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**A MOLECULAR GENETIC ANALYSIS OF L-ASPARAGINASE II
SYNTHESIS IN ERWINIA CHRYSANTHEMI NCPPB 1066.**

by

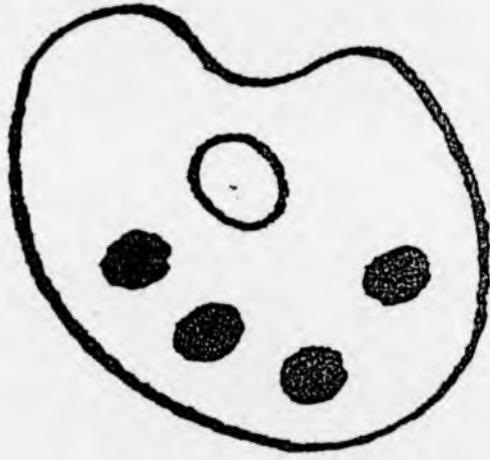
Christopher Martin Kell

B.Sc.(Hons) (Warwick)

A thesis presented for the degree of Doctor of Philosophy.
Department of Biological Sciences, University of Warwick.

September 1991.

NUMEROUS ORIGINALS IN COLOUR



DEDICATION

For my family and Sharon, without whom
none of this would have been possible.

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DECLARATION

I declare that this thesis has been composed by myself, and that none of the work has been used in any previous application for a degree. The results presented here were obtained by myself under the supervision of Dr. G.P.C. Salmond and Dr. N.P. Minton, unless otherwise stated in the text. All sources of information have been specifically acknowledged by means of reference.

A handwritten signature in cursive script that reads "Kell." The signature is written in dark ink and is positioned to the left of the printed name.

CHRISTOPHER M. KELL

SUMMARY

The plant pathogen Erwinia chrysanthemi NCPPB 1066 synthesises large amounts of a high affinity periplasmic L-asparaginase enzyme which has been found to possess antileukaemic properties, and is produced commercially for the treatment of childhood acute lymphoblastic leukaemia.

In the present study, both wild-type Er. chrysanthemi NCPPB 1066 and 5 morphological variants comprising the industrial production culture were characterised physically, biochemically and genetically. EMS mutagenesis was used to isolate LacZ⁻ and PhoA⁻ mutants, and a range of plasmids were successfully introduced into the aforementioned bacteria using either transformation, electroporation or conjugal mobilisation. All isolates were found to be resistant to bacteriophage Mu, phiEC-2, P1 and T4; and lamB⁺ technology failed to generate a lambda-sensitive derivative. Novel bacteriophage for wild-type Er. chrysanthemi NCPPB 1066 were isolated from sewage, but did not appear to be capable of generalised transduction.

Using information from genetic studies, a pBR322-derived ansB expression vector was introduced into the wild-type strain and each of the industrial morphotypes with relatively high efficiency using a pR64drd11/pLVC9 mobilisation system. Small-scale fermentation analysis revealed that L-asparaginase II was overexpressed, but that enzyme levels varied dramatically from isolate to isolate. Plasmid pIC-19H-based ansB expression vectors were also introduced into the wild-type strain and industrial morphotype 2, using an oriT/RP4 mobilisation system at low frequency. Although constructs were stably maintained, no improvement in the level of L-asparaginase II was seen.

Studies performed by Minton *et al.* (1986) had identified a putative -24/-12 promoter consensus sequence within the 5' non-coding region of the ansB structural gene, and evidence suggested that components of the Ntr regulatory system could interact with the ansB promoter in E. coli. Since expression of ansB does not appear to be nitrogen regulated in Er. chrysanthemi NCPPB 1066, the aforementioned workers suggested that Er. chrysanthemi NCPPB 1066 did not possess a functional Ntr system. Heterologous hybridisation studies carried out in the present study revealed the presence of rpoN, ntrB and ntrC homologues within the Er. chrysanthemi NCPPB 1066 genome. These were cloned and shown to complement corresponding lesions in E. coli. Nucleotide sequence analysis of the rpoN locus revealed it to be highly homologous to the K. pneumoniae rpoN locus in terms of nucleotide sequence and gene organisation, and the presence of multiple copies of the former *in trans* to a chromosomal ansB::lacZ fusion, did not appear to affect ansB expression under nitrogen-rich or poor growth conditions. Transcript mapping of the ansB structural gene was also performed with total cellular RNA isolated from cells grown under nitrogen-rich and nitrogen-poor conditions. In all instances, a single mRNA species originating at a cytosine residue, 117 nucleotides from the translational start site, was identified. This did not correspond to the putative -24/-12 promoter consensus sequence. Hence, the evidence suggested that the Er. chrysanthemi NCPPB 1066 ansB structural gene was not RpoN-dependent.

