Isolation, Expression and Purification of the Hydantoin Hydrolysing Enzymes of *Agrobacterium tumefaciens*.

Sally-Ann Clark

Isolation, Expression and Purification of the Hydantoin Hydrolysing Enzymes of *Agrobacterium tumefaciens*.

Thesis submitted in fulfillment of the requirements for the degree of

Master Of Science

of

Rhodes University

by

Sally-Ann Clark

April 2003

ABSTRACT

The production of enantiomerically pure amino acids is of industrial importance as they are used in the synthesis of a number of pharmaceuticals, insecticides and herbicides and biologically active peptides and hormones. A number of microorganisms have been identified which possess hydantoin hydrolysing enzymes that stereoselectively convert racemic hydantoins into enantiomerically pure amino acids. Consequently these microorganisms and their enzymes are sought after as biocatalysts for the production of amino acids.

The isolation of novel hydantoin hydrolysing enzymes with unique or improved biocatalytic characteristics is of importance for the development of potential biocatalysts to be used in the production of enantiomerically pure amino acids. The genes encoding the hydantoin hydrolysing enzymes are linked on the genome. A gene encoding an *N*-carbamoyl-amino acid amidohydrolase, an enzyme involved in the hydrolysis of hydantoin, was isolated by screening a genomic DNA library of *Agrobacterium tumefaciens* RU-AE01. Nucleotide sequence analysis of the region upstream of this gene revealed a fragment of a gene encoding the hydantoinase enzyme. In this study, a DNA probe consisting of the gene encoding the *N*-carbamoyl amino acid amidohydrolase was used to re-isolate the gene encoding the *N*-carbamoyl amino acid amidohydrolase, on a large enough fragment of the genomic DNA library which would allow for the simultaneous isolation the hydantoinase gene located upstream.

Recombinant expression of the genes encoding hydantoin hydrolysing enzymes has been used to facilitate the production and purification of these enzymes for their use as biocatalysts. Two genes (*ncaR1* and *ncaR2*) encoding different *N*-carbamoyl-amino acid amidohydrolases with distinct nucleotide and deduced amino acid sequences were

isolated from the genome of *A. tumefaciens* RU-OR. In this study, the heterologous expression of *ncaR1* and *ncaR2* was explored. Investigation into the optimisation of the heterologous expression of *ncaR1* showed that reducing the growth temperature of the recombinant *E. coli* producing NcaR1 resulted in a two-fold increase in *N*-carbamoyl-amino acid amidohydrolase activity and solubility. Furthermore, NcaR1 was produced with a C-terminal 6xHis tag, but NcaR1-6xHis did not possess *N*-carbamoyl amino acid amidohydrolase activity. Furthermore, purification of NcaR1-6xHis under native conditions using affinity chromatography performed, and used for the production of antibodies.

CONTENTS

TABLE OF CONTENTS

Chapter 1: Literature Review

Chapter 2: Isolation of Genes

Chapter 3: Expression of *ncaR1* and *ncaR1-6xHis*

Chapter 4: Optimisation of Heterologous Expression

Chapter 5: General Conclusions

Appendices

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

bp	base pairs
D-NCAAH	*N*-carbamoyl-D-amino acid amidohydrolase
IPTG	isopropyl-β-D-thiogalactoside
λ	lambda
LB	Luria Bertani
L-NCAAH	*N*-carbamoyl-L-amino acid amidohydrolase
NCAAH	*N*-carbamoyl-amino acid amidohydrolase
Ni-NTA	Nickel-Nitrilotriacetic acid
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamide Gel Electrophoresis
SDS	Sodium dodecyl sulphate

ACKNOWLEDGEMENTS

I would like to thank my project supervisor, Prof. Rosemary Dorrington for her time, patience, encouragement, support and intellectual stimulation during the course of this study. I would also like to thank Dr Stephanie Burton in her capacity as co-supervisor of the Rhodes Hydantoinase Research Group, as well as all the members of the Rhodes Hydantoinase Research Group.

To Lab 471, past and present, thank you for being a wonderful group of individuals. To Arno “Ag-nee” Venter, Anu Idicula and Carol “just-for-one-drink” Kaschula, the senior members of the lab who have already moved on to bigger and better things, thank you for all the help. I have learnt a tremendous amount from you. To Petra Gentz, thank you for all the help and advice with generating the protein purification and antibody production data, and for the beautiful Western Blots we made together. Tho Mesbah Jiwaji and Gwynneth Matcher, thank you for all the advice on the project, and I am so grateful for all the proofreading you did for me. To Keke Mosisili, thank you for not complaining (too much) when I my experiments would spill over onto your bench when mine wasn’t big enough. To Cheryl Walter, Andrew Nel and Michele Tomasacchio, the “babies” of the lab, thank you for making life in the lab interesting. And finally to Val Hogson, the “real boss” of Lab 417, thank you for keeping us all in line and making sure that lab is run as smoothly as it is.

Thank you to Lisa Alexander for being a wonderful friend and listening to all my woes. To Douglas Sanyahumbi and Michael Ludewig, thank you for sharing my writing up experienceand making the worst seem bearable. For all their love and support, I would like to thank my Mom, Keith, Heidi, Carmen and Lynne. To Sean, the love of my life, thank you for standing by me, and particularly looking after me so well for the last few months, despite having your own thesis to write.

For funding this research I gratefully acknowledge The DACST Innovation Fund and the NRF.

CHAPTER 1

Literature Review

CHAPTER 1

Literature Review

1.1. The industrial importance of α-amino acids.

The industrial synthesis of optically pure α-amino acids is lucrative as they have a number purposes (Demain, 2000). Their nutritional value allows them to be used in animal feed supplements and also supplements for human consumption. They are also used as chiral building blocks in the chemical synthesis of other chemicals and molecules that are of importance in the pharmaceutical and agrochemical industries, such as biologically active peptides and hormones, peptide derivatives, semi-synthetic antibiotics and chemical pesticides and herbicides. A few examples of industrially important D- and L-α-amino acids and their uses are listed in Table 1.1.

Table 1.1. Some examples of α-amino acids and their industrial applications.

Amino Acid	Industrial Application		Example	Reference
D,L-methionine	animal feed	feed additive		Syldatk *et al.,* 1990
L-phenylalanine	food supplement	synthetic sweetner	α-aspartame	Kamphuis *et al.,* 1992
L,D-homophenylalanine	pharmaceuticals	component of ACE inhibitors	enalapril benzapril	Kamphuis *et al.,* 1992
L-valine	pharmaceuticals	biulding block of immune suppresant used in transplant surgery	cyclosporin A	Kamphuis *et al.,* 1992
D-citrulline	biologically active peptide	LH-RH antagonist for treating sex-hormone dependant diseases	cetrorlix	Drauz *et al.,* 1991
D-(hydroxy)phenylglycine	semi-synthetic antibiotics	side chain of β-lactam antibiotics	(amoxycillin) ampicillin	Louwrier and Knowles, 1997
D-valine	pesticide	component of pyrethroid insecticide	fluvalinate	Drauz , 1997

1.1.1. The synthesis of amino acids

A number of methods have been used for the synthesis of bulk α-amino acids, such as extracting them from plant and animal tissue hydrolysates (Rosazza, 1995), or from biological fermentation using cheap substrates such as sugars or molasses (Morin *et*

al., 1990). A number of chemical processes have been developed for the synthesis of α-amino acids too, such as the carboxylation of hydantoins (Finkbeiner, 1965), the Strecker synthesis and the chemical alkylation of hydantions (Barret, 1985). But these processes are complex and require extreme reaction conditions such as high temperatures and extreme pH. Furthermore, the synthesis of amino acids by these chemical methods required the optical resolution of the amino acid products once they were synthesised (Ottenheym and Janneskens, 1970; Sano *et al.,* 1976). This is particularly true if they are to be used in the synthesis of fine chemicals, and this often led to a low product yield. So alternative methods for the synthesis of amino acids were highly sought after, and in the late 1970's an awareness of the benefits of biochemical processes using biocatalysts developed.

Biocatalytic processes are defined as the cultivation and use of microorganisms or soluble or immobilised enzymes in media containing organic compounds as substrates. In such processes, enzymes alter the structures of organic substrates to synthesise new compounds (Rosazza, 1995). Tramper (1996) highlighted the benefits of biocatalytic processes in an extensive review of the use of biocatalysts over chemical methods, and a few of them are as follows. The use of biocatalysts often reduces the number of reaction steps in the process, possess faster reaction rates and milder reaction conditions which are considered natural and therefore more "environmentally friendly". Biocatalytic reactions can be manipulated to require fewer and cheaper reagents, and often have higher yields. Moreover, the enantiospecific nature of reactions catalysed by some biocatalysts will often reduce the production of by-products, thereby minimising downstream processing and purification of the product.

1.1.2. Biochemical, biological and enzymatic synthesis of optically active amino acids.

The biochemical productions of a number of optically active amino acids by a variety of hydrolases from a wide range of microorganisms have been developed. Proteases such as chymotrypsin, subtilisin and thermolysin are used for the synthesis of *N*-acyl amino acids for the synthesis of non-natural peptides (Wang *et al.,* 1997; Ishikawa, 1999). Amidases, such as the one from *Ochrobactrum anthropi* have been used for the

synthesis of natural and non-natural amino acids, which are of considerable importance in pharmaceutical applications (Sonke *et al.,* 1999). Acylases, such as the penicillin acylase from *Penicillium* is used for the hydrolysis of penicillin G or V to 6-aminopenicillanic acid, which is used as an important semisynthetic anitibiotic (Bruggink *et al.,* 1998). Oxidoreductases have been applied to the synthesis of non-natural amino acid, such as the production of L-*tert* leucine using leucine dehydrogenase (Krix *et al.,* 1997). But possibly, one of the most successfully applied biocatalysts for the synthesis of optically active amino acids is the use of hydantoin hydrolysing enzymes. They are used to convert the relatively cheap substrate of D,L-5-monosubstituted hydantoins to optically pure natural or unnatural D-and L-amino acids (Syldatk *et al.,* 1992; Volkel and Wagner, 1995; Schulze and Wubbolts, 1999).

1.2. The application of the hydantoin utilising enzymes for the synthesis of optically pure amino acids.

1.2.1. The 'Hydantoinase' process.

Optically active amino acids can be prepared from racemic mixtures of 5-monosubstituted hydantoins by a sequence of two enzyme catalysed reactions, utilising hydantoin hydrolysing enzymes, as illustrated in Figure 1.1. The first enzyme catalysed reaction is the hydrolysis of D,L-5-monosubstituted hydantoins to the corresponding D or L *N*-carbamoyl-amino acid, by a D- or L-stereospecific hydantoinase, or by a non-selective hydantoinase (Syldatk *et al.,* 1990a, 1992, 1999; Drauz, 1995; Ogawa and Shimizu, 1997). The second enzyme catalysed reaction is the stereospecific hydrolysis of the D- or L-*N* carbamoyl-amino acid intermediate to D- or L-amino acids by D- or L-stereospecific *N*-carbamoyl-amino acid amidohydrolase (D-NCAAH or L-NCAAH) (Syldatk *et al.,* 1990, 1992; Drauz, 1995; Ogawa and Shimizu, 1997). This has been referred to as the 'hydantoinase' process (Syldatk *et al.,* 1992, 1999; Drauz, 1995; Ogawa and Shimizu, 1997, Bommarius *et al.,* 1998). Spontaneous racemisation or enzyme catalysed racemisation of the 5-monosubstitued hydantoins also occurs (Syldatk *et al.,* 1990a, 1992, Drauz, 1995; Watabe *et al.,* 1992b,c).

The discovery of the *N*-carbamoyl-amino acid amidohydrolases (NCAAH) only came after the discovery of the hydantoinases (Olivieri *et al.,* 1979), and until then, carbamoyl-amino acids were converted to the corresponding amino acids by chemical methods under conditions where complete retention of configuration was obtained (Cecere, 1975). However chemical hydrolysis of *N*-carbamoyl-derivatives had a number of disadvantages, such as the long reaction time, the high reaction temperature and the low yields and could only be used on *N*-carbamoyl-amino acids, which did not contain oxidisable groups (Olivieri *et al.,* 1981). So, the discovery of the *N*-carbamoyl-amino acid amidohydrolases changed this, allowing for a purely bioenzymatic process (Ogawa and Shimizu, 1999).

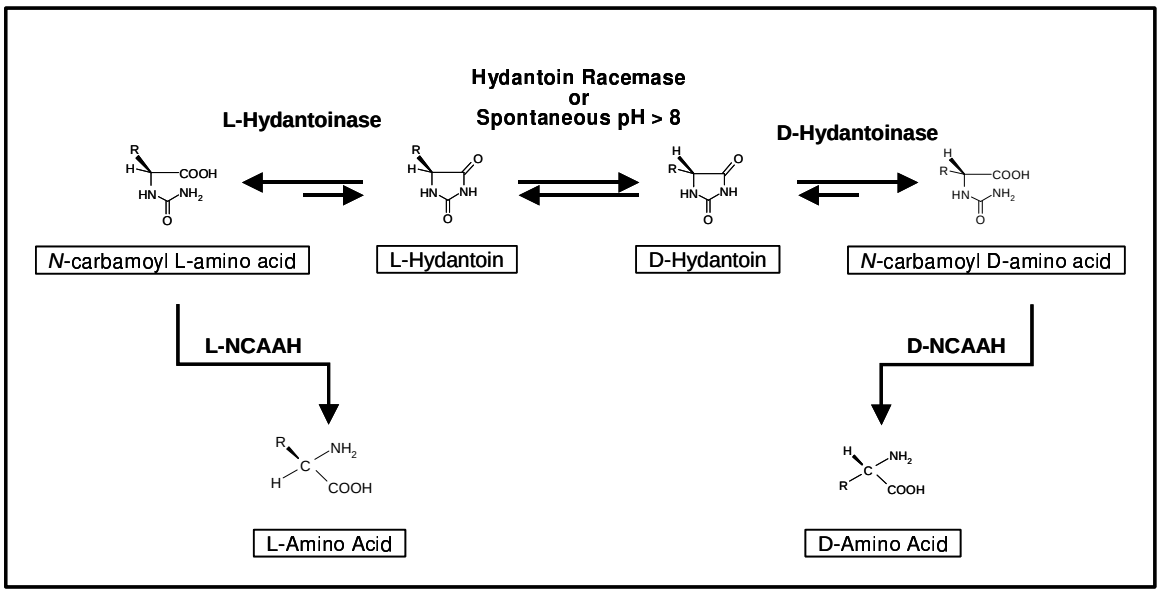


Figure 1.1. Reaction scheme for the enzymatic cleavage of D,L-5-monosubstituted hydantoin derivatives to the corresponding D- or L- amino acids, using the 'hydantoinase' process (adapted from Bommarius *et al.,*1998).

1.2.2. Advantages of the hydantoinase process.

The advantages of using the hydantoinase process for the synthesis of amino acids over other biochemical and chemical method are numerous and have been reviewed extensively (Syldatk *et al.,* 1990a, 1992; Ogawa and Shimizu, 1997*;* Bommarius *et al.,* 1998; Schulze *et al.,* 1999; Altenbuchner 2001).

To begin with, the 5-monosubstitued hydantoins undergo rapid and spontaneous racemisation under certain conditions (Syldatk *et al.,* 1990a, 1992) therefore allowing the complete conversion of the racemic substrate to the D- or L-amino acid product, which gives the process a theoretical yield of 100% (Olivieri *et al.,* 1981, Louwrier and Knowles 1997; Syldatk *et al.,* 1999). Furthermore, enzymatic racemisation of the 5-monosubstituted hydantoins by hydantoin racemases possessed by some microorganisms also act to make the reaction more efficient (Watabe *et al.,* 1992c; Ishikawa *et al.,* 1997; Wiese *et al.,* 2000b).

The advantages of using 5-monosubstituted hydantoins as substrates for the production of amino acids are two-fold. Firstly, the 5-monosubstituted hydantoins can be synthesised relatively cheaply and easily by Bücherer-Bergs synthesis (Bücherer and Stein, 1934) or by the condensation of aldehydes (Syldatk *et al.,* 1992) (Figure 1.2). Secondly, the R-group of the 5-monosubstituted hydantoin can be manipulated and changed, making it possible to synthesise virtually any natural or non-natural α-amino acid (Syldatk *et al.,* 1992, Bommarius *et al.,* 1998, Drauz *et al.,* 1997), including those which would not be possible to synthesise by chemical means (Syldatk *et al.,* 1999).

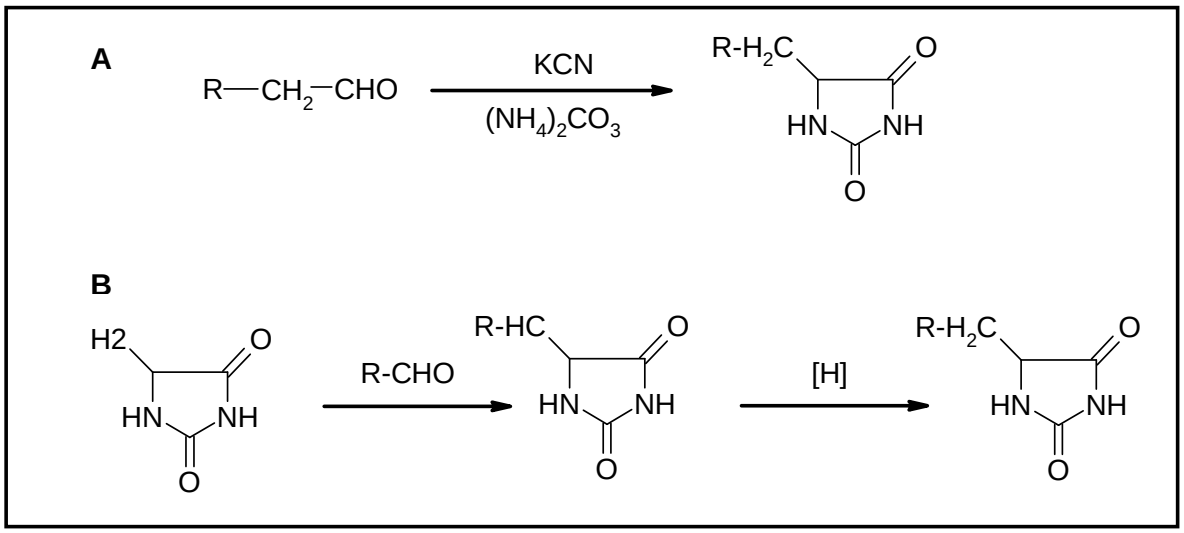


Figure 1.2. Chemical synthesis of 5-monosubstituted hydantoins by Bucherer-Bergs synthesis (A) and condensation of aldehyde (B) (Adapted from Syldatk *et al.,* 1992)

In addition, the use of the hydantoinase process for the synthesis of α-amino acids is a biocatalytic process, and is performed under relatively mild reaction conditions with few

waste products, and therefore is considered “ecologically friendly” when compared to the alternative chemical methods (Syldatk *et al.,* 1999).

1.2.3. The classification of hydantoin hydrolysing enzymes.

Wallach and Grisolia (1957) were the first to propose that hydantoinases are identical to dihydropyrimidinases, and according to EC nomenclature hydantoinase is an alternative name for dihydropyrimidinase (EC 3.5.2.2.), which are enzymes grouped with the cyclic amidases (EC 3.5.2), which are comprised of the enzymes involved in the biosynthesis or degradation of purines and pyrimidines (May *et al.,* 1998d, Syldatk *et al.,* 1999). But this classification is problematic for the following reasons.

While the majority of hydantoinases are functionally similar to the dyhydropyrimidinases, some hydantoinases are not and have been classed in other enzyme groups of the cyclic amidases, such as allantoinase (EC 3.5.2.5), carboxymethylhydantoinase (EC 3.5.2.4) and *N*-methylhydantoinase (EC 3.5.2.14) (May *et al.,* 1998d; Syldatk *et al.,* 1999). Moreover, there are some hydantoinases that have been described but have not yet been classified, such as carboxyethylhydantoinase (Hassel and Greenberg, 1963), a D-hydantoinase isolated and characterised by Runser and Meyer (1993), an ATP-dependant L-hydantoinase isolated by Yamashiro *et al.* (1988) and Ishikawa *et al.* (1997) and a non-specific hydantoinase by May *et al.* (1998a,b,c). Furthermore, phylogenetic analysis shows that these enzymes form separate branches from the hydantoinases classed within the cyclic amidases, which suggests that they are different from these enzymes, but might have evolved from a common ancestor, and are probably product of divergent evolution prior to the formation of Archaea, Eukarya and Bacteria (May *et al.,* 1998).

Amidohydrolases are important in the mineralisation of organic nitrogen in the soil (Acosta-Martinez and Tabatabai, 2001) and *N*-carbamoyl-amino acid amidohydrolases are thought to be similar to β-ureidopropionase (EC 3.1.5.6) from the pyrimidine degradative pathway (Möller *et al.,* 1988; Syldatk *et al.,* 1990a). But it has been shown that D- and L-*N*-carbamoyl-amino acid amidohydrolases are very different from each other, and in some cases show no similarity to the β-ureidopropionases (Ogawa *et al.,* 1995c; Ogawa and Shimizu, 1997). It is interesting to note that putative cyclic

amidohydrolases are ubiquitously distributed in both eukaryotic and prokaryotic cells (Kim *et al.,* 2000c).

1.3. The natural distribution of hydantoinases and *N*-carbamoyl-amino acid amidohydrolases.

The first report of hydantoin hydrolysis in organisms was the metabolism of hydantoin derivatives in dogs (Syldatk *et al.,* 1990a). Subsequent studies showed that hydantoinase activity was also found in plants (Eadie *et al.,* 1949) and in different tissues of certain animals (Bernheim and Bernheim, 1946; Brooks *et al.,* 1983; Syldatk *et al.,* 1990a). More recently, the hydantoinase from various legumes have been discovered (Morin, 1993) and a D-hydantoinase has been isolated and characterised from the Adzuki bean (*Vignia angularis*) (Arcuri *et al.,* 2000; Fan and Lee, 2001).

The most studied hydantoin hydrolysing enzymes are from bacteria, as they provide the most desirable hydantoin hydrolysing biocatalysts for the production of optically active α-amino acids (Ogawa and Shimizu, 1997; Syldatk *et al.,* 1992). This review will focus on these hydantoin hydrolysing microorganisms.

1.3.1. The distribution of hydantoinases and *N*-carbamoyl-amino acid amidohydrolases in bacteria

The distribution of hydantoin utilising enzymes is widespread in both Gram positive and Gram negative bacteria. They have been reported in a number of soil bacteria, such as *Streptomyces griseus* (Gram positive)(Takahashi *et al.,* 1978), *Nocordia coallina* (Gram positive)(Kamphuis *et al.,* 1992), a number of species of *Arthrobacter* (Gram positive)(Möller *et al.,* 1988; Gross *et al.,* 1990; Volkel and Wagner, 1995; Siemann *et al.,* 1999; May *et al.,* 1996, 2000) and a number of *Agrobacterium* species (Olivieri *et al.,* 1981; Meyer and Runser, 1993; Louwrier and Knowles, 1997; Nanba *et al.,* 1998a; Hartley *et al.,* 1998). A large number of Pseudomonads (Gram negative) have also been reported to possess hydantoin hydrolysing enzymes, such as *Pseudomonas* sp. KNK 003A (Ikenaka *et al.,* 1998a), *P. fluorescens* DSM 84 (Morin *et al.,* 1986b), *P. putida* CCRRC 12857 (Chein *et al.,* 1998), *Pseudomonas* sp RU-KM1 and RU-KM3$_S$

(Burton *et al.,* 1998; Buchanan *et al.,* 2001) and *Commamonas* sp. E222c (Ogawa *et al.,* 1993).

There have also been reports of hydantoin hydrolysing enzymes in some endospore-forming bacteria (Gram positive). Dagys *et al.,* (1992) and Syldatk *et al.,* (1990a) reported the presence of a D-hydantoinase in *Bacillus circulans* and *Clostridium uracilicum* respectively. Thermophiles have been the source of thermostable hydantoin hydrolysing enzymes. A number of strains of *B. stearothermophilus* have been shown to possess thermostable D-hydantoinases (Lee *et al.,* 1994; Kim *et al.,* 1997; Keil *et al.,* 1995) and thermostable *N*-carbamoyl-L-amino acid amidohydrolases (Batisse *et al.,* 1997; Mukohara *et al.,* 1993). Also, Abendroth *et al.* (2000a,b) reported the isolation of a thermostable D-hydantoinase from a *Thermus* species.

Furthermore, Morin *et al.* (1992) and Kamphuis *et al.* (1992) reported the detection of D-hydantoinase activity in the anaerobic bacterium *Peptococcus anaerobius* CRDA 303 and *Mycobacterium smegmatis* respectively. More surprisingly, a D-hydantoinase was isolated from *Escherichia coli* K12 (Kim *et al.,* 2000c) that does not possess hydantoinase activity.

Generally, hydantoinases and *N*-carbamoyl-amino acid amidohydrolases are classified according to their substrate specificity or their stereoselectivity (Syldatk, 1999). Here, they will be divided up according to the stereoselectivity with which the bacteria utilise the hydantoins, that is, L-selective bacterial systems (Table 1.1) and D-selective bacterial systems (Table 1.2).

Some of the bacteria that are L-specific for hydantoin hydrolysis possess an L-hydantoinase and an L-NCAAH (Table 1.1), while some only possess an L-hydantoinase or an L-NCAAH (Table 1.1). There are also some that possess a non-specific or an L-"preferential" hydantoinase, but when coupled with an L-NCAAH, they are still L-specific (Ishikawa *et al.,* 1993, 1994).

Table 1.1. A list of L-specific hydantoin hydrolysing bacteria and the hydantoin hydrolysing enzymes they possess.

Microorganism	Enzymes	Reference
Alcaligenes xylooxidans	L-NCAAH	Ogawa *et al.,* 1995a; 2001
Arthrobacter aurescens DSM 3745	L-hydantoinase and L-NCAAH	May *et al.,* 1996; 1998a,b,c; Wiese *et al.,* 2001a
Arthrobacter aurescens DSM 3747	L-hydantoinase and L-NCAAH	Gross *et al.,* 1990; Syldatk *et al.,* 1990b; Siemann *et al.,* 1993; Pietzsch *et al.,* 2000; Ragnitz *et al.,* 2001a,b; Wilms *et al.,* 1999; Wiese *et al.,* 2000; 2001a,b
Arthrobacter sp. DSM 7330	L-Hydantoinase	Volkel and Wagner 1995; Wagner *et al.,* 1996
Bacillus brevis AJ 12299	L-hydantoinase and L-NCAAH	Yamashiro *et al.,* 1988; Syldatk *et al.,* 1990;
Bacillus brevis ATCC 8185	L-Hydantoinase	Tsugawa *et al.,* 1966
Bacillus brevis sp.	L-Hydantoinase	Kamphuis *et al.,* 1990
Bacillus stearothermophilus NCIB 8224	L-NCAAH	Batisse *et al.,* 1997
Bacillus stearothermophilus NS1122A	Non-specific Hydantoinase and L-NCAAH	Ishikawa *et al.,* 1994; Mukohara *et al.,* 1993; 1994
Blastobacter sp. A17p-4	L-hydantoinase and L-NCAAH	Tsuji *et al.,* 1997; Ogawa 1995c
Clostridium oroticum	L-hydantoinase and L-NCAAH	Lieberman and Kornberg, 1955; Syldatk *et al.,* 1990;
Flavobacterium ammonigemes	L-Hydantoinase	Kamphuis *et al.,* 1990
Flavobacterium sp AJ 3912	L-hydantoinase and L-NCAAH	Yokozeki *et al.,* 1987d; Syldatk *et al.,* 1988; 1990 ;
Flavobacterium sp I-3	L-hydantoinase and L-NCAAH	Nishida *et al.,* 1987;
Pseudomonas putida RU-KM3$_S$	Non-specific Hydantoinase and L-NCAAH	Buchanan *et al.,* 2001; Burton *et al.,* 1998
Pseudomonas putida 77	L-Hydantoinase	Kim *et al.,* 1987; Ogawa *et al.,* 1995c
Pseudomonas sp. AJ 11220	L-NCAAH	Yokozeki *et al.,* 1987; Syldatk *et al.,* 1990;
Pseudomonas sp. NS 671	Non-specific Hydantoinase and L-NCAAH	Ishikawa *et al.,* 1993; 1996; 1997; Watabe *et al* 1992a,b,c

Since the synthesis of D-amino acids has been found to be more lucrative than the synthesis of L-amino acids, the number of D-specific hydantoinase systems revealed in bacteria outnumbers those of the L-specific systems (Bommarius *et al.,* 1998). Some of the bacteria that possess D-specific hydantoin utilising enzymes have been found to possess a D-hydantoinase and a D-NCAAH, or only the D-hydantoinase or a D-NCAAH (Table 2.1).

1.3.2. The distribution of hydantoin racemases in bacteria.

Racemisation of 5-monosubstituted hydantoins occurs through keto-enol tautomerism (Figure 1.3) and the rate of racemisation is greatly affected by the electronegativity of the substituent R-group. More electronegative substituents as well as basic pH and increasing temperatures speed up racemisation rates (Syldatk *et al.,* 1992b). Hydantoin racemase enzymes increase the rate of racemisation considerably, and have been detected in a number of bacteria, but have only been isolated and characterised from *Arthrobacter aurescens* DSM 3747 (Wiese *et al.,* 2000), *Arthrobacter aurescens* DSM 3745 (Wilms *et al.,* 2001), *Pseudomonas* sp. NS 671 (Watabe *et al.,* 1992a,b,c) and *Agrobacterium sp.* IP I-671 (Hils *et al.,* 2001). The hydantoin racemases are discussed further in Section 1.3.3.

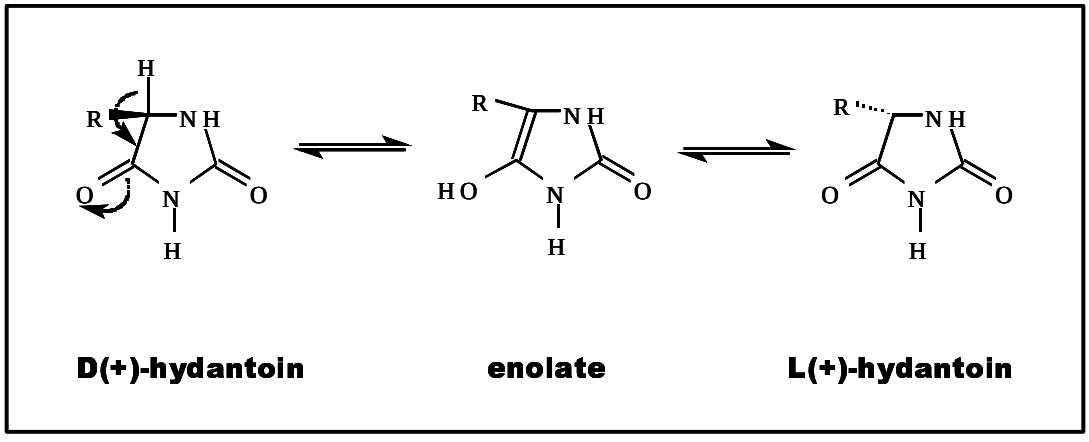


Figure 1.3. Keto-enol tautomerism of 5-monosubstituted hydantoin derivatives (Adapted from Lee and Fan, 1999).

Table 1.2. A list of D-specific hydantoin hydrolysing bacteria that possess and the hydantoin hydrolysing enzymes they possess.

Microorganism	Enzymes	References
Aerobacter cloacae IAM 1221	D-Hydantoinase	Takahashi *et al.,* 1978; Yamada *et al.,* 1980
Agrobacterium radiobacter CCRC 14924	D-NCAAH	Hsu *et al.,* 1999, Wang *et al.,* 2001
Agrobacterium radiobacter KNK 712	D-NCAAH	Ikenaka *et al.,* 1998a,b,c; 1999; Nanba *et al.,* 1998a,b; 1999; Ogawa *et al.,* 1999; Nakai *et al* , 2000
Agrobacterium radiobacter sp. SB	D-NCAAH	Louwrier and Knowles, 1996; 1997
Agrobacterium rhizogenes IFO 13259	D-Hydantoinase	Yamada *et al.,* 1980
Agrobacterium sp IP I-671	D-Hydantoinase and D-NCAAH	Runser and Ohleyer, 1990; Runser *et al.,* 1990; Meyer and Runser, 1993
Agrobacterium tumefaciens 47C	D-Hydantoinase	Durham and Weber, 1995; 1996
Agrobacterium tumefaciens AM10	D-NCAAH	Sareen *et al.,* 2001a,b
Agrobacterium tumefaciens NRRL B1129	D-Hydantoinase and D-NCAAH	Olivieri *et al.,* 1979; 1981
Agrobacterium tumefaciens RU-AE01	D-NCAAH	Herrera, Unpublished data
Agrobacterium tumefaciens RU-OR	D-Hydantoinase and D-NCAAH	Burton *et al.,* 1998; Hartley *et al.,* 1998; 2001
Arthrobacter crystallopoietes AM2	D-Hydantoinase and D-NCAAH	Moller *et al.,* 1988; Syldatk *et al.,* 1990;
Arthrobacter crystallopoietes DSM 20117	D-Hydantoinase	Siemann *et al.,* 1999
Arthrobacter sp. DSM 9771	D-Hydantoinase	May *et al.,* 1998
Bacillus circulans	D-Hydantoinase	Dagys *et al.,* 1992; Luksa *et al.,* 1997
Bacillus sp. AR9	D-Hydantoinase	Sharma and Vohra, 1997
Bacillus stearothermophilus SD1	D-Hydantoinase	Lee *et al.,* 1994; 1996a,b; Kim *et al.,* 1997a,b; 1998; Kim and Kim, 1998a; Park *et al.,* 2000; Cheon *et al.,* 2000; 2002; Lee and Kim, 1998
Bacillus thermocatenulatus GH2	D-Hydantoinase	Park *et al.,* 1998; Kim *et al.,* 1997a; 1999; 2000a,b
Bacillus thermoglucosidans	D-Hydantoinase	Keil *et al.,* 1995
Blastobacter sp. A17p-4	D-Hydantoinase and D-NCAAH	Ogawa *et al.,* 1994a,b; 1995c
Clostridium uracilicum	D-NCAAH	Syldatk *et al.,* 1990
Comamonas sp. E222c	D-NCAAH	Ogawa *et al.,* 1993; 1994b

Table 1.2.cont. A list of D-specific hydantoin hydrolysing bacteria and the hydantoin hydrolysing enzymes they possess.

Microorganism	Enzymes	References
Corneybacterium pseudodiptheriticum 14.10	D-Hydantoinase	Morin *et al.,* 1990
Corneybacterium sepedonicum IFO 3306	D-Hydantoinase	Takahashi *et al.,* 1978; Yamada *et al.,* 1980
Esherichia coli K12	D-Hydantoinase	Kim *et al.,* 2000
Mycobacterium smegmatic	D-Hydantoinase	Kamphuis *et al.,* 1990
Nocardia coallina	D-Hydantoinase	Kamphuis *et al.,* 1990
Peptococcus anaerobius CRDA 303	D-Hydantoinase	Morin *et al.,* 1992
Pseudomonas desmolyticum NCIM 2112	D-Hydantoinase	Gokhale *et al.,* 1996
Pseudomonas fluorescens DSM 84	D-Hydantoinase	Morin *et al.,* 1986b; 1995a; Chevalier *et al.,* 1989; Syldatk *et al.,* 1990a; LaPointe *et al.,* 1994; 1995
Pseudomonas fluorescens strains 1.2 and 1.9	D-Hydantoinase	Morin *et al.,* 1990
Pseudomonas putida 77	D-NCAAH	Kim *et al.,* 1987; Syldatk *et al.,* 1990
Pseudomonas putida 7711-2	D-Hydantoinase	Chen and Tsai, 1996
Pseudomonas putida CCRC 12857	D-Hydantoinase	Chien *et al.,* 1998; Chen *et al.,* 1999
Pseudomonas sp KNK 003A	D-NCAAH	Ikenaka *et al.,* 1998a; Nanba *et al.,* 1998b
Pseudomonas sp KNK 505	D-NCAAH	Ikenaka *et al.,* 1998a; Nanba *et al.,* 1998b
Pseudomonas sp RU-KM1	D-Hydantoinase	Burton *et al.,* 1998
Pseudomonas sp. AJ11220	D-Hydantoinase and D-NCAAH	Yokozeki *et al.,* 1987a,b,c; Syldatk *et al.,* 1990
Pseudomonas sp. KBEL 101	D-Hydantoinase	Kim and Kim, 1993; Kim *et al.,* 1994;
Pseudomonas sp. NCIM 5109	D-Hydantoinase	Sudge *et al.,* 1998
Pseudomonas striata (*putida*) IFO 12996	D-Hydantoinase	Yamada *et al.,* 1978; 1980; Takahashi *et al.,* 1978; Shimizu *et al.,* 1980; Syldatk *et al.,* 1990a; Ogawa *et al.,* 1994a
Serratia liquefans 1.15	D-Hydantoinase	Morin *et al.,* 1990
Streptomyces griseus	D-Hydantoinase	Takahashi *et al.,* 1978;
Thermus sp.	D-Hydantoinase	Abendroth *et al.,* 2000a,b; 2002 ; Lee and Lin, 1996; Garcia and Azerad, 1997; Lee and Fan, 1999

1.3.3. The genetic organisation of hydantoin utilising genes in L-specific and D-specific bacteria.

Gene clusters encoding hydantoin utilising proteins (*hyu)* have been identified in L-specific (Watabe *et al.,* 1992a, b; Wiese *et al.,* 2001b) and D-specific hydantoin hydrolysing bacteria (Grifantini *et al.,* 1998; Hils *et al.,* 2001). These *hyu* gene clusters are represented diagrammatically in Figure 1.4.

L-specifice gene clusters.

In *Pseudomonas* sp. NS671, a L-specific hydantoin hydrolysing bacterium, the *hyu* genes were isolated from the native 172kb plasmid (Watabe *et al.,* 1992a, b)(Figure 1.4, Panel A). The genes encoding an L-"preferential" hydantoinase (*hyuA* and *hyuB*), a L-NCAAH (*hyuC*), a hydantoin racemase (*hyuE*) and a putative permease (ORF5) were identified (Watabe *et al.,* 1992a, b; Wiese *et al.,* 2001b).

The *hyu* gene cluster was isolated from *Arthrobacter aurescens* DSM 3747, another L-specific hydantoin hydrolysing microorganism, by screening a genomic DNA library (Wiese *et al,* 2001a)(Figure 1.4, Panel A). The genes encoding a hydantoinase (*hyuH*), a L-NCAAH (*hyuC*), a racemase (*hyuA*) and a putative permease (*hyuP*) were identified (Wiese *et al.,* 2001a). Additional open reading frames were found in close proximity, but in opposite orientations, to the *hyu* genes. Upstream of *hyuP*, three open reading frames were found, encoding proteins with similarity to a cytochrome P450 monooxygenase (ORF1, incomplete), a putative membrane protein (ORF2) and a ferrodoxin (ORF3). Downstream of *hyuC*, a gene that encodes a protein similar to that of a LacI/GalR transcriptional regulator (ORF8) was found. These genes are not thought to be involved in hydantoin hydrolysis (Wiese *et al.,* 2001a).

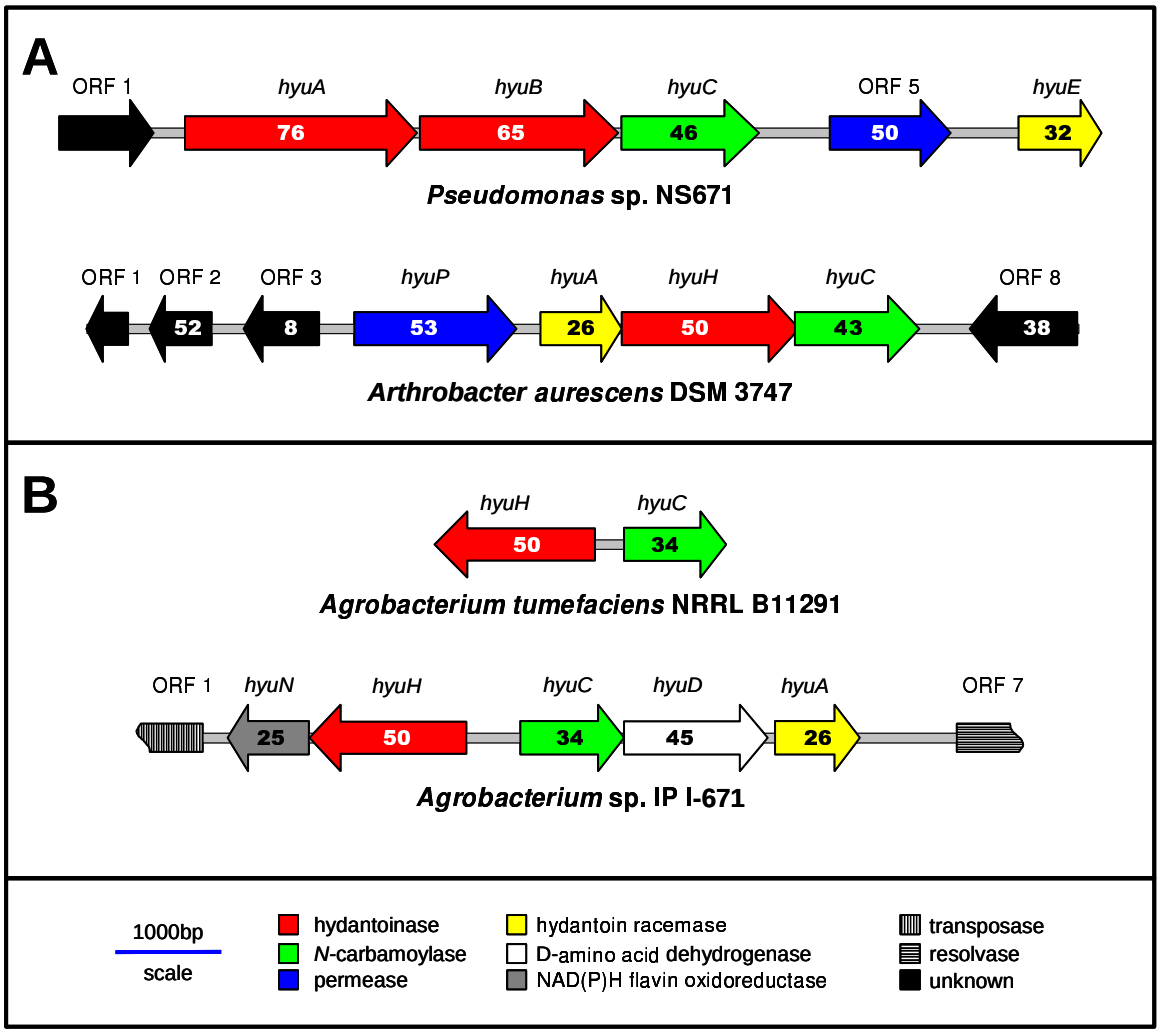


Figure 1.4. The genetic organisation of the *hyu* gene clusters from L-specific (Panel A) and D-specific (Panel B) hydantoin hydrolysing bacteria. The genes and their transcriptional orientation are symbolised by arrows. Genes encoding related proteins are represented with identical colours. The numbers within the arrows indicate the molecular mass (in kDa) of the corresponding deduced proteins. (adapted from Wiese *et al.*, 2001a; Hils *et al.*, 2001).

A comparison of the *Pseudomonas* sp. NS671 and *Arthrobacter aurescens* DSM 3747 *hyu* gene clusters shows that they are very similar. The *hyu* genes in each cluster were in the same orientation, which suggests that they constitute an operon. Furthermore, both contain a putative hydantoin transport protein, a hydantoinase, a L-NCAAH and a racemase. But the orders of the *hyu* genes in these two species differs considerably, and while the transport proteins, the *N*-carbamoyl-L-amino acid amidohydrolase and the racemase all share significant identities in amino acid sequence, the hydantoinases are very different. The hydantoinase of *Pseudomonas* sp. NS671 which is non-selective,

but L-"preferential" consists of two different subunits encoded by two genes (Watabe *et al.,* 1992a), while the L-selective hydantoinase of *Arthrobacter aurescens* DSM 3747 is encoded by one gene (Wiese *et al.,* 2001a). Both these hydantoinases have very different amino acid sequences, which is suggested as an explanation for the differing substrate selectivity of these two organisms (Wiese *et al.,* 2001a).

D-specific gene clusters.

Hils *et al* (2001) reported the isolation of five hydantoin utilising genes clustered on a region of the 190kb plasmid of *Agrobacterium* sp. IP I-671, (Figure 1.4, Panel B). The divergently orientated genes encoding a D-hydantoinase (*hyuH*) and a D-NCAAH (*hyuC*) were identified. A hydantoin racemase (*hyuA*) and a D-amino acid dehydrogenase (*hyuD*) were situated upstream of *hyuC*, and a gene encoding a protein homologous to a NAD(P)H flavin oxidoreductase (*hyuN*) was situated downstream of *hyuH*.

This was the first report of a hydantoin racemase being isolated from a D-specific bacterium, and it shared 46% identity with the amino acid sequence of the racemase from *Pseudomonas* sp. NS671 (Hils *et al.,* 2001). The D-amino acid dehydrogenase (HyuD) was proposed to be similar to those of *E. coli* and *Klebsiella aerogenes*, which are responsible for the catabolism of alanine into pyruvate and ammonium, and these enzymes contain a flavin adenine dinucleotide and a non-heame iron in their active centres (Lobocka *et al.,* 1994; Janes *et al.,* 1998). The NAD(P)H flavin oxidoreductase catalyses the reduction of flavin by NAD(P)H and is believed to produce FMN for luciferase (Lyi and Winans, 1999).

