysis of recombinant clones in *E. coli*, *BioTechniques 14*, 527-528.

Beynon, J.L., Williams, M.K. and Cannon, F.C. (1988) Expression and functional analysis of the *Rhizobium meliloti nifA* gene, *The EMBO Journal 7*, 7-14.

Blasina, A., Kittell, B., Toukdarian, A. amd Helinski, D. (1996) Copy-up mutants of the plasmid RK2 replication initiation protein are defective in coupling RK2 replication origins, *Proceedings of the National Academy of Science USA 93,* 3559-3564.

Blattner, F., Plunkett III, G., Bloch, C., Perna, N., Burland, V., Riley, M., Collado-Vides, J., Glasner, J., Rode, C., Mayhew, G., Gregor, J., Davis, N., Kirkpatrick, H., Goeden, M., Rose, D., Mau, B., and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12, *Science 277*, 1453-1474.

Bolivar, F. (1978) Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco* RI sites for selection of *Eco* RI-generated recombinant DNA molecules, *Gene 4*, 136.

Bommarius, A., Schwarm, M. and Drauz, K. (1998) Biocatalysis to amino acid based chiral pharmaceuticals-examples and perspectives, *Journal of Molecular Catalysis B: Enzymatic 5*, 1-11.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein for utilizing the principle of protein-dye binding, *Analytical Biochemistry 72*, 248-254.

Brenner, C. (2002) Catalysis in the nitrilase superfamily, *Current Opinion in Structural Biology 12,* 775-782.

Brosius, J. (1984) Plasmid vectors for the selection of promoters, *Gene 27*, 151-160.

Brown, M., Segal, A. and Stadtman, E. (1971) Modulation of glutamine synthetase adenylation and deadenylation is mediated by metabolic transformation of the PII regulatory protein, *Proceedings of the National Academy of Science USA 68,* 2949-2953.

Brychem Business Consulting (1999) Global production of amino acids, A review prepared for AECI Ltd, South Africa, July 1999.

Buchanan, K., Burton, S., Dorrington, R., Matcher, G., and Skepu, Z. (2001) A novel *Pseudomonas putida* strain with high levels of hydantoin converting activity producing L-amino acids, *Journal of Molecular Catalysis B: Enzymatic 11*, 397-406.

Burkovski, A. (2003) I do it my way: regulation of ammonium uptake and ammonium assimilation in *Corneybacterium glutamicum*, *Archives of Microbiology 179*, 75-82.

Burton, S., Dorrington, R., Hartley, C., Kirchmann, S., Matcher, G., and Phehane, V. (1998) Production of enantiomerically pure amino acids: characterisation of South African hydantoinases and hydantoinase-producing bacteria, *Journal of Molecular Catalysis B: Enzymatic 5*, 301-305.

Caban, C. and Ginsburg, A. (1976) Glutamine synthetase adenyltransferase from *Escherichia coli:* purification and physical and chemical properties, *Biochemistry 15,* 1569-1580.

Carbonelli, D., Corley, E., Seigelchifer, M., and Zorzopulos, J. (1999) A plasmid vector for isolation of strong promoters in *Escherichia coli*, *FEMS Microbiological Letters 177*, 75-82.

Carlson, T. and Chelm, B. (1986) Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum, Nature 322*, 568-570.

Carmona, M., Claverie-Martin, F., and Magasanik, B. (1997) DNA-bending and the initiation of transcription at σ^{54}-dependent bacterial promoters, *Proceedings of the National Academy of Science USA 94*, 9568-9572.

Cases, I., de Lorenzo, V., and Ouzounis, C. (2003) Transcriptional regulation and environmental adaptation in bacteria, *Trends in Microbiology 11*, 248-253.

Cases, I. and de Lorenzo, V. (1998) Expression systems and physiological control of promoter activity in bacteria, *Current Opinion in Microbiology 1*, 303-310.

Chao, Y-P., Juang, T-Y., Chern, J-T. and Lee, C-K. (1999) Production of D-*p*-hydroxyphenylglycine by *N*-carbamoyl-D-amino acid amidohydrolase-overproducing *Escherichia coli* strains, *Biotechnological Progress* 15, 603-607.

Chao, Y.-P., Chiang, C.-J., Lo, T.-E. and Fu, H. (2000) Overproduction of D-hydantoinase and carbamoylase in a soluble form in *Escherichia coli, Applied Microbiology and Biotechnology 54*, 348-353.

Chen, H., Bjerknes, M., Kumar, R. and Jay, E. (1994) Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs, *Nucleic Acids Research 22,* 4953-4957.

Chen, H.-Y. and Tsai, H. (1996) Cloning, sequencing and expression in *Escherichia coli* of D-hydantoinase gene from *Pseudomonas putida*, *Annals New York Academy of Sciences 864,* 234-237.

Chien, H.-R., Jih, Y.-L., Yang, W.-Y. and Hsu, W.-H. (1998) Identification of the open reading frame for the *Pseudomonas putida* D-hydantoinase gene and expression of the gene in *Escherichia coli, Biochimica et Biophysica Acta 1395*, 68-77.

Cho, K., Fuqua, C., Martin, B. and Winans, S. (1996) Identification of *Agrobacterium tumefaciens* genes that direct the complete catabolism of octopine, *Journal of Bacteriology 178*, 1872-1880.

Chung, H.K. and Rhee, S.G. (1984) Separation of glutamine synthetase species with different states of adenylation by chromatography in monoclonal anti-AMP antibody affinity columns, *Proceedings of the National Academy of Science USA 81,* 4677-4681.

Clark, S.A. (2003) Isolation, expression and purification of the hydantoin-hydrolyzing enzymes of *Agrobacterium tumefaciens,* pp 1-157, MSc Thesis, Rhodes University.

Clemente-Jiminez, J., Martinez-Rodriguez, S., Mingorance-Cazorla, L., de la Escalera-Huesa, S., Las Heras-Vasquez, F., and Rodriguez-Vico, F. (2003) Catalytic analysis of a recombinant D-hydantoinase from *Agrobacterium tumefaciens*, *Biotechnology Letters 25*, 1067-1073.

Contreras, A. and Drummond, M. (1988) The effect on the function of the transcriptional activator NtrC from *Klebsiella pneumoniae* of mutations in the DNA-recognition helix, *Nucleic Acids Research 16*, 4025-4039.

Currier, T. and Nester, E. (1976) Evidence for diverse types of large plasmids in tumor-inducing strains of *Agrobacterium, Journal of Bacteriology 126,* 157-165.

de Bruijn, F., Rossbach, S., Schneider, M., Ratet, P., Messmer, S., Szeto, W., Ausubel, F. and Schell, J. (1989) *Rhizobium meliloti* 1021 has three differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation, *Journal of Bacteriology 171,* 1673-1682.

de la Riva, G., Gonzalez-Cabrera, J., Vazquez-Padron, R. and Ayra-Pardo, C. (1998) *Agrobacterium tumefaciens:* a natural tool for plant transformation, *Electronic Journal of Biotechnology 1*, 1-16.

de Zamaroczy, M., Delorme, F., and Elmerich, C. (1990) Characterisation of three different nitrogen regulated promoter regions for the expression of *glnB* and *glnA* in *Azospirillum brasilense*, *Molecular Genetics and Genomics 224*, 421-430.

Deepa, S., Sivasankar, B., and Jayaraman, K. (1993) Enzymatic production and isolation of D-amino acids from the corresponding 5-substituted hydantoins, *Process Biochemistry 28*, 447-452.

Demain, A. (2000) Small bugs, big business: The economic power of the microbe, *Biotechnological Advances 18*, 499-514.

Dennis, J. and Zylstra, G. (1998) Plasposons: Modular self cloning mini-transposon derivatives for rapid genetic analysis of gram-negative bacterial genomes, *Applied and Environmental Microbiology 64*, 2710-2715.

Dhandayuthapani, S., Rasmussen, W., and Baseman, J. (1998) Identification of mycoplasmal promoters in *Escherichia coli* using a promoter-probe vector with green fluorescent protein as a reporter system, *Gene 215*, 213-222.

Diaz, E. and Prieto, M. (2000) Bacterial promoters triggering biodegradation of aromatic pollutants, *Current opinion in Biotechnology 11*, 467-475.

Dorrington, R. and Rawlings, D. (1989) Identification and sequence of the basic replication region of a broad host range plasmid isolated from *Thiobacillus ferrooxidans*, *Journal of Bacteriology 171*, 2735-2739.

Dorrington, R. and Rawlings, D. (1990) Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids, *Journal of Bacteriology 172*, 5697-5705.

Dorrington, R., Bardien, S., and Rawlings, D. (1991) The broad host range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*, *Gene 108*, 7-14.

Drauz, K. (1997) Chiral amino acids: A versatile tool in the synthesis of pharmaceuticals and fine chemicals, *Chimia 51*, 310-314.