Finally, ansB::phoA and ansB::lacZ gene fusions were generated both *in vivo* and *in vitro*. The ansB::lacZ transcriptional fusion plasmid, pCK11, was introduced a LacZ⁻ mutant of wild-type Er. chrysanthemi NCPPB 1066, and a single copy fusion derivative constructed by marker-exchange mutagenesis. EMS mutagenesis of the latter led to the isolation of 24 lacZ 'down' expression mutants. Characterisation of such mutants, using Southern and Western blotting, revealed that 5 were LacZ catalytic mutants; 8 synthesised a truncated inactive LacZ polypeptide, and the remaining 11 were due to some other cause.

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ABBREVIATIONS

ATP	Adenosine 5' triphosphate
bp	base pair
BSA	Bovine serum albumin
°C	Degree centigrade
ca.	Approximately
cAMP	3'5'-adenosine monophosphate
C.A.M.R.	Centre for applied microbiological research
Cel	Cellulase
cfu	Colony-forming units
Ci	Curie
CIP	Calf intestinal phosphatase
cm	Centimetre
cpm	Counts per minute
Crp	cAMP receptor protein
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytosine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
dTTP	Deoxythymine 5'-triphosphate
DD	Double Difco
DEPC	Diethylpyrocarbonate
DON	6-diazo-5-oxonorvaline
DONV	5-diazo-4-oxonorvaline
DOT	Dissolved oxygen tension
2,5-DKG	2,5-diketo-D-gluconic acid
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dNTP's	Deoxyribonucleoside 5'-triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethane sulphonate

g	Gram
HCl	Hydrochloric acid
Hfr	High frequency of recombination
IPTG	Isopropyl B-D-thiogalactopyranoside
IS	Transposable insertion element
IU	International unit
k	Kilo
kb	Kilobase
kDa	Kilo Dalton
kg	Kilogram
2-KLG	2,-keto-L-gulonic acid
K_m	Michaelis-Menten constant
kv	Kilovolt
LB	Luria broth
LPS	Lipopolysaccharide
M	Molar
mA	Milliamperes
mg	Milligram
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
MMS	Methylmethane sulphonate
min	Minute
NB	Nutrient broth
nm	Nanometer
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
Ntr	Nitrogen regulation
OD	Optical density at wavelength λ
ONPG	O-nitrophenol-B-D-galactoside
ORD	Optical rotary dispersion
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PEB	PIPES/EDTA/NaCl buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

Pel	Pectin lyase
pfu	Plaque-forming units
pl	Isoelectric point
ppt	Precipitate
Prt	Protease
psi	Pounds per square inch
(^r)	Resistant
R _I	Refractory index
RNA	Ribonucleic acid
RNAase	Ribonuclease
rpm	Revolutions per minute
(^s)	Sensitive
SAE	SDS/sodium acetate/EDTA buffer
SD	Shine-Dalgarno
SDS	Sodium dodecyl sulphate
sec	Second
SSC	Sodium chloride/sodium citrate
subsp.	Sub-species
t	Time of reaction in minutes
TAE	Tris/Acetate/EDTA
TBE	Tris/Borate/EDTA
TBS	Tris buffered saline
TCA	Tricarboxylic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFB	Transformation buffer
TPR	Transfer per recipient
Tris	Tris(hydroxymethyl)aminoethane
IU	International unit
UAS	Upstream activator sequence
UF	Ultrafiltration
uF	MicroFaraday
ug	Microgram
ul	Microlitre
uM	Micromolar
UV.	Ultraviolet light
v	Volume

w/v	Weight/volume
v/v	Volume/volume
X-gal	5-bromo-4-chloro-3-indoyl-B-D-galactoside
X-P	5-bromo-4-chloro-3-indoyl phosphate

Amino acid residues

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Nucleotide bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Antibiotic abbreviations are listed in Table 2.5.

