Elucidation and Manipulation of the Hydantoin-Hydrolysing Enzyme System of *Agrobacterium tumefaciens* RU-OR for the Biocatalytic Production of D-amino acids

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by

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Abstract

There is widespread interest in the biocatalytic production of enantiomerically pure D-amino acids for use in the synthesis of antibiotics, insecticides, herbicides, drug carriers and many other pharmaceuticals. Hydantoin-hydrolysing enzyme systems can be successfully utilised to stereoselectively convert racemic hydantoins into enantiomerically pure amino acid products. In fact, the use of microbial D-hydantoinase and D-stereoselective *N*-carbamoyl amino acid amidohydrolase activity to produce D-*p*-hydroxyphenylglycine from D,L-5-*p*-hydroxyphenylhydantoin has been described as one of the most successful biotechnological applications of enzyme technology developed to date.

A need to utilise the novel biodiversity of South African microorganisms for the development of an indigenous process to produce enantiomerically pure amino acids was identified in 1995. Subsequently, the Rhodes Hydantoinase Group was established and several local hydantoin-hydrolysing microorganisms were isolated. The research in this study describes the isolation and selection of *Agrobacterium tumefaciens* RU-OR, which produced D-stereoselective hydantoin-hydrolysing activity. Characterisation of the hydantoin-hydrolysing enzyme system of RU-OR revealed novel biocatalytic properties, and potential for the application of this strain for the biocatalytic production of D-amino acids.

A fundamental understanding of the regulation of hydantoin-hydrolysing enzyme activity in *A. tumefaciens* RU-OR was established, and utilised to produce mutant strains with altered regulation of hydantoin-hydrolysing activity. These strains were used to further elucidate the mechanisms regulating the production of hydantoins-hydrolysing activity in *A. tumefaciens* RU-OR cells. Overproduction of hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase activity in selected mutant strains resulted in efficient conversion of D,L-5-p-hydroxyphenylhydantoin

to D-*p*-hydroxyphenylglycine. Thus the establishment of a primary understanding of the hydantoin-hydrolysing enzyme system in *A. tumefaciens* RU-OR could be used to manipulate the hydantoin-hydrolysing activity in RU-OR cells to produce an improved biocatalyst.

The isolation of *A. tumfecaiens* RU-OR genes encoding for hydantoin-hydrolysing activity revealed two separate *N*-carbamoyl-D-amino acid amidohydrolase-encoding genes (*ncaR1* and *ncaR2)* in this bacterium with distinct chromosomal locations, nucleotide coding sequence and predicted primary amino acid sequence.

The novel biocatalytic properties of the hydantoin-hydrolysing enzyme system in *A. tumefaciens* RU-OR and mutant derivatives present fascinating opportunities for further elucidation of the natural function, regulation and biocatalytic potential of hydantoin-hydrolysing enzymes.

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List of Abbreviations

AGE:	agarose gel electrophoresis
bp	base pairs
chromat.	chromatography
5-FU	5-fluorouracil
GS:	glutamine syntheatse
GT:	γ-glutamyl transferase
HMM:	hydantoin minial medium
HPG:	*p*-hydroxyphenylglycine
HPLC:	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactoside
λ	lambda
LB	Luria Bertani medium
MBP:	Maltose binding protein
MM:	minimal medium
MW:	molecular weight
NCA:	*N*-carbamoyl alanine
NCAAH:	*N*-carbamoyl amino acid amidohydrolase
N-C-D-HPG	*N*-carbamoyl-D-hydroxyphenylglycine
NCDAAH:	*N*-carbamoyl –D-amino acid amidohydrolase
NCLAAH:	*N*-carbamoyl –L-amino acid amidohydrolase
NCG:	*N*-carbamoyl glycine

PCR:	polymerase chain reaction
PAGE:	polyacrylamide gel electrophoresis
Temp.	temperature
TLC:	thin layer chromatography
2-TU	2,4-thiouracil
WCM:	wet cell mass
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Research Outputs

A. Journal Publications

1 **Burton, SG, Dorrington, RA, Hartley, CJ, Kirchmann, S, Buchanan, K and V Phehane.** 1998. Production of enantiomerically pure amino acids: characterisation of South African hydantoinases and hydantoinase-producing bacteria. *J. Molec. Catal. (B: Enzymic)* **224**:301 - 305.

2 **Hartley, CJ, Kirchmann, S, Burton SG and RA Dorrington**. 1998. Characterisation of hydantoin hydrolysis by *Agrobacterium tumefaciens* RU-OR and isolation of a novel constitutive mutant. *Biotechnol. Lett.* **20(7):** 67-72.

3 **Hartley, C.J., Manford, F., Burton, S.G. and R.A. Dorrington**. 2001. Overproduction of hydantoinase and *N*-carbamoylase enzymes by regulatory mutants of *Agrobacterium tumefaciens*. *Appl. Microbiol. Biotechnol.* Accepted for publication March 2001.

B. Patents

1 **AECI Limited, Dorrington, RA, Burton, SG and CJ Hartley.** 1999. Novel microorganisms, their use, and method for producing D-amino acids. South African Provisional Patent ZA99/5981. Currently in application process for full South African Patent.

C. International Conference Proceedings

1 **Burton, SG, Dorrington, RA, Gardner, MN, Hartley, C and S Kirchmann.** 1995. Isolation and characterisation of South African hydantoinase-producing bacterial strains. Joint ASM/SGM symposium on Bioremediation, Aberdeen Scotland. September,

2 **Burton, SG, Dorrington, RA, Gardner, MN, Hartley, C and S Kirchmann. 1995.** Hydantoin-hydrolysing activity of microbial enzymes. Biotrans'95, Warwick, Great Britain. September

3 **Burton, SG, Dorrington, R A, Hartley, CJ, Kirchmann, S, Matcher, G and V Phehane**. 1998. Characterisation and application of hydantoinases and hydantoinase-producing bacteria. 20th Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, Tenn., USA,.

4 **Hartley, CJ and RA Dorrington.** 1999. Biocatalytic production of D-amino acids - the selection and application of *Agrobacterium tumefaciens* RU-OR mutants which over-produce hydantoinase and *N*-carbamyl amino acid amidohydrolase. Fourth International Symposium on Biocatalysis and Biotransformations, Giardini-Naxos (Taormina), Italy.

5 **Hartley, CJ and RA Dorrington**. 1999. Regulatory and molecular characterisation of the D-selective hydantoin-hydrolysing enzyme system of *Agrobacterium tumefaciens* RU-OR. Fourth International Symposium on Biocatalysis and Biotransformations, Giardini-Naxos (Taormina), Italy.

D. 13 National Conference Presentations.

Chapter 1

General Introduction

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1.1 Introduction

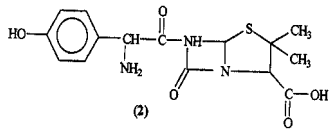
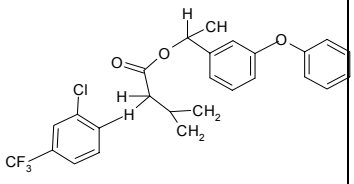
Traditionally, amino acids have been utilized as ingredients in feed and food manufacture, as pharmaceuticals and as chiral building blocks for chemical synthesis. However, enantiomerically pure D- and L-α-amino acids are becoming increasingly important as precursors for new insecticides, herbicides, semi-synthetic antibiotics and physiologically active peptides (Syldatk *et al*., 1990b; Durham & Weber, 1996, Ogawa and Shimizu, 1999). Thus they are of great interest to the food ingredients, pharmaceutical and agrochemical industries (Syldatk and Pietzsch, 1995). Amino acids for bulk use, such as L-glutamine, L-lysine and D,L-methionine are produced by bulk biological and chemical processes. Enzymatic and whole cell processes for the production of specialty amino acids for the fine chemical industry have been explored over the last thirty years, and many systems have been patented, particularly for the production of D-*p*-hydroxyphenylglycine. This amino acid is in great demand for the synthesis of amoxycillin, a semi-synthetic cephalosporin antibiotic (Drauz *et al*., 1997; Bommarius *et al*., 1998).

D-amino acids

Although most amino acid metabolism in bacterial cells involves the natural L enantiomers of amino acids, bacterial cells naturally use D-amino acids as important components of the peptidoglycan cell wall, and in other physiological roles such as the production of anti-bacterial compounds. The biological activity of D-amino acid containing molecules, attributable to the non-natural D-enantiomeric form, has been harnessed for various health and pharmaceutical applications. Another advantage of D-amino acid-containing pharmaceuticals is greater stability, due to decreased decomposition in the liver, kidneys and bloodstream (Bommarius *et al*., 1998). Commercially, D-amino acids have wide application in the synthesis of biologically active molecules such as semi synthetic antibiotics, peptide hormones and pyrethoid insecticides (Syldatk *et al*., 1990b, Wakayama *et al.*, 1996). They are also used as building blocks for the synthesis of sweeteners, and are mostly produced from 5-

substituted hydantoins using microbial systems involving D-hydantoinases and/or *N*-carbamoyl-D-amino acid amidohdyrolase (NCDAAH) enzymes (Syldatk *et al.*, 1990b; Syldakt and Pietzsch, 1995; Ogawa and Shimizu, 1997). Some examples of the commercial use of D-amino acids are listed in Table 1.1.

Table 1.1: Some examples of D-amino acids and their industrial applications (structures adapted from Bommarius *et al.,* 1998)

D-amino acid	Industrial Application	Structure
D-phenylglycine	Ampicillin	
D-hydroxyphenylglycine	Amoxicillin	
D-cysteine D-aspartic acid	Side chains of β-lactam antibiotics	e.g. Amoxicillin
D-citrulline	Analogue of lutenising hormone LHRH-antagonist	Ac-D-Nal-D-(p-Cl)-Phe-D-Pal-Ser-Tyr-D-Cit-Leu-Arg-Pro-D-ala-NH_2 e.g Cetrorellx
Poly-D-lysine	Drug carrier	
D-valine	Pyrethroid insecticide	 e.g. fluvalinate
D-glutamate D-proline	Synthetic pharmaceuticals	
D-alanine	synthetic sweeteners	

D-*p*-hydroxyphenylglycine (D-HPG) currently has the largest commercial demand of the D-amino acids as it used for the synthesis of the valuable semi-synthetic cephalosporin, Amoxycillin (Lee & Kim, 1998). Amoxycillin is formed from two fundamental molecules: a β-lactam moiety (6-aminopenicillinic acid) and a side chain moiety, such as D-HPG. Although the β-lactam moiety is what provides the antibacterial activity of the compound by disrupting the catalytic site of a transpeptidase enzyme involved in cell wall biosynthesis, the side chain determines the amount of bacterial resistance to the

antibiotic (Louwrier and Knowles, 1997). Non-natural amino acids, such as D-HPG, are therefore extremely useful as potent side chains to which bacteria cannot easily develop resistance (Table 1.1). D-Phenylgycine is used to produce ampicillin in a similar manner, whilst D-cysteine and D-aspartic acid also have commercial applications as side chains of β-lactam antibiotics (Louwrier and Knowles, 1997).

L-amino acids

Optically active L-amino acid enantiomers are also valuable commodities for both feed stock and pharmaceutical applications (Syldatk and Pietzsch, 1995). Natural amino acids are utilised in human health applications such as nutritional supplementation and infusion solutions. Essential amino acids like L-lysine, D,L-methionine and L-threonine have become of great commercial significance for the production of animal feed additives, whilst non-natural L-amino acids such as L-*t*-leucine and L-*t*-butylgycine have recently become of commercial interest for applications in the synthesis of antiviral and anti-tumorigenic pharmaceuticals (Drauz *et al*., 1997; Bommarius *et al*., 1998). L-aspartic acid is used to synthesize aspartame, which has industrial application as the main precursor for the production of polyaspartate, a chelant utilised in detergent manufacture (Brychem Business Consulting, 1999).

1.2 Biocatalytic production of optically active amino acids

Chiral intermediates constitute a large part of the fine chemicals market, and enantiomerically pure compounds are projected to form 70% of industrial pharmaceuticals in this century, as opposed to 25% in 1999 (Schulze and Wubbolts, 1999). The chemical synthesis of chiral molecules often involves resolution of racemic mixtures, which is time-consuming and expensive, with generally low yields of the desired product. Biocatalytic reactions involving dynamic kinetic resolution or asymmetric synthesis have therefore become a valuable tool for the synthesis of chiral compounds, such as enantiomerically pure amino acids (Schulze and Wubbolts, 1999). Microbial processes are the industrial production methods of choice for the synthesis of naturally occurring L-α-amino acids, and chemoenzymatic methods are very competitive with classical chemical resolution methods for the synthesis of D- or L-amino acids (Kamphius *et al*., 1990). A variety of cyclic ureide-hydrolyzing enzymes

catalyze the hydrolysis of 5-monosubstituted hydantoin to form *N*-carbamoyl amino acid, which can be cleaved by *N*-carbamoyl amino acid hydrolyzing enzymes or chemically decarbamoylated to produce amino acid (Figure 1.1). The strict stereoselectivity of many of these enzymes, and spontaneous or enzymatic racemization of the hydantoin substrate, allows for 100% yield of the desired enantiomerically pure amino acid.

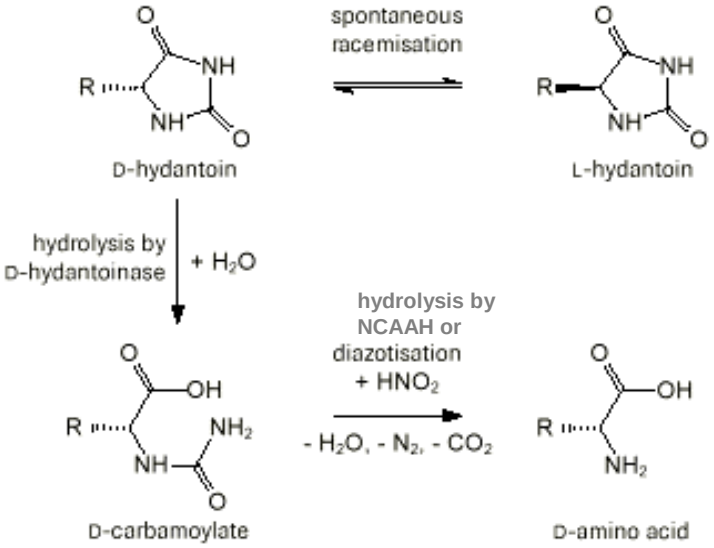


Figure 1.1: Reaction pathway for the hydrolysis of hydantoin to form enantiomerically pure amino acids (adapted from Bommarius *et al*., 1998).

The main applications of hydantoin-hydrolysing enzyme systems are in the production of enantiomerically pure amino acids, of which a bewildering variety can potentially be produced by using dynamic resolution with D-or L-selective hydantoin hydrolyzing enzymes (Schulze and Wubbolts, 1998). The advantages of utilizing hydantoin-hydrolysing enzyme systems include:

- low cost of substrate synthesis,
- wide substrate specificity for both natural and non-natural substrates yields a wide diversity of potential amino acid products,
- racemic substrates can be converted to 100% enantiomerically pure amino acid with either chemical or enzymic racemization,

The chemoselectivity, regioselectivity, stereoselectivity and diversity of hydantoin-hydrolysing enzymes offer the potential for the production a plethora of potentially industrially important amino acids from the hydrolysis of 5-monosubstituted hydantoins

(Figure 1.2). This has made dynamic kinetic resolution by hydantoin-hydrolysing enzymes a powerful tool in the biocatalytic production of enantiomerically pure amino acids such as D-HPG (Bommarius *et al*., 1998). Although side chains such as D-HPG can be chemically synthesised from the corresponding aldehyde or cyanic acid molecule, and the enantiomers separated by chiral resolution, the process is expensive and low yields make it unsuitable for the industrial synthesis of products such as amoxycillin and ampicillin. An alternative biotransformation route has been developed in which hydantoins synthesised by the Bucherer method (Bucherer and Steiner, 1976) are selectively cleaved by a D-hydantoinase to produce *N*-carbamoyl amino acid, and then cleaved by a non- or D-stereoselective *N*-carbamoyl amino acid amidohydrolase (NCAAH) enzyme to produce the desired D-amino acid as illustrated in Figure 1.2 (Yokozeki et al, 1987; Olivieri et al., 1981; Möller et al., 1988; Runser et al., 1990 for review see Syldakt and Pietzsch, 1995a).

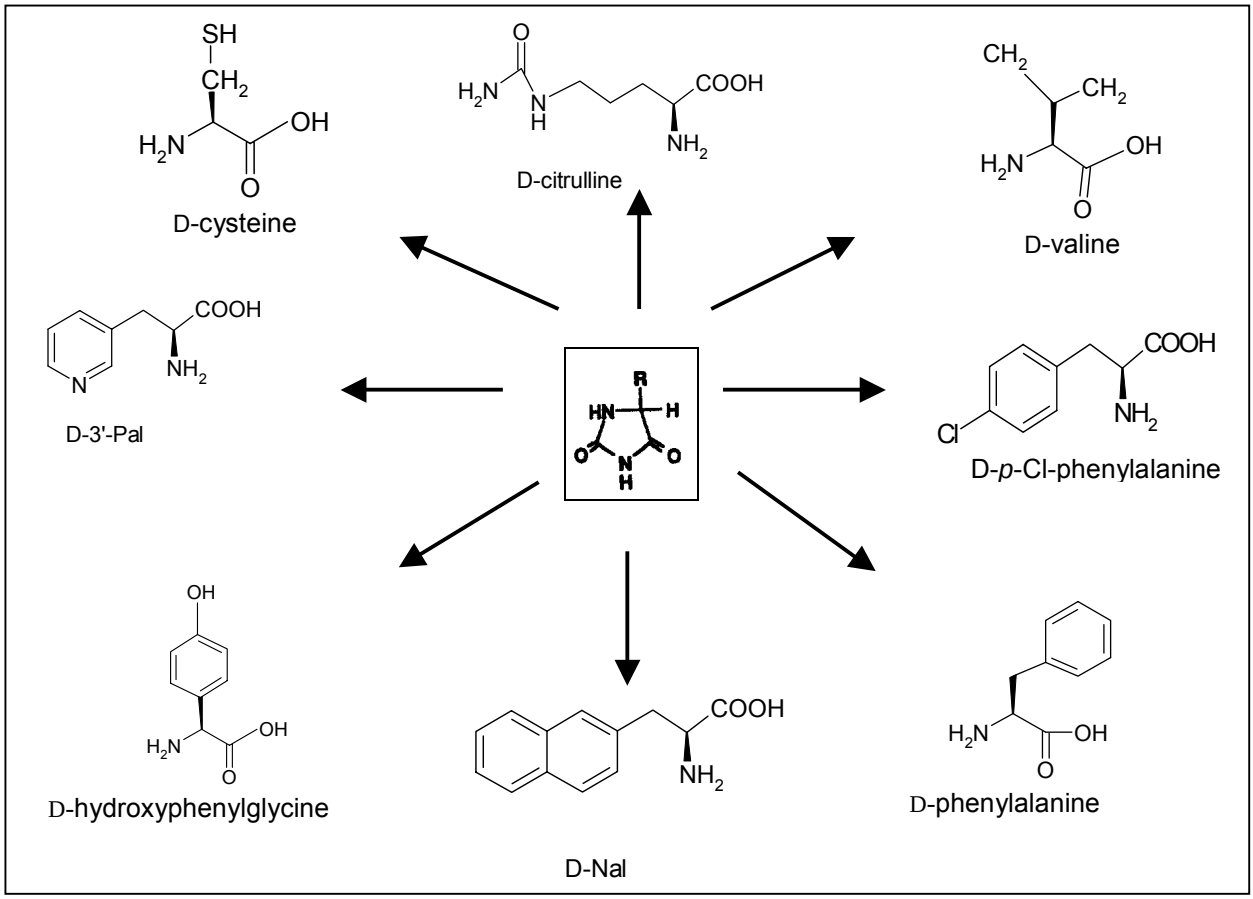


Figure 1.2: Some examples of the wide range of enantiomerically pure amino acids that can be produced by hydrolysis of hydantoin derivatives, with different R groups altering the amino acid product. (Structures adapted from Drauz, 1997; Bommarius *et al*., 1998).

Due to the advantage that spontaneous or enzymic racemization of the hydantoin substrate allows for theoretical yields of 100% D-amino acid, several optically active

amino acids are currently synthesised by this method, and numerous patents for the enzyme systems have been filed in collaboration with large industrial companies (Jacob et al., 1987; Siedel et al., 1989; Burtscher *et al*., 1997; Neal *et al*., 1999-; Grifantini *et al*., 1999; Galli *et al*., 1999; Nanba *et al*., 1999b; Gokhale *et al*., 2000). In fact, the D-hydantoinase plus NCAAH two-enzyme system for the production of optically active amino acids is purported to be one of the most successful industrial applications of enzyme technology thus far (Grifantini *et al*., 1996).

In the same manner as the D-hydantoinase method outlined above, L-hydantoinase and *N*-carbamoyl-L-amino acid amidohydrolase (NCLAAH) activities can be used to produce the desired L-enantiomer from a racemic hydantoin substrate. Whole cell biocatalysts with hydantoin-hydrolysing enzyme systems suitable for this application have been described for a variety of bacterial genera.

Current industrial processes concentrate on the use of whole or immobilized cells due to the instability of NCAAH enzymes. The only commercially available microbial hydantoin-hydrolysing enzymes are two thermostable recombinant hydantoinase enzymes, D-HYD1 and D-HYD2 marketed by Roche Molecular Biochemicals (Kiel *et al*., 1995), although Sigma-Aldrich markets a D-hydantoinase enzyme extracted from Aduki beans (Morin *et al*., 1993). However recent advances in both heterologous and homologous expression, purification and manipulation of recombinant hydantoin-hydrolyzing enzymes should result in advanced process designs for the production of enantiomerically pure amino acids (Kim *et al*., 1999, 2000, May *et al*., 2000, Ikenaka *et al*., 1998b,c, 1999). Immobilization techniques for enzymes as well as whole cells are also emerging (Nanba *et al*., 1998b), and may contribute to the expansion of industrial applications for hydantoin-hydrolyzing enzymes. A number of patents describing potential industrial applications of hydantoin-hydrolysing enzymes attest to the commercial value of this process (Burtscher *et al*., 1997; Drauz *et al*., 1998; Galli et al., 1999; Gokhale *et al*., 2000; Grifantini *et al*., 1999; Jacob *et al*., 1993; Kiel *et al.,* 1998; Morin *et al*., 1993; Nanba et al., 1999; Neal *et al*.,1999; Siedel *et al*., 1989; Wagner *et al*., 1998; Yamada *et al*., 1999).

1.2.1 Biocatalytic production using L-selective hydantoin hydrolysis

Until recently, only L-tryptophan and L-phenylalanine had major industrial applications as feed supplements or artificial sweeteners, respectively. However, a burgeoning interest in the application of non-natural L-amino acids, such as L-*t*-leucine has expanded the potential industrial applications of L-hydantoin hydrolyzing enzymes (Drauz *et al*., 1997; Brychem Business Consulting, 1999). Process design for the use of L-hydantoin-hydrolysing enzymes to produce L-tryptophan from D,L-5-indolylmethylhydantoin would require the use of immobilized cells with high L-hydantoinase, NCLAAH and hydantoin racemase activity, or the use of immobilized enzymes (Syldatk *et al*., 1992b).

1.2.2 Biocatalytic production using D-selective hydantoin hydrolysis

The use of hydantoin-hydrolysing enzyme systems for the production of D-amino acids has several advantages over other production systems. In addition to the general factors listed above, the wide substrate selectivity of the D-strereoselective hydantoin-hydrolysing has been advantageous, particularly for the production of non-natural amino acids such as D-*p*-hydroxyphenylglycine (D-HPG) and D-citrulline (Bommarius *et al*., 1998). The Italian company, Snamprogetti, first harnessed immobilized dihydropyrimidinase for the conversion of D,L-5-p-hydroxyphenylhydantoin (D,L-5-HPH) to *N*-carbamoyl-D-hydroxyphenylglycine (D-NCHPG), using chemical diazotization to convert the intermediate to D-p-hydroxyphenylglycine (D-HPG). Two Japanese companies, Kanegafuchi and Ajinomoto now produce D-HPG using immobilized resting cells with D-hydantoinase (*Pseudomonas* sp.) or both D-hydantoinase and NCDAAH activity (*Bacillus brevis* ATCC 8185), respectively. Immobilized *Agrobacterium radiobacter* NRRL B11292 resting cells are utilized for the production of D-HPG by another Italian company, Recordati (Syldatk and Pietszch, 1995; Bommarius *et al*., 1998).

Several problems are associated with the above methods for the production of D-amino acids. Firstly, the pH optima of the two enzymes involved in the conversion differ, and the production of NCDAAH activity in whole cells is more recalcitrant than hydantoinase activity. Native NCDAAH enzymes are not very stable, making separate immobilization

of the enzymes difficult too (Syldatk and Pietszch, 1995). Utilizing chemical methods to convert the *N*-carbamoyl amino acid to amino acid is successful but obviates the advantages associated with bioconversion. In addition, many of the desired substrates for industrial processes are only poorly soluble in aqueous systems, and at high temperatures. Some exploration has been made into the use of organic solvents as the medium for biocatalysis of hydantoins (Kim and Kim, 1993) with heterologous immobilized D-hydantoinase enzymes from *Bacillus* spp. Immobilized recombinant cells expressing the enzymes are able to convert 96% of 5g/L D, L-5-HPH to D-NCHPG in a heterogenous reaction systems (Lee and Kim, 1998). Recent advances in the production of recombinant hydantoin-hydrolysing enzymes and the exploration of structure-function relationships of the proteins have suggested that recombinant systems may provide solutions to many of the problems associated with bioconversion using hydantoin-hydrolysing enzymes (Chao *et al*., 1999a,b, 2000; Ikenaka *et al*., 1999; May *et al.,* 2000; Pietzsch *et al.,* 2000).

The following sections of this chapter will review the microbial and enzymatic production of D- and L- amino acids from D,L-5-monosubstituted hydantoins, focusing on hydantoinase and NCAAH activity, including whole cell activity, enzyme characteristics, molecular biology of these enzymes, regulation of enzyme production in bacteria and possible evolutionary relationships between the enzymes.

1.3 Chemical synthesis and properties of 5-monosubstituted hydantoin derivatives.

Hydantoins belong to the family of compounds known as pyrimidines, nuceleotide derivatives with a 5-membered ring structure (Ware, 1950). The term hydantoin is derived from the **hyd**rogenation of all**antion**, the reaction by which von Baeyer first crystallized this cyclic ureide compound in 1861 (Syldatk *et al*., 1992a). Other systematic names for hydantoin include imidazoline-2,4-dione or 2,4-diketotetrahydroimidazole. The term hydantoin is nowadays more generally applied to the family of hydantoin derivatives, including hydantoin, 5-monosubstituted hydantoin and 5,5-disubstituted hydantoins (Syldatk *et al*., 1992a,b; Syldakt and Pietzsch, 1995). A variety of methods are used for the chemical synthesis of 5-monosubstituted

hydantoins, including Bucherer-Bergs synthesis from carbonyl compounds (Bucherer and Steiner, 1934) and the condensation of aldehydes followed by reduction of the 5-arylkylidine hydantoin product, as illustrated in Figure 1.3 (Syldatk *et al*., 1992a). The methods above have been modified for the synthesis of non-natural hydantoin derivatives. Ohashi *et al.* (1981) synthesized 5-(4-hydroxyphenyl)-hydantoin (p-HPH) by amidoalkylation of phenol with glyoxylic acid and urea under acidic conditions. The advantage of this method for the synthesis of hydantoins is the elimination of toxic reactants such as sodium cyanide and 4-hydroxybenzaldehyde that also undergo product-contaminating side reactions. This method is now commonly used in the synthesis of p-HPH, which has important industrial application as the substrate for stereo-selective cleavage by hydantoin-hydrolysing enzymes to produce D-p-HPG.

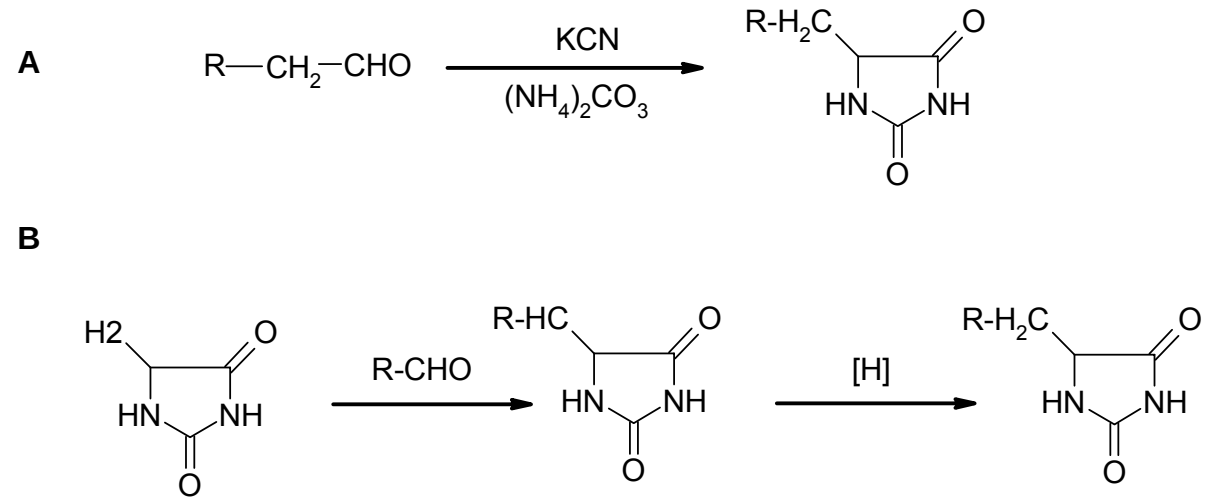


Figure 1.3: Chemical synthesis of C-5-monosubstituted hydantoins by Bucherer-Bergs synthesis (A) and condensation of aldehyde (B) (adapted from Syldatk *et al*., 1992a).

1.4 Natural biological distribution of microbial hydantoin cleavage

Hydantoins occur naturally as the breakdown products of purine and pyrimidine metabolism, and are therefore components of nucleotide metabolism in bacteria. Many of the compounds classified as hydantoins are accepted as substrates for enzymatic reactions, and the ability of certain microorganisms to grow using D,L-5-monosubstituted hydantoins as a sole nitrogen or carbon source was established in the 1940s (Syldatk and Pietzsch, 1995). Hydantoinase enzymes hydrolyse the hydantoin ring structure to form *N*-carbamoyl amino acids. A variety of different substrate selectivites are exhibited for hydantoinase enzymes, and although the reaction is generally stereoselective, non-selective hydrolysis also occurs. Stereoselective

NCAAH enzymes that irreversibly catalyze the hydrolysis of the *N*-carbamolylamino acids to form enantiomerically pure free amino acids often naturally accompany hydantoinase enzymes (Syldatk *et al*., 1992a,b; Ogawa and Shimizu 1997). A third enzyme, hydantoin racemase (Syldatk *et al*., 1990b, 1992b; Watabe *et al*., 1992c), may also be involved in the hydrolysis of hydantoin, acting to convert racemic hydantoins to a single enantiomer.

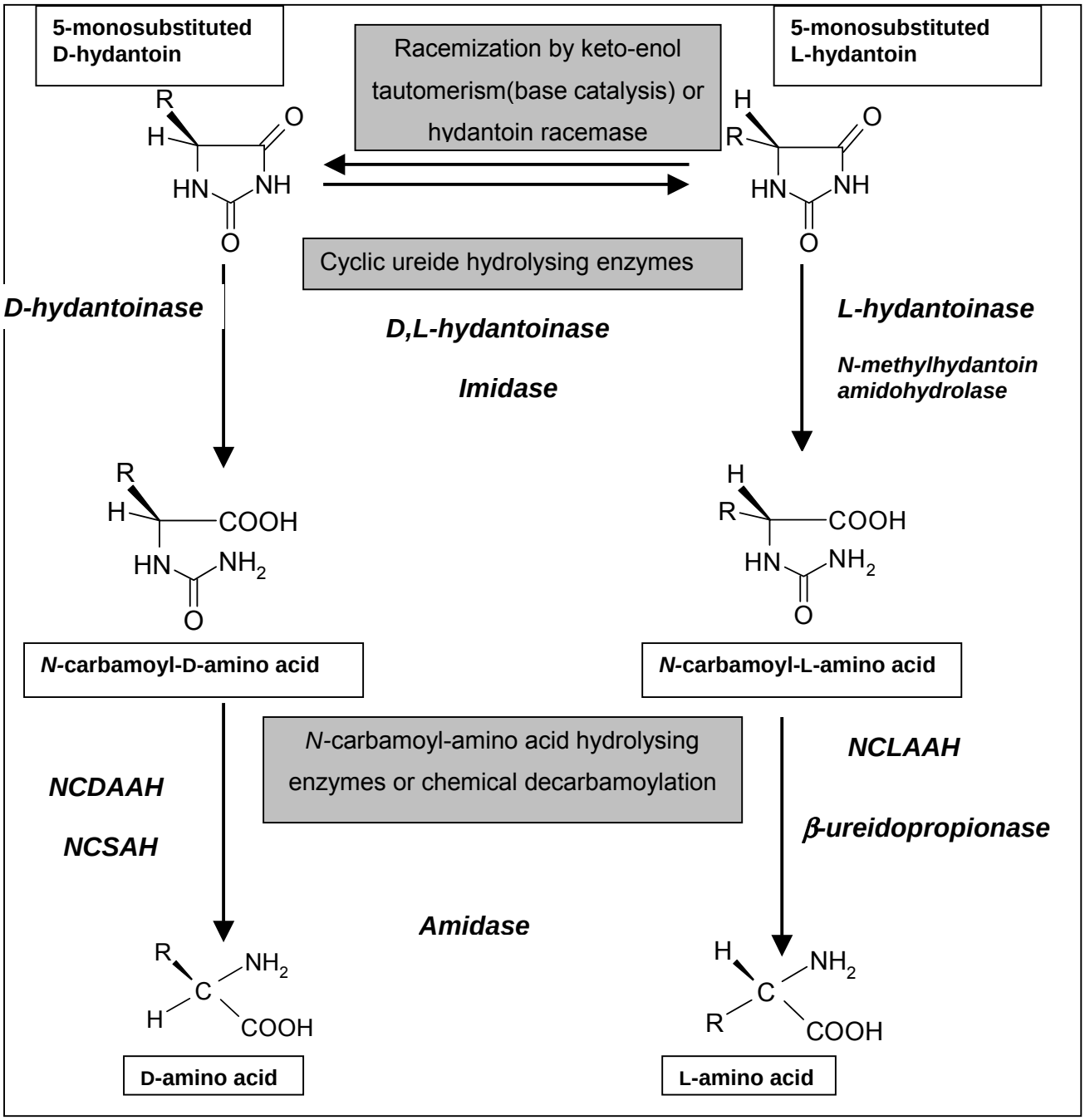


Figure 1.4: The hydrolysis of hydantoin to amino acid via *N*-carbamoyl amino acid by cyclic ureide-hydrolysing and *N*-carbamoyl-amino acid hydrolysing enzymes. NCSAH – *N*-carbamoylsarcosine amidohydrolase; NCDAAH –*N*-carbamoyl amino acid amidohydrolase; NCLAAH – *N*-carbamoyl amino acid amidohydrolase. (adapted from Ogawa and Shimizu, 1997).

The combination of these three enzymes results in the total conversion of D,L-5-monosubstituted hydantoins to enantiomerically pure amino acids. Other related cyclic ureide-hydrolyzing and *N*-carbamoyl amino acid hydrolyzing enzymes also catalyze the cleavage hydantoin and *N*-carbamoyl amino acids as illustrated in Figure 1.4. Among the cyclic ureide-hydrolyzing enzymes, D-hydantoinase is often regarded as being identical to dihydropyrimidinase and this led to the proposal that hydantoin-hydrolyzing enzymes involved in the production of D-amino acids from D,L-5-substituted hydantoins were in fact the enzymes involved in the pyrimidine degradation pathway (Yokozeki *et al*., 1987c; Runser *et al*., 1990b). Much contention surrounds this theory, however, and Runser and Meyer (1993) and Ogawa and Shimizu (1997) have demonstrated that the same enzyme does not necessarily catalyze co-existing cyclic ureide hydrolyzing and *N*-carbamoyl amino acid hydrolyzing activities in bacteria. Cyclic-ureide hydrolysis has also been linked to cyclic imide hydrolysis in eukaryotic cells. Hydantoinase enzyme activity does not generally correlate with imidase activity in bacteria, suggesting that different enzyme systems are involved in the two reactions (Ogawa *et al*., 1997a; Soong *et al*., 1998). However, overlapping hydantoinase and imidase activities for the D-hydantoinase enzyme from *Blastobacter* sp. A17p-4 (Ogawa *et al*., 1997b; Ogawa and Shimizu, 1997) belie this, and suggest a natural function for prokaryotic hydantoinases in cyclic-imide metabolism as well as pyrimidine metabolism (Soong *et al.,* 1999). Precise natural roles for all of the widely diverse hydantoin-transforming enzymes have not yet been elucidated. Accumulation of purified enzyme characteristics, molecular characterization of the genes encoding these enzymes and genetic *in vivo* studies should lead to a clearer understanding of the physiological role of hydantoin-hydrolyzing enzymes and the evolutionary relationships between them (Kim *et al.,* 2000b).

1.4.1 Microbial L-selective hydantoin hydrolysing enzyme systems

L-selective hydantoin cleavage seems to be less abundant than D-selective hydantoin cleavage (Section 1.5.2), as the ability to produce L-amino acids from D,L-5-monosubstituted hydantoins has only been reported for bacteria from the genera of *Arthrobacte*r (Guivarch *et al*., 1987; Gross et al., 1987;), *Bacillus* (Tsugawa *et al.,* 1966; Yamashiro *et al*., 1988), *Clostridium* (Liebermann and Kornberg, 1954, cited in Syldatk

et al., 1992b), *Flavobacterium* (Nishida *et al*., 1987; Yokozeki *et al*., 1987b), *Nocardia* (Klages *et al*., 1988) and *Pseudomonas* (Watabe *et al*., 1992a; Ogawa *et al*., 1995b; Buchanan *et al*., 2001). The enzymes involved in L-selective hydantoin hydrolysis from *Arthrobacter* spp. and *Flavobacterium* spp. have distinctly different substrate specificities and biochemical properties to those found in other genera (Syldatk *et al*., 1992b).

Induction with hydantoin analogues, such as 5-isopropylhydantoin (Yamashiro *et al*., 1988) or 5-indolylmethydantoin (Nishida et al., 1987; Syldatk *et al*., 1990a), is required for the production of L-selective hydantoin-hydrolyzing activity, and the production of these enzymes is also affected by the carbon and nitrogen sources utilized in the growth medium (Nishida *et al*., 1987; Yamashiro et al., 1988; Syldatk *et al*., 1992b;). Divalent manganese ions are also strictly required for the production of L-selective hydantoin-hydrolysing activity, but not cell growth (Syldatk *et al*., 1992b).

1.4.2 Microbial D-selective hydantoin hydrolysis

Microbial cells were first demonstrated to be good catalysts for the production of D-amino acids from racemic D,L-5-monosubstituted hydantoins by the group of Yamada in 1978 (Yamada *et al*., 1978a,b,c; Takahashi *et al*., 1978, 1979). Since then several bacteria able to hydrolyze D,L-5-monosubstituted hydantoins to D-amino acids have been described including *Arthrobacter crystallopoites* AM2 (Möller *et al*., 1988), *Blastobacter* sp. A17p-4 (Ogawa *et al*., 1994a), *Pseudomonas fluorescens* DSM 84 (Morin *et al.*, 1986a), *Pseudomonas striata* (Dinelli *et al.,* 1978), *Pseudomonas* sp. AJ-11220 (Yokozeki *et al*., 1987a), *Pseudomonas* sp. KNK0003A (Ikenaka *et al*., 1998a), *A. radiobacter* NRRL B11291 (Olivieri *et al*., 1981), *Agrobacterium* sp. IP 671 (Runser *et al*., 1990a), *Agrobacterium* sp. KNK712 (Nanba *et al*., 1998) and *Agrobacterium* sp. 80/44-2A (Neal *et al*., 1999). Several other microorganisms exhibit only D-hydantoinase or NCDAAH activity in isolation (Dagys *et al*., 1992; Lee *et al*., 1995, 1999; Lukša *et al*., 1997; Ogawa *et al*., 1994a, b) (Table1.2).

The production of D-hydantoin-hydrolysing activity in microorganisms is regulated by induction, and is normally expressed at highest levels after late exponential growth

phase in complete media (Möller et al., 1988; Meyer & Runser, 1993; Syldatk *et al*., 1990). Various different hydantoin and pyrimidine analogues are able to induce D-hydantoin-hydrolysing activity, and optimal inducers vary widely between species and strains, illustrating the diversity of D-selective hydantoin-hydrolyzing enzymes that occur in nature. Thiolated hydantoins and pyrimidine analogues are often utilized for the induction of D-hydantoin-hydrolysing in *Agrobacterium* species (Meyer and Runser, 1993), although *A. radiobacter* NRRL B11291 does not seem to require induction to produce hydantoin-hydrolyzing activity (Olivieri *et al*., 1979; Deepa *et al*., 1993).

Table 1.2: D-selective hydantoin-hydrolysing microorganisms

Strain	Enzymes (1,2,3)	Inducer	Substrate Preference	Reference
A. radiobacter NRRL B11291	(1) (2)	none	5-monosubstituted hydantoins with aromatic substituents	Olivieri *et al*. (1981)
Agrobacterium sp. IP 671	(1) (2)	2-thiouracil		Runser *et al*. (1990a), Meyer and Runser (1993)
Agrobacterium sp. KNK712	(1) (2)	-		Nanba *et al*. (1998)
Agrobacterium sp. 80/44-2A	(1) (2)	-		Neal *et al*. (1999)
Arthrobacter crystallopoites AM2	(1) (2)	dihydrouracil, hydantoin	D,L-methylhydantoin	Möller *et al*. (1988)
B. stearothermophilus SD-1	(1)	-	-	Lee *et al*. (1994, 1995)
B. thermocatenulatus GH-2	(1)	-	-	Lee *et al*. (1995)
B. circulans	(1)	-	-	Luksa *et al*., (1998)
Blastobacter sp. A17p-4	(1) (2)	dihydrouracil	dihydropyrimidines, 5-monosubstituted hydantoins	Ogawa *et al*. (1994) Ogawa and Shimizu (1997)
Comamonas sp. E222C	(2)	*N*-carbamoyl-amino acids	*N*-carbamoyl-D-amino acids	Ogawa *et al*. (1993)
Pseudomonas striata	(1) (2)	hydantoin		Dinelli *et al*. (1978)
Pseudomonas sp. AJ-11220	(1) (2)	5-cyanoethylhydantoin		Yokozeki *et al*. (1987a)
P. fluorescens DSM 84	(1)	hydantoin isopropylhydantoin	dihydrouracil	Morin *et al*. (1986a)
Pseudomonas sp. KNK0003A	(1) (2)	-	-	Ikenaka *et al*. (1998a)
Pseudomonas sp. RUKM1	(1) (2)	hydantoin	5-monosubstituted hydantoins with aromatic substituents	Burton *et al*.(1998)

Key: Enzymes: 1-hydantoinase, 2-NCAAH, 3-hydantoin racemase.

1.5 Hydantoinase enzymes

A variety of different types of hydantoinase enzymes occur which are able to hydrolyse 5-substituted hydantoins. Hydantoinase enzymes are classified as belonging to the EC 3.5.2 group of cyclic amidase enzymes, which also includes allantoinase, barbiturase, carboxymethylenehydantoinase, dihydropyrimidinase, dihydroorotase, penicillinase, imidazolonepropionase, 5-oxoprolinase, creatinase, indolylmethylhydantoinase and L-methylhydantoinase (Syldatk *et al*., 1990b). Webb (1992) classifies hydantoinase as an alternative name for the enzyme dihydropyrimidinase, which is involved in the catalysis of 5,6-dihydrouracil to 3-ureidopropionate. Although dihydropyrimidinase is able to hydrolyse hydantoins, many hydantoinases are not identical to dihydropyrimidinase, and so the term hydantoinase is now more broadly used to describe all enzymes which can hydrolyse hydantoin or 5-monosubstituted compounds (Syldatk *et al*., 1999). Recently, this group of enzymes was linked to a new superfamily of urease-related amidohydrolase enzymes, through computational determination of sequence homology, showing conserved substructures and catalytic principles within the proteins (Holm and Sander, 1997; Syldatk *et al*., 1999). All the members of the cyclic amidohydrolase group are involved in the biosynthesis or degradation of purines or pyrimidines. A new branch to this superfamily was recently described for the hydantoinase enzyme from *A. aurescens* DSM 3745 which exhibits surprising substrate-dependent enantioselectivity (May *et al*., 1998a, b). Certain other hydantoinase enzymes have not yet been classified, whilst *N*-methylhydantoinase, 5-oxoprolinase and the non-enantiospecific hydantoinase from *Pseudomonas* sp. NS671 do not belong to the amidohydrolase superfamily (May *et al*., 1998a). Thus the classification of hydantoinase enzymes by either phylogenetic relationships or enantioselectivity is not rigorous at present, although they share similar biochemical and structural properties regarding broad substrate selectivity, catalytic principles, quaternary structure, oligomerization and metal dependency (Kim *et al*., 2000b).

Table 1.3: Biochemical and genetic properties of L-selective and non-selective hydantoinase enzymes

Source	Substrate Selectivity	Stereo-selectivity	Optimal temp.	Temp. stability	Optimal pH	Metal Ion / Other Requirements	Gene (kb) [IM]	Protein (Subunit MW)	Reference
A. aurescens DSM 3747	BH, HBH, IPH, BMH, BMMH	substrate dependent	50	<46°C	8.8-9.25	Mn^{2+}, Co^{2+}	*hyuH* (2.55) [2]	tetramer (55.4 kDa)	Syldatk et al. (1992b), Pietzsch et al. (2000)
A. aurescens DSM 3745	D,L-ClBH>L-IMH	L	37°C	-	8.5	Zn^{2+} (catalytic) (4° structure)	-	tetramer (49.67 kDa)	May et al. (1998b,d,e)
Arthrobacter sp. DSM 7330	BeH>IMH>MTEH>H>PH	non	40°C	<47°C	8-9.4	Co^{2+} , Zn^{2+}	-	dimer (58kDa)	Volkel and Wagner (1995)
Arthrobacter sp. BH 20	arylalkylhydantoins	non	27°C		7.5	Mn^{2+}			Syldatk et al. (1987)
Bacillus brevis AJ 2299	BeH, IPH, MH, MTEH	L	50°C	-	8.0	ATP, $Mg^{2+,}$ Mn^{2+} , K^{+}	-	-	Yamashiro et al. (1988b)
Flavobacterium sp. AJ 3912	aromatic substituents	L	40°C	-	9.7	-	-	-	Yokozeki et al. (1987)
Pseudomonas putida 77	NMH>U>DHU>H	L	25-50	<60°C	6-10	ATP, Mg^{2+} , K^{+}	-	tetramer (70, 80 kDa)	Ogawa et al. (1995)
Pseudomonas sp. $KM3_S$*	n-BH>HPH>t-BH>MH	non	40°C		8	Mg^{2+} , Co^{2+}, Zn^{2+}, Mn^{2+}	-	-	Buchanan *et al.* (2001)
Pseudomonas sp. NS671*	LMTEH=IBH>BeH>BH>IPH	non	30°C	-	7.5	ATP	*hyuA, B*	dimer (76, 66kDa)	Ishikawa *et al*. (1997), Watabe *et al*. (1992a,b,c)

Key: * overall conversion, not just hydantoinase activity, MW- molecular weight, kb-kilobases. BH-butylhydantoin, BeH-benzylhydantoin, BMH-benzoylmethylenehydntoin, BMMH- 5-(s-benzoylmercaptomethylene)hydantoin, ClBH-chlorobenzylhydantoin, DHU-dihydrouracil H-hydantoin, IBH-isobutylhydantoin, IMH-indolylmethylhydantoin, MH- methylhydantoin, MTEH-5-(2-methylthioethyl)hydantoin, NMH-N-methylhydantoin PH-phenylhyantoin, HPH-hydroxyphenylhydantoin, U-uracil. IM- Isolation Method: 1 – selected from a genomic DNA library; 2 - isolated using PCR or degenerate oligos; 3 - found up/downstream of a previously isolated ORF

The first isolation and sequencing of a gene encoding hydantoinase activity was published in a patent filed by BASF, describing the isolation of the D-hydantoinase gene from a thermophilic *Bacillus* sp. LU 1220 (Jacob *et al*., 1987, 1993). Next, Watabe and colleagues isolated and sequenced the hydantoinase-, NCAAH- and racemase-encoding genes involved in L-selective hydantoin-hydrolysis by *Pseudomonas* sp. NS671 (Watabe *et al.*, 1992a,b). Since then several hydantoinases with D-, non- and L-stereo selectivity have been isolated and sequenced, and preliminary crystallisation data is available for the hydantoinase enzymes from *A. aurescens* DSM 3745 and *Thermus* sp. (May *et al*., 1996; Abendroth *et al*., 2000). The use of modern recombinant DNA technologies has allowed for many exciting advances in the hydantoinase field - recent groundbreaking publications include the fusion of genes encoding hydantoinase and NCAAH with other proteins to facilitate purification and immobilisation of these enzymes (Pietzsch *et al*., 2000; Kim *et al*, 2000a), and the use of directed evolution to invert the enantioselectivity of D-hydantoinase to L-hydantoinase (May *et al*, 2000).

1.5.1 L-selective and non-selective hydantoinase enzymes

The L-selective hydantoin-hydrolysing enzyme systems normally comprise an L-stereoselective or non-stereoselective hydantoinase enzyme coupled with an L-selective NCAAH and possibly a racemase (Volkel & Wagner, 1995). However, both L-selective and non-selective hydantoinase enzymes have been partially or fully purified from a variety of *Arthrobacter*, *Bacillus, Flavobacterium* and *Pseudomona*s species (Table 1.3). All the L-hydantoinases in which protein structure has been established comprise tetrameric or dimeric proteins with subunits of varying molecular weight. The two L-selective hydantoinases from the *Arthrobacter* species and *Flavobacterium* sp. AJ3912 do not require ATP for activity, and show a substrate preference for 5-arylalkylhydantoins, such as D- or L-methylthioethylhydantoin, as does the non-stereoselective hydantoinase from *Arthrobacter* sp. BH 20. However, the N-methylhydantoinase enzyme from *Pseudomonas putida* 77 (Ogawa *et al*., 1995b) and the non-enantiospecific hydantoinase enzyme isolated from *Pseudomonas* sp. NS 671 (Ishikawa *et* al., 1997) do not share this common substrate selectivity or primary amino acid homology with the other hydantoinase enzymes, and do not form part of the

urease-related amidohydrolase superfamily described previously (May *et al.*, 1998a). Two L-selective hydantoinase enzymes have been isolated from *Pseudomonas* sp. $KM3_S$ and *Pseudomonas* sp. $KM3_L$ (Burton *et al.,* 1998). The hydantoinase enzyme from *Pseudomonas* sp. $KM3_L$ is of particular interest as hydantoin-hydrolysing activity in crude extracts of this strain are optimum at 50°C, and the hydantoinase enzyme may be thermostable. The hydantoinase enzyme form *Pseudomonas* sp. $KM3_S$ exhibited non-stereoselective activity which is dependent upon the presence of divalent metal ions, such as Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, but is significantly inhibited by Cu^{2+} ions, and was able to efficiently convert aliphatic substrates into *N*-carbamoyl amino acids (Buchanan *et al.*, 2000).

The pH optima of the L- and non-selective hydanoinases vary widely, but are mostly in the slightly alkaline to alkaline range, whilst temperature optima are between 25°C and 50°C, no thermostable hydantoinases have been found to occur in this group.

L-selective hydantoinase enzymes

Two L-stereoselective hydantoinases have been isolated from the L-selective hydantoin-hydrolysing systems of *Arthrobacter aurescens* DSM 3747 and *Arthrobacter* sp. DSM 9771 (May *et al*., 2000; Pietzsch *et al*, 2000). Both genes, designated *hyu*H, are 2555 bp in size and encode 50kDa subunits of the tetrameric hydantoinase proteins (Table 1.3). The primary amino acid sequence of these enzymes is remarkably dissimilar to that of D-selective hydantoinases, although they share the common characteristics of the urease-related amidohydrolase protein superfamily. The hydantoinase gene products above, although classified as L-hydantoinases, actually show D-enantioselectivity towards (2-methylthioethyl)hydantoin as a substrate. May *et al*. (2000) reported the use of directed evolution of the *A. aurescens* DSM 9771 hydantoinase gene to change the enantioselectivity of the encoded hydantoinase enzyme. Random mutagenesis of the *Arthrobacter* sp. DSM 9771 hydantoinase using two rounds of error-prone PCR and screening followed by saturation mutagenesis produced a library of twenty thousand clones. These were screened for altered enantioselectivity first by microtitre plate assays comparing conversion of D- or L-5(2-methylthioethyl)hydantoin using cresol red as a pH indicator to detect a decrease in pH due to *N*-carbamoyl methionine production. Selected mutant clones were confirmed

using chiral HPLC analysis of the *N*-carbamoyl methionine product from a racemic substrate and a final evolved mutant hydantoinase that was L-selective towards 5-(2-methylthioethyl)hydantoin as a substrate, and fivefold more active than the original hydantoinase enzyme. Astonishingly, a single amino acid substitution of isoleucine at amino acid residue 95 to phenylalanine (I95F) was able to invert enantioselectivity (May *et al.*, 2000). Overall activity was also improved dramatically during the directed evolution process and this technology has enormous potential for the production of improved hydantoin-hydrolysing biocatalysts. Although the L-hydantoinase enzyme from *A. aurescens* DSM 3745 has been crystallised, molecular models of the protein and a detailed reaction mechanism have not yet been described (May *et al.,* 1996).

Non-stereoselective hydantoinase enzymes

Non-stereoselective hydantoinase enzymes have also been isolated from several microorganisms that produce L-amino acids from racemic hydantoins, including *Arthrobacter* sp. DSM 7330, *Arthrobacter* sp. BH 20, *Pseudomonas* sp. NS671 and *Pseudomonas* sp. $KM3_S$ (Table 1.3). The mechanism of conversion of the racemic hydantoin to enantiomerically pure amino acids in this instance is proposed to involve reversible, non-stereoselective cleavage of the hydantoin to form both D- and L-*N*-carbamoyl amino acids (Figure 1.5).

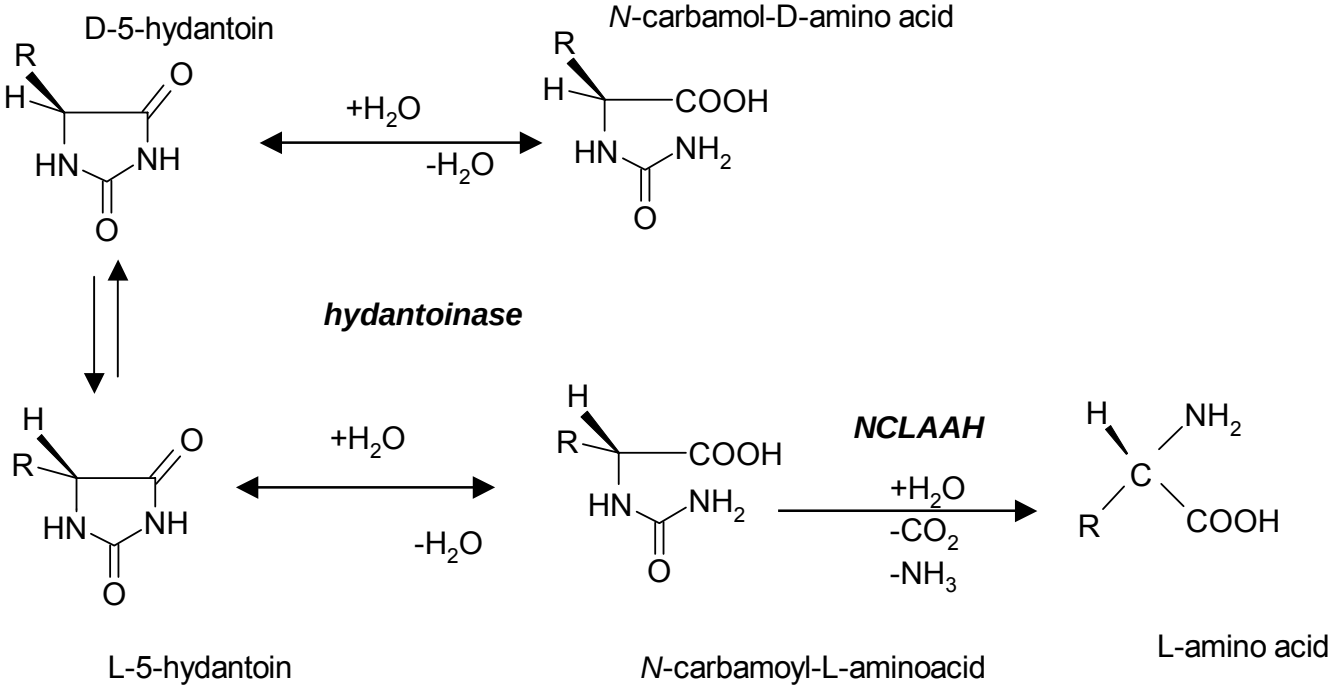


Figure 1.5: Proposed mechanism of conversion of D, L-hydantoin derivatives to enantiomerically pure L-amino acid by Arthrobacter sp. DSM 7330. Adapted from Volkel and Wagner (1995).

In fact, May *et al.* (1998b) illustrated that the hydantoinase from *A. aurescens* DSM 3745 shows substrate-dependent enantioselectivity, and the mechanism of conversion illustrated above may apply to other hydantoinase enzyme-substrate interactions. The biochemical characteristics and substrate specificities of these enzymes were similar to other L-hydantoinase enzymes, with pH and temperature optima between 8 -10, and 37°C - 50°C.

In the first reported cloning of a hydantoinase gene, the native plasmid pHN671 (172 kb) was isolated from *Pseudomonas* sp. NS671. A partial *Mbo* I library of pHN671 was constructed and clones were screened for the ability to grow using D,L-5-(2-methyltioethyl)hydantoin as a sole nitrogen source (Watabe *et al*, 1992a). The smallest selected plasmid, pHPB12 (7.5 kb) was found to encode four open-reading frames (ORFs) involved in the conversion of racemic 5-substituted hydantoins to L-amino acids (Watabe *et al*., 1992a,b). These four ORFs were designated *hyuA, B, C* and *E* and identified as encoding a non-stereoselective hydantoinase, an L-selective NCAAH and a racemase enzyme, respectively (Table 1). Both the ORFs *hyuA* and *hyuB* are required for expression of active non-stereoselective hydantoinase enzyme, and the conversion of D,L-5-(2-methylthioethyl)hydantoin to *N*-carbamoyl-D-methionine or *N*-carbamoyl-L-methionine. The deduced amino acid sequences of HyuA and HyuB share significant identity (40-47%), with two catalytic enzymes involved in the degradation of agropinnic acid, AgaF and AgaG (40-47%) (Lyi *et al.,* 1999), but not with other L-selective hydantoinases (May *et al.*, 1998a).

Mukohara *et al.* (1994) isolated a thermostable hydantoinase from *Bacillus stearothermophilus* NS1122A, selecting for growth with hydantoin as a sole nitrogen source. To allow growth of the *E. coli* clones, the library was constructed in a vector pAHA30 that already harboured the gene for *N*-carbamoyl L-amino acid amidohydrolase, and could therefore produce glycine and ammonia from the *N*-carbamoylglycine produced by the recombinant hydantoinase gene. The gene, *ORF(HN),* encoded a protein with a predicted molecular weight of 44 kDa which shared 91% amino acid sequence identity with a patented thermostable hydantoinase (Jacob *et al*, 1987). The gene had an abnormally low G+C content for a thermophilic enzyme-encoding gene. This reduced G+C content is reputed to be characteristic of

hydantoinases found in thermophilic bacteria (Mukohara *et al.*, 1994). The active homotetrameric hydantoinase enzyme encoded by *ORF(HN)* could be purified from the pelleted insoluble aggregates by solubilization and refolding of the protein, and was found to be a metalloenzyme, although not a Zn-metalloenzyme, as has been reported for other hydantoinase enzymes (May *et al.,* 1998d). The thermostability of the hydantoinase enzyme was related to the oligomeric structure of the protein, and could not be recovered from monomers after twenty-five minutes at 70°C (Mukohara *et al.*, 1994). The expressed hydantoinase enzyme was non-stereospecific but ten times more active towards the D-isomer of D,L-5-methylhydantoin than the L-isomer.

1.5.2 D-selective hydantoinase enzymes

The D-hydantoinase proteins share remarkable similarity in primary and secondary amino acid structure (Kim *et al*., 2000b). A number of D-hydantoinase enzymes from the microorganisms described in Table 1.2 have been purified and characterized (Table 1.4). D-hydantoinases have in common a wide substrate specificity and strict D-stereoselectivity (Pietzsch, and Syldatk, 1995). They mostly share similar biochemical characteristics such as optimal reaction temperatures between 45-60°C, pH optima of 8-9, and a requirement for divalent cations and a metal binding motif (GXXDXHXH) has been identified within the protein structure of a number of hydantoinases and other members of the amidohydrolase superfamily (May *et al*., 1998a). For this reason, chelating agents and sulfhydryl reagents, due to the involvement of cysteine residues in the active mechanism and the metal requirements of the enzyme, often inhibit the D-hydantoinase enzymes.

Morin *et al.* (1990) made a comparison of seven different D-hydantoinases isolated from various strains of *Corynebacterum pseudodiptheriticum*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Serratia liquefaciens*. All the hydantoinases except that isolated from *Corynebacterum pseudodiptheriticum* 14.10 were relatively unstable, sharing low purification recovery yields and stability up to 37°C and pH 5. Purification of the hydantoinase from *Corynebacterum pseudodiptheriticum* 14.10 yielded 75-85% recovery of the enzyme, which was stable at temperatures up to 57°C and at pH 8 or higher.

Table 1.4: Biochemical and Genetic Properties of D-hydantoinases enzymes.

Source	Substrate Selectivity	Optimal temp.	Temp. stability	Optimal pH	pH Stability	Metal Ion/Other Requirements	Gene (size in kb) [IM]	Protein Composition (Subunit MW)	Reference
A. radiobacter NRRL B11291	HPH>H	60°C		9	7.5-9	-	*ORF 2* (1.37) [3]	tetramer (50 kDa)	Olivieri *et al.* (1981), Grifantini *et al*. (1998), Chao *et al.* (1999a,b, 2000)
A. tumefaciens 47C	DHU>MH>BeH	70°C	<70°C	10	-	none	-	-	Durham and Weber (1995)
Agrobacterium sp. IP 671	BeH>HPH>IPH>IBH>DHU	60°C	<70°C	10	7.5-10.5	Co^{2+} Ni^{2+} Mg^{2+}	-	tetramer (62.5 kDa) [2]	Kim and Kim (1995) [1] Runser & Meyer (1993) [2]
Arthrobacter crystallopoites AM2	H>MH>DHU>BeH	50-60°C	<50°C	8.2-9.2	6.5	none	-	-	Möller *et al*. (1988)
Bacillus sp. AR9	H>HPH>aliphatic hydantoins	65°C	<70°C	9.5	>6.0	Co^{2+} Ni^{2+} Mg^{2+} Mn^{2+}	-	-	Sharma and Vohra (1997)
Bacillus sp. LU1220									Jacob *et al*. (1987)
B. stearothermophilus NS1122A	-	50°C	<70°C oligomer	8.8	>6.0	Mn^{2+}	*ORF HN* (1.416) [1]	tetramer (52 kDa)	Mukohara *et al.* (1 (1994)
B. stearothermophilus SD-1	H>HPH	65°C	<60°C	8	-	Mn^{2+}	*dht* (1.3) [1]	dimer (54 kDa)	Lee *et al.* (1995, 1996 b,c; 1997a,b)

Table 1.4: Biochemical and Genetic Properties of D-hydantoinases enzymes continued

Source	Substrate Selectivity	Optimal temp.	Temp. stability	Optimal pH	pH Stability	Metal Ion/Other Requirements	Gene (size in kb) [IM]	Protein Composition (Subunit MW)	Reference
B. thermocatenulatus GH-2	HPH>H	65°C	<75°C	7.5	6.0-9.5	Mn^{2+}	*dht* (1.416) [1]	tetramer (56 kDa)	Park *et al*. (1998), Kim *et al.* (1997)
B. circulans	H≡PH> long aliphatic side chains	75°C	<60°C	8-10	8.5-9.5	Co^{2+} Ni^{2+} Mn^{2+}		tetramer (53 kDa)	Lukša *et al*., (1998)
B. thermoglucosidans	PH>TEH> HPH>MH	80°C	<80°C	8.5	-	-	*D-HYD1*	tetramer (52 kDa)	Keil *et al*. (1995)
Blastobacter sp. A17p-4	H>DHU>DHT>MH	60°C	<60°C	9-10	5-8.5	Co^{2+} Ni^{2+} Mn^{2+}	-	tetramer (53 kDa)	Ogawa *et al*. (1995a)
Corynebacterium pseudodiphteriticum 14.10		57°C	<57°C						Morin *et al*. (1990)
Escherichia coli K-12	HPH>PH>IPH>H	45-50°C	<55°C	8-8.5	-	Co^{2+} Ni^{2+} Mn^{2+}	*hyuA* (1.395) [3]	tetramer (52 kDa)	Kim *et al*. (2000)
Pseudomonas sp. AJ-11220		55°C	-	8.5	-	-	-	-	Yokozeki *et al*. (1987d)
P. fluorescens 1.2		45-55°C	<45°C						Morin *et al*. (1990)
P. fluorescens 1.9		57°C	<45°C						

Table 1.4: Biochemical and Genetic Properties of D-hydantoinases enzymes continued

Source	Substrate Selectivity	Optimal temp.	Temp. stability	Optimal pH	pH Stability	Metal Ion/Other Requirements	Gene (size in kb) [IM]	Protein Composition (Subunit MW)	Reference
P. putida DSM 84	DHU>MTEH>IPH >BH≡H	45-50°C	<50°C	7.5	5.5-8.5	Fe^{2+} Mn^{2+}	*ORF 1* (1.104) [1]	tetramer (~60 kDa)	Morin *et al*. (1986a, 1990) LaPointe *et al*. (1994), Chein *et al*. (1998)
P. putida 2.2		45-50°c	<30°C						Morin *et al.* (1990)
P. putida 2.5		45-55°C	<37°C						
P. putida CCRC 12857	-						*dht* (1.485) [2]	tetramer (53.4 kDa)	Chein *et al*. (1998)
P. putida 7711-2	-						*dht* (1.44) [2]	tetramer (52.5 kDa)	Chen and Tsai (199)
P. striata	-	45-55°C	<60°C	8-9	6-7	Fe^{2+} Co^{2+}	-	oligomer 190 kDa	Takahashi *et al.* (1978)
Serratia liquefaciens	-								Morin *et al*. (1990)
Thermus sp.	TEH>PH>MH> MTEH	80°C	<80°C	8.5	-	-	*D-HYD2*	tetramer (52 kDa)	Lee and Lin (1996)

Key: MW-molecular weight, kb-kilobases. BH-butylhydantoin, BeH-benzylhydantoin, BMH-benzoylmethylenehydntoin, BMMH- 5-(s-benzoylmercaptomethylene)hydantoin, ClBH-chlorobenzylhydantoin, DHU-dihydrouracil H-hydantoin, IBH-isobutylhydantoin, IMH-indolylmethylhydantoin, MH- methylhydantoin, MTEH-5-(2-methylthioethyl)hydantoin, NMH-N-methylhydantoin PH-phenylhyantoin, HPH-hydroxyphenylhydantoin, THE-5-(2-thienyl)hydantoin, U-uracil. [IM]- Isolation Method: 1 – selected from a genomic DNA library; 2 - isolated using PCR or degenerate oligos; 3 - found up/downstream of a previously isolated ORF.

Alll the hydantoinases exhibited maximum hydantoinase activity between 45-55°C, and at alkaline pH values (greater than pH 8). Temperature and pH characteristics vary more widely amongst other purified D-hydantoinases, with several D-hydantoinase enzymes from *Agrobacterium* and *Bacillus* species having temperature optima of 65°C or higher, and stability at up to 70°C, and at higher pH values (Table 1.4). It is of interest to note that D-hydantoinase activity in bacteria is not always accompanied by NCAAH enzyme activity (Durham and Weber, 1995; Syldatk *et al*., 1992a; Pietzsch and Syldatk, 1995; Ogawa and Shimizu, 1997).