Drauz, K., Kottenhahn, M., Makryaleas, K., Klenk, H., and Bernd, M. (1991) Chemoenzymatic synthesis of ω-ureido D-amino acids, *Angewandte Chemie International Edition English 30*, 712-714.

Durham, D. and Weber, J. (1995) Properties of D-hydantoinase from *Agrobacterium tumefaciens* and its use for the preparation of *N*-carbamyl D-amino acids, *Biochemical and Biophysical Research Communications 216*, 1095-1100.

Durham, D. and Weber, J. (1996) Stereospecific preparation of an excitory amino acid antagonist with D-hydantoinase from *Agrobacterium tumefaciens* as a biocatalyst, *Applied and Environmental Microbiology 62*, 739-742.

Eadie, G., Bernheim, F., and Bernheim, M. (1949) The partial purification and properties of animal and plant hydantoinases, *Journal of Biological Chemistry 181*, 449-458.

Engleman, E. and Francis, S. (1978) Cascade control of *E. coli* glutamine synthetase, *Archives of Biochemistry and Biophysics 191*, 602-612.

Erickson, R. and Steers Jr, E. (1970) Comparative study of isoenzyme formation of bacterial β-galactosidase, *Journal of Bacteriology 102,* 79-84.

Feng, J., Goss, T. J., Bender, R. A. and Ninfa, A. J. (1995a) Activation of transcription initiation from the *nac* promoter of *Klebsiella aerogenes*, *Journal of Bacteriology 177,* 5523-5534.

Feng, J., Goss, T. J., Bender, R. A. and Ninfa, A. J. (1995b) Repression of the *Klebsiella aerogenes nac* promoter, *Journal of Bacteriology 177,* 5535-5538.

Figurski, D. H. and Helinski, D. R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans, *Proceedings of the National Academy of Science USA 76,* 1648-1652.

Foor, F., Janssen, K. and Magasanik, B. (1975) Regulation of synthesis of glutamine synthetase by adenylated glutamine synthetase, *Proceedings of the National Academy of Science USA 72,* 4844-4848.

Fotheringham, I. (2000) Engineering biosynthetic pathways: new routes to chiral amino acids, *Current Opinion in Chemical Biology 4*, 120-124.

Forchhammer, K., Hedler, A., Strobel, H. and Weiss, V. (1999) Heterotrimerization of PII-like signaling proteins: implications for PII-mediated signal transduction systems, *Molecular Microbiology 33,* 337-349.

Fu, H., Burris, R. H., and Roberts, G. P. (1990) Reversible ADP-ribosylation is demonstrated to be a regulatory mechanism in prokaryotes by heterologous expression, *Proceedings of the National Academy of Science USA 87,* 1720-1724.

Fuchs, R. and Keister, D. (1980a) Identification of two glutamine synthetases in *Agrobacterium*, *Journal of Bacteriology 141*, 996-998.

Fuchs, R. and Keister, D. (1980b) Comparative properties of glutamine synthetases I and II in *Rhizobium* and *Agrobacterium* spp., *Journal of Bacteriology 144*, 641-648.

Galli, G., Grifantini, R. and Grandi, G. (1995) Stable mutants of D-*N*-α-carbamoylase and process for preparing D-α-amino acids, US Patent 5869298.

Gelvin, S. B. (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration, *Annual Review of Plant Physiology and Plant Molecular Biology 51,* 223-256.

Ginsburg, A. and Stadtman, E. (1973) Regulation of glutamine synthetase in *Escherichia coli* (pgs 9-25) In The Enzymes of Glutamine Metabolism, Editors: Prusiner, S.B. and Stadtman, E.R., Academic Press, New York.

Girbal, L., Mortier-Barriere, I., Raynaud, F., Rouanet, C., Croux, C. and Soucaille, P. (2003) Development of a sensitive gene expression reporter system and an inducible promoter-repressor system for *Clostridium acetobutylicum, Applied and Environmental Microbiology 69,* 4985-4988.

Glanemann, C., Loos, A., Gorret, N., Willis, L. B., O'Brien, X. M., Lessard, P. A. amd Sinskey, A. J. (2003) Disparity between changes in mRNA abundance and enzyme activity in *Corynebacterium glutamicum*: implications for DNA microarray analysis*, Applied Microbiology and Biotechnology 61*, 61-68.

Gokhale, D., Bastawde, K., Patil, S., Kalkote, U., Joshi, R., Joshi, R., Ravindranathan, T., Gaikwad, B., Jogdand, V., and Nene, S. (1996) Chemoenzymatic synthesis of D-(-)-phenylglycine using hydantoinase of *Pseudomonas desmolyticum* resting cells, *Enzyme and Microbial Technology 18*, 353-357.

Gokhale, D., Bastawde, K., Patil, S., Kalkote, U., Joshi, R.R, Joshi, R.A., Ravindranathan, T., Jogdand, V., Gaikwad, B. and Nene, S. (1997) Microbial process for the production of D-(-)-*N*-carbamoylphenylglycine, US Patent 6087136.

Goodner, B., Markelz, B., Flanagan, M., Crowell Jr, C., Racette, J., Schilling, B., Halfohn, L., Mellors, J. and Grabowski, G. (1999) Combined genetic and physical map of the complex genome of *Agrobacterium tumefaciens*, *Journal of Bacteriology 181*, 5160-5166.

Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B., Goldman, B., Cao, Y., Askenazi, M., Halling, C., Mullin, L., Houmiel, K., Gordon, J., Vaudin, M., Iartchouk, O., Epp, A., Liu, F., Wollam, C., Allinger, M., Doughty, D., Scott, C., Lappas, C., Markelz, B., Flanagan, C., Crowell, C., Gurson, J., Lomo, C., Sear, C., Strub, G., Cielo, C. and Slater, S. (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58, *Science 294,* 2323-2328.

Goss, T., Perez-Matos, A. and Bender, R. (2001) Roles of glutamate synthetase, *gltBD* and *gltF* in nitrogen metabolism of *Escherichia coli* and *Klebsiella aerogenes, Journal of Bacteriology 183,* 6607-6619.

Gottschalk, G. (1986) Nutrition of bacteria In *Bacterial metabolism* (2nd Edition), Springer-Verlag.

Gouka, R., Gerk, C., Hooykaas, P., Bundock, P., Musters, W., Verrips, C. and de Groot, M. (1999) Transformation of *Aspergillus awamori* by *Agrobacterium tumefaciens*-mediated homologous recombination, *Nature Biotechnology 17*, 598-601.

Griffantini, R., Galli, G., Carpani, G., Pratesi, C., Frascotti, G., and Grandi, C. (1998) Efficient conversion of 5-substituted hydantoins to D-α-amino acid using recombinant *Escherichia coli* strains, *Microbiology 144*, 947-954.

Gross, C., Syldatk, C., and Wagner, F. (1987) Screening method for microorganisms producing L-amino acids from D, L-substituted hydantoins, *Biotechnology Techniques 1*, 85-90.

Gross, C., Syldatk, C., Mackowiak, V., and Wagner, F. (1990) Production of L-tryptophan from D, L-5-indolylmethylhydantoin by resting cells of a mutant of *Arthrobacter* species (DSM 3747), *Journal of Biotechnology 14*, 363-376.

Hahn, F., Wisseman Jr, C. and H Hopps (1954) Mode of action of chloramphenicol, *Journal of Bacteriology 67,* 662-673.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids, *Journal of Molecular Biology 166*, 557-580.

Hanau, R., Koduri, R., Ho, N. and Brenchley, J. (1983) Nucleotide sequence of the control regions for the *glnA* and *glnL* genes of *Salmonella typhimurium, Journal of Bacteriology 155,* 82-89.

Harley, C. and Reynolds, R. (1987) Analysis of *E. coli* promoter sequences, *Nucleic Acids Research* 15, 2343-2361.

Hartley, C. (1995) Examination of the production of optically active amino acids by various selected soil microorganisms, pp 1-122, Honours Thesis, Rhodes Univeristy.

Hartley, C. (2001) Elucidation and manipulation of the hydantoin-hydrolyzing enzyme system of *Agrobacterium tumefaciens* RU-OR for the biocatalytic production of D-amino acids, pp 1-242, PhD Thesis, Rhodes University.

Hartley, C., Kirchmann, S., Burton, S., and Dorrington, R. (1998) Production of D-amino acids from D, L-substituted hydantoins by an *Agrobacterium tumefaciens* strain and isolation of a mutant with inducer-independent expression of hydantoin-hydrolyzing activity, *Biotechnology Letters 20*, 707-711.

Hartley, C., Manford, F., Burton, S., and Dorrington, R. (2001) Over-production of hydantoinase and *N*-carbamoylamino acid amidohydrolase enzymes by regulatory mutants of *Agrobacterium tumefaciens*, *Applied Microbiology and Biotechnology 57*, 43-49.