The C-terminal truncated ORF1 and ORF7 flanking the *hyu* gene cluster of *Agrobacterium* sp. IP I-671 showed similarity to transposases and resolvases respectively. They were recognised not to be part of the *hyu* gene cluster, but are thought to be part of a transposon carrying the *hyu* genes (Hils *et al.,* 2001).

In other *Agrobacteria* only *hyuC* (Nanba *et al*., 1998a,b; Hsu *et al*., 1999, Sareen *et al.,* 2001a,b) or *hyuC* and *hyuH* (Grifantini *et al.,* 1998, Hils *et al.,* 2001) have been found. In cases where both genes are present, they are always orientated divergently, but the

length of the intergenic regions vary, being 470bp in *Agrobacterium* sp. IP I-671 (Hils *et al.,* 2001) and 214bp in *A. tumefaciens* NRRL B11291 (Figure 1.4, Panel B). It has been proposed that *hyuH* and *hyuC* are transcribed from a common inter-cistronic promoter region that is arranged in opposite orientations (Grifantini *et al.,* 1998; Wiese *et al.,* 2001a).

In *Agrobacterium* sp. KNK712, which only has a D-NCAAH (Nanba *et al.,* 1998a), regions of the sequence downstream of the gene encoding the D-NCAAH show a significant level of sequence identity with the non-coding sequence between *hyuA* and ORF 7 of *Agrobacterium sp* IP I-671, which suggests that *hyuD* and *hyuA* were deleted in *Agrobacterium* sp. KNK712 (Hils *et al.,* 2001). No genes for the transport of hydantoins have been reported in *Agrobacterium* spp.

HyuH and *hyuC*, of *Agrobacterium sp* IP I-671 and *A. tumefaciens* NRRL B11291 were both found on native plasmids (190kb and 160kb respectively) (Hils *et al.,* 2001). Inactivation of *hyuC* in *Agrobacterium sp* IP I-671 resulted in total loss of D-NCAAH activity, suggesting that only one NCAAH encoding gene exists in *Agrobacterium sp* IP I-671. However, inactivation of *hyuH* only resulted in reduced hydantoinase activity, suggesting the presence of a second hydantoinase (Hils *et al.,* 2001).

The natural function of hydantoinases and *N*-carbamoyl-amino acid amidohydrolases.

While the natural function and substrates of the *hyu* genes remains mostly undetermined, the study of the enzymes produced in heterologous hosts and in the wild type strains has helped to elucidate this partially.

Based on a number of studies (Watabe *et al.,* 1992c; Ishikawa *et al.,* 1993, 1996, 1997) the protein products of the *Pseudomonas* sp. NS671 *hyu* gene cluster are proposed to act together as follows. D, L-5-monosubstituted hydantoins are converted exclusively to the L-forms of the corresponding *N*-carbamoyl-amino acid by the hydantoinase (*hyuA* and *hyuB*) in combination with the hydantoin racemase (*hyuE*). The *N*-carbamoyl-L-amino acid is then converted to L-amino acids by L-NCAAH (*hyuC*). The putative

permease (*hyuP*) is thought to play a role in the transport of the substrate into the cell (Wiese *et al.,* 2001a).

The protein products of the *hyu* gene cluster of *Arthrobacter aurescens* DSM 3747 are thought to operate in the same manner (Wiese *et al.,* 2001a). A study of the induction of the *hyu* genes of *Arthrobacter aurescens* DSM 3747 with D,L-5-(3-indolymethyl)-3-*N*-methylhydantoin, a synthetic compound, suggests that the natural substrates of these enzymes may be similar compounds which exist naturally, such as the secondary metabolites of plants or bacteria, which would act as a source of nitrogen (Wiese *et al.,* 2001a).

Hils *et al* (2001) speculate that the protein products of the *hyu* gene cluster of *Agrobacterium sp* IP I-671 act together as follows: the D,L-5-monosubstituded hydantoins are converted enantioselectively into D-amino acids via the *N*-carbamoyl-amino acid intermediate by HyuH and HyuC respectively. HyuD then deaminates the amino acids to a α-keto acid while HyuN keeps the flavin of HyuD in a reduced state. Continuous racemisation of hydantoin by HyuA ensures that the hydantoin is completely converted into the keto acid. The natural substrates of the hydantoin metabolising enzymes of *Agrobacteria* are thought to be plant secondary metabolites, since *Agrobacteria* interact intimately with plants (Hils *et al.,* 2001).

1.4. Strategies used to improve the productivity of the hydantoinase system.

A number of methods have been employed to improve the hydantoin hydrolysing microorganisms, or the hydantoin hydrolysing enzymes used as biocatalysts for the production of optically active α-amino acids. These approaches will be discussed in this section.

1.4.1. Screening for new hydantoin hydrolysing microorganisms.

The first route is to isolate hydantoin hydrolysing microorganisms that possess more robust enzymes, and in order to achieve this, the screening methods used for isolation

need to be improved. Many different screening methods have been developed and applied for the isolation of strains that possess hydantoin hydrolysing microorganisms. One extensively used method is the use of hydantoin as a sole carbon or nitrogen source (Akamatsu, 1960; Hassal and Greenberg, 1963; Sano *et al.,* 1976; Moller *et al.,* 1988). Others use pH dependent colour reactions to detect hydantoin hydrolysis to screen microorganisms growing on agar plates (Morin *et al.,* 1986a; Kim *et al.,* 1997a) or in microtitre plates (Chien and Hsu, 1996). Grob *et al* (1987) used the production of 'clear hydantoin hydrolysing zones' to detect positive strains. DNA probes and polyclonal antibodies have also been used for screening genomic DNA or protein extracts of microorganisms to identify hydantoinase positive strains (LaPointe *et al.,* 1995; Siemann *et al.,* 1993).

The sequences of whole genomes will be an increasingly important source of information to assist the isolation of new enzymes as discovered by Kim *et al* (2000), who identified a putative allantoinase and hydantoinase gene from the *E. coli* genome database, which they then isolated by polymerase chain reaction amplification, and after high-level expression were able to prove that these enzymes possessed allantoinase and hydantoinase activity. Since *E. coli* has never been reported to possess hydantoinase or allantoinase activity, it would not have been considered as a candidate for isolation of such enzymes (Altenbuchner *et al.,* 2001).

1.4.2. Manipulation of media and growth conditions.

A number of studies have also reported the optimisation of the growth medium for hydantoinase producing bacteria, and different bacteria benefit from carbon and nitrogen sources (Deepa *et al.,* 1993; Meyer and Runser, 1993; Kim and Kim, 1995; George *et al.,* 1996; Achary *et al.,* 1997). Supplementation of the culture medium with inducers of hydantoin hydrolysing enzymes has also been shown to improve the efficiency of the hydantoinase system (Olivieri *et al.,* 1981; Runser *et al.,* 1990, Syldatk *et al.,* 1990b; Meyer and Runser, 1993; Durham and Weber, 1995).

1.4.3. Immobilisation of whole cells or purified enzymes.

Comparisons of free or immobilised whole cells expressing hydantoin hydrolysing enzymes (Chevalier *et al.,* 1989;Kim and Kim, 1993) and purified hydantoin hydrolysing enzymes (Lee and Lin, 1996; Lee *et al.,* 1996a; Nanba *et al.,*1998b, 1999; Fan and Lee, 2000; Cheon *et al.,* 2000; Ragnitz *et al.,* 2001a, b) have shown that immobilisation improves the efficiency of the biocatalyst, as well as the reusability of the biocatalyst for the production of amino acids.

1.4.4. Mutagenesis to improve hydantoin hydrolysing enzymes in bacteria.

Chemical mutagenesis has been used to isolate microorganisms with improved hydantoin hydrolysing abilities. Inducer independent mutants have been isolated from *Arthrobacter* strains and *Agrobacterium tumefaciens* RU-OR (Gross *et al.,* 1990; Syldatk *et al.,* 1990; Hartley *et al.,* 1998, 2001). Wagner *et al.,* (1996) used selective pressure to isolate more robust mutant strain of *Arthrobacter* sp. DSM 7330 for the production of L-methionine, and Morin *et al.,* (1995a) used a continuous cultivation system with selective pressure to isolate better D-hydantoinase producing microorganisms.

1.4.5. Heterologous expression of hydantoin hydrolysing enzymes.

The heterologous expression of hydantoin hydrolysing enzymes has been employed extensively (Table 1.3). It has been used to create recombinant *E. coli* strains which overproduce the hydantoin hydrolysing enzymes, to be used as whole biocatalysts (Chao *et al.,* 1999a,b, 2000a; Wilms *et al.,* 2001a,b). In addition, it has been used for the overproduction of the enzymes, which can be purified and used as free or immobilised enzyme biocatalysts (Nanba *et al.,* 1998b, 1999; Ragnitz *et al.,* 2001a,b; Sareen *et al.,* 2001b).

Table 1.3. Hydantoin hydrolysing enzymes that have been expressed heterologously in *E. coli.*

Microbial strain	Enzyme	Reference
A. aurescens DSM 3747	Non-specific hydantoinase, L-NCAAH and Racemase	Pietzsch *et al.,* 2000; Ragnitz *et al.,* 2001a,b Wiese *et al.*, 2000, 2001a,b; Wilms *et al.,* 1999, 2001a,b
A. radiobacter (Gen bank X91070)	D-hydantoinase	Chen and Tsai, 1996
A. radiobacter CCRC 14924	D-NCAAH	Hsu *et al.,* 1999
A. tumefaciens NRRL B11291	D-hydantoinase and D-NCAAH	Buson *et al.,* 1996; Grifantini *et al.,* 1996, 1998; Chao *et al.,* 1999a,b; Fan *et al.,* 2000
Agrobacterium sp. IP-671	D-hydantoinase, D-NCAAH and racemase	Hils *et al.,* 2001
Agrobacterium sp. KNK 712	D-NCAAH	Nanba *et al.,* 1998a,b, 1999; Ikenaka *et al.,* 1998b,c, 1999;
Agrobacterium tumefaciens AM10	D-NCAAH	Sareen *et al.,* 2001b
B. stearothermophilus	D-hydantoinase	Chen and Tsai, 1996
B. stearothermophilus NS1122A	L-hydantoinase and L-NCAAH	Mukohara *et al.,* 1993, 1994
B. stearothermophilus SD-1	D-hydantoinase	Kim *et al.,* 1997b, 1999, 2000a,b; Lee *et al.,* 1996a,b, 1994,1995; Park *et al.,* 2000
B. thermoogluosidasius	D-hydantoinase	Chen and Tsai, 1996
E. coli K12	D- hydantoinase	Kim *et al.,* 2000c
P. putida	D- hydantoinase	Yin *et al.,* 2000
P. putida 7711-2	D- hydantoinase	Chen and Tsai, 1996
P. putida CCRC 12857	D- hydantoinase	Chien *et al.,* 1998
P. putida DSM 84	D- hydantoinase	LaPointe *et al.,* 1994
Pseudomonas sp. KNK003A	D-NCAAH	Ikenaka *et al.,* 1998a,
Pseudomonas sp. NS 671	Non-specific Hydantoinase, L-NCAAH and racemase	Ishikawa *et al.,* 1996; Watabe *et al.,* 1992a,b,c
Recombinant commercial *E. coli* (BM)	D- hydantoinase	Lee and Lin, 1996

The use of heterologous expression systems introduces new problems, such as the formation of insoluble protein aggregates. Problems associated with heterologous expression have been reviewed extensively, and strategies to overcome these problems have been suggested. These include the use of alternative expression vectors with various promoters in alternative expression hosts, the use of reduced temperatures for expression, the co-expression of chaperone proteins and the use of fusion proteins or tags (Hockney, 1994; Hannig and Makrides, 1998; Weickert *et al.,* 1996; Baneyx, 1999).

Some of these problems have been experienced with the heterologous expression of some hydantoin hydrolysing enzymes, particularly the *N*-carbamoyl-D-amino acid amidohydrolases (Buson *et al.,* 1996; Grifantini *et al.,* 1998, Chao *et al.,* 2000b). The use of a few of the previously mentioned strategies have been employed to overcome these problems, and are discussed further with regards to the heterologous expression of *N*-carbamoyl-amino acid amidohydrolases in Section 1.6.

One of the methods used worth a mention is the use of protein fusions or tagging of proteins to improve heterologous expression. The use of fusion proteins or amino acid tags were originally constructed to facilitate protein purification and immobilisation of heterologously produced proteins (Baneyx, 1999), and has been applied for these purposes with various D- and L-specific hydantoin utilising enzymes (Chien *et al.,* 1998; Pietzsch *et al.,* 2000; Wiese *et al.,* 2001b; Hsu *et al.,*1999) as listed in Table 1.4. In contrast it has also been found that certain fusion partners and tags greatly improve the solubility of the proteins that would otherwise accumulate within inclusion bodies in the cell cytoplasm (Baneyx, 1999) as has been observed with a fusion of maltose binding protein to the N-terminus of an L-hydantoinase (Wiese *et al.,* 2001b). However, the most important advantage of using fusion proteins or tags has been the potential to increase the enzyme activity of the purified enzyme, such as the remarkable 600-fold increase in activity that was obtained with the *E. coli* K12 D-phenylhydantoinase when fused to maltose binding protein (Kim *et al.,* 2000c).

Table 1.4. Amino acid tags or protein fusions used to improve the heterologous production of hydantoin hydrolysing enzymes.

Enzyme	Amino acid Tag or Protein Fusion	Effect on activity, solubility and ease of purification	Species, Strain	Reference
D- hydantoinase	N-Term His_6 Tag	No improvement in activity or solubility, but more easily purified	*P. putida* CCRC 12857	Chien *et al.,* 1998
D- hydantoinase	C-Term His_6 Tag	No improvement in activity or solubility, but more easily purified	*P. putida* CCRC 12857	Chien *et al.,* 1998
D- hydantoinase D-NCAAH	Fusion to form bifunctional D-specific NCAAH-hydantoinase	No change in enzyme acitivity, but insoluble aggregates formed that are prone to proteolysis	*B. stearothermophilus* SD1 and *A. tumefaciens* NRRL B11291	Kim *et al.,* 2000a,b
D- hydantoinase D-NCAAH	Fusion to form bifunctional enzyme (D-NCAAH-D-Hyd)	No change in enzyme activity, but fusions protein is more soluble	*B. thermocatenulanus* GH2 and *A. tumefaciens* NRRL B11291	Kim *et al.,* 2000a,b
D- hydantoinase	N-terminal MalE fusion	No change in enzyme activity	*B. stearothermophilus* SD1	Kim *et al.,* 2000a,b
D-NCAAH	N-term His_6 Tag	More easily purified, activity not determined	*A. radiobacter* CCRC 14924	Hsu *et al.,* 1999
D-NCAAH	N-terminal His_6 tag	No change in enzyme activity	*A. tumefaciens* AM10	Sareen *et al.,* 2001b
L- hydantoinase	N-terminal MalE fusion	Improves enzyme activity and solubility and more readily purified	*A. aurescens* DSM 3747	Wiese *et al.*, 2001b
L- hydantoinase	C-terminal intein-CBD fusion	No improvement in enzyme activity, inclusion bodies still formed	*A. aurescens* DSM 3747	Wiese *et al.*, 2001b
L- hydantoinase	C-term His_6 tag	No improvement in enzyme activity, inclusion bodies still formed	*A. aurescens* DSM 3747	Pietzsch *et al.,* 2000
L-NCAAH	C-term Asp_6 tag	More easily purified, no effect on enzyme activity	*A. aurescens* DSM 3747	Pietzsch *et al.,* 2000
L-NCAAH	C-term His_6 tag	Slight improvement in enzyme activity and more easily purified	*A. aurescens* DSM 3747	Pietzsch *et al.,* 2000
phynyl-hydantoinase	N-terminal MBP fusion	Improved expression levels, and up to 600-fold increase in enzyme activity	*E. coli* K12	Kim *et al.,* 2000c
Racemase	C-term His_6 tag	Complete loss of activity	*A. aurescens* DSM 3747	Wiese *et al.*, 2000

An interesting fusion was that of the D-NCAAH from *Agrobacterium tumefaciens* NRRL B11291 fused to the thermostable D-hydantoinases from *Bacillus stearothermophilus* SD1 and *B. thermocatenulatus* GH2. The result was the formation of a bifunctional enzyme that possessed both D-hydantoinase and D-NCAAH activity, and in the case of the NRRL B11291-GH2 fusion, a more soluble protein product was obtained (Kim *et al.,* 2000a,b).

The use of protein fusions or tags however, can also affect the heterologously produced enzyme adversely, as in the case of the expression of the hydantoin racemase of *Arthrobacter aurescens* DSM 3747 with a C-terminal His-tag, where the racemase was completely inactivated (Wiese *et al.,* 2000).

1.4.6. Mutagenesis, rational protein design and directed evolution for the improvement of hydantoin hydrolysing proteins.

Random mutagenesis, saturated mutagenesis and high throughput screening has been used to improve the L-selectivity of a L-hydantoinase used for production of L-methionine (May *et al.,* 2000). Random mutagenesis and site-directed mutagenesis was used improve the thermostability and enzyme activity of the D-NCAAH from *A. tumefaciens* NRRL B11291 (Buson *et al.,* 1996) and *Agrobacterium* sp KNK712 (Ikenaka *et al.,* 1998a,b, 1999), but these were carried out with little knowledge regarding the structure of these enzymes.

The crystal structures of a two D-hydantoinases and two D-NCAAHs have been determined (Nakai *et al.,* 2000; Wang *et al.,* 2001; Cheon et al., 2002; Abendroth *et al.,* 2002) revealing the mechanisms of hydantoin and *N*-carbamoyl-amino acid hydrolysis. Information derived from the elucidation of these crystal structures and the crystal structures of other enzymes, will allow for rational protein design to be used to improve these enzymes. Also, the crystal structures will make it possible to solve the structures of closely related enzymes (Syldatk *et al.,* 1999).

Directed evolution has also been used to improve a number of enzymes with industrial applications (Arnold and Moore, 1997). In the case of hydantoin hydrolysing enzymes,

it has been used to improve the oxidative and thermal stability of an D-NCAAH (Oh *et al.,* 2002a,b) and changed the stereoselectivity of a D-hydantoinase to an L-selective enzyme (May *et al.,* 2000). Furthermore gene shuffling of the novel bi-functional D-hydantoinase-*N*-carbamoyl-amino acid amidohydrolase created by Kim *et al.,* (2000a,b) that was prone to the formation of inclusion bodies and proteolysis, resulted in the production of a more soluble, stable enzyme that did not undergo proteolysis as readily (Kim *et al.,* 2000a).

1.5. The distribution and properties of hydantoin utilising enzymes in *Agrobacteria*.

This study will focus on the hydantoin hydrolysing enzymes present in various species of *Agrobacteria*, more specifically the D-NCAAH of *Agrobacteria.* Here, the hydantoin hydrolysing enzymes of *Agrobacteria*, all of which are D-specific, will be discussed briefly.

The *Agrobacteria* that have been reported to possess hydantoin hydrolysing enzymes are listed in Table 1.5. As discussed previously, some of the *Agrobacteria* possess a D-hydantoinase and an *N*-carbamoyl-amino acid amidohydrolase, while others posses either one or the other. Furthermore, *Agrobacterium* sp. IP I-671 possesses the only racemase that has been isolated in D-specific microorganisms (Hils *et al.,* 2001).

The nucleotide and predicted amino acid sequences of the D-hydantoinases of *Agrobacterium* sp. IP I-671 and *A. tumefaciens* NRRL B11291 show that they share 87% nucleotide sequence identity and 90% amino acid sequence identity (Hils *et al.,* 2001) and both have a subunit size of approximately 50kDa (Runser *et al.,* 1990; Grifantini *et al.,* 1998). The chemical characterisation of all the D-hydantoinases from *Agrobacteria* shows that they are all similar, and is further evidence that they are all related (Yamada *et al.,* 1980; Runser and Ohleyer, 1990; Durham and Weber, 1995; Burton *et al.,* 1998; Grifantini *et al.,* 1998; Hartley *et al.,* 2001; Hils *et al.,* 2001).

The D-hydantoinases from *Agrobacteria* are stable at temperatures up to 70°C at alkaline pH (pH 7-10), but the optimum reaction conditions are usually between 40°C to

60°C at a pH of 10 (Durham and Weber, 1995; Runser and Ohleyer, 1990; Burton *et al.,* 1998, Olivieri *et al.,* 1981). This correlates with the pH and temperature optima and stabilities of other D-hydantoinases, except for some which have slightly lower pH optima (Lee *et al.,* 1995; Park *et al.,* 1998). In addition, they are particularly suitable for industrial application as they exhibit a high substrate selectivity towards hydantoins with 5'-aromatic substituents such as D,L-5-hydoxyphenylhydantoin and D,L-5-methylhydantoin (Runser and Meyer, 1993; Durham and Weber, 1995).

Of all the D-*N*-carbamoyl-amino acid amidohydrolases that have been reported (Table 1.2), the majority have been isolated from *Agrobacterium* species (Table 1.5). Often they are associated with a D-hydantoinase. Of particular significance, *A. tumefaciens* RU-OR was found to possess two different D-NCAAHs, NcaR1 and NcaR2. The nucleotide and amino acid sequence have been determined for a nearly all of these D-*N*-carbamoyl-amino acid amidohydrolases (Grifantini *et al.,* 1996; Nanba *et al.,* 1998a; Hsu *et al.,* 1999; Hils *et al.,* 2001; Hartley, 2001), and all of them share greater than 90% nucleotide and amino acid sequence identity (Nanba *et al.,* 1998a; Hartley, 2001; Hils *et al.,* 2001), except for NcaR2 which shares between 62% to 65% nucleotide sequence identity and between 57% to 59% amino acid sequence identity with the other D-*N*-carbamoyl-amino acid amidohydrolases isolated from *Agrobacteria* (Hartley, 2001).

Analysis of the deduced amino acid sequence of the D-NCAAH, from *A. tumefaciens* NRRL B11291, revealed the presence of 5 cysteine residues which were thought to play a role in the activity of the D-NCAAHs and site directed mutagenesis of each of these showed that Cys171 was found to be essential for enzyme activity (Grifantini *et al.,* 1996). A multiple alignment of the deduced amino acid sequences of a number of other D-*N*-carbamoyl-amino acid amidohydrolases revealed 6 conserved amino acids within this group of enzymes, and they were Gly118, Arg125, Lys126, Glu145, Gly152 as well as the previously mentioned Cys171 (Ikenaka *et al.,* 1998c).

The crystal structures of the D-*N*-carbamoyl-amino acid amidohydrolases from *Agrobacterium* sp. KNK712 and *A. radiobacter* CCRC 14924 have been determined, which confirmed that Cys171 is indeed in the catalytic centre of the enzyme, together with Lys126 and Glu46 (Nakai *et al.,* 2000; Wang *et al.,* 2001).

Table 1.5. The distribution of hydantoin utilising enzymes in *Agrobacteria*.

Species, Strain	Enzymes	Size		Molecular Weight (kDa)		References
		Gene (bp)	Subunit (aa)	Total	Subunit	
A. radiobacter CCRC 14924	D-NCAAH	912	304	-	36 (dimer)	Hsu *et al.,* 1999, Wang *et al.,* 2001
A. radiobacter KNK 712	D-NCAAH	912	304	-	34 (dimer)	Nanba *et al.,* 1998a
A. radiobacter sp. SB	D-NCAAH	912	304	84	38 (dimer)	Louwrier and Knowles, 1996
A. rhizogenes IFO 13259	D-hydantoinase	-	-	-	-	Yamada *et al.,* 1980; Kamphuis *et al.,* 1990
Agrobacterium sp IP I-671	D-hydantoinase	1374	457	250	50 (tetramer)	Runser and Ohleyer, 1990; Runser *et al.,* 1990; Hils *et al.,* 2001
	D-NCAAH	912	304	-	34 (dimer)	Runser *et al.,* 1990; Hils *et al.,* 2001
	Racemase	744	248	-	26	Hils *et al.,* 2001
A. tumefaciens 47C	D-hydantoinase	-	-	-	-	Durham and Weber, 1995
A. tumefaciens AM10	D-NCAAH	-	-	67	38 (dimer)	Sareen *et al.,* 2001a
A. tumefaciens NRRL B1129	D-hydantoinase	1374	457	-	50 (tetramer)	Grifantini *et al.,* 1998
	D-NCAAH	912	304	-	34.3 (dimer)	Grifantini *et al.,* 1996
A. tumefaciens RU-AE01	D-hydantoinase	-	-	-	-	Herrera, unpublished; This study
	D-NCAAH	-	-	-	-	Herrera, unpublished; This study
A. tumefaciens RU-OR	D-hydantoinase	-	-	-	-	Hartley *et al.,* 1998; 2001
	D-NCAAH: (*NcaR1*)	909	303	-	34	Hartley *et al.,* 1998; 2001; Hartley 2001
	D-NCAAH: (*NcaR2*)	912	304	-	34	

The mechanism of *N*-carbamoyl-D-amino acids hydrolysis by the amino acids in the catalytic centre, and the importance of other amino acid residues in these D-NCAAHs were discussed based on these crystal structures (Nakai *et al.,* 2000; Wang *et al.,* 2001).

The *N*-carbamoyl-amino acid amidohydrolases from *Agrobacteria* are stable at temperatures up to 60°C at alkaline pH (pH 7-9), but the optimum reaction conditions are usually at pH 7 or 8, and varies from temperatures of 40°C to 60°C (Olivieri *et al.,* 1981; Runser *et al.,* 1990; Louwrier and Knowles, 1996; Nanba *et al.,* 1998a; Burton *et al.,* 1998). This correlates with the pH and temperature optima and stabilities of other D-NCAAHs, except for that of *A. tumefaciens* RU-OR, which has been reported to be stable up to pH 11 (Hartley *et al,* 1998).

The racemase of *Agrobacterium* sp. IP I-671 is the only hydantoin racemase to be reported in a bacterium possessing D-specific hydantoin hydrolysing activity (Hils *et al.,* 2001) and its isolation was surprising as *Agrobacterium* sp. IP I-671 was not known to possess racemase activity. It has been suggested that other bacteria with D-specific hydantoin hydrolysing activity may possess hydantoin racemases, but the high rate of spontaneous racemisation of D,L-5-monosubstituted hydantoins (Lee and Fan, 1999) may mask the activity of the racemases (Hils *et al.,* 2001). The hydantoin racemase of *Agrobacterium* sp. IP I-671 shares 46% amino acid sequence identity with the racemase of *Pseudomonas* sp. NS671 (Watabe *et al.,* 1992b).

1.6. The heterologous expression of *N*-carbamoyl-amino acid amidohydrolases in *E. coli*.

The heterologous expression of D-NCAAH in *E. coli* has been investigated extensively, and earlier studies showed that these enzymes were prone to the formation of insoluble, inactive protein aggregates (Buson *et al.,* 1996; Grifantini *et al.,* 1998; Chao *et al.,* 1999a, 2000a; Hils *et al* 1999; Sareen *et al.,* 2001b). Since, a number of approaches have been used to

Table 1.6. Approaches used to improve the heterologous expression of D-NCAAH. Items in bold indicate negative, or reduced D-NCAAH solubility and activity.

Strain of Origin	Vector and Promoter	Strain of *E. coli*	Fusions or Tags	Temperature	Chaperone Co-expression	Reference
A. tumefaciens NRRL B11291	P_{T7} promoter pRSETA	BL21 (DE3)	none	30°C and **37°C**	none	Buson *et al.*, 1996
A. tumefaciens NRRL B11291	endogenous promoter pBluescriptII SK$^+$, **pSY343, pBR322, pGB2**	**JCL1258**, ATCC11303	none	37°C	none	Chao *et al.*, 1999a
A. tumefaciens NRRL B11291	**separately** vs polycistronicly with D-hydantoinase	**71/18**	none	25°C and **37°C**	none	Grifantini *et al.*, 1998
A. radiobacter CCRC 1492 *	P_{T7} promoter pQE30	JM 109	N-terminal 6xHis Tag	not specified	none	Hsu *et al.*, 1999
A. tumefaciens NRRL B11291	P_{tac} promoter **separately** vs polycistronicly with D-hydantoinase	BL21 (DE3)	none	37°C	none	Chao *et al.*, 2000a
A. tumefaciens NRRL B11291	P_{trc} promoter	BL21 (DE3)	none	25°C and **37°C**	GroEL/ES	Chao *et al.*, 2000b
A. tumefaciens NRRL B11291	promoter from hydantoinase of *B. stearothermophilus* SD1	XL1-Blue	none	not specified	none	Park *et al.*, 2000
A. tumefaciens NRRL B11291	P_{trc} promoter **P_{BAD} promoter**	JM 109	C-Terminal of NCAAH fused to N-terminal of hydantoinase	30°C	none	Kim *et al.*, 2000a
A. tumefaciens AM10	P_{T7} promoter pET-28a	**BL21 (DE3)** JM 109 (DE3) **Origami (DE3)**	N-terminal 6xHis Tag	18°, 28°C and **37°C**	GroEL/ES	Sareen *et al.*, 2001b

* enzyme activity and solubility is not specified

improve the heterologous expression of these enzymes so that they are soluble and active, as listed in Table 1.6, which shows that the ability to achieve this is dependant on a number of factors.

It has been found that the accomplishment of heterologous expression can be dependant on the expression vector and the host strain of *E. coli* used (Chao *et al.,* 1999a; Sareen *et al.,* 2001b). For example, *E. coli* BL21 (DE3) was used successfully as a host strain for the expression of D-NCAAH using a number of different expression vectors (Buson *et al.,* 1996; Chao *et al.,* 2000a,b), but Sareen *et al.* (2001b) failed to obtain any expression of D-NCAAH using this strain.

One of the most important factors though, is the temperature at which the recombinant *E. coli* is grown after expression of the genes has been induced. Lower temperatures have been found to give consistently better results for the expression of D-NCAAH (Buson *et al.,* 1996; Grifantini *et al.,* 1998, Chao *et al.,* 2000a,b; Sareen *et al.,* 2001b). It has also been found that solubility and activity of the heterologously expressed D-NCAAH can be improved when co-expression with GroEL/ES chaperone proteins (Chao *et al.,* 2000b; Sareen *et al.,* 2001b).

1.7. Research Proposal

The gene encoding an *N*-carbamoyl-amino acid amidohydrolase has been isolated from a genomic DNA library of *Agrobacterium tumefaciens* RU-AE01. Hydantoin hydrolysis genes are associated in gene clusters on the genomes of bacteria. Therefore the isolation of a large enough DNA fragment, encoding the *N*-carbamoyl-amino acid amidohydrolase, from genomic DNA library of *A. tumefaciens* RU-AE01 using a DNA probe should enable the isolation of genes encoding other proteins involved in the hydrolysis of hydantoins, such as the hydantoinase, the hydantoin racemase and the hydantoin permease.

A. tumefaciens RU-OR has novel *N*-carbamoyl-amino acid amidohydrolase activity, which may be accounted by for the presence of two different genes encoding N-carbamoyl-amino acid amidohydrolases, *ncaR1* and *ncaR2*. Heterologous expression of *ncaR1* and *ncaR2* with 6xHis tags in *E. coli* will facilitate the production and purification of NcaR1 and NcaR2, to characterise each enzyme separately. Furthermore, polyclonal antibodies raised against each of these enzymes can be used to quantify the expression of each enzyme in protein extract of *A. tumefaciens* RU-OR.

1.8. Research Objectives

1. To isolate genes responsible for hydantoin hydrolysis in *A. tumefaciens* RU-AE01.
2. To express *ncaR1* and *ncaR2,* the genes encoding two different D-NCAAHs in *A. tumefaciens* RU-OR, in *E. coli.*
3. To purify 6xHis tagged NcaR1 and NcaR2 for the production of polyclonal antibodies.

CHAPTER 2

Isolation of the genes encoding enzymes involved in hydantoin hydrolysis from *Agrobacterium tumefaciens* strains.

CHAPTER 2

Isolation of the genes encoding enzymes involved in hydantoin hydrolysis from *Agrobacterium tumefaciens* strains.

2.1 Introduction

2.1.1. Isolation of genes encoding enzymes involved in hydantoin hydrolysis.

Two approaches have been used for the isolation of hydantoin hydrolysing enzymes. The first is the PCR amplification from the genomic DNA using primers based on published sequences (Wilms *et al.,* 1999; Chao *et al.,* 1999a; Hsu *et al.,* 1999 and Sareen *et al.,* 2001a) and the second being the screening of genomic DNA libraries (Watabe *et al.,* 1992a; Mukohara *et al.,* 1993; LaPointe *et al.,* 1994; Chen and Tsai, 1996; Buson *et al.,* 1996; Kim *et al.,* 1997b; Nanba *et al.,* 1998a, Ikenaka *et al.,* 1998a; Grifantini *et al.,* 1998; Park *et al.,* 2000 and Hartley 2001).

A number of techniques have been developed for screening genomic DNA libraries for the isolation of hydantoin hydrolysing enzymes, including growth with hydantoins and *N*-carbamoylamino acids as sole nitrogen sources (Watabe *et al.,* 1992a; Mukohara *et al.,* 1993; Kim *et al.,* 1997b; Nanba *et al.,* 1998a and Ikenaka *et al.,* 1998a), screening with DNA probes (Chen and Tsai, 1996; Grifantini *et al.,* 1998 and Park *et al.,* 2000) and detection of pH change due to production of *N*-carbamoylamino acids and amino acids (Park *et al.,* 2000). In some cases the entire set of library clones were screened for *N*-carbamoylglycine, glycine or ammonium production in resting cells or sonicated cell-free extracts (LaPointe *et al.,* 1994; Buson *et al.,* 1996; Hartley, 2001).

Hydantoin hydrolysing enzymes are often closely linked on the genome, and it has been found that once the location of one hydantoin hydrolysing enzyme has been established, others have been located by examination of open reading frames in the vicinity (Watabe *et al.,* 1992a,b; Wiese *et al.,* 2001a; Grifantini *et al.,* 1998 and Hils *et al.,* 2001). In the case of *A. tumefaciens* NRRL B11291 and *Agrobacterium* sp. IP I-671, the hydantoin utilising genes were located on 160kb and 190kb plasmids respectively (Hils *et al.,* 2001).

Several genes encoding *N*-carbamoyl-amino acid amidohydrolases have been isolated from the genomic libraries of *Agrobacterium* species, using DNA probes (Grifantini *et al.,* 1998; Buson *et al.,* 1995; Nanba *et al.,* 1998a). However, no hydantoinases have been isolated from *Agrobacterium* in this manner. The only hydantoinases that have been isolated from this genus were discovered upstream of NCAAH genes (Grifantini *et al.,* 1998; Hils *et al.,* 2001). The difficulties experienced with the isolation of the hydantoinase gene from *Agrobacterium* species has been ascribed to the recalcitrant nature of heterologous expression of the enzymes in *E. coli* (Grifantini *et al.,* 1998 and Chao *et al.,* 2000b).

2.1.2. Genes encoding *N*-carbamoyl-amino acid amidohydrolases used in this study.

A. tumefaciens RU-AE01 was isolated from soil and was shown to possess good hydantoin hydrolysing activity. Screening of a genomic DNA library using phenol red to detect the increase in pH produced by conversion of *N*-carbamoylamino acids to amino acids resulted in the isolation of the recombinant plasmid pGH45 (Figure 2.1), containing the gene encoding a D-NCAAH (Harrera, unpublished results). Partial sequencing of pGH45 revealed that the 5' end of the hydantoinase gene was also included on this plasmid (unpublished results) which suggested that the hydantoinase and D-NCAAH of *A. tumefaciens* RU-AE01 were arranged much like those described previously (Grifantini *et al.,* 1998 and Hils *et al.,* 2001). The plasmid pGH45PROM (Figure 2.2) carries the divergent promoter from pGH45 flanked by 5' regions of the hydantoinase and *N*-carbamoyl-amino acid amidohydrolase genes, which was inserted into pGEM-T Easy (Jiwaji, unpublished results).

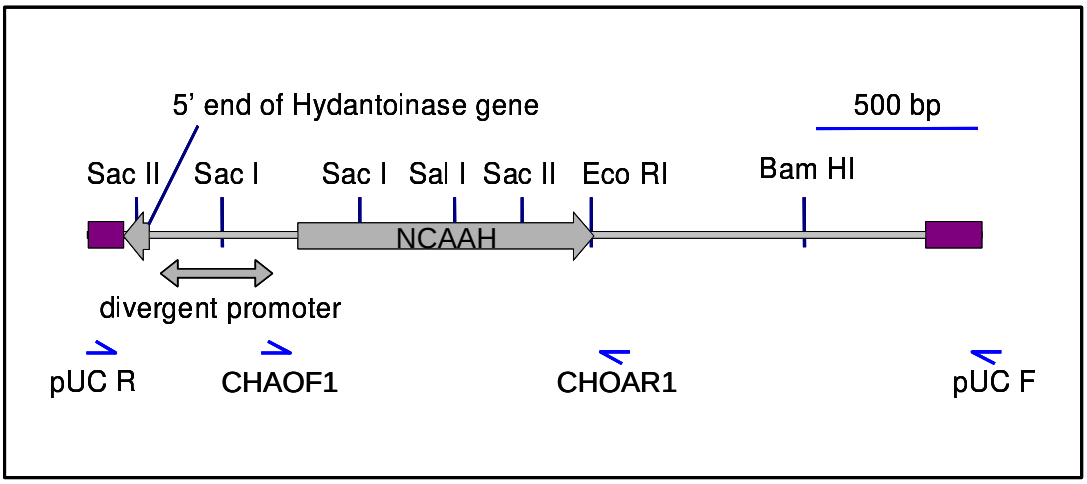


Figure 2.1. A diagram of the genomic DNA insert of pGH45, showing the position of the gene encoding an NCAAH and the 5' region of the gene encoding a Hydantoinase and the divergent promoter. Sense (>) and antisense (<) primers used for PCR amplification and sequencing are indicated.

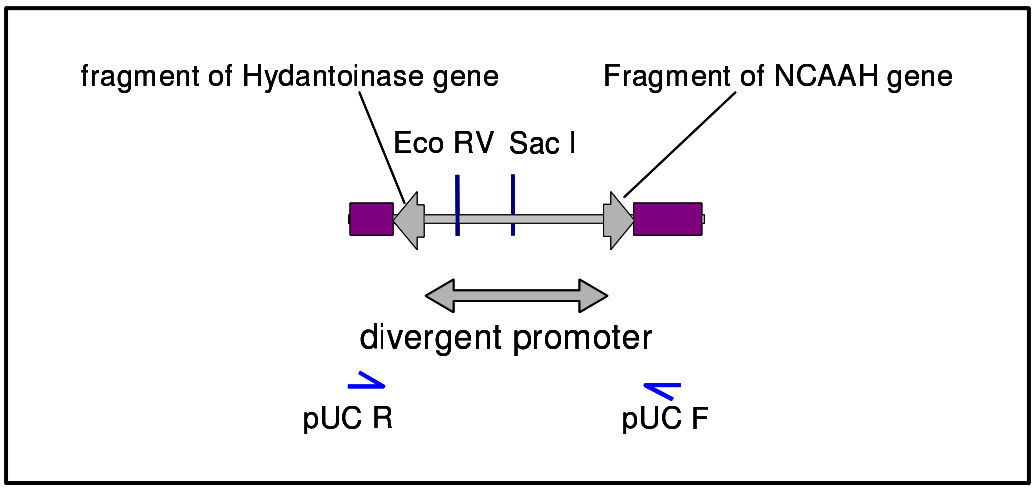


Figure 2.2. A diagram of the genomic DNA insert of pGH45 PROM carrying the divergent promoter that lies between the genes encoding the hydantoinase and NCAAH enzymes. The primers pUCF and pUCR are shown.

A. tumefaciens RU-OR was isolated from soil and possesses D-stereoselective hydantoin-hydrolysing activity (Burton *et al.,*1998 and Hartley *et al.,*1998). Screening the genomic library of *A. tumefaciens* RU-OR genes encoding enzymes for the hydrolysis of hydantoin failed to isolate a gene encoding a hydantoinase. However two separate genes encoding *N*-carbamoyl-amino acid amidohydrolase (*ncaR1* and *ncaR2*) were discovered with distinct chromosomal locations, nucleotide coding sequences, and predicted primary amino acid sequences (Hartley, 2001).

The plasmid pG4 (Figure 2.3) consists of a 1kb PCR amplification product derived from *A. tumefaciens* RU-OR genomic DNA, using primers CHAOF1 and CHAOR1, cloned into the vector pGEM-T Easy (Promega). The PCR fragment carries the promoter region and coding sequence of *ncaR1* (Hartley, 2001). pG4 was shown to possess *N*-carbamoyl-amino acid amidohydrolase activity in *E. coli* (Hartley, 2001)

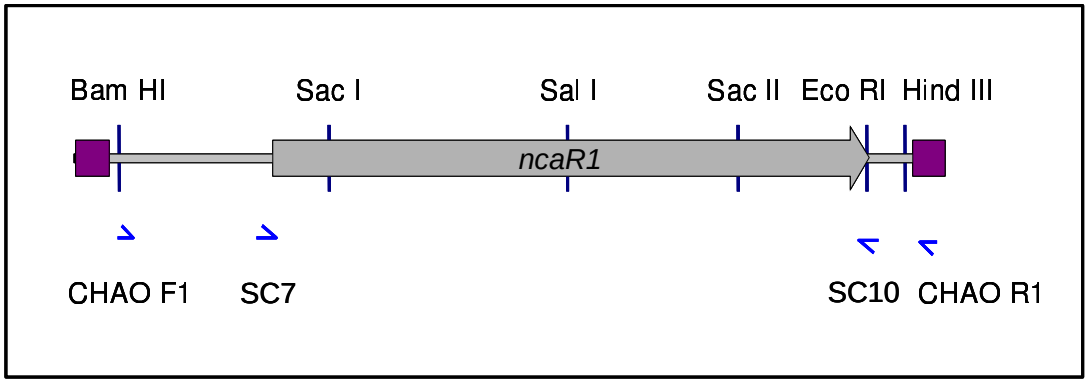


Figure 2.3. An illustration of pG4 showing the 1 kb PCR amplification product from *A. tumefaciens* RU-OR genomic DNA. A partial restriction enzyme maps is given. A full restriction enzyme map is available in Appendix 2c. Sense (>) and antisense (<) primers are indicated.

The plasmid p6B3-1a (Figure 2.4) contains a 3.4kb *Sau* 3AI genomic DNA fragment from *A. tumefaciens* RU-OR, which carries the gene *ncaR2*, inserted into the *Bam* HI site of pBK-CMV. Three plasmids, p6B3-1a dX, p6B3-1a dC and p6B3-1a dH were created by performing *Xho* I, *Cla* I and *Hind* III deletions of p6B3-1a. p6B3-1a, p6B3-1a dX and p6B3-1a dC were shown to possess *N*-carbamoyl-amino acid amidohydrolase activity in *E. coli,* while p6B3-1a dH did not. The complete sequence for the genomic DNA insert was obtained by sequencing nested deletions of p6B3-1a produced using exonuclease III digestions of p6B3-1a insert DNA (Hartley, 2001).

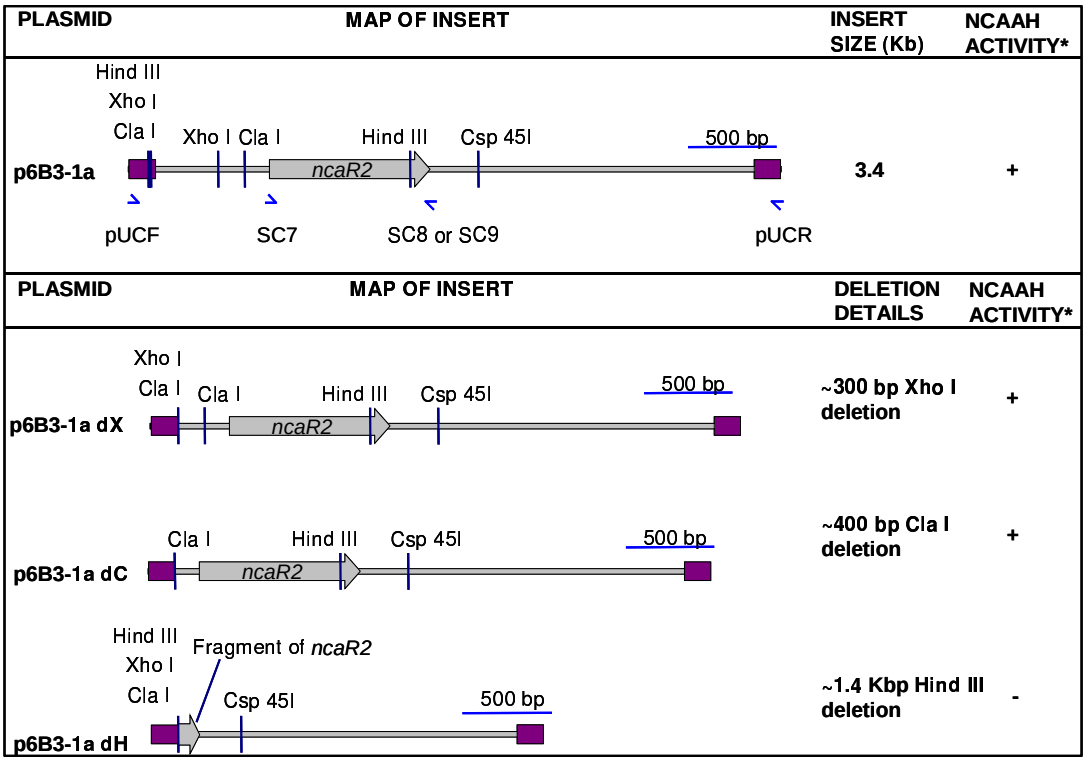


Figure 2.4. p6B3-1a and the *Xho* I, *Cla* I and *Hind* III deletions of p6B3-1a. Partial restriction enzyme maps are shown. Full restriction enzyme maps are available in Appendix 2c. Sense (>) and antisense (<) primers used for sequencing and PCR amplification are indicated. *NCAAH activity as determined by Hartley (2001).