CHAPTER ONE

LITERATURE REVIEW

1. Literature review

PREFACE

The enzyme L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) has been shown to possess antitumour activity and has been extensively used over many years for the treatment of childhood acute lymphoblastic leukaemia. Today, this enzyme is produced commercially from either the enteric bacterium Escherichia coli or the plant pathogen Erwinia chrysanthemi NCPPB 1066. The following sections of this chapter have been written in the form of a review which summarises the available literature concerning L-asparaginase. The clinical, physical, biochemical, commercial and regulatory aspects of this enzyme are discussed, with particular emphasis on the chemotherapeutic L-asparaginase from Er. chrysanthemi NCPPB 1066, since this is the subject of the present study. In addition, the reader is introduced to the genus Erwinia and to the variety of tools that have been developed for the genetic analysis of a number of Erwinia species.

1. Literature review

1.1 INTRODUCTION

For centuries, cancer has been a major and often fatal disease of mankind. Leukaemia, one of the so-called 'blood cancers', accounts for approximately four per cent of all cancer mortality, and is the commonest neoplasm observed in children in the United Kingdom today. It is, as its name implies, a group of malignant diseases of the blood-forming organs, in which there is an uncontrolled clonal proliferation of haemopoietic cells derived from a malignantly transformed progenitor (Lascardi, 1973). During the course of the disease, cancerous cells replace the normal bone marrow and often infiltrate other organs (Lascardi, 1973). This causes a variety of clinical symptoms such as breathlessness, haemorrhaging, bone and joint pain, anaemia and enlargement of lymphoreticular structures such as the lymph nodes, liver and spleen, and eventually leads to death (Whittaker and Delamore, 1987).

On average, five people in one hundred thousand develop leukaemia per annum. In the majority of cases there is no clue as to the aetiology of the condition, although an increased incidence has been found in relation to certain factors such as viral infection (Jarrett, 1987), ionising radiation, chemical agents and genetic predisposition (Cartwright and Bernard, 1987).

The leukaemias are conventionally classified on the basis of the dominant cell type involved in the disorder, and are described as either acute or chronic depending upon whether the course followed is short and relatively severe, or persists over a long period. A large proportion of patients with leukaemia are those with the acute lymphoblastic type. This form of the disease is characterised by an abnormal multiplication of poorly differentiated variant lymphoblastic cells, and arises predominantly in male children around

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the age of two to three years (Figure 1.1). This contrasts with other types of leukaemia, such as acute myeloblastic leukaemia or chronic myeloid, granulocyte and lymphoblastic leukaemias, which mostly occur in individuals of either sex over the age of forty years, and involve the excessive proliferation of cells at a relatively late stage of differentiation.

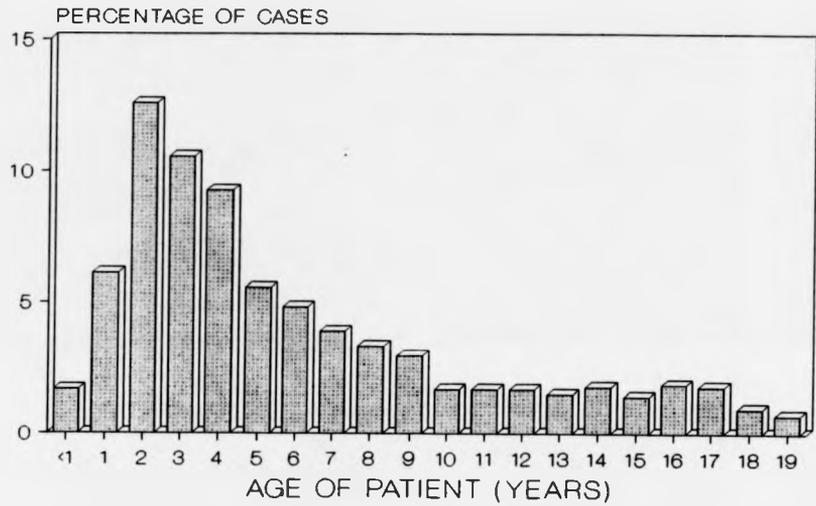
1.1.1 L-asparaginase and the treatment of leukaemia

Forty years ago, a child recognised as having developed acute lymphoblastic leukaemia would have had at most a few months to live. Today, the patient has a high likelihood of recovery and of leading a normal life. This drastic improvement has resulted from treatment of the disease with a variety of drugs, including the enzyme L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1).