Hauptmann, S. and Schmitt, W. (2006) Transposable elements- is there a link between evolution and cancer?, *Medical Hypotheses 66,* 580-591.

Helmann, J. and Chamberlin, M. (1988) Structure and function of bacterial sigma factors, *Annual Review of Biochemistry* 57, 839-872.

Herrera, G. (1995) Cloning and initial characterisation of a gene encoding *N*-carbamoyl-D-amino acid amidohydrolase activity from *Pseudomonas paucimobilis*, RN1905/C ed., pp 1-8, AECI Limited Research and Development Department.

Hester, K., Lehman, J., Najar, F., Song, L., Roe, B., MacGregor, C., Hager, P., Phibbs Jr, P., and Sokatch, J. (2000) Crc is involved in catabolite repression control of the *bkd* operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*, *Journal of Bacteriology 182*, 1144-1149.

Hill, R., Parker, J., Goodman, H., Jones, D. and Woods, D. (1989) Molecular analysis of a novel glutamine synthetase of the anaerobe *Bacteroides fragilis, Journal of General Microbiology 135,* 3271-3279.

Hils, M., Munch, P., Altenbuchner, J., Syldatk, C., and Mattes, R. (2001) Cloning and characterization of genes from *Agrobacterium* sp. IP I-671 involved in hydantoin degradation, *Applied Microbiology and Biotechnology 57*, 680-688.

Holm, L. and Sander, C. (1997) An evolutionary treasure: Unification of a broad set of amidohydrolases related to urease, *PROTEINS: Structure, Functions and Genetics 28*, 72-82.

Hols, P., Kleerebezem, M., Schanck, A., Ferain, T., Hugenholtz, J., Delcour, J., and de Vos, Q. (1999) Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering, *Nature Biotechnology 17*, 588-592.

Holsters, M., Silva, B., Van Vliet, F. Genetello, C., De Block, M., Dhaese, P., Depicker, A., Inze, D., Engler, G., Villarroel, R., Van Montagu, M. and Schell, J. (1980) The functional organization of the nopaline A. tumefaciens plasmid pTiC58, *Plasmid 3(2)*, 212-230.

Hsu, W.-H., Chien, F.-T., Hsu, C.-L., Wang, T.-C., Yuan, H., and Wang, W.-C. (1999) Expression, crystallisation and preliminary X-ray diffraction studies of *N*-carbamyl-D-amino acid amidohydrolase from *Agrobacterium radiobacter*, *Acta Crystallographica Section D D55*, 694-695.

Huerta, A. and Collado-Vides, J. (2003) Sigma-70 promoters in *Escherichia coli:* specific transcription in dense regions of overlapping promoter-like signals, *Journal of Molecular Biology 333,* 261-278.

Hwang, I., Thorgeirsson, T., Lee, J., Kustu, S. and Shin, Y-K. (1999) Physical evidence for a phosphorylation-dependent conformational change in the enhancer-binding protein NtrC, *Proceedings of the National Academy of Science USA 96,* 4880-4885.

Ikenaka, Y., Nanba, H., Yamada, Y., Yajima, K., Takano, M., and Takahashi, S. (1998) Screening, characterisation and cloning of the gene for *N*-carbamyl-D-amino acid amidohydrolase from thermotolerant soil bacteria, *Bioscience Biotechnology and Biochemistry 62*, 882-886.

Inouye, S., Nakazawa, A. and Nakazawa, T. (1987) Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product, *Biochemistry 84,* 5182-5286.

Jaeger, K., Reetz, M., and Dijkstra, B. (2002) Directed evolution to produce enantioselective biocatalysts, *ASM News 68*, 556-562.

Jaggi, R., van Heeswijk, W., Westerhoff, H., Ollis, D. and Vasudevan, S. (1990) The two opposing activities of adenyltransferase reside in distinct homologous domains, with intramolecular signal transduction, *The EMBO Journal 16,* 5562-5571.

Javelle, A., Severi, E., Thornton, J. and Merrick, M. (2004) Ammonium sensing in *Escherichia coli, Journal of Biological Chemistry 279,* 8530-8538.

Jefferson, R., Burgess, S., and Hirsh, D. (1986) β-glucuronidase from *Escherichia coli* as a gene fusion vector, *Proceedings of the National Academy of Science USA 83*, 8447-8451.

Jensen, L., Friis, C. and Ussery, D. (1999) Three views of microbial genomes, *Research in Microbiology 150,* 773-777.

Jiang, P., Peliska, J. and Ninfa, A. (1998) Enzymological characterization of the signal transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein, *Biochemistry 37,* 12782-12794.

Jiang, J., Atkinson, M., Srisawat, C., Sun, Q. and Ninfa, A. (2000) Functional dissection of the dimerization and enzymatic activities of *Escherichia coli* nitrogen regulator II and their regulation by the PII protein, *Biochemistry 39,* 13433-13449.

Kadonaga, J. and Tjian, R. (1986) Affinity purification of sequence-specific DNA binding proteins, *Proceedings of the National Academy of Science USA 83,* 5889-5893.

Kamberov, E., Atkinson, M. and Ninfa, A. (1995) The *Escherichia coli* PII signal transduction protein is activated upon binding 2-ketoglutarate and ATP, *Journal of Biological Chemistry 270,* 17797-17807.

Kamphuis, J., Boesten, W., Broxterman, Q., Hermes, H., van Balken, J., Meijer, E. and Schoemaker, H. (1990) New developments in the chemo-enzymatic production of amino acids, *Advances in Biochemical Engineering/Biotechnology 42*, 133-186.

Kamphuis, J., Meijer, E., Boesten, W., Broxterman, Q., Kaptein, B., Hermes, H. and Schoemaker, H. (1992) Production of natural and synthetic L- and D-amino acids by aminopeptidases and amino amidases (pgs 177-206) In Biocatalytic production of amino acids and derivatives, Editors: Rozzell, D. and Wagner, F., Hanser.

Kao, C. and Hsu, W. (2003) A gene cluster involved in pyrimidine reductive catabolism from *Brevibacillus agri* NCHU 1002, *Biochemical and Biophysical Research Communications 303*, 848-854.

Keener, J. and Kustu, S. (1988) Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NtrB and NtrC of enteric bacteria: roles of the conserved amino-terminal domain of NtrC, *Proceedings of the National Academy of Science USA 85,* 4976-4980.

Kenealy, W. R., Thompson, T. E., Schubert, K.R. and Zeikus, J.G. (1982) Ammonia assimilation and synthesis of alanine, aspartate and glutamate in *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum*, *Journal of Bacteriology 150*, 1357-1365.

Kern, D., Volkman, B., Luginbuhl, P., Nohaile, M., Kustu, S. and Wemmer, D. (1999) Structure of a transiently phosphorylated switch in bacterial signal transduction, *Nature 402*, 894-898.

Kim, D.-M. and Kim, H.-S. (1993) Enzymatic synthesis of D-*p*-hydroxyphenylglycine from D, L-*p*-hydroxyphenylhydantoin in the presence of organic cosolvent, *Enzyme and Microbial Technology 15*, 530-534.

Kim, G.-J. and Kim, H.-S. (1994) Adsorptive removal of inhibitory by-product in the enzymatic production of optically active D-*p*-hydroxyphenylglycine from 5-substituted hydantoin, *Biotechnology Letters 16*, 17-22.

Kim, G.-J. and Kim, H.-S. (1998) Identification of the structural similarity in the functionally related amidohydrolases acting on the cyclic amide ring, *Journal of Biochemistry 330*, 295-302.

Kim, G.-J., Park, J.-H., Lee, D.-C., Ro, H.-S., and Kim, H.-S. (1997) Primary structure, sequence analysis, and expression of the thermostable D-hydantoinase from *Bacillus stearothermophilus* SD1, *Molecular and General Genetics 255*, 152-156.

Kim, G.-J. and Kim, H.-S. (1995) Optimal reaction conditions for the enzymatic synthesis of optically active D-*p*-hydroxyphenylglycine from 5-substituted hydantoin using D-hydantoinase and *N*-carbamoylase, *Annals New York Academy of Sciences 750*, 185-189.

Knauf, V. C. and Nester, E. W. (1982) Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid, *Plasmid 8*, 45-54.

Koeller, K. and Wong, C.-H. (2001) Enzymes for chemical synthesis, *Nature 409*, 232-240.

Kumada, Y., Benson, D., Hillemann, D., Hosted, T., Rochefort, D., Thompson, C., Wohlleben, W. and Tateno, Y. (1993) Evolution of the glutamine synthetase gene, one of the oldest existing and functioning genes, *Proceedings of the National Academy of Science USA 93,* 3009-3013.