2.1.3. Objectives.

This chapter describes attempts to isolate genes encoding enzymes involved in hydantoin hydrolysis by screening a genomic DNA library of *A. tumefaciens* RU-AE01. A DNA probe prepared from the gene encoding the *N*-carbamoyl-amino acid amidohydrolase from *A. tumefaciens* RU-AE01 will be used to isolate fragments of genomic DNA which contain this gene, and consequently any other genes involved in hydantoin hydrolysis which may be closely linked with it. In addition *ncaR1* and *ncaR2,* the two genes encoding the two *N*-carbamoyl-amino acid amidohydrolases, from *A. tumefaciens* RU-OR are to be prepared to construct vectors for heterologous expression.

2.2. Materials and Methods

2.2.1. Bacterial strains, plasmids and culture conditions.

Recombinant plasmids were hosted in either *E. coli* DH5α or *E. coli* XLOLR (Table 2.1), which were grown a 37°C in LB or on LA plates (Appendix 1) supplemented with an antibiotic to which the recombinant plasmid conferred resistance (100μg/ml ampicillin or 50mg/ml kanamycin). The plasmids used in this chapter are listed in Table 2.2, where the parental vector, the origin of the DNA insert and the antibiotic resistance marker is indicated.

Table 2.1. *E. coli* host strains used in this chapter.

***E. coli* Strain**	**Genotype**	**Reference**
DH5α	*F⁻Φ80dlacZΔM15Δ(lacZYA-argF)U169 endA1 recA1 hsdR17* ($r_k^- m_k^-$) *deoR thi-1 supE44* λ^- *gyrA96 relA1*	Hanahan, 1983
XLOLR	*Δ(mrcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac(F' proAB* $lacI^q$ *ZΔM15 Tn10* [Tet^r]) Su^-, λ^r	Stratagene

Table 2.2. Plasmids used in this chapter.

Plasmid	**Origin of insert**	**Antibiotic Resistance**	**Reference**
pBK-CMV	cloning vector	kanamycin	Stratagene
pGEM-T Easy	cloning vector	ampicillin	Promega
pGH45	genomic DNA from RU-AE01 in pBK-CMV	kanamycin	Harrera, Unpublished
pGH45-PROM	divergent promoter region for NCAAH and Hydantoinase gene from pGH45 in pGEM-T Easy	ampicillin	Jiwaji, Unpublished
p6B3-1a	genomic DNA from RU-OR in pBK-CMV	kanamycin	Hartley, 2001
p6B3-1a dH	deletion of 1374 bp *Hind* III fragment from p6B3-1a in pBK-CMV	kanamycin	Hartley, 2001
p6B3-1a dC	deletion of 416 bp *Cla* I fragment from p6B3-1a in pBK-CMV	kanamycin	Hartley, 2001
p6B3-1a dX	deletion of 1300 bp *Xho* I fragment from p6B3-1a in pBK-CMV	kanamycin	Hartley, 2001
pG4	1kb PCR amplification product from RU-OR genomic DNA using primers CHAOF1 & CHAOR1 in pGEM-T Easy	ampicillin	Hartley, 2001
p7A9E	genomic DNA from RU-AE01 in pBK-CMV	kanamycin	This study
p7A8F	genomic DNA from RU-AE01 in pBK-CMV	kanamycin	This study
p7A8F Δ *Sal* I	genomic DNA from RU-AE01 in pBK-CMV	kanamycin	This study
p1B1F (1-30)	genomic DNA from RU-AE01 in pBK-CMV	kanamycin	This study

2.2.2. Recombinant DNA techniques.

Isolation of recombinant plasmid DNA from *E. coli* DH5α for diagnostic purposes was performed using an easy plasmid preparation protocol (Appendix 2) based on the method of Smart (1993), while that used for sequencing was isolated using the High Pure Plasmid Isolation Kit (Roche) or the QIAprep Spin Miniprep Kit (Qiagen). Restriction analysis was performed by first digesting the plasmids according to the manufacturers instructions, and the resultant restriction fragments were separated by electrophoresis on a 1% agarose gel containing 0.5µg/ml ethidium bromide, and visualised using the Kodak DC120 gel imaging system.

2.2.3. PCR amplification of *ncaR1* and *ncaR2*.

PCR amplification of *ncaR1* and *ncaR2* was carried out using the primers SC7, SC8 and CHAOR1 (Appendix: 3) that were designed for the amplification of *ncaR1* and *ncaR2* as outlined in Table 2.3. PCR amplification was performed with the Expand High Fidelity PCR System (Roche) using thermal cycling programme 1 (Appendix 4)

Table 2.3. Primers SC7, CHAOR1 and SC8 used for the PCR amplification of *ncaR1* and *ncaR2*.

Primer	Description	Sequence
SC7	primer corresponding to the 5' sequence of *ncaR1* and *ncaR2*	CATATGACACGTCAGATGATACTTGC
CHAOR1	primer corresponding to the 3' sequence of *ncaR1*	CCCAAGCTTTCTCGATCGGATAGG
SC8	primer corresponding to the 3' sequence of *ncaR2*	CCCGGGCTACAATTCGGCGATCAGG

The PCR amplification products were analysed by electrophoresis on a 1% agarose gel containing 0.5µg/ml ethidium bromide, and visualised using the Kodak DC120 gel imaging system. The PCR amplification products were inserted into plasmid vectors for heterologous production of NcaR1 and NcaR2, in *E. coli* as discussed in Chapter 3.

2.2.4. DNA Sequencing and Sequence Analysis.

Nucleotide sequence was determined from double stranded plasmid DNA using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.0, v2.0 or v3.0 (PE Applied Biosystems). Sequencing primers pUC F and pUC R (Appendix 3) were used for sequencing of genomic DNA in the phagemid vector pBK-CMV. Additional primers SC5, SC6, SC11 and SC14 to SC20 (Appendix 3) were designed for sequencing p6B3-1a. Sequencing primers pUCF, pUCR, SC12 and SC13 (Appendix 3) were used for sequencing the insert of pG4.

Cycle sequencing was performed according to manufacturers instructions using the GeneAmp PCR System 9700 thermal cycler. Products of the cycle sequencing reactions were purified using the DNA Clean and Concentrator Kit (Zymo), and were dried using vacuum centrifugation with the Speedvac Concentrator (Savant). Pellets were resuspended in Template Suppression Reagent and analysed using an ABI Prism 3100 Genetic Analyser (PE Applied Biosystems). Preliminary analysis of the extracted sequence data was performed using Vector NTI DeLuxe v4.0 or v5.0 (Informax Inc.). Sequences with identity to known sequences from numerous databases was determined using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1990) hosted by the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Sequence alignments were carried out using ClustalW (Thompson *et al.,*1994) hosted by the BCM Search Launcher website (http://searchlauncher.bcm.tmc.edu).

2.2.5. Preparation of genomic DNA library for screening.

A lambda-phage based library of genomic DNA from *A. tumefaciens* RU-AE01 was constructed using the ZAP Express Predigested Vector Kit and Gigapack Cloning Kit (Stratagene) by AECI researchers (Harrera, unpublished results). Glycerol stocks of *E. coli* XLOLR transformed with the mass excision supernatant of the genomic DNA library were used for screening for genes encoding enzymes involved in hydantoin hydrolysis in *A. tumefaciens* RU-AE01.

The average genomic DNA insert size was estimated, by restriction enzyme analysis of the plasmid DNA isolated from 30 single colonies obtained from the glycerol stocks of the genomic DNA library of *A. tumefaciens* RU-AE01. This was necessary in order to evaluate the clonal representation within the genomic DNA library, so that the number of individual insert-containing colonies that would have to be screened to adequately cover the entire genome of *A. tumefaciens* RU-AE01 could be determined. This was determined using them formula of Ausubel *et al.,* (1983):

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

Where *l* is the average insert size, *G* the genome size in base pairs, *P* the probability of isolating an individual sequence.

Microtitre plates (96-well) were used for growing, replicating and storing the RU-AE01 genomic library for screening. Appropriate dilutions of the glycerol stock of *E. coli* XLOLR containing the RU-AE01 genomic DNA library were made such that approximately 20 cells harbouring different insert-containing plasmids were inoculated into each well of the microtitre plates. These were grown for 48 hours at 37°C in 200µl of LB containing 50µg/ml Kanamycin. Glycerol stocks of each microtitre plate were made, by transferring 100µl of each well to another microtitre plate to which 30µl of sterile glycerol was added. These microtitre plates were sealed and stored at -70°C as glycerol stocks. The duplicated plates were used for screening.

For each set of plates prepared, a small volume of a cells from a microtitre plate well chosen at random were streaked onto LA plates containing kanamycin (50 µg/ml), and up to 30 colonies were selected. The size of the inserts of the recombinant plasmids from these isolates were determined by agarose gel electrophoresis of the plasmid DNA digested with *Eco* R1 and *Pst* I in order to establish the average number of different insert-containing plasmids that were present in each well.

2.2.6. Screening the genomic DNA library of *A. tumefaciens* RU-AE01 by Southern Hybridisation.

The isolation of recombinant plasmid DNA and transfer to nylon membranes.

Three methods were used for transferring plasmid DNA to nylon membrane: dot blotting, colony blotting and capillary transfer of plasmid DNA that had been digested with restriction enzymes and subjected to agarose gel electrophoresis.

Dot blotting involved the isolation of plasmid DNA from the *E. coli* grown in microtitre plates by alkaline lysis, which was performed in the microtitre plates based on the method described by Ausubel *et al.,* (1983). The plasmid DNA was applied to Hybond N+ (Amersham) nylon membranes using a dot-blot filtration manifold (Appendix 5).

For colony blotting, the microtitre plates of cells were replicated onto 14mm LA plates containing 50µg/ml kanamycin by transferring 5µl of the cells that were grown in the microtitre plates with a multiwell pipette. The plates were dried for 3 day to ensure that they would absorb the liquid from the growth medium in order to prevent the cells from adjacent wells running into one another. The plates were incubated at 37°C until the colonies grew to 2-3mm. The colonies were transferred to circular nylon membranes (Millipore) and lysed using the method described by Sambrook *et al.,* (1989) (Appendix 6).

The capillary transfer of plasmid DNA from an agarose gel electrophoresis to Hybond N+ (Amersham) nylon membranes was performed based on the method described by Sambrook *et.al.* (1989), and is outlined in Appendix 7. Plasmid DNA of pBK-CMV and pGH45 was applied to all of the membranes as negative and positive controls for the Southern hybridisation.

Crosslinking of the plasmid DNA to the nylon membranes using short wave length UV light was performed by placing the membranes DNA side down on a transilluminator for 5 minutes.

Southern Hybridisation.

The plasmid DNA on the nylon membranes with homology to the gene encoding the *N*-carbamoyl-amino acid amidohydrolase or with homology to the divergent promoter of pGH45 was detected using radioactively labelled DNA probes, which were prepared by PCR amplification. Probe 1, which consists of the gene encoding the NCAAH, was PCR amplified (Thermal Cycling Programmme 2, Appendix 4) using primers CHAOF1 and CHAOR1 (Appendix 3). The primers pUCF and pUCR (Appendix 3) were used to amplify Probe2, consisting of the divergent promoter, from pGH45-PROM using thermal cycling programme 3 (Appendix 4). 50μCi of α-^{32}P-dCTP (NEN Life Science Products) was added to the reaction, and the resulting product purified using the High Pure PCR Product Purification Kit (Roche). Non-radioactive PCR reactions were carried out concomitantly and analysed by gel electrophoresis to verify amplification of the correct fragment.

Hybridisation of the radioactive probe to the plasmid DNA bound to the nylon was performed at 42°C and highly stringent washes were carried out to remove unbound and non-specifically bound radioactively labelled probe. The hybridisation and wash steps were performed based on the methods discussed by Ausubel *et.al.* (1983) as outlined in Appendix 13. The detection of binding of radioactively labelled probe was detected by incubation of the membranes under film (Hyperfilm MP, Amersham or Biomax MS, Kodak) at -70°C with an intensifying screen for various lengths of time. Standard photographic techniques and solutions (Kodak) were used to visualise the radioactive exposure.

2.2.7. Biocatalytic reactions for the detection of *N*-carbamoyl-amino acid amidohydrolase activity.

Biocatalytic reactions of resting *E. coli* cells harbouring recombinant plasmids were performed for the detection of *N*-carbamoyl-amino acid amidohydrolase activity as follows. A stationary phase starter culture grown in LB containing the appropriate antibiotic was diluted to OD_{600}=0.02 in LB containing the appropriate antibiotic, and grown at 37°C with shaking at 200rpm until stationary growth phase was reached. The cells were harvested by centrifugation at 7000rpm for 10 minutes in a Beckman JA14

rotar (Beckman J2-21) and resuspended to a final concentration of 20 mg/ml wet cell mass in 0.1M potassium phosphate buffer (pH8) (Appendix 1) containing 25mM *N*-carbamoyl glycine.

The reaction was carried out at 40°C with shaking at 200rpm for 6 hours, after which the samples were microfuged to pellet the cells and the supernatant analysed. Any *N*-carbamoyl glycine converted to glycine by NCAAH was quantified using the ninhydrin assay adapted from Plummer (1971) as follows.

For each sample, 20µl of the reaction supernatant was added to 980µl 0.1M potassium phosphate buffer in a test tube. A standard curve of 0 to 0.5mM glycine in a reaction volume of 1ml was prepared from a 1mM stock as shown in Table 2.4. 1 ml ninhydrin reagent (Appendix 1) was added to the standard curve and samples. The test tubes were capped and boiled for 15 minutes. After cooling 3ml of 50% ethanol was added and the $OD_{570\ nm}$ was read versus the standard curve. Fresh ninhydrin reagent and standard curves were prepared for each assay.

Table 2.4. Preparation of a standard curve of 0 to 0.5mM glycine

Concentration (mM)	0.1M PO_4 buffer (ml)	1mM glycine in 0.1M PO_4 buffer (ml)
0	1.0	0
0.1	0.9	0.1
0.2	0.8	0.2
0.3	0.7	0.3
0.4	0.6	0.4
0.5	0.5	0.5

One unit of NCAAH (U) activity was calculated as µmol/ml glycine produced from *N*-carbamoyl glycine. Spreadsheets with standard curve regression (Microsoft Excel) were used to calculate the µmol/ml amino acid produced for each sample, and to calculate the averages and SEM values for the triplicate reactions.

2.3. Results

2.3.1. Screening the genomic DNA library of *A. tumefaciens* RU-AE01.

Preparation of Probe 1 and Probe 2 for Southern hybridisation.

Radioactively labelled (α-^{32}P-dCTP) DNA probes, Probe 1 and Probe 2 were prepared by PCR amplification from the plasmids pGH45 and pGH45-PROM respectively, using the primers CHAOF1 and CHAOR1 or pUCF and pUCR as indicated in Figure 2.5.

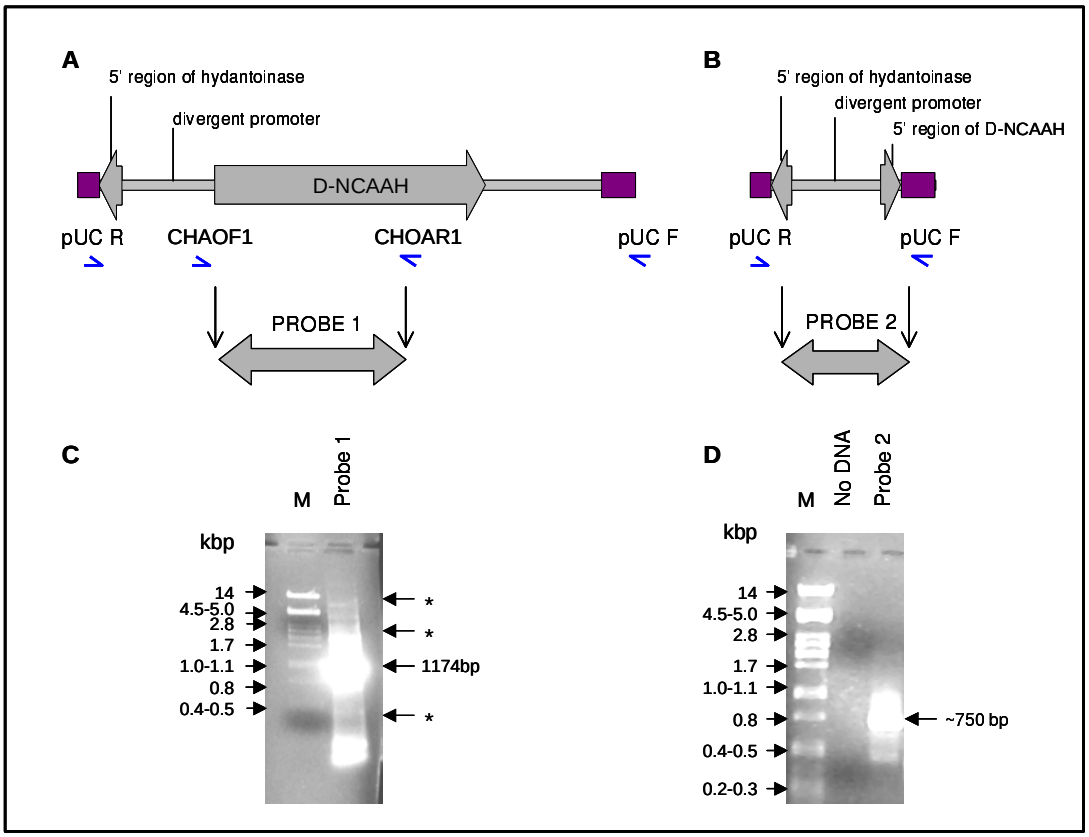


Figure 2.5. The PCR amplification of Probe 1 and Probe 2. Probe 1 was prepared from pGH45 as template DNA, with primers CHAOF1 and CHAOR1 (Panel A) and Probe 2 was prepared from pGH45-PROM as template DNA, with primers pUCF and pUCR. Agarose gel electrophoresis of non-radioactive preparations of Probe 1 and Probe 2 are shown in Panel C and Panel D respectively. Lambda DNA digested with *Pst* I was used as a molecular weight marker (M). The molecular weight of the products of PCR amplification are indicated on the right hand side of the gel, while the molecular weights of the molecular weight marker are indicated on the left hand side of the gel. Non-specific PCR amplification products are indicated by (*).

The analysis of the PCR amplifications of Probe 1 and Probe 2 by agarose gel electrophoresis (Figure 2.5, C and D) verified the amplification of the correct fragments (1174bp and 750bp) respectively. Some non-specific PCR amplification products and unincorporated nucleotides were observed in the PCR amplification reaction of Probe 1.

Screening the genomic DNA library of *A. tumefaciens* RU-AE01

The typical genome of *A. tumefaciens* is approximately 5.67 million base pairs (Wood *et al.,* 2001). The average insert size of the *A. tumefaciens* RU-AE01 genomic DNA library was found to be approximately 4700bp. So according to the formula of Ausubel *et al.,*(1983), in order to have a 99% probability of isolating the gene of interest, approximately 5000 individual insert-containing plasmids had to be screened in order to adequately cover the whole *A. tumefaciens* RU-AE01 genome.

In the first round of screening, 4 microtitre plates were prepared which unexpectedly turned out to have an average of 1 colony per well, despite having prepared the dilution of the glycerol stock such that there would be approximately 20 colonies per well. The plasmid DNA of these microtitre plates was prepared by alkaline lysis, transferred to the nylon membranes with a dot blot manifold. Southern hybridisation of these membranes with Probe 1 was carried out, and a sample of the results that were obtained is shown in Figure 2.6. No reaction observed between Probe 1 and the negative control, pBK-CMV, and a positive reaction observed between Probe 1with pGH45. There was some non-specific binding of Probe 1 to most of the wells, but positive colonies equivalent to the signals obtained with the positive controls were not identified.

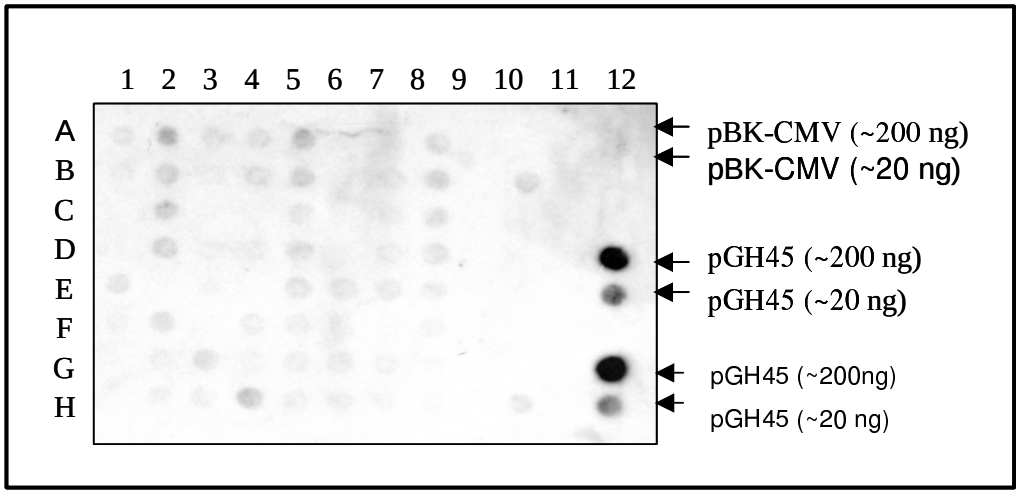


Figure 2.6. Southern Hybridisation of plasmid DNA prepared in microtitre plates, and transferred to nylon membranes with a dot blot manifold. Plasmid DNA of pBK-CMV was placed in wells12A and 12B, while plasmid DNA of pGH45 was placed in wells 12D and 12E.

An alternative method for the transfer of DNA to the nylon membranes was used in the second round of screening where 2 sets of microtitre plates were prepared due to the low insert-containing plasmid per well ratio of the first set of plates. The DNA was transferred to nylon membranes using the technique of colony blots. The first set of plates (Set A) consisted of 10 plates (1-10), which contained an average of 4 insert-containing plasmids per well, while the second set of plates (Set B) consisted of 5 plates (1-5), which contained an average of 30 insert-containing plasmids per well. A total of approximately 340 000 inserts were screened in these experiments.

Probe 1 reacted positively with the plasmid DNA from two wells of Set A microtitre plates, and with the plasmid DNA from one well of Set B microtitre plates. Cells isolated from these wells were plated onto LA plates containing 50µg/ml kanamycin, and a representative number of colonies (Table 1.5) were picked for further analysis of the recombinant plasmids harboured by those cells.

Table 2.5. Results of Southern Blot Hybridisation.

Set A or B	Plate Number	Well Co-ordinates	Number of Colonies Picked	Recombinant Plasmid
A	7	9E	5	p7A9E (1-8)
A	7	8F	8	p7A8F (1-8)
B	1	1F	30	p1B1F (1-30)

Isolation and analysis of p7A9E and p7A8F

The recombinant plasmids p7A9E (1-5) and p7A8F (1-8), together with pBK-CMV and pGH45 were analysed by gel electrophoresis after being digested with *Eco* RV, *Nru* I and *Eco* RI in combination with *Sal* I which showed that only one insert-containing plasmid was present in each well. The plasmid p7A9E was isolated from well 9E and had a genomic DNA insert of approximately 5100bp, and the plasmid p7A8F was isolated from well 8F and had a genomic DNA insert of approximately 4800bp.

The results of the agarose gel electrophoresis of *Eco* RI, E*co* RV and *Sal* I restriction enzyme digests of p7A9E and p7A8F was used to produce restriction enzyme maps (Figure 2.7), which showed that the inserts of p7A9E and p7A8F shared similar restriction fragments suggesting that they encode common DNA sequences. A 2100 bp *Sal* I-*Sal* I fragment was deleted from p7A8F, and the resulting plasmid was named p7A8FdS.

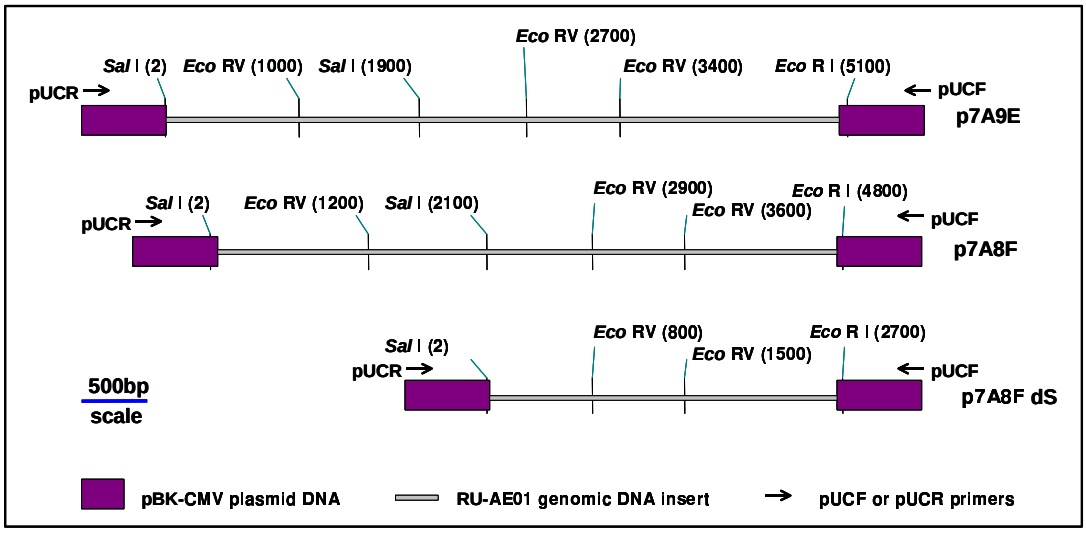


Figure 2.7. Restriction enzyme maps of the RU-AE01 genomic DNA inserts of p7A9E, p7A8F and p7A8FΔ*Sal* I, showing *Sal* I, *Eco* RI and *Eco* RV restriction enzyme sites. The positions of sequencing primers pUCF and pUCR are indicated.

The nucleotide sequences of the 5' and 3' regions of the genomic DNA inserts of p7A9e, p7A8F and p7A8FdS were determined with the primers pUCF and pUCR. Alignments of these nucleotide sequences with the nucleotide sequence of genes encoding hydantoin hydrolysing enzymes yielded no positive results. The nucleotide sequences were aligned with the nucleotide sequences of the *A. tumefaciens* C58 genome, generated by Cereon Genomics (Goodner *et al.,*2001) or the University of Washington (Wood *et al.,*2001). Similar alignment results were obtained for both *A. tumefaciens* C58 genome sequences, so further analysis was carried out using the *A. tumefaciens* C58 genome sequence generated by the university of Washington (Wood *et al.,*2001).

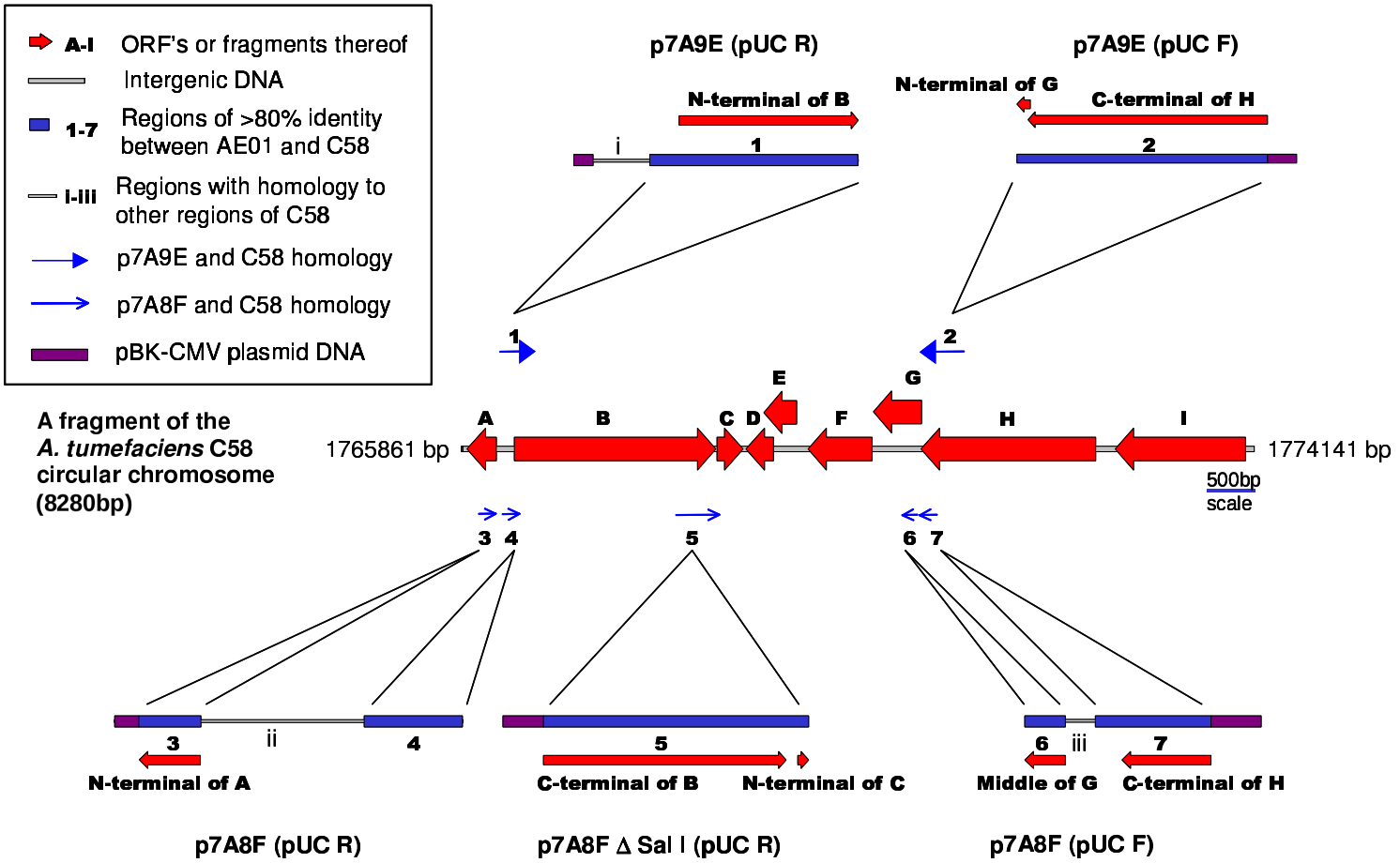


Figure 2.8. The region of the *A. tumefaciens* C58 circular chromosome (1765861bp774141bp) encoding nine open reading frames that share nucleotide sequence homology with the genomic DNA inserts of p7A9E and p7A8F. The manner in which the nucleotide sequence of the 5' and 3' regions of the *A. tumefaciens* RU-AE01 genomic DNA inserts of p7A9E, p7A8F and p7A8FdS align with the nucleotide sequence of the *A. tumefaciens* circular chromosome are shown.

Seven regions (1-7) of the nucleotide sequences generated from p7A9E, p7A8F and p7A8FdS were found to match within the same region of the *A. tumefaciens* C58 circular chromosome, spanning approximately 5000bp (Figure 2.8), where 9 open reading frames (A-I) are located. This indicated that the genomic DNA inserts of p7A9E and p7A8F were from a region of the *A. tumefaciens* RU-AE01 genome that corresponds to this region of the *A. tumefaciens* C58 circular chromosome. Table 2.6 lists the genes encoded by these open reading frames of the *A. tumefaciens* C58 circular chromosome, none of which encode enzymes involved in the hydrolysis of hydantoins.

Table 2.6. The genes surrounding the area on the *A. tumefaciens* C58 circular chromosome where p7A9E and p7A8F show homology. The proteins encoded by these open reading frames are listed.

Open Reading Frame	Location on C58 chromosome	Strand	Gene	Length (nt)	Protein	Length (aa)
A: Atu 1789	1765905-1766225	-		312	Hypothetical protein	104
B: Atu 1780	1766404-1768509	+	recG	2106	ATP dependant DNA helicase	702
C: Atu 1781	1768522-1768794	+		273	Hypothetical protein	91
D: Atu 1782	1768822-1769112	-		291	Hypothetical protein	97
E: Atu 1783	1769109-1769354	-		345	Hypothetical protein	115
F: Atu 1784	1769474-1770142	-		669	Hypothetical protein	223
G: Atu 1785	1770156-1770659	-		504	Acetyltransferase	168
H: Atu 1786	1770656-1772482	-	glmS	1827	Glucosamine-fructose-6-phosphate aminotransferase	609
I: Atu 1787	1772687-1774048	-	glmU	1362	UDP-N-acetylglucosamine phosphorylase	454

Three regions (i-iii) within the nucleotide sequences generated from p7A9E and p7A8F were found to bear no significant similarity to this region of the *A. tumefaciens* C58 circular chromosome, but instead appeared to match randomly distributed intergenic regions of the *A. tumefaciens* C58 genome.

Analysis of recombinants in well 1B1F.

Thirty plasmids from colonies isolated from well 1B1F were isolated and mapped with *Eco* RI and *Pst* I, which showed that at least 18 different recombinant plasmids were present in this well. In order to determine which of these recombinant plasmids were

responsible for the positive reaction with Probe 1, Southern blot analysis of the *Eco* RV digests of these plasmids was carried out with Probe 1 and Probe 2 (Figure 2.9).

Plasmid DNA of pBK-CMV and pGH45 were included as negative and positive controls respectively. The *Eco* RV digests of plasmid DNA of p7A9E, p7A8F and p7A8FdS were also included to establish which regions of the *A. tumefaciens* RU-AE01 genomic DNA insert reacted with Probe 1 and Probe 2

Probe 1 and Probe 2 were found to bind to all the *Eco* RV fragments of pGH45, but were found to bind only to the *Eco* RV fragments corresponding to the vector DNA of p7A9E, p7A8F and all the genomic DNA library recombinants isolated from well 1B1F. No reaction of Probe 1 or Probe 2 with the lambda DNA molecular weight marker indicated that Probe 1 and 2 were not binding non-specifically to all DNA. However, binding of Probe 1 and Probe 2 to the vector DNA of the pBK-CMV recombinants in addition to the inserts of pGH45 made them ineffective as DNA probes for screening the genomic DNA library within pBK-CMV.

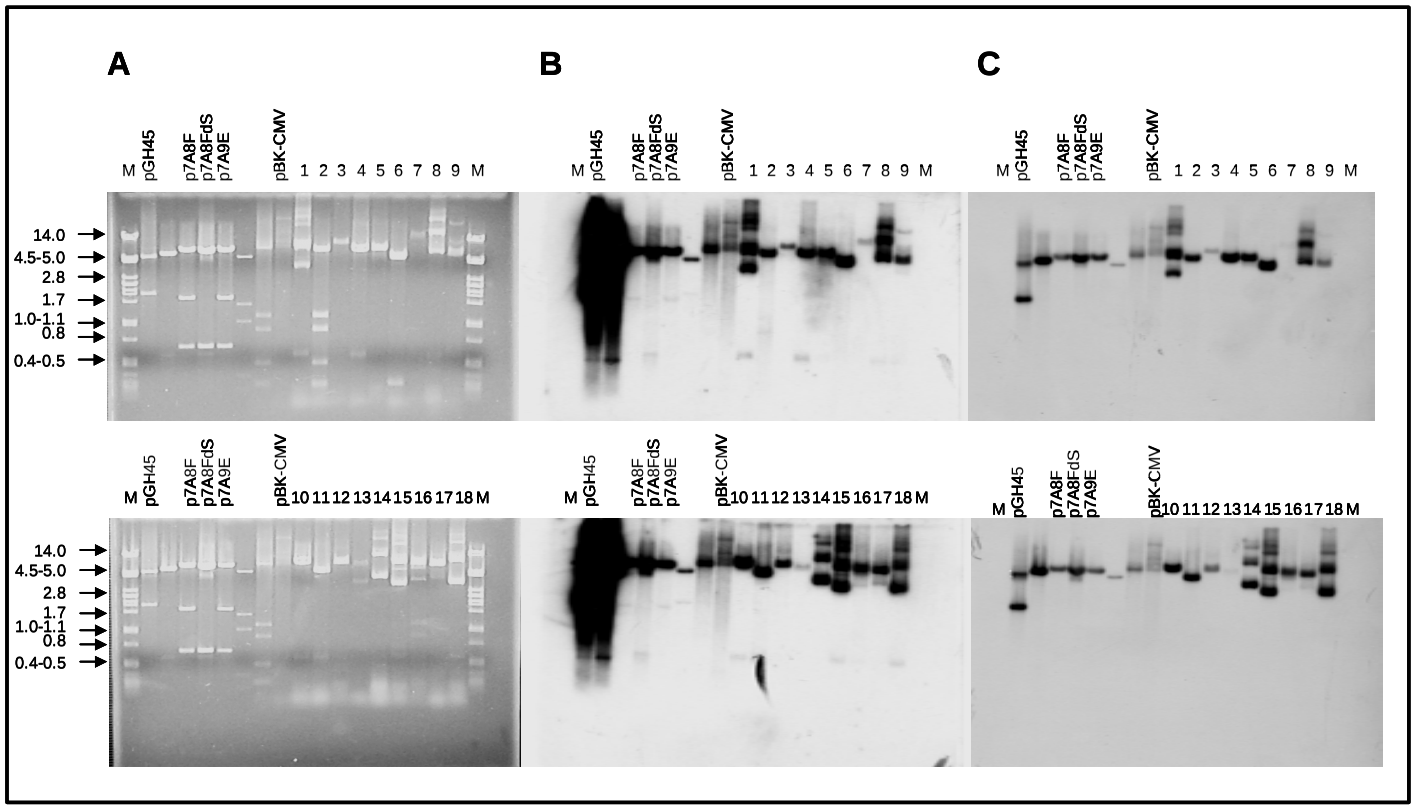


Figure 2.9. Southern hybridisation of recombinant plasmids (1-18) isolated from well 1B1F. Panel A: Agarose gel electrophoresis of the *Eco* RV digested plasmids isolated from well 1B1F, before transfer to a nylon membrane. Southern hybridisation of the DNA on the nylon membranes with Probe 1 (Panel B) and Probe 2 (Panel C) are shown. Southern hybridisation of *Eco* RV digested plasmid DNA of pGH45, p7A9E, p7A8F, p7A8FdS and pBK-CMV was also performed. Lambda DNA digested with *Pst* I was used as the molecular weight marker (M) and the sizes (kb) are shown on the left hand side.

2.3.2. PCR amplification of *ncaR1* and *ncaR2*.

To prepare *ncaR1* and *ncaR2* for cloning into expression vectors, PCR amplification from pG4 and p6B3-1a, respectively, was performed with SC7, SC8 and CHAOR1 as indicated in Figure 2.10.

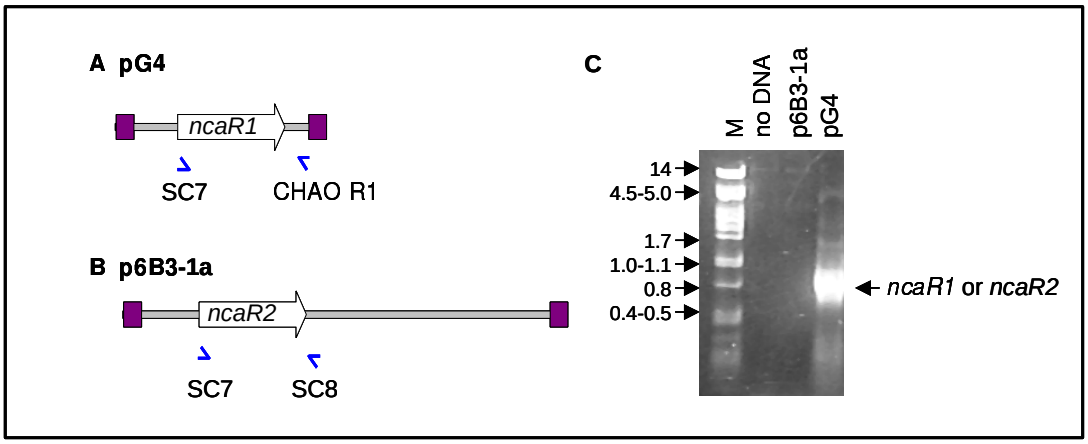


Figure 2.10. PCR amplification of *ncaR1* and *ncaR2*. Panel A shows the primers used to amplify *ncaR1* from pG4, and Panel B shows the primers used to amplify *ncaR2* from p6B3-1a. Panel C shows the agarose gel electrophoresis of the PCR amplification products. The position of *ncaR1* and *ncaR2* is indicated on the right hand. Lambda DNA digested with *Pst* I (M) is the molecular weight marker, the sizes of which (in kb) are indicated on the left hand side.

Agarose gel electrophoresis (Figure 2.10.C) showed that PCR amplification of *ncaR1* was obtained, while PCR amplification of *ncaR2* was not. Further attempts to isolate *ncaR2* by altering the PCR amplification conditions, such as lowering the annealing temperature and varying the concentration of template DNA, proved to be unsuccessful (data not shown).

Routine sequence analysis during the course of this study revealed some discrepancies with the previously determined nucleotide sequence of *ncaR1*. This, together with the inability to PCR amplify *ncaR2* resulted in the necessity to confirm the sequence of *ncaR1* and the presence of *ncaR2* in the plasmid constructs used in this study.

2.3.3 The nucleotide sequence of pG4.

The strategy for obtaining the complete nucleotide sequence of the DNA insert of pG4 with primers pUCF, pUCR, SC12 and SC13 is outlined in Figure 2.11.

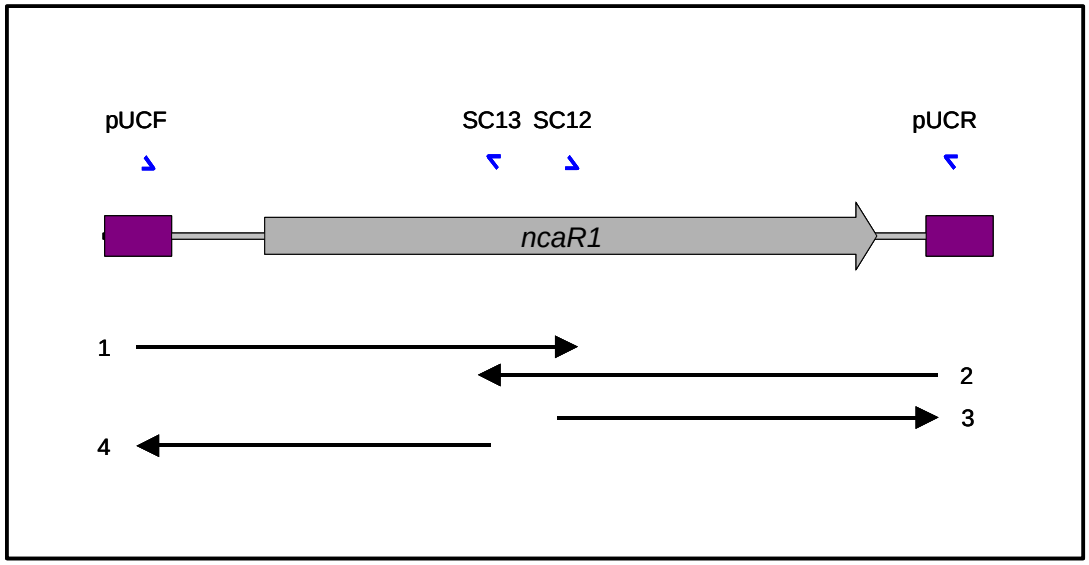


Figure 2.11. The strategy for sequencing the insert of pG4 where primers pUCF and pUCR were used to obtain preliminary sequence (1 and 2 respectively), and primers SC12 and SC13 were used complete the sequence in both directions (3 and 4 respectively).

The nucleotide sequences generated with these primers were used to compile a revised sequence of pG4, named pG4 (SAC) (Appendix 8a). The original nucleotide sequence of pG4 was aligned with pG4 (SAC) (Appendix 8b) and 33 nucleotides of the original pG4 sequence were found to be incorrect. Some of these nucleotide sequence errors resulted in changes in the deduced amino acid sequence of pG4 as revealed when alignments of the deduced amino acid sequence of pG4 and pG4 (SAC) were carried out (Figure 2.12). An alignment of the revised amino acid sequence of pG4 (SAC) with the available amino acid sequences of other *N*-carbamoyl-D-amino acid amidohydrolases (Figure 2.13), revealed that the *N*-carbamoyl-D-amino acid amidohydrolases *ncaR1* was more similar to previously isolated *N*-carbamoyl-D-amino acid amidohydrolases than previously thought.

Figure 2.12. An alignment of the deduced amino acid sequence of the original sequence of pG4, and pG4 (SAC). Dark shading represents residues identical in both sequences, while grey shading represents similar residues.