Webb (1992) classified microbial hydantoinases as synonymous with the enzyme dihydropyrimidinase. Although D-hydantoinases exhibit several biochemical properties similar to mammalian dihydropyrimidinase (Syldatk and Pietzsch, 1995), not all D-selective hydantoinases exhibit dihydropyrimidinase activity (Runser and Meyer, 1993; May *et al*., 1998a; Kim *et al*., 2000b). Co-existing D-hydantoinase and dihydropyrimidinase activities within bacteria are therefore not always catalyzed by the same enzyme (Ogawa and Shimizu, 1997), and D-hydantoinases cannot be regarded entirely as the microbial counterpart of the mammalian dihydropyrimidinase. Recently, Kim *et al.* (2000b) isolated a novel phenylhydantoinase from within a cluster of genes in *E. coli*.All the clustered genes were found to be involved in purine metabolism, and the expression of the phenylhydantoinase enzyme was discovered to strongly stimulate the expression of *tna A*, encoding tryptophanase activity in *E. coli* K-12. Further examination of the effect of gene deletions in this and other wildtype organisms should further elucidate the physiological and biochemical role of hydantoinase enzymes.

The German company, BASF, who published a patent involving the cloning of D-hydantoinase from *Bacillus* sp. LU1220 (Jacob *et al*., 1987, 1993), initiated genetic engineering of D-hydantoinases. A further eleven D-hydantoinases have been isolated and sequenced subsequently (Table 1.4), and several of these have been patented for industrial application (Jacob *et al*., 1987; Neal *et al*., 1999; Burtscher *et al*.,1997; Grifantini *et al.*, 1998).

The “workhorse” for research into the production of D-amino acids from D,L-5-substituted hydantoins has been *A. tumefaciens (radiobacter)* NRRLB11291 and the hydantoin-

hydrolysing enzymes from this strain are used in commercial applications for the production of D-*p*-hydroxyphenylglycine as the D-hydantoinase and D-NCAAH enzymes exhibit a high specificity for D,L-5-*p*-hydroxyphenylhydantoin as a substrate (Syldatk *et al*., 1992a). Grifantini *et al.* (1998) isolated the 1.37 kb D-hydantoinase gene approximately 100 base pairs upstream of the NCAAH gene from *A.radiobacter* NRRL B11291. The D-hydantoinase from *A. radiobacter* NRRL B11291, and indeed other *Agrobacterium* species are particularly suitable for industrial application as the D-hydantoinase enzymes exhibit a high substrate selectivity towards hydantoins with 5'-aromatic substituents such as D,L-5-HPH. These enzymes also have high optimal reaction temperatures and are fairly high degree of temperature stablity (Table 1.4). The gene encoding D-hydantoinase activity from *A. radiobacter* NRRL B11291 has been utilised in the heterologous production of D-HPG from D,L-5-HPH as will be discussed in section 1.11 (Chao *et al.,* 1999a,b, 2000).

The D-hydantoinase gene from *Pseudomonas putida* DSM 84 has been isolated and sequenced and showed three regions of high scoring sequence homology with other amidohydrolase enzymes, but shared no homology with the non-specific hydantoinase from *Pseudomonas* sp. NS671 and very low homology with the D-hydantoinases iolated from thermophilic microorganisms by Jacob *et al.* (1987) (LaPointe *et al*., 1994). This is consistent with the model of molecular evolution of hydantoinases proposed by May *et al*. (1998a) and Syldatk *et* al. (1999), which places most hydantoinases within the protein superfamily of amidohydrolases related to ureases but divides the family into a number of different gene families (see section 1.10). The *Pseudomonas putida* DSM 84 D-hydantoinase was used as a probe to detect hydantoinase genes from total DNA of other bacterial strains, but was only effective for the detection of D-hydantoinases from *Pseudomonas* strains of rRNA group I (LaPointe *et al*., 1994). A PCR probe based on the N-terminal region of the D-hydantoinase gene was then developed and found to amplify a 122bp fragment in bacteria with D-hydantoinase activity belonging to all *Pseudomonas* rRNA groups, or to the genera *Agrobacterium, Serratia, Corynebacterium and Arthrobacter,* under conditions of low stringency (LaPointe *et al*., 1995).

A "cocktail" of similar oligonucleotides synthesised to match the N-terminal region of D-hydantoinase was used to isolate the *dht* gene by Southern hybridisation to an *Eco* RI gene bank from *Pseudomonas putida* 7711-2 (Chen & Tsai, 1997). Whilst the D-hydantoinase genes from *Pseudomonas putida* CCRC 12857 and *Pseudomonas putida*

DSM 84 are identical and share significant identity with other reported D-hydantoinases (Chein *et al.*, 1998), differences were noted between the D-hydantoinase gene from *Pseudomonas putida* 7711-2 and these two D-hydantoinases, mainly in the C-terminal region of the coding sequence (Chen & Tsai, 1997).

Thermostable D-Hydantoinase Genes.

Thermostable D-hydantoinase are obviously advantageous for the conversion of D,L-5-hydroxyphenylhydantoin to *N*-carbamoyl-D-hydroxyphenylglycine due to the increased solubility of the substrate at higher temperatures. Thus, the cloning, overproduction and genetic engineering of thermostable D-hydantoinases has been an important goal for hydantoinase research.

The D-hydantoinase from *Bacillus stearothermophilus* SD1 was isolated and characterised. This D-hydantoinase enzyme exhibited low substrate specificity towards hydantoin derivatives with an aromatic substituent at the 5' –position, but the D-hydantoinase from *B. thermocatenulatus* GH2 exhibited high specificity for aromatically substituted hydantoins. The two enzymes differ in oligomeric structure but shared 100% sequence homology and 92% amino acid homology (Kim *et al.,* 1997). Only in the C-terminal regions of the two hydantoinases were amino acids completely mismatched, suggesting that the N-terminal region was the functional domain of the enzyme. PCR was used to delete 11 and 12 amino acid residues from the genes and construct mutant hydantoinase genes with truncated C-terminal regions. The truncated enzymes displayed higher thermostability than the wild-type enzymes and hybrid enzymes constructed by swopping the C-terminal regions of the two different D-hydantoinases with each other resulted in slightly improved specific activity towards aromatically-substituted hydantoins (Kim *et al.*, 1997).

1.6 *N*-carbamoyl amino acid amidohydrolase enzymes

Many kinds of enzymes with different stereoselectivities and regiospecificties are involved in *N*-carbmoyl amino acid hydrolysis. Both D- and L-stereoselective NCAAH enzymes occur in combination with D-, L- and non-selective hydantoinase enzymes in bacteria, and even an NCAAH enzyme independent of hydantoin-hydrolysing activity has been isolated (Ogawa *et al.,* 1993). Although most NCAAH enzymes consist of dimeric protein structures, trimeric enzymes have also been isolated, and there is a large amount of

variation in the biochemical properties of different NCAAH enzymes. No NCAAH enzymes with non-stereoselectivity or substrate-dependent stereoselectivity have been reported to date.

1.6.1 *N*-Carbamoyl-L-amino acid amidohydrolase enzymes

Nine different NCLAAH enzymes have been isolated and characterized from a similar diversity of bacterial strains as was described for L-hydantoinase activity. A natural physiological role has been described for only one of these enzymes, the L-selective ureidosuccinase from *Clostridium oroticum*, which converts *N*-carbamoyl aspartic acid to L-aspartic acid (Syldatk *et al*., 1992b). NCLAAH enzymes accept a wide range of substrates with both aliphatic and aromatic substituents. Some enzymes show a preference for aliphatic *N*-carbamoyl amino acid substrates (Yokozeki *et al.,* 1987b; Buchanan *et al*., 2001), whilst others exhibit faster hydrolysis rates with aromatic *N*-carbamoyl amino acids as substrates (Yokozeki *et al*., 1987b; Yamashiro *et al*., 1987a,b; Syldatk and Pietzsch, 1995). Yokozeki *et al.* (1987d) also examined crude NCLAAH activity in *Pseudomonas* sp. AJ-12299, and found that most rapid hydrolysis occurred with *N*-carbamoyl valine and *N*-carbamoyl leucine as substrates. A different NCLAAH enzyme has been described from *Pseudomonas* sp. $KM3_s$, with L-stereoselectivity, which also showed a high substrate affinity for *N*-carbamoyl-L-*t*-leucine and *N*-carbamoyl valine as substrates (Buchanan *et al.,* 2001).

The NCLAAH enzymes from *Flavobacterium* sp. AJ 3912 and *A. aurescens* DSM 3747 shared similar protein structures comprising two subunits of ~32 kDa each, and identical substrate specificities for a variety of aliphatic and aromatic *N*-carbamoyl-L-amino acids, Only *N*-carbamoyl-L-amino acids with an aromatic residue bound via a spacer to the alpha-carbon were accepted as substrates for these two NCLAAH enzymes (Syldatk and Pietzsch, 1995). An NCLAAH enzyme from *P. putida* IFO 12996 was purified and identified as being a β-ureidopropionase enzyme, and was able to hydrolyse *N*-carbamoyl-β-amino acids and *N*-carbamoyl-γ-amino acids as well as *N*-carbamoyl-α-amino acids, unlike β-ureidopropionase enzymes from mammals, protozoa and anaerobic bacteria which only hydrolyse *N*-carbamoyl-β-amino acids. The *P. putida* β-ureidopropionase enzyme shows strict L-stereoselectivity for *N*-carbamoyl-α-amino acids (Ogawa and Shimizu, 1997).

Table 1.5: Biochemical and genetic properties NCLAAH enzymes

Source	Substrate Selectivity	Optimal temp.	Temp. stability	Optimal pH	pH Stability	Metal Ion/Other Requirement	Gene (size in kb) [IM]	Protein Structure (Subunit MW)	Reference
Alcaligenes xylooxidans	N-carbamoyl-L-α-amino acids, long chain aliphatic or aromatic side chains	35°C	<30°C	8-8.3	6-9.5	Co^{2+}, Mn^{2+}, Ni^{2+}	-	dimer (65 kDa)	Ogawa *et al.* (1994)
Arthrobacter aurescens DSM 3747	β-substituted N-carbamoyl-α-alanines e.g. N-C-L-Trp	45°C	<35°C	9	6-6.5	Mn^{2+}, Fe^{2+}	*hyuC* (1.09) [2]	dimer (44kDa)	Syldatk *et al*. (1992b) Wilms *et al*. (1999)
Bacillus brevis AJ-12299	N-C-L-Ala, N-C-L-Val, N-C-L-Leu, N-C-L-Met, N-C-L-Phe, N-C-L-Tyr	40°C	-	7.5	-	Mn^{2+}, Fe^{2}	-	-	Yamashiro *et al.* (1988a,b)
Bacillus stearothermophilus NCIB8224	N-C-L-Ala>N-C-L-Glu>N-C-L-Met>N-C-L-Gly> N-C-L-Leu, N-acetyl-L-amino acids	-	-	-	-	-	*amaA* ( ) [3]	dimer (44 kDa)	Batisse *et al.* (1997)
Bacillus stearothermophilus NS1122A	-	-	-	-	-	-	*ORFNC* (1.230) [1]	dimer (44 kDa)	Mukohara *et al*. (1993)
Clostridium oroticum (ureidosuccinase)	N-C-L-Asp	-	-	7.8-8.5	-	Mn^{2+}, Fe^{2}	-	-	Liebermann & Kornberg(1955)

Table 1.5: Biochemical and genetic properties NCLAAH enzymes continued

Source	Substrate Selectivity	Optimal temp.	Temp. stability	Optimal pH	pH Stability	Metal Ion/Other Requirement	Gene (size in kb) [IM]	Protein Structure (Subunit MW)	Reference
Flavobacterium sp. AJ-3912	N-C-L-Gly, N-C-L-Ala, N-C-L-Val, N-C-L-Leu, N-C-L-Ile, N-C-L-Ser, N-C-L-Met, N-C-L-Asp, N-C-L-Ala, N-C-L-Phe, N-C-L-Trp, N-C-L-Tyr, N-C-L-BeSer, N-C-L-MSer	40°C	-	8.0	-	-	-	-	Yokozeki et al. (1987b)
Pseudomonas sp. AJ 11220	N-C-L-Val, N-C-L-Leu >N-C-L-Met, N-C-L-Asp, N-C-L-Phe, N-C-L-Tyr								Yokozeki et al. (1987d)
P . putida IFO 12996 (β-ureidopropionase)	N-carbamoyl-α-, N-carbamoyl-β and N-carbamoyl-γ-amino acids	60°C	<65°C	7.5-8.2	6-8.5	Co^{2+}, Fe^{2+} Mn^{2+}, Ni^{2}	-	dimer (44 kDa)	Ogawa and Shimizu (1997)
Pseudomonas sp. $KM3_s$	N-C-L-t-Leu> N-C-HPG>N-C-L-val	40°C		9-10		Co^{2+}, Mn^{2+}	-	-	Buchanan *et al*. (2001)

Key: MW- molecular weight, kb-kilobases. Substrates: *N*-C-L-amino acid -*N*-carbamoyl-L-amino acid. Amino acids abbreviated according to standard 3-letter code. Be –benzyl, M-methyl. [IM]- Isolation Method: 1 – selected from a genomic DNA library; 2 - isolated using PCR or degenerate oligos; 3 - found up/downstream of a previously isolated ORF.

L-selective hydantoin-hydrolysing activity has been identified in a variety of bacterial species, and L-stereoselective *N*-carbamoyl amino acid amidohydrolase encoding genes have been isolated and sequenced from *Arthrobacter aurescens* DSM 3747, *Bacillus stearothermophilus* NCIB8224 and *Pseudomonas* sp. NS671 (Table 1.5).The NCLAAH gene from *Arthrobacter aurescens* DSM 3747, designated *hyuC* was isolated and the protein shared 35-38% amino acid identity with other NCAAH enzymes. The recombinant enzyme was also strictly L-stereospecific but differed in a high substrate specificity for β-aryl substituted hydantoin derivatives (Wilms *et al*., 1999).

The native plasmid pHN671 of *Pseudomonas* sp. NS671 encoded an NCLAAH enzyme, a homodimer of 45.7 kDA, designated HyuC (Watabe *et al*., 1992a). The gene shares some sequence identity with the *hyuA* and *hyuB* genes of this strain (43-46%), which encode a non-specific hydantoinase enzyme and it was proposed that they may have evolved from a common ancestor by gene duplication events (Watabe *et al*., 1992a), although this seems unlikely in the light of subsequent analysis of NCLAAH-encoding genes (Wilms *et al.,* 1999). The *Pseudomonas* sp. NS671 *hyuC* gene has been expressed in *E.coli* and the purified protein shown to be strictly L-stereospecific, with broad substrate selectivity. The recombinant enzyme is also inhibited by ATP and sulphydryl groups, as is the wild-type enzyme (Ishikawa *et al.*, 1996). The only other reported NCAAH gene from a *Pseudomonas* strain is D-stereospecific (Ikenaka *et al*., 1998, see section 5.3.1).

The thermostable NCLAAH gene from *Bacillus stearothermophilus* NS1122A was isolated using growth with *N*-carbamoyl-L-methionine as sole nitrogen source as a selection criterion (Mukohara *et al*., 1993). The deduced amino acid sequence of this gene shared 46% homology with the mesophilic NCLAAH gene *hyuC* from *Pseudomonas* sp. NS671, but was active at temperatures 20°C higher than this enzyme. A comparison of the amino acid substitutions between the two genes suggested that the increased thermostability might be accompanied by high frequency of codons with a G or a C in the first position of the codon, and that a large number of Pro (proline) substitutions over the whole polypeptide chain may also enhance thermostability of the NCLAAH enzyme (Mukohara *et al*., 1993).

The cloning and sequencing of a second NCLAAH gene from *Bacillus stearothermophilus* NCIB8224 (*amaB)* showed that the gene shares an operon with an L-stereospecific

aminoacylase (*amaA)* and that the two genes operate off a single shared promotor. Southern blot analysis using PCR-amplified *amaA* and *amaB* genes as probes showed that this common operon structure is shared by several *B. stearothermophilus* strains. This was confirmed by PCR amplification and sequencing of the two genes from four different *B*. *stearothermophilus* strains (Batisse *et al*., 1997).

1.6.2 D-selective *N*-carbamoyl-amino acid amidohydrolase enzymes

D-hydantoinase enzymes are frequently associated with D-stereoselective NCAAH (NCDAAH) enzymes. Unlike D-hydantoinases, which are often similar or identical to dihydropyrimidinase, most NCDAAH enzymes do not resemble β-ureidopropionase (E.C. 3.5.1.6) as they cannot hydrolyse *N*-carbamoyl-β-amino acids, and show strictly D-stereoselective cleavage of *N*-carbamoyl-α-amino acids (Ogawa and Shimizu, 1997). The NCDAAH enzyme from *Arthrobacter crsytallopoites* AM 2 provides an exception to this rule (Möller *et al*., 1988). Recent information on the biochemical characteristics of purified NCDAAH and β-ureidopropionase proteins and amino-terminal amino acid sequence comparisons also illustrate that two different enzymes may catalyze the two reactions (Ogawa and Shimizu, 1997). Interestingly, Ogawa *et al*. (1993) isolated and purified an NCDAAH enzyme from *Comamona*s sp. E222C without associated hydantoinase activity.

Microbial NCDAAH activity is less stable than D-hydantoinase activity, and may be subject to product inhibition by ammonium ions (Runser *et al*., 1990b; Kim *et al*., 1994a; Ogawa and Shimizu, 1997; Nanba *et al*., 1998). The production of NCDAAH activity in bacterial cells can be induced by both hydantoin and *N*-carbamoyl amino acid derivatives in the growth medium (Syldatk and Pietzsch, 1995; Ogawa and Shimizu, 1997), and is also limited to high levels of expression until late exponential growth phase in complete medium (Möller *et al*., 1988; Syldatk *et al*., 1990a; Meyer & Runser, 1993).

NCDAAH enzymes generally require reducing agents to maintain the active enzyme (Louwrier and Knowles, 1997). Unlike the NCLAAH enzymes, some NCDAAH enzymes do not require metal ions for activity (Louwrier and Knowles, 1997, Ogawa *et al*., 1993,1994b; Olivieri *et al*., 1979), in common with β-ureidopropionase and *N*-carbamoyl

sarcosine amidohydrolase enzymes (Kim *et al*., 1987). NCDAAH enzymes catalyze the hydrolysis of a wide of variety of *N*-carbamoyl-D-amino acids to form D-amino acids. The enzymes are strictly D-stereoselective and are frequently inhibited by L-isomers of the *N*-carbamoyl amino acid substrate. Several NCDAAH enzymes and genes have been isolated and characterized from D-selective hydantoin-hydrolyzing microorganisms (Table 1.6).

Nine NCDAAH genes have been isolated and characterised from bacteria belonging to the *Agrobacterium, Arthrobacter, Pseudomonas* and *Bacillus* species groups (Table 1.6). Many of the reported NCDAAH genes are from *Agrobacterium* species. This is probably due to the industrial application of these particular enzymes for D-hydroxyphenylglycine production, as they all exhibit a high relative activity towards D,L-*N*-carbamoylhydroxyphenylhydantoin (Louwrier & Knowles, 1996). The British company, Smithkline Beecham, patented the cloning of the NCDAAH gene from *A. tumefaciens (radiobacter)* NRRLB112921, and the mating of this gene back into the parent *Agrobacterium* strain for efficient production of large amounts of NCAAH protein (Neal *et al*., 1999).A similar gene was obtained by screening clones from a genomic library of *A. radiobacter* NRRLB11291 for the production of ammonium from hydantoin, using the Berthelot assay (Buson *et al*., 1996) and again by screening a genomic library of *A. tumefaciens (radiobacter)* NRRLB11291 using degenerate oligonucleotides based on the N-terminal amino acid sequence of the *Comamonas* sp. E222c NCDAAH gene (Grifantini *et al*., 1998). A fourth NCDAAH gene was isolated from *Agrobacterium* sp. KNK712 by selection for recombinant plasmids from a genomic DNA library, which conferred the ability to utilise 5-methylhydantoin as a sole nitrogen source (Nanba *et al*., 1998). The deduced amino acid sequence of this NCDAAH gene was similar but not identical to the *A. radiobacter* NRRLB11291 gene, differing by nine amino acid residues.

Five cysteine residues were found in the NCDAAH amino acid sequence and analysed by site-directed mutagenesis and chemical derivatization methods (Grifantini *et al*., 1996). This analysis showed that only Cys172 is crucial for enzyme activity, and that this residue is exposed to solvent, and therefore probably lies on the outer surface of the tertiary protein structure.

Table 1.6: Biochemical and Genetic Properties of *N*-carbamoyl-D-amino acid amidohydrolase enzymes

Source	Substrate Selectivity	Optimal temp.	Temp. stability	Optimal pH	pH stability	Metal Ion/Other Requirements	Gene (size) [IM]	Protein Composition (Subunit MW)	Reference
A. radiobacter NRRL B11291	N-C-D-PG>N-C-D-HPG>N-C-D-ClPG	60°C	<40°C	7	7-9	none	*ORF1* (914 bp) [2]	dimer (34 kDa)	Olivieri et al. (1979), Grifantini et al., 1996; 1998)
							cauA (914 bp) [1]	dimer (34.3 kDa)	Buson et al. (1996)
Agrobacterium sp. KNK712	N-C-D-Met>N-C-D-PG≡ N-C-D-amino butyric acid> N-C-D-Leu> N-C-D-NorVal	65°C	<55°C	7	7	none	DCase (914 bp) [1]	dimer (34.3 kDa)	Nanba et al. (1998)
Agrobacterium sp.SB	N-C-D-Met,>N-C-D-Val> N-C-D-PG> N-C-D-HPG	70°C	<64°C	7.4-7.6	5.8-9	none	*car* (911bp) [1]	dimer (38 kDa)	Louwrier and Knowles (1996) Neal et al. (1999)
Arthrobacter crystallopoites AM2	N-C-D-amino acids (incl. with charged sidechains)	50°C	-	9.2	-	none	-	-	Möller et al. (1988)
Blastobacter sp. A17p-4	N-C-D-PG> N-C-D-HPG≡ N-C-D,L-HPG> N-C-D-Phe≡ N-C-D,L-Phe> N-C-D,L-NorLeu>	55°C	<50°C	8-9	6-9	none	-	trimer (40 kDa)	Ogawa et al. (1994b)

Table 1.6: Biochemical and Genetic Properties of *N*-carbamoyl-D-amino acid amidohydrolase enzymes continued

Source	Substrate Selectivity	Optimal temp.	Temp. stability	Optimal pH	pH stability	Metal Ion/Other Requirements	Gene (size) [IM]	Protein Composition (Subunit MW)	Reference
Comamonas sp. E222C	N-C-D-PG,>N-C-D-Met,>N-C-D-NorLeu> N-C-D-Phe> N-C-D-HPG	40°C	<40°C	8-9	7-9	none	-	trimer (40 kDa)	Ogawa et al. (1993)
P. putida 77 (N-carbamoylsarcosine amidohydrolase)	creatine metabolism	37°C	<40°C	7-8	6-7	-	-	tetramer (27 kDa)	Kim et al. (1986), cited In Syldatk and Pietzsch, 1995
Pseudomonas sp. KNK0003A	Non-aromatic sidechains e.g. *N*-C-D-Ala 10 fold> *N*-C-D-HPG	-	<73°C	7	7	-	Dcase (936 bp) [1]	dimer (38 kDa)	Ikenaka et al. (1998a)

Key: MW- molecular weight, kb-kilobases. Substrates: *N*-C-L-amino acid -*N*-carbamoyl-L-amino acid. Amino acids abbreviated according to standard 3-letter code. ClPG-chloro-phenylglycine, HPG-hydroxyphenylglycine, PG –phenylglycine, Be –benzyl, M-methyl. [IM]-Isolation Method: 1 – selected from a genomic DNA library; 2 - isolated using PCR or degenerate oligos; 3 - found up/downstream of a previously isolated ORF.IM- Isolation Method: 1 – selected from a genomic DNA library; 2 - isolated using PCR or degenerate oligos; 3 - found up/downstream of a previously isolated ORF.

Site-directed mutagenesis of the NCDAAH gene from *Agrobacterium* sp. KNK003A was used to produce increased thermostability and pH stability in the protein. Three amino acids substitutions produced a more stable mutant NCDAAH enzyme (455M): His57Tyr, Pro203Glu/Leu, Val236Ala (Ikenaka *et al*., 1998b, 1999). Thermostability was postulated to have increased due to the conformational changes caused by these mutations, removing positive charges and changing the stearic configuration of the protein structure (Ikenaka *et al*., 1998b, 1999). The thermostability of the mutagenized enzyme was increased by 5°C or 10°C and immobilization of the mutagenized enzyme using a phenol formaldehyde resin revealed that the half-life of the immobilized mutant recombinant enzyme was twice that of the immobilized native NCDAAH enzyme (Nanba *et al*., 1999a), making the mutant enzymes promising candidates for the production of D-amino acids.

A thermotolerant NCDAAH gene was selected from a genomic library of *Pseudomonas* sp. KNK003A, isolated, sequenced and the recombinant enzyme expressed in *E.coli* (Ikenaka *et al*., 1998a). The gene shared 62 % and 65 % sequence identity with the NCDAAH genes from *A. radiobacter* NRRLB11291 and *Agrobacterium* sp. KNK712, respectively, with only eight amino acid differences between these three NCDAAH enzymes in the N-terminal region of the protein. The C-terminal region of the *Pseudomonas* sp. KNK003A NCDAAH gene showed highest amino acid similarity (57%) with the C-terminus of the NCDAAH gene of *Comamonas* sp. E222c (Ikenaka *et al*., 1998a). Alignment of the gene with 10 known related enzyme sequences revealed six amino acids that are conserved, including Cys-172 which appears to be closely involved in enzyme activity as an Ala172Cys replacement in *Agrobacterium* sp KNK712 results in loss of enzyme activity (Ikenaka *et al*., 1998a).

Crystal Structure and Molecular Modelling of NCDAAH enzymes.

Hsu *et al.* (1998) performed preliminary crystallographic studies on the NCDAAH enzyme from *A. radiobacter* NRRL B11291 and recently published the crystal structure of the enzyme resolved to 1.95 Å using multiple isomorphous replacement (Wang *et al.,* 2001). Nakai *et al.* (2000) also recently resolved the crystal structure of the NCDAAH enzyme from *Agrobacterium* sp. KNK712 to 1.7Å. For both enzymes, protein structure was homotetrameric, with each monomer composed of a variant of α helix + β fold structure. The NCDAAH enzyme from *Agrobacterium* sp. KNK712 has a topology

unique among amidohydrolase enzymes, with a sandwich of β sheets surrounded by two layers of α-helices (Figure 1.6). In confirmation of the proposed role of Cys 172 (171) in the mechanism of action of NCDAAH enzymes, each monomer was proposed to have an active site pocket comprising the catalytic residues Glu 46, Lys 126 and Cys 171. The position of these residues on the edges of three β-strands (β2, β5, β7) stabilizes the geometry of the active site, whilst the pocket structure is formed by two α helices (α2, α6) and the loops connecting several strands (β4 and α5, β7 and α5, β8 and α6) (Nakai *et al.*, 2000). The gate to the active site pocket is narrowed by a covering α helix (α7). Wang *et al.* (2001) have further demonstrated that three histidine residues (His129, His144 and His215) have strict geometric configurations required to maintain stable conformation of the putative catalytic cleft.

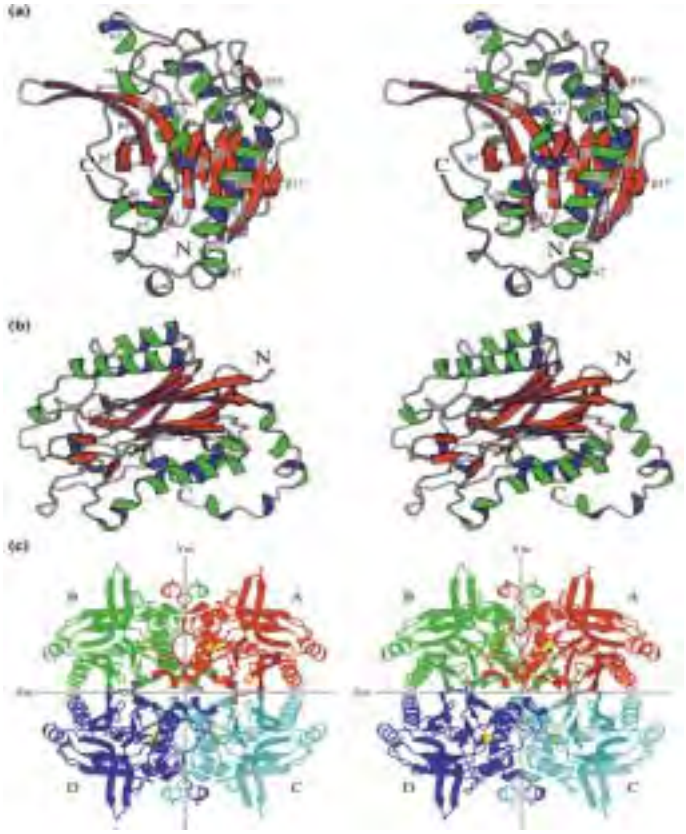


Figure 1.6: Schematic ribbon diagram of the structure of NCDAAH from *Agrobacterium* sp. KNK712. (A) Front view with secondary structure elements shown in green/blue (α helices) and red/purple (β strands).(B) Side view of secondary structure. (C) Schematic ribbon diagram along the X_m axis with the 4 monomers A,B,C, and D coloured red, green, cyan and blue respectively. (Reproduced by kind permission of H. Nakai from Nakai *et al*., 2000).

- The NCDAAH enzyme from *Agrobacterium* sp. KNK 712 shares a similar 4-layer sandwich architecture to several other amidohydrolases belonging to the

superfamily of N-terminal nucleophile (Ntn) hydrolases, but differs to these enzymes in topological structure and active site organization (Nakai *et al.,* 2000). A model of the active site of the NCDAAH enzyme from *Agrobacterium* sp. KNK 712 (Figure 1.7) shows the amino acid residues involved in binding and catalysis of *N*-carbamoyl amino acid substrates.

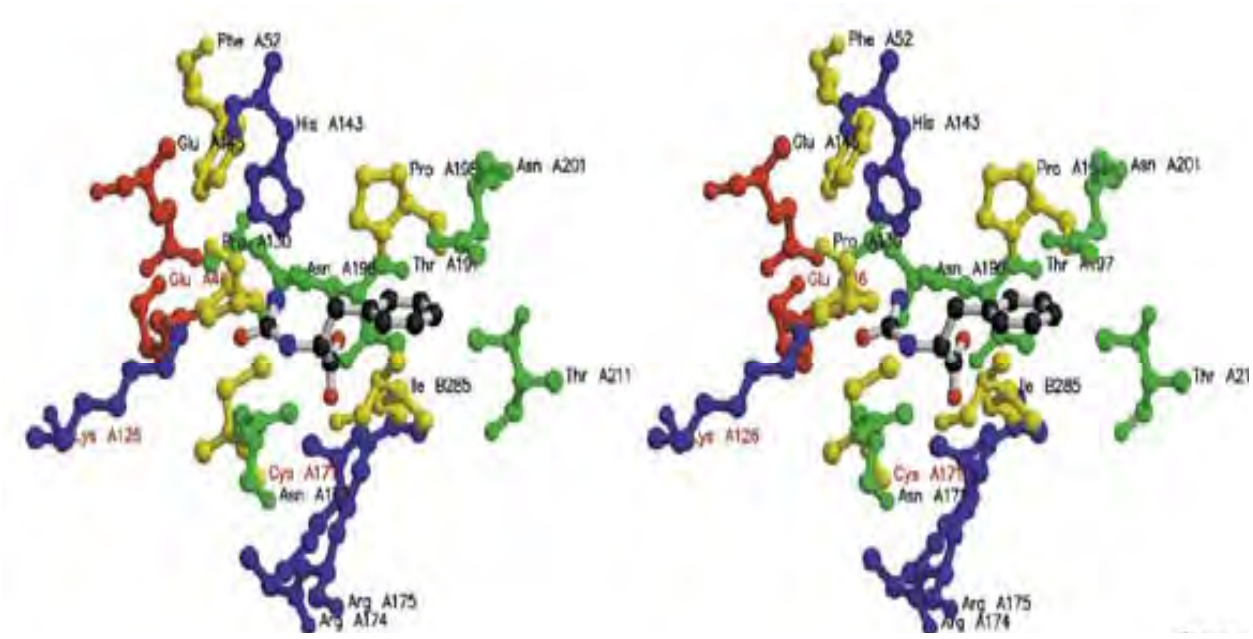


Figure 1.7: Key amino acids that are involved in the interaction of the DCase (NCDAAH) from *A*. sp. KNK712 enzyme with the substrate *N*-carbamoyl-D-phenylalanine. The amino acid residues are coloured by residue type: acidic in green, basic in blue, polar in green and hydrophobic in yellow. The substrate is coloured by atom type: carbon in grey, nitrogen in blue and oxygen in red. The three catalytically important residues (Glu 46 [47], Lys 126 [127], Cys 171[172]) are coloured in red. Reproduced by kind permission of Nakai et al. (2000).

The two arginine residues Arg 174, 175 are proposed to be essential for substrate recognition. The mechanism of action of both *Agrobacterium* NCDAAH enzymes is suggested to involve the following mechanisms:

- the carboxyl group of Glu 46 enhances the nucleophilicity of Cys 171, by abstracting the proton from the thiol group
- the activated nucleophile then attacks the amide carbon of substrate to form a tetrahedral intermediate
- the ε-amino group of Lys 126 stabilizes the intermediate

- the intermediate collapses to form an acyl-enzyme complex, releasing ammonia
- nucleophilic attack by a water molecule that replaces ammonia and is activated by Glu 46 results in deacylation of the complex
- the released *N*-carboxy-amino acid spontaneously collapses to from the corresponding D-amino acid plus carbon dioxide (Nakai *et al*., 2000).

The active site structure of the NCDAAH enzymes is similar to the Cys-Asp-Lys site of *N*-carbamoylsarcosine amidohydrolase (Wang *et al*., 2001), and the homology within the active-site framework of these enzymes with distinct protein structures suggests convergent evolution of a common catalytic mechanism (Wang *et al*., 2001).

1.7 Hydantoinase-NCAAH Gene Fusions

Bifunctional enzymes increasingly provide alternative solutions to the limits of naturally occurring biocatalysts and the construction of an active bifunctional D-hydantoinase/NCDAAH enzyme has shown that this technology is applicable with hydantoin-hydrolysing biocatalysts too (Kim *et al*., 2000a,c). As shown in Table 1.7, a maltose binding protein fusion with D-hydantoinase from *Bacillus stearothermophilus* SD-1 was first tested to ensure that fusion with the N-terminus would not result in loss of hydantoinase activity. A fusion construct was then designed with an end-to-end fusion of the D-hydantoinase gene from either *B. stearothermophilus* SD-1 or *B. thermocatenulatus* GH2 and the NCDAAH gene from *A. radiobacter* NRRLB11291 (Table 1.7). CAB-HYD1, the latter construct, showed superior bifunctional activity compared to the first CAB-HYD construct. The activity of CAB-HYD1 was higher than that of the two enzymes expressed separately in *E. coli* and equal to that of the two enzymes co-expressed on separate plasmids in *E. coli.* (Kim *et al*., 2000c). Initially, difficulties with the susceptibility of CAB-HYD construct to proteolysis were experienced. Directed evolution of the fusion construct in order to increase the stability of the construct (with a specific primer to maintain domains essential for catalytic activity) was attempted, but with little success even after three rounds of random mutagenesis and screening (Kim *et al.*, 2000a).

Table 1.7: Fusion proteins constructed with D-hydantoinase and NCDAAH genes (Kim *et al*., 2000c)

Source	Enzyme1	Enzyme 2	Fusion
	Gene Product Size	Gene Product Size	Gene Product Size
B. stearothermophilus SD-1	ORF 1 52 kDa	MalE 43 kDa	MBP-HYD 95 kDa
B. stearothermophilus SD-1	ORF 1 54 kDa		CAB-HYD 86 kDa
A. radiobacter NRRL B11291		ORF 1 34 kDa	
B. thermocatenulatus GH2	dht 52 kDa		CAB-HYD1 86 kDa
A. radiobacter NRRL B11291		ORF 1 34,2 kDa	

1.8 Hydantoin racemase enzymes

From an economical perspective the racemization of hydantoin derivatives is crucial for the competitiveness of the processes for the production of enantiomerically pure amino acids. Racemizing activity is widespread in nature, and a variety of amino acid racemases occur, and their use for the production of L-amino acids has been examined. Although most racemase enzymes require co-factors such as pyridoxal 5'-phosphate and ATP, this is not a prerequisite for racemase activity (Syldatk *et al*., 1992b). Racemization of 5-monosubstituted hydantoins occurs through keto-enol tautomerism or base substitution (Figure 1.8) and the rate of racemization is greatly affected by the electronegativity of the substituent R-group (Syldatk et al., 1992b).

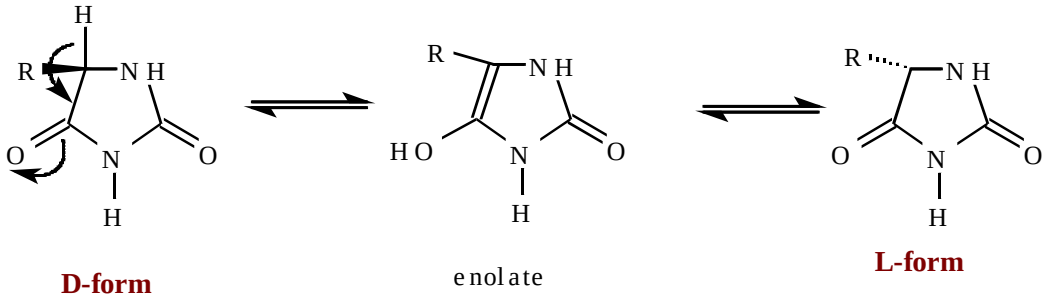


Figure 1.8: Keto-enol tautomerism of 5-monosubstituted hydantoin derivatives. (Adapted from Syldatk and Pietzsch 1995; Ogawa and Shimizu, 1997).

More electronegative substituents as well as basic pH and increasing temperatures speed up racemization rates. Hydantoin racemase enzymes increase the rate of racemization considerably, and hydantoin racemase activity has been detected in *Pseudomonas, A.grobacterium* and *Arthrobacter* species (Syldatk *et al*., 1992b). The substrate selectivity of the hydantoin racemases isolated from these organisms differs widely (Table 1.8).

Table 1.8: Substrate selectivity of hydantoin racemase enzymes.

Source	Substrate preference	Reference
Arthrobacter aurescens 3747	5-indolylmethylenehydantoin, 5-indolylmethylene-N-3-hydantoin, 5-benzylhydantoin, 5-(2'-methylthioethyl)hydantoin, 5-(*p*-hydroxybenzyl)hydantoin, 5-*i*-butylhydantoin	Syldatk and Pietzsch (1995); Wiese *et al*.(2000)
Arthrobacter aurescens 3745	5-monosubstituted hydantoins with aliphatic side chains	Syldatk and Pietzsch (1995);
Pseudomonas sp. NS 671	5-(2'-methylthioethyl)hydantoin, 5-isopropylhydantoin, 5-isobutylhydantoin, 5-benzylhydantoin	Watabe *et al*. (1992 b,c)

Hydantoin racemase enzymes have been purified from *Pseudomonas* sp. NS 671 and *Arthrobacter aurescens* DSM 3747, and are most active at 55°C, and pH 8.5. (*A. aurescens* DSM 3747) or 45°C, pH 9.5 (*Pseudomonas* sp. NS 671). Both hydantoin racemase enzymes were stimulated by reducing agents such as dithiothreitol and sulfhydryl reagents, suggesting that cysteines might be involved in the active site of the enzyme.(Syldatk *et al*., 1992b). A racemase gene from *Arthrobacter aurescens* DSM 3747, referred to as *hyuR* or *hyuA*, has been isolated and expressed in *E. coli*. The gene encoding the hydantoin racemase enzyme in *A. aurescens* DSM 3747 was situated upstream of the hydantoinase and NCLAAH-encoding genes in *A. aurescens* DSM 3747, showing clustering of genes encoding the proteins involved in hydantoin

hydrolysis in this bacterium (Wiese *et al*., 2000). *HyuR* encoded a protein of 236 amino acids, with a calculated molecular weight of 25.1 kDa (May *et al*., 2000; Wiese *et al*., 2000). In contrast, the hydantoin racemase protein purified from *A. aurescens* DSM 3747 cells was 84 kDa in size. When the racemase-encoding gene was expressed in *E. coli*, the purified recombinant protein had a molecular weight of 25.1 kDa, and showed different substrate selectivity to the enzyme purified from *Arthrobacter aurescens* DSM 3747. Two different hydantoin racemase enzymes may therefore exist in this particular *Arthrobacter* strain (Wiese *et al*., 2000). Both types of racemase enzymes from *A. aurescens* DSM 3747 were inhibited by mercuric chloride and iodoacetamide, suggesting that cysteines might be involved in the active mechanism of racemization, as has been reported for other types of racemase enzymes. The purported role for cysteine amino acid residues in the mechanism of racemization was later confirmed by comparison of the primary amino acid sequence from several known and putative hydantoin racemase coding sequences (Weise *et al*., 2000).

The gene product of *hyuE*, which encodes an hydantoin racemase capable of racemizing either enantiomer of racemic 5-substituted hydantoins was isolated from *Pseudomonas* sp. NS671 and found to encode a protein of 32.1 kDa, occurring in hexameric form (Watabe *et al.,* 1992b). This recombinant racemase was inactivated by 5-isopropylhydantoin but could be protected from this effect by divalent-sulphur-containing compounds (Watabe *et al*., 1992c).

Alignment of the deduced primary amino acid sequence from the HyuR (HyuA) hydantoin racemase enzyme from *A. aurescens* 3747, with that from *Pseudomonas* sp. NS 671and with two putative hydantoin racemases from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* revealed 47.2 % homology between the two bacterial hydantoin racemases, with slightly lower identity to the yeast enzymes. All four shared significant similarity in the N-terminal region, and two conserved cysteine residues supported the theory that cysteine residues might be involved in the mechanism of action of hydantoin racemases (Wiese *et al*., 2000).

1.9 Regulation of the production of hydantoin-hydrolysing enzymes

The production of hydantoin-hydrolysing activity in bacterial cells is regulated by at least two different components of the growth medium. Firstly, the presence of hydantoin or hydantoin analogues is required for the induction of enzyme activity in most bacteria. Secondly, the available carbon and nitrogen sources for the cell affect the production of hydantoin-hydrolysing enzymes (Nishida *et al*., 1987; Yamashiro *et al*., 1988a,b; Syldatk *et al*., 1992b).

1.9.1 Induction

Various different hydantoin and pyrimidine analogues are able to induce hydantoin-hydrolysing activity, and optimal inducers vary widely between species and strains, illustrating the diversity of hydantoin-hydrolyzing enzymes that occur in nature (Syldatk *et al*., 1992a,b; Ogawa and Shimizu, 1997; Table 1.9).

Induction with hydantoin analogues, such as 5-isopropylhydantoin (Yamashiro *et al*., 1988) or 5-indolylmethydantoin (Nishida *et al*., 1987; Syldatk *et al*., 1990a), is required for the production of L-selective hydantoin-hydrolysing activity. Interestingly, the production of hydantoinase from *Arthrobacter* sp. BH 20 was induced only by D-, L- or D,L-indolymethylhydantoin, and highest levels of production were induced by the racemic 5-indolylmethylhydantoin as opposed to either enantiomer (Syldatk *et al*, 1992b). Thiolated hydantoins and pyrimidine analogues are utilized for the induction of D-hydantoin-hydrolysing activity in *Agrobacterium* species (Meyer and Runser, 1993, Table 1.2), although *A. radiobacter* NRRL B11291 does not seem to require induction to produce hydantoin-hydrolyzing activity (Olivieri *et al*., 1979; Deepa *et al*., 1993). The choice of inducer may significantly affect the total hydantoin-cleaving activity as well as the ratio of *N*-carbamoyl amino acid : amino acid produced from the hydantoin substrate, as demonstrated by Möller *et al*. (1988).

Table 1.9: Inducers of hydantoin-hydrolysing activity in bacteria

Source	Inducers	Stereo-selectivity	Reference
A. radiobacter NRRL B11291	none	D	Olivieri *et al*. (1981), Deepa et al. (1993)
Agrobacterium sp. IP 671	2-thiouracil	D	Meyer and Runser (1993)
Arthrobacter crystallopoites AM2	dihydrouracil, hydantoin	D	Möller *et al.* (1988)
Arthrobacter sp. BH 20	D,L-indolylmethylhydantoin	non	Syldatk et al. (1992b)
Arthrobacter aurescens 3747	D,L-indolylmethylhydantoin	L	Syldatk and Pietzsch (1995);
Arthrobacter aurescens 3745	D,L-indolylmethylhydantoin	L	Syldatk and Pietzsch (1995);
Blastobacter sp. A17p-4	dihydrouracil	D	Ogawa *et al*. (1994)
Comamonas sp. E222C (no hydantoinase)	N-carbamoyl amino acids	D	Ogawa *et al*. (1993)
Pseudomonas striata	hydantoin	D	Dinelli *et al*. (1978)
Pseudomonas sp. AJ-11220	5-cyanoethylhydantoin	D	Yokozeki *et al*. (1987d)
P. fluorescens DSM 84	Hydantoin, isopropylhydantoin	D	Morin *et al*. (1986a)
Pseudomonas sp. NS 671		Non	Watabe *et al.* (1992b,c)
Pseudomonas sp. RUKM1 *Pseudomonas* sp. RUKM3$_S$	hydantoin hydantoin, dihydrouracil, thiouracil	D L	Burton *et al*. (1998)

The addition of inducer to the growth medium for the production of active hydantoin-hydrolysing biocatalysts is obviously an additional expense, and depletion of the inducer by cell metabolism aggravates this, necessitating step-wise addition of inducer throughout fermentation to maintain hydantoins-hydrolysing activity (Syldatk *et al.,* 1990a). Growth of cells to the desired biomass density followed by induction for a short period of time was found to efficiently induce hydantoins-hydrolysing activity in *A. aurescens* DSM 3747 (Syldatk *et al.*, 1990a), but still required feeding of inducer at a certain time. Non-metabolizable inducers such as 2-thiouracil (Meyer and Runser, 1993), or chemically modified inducers such as D,L-5-(indolylmethyl)-N-3-hydantoin (Syldatk *et al.,*1990a) can be used to alleviate the problem. Wagner *et al*. (1996)

isolated a mutant strain of *A.* aurescens DSM 7330 which was selected for constitutive production of L-stereoselective hydantoin-hydrolysing activity. The isolated mutant strain, *Arthrobacter* sp. 9771 produced inducer-independent hydantoinase activity at higher levels than the wild-type strain, and also exhibited elevated levels of hydantoinase and NCAAH activity (by a factor of 2.3) in the presence of inducer, when compared to wildtype induced levels. Inducer-independent mutants therefore also provide a satisfactory means of reducing the cost of producing hydantoin-hydrolysing activity in bacteria (Wagner *et al*., 1996).

1.9.2 Catabolite repression

The production of both D- and L-hydantoin-hydrolysing enzymes is affected by both the carbon and nitrogen sources utilized in the growth medium (Nishida *et al*., 1987; Möller *et al*., 1988; Meyer & Runser, 1993; Syldatk *et. al*., 1990a, Syldatk *et al*., 1992b; Yamashiro *et al*., 1988a). Hydantoin-hydrolysing activity in microorganisms is normally produced at highest levels after late exponential growth phase in complete media (Möller *et al*., 1988; Meyer & Runser, 1993; Syldatk *et. al*., 1990a), suggesting that the expression of these enzymes is subject to catabolite repression. Catabolite repression involves the preferential expression of metabolic enzyme systems, such that non-growth-rate limiting or “good” nitrogen and carbon sources are effectively utilised for rapid cell growth before utilisation of growth-rate-limiting or “poor” nitrogen and carbon sources (Alberts *et al.*, 1994). As hydantoin provides a poor or growth-rate limiting nitrogen or carbon source for bacterial cells, it is probable that nitrogen or carbon catabolite repression mechanisms regulate the expression of hydantoin-hydrolysing enzymes in bacteria. Putative binding sites upstream of the genes encoding hydantoin-hydrolysing enzymes that are specific for sigma transcription factors (such as sigma 54) that are involved in the expression of nitrogen-regulated promoters, suggest the possible involvement of *ntr*-type nitrogen catabolite repression (LaPointe *et al*., 1994; Watabe *et al.*, 1992a). Sequences upstream of the *hyuA* and *hyuB* genes from *Pseudomonas* sp. NS671 resemble the consensus sequence for nitrogen-related or nitrogen fixation promoters, such as Ntr or Nif promoters (Watabe *et al*., 1992a). Interestingly, the deduced amino acid sequences of HyuA and HyuB share significant homology (40-47%) with two catalytic enzymes involved in the degradation of agropinnic acid, AgaF and AgaG, which are strictly regulated in accordance with both nitrogen and carbon catabolite repression (Lyi *et al*., 1999). Sequences resembling

promotor-binding sequence for σ transcription factors involved in regulation of gene expression were found in the overlapping promotor region of the D-hydantoinase and NCDAAH-encoding genes from *A. radiobacter* NRRL B11291 (Grifantini *et al*., 1998), as well as upstream of the D-hydantoinase open reading frame (ORF) from *P. putida* DSM 84. This provides further support for the possible regulation of hydantoin-hydrolysing enzyme expression in accordance with ntr-type catabolite repression (LaPointe *et* al., 1994; Merrick & Edwards, 1995).

1.10 Evolution of hydantoin-hydrolysing enzymes systems

Hydantoinases have recently been demonstrated to belong to the superfamily of cyclic amidohydrolase enzymes described by Holm and Sander (1997). May *et al*. (1998a) used phylogenetic analysis of selected portions of members of the superfamily to illustrate that the cyclic amidohydrolases probably evolved by divergent evolution from a common ancestor, and form five different groups (Table 1.10).

Table 1.10: Phylogenetic groupings of hydantoinases within the cyclic amidohydrolase superfamily

Group	Proteins	Sources	% Homology to Group A
A	L-hydantoinase	*A. aurescens* DSM 3747	100
B	Collapsing response mediator proteins dihydropyrimidinase	*Gallus gallus*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* *Homo sapiens, Rattus norvegicus, A. radiobacter , B. stearothermophilus*	30
C	allantoinase	*Rana cateisbeiana, S. cerevisiae*	24
D	urease	*Ureaplasma urealyticum, Helicobacter pylori, Helicobacter felis, Bacillus pasteurii, Klebsiella aerogenes*	15
E	dihydroorotase	*Thermus aquaticus, Lysteria leichmannii, Bacillus cadolyticus, Bacillus subtilis, Methanococcus jannaschii, Methanococcus thermoautotrophicum, Cricetulus sp., Homo sapiens*	20

Interestingly, the N-methylhydantoinase-type enzymes, including the hydantoinase from *Pseudomonas* sp. NS671 do not belong to this superfamily. However, all the hydantoinases related to urease share only low amino acid sequence similarity. Syldatk *et al.* (1999) therefore suggests that these hydantoinase enzymes are all members of a very old protein family, and have evolved from a common ancestor. Unfortunately, no sequence data is available for imidase and carboxymethylhydantoinase enzymes, so their relationship to the urease-related hydantoinase enzymes cannot be established as yet.

The phylogenetic data also shows that the term “hydantoinase” cannot be applied to one single group of the family. Separation of hydantoinase enzymes on the basis of enantioselectivity is not supported by molecular data either, rather hydantoinases are distributed throughout the groups of the cyclic amidohydrolase superfamily, which probably diverged very early in evolution, possibly prior to the formation of the Archaea, Eucarya and Prokaryota domains, as enzymes from within these domains group randomly throughout the superfamily (May *et al*., 1998a; Syldatk *et al*., 1999).

The molecular evolutionary relationships between NCAAH enzymes have not been examined in detail, although Ikenaka *et al.* (1998a) illustrated a fairly high level of similarity between NCDAAH enzymes, aliphatic amidases, β-alanine synthetases, nitrilases and a cyanide hydratase enzyme, all from microbial or plant sources.

1.11 Heterologous production of hydantoin-hydrolysing enzymes

Naturally occurring hydantoin-hydrolysing microorganisms provide an abundant pool of hydantoin-hydrolysing enzymes with a variety of characteristics, and this resource remains substantially unexplored. However, the process of evolution has ensured that the naturally occurring enzymes function within strict limits of existing conditions and within a limited range of substrate specificities (Kim *et al*., 2000a). There is widespread recognition that genetic engineering methods reported in the literature have an application in the future development of biotechnological processes.

Since 1992 (Syldatk *et* al., 1990b.), recombinant DNA technology has been used for the overproduction of hydantoinases and NCAAH enzymes, to facilitate isolation and characterisation of enzymes, to manipulate enzyme characteristics outside of the natural environment and even to direct the evolution of superior enzymes (Headon & Walsh, 1994; Syldatk and Pietzsch, 1995; Chao *et al*., 1999a,b, 2000; Kim *et al*., 2000a,c; May *et al*., 2000). In addition, *E. coli* provides a convenient host for the production of recombinant heterologous proteins (Baneyx, 1999). This has allowed for rapid advances in the industrial production of enantiomerically pure amino acids from 5-monosubstituted hydantoins using hydantoinase and *N*-carbamoyl amino acid amidohydrolase enzymes (Schulze & Wubbolts, 1999). Insoluble aggregates formed during recombinant hydantoinase expression frequently pose a problem to the mass production of recombinant D-hydantoinase in *E. coli* but some methods for circumventing this problem are presented in the following sections (Lee *et al*., 1997a,b, 1998; Grifantini *et al*, 1998; Ikenaka *et al*, 1998).

Much of the initial research into heterologous production of hydantoinase enzymes was done using thermostable hydantoinase genes from *B. stearothermophilus* SD1expressed in *E. coli*. Constitutive expression of D-hydantoinase from *B. stearothermophilus* SD1 using the native promoter, glycerol as a sole carbon and at low temperatures was used by Lee *et al*. (1997a,b) for mass recombinant expression of the enzyme without the formation of insoluble aggregates. Low temperatures resulted in much lower growth rate, but the combination of glycerol as a carbon source and slow accumulation of the D-hydantoinase due to constitutive expression from the native promotor resulted in cost-effective production of the D-hydantoinase in a batch system, yielding 50g/L dry cell weight (DCW) with a specific activity of 38 000 U (µmol/min) per gram DCW. This heterologous production system was successfully used in a heterogenous reaction system to convert up to 96% of 300g/L D,L-HPH substrate to *N*-C-D-HPG (Lee *et al.*, 1998, 1999). The reverse reaction of this enzyme system was also utilised as a novel mild enzymatic reaction process for the production of enantiomerically pure hydantoins from racemic hydantoins mixtures. The D-hydantoinase first stereoselectively converted D,L-HPH substrates to *N*-carbamoyl-D-amino acid. By altering the reaction pH, the D-hydantoinase enzyme could then be

used in the reverse reaction, to convert the *N*-C-D-HPG to enantiomerically pure D-HPH (Lee and Fan, 1999).

Mukohara *et al*. (1994) reported that recombinant production of the thermostable hydantoinase from *B. stearothermophilus* NS1122A caused poor growth of the host *E. coli* cells when the *ORF(HN)* was placed under the more efficient promotor of the NCLAAH from *B. stearothermophilus* NS1122A, suggesting that overexpression of the gene during log phase was toxic. ORF(HN) was therefore placed under the control of an inducible *lac* promotor-operator system which would ensure expression only in late logarithmic or early stationary growth phase. This prevented the depletion of pyrimidines during logarithmic growth phase, and allowed for normal growth of the host *E. coli* strain. However, the high expression of the hydantoinase gene still led to the formation insoluble aggregates, from which the active homotetrameric hydantoinase enzyme encoded by *ORF(HN)* could be purified by solubilization and refolding of the protein (Mukohara *et al*., 1993, 1994).

The heterologous production of the hydantoin-hydrolysing enzymes from various *Agrobacterium* strains has also been explored. Grifantini *et al*. (1998) showed stable, constitutive expression of the NCDAAH and D-hydantoinase genes from *A. radiobacter* NRRL B11291 in *E.coli.* When placed under the control of the T7 RNA-dependent promotor and expressed in *E. coli* BL21, the NCDAAH-encoding *cau A* gene product from *A. radiobacter* NRRL B11291 reached 40 % of total cell protein and was more stable then the wild-type enzyme (Buson *et al*., 1996). Arrangement of the D-hydantoinase and NCDAAH-encoding genes in an operon structure with the NCDAAH gene preceding the D-hydantoinase gene, and growth of the cells at 28°C resulted in maximum conversion of racemic hydantoin to D-amino acid (Grifantini *et al*., 1998). Both the D-hydantoinase and NCDAAH genes from *A. radiobacter* NRRL B11291 were isolated using PCR amplification by Chao *et al*. (1999a,b). Different types of vectors, *E. coli* host stains and immobilization of the recombinant cells in κ-carrageenan, were used to achieve one-step heterologous production of high levels of D-HPG (Chao *et al*., 1999). However, immobilization methods resulted in poor substrate diffusion across the interface, and poor reusability due to surface fouling (Nanba *et al*., 1999a).

Overexpression of the *Agrobacterium* sp. KNK712 NCDAAH in *E. coli* resulted in a specific activity five times higher than that reported for recombinant expression of the *A. radiobacter* NRRLB11291 gene, probably due to more suitable host-vector systems and culture conditions of the recombinant *E. coli*, rather than differences between the properties of the two enzymes (Nanba *et al*., 1998). NCDAAH activity of the recombinant *E. coli* strain expressing the NCDAAH gene from *Pseudomonas* sp. KNK003A was 40-fold that of the native strain and the recombinant enzyme was five-fold more stable after reuse than the recombinant NCDAAH from *Agrobacterium* sp. KNK712, making it a suitable candidate for industrial production of D-amino acids (Ikenaka *et al*., 1998a).

Overexpression of the D-hydantoinase and NCDAAH genes from *A. radiobacter* NRRL B11291 in *E. coli* also produced insoluble aggregates forming inclusion bodies within the bacterial cytoplasm (Chao *et al*., 2000). Although these can be useful for the isolation of the enzyme from other cytosolic components, the *in vitro* refolding of the protein aggregates to produce active soluble enzyme is variable from case-to-case, expensive and usually results in low yields (Hockney, 1994). Various factors enhance the formation of correctly folded proteins in bacteria, including molecular chaperones, fermentation temperature, redox state modulators such as thioredoxin and fusion of the desired protein with another protein. Chao *et al*. (2000) systematically tested combinations of these factors on the formation of active recombinant D-hydantoinase and NCDAAH enzymes in *E. coli*. Co-production of *E. coli* molecular chaperones produced active hydantoin-hydrolysing enzyme activity. Interestingly, different *E. coli* molecular chaperones mediated correct folding into the native conformation for each enzyme: co-production of DnaJ/DnaK alleviated the formation of insoluble aggregates of D-hydantoinase protein, whilst co-production of GroEL/GroES alleviated the formation of insoluble aggregates of recombinant NCDAAH protein. This suggests that the limiting step in the protein-folding pathway is different for each of the enzymes (Chao *et al*., 2000). Fusion of the D-hydantoinase and NCDAAH proteins with thioredoxin did not positively influence correct folding, and in fact seemed to increase proteolytic attack of the NCDAAH folding intermediate. Lower fermentation temperatures alleviated the formation of insoluble protein aggregates of D-hydantoinase, similar to the results reported by Lee *et al*. (1999) above, but did not alleviate formation of insoluble protein aggregates of NCDAAH.

Fusion proteins have also been used in attempts to prevent the incorrect folding of other heterologously expressed hydantoins-hydrolysing enzymes. Expression of the *dht* gene from *Pseudomonas putida* CCRC 12857 with 6 histidine tags on either the carboxy terminal or the amino-terminus of the protein did not affect hydantoinase activity, but also did not reduce the formation of insoluble aggregates in inclusion bodies when high levels of protein expression were induced in *E. coli*. Lowering the growth temperature did not significantly reduce inclusion body formation. However, recombinant *E. coli* harbouring plasmid expressing the D-hydantoinase gene under control of the T5*lac* promotor, induced with 5 mM lactose exhibited 20 fold higher D-hydantoinase activity than the gene donor strain *P. putida* CCRC12857 (Chein *et al*., 1998). Immobilization of these recombinant cells in calcium alginate matrix increased the optimal temperature by 20-60°C and significantly increased the reusability of the cells. Unfortunately, the D-hydantoinase activity of immobilized cells was lower than that of free cells due to diffusion limitations of the alginate matrix and therefore matrices with lower mass transfer resistance will be required to improve the use of recombinant immobilized cells for *N*-carbamoyl-D-amino acid amiodhydrolase production (Chen *et al*., 1999).

A fusion protein with the NCLAAH gene from *A. aurescens* DSM 3747 and six aspartate residues, HyuC-Asp_6, was created by oligonucleotides insertion (Pietzsch *et al*., 2000). The Asp-tag decreased the isoelectric point of the protein and allowed for easier purification by shifting the fusion protein out of the elution range of contaminating proteins. The HyuC-Asp_6 fusion enzyme could be immobilized without removal of the aspartate residues as only the C-terminal region of the protein was involved in immobilization. The hydantoinase enzyme from *A. aurescens* DSM 3747 (HyuH) was fused to six histidine residues. Active His-tagged hydantoinase hyantoinase enzyme (HyuH) could also be purified to homogeneity using metal affinity chromatography and thus purification and stability of hydantoins-hydrolysing proteins was greatly enhanced using fusion technology (Pietzsch *et al*., 2000).

Processes using heterologous co-production of the D-hydantoinase gene from *B. stearothermophilus* SD1 and the NCDAAH gene from *A. radiobacter* NRRL B11291 have also been examined for the production of D-HPG from D,L-HPH. Chao *et al.* (2000a) reported a 98% product yield of D-HPG from 15mM D,L-5-HPH in 15 hours,

using *E. coli* cells co-expressing these enzymes. Site-directed mutagenesis has also been utilized to produce increased thermostability in recombinant NCDAAH enzymes from *Agrobacterium* sp. KNK712 (Ikenaka *et al*., 1999). Immobilization of crude extracts of these mutant recombinant NCDAAH enzymes was used to provide increased reaction stability and reusability (Nanba *et al*., 1999a). Directed evolution and fusion enzymes have also been utilized to manipulate recombinant hydantoin-hydrolysing enzymes for improved biocatalytic properties applicable to the production of D-amino acids (May *et al*., 2000; Kim *et al.,* 2000a).

1.12 Research Proposal

In 1995, a need to exploit the novel biodiversity of South African microorganisms for the production of enantiomerically pure amino acids for fine chemical industry applications was identified. Consequently, the Rhodes Hydantoinase Group was formed in alliance with AECI (Pty) Ltd. (South Africa), and research into the development of biocatalytic processes for the production of both D-and L-amino acids using novel indigenous microorganisms was initiated.

The research described in this thesis focused on the development of a novel biocatalytic process for the production of enantiomerically pure D-HPG from D,L-5-HPH. The principal goal of the research was to develop a fundamental understanding of an hydantoin-hydrolysing enzyme system, and then apply this knowledge to engineer an improved biocatalyst.

Research Objectives:

- Isolation and identification of hydantoin-hydrolysing soil isolates and evaluation of hydantoin-hydrolysing activity. Selection of a suitable D-stereoselective hydantoin-hydrolysing bacterial strain.
- Characterization and optimization of the hydantoin-hydrolysing activity of the selected strain in biocatalytic resting cell reactions, with the aim of producing D-hydroxyphenylglycine from D,L-5-hydroxyphenylhydantoin.

- Investigation into the regulation of the production of hydantoin-hydrolysing activity in the selected strain cells.
- Manipulation of the production of hydantoin-hydrolysing activity in the selected strain cells using regulatory mutants.
- Isolation and characterization of the genes encoding hydantoin-hydrolysing activity in the selected strain.

Chapter 2

Isolation of *Agrobacterium tumefaciens* RU-OR and optimization of resting cell biocatalytic reactions

Chapter 2

Isolation of *Agrobacterium tumefaciens* RU-OR and optimization of resting cell biocatalytic reactions

2.1 Introduction

New biocatalytic processes may be based on either available enzymes or organisms, but many start with the process of screening for an organism or enzyme that is capable of carrying out the desired reaction (Figure 2.1). Once a suitable enzyme or organism has been isolated and identified, the optimal reaction conditions for maximum function and reactivity of the biocatalyst must be selected (Schmid *et al.*, 2001).

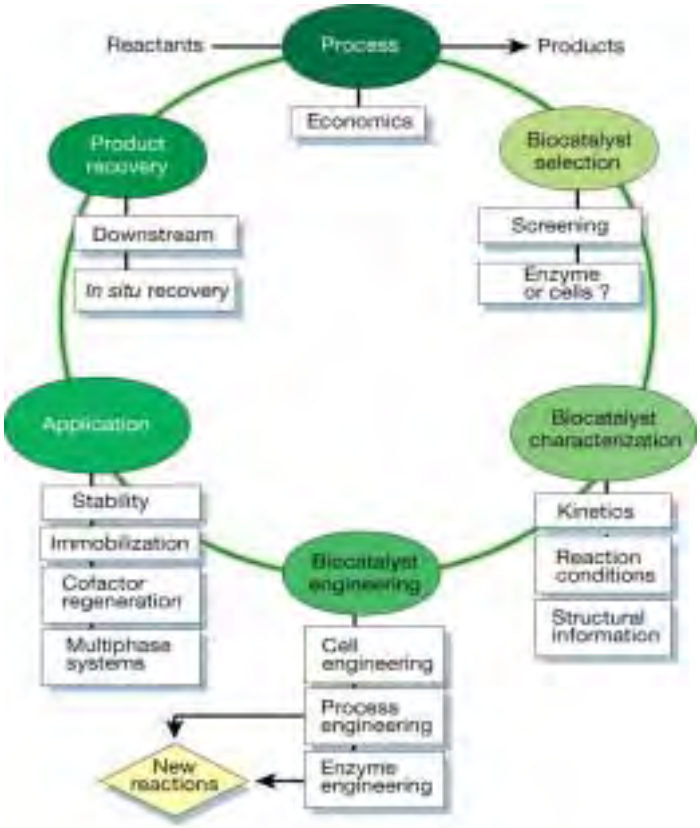


Figure 2.1: The cycle of processes involved in the selection and development of a biocatalyst (from Schmid et al, 2001).

Screening techniques for the isolation of hydantoin-hydrolysing bacteria mostly rely on the selection of bacteria from enriched soil. Selection criteria are normally based on the use of hydantoin as a sole nitrogen source (Syldatk *et al.*, 1999); a spot assay for NCG production using Ehrlich's reagent (Morin *et al.*, 1986b); thin layer chromatographic (TLC) analysis of the reaction products after incubation with hydantoins; or detection with DNA probes (La Pointe *et al*., 1995, for reviews see Syldatk *et al.*, 1992a, 1999). A rapid screening procedure using antibodies raised against the hydantoinase enzyme of an *Arthrobacter* sp. has been used for the isolation of microorganisms with L-selective hydantoin-hydrolysing activity (Siemann *et al.*, 1993a, b). More recently, microtitre plate assays have been developed for rapid large-scale screening (Chein & Hsu, 1996).

Screening processes must also assess the enantiomeric properties of the products (Schmid *et al.*, 2001). Classical methods for the measurement of enantiomeric purity, such as optical rotation in comparison with optically pure D- or L- compounds, are not ideal for analysis of hydantoin-hydrolysis products as they require prior separation and purification of the optically active hydantoins, *N*-carbamoyl amino acids and the amino acids (Syldatk *et al.*, 1992a). Commercially available D- and L-amino acid oxidases can be used to detect enantiomeric purity, but this method is relatively expensive. Chiral HPLC using chiral columns or chiral supports (Syldatk *et al.*, 1995), and chiral TLC using similar supports, provide efficient methods for enantiomeric analysis of hydantoin-hydrolysing reaction products (Morin *et al.*, 1987; Martens and Bhushan, 1993).

Once a suitable hydantoin-hydrolysing strain has been isolated, specific identification provides further information on the potential novelty of the enzyme system. Biochemical identification using a standard range of metabolic and biochemical reaction tests can provide an initial identification, but such methods are labour intensive and have limited specificity (Brunk *et al*., 1996). The prokaryotic 16S rRNA gene provides a useful molecular identification tool, as both widely conserved and divergent regions occur within the gene sequence (Avaniss-Aghajani *et al.*, 1999).

The nucleotide sequence of the divergent regions provides distinctive characteristics that can be used for identification of bacterial species, whilst the conserved regions allow for easy polymerase chain reaction (PCR) amplification using universal primers. The substantial database of large and small subunit ribosomal RNA sequences available through the Ribosomal Database Project facilitates rapid and accurate identification of bacterial species (Maidak *et al.*, 2000, 2001).

This chapter describes the screening and isolation procedures that resulted in the selection of *A. tumefaciens* RU-OR and the characterization of its novel hydantoin-hydrolysing enzyme system, as published in Burton *et al.* (1998) and Hartley *et al.* (1998).