Kunik, T., Tzfira, T., Kapulnik, Y., Gafni, Y., Dingwall, C. and Citovsky, V. (2001) Genetic transformation of HeLa cells by *Agrobacterium, Proceedings of the National Academy of Science USA 98*, 1871-1876.

Kustu, S., Santero, E., Keener, J., Popham, D., and Weiss, D. (1989) Expression of σ^{54} (*ntrA)*-dependant genes is probably united by a common mechanism, *Microbiological Reviews 53*, 367-376.

Kustu, S. and McKereghan, K. (1975) Mutations affecting glutamine synthetase activity in *Salmonella typhimurium*, *Journal of Bacteriology 122*, 1006-1016.

Las Heras-Vasquez, F., Martinez-Rodriguez, S., Mingorance-Cazorla, L., Clemente-Jiminez, J., and Rodriguez-Vico, F. (2003) Over-expression and characterization of hydantoin racemase from *Agrobacterium tumefaciens* C58, *Biochemical and Biophysical Research Communications 303*, 541-547.

LeBlond-Francillard, M., Dreyfus, M. and Rougeon, F. (1987) Isolation of DNA-protein complexes based on streptavidin and biotin interactions, *European Journal of Biochemistry 166,* 351-355.

Lee, S.-G., Lee, D.-C., Sung, M.-H., and Kim, H.-S. (1994a) Isolation of thermostable D-hydantoinase producing thermophilic *Bacillus* sp. SD-1, *Biotechnology Letters 16*, 461-466.

Lee, J.-H., Scholl, D., Nixon, T. and Hoover, T. (1994b) Constitutive ATP hydrolysis and transcription activation by a stable truncated form of *Rhizobium melitoti* DCTD, a σ^{54}-dependent transcriptional activator, *Journal of Biological Chemistry 269*, 20401-20409.

Lee, D.-C., Kim, G.-J., Cha, Y.-K., Lee, C.-Y., and Kim, H.-S. (1997) A cultivation strategy of recombinant *Escherichia coli* for mass production of thermostable D-hydantoinase, *Annals New York Academy of Sciences 864*, 371-374.

Lee, C.-K., Lee, Z.-S., and Yang, P.-F. (2001) Effect of cell membrane of *Agrobacterium radiobacter* on enhancing D-amino acid production from racemic hydantoins, *Enzyme and Microbial Technology 28*, 806-814.

Li, P-L. and Farrand, S. (2000) The replicator of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system, *Journal of Bacteriology 182,* 179-188.

Linn, T. and St Pierre, R. (1990) Improved vector system for constructing transcriptional fusions that ensures independent translation of *lacZ*, *Journal of Bacteriology 172*, 1077-1084.

Liu, Y. and Kahn, M. (1995) ADP-ribosylatoin of *Rhizobium meliloti* glutamine synthetase III *in vivo, Journal of Biological Chemistry 270,* 1624-1628.

Louwrier, A. and Knowles, C. (1996) The purification and characterisation of a novel D(-)-specific carbamoylase enzyme from an *Agrobacterium* sp., *Enzyme and Microbial Technology 19*, 562-571.

Lowery, R. G., Saari, L. L. and Ludden, P. W. (1986) Reversible regulation of the nitrogenase iron protein from *Rhodospirillum rubrum* by ADP-ribosylation in vitro, *Journal of Bacteriology 166*, 513-518.

Lu, C.-D. and Abdelal, A. (2001) The *gdhB* gene of *Pseudomonas aeruginosa* encodes an arginine inducible NAD^+ dependent glutamate dehydrogenase which is subject to allosteric regulation, *Journal of Bacteriology* 183, 490-499.

Luksa, V., Starkuviene, V., Starkuviene, B., and Dagys, R. (1997) Purification and characterisation of the D-hydantoinase from *Bacillus circulans*, *Applied Biochemistry and Biotechnology 62*, 219-231.

Luo, Z.-Q. and Farrand, S. (1999) Cloning and characterisation of a tetracycline resistance determinant present in *Agrobacterium tumefaciens* C58, *Journal of Bacteriology 181*, 618-626.

Lyi, S., Jafri, S. and Winans, S. (1999) Mannopinic acid and agropinic acid catabolism region of the octopine-type Ti plasmid pTi15955, *Molecular Microbiology 31*, 339-347.

Ma, Y. and Ludden, P. W. (2001) Role of dinitrogenase reductase arginine 101 residue in dinitrogenase reductase ADP-ribosyltransferase binding, NAD binding and cleavage, *Journal of Bacteriology 183*, 250-256.

Macaluso, A., Best, E. A. and Bender, R. A. (1990) Role of the *nac* gene product in the nitrogen regulation of some Ntr-regulated operons of *Klebsiella aerogenes*, *Journal of Bacteriology 172,* 7249-7255.

MacFarlane, S. and Merrick, M. (1985) The nucleotide sequence of the nitrogen regulation gene *ntrB* and the *glnA-ntrBC* intergenic region of *Klebsiella pneumoniae*, *Nucleic Acids Research 13*, 7591-7606.

MacFarland, N., McCarter, L., Artz, S. and Kustu, S. (1981) Nitrogen regulatory locus '*glnR*' of enteric bacteria is composed of cistrons *ntrB* and *ntrC:* identification of their protein products, *Proceedings of the National Academy of Science USA 78,* 2135-2139.

MacGregor, C., Arora, S., Hager, P., Dail, M. and Phibbs Jr, P. (1996) The nucleotide sequence of the *Pseudomonas aeruginosa pyre-crc-rph* region and the purification of the *crc* protein, *Journal of Bacteriology 178,* 5627-5635.

Magasanik, B. (1982) Genetic control of nitrogen assimilation in bacteria, *Annual Review of Genetics 16*, 135-168.

Magasanik, B. (2000) Global regulation of gene expression, *Proceedings of the National Academy of Science USA 97*, 14044-14045.

Maheswaran, M. and Forchhammer, K. (2003) Carbon-source-dependent nitrogen regulation in *Escherichia coli* is mediated through glutamine-dependent GlnB signalling, *Microbiology 149,* 2163-2172.

Maniatis, T., Sambrook, J., and Fritsch, E. (1982) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Press, New York.

Martinez-Rodriguez, S., Las Heras-Vasquez, F., Clemente-Jiminez, J., and Rodriguez-Vico, F. (2004) Biochemical characterisation of a novel hydantoin racemase from *Agrobacterium tumefaciens* C58, *Biochimie 86*, 77-81.

Matcher, G. (2004) Characterization of the hydantoin-hydrolysing system of *Pseudomonas putida* RU-KM3$_S$, pp 1-189, PhD Thesis, Rhodes University.

Matcher, G., Burton, S., and Dorrington, R. (2004) Mutational analysis of the hydantoin hydrolysis pathway in *Pseudomonas aeruginosa* KM3$_S$, *Applied Microbiology and Biotechnology 65*, 391-400.

Matin, A. (1996) Role of alternative sigma factors in starvation protein synthesis-novel mechanisms of catabolite repression, *Research in Microbiology* 147, 494-505.

May, O., Siemann, M., Pietzsch, M., Kiess, M., Mattes, R., and Syldatk, C. (1998a) Substrate dependent enantioselectivity of a novel hydantoinase from *Arthrobacter aurescens* DSM3745: Purification and characterisation as a new member of cyclic amidases, *Journal of Biotechnology 61*, 1-13.

May, O., Habenicht, A., Mattes, R., Syldatk, C. and Siemann, M. (1998b) Molecular evolution of hydantoinases, *Biological Chemistry 379,* 743-747.

May, O., Nguyen, P. and Arnold, F. (2000) Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine, *Nature Biotechnology 18*, 317-320.

McPherson, M. and Wootton, J. (1983) Complete nucleotide sequence of the *E. coli gdhA* gene, *Nucleic Acids Research 11,* 5257-5266.

Mettke, I., Fiedler, U., and Weiss, V. (1995) Mechanism of activation of a response regulator: Interaction of NtrC-P dimers induces ATPase activity, *Journal of Bacteriology 177*, 5056-5061.

Merrick, M. and Edwards, R. (1995) Nitrogen control in bacteria, *Microbiological Reviews 59*, 604-622.

Meyer, P. and Runser, S. (1993) Efficient production of the industrial biocatalysts hydantoinase and *N*-carbamyl amino acid amidohydrolase: Novel non-metabolisable inducers, *FEMS Microbiological Letters 109*, 67-74.

Miller, R. (1973) Glutamate synthase from *Escherichia coli:* an iron-sulphide flavoprotein (pgs 183-203) In The Enzymes of Glutamine Metabolism, Editors: Prusiner, S.B. and Stadtman, E.R., Academic Press, New York.

Miller, J. (1992) A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria, Cold Spring Harbor Laboratory Press, USA.

Mizuno, T. (1998) His-Asp phosphotransfer signal transduction, *Journal of Biochemistry 123,* 555-563.