The chemotherapeutic use of the aforementioned enzyme represents a unique development in the field of cancer treatment, and is probably the most important factor which accounts for the improved rates of survival. To date, acute lymphoblastic leukaemia appears to be almost uniquely sensitive to L-asparaginase treatment. In this instance, complete remissions can be obtained in 60 to 80 per cent of the patients treated, and cure achieved in approximately 50 per cent of cases. In contrast, acute myelocytic and granulocytic leukaemias are less responsive, resulting in below 20 per cent remission rates, and in general require larger doses of enzyme. Inconsistent responses are seen with solid tumours, even though cultures of cells derived from the tumour, such as those from malignant myelomas, are L-asparaginase-sensitive.

1. Literature review

Figure 1.1 The incidence of acute lymphoblastic leukaemia



Histogram showing the percentage distribution of acute lymphoblastic leukaemia in patients of both sexes aged up to 19 years. From a survey of 873 cases by Zippin *et al.* (1971).

1. Literature review

1.1.2 Uses of L-asparaginase in the treatment of acute lymphoblastic leukaemia

Clinically, L-asparaginase is used for the induction of initial remission of acute lymphoblastic leukaemia and for maintenance therapy (Gallagher *et al.*, 1989). Moreover, in certain circumstances, the enzyme can also be employed for the re-induction of remission, for those patients in relapse of the disease (Rivera *et al.*, 1986).

Even though L-asparaginase has substantial activity as a single agent, it has proven more successful to treat patients with a combination of drugs (eg. vincristine, methotrexate and prednisone), in concert with L-asparaginase, rather than using the enzyme alone (Gallagher *et al.*, 1989). During treatment, a typical dosage of L-asparaginase would be 200 international units (IU) kg^{-1} of body weight ($5-6 \times 10^3$ IU m^{-2} body surface area), administered either intravenously, subcutaneously or intramuscularly.

1.1.3 Medical applications of other purified enzymes

L-asparaginase is just one example of the variety of highly purified enzymes now widely used in medicine, both locally and systemically, for the treatment of a range of disorders (see Bohak and Sharon, 1977; Chang, 1977; Price and Stephens, 1989). Microbial hydrolases for instance, can be used to supplement a deficiency in the production of digestive enzymes. In this respect, lipases produced by *Aspergillus oryzae* or *Candida lipolytica* are sometimes used to compensate for a deficiency in pancreatic lipase. Acid-resistant amylases from *Aspergillus oryzae* and *Aspergillus niger*, alpha-amylase from *Bacillus subtilis*, and cellulases from *Trichoderma viride* are also employed as digestive aids.

The use of microbial enzymes for the treatment of disorders such as

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thrombosis has also been investigated. Streptokinase, an enzyme produced by some beta-haemolytic streptococci, can be injected intravenously to activate plasminogen, thereby transforming it to plasmin (fibrinolysin), which in turn causes digestion and solubilisation of the fibrin network comprising the clot. Streptodornase is another enzyme of medical usefulness produced by haemolytic streptococci. Its substrate is deoxyribonucleic acid (DNA) or deoxyribonucleoproteins, which are responsible for much of the viscidness of purulent exudates.

A pure toxin-free collagenase from Clostridium histolyticum has been used for the enzymic debridement of necrotic infected tissue in burns or certain forms of skin ulcer. Penicillinase obtained from Bacillus cereus hydrolyses penicillin to biologically inactive penicilloic acid, and is used to overcome allergic reactions in hypersensitive patients dosed with the antibiotic. Finally, a urate oxidase from a strain of Aspergillus flavus has been used in the treatment of gout where it apparently aids the dispersal of uric acid crystals.

1.2 L-ASPARAGINASE; DISCOVERY, CYTOTOXICITY, PRODUCTION AND PURIFICATION

1.2.1 The discovery of L-asparaginase cytotoxicity

The discovery of the antileukaemic action of L-asparaginase developed from an unexpected but highly astute observation made by Kidd (1953 a,b), during a series of experiments aimed at investigating the immunology of murine leukaemia. In the course of this study, Kidd clearly demonstrated that normal guinea pig serum could cause regression of established 8C3HED lymphomas in 8C3 mice, whereas the sera from horse, rabbit, cat, dog, rat

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and cattle were ineffective. Further experimentation revealed that very few other types of established lymphomas regressed when guinea pig serum was injected into animals bearing them. Moreover, the unique antitumour property of guinea pig serum (or "Kidd Phenomenon") did not appear to arise from complement activity or other immunological factors, but resulted instead from the action of a heat-labile protein.