2.2 Materials and Methods

2.2.1 Isolation of hydantoin-hydrolysing bacterial strains

Hydantoin-hydrolysing bacteria were isolated from local soil samples, which were enriched by overnight culture in minimal medium (MM) medium (Appendix 2) containing hydantoin as a sole nitrogen source (HMM), shaking at 200rpm, 25°C. Dilutions of the enriched medium were then selected for the ability to grow on HMM agar plates at 25°C (Gardner, 1995). A total of 146 isolates were obtained and and examined for the ability to use various hydantoin derivatives as nitrogen sources. Hydantoin-hydrolysing isolates were streaked to single colonies on MM agar plates containing 1% glucose as a carbon source, and 1% hydantoin, methylhydantoin, *N*-carbamoylglycine or *N*-carbamoylalanine as a sole nitrogen source. Growth was qualitatively rated on a scale of 1 to 5, with 1 representing slight growth and 5 representing rapid, dense growth. Resting cell biocatalytic assays of selected strains were performed as described below using cultures grown to confluence in nutrient broth containing 1% hydantoin.

2.2.2 Culture conditions

Unless otherwise stated, a stationary phase starter culture in HMM broth was diluted to OD_{600nm} = 0.02 in nutrient broth (Merck) supplemented with inducer, and grown at 25^{o}C, shaking at 200rpm until stationary growth phase was reached (approximately 24 h for strain RU-OR).

2.2.3 Optimization of resting cell biocatalytic reactions

Initial resting cell biocatalytic reactions were performed as follows. Unless otherwise stated, cells were harvested by centrifugation (7000rpm, Beckman JA 14 rotor, Beckman J2-21), washed with 0.1 M potassium phosphate buffer pH 8.0, and resuspended at a final concentration of 20 mg/ml wet cell mass (WCM) in 0.1 M potassium phosphate buffer (pH 8.0) containing 50 mM of hydantoin or methylhydantoin, 30 mM *p*-hydroxyphenylhydantoin, or 25 mM of the appropriate *N*-carbamoyl amino acid substrates. The reaction was carried out at 40°C, shaking at 200rpm for 6 h after which samples were microfuged to pellet the cells and the supernatant analysed for *N*-carbamoyl amino acids and amino acids by colorimetric assays or TLC as described in Appendix 4. For the initial measurement of hydantoin-hydrolysing conversion and the optimization of reaction parameters in resting cell biocatalytic reactions, the production of *N*-carbamoyl amino acid and amino acid from hydantoin was measured. Production was measured as the concentration of product (µmol/ml) detected in the supernatant. The optimization of resting cell biocatalytic reactions was later refined to measure both hydantoinase and NCAAH activity in the cells. Hydantoinase activity in resting cell reactions was then expressed as the total *N*-carbamoyl amino acid and amino acid produced from hydantoin substrate (µmol/20 mg WCM/ml). NCAAH activity was expressed as amino acid produced from an *N*-carbamoyl amino-acid substrate (µmol/ 20 mg WCM/ml).

2.2.4 Enantiomeric resolution

The enantiomeric form of the amino acids produced from hydantoin substrates was analysed by chiral TLC (Appendix 4). After testing a variety of different mobile

phases, acetone: methanol: water (10:5:2) was selected as the optimum mobile phase for the separation of D- and L- enatiomers of alanine and HPG. Relative migration (R_f) values were calculated using the following formula:

R_f = distance migrated / distance to solvent front (Heftmann, 1975).

2.2.5 Statistical Analysis

Where necessary, statistical analyses of data obtained, such as t-test or ANOVA analysis for comparison of means was used to assess statistical differences and similarities (Maxwell & Delaney, 1989; Trochim, 2000). Standard error of the mean was determined for *n* number of repeats determined over three or four replicate experiments with 3 samples in each.

2.2.6 Strain Identification

General recombinant techniques were performed as described by Sambrook *et al.* (1989), or according to manufacturers instructions. The selected hydantoin-hydrolysing strains were initially identified biochemically by CSIR (Pretoria, RSA) using the API system and other standard metabolic tests. Biochemical identification of strain RU-OR was confirmed using analysis of the 16S rRNA gene. Chromosomal DNA was extracted from RU-OR cells by the method of Ausubel *et al.* (1983), and PCR amplification of 16S rRNA performed using universal primers for the 16S rRNA coding region, CH1 and CH3 (Appendix 6). Vent™ DNA Polymerase was used under standard cycling conditions: 95°C, 90 seconds; thirty cycles of 95°C, 45 seconds; 58°C, 45seconds, 72°C, 60 seconds, followed by a final extension at 72°C, 120 seconds. Subsequent to restriction enzyme mapping, the resultant 1.2 kb PCR product was then sub-cloned into the plasmid vector, pUC 18 (Appendix 7). *Bam* HI digested pUC 18 vector DNA was dephosphorylated using shrimp alkaline phosphatase (USB) and ligated according to manufacturers instructions (T4 DNA ligase, Promega) with the *Bam* HI digested PCR product, using the *Bam* HI sites engineered into primers CH1 and CH3. White insert-containing colonies were selected using blue/white selection on Luria agar plates containing 100µg/ml

ampicillin, 40µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 50µg/ml isopropyl-β-D-thiogalactoside (IPTG) (Sambrook *et al.*, 1989). The identification of strain RU-OR was confirmed by sequencing the 16SrRNA gene insert of pCH1 in both directions using the ALF-express automated DNA sequencing service (University of Cape Town, South Africa). Two constructs based on pCH1 (Figure 2.2) were created to obtain complete sequence of the gene from both strands of DNA. In one construct, pCH2, the unique *Eco* RI fragment (1006-1709 bp) was deleted from pCH1, whilst in the other construct, pCH3, the same *Eco* RI fragment was excised and inserted back into pUC18 vector digested with *Eco* RI (Appendix 7).

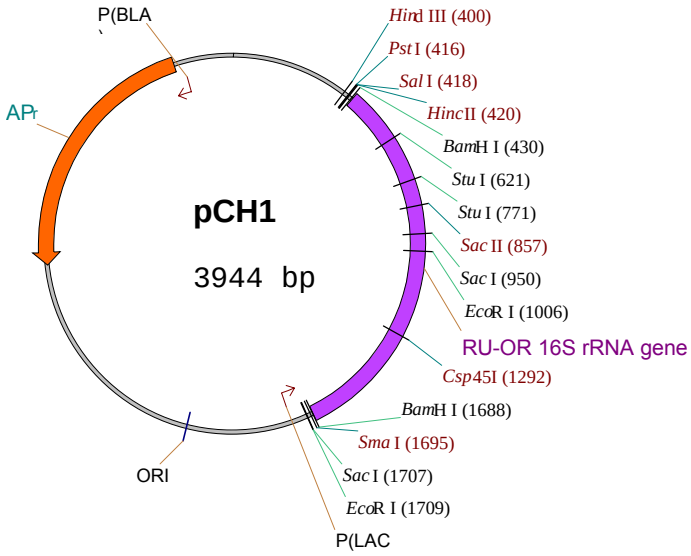


Figure 2.2: Restriction map of pCH1. The plasmid was constructed by cloning the 1.2 kb Bam HI fragment generated by PCR amplification of the 16SrRNA gene from chromosomal DNA of strain RU-OR into the Bam HI site of pUC18.

2.3 Results

2.3.1 Isolation of hydantoin-hydrolysing bacterial strains

In this instance, the selection of a suitable biocatalyst for the production of D-amino acids began with the screening of South African soil isolates for the ability to grow

utilizing hydantoins or *N*-carbamoyl amino acids as sole nitrogen sources. A strain collection of 146 isolates was obtained (Gardner, 1995) and these were then screened further using plate growth tests and whole cell biocatalytic assays (Hartley, 1995). Six isolates selected for a variety of growth characteristics, including a score of > 4 for at least one of the selection media, were identified using the API bacterial identification system (Table 2.1). RU-KM1 was selected for good growth with both NCG and hydantoin as sole nitrogen sources, although this strain did not grow very well with NCA or methylhydantoin as nitrogen sources. RU-OG_A, RU-OG_B and RU-1L were all selected for good growth characteristics with NCG as a sole nitrogen source. Strain RU-OR showed particularly good growth with NCA or methylhydantoin as sole nitrogen sources.

Table 2.1: Growth of selected strains on MM agar plates containing various hydantoin derivatives as a sole nitrogen source.

Strain	Sole Nitrogen Source			
	Hydantoin	**NCG**	**Methylhydantoin**	**NCA**
Chrysomonas luteola RU-KM1	4	4	2	2
Pseudomonas sp. RU-OG_A	1	4	1	2
Pseudomonas sp. RU-OG_B	1	4	2	2
Burkholderia cepacia RU-OR	2	3	5	4
Pseudomonas sp. RU-1L	2	4	2	2
Pseudomonas sp. RU-KM3	1	1	2	4

Key: NCG – *N*-carbamoyl glycine, NCA –*N*-carbamoyl alanine. Growth was rated visually from 0 –5, where 0 represents zero growth, 5 represents rapid dense growth.

Resting cell biocatalytic assays of the selected strains were used to confirm the enzyme activity observed on agar plates (Table 2.2). RU-OR cells were able to convert 23 % of 50mM methylhydantoin into alanine in resting cell biocatalytic reactions. RU-OG_A and RUKM1 produced only NCG from hydantoin, suggesting that these strains lack the presence of a functional hydantoinase enzyme. RU-OG_B produced large amounts of NCG from hydantoin, but produced fairly low levels of amino acid from both hydantoin and methylhydantoin. RU-1L produced large amounts of both NCG and NCA from hydantoin and methylhydantoin respectively, but produced very low levels of alanine from methylhydantoin.

Table 2.2: Production of *N*-carbamoyl amino acid and amino acid from hydantoin and methylhydantoin by selected soil isolates.

Strain	Hydantoin as substrate		Methylhydantoin as substrate	
	NCG (µmol/ml)	Glycine (µmol/ml)	NCA (µmol/ml)	Alanine (µmol/ml)
RU-KM1	28.5 ± 1.00	0	23.1 ± 0.78	0
RU-OG_A	21.7 ± 0.34	0	9.84 ± 0.70	0
RU-OG_B	23.7 ± 0.76	5.81 ± 0.10	5.60 ± 0.14	5.7 ± 0.37
RU-OR	2.64 ± 0.06	4.32 ± 0.17	6.54 ± 0.18	10.7 ± 0.50
RU-1L	23.0 ± 1.66	5.50 ± 0.26	20.6 ± 0.49	1.73 ± 0.09

Key: NCG – *N*-carbamoylglycine, NCA –*N*-carbamoylalanine. ± SEM (n=9).

Only resting cells from strain RU-OR produced higher levels of *N*-carbamoyl alanine and alanine with methylhydantoin as a substrate, than with hydantoin as a substrate. Resting cells from the other strains all exhibited higher *N*-carbamoyl amino acid and amino acid production with unsubstituted hydantoin as compared with 5-substituted methylhydantoin (Table 2.2). Since this research project was directed towards the production of D-amino acids from D,L-5-substituted hydantoins, such as D-HPG from D,L-*p*-hydroxyphenylhydantoin applications, strain RU-OR was chosen for further characterization of hydantoin-hydrolysing activity and development as a biocatalyst.

2.3.2 Optimization of resting cell biocatalytic reactions

Growth phase for harvesting cells

The changes in hydantoin-hydrolysing activity during a batch culture of strain RU-OR were studied in order to determine at which stage of growth relative enzyme activity was highest in complete medium (nutrient broth). During logarithmic phase, relatively low levels of NCG and glycine production from hydantoin were detected (Figure 2.3).

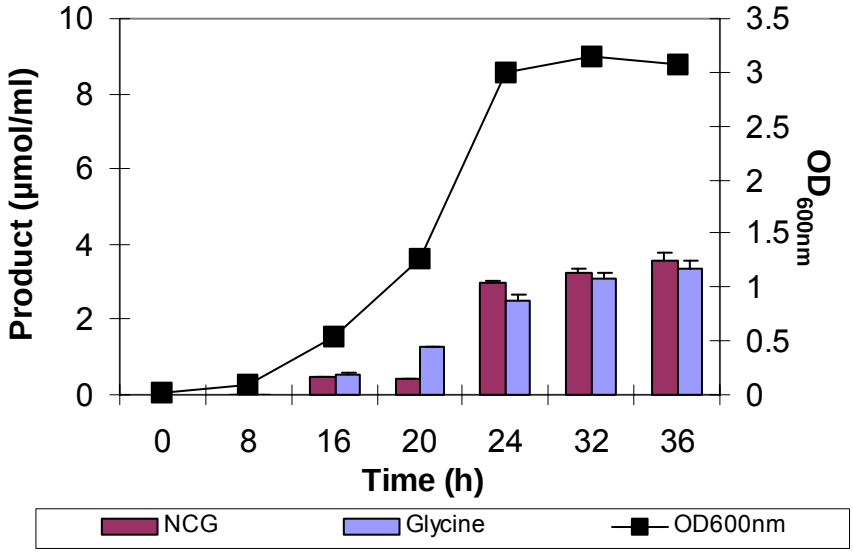


Figure 2.3: Growth and resting cell biocatalytic activity in complete medium. NCG – *N*-carbamoylglycine. Error bars represent SEM (n=12).

Conversion of hydantoin to *N*-carbamoyl amino acid and amino acid was highest from early stationary growth phase onwards, after 24 hours of growth. Little change in product levels was seen from early to late stationary growth. In the light of this data, subsequent resting cell biocatalytic assays described in this chapter were performed using cells harvested in early stationary growth phase, after growth for 24 hours in nutrient broth.

Induction of hydantoin-hydrolysing activity

It has previously been reported that hydantoinase and NCAAH activities are induced to high levels when cells are grown in the presence of hydantoins or their structural analogs (Meyer & Runser, 1993; Ogawa and Shimizu, 1997; Syldatk *et al.*, 1990b). To test the inducibility of hydantoin-hydrolysing activity in strain RU-OR, cells were cultured in the presence of inducers and assayed for hydantoinase and NCAAH activity. No hydantoinase activity was detected in cells grown without inducer, whilst enzyme activities in strain RU-OR were elevated above uninduced levels by all four inducers (Table 2.3).

***Table 2.3**:* Hydantoin-hydrolysing enzyme activity with cells cultured in media containing hydantoin or hydantoin analogues as inducers.

Inducer (0.1%)	Hydantoinase Activity (µmol/ml/20mg WCM)	NCAAH activity (µmol/ml/20mgWCM)
-	0.95 ± 0.04	1.05 ± 0.02
Hydantoin	4.10 ± 0.21	5.68 ± 0.28
Dihydrouracil	1.89 ± 0.05	3.06 ± 0.10
Dimethylhydantoin	2.44 ± 0.14	3.28 ± 0.15
2-Thiouracil	5.72 ± 0.14	9.70 ± 0.33

Key: NCAAH – *N*-carbamoyl amino acid amidohydrolase. WCM- wet cell mass. ± SEM (n=12). Comparison of independent means for all induced activities versus uninduced levels using t-test analysis showed that p>0.1 in all cases.

Dihydrouracil was a poor inducer of hydantoinase activity, resulting in less than two-fold increase in hydantoinase activity. Induction with 0.1% dimethylhydantoin did produce hydantoinase and NCAAH activity at least two- to three-fold greater than that of uninduced cells, but the highest levels of activity were induced by the presence of hydantoin or 2-thiouracil (2-TU). There was a five-fold increase in hydantoinase activity and a ten-fold increase in *N*-carbamoyl amino acid amidohydrolase activity when cells were induced with 2-TU. Therefore, cells for subsequent resting cell biocatalytic reactions were cultured to early stationary growth phase in nutrient broth containing 0.1% 2-TU.

Resting cell biocatalytic reaction conditions

The reaction conditions for maximum hydantoin-hydrolysing activity in biocatalytic reactions were optimised by first changing one individual variable in each experiment and then by altering combinations of different variables.

The concentration of biocatalyst (measured as mg WCM per ml of reaction mixture) was optimized for maximum production of NCG and glycine from hydantoin. Although variations in biocatalyst concentration did not appear to have a profound effect on hydantoin-hydrolysing activity, the best production levels occurred using biocatalyst concentrations between 20 and 30 mg/ml (Figure 2.4). Concentrations greater than 30 mg/ml resulted in decreased conversion of hydantoin to NCG and

glycine. The optimum biocatalyst concentration for biocatalytic reactions was therefore standardized at 20 mg/ml wet cell mass for subsequent experiments.

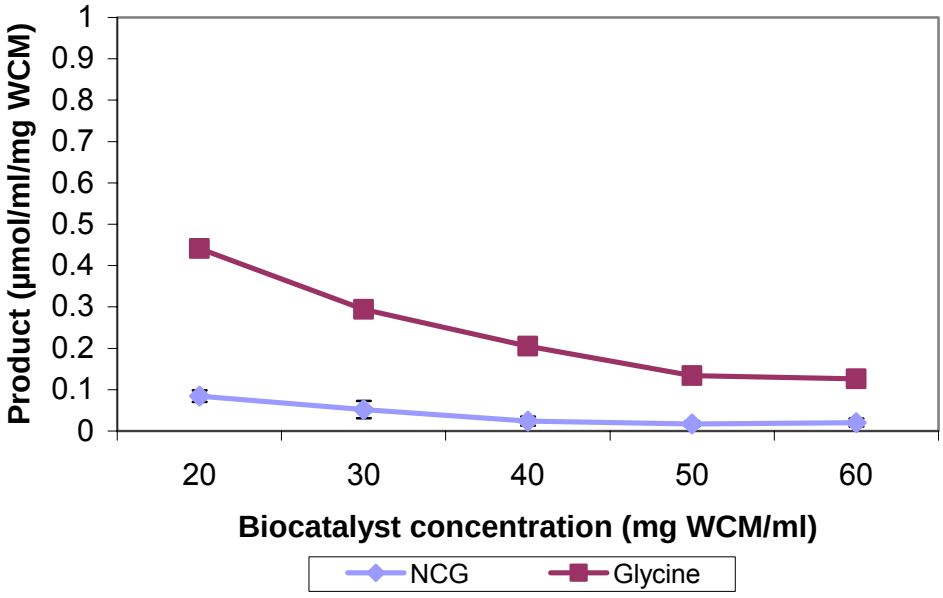


Figure 2.4: Optimization of the amount of wet cell mass in hydantoin-hydrolysing biocatalytic reactions. Error bars represent SEM (n=12). Error bars not visible where SEM < 0.01. Reactions were performed at 40°C, 200rpm, and pH 8.0, for 6 hours.

Having established saturation levels for the biocatalyst concentration, the ratio of substrate to biocatalyst concentration was optimized using a constant biocatalyst concentration of 20mg/ml WCM. The biocatalyst to substrate ratio in the biocatalytic reactions did not have a dramatic effect on the production of NCG and glycine by RU-OR cells. There was a gradual increase in total conversion of hydantoin from 3.62 µmol/ml to 5.56 µmol/ml from concentrations of 10mM to 50mM (Figure 2.5). . Product formation decreased slightly with the increase of substrate concentration to 100mM substrate, and increased to slightly higher levels when using 150mM hydantoin. For maximum hydantoin-hydrolysing activity, 50 mM hydantoin was used for all further resting cell biocatalytic reactions

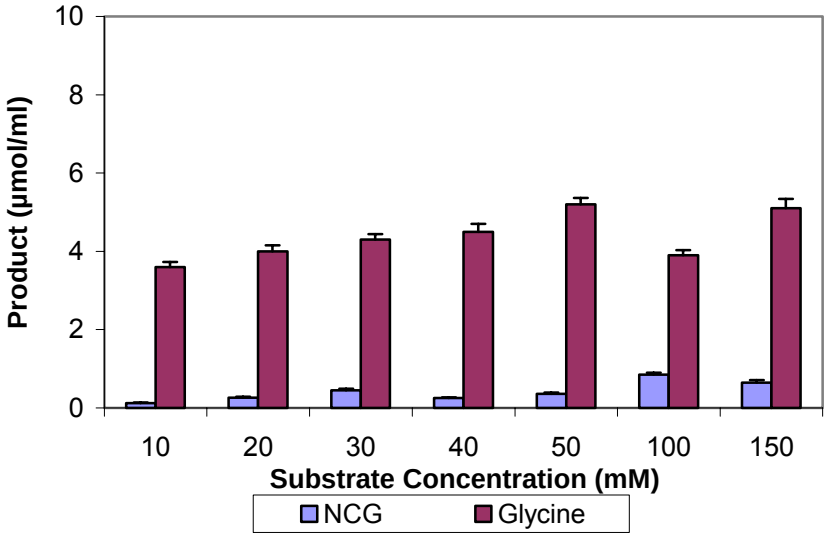


Figure 2.5: The effect of substrate concentration on the production of NCG and glycine from hydantoin by STRAIN RU-OR resting cells. Error bars represent SEM (n=12).

Using the optimised biocatalyst to substrate ratio of 20mg/ml WCM: 50mM hydantoin, hydantoin-hydrolysing activity in biocatalytic reactions was measured at time intervals from 0 to 24 hours (Figure 2.6). There was a gradual increase in NCG and glycine production up to 6 hours, after which the activity was fairly constant, only increasing slightly between 6 and 24 hours. Rate of conversion was highest at 6 hours, slowing considerably after this time. Subsequent biocatalytic reactions with strain RU-OR resting cells were standardized to a reaction time of 6 hours.

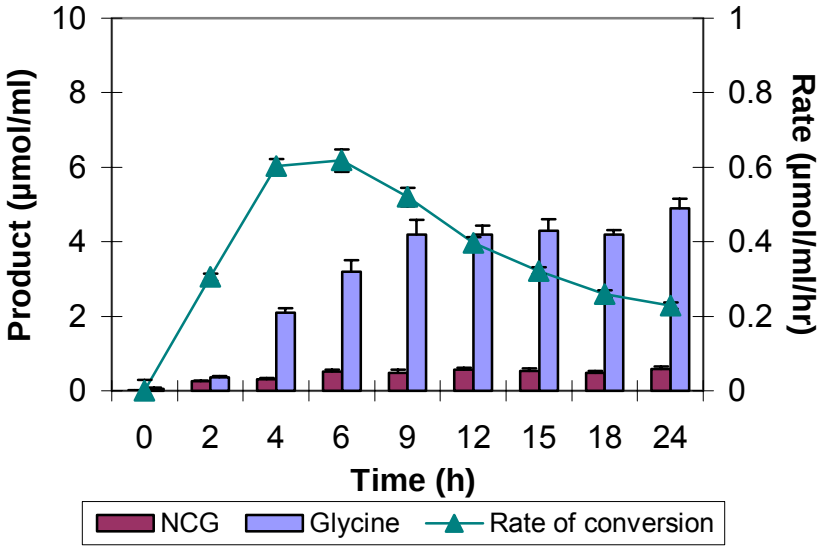


Figure 2.6: The production of NCG and glycine, and rate of conversion of hydantoin over time by strain RU-OR in resting cell biocatalytic reactions. Error bars represent SEM (n=9).

In summary, this data indicates that maximum biocatalytic activity in resting cell reactions was produced using strain RU-OR cells harvested after growth to early stationary growth phase in nutrient broth containing 0.1% 2-TU as an inducer. Optimum reaction conditions were obtained using a biocatalyst: substrate ratio of 20mg/ml WCM: 50mM hydantoin, for a reaction time of 6 hours.

Having established the basic parameters for maximum production from hydantoin in resting cell biocatalytic reactions, the optimum conditions for hydantoinase (using hydantoin as a substrate) and NCAAH activity (using *N*-carbamoyl amino acids as substrates) of strain RU-OR resting cells were refined. Similar levels of hydantoinase and NCAAH activities were measured in biocatalytic resting cell reactions performed over a range of temperatures. Comparable levels of hydantoinase activity were observed between 30°C to 70°C, with maximum activity levels at 40°C – 60°C. NCAAH activity was highest at 60°C, but was relatively constant between 40°C and 60°C (Figure 2.7).

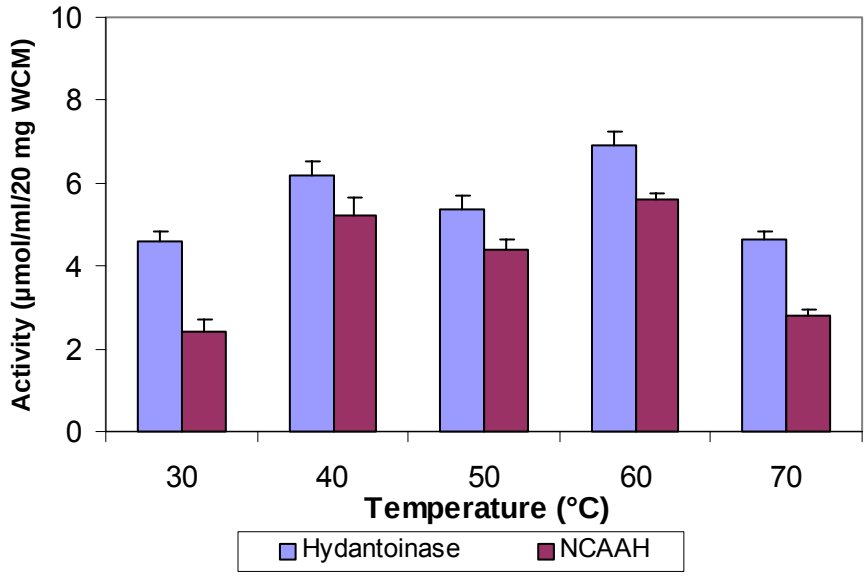


Figure 2.7: The effect of reaction temperature on the hydantoin-hydrolysing biocatalytic activity of strain RU-OR resting cells. Error bars represent SEM (n=12). WCM- wet cell mass. NCAAH – *N*-carbamoyl amino acid amidohydrolase.

Investigation into the influence of reaction pH on hydantoin-hydrolysing activity revealed a wide pH range for NCAAH activity from pH 6-pH10, but distinct maximum hydantoinase activity at pH 9.0 (Figure 2.8).

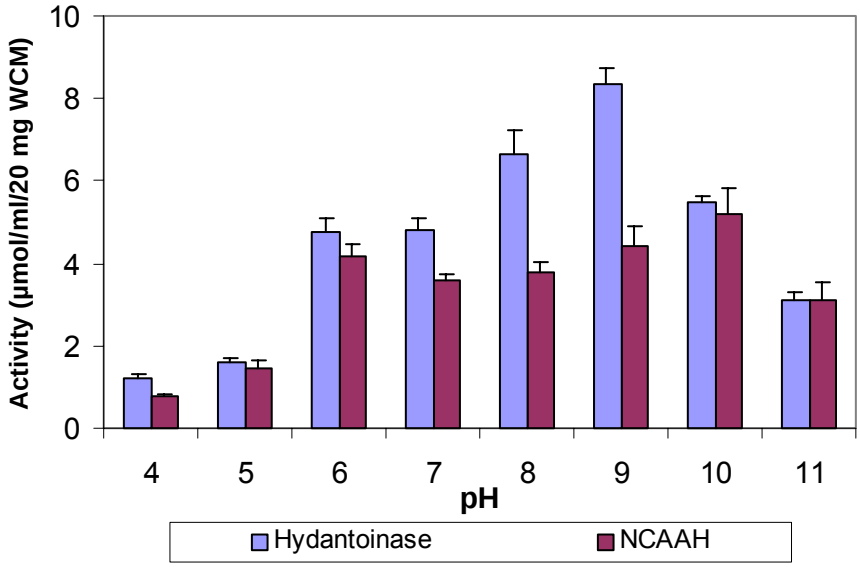


Figure 2.8: The effect of reaction pH on the hydantoin-hydrolysing biocatalytic activity of strain RU-OR resting cells. Error bars represent SEM (n=12). WCM- wet cell mass.

In order to increase the availability of substrate and release of products, strain RU-OR cells were treated with various detergents in order to permeabilise the cell membrane and possibly increase the diffusion/uptake of substrates and products during the catalysis reaction (Olivieri *et al*., 1981; Gross *et al*., 1990). Hydantoinase and NCAAH activity of RU-OR cells actually decreased when treated with a 0.1% or 1% final concentration (v/v) of most of the detergents used as permeabilising agents (Figure 2.9). The use of 0.1% Tween 20 did produce an interesting increase in hydantoinase activity (~ 1.5 fold, Figure 2.9) but as NCAAH activity decreased as a result of treatment with this and most other detergents tested (data not shown), the use of detergents to permeabilise the cell membrane was not pursued further.

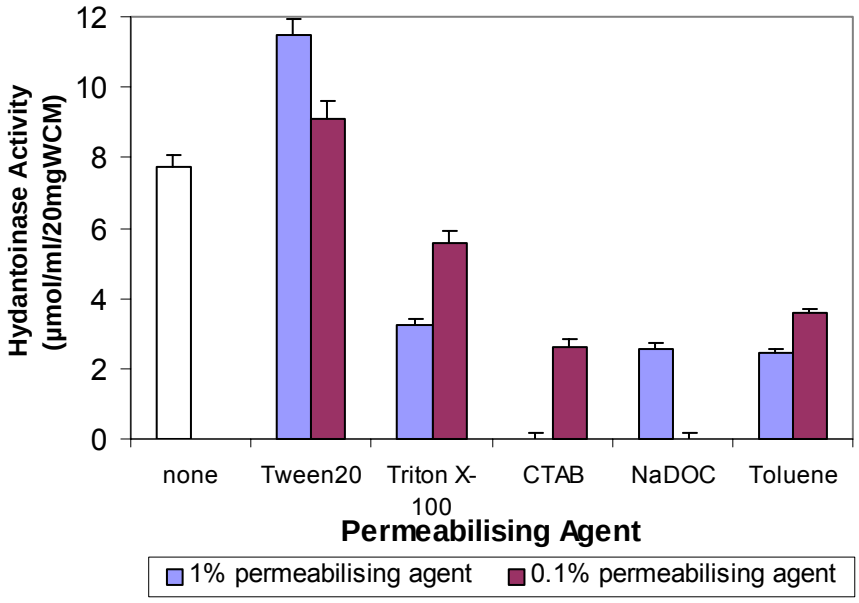


Figure 2.9: Permeabilisation of RU-OR cells prior to resting cell biocatalytic reactions. Error bars represent ± SEM (n=12). Key: CTAB- cetyltrimethylammonium bromide, NaDOC – sodium desoxycholate.

Substrate selectivity

Having optimised resting cell biocatalytic reaction conditions for production of NCG and glycine from hydantoin, and maximum hydantoinase and NCAAH activity in strain RU-OR, the substrate selectivity of the hydantoin-hydrolysing enzyme system in RU-OR cells was re-examined. For the purposes of examining substrate specificity in more detail, enzyme activity was measured as the amount of product formed (µmol/ml), and not as total hydantoinase or NCAAH activity. The ability of RU-OR cells to hydrolyse racemic hydantoins was tested using a combination of reaction temperature (40^{o}C; 60^{o}C) and buffer pH conditions (8.0; 9.0). Resting cells showed higher conversion levels with D,L-methylhydantoin and D,L-*p*-hydroxyphenylhydantoin than with hydantoin, both substrates generally yielding twice as much amino acid as was obtained from hydantoin (Table 2.4). Overall conversion was highest at pH 9.0 for all substrates. Whilst more glycine was generally produced from hydantoin and NCG at a reaction temperature of 40^{o}C, a reaction temperature of 60^{o}C resulted in the production of more alanine and *p*-hydroxyphenylglycine from the respective substrates. Analysis of alanine and *p*-

hydroxyphenylglycine by chiral TLC indicated that the RU-OR enzyme system was D-stereoselective (Figure 2.10).

Table 2.4: Effect of pH and temperature on conversion of D,L-hydantoin, D,L-methylhydantoin and D,L-hydroxyphenylhydantoin.

Substrate	Reaction pH	Product (µmol/ml)			
		40°C		60°C	
		N-carbamoyl amino acid	Amino acid	*N*-carbamoyl Amino acid	Amino acid
Hydantoin	8.0	0.94 ± 0.07	2.84 ± 0.16	1.29 ± 0.29	5.26 ± 0.42
	9.0	3.80 ± 0.11	4.74 ± 0.15	4.64 ± 0.22	1.87 ± 0.32
N-Carbamoyl glycine	8.0	-	3.25 ± 0.13	-	5.10 ± 0.25
	9.0	-	4.80 ± 0.23	-	5.96 ± 0.23
D,L-Methylhydantoin	8.0	6.15 ± 0.25	4.83 ± 0.15	6.62 ± 0.15	6.79 ± 0.26
	9.0	3.98 ± 0.16	7.52 ± 0.36	3.85 ± 0.19	8.85 ± 0.34
N-Carbamoyl alanine	8.0	-	4.10 ± 0.19	-	6.98 ± 0.24
	9.0	-	6.98 ± 0.23	-	8.95 ± 0.35
D,L-Hydroxyphenylhydantoin	8.0	1.88 ± 0.06	7.77 ± 0.34	5.42 ± 0.18	4.04 ± 0.24
	9.0	2.15 ±0.09	5.54 ± 0.17	2.65 ± 0.18	7.66 ± 0.23
N-Carbamoyl-*p*-hydroxyphenylglycine	8.0	-	8.10 ± 0.29	-	6.15 ± 0.23
	9.0	-	7.92 ± 0.26	-	7.95 ± 0.31

Key: ± SEM (n=9). – not done.

Substrate stereoselectivity

Initially, chiral TLC was used to analyze the enantiomeric purity of the reaction products from hydantoin-hydrolysing reactions using RU-OR cells. Subsequently, chiral HPLC analysis was developed for improved quantitative analysis of the reaction products. The alanine and *p*-hydroxyphenylglycine produced from the reactions in Table 2.4 were analyzed by chiral TLC (Figure 2.10). Only D-alanine and D-HPG were produced from both hydantoin and *N*-carbamoyl amino acid substrates, indicating that the enzyme system of strain RU-OR is D-stereoselective.

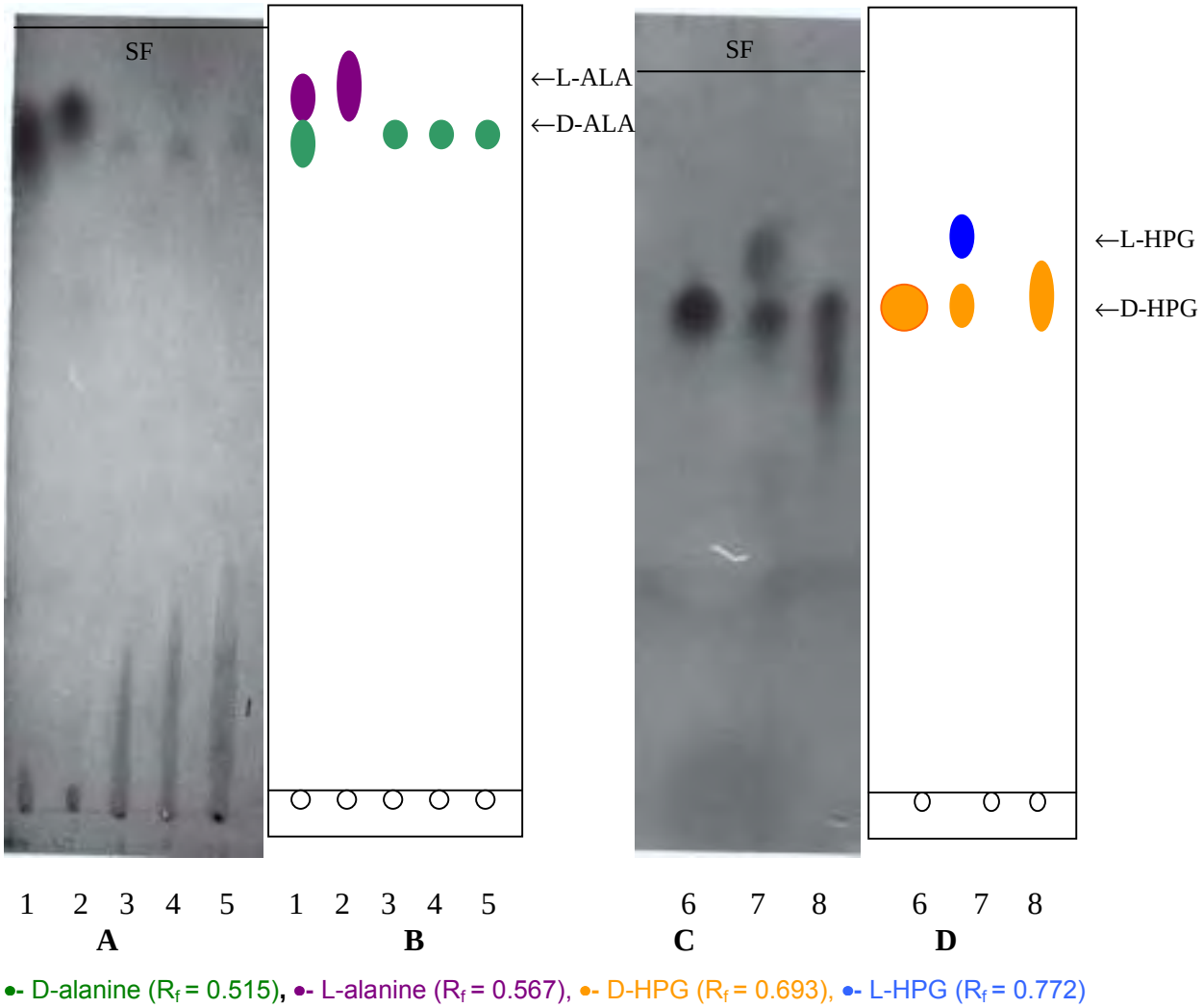


Figure 2.10: Chiral TLC analysis of amino acids produced by strain RU-OR. A, Photograph of TLC illustrating separation of D-and L- alanine. B, graphical depiction of A. C, Photograph of TLC illustrating separation of D-and L-p-hydroxyphenylglycine. D, graphical depiction of C. Each lane was loaded with 20µl of standard or sample (reaction supernatant). Lanes: 1, 10mM D,L-alanine; 2, 10mM L-alanine; 3-5 supernatant from reaction of RU-OR cells with: 3, D-methylhydantoin; 4, L-methylhydantoin; 5, *N*-carbamoyl-D,L-alanine; 6, 10 mM D,L-p-hydroxyphenylglycine; 7, D-p-hydroxyphenylglycine; 8 supernatant from reaction of RU-OR with *N*-carbamoyl-D,L-p-hydroxyphenylglycine. SF-solvent front. HPG – p-hydroxyphenylglycine.

Experiments were conducted to further examine the stereoselectivity of the hydantoinase and *N*-carbamoyl amino acid amidohydrolase reactions. Biocatalytic assays were performed using D-methylhydantoin, L-methylhydantoin and D,L-methylhydantoin (a 1:1 mixture of 50 mM D- and L-methylhydantoin), as substrates. Reactions were performed in 0.1M phosphate buffer pH 7.0 to prevent the spontaneous racemization of D,L-hydantoin to D-hydantoin which occurs under alkaline conditions (Pietzsch and Syldatk, 1995). In all three cases, similar amounts of *N*-carbamoylalanine and alanine were produced, irrespective of the chirality of the methylhydantoin, showng that the hydantoin-hydrolysing enzymes of strain RU-OR

were not inhibited by the L-isomers of the substrate (Table 2.5). When analysed by chiral TLC, only D-alanine was detected in all three reactions.

Table 2.5: Stereoselectivity of hydantoin-hydrolysing activity in resting cell assay (pH 7.0, 40°C).

Substrate	Product (µmol/ml)	
	***N*-carbamoyl amino acid**	**D-Amino acid**
D,L-*N*-Carbamoyl alanine	-	6.33 ± 0.21
D,L-*N*-Carbamoyl hydroxyphenylglycine	-	7.11 ± 0.35
D-Methylhydantoin	2.26 ± 0.03	4.52 ± 0.59
L-Methylhydantoin	2.36 ± 0.19	4.37 ± 0.03
D,L-Methylhydantoin	2.28 ±0.30	4.80 ± 0.20

Key: - not done. ± SEM (n=9). Activity for this experiment is measured as the individual production of *N*-carbamoyl amino acid or amino acid. The chirality of the amino acid produced was determined using chiral TLC**.** P values for t-test analysis of the values obtained from D-, L- and D,L-methylhydantoin <0.006.

To examine the stereoselectivity of the *N*-carbamoyl amino acid amidohydrolase, biocatalytic assays were performed using *N*-carbamoyl-D,L-alanine and *N*-carbamoyl-D,L-*p*-hydroxyphenylglycine as substrates. Analysis of the amino acids produced again resulted in detection of only the D-enantiomer (Figure 2.10). This data led to the conclusion that the NCAAH enzyme of RU-OR was D-stereoselective. Conversion of L-methylhydantoin to D-alanine at pH 7.0 suggests the presence of a D-hydantoin racemase. However, the data could not provide conclusive evidence of the stereoselectivity of the hydantoinase enzyme from strain RU-OR.

2.3.3 Identification of Strain RU-OR

The isolate RU-OR was initially identified as a possible strain of *Burkholderia* (formerly *Pseudomonas*) *cepacia* using API biochemical identification (Table 2.1). PCR amplification of the 16S rRNA gene from strain RU-OR was used for restriction mapping analysis to confirm this identification. The data showed that the 16S rRNA gene from RU-OR (Figure 2.11B) did not closely resemble the expected *Burkholderia cepacia* 16S rRNA gene retrieved from the Ribosomal Database (Maidek *et al.,* 2000) (Figure 2.10A).

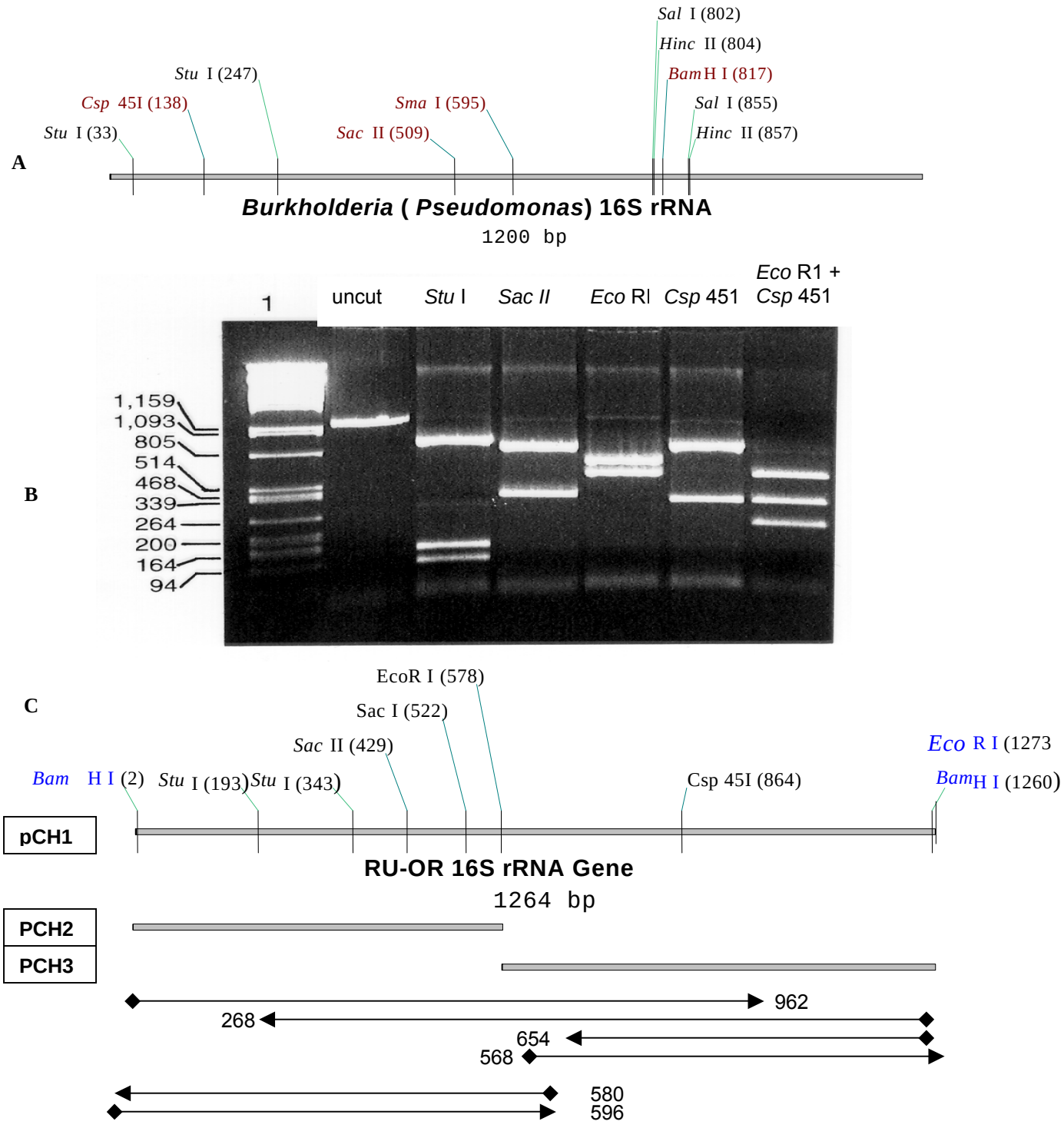


Figure 2.11: Restriction map and RFLP analysis of the 16S rRNA gene from strain RU-OR. **A**, map of a typical *Burkholderia* sp. 16S rRNA gene derived from sequence data obtained from the RDP; **B**, Restriction mapping of the 16S rRNA gene from strain RU-OR. Lanes: 1-Lambda DNA digested with *Pst* I, 2-Uncut PCR fragment, (3-7) PCR fragment digested with: 3-*Stu* I, 4-*Sac* II, 5-*Eco* RI, 6-*Csp* 451,7-*Eco* RI & *Csp* 451. **C**, map of RU-OR 16S rRNA gene and diagram of the sequence fragments generated from pCH1, pCH2 and pCH3 (Appendix 7) that were used to construct the full-length 16S rRNA sequence for RU-OR. For primer sequences see Appendix 6, for full RU-OR 16S rRNA sequence and alignment with *A. tumefaciens* IAM 13129 see Appendix 5.

For example, restriction mapping identified an *Eco* RI site at position 570 in the RU-OR 16S rRNA gene which was absent in the 16S rRNA gene from the *B. cepacia* type strain. Other key inconsistencies included the difference in *Csp* 451 and *Stu* I sites between the two 16S rRNA genes, and the lack of *Sma* I and *Sal* I sites in the 16S rDNA of strain RU-OR that are present in the *B. cepacia* 16S rRNA gene.

pCH1 and two *Eco* RI subclones (pCH2 and pCH3) were used to obtain full sequence of both strands of the 16S rRNA gene (Figure 2.11). Sequence data was aligned using Vector NTI (Informax) and Clustal W Sequence Alignment (Thomson *et al*., 1994). The completed sequence (Appendix 5) was aligned with 16S rRNA sequences from the Internet Ribosomal Database Project (Maidek *et al*., 2000) and found to have 96% sequence identity with strain *A. tumefaciens* IAM 11391 (Yanagi *et al*., 1993). The predicted restriction map derived from the DNA sequence of the RU-OR 16S rRNA gene correlated with the mapping of the 16S rRNA PCR product (Figure 2.11), therefore positively identifying strain RU-OR as a strain of *A. tumefaciens*. *A. tumefaciens* RU-OR has been submitted to the ATCC strain collection (Accession Number: BAA-174) (Virginia, USA).

2.4 Discussion

Selection of hydantoin-hydrolysing microorganisms by assessing growth with hydantoin as a sole nitrogen source resulted in the successful establishment of the Rhodes Hydantoinase Research Group strain, of which strain RU-OR was selected for further characterisation based on the observed conversion of methylhydantoin to alanine by RU-OR cells.

Strain RU-OR was identified as a strain of *A. tumefaciens* using restriction mapping and sequence analysis of the 16S rRNA gene. The misidentification of strain RU-OR as a strain of *Burkholderia cepacia* by biochemical tests illustrates the importance of molecular identification using tools such as sequence analysis of 16S rRNA genes. PCR amplification of small subunit rRNA genes provides the ideal bacterial

identification system, as it is sensitive, rapid, and can be used to discriminate among a wide variety of bacterial taxa (Brunk *et al*., 1996). This technique was routinely used in subsequent research for the confirmation of the strain identity of RU-OR, derivatives strains described in subsequent chapters and the identification of other hydantoin-hydrolysing bacterial strains currently being researched by the Rhodes Hydantoinase Research Group (Hartley *et* al., 1998; Burton *et al.,* 1997; Buchanan *et al*., 2001). Within the large selection of reported hydantoin-hydrolysing bacteria, the characteristics of hydantoin-hydrolysing activity vary considerably between species and even strains. Several Agrobacteria with hydantoin-hydrolysing activities have been isolated and *A. radiobacter* (sometimes referred to as *A. tumefaciens*) NRRL B11291 is arguably the most abundantly used and well-characterized biocatalyst for the production of D-*p*-hydroxyphenylglycine.

Optimization of enzyme production during batch culture in complete medium showed that hydantoin-hydrolysing activity was highest when *A. tumefaciens* RU-OR cells were harvested in early stationary growth phase. This was consistent with enzyme production reported for other hydantoin-hydrolysing *Agrobacterium* species (Durham & Weber, 1995; Möller *et al.*, 1988; Runser *et al.*, 1990). Also consistent with enzyme production data for *Agrobacterium* IP-671, was the high level of induction of enzyme activity by 2-thiouracil (Meyer & Runser, 1993). This was dissimilar to enzyme production by *A. tumefaciens* 47 C cells, which is best induced by methylhydantoin (Durham & Weber, 1995). Interestingly, NCAAH activity in *A. tumefaciens* RU-OR cells induced by 2-TU was two-fold higher than in cells induced by hydantoin. This increase in activity was not apparent when using hydantoin as a substrate. Therefore, in addition to 2-TU acting as an inducer of hydantoin-hydrolysing activity, the sulfhydryl groups of 2-TU may also enhance the NCAAH activity of *A. tumefaciens* RU-OR. In addition to inducing high levels of hydantoin-hydrolysing activity, 2-TU is non-metabolizable which is advantageous for industrial applications (Meyer *et* al., 1993). This property also allows for differentiation between of the effect of induction and nitrogen source effects, which is not possible when using a metabolizable compound as an inducer. Preferential induction of hydantoin-hydrolysing activity by six-membered cyclic amides in these strains, and not in other *Agrobacterium* strains, lends support to the hypothesis that hydantoin-hydrolysing

enzymes are involved in different catabolic pathways in different bacterial strains (Ogawa *et al*., 1994a; Pietzsch and Syldatk, 1995), and may have evolved from a common ancestry in the primitive hydrosphere, where hydantoins and *N*-carbamoyl amino acids were more prevalent (May *et al*., 1998a; Syldatk *et al*., 1999).

Among the hydantoin-hydrolysing Agrobacteria, the pH optima and temperature optima differ between strains of the same species, although they share similar substrate- and stereo-selectivities (Hartley *et al.*, 1998; Olivieri *et al.*, 1980; Nanba *et al.*, 1999; Runser *et al.*, 1991). The biochemical characteristics of the RU-OR hydantoin-hydrolysing enzyme system as observed during resting cell biocatalytic reactions are summarized in Table 2.6.

Table 2.6: Summary of the optimization of hydantoin-hydrolysing activity in biocatalytic reactions with *A. tumefaciens* RU-OR cells.

Variable	**Optimal Condition**	
	Hydantoinase	**NCAAH**
Time	6 hours	
Biocatalyst Concentration	20 mg/ml WCM	
Substrate Concentration	50 mM hydantoins	25 mM NCAA
Reaction Temperature	40°C-60°C	
Reaction pH	pH 9	pH 6-pH 10
Substrate Affinity	D,L-*p*-HPH	*N*-carbamoyl-D,L-HPG
Stereoselectivity	D- or non-selective, possible racemase	D-selective

Key: HPH-hydroxyphenylhydantoin, HPG-hydroxyphenylglycine, NCAA- *N*-carbamoyl-amino acids. WCM – wet cell mass.

Hydantoinase activity in RU-OR resting cells was highest at reaction temperatures between 40 ^{o}C and 60 ^{o}C and at pH 9, dependent upon the substrate. The activity of

the RU-OR hydantoinase was similar to that of hydantoinases characterized from *A. radiobacter* NRRL B11291, *A. tumefaciens* 47C and *Agrobacterium* sp. IP-671 (Durham & Weber, 1995; Olivieri *et al.*, 1981; Runser *et al.*, 1990; Runser & Ohleyer, 1990). NCAAH activity was also highest between temperatures of 40 ^{o}C and 60 ^{o}C, and over a broad pH range (pH 6 - 11). Temperature optima in this range have been published for several NCDAAH enzymes (Olivieri *et al.*, 1979; Yokozeki *et al*., 1987; Ogawa *et al*., 1993, 1994; Louwrier and Knowles, 1996b). However, the NCAAH activity in *A. tumefaciens* RU-OR was distinguished from that of *Agrobacterium* IP-671, *A. radiobacter* NRRL B11291 and *Agrobacterium* sp. 80/44-2A enzymes by its activity under high pH conditions, up to pH 11 (the latter three enzymes are virtually inactive at pH 8.5 and higher: Olivieri *et al.*, 1978; Olivieri *et al.*, 1981; Runser *et al.*, 1990; Runser & Ohleyer, 1990; Louwrier and Knowles, 1996a; Neal *et al*., 1999).

The addition of ionic and non-ionic detergents to the buffered wash mixtures was used to determine if disruption of the resting cell membrane might increase the availability of substrate for enzyme catalysis, and diffusion of the product into the supernatant, thus increasing the overall production and detection of *N*-carbamoyl amino acids and amino acids. Olivieri *et al*. (1981) reported increased hydantoinase activity after permeabilisation of *A. radiobacter* NRRL B11291 cells with 0.1% toluene. Although the addition of 0.1% Tween 20 did result in an increase in hydantoinase activity, a concomitant increase in NCAAH activity in RU-OR cells was not observed. Sodium desoxycholate has also been used successfully to increase the availability of D,L-5-indolylmethylhydantoin as a substrate for *Arthrobacter* sp. DSM 3747 cells (Syldatk *et al*., 1990a), but a similar effect was not observed for RU-OR cells with hydantoin as a substrate. In general, hydantoin-hydrolysing activity in RU-OR cells decreased as a result of treatment with the detergents tested, and the addition of detergents to the reaction mixtures also interfered with product detection in colourimetric assays.

Strain RU-OR cells produced only D-alanine from both racemic methylhydantoin and L-methylhydantoin, suggesting a D-selective or non-stereoselective hydantoinase. The enantioselectivity of the hydantoinase activity in RU-OR cells remains

unresolved as low levels of production of the *N*-carbamoyl amino acid intermediate during biocatalytic reactions at pH 7 made differentiation between the D- and L-enantiomers of the intermediate difficult. However, the production of equal amounts of D-alanine from D-, L- or D,L-MH was consistent with the activity of a D-hydantoinase combined with racemase activity. The conversion of L-methylhydantoin to D-alanine at pH 7.0 also indicated the presence of a D-hydantoin racemase.

Conversion of a racemic mixture of *N*-carbamoyl-D,L-hydroxyphenylglycine or *N*-carbamoyl-D,L-*p*-hydroxyphenylglycine by *A. tumefaciens* RU-OR cells resulted in the production of only D-alanine or D-*p*-hydroxyphenylglycine, providing evidence for D-stereoselective NCAAH enzyme activity. The NCAAH activity in *A. tumefaciens* RU-OR was further distinguished by the fact that the presence of *N*-carbamoyl-L-amino acids did not appear to affect its activity. Other *Agrobacterium* NCAAH enzymes are strongly inhibited by *N*-carbamoyl-L-amino acids (Olivieri *et al.*, 1978; Nanba *et al*., 1998; Runser *et al.*, 1990).

In conclusion, characterization of the hydantoin-hydrolysing enzyme system of *A. tumefaciens* RU-OR in resting cell biocatalytic reactions proved that this strain can be induced by 2-TU to convert racemic mixtures of 5-substituted hydantoins to their respective D-amino acids, and that NCAAH activity in this strain is not inhibited by L-isomers of *N*-carbamoyl amino acids. Thus, whilst hydantoinase activity in RU-OR is very similar to that reported for other Agrobacteria, the NCAAH activity of *A. tumefaciens* RU-OR resting cells shows some novel characteristics.

Together with a high substrate selectivity for D,L-5-HPH, the novel biocatalytic properties of the RU-OR hydantoin-hydrolysing enzyme system indicated potential for the application of RU-OR cells in a biocatalytic system for the production of D-HPG from D,L-5-HPH. However, the characterisation of activity in resting cell biocatalytic reactions suggested that enzyme production and activity depended on

both the presence of an inducer and the growth phase of RU-OR cells. As these factors posed limitations for the economic viability of a biocataltyic process using strain RU-OR, the regulation of hydantoin-hydrolysing enzyme production in RU-OR cells was explored further.

Chapter 3

Regulation of hydantoin-hydrolysing activity in *Agrobacterium tumefaciens* RU-OR

Chapter 3

Regulation of hydantoin-hydrolysing activity in *A. tumefaciens* RU-OR

3.1 Introduction

Once a bacterial strain that produces hydantoinase or NCAAH activity with potential industrial application has been isolated and characterized, the optimization of enzyme production is the next logical step in the development of a biocatalyst (Schmid *et al*., 2001). Several reports in the literature have indicated that the production of the enzymes involved in hydantoin-hydrolysis in *Agrobacterium* cells is regulated by growth conditions. Maximum enzyme activity in complex growth medium is detected during late logarithmic or early stationary phase and is dependent upon the presence of hydantoins or hydantoin analogues in the growth medium (Möller *et al*., 1988; Meyer & Runser, 1993; Syldatk *et al.*, 1990a), suggesting that the two main factors regulating the production of hydantoin-hydrolysing enzyme activity are nitrogen control and induction.

Meyer and Runser (1993) reported optimal induction of hydantoinase and NCAAH activity in *Agrobacterium* sp. IP 671 with the hydantoin analogue 2-thiouracil. Hydantoin–hydrolysing activity in other *Agrobacterium* species is optimally induced by different compounds, such as methylhydantoin (Louwrier and Knowles, 1996, Durham and Weber, 1997). Although this is not universally apparent (e.g. Runser *et al*., 1990a), D-Hydantoinase activity has often been regarded as being identical to dihydropyrimidinase activity in bacteria (Syldatk and Pietzsch, 1995), and can also be induced by pyrimidine analogues. The presence of uracil in the growth medium has been reported to induce high levels of hydantoinase and NCAAH activity in *A. radiobacter* NRRL B11291 cells (Olivieri *et al*., 1981). In contrast, Deepa *et al*. (1993) reported constitutive expression of hydantoin-hydrolysing activity in the same strain, without the need for induction, so the induction of hydantoinase and NCAAH activity in this particular strain remains unclear. However, the general consensus of

reported literature is that the production of hydantoin-hydrolysing activity in *Agrobacterium* strains requires induction by hydantoin analogues in the growth medium.

Although hydantoin can serve as a source of both carbon and nitrogen for metabolism, D-stereoselective hydantoin hydrolysis has been chiefly linked to nitrogen metabolism in bacteria, through the dihydropyrimidinase pathway (Vogels and Van der Drift, 1976; Yokozeki *et al.,* 1987a; Ogawa and Shimizu, 1997; Runser *et al.*, 1990a). Carbon catabolite repression of hydantoin-hydrolysing enzyme production by non-growth-rate-limiting carbon sources has not been reported to date. In fact, glucose is frequently utilized as the optimum carbon source in growth media for the production of hydantoin-hydrolysing activity (Syldatk *et al*., 1990a; Deepa *et al*., 1993), hence hydantoin-hydrolysing activity seems to be regulated principally by nitrogen availability in the cell.

Research into the best synthetic medium for growth and production of hydantoin-hydrolysing activity in batch culture of *A. radiobacter* NRRL B11291 showed that ammonium was not the optimal nitrogen source for enzyme production, and that non-preferential nitrogen sources provided better hydantoin-hydrolysing enzyme activity (Deepa *et al.*, 1993; George and Sadhukhan., 1996; Achary *et al*., 1997). This suggested the regulation of hydantoin-hydrolysing activity by nitrogen catabolite repression.

Having established the commercial potential of the D-stereoselective hydantoin-hydrolysing enzyme system of RU-OR (Chapter 2), the development of a production process was required. Understanding the mechanisms that regulated hydantoin-hydrolysing enzyme production in RU-OR was necessary for both optimisation of growth conditions, and for the physiological and genetic manipulation of the regulatory systems for enhanced enzyme biocatalyst production and activity. The previous chapter illustrated that the production of hydantoinase and NCAAH activity in RU-OR only reached high levels after early stationary growth phase in complete medium, and required induction by hydantoin analogues such as 2-thiouracil.

Together with the observed regulationof hydantoin-hydrolysing enzyme production in other *Agrobacterium* strains, this led to the hypothesis that the hydantoin-hydrolysing enzyme systems of RU–OR might be regulated by nitrogen control pathways and an induction system. The aim of this section of work was to examine the regulation of hydantoin-hydrolysing activity in RU-OR cells and to explore the possible involvement of global regulatory pathways, such as the *ntr*-nitrogen regulatory system, in the regulation of hydantoin-hydrolysing activity in strain RU-OR. This chapter describes the effect of induction, carbon and nitrogen sources on hydantoin-hydrolysing activity in RU-OR cells. The three main mechanisms controlling hydantoin-hydrolysing activity in RU-OR were examined and described. The results support the proposed hypothesis and indicate that a complex regulatory network controls the production of hydantoinase and NCAAH enzyme activity in RU-OR cells.

3.2 Methods and Materials

3.2.1 Media and culture conditions

Starter cultures were grown to confluence in HMM. Subsequent culture media consisted of basic M9 minimal (MM) medium supplemented with 200 μM magnesium chloride and calcium chloride, trace elements (Sambrook *et al.*, 1989) and the appropriate carbon and nitrogen source, as described in Appendix 2. Unless otherwise stated, all cultures were routinely grown, shaking at 28^{o}C, in minimal medium (MM) supplemented with 0.01% casamino acids (CAAMM). For induction of hydantoin-hydrolysing enzymes in RU-OR, cells were grown in induction medium (CAAMM + 2-TU) to OD_{600} = 0.5 - 0.8 (Dry cell mass = 20-30gL^{-1}). Cultures were routinely grown to mid-exponential growth phase (OD_{600}=0.5 - 0.8, dry cell mass = 20-30gL^{-1}) for resting cell biocatalytic assays. The growth rate constant (μ) of all cultures was calculated using the formula as described below:

$$\mu = (\log_{10}Z - \log_{10}Z_0)\ 2.303/\ (t\text{-}t_0),$$

where Z is the amount of any cellular component: *t* is time (Stanier *et al.*, 1976). For Z, optical density measured as OD_{600nm} was calibrated with dry cell mass as described in Appendix 4.

3.2.2 Ammonium shock assays.

Induced cells grown inCAAMM + 2-TU, were harvested by centrifugation, washed in 0.1 M potassium phosphate buffer pH 8.0 and resuspended in ammonium shock medium at an OD_{600nm} = 0.5 - 0.8 (ammonium shock medium: MM containing 0.1% $(NH_4)_2SO_4$ and 0.1% 2-thiouracil). Unless otherwise specified, the cells were incubated shaking at 200rpm, 28^{o}C for 30 minutes before being assayed for enzyme activity under standard resting cell biocatalytic reaction conditions. No appreciable increase in optical density (OD600nm ≤ 0.1) was detected over this time period.

3.2.3 Ammonium shock recovery.

Induced cells were subjected to ammonium shock as described above, harvested by centrifugation, washed in a 0.1 M phosphate buffer pH 8.0 and resuspended at an OD_{600nm} = 0.5 - 0.8 in induction medium (CAAMM + 2-TU) or CAAMM. After one hour of incubation with shaking at 28°C, enzyme activity was determined. A small increase in optical density was detected during this period (OD600nm increase ≤ 0.2).

3.2.4 Resting cell biocatalytic assays

Resting cell reactions were performed in accordance with the optimization of resting cell biocatalytic reaction conditions in Chapter 2. The reaction was carried out at 40 °C for 6 h after which samples were microfuged to pellet the cells and the supernatant analysed by colorimetric assays as described in Appendix 4. Units of activity were measured as the *μ*mol/ml of product detected in the supernatant per 20 mg WCM per ml, and are expressed as the average of *n* samples, determined over 3 or 4 replicate experiments. One unit of hydantoinase activity (U) in resting cell reactions was expressed as the total *μ*mol/ml of *N*-carbamoylamino acid and amino acid produced from hydantoin substrate. One unit of *N*-carbamoylamino-acid amidohydrolase activity (U) was expressed as *μ*mol/ml amino acid produced from *N*-carbamoylamino-acid substrate. Statistical analysis was performed as described in Chapter 2.

3.3. Results

3.3.1 Regulation of hydantoin-hydrolysing activity by carbon source

The effect of carbon source upon enzyme activity was investigated by assaying cells grown in HMM with glucose, glycerol or mannitol as carbon sources (Table 3.1). Growth rate of the cells was slow in all the media with hydantoin as a sole nitrogen source (compare to Table 3.2), but significantly slower with mannitol as a carbon source than with glucose or glycerol. Growth with either glucose, glycerol or mannitol as sole carbon sources appeared to have no effect upon hydantoinase activity. However, cells grown in medium containing 1% mannitol as a sole carbon source, showed a significant increase in NCAAH activity. RU-OR cells were also unable to grow in medium containing hydantoin as both sole nitrogen and carbon source.

Table 3.1: Effect of different carbon sources on hydantoin-hydrolysing activity

Carbon Source	Growth Rate Constant (μ)	Hydantoinase Activity (U)	NCAAH Activity (U)
1 % glucose	0.043 ± 0.001	4.87 ± 0.20	5.77 ± 0.15
1 % glycerol	0.047 ± 0.004	3.97 ± 0.18	5.85 ± 0.18
1 % mannitol	0.037 ± 0.006	4.51 ± 0.11	9.89 ± 0.20

Key: Cells were cultured in medium containing 1% glucose as a carbon a source, and the appropriate nitrogen source. Growth rate (μ) was calculated as the difference in optical density (OD_{600nm}) per unit time (h). ± SEM (n=9).

3.3.2 Regulation of hydantoin-hydrolysing activity by nitrogen

Previous experiments showed that hydantoinase and NCAAH expression in RU-OR cells could be detected only in late exponential to early stationary phase during batch culture in a complex growth medium (Chapter 2, Figure 2.3). Further identification and understanding of the regulation of enzyme expression was needed to enable the manipulation of growth conditions for maximum hydantoin-hydrolysing enzyme production and activity in RU-OR cells. To establish a baseline from which to work hydantoinase and NCAAH activities were measured during growth of RU-OR cells in HMM medium containing hydantoin and glucose as nitrogen and carbon sources respectively. Activity of both enzymes was low in early to middle exponential growth

phase, and after the cells reached stationary phase, with highest activity detected during mid to late exponential growth phase (Figure 3.1).

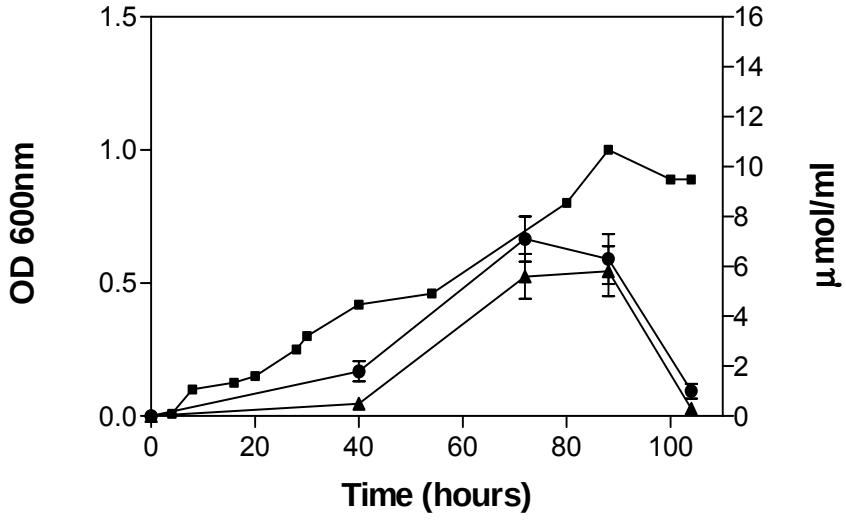


Figure 3.1: Growth and hydantoin–hydrolysing activity of RU-OR containing hydantoin and glucose as nitrogen and carbon sources.■ - OD_{600nm}; ▲ - hydantoinase; ● - *N*-carbomoyl amino acid amidohydrolase.

To assess the effect of different nitrogen sources upon growth rate and enzyme activity, cells were grown in MM medium with either 0.1% ammonium sulfate, 0.1% serine, 0.5% or 0.01% casamino acids or 1% hydantoin as nitrogen sources. Growth rate was measured in ODU/hr after calibration with dry cell mass as described in appendix 4 (Table 3.2).

Table 3.2: Effect of growth in different nitrogen sources on hydantoin-hydrolysing activity in RU-OR.