Moller, A., Syldatk, C., Schulze, M., and Wagner, F. (1988) Stereo- and substrate-specificity of a D-hydantoinase and a D-*N*-carbamyl amino acid amidohydrolase of *Arthrobacter crystallopoietes* AM2, *Enzyme and Microbial Technology 10*, 618-625.

More, N., Daniel, R. M. and Petach, H. H. (1995) The effect of low temperatures on enzyme activity, *Biochemical Journal 305*, 17-20.

Morin, A. (1993) Use of a D-hydantoinase extracted from legumes to produce *N*-carbamyl D-amino acids, *Enzyme and Microbial Technology 15*, 208-214.

Morin, A., Tran Trung, N.-H., LaPointe, G., and Dubeau, H. (1995) Conditions used with a continuous cultivation system to screen for D-hydantoinase producing microorganism, *Applied Microbiology and Biotechnology 43*, 259-266.

Morin, A., Hummel, W., Schutte, H., and Kula, M.-R. (1986) Characterization of hydantoinase from *Pseudomonas fluorescens* strain DSM84, *Biotechnology and Applied Biochemistry 8*, 564-574.

Moss, J., Stanley, S. and Levine, R. (1990) Inactivation of bacterial glutamine synthetase by ADP-ribosylation, *Journal of Biological Chemistry 265,* 21056-21060.

Mukohara, Y., Ishikawa, T., Watabe, K., and Nakamura, H. (1993) Molecular cloning and sequencing of the gene for a thermostable *N*-carbamyl-L-amino acid amidohydrolase from *Bacillus stearothermophilus* strain NS1122A, *Bioscience Biotechnology and Biochemistry 57*, 1935-1937.

Mukohara, Y., Ishikawa, T., Watabe, K., and Nakamura, H. (1994) A thermostable hydantoinase of *Bacillus stearothermophilus* NS1122A: Cloning, sequencing, and high expression of the enzyme gene, and some properties of the expressed enzyme, *Bioscience Biotechnology and Biochemistry 58*, 1621-1626.

Muse, W. B. and Bender, R. A. (1998) The *nac* (nitrogen assimilation control) gene from *Escherichia coli*, *Journal of Bacteriology 180,* 1166-1173.

Nagy, Z. and Chandler, M. (2004) Regulation of transposition in bacteria, *Research in Microbiology 155,* 387-398.

Nanba, H., Yamada, Y., Yajima, K., Takano, M., Ikenaka, Y., Takahashi, S. and Ohashi, T. (1996) Process for producing D-amino acids with composite immobilized enzyme preparation, US Patent 5962279.

Nanba, H., Ikenaka, Y., Yamada, H., Yajima, K., Takano, M., and Takahashi, S. (1998) Isolation of *Agrobacterium* sp. strain KNK712 that produces *N*-carbamyl-D-amino acid amidohydrolase, cloning of the gene for this enzyme, and properties of the enzyme, *Bioscience Biotechnology and Biochemistry 62*, 875-881.

Nakai, T., Hasegawa, T., Yamashita, E., Yamamoto, M., Kumasaka, T., Ueki, T., Nanba, H., Ikenaka, Y., Takahashi, S., Sato, M. and Tsukihara, T. (2000) Crystal structure of *N*-carbamyl D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases, *Structure* 8, 729-739.

Neal, R., Griffin, A., Scott, M., Shatzman, A. and Gorham, H. (1999) D-*N*-carbamoyl amino acid amidohydrolases and hydantoinase, US patent 5858759 awarded to Smithkline Beecham.

Neidhardt, F. C., Ingraham, J. L. and Schaechter, M. (1990) Physiology of the bacterial cell, Sinauer, Sunderland, Massachusetts.

Ninfa, A. and Atkinson, M. (2000) PII signal transduction proteins, *Trends in Microbiology 8,* 172-179.

Ninfa, A. and Bennett, R. (1991) Identification of the site of autophosphorylation of the bacterial protein kinase/phosphatase NRII, *Journal of Biological Chemistry 266,* 6888-6893.

Ninfa, A. and Magasanik, B. (1986) Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli, Proceedings of the National Academy of Science USA 83,* 5909-5913.

Nishida, Y., Nakamichi, K., Nabe, K. and Tosa, T. (1987) Enzymatic production of L-tryptophan from D, L-5-indolylmethylhydantoin by *Flavobacterium* sp., *Enzyme and Microbial Technology 9*, 721-725.

Nohaile, M. Kern, D., Wemmer, D., Stedman, K. and Kustu, S. (1997) Structural and functional analyses of activating amino acid substitutions in the receiver domain of NtrC: Evidence for an activating surface, *Journal of Molecular Biology 273,* 299-316.

Oakes, J., Castro, C. and Britt, M.B. (2001) Galactosylation thermodynamics of *E. coli* β-glactosidase by ONPG and PNPG, *Protein and Peptide Letters 8,* 303-306.

O'Donnell, S. M. and Janssen, G. R. (2001) The initiation codon affects ribosome binding and translational efficiency in *Escherichia coli* of *c*I mRNA with or without the 5' untranslated leader*, Journal of Bacteriology 183,* 1277-1283.

Ogawa, J. and Shimizu, S. (1994) β̃-ureidopropionase with *N*-carbamyl-α-L-amino acid amidohydrolase activity from an aerobic bacterium *Pseudomonas putida* IFO 12996, *European Journal of Biochemistry 223*, 625-630.

Ogawa, J. and Shimizu, S. (1997) Diversity and versatility of microbial hydantoin transforming enzymes, *Journal of Molecular Catalysis B: Enzymatic 2*, 163-176.

Ogawa, J. and Shimizu, S. (2002) Industrial microbial enzymes: their discovery by screening and use in large-scale production of useful chemicals in Japan, *Current opinion in Biotechnology 13*, 367-375.

Ogawa, J., Shimizu, S. and Yamada, H. (1993) *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. E222c purification and characterization, *European Journal of Biochemistry 212*, 684-691.

Ogawa, J., Chung, M.C.-M., Hida, S., Yamada, H. and Shimizu, S. (1994a) Thermostable *N*-carbamoyl-D-amino acid amidohydrolase: screening, purification and characterization, *Journal of Biotechnology 38*, 11-19.

Ogawa, J., Kaimura, T., Yamada, H. and Shimizu, S. (1994b) Evaluation of pyrimidine and hydantoin degrading enzyme activities in aerobic bacteria, *FEMS Microbiological Letters 122*, 55-60.

Ogawa, J., Min Kim, J., Nirdnoy, W., Amano, Y., Yamada, H. and Shimizu, S. (1995a) Purification and characterization of an ATP-dependent amidohydrolase, *N*-methylhydantoin amidohydrolase from *Pseudomonas putida* 77, *European Journal of Biochemistry 229*, 684-691.

Ogawa, J., Miyake, H. and Shimizu, S. (1995b) Purification and characterisation of *N*-carbamoyl-L-amino acid amidohydrolase with broad substrate specificity from *Alcaligenes xylosoxidans, Applied Microbiology and Biotechnology 43*, 1039-1043.

Oh, K.-H., Nam, S.-H. and Kim, H.-S. (2002) Improvement of oxidative and thermostability of *N*-carbamyl-D-amino acid amidohydrolase by directed evolution, *Protein Engineering 15*, 689-695.

Ohmachi, T., Nishino, M., Kawata, M., Edo, N., Funaki, H., Narita, M., Mori, K., Tamura, Y. and Asada, Y. (2002) Identification, cloning and sequencing of the genes involved in the conversion of D, L-2-amino-Δ^2-thiazoline-4-carboxylic acid to L-cysteine in *Pseudomonas* sp. strain ON-4a, *Bioscience Biotechnology and Biochemistry 66,* 1097-1104.

Olivieri, R., Fascetti, E., Angelini, L., and Degen, L. (1979) Enzymatic conversion of *N*-carbamoyl-D-amino acids to D-amino acids, *Enzyme and Microbial Technology 1*, 201-204.

Olivieri, R., Fascetti, E., Angelini, L., and Degen, L. (1981) Microbial transformation of racemic hydantoins to D-amino acids, *Biotechnology and Bioengineering 23*, 2173-2183.

Osuna, R., Boylan, S. A. and Bender, R. A. (1991) In vitro transcription of the histidine utilization (*hutUH*) operon from *Klebsiella aerogenes*, *Journal of Bacteriology 173,* 116-123.

Ottenheym, H. and Jenneskens, P. (1970) Synthetic amino acids and their use in fortifying foods, *Journal of Agricultural and Food Chemistry 18*, 1010-1014.

Pace, H. and Brenner, C. (2001) The nitrilase superfamily: classification, structure and function, *Genome Biology 2,* 1-9.

Pahel, G., Rothstein, D. and Magasanik, B. (1982) Complex *glnA-glnL-glnG* operon of *Escherichia coli, Journal of Bacteriology 150,* 202-213.