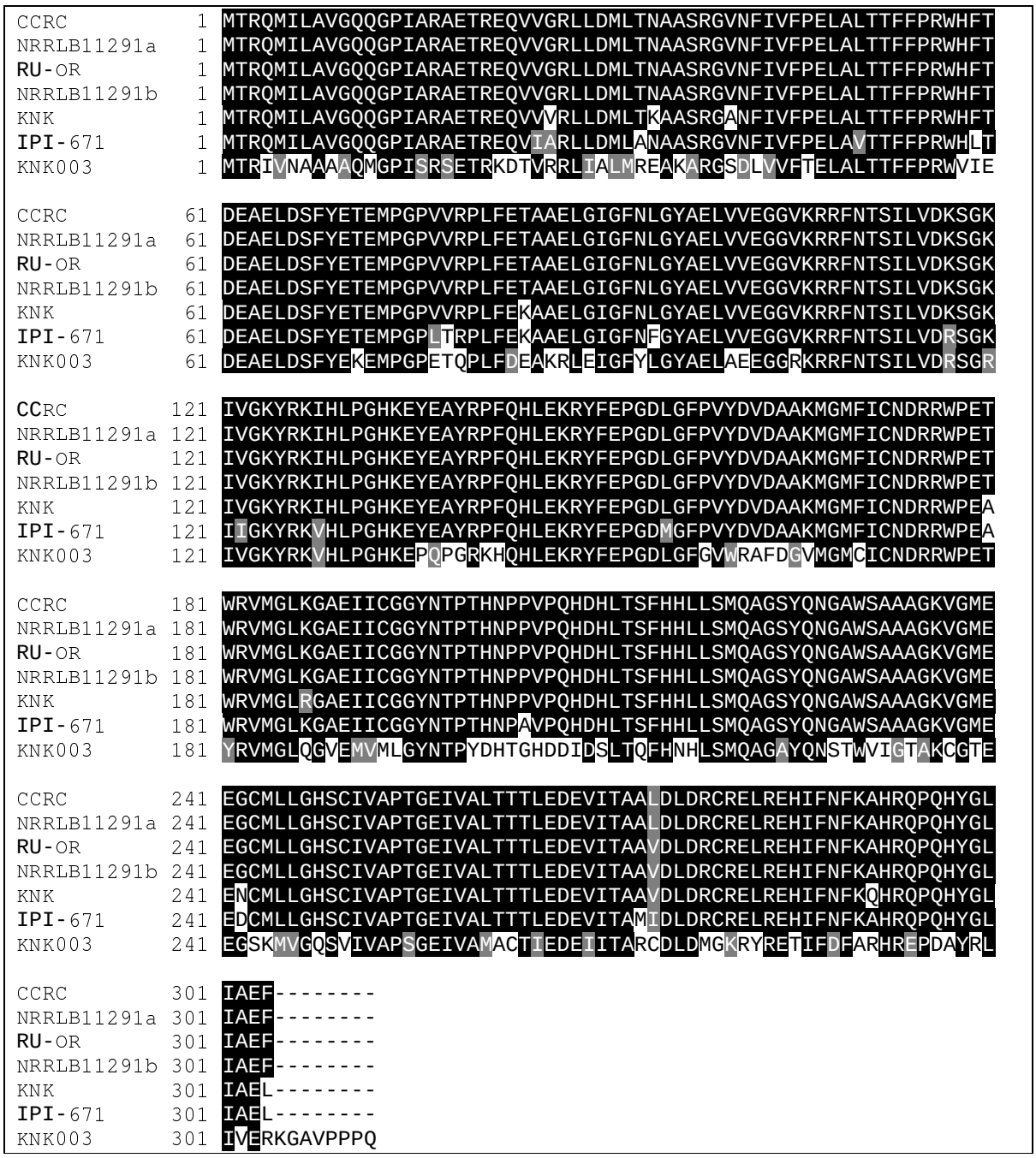
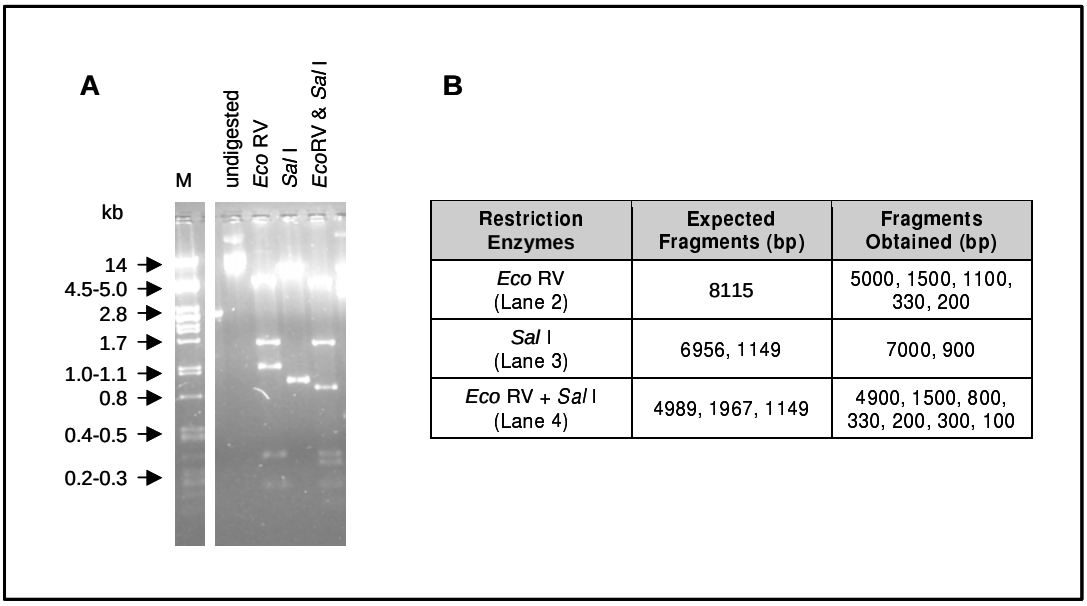


Figure 2.13. Multiple alignment (BOXSHADE) of the deduced amino acid sequence of NcaR1 from *A. tumefaciens* RU-OR (RU-OR) with the *N*-carbamoyl-amino acid amidohydrolases of *A. radiobacter* CCRC 14924 (CCRC14924), *A. radiobacter* KNK 712 (KNK712), *A. tumefaciens* NRRL B11291 (NRRLB11291a and NRRLB11291b), *Agrobacterium* sp. IP I-671 (IPI-671) and *Pseudomonas* sp. KNK 003A (KNK003). Dark shading represents residues identical in all the sequences, while grey shading represents similar residues.

2.3.4 The nucleotide sequence of p6B3-1a.

Restriction enzyme mapping of p6B3-1a (Figure 2.14), revealed several discrepancies which indicated that the plasmid DNA being used for PCR amplification (Section 2.2.2) did not correspond to the p6B3-1a, which was originally isolated.



Restriction Enzymes	Expected Fragments (bp)	Fragments Obtained (bp)
Eco RV (Lane 2)	8115	5000, 1500, 1100, 330, 200
Sal I (Lane 3)	6956, 1149	7000, 900
Eco RV + *Sal* I (Lane 4)	4989, 1967, 1149	4900, 1500, 800, 330, 200, 300, 100

Figure 2.14. Analysis of restriction enzyme fragments of p6B3-1a. Panel A shows the agarose gel electrophoresis of the restriction enzyme digests of p6B3-1a. The sizes of the Lambda DNA molecular weight marker (M) are indicated of the left hand side of the gel. Panel B shows the expected sizes and actual sizes of fragments of p6B3-1a obtained when digested with *Eco* RV, *Sal* I.

Numerous attempts to re-isolate the original p6B3-1a from glycerol stocks were unsuccessful. This, together with the inability to PCR amplify *ncaR2* (Section 2.2.2) resulted in the necessity to confirm the presence of *ncaR2* on the genomic DNA insert of p6B3-1a. The strategy used to sequence the genomic DNA insert of p6B3-1a involved determining the nucleotide sequence of 5' and 3' regions of the genomic DNA insert with the primers pUCF and pUCR, and then designing nested primers based on the sequence generated from pUCF and pUCR, which would be used to sequence further into the genomic DNA fragment. Then in turn, the nucleotide sequence generated from those primers would be used to design more primers for further sequencing. Figure 2.15 shows the primers that were designed, and the sequence that was generated in this manner.

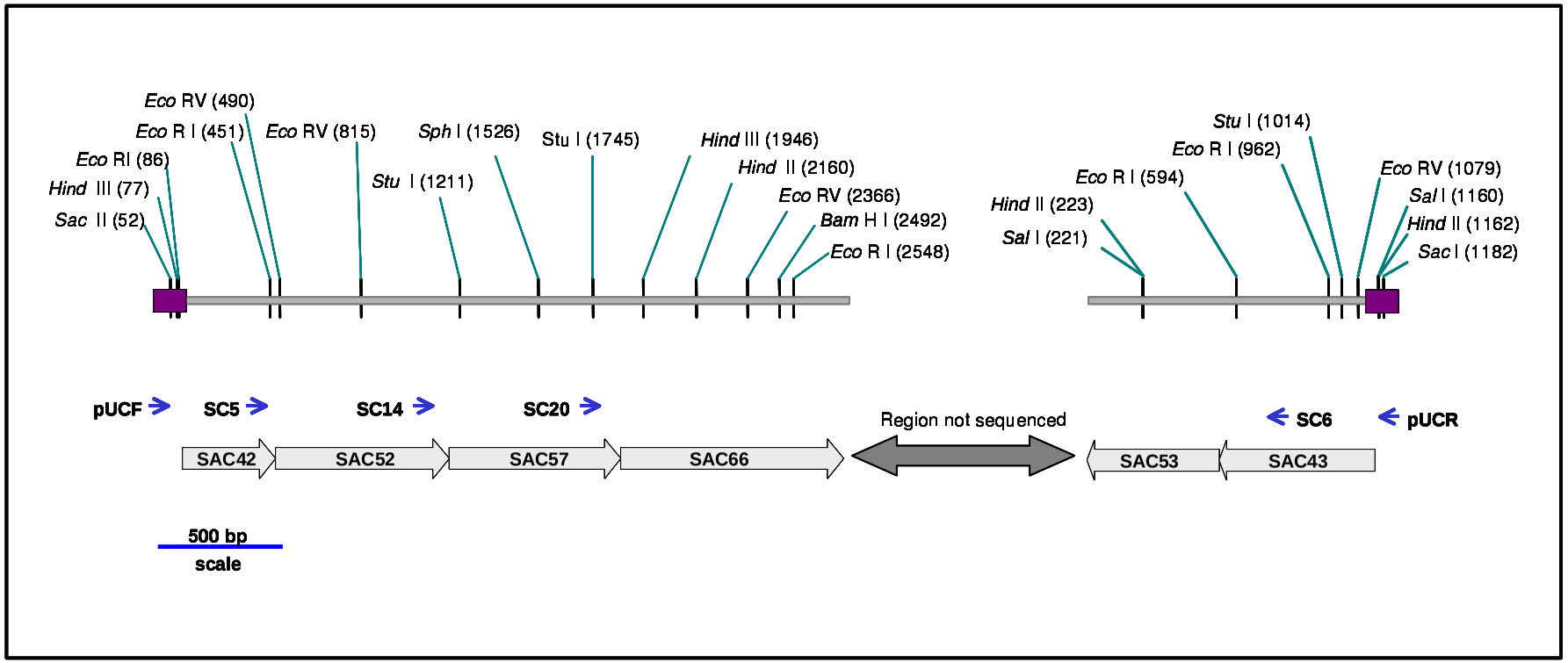


Figure 2.15. Sequencing the genomic DNA insert of p6B3-1a (SAC). A series of nested primers (indicated by →) were used to sequence p6B3-1a.

An alignment of the revised nucleotide sequence, p6B3-1a (SAC), with the original sequence of p6B3-1a showed that these sequences shared no significant nucleotide sequence similarity (data not shown). The nucleotide sequence of p6B3-1a (SAC) was analysed with Vector NTI and ten possible open reading frames were identified, as shown in Figure 2.16. Alignments of the nucleotide sequences of these open reading frames with the nucleotide sequence of *ncaR2* showed no significant similarity.

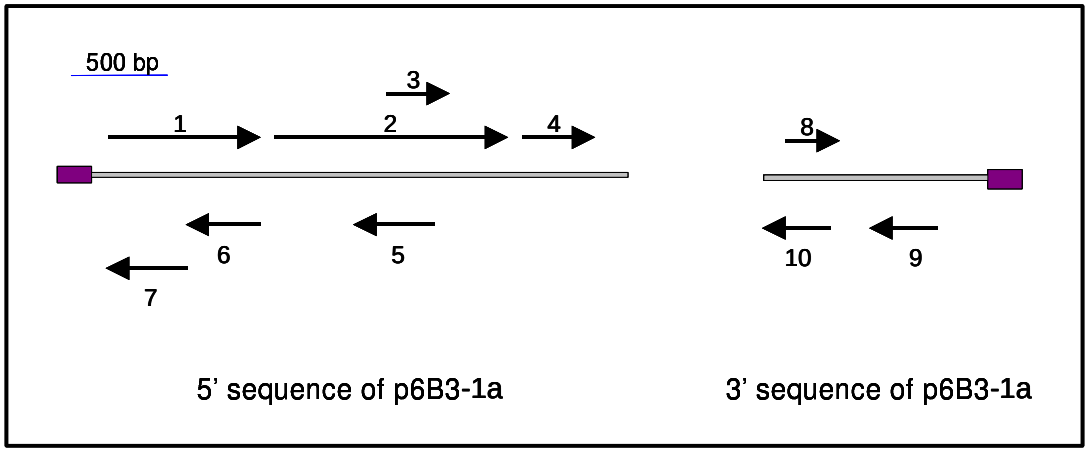


Figure 2.16. The positions of the predicted open reading frames in the 5' (1-7) and 3' (8-10) sequences of p6B3-1a (SAC).

Furthermore, alignments of the nucleotide sequences of these open reading frames with the nucleotide sequence of genes encoding other *N*-carbamoyl-amino acid amidohydrolases or related enzymes showed no significant nucleotide sequence similarity. The nucleotide and deduced amino acid sequences of the open reading frames of p6B3-1a (SAC) were compared with nucleotide and amino acid sequences in various databases, including the database of the *A. tumefaciens* C58 genome. These results are summarised in Table 2.7 and show that these open reading frames encode proteins that are not related to the enzymes involved in the utilisation of hydantoin.

Table 2.7. The proteins that show homology with the predicted open reading frames of p6B3-1a (SAC).

Predicted ORF	Length (nt)	Protein Encoded	Organism	Identity (%)
1	726	(vrlR)	*Dichelobacter nodosus*	44
2	1170	DEAH ATP-dependant helicase (vrlS)	*Dichelobacter nodosus*	41
3	318	D-lactate dehydrogenase like protein	*Arabidopsis thaliana*	44
4	366	no significant similarity		
5	414	putative sugar ABC transporter	*Sinorhizobium meliloti*	34
6	369	putative tRNA synthetase.	*Rhizobium etli*	32
7	414	cyclin B	*Dreissena polymorpha*	37
8	348	no significant similarity		
9	438	no significant similarity		
10	429	no significant similarity		

The nucleotide sequence of the plasmids p6B3-1a dX, p6B3-1a dC and p6B3-1a dH was performed with primers pUCF and pUCR. Alignments of these sequences with the original nucleotide sequence of p6B3-1a, and the nucleotide sequence p6B3-1a (SAC) were carried out. Since these plasmids were derived from p6B3-1a from which the original nucleotide sequence was obtained, it was expected that they would match with the original nucleotide sequence of p6B3-1a.

However, it was found that these sequences matched with the nucleotide sequence of p6B3-1a (SAC) (Figure 2.17), and not with the original sequence of p6B3-1a. This indicated that the nucleotide sequence of p6B3-1a (SAC) is the correct existing sequence of p6B3-1a.

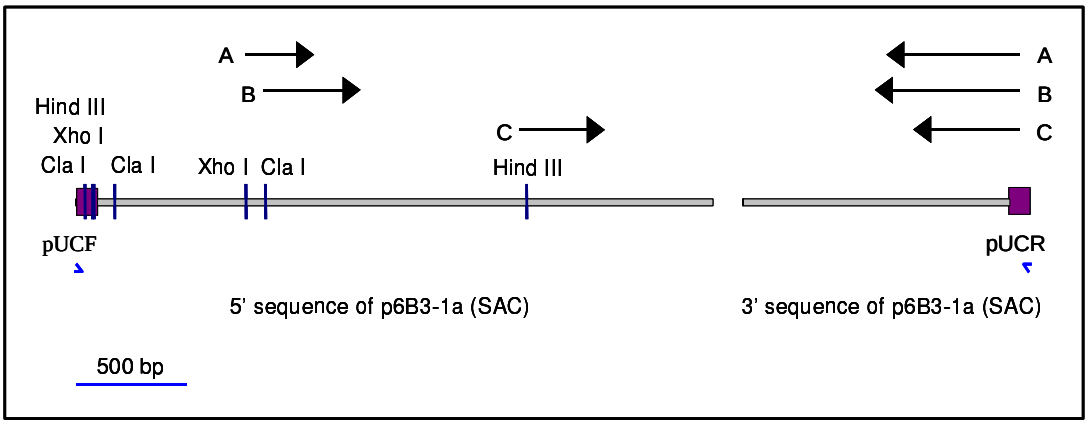


Figure 2.17. p6B3-1a deletions alignment with p6B3-1a (SAC). The positions of the sequences of p6B3-1a dX, p6B3-1a dC and p6B3-1a dH which bear sequence similarity to p6B3-1a (SAC) are indicated by arrows A, B and C respectively.

N-carbamoyl-amino acid amidohydrolase assays were carried out on p6B3-1a, p6B3-1a dX, p6B3-1a dC and p6B3-1a dH. None were found to possess significant *N*-carbamoyl-amino acid amidohydrolase activity (data not shown) despite being shown to possess *N*-carbamoyl-amino acid amidohydrolase activity in the original study (Hartley, 2001, Figure 2.4).

2.4. Discussion

2.4.1. Screening the genomic DNA library of *A. tumefaciens* RU-OR.

According to the formula of Ausubel *et al.* (1983), it was calculated that a total of 5500 insert-containing plasmids from the genomic library of *A. tumefaciens* RU-AE01 needed to be screened in order to have a 99% probably of isolating the gene of interest. It was calculated that a total of 300 000 insert-containing plasmids were screened, which is 54 times more than were required to be screened. Since two of the wells from Library Set A only contained 1 insert-containing plasmids per well, and Library Set B, only contained 18 insert-containing plasmids per well, it could be calculated that approximately 180 000 plasmids were screened. However, this value is still 32 times greater than 5500, so the number of insert-containing plasmids which were screened was excessive, but ensured a greater chance of isolating the genes of interest.

Despite this, no clones carrying genes encoding proteins related to the enzymes involved in hydantoin utilisation were isolated. The partial sequences of plasmids isolated from well 8F and 9E were shown to fall within the same region of the *A. tumefaciens* C58 circular chromosome, and the open reading frames encoded by this region show no homology to hydantoin-hydrolysing enzymes. Since the sequence of this region of the *A. tumefaciens* C58 circular chromosome also bears no homology to the sequence of Probe 1 that was used for screening, the reason for a positive result in the Southern blot is unexplained.

Binding of Probe 1 and Probe 2 to pBK-CMV vector DNA (Figure 2.9) was observed, and since the entire library of clones were recombinants of pBK-CMV, the DNA probes were binding to all of them. The absence of hybridisation of the probe to the DNA molecular weight marker suggested that the DNA probe was not hybridising randomly with any DNA, and supports the proposal that the probes were binding to pBK-CMV vector DNA. Since Probe 1 does not bear any sequence similarity to pBK-CMV, it is unclear why this hybridisation occurred. Repetition of the experiment with more stringent hybridisation conditions did not eliminate the binding of Probe 1 to pBK-CMV.

During the synthesis of Probe 1, there was evidence of non-specific PCR amplification products (Figure 2.5). If sufficiently radiolabelled, binding of these non-specific PCR amplification products to pBK-CMV vector DNA may account for what was mistaken for the hybridisation of Probe 1 to *A. tumefaciens* RU-AE01 genomic DNA.

2.4.2. PCR amplification and sequence analysis of *ncaR1* and *ncaR2.*

The failure to isolate any genes encoding proteins involved in the hydrolysis of hydantoins from *A. tumefaciens* RU-AE01 led to the pursuit of the study of *ncaR1* and *ncaR2* from *A. tumefaciens* RU-OR, with the objective to express and purify these enzymes for biochemical characterisation. PCR amplification of *ncaR1* was carried out, and sequencing revealed discrepancies between the PCR amplified *ncaR1* and the original sequence as encoded by pG4. Sequencing of pG4 was repeated, and the revised sequence, pG4 (SAC) showed that *ncaR1* encoded an *N*-carbamoyl-amino acid amidohydrolase that was more similar to previously isolated *N*-carbamoyl-amino acid amidohydrolases than originally thought by Hartley (2001).

PCR amplification of *ncaR2* was unsuccessful and restriction enzyme mapping of p6B3-1a revealed inconsistencies with the original sequence of p6B3-1a. Sequencing of p6B3-1a was repeated with a series of nested primers and the resulting sequence p6B3-1a (SAC) showed no sequence similarity to original p6B3-1a nucleotide sequence. The open reading frames of p6B3-1a (SAC) bore no similarity to *ncaR2* or any other *N*-carbamoyl-amino acid amidohydrolases.

Partial sequences of p6B3-1a dX, p6B3-1a dC and p6B3-1a dH were found to match with the sequence of p6B3-1a (SAC), which indicated that this was the original nucleotide sequence of p6B3-1a. In addition, p6B3-1a, p6B3-1a dX, p6B3-1a dC and p6B3-1a dH were shown to possess no NCAAH activity, where previously all except p6B3-1a dH were found to possess NCAAH activity (Hartley, 2001).

Plasmid rearrangements or the phenomenon of “looping out” of potentially toxic genes in recombinant plasmids (Banyex, 1999) may provide an explanation for the loss of *ncaR2* from p6B3-1a. Also, Hils *et al* (2001) showed that the hydantoin utilising gene

cluster of *Agrobacterium* sp. IP I-671 was flanked by a putative transposase and resolvase, which were suggested to be part of a transposon carrying the hydantoin utilising genes of *Agrobacterium* sp. IP I-671. Therefore it is possible that *ncaR2* was similarly associated with other hydantoin utilisation genes in a transposon in *A. tumefaciens* RU-OR, and a transposition event may have resulted in the loss of *ncaR2* from p6B3-1a. However, if this were the case, then certain regions of p6B3-1a (SAC) should have show homology to the original sequence of p6B3-1a.

Attempts to isolate *ncaR2* from the genomic DNA of *A. tumefaciens* RU-OR by PCR amplification was also found to be unsuccessful (data not shown) and brought to light the possibility that *ncaR2* was no longer present on the chromosome of *A. tumefaciens* RU-OR. A recent study showed that the *N*-carbamoyl-amino acid amidohydrolases of *A. tumefaciens* NRRL B11291 and *Agrobacterium* sp. IP I-671 were located on 160kb and 190kb plasmids respectively (Hils *et al.,* 2001). This may have been the case in *A. tumefaciens* RU-OR. There is a possibility that this plasmid have been cured from this strain with repeated sub culturing resulting in the loss of *ncaR2*. However it was shown that *A. tumefaciens* RU-OR still possessed *N*-carbamoyl-amino acid amidohydrolases (data not shown) and this *N*-carbamoyl-amino acid amidohydrolase activity may be encoded by *ncaR1* elsewhere on the genome of *A. tumefaciens* RU-OR.

CHAPTER 3

Expression of *ncaR1* and *ncaR1-6xHis*

CHAPTER 3

Expression of *ncaR1* and *ncaR1-6xHis*

3.1. Introduction.

E. coli is one of the most widely used hosts for the production of heterologous proteins. Of the many systems available, *E. coli* remains one of the most attractive for a number of reasons. The genetics are well characterised making it easier to manipulate from the natural host, and there are well understood mutants of *E. coli* already available. *E. coli* also has the ability to grow rapidly on many substrates (Banyex 1999; Hannig and Makrides, 1998; Hockney, 1994; Weickert *et al.,* 1996).

One of the advantages of heterologous expression systems is that instead of using the native promoter for the expression of the gene of interest, it is placed under the control of an alternative promoter whose activity may easily be regulated. The cloning vectors that are available for heterologous expression in *E. coli* have a wide range of promoters (Table 3.1.), that have been reviewed extensively (Hannig and Makrides, 1998; Weickert *et al.,* 1996), and can be chosen to suit the needs of the gene of interest.

Table 3.1. Some commonly used promoters used in *E. coli* heterologous expression systems. (adapted from Weickert *et al.,* 1996).

Promoter	Induction	Advantages	Disadvantages
lac	IPTG, high temperature	wide range of induction levels	low level expression, leaky expression
T7	IPTG, high temperature	wide range of induction levels, many vectors available	leaky expression, difficult to achieve high expression
trp	tryptophan starvation, indole acrylic acid	high level expression, many vectors available, can be titrated	leaky expression
ara	arabinose	tight regulation, wide range of induction levels, rapid induction/expression	few vectors available, catabolite repression by glucose
recA	nalidixic acid	high level expression, not host specific	not titratable

3.1.1. T7 RNA Polymerase Heterologous Expression System.

The T7 polymerase system was selected for heterologous production of NcaR1 in *E. coli*, using the expression vector pT7-7 (Tabor, 1983), which possesses the T7 RNA polymerase promoter. The highly processive T7 RNA polymerase is supplied in *trans*, where the host contains a prophage (λDE3) encoding the enzyme under the control of the IPTG inducible *lac* promoter (Baneyx, 1999).

This is a highly efficient expression system, leading to an accumulation of the desired protein up to 40-50% of total cell protein (Baneyx, 1999). However, it has a number of disadvantages. The leaky expression of the T7 RNA polymerase may result in plasmid or expression instability (Baneyx, 1999), and it has been shown that other expression vectors based on the T7 polymerase system are toxic to the cell, even when they contain no insert (Miroux, 1996). In addition, the strong promoter system results in the synthesis of proteins, which do not fold correctly and aggregate to form inclusion bodies. This problem may be overcome by the co-expression of chaperone proteins, which assist the correct folding of these proteins (Weickert *et al.*., 1996).

3.1.2. Fusion Proteins and Affinity Tags to Improve Heterologous Expression

The development of the technology for the expression of fusion proteins and affinity tags has facilitated high-level production and purification of recombinant proteins in *E. coli.* The advantages of expression of a protein fused to another protein, protein fragment or short polypeptide tag are numerous. They can increase the accumulation of the desired protein and they often prevent inclusion body formation by improving the folding characteristics of the protein of interest (Guan *et al.,* 1991; Kamitani *et al.,* 1992). Fusion proteins provide a means by which the protein of interest can be readily purified and or immobilised (Ostermeier *et al.,* 1996; Forrer *et al.,* 1998 and Pryor *et al.,* 1997). They also provide a means by which the expression of the protein of interest can be detected (Ellsworth *et al.,* 2000 and Hernan *et al.,* 2000). In addition, they curb proteolysis of the protein of interest (Hannig and Makrides, 1998, Hockney, 1994).

A few examples of widely used fusion proteins and affinity tags include maltose-binding proteins (MBP), green fluorescent protein (GFP), glutothione-S-transferase (GST) and hexa-Histidine tags (6xHis) (reviewed by Hannig and Makrides, 1998). Vectors for creating these protein fusions are described widely in the literature (Bedouelle and Duplay, 1998; Smith and Johnson, 1988; Hochuli *et al.,* 1987), and are freely available commercially.

3.1.3. *In vitro* expression systems.

The Rapid Translation System (RTS) (Roche) is an *in vitro* protein synthesis system, where transcription and translation take place simultaneously in an E. *coli* HY cell-free lysate, producing functionally active protein. The vectors that are provided with this system are designed for high-level expression of His-tagged proteins in the cell free RTS *E. coli* system. Two types of the vectors supplied with the RTS, introduce either an amino- or a carboxy-terminal (N-terminal or C-terminal) hexa-histidine tag, which provides a rapid method for detection and purification, by nickel affinity chromatography, of the protein of interest. The target gene is placed under the control of the T7 promoter located downstream of a ribosomal binding sequence.

The RTS 100 system has a number of advantages for the expression of *ncaR1*. It is a rapid protein synthesis system that can easily be optimised. The cell free nature of the reaction allows for the expression of toxic gene products, which is of particular use for the expression of *ncaR1*, as it has been suggested that previous attempts at heterologous expression and isolation of other N-carbamoyl-amino acid amidohydrolases has been unsuccessful due to the toxicity of the protein which leads to the formation of insoluble aggregates (Buson *et al.,* 1996; Chao *et al.,* 1999a; Grifantini *et al.,* 1998; Hils *et al.,* 2001, Sareen *et al.,* 2001b). In addition, the reaction conditions of the i*n vitro* system are easily altered to overcome insolubility or folding problems commonly associated with bacterial systems. Furthermore, the expression of proteins with tags or reporter genes facilitates easy purification and detection.

3.1.4. Objectives.

The research performed in this chapter describes the production of NcaR1 using recombinant DNA technology. Two approaches were used for two different purposes. The first approach was to determine if it was possible to heterologously express *ncaR1* in *E. coli* BL21 (DE3) to produce NcaR1 using the T7 RNA polymerase promoter system (Tabor, 1983). The second was the *in vitro* synthesis of NcaR1 with a C-terminal His-tag (NcaR1-6xHis) using the RTS-100 *E. coli* HY system (Roche). The His-tag provided a means to purify the protein using His-tag affinity columns (Ni-NTA) and allowed for simplified detection of NcaR1-6xHis. The purified NcaR1-6xHis would then be used for the synthesis of anti-NcaR1-6xHis antibodies, which would provide a tool for the detection of NcaR1 in *A. tumefaciens* RU-OR, and to assess the level of NcaR1 expression in *A. tumefaciens* RU-OR and various regulatory mutants of RU-OR under different growth conditions.

3.2. Materials and Methods.

3.2.1. Bacterial strains, plasmids and culture conditions.

Recombinant plasmids were hosted in *E. coli* DH5α, and heterologous expression was carried out in *E. coli* BL21 (DE3) (Table 3.2). Growth of *E. coli* was carried out as described in section 2.2.1. The plasmids used in this chapter are listed in Table 3.3, where the parental vector and the origin of the DNA insert is indicated.

Table 3.2. *E. coli* host strains used in this chapter.

Strain	Genotype	Reference
DH5α	*F⁻Φ80dlacZΔM15Δ(lacZYA-argF)U169 endA1 recA1 hsdR17* ($r_k^-m_k^-$) *deoR thi-1 supE44 λ⁻gyrA96 relA1*	Hanahan, 1983
BL21 (DE3)	*F⁻ dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ[DE3]	Studier and Moffat, 1986

Table 3.3. Plasmids used in this chapter.

Plasmid	Origin of Insert	Reference
pGEM-T Easy	cloning vector	Promega
pT7-7	expression vector	Tabor, 1983
pIVEX2.3 MCS	expression vector	Roche
pRD106	expression vector, derived from pT7-7	Idicula *et al.,* 2002
pGFP	contains the gene encoding Green Fluorescent Protein (GFP), in pIVEX	Prasher *et al.,* 1992
pG4	1179bp PCR amplification product from RU-OR genomic DNA using primers CHAOF1 & CHAOR1 in pGEM-T Easy	Hartley, 2001
pB+M	993bp PCR amplification product from RU-OR genomic DNA using primers CH7 & CHAOR1 in pGEM-T Easy	This study
pBM	986 bp *Nde* I – *Hind* III fragment from pB+M in pT7-7	This study
pT7G4	1171 bp *Bam* HI – *Hind* III fragment from pG4 in pT7-7	This study
pT7G4-CDS	195bp *Nde* I -*Sac* I fragment from pBM1 inserted into pT7G4 in pT7-7	This study
pT7G4-ΔCDS	423bp *Eco* RI – *Sal* I fragment deleted from insert of pT7G4-CDS in pT7-7	This study
pG4-7/10	920 bp PCR amplification product from RU-OR genomic DNA using primers SC7 & SC10 in pGEM-T Easy	This study
pG4-7/10a	921 bp PCR amplification product from RU-OR genomic DNA using primers SC7 & SC10a in pGEM-T Easy	This study
pIVEXG4-CDS-N	915bp *Nde* I–*Sma* I fragment from pG4-7/10 in pIVEX2.3 MCS	This study
pIVEXG4-CDS	721 bp *Sac* I-*Sma* I fragment from pG4-7/10a inserted into pIVEXG4-CDS-N in pIVEX2.3 MCS	This study

3.2.2. Recombinant DNA techniques.

Recombinant plasmid DNA from *E. coli* was isolated as described in Section 2.1.2. Restriction analysis of plasmid DNA was performed as described in Section 2.1.2. DNA modifying enzymes (Appendix 9) were used as recommended by the manufacturers. DNA was concentrated and purified by phenol extraction and ethanol precipitation as described by Sambrook *et al* (1989) or using the DNA Clean and Concentrator Kit (Zymo).

3.2.3. PCR amplification of *ncaR1* and *ncaR1*-6xHis.

PCR amplification of *ncaR1* and *ncaR1*-6xHis was carried out using the primers CH7, SC7, CHAO R1, SC10 and SC10a that were designed for the amplification of *ncaR1* as outlined in Table 3.4. PCR amplification was performed with Taq RNA polymerase (Roche) or with the Expand High Fidelity PCR System (Roche) using thermal cycling programme 1 (Appendix 4). The PCR amplification products were analysed as described previously (Section 2.1.3).

Table 3.4. Primers used for the PCR amplification of *ncaR1* and *ncaR1-6xHis*.

Primer	Description	Primer
CH7	primer corresponding to the 5' sequence of *ncaR1*, introduces an *Nde* I restriction enzyme site	GGAATCCCATATGACACGTCAGATGATACTTGCTGTCG
SC7	primer corresponding to the 5' sequence of *ncaR1*, introduces an *Nde* I restriction enzyme site	CATATGACACGTCAGATGATACTTGC
CHAOR1	primer corresponding to the 3' sequence of *ncaR1*, introduces a *Bam* HI restriction enzyme site	CCCAAGCTTTCTCGATCGGATAGG
SC10	primer corresponding to the 3' sequence of *ncaR1*, introduces a *Sma* I restriction enzyme site	CCCGGGAATTCCGCGATCAGACC
SC10a	primer corresponding to the 3' sequence of *ncaR1*, introduces a *Sma* I restriction enzyme site an in-frame fusion to His-tag encoded by pIVEXG4-CDS	CCCGGGGAATTCCGCGATCAGACC

3.2.4. DNA sequencing and sequence analysis.

DNA sequencing and sequence analysis was carried out as described in Section 2.1.6. Unless otherwise stated the primers used for sequencing of recombinants of pGEM-T Easy were pUCF and pUCR, those for sequencing recombinants for pT7-7 were T7-7F and T7-7R, and those for sequencing recombinants of pIVEX2.3 MCS were T7-7F and T7-R (Appendix 3).

3.2.5. Construction of vectors for heterologous expression of *ncaR1*.

A 1171bp *Bam* HI – *Hind* III fragment, carrying the promoter and coding sequence for *ncaR1*, was excised from pG4 (Figure 3.1, Step II) and inserted between the *Bam* HI and *Hind* III restriction enzyme sites of the multiple cloning site (MCS) of pT7-7 by ligation with T4 DNA Ligase (Promega) (Figure 3.1, Step I and III). Recombinants with the correct insert were selected by restriction enzyme mapping. DNA sequencing with primers T7-7F and T7-7R was carried out to confirm the presence of the correct insert. The resulting plasmid, pT7G4 carried the *ncaR1* gene and its endogenous promoter downstream of the T7 polymerase promoter and ribosomal binding site.

The coding sequence of *ncaR1* was amplified from pG4 by PCR amplification with CH7 and CHAOR1 (Figure 3.2, Step I), using Taq DNA polymerase (Roche), as described in Section 3.2.3. The 1003bp PCR amplification product, carrying *ncaR1* was ligated into pGEM-T Easy Cloning Vector (Figure 3.2, Step II), to generate pB+M. The primers CH7 and CHAO R1 introduced *Nde* I and *Hind* III restriction enzyme sites flanking *ncaR1*, which were used to excise a 986bp *Nde* I -*Hind* III fragment from pB+M (Figure 3.2, Step III) that was ligated into the *Nde* I – *Hind* III restriction enzyme sites of pRD106 (Figure 3.2. Step IV and V). The presence of the correct insert was confirmed by restriction mapping, and the resultant construct pBM1, was sequenced using T7-7F and T7-7R (Appendix 3) to confirm the absence of point mutations in *ncaR1* that may have arisen during the PCR amplification.

A 195 bp *Nde* I - *Sac* I fragment was excised from pBM1 (Figure 3.3, Step II) and inserted into the corresponding *Nde* I – *Sac* I cohesive ends of pT7G4 (Figure 3.3, Step

I and III). The insertion of the correct insert was confirmed by restriction mapping. DNA sequencing was carried out using T7-7F and T7-7R (Primers 3) for further confirmation. The resultant construct, pT7G4-CDS, was used for expression of *ncaR1* in *E. coli* BL21 (DE3).

A construct for the expression of a truncated *ncaR1* was generated by deleting a 423 bp *Eco* RI – *Sal* I fragment from the coding sequence of *ncaR1* in pT7G4-CDS (Figure 3.3, Step IV). This was achieved by digesting pT7G4-CDS with *Eco* RI and *Sal* I, filling in the 5' overhangs with Klenow (DNA Polymerase I Large Fragment) and dNTPs. The plasmid was self ligated causing re-circularisation of the 2998bp fragment generating pT7G4-ΔCDS.

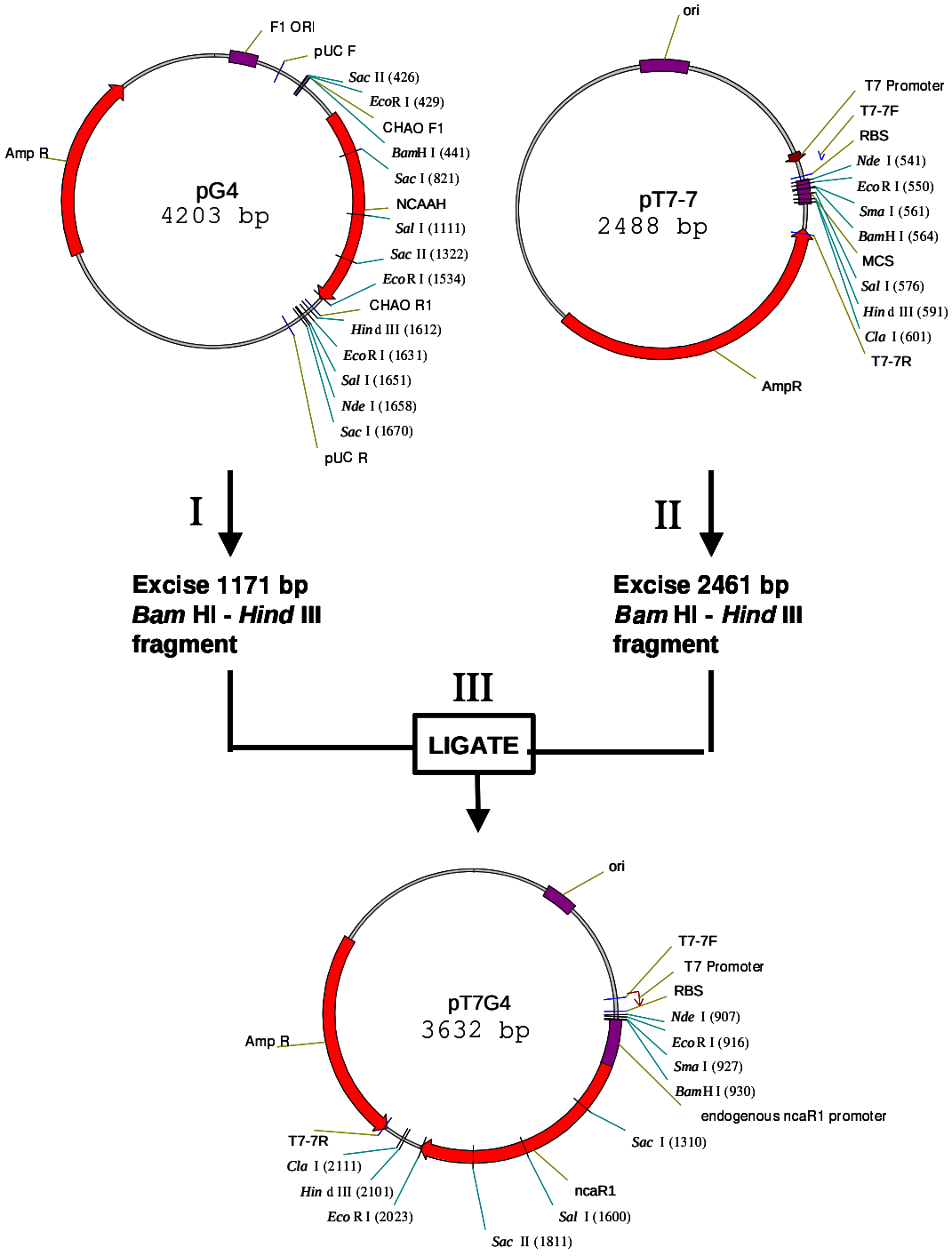


Figure 3.1. A flow diagram showing the approach used for the construction of pT7G4.

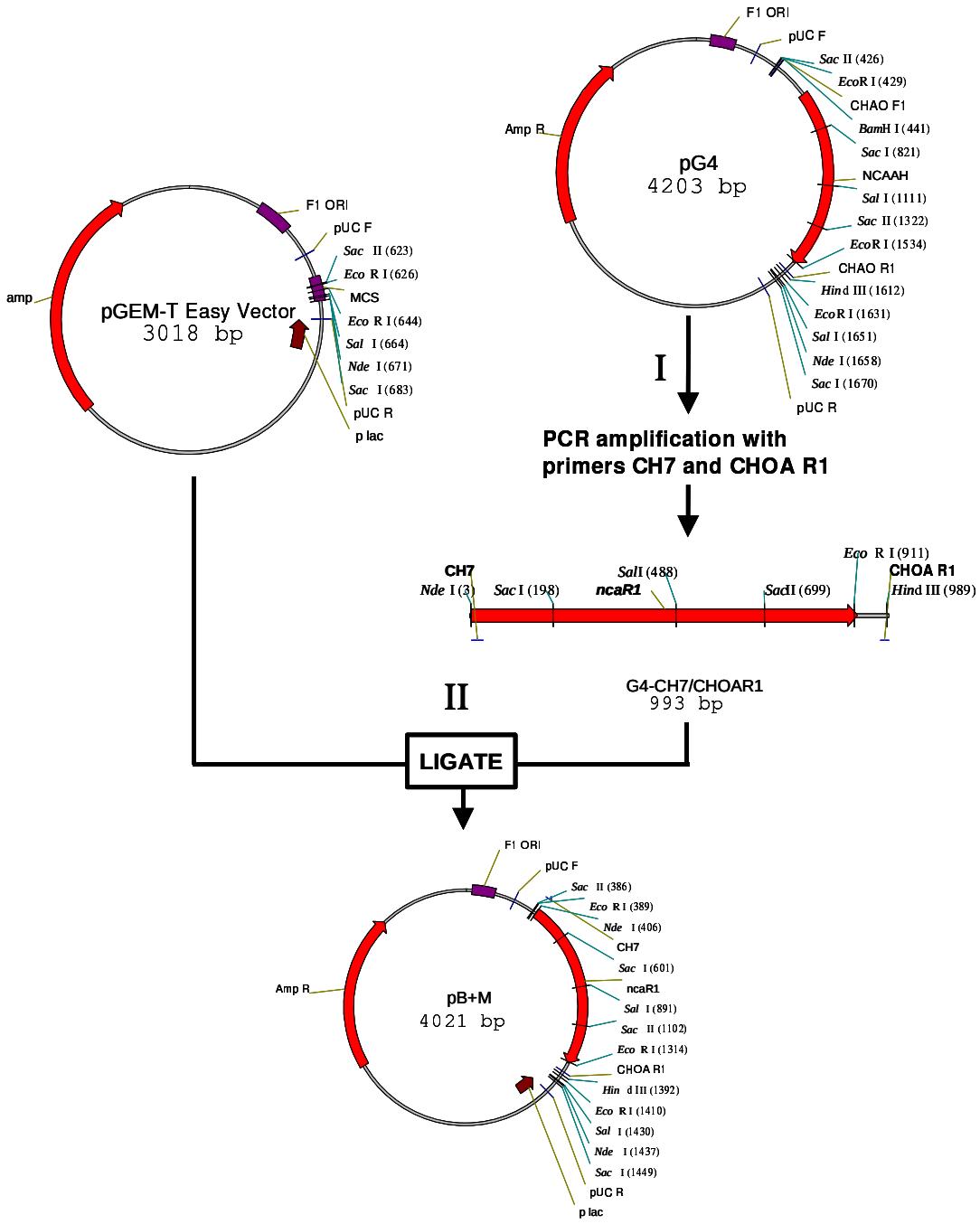


Figure 3.2. A flow diagram showing the approach for the construction of pBM1.