Nitrogen Source	Growth Rate (μ_{max})	Hydantoinase Activity (U)	NCAAH Activity (U)
1 % hydantoin	0.043 ± 0.001	4.87 ± 0.19	5.77 ± 0.15
0.5% CAA	0.147 ± 0.004	2.07 ± 0.05	1.85 ± 0.08
0.1% $(NH_4)_2SO_4$	0.111 ± 0.018	1.15 ± 0.09	1.09 ± 0.06
0.1% serine	0.089 ± 0.005	4.70 ± 0.26	3.70 ± 0.16*
0.01% CAA	0.060 ± 0.004	10.87 ± 0.43	8.68 ± 0.31

Key: ± - SEM (n = 12). * Measured as the amount of glycine generated from hydantoin as substrate. CAA - casamino acids. Growth rate (μ) was calculated as the difference in optical density OD_{600nm} per unit time (h).

Both 0.1 % ammonium sulfate and 0.5% casamino acids were non-growth-rate-limiting nitrogen sources for RU-OR cells (μ > 0.1 $ODUhr^{-1}$), whilst hydantoin and

0.01% casamino acids were growth-rate-limiting (μ < 0.05 ODUhr^{-1}). Serine as nitrogen source, at a concentration of 0.1%, resulted in an intermediate growth rate for RU-OR cells. Cells were harvested in mid-exponential phase and assayed for hydantoinase and NCAAH activity. High levels of activity were detected for both hydantoinase and NCAAH activity when cells were grown in media containing either hydantoin or 0.01% casamino acids as nitrogen sources, while activity of both enzymes was repressed during growth with ammonium sulfate or 0.5% casamino acids (Figure 3.2, Table 3.2). An intermediate level of hydantoinase and NCAAH activity was present in cells grown with 0.1 % serine, which also showed an intermediate growth rate of ~ 0.09 ODUhr^{-1}.

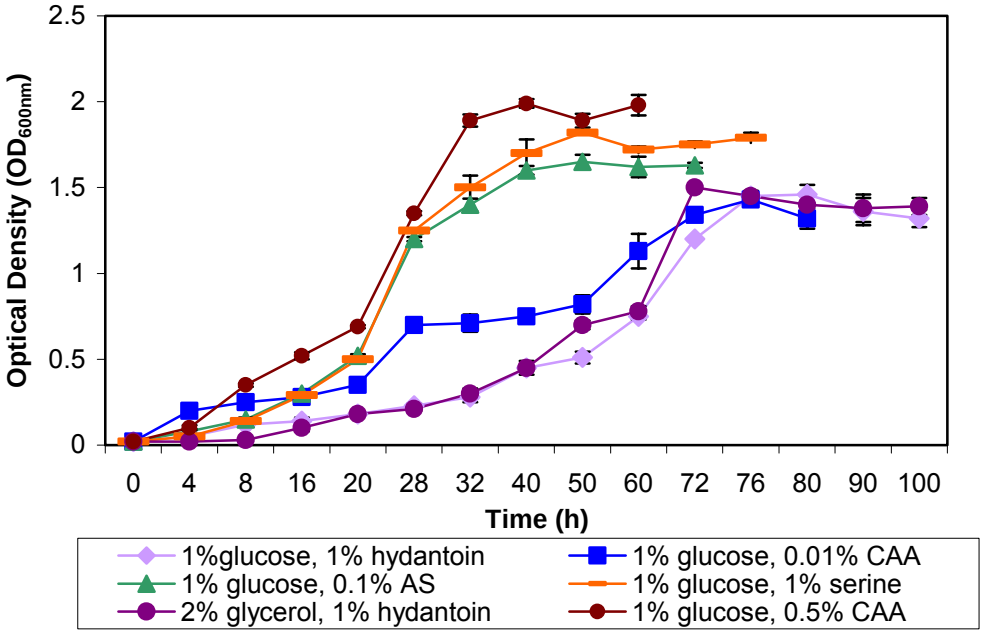


Figure 3.2: Growth of RU-OR cells with different carbon and nitrogen sources. Error bars represent standard error of the mean (n=12). Error bars not visible where SEM< 0.01.

Although, the growth rate of RU-OR cells is highest with 0.5% CAA as a sole nitrogen source, cells grown with 0.1% $(NH4)_2SO_4$ had the lowest hydantoinase and NCAAH activities. This data indicates that hydantoinase and NCAAH activity in RU-OR cells is approximately inversely proportional to the growth rate of the cells. However, the correlation is not absolute, as the most growth-rate-limiting nitrogen source (hydantoin) did not produce the highest hydantoinase and NCAAH activity. Growth with 0.01% CAA as sole nitrogen source produced higher hydantoinase and NCAAH activity. Other factors, such as the level of starvation of the cells may influence the hydantoinase and NCAAH activity levels. It is clear, though, that rapid

growth of RU-OR cells (μ > 0.1 ODUhr^{-}1) results in reduced hydantoinase and NCAAH activity. Thus non-growth-rate-limiting nitrogen sources such as 0.1% NH_4^+ and 0.5% casamino acids exerted a repressive effect on the hydantoin-hydrolysing enzyme system in mid-exponential growth phase.

To further investigate the effect of nitrogen source upon enzyme activity, cells were subjected to ammonium shock, a situation in which cells are rapidly transferred from medium with a growth-rate limiting nitrogen source to one containing ammonium sulphate. RU-OR cells were grown to mid-exponential phase in CAAMM plus 0.1% 2-TU to induce enzyme activity, washed in phosphate buffer, and then resuspended in either CAAMM or NH_4MM medium containing 2-TU. Hydantoinase and NCAAH activities were determined after incubation for a further thirty minutes. There was an eleven fold drop in hydantoinase activity after 20-30 minutes ammonium shock, as compared with activity in 0.01% casamino acids. There was a corresponding three-fold drop in NCAAH activity in ammonium-shocked cells (Table 3.3). In contrast, when cells were shocked with 0.5% casamino acids instead of NH_4^+, there was no inhibition of either hydantoinase or *N*-carbamyl amino acid amidohydrolase activities, even after incubation for 2 hours (Table 3.3).

Table 3.3: Ammonium shock effect upon hydantoin-hydrolysing enzyme activity.

Nitrogen Source	Hydantoinase (U)			*N*-carbamoylamino acid amidohydrolase (U)		
	Time (min)					
	30	60	120	30	60	120
0.01% CAA	11.11 ± 1.06	10.65 ± 0.44	11.85 ± 0.33	12.53 ± 0.12	9.66 ± 0.27	10.2 ± 0.23
0.1% NH_4^+	1.06 ± 0.05	1.14 ± 0.07	1.67 ± 0.02	4.32 ± 0.12	4.29 ± 0.18	2.92 ± 0.11
0.5% CAA	nd	9.32 ± 0.23	11.63 ± 0.29	nd	8.21 ± 0.24	10.38 ± 0.44

Key: Cells were induced by growth to mid-exponential growth phase in medium containing 0.01% CAA +2-TU, before being washed and resuspended in the appropriate medium. Initial starting activity of all cultures after 24hr growth in 0.01% casamino acids with 0.1% 2-thiouracil was 9.20U (hydantoinase) and 10.1 U(*N*-carbamylamino acid amidohydrolase). Key: ± SEM (n=12). CAA – casamino acids; nd – not determined.

This data indicated that not only did non-growth-rate-limiting nitrogen sources such as 0.1% NH_4^+ and 0.5% casamino acids repress both enzymes, but NH_4^+(and not 0.5% casamino acids) had a separate and distinct inhibitory effect upon the hydantoinase activity in cells already expressing the enzymes. When ammonium

shocked RU-OR cells were resuspended in CAAMM containing no ammonium sulphate, the hydantoinase activity recovered to induced levels within 60 minutes (Figure 3.3), indicating that the ammonium shock effect was reversible.

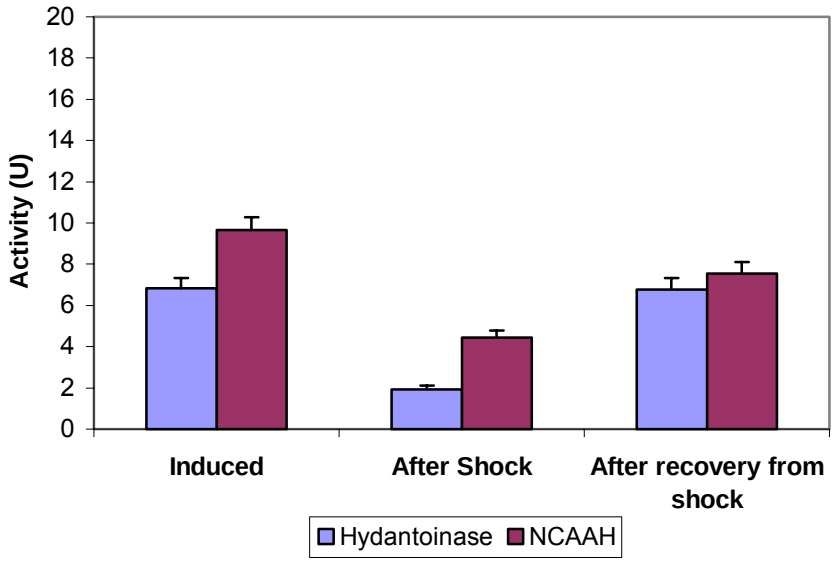


Figure 3.3: Ammonium shock effect upon hydantoinase and NCAAH after 20 minutes, and the reversal of this within 60 minutes. Error bars represent SEM (n=9).

Virtually all cells utilise glutamate and glutamine as key nitrogen donors for biosynthetic reactions, and the most important pathway for the assimilation of ammonia into glutamate and glutamine involves the conversion of ammonia plus glutamate to glutamine by glutamine synthetase (GS), followed by transfer of the amide group from glutamine to 2-ketoglutarate by glutamate syntheatase (GOGAT) to form two molecules of glutamate. This pathway is ubiquitous in bacteria (Merrick and Edwards, 1995). Studies in *E. coli* have shown that the regulation of ammonia assimilation by glutamine synthetase I, encoded by the *gln* A gene is regulated by the cellular ratio of 2-ketoglutarate: glutamine through the *ntr* system (for review see Merrick and Edwards, 1995). High concentrations of ammonia (> 1mM) alter the 2-ketoglutarate: glutamine cellular ratio and activate the phosphorylation of NTR B/C transcriptional factors to prevent the transcription of *ntr*-regulated genes such as *glnA*. In addition, an adenyltransferase enzyme is activated to adenylate a unique tyrosyl group on the Gln A glutamine synthetase protein (Stadman, 1990). This immediately inactivates the enzyme, which in turn triggers the shutdown of several *ntr*-regulated nitrogen metabolism pathways.

The results above indicated that a similar rapid inactivation mechanism might be responsible for the ammonium shock effect on hydantoinase activity in strain RU-OR, possibly mediated through the conversion of ammonium to glutamine by GS. To test this hypothesis, a glutamine synthetase inhibitor, D,L-methionine-D,L-sulfoximine (MSX), was added to RU-OR cells during ammonia shock, and hydantoinase and NCAAH activity determined. Under these conditions, hydantoinase and NCAAH activity were unaffected by ammonium shock (Figure 3.4).

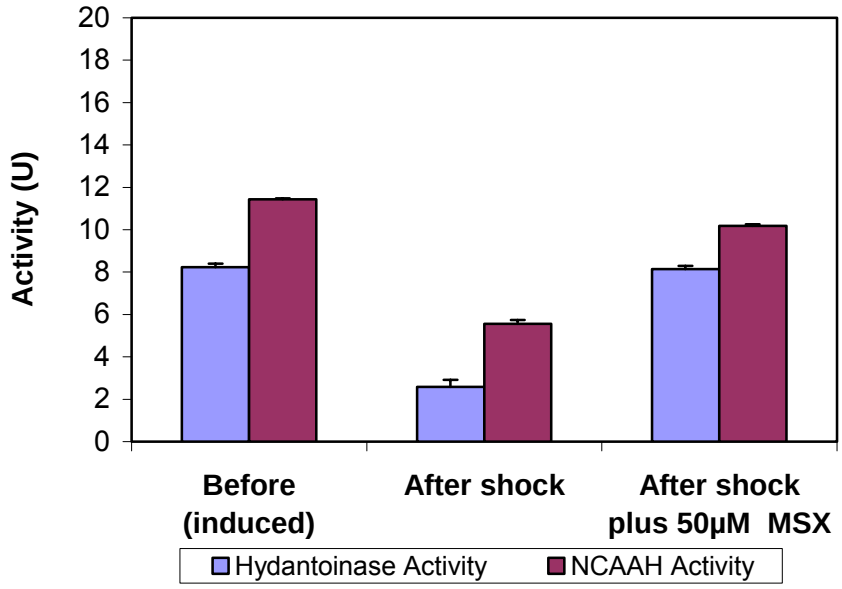


Figure 3.4: The inhibition of ammonium shock effect by the glutamine synthetase inhibitor D,L-methionine-D,L-sulfoximine (MSX). Error bars represent SEM (n=9).

The conversion of hydantoin to glycine produces CO_2 and NH_4^+ and there have been several reports in the literature that NCAAH enzymes are sometimes subject to product inhibition by NH_4^+ ions (Runser *et al.*, 1990a; Kim *et* al., 1994a; Ogawa and Shimizu, 1997). To determine whether or not the ammonium shock effect could be accounted for by product inhibition during biocatalytic reactions, cells were grown in CAAMM supplemented with 2-TU and then assayed for enzyme activity in the presence of varying concentrations of ammonium sulphate. Concentrations of ammonium sulphate above 1mM within the reaction mixture had a significant inhibitory effect on both the hydantoinase and *N*-carbamoylamino acid amidohydrolase activity of RU-OR resting cells (Table 3.4).

Table 3.4: Inhibition of hydantoinase and NCAAH activity in crude enzyme extracts of RU-OR by ammonium sulphate.

$[NH_4^+]$ (mM)	Hydantoinase Activity (U)		NCAAH Activity (U)	
	Crude extract	Resting cells	Crude extract	Resting cells
0	10.46 ± 0.33	6.88 ± 0.26	15.17 ± 0.58	7.59 ± 0.19
1	16.01 ± 0.77	7.01 ± 0.34	10.98 ± 0.59	8.11 ± 0.39
5	3.05 ± 0.06	4.65 ± 0.21	2.026 ± 0.04	6.11 ± 0.29
10	2.36 ± 0.01	4.37 ± 0.05	0.22 ± 0.02	4.9 ± 0.12

Key: ± SEM (n=9).

However, the inhibitory effect of NH_4^+ ions was much more pronounced in crude extract preparations than in whole cells. 10mM ammonium sulphate is approximately equivalent to 0.1% ammonium sulphate (7.5mM), the concentration observed to cause ammonium shock. Inhibition by 10mM NH_4^+ ions produced a 44% reduction in hydantoinase activity (Table 3.4.), whereas ammonium shock produced an 80-90% reduction in hydantoinase activity (Table 3.3, Figures 3.3, 3.4).

3.3.3 Induction of hydantoin-hydrolysing activity in RU-OR

To investigate the mechanism by which hydantoinase and NCAAH activities are induced by 2-TU, cells were first grown to mid-exponential growth phase in CAAMM. The cells were then transferred to the same medium containing either 2-TU, 2-TU plus the transcriptional inhibitor, rifampicin, or no inducer. Cells were assayed over six hours for hydantoinase and NCAAH activity. The presence of inducer resulted in a four-fold increase in hydantoinase activity over six hours while there was very little activity in cells grown in the absence of inducer (Figure 3.5, panel A). When rifampicin was included in the induction medium containing 2-TU, there was no increase in hydantoinase activity. A corresponding increase in NCAAH activity, which was inhibited in the presence of rifampicin, was also observed in the presence of 2-TU, although the level of induction was lower than that for the hydantoinase (Figure 3.7, panel B). The inhibition of induction by rifampicin suggests that the activation of transcription is involved in the mechanism of induction of hydantoin-hydrolysing enzymes in RU-OR cells.

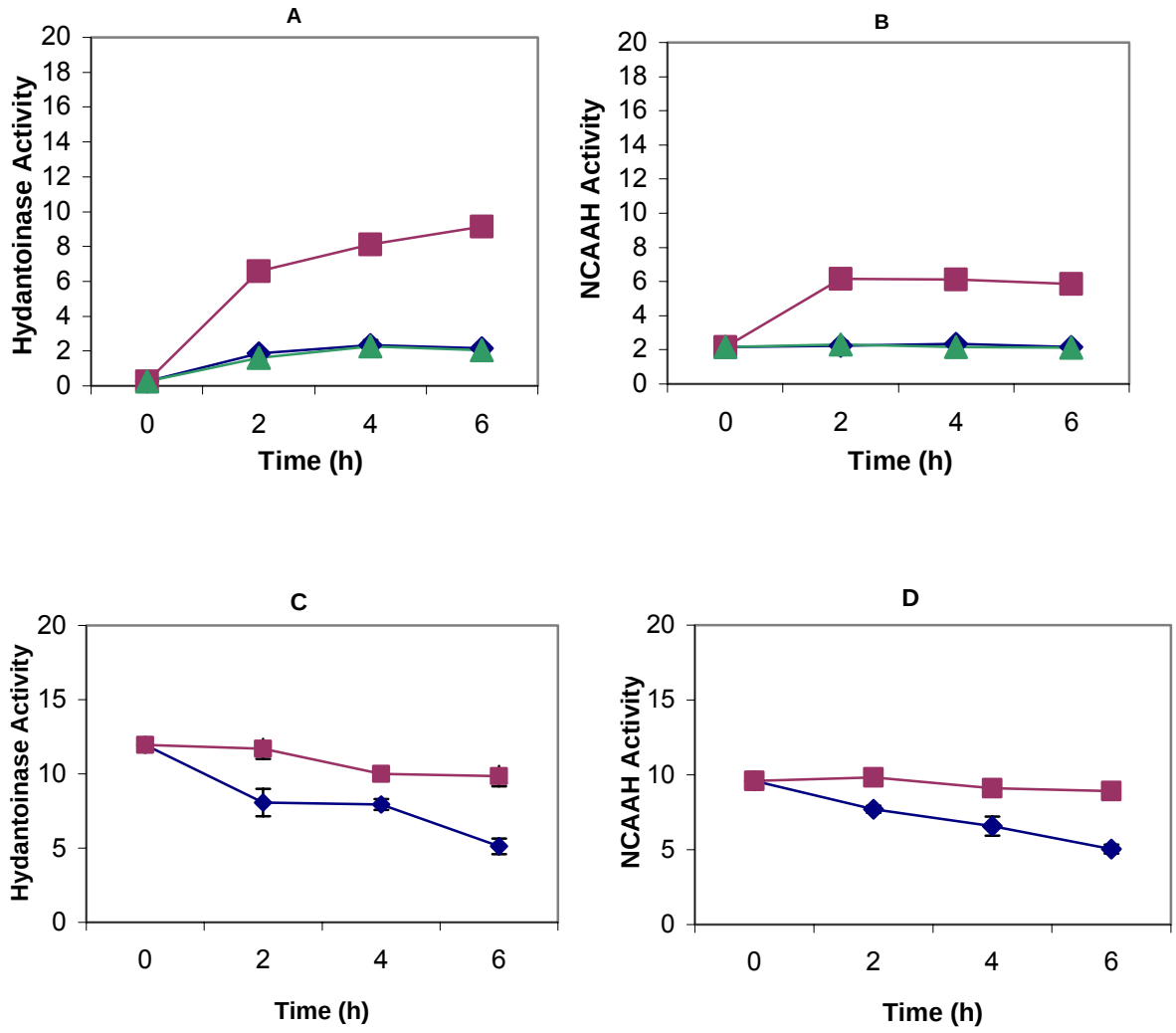


Figure 3.5: Induction of hydantoinase (A) and NCAAH activity (B) in RU-OR cells by 2-thiouracil. •- Cells suspended in 0.01% casamino acids; • - cells suspended in 0.01% casamino acids plus 0.1% 2-thiouracil; ♦- cells suspended in 0.01% casamino acids, 0.1% 2-thiouracil and 200 µg/ml rifampicin. Duration of induction of hydantoinase (C) and NCAAH activity (D) in RU-OR cells after removal of 2-thiouracil inducer. Induced cells were resuspended in: •- 0.01% casamino acids; ♦ - 0.01% casamino acids plus 0.1% 2-thiouracil. Error bars represent the standard error of the mean (n = 9).

Determination of the duration of induction after removal of 2-TU from the growth medium showed that hydantoinase and NCAAH activity decreased gradually by about 50% over six hours after removal of inducer, rather than rapidly dissipating as observed during ammonium shock (Figure 3.5 panels C and D, as compared to Figure 3.3).

3.4 Discussion

The choice of carbon source used in growth medium, and the relative growth rate of the cells had no significant effect on hydantoinase activity in *A. tumefaciens* RU-OR. However, growth in MM medium containing 1% mannitol as a carbon source resulted in increased NCAAH activity. This two-fold increase in NCAAH activity with 1% mannitol as a sole carbon source is intriguing and could indicate some form of carbon regulation of NCAAH activity in RU-OR cells. Alternatively, the increased NCAAH activity could be related to an observed decrease in exopolysaccharide production by RU-OR cells when cultivated with mannitol as a sole carbon source. Increased transport of the glycine product out of the cell in the absence of exopolysaccharide might explain the higher levels of NCAAH activity observed. Unfortunately, the use of MM medium containing 1% mannitol as a sole carbon source for industrial applications would not be viable due to the exceptionally slow growth rate of RU-OR cells in this medium.

Reports in the literature to date have not focused a great deal on the regulation of hydantoin-hydrolysing enzymes by nitrogen catabolite repression (NCR) mechanisms. However, the delayed onset of activity in non-growth-rate-limiting culture media (Möller *et al*., 1988; Syldatk *et. al.*, 1990; Meyer & Runser, 1993) and the presence of sequences that resemble sigma (σ) factor binding sites upstream of genes encoding hydantoin-hydrolysing enzymes (Watabe *et al.*, 1992a; LaPointe *et al.,* 1994) implicate *ntr*-type regulation of transcription. The low levels of hydantoin-hydrolysing activity during exponential growth in non-growth-rate-limiting nitrogen sources, but optimal hydantoin-hydrolysing activity in mid- to late-exponential phase when grown with growth-rate-limiting nitrogen sources (Figure 3.2) suggests the involvement of nitrogen catabolite repression in the regulation of hydantoin-hydrolysing activity in *A. tumefaciens* RU-OR.

Further investigation of effect of non-growth-rate-limiting (repressive) nitrogen sources in the growth medium on the production of hydantoin-hydrolysing activity showed at least two, and possibly three separate mechanisms of repression. The first mechanism involves direct repression of the hydantoin-hydrolysing enzymes,

probably by transcriptional regulation through global nitrogen catabolite (*NTR*) repression, when grown in media containing non-growth-rate-limiting or repressive nitrogen sources (Figure 3.2, Table 3.2).

The second mechanism is specific to ammonium ions, which caused a "shock" effect, resulting in a rapid decrease in hydantoin-hydrolysing activity in RU-OR cells(Table 3.3, Figures 3.3, 3.4). Ammonium shock of hydantoinase activity may be similar to the ammonium shock effect experienced by other enzymes regulated by *ntr* regulatory systems , such as glutamine synthetase (Magasanik *et al.*, 1995; Merrick & Edwards, 1995). This regulatory mechanism serves to protect the cell from rapid depletion of energy when the efficient enzyme systems involved in ammonium metabolism immediately begin to operate at a maximal level in the presence of ammonium, by rapidly "switching off" all other nitrogen catabolic pathways (Merrick & Edwards, 1995). Ammonium shock normally involves post-translational modification of the enzymes involved, such as adenylation or deadenylation of glutamine synthetase (Merrick & Edwards, 1995). The reversibility of the ammonium shock effect in RU-OR cells within sixty minutes is consistent with a modification at the post-translational level.

A third regulatory mechanism, product inhibition by ammonium ions, particularly of the NCAAH enzyme occurs in the resting biocatalytic cell reactions, as previously reported (Runser *et al.*, 1990a; Kim *et al.,* 1994a; Ogawa and Shimizu, 1997). Although product inhibition by ammonium ions was very pronounced for crude extracts of RU-OR cells, hydantoin-hydrolysing activity in whole cells was less inhibited by the presence of ammonium ions (Table 3.3). This suggests that the ammonium shock effect might be separate from the product inhibition effect seen within the resting cell biocatalytic reactions. Product inhibition by ammonium ions caused only a 44% reduction in hydantoinase activity (Table 3.4.), whereas ammonium shock produced an 80-90% reduction in hydantoinase activity (Table 3.3, Figures 3.3, 3.4). Therefore, although it is undoubtedly a contributory factor, product inhibition by $NH4^+$ ions does not account entirely for the ammonium shock of hydantoinase activity observed in RU-OR cells. More evidence, such as measurements of the levels of mRNA levels, protein concentration and activity under

ammonium shock conditions would be necessary to establish the exact mechanism of ammonium shock conclusively.

The inhibition of induction by a transcriptional inhibitor, rifampicin, indicates that regulation of hydantoin-hydrolysing activity in RU-OR occurred at a transcriptional level. Similarly regulated inducible enzyme systems are common in prokaryote bacteria, and have been reported previously in *Agrobacterium tumefaciens* species for opine and octopine catabolic enzyme pathways (Hong *et al.*, 1993; Cho *et al.*, 1996; Cho *et al.*, 1997). This data raises the possibility of generating mutant strains of strain RU-OR with inducer-independent hydantoin-hydrolysing enzyme production. Such mutant strains would be advantageous in the development of an industrial process for the production of optically active D-amino acids.

The results presented in this chapter show that hydantoinase and NCAAH activity in RU-OR cells is tightly regulated by the available nitrogen source, through both catabolite repression, and by a distinct ammonium shock effect. Product inhibition of the hydantoin-hydrolysing reaction, particularly the NCAAH enzyme, by ammonium ions also regulated hydantoin-hydrolysing activity RU-OR cells. The dependence of hydantoinase and NCAAH activity in RU-OR cells upon growth-rate-limiting conditions had important implications for the economic feasibility of RU-OR biocatalyst production on an industrial scale, where the production of maximum active biomass in the minimum time is optimal. Ammonium shock further complicated the successful production of hydantoin-hydrolysing activity in RU-OR cells. Production of regulatory mutants of strain RU-OR that were insensitive to catabolite repression and ammonium shock presented a potential solution to these problems.

The different regulatory effects described above illustrate that several complex mechanisms are involved in the regulation of hydantoin-hydrolysing activity in RU-OR. The production of regulatory mutants not only presented opportunities for the manipulation of strain RU-OR to produce improved biocatalytic properties, but also provided the prospect of further elucidating the mechanisms controlling hydantoin-hydrolysing activity in RU-OR.

Chapter 4

Mutational analysis of hydantoin-hydrolysing activity in *Agrobacterium tumefaciens* RU-OR

Chapter 4

Mutational analysis of hydantoin-hydrolysing activity in *Agrobacterium tumefaciens* RU-OR

4.1 Introduction

The three main mechanisms of regulation of hydantoin-hydrolysing activity in bacterial cells described in the previous chapter, induction, nitrogen control and product inhibition present major limitations to the application of whole cell biocatalysts for the production of enantiomerically pure amino acids.

The induction of hydantoinase and NCAAH activity in bacteria by hydantoin and hydantoin analogues has been well documented (Möller *et al*., 1988; Meyer and Runser, 1993; Ogawa and Shimizu, 1997; Pietzsch and Syldatk *et al*., 1995*;* Syldatk *et al*., 1990a) and was described for RU-OR cells in Chapters 2 and 3. Obviously, the addition of inducer to the growth medium in large-scale industrial processes for the production of D-amino acids is uneconomical. Syldatk *et al.* (1990b) found that the concentration of inducer in the growth medium inducer needed to be kept at high levels to maintain hydantoinase and NCAAH activity in *Arthrobacter* sp. DSM 7330 cells during culture. However, this could be alleviated by the use of modified non-metabolizable inducers, by addition of inducer after cell growth had reached late exponential growth phase or through use of a mutant strain of *Arthrobacter* sp. DSM 7330 selected for constitutive production of L-stereoselective hydantoin-hydrolysing activity. The isolated mutant strain, *Arthrobacter* sp*.* 9771 produced inducer-independent hydantoinase activity at higher levels than the wild-type strain, and also exhibited elevated levels of hydantoinase and NCAAH activity (by a factor of 2.3) in the presence of inducer, when compared to wildtype induced levels (Wagner *et al*., 1996). Meyer and Runser (1993) utilised the non-metabolizable hydantoin analogue, 2,4-thiouracil to induce hydantoinase and NCAAH activity in *Agrobacterium* sp. IP-671 over a six-hour period, and 2-TU has been used subsequently for the induction

of hydantoin-hydrolysing activity in other *Agrobacterium* strains, including *A. tumefaciens* RU-OR.

The second control mechanism involved in the regulation of hydantoin-hydrolysing activity is that of nitrogen control. Hydantoinase and NCAAH activity was not produced in RU-IOR cells until late exponential or stationary growth when cells are cultured in complete media, as has been reported for other bacterial strains (Möller *et al*., 1988; Syldatk *et* al., 1990a, b; Deepa *et al*., 1993; George and Sadhukhan, 1993; Ogawa and Shimizu, 1997). Nitrogen sources that allow rapid cell growth prevent high levels of hydantoinase and NCAAH activity (Möller *et al*., 1988; Syldatk *et* al., 1990a; Deepa *et al*., 1993; George and Sadhukhan, 1993; Chapter 3, Table 3.2). This data is suggestive of regulation by an *ntr*-type regulatory pathway.

The regulation of nitrogen metabolism in prokaryotes is a complex process but in recent years, several nitrogen metabolic pathways have been linked to the global *ntr* nitrogen regulatory pathway (Wardhan *et al*., 1989; Maloy *et al*., 1994). The complex network involved in bacterial nitrogen control illustrates the key role of glutamine and α-2-ketoglutarate metabolites in the regulation of nitrogen-metabolism in enteric bacteria (Figure 4.1.2). The ratio of glutamine : 2-ketoglutarate in the cell reflects the availability of fixed nitrogen in the cell. High ammonia concentrations result in a high glutamine : 2-ketoglutarate ratio, and increasing levels of ammonia defiency result in the lowering of this ratio (Stadman and Prusiner, 1976; Merrick & Edwards, 1995). As the Utase activity is stimulated by 2-ketoglutarate, and inhibited by glutamine, the uridylation state of P_{II} reflects the glutamine: 2-ketoglutarate ratio, and hence the nitrogen status of the cell. In the regulation of Ntr-dependent genes, the uridylation state of P_{II} triggers the phoshorylation or dephosphorylation of Ntr C, a DNA binding protein involved in the regulation of *ntr*-dependent gene transcription, in response to the nitrogen status of the cell (Merrick & Edwards, 1995). Glutamine levels and glutamine synthetase activity are therefore pivotal to the regulation of *ntr*-dependent nitrogen metabolising systems.

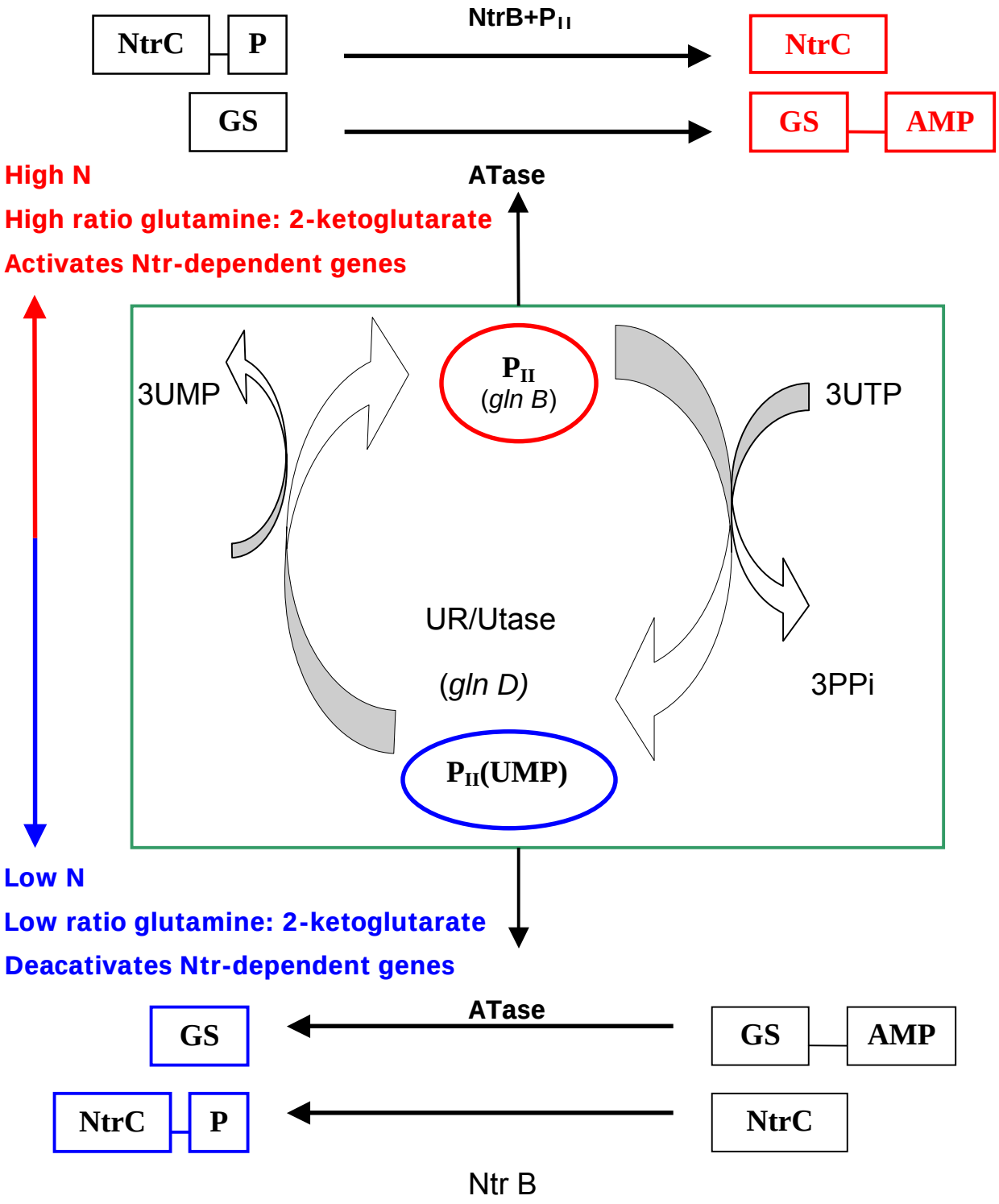


Figure 4.1: Model for the regulation of GS activity and NtrC in response to nitrogen status. The uridylation and deuridylation of P_{II} system through UR/Utase (green box) acts as the mechanism for sensing intracellular nitrogen status. ATase catalyses the adenylation /deadenylation of GS. NtrB protein catalyses the phsophorylation/dephosphorylation of NtrC protein. Key: GS – glutamine synthetase, UTase/UR-uridyltransferase/uridyly-removing enzyme, ATase-adenyltransferase, AMP-adenosinemonophosphate, UMP-uridyl-monophosphate. P-phosphate. N –nitrogen status. Adapted from Merrick and Edwards, 1995).

In addition to a central role in this regulatory pathway, GS is the enzyme responsible for the assimilation of ammonium at low levels of ammonium sufficiency, and therefore enzymes converting various nitrogen sources to ammonium are only useful if there is sufficient GS activity to assimilate this ammonia (Friefelder *et al*., 1987; Brock & Madigan, 1996). Hence, NtrC will not activate the transcription of the genes encoding these pathways until the uridylation of P_{II} triggers the deadenylation of GS, and the phosphorylation of NtrC by NtrB. NtrC is thought to activate transcription by blocking the binding of RNA polymerase containing subunits other than σ^N or σ^{54} subunits (Merrick & Edwards, 1995), and allowing σ^N or σ^{54} polymerases to bind and transcribe *ntr*-dependent genes. GS activity is obviously directly involved in the regulation of cellular glutamine levels, and thus is an important link in the network described above. Although this network has only been defined for enteric bacteria, glutamine levels have also been established as key metabolites in the control of similar enzymes involved in nitrogen assimilation pathways e.g. allantoinase activity in *Pseudomonas aeruginosa* (Janssen *&* Van der Drift, 1983).

Although a complete *ntr* regulatory pathway for *A. tumefaciens* has not yet been described, a common *ntr* regulatory factor (*ntr*C) has been isolated from *A. tumefaciens* and sequenced. (Rossbach *et al.,* 1987; Wardhan *et al.,* 1989; Merrick and Edwards, 1995). This provided evidence of that a similar global regulatory system to the well-characterised *ntr* gene regulation system of enteric bacteria exists in *A. tumefaciens* cells *.* The global *ntr* regulatory system has also been linked to the regulation of certain nitrogen-metabolising enzymes in *A. tumefaciens,* such as the enzymes involved in malopinnic and agropinnic acid utilisation, which have significant sequence identity to some hydantoin-hydrolysing enzymes (Lyi *et al*., 1999). The data represented in Chapter 3 also implicated the regulation of hydantoin-hydrolysing activity by a similar nitrogen control pathway in *Agrobacterium tumefaciens* RU-OR*.* 0.1% ammonia sulphate both repressed hydantoin-hydrolysing activity when used as a sole nitrogen source (Table 3.1), and inactivated hydantoinase activity at a post-translational level (Table 3.2, Figure 3.3), which was consistent with the effect of ammonia on the regulation of *ntr*-dependent genes. In addition to this, inhibition of GS activity using MSX deactivated the ammonium shock effect (Figure 3.4).

The recent introduction of recombinant systems for the production of hydantoinase and NCAAH activity may alleviate the problems encountered in biocatalytic production of D-amino acids using native hydantoin-hydrolysing enzyme systems. However, recombinant enzyme systems present other difficulties such as toxicity of high levels of recombinant gene expression and the formation of insoluble aggregates of improperly folded heterologous protein, and are not yet ideal systems for the production of hydantoinase and NCAAH activity (Chao *et al*., 2000). Thus, the first step towards improving the industrial potential of *A. tumefaciens* RU-OR for the production of D-amino acids, involved the manipulation of *A. tumefaciens* RU-OR cells to produce mutant strains with improved biocatalytic potential. The development of an understanding of the mechanisms that regulate hydantoin-hydrolysing enzyme activity in RU-OR as presented in Chapter 3 facilitated the formation of selection criteria for the isolation of regulatory mutant strains of RU-OR. Desirable mutant phenotypes therefore included: inducer-independent hydantoinase and NCAAH activity with the potential for elevated enzyme production, *ntr*-insensitive hydantoin hydrolysing enzyme expression and ammonia shock resistant hydantoinase activity. In addition to improved biocatalytic potential, characterization of mutants such as these would allow for further elucidate the regulatory mechanisms involved in controlling the production and activity of hydantoin-hydrolysing enzymes in RU-OR cells.

This chapter describes the isolation and characterisation of mutant strains of *A. tumefaciens* RU-OR in which the regulation of hydantoin-hydrolysing activity by both induction and nitrogen control had been altered. The biocatalytic activity of the mutant strains was assessed with a view to potential industrial application as biocatalysts for the production of enantiomerically pure amino acids such as D-HPG, as described in Hartley *et al*. (2001).

4.2 Methods and Materials

4.2.1 Bacterial strains and culture conditions.

All strains used in this chapter were either *A. tumefaciens* RU-OR or mutants derived from this strain using ethylmethane sulfonate (EMS). The identity of all mutant strains isolated was routinely confirmed by restriction mapping of PCR fragments generated from the 16S rRNA gene (Chapter 2*)*. Unless otherwise stated, all cultures were routinely grown, shaking at 200 rpm, 28^{o}C, in CAAMM medium. For induction of hydantoin-hydrolyzing enzymes in RU-OR, cells were grown in induction medium (CAAMM + 2-TU) to OD_{600} = 0.5 - 0.8 (Dry cell mass = 20-30gL^{-1}). Mutant strains RU-ORF1 and RU-ORF9 were cultured in medium supplemented with 0.002% glutamine where necessary.

4.2.2 Mutagenesis of *Agrobacterium tumefaciens* RU-OR

Cells were mutagenised with EMS according to the method of Miller (1992). 5ml cultures of cells were grown to saturation at 25°C, 200rpm in nutrient broth, diluted 1:50 into 30 ml nutrient broth, and grown at 25°C, 200rpm, for approximately 2 hrs to a cell density of approximately 1-2 x 10^8 cells/ml. Cells were then centrifuged for 5 minutes, 8000 rpm (Beckman J2-21 centrifuge, Beckman JA 20 rotor), before washing twice with 30 ml minimal A buffer (per litre: 10.5g K_2HPO_4, 4.5g KH_2PO_4, 1g $(NH_4)_2SO_4$, 0.5g sodium citrate.$2H_2O$). Washed cells were resuspended in 15 ml (half-volume) minimal A buffer. 2 ml aliquots of the cells were incubated at 28°C, shaking at 100rpm. 0.03ml of EMS was added to each and the tubes were incubated for varying time intervals of 5,10, 15, 20, 25, 30, 45 minutes. An untreated control was included. After incubation, cells were centrifuged for 5 minutes, 8000 rpm (Beckmam J2-21 centrifuge, JA 20 rotor), and washed twice in 5 ml minimal A buffer before resuspension in 2 ml minimal A buffer (could be stored at –20°C). Plating out 10^{-4} and 10^{-5} dilutions of the mutagenised cells, and 10^{-5} and 10^{-6} dilutions of the control cells onto nutrient agar plates for incubation overnight at 28°C was used to test viability. Mutagenised cells were then diluted 1:20 in 5 ml nutrient broth and grown overnight at 25°C, 200 rpm. This outgrowth was used to test for viability and mutation frequency, and was subsequently used for selection of the desired mutants.

Mutation frequency was estimated using selection for resistance to rifampicin as a marker chromosomal mutation (Miller, 1992).

4.2.3 Isolation of inducer-independent mutant strains

Serial dilutions of outgrowth from mutagenised cells were plated onto nutrient agar plates containing 0.01% 5-fluorouracil (5-FU). After incubation at 28°C for 48 hrs, 5-FU resistant colonies were isolated and re-streaked onto nutrient agar containing 0.01% 5-fluorouracil. Isolated colonies were then assayed for hydantoinase activity after growth in nutrient broth containing no inducer. Mutant strains RU-ORL5 and RU-ORLB3 were selected for further characterization.

4.2.4 Isolation of inducer-independent mutant strains with altered nitrogen regulation

Serial dilutions of outgrowth from mutagenised cells (section 4.2.3) were plated onto MM agar plates containing 0.1% ammonium sulphate as a sole nitrogen source, and 0.2% 5-FU. After 48 hrs growth at 28°C, 5-FU resistant colonies were isolated and re-streaked. Selected colonies were then assayed for hydantoinase activity after growth to mid-log growth phase in MM broth containing 0.1% ammonium sulphate as a sole nitrogen source, without inducer, and RU-ORPN1 was selected for further characterization.

4.2.5 Isolation of glutamine-dependent mutant strains

Isolation of glutamine-requiring mutants of RU-ORPN1 was performed as described by Janssen *et al.* (1981). Outgrowth (supplemented with 0.002% glutamine) of mutagenised cells (section 4.2.3) was washed twice, and then nitrogen-starved for 3 hrs at 25°C, before counter selection for 16 hrs at 25°C, 200rpm in medium containing 0.1% $(NH_4)_2SO_4$, 0.2% glutamate and 100µg/ml ampicillin. Cells were then washed and grown in medium containing 0.1% citrate, 0.2% ammonium, 0.2% glutamate and 0.002% glutamine. After repeating this enrichment cycle, cells were plated on MM agar plates containing 0.1% $(NH_4)_2SO_4$, supplemented with 0.002% glutamine. Glutamine-dependent colonies were identified by their inability to grow on MM agar plates containing 0.1% $(NH_4)_2SO_4$ without glutamine supplementation.

4.2.6 Ammonia shock and glutamine shock assays.

Ammonia shock assays were performed as described in Chater 3 (Section 3.2.2). Glutamine shock conditions were the same as described for ammonium shock, but using MM containing 0.1% glutamine instead of 0.1% $(NH_4)_2SO_4$.

4.2.7 Ammonia shock recovery.

Ammonia shock recovery was performed as described in Chapter 3 (Section 3.2.3).

4.2.8 Biocatalytic resting cell reactions.

Biocatalytic resting cell reactions were performed as described in Chapter 3. The reaction was carried out, with shaking, at 40°C for 6 h, after which samples were microfuged to pellet the cells and the supernatant analysed for biocatalytic reaction products, by colorimetric assays or HPLC analysis. *N*-carbamoylamino acid and amino acid production was detected using colourimetric assays as described in Appendix 4. D,L-5-*p*-hydroxyphenylhydantoin(HPH), D,L-5-N-carbamoyl-hydroxyphenylglycine (NCHPG) and D,L-5-*p*-hydroxyphenylglycine (HPG) were also measured using HPLC analysis of 20µl of reaction supernatant, also as described in Appendix 4. One unit of hydantoinase activity (U) was defined as the sum of *N*-carbamoylamino acid (µmol) and amino acids (µmol) produced under optimised reaction conditions as described in Chapter 2. One unit of NCAAH activity (U) was defined as the amount of glycine (µmol) produced under optimised reaction conditions as described in Chapter 2. All enzyme activity values were reported as the average of *n* repeats. Statistical analysis, where appropriate, was performed as described in Chapter 2.

4.2.9 Enantiomeric Resolution.

The reaction supernatant from assays using D,L-HPH and D,L-NCHPG as a substrate were analysed to determine the enantiomeric ratio of D-HPG:L-HPG, using chiral HPLC analysis as described in Appendix 4.

4.2.10 Glutamine Synthetase Enzyme Assays

Total glutamine synthetase activity was measured using the γ-glutamyl transferase assay. Cells were prepared by treatment with 0.01% cetyl-trimethylammonium bromide for 10 minutes before harvesting. The cells were then washed twice with 0.1M phosphate buffer pH 9.0 before being suspended in 50 times less volume of resuspension buffer, and assayed according to the method of Bender *et al*. (1977). Protein concentration of solubilised cells was measured using the method of Bradford (1976). Activity was expressed as μmoles of γ-glutamyl hydroxamate generated per minute per milligram protein. The percentage adenylation of the GS enzyme subunits was measured using the method of Magasanik *et al.* (1976), which compares γ-glutamyl transferase in the presence and absence of magnesium ions. Magnesium ions inhibit the activity of adenylated enzyme subunits and the difference can then be used to calculate the percentage adenylation of the GS enzyme.

4.3 Results

4.3.1. Mutagenesis of *Agrobacterium tumefaciens* RU-OR

The percentage survival of cells after mutagenesis treatment was calculated by comparison with untreated control cells (Miller, 1992). Mutation frequency was calculated by determining the frequency of mutations causing rifampicin resistance (caused by a single mutation in the β subunit of RNA polymerase structure). Optimal mutagenesis time with EMS was 20 minutes for RU-OR cells, where a 19.8% survival frequency corresponded with a mutation frequency of eight mutant cells for every hundred thousand cells (Figure 4.2). These conditions were used for all subsequent EMS treatments of RU-OR cells.

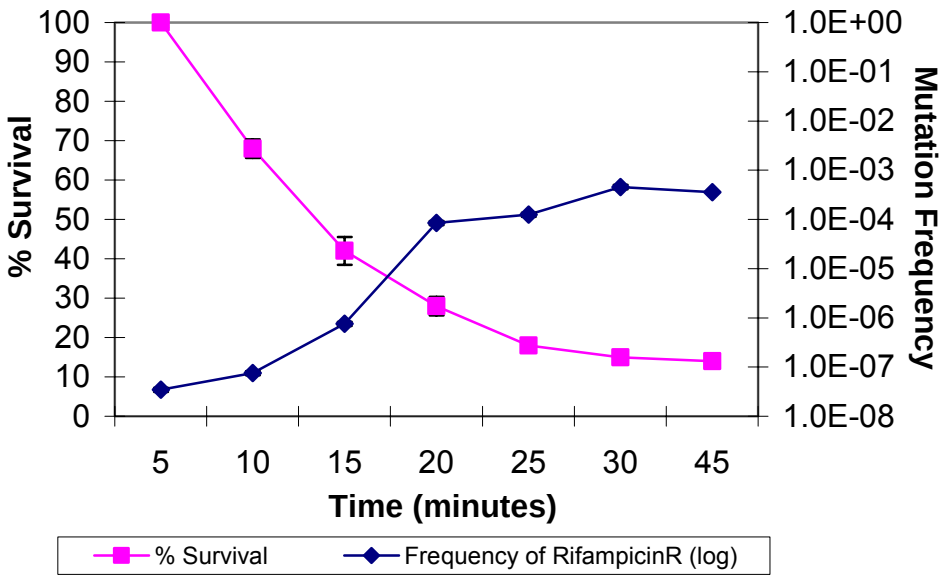


Figure 4.2: Optimisation of EMS mutagenesis conditions for RU-OR cells. Twenty minutes was selected as the optimal mutagenesis time for RU-OR cells. Error bars represent SEM (n=5).

4.3.2 Selection and characterization of inducer-independent mutant strains

Mutagenised cells were screened for inducer-independent hydantoinase activity using a selection system based on resistance to the toxic hydantoin analogue, 5-FU (Hartley *et al.,* 1998). 5-FU has a pyrimidine ring structure, which matches the dihydropyrimidine substrate configuration of most D-hydantoinases (Syldatk *et al*., 1999). 5-FU is toxic to bacterial cells and is in fact used for the treatment of certain cancers (Naguib *et al*., 1985). Cleavage of the molecule at the α-carbon bond by D-hydantoinase should produce 5-fluoroureidopropionic acid, which is not toxic to the cells (Figure 4.3).

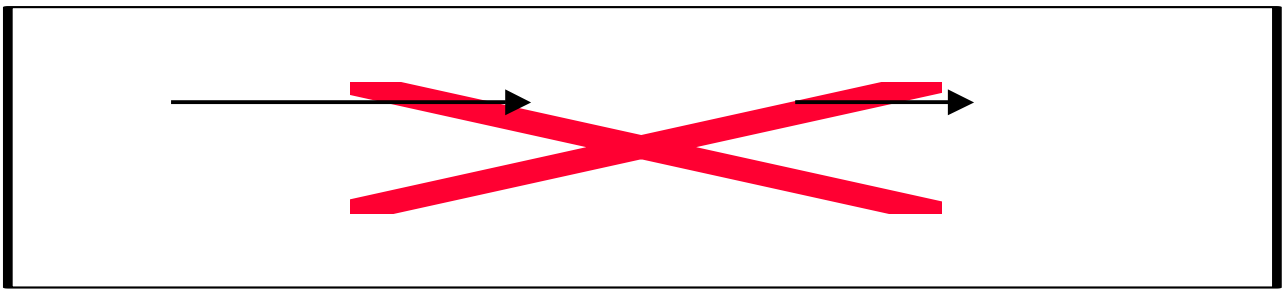


Figure 4.3: The structure of 5-fluorouracil and the proposed mechanism of breakdown by hydantoinase activity.

5-FU was observed to be toxic to wild-type RU-OR cells, where uninduced cells were unable to grow in the presence of 0.01% 5-FU unless hydantoinase activity was induced by 0.1% 2-TU (data not shown). Having established the dependence of 5-FU resistance upon hydantoinase activity in RU-OR cells, several mutant strains were selected for resistance to varying concentrations of 5-FU in the absence of inducer (Figure 4.4). It was hypothesised that only cells which could produce inducer-independent hydantoinase activity would be able to grow in the presence of 5-FU, without inducer.

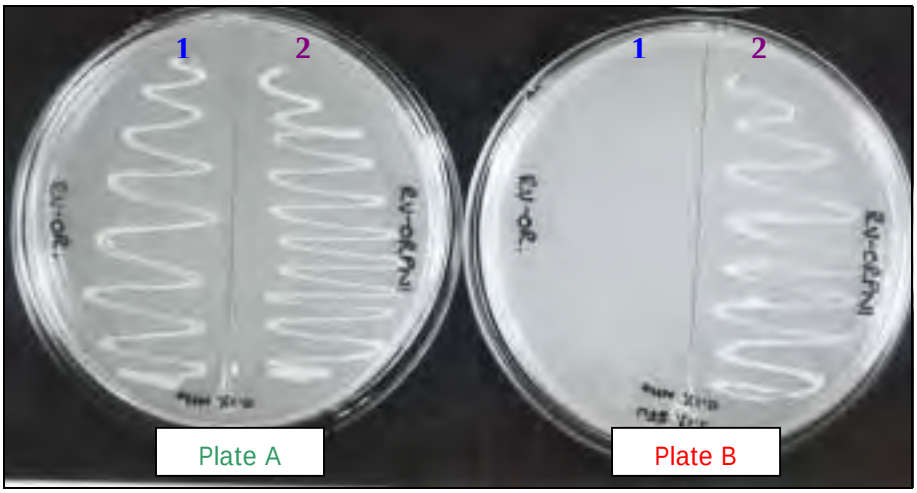


Figure 4.4: Illustration of 5-FU resistance, showing growth of RU-ORPN1 on 0.1% ammonium sulphate MM agar containing 0.2% 5-fluorouracil. Key: Plate A: MM agar with 0.1% ammonium sulphate as sole nitrogen source, Plate B: Plate A containing 0.2% 5-fluoruracil. 1 – RU-OR, 2- RU-ORPN1. (Photograph courtesy of F. Manford).

The hydantoin-hydrolysing activity of mutant colonies that were able to grow on agar plates containing 5-FU, such as RU-ORPN1 illustrated in Figure 4.4. above, was assayed in resting cell reactions and compared to that of RU-OR.

A variety of mutant strains with altered patterns of hydantoin-hydrolysing activity were identified. These fell into four distinct classes:

- Those that were no longer able to hydrolyse hydantoin in whole cell assays, termed null mutants (data not shown).
- Those which exhibited inducer-independent hydantoinase activity, but which retained inducer-dependent NCAAH activity (represented by RU-ORLB3, Table 4.1)

- Those which showed both inducer-independent hydantoinase and inducer-independent NCAAH activity (represented by RU-ORL5, Table 4.1)
- Those which were similar to RU-ORL5, but with elevated inducer-independent hydantoinase activity (represented by RU-ORPN1, Table 4.1).

The capacity of the mutant strains for inducer-independent production of hydantoinase and NCAAH was assessed by assaying enzyme activity in cells grown in CAAMM with and without 0.1% 2-TU. All three mutant strains showed elevated hydantoinase activity as compared to wild-type cells in the absence of inducer (Table 4.1). Hydantoinase activity in two mutant strains (RU-ORL5 and RU-ORLB3) was equivalent to that observed in induced wild-type cells, while strain RU-ORPN1 showed a significant (three-fold) increase in activity.

Not all three strains showed concomitant inducer-independent expression of NCAAH activity. In strains RU-ORL5 and RU-ORPN1, NCAAH activity was equivalent to induced levels in wild-type cells, but there was no inducer independent NCAAH activity in mutant strain RU-ORLB3. However, the levels of NCAAH activity increased significantly when RU-ORLB3 cells were grown in the presence of inducer, indictaing that while hydantoinase activity in this strain was inducer-independent, NCAAH activity was still dependent upon growth in medium containing an inducer such as 2-TU (Table 4.1).

Table 4.1. Hydantoin-hydrolysing activity of mutant Agrobacterium tumefaciens RU-OR strains

Strain	**Hydantoinase Activity**		**NCAAH Activity**	
	no inducer	2-thiouracil	no inducer	2-thiouracil
RU-OR (wt)	1.98±0.05	7.51±0.27	2.62±0.15	11.74±0.50
RU-ORL5	7.64±0.33	9.07±0.13	9.18±0.35	7.07±0.24
RU-ORLB3	8.70±0.14	6.57±0.21	0.70±0.05	5.89±0.12
RU-ORPN1	21.8±0.78	20.9 ±0.89	8.04±0.35	6.78±0.36

± - SEM (n = 12).

Previous data has indicated the presence of D-hydantoin racemase activity in *A. tumefaciens* RU-OR (Chapter 2, Table 2.5). The effect of the mutations in strains RU-ORLB3 and RU-ORL5 upon racemase activity was determined in resting cell biocatalytic reactions using D- and L-methylhydantoin as substrates. As in wildtype cells, strain RU-ORL5 produced equivalent amounts of hydantoinase activity from either D- or L-methylhydantoin. However, examination of the induced and uninduced hydrolysis of D, L, and D,L-methylhydantoin by RU-ORLB3 showed that the racemization of L-methylhydantoin to D-methylhydantoin is increased in the presence of inducer (Figure 4.5).

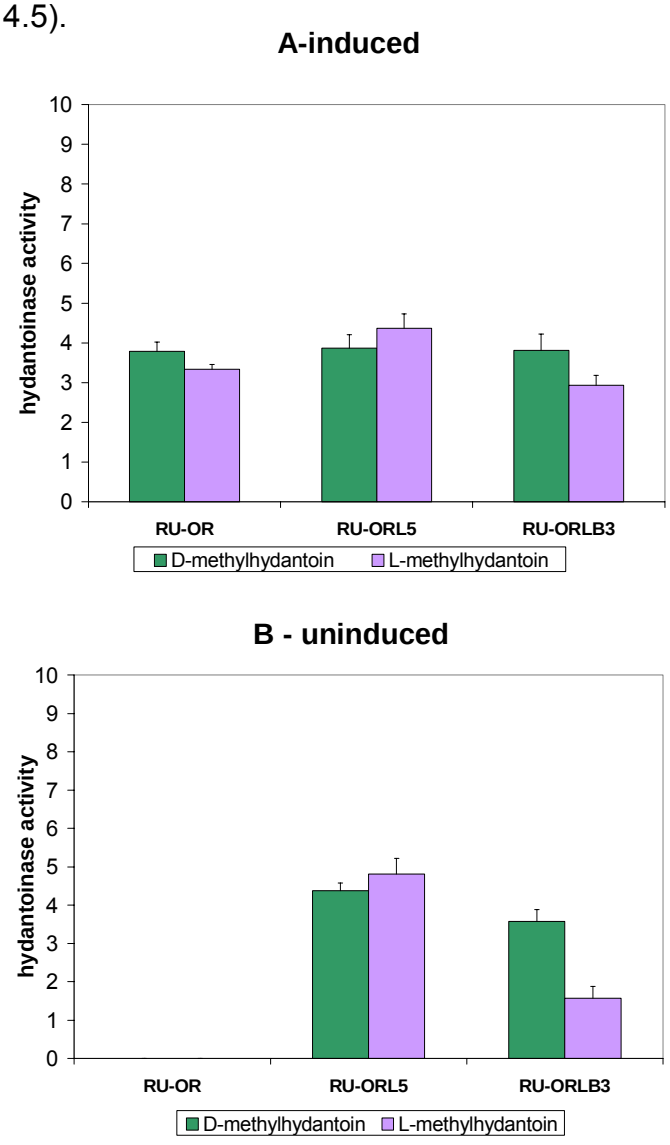


Figure 4.5: Effect of induction of the conversion of D-and L- methylhydantoin enantiomers by the wildtype RU-OR and mutant hydantoinases. (A) induced by 0.1% 2-thiouracil (B) uninduced. Error bars represent SEM (n=6).

T-test analysis showed that induced hydantoinase activity with L-methylhydantoin as a substrate was significantly greater than uninduced hydantoinase activity for RU-

ORLB3 (t >2.36, df = 10, α=0.05). The same test showed that this was not true for hydantoinase activity of RU-ORL5 with L-methylhydantoin as a substrate (t<2.36, df = 10, α=0.05), or for induced and uninduced hydantoinase activity of RU-ORLB3 with D-methylhydantoin as a substrate (t<2.36, df=10, α=0.05). This suggested that an inducible racemase might be involved in the hydantoin-hydrolysing system of RU-OR cells.

4.3.3. Selection and characterization of inducer-independent mutant strains with altered nitrogen regulation

Strains RU-ORL5 and RU-ORPN1, which exhibited inducer-independent production of both hydantoinase and NCAAH enzymes, were examined to determine whether enzyme activity was still sensitive to nitrogen repression. As with the wild-type RU-OR cells, very low levels of hydantoinase activity were observed when RU-ORL5 and RU-ORPN1 cells were grown in minimal medium with 0.1% $(NH_4)_2SO_4$ as nitrogen source (Figure 4.6, Panel A). Unexpectedly, in contrast to the wild-type and RU-ORL5 cells, NCAAH activity was elevated to wild-type, induced levels in RU-ORPN1 cells, even though hydantoinase activity appeared to be repressed during growth in minimal medium with 0.1% $(NH_4)_2SO_4$ as nitrogen source (Figure 4.6, Panel B vs. Table 4.1).

The sensitivity of hydantoinase activity in RU-OR cells to ammonium shock and the observation that NCAAH activity in RU-ORPN1 cells appeared to be insensitive to nitrogen repression, raised the possibility that the lack of hydantoinase activity in this strain might be due to ammonia shock of this enzyme, rather than transcriptional nitrogen repression. To test this hypothesis, cells grown in minimal medium with 0.1% $(NH_4)_2SO_4$ as nitrogen source, were resuspended in CAAMM (plus 2-thiouracil inducer in the case of the wild-type) and grown for a further 50 minutes, before assaying for enzyme activity. The result was a four-fold increase in hydantoinase activity in RU-ORPN1 cells with a less significant increase (two-fold) in the hydantoinase activity of wildtype and RU-ORL5 cells (Figure 4.6, Panel B). This result suggested that hydantoinase and NCAAH enzyme production in RU-ORPN1 cells was no longer sensitive to nitrogen repression, but that the lack of detectable hydantoinase activity was probably due to ammonia shock.

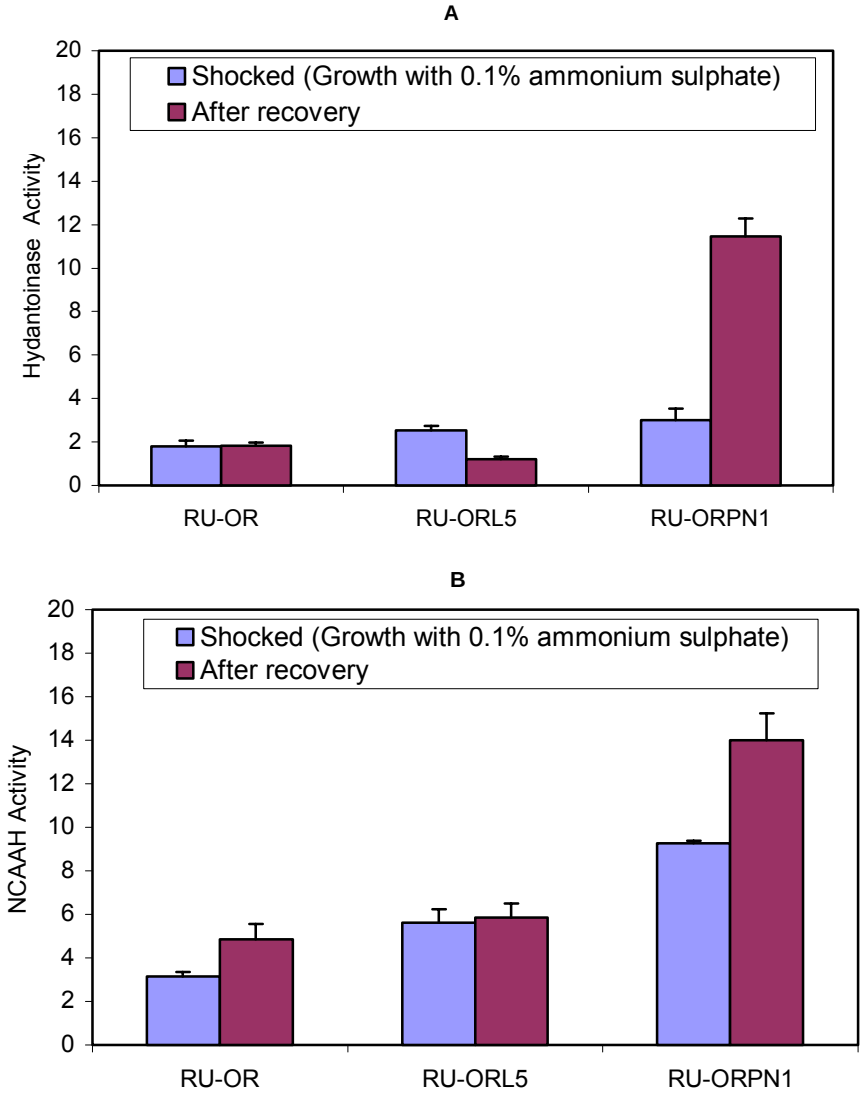


Figure 4.6. Nitrogen control of hydantoinase (A) and *N*-carbamoyl amino acid amidohydrolase (B) enzymes in wildtype and mutant *A. tumefaciens* RU-OR strains. Cells were cultured in repressive medium, with 0.1% ammonium sulphate as a nitrogen source, and then resuspended in non-repressive medium, with 0.01% casamino acids as a nitrogen source for 60 minutes. Error bars represent standard error of the mean (n=9).

4.3.4. Hydantoinase and NCAAH activity of glutamine auxotrophic mutant strains.

The inhibition of GS activity reduced the sensitivity of hydantoinase activity to ammonia shock in wildtype RU-OR cells (Chapter 3, Figure 3.4), raising the possibility that *gln*$^-$ auxotrophic mutants of strain RU-ORPN1 might show elevated levels of hydantoinase activity in addition to the high levels of NCAAH, when grown with 0.1% $(NH_4)_2SO_4$ as nitrogen source. Accordingly, several mutants of RU-ORPN1, with varying degrees of glutamine-dependent growth, were isolated by

Manford (1999). Of these, two strains termed RU-ORPN1F1 and RU-ORPN1F9 were unable to grow in the absence of glutamine supplementation (Figure 4.7). This suggested possible alterations in the nitrogen control pathways of these two strains, and they were selected for the purposes of testing the above hypothesis.

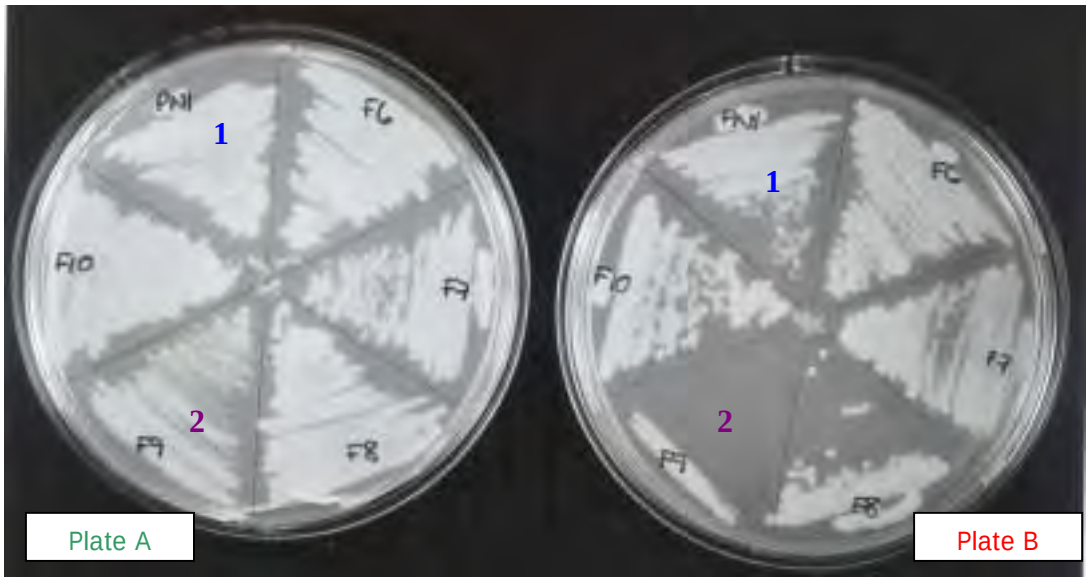


Figure 4.7: Illustration of 5-fluorouracil resistant, glutamine-dependent mutants strains showing growth of RU-ORPN1 and mutant strains on 0.1% ammonium sulphate MM plate containing 5-FU supplemented with glutamine, and the inability of RU-ORF9 to grow on the same medium without glutamine supplementation. Key: Plate A: MM agar with 0.1% ammonium sulphate as sole nitrogen source, 0.2% 5-fluoruracil and 0.002% glutamine, Plate B: Plate A without glutamine supplementation. -RU-ORPN1, 2-RU-ORF1. (Photograph courtesy of F. Manford).

Firstly, the effect of ammonium shock on hydantoinase in the *gln*$^-$ strains was examined. The results showed a significant drop in activity in wild-type and RU-ORPN1 cells, but no reduction in hydantoinase activity in RU-ORF9 and RU-ORF1 cells after ammonia shock (Figure 4.8A). The nature of the mutation conferring the *gln*$^-$ phenotype upon strain RU-ORF9 was examined by measuring GS activity before and after ammonia shock (Figure 4.8B).