Parales, R. and Harwood, C. (1993) Construction and use of a new broad host range *lacZ* transcriptional fusion vector pHRP309 for gram-negative bacteria, *Gene 133*, 23-30.

Park, H., Luche, R., and Cooper, T. (1992) The yeast UME6 gene product is required for transcriptional repression mediated by the CAR1 URS1 repressor binding site, *Nucleic Acids Research 20*, 1909-1915.

Park, J.-H., Kim, G.-J., Lee, S.-G., and Kim, H.-S. (1998) Biochemical properties of thermostable D-hydantoinase from *Bacillus thermocatenulatus* GH-2, *Annals New York Academy of Sciences 864*, 337-340.

Pedersen, H. and Valentin-Hansen, P. (1997) Protein-induced fit: the CRP activator protein changes sequence-specific DNA recognition by the CytR repressor, a highly flexible LacI member, *The EMBO Journal 16,* 2108-2118.

Pesole, G., Gissi, C., Lanava, C. and Saccone, C. (1995) Glutamine synthetase gene evolution in bacteria, *Molecular Biology and Evolution 40,*189-196.

Pioszak, A. and Ninfa, A. (2003) Genetic and biochemical analysis of phophatase activity of *Escherichia coli* NRII (NtrB) and its regulation by the PII signal transduction protein, *Journal of Bacteriology 185,* 1299-1315.

Plummer, D. (1987) The quantitative estimation of amino acids using the ninhydrin reaction (pg 158) In Practical Biochemistry (3rd Edition), Editor: Plummer, D., Wiley-Interscience, New York.

Pope, M. R., Murrell, S. A. and Ludden, P. W. (1985) Covalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginine residue, *Proceedings of the National Academy Science USA 82,* 3173-3177.

Pribnow, D. (1975) Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter, *Proceedings of the National Academy Science USA 72,* 784-788.

Priefer, U. B., Simon, R. and Puhler, A. (1985) Extension of the host range of *Escerichia coli* vectors by incorporation of RSF1010 replication and mobilization functions, *Journal of Bacteriology 163,* 324-330.

Rawlings, D., Sewcheran, R. and Woods, D. (1986) Characterization of a broad host range mobilizable *Thiobacillus ferrooxidans* plasmid and the construction of *Thiobacillus* cloning vectors In Fundamental and applied biohydrometallurgy, Editors: Lawrence, R. W., Branion, R. M. R. and Ebnes, H. G., Elsevier Biomedical Press, Amsterdam.

Rawlings, D. and Kusano, T. (1994) Molecular genetics of *Thiobacillus ferrooxidans*, *Microbiological Reviews 58*, 39-55.

Rawlings, D. and Tietze, E. (2001) Comparative biology of IncQ and IncQ-like plasmids, *Microbiology and Molecular Biology Reviews 65*, 481-496.

Reitzer, L. (2003) Nitrogen assimilation and global regulation in *Escherichia coli*, *Annual Review of Microbiology 57*, 155-176.

Reitzer, L. and Magasanik, B. (1983) Isolation of the nitrogen assimilation regulator NR_I, the product of the *glnG* gene of *Escherichia coli*, *Proceedings of the National Academy of Science USA 80*, 5554-5558.

Reitzer, L. and Magasanik, B. (1985) Expression of *glnA* in *Escherichia coli* is regulated at tandem promoters, *Proceedings of the National Academy of Science USA 82*, 1979-1983.

Rice, E., Allen, M., Brenner, D. and Edberg, S. (1991) Assay for β-glucuronidase in species of the genus *Escherichia* and its applications for drinking-water analysis, *Applied and Environmental Microbiology 57,* 592-593.

Rippe, K., Mucke, N., and Schulze, A. (1998) Association states of the transcription activator protein NtrC from *E. coli* determined by analytical ultracentrifugation, *Journal of Molecular Biology 278*, 933.

Robison, K., McGuire, A., and Church, G. (1998) A comprehensive library of DNA binding site matrices for 55 proteins applied to the complete *E. coli* K12 genome, *Journal of Molecular Biology 284*, 241-254.

Rossbach, S., Schell, J., and de Bruijn, F. (1987) The *ntr*C gene of *Agrobacterium tumefaciens* controls glutamine synthetase (GSII) activity, growth on nitrate and chromosomal but not Ti-encoded arginine catabolism pathway, *Molecular Genetics and Genomics 209*, 419-426.

Rossbach, S, Schell, J, and de Bruijn, F, J (1988) Cloning and analysis of *Agrobacterium tumefaciens* C58 loci involved in glutamine biosynthesis; neither the *glnA* (GSI) nor the *glnII* (GSII) gene plays a special role in virulence, *Molecular Genetics and Genomics 212*, 38-47

Rothman, N., Rothstein, D., Foor, F and Magasanik, B. (1982) Role of *glnA*-linked genes in regulation of glutamine synthetsae and histidine formation in *Klebsiella aerogenes, Journal of Bacteriology 150,* 221-230.

Runser, S. and Meyer, P. (1993) Purification and biochemical characterization of the hydantoin-hydrolysing enzyme from *Agrobacterium species*: A hydantoinase with no 5, 6-dihydropyrimidine amidohydrolase activity, *European Journal of Biochemistry 213*, 1315-1324.

Runser, S. and Ohleyer, E. (1990) Properties of the hydantoinase from *Agrobacterium* sp. IP I-671, *Biotechnology Letters 12*, 259-264.

Runser, S., Chinski, N., and Ohleyer, E. (1990) D-*p*-hydroxyphenylglycine production from D, L-5-*p*-hydroxyphenylhydantoin by *Agrobacterium* sp., *Applied Microbiology and Biotechnology 33*, 382-388.

Runyen-Janecky, L., Sample, A., Maleniak, T. and West, S. (1997) A divergently transcribed open reading frame is located upstream of the *Pseudomonas aeruginosa vfr* gene, a homolog of *Escherichia coli crp, Journal of Bacteriology 179,* 2802-2809.

Sage, A., Proctor, W. and Phibbs, P. (1996) A two-component response regulator, *gltR,* is required for glucose transport activity in *Pseudomonas aeruginosa* PAO1, *Journal of Bacteriology 178,* 6064-6066.

Salinovich, O. and Montelaro, R. (1986) Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulphate-polyacrylamide gene electrophoresis, *Analytical Biochemistry 156,* 341-347.

Sambrook, J., Margolin, A., Russell, A., and Klibanov, A. (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Press, New York.

Sanchez, S. and Demain, A. (2002) Metabolic regulation of fermentation processes, *Enzyme and Microbial Technology 31*, 895-906.

Sanders, D., Gillece-Castro, B., Burlingame, A. and Koshland (Jnr), D. (1992) Phosphorylation site of NtrC, a protein phosphatase whose covalent intermediate activates transcription, *Journal of Bacteriology* 174, 5117-5122.

Sanger, F., Nicklen, S., and Coulson, A. (1977) DNA sequencing with chain terminating inhibitors, *Proceedings of the National Academy of Science USA 74*, 5463-5467.

Sareen, D., Sharma, R., Nandanwar, H., and Vohra, R. (2001) Two-step purification of a D-(-)-specific carbamoylase from *Agrobacterium tumefaciens* AM10, *Protein Expression and Purification 21*, 170-175.

Schleissner, C., Reglero, A. and Luengo, J. (1997) Catabolism of D-glucose by *Pseudomonas putida* U occurs via extracellular transformation into D-gluconic acid and induction of a specific gluconate transport system, *Microbiology 143,* 1595-1603.

Schulze, B. and Wubbolts, M. (1999) Biocatalysis for industrial production of fine chemicals, *Current opinion in Biotechnology 10*, 609-615.

Schwacha, A. and Bender, R. A. (1993) The *nac* (nitrogen assimilation control) gene from *Klebsiella aerogenes*, *Journal of Bacteriology 175,* 2107-2115.

Seeburg, P., Nusslein, C. and Schaller, H. (1977) Interaction of RNA polymerase with promoters from bacteriophage fd, *European Journal of Biochemistry 74,* 107-113.

Shahjee, H., Banerjee, K. and Ahmad, F. (2002) Comparative analysis of naturally occurring L-amino acid osmolytes and their D-isomers on protection of *Escherichia coli* against environmental stresses, *Journal of Biosciences 27,* 515-520.

Sharma, R. and Vohra, R. (1997) A thermostable D-hydantoinase isolated from a mesophilic *Bacillus* sp. AR9, *Biochemical and Biophysical Research Communications 234*, 485-488.

Shatters, R., Liu, Y. and Kahn, M. (1993) Isolation and characterization of a novel glutamine synthetase from *Rhizobium meliloti, Journal of Biological Chemistry 268,* 469-475.