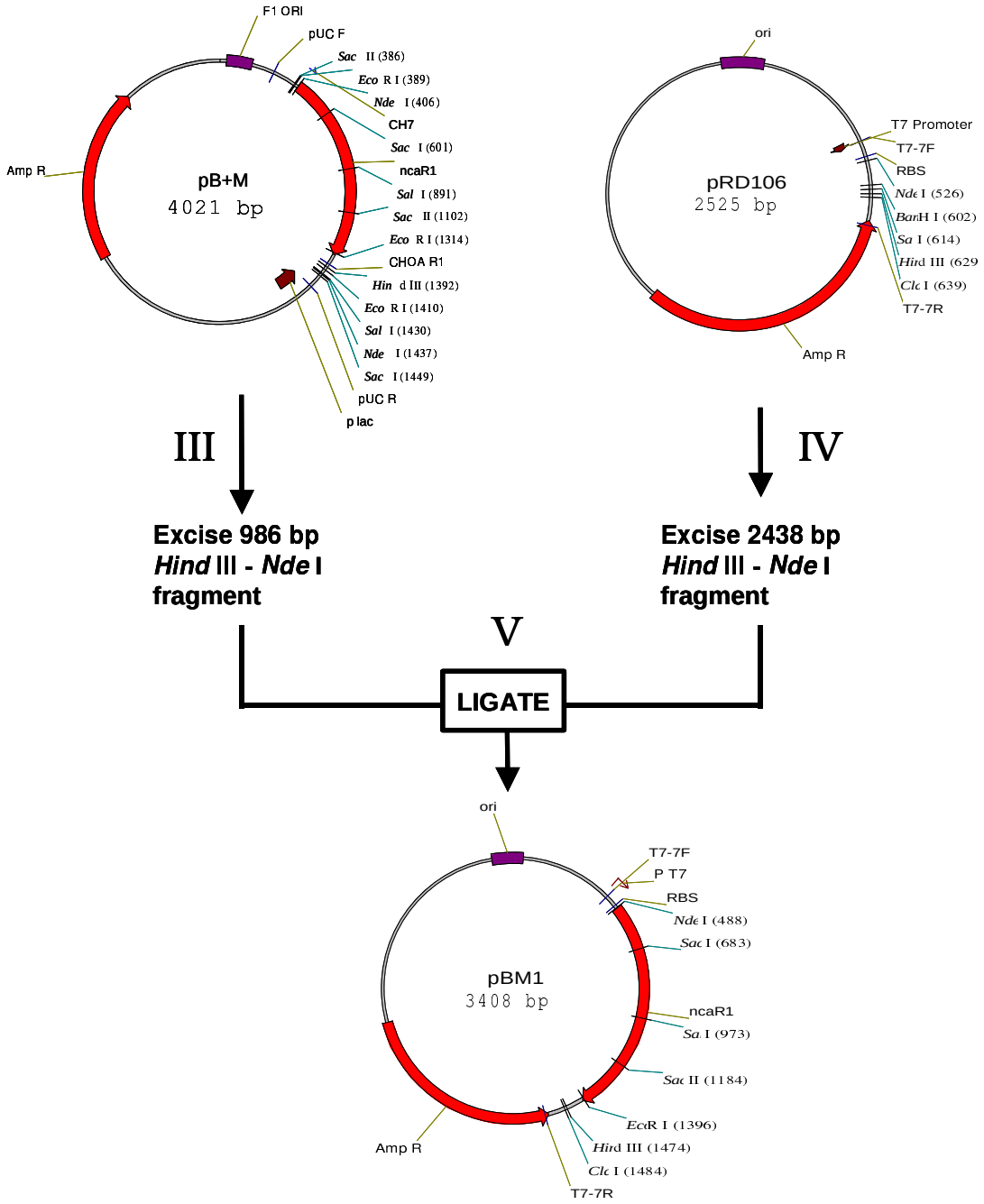


Figure 3.2 contd. A flow diagram showing the approach for the construction of pBM1.

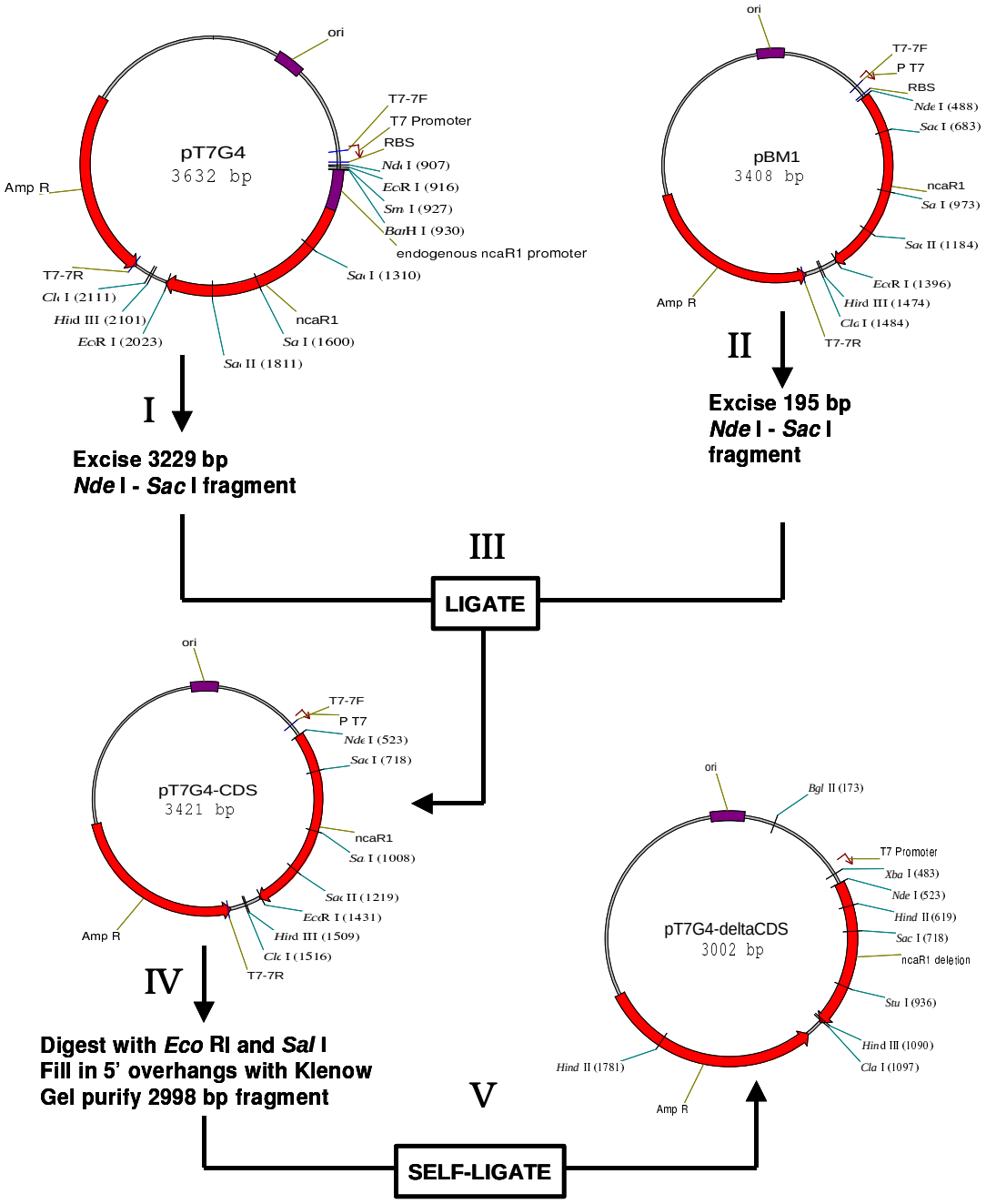


Figure 3.3. A flow diagram showing the approach used for the construction of pT7G4-CDS and pT7G4-ΔCDS.

3.2.6. Construction of C-terminal His-tagged NcaR1 vector.

The coding sequence of *ncaR1* was amplified by PCR from pG4 (Figure 3.4, Step I) using the Expand High Fidelity PCR System (Roche) with primers SC7 and SC10 as described in Section 3.2.3. The 920bp PCR amplification product carrying the NcaR1 coding sequence flanked by *Nde* I and *Sma* I was ligated into the pGEM-T Easy cloning vector (Figure 3.4, Step II), resulting in pG4-7/10. Sequencing of pG4-7/10 was carried out with primers pUC F and pUC R (Appendix 3) to confirm the absence of point mutations in *ncaR1*, which may have been introduced by PCR amplification. A 915bp *Nde* I -*Sma* I fragment was excised from pG4-7/10 (Figure 3.4, Step III) and inserted between the *Nde* I – *Sma* I restriction enzyme sites of the vector pIVEX 2.3 MCS (Figure 3.4. Step IV and V). The fusion of *ncaR1* with a C-terminal His-tag in the resulting construct, pIVEX G4-CDS-N, was determined by sequencing with T7 Prom and T7 Term (Appendix 3).

An error in the design of the primer SC10, which introduced a frame shift in the sequence encoding the His-tag and resulted in the incorrect fusion of *ncaR1* with the His-tag, was discovered. SC10 was redesigned to correct this frame shift, and SC10a, was used in combination with SC7 to repeat the PCR amplification of *ncaR1* (Figure 3.5, Step I) as described previously. The 921bp PCR amplification product was inserted into the pGEM-T Easy cloning vector (Figure 3.5, Step II), resulting in pG4-7/10a. Sequencing of pG4-7/10a was carried out with primers pUC F and pUC R (Appendix 3) to confirm the absence of point mutations in *ncaR1*, which may have arisen during PCR amplification. The 721 bp *Sac* I – *Sma* I fragment, carrying the sequence for the correct fusion between *ncaR1* and the His-tag, was excised from pG4-7/10a (Figure 3.5, Step III) and inserted into the *Sac* I – *Sma* I sites of pIVEX G4-CDS-N, to replace the 720 bp *Sac* I – *Sma* I fragment with the frame shift mutation generated by primer SC10 (Figure 3.5, Step IV and V). DNA sequencing with primers T7-7F and T7 R (Appendix 3) was carried out to verify the correct fusion of NcaR1 with a C-terminal His-tag in the resulting construct, pIVEX G4-CDS. pIVEX G4-CDS was used for *in vitro* expression of *ncaR1* using the RTS 100 *E. coli* HY Kit, and also for heterologous expression of *ncaR1* in *E. coli* BL21(DE3) cells.

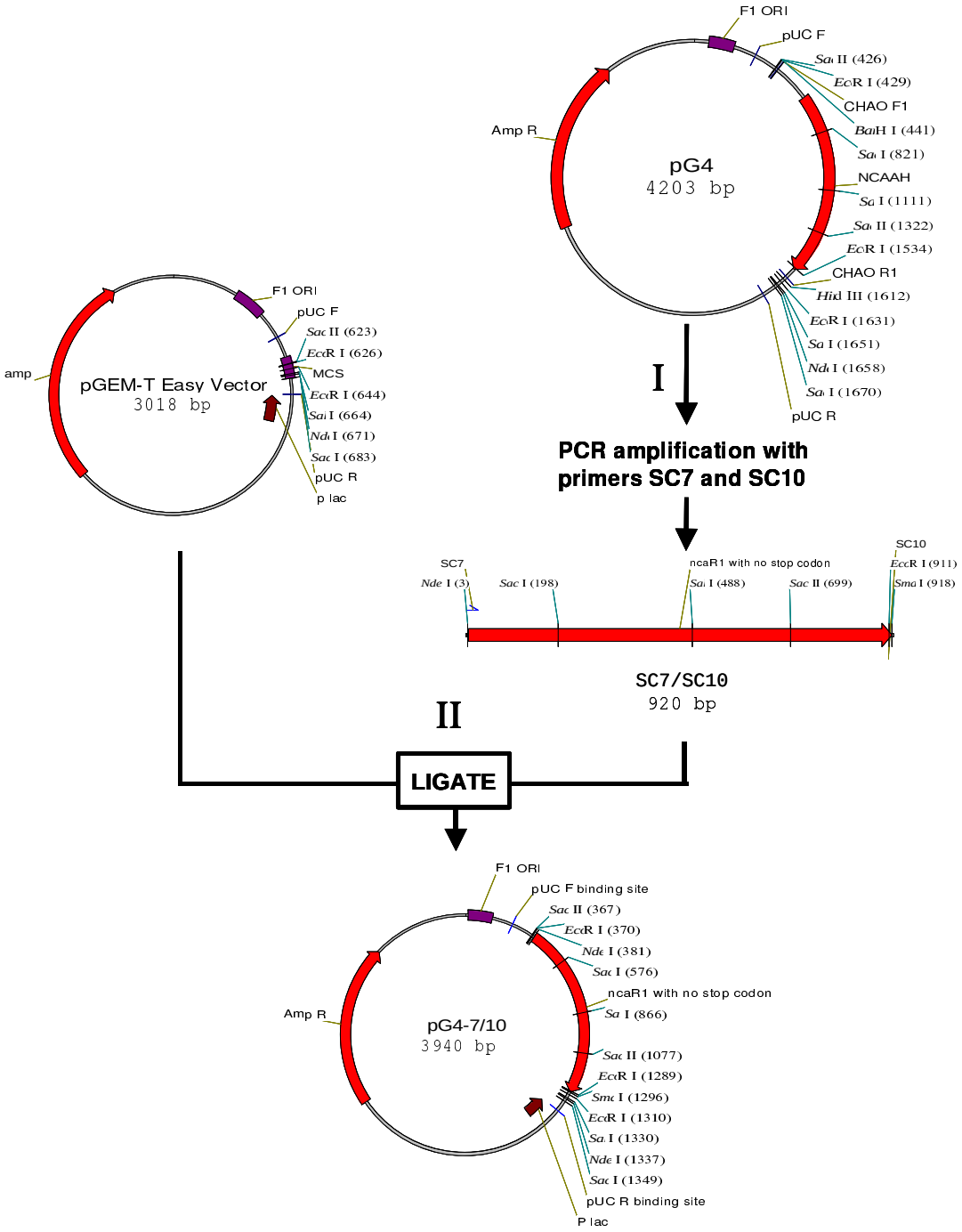


Figure 3.4. Construction of pIVEX-G4-CDS-N**.**

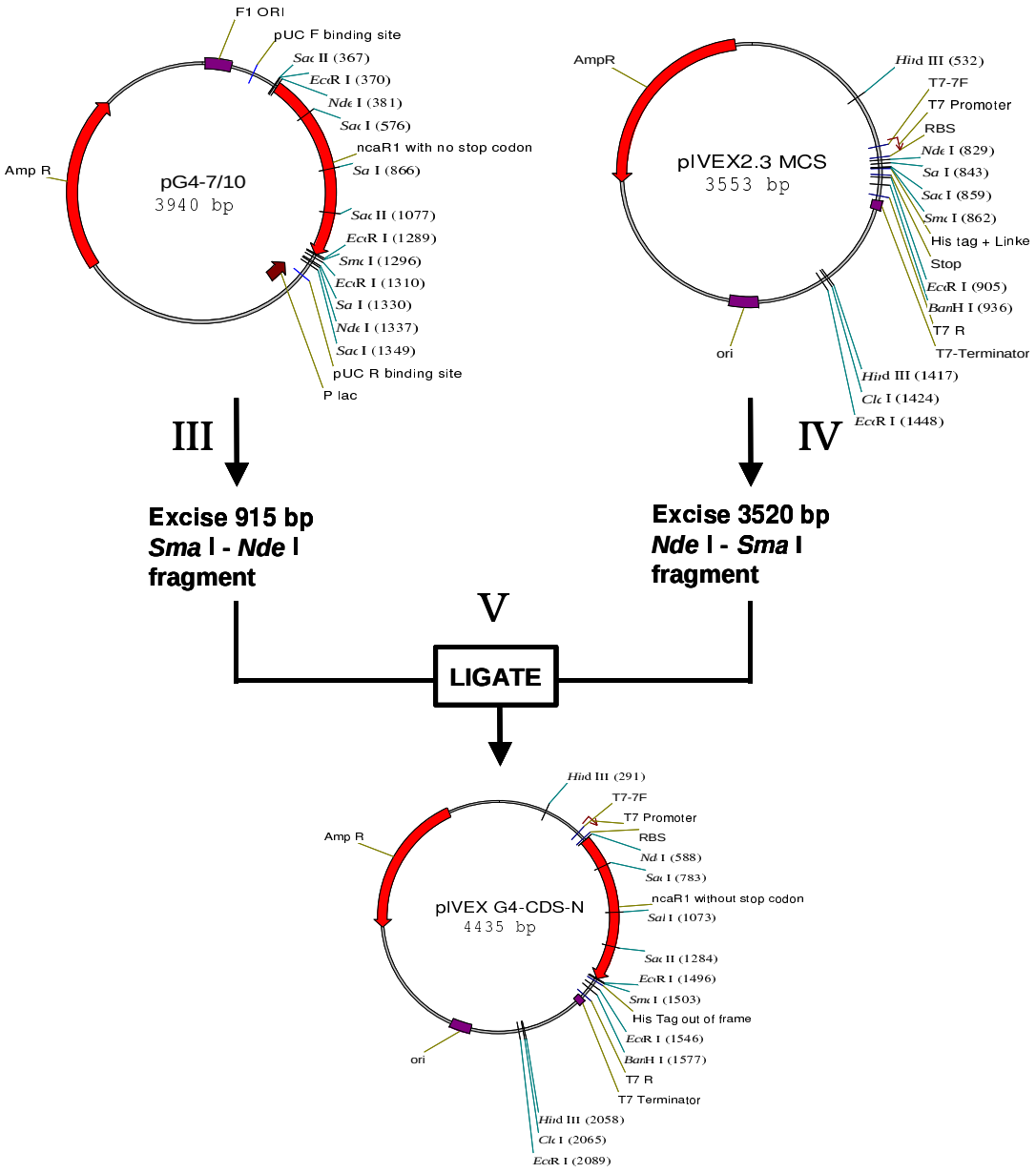


Figure 3.4 contd. Construction of pIVEX-G4-CDS-N.

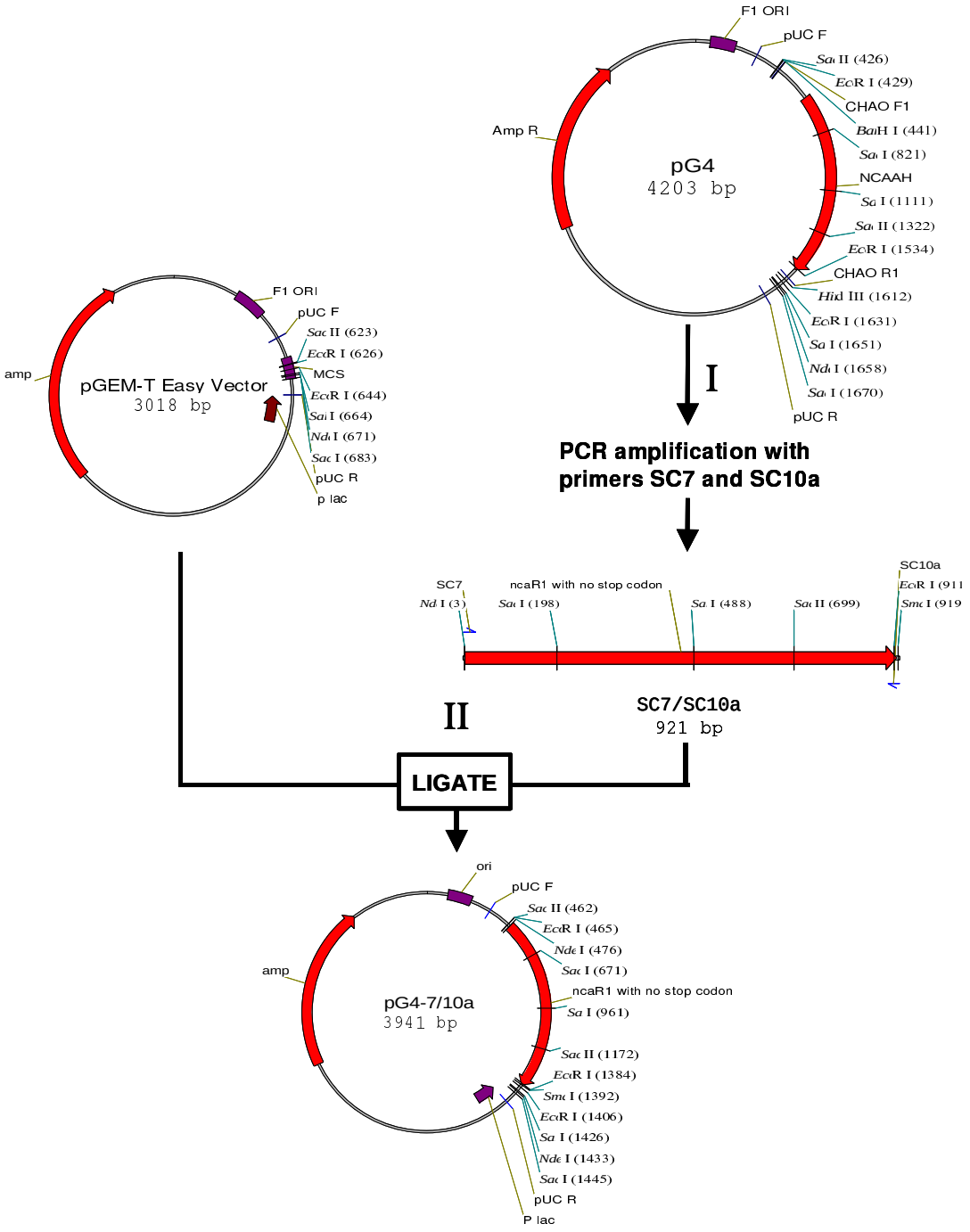


Figure 3.5. Construction of pIVEX-G4-CDS

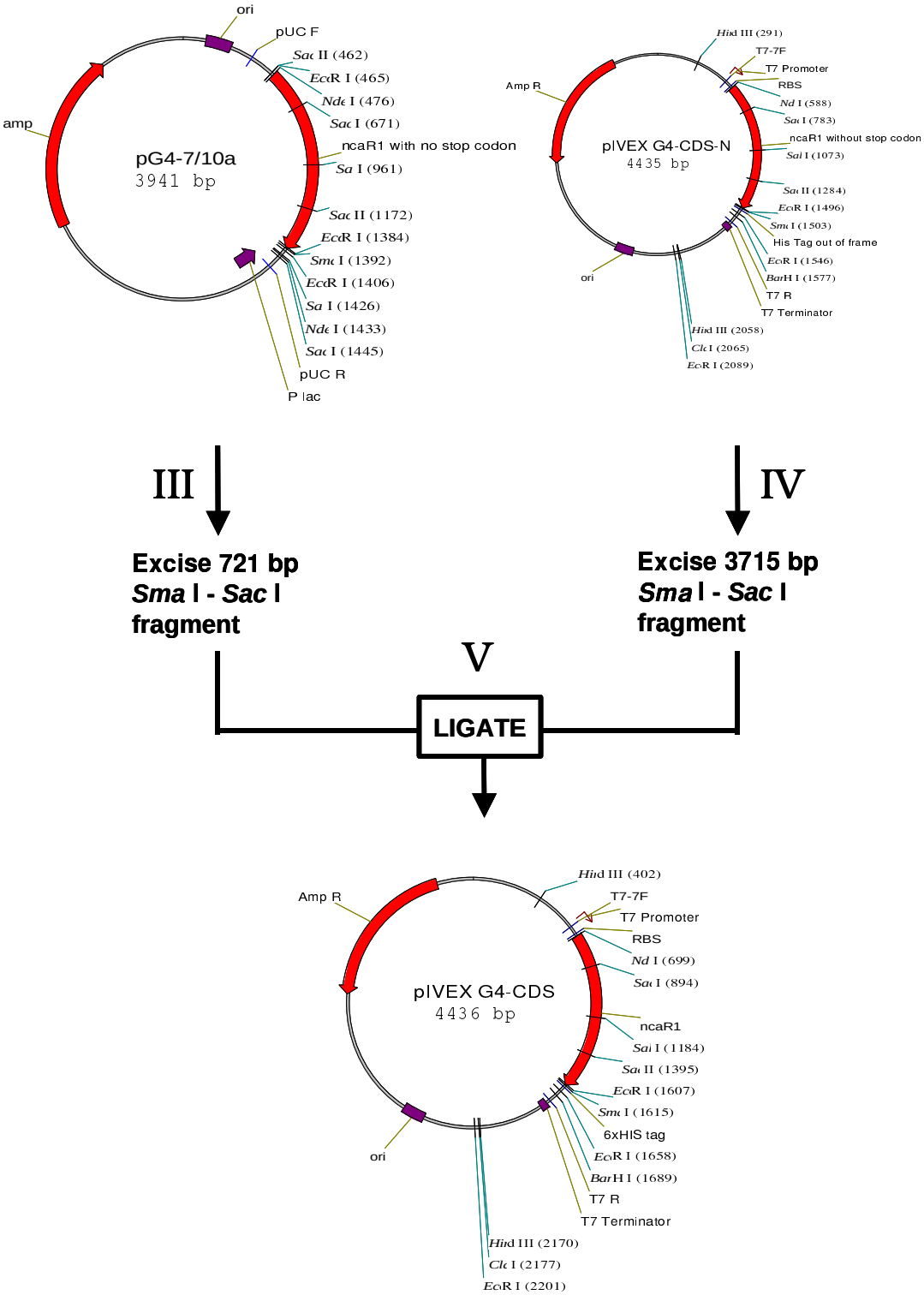


Figure 3.5 contd. Construction of pIVEX-G4-CDS

3.2.7. Heterologous expression in *E. coli* BL21 (DE3).

The recombinant plasmids were freshly transformed into *E.coli* BL21 (DE3) for the heterologous expression of *ncaR1* and *ncaR1-6xHis*. The cells were grown and induced for expression as described in Appendix 10, which is an adaptation of the method described by Ausubel *et al.,* (1983). Expression experiments with cells harbouring the plasmids pT7-7 and pIVEX2.3 MCS were carried out in parallel as negative controls for the expression of *ncaR1* and *ncaR1-6xHis.* At hourly intervals after induction with IPTG (1mM), the OD_{600} of the culture was measured. Sample of 1ml of the culture were collected in a microfuge tube and were centrifuged in a microfuge at 13000rpm for 3 minutes. The pellets were resuspended in 0.1M potassium phosphate buffer pH8.0 (Appendix 1) based on the OD_{600} at the time of harvest, where 1 ml of cells with an OD_{600} = 1 were resuspended in 250µl of buffer. An equal volume of 2xSDS-PAGE sample buffer (Appendix 1) was added, and the samples were stored at -20°C until analysis by SDS-PAGE or Western Blotting was carried out.

For the large-scale production of NcaR1-6xHis, the plasmid pIVEXG4-CDS was freshly transformed into *E. coli* BL21 (DE3). The cells were grown and induced for expression as described in Appendix 15. After 6 hours of induction, the cells were harvested by centrifugation (7000 rpm for 10 minutes in a JA14 rotar using a Beckman J2-21) and the pellets were stored at -70°C until purification was carried out.

3.2.8. Optimisation of an *in vitro* system for the synthesis of protein.

The plasmid pIVEX-GFP, which carries the gene that encodes a green florescent protein (GFP, 27 kDa) fused to a His tag, was provided with the RTS 100 E*. coli* HY Kit (Roche). The *in vitro* synthesis of the His-tagged GFP (GFP-6xHis) from pIVEX-GFP was used to optimise the experimental conditions for this system, which were applied to the synthesis of NcaR1-6xHis from pIVEX G4-CDS. The High Pure Plasmid Isolation Kit (Roche) and the QIAprep Spin Miniprep Kit (Qiagen) were used for the isolation of pIVEX-GFP DNA from *E. coli* DH5α, which was used as template DNA for the reaction. The DNA Clean and Concentrator Kit (Zymo) or phenol extraction of the plasmid DNA

(Sambrook *et al.,* 1989) was used to purify the plasmid DNA to be used as the template for *in vitro* expression if *ncaR1-6xHis*.

3.2.9. *In vitro* expression of NcaR1-6xHis.

The plasmid pIVEX G4-CDS was used for the *in vitro* expression of *NcaR1-6xHis* using The Rapid Translation System: RTS 100, *E.coli* HY Kit (Roche) as described by the manufacturers. The plasmid DNA that was used as the template for the synthesis was either that provided with kit, or prepared using the High Pure Plasmid Isolation Kit (Roche), and purified using the DNA Clean and Concentrator Kit (Zymo). The reaction was carried out at 30°C for 8 hours.

3.2.10. Purification of NcaR1-6xHis and the production of anti-NcaR1-6xHis antibodies.

Purification of NcaR1-6xHis from *E. coli* was performed under native conditions using the Ni-NTA Spin Kit and Columns (Qiagen) as outlined by the manufacturers (Appendix 11). Polyclonal anti-NcaR1-6xHis antibodies were raised in rabbits by Prof. DU Bellstedt (Department of Biochemistry, University of Stellenbosh). Serum was collected from the rabbit before inoculation (Day 0) and on the 28th day (Day 28) after inoculation with purified NcaR1-6xHis. In addition, on the 28th, 30th and 32nd day, boosts of purified NcaR1-6xHis were administered and further serum was collected from the rabbit on the 39th day (Day 39). To calibrate the sera, 1: 1000000, 1: 500000, 1:100000 and 1:50000 dilutions of the sera (Day 0, Day 28 and Day 39) were prepared and used for Western blot analysis of the whole cell lysates of *E. coli* [pIVEX2.3 MCS] and *E. coli* [pIVEXG4-CDS].

3.2.11. Analysis and detection of NcaR1 and NcaR1-6xHis by SDS-PAGE and Western Blot.

Protein samples were resolved by SDS-PAGE using 12.5% SDS-polyacrylamide gels. Premixed Protein Molecular Weight Markers (phosphorylase B: 97.4kDa, bovine serum albumin: 66.2 kDA, Aldolase: 39.2 kDa, triose phosphate isomerase: 21.5 kDa,

Lysosyme: 14.4 kDa, manufactured by Roche) were used for molecular weight estimation of bands visualised on gels. Electrophoresis was carried out using the Mini-PROTEAN 3 Electrophoresis System (BioRad) with bath buffer at approximately 100V until the desired separation had been achieved. Bands of protein were visualised on the SDS-PAGE gels by staining with Coomassie Brilliant Blue staining solution for 2 hours, followed by destaining with destain solutions. All solutions used for SDS-PAGE analysis of proteins are listed in Appendix 1.

For Western Blot analysis, electrophoretic transfer of protein samples from SDS-PAGE gels to Hybond-C+ nylon membranes (Amersham) at 100 volts for 1 hour in transfer buffer was performed using the Mini-PROTEAN 3 Mini Trans-Blot module (BioRad). Membranes were stained with Ponceau-S for 1 minute to confirm the transfer of proteins and to demarcate the position of the protein molecular weight markers.

The membrane was incubated in 5% milk powder (in TBS-Tween) overnight to block non-specific binding sites. Once thoroughly washed with TBS-Tween, the membrane was incubated with the primary antibody (in 1% BSA in TBS-Tween) for 1 hour. A 1:300 dilution of anti-His antibodies (Sigma) or antibodies raised against purified NcaR1-6xHis from rabbit serum (Section 3.1.9) were used as primary antibody.

The membranes were washed (one 15-minute and four 5-minute washes in TBS-Tween) and then incubated with the secondary antibody (anti-rabbit or anti -mouse IgG linked to horseradish peroxidase) provided with the Chemiluminescence Western Blotting Kit (Roche) for half an hour. Further washes in TBS-Tween (one 15-minutes and four 5-minute washes) were performed before the membrane was placed in a solution of luminol provided with the Chemiluminescence Western Blotting Kit (Roche). The chemiluminescence produced by the oxidation of luminol by the horseradish peroxidase was detected with X-ray film (Amersham or Kodak). Standard photographic techniques and solutions (Kodak) were used to visualise the chemiluminescent exposure of the X-Ray film. All solutions used for Western Blot analysis of proteins are listed in Appendix 1.

3.3. Results.

3.3.1. Optimisation of the i*n vitro* expression system and *in vitro* synthesis of NcaR1-6xHis.

The synthesis of GFP-6xHis from pGFP-6xHis plasmid DNA was used to optimise the use of the RTS-100 *in vitro* protein synthesis system. Plasmid DNA of pGFP-6xHis provided with the kit together with pGFP-6xHis prepared from *E. coli* by various means was used as the template DNA for the *in vitro* reaction, which was carried out at 30°C for 6 hours. The products of the *in vitro* protein synthesis were analysed by SDS-PAGE and Western Blot with anti-His antibodies as the primary antibody to detect GFP-6xHis (27kDa)(Figure 3.6).

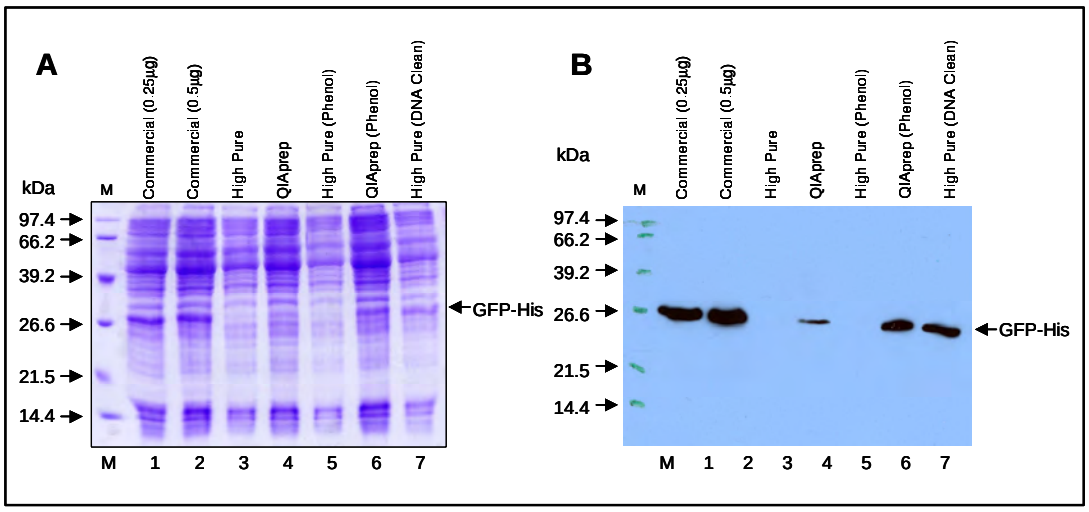


Figure 3.6. SDS-PAGE (A) and Western Blot (B) analysis of GFP-6xHis synthesised with the RTS-100 *in vitro* protein synthesis system. Each *in vitro* reaction was carried out with different preparations of template DNA. The sizes of the molecular weight markers are indicated on the left hand side of the gel, while the position of GFP-6xHis is indicated on the right hand side of the gel.

Synthesis of GFP-6xHis was most efficient when carried out using the pGFP-6xHis plasmid DNA provided with the kit (Figure 3.6, Lane 1 and 2). GFP-6xHis synthesis from the template DNA prepared with the High Pure Plasmid Isolation Kit was undetectable (Figure 3.6, Lane 3), and barely detectable when using pGFP-6xHis prepared using the QIAprep Spin Miniprep Kit (Figure 3.6, Lane 4). Phenol extraction of

the template DNA prepared with these two kits yielded no results for the DNA prepared with the High Pure Plasmid Isolation Kit (Figure 3.6, Lane 5), but improved the synthesis of GFP-6xHis for the DNA prepared with the QIAprep Spin Miniprep Kit (Figure 3.6, Lane 6) to levels comparative to that obtained when using the pGFP-6xHis provided with the kit. The synthesis of GFP-6xHis from pGFP-6xHis prepared with the High Pure Plasmid Isolation Kit, and then purified with the DNA clean and concentrator kit, also yielded results comparative to levels obtained when using the pGFP-6xHis provided with the kit (Figure 3.6, Lane 7). This method was chosen as the preferred method for template DNA preparation for future *in vitro* synthesis reactions. *In vitro* synthesis of protein from pIVEXG4-CDS was performed, but a protein corresponding to NcaR1-6xHis was not observed (data not shown).

3.3.2. Heterologous expression of *ncaR1* and *NcaR1-6xHis*.

The heterologous expression of *ncaR1* or *ncaR1-6xHis* was carried out in *E. coli* BL21 (DE3) with the constructs pT7G4-CDS and pIVEXG4-CDS respectively. The predicted molecular mass of NcaR1 is 34.2kDa and of NcaR1-6xHis is 35.4kDa respectively. The heterologous expression of a truncated NcaR1 called ΔNcaR1, (with a predicted molecular mass of 22kDa) was carried out with the construct of pT7G4-ΔCDS.

The heterologous expression of *ncaR1* in *E. coli* (pT7G4-CDS) induced with IPTG (1mM) at 30°C and 37°C was carried out. Heterologous expression of Δ*ncaR1* was performed simultaneously to ascertain whether the T7 RNA polymerase system was functional. SDS-PAGE analysis of the protein extracts from *E. coli* (pT7G4-CDS) before and 1, 2, 3, 4 and 18 hours post induction (Figure 3.7, Lanes 2-6, 9) showed that a protein migrating at approximately the molecular mass of NcaR1 was present. This protein was absent from the protein extracts of *E. coli* (pT7-7) (Figure 3.7, Lanes 1 and 8). The presence of a protein migrating at approximately the molecular mass of ΔNcaR1 in the cell extracts of *E. coli* (pT7G4-ΔCDS) confirmed that the T7 RNA polymerase system was functional (Figure 3.7, Lane 7).

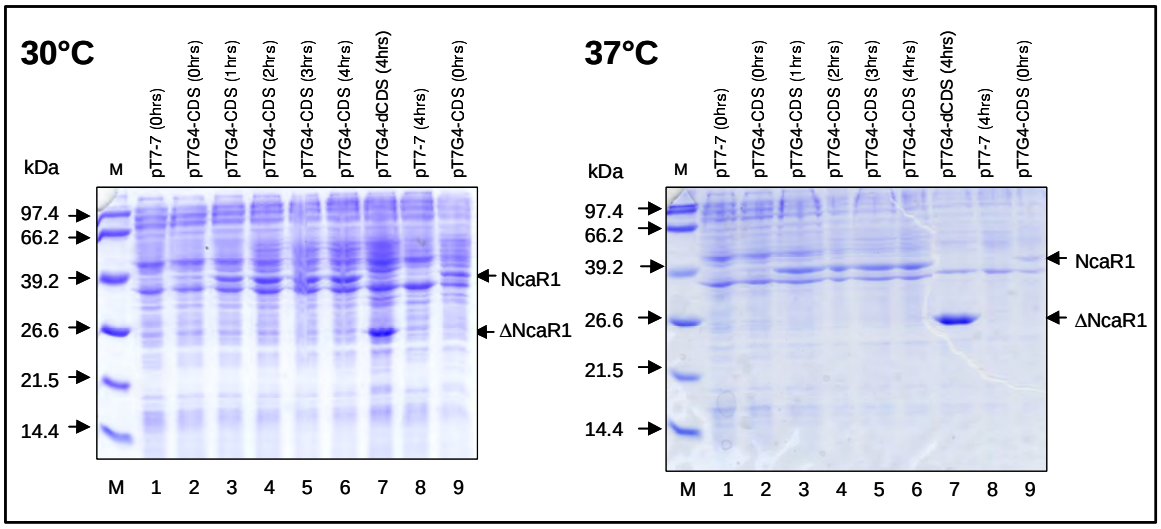


Figure 3.7. SDS-PAGE analysis of proteins produced by *E. coli* BL21 (DE3) harbouring pT7-7, pT7G4-CDS or pT7G4-ΔCDS at 30°C or 37°C. The sizes of the molecular weight markers are indicated on the left hand side of each gel, while the position of NcaR1 and ΔNcaR1 are indicated on the right hand side of each gel.

A comparison of NcaR1 produced at 30°C and 37°C showed that similar results were obtained at these temperatures. A low level of NcaR1 was present in *E. coli* (pT7G4-CDS) before induction. From 1 hour post induction an increase in NcaR1 was observed (Figure 3.7, Lanes 2-6, 30°C versus 37°C). However, at 18 hours post induction, there was a reduction in the amount of NcaR1 in the cells grown at 37°C when compared to the cells grown at 30°C (Figure 3.7, Lane 9 (30°C) versus Lane 9 (37°C)).

The heterologous production of *ncaR1-6xHis* in *E. coli* (pIVEXG4-CDS) induced with IPTG (1mM) at 30°C and 37°C was performed. SDS-PAGE analysis of the protein extracts from these cells before induction and at various time intervals post induction (Figure 3.8, 30°C: Lanes 2-5 versus 37°C: Lanes2-6) showed that a protein migrating at approximately the molecular mass of NcaR1-6xHis was present.

A comparison of the cell extracts of *E. coli* (pIVEXG4-CDS) grown at 30°C and 37°C post induction showed that the production of NcaR1-6xHis differed at these temperatures. A low level of NcaR1-6xHis was present before induction (Figure 3.8, Lane 2) at both temperatures, but the production of NcaR1-6xHis post induction proceeded more rapidly at 37°C (Figure 3.8, 37°C: Lanes 2-6) than at 30°C (Figure 3.8, 30°C: Lanes 2-5). At 30°C the greatest of amount of NcaR1-6xHis was only produced

after 6 hours of induction (Figure 3.8, 30°C: Lane 5) while at 37°C the greatest amount of NcaR1-6xHis was produced after 3 hours (Figure 3.8, 37°C: Lane 5).

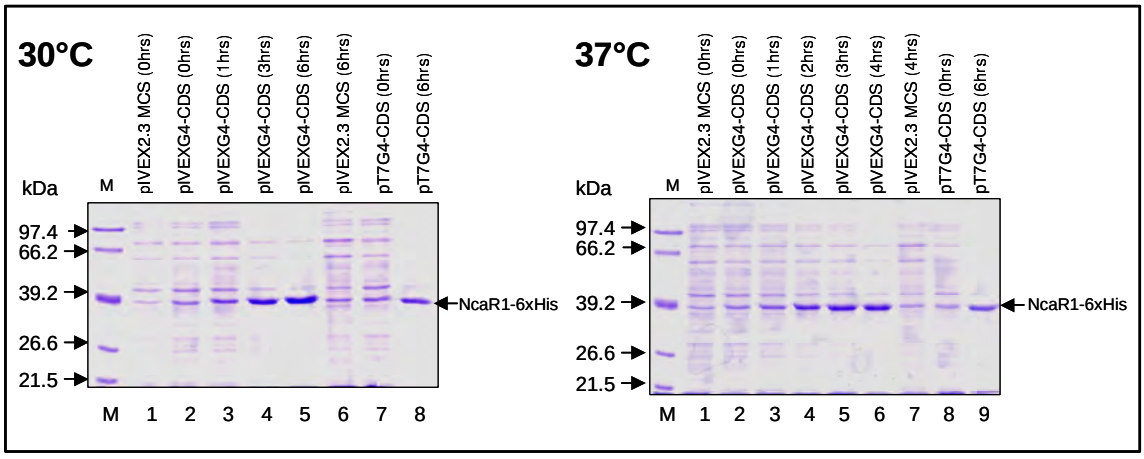


Figure 3.8. SDS-PAGE analysis of proteins produced by *E. coli* BL21 (DE3) harbouring pIVEX 2.3 MCS, pIVEX G4-CDS and pT7G4-CDS at 30°C or 37°C. The sizes and positions of the molecular weight markers are indicated on the left hand side of the gel, while the position of NcaR1-6xHis is indicated on the right hand side of the gel.

A protein migrating at approximately the same molecular mass of NcaR1-6xHis was present in *E. coli* (pIVEX2.3 MCS) (Figure 3.8, 30°C: Lanes 1and 6, 37°C: Lanes 1 and 7), which should not produce NcaR1-6xHis. However, a comparison of the cell extracts of *E. coli* (pIVEX2.3 MCS) before induction (Figure 3.8, 30°C: Lane 1, 37°C: Lane 1) and post induction (Figure 3.8, 30°C: Lane 6, 37°C: Lane 7) did not show an increase in the production of this protein, as was observed in the cell extracts of *E. coli* (pIVEXG4-CDS), suggesting that it is an *E. coli* protein. The migration of NcaR1-6xHis produced in *E. coli* (pIVEXG4-CDS) corresponds to the migration of NcaR1 produced in *E. coli* (pT7G4-CDS) (Figure 3.8, 30°C: Lane 5 versus 8; 37°C: Lane 6 versus 9).

3.3.3. The affinity purification of NcaR1-6xHis.

The heterologous expression system was used for the production of NcaR1-6xHis for purification. Purification of NcaR1-6xHis was carried out under native conditions using Ni-NTA spin columns, which possess nitrilotriacetic acid chromatagraphy matriceswhich have an affinity for 6xHis tags.