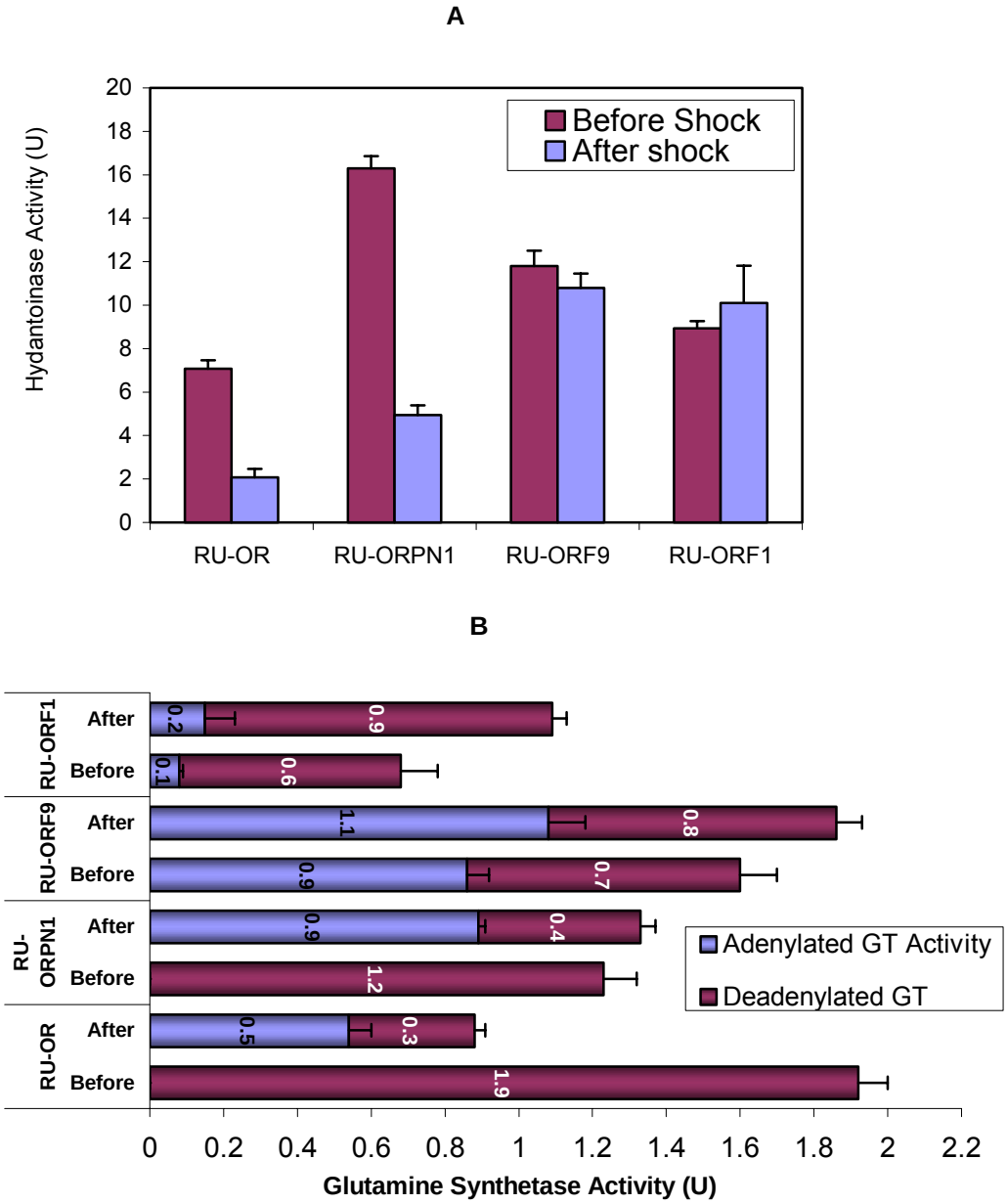


Figure 4.8: (A) Ammonium shock effect on hydantoinase activity of wildtype RU-OR and mutant strains. Error bars represent SEM (n=12). (B) Glutamine synthetase activity of RU-OR and mutant strains measured before and after ammonium shock. GS activity was measured by comparison of deadenylated and adenylated GT activity. The deadenylated GT activity represents the proportion of active GS. Error bars represent SEM (n=3). Before – before ammonium shock treatment. After – after ammonium shock treatment.

There was a two-fold drop in GS activity after ammonia shock in the wild-type cells, but no significant drop in either RU-ORPN1 or RU-ORF9 cells after ammonia shock (Figure 4.8B). Since this GS enzyme assay measures the γ-glutamyl transferase (GT) activity, the degree of GS adenylation was used as an indication of glutamine synthesis before and after ammonia shock. It is generally accepted that the higher the degree of adenylation of the GS enzyme, the lower the level of GS activity

(Magasanik *et al*., 1976). There was no adenylation of GS in wild-type and RU-ORPN1 cells prior to ammonia shock, while the post-ammonia shock cells in both strains showed 40 – 60 % adenylation of the enzyme (Figure 4.9). This was not the case in mutant strain RU-ORF9, where the level of adenylation prior to and post-ammonia shock was elevated to post-shock wild-type levels. The *gln*$^-$ phenotype in RU-ORF9 could therefore be postulated to be due to abnormally high levels of adenylation of the GS enzyme and thus, low levels of glutamine synthesis, in RU-ORF9 cells. Strain RU-ORPN1 appeared to have low levels of GS activity, irrespective of the nitrogen status of the cells, ad there was no apparent adenylation of GS after ammonium shock.

The correlation between ammonium shock and low levels of glutamine synthesis in the mutant strains RU-ORF1 and RU-ORF9, led to the prediction that ammonium shock in the wildtype RU-OR cells was mediated through the cellular concentration of glutamine. To test the hypothesis that glutamine levels triggered the ammonium shock effect, mutant strains RU-OR, RU-ORPN1, RU-ORF1 and RU-ORF9 were shocked with glutamine. Hydantoinase, NCAAH and GS activities were measured before and after glutamine shock (Figure 4.9). A similar decrease in hydantoinase activity to that observed under ammonium shock conditions was observed when both wildtype RU-OR and RU-ORPN1 where subjected to glutamine shock for 30 minutes (Figure 4.9A). This supported the theory that glutamine itself mediated the ammonium shock effect. In addition, hydantoinase activity in RU-ORF9 was also significantly reduced by glutamine shock, although hydantoinase activity of RU-ORF1 cells was not affected (Figure 4.9A).

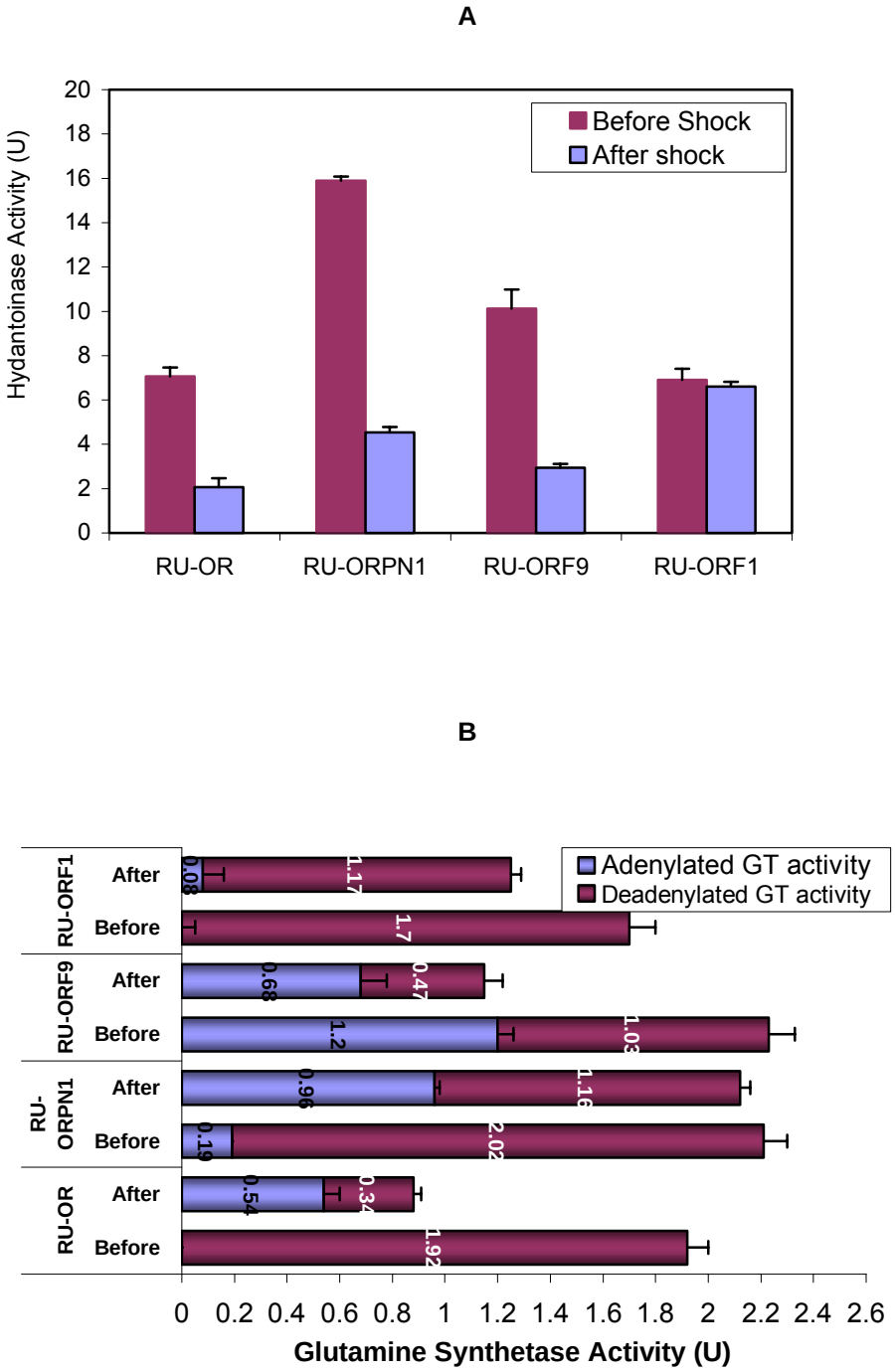


Figure 4.9: (A) Glutamine shock effect on hydantoinase activity of wildtype RU-OR and mutant strains. Error bars represent SEM (n=12). (B) Glutamine synthetase activity of RU-OR and mutant strains measured before and after glutamine shock. GS activity was measured by comparison of deadenylated and adenylated GT activity. The deadenylated GT activity represents the proportion of active GS. Error bars represent SEM (n=3). Before – before glutamine shock treatment. After – after glutamine shock treatment.

As was observed under ammonium shock conditions, GS activity in RU-ORF9 showed a high percentage of adenylated subunits before and after glutamine shock (Figure 4.9B). In contrast, hydantoinase activity in RU-ORF1 remained unaffected by

glutamine shock, and GS activity in RU-ORF1 cells also remained deadenylated under shock conditions that would normally result in adenylation of the enzyme (Figures 4.8B and 4.9B). Interestingly, the level of GS activity in RU-ORPN1 cells remained high after ammonium or glutamine shock, when compared to the wildtype RU-OR cells (Figure 4.8B and Figure 4.9B). RU-ORF9 was selected as the model glutamine-dependent mutant for subsequent experiments.

4.3.4 Biomass yield, specific hydantoinase and specific NCAAH activity in regulatory mutants during growth in $(NH_4)_2SO_4$.

A major goal for improved biotechnological application was to develop *A. tumefaciens* strains with high levels of hydantoin-hydrolyzing enzyme activity irrespective of growth rate or the presence of inducer in the medium. Results for experiments thus far indicated that strain RU-ORF9 might fulfil these requirements, and the ability of this strain to constitutively overproduce hydantoinase and NCAAH activity was examined. The growth phases, biomass yield and productivity (specific hydantoinase and NCAAH activity per unit of dry cell mass) were monitored during batch culture of RU-ORPN1 and RU-ORF9 cells in MM medium with 0.1% $(NH_4)_2SO_4$ as sole nitrogen source. This data was compared to the productivity and biomass yield of wild-type RU-OR cells grown in the same medium, supplemented with 2-TU as inducer (Figures 4.10 and 4.11).

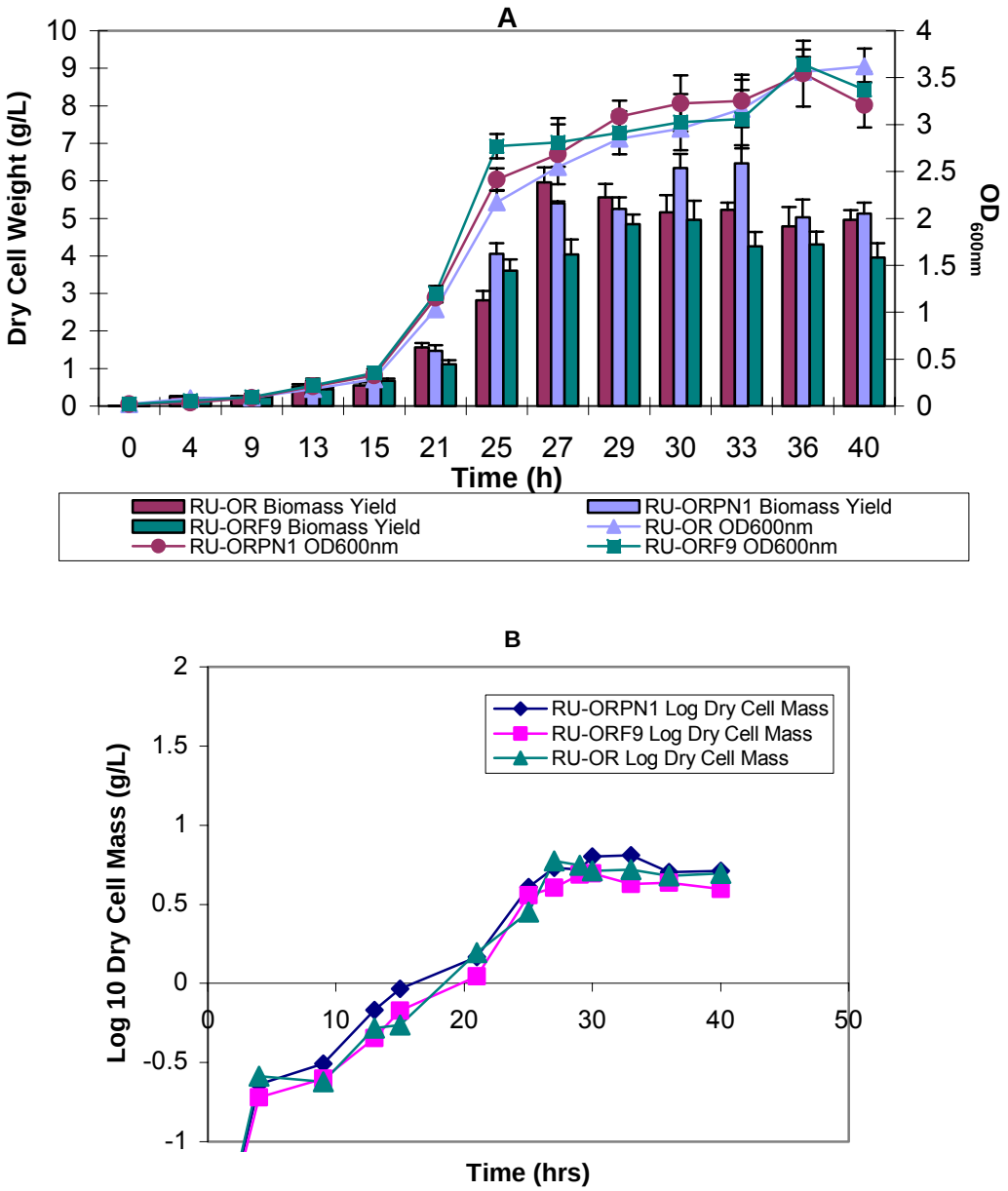


Figure 4.10: Biomass yield of RU-OR and mutant strains during batch culture. (A) Biomass yield and optical density over time. Error bars represent SEM (n=12). (B) Exponential or balanced growth occurs when there is a linear increase in log dry cell mass over time. Log phase growth for all strains occurred between 9-29 hrs. Biomass yield in mid-log growth phase for RU-ORF9 was 3.61g dry cell weight (DCW) per litre.

-

Figure 4.10 illustrates that the biomass yield from wildtype and mutant strains is similar and that the mutations which produce improved hydantoin-hydrolysing activity in exponential growth phase do not affect the growth and biomass yield of the cells. As observed previously (Chapters 2and 3), both hydantoinase and NCAAH activities in the wild-type RU-OR were highest during stationary phase, with low levels of activity during exponential growth phase (Figure 4.11, Panels A and B).

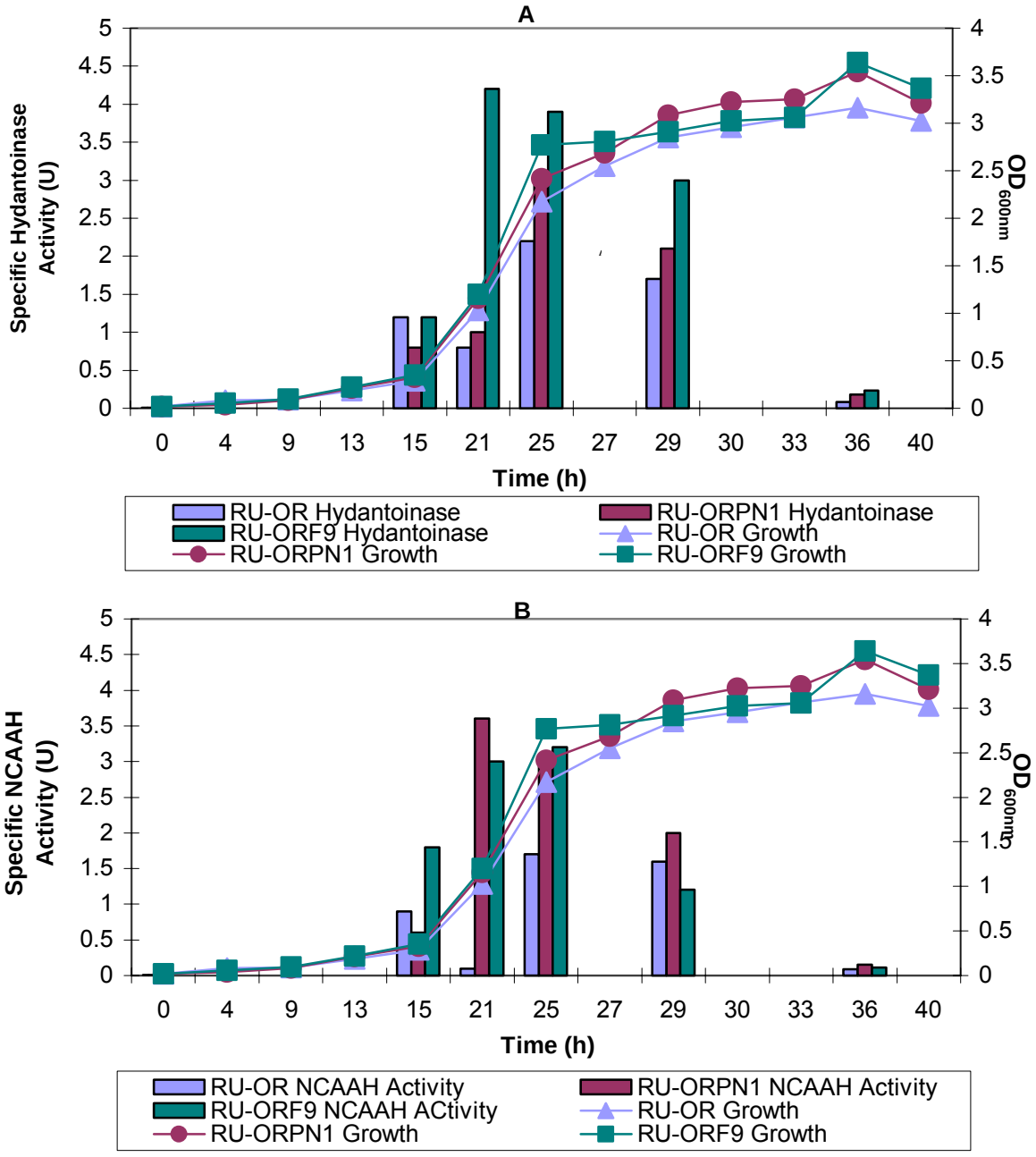


Figure 4.11: Specific hydantoinase (A) and *N*-carbamyl amino acid amidohydrolase (B) activity of wild-type and mutant *Agrobacterium tumefaciens* strains during growth with 0.1% ammonium sulphate as a sole nitrogen source. Specific activity is reported as µmol per mg dry cell mass per hour. Standard error of the mean for all specific hydantoin-hydrolysing activity < 0.01 (n=12).

Hydantoinase activity in mutant strain RU-ORPN1 followed the same trend as the wild-type (Figure 4.11, Panel A), but high levels of NCAAH activity were detected in exponential growth phase, in contrast with the wild-type (Figure 4.11, Panel B). In RU-ORF9 cells, hydantoinase and NCAAH activities were highest in exponential growth phase and the levels declined during stationary phase. In strain RU-ORF9, both hydantoinase and NCAAH activity during exponential growth phase exceeded that detected in either wild type or RU-ORPN1 cells, proving that the desired

biocatalyst characteristics had been achieved. After 21 hours growth, the biomass yield of RU-ORF9 was 3.61 g DCW/L, with specific hydantoinase activity of 4U, and specific NCAAH activity of 3U.

Since *A. tumefaciens* RU-OR was originally selected for its efficient conversion of D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine, it was important to determine whether the high levels of hydantoin-hydrolysis by RU-ORF9 cells during exponential growth in medium containing $(NH_4)_2SO_4$ would also result in high levels of activity with D,L-*p*-hydroxyphenylhydantoin as a substrate. Specific hydantoinase and NCAAH activity levels per milligram dry cell mass were similar to those obtained with hydantoin as substrate. As with hydantoin as substrate, the highest D,L-*p*-hydroxyphenylhydantoin conversion by the wild-type and RU-ORPN1 cells was detected during stationary growth phase (Figure 4.12).

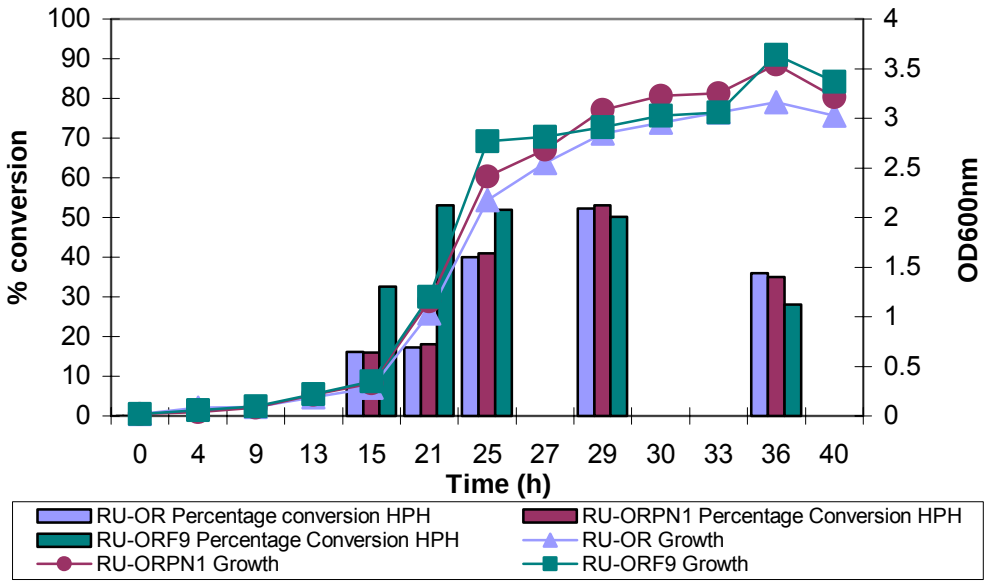


Figure 4.12: Hydrolysis of D,L-5-*p*-hydroxyphenylhydantoin during growth with 0.1% ammonium as a sole nitrogen source. Percentage conversion of 25 mM D,L-5-p-hydroxyphenylhydantoin to N-carbamyl-p-hydroxyphenylglycine and D-p-hydroxyphenylglycine. SEM for all conversion values < 0.01(n=12).

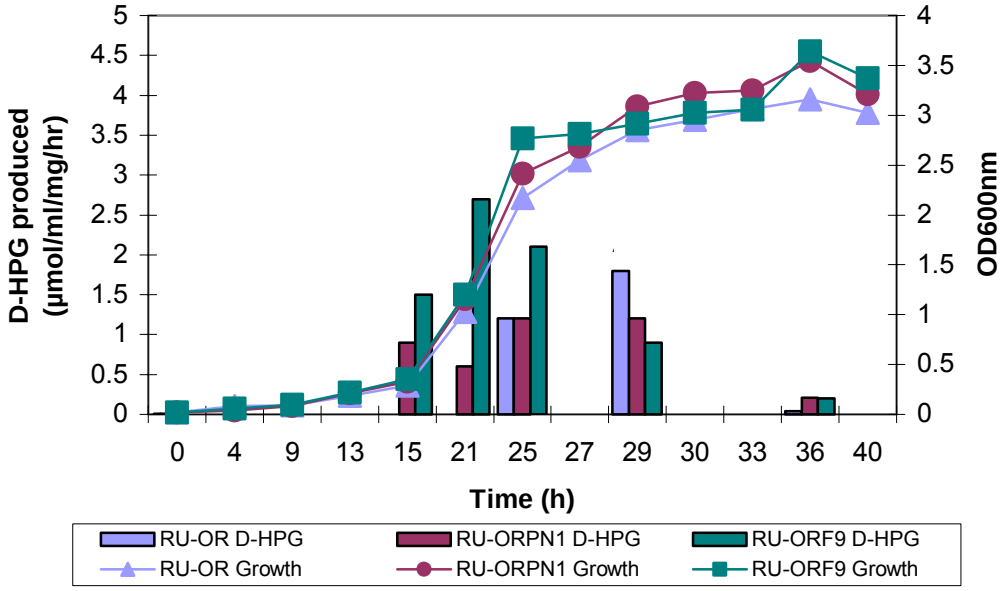


Figure 4.13: Hydrolysis of D,L-5-*p*-hydroxyphenylhydantoin during growth with 0.1% ammonium as a sole nitrogen source: production of D-*p*-hydroxyphenylglycine from 25 mM D,L-5-*p*-hydroxyphenylhydantoin over growth period of wild-type and mutant *Agrobacterium tumefaciens* strains. SEM < 0.05 (n=12).

Up to 45 % of D,L-*p*-hydroxyphenylhydantoin was converted to either *N*-carbamoyl-*p*-hydroxyphenylglycine or D-*p*-hydroxyphenylglycine by RU-ORF9 cells within six hours (Figure 4.12). RU-ORF9 cells produced approximately 6 µmoles/ml D-*p*-hydroxyphenylglycine after six hours, which corresponds to 25 % conversion of D,L-*p*-hydroxyphenylhydantoin substrate (Figure 4.13). The enantiomeric purity of the *p*-hydroxyphenylglycine produced by RU-ORF9 was also of great importance for the potential application as a biocatalyst. Chiral HPLC analysis was used for the enantiomeric resolution and quantitation of the D-*p*-hydroxyphenylglycine produced in the experement illustrated in Figure 4.13. The enantiomeric excess of D-*p*-hydroxyphenylglycine in the reaction supernatant was calculated at 99.6%, and a typical chromatogram analysis of the amino acid product is illustrated in Figure 4.14.

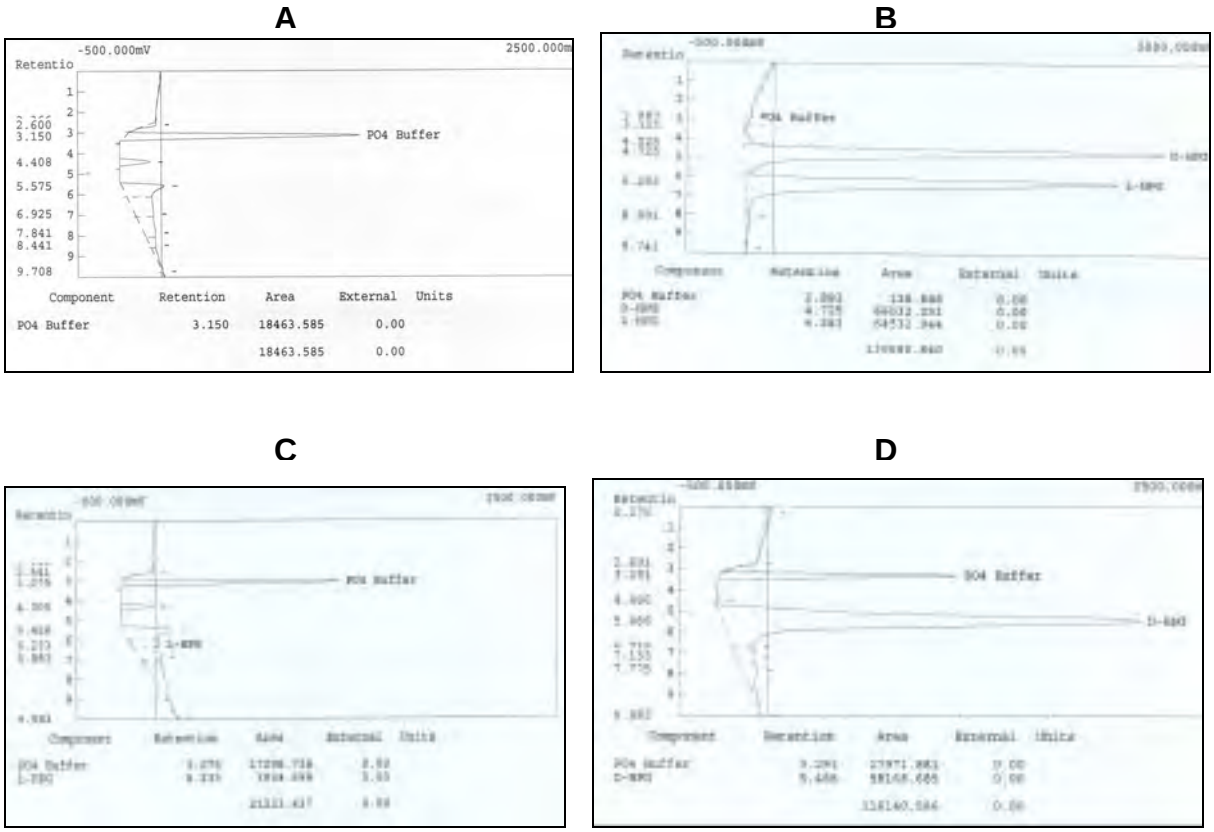


Figure 4.14: Typical enantiomeric analysis of D-and L-*p*-HPG produced by RU-OR, RU-ORPN1 and RU-ORF9 during growth in 0.1% ammonium sulphate.

4.4 Discussion

Three different mechanisms of regulating hydantoin-hydrolysing activity in *A. tumefaciens* RU-OR through induction, nitrogen control and product inhibition were revealed in the previous chapter. In order to develop improved biocatalysts and further examine these regulatory mechanisms, mutants of *A. tumefaciens* RU-OR with altered regulatory characteristics were generated (Table 4.2).

Table 4.2: Characteristics of the mutant strains with altered regulation of hydantoin-hydrolysing activity derived from *A. tumefaciens* RU-OR.

Strain	Induction (I)		NCR (N)	Ammonium shock (AS)
	Hydantoinase	NCAAH		
RU-OR	I^+	I^+	N^+	AS^+
RU-ORLB3	I^-	I^+	N^+	AS^+
RU-ORL5	I^-	I^-	N^+	AS^+
RU-ORPN1	I^-	I^-	N^-	AS^+
RU-ORF1	I^-	I^-	N^-	AS^-
RU-ORF9	I^-	I^-	N^-	AS^-

Key: the superscript + denotes susceptibility to a characteristic, superscript – denotes independence or resistance to a particular characteristic.

If the wildtype strain was designated I^+, N^+, AS^+ based on the nomenclature in Table 4.2, the mutants could be divide into three main categories: I^-, N^+, AS^+ (RU-ORLB3, RU-ORL5); I^-, N^-, AS^+ (RU-ORPN1); I^-, N^-, AS^- (RU-ORF1, RU-ORF9). In all three classes, hydantoinase and/or NCAAH activity in the mutant strains was no longer inducer-dependent. In addition, strain RU-ORPN1 appeared no longer sensitive to transcriptional nitrogen repression, and two mutant derivatives of this strain, RU-ORF1 and RU-ORF9 were resistant to ammonium shock of hydantoinase activity. An examination of the possible origin of these mutations, and the implications for the mechanisms controlling hydantoin-hydrolysing activity in RU-OR cells are discussed in the following sections.

Induction

Combined wildtype NCAAH activity with inducer-independent hydantoinase activity in the mutant strain RU-ORLB3 (Table 4.1), suggested that the expression of the hydantoinase- and NCAAH-encoding genes in *A. tumefaciens* RU-OR was probably functionally independent. This indicated that separate promotor binding sites might govern the induction of the two genes encoding hydantoinase and NCAAH activity in RU-OR cells i.e. the genes might be independently transcribed. This may be related to the higher levels of NCAAH activity in RU-OR cells produce in response to growth

with mannitol as a sole carbon source (Chapter 3), and the presence of independent promotor regions for two different NCDAAH-encoding genes isolated from RU-OR as will be described in Chapter 6. The mutational alteration in strain RU-ORLB3 was of great interest due to the apparent decrease in hydantoin racemase activity in uninduced RU-ORLB3 cells, which suggested that racemase activity in *A. tumefaciens* RU-OR may only be active when hydantoinase activity is induced.

The inducer-independence of hydantoinase, NCAAH and possibly hydantoin racemase activity in strain RU-ORL5 supports the presence of a common regulatory factor involved in the transcriptional activation of hydantoin-hydrolysing gene expression in the presence of an inducer. The mutation in strains RU-ORL5 and R-ORPN1 could be explained by a mutation in this common factor, which would prevent induction of genes encoding hydantoin-hydrolysing activity in *A. tumefaciens* RU-OR. A frequent scenario for induction of gene expression in prokaryotic systems involves the binding of inducer molecules to a repressor molecule, thus preventing the repressor from binding to the promotor region and inhibiting transcription. A mutation in the repressor molecule that prevents the repressor binding to the promotor region even in the absence of inducer is the most common mutation involved in inducer–independent gene expression (Maloy *et al*., 1994). Assuming induction in *A. tumefaciens* RU-OR to operate in a similar manner, such a mutation may have occurred in strain RU-ORL5. Screening mutants for 5-FU resistance selected specifically for overexpression of hydantoinase activity, (as seen in RU-ORPN1) and not necessarily NCAAH activity in *A. tumefaciens* RU-OR. The isolation of strains RU-ORL5 and RU-ORPN1, which exhibited inducer-independent expression of both hydantoinase and NCAAH activity without selection pressure for the latter, provided further evidence to support a common transcriptional regulatory factor being involved in the induction of hydantoin-hydrolysing activity in *A. tumefaciens* RU-OR.

Nitrogen catabolite repression

The production of hydantoinase and NCAAH activity in RU-OR cells appeared to be regulated by nitrogen catabolite repression, as non-growth-rate-limiting nitrogen

sources repressed enzyme activity. It is likely that nitrogen catabolite repression prevents the expression of the hydantoinase- and NCAAH-encoding genes when RU-OR cells are cultured in medium containing repressive nitrogen sources. This effect is probably mediated through the action of a global transcriptional regulatory factor, as evidenced by increased levels of both hydantoinase and NCAAH activity in RU-ORPN1 cells when grown with $(NH_4)_2SO_4$ as a nitrogen source (after reversal of the ammonium shock effect on hydantoinase activity). The regulation of hydantoin-hydrolysing enzyme expression by a global regulatory factor such as the σ^{54} transcriptional factor is further supported by the presence of sequences resembling σ^{54}-binding sites located upstream of genes encoding hydantoin-hydrolysing activity in other bacterial strains. (Watabe *et al*., 1992a, Grifantini *et al*., 1998). *Ntr*-type regulation of other nitrogen metabolic enzyme systems in *A. tumefaciens* cells, which share significant sequence identity with certain hydantoin-hydrolysing enzymes has also been demonstrated (Lyi *et al.,* 1999). Analysis of the promotor regions upstream of genes encoding hydantoin-hydrolysing activity in RU-OR provided more evidence for this hypothesis, as will be described in Chapter 6. The involvement of cellular glutamine levels and GS activity in the regulation of hydantoinase and NCAAH activity in RU-OR cells also implicates *ntr*-type nitrogen control based on the cellular ratio of glutamine : 2-ketoglutarate, as described in the introduction to this chapter (Merrick and Edwards, 1995).

Ammonium shock

The data presented in Chapter 3 and this chapter illustrated that ammonium shock of hydantoinase activity in wildtype RU-OR cells is mediate through the conversion of 2-ketoglutarate and ammonia to glutamine by GS, as both ammonium sulphate and glutamine produce ammonium shock of hydantoinase activity. Alterations to the cellular ratio of glutamine : 2-ketoglutarate are reported to produce an ammonium shock effect on GS activity (Magasanik *et al.*, 1976) , as was observed in RU-OR cells. This alteration to the glutamine : 2-ketoglutarate cellular ratio seemed to trigger a similar ammonium shock effect on hydantoinase activity in RU-OR cells.

The glutamine-dependent phenotype of RU-ORF9 cells resulted in abnormally high levels of adenylation of GS, and concomitant low levels of glutamine synthesis. This would result in reduced conversion of ammonium to glutamine, and thus high concentrations of ammonium would not dramatically alter the cellular ratio of glutamine : 2-ketoglutarate. The data in this chapter indicates that the mutation in strain RU-ORF9 results in an inability of the cells to detect cellular nitrogen levels due to the abnormal adenylation of GS. Post-translational modification of the subunits of the GS enzyme occurs in the presence of ammonium ions, and results in rapid deactivation of GS enzyme activity by adenylation. This affects the cellular ratio of 2-ketoglutarate to glutamine, too, and adenylation of GS therefore forms part of the mechanism by which enteric bacteria respond to changes in cellular nitrogen levels (Merrick and Edwards, 1995, Figure 4.1). The abnormal adenylation of GS activity in RU-ORF9 would therefore disable the ability of the cell to detect cellular nitrogen levels through the cellular ratio of 2-ketoglutarate to glutamine, and may explain why RU-ORF9 cells were not able to respond to ammonium shock in a normal manner. Ammonium shock of hydantoinase activity was inactivated by the mutation in strain RU-ORF9.

Strain RU-ORF1 exhibited hydantoin-hydrolysing activity that was resitant to both ammonium and glutamine shock. GS activity in this strain was generally lower than observed for the wildtype strain RU-OR at all times, and was also resistant to shock by both ammonium and glutamine. The lack of adenylation of GS even after glutamine shock in RU-ORF1 suggested that the mechanism for sensing the cellular concentrations of glutamine might be mutated in this strain. The concomitant lack of ammonium shock of hydantoin-hydrolysing activity in RUORF1 also supported the link between *ntr*-type regulation of GS and hydantoin-hydrolysing activity in *A. tumefaciens* RU-OR cells. A possible explanation for the mutation in RU-ORF1 could be the mutation of a factor involved in the signal transduction pathway associate with *ntr*-type regulation in enteric cells (such as PII in Figure 4.1), which normally enables the cell to respond increases in the cellular nitrogen levels.

Together with the effect of the GS inhibitor, MSX, on ammonium shock, the altered phenotype of glutamine-dependent mutants RU-ORF9 and RU-ORF1 under ammonium shock conditions illustrated conclusively that the *ntr-gln* regulatory network and GS activity is linked to the regulation of hydantoin. Glutamine shock inhibition of hydantoin-hydrolysing activity in RU-OR and the mutant strains corroborated the postulate that cellular glutamine levels affect the regulation of hydantoin-hydrolysing activity in RU-OR. The role of GS in ammonia shock was not unexpected because conversion of glutamate plus NH_4^+ to glutamine, catalysed by glutamine synthetase, is the pathway through which many gram-negative cells assimilate nitrogen under high-nitrogen conditions (Merrick & Edwards, 1995). Furthermore, the cellular ratio of α-2-ketoglutarate: glutamine often acts as a sensor for nitrogen status and hence catabolite repression.

One of the major disadvantages of using *Agrobacterium* cells for the production of hydantoinase and NCAAH activity has been the relatively low levels of enzyme production per unit biomass (Chao *et al.*, 1999a, 2000). This is mostly due to the fact that in the wild-type cells, maximum enzyme activity is observed when cells are grown in media with growth-rate-limiting nitrogen sources. In addition to the slow growth rate, these fermentation conditions also limit biomass production (Chao *et al.,* 1999a). Other than the obvious economic implications of inducer-independence, the advantage of using mutant strains such as RU-OF9 was that biomass production could be maximized without compromising enzyme production or activity. The mutant strains resulting from this study, and RU-ORF9 in particular, offered a viable alternative to heterologous overproduction of these industrially important enzymes. The growth rate and biomass yield of the mutant strains RU-ORPN1 and RU-ORF9 was equivalent to that of the wildtype RU-OR cells, suggesting that overexpression of hydantoin-hydrolysing enzymes in *A. tumefaciens* cells did not produce toxicity or protein aggregation as is experienced in heterologous overproduction of hydantoin-hydrolysing enzymes in *E. coli*. Use of these strains therefore eliminated the problems of protein instability and toxicity in *E. coli*, whilst offering the potential for high levels of enzyme activity per unit biomass.

D,L-*p*-hydroxyphenylhydantoin conversion levels by RU-ORF9 cells grown in $(NH_4)_2SO_4$, were extremely encouraging, given the fact that growth conditions and biocatalytic reactions have not yet been fully optimised for a commercial production system. Production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine and D-*p*-hydroxyphenylglycine by RU-ORF9 cells from D,L-*p*-hydroxyphenylhydantoin compares favourably with levels achieved by Chao *et al.* (1999a,b) using recombinant enzyme systems: 25% molar conversion of D,L-*p*-HPH to D-HPG in 6 hours by RU-OR cells, as opposed to 61% molar conversion by recombinant *E. coli* cells after 18 hours (Table 4.3).

Table 4.3: Comparison of conversion yield, hydantoinase and NCAAH activity between RU-ORF9 cells and recombinant *E. coli* cells.

Whole Cell System	Molar conversion of D,L-p-HPH to D-HPG (Time)	Hydantoinase Activity (µmol/min/mgDCW/ml)	NCAAH Activity (µmol/min/mgDCW/ml)	Reference
A. radiobacter NRRL B11291	8% (18h)	Not specified	Not specified	Chao *et al*., 1999a
A. tumefaciens RU-ORF9	61% (18h)	0.076	0.06	Hartley *et al*., 2001
Recombinant *E. coli*	25% (6h)	0.054	0.075	Chao *et al*., 1999a

Specific hydantoinase activity per unit biomass was also higher for RU-ORF9 cells than the unoptimised recombinant cells, although specific NCAAH activity was slightly lower. These conversion levels increased considerably with optimisation of the reaction conditions for the recombinant *E. coli* (Chao *et al.*, 1999a,b, 2000a,b), and it is expected that similar optimisation of the reaction parameters for RU-ORF9 will produce a similar increase in molar conversion yield. This work also resulted in significant progress towards understanding the factors that are responsible for regulating the production and activity of the hydantoinase and NCAAH enzymes in *A. tumefaciens*.

For the purposes of obtaining molecular data to support and further elucidate the mechanisms involved in the regulation of hydantoin-hydrolysing activity, it was necessary to isolate the genes encoding hydantoin-hydrolysing activity in RU-OR, and to examine the heterologous expression of these genes.

Chapter 5

Isolation of *A. tumefaciens* RU-OR genes encoding hydantoin-hydrolysing activity

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Isolation of *A. tumefaciens* RU-OR genes encoding hydantoin-hydrolysing activity

5.1 Introduction

Recombinant gene expression and manipulation is mainly used to improve either the production capabilities or the catalytic abilities of a specific biocatalyst. Genetic engineering for improved production can involve the manipulation of the chromosomal regions regulating enzyme expression in the natural host either by mutagenesis or recombinant technology (this work, Neal *et al.,* 1999; Lee *et al*., 1998), or isolation of the gene encoding the enzyme from the chromosomal region into an expression vector for overproduction of the protein (Syldatk *et al*., 1992a). There are several advantages to recombinant expression of hydantoinase and NCAAH enzymes, including decreased cost of producing an effective biocatalyst through improved biomass yield and productivity (Ikenaka *et al.*, 1997, 1998a; Nanba *et al*., 1998; Grifantini *et al*., 1998; Chao *et* al., 1999a,b; Syldatk *et al*, 1999).

The cloning and recombinant expression of many hydantoin-hydrolysing enzymes has been reported, particularly over the last three years. Recombinant expression of D-hydantoinase and NCDAAH enzymes in *E. coli* has been utilized to improve productivity and biomass yield (Nanba *et* al., 1998; Chao *et al*., 1999a,b; Ikenaka *et al*, 1999; Neal *et* al., 1999; Wilms *et al*., 1999; Park *et al.*, 2000), and recombinant systems are able to produce soluble hydantoin-hydrolysing enzymes at levels of up to 40% of total soluble protein in the *E. coli* cells (Park *et al*., 2000). Catalytic properties such as stability (Chein *et al*., 1998; Grifantini *et al.*, 1998; Kim *et al.*, 1999; 2000a), thermostability (Ikenaka *et al*., 1998b,1999; Nanba *et al*, 1999a) and stereoselectivity (May *et al*., 2000) were improved by site-directed mutagenesis and directed evolution. Gene fusion expression methods can also be used to enhance and simplify enzyme purification (Pietzsch *et al*., 2000).

Recombinant plasmids expressing hydantoin-hydrolysing genes can be isolated from genomic DNA by PCR amplification, or through the screening of genomic DNA libraries. Developing effective methods to screen genomic libraries for the desired genes is frequently a major obstacle in the isolating of the desired gene. Many different methods have been used for the isolation of genes encoding hydantoinase and NCAAH enzymes from genomic DNA libraries (Table 1.5.1; Table 1.5.2). These include growth with hydantoins or *N*-carbamoyl amino acids as sole nitrogen sources (Watabe *et al*, 1992a; Mukohara *et al.*, 1993; Kim *et al*., 1997; Nanba *et al.*, 1998; Ikenaka *et al*, 1998a), screening with degenerate or non-degenerate DNA probes (Chen & Tsai, 1996; Grifantini *et al*, 1998; Park *et al*., 2000) and detection of pH change due to the production of *N*-carbamoyl amino acids or amino acids (Park *et al.*, 2000). La Pointe *et al.* (1994), Buson *et al.* (1996) and Neal *et al.* (1999) simply screened the entire set of library clones for NCG, glycine or ammonium production in resting cell or sonicated cell-free extract reactions. Other hydantoin-hydrolysing genes have been located by examination of open reading frames in the vicinity of the known hydantoin-hydrolysing genes (Watabe *et al.*, 1992c; Batisse *et al*., 1997; Grifantini *et al.*, 1998).

Several methods have been utilised to isolate NCAAH genes from the genomic DNA of *Agrobacterium* spp. These include screening genomic DNA libraries expressed in *E. coli* for hybridisation to degenerative probes (Grifantini *et al.*, 1998) or for the expression of NCAAH activity. Heterologous expression of NCAAH activity has been detected using ammonium production or pH change in biocatalytic reactions with NCG as a substrate (Herrera, 1995; Buson *et al*., 1996; Neal *et al*., 1999) and by selection for growth in broth culture with methylhydantoin as a sole nitrogen source (Nanba *et al.*, 1998). Screening of the genomic DNA library of *A. tumefaciens* AE-01 using phenol red to detect the increase in pH produced by conversion of *N*-carbamoyl amino acid to amino acid resulted in the isolation of the recombinant plasmid pGH45 containing the gene encoding NCDAAH activity from *A. tumefaciens* AE-01 (Herrera, 1995).

Although several hydantoinase-encoding genes have been isolated from other bacteria by screening for activity/growth with hydantoin, no genes encoding

hydantoinase activity have been isolated from *Agrobacterium* genomic DNA libraries has been directly isolated in this way. The only *Agrobacterium* hydantoinase genes isolated from genomic libraries were discovered upstream of previously isolated NCAAH genes in *A. radiobacter* NRRL B11291 (Grifantini *et al*, 1998) and *Agrobacterium* sp. 80-44-2A (Neal *et al*., 1999). The hydantoinase-encoding gene from *A. radiobacter* NRRL B11291 has also been isolated from this strain by PCR amplification (Chao *et al*., 1999a,b), but this method does not seem to extend to hydantoinase-encoding genes from other *Agrobacterium* species, as PCR amplification of the hydantoinase-encoding gene from *Agrobacterium* sp. KNK712 has not been reported. The difficulties with isolation of the hydantoinase genes from *Agrobacterium* strains are probably due to the recalcitrant nature of heterologous expression of the enzyme in *E. coli* (Grifantini *et al*, 1998; Chao *et al*., 2000).

Isolation and characterisation of the genes encoding hydantoin-hydrolysing in *A. tumefaciens* RU-OR was desirable for several reasons. Firstly, the unique biocatalytic properties of hydantoin-hydrolysing activity in RU-OR cells suggested that the hydantoin-hydrolysing enzymes of RU-OR might have different properties to those previously isolated from *Agrobacterium* strains. Resolution of the nucleotide and primary amino acid sequence of the genes encoding hydantoin-hydrolysing activity in RU-OR was needed to allow comparison with the published nucleotide, primary amino acid sequence and molecular models of other hydantoin-hydrolysing enzymes.

Secondly, the analysis of mutant strains of RU-OR described in the previous chapter resulted in significant progress towards an understanding of the factors regulating hydantoin-hydrolysing activity, but molecular evidence for the mechanisms involved was still elusive. Data from the coding sequences and promotor regions of the genes encoding hydantoin-hydrolysing activity in both wildtype and mutant strains of RU-OR was required for further characterization of the mutations producing altered regulation of hydantoin-hydrolysing activity.

Recombinant expression systems also provide many advantages for the production of hydantoin-hydrolysing enzymes for biocatalytic applications (Grifantini *et al.,* 1998;

Baneyx, 1999; Chao *et al*., 1999b). Isolation, characterisation and manipulation of the genes encoding hydantoin-hydrolysing activity in RU-OR would therefore provide important options in the development of a biocatalytic system for the production of D-amino acids, including genetic engineering of hydantoinase and NCAAH activity in RU-OR for improved biocatalytic potential.

Two recombinant plasmids encoding hydantoinase and NCAAH activity, respectively, were used as positive controls for the isolation of the RU-OR genes encoding hydantoin-hydrolysing activity. *E. coli* [pGH45], producing NCDAAH activity was isolated from the genomic DNA library of *A. tumefaciens* AE-01 (Herrera, 1995), whilst *E. coli* [pGES19] producing D-hydantoinase activity was isolated from *P. putida* DSM 86 (LaPointe *et al*., 1994).

This chapter describes the construction of a genomic library of *A. tumefaciens* RU-OR, and the screening of this library for the isolation of clones encoding hydantoin-hydrolysing activity. PCR amplification of a gene encoding NCAAH activity from chromosomal DNA of *A. tumefaciens* RU-OR is also described. The heterologous production of hydantoin-hydrolysing activity in the recombinant *E. coli* cells *was* analysed, and the substrate and stereo-selectivity of the encoded enzymes ascertained.

5.2 Materials and Methods

5.2.1 Isolation and calibration of chromosomal DNA

RU-OR cells were grown to confluence in HMM broth and total genomic DNA extracted from the harvested cells using the lysosyme lysis/organic extraction method of Ausubel *et al*. (1983). Isolated chromosomal DNA was purified using phenol: chloroform: isoamylalcohol (25:24:1) extraction, precipitation with 0.1 volume 4M sodium acetate and 2.5 volume 96% rectified ethanol at -20°C, 18h, followed by three to four washes with 0.1 volume 70% ethanol. Chromosomal DNA concentration was estimated by comparison to lambda DNA standards after electrophoresis in a 0.6% agarose gel containing 0.5 µg/ml ethidium bromide, or calculated using the

GeneQuant RNA/DNA calculator (Pharmacia Biotech). Genomic DNA was partially digested using *Sau* 3AI restriction enzyme and calibrated to yield fragments of DNA between 1 kilobase and 10 kilobases in size (Ausubel *et al.*, 1983) and then extracted again as described above.

5.2.2 Construction of RU-OR genomic DNA library

A lambda-phage based library of genomic DNA from RU-OR was constructed using a pre-digested *Bam* HI/ CIAP treated vector (ZAP Express® Predigested Vector Kit, Short *et al.,* 1988). The calibrated genomic DNA from RU-OR was ligated with the vector DNA in a molar ratio of 3:1 using T4 DNA ligase, before being packaged into lambda phage particles using the ZAP Express™ Gigapack® III Gold packaging extract. Packaging was carried out for 2 hours at room temperature. Packaged phage particles were then amplified to create a phage library with an estimated concentration of 10^9pfu/ml (Sambrook *et al.*, 1989). For screening and analysis of the library, *in vivo* mass-excision techniques were used to yield a library carried in the phagemid expression vector pBK-CMV (Short and Sorge, 1992). The mass-excision supernatant could be stored for short periods at –20°C or –70°C.

Mass-excision supernatant was then transformed into *E. coli* cells and plated onto LB agar containing 50µg/ml kanamycin, 5 µg/ml isopropyl-β-D-thiogalactoside (IPTG) and 80µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for selection of white insert-containing colonies. Restriction enzyme digests of a representative selection of insert-containing plasmids were electrophoresed (1% agarose gel containing 0.5 µg/ml ethidium bromide, 1xTBE buffer {Sambrook *et al*., 1989}) and analysed using the Kodak DC120 Digital Imaging System. The average insert size was estimated to evaluate the clonal representation within the genomic DNA library. According to the formula of Ausubel *et al*. (1983), an average insert size of 5 kb would require screening of approximately 5000 individual insert-containing colonies to adequately represent the typical genome of *Agrobacterium tumefaciens* of approximately 5.1 million base pairs (Goodner *et al.*, 1999), with a 99% chance of isolating an individual sequence:

$$N = \ln(1-P)/\ln[1-(I/G)]$$

Where *I* is the average insert size; *G* the genome size in base pairs; *P* the probability of isolating an individual sequence. This value also complies with the rule of thumb that the size of the library in basepairs should be 4.6 fold higher than the size of the genome to be sequenced (Ausubel *et al*., 1983).

Screening of the RU-OR genomic library for hydantoinase and NCAAH activity using microtitre plates involved first creating a set of master plates representative of the genomic library. A set of ten 96 well culture cluster plates containing ~ 40 insert-containing plasmids per well generated through appropriate dilutions of mass-excision supernatant aliquoted into the wells, and grown overnight at 37°C in 200µL LB broth plus 50µg/ml kanamycin. One well (A,12) in each plates was left uninoculated to monitor sterility. After addition of 60µl sterile glycerol to each well, the plates were frozen and stored at –70°C. Randomly selected wells were then diluted, plated out and the insert size and efficiency of the duplicate library rechecked as described above.

5.2.3 Genomic DNA library screening (Agar plate method)

The recombinant genes expressed in *E. coli* DH5α (*sup*E44Δ*la c*U169[Φ80*lacZ*ΔM15]*hsd*R17*rec*A1*end*A1*gyr*A96*thi*-1*rel*A1) or *E. coli* XLOLR (Δ[*mrcA]183*Δ[mcrCB-hsdMR-mrr]173endA1 thi-1 recA1 gyrA96 relA1 lac[F'proAB $lacI^qZ\Delta M15Tn10$ {Tet^r}] Su^- [nonsuppressing] λ^R [lambda resistant]) were screened for growth on MM agar containing 1% hydantoin or 1% NCG as a sole nitrogen source and supplemented with 0.01% thiamine hydrochloride, or nutrient agar plates containing 0.01% 5-fluorouracil. MM agar plates containing 0.001% phenol red as a pH indicator were also used to detect hydantoinase or NCAAH activity through changes in the pH of the medium. *A. tumefaciens* RU-OR, *Pseudomonas putida* $RUKM3_S$ (a bacterial strain which produces higher amounts of NCG from hydantoin than RU-OR) and two previously isolated plasmids transformed into *E. coli* DH5α were used to pre-test this screening method. The two plasmids included pGH45, a previously isolated NCAAH gene inserted into pBK-CMV, from *A. tumefaciens* AE-01 (Herrera, 1995) and pGES19, containing a previously isolated hydantoinase-encoding gene from *Pseudomonas putida* DSM 84 (LaPointe *et al*., 1994, supplied

by Andre Morin). For complete restriction maps see Appendix 7. All the transformants were grown on hydantoin minimal agar and minimal agar containing casamino acids as a nitrogen source, with 0.001% phenol red dye at pH 6.5 and pH 8.0. The agar containing casamino acids was used as a control to ensure that any observed colour change on hydantoin minimal agar was due solely to hydantoin-hydrolysis and not other reactions that might affect the pH of the medium.

Selected colonies were then cultured overnight in 5 ml LB broth plus 50µg/ml kanamycin. Plasmid DNA was extracted from the cultures by the method of Berhammer and Auer (1993), before dilution of each culture to an OD_{600nm} =0.02 into two flasks containing 100ml LB plus 50µg/ml kanamycin. IPTG (1mM) was added to one of the two flasks, before growth overnight at 37°C. The *E. coli* cells were then harvested and assayed in resting cell biocatalytic reactions.

5.2.4 Genomic DNA library screening (Microtitre Plate Method)

For the screening of the microtitre master plates, each 96 well plate was inoculated into four replicate plates: two containing 180 µl LB plus 50µg/ml kanamycin in each well, two containing 180 µl LB plus 50µg/ml kanamycin plus 1mM IPTG in each well. The frozen master plates were thawed slightly before inoculating 20µl from each well into the appropriate wells of the four replicate plates. The microtitre plate were then incubated shaking at 200rpm, 37°C for ~ 18 hours (overnight) till an OD_{600nm} of >2.0 was reached. The plates were then centrifuged at 240g for 15 minutes (Eppendorf A-2-MTP rotor, Eppendorf Centrifuge 5810R), and the pellet in each well resuspended in 200µl 0.1M phosphate buffer pH 8 containing 50mM hydantoin or 30mM NCG. After incubation at 40°C for 4-24hrs with shaking (200rpm), plates were centrifuged at 240g for 15 minutes, and the supernatant analysed for production of NCG using a modified Ehrlich's reagent assay (Appendix 4), and for production of glycine using a modified ninhydrin assay (Wilms *et al*., 1999; Appendix 4).

After detection of production of either NCG or glycine at least twice in repeated screening assays, each selected well was thawed slightly, 20µl inoculated into 5ml LB plus 50µg/ml kanamycin and grown at 37°C for ~ 5 hours, before dilution and

plating out onto LB plus kanamycin plates containing 1mM IPTG and 40µg/ml X-gal. Selected insert-containing (white) colonies were then grown to stationary growth phase in 5ml LB plus kanamycin, ± IPTG, and re-assayed in microtitre plates using 400µl of culture in each well. Plasmid DNA isolated from each colony was subjected to restriction fragment analysis with a number of different restriction enzymes.

For the third round of screening the remaining positive isolates with appropriate inserts were cultured to stationary growth phase in LB plus kanamycin ± IPTG, and assayed for hydantoinase and NCAAH activity in standard biocatalytic reactions (5.2.8 and 5.2.9). Again, plasmid DNA isolated from each colony was subjected to restriction fragment analysis.

5.2.5 Plasmid Isolation and restriction fragment analysis

Plasmid DNA for initial screening purposes was extracted using "easypreps"(Berghammer & Auer, 1993). For subsequent analysis plasmid DNA was isolated according to manufacturer's instructions using High Pure® Plasmid Isolation, QIAprep® Spin Minipreps or Quantum® Prep Plasmid Midipreps. Restriction enzyme analysis was performed by first digesting the plasmids according to manufacturer's instructions. The restriction fragments were then separated by electrophoresis in 1% agarose gel containing 0.5µg/ml ethidium bromide and visualised using the Kodak DC 120 gel imaging system.

5.2.6 PCR Amplification of NCAAH gene from RU-OR

Primers NCAA F1 and NCAA R1 (Appendix 6) were used to amplify a 1.1kb fragment from chromosomal DNA, isolated according to the lysome lysis/ organic extraction method of Ausubel *et al.* (1983). Primer design was adapted from Chao *et al.* (1999a), based on the similarity in sequence between published NCAAH sequences from *Agrobacterium* spp. The resulting PCR fragments were analysed by electrophoresis on a 1% agarose gel containing 0.5µg/ml ethidium bromide and visualised with UV detection using the Kodak DC 120 Digital Imaging System. The PCR product was then purified (High Pure® PCR Product Purification Kit, Roche) and ligated into the pGEM-T-Easy vector using the AA-overhangs generated by the tailing

action of Taq DNA polymerase in Expand™ High-Fidelity PCR System (Roche). The resulting plasmid was named pG4.

5.2.7 Resting Cell and Sonicated Crude Extract Biocatalytic Reactions

These were performed exactly as described for wildtype hydantoin-hydrolysing activity (Chapter2, Appendix 2), except that 0.1M phosphate buffer pH 9.0 was replaced by 0.1M phosphate buffer pH 8.0, and activity was measured at various time intervals from 3 to 24 hours, with incubation at 40°C, shaking at 200rpm. For sonicated cell extract reactions, the cells were resuspended in 0.1M potassium phosphate buffer pH 8 at a biocatalyst concentration of 20 mg/ml wet cell mass, and then sonicated on ice for 100 seconds at 60kHz. Sonication of the cells was done in bursts of 10 seconds, with 10 second recovery intervals between each burst. The sonicated cell suspension was then assayed for NCAAH activity as described above.

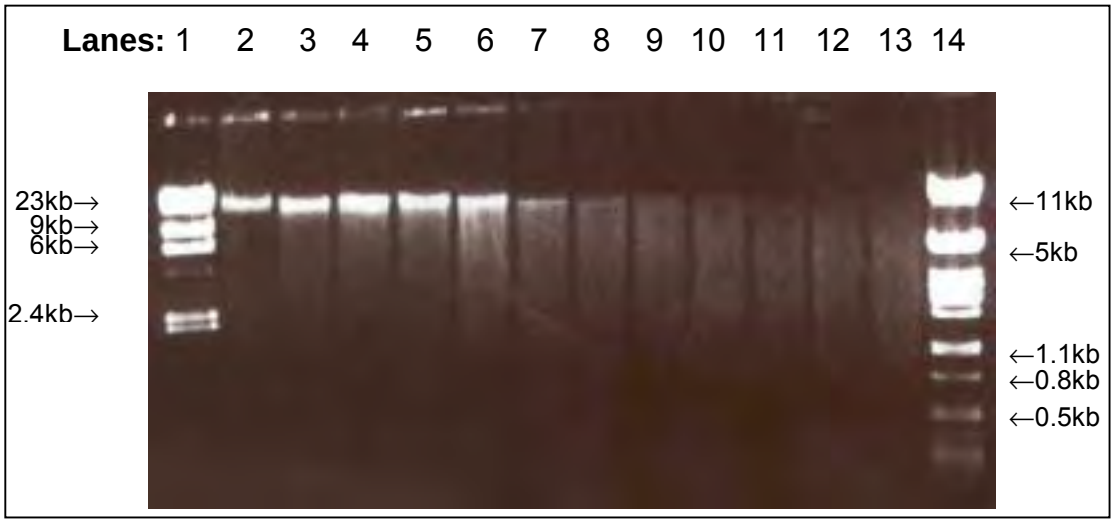
5.2.8 Analytical Methods

The biocatalytic reaction supernatant was analysed for reaction products, by colorimetric assays or HPLC analysis. Amino acid production was detected using the colourimetric ninhydrin assay as described in Appendix 4. D,L-5-*p*-hydroxyphenylglycine (HPG) production was measured using HPLC analysis of 20µl of reaction supernatant, also as described in Appendix 4. One unit of NCAAH activity (U) was defined as the amount of glycine (µmol) produced under optimised reaction conditions as described in Chapter 2. Specific activity in crude enzyme extracts was calculated as µmol glycine produced per minute per mg protein. All enzyme activity values were reported as the average of *n* repeats. For enantiomeric resolution, the reaction supernatant from assays using D,L-HPH and D,L-NCHPG as a substrate were analysed to determine the enantiomeric ratio of D-HPG:L-HPG, using chiral HPLC analysis. Chiral HPLC was carried out using an ODS C 18 column, with a flow rate of 1 ml/minute and an eluent of 2mM N,N-dimethyl-L-phenylalanine, 1mM copper acetate, 5% (v/v) methanol, as described in Appendix 4.

5.3 Results

5.3.1 Construction of a genomic library of RU-OR

Chromosomal DNA from RU-OR was diluted to a concentration of ~ 200ng/µl, and partially digested with *Sau* 3AI for varying time intervals until calibrated to yield partially digested DNA with the majority of fragments in the 1 to 10kb range (Figure 5.1, Lane 8).



Lanes: 1, λ DNA digested with *Hind* III; 2-13, the equivalent of 1µg of RU-OR DNA digested with *Sau* 3A1 for 0-11 minutes, sampling every minute; 14, λ DNA digested with *Pst* I. DNA digested to the same extent as in Lane 8 (6 minutes) was used to produce genomic library.

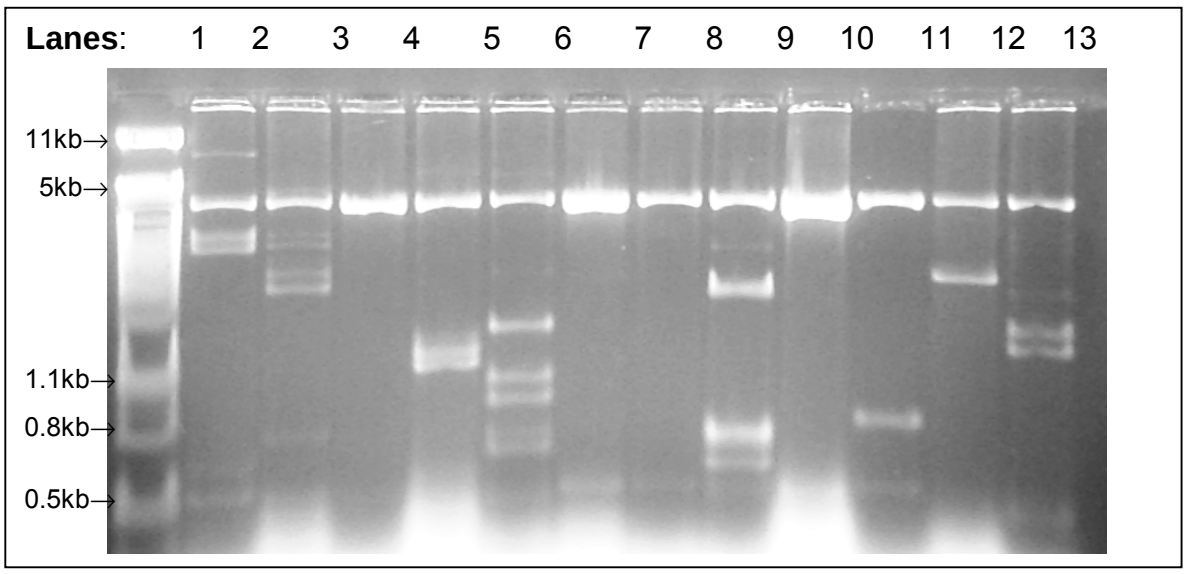


Figure 5.2: Estimation of the insertion frequency and average insert size of plasmids in the RU-OR genomic DNA library. **Lanes:** 1, Lambda *Pst* I marker DNA; 2-13, 12 randomly selected library plasmids digested with *Pst* I and *Hind* III. No insert – lanes 4 and 10. Total insert size of other 10 lanes was ~39.8 kb.

The insertion frequency of the phagemid library after mass excision was estimated at 80%, with an average insert size of ~ 4kb (Figure 5.2), yielding ~10^3 colonies per µl of mass-excision supernatant, which made it feasible to screen the representative number of library plasmids from 200 µl of supernatant.

5.3.2 Screening for genes encoding hydantoin-hydrolysing activity with MM agar plates containing phenol red

Production of *N*-carbamoylamino acids from hydantoin results in a decrease in pH, whilst the conversion of *N*-carbamoylamino acids to amino acids releases ammonia as a by-product, resulting in an increase in pH. Phenol red is a pink-red colour at pH greater than 7.9, but changes to orange-red at a pH less than 7.9 (Plummer, 1987). This colour change can therefore be used to detect the production of both *N*-carbamoylamino acid and amino acid (Gulati *et al*., 1997; Neal *et al.,* 1999). The production of *N*-carbamoylamino acid results in a decrease in pH, and a colour change from pink-red to orange-red. Amino acid production involves an increase in pH, and a colour change from orange-red to pink-red. Both *E. coli* DH5α (pGES19) and *P. putida* RU-KM3$_S$ produced a colour change from pink to yellow-orange when grown on HMM agar plates at pH8.0. However, this pH decrease could not be attributed solely to hydantoin-hydrolysing activity, as the negative control *E. coli* DH5α produced the same colour change, and both the strains *E. coli* DH5α (pGES19) and *P. putida* RU-KM3$_S$ produced the same colour change on control plates containing no hydantoin (Table 5.1). *E. coli* DH5α did not produce an increase in pH when grown on NCMM agar containing phenol red at pH6.5, but both *E. coli* DH5α (pGH45) and *A. tumefaciens* RU-OR did cause the colour change from yellow-orange to dark pink on the same medium. *E. coli* DH5α (pGH45) and *A. tumefaciens* RU-OR did not produce the same colour change on the control 0.01% casamino acid plates, therefore the colour change was assumed to be due to the production of glycine and ammonia from NCG, as expected. The genomic library was therefore plated onto NCMM agar plates containing 0.001% phenol red at pH6.5. However, numerous screenings of library colonies did not isolate any insert-

containing colonies that produced the expected colour change from yellow-orange to dark pink.