A short time later, Neuman and McCoy (1956) discovered that cells of the rat tumour Walker 256 failed to grow in tissue culture medium lacking L-asparagine. Similarly, Fischer (1958) and Haley *et al.* (1961) noted that cells of the transplanted mouse leukaemia L5178Y were unable to grow in culture medium devoid of L-glutamine or L-asparagine. Broome (1961) subsequently correlated these independent, and apparently unconnected observations, with the fact that the guinea pig was the only mammal known at that time to contain L-asparaginase in the blood. Using this knowledge, guinea pig serum was subjected to heat treatment as well as changes in pH, and a direct relationship between the L-asparaginase activity and antilymphoma activity against implanted 6C3HED cells was quickly established. This finding was reinforced by further experimentation, which revealed that the serum from newborn guinea-pigs (in which L-asparaginase is lacking) was devoid of antilymphoma activity. Moreover, fractionation of guinea pig serum for antilymphoma activity was shown to result in a parallel purification of L-asparaginase activity (Broome, 1963 a). Finally, ultimate confirmation came from the studies of Yellin and Wriston (1966 a), who purified L-asparaginase from guinea pig serum to apparent homogeneity and went on to clearly demonstrate its potent *in vivo* antileukaemic action (Yellin and Wriston, 1966 b).

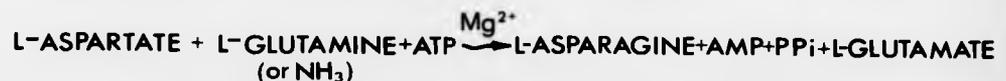
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1.2.2 The basis for L-asparaginase cytotoxicity

L-asparaginase differs from many of the chemotherapeutic agents used in the treatment of human cancer, due to the fact that its action is based on a unique biochemical difference between certain cancer cells (mainly the lymphoid type) and normal cells. The enzyme itself catalyses the non-reversible hydrolysis of L-asparagine to yield L-aspartic acid and ammonia (Wriston, 1971), as shown below.



The most important macromolecular fate of L-asparagine is its incorporation into protein, and as such it is required for protein synthesis to take place within the cell. Following parenteral administration of the L-asparaginase, the plasma is rapidly cleared of circulating L-asparagine, normally present at a concentration of about 40uM (Broome, 1968; Miller et al., 1969). However, most normal tissues are able to tolerate the depletion of this amino acid because they are able to synthesise sufficient L-asparagine in situ through the action of L-asparagine synthetase (EC 6.3.1.1) to maintain protein synthesis (Broome and Schwartz, 1967; Horowitz et al., 1968; Patterson and Orr, 1967, 1968, 1969; Prager and Bachynsky, 1968 a,b).



In contrast to normal cells, a small number of malignant cell types have been found to produce invariantly low or immeasurable levels of the L-asparaginase synthetase enzyme and can therefore only synthesise endogenous L-asparagine slowly, if at all. Consequently, these particular cells are dependent on an extracellular supply of this amino acid for viability and proliferation, and are therefore sensitive to the action of L-asparaginase

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(Boyse et al., 1967; Broome, 1963 b; Sobin and Kidd, 1965,1966).

The relationship between L-asparaginase-sensitivity and the level of L-asparagine synthetase within the cell is further highlighted by the fact that in certain instances, spontaneous mutations can arise within a population of L-asparaginase-sensitive cells treated with sub-lethal doses of L-asparaginase, leading to the formation of L-asparaginase-resistant variants (Broome, 1963 b). Such resistant cells have been found to display higher L-asparagine synthetase levels compared with the parent population and normal tissues, which are inversely related to the exogenous supply of L-asparagine. However, the exact nature of the mutation(s) causing this change has so far not been elucidated.

1.2.3 Events leading to L-asparaginase cytotoxicity

A rapid cessation of protein synthesis has been shown to take place within sensitive neoplastic cells following deprivation of L-asparagine (Saunders, 1972; Sobin and Kidd, 1965; Regan et al., 1969). This is followed by a temporal inhibition of the synthesis of RNA (Becker and Broome, 1969) and DNA (Becker and Broome, 1967; Ellem et al., 1970) and finally culminates in cell death.