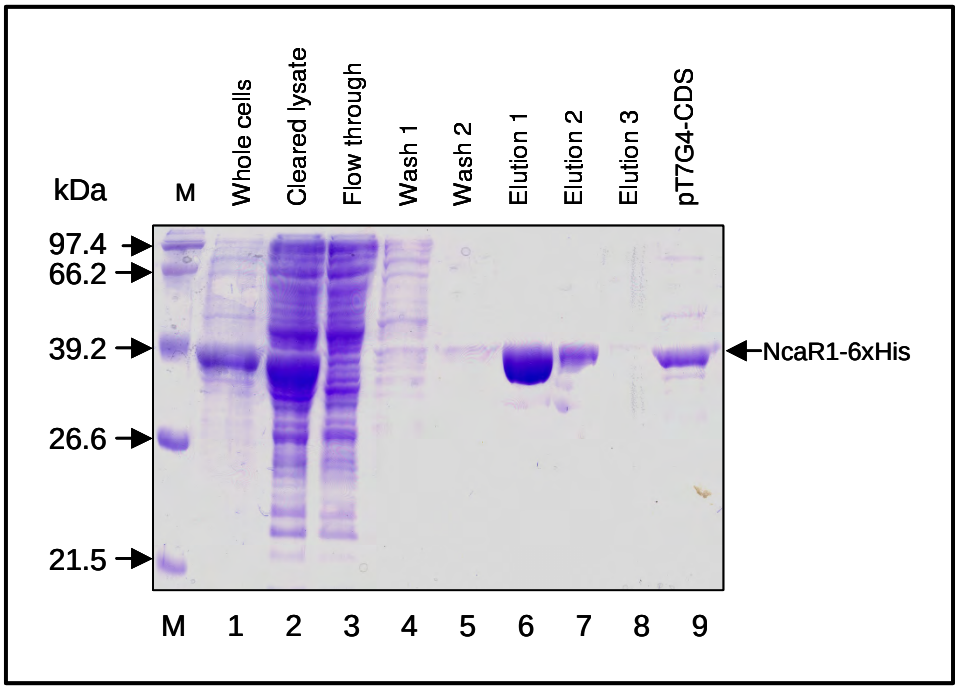


Figure 3.9. SDS-PAGE analysis of the fractions obtained at each step of the native purification of NcaR1-6xHis, which was expressed from pIVEX G4-CDS in *E.coli* BL21 (DE3). The positions and sizes of the molecular weight markers are indicated on the left hand side of the gel, while the position of NcaR1-6xHis is indicated on the right hand side of the gel.

The cleared lysate of *E. coli* (pIVEXG4-CDS) was passed through the Ni-NTA spin columns to allow binding of NcaR1-6xHis to the matrix. The column was then washed with a buffer containing a low concentration imidazole (20mM) to remove the non-specifically bound proteins from the matrix. NcaR1-6xHis was eluted from the column with a buffer containing a high concentration of imidazole (250mM). SDS-Page analysis of the protein eluted from the Ni-NTA column after each purification step showed that a protein corresponding to the molecular mass of NcaR1-6xHis was purified (Figure 3.9).

A number of purifications of NcaR1-6xHis were carried out, and the first and second elution (Elution 1 and Elution 2) of each purification were pooled together. In order to confirm that the protein being purified was NcaR1-6xHis, SDS-PAGE and Western Blot analysis was performed (Figure 3.10). SDS-PAGE analysis showed that both Elution 1 and Elution 2 contained some contaminating proteins in addition to NcaR1-6xHis (Figure 3.10 A: Lanes 2 and 3), but these concentrations were low when compared to the concentration of NcaR1-6xHis. The Western Blot analysis, using anti-His antibodies

as primary antibody, detected NcaR1-6xHis (Figure 3.10.B, Lanes 2 and 3), and GFP-6xHis (Figure 3.10.B, Lane 1).

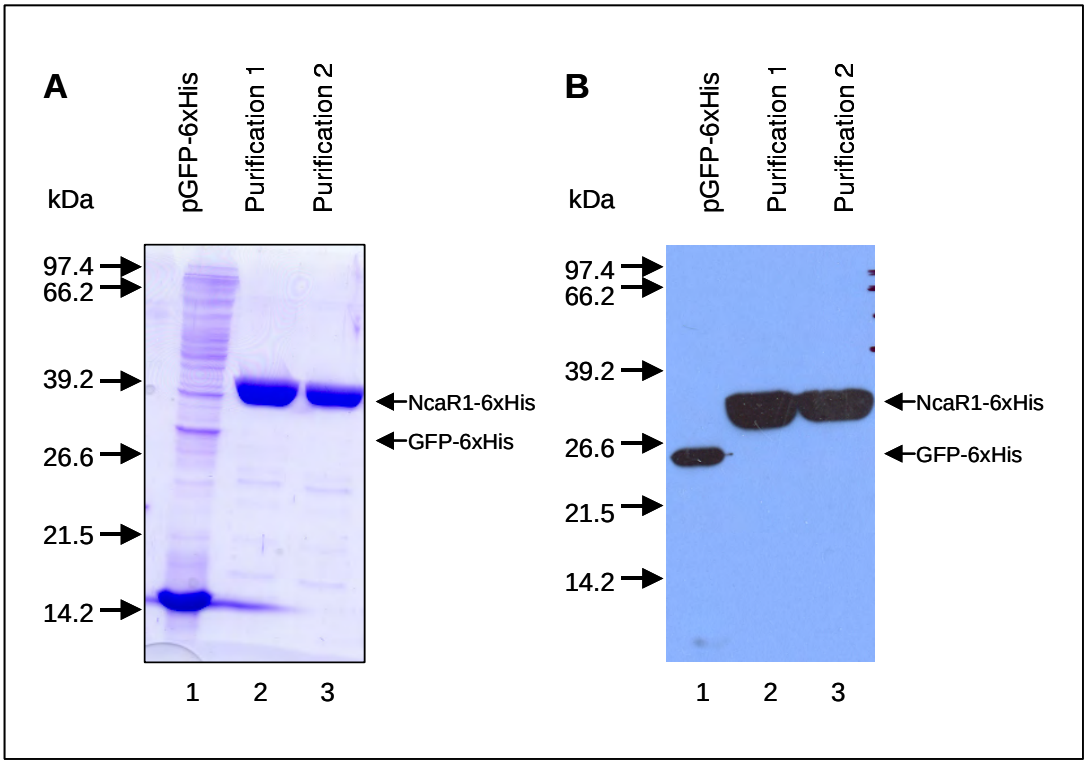


Figure 3.10. SDS-PAGE (A) and Western Blot (B) analysis with anti-His antibodies for the detection of purified NcaR1-6xHis. Two preparations of purified C-terminal His-tagged NcaR1 Elution 1 (2) and Elution 2 (3) are shown, GFP-6xHis expressed in *E. coli* BL21 (DE3) is also shown (1) as a control. The positions and sizes of the molecular weight markers are indicated on the left hand side of each gel, while the position of NcaR1-6xHis and GFP-6xHis are indicated on the right hand side of each gel.

3.3.4. Testing and calibration of antibodies raised against NcaR1-6xHis.

Approximately 1 mg of protein derived from the affinity purification of NcaR1-6xHis was used to immunise a rabbit to raise antibodies against NcaR1-6xHis. Serum was taken before immunisation (Day 0) and 28 days post immunisation (Day 28). A further 3 immunisations were administered on day 28, 30 and 32 to boost antibody production, and on Day 39 further serum was isolated.

The sera from Day 28 and Day 39 were tested for the presence of NcaR1-6xHis antibodies by Western Blot analysis of cell extracts from E. *coli* (pIVEX2.3 MCS) and

E. coli (pIVEXG4-CDS) (Figure 3.11 Panel C to H). The serum from Day 0 was used as a negative control for Western Blot analysis, to show that the immunological reaction observed with the sera isolated after immunisation was not as a result of pre-existing antibodies in the system of the rabbit (Figure 3.11 Panel A and B). It was necessary to determine the optimal dilutions of the sera to be used for Western Blot analysis. Initially 1: 1 000 000 and 1: 500 000 dilutions were used for each of the sera, but no immunological reaction was detected. Consequently 1: 100 000 and 1: 50 000 dilutions of the sera were used. The 1: 50 000 dilution was found to be optimal (Figure 3.11.B, Day 28: Panel D versus Panel G, Day 39: Panel E versus Panel H).

A 1: 50 000 dilution of the serum from Day 0 did not react immunologically with the protein extracts of *E. coli* (pIVEX 2.3 MCS) or *E. coli* (pIVEXG4-CDS) (Figure 3.11, Panel B, Lanes 1 and 2). This confirmed that the immunological reactions observed with the Day 28 and Day 39 sera were as a result of antibodies produced against the protein preparation of NcaR1-6xHis used for the immunisation.

The sera drawn from Day 28 and Day 39 produced an immunological reaction with a protein in *E. coli* (pIVEXG4-CDS) corresponding in molecular mass to NcaR1-6xHis (Figure 3.11, Panel D, E, G and H, Lane 2). However an immunological reaction with the same protein in *E. coli* (pIVEX2.3 MCS) (Figure 3.11, Panel D, E, G and H, Lane 1) suggested that this immunological reaction was not due to antibodies specific to NcaR1-6xHis, as *E. coli* (pIVEX2.3 MCS) does not express NcaR1-6xHis.

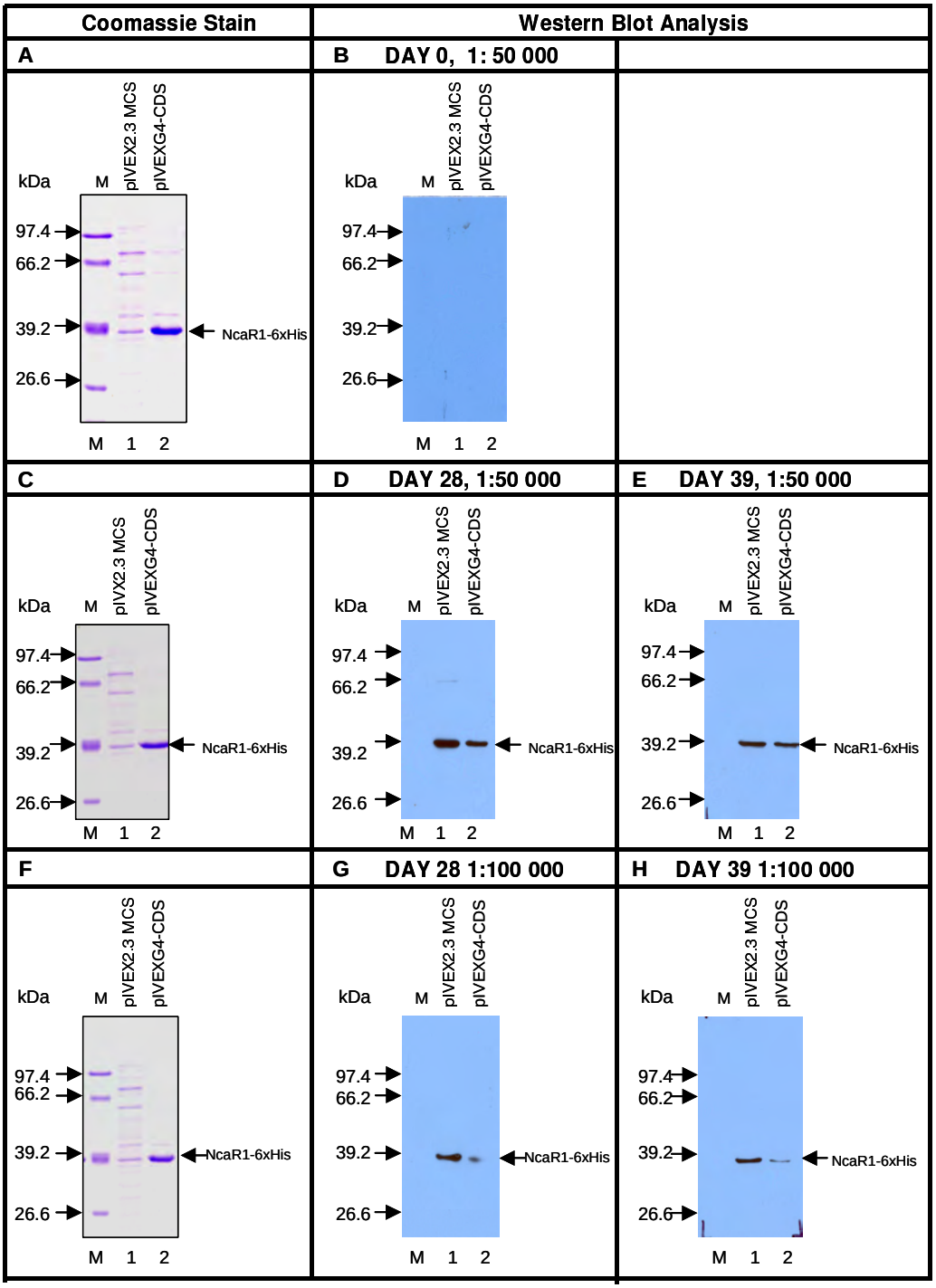


Figure 3.11. Cell extracts from *E. coli* (pIVEX2.3 MCS) and *E. coli* (pIvexG4-CDS) were analysed by SDE PAGE (Panel A, C and F) and by Western Blot with sera drawn on Day 0 (Panel B), Day 28 (Panel D and E) and Day 39 (Panel G and H) for the detection of NcaR1-6xHis. To optimise the concentration of the antibodies in the sera to provide an immunological response, 1: 100 000 or 1: 50 000 dilutions of the sera from Day 28 and Day 39 were used for Western Blot analysis. The positions and sizes of the molecular weight markers are indicated on the left hand side of each gel, the position of NcaR1-6xHis is indicated on the right hand side of each gel.

3.3.5. Detection of heterologously expressed NcaR1 and NcaR1-6xHis by Western blot analysis with anti-NcaR1-6xHis serum.

Due to the immunological reaction that occurred with the negative control cell extract of *E. coli* (pIVEX2.3 MCS), a number of cell extracts of the negative control prepared on different occasions were tested, in order to eliminate the possibility that the previous cell extract from *E. coli* (pIVEX2.3 MCS) was contaminated with NcaR1-6xHis. In addition, the cell extracts of *E. coli* (pT7-7), *E. coli* (pT7G4-CDS) and *E. coli* (pIVEXG4-CDS) were tested to determine whether the cross reaction was specific to the *E. coli* (pIVEX2.3MCS) cell extracts or not.

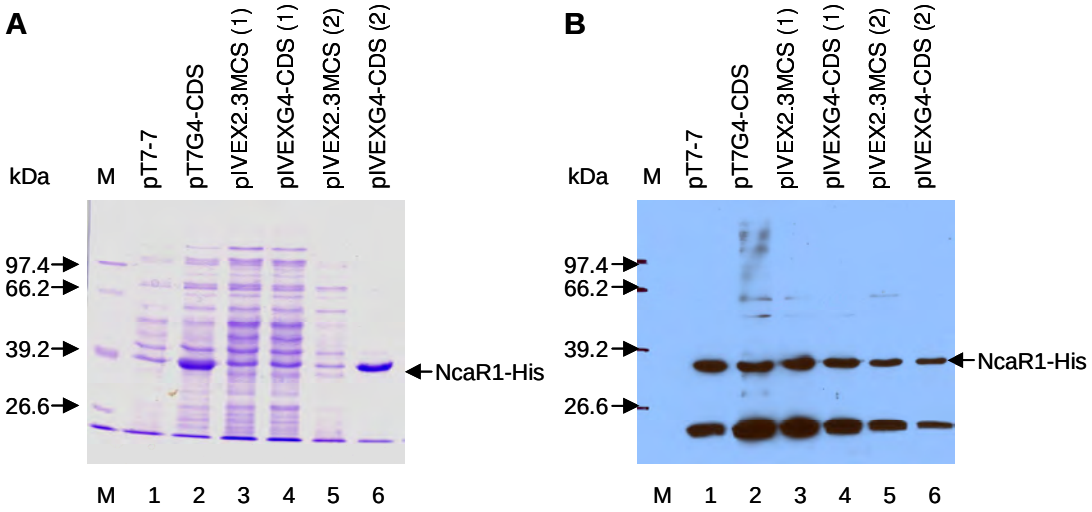


Figure 3.12. SDS-PAGE (A) and Western Blot (B) analysis for the detection of NcaR1 and NcaR1-6xHis heterologously expressed in *E. coli* (pT7G4-CDS) and *E. coli* (pIVEXG4-CDS) with sera drawn on Day 39 (1: 50 000 dilution) as primary antibody. The positions and sizes of the molecular weight markers are indicated on the left hand side of each gel, and the position of NcaR1-6xHis is indicated on the right hand side of each gel.

Western Blot analysis of these samples showed that the serum drawn on Day 39 reacted similarly with all the cell extracts, (Figure 3.12.B, Lanes 1-6) despite NcaR1 or NcaR1-6xHis only being present in the cell extracts of *E. coli* (pT7G4-CDS) and *E. coli* (pIVEXG4-CDS) (Figure 3.12.B, Lanes 2, 4 and 6). This confirmed that the antibodies produced were not specific to NcaR1-6xHis, but instead to an *E. coli* protein, which may have co-purified with NcaR1-6xHis.

3.4. Discussion.

A protein band corresponding to the predicted molecular mass of NcaR1 was observed in SDS-PAGE analysis of cell extracts from *E. coli* (pT7G4-CDS) grown at 30°C and 37°C, but not in cells harbouring the vector plasmid pT7-7. Low levels of NcaR1 was detected in the cell extracts of *E. coli* (pT7G4-CDS) before induction, and could be due to the slightly "leaky" regulation of the *lac* promoter (Tabor, 1983). At eighteen hours post induction, the amount of NcaR1 remaining in the cells grown at 30°C was higher than in the cells grown at 37°C. The expression of ΔNcaR1 from *E. coli* (pT7G4-ΔCDS) provided an excellent tool for testing the efficiency of the *E. coli* BL21 (DE3) heterologous expression system. A protein corresponding to the molecular mass of NcaR1-6xHis was produced in *E. coli* (pIVEXG4-CDS) at 30°C and 37°C.

The use of an *in vitro* system for the production of an *N*-carbamoyl-amino acid amidohydrolase has not been reported before. Initial *in vitro* expression experiments for the synthesis of NcaR1-6xHis were unsuccessful and failed to produce any detectable protein (data not shown). This necessitated some optimisation studies with the RTS 100 system, which were performed with the control plasmid (pGFP-6xHis) for the production of GFP-6xHis, and gave the following results. The recommended method of DNA preparation (High Pure Plasmid Isolation Kit) and an equivalent DNA preparation kit (QIAprep Spin Miniprep Kit) did not produce as much protein as the pGFP DNA provided with the kit. The protein produced was improved by further purification of the DNA by phenol extraction or using the DNA Clean and Concentrator Kit. These results could be due to the improved purity of the DNA, or alternatively due to the increased concentration of the DNA, which resulted in the addition of a smaller volume of DNA thereby reducing the amount of inhibitors such as salts.

NcaR1-6xHis was not produced from pIVEXG4-CDS using the RTS 100 system, but the synthesis possibly requires further optimisation. Since the heterologous expression system produced NcaR1-6xHis, large-scale production of NcaR1-6xHis for purification was carried out using this method. Purification of NcaR1-6xHis using Ni-NTA spin columns under native purification conditions resulted in the purification of a protein with a molecular weight corresponding to NcaR1-6xHis. However some contaminating

proteins were present, despite the use of a wash buffer containing 20mM imidazole, which inhibits the binding of non-specific proteins.

Many proteins exist that possess naturally occurring histidine-rich regions or domains, the purposes of which are numerous. Some act as binding domains for protein-protein interactions (Vanguri et al., 2000), while others act as metal binding domains for the binding of metal cofactors required by certain enzymes (Fu et al., 1995; Hottenrott et al., 1997; Suzuki and Endo, 2002). But most commonly, they act as metal binding domains for proteins involved in the transport of metal ions (Guerinot, 2000; Chen and Morse, 2001, Eng et al., 1998). Proteins such as these may have been bound to the Ni-NTA column, and thus co-purified with NcaR1-6xHis.

The Day 28 and Day 39 sera, isolated from the rabbit after immunisation with purified NcaR1-6xHis, were found to produce an immunological response with a protein with a molecular mass corresponding to NcaR1-6xHis in the Western blot analysis of *E. coli* (pIVEXG4-CDS) cell extracts. However, the same reaction was observed in *E. coli* (pIVEX2.3 MCS) cell extracts that did not contain NcaR1 or NcaR1-6xHis, indicating that the antibodies in the sera were not specific to NcaR1-6xHis.

As the serum isolated before the rabbit was inoculated with the NcaR1-6xHis (Day 0) did not show an immunological reaction with NcaR1-6xHis, pre-existing rabbit antibodies could be eliminated as the cause of the results obtained with the Day 28 and Day 39 sera. It is possible that the reaction was due to antibodies that were produced against the non-specific proteins that were eluted from the Ni-NTA column with NcaR1-6xHis. However this is not a suitable explanation, as the proteins to which the antibodies are reactive do not correspond to the molecular masses of the non-specifically purified protein.

Since native purification conditions were used for the purification of NcaR1-6xHis, it is possible that proteins bound to NcaR1-6xHis, such as chaperone proteins involved in protein folding, were co-purified with NcaR1-6xHis. And it is possible that it is to these proteins that antibodies in the rabbit were formed.

In conclusion, proteins corresponding to the size of NcaR1 and NcaR1-6xHis were produced, but the antibodies that were raised against the protein preparation of NcaR1-6xHis were not specific to NcaR1 or NcaR1-6xHis. This problem may have been overcome if two courses of action had been followed. The first involves the conditions under which the NcaR1-6xHis was purified. If NcaR1-6xHis had been purified under denaturing conditions instead of native conditions, the likelihood of co-purification of NcaR1-6xHis with other proteins such as chaperone proteins or proteins with naturally occurring His-rich regions, may have been reduced thus reducing the presence of non-specific antibodies in the serum. The second involves the pre-adsorption of the anti-NcaR1-6xHis serum with *E. coli* cell lysate to eliminate the non-specific antibodies that were interacting with *E. coli* proteins.

CHAPTER 4

Optimisation of *ncaR1* and *ncaR1-6xHis* heterologous expression.

CHAPTER 4

Optimisation of *ncaR1* and *ncaR1-6xHis* heterologous expression.

4.1. Introduction.

E. coli has been discussed previously as one of the most widely used hosts for the heterologous production of proteins. However, it is a system that is not without its disadvantages, the most common problem being that the proteins expressed in *E. coli* accumulate intracellularly in the form of insoluble, biologically inactive inclusion bodies (Hockney 1994, Weickert *et al.,* 1996) so heterologous expression often needs to be optimised. The insoluble nature of heterologously produced D-NCAAHs has been reported previously (Buson *et al.,* 1996; Grifantini *et al.,* 1998; Chao *et al.,* 1999b; Hils *et al.* 2001; Sareen *et al.,* 2001b) and a number of methods have been used to optimise the heterologous production of NCAAH.

Various studies have shown that the solubility and activity of the D-NCAAH produced is dependant on the promoter used (Chao *et al.,* 2000a; Park *et al.,* 2000; Kim *et al.*, 2000a), the expression vector used (Chao *et al.,* 2000a) and the strain of *E. coli* used (Chao *et al.,* 2000a; Sareen *et al.,* 2001b). In some cases, co-expression of chaperone proteins with D-NCAAH has been shown to bring about an increase in the production of the enzyme in a soluble, active form (Chao *et al.,* 2000a; Sareen *et al.,* 2001b). Most commonly, the approach used to improve the solubility and activity of heterologously produced D-NCAAH is to lower the temperature at which the host strain is grown (Buson *et al.,* 1996; Grifantini *et al.*, 1998; Chao *et al.,* 2000a; Sareen *et al.,* 2001b).

4.1.1. Objectives.

The aim of the research described in this chapter was to determine if NcaR1 and NcaR1-6xHis heterologously produced in *E. coli* possessed *N*-carbamoyl-amino acid amidohydrolase activity. Subsequently it was investigated if the heterologous production of NcaR1 and NcaR1-6xHis could be optimised. In order to achieve this it was necessary to determine which conditions were conducive to NcaR1 or NcaR1-6xHis heterologous production, while at the same time ensuring that the solubility and *N*-carbamoyl-amino acid amidohydrolase activity of NcaR1 and NcaR1-6xHis was not undermined. Parameters such as the optimum period of induction and the temperature used for growth after induction were investigated.

4.2. Materials and Methods.

4.2.1. Bacterial strains, plasmids and culture conditions.

Heterologous expression was carried out in *E. coli* BL21 (DE3) (F^- *dcm ompT* hsdS[r_B^- m_B^-] *gal* λ[DE3]) containing the recombinant plasmid of interest. Unless otherwise stated, growth of *E. coli* was carried out as previously described (Section 2.2.1). The plasmids used in this chapter are listed in Table 4.1, where the parental vector and the origin of the DNA insert is indicated.

Table 4.1. Plasmids used in this chapter.

Plasmid	Origin of Insert	Reference
pGEM-T Easy	Cloning Vector	Promega
pT7-7	Expression Vector	Tabor, 1983
pIVEX2.3 MCS	Expression Vector	Roche
pG4	1179bp PCR amplification product from RU-OR genomic DNA using primers CHAOF1 & CHAOR1 in pGEM-T Easy	Hartley, 2001
pT7G4-CDS	195bp *Nde* I -*Sac* I fragment from pBM1 inserted into pT7G4	This study
pT7G4-ΔCDS	423bp *Eco* RI – *Sal* I fragment deleted from insert of pT7G4-CDS	This study
pIVEXG4-CDS	721 bp *Sac* I-*Sma* I fragment from pG4-7/10a inserted into pIVEXG4-CDS-N	This study

4.2.2. Heterologous expression of NcaR1 and NcaR1-6xHis in *E.coli* BL21 (DE3).

Heterologous production of NcaR1 and NcaR1-6xHis was carried out as outlined in Appendix 10. The cells were harvested by centrifugation at 7000rpm in a Beckman JA14 rotar (Beckman J2-21) for 10 minutes and resuspended to a final concentration of 40 mg/ml wet cell mass in 0.1M potassium phosphate buffer (pH8). Following resuspension, a fraction of cells were set aside for whole cell (WC) biocatalytic assays and protein analysis by SDS-PAGE. The remainder were sonicated for a total of 1 minute on ice (six 10-second pulses with 10-second cooling periods inbetween). A fraction of the sonicated cells (SON) were set aside for SDS-PAGE and biocatalytic assays and the remainder were separated into the soluble and insoluble fractions as follows. A known volume of sonicated cells were centrifuged at 8000rpm in a Beckman JA20 rotar for 10 minutes (Beckman J2-21). The supernatant was poured off and used as the soluble fraction (SOL) for biocatalytic assays or SDS-PAGE analysis. The pellet

was resuspended in an equivalent volume of 0.1M potassium phosphate buffer (pH8) and was used as the insoluble fraction (INS) for biocatalytic assays or SDS-PAGE analysis.

4.2.3. Biocatalytic reactions for the detection of NCAAH activity.

Biocatalytic reactions for the detection of NCAAH activity using the whole cell, sonicated, soluble and insoluble fractions of recombinant *E. coli* cells were performed according to the method of Hartley *et al.,* (1998). A volume of each of the fractions (originating from a cell suspension of 40mg/ml) were added to an equal volume of 0.1M potassium phosphate buffer (pH8) containing 25mM *N*-carbamoyl glycine to the equivalent of a final concentration of 20 mg/ml wet cell mass. Controls consisting of cells (resuspended to 40mg/ml) added to an equal volume of 0.1M potassium phosphate buffer (pH8) (cell blank) or substrate (25 mM *N*-carbamoyl glycine) added to an equal volume of 0.1M potassium phosphate buffer (pH8) (substrate blank) were also prepared. The biocatalytic reaction was carried out at 40°C with shaking at 200rpm for 6 to 18 hours, after which the samples were microfuged to pellet the cells and the supernatant analysed. Any *N*-carbamoyl glycine converted to glycine by NCAAH was quantified using the ninhydrin assay as described in Section 2.1.7. One unit of NCAAH (U) activity was calculated as μmol/ml glycine produced from *N*-carbamoyl glycine.

4.2.4. Detection of NcaR1 and NcaR1-6xHis by SDS-PAGE.

Protein samples were analysed by SDS-PAGE in 12.5% polyacrylamide gels as described in Section 3.2.11.

4.3. Results.

4.3.1. Preliminary analysis of NcaR1 and NcaR1-6xHis enzyme activity.

The NCAAH activity obtained from NcaR1 and NcaR1-6xHis heterologously produced in *E. coli* (pT7G4-CDS) and *E. coli* (pIVEXG4-CDS) after induction at 37°C for 4 hours was determined (Figure 4.1.).

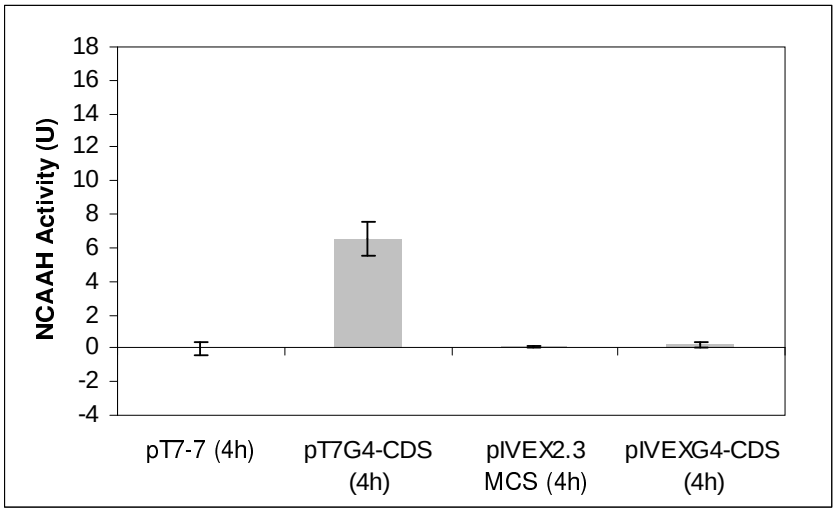


Figure 4.1. NCAAH activity (U) of NcaR1 produced in *E. coli* (pT7G4-CDS) and NcaR1-6xHis produced in *E. coli* (pIVEXG4-CDS) at 37°C, 4 hours after induction with IPTG (1mM).

The negative controls, *E. coli* (pT7-7) and *E. coli* (pIVEX2.3 MCS) showed no detectable NCAAH activity. NCAAH activity was detected in cells containing pT7G4-CDS (6.6 U) whereas the cells containing pIVEXG4-CDS showed no detectable NCAAH activity. The visualisation of NcaR1-6xHis on SDS-PAGE gels (data not shown) indicated that the protein was produced, so it was concluded that the presence of a 6xHis tag at the C-terminal of the NcaR1 protein inhibited NcaR1 activity.

4.3.2. Analysis and Optimisation of NcaR1 enzyme activity.

i) Effect of sonication.

To determine whether NCAAH activity could be improved by using cell free extracts, the NCAAH activity of whole cells was compared to that of sonicated cells (Figure 4.2).

Cells that had been subjected to sonication showed an increase in NCAAH activity by more than a 30% (from 6.6U in whole cells to 8.9U in sonicated cells, Figure 4.2). Further NCAAH activity in subsequent experiments was determined using cell free extracts *E. coli* cells as the increase in activity was significant.

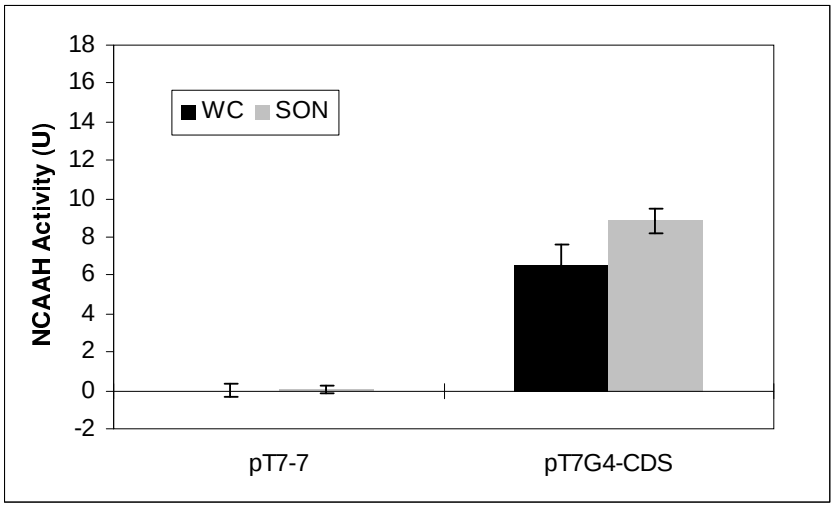


Figure 4.2. A comparison of NCAAH activity of *E. coli* (pT7-7) and *E. coli* (pT7G4-CDS) whole cells (WC) and sonicated (SON) cells.

ii) The T7 RNA polymerase system for the overexpression of *ncaR1*.

The efficiency of active NcaR1 produced from pG4 was compared to the efficiency of active NcaR1 produced in pT7G4-CDS. NCAAH activity of NcaR1 produced in *E. coli* (pG4) and *E. coli* (pT7G4-CDS) by inducing with IPTG for 4 hours at 37°C was determined. *E. coli* (pT7G4-ΔCDS) producing ΔNcaR1, a deletion of *ncaR1* which truncates the protein by 160 amino acids, was also grown and assayed for NCAAH activity (Figure 4.3.A). SDS-PAGE analysis of the protein in the sonicated cells, as well as in the soluble and insoluble fractions was also carried out (Figure 4.3.B).

The NCAAH activity of NcaR1 produced in *E. coli* (pT7G4-CDS) was 60% greater than NCAAH activity of NcaR1 produced in *E. coli* (pG4) (6.9U versus 2.8U respectively Figure 4.3.A). ΔNcaR1 produced in *E. coli* (pT7G4-ΔCDS) did not possess any detectable NCAAH activity when

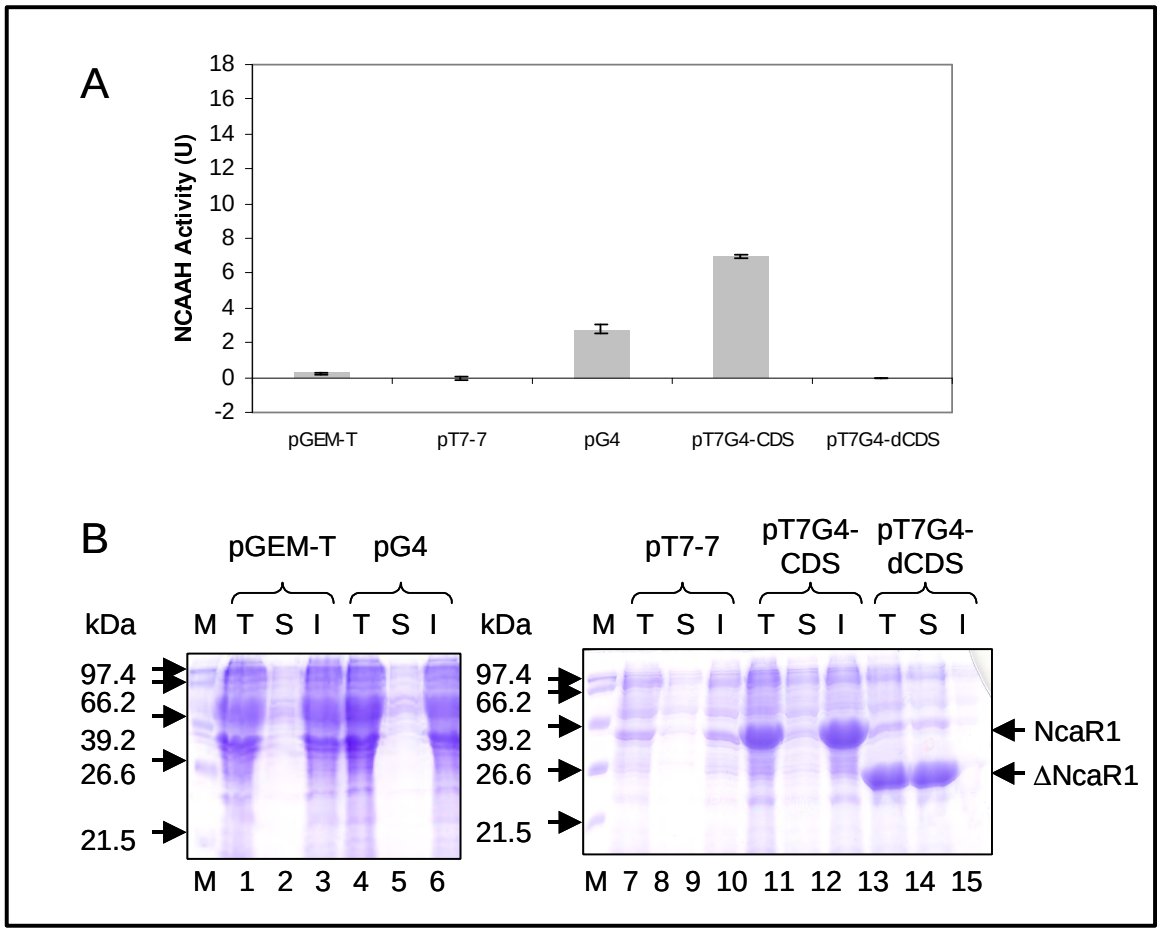


Figure 4.3. Panel A. A comparison of NCAAH activity in cell free extracts of *E. coli* (pGEM-T Easy), *E. coli* (pT7-7), *E. coli* (pG4), *E. coli* (pT7G4-CDS) and *E. coli* (pT7G4-ΔCDS) at 37°C after 4 hours of induction. Panel B. SDS-PAGE analysis of total protein (T) soluble protein (S) and insoluble protein (I) in cell free extracts of *E. coli* (pGEM-T Easy) *E. coli* (pT7-7), *E. coli* (pG4), *E. coli* (pT7G4-CDS) and *E. coli* (pT7G4-ΔCDS) grown at 37°C for 4 hours with induction. The sizes and positions of the molecular weight markers (M) are shown on the left hand side of the gels. The position of NcaR1 and ΔNcaR1 are indicated by shown on the right hand side.

SDS-PAGE analysis showed that NcaR1 could not be detected when produced in *E. coli* (pG4) (Figure 4.3.B, Lanes 5 to 6). A protein corresponding to the molecular mass of NcaR1 was detected in *E. coli* (pT7G4-CDS), and protein corresponding to ΔNcaR1 was detected in *E. coli* (pT7G4-ΔCDS) (Figure 4.3.B, Lanes 12 and 15 respectively). While most of the NcaR1 was distributed in the insoluble protein fraction (Figure 4.3.B, Lanes 13 versus 14), most of the ΔNcaR1 was distributed in the soluble protein fraction (Figure 4.3.B, Lanes 16 versus 17).

iii) The effect of induction on the solubility of NcaR1.

NCAAH activity of NcaR1 produced in uninduced and induced *E. coli* (pT7G4-CDS) grown at 37°C was determined (Figure 4.4A). In addition, SDS-PAGE analysis of the protein of whole cells and the soluble and insoluble protein fractions of cell free extracts was performed (Figure 4.4B).

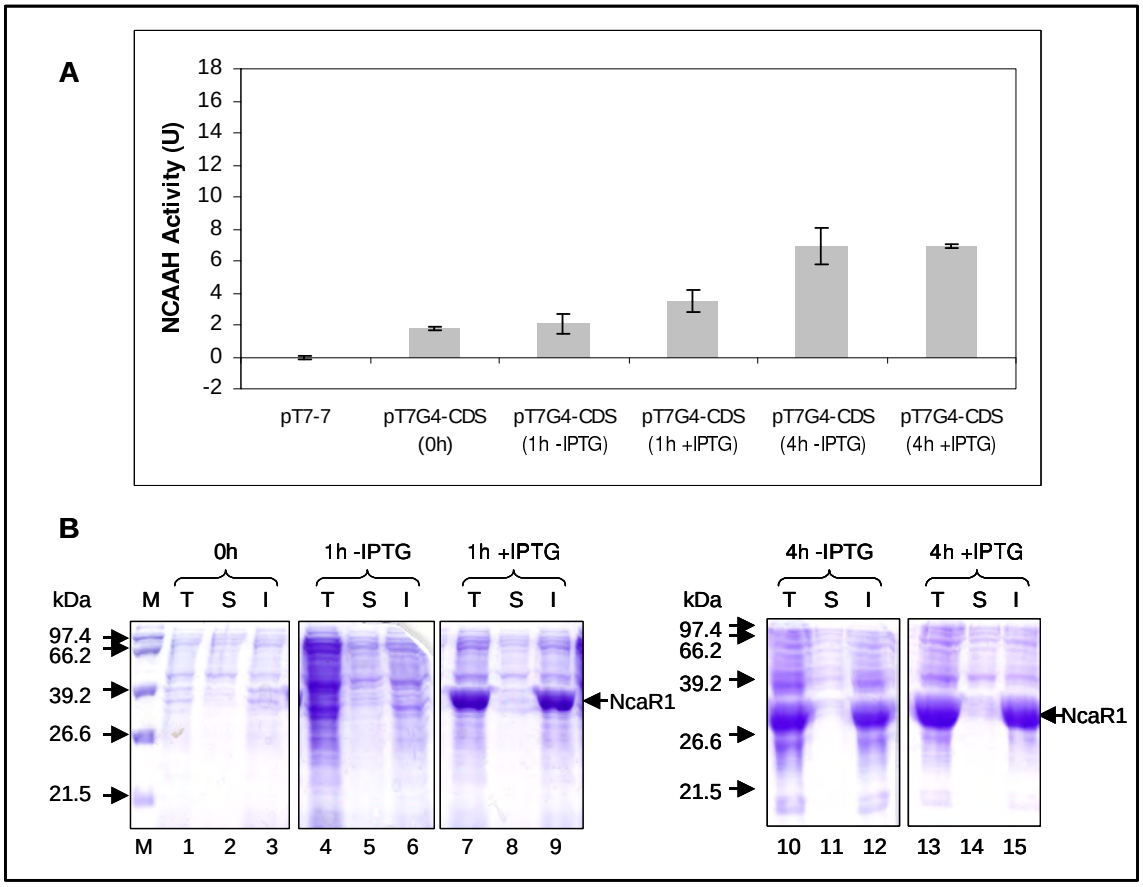


Figure 4.4. Panel A. NCAAH activity in *E. coli* (pT7G4-CDS) induced with IPTG (1mM) for 1 and 4 hours, compared to NCAAH activity in uninduced *E. coli* (pT7G4-CDS). Panel B. SDS-PAGE analysis of NcaR1 produced in induced (+IPTG) and uninduced (-IPTG) *E. coli* (pT7G4-CDS) sampled at 0, 1 and 4 hours post induction. Total protein from the sonicated cells (T) as well as the soluble (S) and insoluble (I) protein fractions of the cells were analysed. The positions and sizes of the molecular weight markers (M) are shown on the left hand side of the gels. The position of NcaR1 is shown on the right hand side of the gels.

Before induction, 1.7U of NCAAH activity was detected in *E. coli* (pT7G4-CDS) (Figure 4.4A) and a protein corresponding to the molecular weight of NcaR1 was detected (Figure 4.4B, Lane 1).

At 1 hour post induction, the induced *E. coli* (pT7G4-CDS) possessed approximately 40% more NCAAH activity than the uninduced cells (Figure 4.4A, 3.5U versus 2.1U). SDS-PAGE analysis of the protein in these cells showed that NcaR1 was over produced in the induced cells (Figure 4.4B, Lane 7). However, in the induced cells, the majority of the NcaR1 produced was in the insoluble fraction (Figure 4.4B, Lane 8 versus Lane 9).

At 4 hours post induction, the total NCAAH activity was almost the same in the induced and uninduced cells (Figure 4.4.A, 6.9U and 6.9U respectively) and SDS-PAGE analysis showed that NcaR1 was over produced in both the induced and uninduced cells (Figure 4.4.B, Lanes 10 and 13). SDS-PAGE analysis shows that the majority of the NcaR1 produced is insoluble in the induced cells (Figure 4.4.B, Lane 14 versus Lane 15) and the uninduced cells (Figure 4.4.B, Lane 11 versus Lane 12).

These results indicate that when NcaR1 is overproduced the majority is insoluble and inactive. A reduction in NcaR1 production was observed when *E.coli* (pT7G4-CDS) was not induced with IPTG, but the solubility and NCAAH activity of NcaR1 was retained. However this was only observed early in mid-log growth phase. Once the cells reached stationary phase, similar results were obtained with cells that were induced and cells that were not induced with IPTG.

iv) The effect of temperature on the solubility of NcaR1 produced in *E. coli*.

The effect of the temperature used for induction on the solubility and NcaR1 production was investigated. NCAAH activity of NcaR1 produced in *E. coli* (pT7G4-CDS) induced with IPTG for 0, 1 and 4 hours at 30°C and 37°C was compared (Figure 4.5A). The distribution of the NCAAH activity in the soluble and insoluble fractions of the cells harvested before induction (0 hours) and at 1 and 4 hours post induction was investigated, and related to the amount of NcaR1 observed in each of these fractions when analysed by SDS-PAGE (Figure 4.5B).

Before induction, cell free extracts of *E.coli* (pT7G4-CDS) possessed 1.7U of NCAAH activity (Figure 4.5A) and a protein corresponding to the molecular mass of NcaR1 was detected (Figure 4.5B, Lane). After 1 hour of induction with IPTG, the NCAAH activity of the cells grown at 30°C was greater than cells grown at 37°C (Figure 4.5A, 4.5U and 3.5U respectively).

After 4 hours of induction with IPTG, the NCAAH activity of the cells grown at 30°C was greater than the NCAAH activity of cells grown at 37°C (Figure 4.5A, 12.9U and 6.9U respectively).

For both of these time intervals, the majority of the NcaR1 being produced was insoluble at 30°C (Figure 4.5A, Lane 5 versus Lane 6 and Lane 8 versus Lane 9) and 37°C (Figure 4.5A, Lane 11 versus Lane 12 and Lane 14 versus Lane 15). Only in cells grown at 30°C for 4 hours showed an increase in the amount of soluble NcaR1 produced (Figure 4.5B, Lane 8).