Table 5.1: Determination of the efficiency of using 0.01% phenol red dye to detect hydantoinase and NCAAH activities.

Model	Colour Change				
	HMM		NCMM	0.01% Casamino Acids Agar	
	pH 6.5#	pH 8.0*	pH 6.5#	pH 6.5#	pH 8.0*
E. coli DH5α	✗	✔	✗	✗	✔
E. coli DH5α [pGES19]	✔	✔	✗	✔	✔
E. coli DH5α [pGH45]	✗	✗	✔	✗	✗
A. tumefaciens RU-OR	✔	✗	✔	✗	✗
P. putida RU-KM3$_S$	✔	✔	✔	✔	✔

Key: ✔ - Detectable colour change; ✗- No detectable colour change. # - Colour change of colonies on plates at pH 6.5 was from yellow-orange to dark pink or red, representing an increase in the pH of the medium.* Colour change of colonies on plates at pH 8.0 was from dark pink to yellow-orange, representing a decrease in pH.

5.3.3 Screening for genes encoding hydantoin-hydrolysing activity using growth on HMM or NCMM agar plates.

The genomic library from RU-OR, containing approximately 38000 individual insert-containing plasmids, was screened for colonies able to grow on MM agar plates containing hydantoin or NCG as sole nitrogen sources. Two transformants were obtained which were able to grow on the selection media and contained sizeable chromosomal DNA inserts: one from hydantoin minimal medium (pCHH1) selection and one from screening for growth on NCG minimal medium (pCHNC2). The plasmids pCHH1 and pCHNC2 were isolated from *E. coli* XLOLR and transformed into *E. coli* DH5α. The hydantoin-hydrolysing activity of these clones in resting cell biocatalytic assay was then compared to that of RU-OR (Table 5.2). Both plasmids appeared to produce hydantoinase and NCAAH activity. Insertion of the genomic DNA into the multiple cloning cassete in pBK-CMV (Appendix 7) placed the insert DNA under the control of the *lac* promotor, which is induced to high levels of expression by IPTG. The highest activity was obtained from the plasmid designated pCHH1, and was induced by IPTG (Table 5.2).

Table 5.2: Hydantoin-hydrolysing activity of selected *E. coli* transformants from a genomic library of RU-OR

Strain or Plasmid	Insert Size (Kb)	Inducer	Assay time (h)	Hydantoinase (µmol/ml)	NCAAH (µmol/ml)
RU-OR	-	0.1% 2-thiouracil	6	**6-8**	**8-10**
Heterologous expression in *E. coli* DH5α					
pBK-CMV	0	+ 1 mM IPTG	2	0	0.42 (± 0.03)
		- IPTG		0.26 (± 0.08)	0.36 (± 0.03)
pCHH1	5	+ 1 mM IPTG	2	11.7 (± 1.05)	10.4 (± 0.48)
		- IPTG		1.69 (± 0.21)	2.68 (± 0.21)
pCHNC2	3	+ 1 mM IPTG	4	6.99 (± 0.45)	7.24 (± 0.69)
		-IPTG		2.25 (± 0.79)	5.89 (± 0.50)

Key: (± SEM, n=6).

This suggested that the genes contained within the plasmid were under control of the IPTG-inducible pBK-CMV *lac* promotor. Although selected only for growth on NCG minimal medium, pCHNNC2 showed both hydantoinase and *N*-carbamoyl amino acid amidohydrolase activity, which did not seem to be induced by IPTG. Limited restriction endonuclease mapping of the two plasmids showed that there were similar regions of DNA between the two plasmids. Despite the apparent hydantoinase and NCAAH activity of *E. coli* [pCHH1] and *E. coli* [pCHNC2], the sequence data from the insert DNA (Appendix 8) did not reveal any open reading frames (ORFs) with significant identity (>10%) to amidohydrolase enzymes. The sequence data was analysed using BLAST searches with nucleotide sequence (BLASTN) and/or predicted protein sequence (BLAST-X, BLAST-P) and a variety of bioinformatics tools provided by HGMP-RC Bioinformatics. None of these provided coding regions that could reasonably be predicted to encode hydantoin-hydrolysing activity, and so a different screening procedure was designed to isolate hydantoin-hydrolysing genes from the genomic library of RU-OR.

5.3.4 Screening for genes encoding hydantoin-hydrolysing activity using microtitre-plate biocatalytic assays

The total genomic library of RU-OR produced was aliquoted into ten 96-well microtitre plates with each well containing bacteria representing approximately 40 insert-containing plasmids (a total representation of 38000 individual plasmids). These master plates were then sub-cultured and screened for hydantoinase and /or NCAAH activity. Each master plate was passed through two rounds of screening, both with and without induction of the library vector promoter using IPTG and the results are summarised in Table 5.3.

Table 5.3: Summary of the selection process

Screening - Part 1	**Round**	**Wells screened**	**Number of wells selected**
Plates screened in microtitre assays.	1-1	3 * 96 (Plates 1-3)	19
	1-2	3 * 96 (Plates 4-6)	18
	1-3	4 * 96 (Plates 7-10)	40
Part 2	**Round**	**Wells screened**	**Number of plasmids selected**
Wells selected in part1 were screened for hydantoinase and NCAAH activity in microtitre plate assays from 5ml overnight cultures.	2-1	77	10
Part 3	**Round**	**Well from which transformants were obtained**	**Number of plasmids selected**
Plasmids isolated from wells in part 2, were transformed into *E. coli* DH5α and assayed in mirotitre plates from 5ml overnight cultures.	3-1	1A6	3
		2A4	2
	3-2	2B4	3
		4A4	1
	3-3	5D12	1
		6B3	2
	3-4	9C1	3
		9C3	2
	3-5	10H5	2
		10G9	2

Wells showing positive results were selected after each round and then diluted and plated onto LB agar plus kanamycin containing 1mM IPTG and 40 μgmL^{-1} X-gal, and individual insert-containing (white) colonies selected. These were screened for insert size and activity in microtitre biocatalytic assays, and then the selected plasmids were transformed from *E. coli* XLOLR into *E. coli* DH5α for further characterisation (Table 5.4). Further screening using larger scale (5ml) resting cell biocatalytic reactions, growth with hydantoin or NCG as sole nitrogen sources and growth in medium containing the toxic hydantoin analogue 5-fluorouracil resulted in the final selection of seven plasmids. None of the isolated plasmids conferred consistent growth of *E. coli* transformants on LB agar plates containing 0.01% 5-FU (data not shown in Table 5.4).

Table 5.4: Characteristics conferred on *E. coli* by recombinant plasmids selected from genomic library of *A. tumefaciens* RU-OR

Plasmid	NCMM		HMM		Hydantoinase Activity *		NCAAH Activity #		Insert Size (kb)
	-	+	-	+	-	+	-	+	
pBK-CMV	0	0	0	0	0.29	0.15	0.03	0.21	0
pGH45	3	4	1	1	-	-	5.34	8.97	2.4
p9C3NC	2	3	3	4	0.96	6.39	4.34	5.70	5.45
p10G9-1c	2	3	3	4	0.96	3.50	2.56	3.52	5.30
p5D12-3a	1	1	0	0	3.32	5.35	5.80	2.32	4.5
p6B3-1a	4	2	3	4	1.92	2.77	8.47	0.73	3.4

Key: *- NCG plus glycine produced from 50mM hydantoin. **#** - glycine produced from 30mM *N*-carbamoylglycine. Growth Rating: 0-no growth; 1-very slight growth; 2-slight growth; 3-fair growth; 4-good growth.

The hydantoin-hydrolysing activity for all of the isolated plasmids in resting cell biocatalytic reactions was inconsistent with standard error of the mean variations sometimes greater then 0,05% of the actual mean, and therefore not reliable (Maxwell & Delaney, 1990). In addition, the use of Ehrlich's reagent produced a pink colour in reaction with the *E. coli* resting cell reaction supernatant, making accurate measurement of the production of small amounts of NCG from hydantoin difficult. Only the NCAAH activity of *E. coli* [p6B3-1] was consistently significant, when expressed in *E. coli* DH5α grown to stationary growth phase in LB plus kanamycin without IPTG induction. This suggested that NCAAH activity in *E.coli* [p6B3-1a] was produced by transcription from the wildtype RU-OR promoter region, rather than from

the pBK-CMV *lac* promoter. Plasmid p6B3-1a was therefore selected for further characterization and sequence analysis.

5.3.5 PCR amplification of NCAAH gene from RU-OR

Primers based on published NCAAH sequences (Chao *et al*., 1999a) were used to amplify a 950 bp region from RU-OR chromosomal DNA (Figure 5.4). The amplified product included the coding region, together with the ribosomal binding site and approximately 100 nucleotides of upstream sequence. This was then gel-purified and ligated into the pGEM-T-Easy expression vector. Both plasmids obtained (pG1, pG4) contained the insert orientated in the opposite direction to the *lac* promoter in pGEM-T-Easy (Appendix 7).

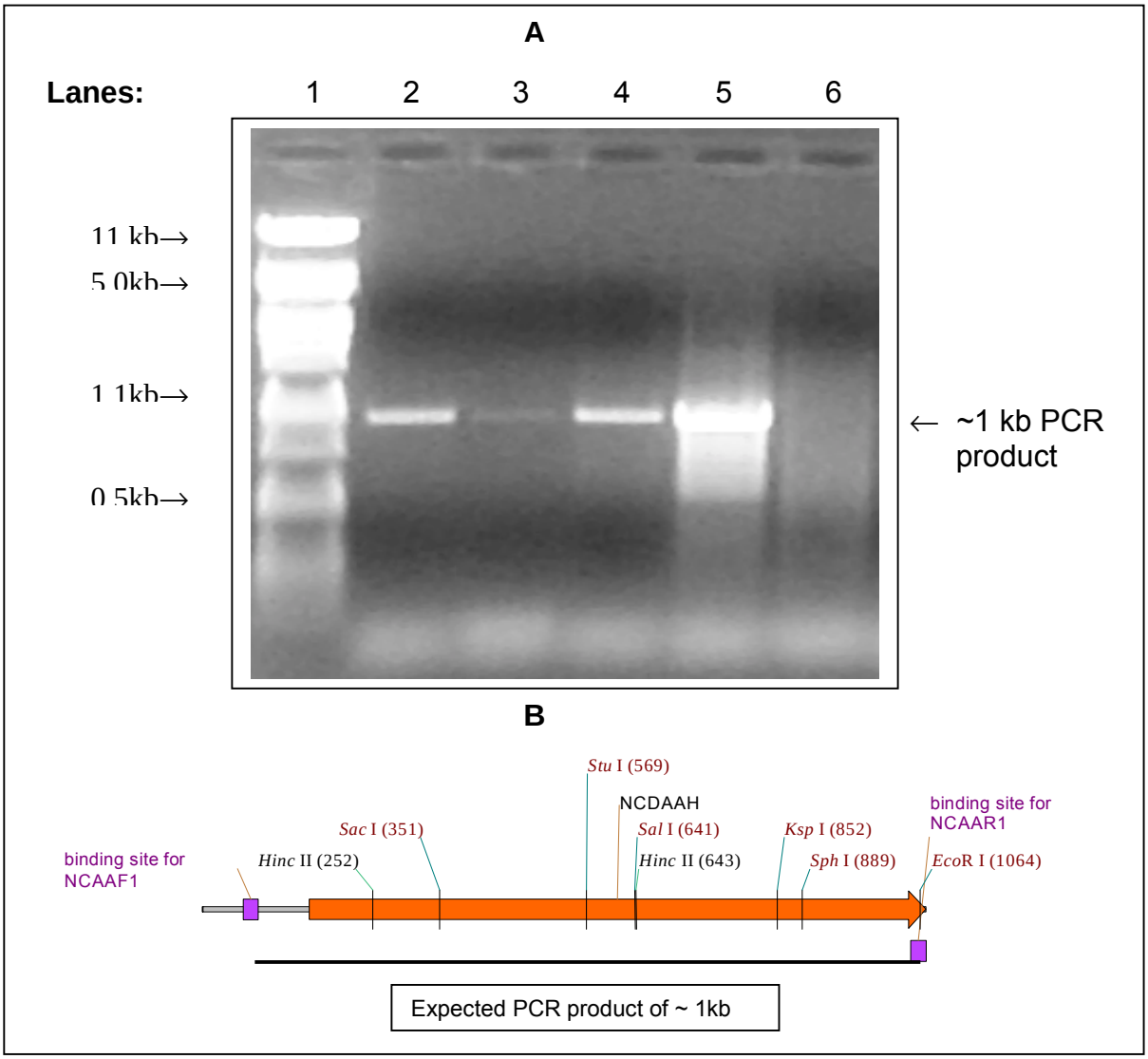


Figure 5.4: (A) PCR products amplified from chromosomal DNA using primers based on known NCAAH sequences. Lanes: 1, Lambda DNA digested with *Pst* I (marker) 2, PCR product from RU-OR 3, PCR product from RU-ORPN1 4, PCR product from *A. tumefaciens* AE-O1 5, Positive control - PCR product from pGH45 6, Negative control (no DNA). (B) Illustration of the expected PCR product and the location of the binding sites of the two primers in the region of the NCDAAH-encoding gene from *A. radiobacter* NRRL B11291 (Grifantini *et al.,* 1998; Genbank Accession Number X91070).

The NCAAH activity of *E. coli* DH5α [pG4] after growth to stationary phase in LB was compared to that of *E. coli* DH5α [pGH45], both uninduced and induced with 1mM IPTG. NCAAH activity with *E. coli* [pG4] was significantly higher than the negative control, pGEM-T-Easy, but lower than that of *E. coli* [pGH45], probably due to the inverted orientation of the ORF in pG4 in relation to the *lac* promoter region.

Table 5.5: Comparison of heterologously produced NCAAH activity of NCAAH-encoding genes from RU-OR (pG4) and *A. tumefaciens* AE-01 (pGH45).

Recombinant Plasmid	**NCAAH Activity**	
	-IPTG	**+IPTG**
E. coli DH5α (pGEM-T-Easy)	0.05 ± 0.002	0.25 ± 0.01
E. coli DH5α (pG4)	4.65 ± 0.21	3.19 ± 0.17
E. coli DH5α (pGH45)	5.62 ± 0.23	8.79 ± 0.33

Key: IPTG- isopropyl-β-D-thiogalactoside. ± SEM (n=9).

5.3.6 Comparison of the biocatalytic activity expressed by recombinant NCAAH genes.

Two different NCAAH-encoding plasmids were thus isolated from RU-OR, and preliminary restriction endonuclease fragment mapping showed that the two were not identical. This suggested the existence of two different enzymes in RU-OR that shared an *N*-carbamoyl amide hydrolysing function. To explore the functions of the two genes, a comparison of the biocatalytic properties of the two recombinant enzymes was undertaken. The NCAAH activities of the different plasmids in *E. coli* resting cell reactions were determined, with pGH45 as a positive control and pBK-CMV as a negative control. *E. coli* transformant cells were grown to early stationary growth phase in LB plus kanamycin, without induction with IPTG, and assayed. The production of D-amino acid from NCG and *N*-carbamoyl-D,L-hydroxyphenylglycine (NCHPG) showed that both of the recombinant NCAAH enzymes from RU-OR could hydrolyse unsubstituted NCG, and *N*-carbamoyl amino acids with aromatic

substituents, such as NCHPG (Figure 5.5). *E. coli* [pGH45] showed the highest levels of NCAAH activity, producing 1.5 times more glycine than *E. coli* [p6B3-1a] or *E. coli* [pG4] and more than twice as much HPG from NCHPG as bacteria containing either of the two plasmids. *E. coli* [pG4] and *E. coli* [p6B3-1a] showed similar levels of activity with both NCG and NCHPG as substrates.

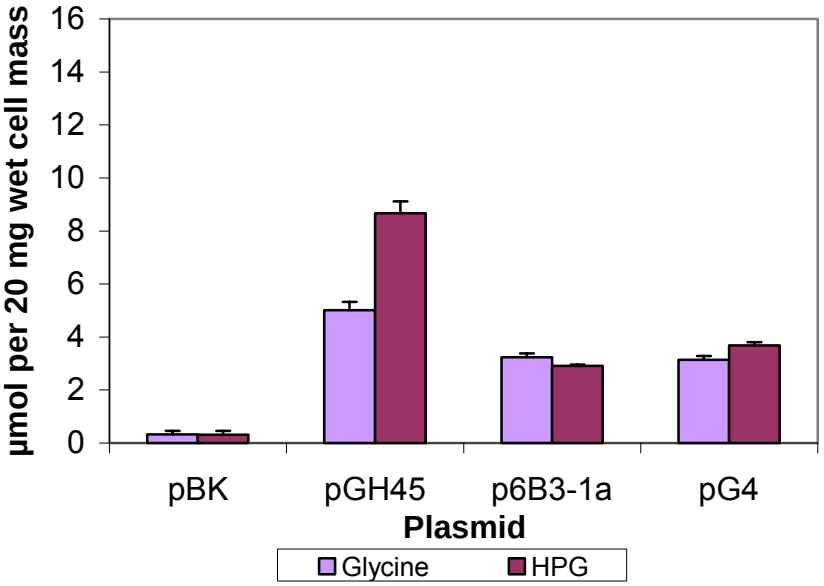


Figure 5.5: NCAAH activity of *E. coli* cells transformed with recombinant plasmids: production of glycine and *p*-hydroxyphenylglycine from NCG and NCHPG in resting cell biocatalytic reactions after 4hrs. Error bars represent SEM (n=9). Recombinant plasmids: pBK-CMV: phagemid vector, negative control, pGH45: NCDAAH gene from *A. tumefaciens* AE-01 inserted into pBK-CMV, p6B3-1a: NCDAAH gene from *A. tumefaciens* RU-OR inserted into pBK-CMV, pG4 –PCR amplified NCDAAH gene from *A. tumefaciens* RU-OR inserted into pGEM-T-Easy.

Crude enzyme preparations from sonicated cell extracts were assayed to determine the specific NCAAH activity of the enzymes in µmol per minute per milligram of protein per minute. Enzyme extracts from *E. coli* [pGH45] had higher specific NCAAH activity than *E. coli* [p6B3-1a] and *E. coli* [pG4] extracts. Concurring with the results of resting cell biocatalytic reactions, specific NCAAH activity was lowest for *E. coli* [p6B3-1a] with both *N*-carbamoyl amino acid substrates tested (Figure 5.6). The lower specific activity of *E. coli* [p6B3-1a] may have been due to the intrinsic natural properties of the NCAAH encoded by p6B3-1a or may have been attributable to differences in expression levels due to the orientation of the gene, and differences between pBK-CMV-and pGEM-T-Easy- based expression systems.

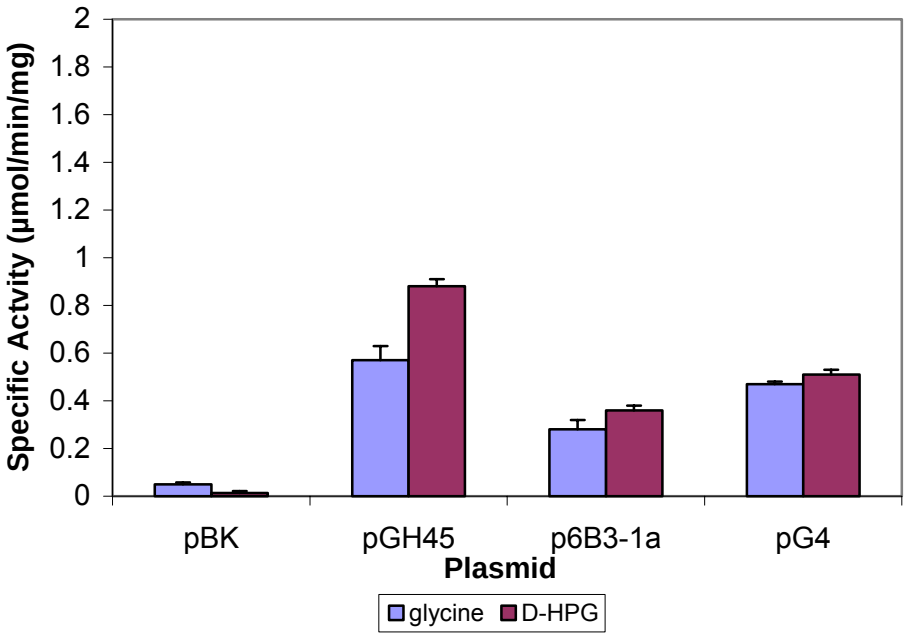


Figure 5.6: Specific NCAAH activity (µmol/min/mg protein) of cell extracts from *E. coli* transformed with recombinant plasmids. Cells were assayed after growth to stationary phase, without induction with IPTG. Error bars represent SEM (n=9). Recombinant plasmids: pBK-CMV: phagemid vector, negative control, pGH45: NCDAAH gene from *A. tumefaciens* AE-01 inserted into pBK-CMV, p6B3-1a: NCAAH gene from *A. tumefaciens* RU-OR inserted into pBK-CMV, pG4: PCR-amplified NCAAH gene from *A. tumefaciens* RU-OR inserted into pGEM-T-Easy.

Recombinant activity of crude enzyme extracts of NCAAH genes from other *Agrobacterium* strains showed similar levels of specific activity to p6B3-1a, from 0.23 to 0.28 µmol/min/mg protein (Chao *et al*., 2000; Grifantini *et al*., 1998). The specific activity of *E.* coli [pGH45] and *E. coli* [pG4] was 1.5 to 3 fold higher than this, without induction with IPTG. All three *E. coli* transformants producing NCAAH activity showed higher specific activity with NCHPG as a substrate than with NCG as a substrate.

The HPG produced from hydrolysis of *N*-carbamoyl-D,L-hydroxyphenlglycine was analysed by chiral HPLC for the enantiomeric resolution of the amino acid produced by the recombinant NCAAH genes. All three recombinant NCAAH enzymes produced D-HPG with enantiomeric excess values of >99%, showing that the encoded NCAAH enzymes were D-stereoselective with regard to the two substrates tested (Table 5.6). Although the enantioselectivity of hydantoins-hydrolysing enzymes can be substrate-dependent (Ogawa and Shimizu, 1997; May *et al*., 1998b;

Syldatk *et al.*, 1999), this suggested that the isolated genes encoded D-stereoselective enzymes (NCDAAH enzymes).

Table 5.6: Production of HPG from N-C-D,L-HPG and enantiomeric resolution of the amino acid product.

Plasmid	D-HPG	L-HPG	Total HPG	% Conversion	EE (D-HPG)
	(µmol/ml as calculated from peak area using standard curve)				
pBK	0	0	0	0	-
pGH45	4.70	0.008	4.71	31.4	99.63
pG4	9.64	0.03	9.68	64.5	99.44
p6B3-1a	3.77	0.004	3.77	25.2	99.78
p6B3dC	2.02	0.008	2.03	13.5	99.91

5.4 Discussion

Despite successful detection of NCAAH activity in *Agrobacterium* cells and control plasmids transformed into *E. coli* using the phenol red plate method, no library plasmids encoding NCAAH activity were isolated using this method. Problems associated with heterologous expression of hydantoin-hydrolysing activity in *E. coli* include improper folding of the foreign peptides, and the formation of insoluble aggregates and inclusion bodies, causing poor growth and enzyme activity when grown at 37°C (Chao *et al*., 1999a, 2000; Grifantini *et al*., 1998; Park *et al*., 2000). Some of these complications may have resulted in the failure of this screening method to select NCAAH-producing plasmids from the genomic library of RU-OR. Specifically, the recombinant hydantoin-hydrolysing enzymes in the library may not have been produced in an active form in *E. coli,* or may not have produced sufficient activity to cause a detectable pH change.

Screening for growth with hydantoin or NCG as a sole nitrogen source produced two plasmids that encoded enzymes able to convert hydantoin and NCG into amino acids in biocatalytic resting cell reactions, but initial activity levels obtained for these two plasmids were not repeatable after the first successful expression experiments. Even reversion to preserved plasmid stocks from initial isolation did not result in the recovery of significant levels of recombinant hydantoinase and NCAAH activity.

Sequence analysis of pCHH1 did not reveal DNA or protein homology to any of the known amidohydrolase genes. One plausible explanation for this is the occurrence of changes in the DNA sequence of the genomic DNA insert after the initial experiments. These alterations were not detected during routine monitoring of gross plasmid integrity using restriction fragment patterns. A third plasmid isolated by the same screening method reverted to the original pBK-CMV cloning vector shortly after detection of hydantoinase activity in resting cell reactions, presumably by "looping out" of the insert DNA (data not shown). This phenomenon has been documented for other plasmids encoding genes that are potentially toxic to the *E. coli* host strain (Banyex, 1999). Another likely explanation, however, is that insufficient stringency in the screening process allowed false positives to be selected.

The third library-screening method involved production of a master copy of each library plasmid in microtitre plates for long-term storage, and subsequent screening of each well for hydantoinase or NCAAH activity in scaled-down 200µl microtitre plate resting cell reactions. Although a similar method has been reported as a rapid way to detect native hydantoinase activity in bacterial strains (Chein & Hsu, 1996), this particular method of screening for hydantoinase and NCAAH activity in microtitre libraries has not been previously reported in literature. Microtitre screening of ~ 40000 individual plasmids resulted in the isolation of at least one plasmid which produced consistent recombinant NCAAH activity in resting cell reactions. Establishment of the genomic DNA library in microtitre plates may have produced more stable recombinants, allowing for the successful isolation of the NCDAAH gene encoded by plasmid p6B3-1a. Maximum NCAAH activity in *E. coli* [p6B3-1a] was produced in the absence of the artificial *lac* inducer, IPTG. Consequently, it was propable that NCAAH activity in this plasmid was not expressed from the *lac* promoter of pBK-CMV, but resulted from transcription and translation directed by an *A. tumefaciens* promoter region contained within the insert DNA of p6B3-1a. An interestingly implication of this was that in the recombinant system inducer molecules such as 2-thiouracil were no longer required to activate expression of the NCAAH gene from its original promoter.

Detection of NCG production by recombinant cells using Ehrlich's reagent was not reliable, due to cross-reaction with an unidentified component of *E. coli* cells that produced a pink colour. The interference of this colour with the absorption at OD_{420nm} prevented accurate determination of hydantoinase activity. Thus, only glycine production from NCG or hydantoin could be reliably detected using colorimetric assays. As the production of glycine from hydantoin required the active presence of both hydantoinase and NCAAH genes within the plasmid insert, the probability of isolating a hydantoinase gene from the genomic library by detection of glycine production was dramatically reduced. This could explain why screening of over 40 000 individual insert-containing plasmids failed to isolate a hydantoinase gene from RU-OR. PCR amplification of the hydantoinase gene from RU-OR chromosomal DNA using primers HYU1 and HYU2 designed from the published hydantoinase sequence of *A. radiobacter* NRRL B11291 (Grifantini *et al*., 1998) failed to produce the desired amplification product even under non-stringent PCR conditions (data not shown). The hydantoinase gene from *A. radiobacter* NRRL B11291 has been re-amplified by PCR with primers based on the same published sequence by Chao *et al*. (1999a). PCR amplification of an NCAAH gene and upstream promoter region from *A. tumefaciens* RU-OR was successful, producing pG4 when inserted into an expression vector. As in p6B3-1a, the NCAAH-encoding ORF in pG4 was transcribed from the original promoter, the amplified ORF being orientated in the opposite direction to the *lac* promoter of the vector. Once again, heterologous production of NCAAH activity from the original promoter did not require induction with hydantoin or 2-thiouracil, suggesting that the induction mechanism that controls hydantoin-hydrolysing expression in RU-OR cells is not active in *E. coli* cells. A possible reason for this could be the absence of a negative regulatory binding factor in *E. coli* that normally binds the activation region of the NCAAH promotor found in RU-OR cells in the absence of inducer.

Having isolated two plasmids encoding NCAAH activity from the genomic DNA of *A. tumefaciens* RU-OR, which appeared to have different coding sequences based on restriction endonuclease mapping, further characterisation of the two plasmids, and comparison with the cloned NCAAH from *A. tumefaciens* AE-01 was undertaken. Comparative analysis of the biocatalytic NCAAH activity of both *E. coli* [pG4] and *E.*

coli [p6B3-1a] showed that both transformants encoded D-stereoselective NCAAH activity, with an enantiomeric excess of >99%. The specific activity of the two NCDAAH clones compared favourably with that of the NCDAAH gene from *A. tumefaciens* AE01, encoded by pGH45 and that reported for other unoptimised recombinant NCDAAH expression systems. However, recent publications indicate that superior levels of NCAAH activity conversion, such as 50-60% conversion of 55mM N-C-D,L-HPG in 18 hours can be obtained with optimisation of the host: vector expression system (Chao *et al.*, 1999a). Problems of toxicity and protein aggregation, which decrease the efficiency of the recombinant enzyme systems, can now be minimized with the use of chaperone molecules. For example, co-expression of NCAAH enzymes with chaperone molecules such as Gro EL and Gro ES may prevent protein aggregation and result in a four-fold increase in recombinant NCAAH enzyme activity (Chao *et al.*, 2000). Similar manipulation of the recombinant expression of the two NCDAAH genes from *A. tumefaciens* RU-OR is expected to provide improved biocatalytic conversion levels. Purification of the recombinant enzymes also results in 10- to 100- fold increase in the specific NCAAH activity (Grifantini *et al.*, 1996, 1998; Chao *et al*., 1999a,b; 2000; Pietzsch *et al*., 2000;). Purification of the two enzymes from RU-OR could improve specific activity levels, and allow more detailed biochemical and biophysical examination of similarities and differences in the properties of the two enzymes. Before purifying the recombinant enzymes, however, detailed sequence and molecular analysis was required to allow further exploration of the properties of the two NCDAAH enzymes from RU-OR.

Chapter 6

Molecular analysis of *A. tumefaciens* RU-OR genes encoding NCDAAH activity

Chapter 6

Molecular analysis of *A. tumefaciens* RU-OR genes encoding NCDAAH activity

6.1 Introduction

The isolation and recombinant expression of several hydantoin-hydrolysing enzymes has been reported, particularly over the last three years. Recombinant expression of D-hydantoinase and NCDAAH enzymes in *E. coli* has been utilized to improve productivity and biomass yield (Chao *et al*., 1999a,b; Nanba *et al*., 1998; Ikenaka *et al.*, 1998a,b,c,1999; Park *et al.*, 2000; Wilms *et al*., 1999). Apart from providing improved biocatalyst yield, the protein and nucleotide sequence data from these clones has also been used to elucidate and manipulate structure-function relationships of the enzymes. In addition, phylogenetic analysis has provided insight into the evolutionary relationships of the hydantoin-hydrolysing enzymes and enabled the identification of novel members of the amidohydrolase superfamily (May *et al*., 1998a; May *et al*., 1998b; Syldatk *et al*., 1999).

A total of nine different NCAAH genes have been isolated and used for recombinant expression in heterologous or homologous host strains (Syldatk *et al*., 1992b; Watabe *et* al., 1992a; Mukohara *et* al., 1993; Buson *et* al., 1996; Batisse *et al*., 1997; Louwrier and Knowles, 1997; Grifantini *et al*., 1998; Ikenaka *et al*., 1998a,b,c,d; Nanba *et al*., 1998). These include five *N*-carbamoyl-D-amino acid amidohydrolase (NCDAAH) enzymes, which hydrolyse *N*-carbamoyl-D-amino acids (Buson *et* al., 1996; Louwrier and Knowles, 1997; Grifantini *et al*., 1998; Ikenaka *et al*., 1998a,b,c,d; Nanba *et al*., 1998; Neal *et al*., 1999). All six published NCDAAH genes were isolated from *Agrobacterium* spp., except for one NCAAH-encoding gene from *Pseudomonas* sp. KNK0003A. Two NCDAAH enzymes have also been purified from *Comamonas* sp. and *Blastobacter* sp., and the amino-terminal amino acid sequence of the protein determined (Ogawa *et al*., 1993, 1994b). Topological mapping, purified enzyme characteristics and amino-terminal protein sequences provided the first leads to unraveling the molecular characteristics of

NCDAAH enzymes. Together with the biochemical data obtained from purified enzymes, nucleotide sequence data, predicted and known protein structure and biochemical analysis of recombinant NCAAH enzymes has been used to provide detailed molecular analysis of NCDAAH enzymes (Nakai *et al*., 2000; Wang *et al*., 2001). Elucidation of structure-function relationships for the enzymes has been utilized for the manipulation of biocatalytic properties such as substrate selectivity, stereoselectivity, stability and thermostability, using site-directed mutagenesis, directed evolution and fusion protein technology (Ikenaka *et al*., 1999; Kim *et al*., 1997, 2000a,b; May *et al.,* 2000). Other interesting data derived from bioinformatic analysis of nucleotide and protein sequence include the identification of crucial active site and thermostability-related protein residues (Nanba *et al*, 1998a,b; Ikenaka *et al*, 1999) and the establishment of phylogenetic relationships. Recent crystallization studies of the NCDAAH enzyme from *A. radiobacter* NRRL B11291 have allowed the proposal of a detailed mechanism of action for hydrolysis of *N*-carbamoyl-D-amino acids by this enzyme, and modelling of the enzyme-substrate interaction at a molecular level (Nakai *et al*., 2000, Figure 6.1).

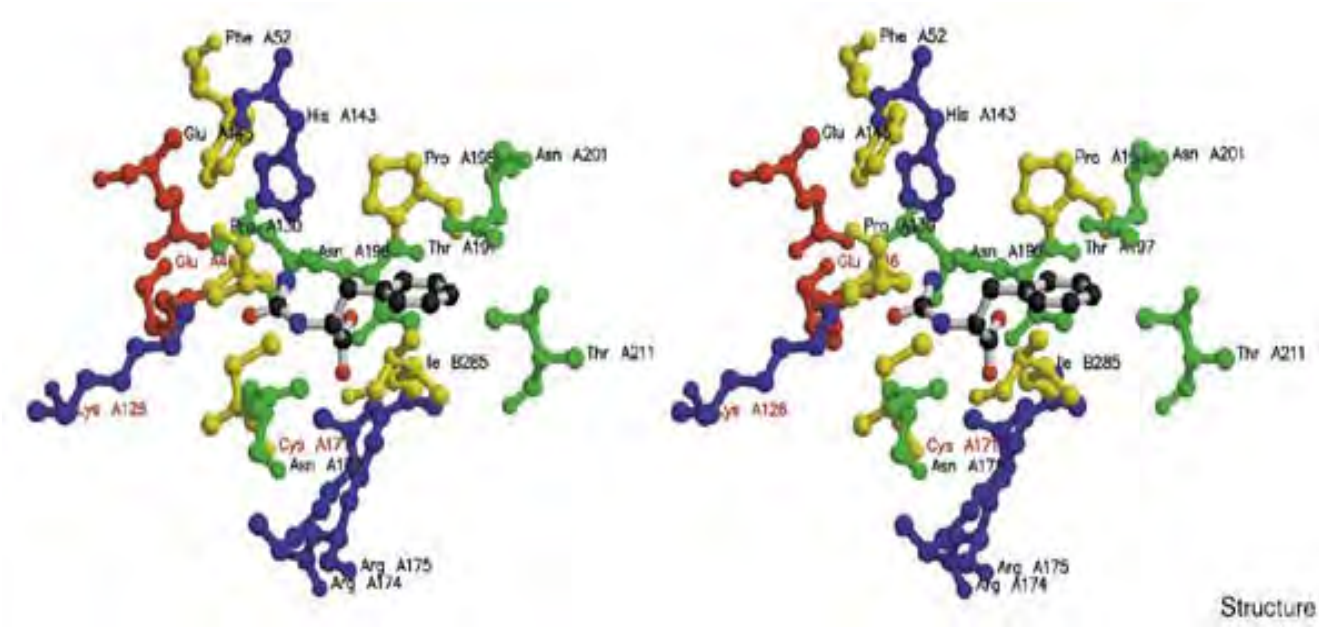


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Figure 6.1: Model of the active site of NCDAAH from *Agrobacterium* sp. KNK712 with bound substrate N-carbamoyl-D-phenylalanine. The residues involved are coloured by residue type (acidic amino acids in red, basic amino acids in blue, polar in green and hydrophobic in yellow). The substrate is coloured by atom type (carbon-grey, nitrogen-blue, oxygen-red). The three catalytically important residues are coloured red. Catalytically important residues: Glu46, Lys126, Cys 171(172).

In addition to NCDAAH, several other enzymes catalyze the hydrolysis of *N*-carbamoylamide compounds, including *N*-carbamoyl-L-amino acid amidohydrolase (NCLAAH) enzymes, β-alanine synthase, citrullinase, ureidosuccinase, *N*-carbamoylsarcosine amidohydrolase, aliphatic amidases and nitrilases (Nanba *et al*., 1999a, Nakai *et al.*, 2000). NCDAAH enzymes from *Agrobacterium* sp. KNK712 and *A. radiobacter* NRRL B11291 share some sequence identity with β-alanine synthase, aliphatic amidases and nitrilases, including strict conservation of the residues proposed to be involved in the catalytic site (Nanba *et al*., 1999a; Nakai *et al*., 2000). These enzymes seem to share a common structural and catalytic framework, but differ in substrate specificity and other properties. Evolutionary relationships between NCDAAH enzymes and other Ntn hydrolases have not been determined, but it seems likely that NCDAAH evolved divergently from other hydrolase enzymes (Nakai *et al*., 2000). Although there is a high degree of sequence similarity between the various NCDAAH enzymes, there seems to be little sequence conservation between NCLAAH and NCDAAH enzymes (Wilms *et al*., 1999).

The previous chapter described the isolation and characterization of recombinant plasmids encoding hydantoin-hydrolysing activity from RU-OR chromosomal DNA. The NCDAAH–encoding plasmids p6B3-1a and pG4 did not appear to share a high level of sequence similarity based on restriction mapping. In addition, the position of the NCAAH genes on the genome of *A. tumefaciens* RU-OR was mapped to elucidate the similarities and differences between the spatial locations of each of the genes. This suggested the existence of two different enzymes in RU-OR that shared *N*-carbamoyl amide hydrolysing activity. In order to explore the functions of the isolated plasmids, the ORFs encoding hydantoin-hydrolysing activity in the isolated plasmids were mapped and the nucleotide sequence determined. Nucleotide sequence data and predicted protein data was used to compare the NCAAH-encoding ORFs to similar genes and gene products, and for phylogenetic analysis of the relationships between related NCAAH enzymes.

6.2 Materials and Methods

6.2.1 Culture conditions

The original p6B3-1a and pG4 plasmids, as well as sub-clones of the two plasmids were maintained in *E. coli* DH5α. Starter cultures produced by inoculation of single *E. coli* colonies into 5ml LB plus the required antibiotic were grown to confluence overnight at 37°C, shaking at 200rpm. These were diluted to an OD_{600nm} of 0.02 into 100mL LB containing the required antibiotic and grown to stationary growth phase (OD_{600nm}>2.5). If required, IPTG was added to the growth medium as an inducer, at a final concentration of 1mM.

6.2.2 Sonicated Crude Extract Biocatalytic Reactions

The cells were resuspended in 0.1M potassium phosphate buffer pH 8 at a biocatalyst concentration of 20 mg/ml wet cell mass, and then sonicated on ice for 100 seconds at 60kHz. Sonication of the cells was done in bursts of 10 seconds, with 10 second recovery intervals between each burst. The sonicated cell suspension was then assayed for NCAAH activity as described in Chapter 5, section 5.2.8.

6.2.3 Analytical Methods

The biocatalytic reaction supernatant was analysed for reaction products, by colorimetric assays or HPLC analysis. Amino acid production was detected using the colourimetric ninhydrin assay as described in Appendix 4. D,L-5-*p*-hydroxyphenylglycine (HPG) production was measured using HPLC analysis of 20µl of reaction supernatant, as described in the previous chapter and in Appendix 4. One unit of specific NCAAH activity (U) was defined as µmol glycine produced per minute per mg protein. All enzyme activity values were reported as the average of *n* repeats.

6.2.4 Molecular Techniques

Standard molecular techniques were performed as published by Ausubel *et al* (1983) and Sambrook *et al*. (1989). Sub-clones for the sequencing of p6B3-1a and pG4 were produced by deletion of selected restriction fragments: a 1379 bp *Hind* III fragment, and

a 405 bp *Cla* I fragment in p6B3-1a; *Sac* I (299 bp) and S*ac* II (227 bp) fragments in pG4. 2.5 ng of digested plasmid was ligated using standard T4 DNA ligase (Promega). Transformants were screened using restriction mapping, and assayed for NCAAH activity using sonicated crude extract biocatalytic reactions.

6.2.5 DNA: DNA Hybridisation

Chromosomal DNA was digested to completion using a combination of restriction enzymes *Eco* RI, *Bam* HI, *Pst* I, *Eco* RV, *Hind* III, *Hinc* II and *Sal* I. Plasmid DNA for blotting was digested with combinations of *Sal* I, *Eco* RI, *Pst* I and *Bam* HI restriction enzymes. After separation by electrophoresis in 0.7% (chromosomal DNA) or 1% (plasmid DNA) agarose gel, DNA was bound to Hybond N+ nylon membrane by capillary transfer using 0.4M sodium hydroxide. Membranes were dried and stored at 4°C until required. Radioactively labelled DNA probes were prepared by PCR amplification from p6B3-1a or pG4 plasmid DNA using standard cycling conditions with an annealing temperature of 57°C (Appendix 10). Primers NCAAR1 and NCAAF1 (Appendix 6) were used to amplify the probe from pG4, and primers pUCF and pUCR (Appendix 6) were used to amplify the probe from p6B3-1a. 50 µCi of α-P^{32}-dCTP was added to the reaction, and the resulting product purified using a spin column method based on size exclusion matrix separation (High Pure™ PCR Product Purification kit, Roche). Concomitant non-radioactive PCR reactions were analysed by gel electrophoresis to verify amplification of the correct fragment. Southern hybridisation of the probe to the membrane-bound DNA was performed according to the method of Ausubel *et al* (1983), at 42°C in a Hybaid Omnigene Hybridisation Oven. Washes were as described by the manufacturers of Hybond N+ nylon membranes (Amersham), using high stringency washes: two 15 minute washes in 2x SSPE, 0.1% sodium dodecylsulphate (SDS) at room temperature, one 30 minute wash in 1x SSPE, 0.1% SDS at 37°C and two 15 minute washes at 42°C in 0.1 x SSPE, 0.1% SDS. Binding of radioactively labelled probes was detected by incubation of membranes under film (Hyperfilm MP or Kodak Biomax MS, Amersham) at –70°C with an intensifying screen for varying amounts of time. Standard photographic developing techniques and solutions available from Kodak were used to visualise the radioactive exposure.

6.2.6 Sequence analysis

Three deletions of the insert DNA of p6B3-1a in which *Hind* III (1374bp) and *Cla* I (416 bp) restriction fragments were constructed and used for positional mapping and initial nucleotide sequencing of p6B3-1a. Exonuclease III digestion of p6B3-1a insert DNA was then used to produce nested deletions of both strands of insert DNA. A series of nested deletions of p6B3-1a were created by progressive unidirectional digestion with exonuclease III, based on the method of Henikoff (1984) (Erase-A-Base Kit, Promega). For sequencing in the 5'-3' direction, p6B3-1a was digested with *Pst* I to provide a 3' overhang for protection from *Exo* III deletion, then digested with *Bam* HI to provide a 5' overhang susceptible to exonuclease III digestion (Putney *et al*., 1981). For 3' to 5' deletions, p6B3-1a was first protected by digestion with *Kpn* I to produce a 3' overhang, then digested with *Xho* I to provide a 5' overhang for exonuclease III deletion. During digestion at 37°C, samples were collected every 30 seconds for 10 minutes, then treated with SI Nuclease and Klenow fragment (Sambrook et *al.,* 1989) to produce blunt ends for ligation and transformation according to manufacturer's instructions. Ten colonies from each time interval were inoculated into LB plus 50µg/ml kanamycin and grown overnight for plasmid extraction. Plasmid isolations were performed as described in Chapter 5, Section 5.2.6. Restriction analysis of the plasmids placed them in the appropriate region of the insert, and selected deletions were sequenced. The *Csp* 451–*Cla* I (1243 bp) region of p6B3-1a was also inserted into pBK-CMV and deletion sub-clones of this construct were used to obtain complete sequence of both strands of DNA in this region. For complete sequencing of both strands of insert DNA of pG4, two subclones of the plasmids were made by deletion of the unique *Sac* I (299 bp), *Sac* II (227bp) and *Sal* I (600bp) restriction fragments of the plasmid (pG4dSI, pG4dSII and pG4dSal, Appendix 7).

Nucleotide sequence for p6B3-1a and pG4 was determined from double stranded plasmid DNA by the dideoxy-chain termination method (Sanger, 1987) using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequencing primers pUCF and pUCR (Appendix 6), based on standard M13 sequencing primers, were synthesised (Stratagene) and used for sequencing reactions. Reactions were performed according to manufacturers instructions using the

GeneAmp PCR System 9700 thermal cycler. Extension products were purified by passage through DNA Clean and Concentrator columns (Zymo), and the remaining liquid removed by vacuum centrifugation (Speedvac Concentrator, Savant). Pellets were resuspended in Template Suppression Reagent and the nucleotide extension products separated by electrophoresis using an ABI Prism 3100 instrument and POP6 polymer.

The extracted sequence data from all plasmids was aligned using restriction sites, BLAST analysis and visual matching of sequence to obtain the complete sequence in the correct order. Possible open reading frames, promoter regions and areas with identity to known sequences from numerous databases were identified using the BLAST facilities at the National Center for Biotechnology Information, Clustal W Multiple Alignment (Higgins *et al*., 1996), Vector NTI version 5 and the HGMP Bioinformatics Resource Centre. Phylogenetic analyses were performed using Phylogeny Inference Package version 3.5 (PHYLIP), supplied by Joe Felsenstein (Felsenstein, 1993). The program TREEVIEW was used for the production of graphic images of phylogenetic trees (Page, 1996).

6.3 Results

6.3.1 Deletion analysis of p6B3-1a

Two sub-clones of p6B3-1a were constructed in which the regions between the 3' end of the insert and the unique *Cla* I and *Hind* III sites in insert DNA were deleted (Figure 6.2).

The specific NCAAH activity of the deletions was then compared to that of the p6B3-1a, using pBK-CMV as a negative control, and pGH45 and pG4 as positive controls (Figure 6.3). NCAAH activity in p6B3dC remained similar to that of p6B3-1a, whilst p6B3dH produced no significant activity, substantiating the suggested location of the NCAAH ORF in p6B3-1a.

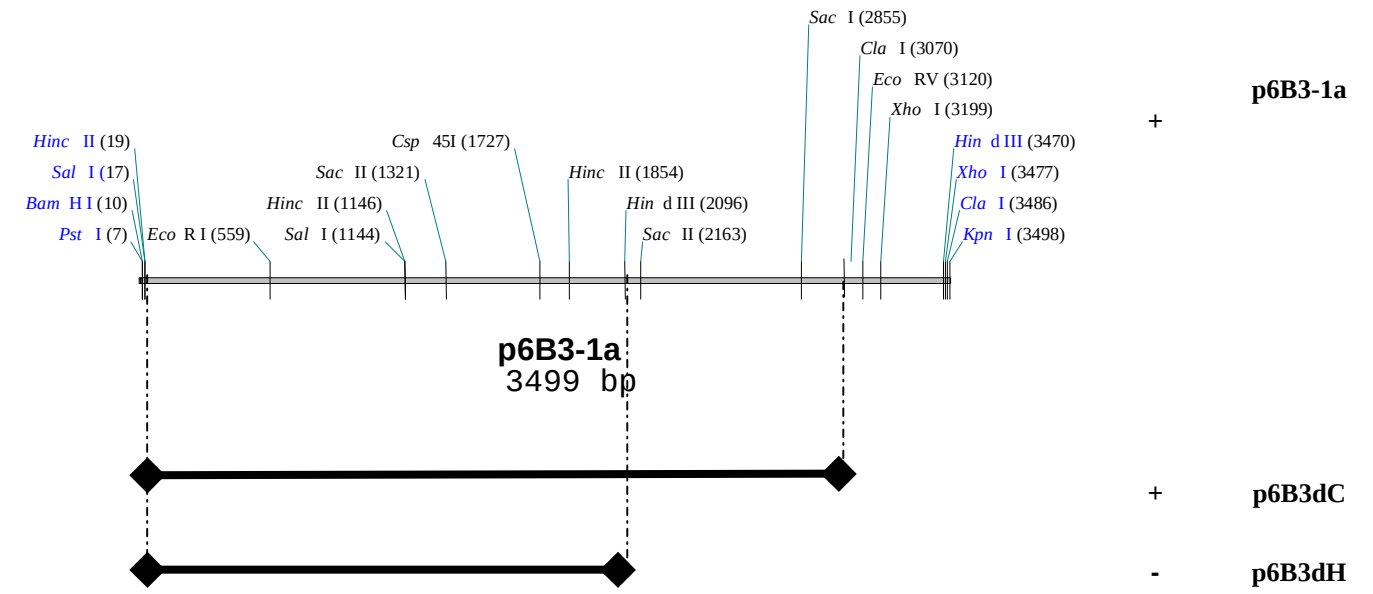


Figure 6.2: Restriction endonuclease map of the insert DNA from p6B3-1a showing deletion analysis of NCAAH activity encoded by p6B3-1a. Restriction sites from within the vector-cloning cassette are illustrated in blue, restriction sites within the insert DNA in black.

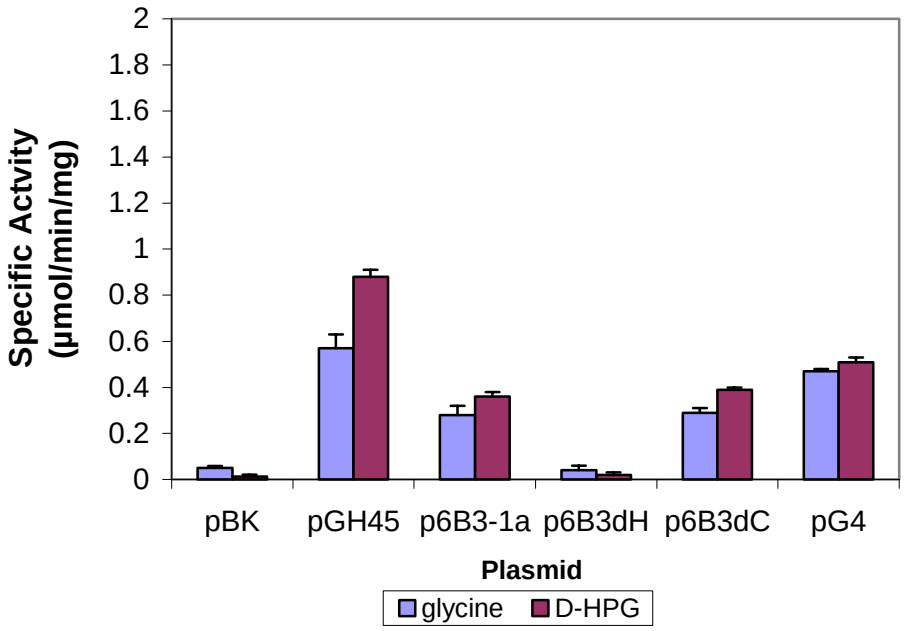


Figure 6.3: Specific NCAAH activity (µmol/min/mg protein) of cell extracts from *E. coli* transformed with recombinant plasmids. Cells were assayed after growth to stationary phase, without induction with IPTG. Error bars represent SEM (n=9).

Positional presence or absence of activity as depicted in Figure 6.2 was then used to map the location of the ORF encoding NCAAH activity in p6B3-1a to between the *Cla* I (3070) site (as this deletion did not affect activity) and the *Sal* I (1144) site, with the *Hind* III (2096) site within the coding sequence (as deletion of this region resulted in loss of activity (Figure 6.4). Preliminary sequence data from pG4 showed that the sequence was almost identical to the NCAAH enzyme from *A. radiobacter* NRRL B11291, and that the gene was orientated as shown in Figure 6.4.

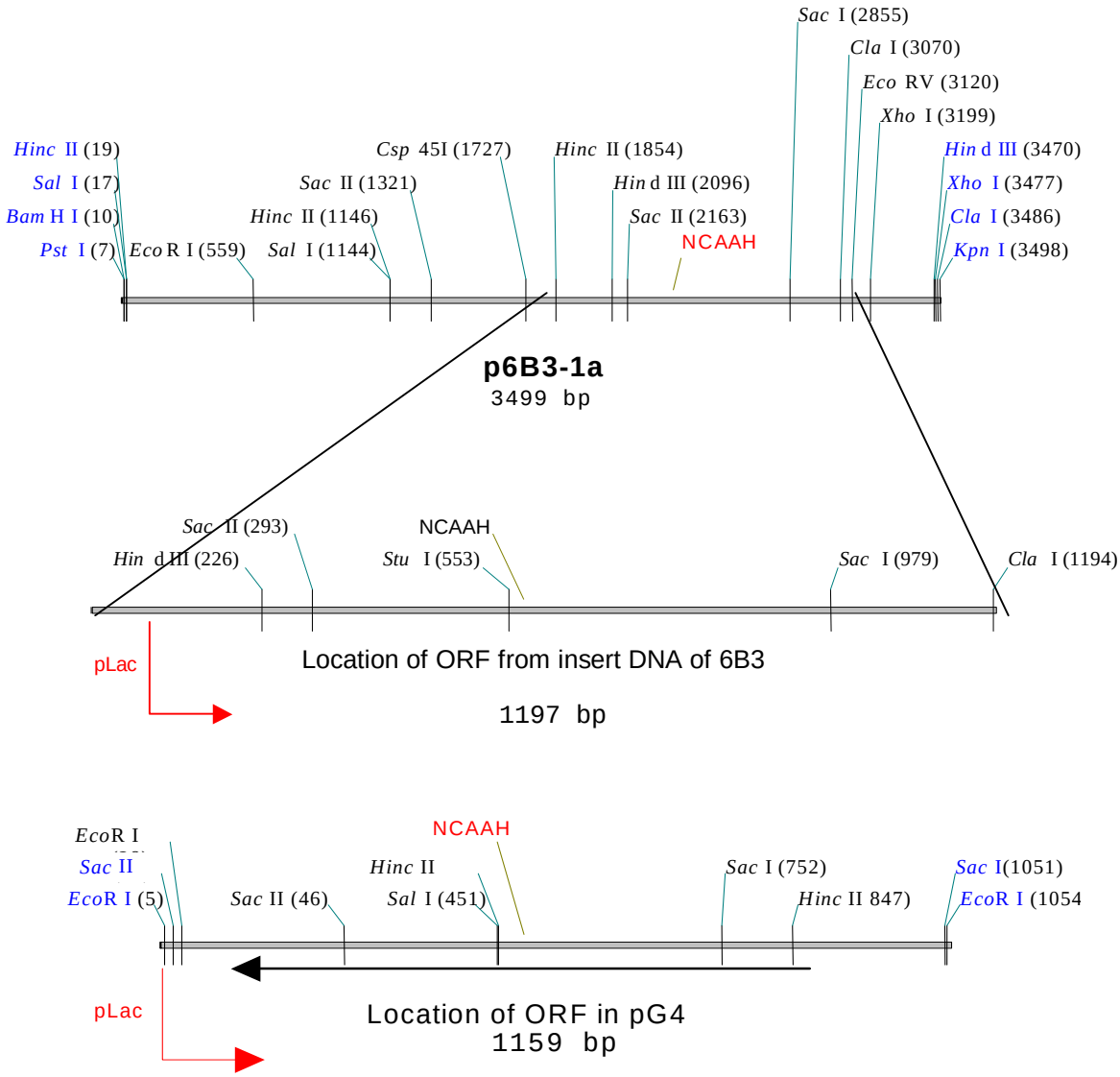


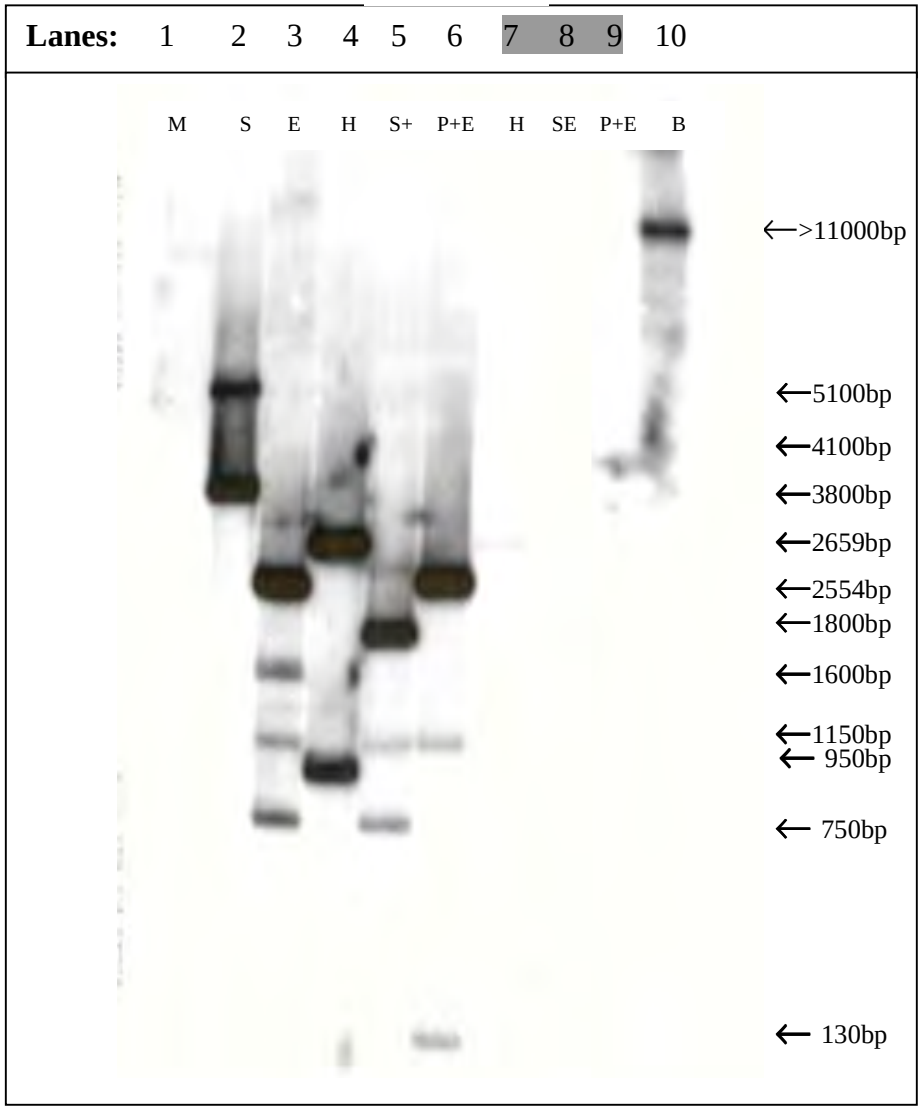
Figure 6.4: The putative location of the NCAAH-encoding regions in p6B3-1a and pG4. The arrow in red illustrates the direction of transcription based on the orientation of the pLac promoter in the vector. Restriction sites from within the vector-cloning cassette are illustrated in blue, restriction sites within the insert DNA in black. The black arrow beneath pG4 illustrates the expected orientation of the amplified NCDAAH ORF based on restriction endonuclease mapping

The insert DNA of the two plasmids pG4 and p6B3-1a conferring NCAAH activity on recombinant *E. coli* cells appeared to be dissimilar based on restriction endonuclease mapping of the insert DNA. This suggested some nucleotide differences between the putative NCDAAH-encoding ORFs in the two plasmids and mapping of their chromosomal location was used to test this and to verify the origin of the insert DNA from *A. tumefaciens* RU-OR.

6.3.2 Chromosomal mapping of DNA encoding NCDAAH activity from *A. tumefaciens* RU-OR

DNA: DNA hybridisation was used to map the chromosomal location of the two plasmids carrying NCAAH-encoding genes from RU-OR (Figures 6.6 - 6.8). The insert from p6B3-1a produced hybridization to several chromosomal fragments with *Sal* I, *Eco* RI, *Hinc* II and *Pst* I restriction sites external to the insert DNA region, mapping to two *Sal* I sites approximately 5100 and 3800 base pairs away from the internal *Sal* I site of p6B3-1a, several *Eco* R I sites within 2 kilobases distance from the insert DNA, and a single external *Hinc* II site approximately 2500 base pairs from one of the internal *Hinc* II sites (Figures 6.6, panel A, lanes 2, 3,4, 5 and Figure 6.7). The radioactively-labeled PCR probe amplified from pG4 hybridized to the chromosomal DNA from both *A. tumefaciens* RU-OR and to a *Hinc* II fragment from *A. tumefaciens* AE-O1 (Figure 6.6 panel B), and the deduced restriction maps of the chromosomal location of pG4 are shown in Figure 6.8. Although there are similar *Hinc* II sites located upstream of the ORF in pG4 (Figure 6.6 panel B, Lanes 4 and 7) on both chromosomes, there are also differences between the restriction maps of the chromosomal location of the insert DNA from pG4 in RU-OR and AE-O1. The hybridization of insert DNA from pG4 to a 4 kb *Sal* I fragment located downstream of the pG4 region on the chromosome of RU-OR was not detected in the hybridization of insert DNA from pG4 to the chromosomal DNA of AE-O1 (Figure 6.6 panel B, Lane 5 vs Lane 8). The pG4-based probe showed no hybridization to other digested chromosomal DNA in *A. tumefaciens* AE-01. However, this may be have been attributable to a lower concentration of *A. tumefaciens* AE-01 chromosomal DNA on the membrane blot, rather than representing no hybridization of the DNA.

A-Nca R1



B-*nca R2*

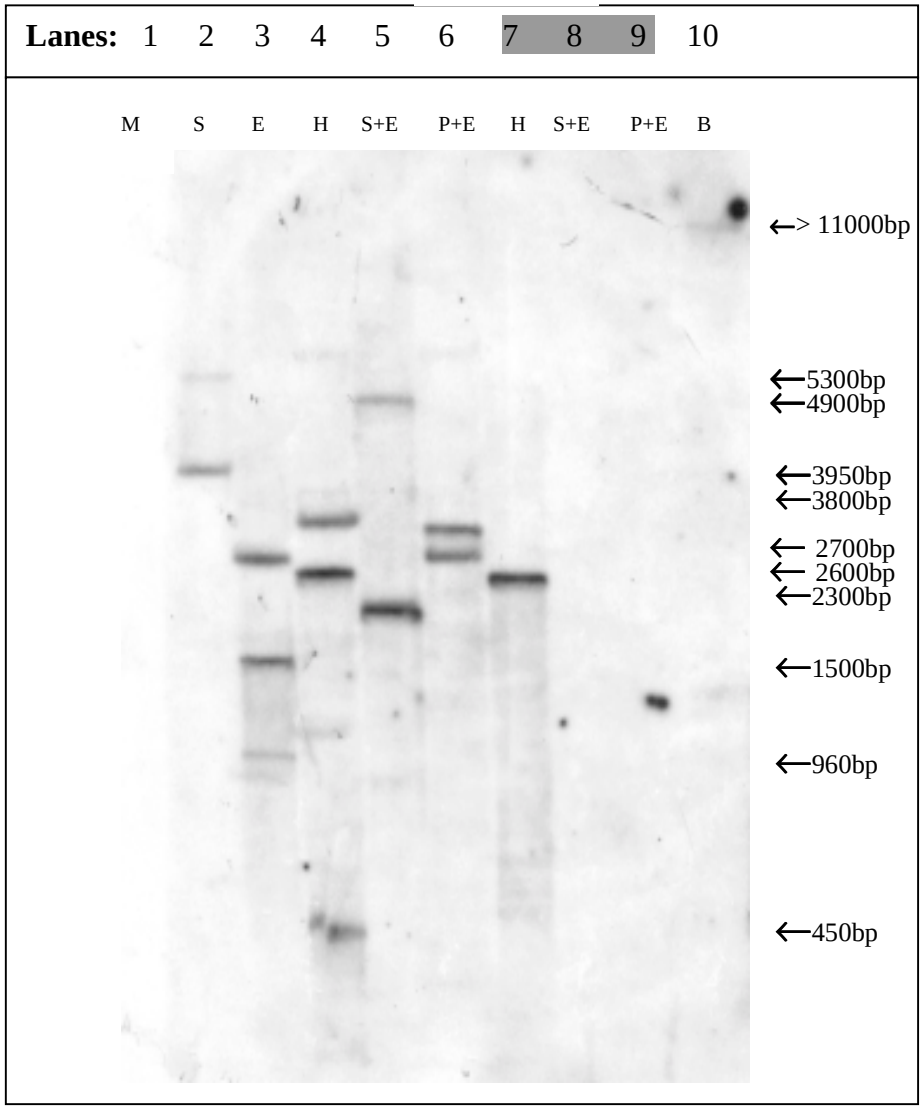
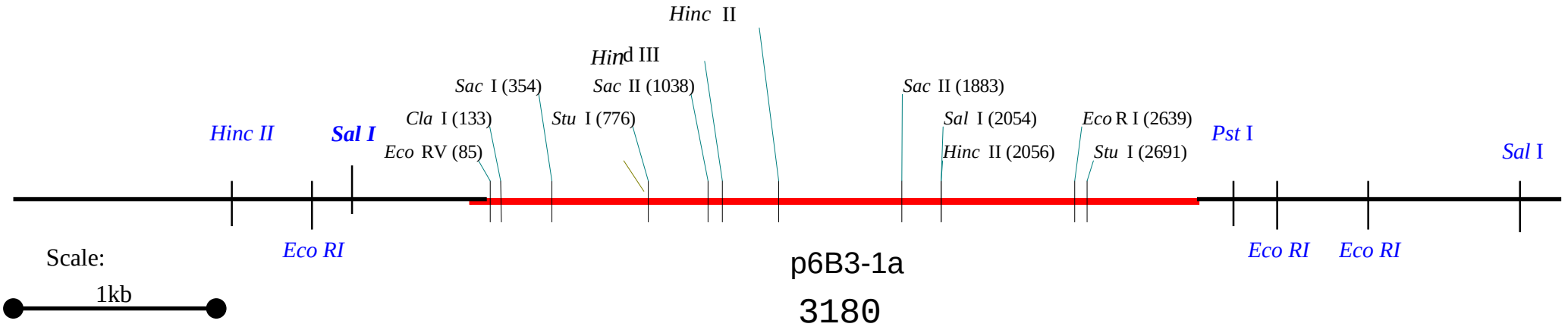
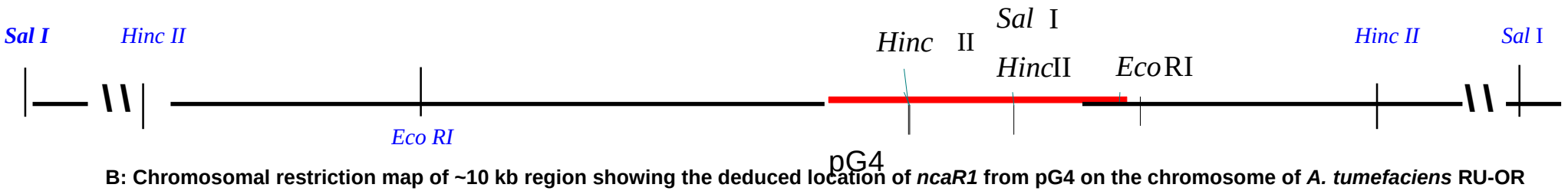


Figure 6.5: Southern blot analysis. DNA: DNA hybridization of insert DNA from p6B3-1a (**A**) and from pG4 (**B**) with chromosomal DNA from *A. tumefaciens* RU-OR. Numbers on the right represent approximate sizes of hybridization. **Lanes:** 1-lambda DNA digested with *Pst* I, 2-6, RU-OR chromosomal DNA digested with 2, *Sal* I (S); 3, *Eco* RI (E); 4, *Hinc* II (H); 5, *Sal* I + *Eco* RI (S+E); 6, *Pst* I + *Eco* RI (P+E); lanes 7-9, AE01 chromosomal DNA digested with 7, *Hinc* II (H); 8, *Sal* I + *Eco* RI (S+E); 9, *Pst* I+ *Eco* RI (P+E); 10, RU-OR chromosomal DNA digested with *Bam* HI (B).



A: Chromosomal restriction map of ~8 kb region showing the deduced location of *ncaR2* from p6B3-1a on the chromosome of *A. tumefaciens* RU-OR



B: Chromosomal restriction map of ~10 kb region showing the deduced location of *ncaR1* from pG4 on the chromosome of *A. tumefaciens* RU-OR

Figure 6.6: Deduced chromosomal location of the insert DNA from p6B3-1a (A) and pG4 (B) based upon restriction mapping of p6B3-1a and the Southern analysis shown in Figure 6.13. The central red bar represents the insert DNA of the plasmids. Restriction sites that have been mapped to p6B3-1a are depicted in black, those based on DNA: DNA hybridization in blue.