Shaw, C. (1995) Introduction of cloning plasmids into *Agrobacterium tumefaciens*, *Methods in Molecular Biology 49: Plant gene transfer and expression protocols,* 33-37.

Shimizu, S., Shimada, H., Takahashi, S., Ohashi, T., Tani, Y. and Yamada, H. (1980) Synthesis of *N*-carbamyl-D-thienylglycine and D-2-thienylglycine by microbial hydantoinase, *Agricultural and Biological Chemistry 44,* 2233-2234.

Shine, J. and Dalgarno, L. (1975) Determinant of cistron specificity in bacterial ribosomes, *Nature 254,* 34-38.

Siemann, M., Alvarado-Marin, A., Pietzsch, M., and Syldatk, C. (1999) A D-specific hydantoin amidohydrolase: properties of the metalloenzyme purified from *Arthrobacter crystallopoietes*, *Journal of Molecular Catalysis B: Enzymatic 6*, 387-397.

Sivaraman, K., Seshasayee, A., Swaminathan, K., Muthukumaran, G. and Pennathur, G. (2005) Promoter addresses: revelations from oligonucleotide profiling applied to the *Escherichia coli* genome, *Theoretical Biology and Medical Modelling 2,* 20-28.

Soupene, E., He, L., Yan, D., and Kustu, S. (1998) Ammonia acquisition in enteric bacteria: Physiological role of the ammonia/methylammonium transport B (AmtB) protein, *Proceedings of the National Academy of Science USA 95*, 7030-7034.

Sperker, B., Tomkiewicz, C., Burk, O., Barouki, R. and Kroemer, H. K. (2001) Regulation of human β-glucuronidase by A23187 and thapsigargin in the hepatoma cell line HepG2, *Molecular Pharmacology 59*, 177-182.

Spinosa, M., Riccio, A., Mandrich, L., Manco, G., Lamberti, A., Iaccarino, M., Merrick, M., and Patriarca, E. (2000) Inhibition of glutamine synthetase II expression by the product of the *gstI* gene, *Molecular Microbiology 37*, 443-452.

Stadtman, E. (1973) A note on the significance of glutamine in intermediary metabolism (pgs 1-6) In The Enzymes of Glutamine Metabolism, Editors: Prusiner, S.B. and Stadtman, E.R., Academic Press, New York.

Stadtman, E. (1990) Discovery of glutamine synthetase cascade, *Methods in Enzymology* 182, 793-809.

Stewart, G. (1993) Catabolite repression in the gram positive bacteria: generation of negative regulators of transcription, *Journal of Cellular Biochemistry 51*, 25-28.

Studier, F. and Moffat, K. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, *Journal of Molecular Biology 189*, 113-130.

Stulke, J. and Hillen, W. (1999) Carbon catabolite repression in bacteria, *Current Opinion in Microbiology 2*, 195-201.

Su, W., Porter, S., Kustu, S. and Echols, H. (1990) DNA looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial *glnA* promoter, *Proceedings of the National Academy of Science USA* 87, 5504-5508.

Sudge, S., Bastawde, K., Gokhale, D., Kalkote, U., and Ravindranathan, T. (1998) Production of D-hydantoinase by halophilic *Pseudomonas* sp. NCIM5109, *Applied Microbiology and Biotechnology 49*, 594-599.

Suzuki, K., Hattori, Y., Uraji, M., Ohta, N., Iwata, K., Murata, K., Kato, A., and Yoshida, K. (2000) Complete nucleotide sequence of a plant tumor-inducing Ti plasmid, *Gene 242*, 331-336.

Syldatk, C., Cotoras, D., Dombach, G., Gross, C., Kallwaß, H., and Wagner, F. (1987) Substrate and stereospecificity, induction and metallodependence of a microbial hydantoinase, *Biotechnology Letters 9*, 25-30.

Syldatk, C., Laufer, A., Muller, R. and Hoke, H. (1990a) Production of optically pure D- and L-α-amino acids by bioconversion of D, L-5-monosubstituted hydantoin derivatives, *Advances in Biochemical Engineering/Biotechnology 41*, 29-75.

Syldatk, C., Mackowiak, V., Hoke, H., Gross, C., Dombach, G., and Wagner, F. (1990b) Cell growth and enzyme synthesis of a mutant of *Arthrobacter* sp. (DSM3747) used for the production of L-amino acids from D, L-5-monosubstituted hydantoins, *Journal of Biotechnology 14*, 345-362.

Syldatk, C., Muller, R., Siemann, M., Krohn, K. and Wagner, F. (1992) Microbial and enzymatic production of D-amino acids from D, L-5-monosubstituted hydantoins (pgs 75-127) In Biocatalytic production of amino acids and derivatives, Editors: Rozzell, D. and Wagner, F., Hanser.

Syldatk, C., May, O., Altenbuchner, J., Mattes, R. and Siemann, M. (1999) Microbial hydantoinases – industrial enzymes from the origin of life?, *Applied Microbiology and Biotechnology 51*, 293-309.

Tabor, S. and Richardson, C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase, *Proceedings of the National Academy of Science USA 84*, 4767-4771.

Taillades, J., Beuzelin, I., Garrel, L., Tabacik, V., Bied, C. and Commeyras, A. (1998) *N*-carbamoyl-α-amino acids rather than free α-amino acids formation in the primitive hydrosphere: a novel proposal for the emergence of prebiotic peptides, *Origins of Life and Evolution of the Biosphere 28,* 61-77.

Tait, R. C., Close, T. J., Lundquist, R. C., Hagiya, M., Rodriguez, R. L. and Kado, C. I. (1983) Construction and characterization of a versatile broad host range DNA cloning system for gram-negative bacteria, *Bio/Technology 1,* 269-275.

Takahashi, S., Kii, Y., Kumagai, H. and Yamada, H. (1978) Purification, crystallisation and properties of hydantoinase from *Pseudomonas striata*, *Journal of Fermentation Technology 56*, 492-498.

Thomas, G., Coutts, G. and Merrick, M. (2000) The *glnKamtB* operon, *Trends in Genetics 16,* 11-14.

Thony, B. and Hennecke, H. (1989) The -24/-12 promoter comes of age, *FEMS Microbiological Reviews 63*, 341-358.

Tian, Z.-X., Li, Q.-S., Buck, M., Kolb, A., and Wang, Y.-P. (2001) The CRP-cAMP complex and downregulation of the *glnAp2* promoter provides a novel regulatory linkage between carbon metabolism and nitrogen assimilation in *Escherichia coli*, *Molecular Microbiology 41*, 911-924.

Tiboni, O., Cammarano, P. and Sanangelantoni, A.M. (1993) Cloning and sequencing of the gene encoding glutamine synthetase I from the archaeum *Pyrococcus woesii*: Anomalous phylogenies inferred from analysis of archaeal and bacterial glutamine synthetase I sequences, *Journal of Bacteriology 175*, 2961-2696.

Tripathi, C., Bihari, V. and Tyagi, R. (2000) Microbial production of D-amino acids, *Process Biochemistry 35*, 1247-1251.

Tyler, B. (1978) Regulation of the assimilation of nitrogen compounds, *Annual Review of Biochemistry 47*, 1127-1162.

Tyler, B. and Magasanik, B. (1962) Molecular basis of transient repression of β-galactosidase in *Escherichia coli, Journal of Bacteriology 97,* 550-556.

Ullmann, A. (1996) Catabolite repression: a story without end, *Research in Microbiology 147,* 455-458.

Ueno-Nishio, S., Mango, S., Reitzer, L. and Magasanik, B. (1984) Identification and regulation of the *glnL* operator-promoter of the complex *glnALG* operon of *Escherichia coli, Journal of Bacteriology 160,* 379-384.

Valsa, J. and Felzenszwalb, I. (2001) Genotoxic evaluation of the effect of *Thuya occidentalys* tinctures, *Revista Brasileira de Biologia 61,* 329-332.

van Heeswijk, W., Rabenberg, M. Westerhoff, H. and Kahn, D. (1993) The genes of the glutamine synthetase adenylylation cascade are not regulated by nitrogen in *Escherichia coli, Molecular Microbiology 9,* 443-457.

van Heeswijk, W., Meinsma, R., Hoving, S., Molenaar, D., Kahnm D. and Westerhoff, H. (1995) An additional PII in *Escherichia coli*: a new regulatory protein in the glutamine synthetase cascade, *FEMS Microbiology Letters 132,* 153-157.

van Heeswijk, W., Wen, D., Clancy, P., Jaggi, R., Ollis, D., Westerhoff, H., and Vasudevan, S. (2000) The *Escherichia coli* signal transducers PII (GlnB) and GlnK form heterotrimers *in vivo*: fine tuning the nitrogen signal cascade, *Proceedings of the National Academy of Science USA 97*, 3942-3947.