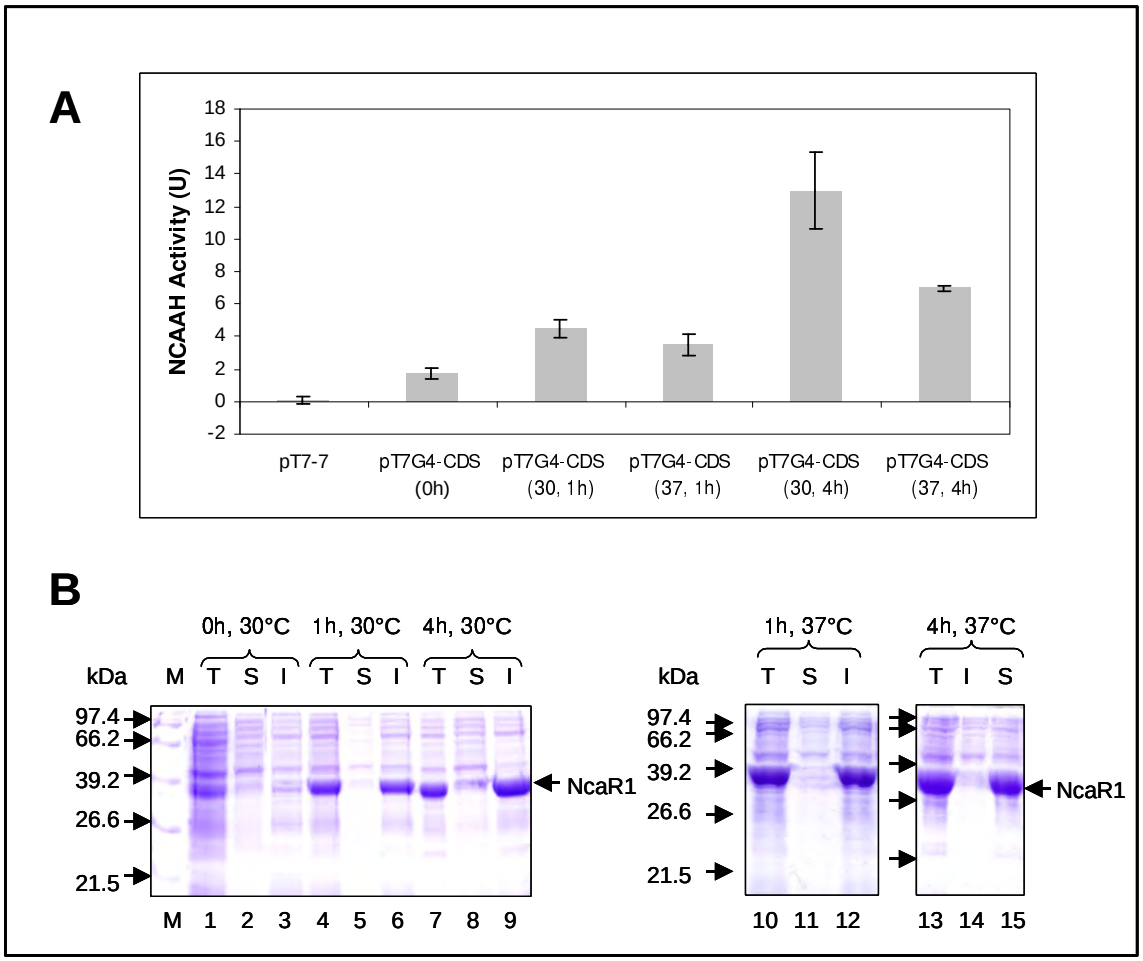


Figure 4.5. Panel A. NCAAH activity in *E. coli* (pT7G4-CDS) grown at 30°C compared to NCAAH activity in *E. coli* (pT7G4-CDS) grown at 37°C. Panel B. SDS-PAGE analysis of protein produced in *E. coli* (pT7G4-CDS) at 30°C and 37°C before induction and 1 and 4 hours post induction. Total protein from the cell free extracts (T), as well as the soluble (S) and insoluble (INS) protein fractions of the cells were analysed. The positions and sizes of the molecular weight markers (M) are shown on the left hand side of the gels. The position of NcaR1 is shown on the right hand side of the gels.

4.4. Discussion.

The research discussed in this chapter set out to determine if NcaR1 and NcaR1-6xHis produced in *E. coli* (pT7G4-CDS) and *E. coli* (pIVEXG4-CDS) possessed NCAAH activity. Furthermore, optimisation of NcaR1 and NcaR1-6xHis production in *E. coli* (pT7G4-CDS) and *E. coli* (pIVEXG4-CDS) was to be optimised.

4.4.1. NCAAH activity of heterologously produced NcaR1 and NcaR1-6xHis.

In this study, NcaR1 produced in *E. coli* (pT7G4-CDS) showed NCAAH activity, but the addition of a C-terminal His-tag resulted in the loss of NCAAH activity. In some cases the use of protein tags have been found to interfere with the function of the heterologously expressed proteins. For example, a hydantoin racemase from *A. aurescens* DSM 3747 produced with a C-terminal His-tag resulted in a complete loss of racemase activity (Wiese *et al.*, 2000). The purification of a heterologously produced D-NCAAH with an N-terminal His-tag has been performed before (Hsu *et al.,* 1999; Sareen *et al.,* 2001a) and was not reported to affect NCAAH activity detrimentally (Sareen *et al.,* 2001a).

There are no reports of the heterologous production of a D-NCAAH with a C-terminal His-tag, however, a study where a D-hydantoinase was fused to the C-terminal of a D-NCAAH to form a bi-functional enzyme did not result in the loss of NCAAH activity (Kim *et al.* 2000a,b), which suggests that the fusion of a His-tag to the C-terminal end would not affect NCAAH activity either.

4.4.2. Optimisation of heterologous production of NcaR1.

The optimisation of production of enzymatically active and soluble NcaR1 was pursued. It has been suggested that *N*-carbamoyl-amino acids, the substrate of NCAAHs, have a low permeability through cell membranes (Olivieri *et al.*, 1981; Lee *et al.,* 1999; Park *et al.,* 2000), which results in low NCAAH activity, as the substrate is not freely available to the enzyme. So the first course of action to increase the activity of NcaR1 was the use of sonication to lyse the cells in which the enzyme was produced, thereby allowing the enzyme to come into contact with the substrate more readily. This approach

resulted in an increase in NCAAH activity of more than a third when compared to that of whole cells (Figure 4.2).

Another factor that was thought to affect the NCAAH activity of the NcaR1 produced was the solubility of the protein, as the insoluble nature of heterologously produced D-NCAAH's has been reported in the past (Buson *et al.,* 1996; Grifantini *et al.,* 1998; Chao *et al.,* 1999; Hils *et al.* 2001; Sareen *et al.,* 2001a). With heterologous expression, it is believed that the rate of expression and protein production plays a role in the quality of the protein product and it is suspected that if the protein is produced too rapidly the chances of protein mis-folding and consequently the formation of insoluble aggregates are increased (Hockney, 1994).

In this study, the T7 RNA polymerase promoter was used for the heterologous expression of *ncaR1.* It is commonly known that the *lac* promoter regulating the expression of the T7 RNA polymerase is "leaky" resulting in low-level expression of the gene of interest before the expression of T7 RNA polymerase is induced (Baneyx, 1999). Here it was observed that this was the case for the expression of *ncaR1* and was reported for another D-NCAAH being expressed under the control of a T7 promoter (Sareen *et al.,* 2001b). To improve the solubility of the NcaR1 being produced by decreasing the rate at which *ncaR1* was being expressed, the "leaky" nature of the *lac* promoter controlling the T7 RNA polymerase was taken advantage of, and the low-level expression of *ncaR1* in uninduced cells was compared to the normal expression of *ncaR1* in induced cells.

It was found that NcaR1 produced before induction possessed NCAAH activity, but only at 20% of that obtained after 4 hours with induction. At 1 hour, an increase in NCAAH activity was observed for both the induced and uninduced cells and very little NcaR1 was observed in the soluble fractions compared to those in the insoluble fractions.

At four hours post induction, the total NCAAH activity for both the induced and uninduced cells was similar. Both possessed higher NCAAH activity than at 1 hour post induction and SDS-PAGE analysis showed that the amount of NcaR1 produced in the induced and uninduced cells was similar, the majority of which was in the insoluble fraction, thus it appeared that induction with IPTG might be unnecessary.

The solubility and activity of heterologously produced NcaR1 was improved further by lowering the temperature used for the growth of the cells. Decreasing the temperature has been employed regularly to reduce the production of insoluble proteins during heterologous production (Surek *et al.,* 1991; Baneyx *et al.,* 1991; Hockney, 1994; Nygaard, 2001) and it has been shown to be successful when applied to the heterologous production of NCAAH's (Buson *et al.,* 1996; Grifantini *et al.,* 1998; Chien *et al.,* 1998; Chao *et al.*, 2000; Sareen *et al.,* 2001). In this study decreasing the temperature from 37°C to 30°C resulted in an almost a two-fold increase of NCAAH activity and an increase in solubility of NcaR1 was observed by SDS-PAGE analysis.

CHAPTER 5
General Conclusions

CHAPTER 5
General Conclusions

The research described in this thesis focused on the isolation and expression of hydantoin hydrolysing enzymes from *A. tumefaciens* RU-AE01 and *A. tumefaciens* RU-OR.

5.1. Isolation of genes encoding hydantoin hydrolysing enzymes from *A. tumefaciens* RU-AE01.

It has been shown that the genes encoding the hydantoin hydrolysing genes of some bacteria are associated in gene clusters on the genome (Watabe *et al.,* 1992a,b; Wiese *et al.,* 2001a,b; Hils *et al*., 2001). Previously, the gene encoding an *N*-carbamoyl-amino acid amidohydrolase was isolated from a genomic DNA library of *Agrobacterium tumefaciens* RU-AE01 (Harrera, unpublished results). Nucleotide sequence analysis of the region upstream of the *N*-carbamoyl-amino acid amidohydrolase of this strain revealed a fragment of an open reading frame that corresponds to the 5' region of the gene encoding a D-hydantoinase (Hartley, unpublished results).

Since the genes encoding the hydantoinase and NCAAH of *A. tumefaciens* RU-AE01 are linked on the genome, the re-isolation of the NCAAH on a large enough fragment from the genomic DNA library could have allowed for the simultaneous isolation of the hydantoinase. Furthermore, other genes encoding proteins involved in the hydrolysis of hydantoins, such as the hydantoin racemase and the hydantoin permease may also have been isolated. To achieve this, a gene library of *A. tumefaciens* RU-AE01 was screened with a DNA probe consisting of the gene encoding the *N*-carbamoyl-amino acid amidohydrolase from this strain.

Nucleotide sequence was generated from the 5' and 3' regions of two genomic DNA inserts that were shown to possess homology to the DNA probe. Nucleotide sequence

analysis showed that both of these genomic DNA fragments shared nucleotide sequence homology to the same region of the *A. tumefaciens* C58 circular chromosome. However, analysis of the open reading frames of this region of the *A. tumefaciens* C58 circular chromosome did not reveal any genes encoding enzymes involved in the hydrolysis of hydantoins. Further Southern Hybridisation of the recombinant plasmids carrying these DNA inserts, and a third set of recombinant genomic DNA library plasmids (that also showed homology to the DNA probe) with the DNA probe was performed. This showed that the DNA probe used for screening the genomic DNA library was binding to the vector DNA in which the genomic DNA library was inserted. The reason for this was undetermined, as they shared no sequence homology. However the implications were that this DNA probe was not suitable for screening the genomic DNA library. As a result, isolation of the hydantoinase gene and other enzymes involved in the hydrolysis of hydantoin was not achieved.

5.2. Expression of *ncaR1* and *ncaR2*.

In bacteria, *N*-carbamoyl-amino acid amidohydrolase activity is usually encoded by one enzyme (Wiese *et al.,* 2001a,b; Watabe *et al,* 1992a,b; Hils *et al,* 2001). However *A. tumefaciens* RU-OR has been shown to possess two genes, *ncaR1* and *ncaR2,* which encode two *N*-carbamoyl-amino acid amidohydrolases with unique deduced amino acid sequences (Hartley, 2001) and probably have distinct NCAAH activity. This may account for the unusual *N*-carbamoyl-amino acid amidohydrolase activity of *A. tumefaciens* RU-OR (Hartley *et al.,* 1998).

One of the objectives of this study was to express *ncaR1* and *ncaR2* separately, and to compare the *N*-carbamoyl-amino acid amidohydrolase activity of each of these enzymes. However the plasmid containing the genomic DNA insert that carries *ncaR2* was found to possess a different nucleotide sequence when compared to the nucleotide sequence that was originally obtained (Hartley, 2001), and the open reading frame of *ncaR2* could not be found. The phenomenon of plasmid rearrangements, or the 'looping out' of potentially toxic genes in recombinant plasmids may provide an explanation for the loss of *ncaR2* from the plasmid on which it was encoded.

Hils *et al.* (2001) showed that the hydantoin utilising gene cluster of *Agrobacterium* sp. IP I-671 was flanked by a putative transposase and a putative resolvase, which were suggested to be part of a transposon carrying the hydantoin utilising genes. If a similar scenario exists in *A. tumefaciens* RU-OR, it is possible that a transposition event may have resulted in the loss of *ncaR2* from the *A. tumefaciens* RU-OR genome, which would explain why PCR amplification of *ncaR2* from the *A. tumefaciens* RU-OR genome using primers specific for this gene failed (Hartley, unpublished results). Consequently the expression of *ncaR2* could not be performed in this study.

The gene encoding *ncaR1* was expressed in *E. coli* with the T7 RNA polymerase promoter and a protein corresponding to the expected molecular mass of NcaR1 was produced. Furthermore, a truncation of *ncaR1* was constructed, and used to produce the protein ΔNcaR1. Enzyme activity assays of cell free extracts of recombinant *E. coli* producing NcaR1 and ΔNcaR1 showed that NcaR1 possessed *N*-carbamoyl-amino acid amidohydrolase activity, while ΔNcaR1 did not. This proved that the NCAAH activity was as a result of the production of NcaR1.

The optimisation of the expression of *ncaR1* was performed. Studies have shown that lowering the temperature used for the growth of the cells producing the *N*-carbamoyl-amino acid amidohydrolases reduces the production of insoluble protein aggregates and increases the amount of active enzyme in the cells (Buson *et al.,* 1996; Grifantini *et al.,* 1998; Chien *et al*., 1998). In this study, decreasing the temperature from 37°C to 30°C improved the solubility of NcaR1, and resulted in almost a two-fold increase in *N*-carbamoyl-amino acid amidohydrolase activity.

5.3. Expression and purification of *ncaR1-6xHis.*

The expression of *ncaR1-6xHis* in *E. coli* to produce NcaR1-6xHis, which possessed a C-terminal 6xHis tag was performed to facilitate the purification of NcaR1. Enzyme activity assays showed that NcaR1-6xHis did not possess *N*-cabamoyl-amino acid amidohydrolase activity, and is thought to be as a result of the addition of the 6xHis tag on the C-terminus of the protein.

NcaR1-6xHis was purified under native conditions using Ni-NTA affinity chromatography spin columns, and was used to raise polyclonal antibodies in a rabbit. However, the serum isolated from the rabbit after immunisation was shown to possess antibodies that were not specific for NcaR1 or NcaR1-6xHis produced in *E. coli*. Since native purification conditions were used to purify NcaR1-6xHis, it is possible that proteins bound to NcaR1-6xHis, such as chaperone proteins, were co-purified with NcaR1-6xHis, and it is to these proteins that antibodies in the rabbit were formed.

5.4. Future Research.

Hils *et al.,* (2001) showed that inactivation of the NCAAH gene in *Agrobacterium* sp. IP I-671 resulted in a total loss of NCAAH activity, proving that NCAAH activity is only encoded by one enzyme in this strain. In the case of *A. tumefaciens* RU-OR, the inactivation of *ncaR1* would result in a mutant that would still possess *ncaR2*. This mutant could be useful for studying the enzyme characteristics, which are proposed to be novel, of NcaR2 in the absence of NcaR1. In addition, the isolation and heterologous expression of *ncaR2* for the purification of NcaR2 is still of interest, so that the enzyme characteristics can be compared to NcaR1.

Furthermore, the isolation of the hydantoinase gene or genes encoding other enzymes involved in the hydrolysis of hydantoin could still be pursued. This would require the development of a new DNA probe for screening the genomic DNA library, or alternatively the application or development of a completely new method for screening the genomic DNA library. Screening methods that have been used before include screening the genomic DNA library for enzyme activity, or for the ability to utilise hydantoin as a sole nitrogen source.

Once *ncaR1* and *ncaR2* have been isolated, directed evolution could be performed by shuffling these genes, to generate a gene encoding an NCAAH with improved characteristics, such as enhanced enzyme activity and improved oxidative stability and thermostability. This would result in an enzyme that would have improved performance as a biocatalyst for the production of amino acids.

With regards to production of antibodies to NcaR1, the isolation of NcaR1-6xHis under denaturing conditions may provide a better method protein preparation for the synthesis of NcaR1-6xHis antibodies. Denaturing conditions will ensure that there are no proteins bound to NcaR1-6xHis, and would eliminate the co-purification of other proteins. The isolation of NcaR1 antibodies will provide a useful tool for the detection of NcaR1 or other *N*-carbamoyl amino acid amidohydrolases. They could be used to quantify the expression of NcaR1 in *A. tumefaciens* RU-OR. Furthermore, antibodies raised against NcaR1 may also react immunogenically with NcaR2, and may provide a tool for the detection of this enzyme too. It might also be of interest to produce NcaR1 with an N-terminal 6xHis tag, and determine if it still possessed NCAAH activity.

APPENDICES

Appendix 1: Media and Buffers

Luria Broth (LB) and Luria Agar (LA)

10g tryptone

5g yeast extract

5g NaCl

Made up to 1L with dH_2O, and autoclaved for 20 minutes.

For Luria agar, 15g agar was added to the above before autoclaving.

Antibiotics were added (if necessary) after autoclaving.

0.1M Potassium Phosphate Buffer

Make stocks of 0.2M potassium hydroxide (KOH) and 0.2 M potassium dihydrogen orthophosphate (KH_2PO_4). Mix to required pH.

40% Acrylamide Stock

38% Acrylamide

2% Bis-Acrylamide

Resolving Gel

3.2 ml 40% Acrylamide stock

3.2 ml water

3.75ml 1M Tris (pH8.7)

100µl 10% SDS

100µl 10% APS

10 µl TEMED

Stacking Gel

1.3ml 40% Acrylamide stock

7.25ml water

1.3ml 1M Tris (pH 6.8)

100µl 10% SDS

50µl 10% APS

10µl TEMED

2x SDS-PAGE Buffer

2.5ml Tris (pH 6.8)

2ml 20% SDS

1ml β-mercaptoethanol

2ml glycerol

0.01% Bromophenol blue

Make up to 10ml with dH_2O

2X Bath Buffer for SDS-PAGE

57.6g glycine

12g Tris (pH 8.3)

4g SDS

Made up to 2L with dH_2O

Transfer Buffer

3.03g Tris

14.4g Glycine

200ml methanol in 1L

Make fresh and chill

TBS-Tween

100ml 1M Tris pH7.5

30ml 5M NaCl

1ml TWEEN-20

Made up to 1L with dH_2O

Coomassie Stain

Dissolve 2g Coomassie in 200ml Destain I, and stir overnight

Filter

Destain I

50% Methanol

10% Glacial acetic acid

Destain II

5% Methanol

7% Glacial acetic acid

Appendix 2: Easy Preps for plasmid DNA Isolation

1. A sterile microfuge is filled with an overnight culture of *E. coli* and centrifuged for 3 minutes at 13000rpm
2. The supernatant is poured off and resuspended in 50µl Smart Buffer, followed by a 30 minute incubation at 37°C.
3. Boil for one minute, then incubate on ice for 5 minutes.
4. Spin for 10 minutes at 13000 rpm. The plasmid DNA is in the supernatant.

SMART BUFFER

10mM TRIS, pH8

1mM EDTA

15% Sucrose

100 µg/ml BSA

200 µg/ml lysosyme

Appendix 3: Oligonucleotides used in this study.

Appendix 8a: PCR Amplification Primers

Primer	Gene	Location	Sense/ Anti-Sense	Sequence
CHOA F1	*ncaR1*	1-24	S	CGGGATCCTTGACCCTGGTCCTTG
CHOA R1	*ncaR1*	1159-1182	A	CCCAAGCTTTCTCGATCGGATAGG
SC7	*ncaR1*	190-212	S	CATATGACACGTCAGATGATACTTGC
SC8	*ncaR2*	190-212	S	CTACAATTCGGCGATCAGG
SC10	*ncaR1*	1084-1101	A	CCCGGGAATTCCGCGATCAGACC
SC10a	*ncaR1*	1084-1101	A	CCCGGGGAATTCCGCGATCAGACC
CH7	*ncaR1*	190-217	S	GGAATCCCATATGACACGTCAGATGATACTTGCTGTCG

Appendix 8b: Sequencing Primers

Primer	Vector	Sense/Anti-Sense	Sequence
pUC F	pGEM-T Easy Vector	S	CGCCAGGGTTTTCCCAGTCACGAC
pUC R	pGEM-T Easy Vector	A	TCACACAGGAAACAGCTATGAC
T7-7F	pT7-7, pIVEX 2.3 MCS	S	GGGAGACCACAACGGTTTCCC
T7-7R	pT7-7	A	CGCTGAGATAGGTGCCTCAC
T7-R	pIVEX 2.3 MCS	A	GCTAGTTATTGCTCAGCGG
SC5	p6B3-1a	S	AAGTCTCCGTTCCGGTGATT
SC6	p6B3-1a	A	GAAATACTGCCACCAGTGCG
SC11	p6B3-1a	S	TACTGGCGGTCCACACGGTT
SC12	pG4	S	CTCGGCTTCCCGGTCTATG
SC13	pG4	A	GACGACGAGTTCGGCGTAG
SC14	p6B3-1a	S	TTGAGTGTATCGTGATCCGC
SC20	p6B3-1a	S	CCTGGCAACCTGCTCTAGCG

Appendix 4: PCR Thermal Cycling Programmes

Programme 1:

1×	94°C for 2 min
25×	94°C for 45 sec 54°C for 45 sec 72°C for 1 min
1×	72°C for 4 min

Programme 2:

1×	94°C for 2 min
25×	94°C for 45 sec 56°C for 45 sec 72°C for 1 min
1×	72°C for 4 min

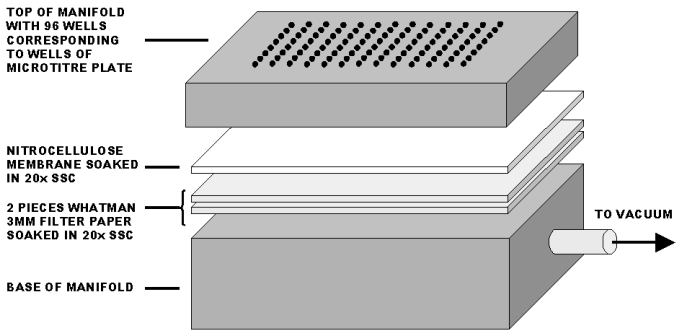
Programme 3:

1×	94°C for 90 sec
25×	94°C for 45 sec 60°C for 60 sec 72°C for 60 sec
1×	72°C for 2 min

Appendix 5: Dot Blotting DNA.

The method used for dot blotting the plasmid DNA prepared by alkaline lysis onto the Hybond N+ nitrocellulose membrane (Amersham) was based on the method suggested by Amersham.

1. Assemble the dot blot manifold with the nitrocellulose membrane and 2 pieces of Whatman 3MM filter paper soaked in 20x SSC as follows:



   Once assembled, attach the dot blot manifold to a vacuum.
2. Starting with the first 8 wells of the microtitre plate, transfer a volume of plasmid DNA from the microtitre plate with a multiwell pipette to an equal volume of 20x SSC buffer on a piece of Parafilm ( ), ensuring that the plasmid DNA does not mix with that from adjacent wells.
3. Apply the plasmid DNA mixed with 20x SSC to the wells of the dot blot manifold corresponding to the well of the microtitre plate where the plasmid DNA was obtained. The sample is drawn through the dot blot manifold by the vacuum, which allows the plasmid DNA to bind to the nitrocellulose membrane.
4. Repeat with the plasmid DNA from the rest of the microtitre plate. Dismantle the dot blot manifold, and transfer the membrane (DNA side up) to a container with Whatman 3MM filter paper soaked in denaturing solution and leave for 5 minutes.
5. Transfer the membrane to a container with Whatman 3MM filter paper soaked in neutralising solution and leave for 1 minute.
6. Transfer the membrane to a dry sheet of Whatman 3MM paper, and air dry for at least 30 minutes.
7. Fix the plasmid DNA onto the membrane by placing it on a UV transilluminator for 5 minutes.

Appendix 6: Colony Blots

This procedure involves

Colonies stuck to nitrocellulose membrane

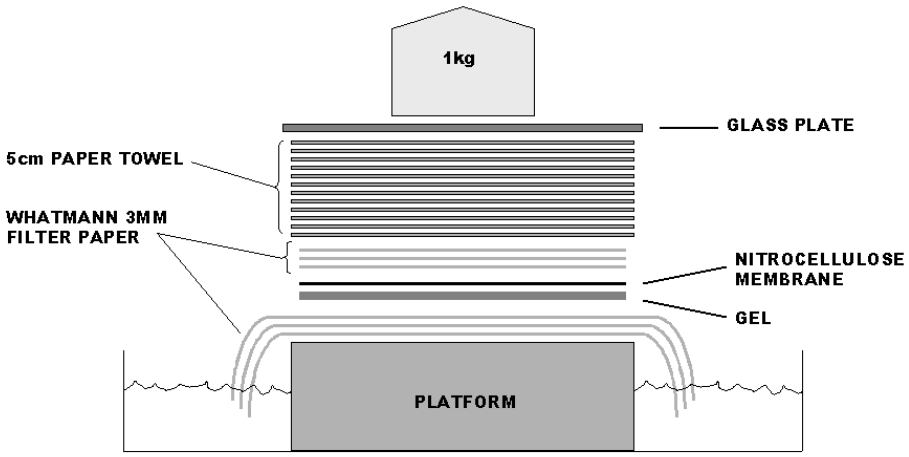
Membrane transferred, colony side up, to a series of Whatman 3MM paper saturated in solutions required for the lysis of the cells

Plasmid DNA then fixed to the membrane using UV light.

1. The nitrocellulose membranes and the 14mm LA plates are marked and numbered for orientation relative to the microtitre plates. The membrane is placed on the surface of the agar medium so as to come into contact with the bacterial colonies and is left until it is completely wet.
2. Using forceps, the membrane is lifted from the LA plate and transferred to a container with Whatman 3MM paper soaked in 10% SDS solution, and leave for 3 minutes.
3. Transfer the membrane to a container with Whatman 3MM paper soaked in denaturing solution and leave for 5 minutes.
4. Transfer the membrane to a container with Whatman 3MM paper soaked in neutralizing solution and leave for 5 minutes.
5. Transfer the membrane to a container with Whatman 3MM paper soaked in 2xSSC and leave for 5 minutes.
6. Transfer the membrane to a dry sheet of Whatman 3MM paper, and air dry for at least 30 minutes.
7. Fix the plasmid DNA onto the membrane by placing it on a UV transilluminator for 5 minutes.

Appendix 7: Capillary transfer of plasmid DNA from an agarose gel to nitrocellulose membranes.

1. The plasmid DNA digested with restriction enzymes was separated on a 1% agarose gel (no ethidium bromide added) using electrophoresis.
2. After electrophoresis the gel was stained with ethidium bromide (2.5μg/ml in TAE) for 10 minutes. After staining, the gel was photographed alongside a transparent ruler so that the distance migrated by any band of DNA on the gel could be determined.
3. The gel was then placed in 0.25M HCl until the xylene cyanol and bromophenol blue (from the loading buffer) changed colour, and then the gel was left for an additional 10 minutes in the HCl.
4. The gel was then rinsed in dH_2O and then soaked in denaturation buffer for 30 minutes with agitation.
5. The gel was then rinsed in dH_2O and soaked in neutralisation buffer for 15 minutes with agitation. This step was repeated.
6. A capillary blot was set up as shown in the following figure:



7. After 12-16 hours, the sandwich was dismantled, and the positions of the wells from the agarose gel were marked on the nitrocellulose membrane with a pencil, and the orientation of the gel was also noted.
8. The membrane was then washed in 2X SSPE to remove any adhering agarose.
9. After transfer, the membrane was air dried and stored in a zip-lock bag until used for hybridisation reactions.

Appendix 7: Southern Hybridisation

All hybridisation and wash steps were carried out in a Hybaid Omnigene Hybridisation Oven.

1. The nitrocellulose membrane was incubated at 42°C in 25ml pre-hybridisation solution containing denatured salmon sperm DNA to block non-specific binding sites for 8 hours.
2. The radioactively labelled DNA probe was denatured at 100°C for 5 minutes on a heating block, and then added to the membrane in the pre-hybridisation solution. The membrane was incubated with the probe at 42°C for at least 12 hours.
3. Highly stringent washes were carried out as follows:
   i) 2x SSPE with 0.1% SDS for 10 minutes at room temperature. Repeat.
   ii) 0.5x SSPE with 0.1% SDS for 15 minutes at room temperature
   iii) 0.5x SSPE with 0.1% SDS for 30 minutes at 42°C
   iv) 0.5x SSPE with 0.1% SDS for 10 minutes at 65°C

20x SSC:
3M NaCl
0.3M Sodium Citrate

Denaturing Solution:
1.5M NaCl
0.5M NaOH

Pre-Hybridisation Solution:
5x SSPE
5X Denhardt's Solution
0.5% (w/v) SDS
50% formamide
To 25ml, add 0.5ml of a 1mg/ml solution of denatured salmon sperm DNA

20x SSPE:
3.6M NaCl
0.2M Sodium Phosphate
0.2M EDTA pH7.7

Neutralising Solution:
1.5M NaCl
0.5M Tris pH 7.2
0.001M EDTA

Denhardt's Solution:
2% (w/v) BSA
2% (w/v) Ficoll
2% (w/v) polyvinylpyrrolidone

Appendix 8: Nucleotide and amino acid sequence analysis of NcaR1.

Appendix 8a: The revised nucleotide and deduced amino acid sequence of pG4.

```
CGGGATCCTT GACCCTGGTC CTTGACAGAT CAAAAGTTTT ACGCCTGTAG TATGAGTACT 60

GCATGTGGCA TTTATCCTTT TTGTAGAACA ATCATTGGCG TGCCAAGCTG AGACGTGTGT 120

TCCTGAAATG TGCATAGCAG CGTTCTCCCG GCCGCGAGGC CGGATTAACT ATCGAAGGAG 180

CAAAGGTTCA TGACACGTCA GATGATACTT GCTGTCGGAC AGCAAGGCCC CATCGCGCGA 240
          M  T  R  Q   M  I  L   A  V  G   Q  Q  G  P   I  A  R  17
GCGGAGACAC GCGAACAGGT GGTTGGCCGC CTCCTCGACA TGTTGACGAA CGCAGCCAGC 300
 A  E  T   R  E  Q  V   V  G  R   L  L  D  M   L  T  N   A  A  S  37
CGGGGCGTGA ACTTCATCGT CTTTCCCGAG CTTGCGCTCA CGACCTTCTT CCCGCGCTGG 360
 R  G  V   N  F  I  V   F  P  E   L  A  L  T   T  F  F   P  R  W  57
CATTTCACCG ACGAGGCCGA GCTCGATAGC TTCTATGAGA CCGAAATGCC CGGCCCGGTG 420
 H  F  T   D  E  A  E   L  D  S   F  Y  E   T  E  M  P   G  P  V  77
GTCCGTCCAC TCTTTGAGAC GGCCGCCGAA CTCGGGATCG GCTTCAATCT GGGCTACGCC 480
 V  R  P   L  F  E  T   A  A  E   L  G  I   G  F  N  L   G  Y  A  97
GAACTCGTCG TCGAAGGCGG CGTCAAGCGT CGCTTCAACA CGTCCATTCT GGTGGATAAG 540
 E  L  V   V  E  G  G   V  K  R   R  F  N   T  S  I  L   V  D  K  117
TCAGGCAAGA TCGTCGGCAA GTATCGTAAG ATCCATTTGC CGGGTCACAA GGAGTACGAG 600
 S  G  K   I  V  G  K   Y  R  K   I  H  L   P  G  H  K   E  Y  E  137
GCCTACCGGC CGTTCCAGCA TCTTGAAAAG CGTTATTTCG AGCCGGGCGA TCTCGGCTTC 660
 A  Y  R   P  F  Q  H   L  E  K   R  Y  F   E  P  G  D   L  G  F  157
CCGGTCTATG ACGTCGACGC CGCGAAAATG GGGATGTTCA TCTGCAACGA TCGCCGCTGG 720
 P  V  Y   D  V  D  A   A  K  M   G  M  F   I  C  N  D   R  R  W  177
CCTGAAACGT GGCGGGTGAT GGGACTTAAG GGCGCCGAGA TCATCTGCGG CGGCTACAAC 780
 P  E  T   W  R  V  M   G  L  K   G  A  E   I  I  C  G   G  Y  N  197
ACGCCGACCC ACAATCCCCC CGTTCCCCAG CACGACCATC TGACGTCCTT CCACCACCTT 840
 T  P  T   H  N  P  P   V  P  Q   H  D  H   L  T  S  F   H  H  L  217
CTGTCGATGC AGGCCGGGTC GTACCAAAAC GGCGCCTGGT CCGCGGCGGC CGGCAAGGTC 900
 L  S  M   Q  A  G  S   Y  Q  N   G  A  W   S  A  A  A   G  K  V  237
GGCATGGAGG AGGGGTGCAT GCTGCTCGGC CATTCGTGCA TCGTGGCGCC GACCGGCGAA 960
 G  M  E   E  G  C  M   L  L  G   H  S  C   I  V  A  P   T  G  E  257
ATCGTTGCCC TGACCACGAC GTTGGAAGAC GAGGTGATCA CCGCCGCCGT CGATCTCGAC 1021
 I  V  A   L  T  T  T   L  E  D   E  V  I   T  A  A  V   D  L  D  277
CGCTGCCGGG AACTGCGCGA ACACATCTTC AATTTCAAAG CCCATCGTCA GCCACAGCAC 1081
 R  C  R   E  L  R  E   H  I  F   N  F  K   A  H  R  Q   P  Q  H  297
TACGGTCTGA TCGCGGAATT CTGAAGGTCA GGCCAAAAAA ACGGATGGGG CTGGGGACGT 1140
 Y  G  L   I  A  E  F   *                                        304
CGAAGCGGCA GCGTTACGCC TATCCGATCG AGAAAGCTTG GG                    1182
```

Appendix 8b: The nucleotide sequence of the ORF encoding *ncaR1* pG4 aligned with the revised sequence of pG4 (SAC).

```
CJH    1 ATGACACGTCAGATGATACTTGCTGTCGGACAGCAAGGCCCCATCGCGCGAGCGGAGACA
SAC    1 ATGACACGTCAGATGATACTTGCTGTCGGACAGCAAGGCCCCATCGCGCGAGCGGAGACA

CJH   61 CGCGAACAGGTGGTTGGCCGCCTCCTCGACATGTTGACGAACGCAGCCAGCCGGGGCGTG
SAC   61 CGCGAACAGGTGGTTGGCCGCCTCCTCGACATGTTGACGAACGCAGCCAGCCGGGGCGTG

CJH  121 AACTTCATCGTCTTTCCCGAGCTTGCGCTCACGACCTTCTTCCCGCGCTGGCATTTCACC
SAC  121 AACTTCATCGTCTTTCCCGAGCTTGCGCTCACGACCTTCTTCCCGCGCTGGCATTTCACC

CJH  181 GACGAGGCCGAGCTCGATAGCTTCTATGAGACCGAAATGCCCGGCCCGGTGGTCCGTCCA
SAC  181 GACGAGGCCGAGCTCGATAGCTTCTATGAGACCGAAATGCCCGGCCCGGTGGTCCGTCCA

CJH  241 CTCTTTGAGACGGCCGCCGAACTCGGGATCGGGTTCAATCTGGGCTACGCCGAACTCGTC
SAC  241 CTCTTTGAGACGGCCGCCGAACTCGGGATCGGCTTCAATCTGGGCTACGCCGAACTCGTC

CJH  301 GTCAGCGGGGGTGTCAAGCGCAGGTTCAACACGTCCATTTTGGTGGATAAGTCAGGCAAG
SAC  301 GTCGAAGGCGGCGTCAAGCGTCGCTTCAACACGTCCATTCTGGTGGATAAGTCAGGCAAG

CJH  361 ATCGTCGGCAAGTGTAGAAAGATCCATTTGCCGGGTCACAAGGAGTACGAAGCCTACCGG
SAC  361 ATCGTCGGCAAGTATCGTAAGATCCATTTGCCGGGTCACAAGGAGTACGAGGCCTACCGG

CJH  421 CCGTTCCAGCTT-TTAAAAAGG--TATTTCGAGCCGGGCGATTTGGGCTTTCCCGTTTAT
SAC  421 CCGTTCCAGCATCTTGAAAAGCGTTATTTCGAGCCGGGCGATCTCGGCTTCCCGGTCTAT

CJH  478 AACGTCGACGCCGCGAAAATGGGAATGTTCATTTGCAACGATCGCCGCTGGCCTGAAACG
SAC  481 GACGTCGACGCCGCGAAAATGGGGATGTTCATCTGCAACGATCGCCGCTGGCCTGAAACG

CJH  538 TGGCGGGTGATGGGACTTAAGGGCGCCGAGATCATCTGCGGCGGTTACAACACGCCGACC
SAC  541 TGGCGGGTGATGGGACTTAAGGGCGCCGAGATCATCTGCGGCGGCTACAACACGCCGACC

CJH  598 CACAATCCCCCCGTTCCCCAGCACGACCATCTGACGTCCTTCCACCACCTCCTGTCGATG
SAC  601 CACAATCCCCCCGTTCCCCAGCACGACCATCTGACGTCCTTCCACCACCTTCTGTCGATG

CJH  658 CAGGCCGGGTCGTACCAAAACGGCGCCTGGTCCGCGGCGGCCGGCAAGGTCGGCATGGAG
SAC  661 CAGGCCGGGTCGTACCAAAACGGCGCCTGGTCCGCGGCGGCCGGCAAGGTCGGCATGGAG

CJH  718 GAGGGGTGCATGTTGCTCGGCCATTCGTGCATCGTGGCGCCGACCGGCGAAATCGTTGCC
SAC  721 GAGGGGTGCATGCTGCTCGGCCATTCGTGCATCGTGGCGCCGACCGGCGAAATCGTTGCC

CJH  778 CTGACCACGACGTTGGAAGACGAGGTGATCACCGCCGCCGTCGATCTCGACCGCTGCCGG
SAC  781 CTGACCACGACGTTGGAAGACGAGGTGATCACCGCCGCCGTCGATCTCGACCGCTGCCGG

CJH  838 GAACTGCGCGAACACATCTTCAATTTCAAAGCCCATCGTCAGCCACAGCACTACGGTCTG
SAC  841 GAACTGCGCGAACACATCTTCAATTTCAAAGCCCATCGTCAGCCACAGCACTACGGTCTG

CJH  898 ATCAAGGAATTCTAA
SAC  901 ATCGCGGAATTCTGA
```

Appendix 9: Restriction enzymes and DNA modifying enzymes

Name	Source
Restriction Enzymes	Roche (SA) Promega (USA) Amersham, USB (USA) New England Biolabs (USA)
T4 DNA Ligase	Promega (USA)
Expand High Fidelity PCR System	Roche (SA)
Klenow	Amersham, USB (USA)
Taq DNA Polymerase	Promega (USA)

Appendix 10: Heterologous Expression in *E.coli* BL21 (DE3)

1. A single colony of freshly transformed *E.coli* BL21 (DE3) harbouring the recombinant plasmid was inoculated into a test tube of 5mL LB containing 100μg/ml ampicillin. The cultures were grown overnight at 37°C on a shaker.
2. The OD_{600} of the overnight culture was measured, and was used it to inoculate 100ml LB containing 100μg/ml ampicillin (in a 500 ml conical flask) to a final OD_{600} of 0.05. Alternatively, 1 ml of the overnight culture was used to inoculate the media.
3. The cultures were grown at 37°C with shaking (200rpm) until mid-log growth phase (OD_{600} between 0.4 and 0.8) was reached. Expression was then induced with the addition of IPTG (final concentration of 1mM) to the culture. After induction, the cultures were grown at 37°C or 28°C.

Appendix 11: Native Purification of NcaR1-His.

1. The pellet from 100ml of cells was thawed on ice and resuspended in 3 ml lysis buffer.
2. After the addition of 1mg/ml lysosyme was, the cells were incubated on ice for 30 minutes.
3. The cell lysate was sonicated on ice for 1 minute, where 6 ten-second bursts were alternated with 6 ten-second cooling periods.
4. The cell debris was separated from the supernatant by centrifugation for 20 minutes at 9000rpm in a JA20 (Beckman J2-21). The supernatant was separated from the pellet, and was used for the purification of NcaR1-His.
5. Before use the Ni-NTA spin columns were equilibrated with 600µl lysis buffer, followed by centrifugation in a microfuge at 2000 rpm for 2 minutes.
6. 600µl of the supernatant from Step 4 was applied to the spin column, followed by centrifugation in a microfuge at 2000 rpm for 2 minutes. The flow-through was collected and stored as Flow-Through in a microfuge tube.
7. The Ni-NTA column was washed twice with 600µl wash buffer, each wash followed by centrifugation in a microfuge at 2000 rpm for 2 minutes. The flow-through of each was collected and stored as Wash 1 and Wash 2 in microfuge tubes.
8. The protein was eluted from the Ni-NTA column with the application of 200µl elution buffer followed by centrifugation in a microfuge at 2000 rpm for 2 minutes. The eluant was collected and stored in a microfuge as Elution 1. This step was repeated up to 3 times, and each subsequent eluant was stored as Elution 2, 3, *etc.*

Lysis Buffer
50 mM NaH_2PO_4
300 mM NaCl
10 mM imidazole, pH 8

Wash Buffer
50 mM NaH_2PO_4
300 mM NaCl
20 mM imidazole, pH 8

Elution Buffer
50 mM NaH_2PO_4
300 mM NaCl
250 mM imidazole, pH 8

REFERENCES

Abendroth, J., S. Chatterjee, and D. Schomburg. 2000a. Purification of a D-hydantoinase using a laboratory-scale Streamline phenyl column as the initial step. Journal of Biochemistry **737**:187-194.

Abendroth, J., K. Niefind, S. Chatterjee, and D. Schomburg . 2000b. Crystallisation, preliminary x-ray analysis of a native and selenomethionine D-hydantoinase from *Thermus* sp. Acta Crystallographica Section D: Biological Crystallography **D56**:1166-1169.

Abendroth, J., K. Niefind, O. May, M. Siemann, C. Syldatk, and D. Schomburg. 2002. The structure of L-hydantoinase from *Arthrobacter aurescen* leads to an understanding of dihydropyrimidinase substrate and enantio specificity. Biochemistry **41**:8589-8597.

Achary, A., K. Hariharan, S. Bandhyopadhyaya, R. Ramachandran, and K. Jayaraman. 1997. Application of numerical modeling for the development of optimised complex medium for D-hydantoinase production from *Agrobacterium radiobacter* NRRL B 11291. Biotechnology and Bioengineering **55**:148-154.

Acosta-Martinez, V. and M. Tabatabai. 2001. Arylamidase activity in soils: effect of trace elements and relationships to soil properties and activities of amidohydrolases. Soil Biology and Biochemistry **33**:17-23.

Akamatsu, N. 1960. Specificity of the hydantoinases, dihydropyrimidinases and decarbamoylases. Journal of Biochemistry **47 (Tokyo)**:809-819.

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

Altschul, S., W. Gish, W. Miller, E. Meyers, and D. Lipman . 1990. Basic local alignment search tool. Journal of Molecular Biology **215**:403-410.

Arcuri, M., O. Antunes, S. Sabino, G. Pinto, and E. Oestreicher. 2000. Resolution of DL-hydantoins by D-hydantoinase from *Vigna angularis*: Production of highly enantioenriched *N*-carbamoyl-D-phenylglycine at 100% conversion. Amino Acids **19**:477-482.

Arnold, F. and J. Moore. 1997. Optimising Industrial Enzymes by Directed Evolution. Advances in Biochemical Engineering/Biotechnology **58**:1-14.

Ausubel, F., R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl. 1983. Current Protocols in Molecular Biology. **Wiley Interscience**, New York.

Baneyx, F. 1999. Recombinant protein expression in *Escherichia coli*. Current opinions in Biotechnology **10**:411-421.

Batisse, N., P. Weigel, M. Lecocq, and V. Sakanyan. 1997. Two amino acid amidohydrolase genes encoding L-stereospecific carbamoylase and aminoacylase are organised in a common operon in *Bacillus stearothermophilus*. Applied and Environmental Microbiology **63**:763-766.

Bedouelle H and Duplay P. 1998. Production in Escherichia coli and one-step purification of bifunctional hybrid proteins which bind maltose. Export of the Klenow polymerase into the periplasmic space. European Journal of Biochemistry **171**:541-549.

Bernheim F and Bernheim M. 1946. The hydrolysis of hydantoin by various tissues. Journal of Biological Chemistry **163**:683-685.

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

Brooks, K., E. Jones, B. Kim, and E. Sander. 1983. Bovine liver dihydropyrimidine amidohydrolase: purification, properties, and characterization as a zinc metalloenzyme. Archives of Biochemistry and Biophysics **226** :469.