The hybridization of insert DNA from both p6B3-1a and pG4 to a large *Bam* HI restriction endonuclease fragment from the chromosomal DNA of RU-OR, and possibly another smaller *Bam* HI restriction endonuclease fragment for the probe from pG4, is important to note (Figure 6.5 A and B, lane 10). As illustrated in Figure 6.7, a *Bam* HI site occurs in the hydantoinase-encoding gene upstream of the NCDAAH gene of *A. radiobacter* NRRL B11291.

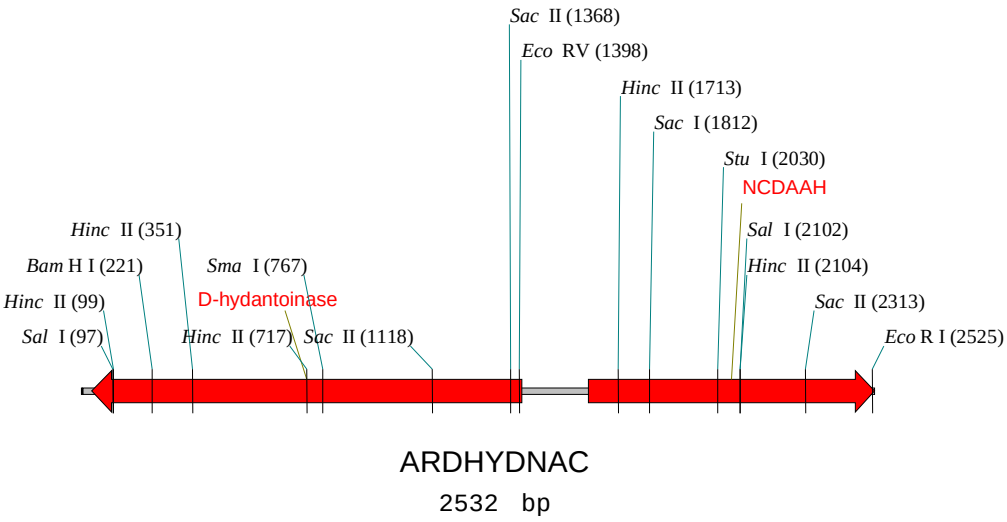


Figure 6.7: The arrangement of the hydantoinase-encoding and NCDAAH-encoding genes from *A. radiobacter* NRRL B11291 (from Grifantini *et al*., 1998). The nucleotide sequence data for the construction of the diagram was obtained from nucleotide data contained in Genbank (Accession No. X91070).

Insert DNA from pG4 also hybridized to a small *Hinc* II fragment of ~450 bp, which corresponds to the *Hinc* II fragment from nucleotide positions 1713 to 2104 in the NCAAH-encoding gene from *A. radiobacter* NRRL B11291 (Figure 6.7). Hybridization of the probe from pG4 to a *Hinc* II chromosomal DNA restriction fragment of approximately 1 kb also corresponds to the *Hinc* II fragment from nucleotide positions 717 to 1713 overlapping both the D-hydantoinase-encoding and NCAAH-encoding genes from *A. radiobacter* NRRL B11291 (Figure 6.5, Panel B, Lane 4 compared to Figure 6.7).

Chromosomal mapping of the location of the insert DNA from pG4 and p6B3-1a therefore illustrated that the two plasmids originate from the chromosomal DNA of strain RU-OR, but occur in different locations on the chromosomal DNA.

6.3.3 Molecular analysis of NCDAAH-encoding genes from *A. tumefaciens* RU-OR

The complete nucleotide sequence of pG4 was obtained by sequencing the original plasmid, and three plasmid constructs in which the *Sac* I (299 bp), *Sac* II (227bp) and *Sal* I (600bp) restriction endonuclease fragments from pG4 had been deleted (pG4dSI, pG4dSII and pG4dSalI, Appendix 7), as illustrated in Figure 6.9.

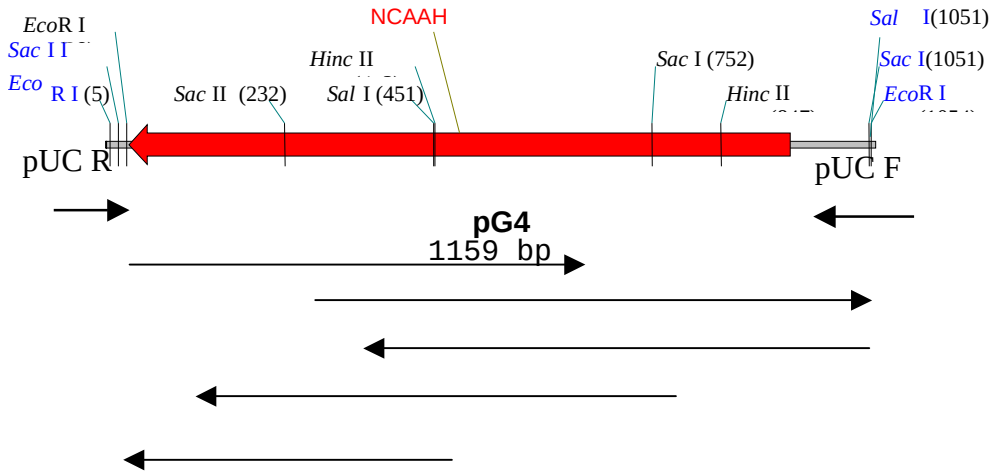


Figure 6.8: Strategy for sequencing of both strands of the insert DNA in pG4. Arrows represent sequenced regions, and direction of sequencing; the primers used for either direction are illustrated above the sequencing arrows. Restriction sites from within the vector-cloning cassette are illustrated in blue, restriction sites within the insert DNA in black.

Bioinformatic analysis of the full sequence of the insert DNA in pG4 revealed an ORF of 912 basepairs, encoding 304 amino acids that was designated *ncaR1* (Figure 6.10). Analysis of the 5' region flanking the ORF in pG4 revealed a putative ribosome-binding site at nucleotides 91-96, a possible Pribnow Box or –10 consensus sequence from

nucleotides 80-86, and sequence similar to the prokaryotic –35 consensus sequence from nucleotides 46-55.

```
   1 gagaacttta attggcgtgc caagctgaga cgtgtgttcc tgaaatgtgc atagcagcgt
  61  tctcccggcc gcgaggccgg attaactatc gaaggagcaa aggttcatga cacgtcagat
                                                    M   T   R   Q   M
 121 gatacttgct gtcggacagc aaggccccat cgcgcgagcg gagacacgcg aacaggtggt
       I   L   A   V   G   Q   Q   G   P   I   A   R   A   E   T   R   E   Q   V   V
 181 tggccgcctc ctcgacatgt tgacgaacgc agccagccgg ggcgtgaact tcatcgtctt
       G   R   L   L   D   M   L   T   N   A   A   S   R   G   V   N   F   I   V   F
 241 tcccgagctt gcgctcacga ccttcttccc gcgctggcat ttcaccgacg aggccgagct
       P   E   L   A   L   T   T   F   F   P   R   W   H   F   T   D   E   A   E   L
 301 cgatagcttc tatgagaccg aaatgcccgg cccggtggtc cgtccactct ttgagacggc
       D   S   F   Y   E   T   E   M   P   G   P   V   V   R   P   L   F   E   T   A
 361 cgccgaactc gggatcgggt tcaatctggg ctacgccgaa ctcgtcgtca gcgggggtgt
       A   E   L   G   I   G   F   N   L   G   Y   A   E   L   V   V   S   G   G   V
 421 caagcgcagg ttcaacacgt ccattttggt ggataagtca ggcaagatcg tcggcaagtg
       K   R   R   F   N   T   S   I   L   V   D   K   S   G   K   I   V   G   K   C
 481 tagaaagatc catttgccgg gtcacaagga gtacgaagcc taccggccgt tccagctttt
       R   K   I   H   L   P   G   H   K   E   Y   E   A   Y   R   P   F   Q   L   L
 541 aaaaaggtat ttcgagccgg gcgatttggg ctttcccgtt tataacgtcg acgccgcgaa
       K   R   Y   F   E   P   G   D   L   G   F   P   V   Y   N   V   D   A   A   K
 601 aatgggaatg ttcatttgca acgatcgccg ctggcctgaa acgtggcggg tgatgggact
       M   G   M   F   I   C   N   D   R   R   W   P   E   T   W   R   V   M   G   L
 661 taagggcgcc gagatcatct gcggcggtta caacacgccg acccacaatc cccccgttcc
       K   G   A   E   I   I   C   G   G   Y   N   T   P   T   H   N   P   P   V   P
 721 ccagcacgac catctgacgt ccttccacca cctcctgtcg atgcaggccg ggtcgtacca
       Q   H   D   H   L   T   S   F   H   H   L   L   S   M   Q   A   G   S   Y   Q
 781 aaacggcgcc tggtccgcgg cggccggcaa ggtcggcatg gaggaggggt gcatgttgct
       N   G   A   W   S   A   A   A   G   K   V   G   M   E   E   G   C   M   L   L
 841 cggccattcg tgcatcgtgg cgccgaccgg cgaaatcgtt gccctgacca cgacgttgga
       G   H   S   C   I   V   A   P   T   G   E   I   V   A   L   T   T   T   L   E
 901 agacgaggtg atcaccgccg ccgtcgatct cgaccgctgc cgggaactgc gcgaacacat
       D   E   V   I   T   A   A   V   D   L   D   R   C   R   E   L   R   E   H   I
 961 cttcaatttc aaagcccatc gtcagccaca gcactacggt ctgatcaagg aattctaagg
       F   N   F   K   A   H   R   Q   P   Q   H   Y   G   L   I   K   E   F   *
1021 tcaggcc
```

Figure 6.9: Complete nucleotide sequence of the insert DNA of pG4 and deduced amino acid sequence of NCAR1 from *A. tumefaciens* RU-OR. The numbers at the left are nucleotide positions. The ORF encoding NCDAAH activity is underlined, and the initiation codon and terminator codon are doubly underlined in black. A putative ribosome binding site (RBS) or Shine-Dalgarno sequence is doubly underlined in blue, and proposed –10 and –35 signals similar to the consensus sequences for *E. coli* promotors are doubly underlined in violet and green respectively.

Nested deletions of the insert DNA from plasmid p6B3-1a were created using the Erase-A-Base Kit, and used to compile sequence data for both strands of DNA (Figure 6.11). A pronounced increase in the rate of exonuclease III digestion in the central region of p6B3-1a prevented isolation of nested deletions to completely cover the sequence of this both strand of DNA in this region. Therefore, the *Csp* 451 (1727) to *Cla* I (3070) fragment of p6B3-1a was isolated and inserted into the *Cla* I site of pBK-CMV vector to produce pBK6B3.

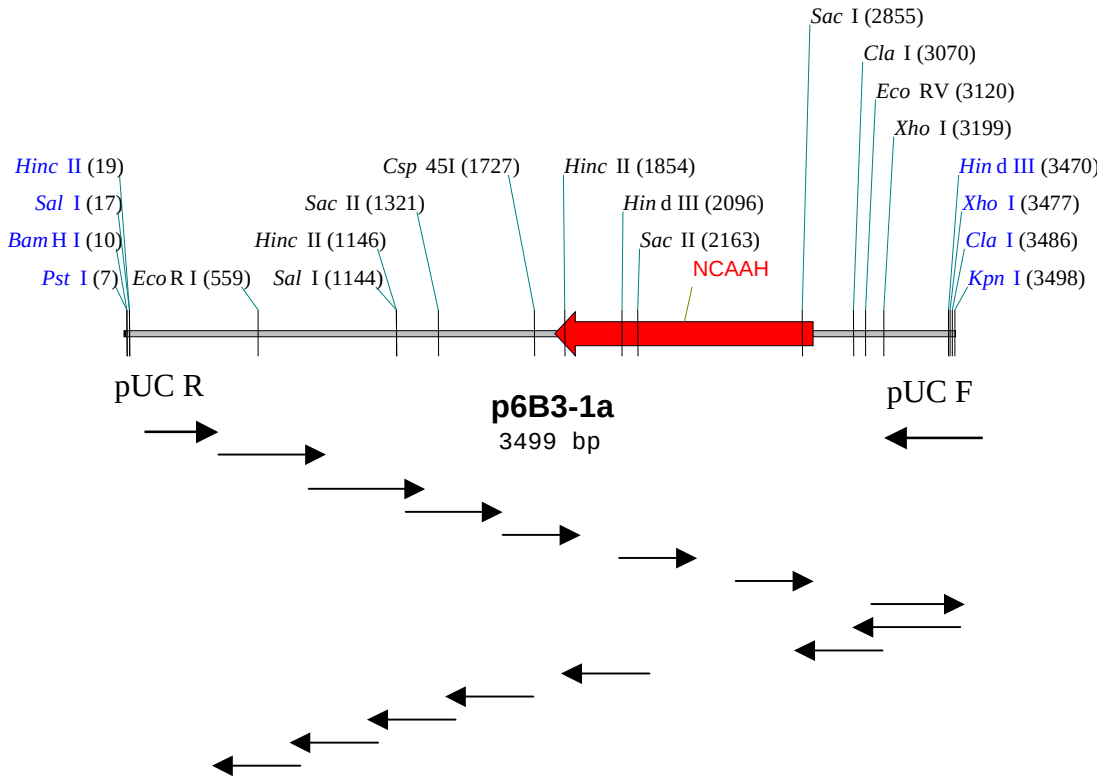


Figure 6.10: Strategy for sequencing of both strands of insert DNA from p6B3-1a. Nested deletions were used to partially sequence the full insert DNA of 6B3-1a. Arrows represent sequenced regions, and direction of sequencing; the primers used for either direction are illustrated above the sequencing arrows. Restriction sites from within the vector-cloning cassette are illustrated in blue, restriction sites within the insert DNA in black.

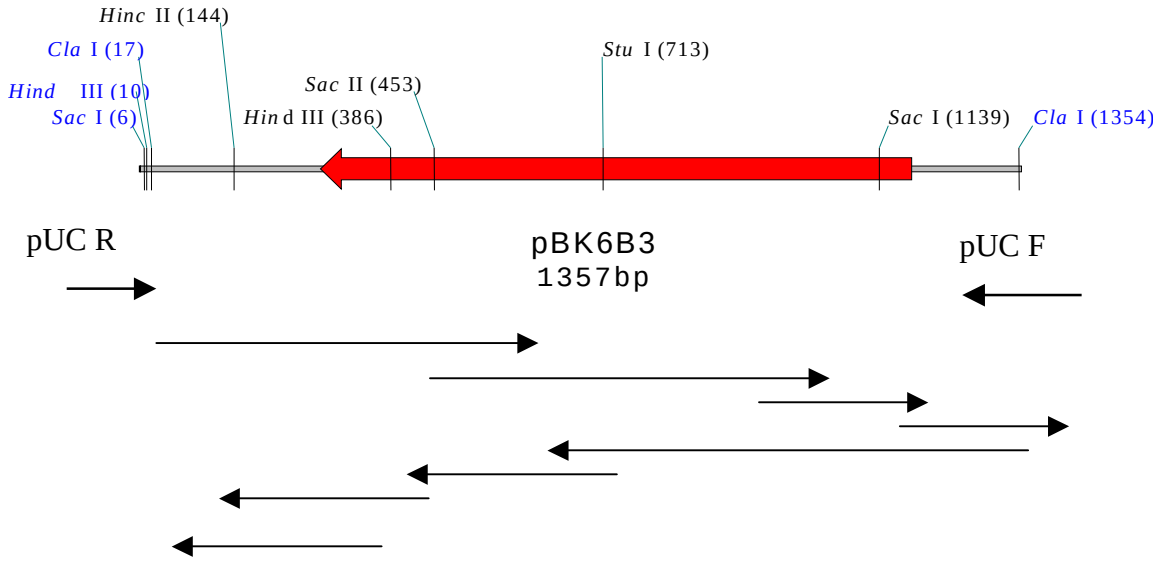


Figure 6.11: Insert DNA of pBK6B3 sub-clone, and the sequencing of both strands of DNA using nested and other restriction fragment deletions. Arrows represent sequenced regions, and direction of sequencing; the primers used for either direction are illustrated above the sequencing arrows.

Constructs derived from this plasmid in which the 376 bp *Hind* III, 453 bp Sac II bp fragment and 1133 bp *Sac* I fragments had been deleted were used together with the

original plasmid in order to obtain DNA sequence for both strands of the insert (Figure 6.12). Bioinformatic analysis of the complete sequence of both strands of insert DNA in pBK6B3 revealed an ORF of 912 base pairs in length, predicted to encode a protein 304 amino acids in length with a predicted molecular mass of approximately 34 kDa (Figure 6.13).

```
   1   atcgatgt cttcatcggg acgaacttgg aggagacgga cactaccggg cataagaacg
  61 cggacaacct gcgtcttctc gtcatccggg ggccttcact catgaagcta gtccacgccc
 121 ttttatgaggg gccgcaatga agccttaact atcgaaggag caaaggttca tgacacgtca
                                                       M   T  R Q
 181 gatgatactt gctgtcgccc agcaaggccc catcagccga gctctggcca ctgccaacag
       M  I  L   A  V  A   Q  Q  G  P   I  S  R   A  R  A   T  A  N  R
 241 gctggtgtcc cgcttgcgcg ctttggcaaa aatggccgaa cagagagggg ttagaacttc
       L  V  S   R  L  R  A   R  A  K   M  A  E   Q  R  G  V   R  T  S
 301 atcggtcttc ccggaattgg ccttgaccac cttcttcccg aggtggattt caccggacga
       S  S  F   P  E  L  A   L  T  T   F  F  P   R  W  I  S   P  D  E
 361 agccgaactg gacagcttct atccgaaatg cccggcccgg tggtccgtcc cgttgttcag
       A  E  L   D  S  F  Y   P  K  C   P  A  R   W  S  V  P   L  F  R
 421 acggccgccg aactcgggaa tcgggcttca atctgggcta gccgaattgc gtgaaggcgg
       R  P  P   N  S  G  S   G  L  Q   S  G  L   A  E  T  R   E  G  G
 481 ccgtcgaagg cggttcaaca cctcgcttca acacgtcaag tctggtggag tcaggcaaaa
       R  R  R   R  F  N  T   S  L  Q   H  V  K   S  G  G  V   R  Q  K
 541 gcgtcggaag gtatcggatc caggccataa ggaaccggaa gcctatcgcc cgcatcagca
       R  R  K   V  S  D  P   G  H  K   E  P  E   A  Y  R  P   H  Q  H
 601 tctggaaaag cgctatttct ttccggggga cctgggattc cgaggcctac cggccgttcc
       L  E  K   R  Y  F  F   P  G  D   L  G  F   R  G  L  P   A  V  P
 661 agcatctatg gcgatgttta tctgcaacga cagacgctgg ccggaactac ggagagtcat
       A  S  M   A  M  F  I   C  N  D   R  R  W   P  E  L  R   R  V  M
 721 gggcttgggg gatgttgaac tgcaacgatc gggctataac accccggcgg gtgatccgcc
       G  L  G   D  V  H  L   Q  R  S   G  Y  N   T  P  A  G   D  P  P
 781 ggtcgggcgc cgagatcatc tgcggctaca acacgccgac ttgagcatgc aggccggccc
       V  G  R   R  D  H  L   R  L  Q   H  A  D   L  S  M  Q   A  G  P
 841 atatcagaac agccgctgga cgtctgacgc cgcgaaaatg gggatggaag aaggctgcga
       Y  Q  N   S  R  W  T   S  D  A   A  K  M   G  M  E  E   G  C  D
 901 tcgccgcggc cctgaatgca tcgtcgcccc gcgaggtgtt aagggcgccc gatcagactc
       R  R  G   P  E  C  I   V  A  P   R  G  V   K  G  A  R   S  D  S
 961 gttggaaaag cttcggattc cttccagttc ggacctggac tgcaagaaat actggcggtc
       L  C  K   L  R  I  P   S  S  S   D  L  D   C  K  K  Y   W  R  S
1021 cacacggttc aacttcaagg cccatcgcga accggaccat tatcgcctga tcgccgaatt
       T  R  F   N  F  K  A   H  R  E   P  D  H   Y  R  L  I   A  E  L
1081 gtagtgctct gaactggtcg agcgtatcgg cctcatcatc
       *
1141 tacgacgagg gccaccagtt tgatggcctg acgcgtggcc caacctacga actacttctg
1201 acgtcgctaa agttaacctt gccagaagcg acgcaggtaa ttctgatgtc ggcggtgatt
1261 ggcaatgcga gcgccgtggc agaatggctg gtaggtgatc ccgacgccgt tatagatgga
1321 gctggcttgc ttccaacttc gaag
```

Figure 6.12: Nucleotide sequence of the 1340 bp *Csp* 451/ *Cla* I region of p6B3-1a and deduced amino acid sequence of Nca R2 from *A. tumefaciens* RU-OR. The numbers at the left are nucleotide positions. The ORF for *ncaR2* is underlined, and the initiation codon and terminator codon are doubly underlined in black. A putative ribosome binding site (RBS) or Shine-Dalgarno sequence is doubly underlined in blue, and proposed –10 and –35 signals similar to the consensus sequences for *E. coli* promotors are doubly underlined in violet and green respectively.

The NCAAH ORF encoded by p6B3-1a was designated *ncaR2* and the NCAAH ORF encoded by pG4 as *ncaR1*, from *A. tumefaciens* RU-OR. Analysis of the 5' region

flanking *ncaR2* revealed sequences similar to the -10 and –35 signals found in *E. coli* for RNA polymerase promotor binding sites, between nucleotides 119-125 and 91-99 respectively. A putative ribosome-binding site (Shine-Dalgarno) was found between nucleotides 154-159 (Figure 6.13). NCDAAH enzymes from several sources were used for comparative analysis of the molecular characteristics of the NCAAH enzymes from RU-OR, as listed in Table 6.1.

Table 6.1: NCDAAH sources used for comparative analysis

Notation	Source Strain	Protein	Functional Features	Reference
Arad-G (X91070) **Arad-1** (U59376)	*A. radiobacter* NRRL B11291	304 aa, dimer 304 aa, dimer	Substrate affinity for NCHPG, thermostability <40C, pH stability <7	Grifantini *et al.* (1998) Buson *et al*. (1996)
NcaR1 **NcaR2**	*A. tumefaciens* RU-OR	304 aa 304 aa	- -	This study
AE01	*A. tumefaciens* AE-01	304 aa, dimer	-	Herrera (1995), This study
Agro SB	*Agrobacterium* sp. 80/44-2A	304aa, dimer	Substrate affinity for NCHPG, thermostability <64C, pH stability < 9	Neal *et al*.(1999)
Agro LK	*Agrobacterium* sp.	20 N-terminal aa	-	Louwrier and Knowles (1996)
IAgro (I27672)	*Agrobacterium* sp. KNK712	304 aa	Substrate affinity for NCHPG, thermostability <55C, pH stability <7	Nanba *et al*. (1998)
IPseud (AR102278)	*Pseudomonas* sp. KNK0003A	312 aa	Affinity for substrates with non-aromatic sidechains, thermostablity <73C, pH stability <7	Ikenaka *et al*. (1998a)
E222C	*Comamonas* sp. E222C	30N-terminal aa, trimer	Substrate affinity for NCHPG, thermostability <40C, pH stability up to pH9	Ogawa *et al.* (1993)
A17p-4	*Blastobacter* sp. A17p-4	30N-terminal aa, trimer	NCHPG, thermostability <50C, pH stability up to 9	Ogawa *et al*. (1994b)

Alignment of the *ncaR1* and *ncaR2* nucleotide sequences with sequences of other NCDAAH enzymes showed that *ncaR2* shared some level of DNA identity with certain blocks of sequence from other NCDAAH genes, particularly in the 5' region of the open reading frame (Appendix 9). The NCDAAH genes isolated from *A. radiobacter* NRRL B11291, *A.* sp KNK712 and *ncaR1* from *A. tumefaciens* RU-OR shared DNA identities of greater than 90% (Table 6.2). The coding sequence for the NCDAAH enzyme from *Agrobacterium* sp. 80/44-2A was identical to the *Arad*G gene from *A. radiobacter* NRRL B11291, and so was not included as a separate comparison in further analyses.

Table 6.2: Matrix of DNA identity (%) between NCDAAH coding sequences.

	Arad-G	**Arad1**	**NcaR1**	**NcaR2**	**IAgro**
Arad-G					
Arad1	99.6				
NCA R1	97.3	97.0			
NCA R2	65.7	65.2	62.4		
IAgro	93.0	92.7	91.5	64.6	
IPseud	65.0	63.0	64.2	51.2	62.0

The NCDAAH gene from *Pseudomonas* sp. KNK003A (IPseud) and the second type of NCDAAH from RU-OR, *ncaR2*, share approximately 60% DNA identity with the type 1 NCDAAH genes, but only 50% identity with each other, therefore the differences between *ncaR2* and the type 1 NCDAAH genes are not shared with the NCDAAH gene from *Pseudomonas* sp. KNK003A.

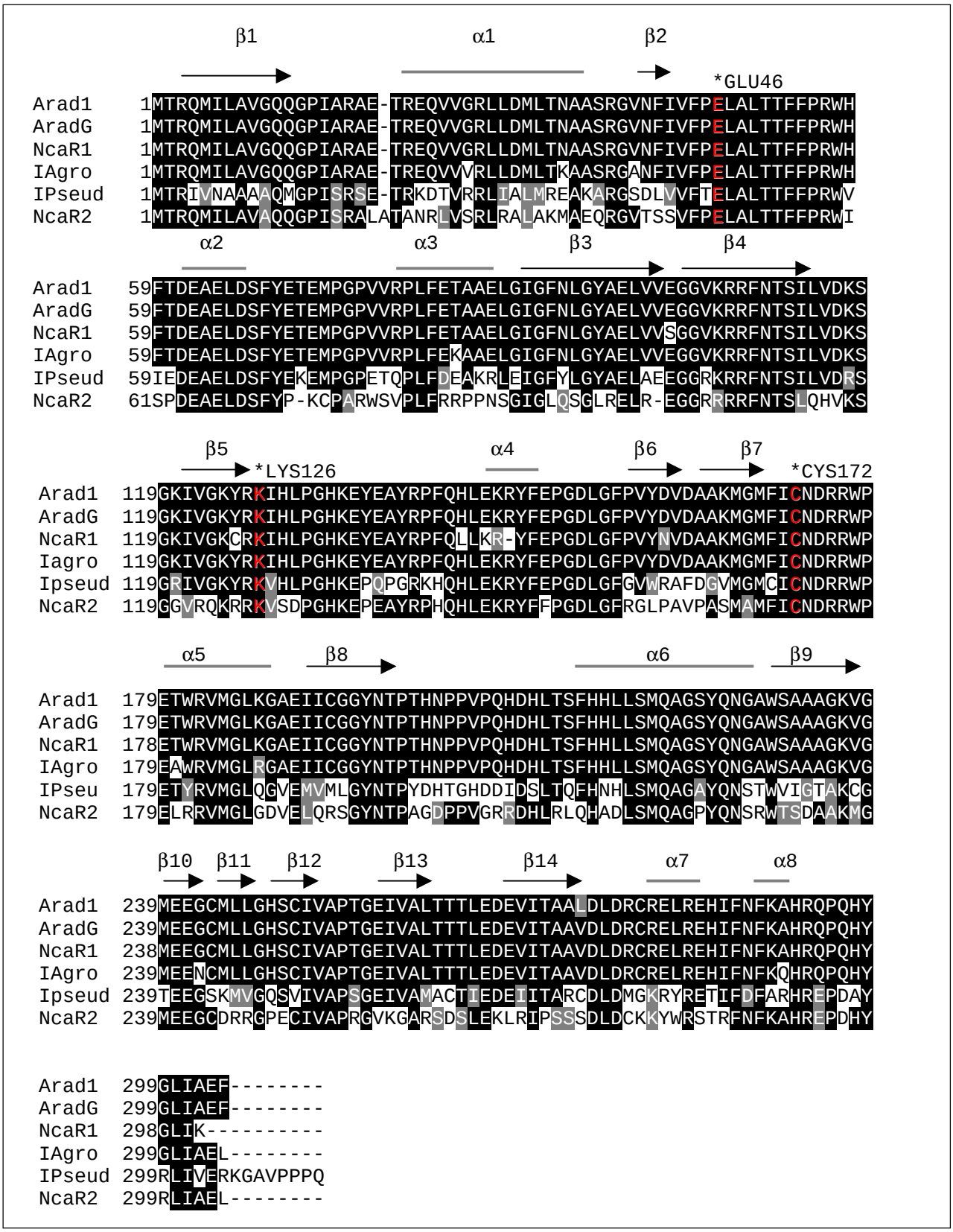


Figure 6.13: Multiple alignment of the available primary amino acid sequence of NCDAAH enzymes (BOXSHADE). **Key:** Numbers on the right are residue positions. The nucleotide sequence of Arad1 was used as a consensus sequence. Dark shading represents residues identical to the consensus. Similar residues are shaded in grey. Residues known to be involved in the active site are coloured red and asterisked. Arrows represent putative α helices and β sheets in the protein structure, based on a known NCDAAH structure (Nakai *et al*., 2000). Amino acid sequence sources are as described in Table 6.1.

The primary amino acid sequence data revealed similar levels of sequence identity between the different NCDAAH enzymes. Alignment of the NcaR2 coding sequence with primary amino acid sequences of other NCDAAH enzymes showed that NcaR2 shared 59.2% amino acid identity with the NCDAAH enzyme from *A. radiobacter* NRRL B11291, 54.6% with the NCDAAH from *Agrobacterium* sp. KNK712 and 48.6% with the NCDAAH from *Pseudomonas* sp. KNK0003A (Figure 6.13, Table 6.3). Based on the identity between the different enzymes, the NCDAAH enzymes from *A. radiobacter* NRRL B11291, *Agrobacterium* sp. KNK712 and NcaR1 from *A. tumefaciens* RU-OR could be classed as "type 1" enzymes, sharing a level of DNA sequence and amino acid identity greater than 90%. Due to the high level of amino acid identity between *ncaR1* and the NCDAAH-encoding genes from other *Agrobacterium* species, it can be concluded that *ncaR1* is most likely analogous to these genes.

Table 6.3: Matrix of primary amino acid sequence identity (%) between NCDAAH proteins.

	Arad-G	**Arad1**	**NcaR1**	**NcaR2**	**IAgro**
Arad-G					
Arad1	99.7				
NcaR1	96.3	96.7			
NcaR2	59.2	59.4	57.4		
IAgro	97.2	97.8	91.5	54.6	
IPseud	61.0	60.8	59.7	48.6	60.0

Significant differences occur between the amino acid sequences of type I NCDAAH enzymes, such as NcaR1, and the amino acid sequence of the second NCDAAH enzyme from RU-OR (NcaR2) and the NCDAAH enzyme from *Pseudomonas* sp. KNK0003A (IPseud) (Figure 6.14). Although small amino acid differences occur throughout the primary amino acid sequence for all three enzymes, there are four

sizeable blocks of conserved amino acid sequence between the enzymes, two at the N-terminal end of the protein (residues 49-59 and residues 63-71), and two closer to the C-terminus (residues 195-199 and residues 218-223). Some regions of the NcaR2 primary amino acid sequence show identity to the NCDAAH enzyme from *Pseudomonas* KNK0003A (IPseud) but not to NcaR1 and the type 1 NCDAAH enzymes e.g. residues 102-105, 142-147, 280, 299-301.

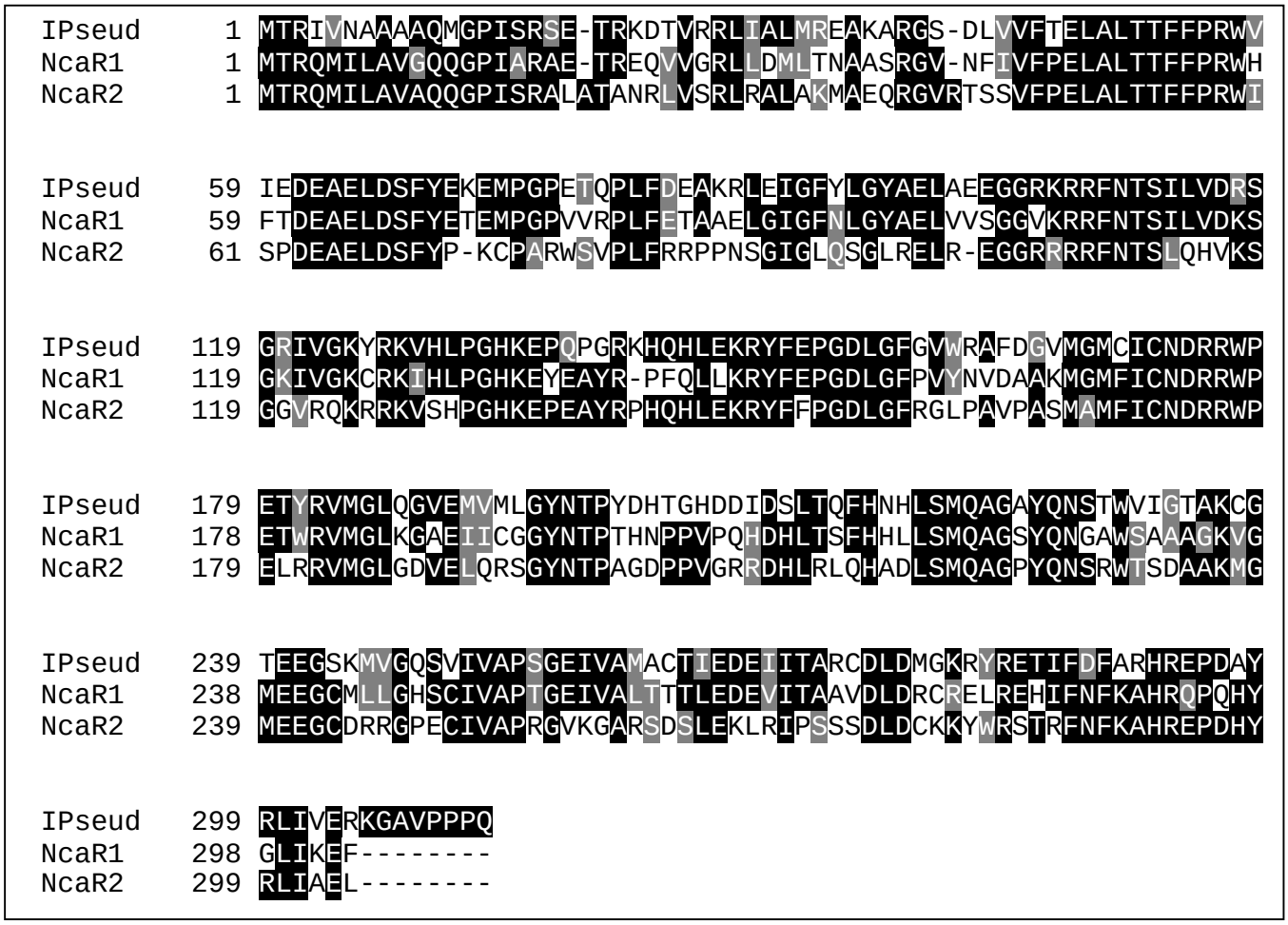


Figure 6.14: Multiple alignment of the available primary amino acid sequence of NCDAAH enzymes from IPseud, NCA R1 and NCA R2 (BOXSHADE). **Key:** Numbers on the right are residue positions. Residues that are identical between any two sequences are shaded black. Similar residues are shaded in grey. Residues known to be involved in the active site are coloured red and asterisked (Nakai *et al*., 2000). Amino acid sequence sources are as described in Table 6.1. No consensus sequence shown.

Phylogenetic analysis of the relationship between the different NCDAAH enzymes was carried out on the aligned primary amino acid sequences shown in Figure 6.14. Programs supplied by PHYLIP (Felsenstein, 1993) were used to build both distance-based and parsimonious phylogenetic trees from maximum likelihood calculations. The

Neighbor-joining method was used to construct the distance-based tree, and bootstrap analysis for both trees was performed as described by Hershkowitz and Leipe (1998).

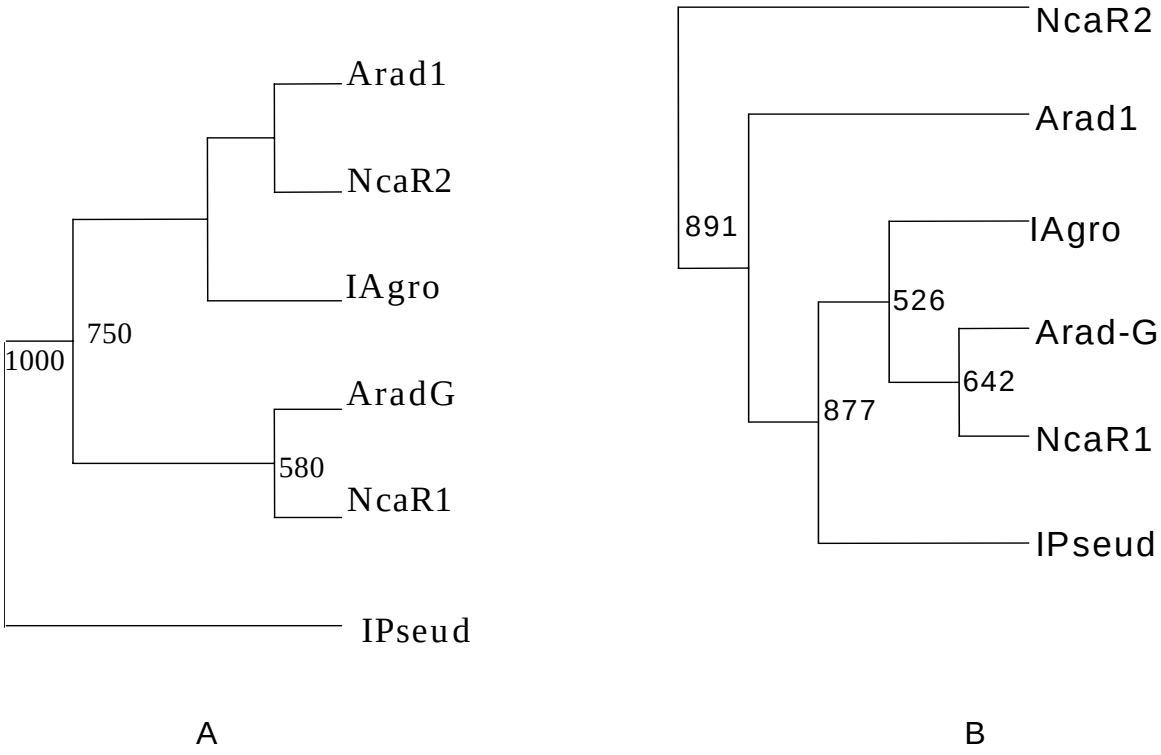


Figure 6.15: Phylogenetic trees of NCDAAH enzymes. Each tree comprises a subset of the protein sequences presented in Figure 6.13. A. The tree was constructed according to the neighbor-joining method with the program PHYLIP (using internal programs SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE). B. The tree was constructed based on protein parsimony using the program PHYLIP (internal programs SEQBOOT, PROTPARS and CONSENSE). Numbers next to the respective nodes indicate the bootstrap value for 1000 replicates. Bootstrap values less than 50% are not shown.

The distance-based bootstrap values (Figure 6.15, panel A) showed that the separation of the NCDAAH from *Pseudomonas* sp. KNK0003A was statistically significant, as was the branching between AradG and NcaR1, and the grouping of the other three NCDAAH enzymes from *Agrobacterium* spp. However, the divisions within these branches are not sufficiently supported to be regarded as true phylogenetic branchings, probably because there is insufficient variation between the sequences to separate them conclusively. Parsimonious methods provided high bootstrap values for the separation of NcaR2 and IPseud as outgroups from the main tree, and also provided a well supported grouping of three of the type 1 NCDAAH enzymes into a clade (Figure 6.15 panel B). Within this type 1 clade, the grouping of AradG and NcaR1 is once again well supported by parsimonious methods, as it was by distance-based methods.

As neither of these trees is rooted, the evolutionary relationships cannot be calculated accurately, but the trees can be used to infer the phylogenetic relationships between the different NCDAAH sequences.

Full sequence is not available for the ORFs encoding NCDAAH activity in *Comamonas* sp. E222C, *Blastobacter* sp. A17p-4 (Ogawa *et* al., 1993, 1994) and *Agrobacterium* sp. SB (Louwrier and Knowles, 1996). However, alignment of 30 amino acids in the N-terminal region of these enzymes with those in Figure 6.13 revealed that all the NCDAAH enzymes share at least 50% similarity in this region (Figure 6.16). Interestingly, the N-terminus of the NCDAAH enzyme from *Blastobacter* sp. A17p-4 shares a higher level of identity with type 1 NCDAAH enzymes than either NcaR2, or the NCDAAH from *Pseudomonas* sp. KNK0003A. As was noted by Ikenaka *et al.* (1998), the NCDAAH enzymes from *Comamonas* sp. E222C and *Pseudomonas* sp. KNK0003A have 57% identity with each other in this region. The first 20 amino acids of the N-terminus for the NCDAAH from *A*. sp. SB (AgroSB) and NcaR2 are very similar to the conserved residues of the type 1 NCDAAH enzymes from *Agrobacterium* spp. with 85% and 90% similarity, respectively.

```
Arad-1          1MTRQMILAVGQQGPIARAETREQVVGRLLD
Arad-G          1MTRQMILAVGQQGPIARAETREQVVGRLLD
NCA R1          1MTRQMILAVGQQGPIARAETREQVVGRLLD
IAgro           1MTRQMILAVGQQGPIARAETREQVVVRLLD
NCA R2          1MTRQMILAVAQQGPISRALATANRLVSRLR
AgroSB          1MTRQMILAVGQQ-PIARAQET----------
A17p-4          1MARKLNLAVAELGPIARAETRDQVVARLMEM
IPseud          1MTRIVNAAAAQMGPISRSETRKDTVRRLIA
E222C           1MSRIVNYAAAELGPIERADSRADVMERLLA
```

Figure 6.16: Alignment of the amino-terminal amino acids of nine different NCDAAH enzymes. Residues that are identical in more than half of the sequences are shaded red, similar residues are shaded blue and dissimilar residues are represented in black. Dashes indicate gaps in the alignment. The sources used are as described in Table 6.1. The alignment was performed using Clustal W version 1.7 (Thomson, 1994).

The alignment of the N-terminal amino acid sequence shows that the NCDAAH enzymes from *Comamonas* sp. E222C, *Pseudomonas* sp. KNK0003Aare significantly different from the other NCDAAH enzymes. The NCDAAH enzyme from *Blastobacter* sp. A17p-4 was more similar to the NCDAAH enzymes from *Agrobacterium* spp., but

still showed distinct differences in primary amino acid sequence in this region. All the NCDAAH enzymes from *Agrobacterium* species exhibited a high degree of sequence identity in this region, particularly within the first twenty amino acids, suggesting that these enzymes are orthologous, and may have been derived from a common ancestor by divergent evolution of the regions outside of this region, or by convergent evolution of different enzymes to produced the conserved N-terminal primary amino acid structure necessary for their function.

Modeling of the 3 dimensional structure of NcaR2 based on the known structure of the DCase (NCAAH) enzyme from *Agrobacterium* sp. KNK712 (Nakai *et al.*, 2000, PDB accession number 1ERZ) and *A. radiobacter* NRRL B11291 (Wang *et al.,* 2001, PDB Accession number 1FO6) was not possible, as these structures are not due for release until after April 2001. Modeling the structure using the SWISS-MODEL program (Guex and Pietsch, 1997) was also unsuccessful due to the unavailability of this structure, as NcaR2 has < 25% global similarity with the primary sequence of any of other the available structures in the Protein Data Bank (PDB). However, at least one of the amino acids represented in the modeling of the *Agrobacterium* sp. KNK712 DCase (NCDAAH) enzyme in interaction with substrate differs to those found in the same position in NcaR2. This may have a significant effect on the activity of the NcaR2 enzyme (Figure 6.17). Nonetheless, the two key residues involved in substrate recognition (Arg174 and Arg175 in Figure 6.17) are identical in all the NCDAAH enzymes illustrated in Figure 6.14 (Arg 175 and Arg 176). Interestingly, despite the high overall similarity between NcaR1 and the type I NCDAAH enzymes, one of these key amino acids, His143, is replaced by leucine in NcaR1. These amino acid replacements alter the ionic nature of the particular amino acid residue, which may have important consequences for enzyme activity in NcaR1 and NcaR2.

It is important to note that the numbering of the residues in Figure 6.17 begins with the methionine as position 0, not position 1 as in Figure 6.13 and other references (Nanba *et al.*, 1998). Hence all references to amino acid residue numbers in Figure 6.17 relate to the residue numbered one position ahead in Figure 6.13.

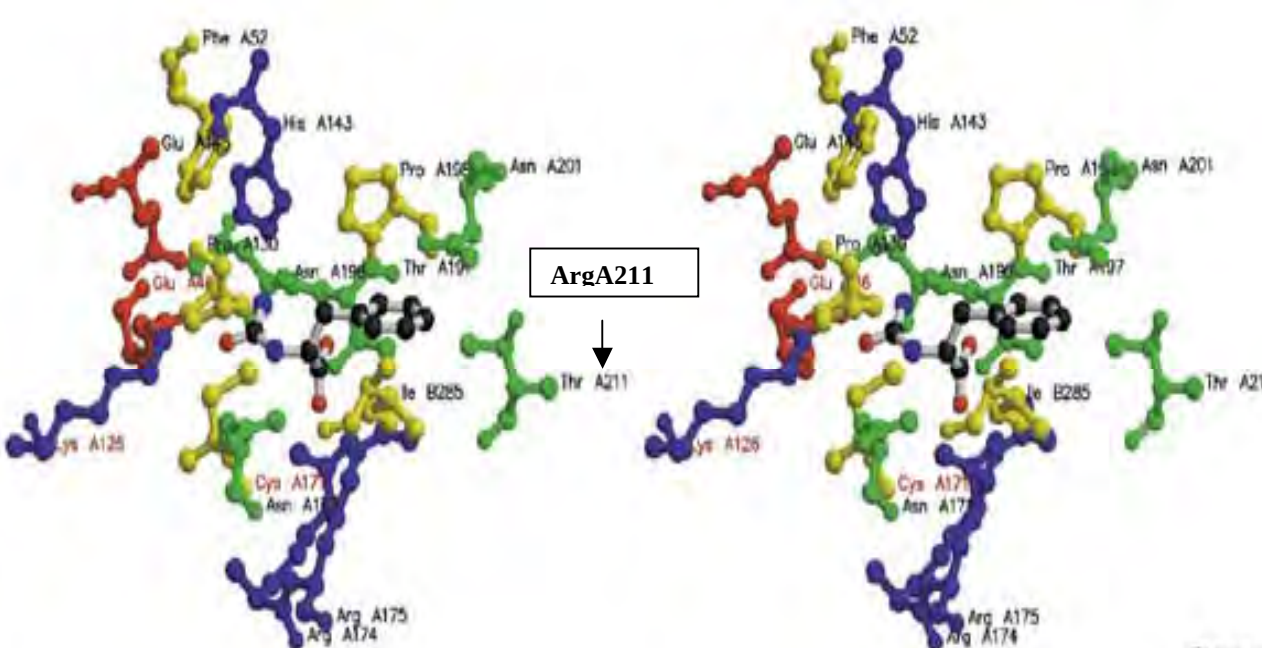


Figure 6.17: Key amino acids that are involved in the interaction of the DCase (NCDAAH) from *A*. sp. KNK712 enzyme with the substrate *N*-carbamoyl-D-phenylalanine, and the different amino acids which occur at two of these positions in NCA R2. The amino acid residues are coloured by residue type: acidic in green, basic in blue, polar in green and hydrophobic in yellow. The substrate is coloured by atom type: carbon in grey, nitrogen in blue and oxygen in red. The three catalytically important residues (Glu 46 [47 in Figure 6.14], Lys 126 [127 in figure 6.14], Cys 171[172 in Figure 6.14]) are labeled in red. Arrows depict the residues that differ in NCA R2. (Adapted from Nakai *et al*., 2000).

Of the three amino acid residue which were mutated to produce increased thermostability in the NCDAAH of *Agrobacterium* sp. KNK712 by Ikenaka *et al*. (1998b,c; 1999), two are replaced by different amino acid residues in NCAR2: His57 with an isoleucine residue and Val236 with methionine (Figure 6.13).

6.3.4 Molecular analysis of the promotor regions upstream of NCDAAH genes

Whilst several ORFs encoding NCAAH enzymes have been characterized, little is known about the promotor sequences upstream of these genes. Shine-Dalgarno ribosome binding sites, -10 and –35 promotor signals similar to those found in *E. coli* have been identified for some genes encoding hydantoin-hydrolysing activity (Grifantini *et* al, 1998; Ikenaka *et* al., 1998a; La Pointe *et* al., 1994; Nanba *et al*., 1998; Watabe *et al*., 1992a,b), as illustrated for *ncaR*1 and *ncaR*2 in Figures 6.8 and 6.10, respectively.

Sigma factor binding sites have also been identified upstream of some NCAAH and hydantoinase genes (La Pointe *et al*., 1994; Watabe *et al*., 1992a,b; Grifantini *et al*., 1998). To examine the sequence conservation between NCDAAH promotor regions, the sequence upstream of the NCDAAH genes was also aligned, based on the region from upstream of the deduced transcription start (+1) nucleotide (Figure 6.18).

CONSENSUS:	**-35**	**-10**
	TTGACA	TATAAT
AE01	GCAGCGCAGCGTTCTCCCGGCCGGGAGGCCGGATTAACTATC**G**	1
Arad1	CATAGCAGCGTTCTCCGGCCGCGAGGCCGGATTAACTATC**G**	1
NCAR1	AATGTGCATAGCAGCGTTCTCCGGCCGCGAGGCCGGATTAACTATC**G**	1
IAgro	ATGTGTTTCACAACGTTTTCCCGGCCGCTGGGCCGGACATCACCTAG**G**	1
Arad-G	TCGGCGCTGCGAGGAACGTATCGAGTTTCGATTAGACGCG**G**	1
IPseud	CCGCTTCCCTGCAGGCGAAAATACCGTTTCAATGGGCAAGG**G**	1
NCA R2	CCACGCCCTTTATGAGGGGCCGCAATGAAGCCTTAACTATC**G**	1

Figure 6.18: Sigma factor consensus binding regions in the upstream sequence from the +1 nucleotide of NCDAAH genes. The putative +1 nucleotide for the transcriptional start site is shaded green. 1 represents the +1 nucleotide.

Although the promotor regions upstream of the NCDAAH enzymes from *A. radiobacter* NRRL B11291 (as described by Buson *et al.*, 1996), *A. tumefaciens* AE-O1, and the NcaR1 enzyme from *A. tumefaciens* RU-OR share a high degree of identity, there are several differences between these promotor regions and those upstream of the NCDAAH coding sequences from *Pseudomonas* sp. KNK712, *Agrobacterium* sp. KNK712, NcaR2 from *A. tumefaciens* RU-OR and the promotor region found between the hydantoinase and NCDAAH enzymes from *A. radiobacter* NRRL B11291, as described by Grifantini *et al.* (1998).

Thus, not only the coding sequences for the different types of NCDAAH enzymes, but also their promotor regions differ. In the case of NcaR2 from *A. tumefaciens* RU-OR this indicates that the two NCDAAH enzymes from RU-OR are probably located in different regions of the *A. tumefaciens* RU-OR chromosome. Significantly, the difference in promotor structure also implies that the regulation of the two types of NCDAAH in RU-OR may be disparate under some or all of the conditions described in previous chapters. Peculiarly, the promotor regions upstream of the coding regions for the two NCDAAH enzymes isolated from *Agrobacterium radiobacter* NRRL B11291, Arad1 (Buson *et al*., 1996) and AradG (Grifantini *et al*., 1998) in Figure 6.19, share no significant sequence similarity, although the open reading frames share 99.6% nucleotide identity. The reason for this is not known and has not been reported in literature. However, the NCDAAH coding region from *Agrobacterium* sp. 80/44-2A (Agro SB, Neal *et al*., 1990) is identical to that of AradG, and the promotor region upstream of this coding region does share significant identity with the promotor region upstream of the ORF encoding AradG.

The minimal consensus sequence for binding of the sigma factor σ^{54} as described by Kustu *et al.* (1989) requires a GC doublet 11-14 base pairs from the transcriptional start +1 nucelotide, and a GG doublet exactly 10 base pairs upstream of this. No sequences resembling the σ^{54} minimal consensus sequence were identified in the upstream region of the NCDAAH genes shown in Figure 6.18. Sigma factor σ^{54} mediates *ntr*-type transcriptional regulation of several other genes whose products are involved in nitrogen assimilation, but may be more involved in the regulation of hydantoinase enzyme expression than NCDAAH expression in Agrobacteria.

6.4 Discussion

The mapping of *ncaR1* and *ncaR2* genes encoded by pG4 and p6B3-1a to the genomic DNA of *A. tumefaciens* RU-OR clearly shows that both genes originate from the genomic DNA of this bacterium.

The sequence identity between *ncaR1* and the genomic DNA of *A. tumefaciens* RU-OR is clear. Some hybridization of pG4 to the genomic DNA of *A. tumefaciens* AE-01 also occurs but the hybridization pattern is not similar. However, some hybridization fragments may not be visible due to a low concentration of DNA on the membrane. The NCDAAH gene carried by plasmid pG4 has a sequence identity of greater than 90 % with the NCDAAH-encoding gene from *A. tumefaciens* AE-01 found in plasmid pGH45, so hybridization of pG4 to chromosomal DNA from *A. tumefaciens* AE-01 was expected. However, the hybridization pattern of the insert DNA from pG4 is not the same for the chromosomal DNA from both strains, and nucleotide sequence differences between the ORFs encoding *ncaR1* and the NCDAAH enzyme from *A. tumefaciens* AE-01 preclude the isolation of *ncaR1* from this bacterium.

NcaR2 clearly shares sequence identity with the genomic DNA of *A. tumefaciens* RU-OR. Interestingly, however, *ncaR2,* as encoded by the insert DNA of p6B3-1a, did not hybridize the chromosomal DNA of *A. tumefaciens* AE-01, suggesting that *ncaR2* and similar NCDAAH enzymes may not be common to all hydantoin-hydrolysing *Agrobacterium* strains. NcaR2 may therefore be a unique acquisition of *A. tumefaciens* RU-OR. However, the method of screening the genomic DNA library of *A. tumefaciens* RU-OR may also have influenced the isolation of the NcaR2 enzyme, as the screening method did not rely on DNA hybridization or growth with hydantoin as a sole nitrogen source, and did not place strong selection pressure for the expression of high levels of hydantoinase or NCAAH activity. Due to the similarity in predicted protein molecular weight, substrate selectivity, and stereoselectivity, the two enzymes might also have been difficult to purify separately from *A. tumefaciens* RU-OR.

Deletion and sequence analysis of p6B3-1a and pG4 identified two ORFs encoding NCDAAH activity, *ncaR1* and *ncaR2*, and showed that these two genes shared only 62% DNA identity and 60% amino acid identity with each other. Both enzymes shared the catalytic triad of crucial active site amino acids identified as being involved in the mechanism of action for cleavage of *N*-carbamoylamides (Figure 6.13; Nakai *et* al., 2000), and also showed similar high levels of identity to other NCDAAH and related enzymes over the region from residues 103-192 as described by Ikenaka *et al*. (1998)

(104 to 193 in Figure 6.10). Although the NcaR1 enzyme encoded by pG4 shared greater than 90% identity at a DNA and primary amino acid sequence level with other type 1 NCDAAH enzymes previously isolated from *Agrobacterium* spp., the NcaR2 enzyme of p6B3-1a showed less identity with type 1 NCDAAH enzymes, and even less with the DCase from *Pseudomonas* sp. KNK0003A, sharing only 51.2 % DNA sequence identity. Eleven extra non-identical regions of primary sequence occur between NcaR2 and the DCase from *Pseudomonas* sp. KNK0003A than occur between NcaR2 and the NCDAAH gene from *Agrobacterium radiobacter* NRRL B11291 (Figure 6.14). Thus, NcaR2 seems to comprise a new type of NCDAAH, sharing common function and a fairly high level of sequence identity with all other reported NCDAAH enzymes, but differing significantly from the type 1 NCDAAH enzymes normally isolated from *Agrobacterium* strains.

Variations in the amino acid residues of the NCDAAH enzymes have been linked to changes in the thermostability of the enzymes, and therefore the differences between NcaR, NcaR2 and other NCDAAH enzymes may have significant effects on the biocatalytic properties of the enzyme. Mutation of three residues (H57Y, P203E, V236A) within the coding sequence of the NCDAAH (DCase) enzyme of *A*. sp. KNK712 produced an increase of 19°C in the thermostability of the enzyme. Ikenaka *et al*. (1999) postulate this increase resulted from the removal of the positive histidine charge from the 57th amino acid, the change in a bend of the enzyme chain caused by removal of Pro203, and the removal of stereo-obstruction by changing the valine at position 236. In the natural coding sequence of NcaR2 and the NCDAAH (DCase) enzyme from *Pseudomonas* sp. KNK0003A, the His57 residue is replaced with isoleucine or valine, respectively, and Val236 is replaced by cysteine or methionine, respectively (Figure 6.9). The native *Pseudomonas* sp. KNK0003A DCase has increased thermostability over the *Agrobacterium* sp. KNK712 DCase. The improved natural thermostability of the NCDAAH or DCase enzyme from *Pseudomonas* sp. KNK0003A may be attributable in part to these alterations, although higher overall G+C content and the eight extra amino acids on the C-terminus of this enzyme should also be considered. The thermostability of the NcaR2, in which His57 is replaced by an isoleucine residue, and Val236 by a methionine residue, has not yet been determined. Purification of the

enzyme and detailed analysis of the thermostability may provide additional information on the role of these particular amino acid changes in alteration of thermostability.

Unfortunately, although the crystal structures for the NCDAAH enzymes from *A.* sp. KNK712 and *A. radiobacter* CCRC 14942 have been resolved and modeled (Hsu *et al*., 1999; Nakai *et al*., 2000; Wang *et al*., 2001), modeling of the primary sequence of NcaR2 to see what effect specific residue changes may have on the active site of the protein was not possible as access to the PDB files describing the crystal structures (PDB 1ERZ and PDB 1FO6, respectively) are not yet available. Substrate recognition may not be affected, as the two conserved arginine residues reputed to be crucial for this function are not altered in NcaR1 or NcaR2. However, the alterations to active site amino acids in both NcaR1 and NcaR2 produce alterations in the ionic environment of these regions of the active site, and may have important implications for the biocatalytic activity of the enzymes.

Examination of the evolutionary relationships between the primary amino acid sequence of different NCDAAH enzymes using distance-based and parsimonious methods to compute the maximum likelihood of various groupings only produced two strongly supported branches in the relationships between the enzymes. NcaR2 and IPseud from *Pseudomonas* sp. KNK0003A both formed strongly supported branches from the other enzymes. Within the type 1 NCDAAH enzymes, grouping of AradG and NcaR1 in the same clade, and Arad-1 with IAgro, although not in the same clade, were often the result of the majority-rule and strict consensus type tree produced by CONSENSE, but bootstrap and support values for these were never greater than 50%. Various other tree-building methods were used to try and obtain a more strongly supported tree, but with little success.

This work represents the first report of the isolation of two different genes encoding NCDAAH activity in a single bacterial strain, and was initially puzzling. The lack of DNA : DNA hybridization of *ncaR2* to the chromosomal DNA of *A. tumefaciens* AE-01 also suggested that NcaR2 might be an unusual feature of RU-OR, not shared with

other *Agrobacterium* strains. There is, however, some variation between the NCDAAH enzymes isolated from different *Agrobacterium* strains, suggesting species level differentiation in the evolution of the enzymes. In addition, Ogawa and Shimizu (1997) reported the isolation of two different *N*-carbamoylamino acid-hydrolyzing enzymes from *Comamonas* sp. E222c and *Blastobacter* sp. A17p-4. In this instance, both strains exhibited NCDAAH enzyme activity as well as β-ureidopropionase activity. Louwrier and Knowles (1996) also did not rule out the possibility of more than one enzyme with *N*-carbamoyl-amino acid-hydrolyzing activity in *Agrobacterium* sp. cell extracts. In the case of NCLAAH enzymes there are pronounced differences between the NCLAAH enzyme isolated from *Arthrobacter aurescens* DSM 3747 (Syldatk *et al*., 1990a,b; Wilms *et al*., 1999), and other NCLAAH enzymes, and this enzyme is classified as a novel member of the amidohydrolase superfamily (Wilms *et al*., 1999). Thus, precedence does exist for the isolation of two *N*-carbamoyl amino acid-hydrolyzing enzymes from a single strain. The reasons for a second type of NCDAAH gene in *A. tumefaciens* RU-OR are not clear, but the diversity and versatility of *N*-carbamoylamide and hydantoin-transforming enzymes is well documented (Syldatk *et al.*, 1999; Ogawa *et al*., 1994a; 1997a; Soong *et al*., 1999; Wilms *et al*., 1999). Purification of the NcaR2 enzyme, and more detailed examination of the properties of the purified enzyme may illuminate the function of NcaR2 in *A. tumefaciens* RU-OR cells more clearly. The genome of *Agrobacterium tumefaciens* comprises two non-homolgous chromosomes: a circular chromosome of 3Mbp, and an additional linear chromosome of 2.1Mbp, with most of the auxotrophic markers located on the circular chromosome (Goodner *et al*., 1999). The positional location of *ncaR1* and *ncaR2* on either of these two chromosomes might also provide insight into the physiological role and the acquisition of two NCDAAH enzymes in RU-OR cells.

Analysis of the differential or similar expression of the two NCDAAH genes from RU-OR, using Northern analysis of mRNA production (Sambrook *et al*., 1983), will also be necessary to determine the contribution of each of these enzymes to the overall NCDAAH activity in RU-OR under various conditions. In particular the differential increase in NCAAH activity, but not hydantoinase activity when RU-OR cells were cultured with 1% mannitol as a sole carbon source (Chapter 3, Section 3.4.1) may be related to an increase in the expression of one of the two enzymes due to release of

carbon catabolite repression of transcription. The phenotype of mutants strain RU-ORLB3, which shows both inducer-independent hydantoinase activity and inducer-dependent NCAAH activity may also be related to a mutation in the promotor region of only one of the NCDAAH enzymes. The differences in the promotor regions of all the NCDAAH enzymes are notable and may be related to the different levels and biochemical properties of NCAAH activity. For this reason, the Rhodes Hydantoinase Research Group is undertaking studies on the structure and function of the two NCDAAH promotor regions upstream of the coding regions for NcaR1 and NcaR2 using reporter gene fusion techniques.

Chapter 7

General Conclusions

Chapter 7
General Conclusions

The research described in this thesis focused on the development of a novel biocatalytic process for the production of enantiomerically pure D-HPG from D,L-5-HPH. The principal goal of the research was to develop a fundamental understanding of an hydantoin-hydrolysing enzyme system, and then apply this knowledge to engineer an improved biocatalyst. The following sections outline how this was achieved through the isolation and characterization of the hydantoin-hydrolysing enzyme system of *A. tumefaciens* RU-OR, and some of the genes encoding hydantoin-hydrolysing activity in this strain. A fundamental understanding of the regulation of hydantoin-hydrolysing activity in RU-OR by at least three different mechanisms was developed, and this was manipulated to produce mutants with altered regulation of hydantoin-hydrolysing activity. Some of these mutant strains showed potential as efficient biocatalysts for the production of D-HPG from D,L-5-HPH. Several hypotheses were evaluated in this thesis and these are also discussed in the following sections.

7.1 The biocatalyst: selection and characterization of the hydantoin-hydrolysing enzyme system of *A. tumefaciens* RU-OR

Firstly, it was necessary to isolate hydantoin-hydrolysing microorganisms and select a suitable bacterial strain with D-stereoselective hydantoin-hydrolysing activity and novel biocatalytic properties. *Agrobacterium tumefaciens* RU-OR was selected from a number of hydantoin-hydrolysing isolates because of potentially unusual hydantoin-hydrolysing characteristics. Further characterization of the hydantoin-hydrolysing enzyme system of RU-OR confirmed the novel biocatalytic properties of this strain.

Hydantoinase activity in RU-OR resting cells was highest at reaction temperatures between 40 ^{o}C and 60 ^{o}C and at pH 9.0. The activity of the RU-OR hydantoinase was similar to that of hydantoinases characterized in *A. radiobacter*, *A. tumefaciens* 47C and *Agrobacterium* IP-671 (Durham & Weber, 1995; Olivieri *et al.*, 1981; Runser *et al.*, 1990; Runser & Ohleyer, 1990). A. *tumefaciens* RU-OR cells produced only D-amino acids from racemic hydantoins substrates, had showed D-stereoselective NCAAH activity. Although this cannot be proven conclusively, this suggested that the hydantoinase from RU-OR was D-selective and might resemble dihydropyrimidinase, as RU-OR cells were able to produce *N*-carbamoyl-β-alanine from dihydrouracil (data not shown).

The NCAAH activity of *A. tumefaciens* RU-OR was strictly D-stereoselective, producing only D-amino acids from racemic *N*-carbamoyl amino acid substrates. However, NCDAAH activity in *A. tumefaciens* RU-OR cells showed some unique characteristics. Activity was apparent over a broad pH range and at very high pH (11), unlike other *Agrobacterium* NCDAAH enzymes. NCDAAH activity in RU-OR cells was also uninhibited by L-isomers of *N*-carbamoyl amino acid substrates, whereas other *Agrobacterium* NCDAAH enzymes are strongly inhibited by *N*-carbamoyl-L-amino acids (Olivieri *et al.*, 1978; Runser *et al.*, 1990).

Due to the probable ancient origin of hydantoin-hydrolysing enzymes, and the divergent characteristics of these enzymes (May *et al.,* 1998a; Ogawa *et al.*, 1997b; Syldatk *et al.*, 1999), the unique characteristics of NCDAAH activity in RU-OR suggested that more than one NCDAAH enzyme might be involved in the NCAAH activity of *A. tumefaciens* RU-OR. This second enzyme did not seem to be the β-ureidopropionase described by Ogawa and Shimizu (1997), which exhibits strict L-stereoselectivity towards *N*-carbamoyl-α-amino acids. The isolation and cloning of two different NCDAAH-encoding genes from *A. tumefaciens* RU-OR confirmed these observations and revealed the presence of a second, novel NCDAAH enzyme, NcaR2, in this bacterial strain. The biochemical properties of purified NcaR2 enzyme are not yet known, but the existence of second dissimilar NCDAAH enzyme may explain the exceptionally broad pH range for NCAAH activity in *A. tumefaciens*

RU-OR, and the unusual lack of inhibition of the enzyme activity by L-isomers of the *N*-carbamoyl amino acid substrate.

Purification of the hydantoinase enzyme from *A. tumefaciens* RU-OR, and genetic studies of the hydantoinase and NCDAAH genes, together with further biochemical and biophysical studies of the enzymes should help resolve the exact nature and physiological role of hydantoinase activity in *A. tumefaciens* RU-OR.

Isolation and characterization of the hydantoin-hydrolysing enzyme system of *A. tumefaciens* RU-OR therefore fulfilled the first requirement of this thesis: the selection of an indigenous South African microorganism which could produce D-amino acids from racemic hydantoins substrates, including D,L-5-HPH, and which exhibited novel biocatalytic properties.

7.2 Regulation of hydantoin-hydrolysing activity in RU-OR

The second crucial aim was to develop a fundamental understanding of the regulation of the production of hydantoin-hydrolysing enzymes in the native strain.

Both induction and cellular nitrogen levels strictly regulated the production of hydantoin-hydrolysing activity by *A. tumefaciens* RU-OR cells. Hydantoinase activity was more tightly regulated than NCAAH activity in the cells. This could be due to a natural emphasis of regulation on the first enzyme in the pathway, rather than the second, or may be attributable to differences in the regulation of the two different NCAAH enzymes in *A. tumefaciens* RU-OR.

The induction effect was shown to be transcriptionally regulated, as transcriptional inhibitors such as rifampicin inhibited it. Based on the differing phenotypes of the inducer-independent mutants, induction is probably mediated by the interaction of an inducer molecule with a repressor molecule causing the release of the binding of the repressor to the promotor region involved in the transcription of hydantoinase

activity. A common scenario for induction of gene expression in prokaryotic systems involves the binding of inducer molecules to a repressor molecule, thus preventing the repressor from binding to the promotor region and inhibiting transcription (Alberts *et* al., 19994; Rojo, 1999). A mutation in the repressor molecule that prevents the repressor binding to the promotor region even in the absence of inducer is the most common mutation involved in inducer–independent gene expression (Maloy *et al*., 1994). Assuming induction in *A. tumefaciens* RU-OR to operate in a similar manner, such a mutation may have occurred in strain RU-ORL5.