Volkel, D. and Wagner, F. (1995) Reaction mechanism for the conversion of 5-monosubstituted hydantoins to enantiomerically pure L-amino acids, *Annals New York Academy of Sciences 750*, 1-9.

Volkman, B., Nohaile, M., Amy, N., Kustu, S. and Wemmer, D. (1995) Three dimensional solution structure of the *N*-terminal reciever domain of NTRC, *Biochemistry 34*, 1413-1424.

Wagner, T., Hantke, B., and Wagner, F. (1996) Production of L-methionine from D, L-5-(2-methylthioethyl)hydantoin by resting cells of a new mutant strain of *Arthrobacter* species DSM 7330, *Journal of Biotechnology 46*, 63-68.

Walshaw, D., Reid, C., and Poole, P. (1997) The general amino acid permease of *Rhizobium leguminosarum* strain 3841 is negatively regulated by the Ntr system, *FEMS Microbiological Letters 152*, 57-64.

Wang, Y.-P., Kolb, A., Buck, M., Wen, J., O'Gara, F., and Buc, H. (1998) CRP interacts with promoter-bound σ^{54}-RNA polymerase and blocks transcriptional activation of the *dctA* promoter, *The EMBO Journal 17*, 786-796.

Wang, W.-C., Hsu, W.-H., Chien, F.-T. and Chen, C.-Y. (2001) Crystal structure and site directed mutagenesis studies of *N*-carbamoyl-D-amino acid amidohydrolase from *Agrobacterium radiobacter* reveals a homotetramer and insight into a catalytic cleft, *Journal of Molecular Biology 306*, 251-261.

Wardhan, H., McPherson, M., and Sastry, G. (1989) Identification, cloning and sequence analysis of the nitrogen regulation gene *ntrC* of *Agrobacterium tumefaciens* C58, *Molecular Plant-Microbe Interactions 2*, 241-248.

Ware, E. (1950) The chemistry of hydantoins, *Chemical Reviews 46*, 403-470.

Warner, J. and Lolkema, J. (2003) CcpA-dependent carbon catabolite repression in bacteria, *Microbiology and Molecular Biology Reviews 67*, 475-490.

Watabe, K., Ishikawa, T., Mukohara, Y., and Nakamura, H. (1992a) Cloning and sequencing of the genes involved in the conversion of 5-substituted hydantoins to the corresponding L-amino acids from the native plasmid of *Pseudomonas* sp. strain NS 671, *Journal of Bacteriology 174*, 962-969.

Watabe, K., Ishikawa, T., Mukohara, Y., and Nakamura, H. (1992b) Identification and sequencing of a gene encoding a hydantoin racemase from the native plasmid of *Pseudomonas* sp. strain NS 671, *Journal of Bacteriology 174*, 3461-3466.

Watabe, K., Ishikawa, T., Mukohara, Y., and Nakamura, H. (1992c) Purification and characterisation of the hydantoin racemase of *Pseudomonas* sp. strain NS 671 expressed in *Escherichia coli*, *Journal of Bacteriology 174*, 7989-7995.

Weiss, V., Claverie-Martin, F., and Magasanik, B. (1992) Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription, *Proceedings of the National Academy of Science USA 89*, 5088-5092.

Weiss, V. and Magasanik, B. (1988) Phosphorylation of nitrogen regulator (NRI) of *Escherichia coli, Proceedings of the National Academy of Science USA 85,* 8919-8923.

White, F. and Nester, E. (1980) Relationship of plasmids responsible for hairy root and crown gall tumorigenicity, *Journal of Bacteriology 144,* 710-720.

Wiese, A., Syldatk, C., Mattes, R., and Altenbuchner, J. (2001) Organization of genes responsible for the stereospecific conversion of hydantoins to α-amino acids in *Arthrobacter aurescens* DSM 3747, *Archives of Microbiology 176*, 187-196.

Woese, C., Kandler, O. and Wheelis, M. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya, *Proceedings of the National Academy of Science USA 87,* 4576-4579.

Wood, D., Setubal, J., Kaul, R., Monks, D., Kitajima, J., Okura, V., Zhou, Y., Chen, L., Wood, G., Almeida Jr, N., Woo, L., Chen, Y., Paulsen, I., Eisen, J., Karp, P., Bovee Sr, D., Chapman, P., Clendenning, J., Deatherage, G., Gillet, W., Grant, C., Kutyavin, T., Levy, R., Li, M-J., McClelland, E., Palmieri, A., Raymond, C., Rouse, G., Saenphimmachak, C., Wu, Z., Romero, P., Gordon, D., Zhang, S., Yoo, H., Tao, Y., Biddle, P., Jung, M., Krespan, W., Perry, M., Gordon-Kamm, B., Liao, L., Kim, S., Hendrick, C., Zhao, Z-Y., Dolan, M., Chumley, F., Tingey, S., Tomb, J-F., Gordon, M., Olson, M. and Nester, E. (2001) The genome of the natural engineer *Agrobacterium tumefaciens* C58, *Science 294,* 2317-2323.

Wosten, M. (1998) Eubacterial sigma factors, *FEMS Microbiological Reviews 22*, 127-150.

Yagasaki, M. and Ozaki, A. (1998) Industrial biotransformations for the production of D-amino acids, *Journal of Molecular Catalysis B: Enzymatic 4*, 1-11.

Yamada, H. and Kumagai, H. (1978) Microbial and enzymatic processes for amino acid production, *Pure and Applied Chemistry 50*, 1117-1127.

Yamada, H., Shimizu, S., Kim, J.-M., Shinmen, Y. and Sakai, T. (1985) A novel metabolic pathway for creatinine degradation in *Pseudomonas putida* 77, *FEMS Microbiology Letters 30*, 337-340.

Yamashiro, A., Yokozeki, K., Kano, H., and Kubota, K. (1988) Enzymatic production of L-amino acids from the corresponding 5-substituted hydantoins by a newly isolated bacterium *Bacillus brevis* AJ12299, *Agricultural and Biological Chemistry 52*, 2851-2856.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, *Gene 33*, 103-119.

Yanofsky, M., Montoya, A., Knauf, V., Lowe, B., Gordon, M. and Nester, E. (1985) Limited-host-range plasmid of *Agrobacterium tumefaciens*: molecular and genetic analyses of transferred DNA, *Journal of Bacteriology 163,* 341-348.

Yanofsky, C. (1992) Transcriptional regulation: elegance in design and discovery (pgs 3-24) In Transcriptional regulation, Editors: McKnight, S. and Yamamoto, K., Cold Spring Harbor Laboratory Press.

Yokozeki, K., Nakamori, S., Eguchi, C., Yamada, K., and Mitsugi, K. (1987a) Screening of microorganisms producing D-*p*-hydroxyphenylglycine from D, L-5-(*p*-hydroxyphenyl) hydantoin, *Agricultural and Biological Chemistry 51*, 355-362.

Yokozeki, K., Nakamori, S., Yamanaka, S., Eguchi, C., Mitsugi, K., and Yoshinaga, F. (1987b) Optimal conditions for the enzymatic production of D-amino acids from the corresponding 5-substituted hydantoins, *Agricultural and Biological Chemistry 51*, 715-719.

Yokozeki, K. and Kubota, K. (1987c) Mechanism of asymmetric production of D-amino acids from the corresponding hydantoins by *Pseudomonas* sp., *Agricultural and Biological Chemistry 51*, 721-728.

Zhu, J., Oger, P., Schrammeijer, B., Hooykaas, P., Farrand, S., and Winans, S. (2000) The bases of crown gall tumorigenesis, *Journal of Bacteriology 182*, 3885-3895.

Zimmer, D., Soupene, E., Lee, H., Wendisch, V., Khodursky, A., Peter, B., Bender, R. and Kustu, S. (2000) Nitrogen regulatory protein C controlled genes of *Escherichia coli:* Scavenging as a defense against nitrogen limitation, *Proceedings of the National Academy of Science USA 97*, 14674-14679.

Zubay, G. (1988) Amino acids and amino acid-derived products (pgs 749-767) In Biochemistry (2nd Edition), Macmillan Publishing Company.

INTERNET REFERENCES

* Printed copies of the websites / homepages of the websites referred to in this work are included after the internet references.

The Institute of Genomic Research website
<http://www.tigr.org/software/TransTermResults/ntat01.html>

***Agrobacterium tumefaciens* C58 genome annotation**
<http://cancer.lbi.ic.unicamp.br/agroC58/>

National Centre for Biotechnology Information (NCBI) homepage
<http://www.ncbi.nlm.nih.gov/>

Brown (2005) B-132R Amino Acids: Highlighting Synthesis Applications
<http://www.bccresearch.com/biotech/B132R.html>

Moss (2006) Enzyme nomenclature: recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes by the Reactions they catalyse
<http://www.chem.qmul.ac.uk/iubmb/enzyme/>