Bruggink, A., E. Roos, and E. de Vroom. 1998. Penicillin acylase in the industrial production of beta-lactam anitibiotics. Organic Process Research and Development **2**:128-133.

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

Bucherer, H. and W. Steiner. 1934. Über reactionene "-oxy-u."-amonitrile Synthese von Hydantoinen. Journal für praktische Chemie **140**:291-316.

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

Buson, A., A. Negro, L. Grassato, M. Tagliaro, M. Basaglia, C. Grandi, A. Fontana, and M. Nuti. 1996. Identification, sequencing and mutagenesis of the gene for a D-carbamoylase from *Agrobacterium radiobacter*. FEMS Microbiology Letters **145**:55-62.

Cecere, F., G. Galli, and F. Morisi. 1975. Substrate and steric specificity of hydropyrimidine hydrase. FEBS Letters **57**:192-194.

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

Chao, Y.-P., H. Fu, T.-E. Lo, P.-T. Chen, and J.-J. Wang. 1999b. One-step production of D-*p*-hydroxyphenylglycine by recombinant *Escherichia coli* strains. Biotechnological Progress **15**:1039-1045.

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

Chao, Y.-P., C.-J. Chiang, and P.-T. Chen. 2000b. Optimum ratio of D-carbamoylase to D-hydantoinase for maximising D-*p*-hydroxyphenylglycine productivity. Biotechnology Letters **22**:99-103.

Chen, C. Y. and S. A. Morse. 2001. Identification and characterization of a high-affinity zinc uptake system in Neisseria gonorrhoeae. FEMS Microbiology Letters **202**:67-71.

Chen, H.-Y. and H. Tsai. 1996. Cloning, sequencing and expression in *Escherichia coli* of D-hydantoinase gene from *Pseudomonas putida*. Annals New York Academy of Sciences234-237.

Chen, Y.-C., B.-D. Yin, S.-C. Lin, and W.-H. Hsu. 1999. Production of N-carbamoyl-D-hydroxyphenylglycine by D-hydantoinase activity of a recombinant *Escherichia coli*. Process Biochemistry **35**:285-290.

Cheon, Y.-H., G.-J. Kim, and H.-S. Kim. 2000. Stabilisation of D-hydantoinase by intersubunit cross-linking. Journal of Molecular Catalysis B: Enzymatic **11**:29-35.

Cheon, Y.-H., H.-S. Kim, K.-H. Han, J. Abendroth, K. Niefind, D. Schomburg, J. Wang, and Y. Kim. 2002. Crystal stuecture of D-Hydantoinase from *Bacillus stearothermophilus*: Insight into the stereochemistry and enantioselectivity. Biochemistry **41**:9410-9417.

Chevalier, P., D. Roy, and A. Morin. 1989. Hydantoinase activity of immobilised non-growing *Pseudomonas putida* cells. Applied Microbiology and Biotechnology **30**:482-486.

Chien, H.-R. and W.-H. Hsu. 1996. Rapid and sensitive detection of D-hydantoinase producing microorganisms by using microtiter plate assay. Biotechnology Techniques **10**:879-882.

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

Dagys, R., S. Zvirblis, V. Luksa, and A. Pauliukonis. 1992. Kinetics of deactivation of L-lysinamidase, L-aminoacylase and D-hydantoinase. Journal of Biotechnology **23**:241-256.

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

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

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

Drauz, K. and H. Wolman. 1995. Hydrolysis and the formation of hydantoins, p. 409-431. Enzyme Catalysis in Organic Synthesis. VCH, Weinheim.

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

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

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

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

Ellsworth, J. L., N. Hamacher, N. Bagwell, and J. W. West. 2000. Detection of Glu-Glu-tagged proteins in mammalian cell culture media by dot immunoblotting. Biotechniques **28**:934-6, 937.

Eng, B. H., M. L. Guerinot, D. Eide, and M. H. Saier, Jr. 1998. Sequence analyses and phylogenetic characterization of the ZIP family of metal ion transport proteins. Journal of Membrame Biology **166**:1-7.

Fan, C.-H., C.-K. Lee, and Y.-P. Chao. 2000. Recombinant *Escherichia coli* cell for D-p-hydroxyphenylglycine production from D-N-carbamoyl-p-hydroxyphenyl glycine. Enzyme and Microbial Technology **26**:222-228.

Fan, C.-H. and C.-K. Lee. 2001. Purification of D-hydantoinase from adzuki bean and its immobilisation for D-carbamoyl-D-phenylglycine production. Biochemical Engineering Journal **8**:157-164.

Finkbeiner, H. 1965. The carboxylation of hydantoins. Journal of Organic Chemistry **30**:3419.

Forrer, P. and R. Jaussi. 1998. High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage lambda head protein D. Gene **224**:45-52.

Fu, C., J. W. Olson, and R. J. Maier. 1995. HypB protein of Bradyrhizobium japonicum is a metal-binding GTPase capable of binding 18 divalent nickel ions per dimer. Proceedings of the National Academy of Science **92**:2333-2337.

Garcia, M. and R. Azerad. 1997. Production of ring-substituted D-phenylglycines by microbial or enzymatic hydrolysis/deracemisation of the corresponding DL-hydantoins. Tetrahedron: Asymmetry **8**:85-92.

George, S. and A. Sadhukhan. 1996. A simple and economically viable medium for the growth of *Agrobacterium radiobacter* for the production of D-amino acid. World Journal of Microbiology and Biotechnology **12**:657-659.

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

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

Griffantini, R., C. Pratesi, G. Galli, and C. Grandi. 1996. Topological mapping of the cysteine residues of N-carbamyl-D-amino acid amidohydrolase and their role in enzymatic activity. Journal of Biological Chemistry **271**:9326-9331.

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

Gross, C., C. Syldatk, V. Mackowiak, and F. Wagner. 1990. Production of L-tryptophan from D,L-5-indolylmethyhydantoin by resting cells of a mutant *Arthrobacter* species (DSM3747). Journal of Biotechnology **14**:363-376.

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

Guan, K. L. and J. E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Analytical Biochemistry**192**:262-267.

Guerinot, M. L. 2000. The ZIP family of metal transporters. Biochim. Biophys Acta **1465**:190-198.

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

Hannig, G. and S. Makrides. 1998. Strategies for optimising heterologous protein expression in *Escherichia coli*. TIBTECH **16**:54-60.

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

Hartley, C. Elucidation and manipulation of the hydantoin hydrolysing enzyme system of *Agrobacterium tumefaciens* RU-OR for the biocatalytic production of D-amino acids. 1-242. 2001. Rhodes University. Thesis/Dissertation

Hartley, C., F. Manford, S. Burton, and G. Douglas. 2001. Over production of hydantoinase and N-carbamoylamino acid amidohydrolase enzymes by regulatory mutants of *Agrobacterium tumefaciens*. Appl. Microbiol. Biotechnol. **57**:43-49.

Hassall, H. and D. Greenberg. 1963. The bacterial metabolism of L-hydantoin-5-propionic acid to carbamylglutamic acid and glutamic acid. Journal of Biological Chemistry **238**:3325-3329.

Hernan, R., K. Heuermann, and B. Brizzard. 2000. Multiple epitope tagging of expressed proteins for enhanced detection. Biotechniques **28**:789-793.

Hils, M., P. Munch, J. Altenbuchner, C. Syldatk, and R. Mattes. 2001. Cloning and characterisation of genes from *Agrobacterium* sp. IP I-671 involved in hydantoin degradation. Appl. Microbiol. Biotechnol. **57**:680-688.

Hochuli, E., H. Dobeli, and A. Schacher. 1987. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. Journal of Chromatography **411**:177-184.

Hockney, R. 1994. Recent developments in heterologous protein production in *Escherichia coli*. TIBTECH **12**:456-463.

Hottenrott, S., T. Schumann, A. Pluckthun, G. Fischer, and J. U. Rahfeld. 1997. The *Escherichia coli* SlyD is a metal ion-regulated peptidyl-prolyl cis/trans-isomerase. Journal of Biological Chemistry **272**:15697-15701.

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

Idicula, A. M., G. L. Blatch, T. G. Cooper, and R. A. Dorrington. 2002. Binding and activation by the zinc cluster transcription factors of *Saccharomyces cerevisiae*. Redefining the UASGABA and its interaction with Uga3p. Journal of Biological Chemistry **277**:45977-45983.

Ikenaka, Y., H. Nanba, K. Yajima, Y. Yamada, M. Takano, and S. Takahashi. 1998c. Relationship between an increase in thermostability and amino acid substitutions in *N*-carbamyl-D-amino acid amidohydrolase. Bioscience Biotechnology and Biochemistry **62**:1672-1675.

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

Ikenaka, Y., H. Nanba, K. Yajima, Y. Yamada, M. Takano, and S. Takahashi. 1998b. Increase in thermostability of *N*-carbamyl-D-amino acid amidohydrolase on amino acid substitutions. Bioscience Biotechnology and Biochemistry **62**:1668-1671.

Ikenaka, Y., H. Nanba, K. Yajima, Y. Yamada, M. Takano, and S. Takahashi. 1999. Thermostability reinforcement through a combination of thermostability related mutations of *N*-carbamyl-D-amino acid amidohydrolase. Bioscience Biotechnology and Biochemistry **63**:91-95.

Ishikawa, H., H. Yamanaka, T. Kawamoto, and A. Tanaka. 1999. Enzymatic synthesis of silicon-containing dipeptides with 3-trimethyl silylalanine. Applied Microbiology and Biotechnology**1999**:470-473.

Ishikawa, T., K. Watabe, Y. Mukohara, S. Kobayashi, and H. Nakamura. 1993. Microbial conversion of DL-5-substituted hydantoins to the corresponding L-amino acids by *Pseudomonas* sp. Strain NS671. Bioscience Biotechnology and Biochemistry **57**:982-986.

Ishikawa, T., Y. Mukohara, K. Watabe, S. Kobayashi, and H. Nakamura. 1994. Microbial conversion of DL-5-substituted hydantoins to the corresponding L-amino acids by *Bacillus stearothermophilus* NS1122A. Biosci. Biotechnol. Biochem. **58**:265-270.

Ishikawa, T., K. Watabe, Y. Mukohara, and H. Nakamura. 1996. N-carbamyl-L-amino acid amidohydrolase of *Pseudomonas* sp. Strain NS67: Purification and some properties of the enzyme expressed in *Escherichia coli*. Bioscience Biotechnology and Biochemistry **60**:612-615.

Ishikawa, T., K. Watabe, Y. Mukohara, and H. Nakamura. 1997. Mechanism of sterospecific conversion of DL-5-substituted hydantoins to the corresponding L-amino acids by *Pseudomonas* sp. Strain NS671. Bioscience Biotechnology and Biochemistry **61**:185-187.

Janes, B. and R. Bender. 1998. Alanine catabolism in *Klebsiella aerogenes*: molecular characterisation of the *dadAB* operon ands its regulation by the nitrogen assimilation control protein. Journal of Bacteriology **180**:563-570.

Kamitani, S., Y. Akiyama, and K. Ito. 1992. Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. The EMBO Journal **11**:57-62.

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

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

Keil, O., M. Schneider, and J. Rasor. 1995. New hydantoinases from thermophilic micro-organisms-synthesis of enatiomerically pure D-amino acids. Tetrahedron: Asymmetry **6**:1257-1260.

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

Kim, D.-M., G.-J. Kim, and H.-S. Kim. 1994. Enhancement of operational stability of immobilised whole cell D-hydantoinase. Biotechnology Letters **16**:11-16.

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

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

Kim, G.-J. and H.-S. Kim. 1995b. Optimisation of the enzymatic synthesis of D-*p*-hydroxyphenylglycine from D, L-5-substituted hydantoin using D-hydantoinase and N-carbamoylase. Enzyme and Microbial Technology **17**:63-67.

Kim, G.-J., J.-H. Park, D.-C. Lee, H.-S. Ro, and H.-S. Kim . 1997b. Primary structure, sequence analysis, and expression of the thermostable D-hydantoinase from *Bacillus stearothermophilus* SD1. Mol. Gen. Genet. **255**:152-156.

Kim, G.-J., S.-G. Lee, J.-H. Park, and H.-S. Kim. 1997a. Direct detection of the hydantoinase activity on solid agar plates and electrophoretic acrylamide gels. Biotechnology Techniques **11**:511-513.

Kim, G.-J. and H.-S. Kim. 1998a. C-terminal regions of D-hydantoinases are nonessential for catalysis, but affest the oligomeric structure. Biochemical and Biophysical Research Communications **243** :96-100.

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

Kim, G.-J., J.-H. Park, D.-C. Lee, and H.-S. Kim. 1999. Engineering the thermostable D-hydantoinases from two thermophilic *Bacilli* based on their primary structures. Annals New York Academy of Sciences **864**:332-336.

Kim, G.-J., D.-E. Lee, and H.-S. Kim. 2000c. Functional expression and characterisation of the two cyclic amidohydrolase enzymes, allantoinase and a novel phenylhydantoinase, from *Escherichia coli*. Journal of Bacteriology **182**:7021-7028.

Kim, G.-J., Y.-H. Cheon, and H.-S. Kim. 2000a. Directed evolution of a novel N-cabamylase/D-hydanoinase fusion enzyme for functional expression with enhanced stability. Biotechnology and Bioengineering **68**:211-217.

Kim, G.-J., D.-E. Lee, and H.-S. Kim. 2000b. Construction and evaluation of a novel bifunctional N-carbamylase -D-hydantoinase fusion enzyme. Applied and Environmental Microbiology **66**:2133-2138.

Kim, G.-J., D.-E. Lee, and H.-S. Kim. 2001. High level expression and one step purification of cyclic amidohydrolase family enzymes. Protein Expression and Purification **23**:128-133.

Kim, J.-M., S. Shimizu, and H. Yamada. 1987. Amidohydrolysis of N-methylhydantoin coupled with ATP hydrolysis. Biochemical and Biophysical Research Communications **142**:1006-1012.

Krix, G., A. Bommarius, W. Hummel, and M.-R. Kula. 1997. Enzymatic reduction of α-keto acids leading to L-amino acids or D-hydroxy acids. Journal of Biotechnology **53**:29-39.

LaPointe, G., S. Viau, D. LeBlanc, N. Robert, and A. Morin . 1994. Cloning, sequencing and expression in *Escherichia coli* of the D-hydantoinase gene from *Pseudomonas putida* and distribution of homologous genes in other microorganisms. Applied and Environmental Microbiology **60**:888-895.

LaPointe, G., D. LeBlanc, and A. Morin. 1995. Use of polymerase chain reaction amplified DNA probe from *Pseudomonas putida* to detect D-hydantoinase producing microorganisms by direct colony hybridisation. Applied and Environmental Microbiology **42**:895-900.

Lee, C.-K. and K.-C. Lin. 1996. N-carbamoyl-D-p-hydroxyphenylglycine production using immobilised D-hydantoinase from recombinant *E. coli*. Enzyme and Microbial Technology **19**:623-627.

Lee, C.-K. and C.-H. Fan. 1999. Enzymatic synthesis and subsequent racemisation rates determination of optically active D-5-phenylhydantoin and D-5-hydroxyphenylhydantoin. Enzyme and Microbial Technology **24**:659-666.

Lee, C.-K., C.-H. Fan, and P.-F. Yang. 2001b. Modelling and simulation of a pressure swing reactor for the conversion of poorly soluble substrate by immobilised enzyme: the case of D-hydantoinase reaction. Biochemical Engineering Journal **7**:233-239.

Lee, C.-K., Z.-S. Lee, and P.-F. Yang. 2001a. Effect of cell membrane of *Agrobacterium radiobacter* on enhancing D-amino acid production from racemic hydantoins. Enzyme and Microbial Technology **28**:806-814.

Lee, D.-C., S.-G. Lee, and H.-S. Kim. 1996a. Production of D-*p*-hydroxyphenylglycine from D, L-5-(4-hydroxyphenyl) hydantoin using immobilised thermostable D-hydantoinase from *Bacillus stearothermophilus* SD-1. Enzyme and Microbial Technology **18**:35-40.

Lee, D.-C., S.-G. Lee, S.-P. Hong, M.-H. Sung, and H.-S. Kim. 1996b. Cloning and overexpression of thermostable D-hydantoinase from thermophile in *E. coli* and its application to the synthesis of optically active D-amino acids. Annals New York Academy of Sciences **799**:401-405.

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

Lee, D.-C., G.-J. Kim, Y.-K. Cha, C.-Y. Lee, and H.-S. Kim . 1997a. Mass production of thermostable D-hydantoinase by batch culture of recombinant *Escherichia coli* with a constitutive expression system. Biotechnology and Bioengineering **56**:449-455.

Lee, D.-C. and H.-S. Kim. 1998. Optimisation of a heterogeneous reaction system for the production of optically active D-amino acids using thermostable D-hydantoinases. Biotechnology and Bioengineering **60**:729-738.

Lee, D.-C., J.-H. Park, G.-J. Kim, and H.-S. Kim. 1999. Modelling, simulation and kinetic analysis of a heterogeneous reaction system for the enzymatic conversion of poorly soluble substrate. Biotechnology and Bioengineering **64**:272-283.

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

Lee, S.-G., D.-C. Lee, S.-P. Hong, M.-H. Sung, and H.-S. Kim. 1995. Thermostable D-hydantoinase from thermophilic *Bacillus stearothermophilus* SD-1: characteristics of purified enzyme. Applied Microbiology and Biotechnology **43**:270-276.

Lobocka, M., J. Hennig, J. Wild, and T. Klopotowski. 1994. Organisation and expression of the *E. coli* K12 *dad* operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase. Journal of Bacteriology**176**:1510.

Louwrier, A. and C. Knowles. 1996. The purification and characterisation of a novel D(-)-specific carbamoylase enzyme from an *Agrobacterium* sp. Enzyme and Microbial Technology **19**:562-571.

Louwrier, A. and C. Knowles. 1997. The aim of industrial enzymic amoxycillin production: characterisation of a novel carbamoylase enzyme in the form of a crude, cell-free extract. Biotechnology and Applied Biochemistry **25**:143-149.

Luksa, V., V. Starkuviene, B. Starkuviene, and R. Dagys. 1997. Purification and characterisation of the D-hydantoinase from *Bacillus circulans*. Applied Biochemistry and Biotechnology **62**:219-231.

Lyi, S. and S. Winans. 1999. Mannopinic acid and agropinnic acid catabolism region of the octopine-type Ti plasmid pTi5955. Mol Microbiol **31**:347.

May, O., M. Siemann, C. Syldatk, K. Niefind, and D. Schomburg. 1996. Crystallisation and preliminary X-ray analysis of a hydantoinase from *Arthrobacter aurescens* DSM3745. Acta Crystallographica Section D: Biological Crystallography **D52**:1209-1210.

May, O, Siemann, M, Pietzsch, M, and Syldatk, C. Hydantoinase of *Arthrobacter aurescens* DSM3745: A new member of the EC 3.5.2 group of cyclic amidases. PII-157 from Biotech 1998 Conference, France , 230. 1998e.

May O, Siemann M, Siemann MG, Syldatk C. 1998a. Catalytic and structural function of zinc for the hydantoinase from *Arthrobacter aurescens* DSM 3745. Journal of Molecular Catalysis E: Enzymatic **4:** 211-218.

May O, Siemann M, Siemann MG, Syldatk C. 1998b. The hydantoin amidohydrolase from *Arthrobacter aurescens* DSM 3745 is a zinc metalloenzyme. Journal of Molecular Catalysis E: Enzymatic **5:** 367-370.

May, O., M. Siemann, M. Pietzsch, M. Kiess, R. Mattes, and C. Syldatk. 1998c. Substrate dependent enantioselectivity of a novel hydantoinase from *Arthrobacter aurescens* DSM3745: Purification and characterisation as a new member of cyclic amidases. Journal of Biotechnology **61**:1-13.

May, O., A. Habenicht, R. Mattes, C. Syldatk, and M. Siemann. 1998d. Molecular evolution of hydantoinases. Biological Chemistry **379**:743-747.

May, O., P. Nguyen, and F. Arnold. 2000. Inverting enantioselectivity by directed evolution of hydantoinase for improves production of L-methionine. Nature Biotechnology **18**:317-320.

Meyer, P. and S. Runser. 1993. Efficient production of the industrial biocatalysts hydantoinase and N-carbamyl amino acid amidohydrolase: Novel non-metabolisable inducers. FEMS Microbiological Letters **109**:67-74.

Miroux, B. and J. E. Walker. 1996. Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. Journal of Molecular Biology **260**:289-298.

Moller, A., C. Syldatk, M. Schulze, and F. Wagner. 1988. Stereo- and substrate-specificity of a D-hydantoinase and a D-N-carbamyl amino acid amidohydrolase of *Arthrobacter crystallopoietes* AM2. Enzyme and Microbial Technology **10**:618-625.

Morin, A., W. Hummel, and M.-R. Kula. 1986a. Rapid detection of microbial hydantoinase on solid medium. Biotechnology Letters **8**:573-576.

Morin, A., W. Hummel, H. Schutte, and M.-R. Kula. 1986b. Characterisation of hydantoinase from *Pseudomonas fluorescens* strain DSM84. Biotechnology and Applied Biochemistry **8**:564-574.

Morin, A., W. Hummel, and M.-R. Kula. 1987. Enrichment and selection of hydantoinase producing microorganisms. Journal of General Microbiology **133**:1201-1207.

Morin, A., D. LeBlanc, A. Paleczek, W. Hummel, and M.-R. Kula. 1990. Comparison of seven microbial D-hydantoinases. Journal of Biotechnology **16**:37-48.

Morin, A. and A. Lafond. 1992. Continuous production of N-carbamyl-D-alanine by *Peptococcus anaerobius* adsorbed on activated charcoal. Biotechnology Letters **14**:117-118.

Morin, A. 1993. Use of a D-hydantoinase extracted from legumes to produce *N*-carbamyl D-amino acids. Enzyme and Microbial Technology **15**:208-214.

Morin, A., N.-H. Tran Trung, G. LaPointe, and H. Dubeau. 1995a. Conditions used with a continuous cultivation system to screen for D-hydantoinase producing microorganism. Applied Microbiology and Biotechnology **43**:259-266.

Morin, A., E. Poitras, C. Moresoli, and F. Brion. 1995b. Extraction of cyclic amide amidohydrolase from green hulls of *Pisum sativum* and its use as a biocatalyst for N-carbamyl amino acids. Bioresource Technology **53**:31-37.

Mukohara, Y., T. Ishikawa, K. Watabe, and H. Nakamura. 1993. Molecular cloning and sequencing of the gene for a thermostable N-carbamyl-L-amino acid amidohydrolase from *Bacillus stearothermophilus* strain NS1122A. Bioscience Biotechnology and Biochemistry **57**:1935-1937.

Mukohara, Y., T. Ishikawa, K. Watabe, and H. Nakamura. 1994. A thermostable hydantoinase of *Bacillus stearothermophilus* NS1122A: Cloning, sequencing, and high expression of the enzyme gene, and some properties of the expressed enzyme. Bioscience Biotechnology and Biochemistry **58**:1621-1626.

Nakai, T., T. Hasegawa, E. Yamashita, M. Yamamoto, T. Kumasaka, T. Ueki, H. Nanba, Y. Ikenaka, S. Takahashi, M. Sato, and T. Tsukihara. 2000. Crystal structure of N-carbamyl D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases. Structure **8**:729-739.

Nanba, H., Y. Ikenaka, H. Yamada, K. Yajima, M. Takano, and S. Takahashi. 1998a. Isolation of *Agrobacterium* sp. strain KNK712 that produces N-carbamyl-D-amino acid amidohydrolase, cloning of the gene for this enzyme, and properties of the enzyme. Bioscience Biotechnology and Biochemistry **62**:875-881.

Nanba, H., Y. Ikenaka, Y. Yamada, K. Yajima, M. Takano, K. Ohkubo, Y. Hiraishi, K. Yamada, and S. Takahashi. 1998b. Immobilisation of N-carbamyl-D-amino acid amidohydrolase. Bioscience Biotechnology and Biochemistry **62**:1839-1844.

Nanba, H., Y. Ikenaka, Y. Yamada, K. Yajima, M. Takano, K. Ohkubo, Y. Hiraishi, K. Yamada, and S. Takahashi. 1999. Immobilisation of thermotolerant N-carbamyl-D-amino acid amidohydrolase. Journal of Molecular Catalysis B: Enzymatic **6**:257-263.

Nygaard, F. B. and K. W. Harlow. 2001. Heterologous expression of soluble, active proteins in *Escherichia coli*: the human estrogen receptor hormone-binding domain as paradigm. Protein Expression and Purification **21**:500-509.

Ogawa, J., S. Shimizu, and H. Yamada. 1993. N-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. E222c purification and characterisation. European Journal of Biochemistry **212**:684-691.

Ogawa, J., M. C.-M. Chung, S. Hida, H. Yamada, and S. Shimizu. 1994b. Thermostable N-carbamoyl-D-amino acid amidohydrolase: screening, purification and characterisation. Journal of Biotechnology **38**:11-19.

Ogawa, J. and S. Shimizu. 1994. B–ureidopropionase with N-carbamyl-α-L-amino acid amidohydrolase activity from an aerobic bacterium, *Pseudomonas putida* IFO 12996. European Journal of Biochemistry**223**:625-630.

Ogawa, J., T. Kaimura, H. Yamada, and S. Shimizu. 1994a. Evaluation of pyrimidine and hydantoin degrading enzyme activities in aerobic bacteria. FEMS Microbiological Letters **122**:55-60.

Ogawa, J., H. Miyake, and S. Shimizu. 1995b. Purification and characterisation of N-carbamoyl-L-amino acid amidohydrolase with broad substrate specificity from *Alcaligenes xylosoxidans*. Applied Microbiology and Biotechnology **43**:1039-1043.

Ogawa, J., M. Honda, C.-L. Soong, and S. Shimizu. 1995c. Diversity of cyclic ureide compound-, dihydropyrimidine-, and hydantoin-hydrolysing enzymes in *Blastobacter* sp. A17p-4. Bioscience Biotechnology and Biochemistry **59**:1960-1962.

Ogawa, J., J. Min Kim, W. Nirdnoy, Y. Amano, H. Yamada, and S. Shimizu. 1995a. Purification and characterisation of an ATP-dependent amidohydrolase, N-methylhydantoin amidohydrolase, from *Pseudomonas putida* 77. European Journal of Biochemistry**229**:684-691.

Ogawa, J. and S. Shimizu. 1997. Diversity and versatility of microbial hydantoin transforming enzymes. Journal of Molecular Catalysis B: Enzymatic **2**:163-176.

Ogawa, J., C.-L. Soong, M. Honda, and S. Shimizu. 1997. Imidase, a dihydropyrimidinase-like enzyme involved in the metabolism of cyclic imides. European Journal of Biochemistry**243**:322-327.

Ogawa, J. and S. Shimizu. 1999. Microbial enzymes: new industrial applications from traditional screening methods. Trends in Biotechnology. **17**:13-21.

Ogawa, J., A. Ryouno, S.-X. Xie, R. Vohra, R. Indrati, H. Miyakawa, T. Ueno, and S. Shimizu. 1999a. Simultaneous recognition of two chiral centres in α, β–diastereomeric amino acids by an N-carbamoyl-L-α-amino acid amidohydrolase from *Alcaligenes xylosoxidans*. Biotechnology Letters **21** :711-713.

Ogawa, J., A. Ryono, S.-X. Xie, R. Vohra, R. Indrati, H. Miyakawa, T. Ueno, Y. Ikenaka, H. Nanba, S. Takahashi, and S. Shimizu. 1999b. β-carbon stereoselectivity of N-carbamoyl-D-α–amino acid amidohydrolase for α, β–diastereomeric amino acids. Applied Microbioliology and Biotechnology **52**:797-801.

Ogawa J, Ryono A, Xie S-X, Vohra RM, Indrati R, Akamatsu M, Miyagawa H, Ueno T, Shimizu S. 2001. Separative preparation of the four stereoisomers of β-methylphenylalanine with *N*-carbamoyl amino acid amidohydrolases. Journal of Molecular Catalysis B: Enzymatic **12**: 71-75

Oh, K.-H., S.-H. Nam, and H.-S. Kim. 2002b. Improvement of oxidative and thermostability of *N*-carbamyl-D-amino acid amidohydrolase by directed evolution. Protein Expression and Purification **15**:689-695.

Oh, K.-H., S.-H. Nam, and H.-S. Kim. 2002a. Directed evolution of *N*-carbamyl-D-amino acid amidohydrolase for simultaneous improvement of oxidative and thermal stability. Biotechnology Progress **18**:417.

Olivieri, R., E. Fascetti, L. Angelini, and L. Degen. 1979. Enzymatic conversion of N-carbamoyl-D-amino acids to D-amino acids. Enzyme and Microbial Technology **1**:201-204.

Olivieri, R., E. Fascetti, L. Angelini, and L. Degen. 1981. Microbial transformation of racemic hydantoins to D-amino acids. Biotechnology and Bioengineering **23**:2173-2183.

Ostermeier, M., K. De Sutter, and G. Georgiou. 1996. Eukaryotic protein disulfide isomerase complements *Escherichia coli dsbA* mutants and increases the yield of a heterologous secreted protein with disulfide bonds. Journal of Biological Chemistry **271**:10616-10622.

Ottenheym, H. and P. Jenneskens. 1970. Synthetic amino acids and their use in fortifying foods. Journal of Agricultural and Food Chemistry **18**:1010-1014.

Park, J.-H., G.-J. Kim, S.-G. Lee, and H.-S. Kim. 1998. Biochemical properties of thermostable D-hydantoinase from *Bacillus thermocatenulatus* GH-2. Annals New York Academy of Sciences **864**:337-340.

Park, J.-H., G.-J. Kim, and H.-S. Kim. 2000. Production of D-amino acid using whole cells of recombinant *Escherichia coli* with separately and coexpressed D-hydantoinase and N-carbamoylase. Biotechnological Progress **16**:564-570.

Pietzsch, M., A. Wiese, K. Ragnitz, B. Wilms, J. Altenbuchner, R. Mattes, and C. Syldatk. 2000. Purification of recombinant hydantoinase and L-N-carbamoylase from *Arthrobacter aurescens* expressed in *Escherichia coli:* comparison of wild type and genetically modified protein. Journal of Chromatography B **737** :179-186.

Plummer, D. T. 1987. The quantitative estimation of amino acids using the ninhydrin reaction, p.158. *In* Plummer, D. T. (Ed.) ***Practical Biochemistry*** 3rd Edition.-1987. Ch. 8. Wiley-Interscience, New York.

Prasher, D. C., V. K. Eckenrode, W. W. Ward, F. G. Prendergast, and M. J. Cormier. 1992. Primary structure of the Aequorea victoria green-fluorescent protein. Gene **111**:229-233.

Pryor, K. D. and B. Leiting. 1997. High-level expression of soluble protein in *Escherichia coli* using a His6-tag and maltose-binding-protein double-affinity fusion system. Protein Expression and Purification **10**:309-319.

Ragnitz, K., C. Syldatk, and M. Pietzsch. 2001b. Optimisation of the immobilisation parameters and operational stability of immobilised hydantoinase and L-N-carbamoylase from *Arthrobacter aurescens* for the production of optically pure L-amino acids. Enzyme and Microbial Technology **28**:713-720.

Ragnitz, K., M. Pietzsch, and C. Syldatk. 2001a. Immobilisation of the hydantoin cleaving enzymes from *Arthrobacter aurescens* DSM 3747. Journal of Biotechnology **92**:179-186.

Rosazza, J. 1995. Biocatalysis, Microbiology and Chemistry: The power of positive linking. ASM News **61**:241-245.

Runser, S. and E. Ohleyer. 1990. Properties of the hydantoinase from *Agrobacterium* sp. IP I-671. Biotechnology Letters **12**:259-264.

Runser, S., N. Chinski, and E. Ohleyer. 1990. D-*p*-hydroxyphenylglycine production from DL-5-*p*-hydroxyphenylhydantion by *Agrobacterium* sp. Applied Microbiology and Biotechnology **33**:382-388.

Runser, S. and P. Meyer. 1993. Purification and biochemical characterisation of the hydantoin hydrolysing enzyme from *Agrobacterium species*: A hydantoinase with no 5,6-dihydropyrimidine amidohydrolase activity. European Journal of Biochemistry **213**:1315-1324.

Rushton, P. and I. Somssich. 1998. Transcriptional control of plant genes responsive to pathogens. Current Opinion in Plant Biology **1**:311-315.

Sambrook, J., A. Margolin, A. Russell, and A. Klibanov. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour, USA.

Sano, K., K. Yokozeki, C. Eguchi, T. Kagawa, I. Noda, and K. Mitsugi. 1976. Enzymatic production of L-tryptophan from L- and DL-5-indolyl-methylhydantoin by newly isolated bacterium. Agricultural Biological Chemistry **41**:819-825.

Sareen, D., R. Sharma, H. Nandanwar, and R. Vohra. 2001a. Two-step purification of a D(-)-specific carbamoylase from *Agrobacterium tumefaciens* AM10. Protein Expression and Purification **21**:170-175.

Sareen, D., R. Sharma, and R. Vohra. 2001b. Chaperone assisted over-expression of an active D-carbamoylase from *Agrobacterium tumefaciens* AM10. Protein Expression and Purification **23**:374-379.

Schulze, B. and M. Wubbolts. 1999. Biocatalysis for industrial production of fine chemicals. Current opinion in Biotechnology **10**:609-615.

Sharma, R. and R. Vohra. 1997. A thermostable D-hydantoinase isolated from a mesophilic *Bacillus* sp. AR9. Biochemical and Biophysical Research Communications **234**:485-488.

Shimizu, S., H. Shimada, S. Takahashi, T. Ohashi, T. Tani, and H. Yamada. 1980. Synthesis of *N*-carbamyl-D-2-thienylglycine and D-2-thienylglycine by microbial hydantoinase. Agric Biol Chem **44**:2233-2234.

Siemann, M., Syldatk, C. and F. Wagner. 1993. Detection and comparison of strains with selective L-hydantoin cleaving activity using polyclonal antibodies. *Biotechnol. Techniques* **7 (5)**: 361-366.

Siemann, M., Syldatk, C. and F. Wagner. 1994. Enhanced stability of an L-hydantoinase mediated by its corresponding polyclonal antibody. *Biotechnol. Letters* **16 (4)**: 349-354.

Siemann, M., A. Alvarado-Marin, M. Pietzsch, and C. Syldatk. 1999. A D-specific hydantoin amidohydrolase: properties of the metalloenzyme purified from *Arthrobacter crystallopoietes*. Journal of Molecular Catalysis B: Enzymatic **6**:387-397.

Smith, D. B. and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67**:31-40.

Sonke, T., B. Kaptein, W. Boesten, Q. Broxterman, J. Kamphuis, F. Formaggio, C. Toniolo, F. Rutjies, and H. Schoemaker. 1999. Amino acid amidase catalysed preparation and further transformations of enantiopure alpha-amino acids., p. 23-58. *In* J. Strubenrauch (ed.), Stereoselective Biocatalysis. Marcel Decker Inc, New York.

Studier, F. W. and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Journal of Molecular Biology **189**:113-130.

Sudge, S., K. Bastawde, D. Gokhale, U. Kalkote, and T. Ravindranathan. 1998. Production of D-hydantoinase by halophilic *Pseudomonas* sp. NCIM5109. Applied Microbioliology and Biotechnology **49**:594-599.

Surek, B., M. Wilhelm, and W. Hillen. 1991. Optimizing the promoter and ribosome binding sequence for expression of human single chain urokinase-like plasminogen activator in *Escherichia coli* and stabilization of the product by avoiding heat shock response. Applied Microbiology and Biotechnology**34**:488-494.

Suzuki, A. and T. Endo. 2002. Ermelin, an endoplasmic reticulum transmembrane protein, contains the novel HELP domain conserved in eukaryotes. Gene **284**:31-40.

Syldatk, C., D. Cotoras, A. Moller, and F. Wagner. 1986. Microbial, enantioselectove hydrolysis of D, L-5-monosubstituted hydantoins for the production of D and L amino acids. Biotechnology Forum **3**:9-15.

Syldatk, C., D. Cotoras, G. Dombach, C. Groß, H. Kallwaß, and F. Wagner. 1987. Substrate and stereospecificity, induction and metallodependence of a microbial hydantoinase. Biotechnology Letters **9**:25-30.

Syldatk, C., A. Laufer, R. Muller, and H. Hoke. 1990a. Production of optically pure D- and L-α-amino acids by bioconversion of D, L-5-monosubstituted hydantoin derivatives. Advances in Biochemical Engineering and Biotechnology **41**:29-75.

Syldatk, C., V. Mackowiak, H. Hoke, C. Groß, G. Dombach, and F. Wagner. 1990b. Cell growth and enzyme synthesis of a mutant of *Arthrobacter* sp. (DSM3747) used for the production of L-amino acids from D,L-5-monosubstituted hydantoins. Journal of Biotechnology **14**:345-362.

Syldatk, C., R. Muller, M. Siemann, K. Krohn, and F. Wagner. 1992. Microbial and enzymatic production of D-amino acids from D, L-5-monosubstituted hydantoins, p. 75-127. *In* D. Rozzell and F. Wagner (eds.), Biocatalytic production of amino acids and derivatives. Hanser.

Syldatk, C., O. May, J. Altenbuchner, R. Mattes, and M. Siemann. 1999. Microbial hydantoinases-industrial enzymes from the origin of life? Applied Microbioliology and Biotechnology **51**:293-309.

Tabor, S. 1983. Expression using the T7 RNA polymerase promoter system, p. 16.2.1-16.2.11. *In* F. e. al. Ausubel (ed.), Current Protocols in Molecular Biology. Wiley Interscience, New York.

Takahashi, S., Y. Kii, H. Kumagai, and H. Yamada. 1978. Purification, crystallisation and properties of hydantoinase from *Pseudomonas striata*. Journal of Fermentation Technology **56**:492-498.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research **22**:4637-4680.

Tramper, J. 1996. Chemical versus biochemical conversion: When and how to use biocatalysts. Biotechnology and Bioengineering **52**:290-295.

Vanguri, V. K., S. Wang, S. Godyna, S. Ranganathan, and G. Liau. 2000. Thrombospondin-1 binds to polyhistidine with high affinity and specificity. Biochem J **347**:469-473.

Volkel, D. and F. Wagner. 1995. Reaction mechanism for the conversion of 5-monosubstituted hydantoins to enantiomerically pure L-amino acids. Annals New York Academy of Sciences **750**:1-9.

Wagner, T., B. Hantke, and F. Wagner. 1996. Production of L-methionine from D, L-5-(2-methylthioethyl)hydantoin by resting cells of a new mutant strain of *Arthrobacter* species DSM7330. Journal of Biotechnology **46**:63-68.

Wallach, D. and S. Grisolia. 1957. The purification and properties of hydropyrimidine hydrase. Journal of Biological Chemistry **226**:277-288.

Wang, W.-C., W.-H. Hsu, F.-T. Chien, and C.-Y. Chen. 2001. Crystal structure and site directed mutagenesis studies of N-carbamoyl-D-amino acid amidohydrolase from *Agrobacterium radiobacter* reveals a homotetramer and insight into a catalytic cleft. Journal of Molecular Biology **306**:251-261.

Wang, Y., A. Mukhopadhyay, V. Howitz, A. Binns, and D. Lynn. 2000. Construction of an efficient expression system for *Agrobacterium tumefaciens* based on the coliphage T5 promoter. Gene **242**:105-114.

Wang, Y., K. Yakovlevsky, B. Zhang, and A. Margolin. 1997. Cross-linked crystals of subtilisin-versatile catalyst for organic synthesis. Journal of Organic Chemistry**62**:3488-3495.

Watabe, K., T. Ishikawa, Y. Mukohara, and H. Nakamura. 1992c. Purification and characterisation of the hydantoin racemase of *Pseudomonas* sp. strain NS671 expressed in *Escherichia coli*. Journal of Bacteriology **174**:7989-7995.

Watabe, K., T. Ishikawa, Y. Mukohara, and H. Nakamura. 1992a. Cloning and sequencing of the genes involved in the conversion of 5-substituted hydantoins to the corresponding L-amino acids from the native plasmid of *Pseudomonas* sp. strain NS671. Journal of Bacteriology **174**:962-969.

Watabe, K., T. Ishikawa, Y. Mukohara, and H. Nakamura. 1992b. Identification and sequencing of a gene encoding a hydantoin racemase from the native plasmid of *Pseudomonas* sp. strain NS671. Journal of Bacteriology **174**:3461-3466.

Weickert, M., D. Doherty, E. Best, and P. Olins. 1996. Optimisation of heterologous protein production in *Escherichia coli*. Current opinion in Biotechnology **7**:494-499.

Wiese, A., M. Pietzsch, C. Syldatk, R. Mattes, and J. Altenbuchner. 2000. Hydantoin racemase from *Arthrobacter aurescens* DSM 3747: heterologous expression, purification and characterisation. Journal of Biotechnology **80**:217-230.

Wiese, A., B. Wilms, C. Syldatk, R. Mattes, and J. Altenbuchner. 2001b. Cloning, nucleotide sequence and expression of a hydantoinase and carbamoylase gene from *Arthrobacter aurescens* DSM 3745 in *Escherichia coli* and comparison with the corresponding genes from *Arthrobacter aurescens* DSM 3747. Applied Microbioliology and Biotechnology **55**:750-757.

Wiese, A., C. Syldatk, R. Mattes, and J. Altenbuchner. 2001a. Organisation of genes responsible for the stereospecific conversion of hydantoins to α amino acids in *Arthrobacter aurescens* DSM 3747. Archives of Microbiology **176**:187-196.

Wilms, B., A. Wiese, C. Syldatk, R. Mattes, J. Altenbuchner, and M. Pietzsch. 1999. Cloning, nucleotide sequence and expression of a new L-N-carbamoylase gene from *Arthrobacter aurescens* DSM3747 in *E. coli*. Journal of Biotechnology **68**:101-113.

Wilms, B., A. Wiese, C. Syldatk, R. Mattes, and J. Altenbuchner. 2001a. Development of an *Escherichia coli* whole cell biocatalyt for the production of L-amino acids. Journal of Biotechnology **86**:19-30.

Wilms, B., A. Hauck, M. Reuss, C. Syldatk, R. Mattes, M. Siemann, and J. Altenbuchner. 2001b. High cell density fermentation for production of L-N-carbamoylase using an expression system based on the *Escherichia coli rhaBAD* promoter. Biotechnology and Bioengineering **73**:95-103.

Wood, D. W., J. C. Setubal, R. Kaul, D. E. Monks, J. P. Kitajima, V. K. Okura, Y. Zhou, L. Chen, G. E. Wood, N. F. Almeida, Jr., L. Woo, Y. Chen, I. T. Paulsen, J. A. Eisen, P. D. Karp, D. Bovee, Sr., P. Chapman, J. Clendenning, G. Deatherage, W. Gillet, C. Grant, T. Kutyavin, R. Levy, M. J. Li, E. McClelland, A. Palmieri, C. Raymond, G. Rouse, C. Saenphimmachak, Z. Wu, P. Romero, D. Gordon, S. Zhang, H. Yoo, Y. Tao, P. Biddle, M. Jung, W. Krespan, M. Perry, B. Gordon-Kamm, L. Liao, S. Kim, C. Hendrick, Z. Y. Zhao, M. Dolan, F. Chumley, S. V. Tingey, J. F. Tomb, M. P. Gordon, M. V. Olson, and E. W. Nester. 2001. The genome of the natural genetic engineer Agrobacterium tumefaciens C58. Science **294**:2317-2323.

Yamada, H. and H. Kumagai. 1978. Microbial and enzymatic processes for amino acid production. Pure and Applied Chemistry **50** :1117-1127.

Yamada, H., S. Shimizu, H. Shimada, Y. Tani, S. Takahashi, and T. Ohashi. 1980. Production of D-phenylglycine related amino acids by immobilised microbial cells. Biochimie **62**:395-399.

Yamashiro, A., K. Yokozeki, H. Kano, and K. Kubota. 1988. Enzymatic production of L-amino acids from the corresponding 5-substituted hydantoins by a newly isolated bacterium, *Bacillus brevis* AJ12299. Agricultural Biological Chemistry **52**:2851-2856.

Yin, B.-D., Y.-C. Chen, S.-C. Lin, and W.-H. Hsu. 2000. Production of D-amino acid precursors with permeabilised recombinant *Escherichia coli* with D-hydantoinase activity. Process Biochemistry **35**:915-921.

Yokozeki, K., K. Sano, C. Eguchi, K. Yamada, and K. Mitsugi. 1987d. Enzymatic production of L-tryptophan from DL-5-indolylmethylhydantoin by mutants of *Flavobacterium* sp. T-523. Agricultural Biological Chemistry **51**:363-369.

Yokozeki, K. and K. Kubota. 1987c. Mechanism of asymmetric production of D-amino acids from the corresponding hydantoins by *Pseudomonas* sp. Agricultural Biological Chemistry **51**:721-728.

Yokozeki, K., S. Nakamori, C. Eguchi, K. Yamada, and K. Mitsugi. 1987a. Screening of microorganisms producing D-*p*-hydroxyphenylglycine from DL-5-(*p*-hydroxyphenyl) hydantoin. Agricultural Biological Chemistry **51**:355-362.

Yokozeki, K., Y. Hirose, and K. Kubota. 1987c. Mechanism of assymetric production of L-aromatic amino acids from corresponding hydantoins by *Flavobacterium* sp. Agric Biol Chem **51**:737.