In addition to the effects on protein and nucleic acid metabolism, the administration of L-asparaginase also causes a number of other biochemical alterations that are potentially detrimental to neoplastic cells, and could contribute to the cytotoxic action of this enzyme. These include changes in the levels of free amino acids (Broome, 1968), and an increase in acid and alkaline ribonuclease activity (Mashburn and Gordon, 1968; Mashburn and Landin, 1968; Mashburn and Wriston, 1968). Moreover, an inhibition of

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glycoprotein synthesis (Bossman and Kessel, 1970) and alterations in membrane structure (Han and Ohnuma, 1974) have been reported. This goes some way to explaining why L-asparaginase treatment can result in the rapid lysis of sensitive experimental and human lymphoblastic cells (Summers and Handschumacher, 1971), as well as causing the dissolution of large tumour masses in animals (Old et al., 1967) and humans (Capizzi et al., 1970). It must be noted at this point, however, that the oncolytic action of L-asparaginase does not appear to be a result of direct enzymatic attack on cell membranes, but is probably a consequence of lowered L-asparagine levels, since sensitive-cells grown in medium devoid of L-asparagine can have equivalent rates of lysis (Summers and Handschumacher, 1973).

1.2.4 The development of L-asparaginase as an anti-leukaemic drug

Dolowy and colleagues (1966) were one of the first group of workers to report on the clinical application of crude (four-fold purified) L-asparaginase from guinea pig serum to induce transient remission of advanced acute lymphoblastic leukaemia in an 8 year old boy. Although the initial results were encouraging, it was calculated that the amount of enzyme required for daily treatment could only be obtained from 2 to 6 litres of guinea pig serum. This immediately relegated L-asparaginase treatment to a scientific curiosity rather than a practicality, and it became clear that a more plentiful source of enzyme would be required for full exploitation and clinical evaluation (Burchenal and Karnofsky, 1970).

It had been known for some time that microorganisms, such as yeasts (Broome, 1965) and fungi (Cooney and Handschumacher, 1970), as well as a number of bacteria, including species of Bacillus (Manning and Campbell, 1957), Pseudomonas (De Groot and Lichtenstein, 1960), Mycobacterium

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(Halpern and Grossowicz, 1957) and Escherichia coli (Tsuji, 1957), displayed L-asparaginase activity. Attention subsequently became focused on E. coli as a source of large-scale production of L-asparaginase, following the work of Mashburn and Wriston (1964) who discovered that this particular bacterium synthesised an L-asparaginase that had antitumour activity comparable to that of guinea pig serum (Mashburn and Wriston, 1964). It was subsequently demonstrated that the L-asparaginase enzyme from E. coli B (Campbell et al., 1967; Roberts et al., 1966), and E. coli K12 (Schwartz et al., 1966) actually existed in two forms which could be distinguished in terms of their regulation, activity at different values of pH, and by markedly different affinities for L-asparagine. The first form was a constitutively expressed low affinity L-asparaginase I enzyme (EC-1; K_m value of 3.5mM) which resided in the cytoplasm and displayed no activity at pH 5 to 6. The second form was a high affinity L-asparaginase II enzyme (EC-2; K_m value of 1.15 μ M) located within the periplasmic space, which displayed activity at acid pH. Synthesis of this enzyme was found to be markedly induced under anaerobic conditions in medium containing a high concentration of amino acids and little or no sugar (Cedar and Schwartz, 1968; Schwartz, 1971; Willis and Woolfolk, 1974). Of the two forms, only the periplasmic L-asparaginase II enzyme displayed the capacity to inhibit the synthesis of protein under the direction of bacteriophage f2 RNA in cell-free extracts of E. coli (Schwartz, 1965; Schwartz et al., 1966), and was capable of interfering with the incorporation of amino acids into tumour protein in cell cultures (Broome, 1968). More importantly, unlike L-asparaginase I (Schwartz et al., 1966), the L-asparaginase II enzyme could effectively inhibit the growth of transplantable mouse and rat tumours (Campbell et al., 1967; Scharzt et al., 1966), as well as lymphoma in the dog (Old et al., 1967,1968).

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Early clinical trials soon indicated that L-asparaginase II was also effective for the treatment of human lymphoblastic leukaemia (Adamson and Fabro, 1968; Grundmann and Oettgen, 1970; Hill et al., 1967; Oettgen et al., 1967, 1970; Old et al., 1968), and finally stimulated the development of practical techniques (Roberts et al., 1968) allowing the large-scale production of purified enzyme from a variety of E. coli strains (eg. ATCC 11303, ATCC9637 and ATCC 13706) by a number of pharmaceutical manufacturers. An early demonstration of the antitumour activity of L-asparaginase from Serratia marcescens around this time (Rowley and Wriston, 1967), also prompted the large-scale purification of the enzyme from this bacterial source (Heinemann and Howard, 1969).