The differential inducer-independence and dependence of hydantoinase and NCDAAH activity in mutant strain RU-ORLB3 may be related to the presence of a second type of NCDAAH enzyme (NcaR2) in RU-OR cells which, for instance, may not be regulated by the same mechanisms as the hydantoinase and NcaR1. The unusually high levels of NCAAH activity in RU-OR cells when cultured with 0.1% mannitol as a sole carbon source may also be attributable to the differences in the expression of *ncaR1* and *ncaR2* under these conditions.

Three different mechanisms regulated the production of hydantoin-hydrolysing activity in relation to cellular nitrogen levels. Transcriptional catabolite repression prevented the expression of hydantoinase and NCDAAH activity till late exponential growth phase. In addition, ammonium ions produced a separate shock effect that resulted in rapid inhibition of hydantoinase and NCDAAH activity. Both of these mechanisms are consistent with regulation by a system similar to the *ntr*-type regulatory system reported in enteric bacteria (for review see Merrick and Edwards, 1995), and previous evidence of *ntr* -type regulatory proteins in *A. tumefaciens* cells supported this (Rossbach *et* al., 1987; Wardhan *et al*., 1989; Lyi *et al*., 1999). Inhibition of glutamine synthetase activity in *A. tumefaciens* RU-OR resulted in the prevention of ammonia shock. This was consistent with regulation of *ntr* -dependent genes in correlation with the cellular ration of α-2-ketglutarate to glutamine, and a similar glutamine shock experiment confirmed this. Mutant strains of *A. tumefaciens* RU-OR in which chromosomal mutations using EMS produced altered induction and regulation of hydantoin-hydrolysing activity also supported the involvement of these mechanisms.

A third mechanism involved in the regulation of hydantoin-hydrolysing activity is product inhibition of the NCDAAH enzymes by ammonium ions. This was quite pronounced when assaying crude extracts of the hydantoin-hydrolysing enzymes in *A. tumefaciens* RU-OR, but less significant in resting cell biocatalytic reactions. The discrepancy in inhibition levels between product inhibition of hydantoin-hydrolysing activity resting cells by ammonium and the ammonium shock effect precluded the possibility that product inhibition was responsible for the ammonia shock effect. The pronounced inhibition of hydantoinase activity, rather than NCDAAH activity, under ammonium shock conditions also refuted this theory.

The data presented clearly illustrated the development of a fundamental understanding of the hydantoin-hydrolysing enzyme system of RU-OR cells. Further characterisation of the mutations that produce altered regulation of hydantoin-hydrolysing activity in *A. tumefaciens* RU-OR should elucidate the molecular mechanisms involved in the regulatory process. The differences between the putative promotor regions upstream of NcaR1 and NcaR2 already suggest that the regulation of the two enzymes may be dissimilar, and promotor sequence analysis and gene fusion studies are underway to explore the promotor regions of the mutant strains. The Rhodes Hydantoinase Research Group is currently conducting research toward this end.

7.3 Mutational analysis of hydantoin-hydrolysing activity in RU-OR and overproduction of hydantoin-hydrolysing enzymes

The next hypothesis evaluated was that the development of a fundamental understanding of the regulation of the hydantoin-hydrolysing enzyme system could be applied to the genetic manipulation of the hydantoin-hydrolysing enzyme system to engineer improved biocatalytic production of D-amino acids. Subsequent to the evaluation of the factors regulating the production of hydantoin-hydrolysing activity in RU-OR cells, the genetic manipulation of RU-OR using mutagenesis and various selection systems was used to produce several mutants with altered regulation. The elevated hydantoinase activity and inducer-independent production of hydantoin-hydrolysing enzymes in mutant strain RU-ORPN1 showed potential as an improved biocatalyst for the production of D-amino acids using native cells. This was

enhanced by the mutational alterations achieved in strain RU-ORF9, which not only produced similar levels of inducer-independent expression of hydantoins-hydrolysing, but also produced active hydantoinase and NCAAH activity in the presence of repressive nitrogen sources during exponential growth. In addition, mutational alteration had removed the ammonium shock of hydantoinase activity in this strain. Strain RU-ORF9 therefore presents excellent potential for the continuous production of high levels of D-amino acids from racemic hydantoins during rapid growth in non-growth-rate-limiting conditions. The continuous production of hydantoinase and NCDAAH activity during exponential growth phase in complete medium without the addition of inducer by A. tumefaciens RU-ORPN1F9 cells could be harnessed for a simplified "one-pot" whole cell biocatalytic system to produce D-HPG from D,L-5-HPH without the need for batch culture and growth to stationary phase.

Hence, the production of regulatory mutants of *A. tumefaciens* RU-OR provided two strains with potential for application as biocatalysts. *A. tumefaciens* RU-ORPN1 and RU-ORF9 produced greater conversion of D,L-5-HPH to D-HPG than reported for other wildtype *Agrobacterium* strains, and comparable conversion levels to those produced by recombinant expression of *Agrobacterium* genes encoding hydantoin-hydrolysing activity (Chao *et al.,* 1999a). Optimisation of the D,L-5-HPH conversion in these strains should increase their potential for industrial application. Research into scaled–up fermentation and immobilization studies utilizing these organisms is in progress.

7.4 NcaR1 and NcaR2: Implications and Applications

The isolation of genes encoding two NCAAH enzymes from *A. tumefaciens* RU-OR initially suggested that one might be an amidase or β-ureidopropionase enzyme. However, the strict D-stereoselective cleavage of *N*-carbamoyl-α-amino acids by both enzymes does not support this proposal. The primary amino acid sequence of NcaR1 and NcaR2 also shows a high level of similarity to other NCDAAH enzymes, and NcaR1 is typical of the type 1 NCDAAH enzymes, which have previously been isolated from *Agrobacterium* strains. The dissimilarity between NcaR1 and NcaR2 suggests that they must have evolved divergently. It is possible that this may have occurred within the evolution of *A. tumefaciens* RU-OR, or this gene could have

been acquired from another source. The similarity between the highly conserved N-terminal regions of all other *Agrobacterium* NCDAAH genes and NcaR2 allows for the speculation that these enzymes all evolved from a similar ancestor. If this was congruent with the proposed evolution of cyclic-ureide hydrolyzing enzymes such as hydantoinases, it is likely that the divergence occurred at a distant time in evolutionary history (May *et al*., 1998a). Hydantoin derivatives and *N*-carbamoyl amino acids are reported to have been far more abundant at other times during evolution history than today (May *et al.,* 1998a; Syldatk *et al*., 1999). This may account for the wide diversity of hydantoin-hydrolysing enzymes and the lack of clarity as to their physiological role in the cell.

The dissimilarity between the primary amino acid sequences of NcaR2 and type 1 NCDAAH enzymes may have important implications for the biocatalytic properties of the enzyme. Purification and comparison of the biochemical properties of both NCA R1 and NcaR2 will provide further insight into the structure-function relationships of NCDAAH enzymes, particularly with regard to the amino acid residues involved in the active site as proposed and modeled by Nakai *et al*. (2000).

In addition, the divergence between the two NCDAAH enzymes presents interesting possibilities for gene shuffling and directed evolution of the two genes and their enzyme products. The use of *in vitro* evolution to produce and refine new non-natural enzymes is an important tool of modern biocatalysis (Arnold, 2001), and has already been used successfully in the production of improved hydantoin-hydrolysing enzymes (May *et al.,* 2000*,* Kim *et al*., 2000a).

7.5 Future Research

The genetic characterisation of the hydantoin-hydrolysing enzyme system of *A. tumefaciens* would not be complete without the isolation and chromosomal mapping of the hydantoinase gene. Due to the predicted occurrence of the D-hydantoinase gene within the vicinity of the NCDAAH type 1 ORF in most *Agrobacterium* species, isolation of this gene by screening of the *A. tumefaciens* RU-OR genomic library using the *ncaR1* gene as a probe may provide an effective means of isolating the

hydantoinase gene. Some DNA homology was detected between p6B3-1a and other isolates selected from the RU-OR genomic library, and these are being further investigated for hydantoinase activity and ORFs encoding cyclic-ureide hydrolysing enzymes.

The possible presence of a hydantoin racemase in RU-OR cells has not been investigated further. Purification of the enzyme from native cells, or isolation of the racemase-encoding gene from RU-OR would be desirable as no hydantoin racemase enzymes have previously been isolated from *Agrobacterium* cells. In addition, racemase activity is central to the efficient production of enantiomerically pure amino acids by hydantoin-hydrolysing enzyme systems and an isolated racemase would have potential for application in biocatalytic production systems. Further optimisation and characterisation of the *A. tumefaciens* RU-OR hydantoin-hydrolysing enzymes in both native and recombinant expression systems is required for the utilisation of this hydantoin-hydrolysing enzyme system for the biocatalytic production of D-amino acids.

In addition to applications involving hydantoin-hydrolysing activity, *Agrobacterium tumefaciens* cells are able to transfect DNA into plant cells, and have important applications in plant biotechnology. The chromosome structure of *A. tumefaciens* is also unique, and for this reason there is an interest in the complete sequencing of the genome of this bacterium. The isolation of a novel NCDAAH enzyme from *A. tumefaciens* RU-OR, and the presence of other enzyme activities with potential industrial application, such as imidase, amidase and lipase activity, in RU-OR cells (Gentz, 2000; Hartley, 1995) suggested that RU-OR would present a suitable candidate for genomic analysis. The Rhodes Hydantoinase Research Group has therefore initiated a project with the goal of mapping and obtaining complete sequence of the genome of *A. tumefaciens* RU-OR. It is hoped that this may lead to the isolation of further uncharacterised enzymes.

In conclusion, the research presented in this thesis demonstrated the isolation and characterization of an hydantoin-hydrolysing enzyme system with novel biocatalytic

properties from an indigenous South African bacterium. A fundamental understanding of the factors regulating hydantoin-hydrolysing activity in RU-OR cells was established, and utilized for the genetic manipulation of RU-OR to produce regulatory mutants strains with novel biocatalytic potential. The isolation of a two NCDAAH-encoding genes from RU-OR, and the illumination of certain novel characteristics in the predicted primary amino acid sequence of NcaR2 provided further evidence of a novel hydantoin-hydrolysing enzyme system presenting potential opportunities for further elucidation of the natural function and evolutionary relationships of hydantoin-hydrolysing enzymes in *A. tumefaciens*, and application in the biocatalytic production of D-amino acids. The novel biocatalytic properties of the hydantoin-hydrolysing enzyme system in *A. tumefaciens* RU-OR and mutant derivatives present fascinating opportunities for further elucidation of the natural function, regulation by cell metabolism and biocatalytic potential of hydantoin-hydrolysing enzymes.

Appendices

Appendix 1: List of materials

Standard Chemicals

Chemical	Source
acetic acid (glacial)	SaarChem (South Africa)
acrylamide	Sigma (South Africa)
bis-acrylamide	Sigma (South Africa)
boric acid	SaarChem (South Africa)
chloroform	SaarChem (South Africa)
copper (II) acetate	SaarChem (South Africa)
4-dimethylaminobenzaldehyde	Merck (South Africa)
Ethylenediaminetetra-acetic acid (EDTA)	Merck (South Africa)
hydantoin	Sigma, Aldrich (South Africa, Germany)
hydantoic acid (N-carbamoylglycine)	Sigma (South Africa)
hydrindantin	Fluka (South Africa, Germany)
hydrochloric acid	NT, Merck (South Africa)
isoamylalcohol	SaarChem (South Africa)
D-methylhydantoin	Toronto Research Chemicals (Canada)
L-methylhydantoin	Toronto Research Chemicals (Canada)
methanol	SaarChem (South Africa)
2-methoxyethanol (methylcellusolve)	SaarChem (South Africa)
N,N-dimethyl-L-pheynylalanine	Sigma, Aldrich (South Africa, Germany)
ninhydrin	Merck (South Africa)
sodium dodecyl sulphate	Merck (South Africa)
thiamine hydrochloride	SaarChem (South Africa)
tris(hydroxymethyl)aminomethane	Merck (South Africa, Germany)
Tris (hydroxymethyl)aminomethane	Merck (South Africa, Germany)

Molecular Reagents

agarose D1-LE	Whitehead Scientific (South Africa)
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	Roche Molecular Biochemicals (South Africa, Germany)

Hybond™ N+ nylon membrane	Amersham, USB (USA)
Hyperfilm MP maximum performance autoradiography film	Amersham, USB (USA)
isopropyl-β-D-thiogalactoside (IPTG),	Roche Molecular Biochemicals (South Africa, Germany)
Kodak Biomax MS autoradiography film	Amersham, USB (USA)

Enzymes and Kits

Restriction enzymes	Roche Molecular Biochemicals (South Africa, Germany) Amersham, USB (USA) Promega (USA)
ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit	Perkin-Elmer Applied Biosystems (USA)
DNA Clean and Concentrator Columns	Zymo (USA)
Erase-A-Base System	Promega (USA)
Expand™ Hi-Fidelity PCR System	Roche Molecular Biochemicals (South Africa, Germany)
pGEM -T-Easy Vector System	Promega (USA)
High Pure Plasmid Isolation Kit	Roche Molecular Biochemicals (South Africa, Germany)
High Pure PCR Product Purification Kit	Roche Molecular Biochemicals (South Africa, Germany)
Quantum Prep Plasmid Midipreps	Bio-rad (USA)
QIAprep® Spin Miniprep Kit	Qiagen (USA)
T_4 DNA ligase	Promega (USA)
Vent™ DNA Polymerase	New England Biolabs (USA)
ZAP Express™ Predigested Vector Kit	Stratagene (USA)
ZAP Express™ Predigested Cloning Kit	Stratagene (USA)

Appendix 2: Media

Minimal Medium (MM)

Basic minimal medium (MM), based on the M9 minimal medium recipe from Sambrook *et al.* (1989) was composed of the following:

per litre: 10g glucose; 0.0011g $CaCl_2$; 0.002g $MgCl_2$; 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 0.004g boric acid, 0.004g $MnSO_4$, 0.004g $ZnSO_4$, 0.002g $(NH_4)_6Mo_2O_{24}.4H_2O$, 0.001g KI, 0.0004g $CuSO_4$.

MM medium was supplemented with 20g agar per litre for agar plates. Appropriate amounts of nitrogen sources and inducer were added as required. Several commonly used variations are listed below:

Table A2.1: Commonly used variations of the basic MM medium

Medium Name	**MM medium supplemented with**	
	Nitrogen Source	**Inducer**
HMM	1% hydantoin	-
CAAMM	0.01% casamino acids	-
CAAMM + 2-TU	0.01% casamino acids	0.1% 2-thiouracil
NH_4MM	0.1% $(NH_4)_2SO_4$	-
NH_4MM + 2-TU	0.1% $(NH_4)_2SO_4$	0.1% 2-thiouracil

A typical recipe for making 1 litre of hydantoin minimal medium is described below.

Hydantoin Minimal Medium (HMM)
(for liquid culture or plates)

Stock Solutions (All autoclaved separately.)	Volume/ 1litre	Final Concentration
40% Glucose	25 ml	1 %
10 x M9 salts†	100 ml	1X
trace elements *	10 ml	
1M $MgCl_2$	200 µl	100 µM
1M $CaCl_2$	200 µl	100 µM
4% hydantoin	250 ml	1 %
dH_2O		
agar (if plates)	20g	2 %

†**10 x M9 Salts (per litre)**

Na_2HPO_4	60g
KH_2PO_4	30g
NaCl	5g

Trace Elements (per litre)

- Boric Acid 50 mg
- $MNSO_4.7H_2O$ 40 mg
- $ZnSO_4$ 40 mg
- $(NH_4)_6MO_2O_{24}$ 20 mg
- KI 10 mg
- $CuSO_4$ 4 mg
- $FeCl_3$ 12 mg

Appendix 3: Standard procedure for growth, harvesting and resting cell biocatalytic reactions with *A. tumefaciens* RU-OR

Day 1: Starter Culture Inoculation
Inoculate a single colony from HMM (see appendix 2) agar plate into HMM broth (50 ml) and grow 3-4 days to stationary phase at 25 – 30°C.
Day 4/5: Growth in Required Culture Medium
Measure OD_{600} of starter culture – dilute to OD_{600nm} ~ 0.02 in required medium e.g. CAAMM plus inducer (0.1% 2-thiouracil) for RU-OR (see appendix 2). Grow to mid-log (or other required) growth phase – approx. OD_{600} = 0.5-0.8 (~ 18-24h in CAAMM).
Day 6/7: Harvesting and Resting Cell Biocatalytic Assays
Harvest cells by centrifugation in pre-weighed centrifuge tubes. (7000 rpm, 10 min). Empty off supernatant into biocide container, wash in ~ half volume of 0.1 M potassium phosphate buffer pH 9.0 (unless otherwise required) – vortex or shake vigorously to resuspend. Whilst centrifuging, organise small bottles with a cell blank; substrate blank and at least 3 replicates of reaction (cells + substrate). Centrifuge again (7000 rpm, 10 min). Weigh drained tubes on same balance as before. Calculate the wet cell mass (X mg = final mass- pre-weight of tube) and resuspend the pellet in the required volume (Y ml) of 0.1M potassium phosphate buffer pH 9.0 to give 100mg/2.5ml reaction (40mg/ml): ***e.g.*** (X mg /100)*2.5 = Y ml <u>or</u> X mg /40 = Y ml Add 2.5 ml of cells in buffer to each reaction bottle, and the cell blank bottle. Cap and incubate for 6 h @ 40°C, 200rpm.
Day 6/7: Colourimetric Assay of Reaction Supernatant
Collect supernatant from reaction bottles by microfuging an eppendorf-ful for 3 min at maximum speed. Use this for analysis to determine the amount of *N*-carbamoyl amino acid and amino acid in the sample.

Appendix 4: Analytical methods

A4.1 Colourimetric assays for detection of *N*-carbamoyl amino acids and amino acids

The presence of N-carbamoylamino acids and amino acids in resting cell reaction supernatant was quantified colorimetrically with Ehrlich's reagent based on the method of Morin et al. (1986), using the procedure outlined below.

Ehrlich's Assay procedure

For each sample prepare a reaction as follows :

0.5ml 12% trichloro-acetic acid

1.0 ml reaction supernatant

0.5 ml Ehrlich's reagent (10% dimethylaminobenzaldehyde in 6N HCl)

3.0 ml dH_2O

Vortex, leave to react for 15minutes, and read versus a standard curve of 0-50mM NCG (see appendix 5) at $OD_{420\ nm}$. Use a spreadsheet with standard curve regression (Quattro Pro, Excel) to calculate the µmol/ml NCG produced for each sample, average and calculate SEM. Typical standards, absorbance values and a standard curve are illustrated in Table1 A41 and Figure A4.1. Fresh standard curves were prepared for each assay.

Table A4.1: Standard Curve

***N*-carbamoyl amino acid (0-50mM) – Final Volume 1 ml**			
Standard (mM)	**0.1M PO_4 Buffer (ml)**	**100mM NCG in 0.1M PO_4 Buffer (ml)**	**OD $_{420\ nm}$ after Ehrlich's reaction**
0	**1.0**	**0**	**0**
10	**0.9**	**0.1**	**0.253**
20	**0.8**	**0.2**	**0.465**
30	**0.7**	**0.3**	**0.658**
40	**0.6**	**0.4**	**0.824**
50	**0.5**	**0.5**	**1.125**

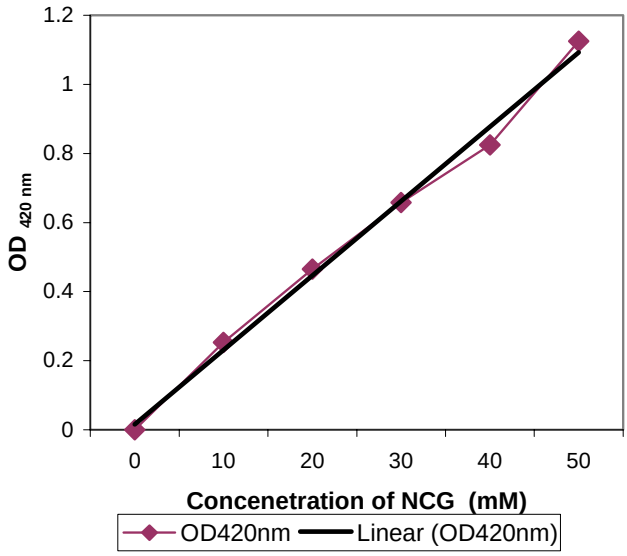


Figure A4.1: Standard curve for reaction of 0-50 mM NCG with Ehrlich's reagent.

Ninhydrin Assay Procedure

- A standard curve was prepared from 0 to 0.5mM glycine using a 1mM stock.
- For each sample test-tube reaction was prepared as follows:
- 0.998 ml 0.1M potassium phosphate buffer
- 0.020 ml reaction supernatant

- ml ninhydrin reagent (0.8 g ninhyrin; 0.12g hydrindantin dissolved in 30 ml methylcellusolve, add 10 ml 4M sodium acetate buffer (Plummer, 1987)

Test tubes were then capped, and boiled for 15 minutes. After cooling, 3.0 ml 50% ethanol was added and the $OD_{595\ nm}$ read versus the standard curve. Spreadsheets with standard curve regression (Quattro Pro, Excel) were used to to calculate the µmol/ml amino acid produced for each sample, average and calculate SEM values. Typical standards, absorbance values and a standard curve are illustrated in Table1 A4.2 and Figure A4.2. Fresh standard curves were prepared for each assay.

Table A4.2: Standard Curve

***N*-carbamoyl amino acid (0-50mM) – Final Volume 1 ml**			
Standard (mM)	**0.1M PO_4 Buffer (ml)**	**1mM glycine in 0.1M PO_4 Buffer (ml)**	**OD $_{420\ nm}$ after Ehrlich's reaction**
0	**1.0**	**0**	**0**
0.1	**0.9**	**0.1**	**0.198**
0.2	**0.8**	**0.2**	**0.412**
0.3	**0.7**	**0.3**	**0.673**
0.4	**0.6**	**0.4**	**0.925**
0.5	**0.5**	**0.5**	**1.143**

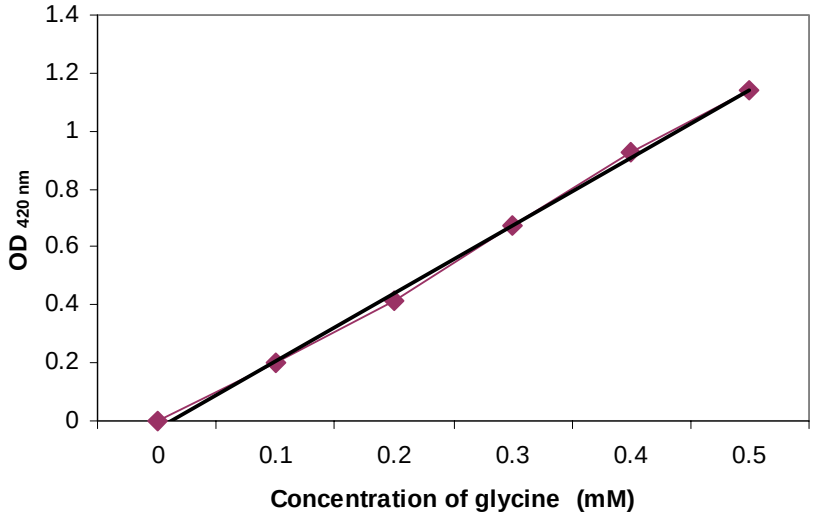


Figure A4.2: Standard curve for reaction of 0-0.5 mM glycine with ninhydrin reagent.

These methods were adapted for the analysis of *N*-carbamoyl-amino acids and amino acids produced from small-scale microtitre resting cell reactions.

Resting Cell Reaction for Hydantoinase Activity of Microtitre Plate Cultures

Centrifuge plate(s) at 4000 rpm for 15 minutes.

Pour off supernatant (flick gently if necessary).

Resuspend each well pellet in 200µl 0.1M phosphate buffer pH 8.0 containing 50 mM hydantoin.

Use the supernatant from each well to perform both an Ehrlich's assay for analysis of *N*-carbamoyl amino acid content and a ninhydrin assay for analysis of amino acid content.

Resting Cell Reaction for *N*-carbamyl amino acid amidohydrolase Activity in Microtitre Plate Cultures

Centrifuge plate(s) at 4000 rpm for 15 minutes.

Pour off supernatant (flick gently if necessary).

Resuspend each well pellet in 200µl 0.1M phosphate buffer pH 8.0 containing 25 mM N-carbamyl glycine.

React plate at 40°C for 4-24hrs with shaking (200rpm).

Centrifuge plate at 4000rpm for 15 minutes.

Use the supernatant from each well to perform a ninhydrin assay for analysis of amino acid content.

Ehrlich's Assay in Microtitre Plate

Remove 10µl of supernatant to a separate microtitre plate (can be a cheaper, non-sterile "ELISA" microtitre plate) for the ninhydrin assay (see below).

Add 30µl of Ehrlichs Reagent (10% p-dimethylaminobenzaldehyde in 6N HCl) to each well.

Dilute with 100µl distilled water.

Read OD_{420nm} on Powerwave Microtitre Plate Reader and compare to a standard curve from 0-50mM N-carbamylglycine prepared in the same volume as the samples (190µL) and treated in the same way as the samples.

Calculate the N-carbamylglycine produced as µmol/ml (same as mM in this instance) and use these values to select microtitre wells containing cultures with hydantoinase activity from the genomic library. Each well contains more than one plasmid-containing cell type,so the master plate must then be streaked to single colonies and these can then be further assayed to select the hydantoinase-producing plasmids.

Ninhydrin Assay in Microtitre Plate (adapted from Wilms et al., 1998).

Aliquot 40µl of 0.1M phosphate buffer pH 8.0 into each well of a duplicate microtitre plate for each plate to be assayed.

Add 10µl of supernatant from the resting cell reactions to the appropriate well, as described above in D. Make up a standard curve of 0.1 to 0.5 mM glycine in a 50µl volume in microtitre wells of a similar microtitre plate.

Add 50µl 4M sodium acetate buffer pH 5.5.

Incubate at 60°C for 5 minutes.

Add 50µl of ninhydrin A solution and react at 60°C for a further 20 minutes.

Add 100µl of 50% ethanol (chemical grade, obtained in this case from Rhodes University Chemistry Stores).

Read the OD_{570nm} using the Powerwave X microtitre plate reader, and compare with the standard curve to calculate the amount of glycine in each well as mM glycine or µmol/ml.

Ninhydrin A Reagent

0.17 g ninhydrin (Fluka, Cat No. 72491)

0.17g hydrindantin (Sigma, Cat No. H-2003)

Dissolve in 20 ml of 2-methoxyethanol (SAARchem, Merck).
Store in the fridge when not in use and make up fresh every day.

A4.2 HPLC detection of *N*-carbamoyl-hydroxyphenylglycine and *p*-hydroxyphenylglycine.

N-Carbamoyl-*p*-hydroxyphenylglycine (NCHPG) and *p*-hydroxyphenylglycine (HPG) were analysed by HPLC using a Macherey-Nagel Nucleosil 100-5 C18 column, 0.01% phosphoric acid mobile phase, and a flow rate of 1.1 ml/min. Retention times: D,L-*p*-hydroxyphenylhydantoin, 4.6 min, *N*-carbamoyl-D,L-*p*-hydroxyphenylglycine, 12.6 min, D,L-*p*-hydroxyphenylglycine, 17.8 min). The amount of NCHPG and HPG produced was calculated by comparison with a standard curve derived from peak areas produced by known concentrations of the compounds.

A4.3 Chiral TLC

Chiral TLC with Macherey-Nagel Chiralplate was used to determine the chirality of alanine and *p*-hydroxyphenylglycine in a mobile phase of acetone/methanol/water (10:5:2), and visualized using ninhydrin reagent B as described in Hartley (1995).

A4.4 Chiral HPLC

Chiral HPLC analysis on reaction supernatants was performed using Beckman System Gold with an ODS C18 coulumn. An eluent of 2mM N, N-dimethyl-L-phenylalanine, 1mM copper acetate, 5% (v/v) methanol was used at a flow rate of 1mLmin^{-1}. Retention times: D-HPG, 5.45 minutes; L-HPG, 7.05minutes. Enantiomeric excess of each enantiomer was calculated as a percentage of the total peak area of both two enantiomers.

A4.5 Calibration of dry cell mass and optical density for growth of RU-OR in MM medium

In order to calibrate the equivalency between cell growth, increase in optical density and dry cell mass, a calibration curve was produced to demonstrate the relationship between OD_{600nm} and dry cell mass (Figure A4.3).

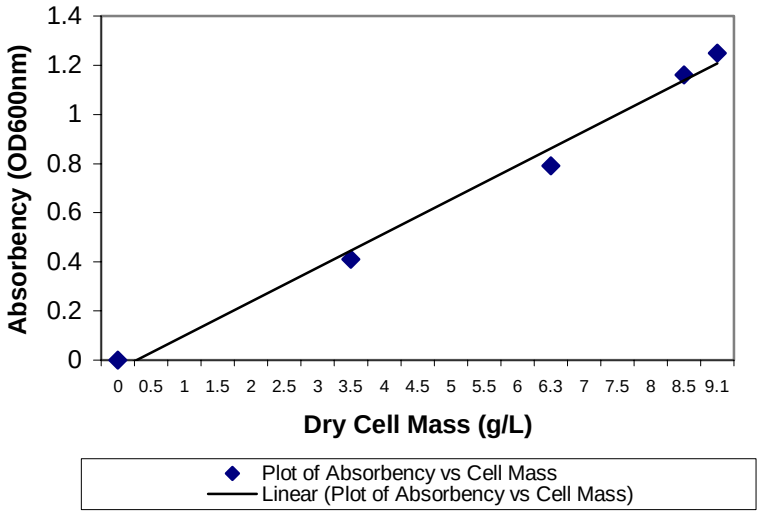


Figure A4.3: Calibration of absorbency with dry cell mass to illustrate OD600nm equates to cell growth. Growth rate average $\mu=0.101\pm0.02$ g/h.

Appendix 5: Alignment of 16S rRNA gene sequences

The complete nucleotide sequence from both strands of DNA encoding the 16 S rRNA gene in *A. tumefaciens* RU-OR was aligned with the closest matching sequence in the small subunit ribosomal database, from *A. tumefaciens* IAM 11391 (Yanagi *et al*., 1993).

```
r16sinsert          ----------------------------------------------CCGCAAGGGAGTGGC
IAM131291           GAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGCCCCGCAAGGGGAGTGGC

r16sinsert        16AGACGGGTGAGTAACGCGTGGGAACATACCCTTTCCTGCGGAATAGCTCCGGGAAACTGG
IAM13129          61AGACGGGTGAGTAACGCGTGGGAACATACCCTTTCCTGCGGAATAGCTCCGGGAAACTGG

r16sinsert        76AATTAATACCGCATACGCCCTACGGGGGGAAAGATTTATCGGGGAAGGATTGGCCCGCGT
IAM13129         121AATTAATACCGCATACGCCCTACGGGGG-AAAGATTTATCGGGGAAGGATTGGCCCGCGT

r16sinsert       136TGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAG
IAM13129         180TGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAG

r16sinsert       196AGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTG
IAM13129         240AGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTG

r16sinsert       256GGGAATATTGGACAATGGGCGCAAGCTG-ATCCAGCCATGCCGCGTGAGTGATGAAGGCC
IAM13129         300GGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCC

r16sinsert       315TTAGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCC
IAM13129         360TTAGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCC

r16sinsert       375GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGGCTAGCGTTGTTCGGAATTA
IAM13129         420GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGG-CTAGCGTTGTTCGGAATTA

r16sinsert       435CTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAATCCCAAGAGCTCAA
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r16sinsert       495CTCTGGAAGGCTGCCTTTGATACTGGGTATCTTGAGTATGGAAGAGGTAAGTGGAATTCC
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```

Appendix 6: Primers used in this study

Table A6.1 describes the primers used in this study for the amplification of DNA. All primers were synthesised by custom oligonucleotide syntheses by either Roche Molecular Biochemicals (South Africa, Germany) or Ransom Hill Bioscience (USA).

Table A6.1: Description of primers used in this study

Primer	Orientation	Length	Sequence (5'-3')	T_m (°C)
PCH1	forward	32	GCGGATCCGCCTAACACATGCAAGTCGAACGG	76.0
PCH3	reverse	32	CGGGATCCCGACGATTACTAGCGATTCCAGCT	72.3
CHAOF1	forward	24	CGGGATCCTTGACCCTGGTCCTTG	65.2
CHAOR1	reverse	24	CCCAAGCTTTCTCGATCGGATAGG	61.0
pUCF	forward	24	CGCCAGGGTTTTCCCAGTCACGAC	66.1
pUCR	reverse	22	TCACACAGGAAACAGCTATGAC	59.5

Appendix 7: Plasmids used in this study

Table A7.1: Plasmids generated during the course of this research

Plasmid	Origin of insert	Vector	Antibiotic resistance marker	Section Reference
pCHH1	Genomic DNA from RU-OR	pBK-CMV	Kanamycin	
pCH1	1.2 kb 16 S rRNA PCR product from A. tumefaciens RU-OR	pUC18	Ampicillin	2.3.3
pCH2	606 bp fragment from Eco R1 (1006) to cloning cassette Hind II (420) in pCH1	pUC18	Ampicillin	2.3.3
pCH3	700 bp fragment from Eco R1 (1006) to cloning cassette Eco RI (1709) in pCH1	pUC18	Ampicillin	2.3.3
pG4	1 kb PCR product amplified from RU-OR genomic DNA using primers NCAA R1 and NCAA F1	pGEM –T-Easy	Ampicillin	5.3.5, 6.3.3
pG4dSI	deletion of 299 bp Sac I fragment from pG4	pGEM –T-Easy	Ampicillin	6.3.3
pG4dSII	deletion of 227 bp Sac II fragment from pG4	pGEM –T-Easy	Ampicillin	6.3.3
pG4dSalI	deletion of 600 bp Sal I fragment from pG4	pGEM –T-Easy	Ampicillin	6.3.3
p6B3-1a	genomic DNA from RU-OR	pBK-CMV	Kanamycin	5.3.4, 6.3.1
p6B3dH	deletion of 1374 bp Hind III fragment from p6B3-1a	pBK-CMV	Kanamycin	6.3.1
p6B3dC	deletion of 416 bp Cla I fragment from p6B3-1a	pBK-CMV	Kanamycin	6.3.1
pBK6B3	1.3 bp Csp451-ClaI fragment from p6B3-1a	pBK-CMV	Kanamycin	6.3.3
pBK6B3dH	deletion of the 376 bp Hind III fragment from pBK6B3	pBK-CMV	Kanamycin	6.3.3
pBK6B3dS	deletion of the 1138 bp Sac I fragment from pBK6B3	pBK-CMV	Kanamycin	6.3.3
pBK6B3dSII	deletion of the 453 bp Sac II fragment from pBK6B3	pBK-CMV	Kanamycin	6.3.3

Plasmids generated from nested deletions of p6B3-1a 5’ to 3’ direction:				
Plasmid	Basepairs	Vector	Antibiotic resistance marker	Section Reference
p6B3-1a	1-3494	pBK-CMV	Kanamycin	6.3.3
PB 3	296-3494			
PB 4.1	357-3494			
PB 5.1	768-3494			
PB 5.3	890-3494			
PB 6	1120-3494			
PB 7.5	1430-3494			
PB 8.2	1854-3494			
PB 12.2	2567-3494			
PB 12.1	2617-3494			
PB 13.4	2956-3494			
PB 12.3	3018-3494			
PB 14.3	3256-3494			
PB 14.7	3289-3494			
PB 20.1	3316-3494			
Plasmids generated from nested deletions of p6B3-1a 3’ to 5’direction:				
Plasmid	Basepairs	Vector	Antibiotic resistance marker	Section Reference
p6B3-1a	1-3494	pBK-CMV	Kanamycin	6.3.3
KX 3.1	1-3120			
KX 5.1	1-2156			
KX 5.3	1-1865			
KX 5.4	1-1756			
KX 6.1	1-1436			
KX 12.1	1-1127			
KX 12.4	1-980			
KX 13.1	1-760			
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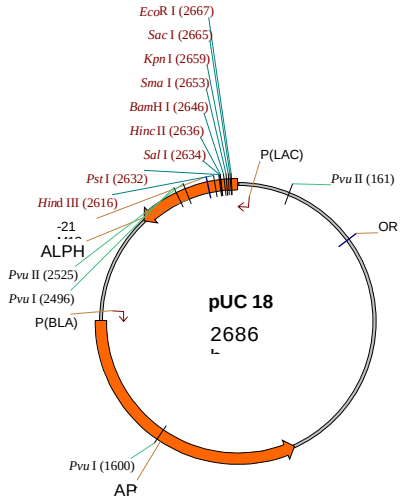
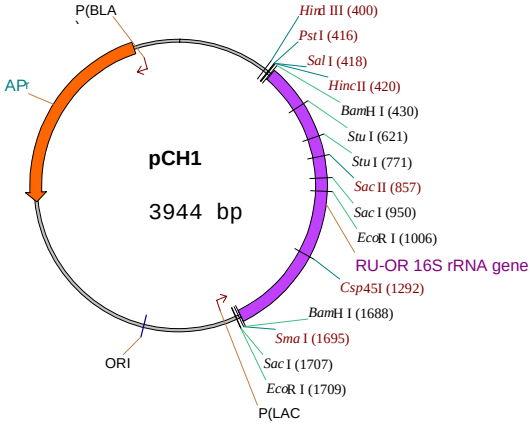
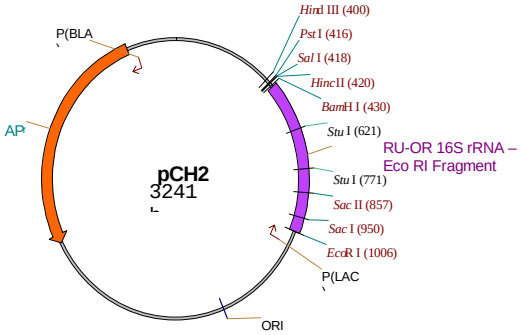
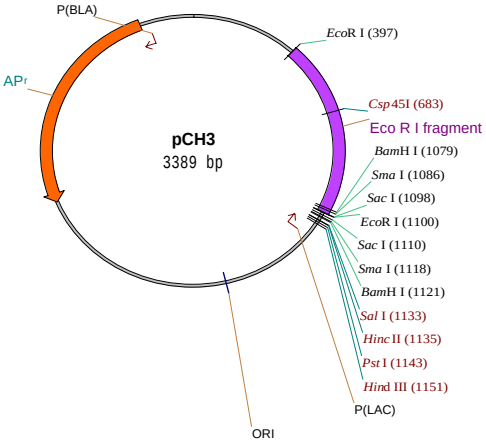
KX 16.2	1-239			
KX 16.3	1-218			

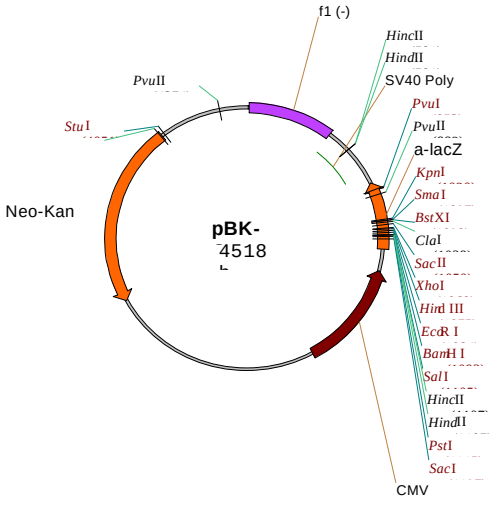
Plasmid maps of some of the relevant plasmids used in this study:

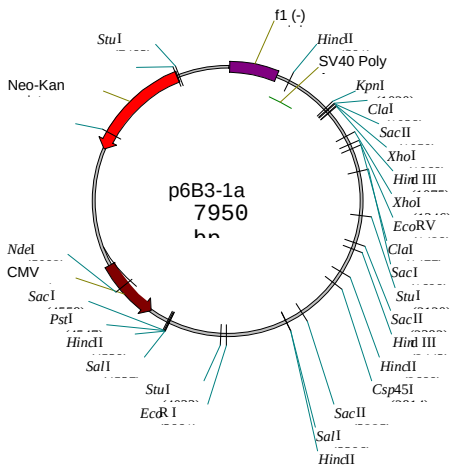


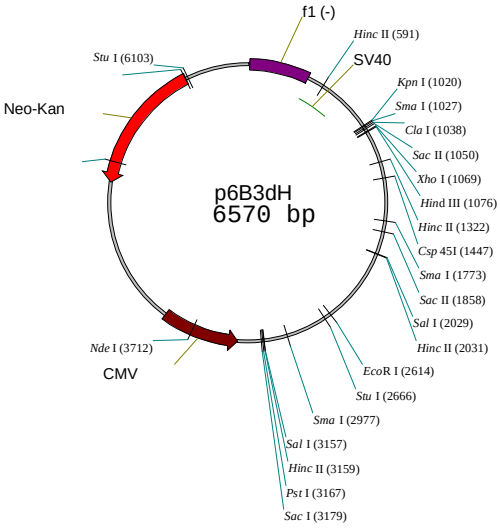








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Xho I (1069)
Hind III (1076)
p6B3dH
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Hinc II (1322)
Csp 45I (1447)
Sma I (1773)
Sac II (1858)
Sal I (2029)
Hinc II (2031)
Nde I (3712)
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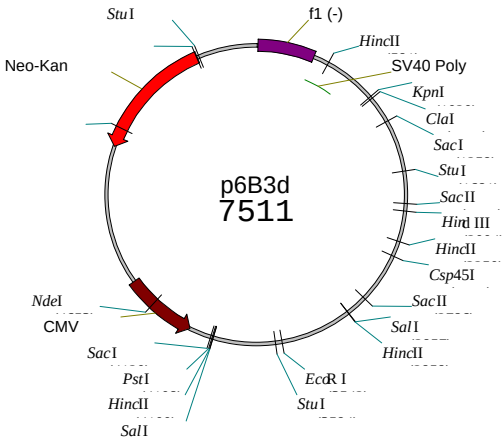
Sac I (3179)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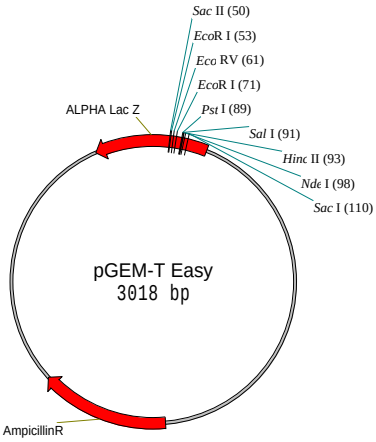
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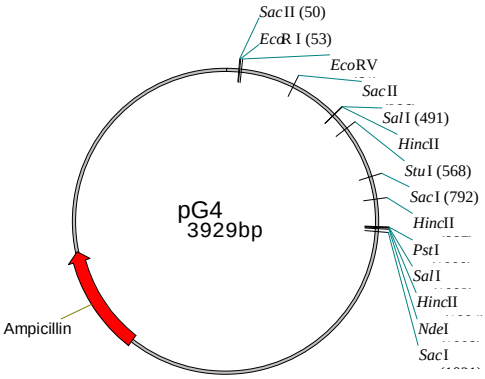
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HincII
NdeI
SacI
Ampicillin

Appendix 8:

Sequence analysis of pGEMH1 using nested deletions and partial DNA sequence of 5'-3' strand of pGEMH1

pCHH1 was sub-cloned into pGEM-T-Easy using tailing of the gel-eluted insert from pCHH1 of to create pGEMH1. Nested deletions of pGEMH1 were selected and sequenced.

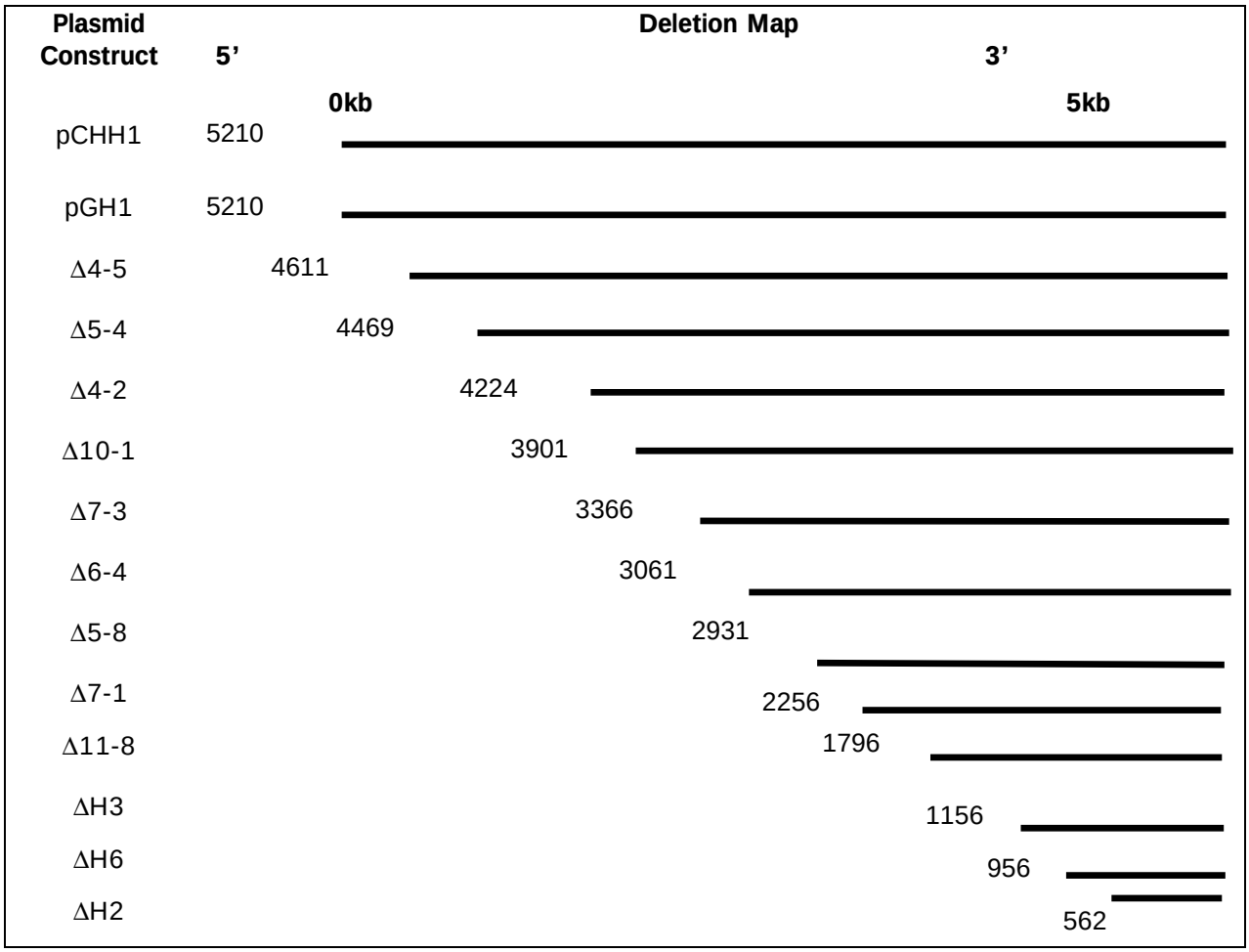


Figure A8.1: Nested deletions of pGH1 used for the compilation of the complete sequence of one strand of the inserted DNA. Numbers represent base pairs in 3' to 5' direction.

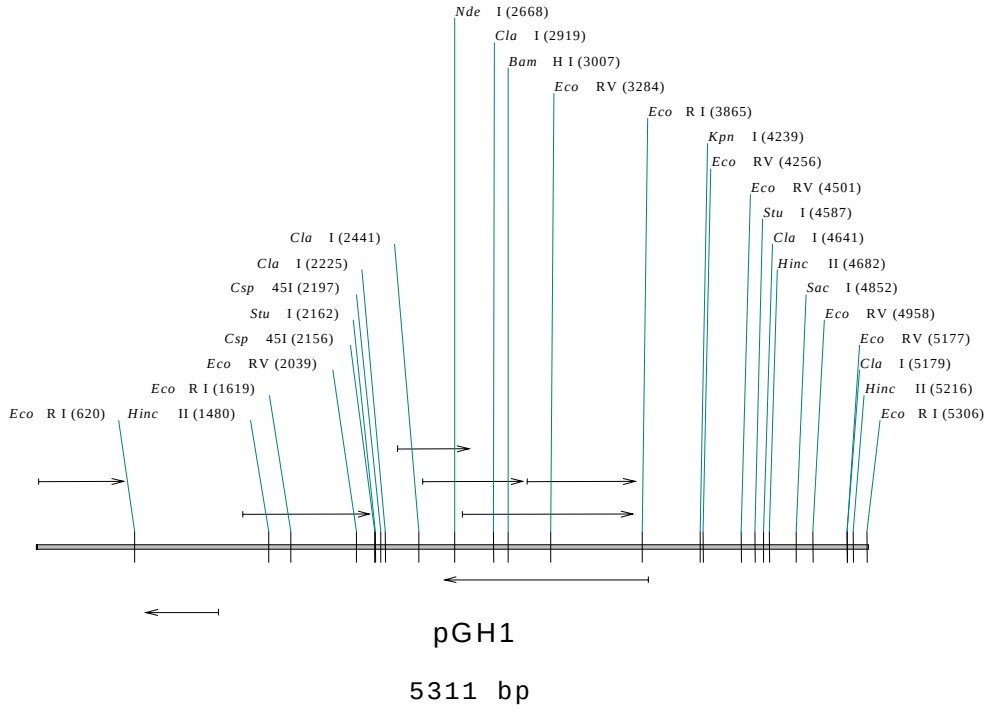
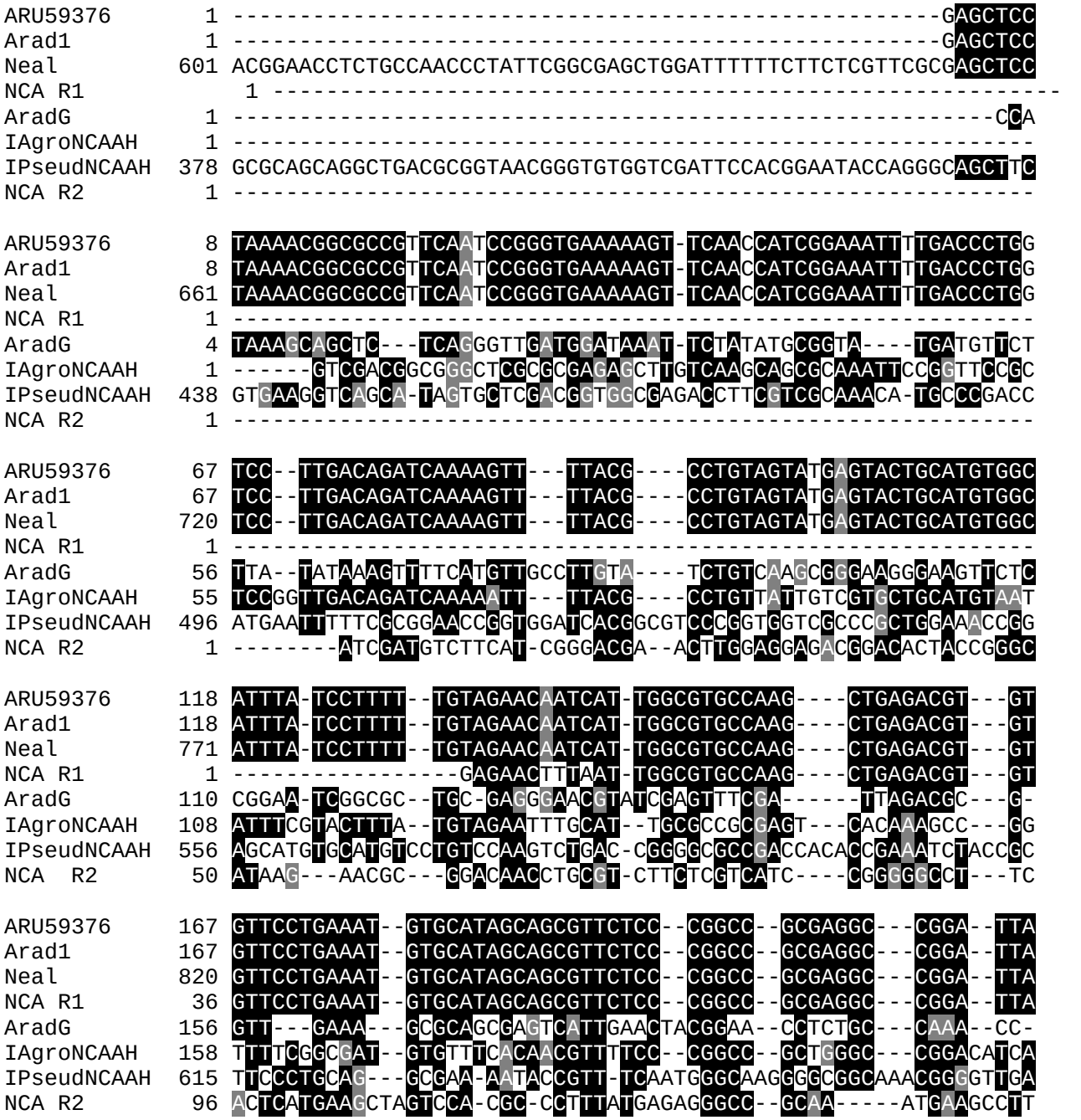
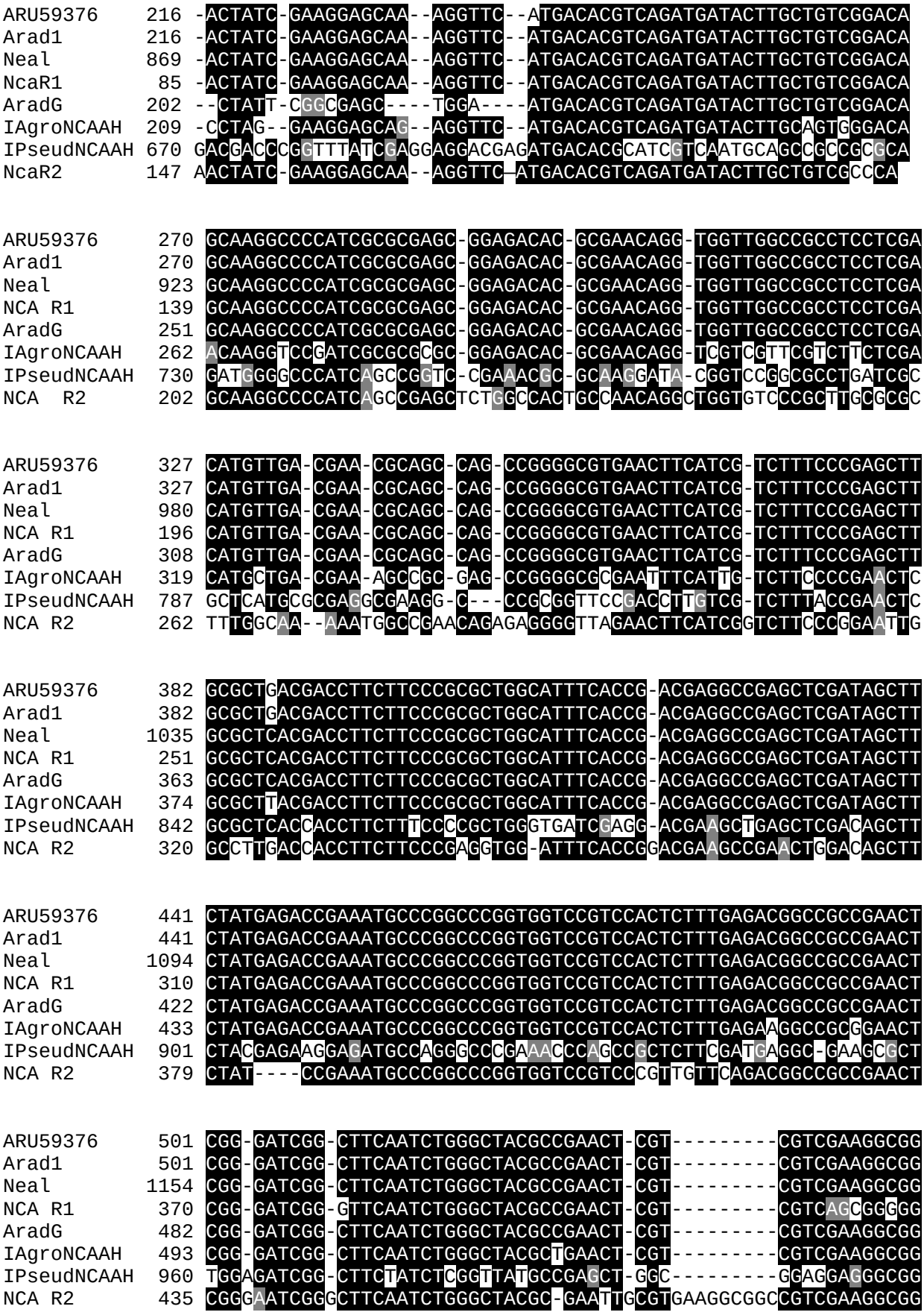


Figure A8.2: Restriction endonuclease map of pGEMH1, showing possible ORFs as predicted by Vector NTI deluxe. None of the suggested ORFs, or other parts of the sequence showed significant identity to amidohydrolase-type enzymes in the BLAST databases.

Appendix 9: Nucleotide sequence alignment of the NCDAAH coding sequences listed in table 6.1

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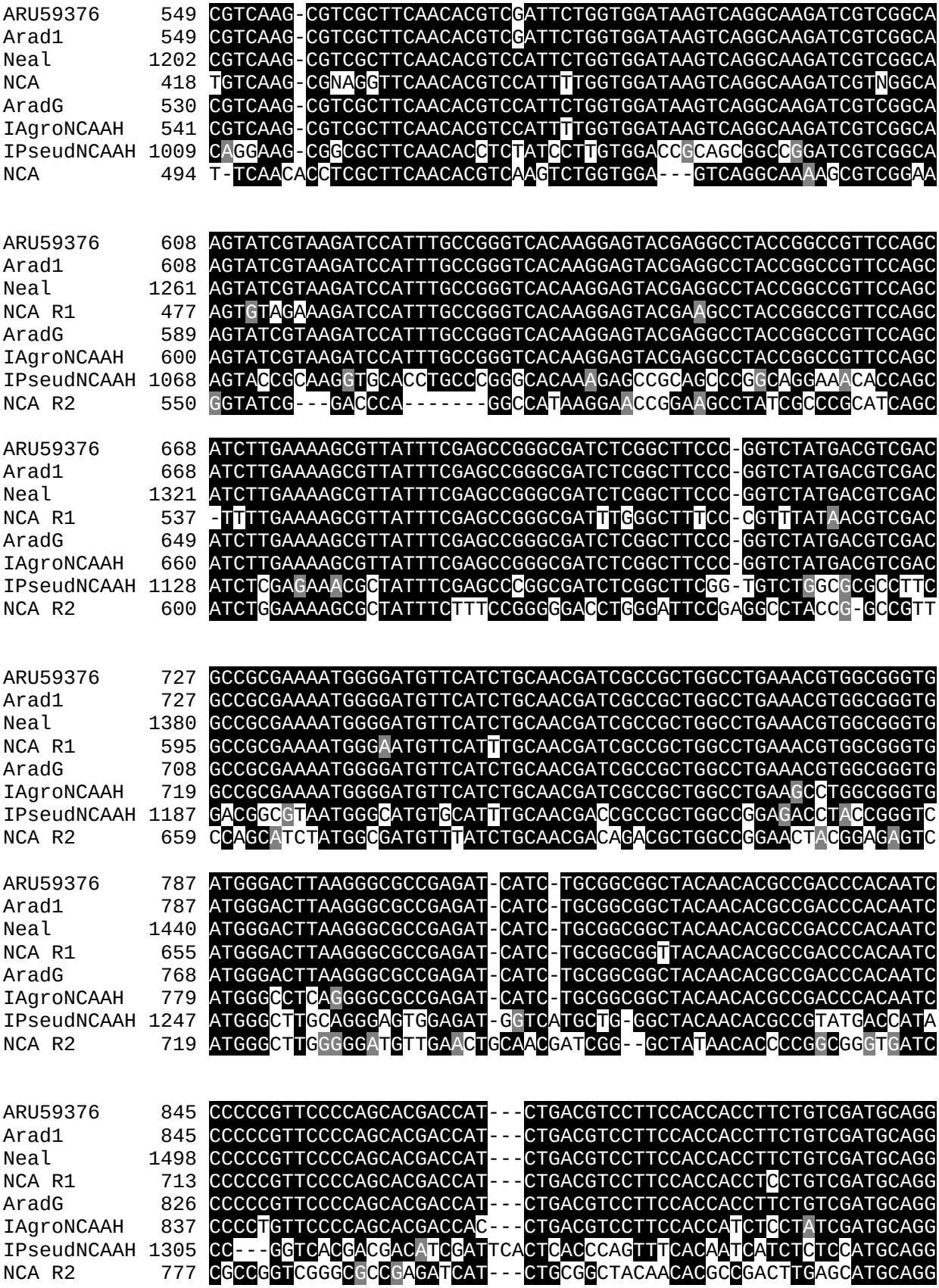
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NCA R1 655 ATGGGACTTAAGGGCGCCGAGAT-CATC-TGCGGCGGTTACAACACGCCGACCCACAATC
AradG 768 ATGGGACTTAAGGGCGCCGAGAT-CATC-TGCGGCGGCTACAACACGCCGACCCACAATC
IAgroNCAAH 779 ATGGGCCTCAGGGGCGCCGAGAT-CATC-TGCGGCGGCTACAACACGCCGACCCACAATC
IPseudNCAAH 1247 ATGGGCTTGCAGGGAGTGGAGAT-GGTCATGCTG-GGCTACAACACGCCGTATGACCATA
NCA R2 719 ATGGGCTTGGGGGATGTTGAACTGCAACGATCGG--GCTATAACACCCGGCGGGTGATC
ARU59376 845 CCCCCGTTCCCCAGCACGACCAT---CTGACGTCCTTCCACCACCTTCTGTCGATGCAGG
Arad1 845 CCCCCGTTCCCCAGCACGACCAT---CTGACGTCCTTCCACCACCTTCTGTCGATGCAGG
Neal 1498 CCCCCGTTCCCCAGCACGACCAT---CTGACGTCCTTCCACCACCTTCTGTCGATGCAGG
NCA R1 713 CCCCCGTTCCCCAGCACGACCAT---CTGACGTCCTTCCACCACCTCCTGTCGATGCAGG
AradG 826 CCCCCGTTCCCCAGCACGACCAT---CTGACGTCCTTCCACCACCTTCTGTCGATGCAGG
IAgroNCAAH 837 CCCCTGTTCCCCAGCACGACCAC---CTGACGTCCTTCCACCATCTCCTATCGATGCAGG
IPseudNCAAH 1305 CC---GGTCACGACGACATCGATTCACTCACCCAGTTTCACAATCATCTCTCCATGCAGG
NCA R2 777 CGCCGGTCGGGCGCCGAGATCAT---CTGCGGCTACAACACGCCGACTTGAGCATGCAGG

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ARU59376      902 CCGGGTCGTACCAAAACGGCGCCTGG-TCCGCGGCGGCCGGCAAGGTCGGCATGGAGGAG
Arad1         902 CCGGGTCGTACCAAAACGGCGCCTGG-TCCGCGGCGGCCGGCAAGGTCGGCATGGAGGAG
Neal         1555 CCGGGTCGTACCAAAACGGCGCCTGG-TCCGCGGCGGCCGGCAAGGTCGGCATGGAGGAG
NCA R1        770 CCGGGTCGTACCAAAACGGCGCCTGG-TCCGCGGCGGCCGGCAAGGTCGGCATGGAGGAG
AradG         883 CCGGGTCGTACCAAAACGGCGCCTGG-TCCGCGGCGGCCGGCAAGGTCGGCATGGAGGAG
IAgroNCAAH    894 CCGGGTCTTATCAGAACGGGGCCTGG-TCCGCGGCCGCGGGCAAGGTGGGCATGGAGGAG
IPseudNCAAH  1362 CGGGCGCCTACCAGAATTCGACCTGGGTGATCGGCA-CCGCCAAATGCGGCACCGAGGAG
NCA R2        834 CCGGCCCATATCAGAACAGCCGCTGG-ACGTCTGACGCCGCGAAAATGGGGATGGAAGAA

ARU59376      961 GGGTGCATGCTGCTCGGCCATT-CGTGCATCGTGGCGCCGACCGGCGAAATCGTTGCCCT
Arad1         961 GGGTGCATGCTGCTCGGCCATT-CGTGCATCGTGGCGCCGACCGGCGAAATCGTTGCCCT
Neal         1614 GGGTGCATGCTGCTCGGCCATT-CGTGCATCGTGGCGCCGACCGGCGAAATCGTTGCCCT
NCA R1        829 GGGTGCATGTTGCTCGGCCATT-CGTGCATCGTGGCGCCGACCGGCGAAATCGTTGCCCT
AradG         942 GGGTGCATGCTGCTCGGCCATT-CGTGCATCGTGGCGCCGACCGGCGAAATCGTTGCCCT
IAgroNCAAH    953 AACTGCATGCTGCTCGGCCACT-CCTGCATCGTGGCGCCGACCGGGGAAATCGTCGCTCT
IPseudNCAAH  1421 GGCTCCAAAATGGTGGGGCAGAGCGTG-ATCGTTGCGCCCTCGGGCGAGATCGTCGCTAT
NCA R2        893 GGCTGCGATCGCCGCGGCCCTG-AATGCATCGTCGCCCCGCGAGGTGTTAAGGGCGCCCG

ARU59376     1020 GACCACGACGTTGGAAGACGAGGTGATCACCGC-CGCCCTCGATCTCGACCGCTGCCGGG
Arad1        1020 GACCACGACGTTGGAAGACGAGGTGATCACCGC-CGCCCTCGATCTCGACCGCTGCCGGG
Neal         1673 GACCACGACGTTGGAAGACGAGGTGATCACCGC-CGCCGTCGATCTCGACCGCTGCCGGG
NCA R1        888 GACCACGACGTTGGAAGACGAGGTGATCACCGC-CGCCGTCGATCTCGACCGCTGCCGGG
AradG        1001 GACCACGACGTTGGAAGACGAGGTGATCACCGC-CGCCGTCGATCTCGACCGCTGCCGGG
IAgroNCAAH   1012 CACTACGACGCTGGAAGACGAGGTGATCACCGC-CGCCGTCGATCTCGATCGCTGCCGGG
IPseudNCAAH  1480 GGCCTGCACGATCGAGGACGAGATCATCACCGCACGCTG-CGATCTCGACATGGGCAAGC
NCA R2        952 ATCAGACTCGTTGGAAAAGCTTCGGATTCCTTCCAGTTC-GGACCTGGAC---TGCAAGA

ARU59376     1079 AA--CTGCGCGAACACATCTTCAATTTCAAAGCCCATCGTCAGCCAC-AGCACTACGGTC
Arad1        1079 AA--CTGCGCGAACACATCTTCAATTTCAAAGCCCATCGTCAGCCAC-AGCACTACGGTC
Neal         1732 AA--CTGCGCGAACACATCTTCAATTTCAAAGCCCATCGTCAGCCAC-AGCACTACGGTC
NCA R1        947 AA--CTGCGCGAACACATCTTCAATTTCAAAGCCCATCGTCAGCCAC-AGCACTACGGTC
AradG        1060 AA--CTGCGCGAACACATCTTCAATTTCAAAGCCCATCGTCAGCCAC-AGCACTACGGTC
IAgroNCAAH   1071 AA--CTGCGTGAACACATCTTCAACTTCAAGCAGCATCGTCAGCCCC-AGCACTATGGTC
IPseudNCAAH  1539 GC--TACCGCGAGACCATCTTCGATTTCGCCCGCCATCGCGAGCCCG-ACGCCTATCGCC
NCA R2       1008 AATACTGGCGGTCCACACGGTTCTTATCGTTACGCCCGGTAGGCCCGGACCATTATCGCC

ARU59376     1136 TGATCGCGGAATTCTGAAGGTCAG-GCCAAAA--AAACGGATGGGGCTGGGGACG-TCGA
Arad1        1136 TGATCGCGGAATTCTGAAGGTCAG-GCCAAAA--AAACGGATGGGGCTGGGGACG-TCGA
Neal         1789 TGATCGCGGAATTCTGAAGGTCAG-GCCAAAA--AAACGGATGGGGCTGGGGACG-TCGA
NCA R1       1004 TGATCAAGGAATTCT-AAGGTCAG-GCC--------------------------------
AradG        1117 TGATCGCGGAATTCTGA-----------------------------------------
IAgroNCAAH   1128 TGATCGCGGAACTCTGAGGTT--G-CCGAAAA--GCATGTGTGTCGTTGT------TCTC
IPseudNCAAH  1596 TGATCGTCGAACGCAAAGGGGCTGTGCCGCCGCCGCAGTGATCGGAACCTGAAAA-CGAA
NCA R2       1068 TGATCGCCGAATTGT--AGTGCTCTACATGCTTCGTCGTGCGCCGGAACTGGTCGAGCGT
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