1.2.5 The production of L-asparaginase from Erwinia

In 1967, the commercial production of L-asparaginase from E. coli was being hampered by the relatively low level of activity of the enzyme in bacterial cultures, and difficulties associated with enzyme extraction. As a result, the demand for E. coli L-asparaginase II far exceeded its availability and fears were expressed by the Department of Health that there would be insufficient stocks of the enzyme for the United Kingdom. In an attempt to remedy this situation, Wade and colleagues at the Microbiological Research Establishment (now the Centre for Applied Microbiological Research (C.A.M.R)) directed efforts towards finding a richer bacterial source of L-asparaginase. Ideally this was to be immunologically distinct from that of E. coli, since it was anticipated that such an enzyme would be of use in the treatment of patients who had become sensitised to the E. coli L-asparaginase II (see Section 1.2.8.2).

The search for a new L-asparaginase concentrated on organisms having

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only remote association with man to minimise his initial immunological sensitivity. In this respect, plant pathogens were placed under particular scrutiny, due to the fact that many plants contain substantial amounts of L-asparagine as an important storage product, and such bacteria were anticipated to have evolved a high affinity L-asparaginase for harnessing this convenient source of energy and nitrogen (Wade and Rutter, 1970).

The results of an initial screen of a range of bacteria carried out by Herbert and Wade (1968) revealed the presence of significant levels of L-asparaginase in many strains of the genus Erwinia (for more detail see Section 1.5), in particular Erwinia carotovora, which contained the enzyme at specific activities over ten times higher than one of the E. coli L-asparaginase II production strains (ATCC 11303), being used at that time (Table 1.1). Overall, the highest levels of L-asparaginase were measured within cells of Er. carotovora NCPPB 1066 (later re-classified as Er. chrysanthemi NCPPB 1066) which was a pathogen of maize (Zea mays L.) originally isolated from Egypt by Sabet (Dickey, 1979).

Subsequent experimentation indicated that the purified L-asparaginase II enzyme from this particular strain of Erwinia had the capacity to inhibit tumour formation in 6C3 mice injected with cells of the 6C3HED lymphoma (Wade et al., 1968). Hence, work was restricted to this organism in order to produce material for clinical trials quickly. Subsequent to this, a further screen of over 200 strains from 78 species was carried out by Wade et al. (1971 a,b) using an automated assay system developed by Wade and Phillips (1971). However, this failed to identify a bacterium superior to Er. chrysanthemi NCPPB 1066 in terms of L-asparaginase productivity.

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Table 1.1 L-asparaginase levels in a range of bacteria

Bacterium	Collection number	Specific activity (IU mg of protein ⁻¹)
<u>Escherichia coli</u>	ATCC 110303	0.48
	MRE 163	0.27
	MRE 164	0.65
	NCTC 8164	0.46
	NCTC 8196	0.36
	NCTC 9001	0.47
<u>Serratia marcescens</u>	NCIB 1377	<0.05
	NCIB 4612	<0.05
	NCIB 8266	0.20
	NCIB 8889	0.15
	NCIB 9155	0.15
	MRE UK/8	0.26
<u>Erwinia aroideae</u>	NCPPB 1274	0.54
	NCPPB 1380	1.15
<u>Erwinia carotovora</u> subsp. <u>atroseptica</u>	NCPPB 549	0.54
<u>Erwinia carotovora</u> subsp. <u>carotovora</u>	NCPPB 31	0.53
	NCPPB 312	1.50
	NCPPB 392	0.60
	NCPPB 438	1.00
	NCPPB 468	0.51
	NCPPB 491	0.95
	NCPPB 569	0.79
	NCPPB 708	4.90
	NCPPB 898	1.20
	NCPPB 1065	4.10
	NCPPB 1066	6.30
NCPPB 1120	3.00	
NCPPB 1280	1.50	
<u>Erwinia chrysanthemi</u>	NCPPB 516	0.68

Data reproduced from Herbert and Wade (1968).

ATCC American Type Culture Collection, USA.
 MRE Microbiological Research Establishment, England.
 NCCPB National Collection of Plant Pathogenic Bacteria, England.
 NCIB National Collection of Industrial Bacteria, Scotland.
 NCTC National Collection of Type Cultures, USA.

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1.2.6 The commercial production of the antileukaemic L-asparaginase II from Er. chrysanthemi NCPPB 1066

For the last twenty years, the large-scale production and purification of the Er. chrysanthemi NCPPB 1066 antileukaemic L-asparaginase has been carried out at C.A.M.R. Porton Down; and today, approximately 700 grams of enzyme are purified on an annual basis. This is subsequently sold on a world-wide basis by Porton Products Ltd., under the trade name 'Erwinase', at a market value of around 1 million pounds (sterling) per annum.

1.2.6.1 The production process

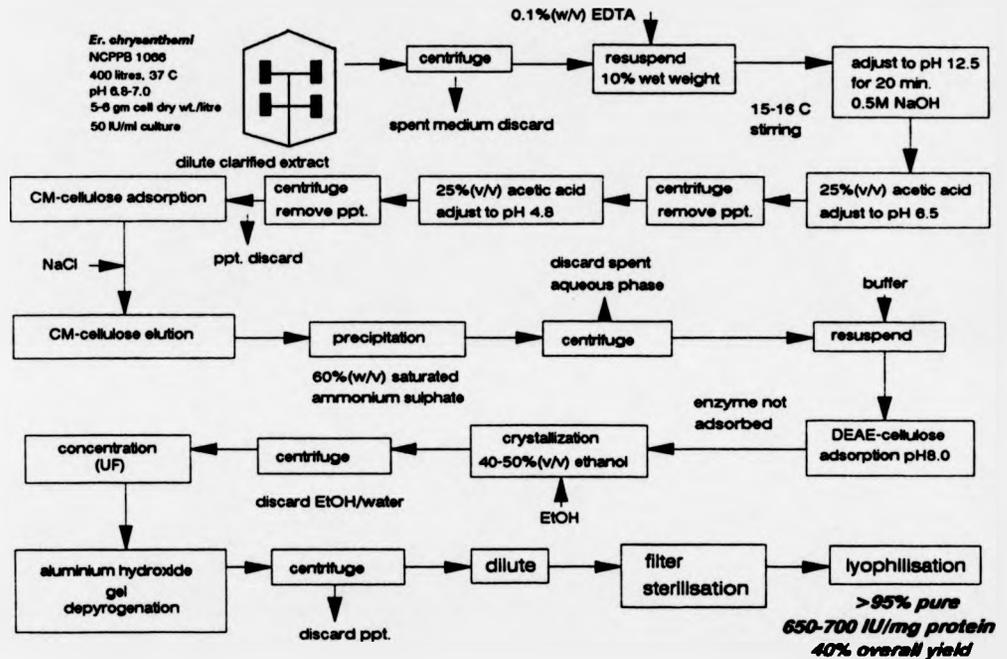
Although a continuous culture process for the production of L-asparaginase II, yielding titres from 80 to 200 IU per ml, has been reported (Callow *et al.*, 1971,1974), the industrial manufacture of this enzyme is now limited to a batch process (Buck *et al.*, 1971), the details of which are summarised in Figure 1.2.

The initial enzyme production process involved culturing of the wild-type Er. chrysanthemi NCPPB 1066 strain in a 400 litre stirred, aerated fermenter, using 4.5% (w/v) yeast extract adjusted to pH 6.8-7.0 as growth medium. Under these conditions, the rate of bacterial growth was found to fall sharply after a period of around 8 hours, at which point L-asparaginase II accumulation ceased. The average yield of enzyme per ml was 8.2 IU, whilst the specific activity of the cell paste was 4.2 IU per mg of protein (Sargeant, 1973). The currently obtainable levels of L-asparaginase have resulted from a series of improvements to the original fermentation process, and these will now be described briefly below.

The first beneficial alteration to the fermentation process stemmed from the observation of Elsworth and coworkers (1969), who noted that the

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Figure 1.2 Process for the commercial production of L-asparaginase II from *Er. chrysanthemi* NCPPB 1066



Adapted from Flickinger (1985).

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amino acids, L-glutamate, L-serine, L-threonine and L-aspartate, had been completely removed from the culture medium towards the latter stages of bacterial growth. In an attempt to prolong bacterial growth, and hence increase the yield of L-asparaginase, the least expensive of these, L-glutamate, was added to the medium. This was subsequently found to improve bacterial growth and caused an even sharper increase in the enzyme content of the culture. It was therefore suggested that L-glutamate was acting not only as a nutrient for bacterial cells, but also as an L-asparaginase inducer. In this instance, batch cultures grown in 4.5% (w/v) yeast extract supplemented with 1.75% (w/v) sodium glutamate monohydrate gave an average yield per ml of 44.5 IU and a specific activity of 7.6 IU mg protein⁻¹.

The second improvement in L-asparaginase II yields followed the isolation of a hyper-producing strain of Er. chrysanthemi NCPPB 1066. This derivative, designated S10, resulted from long-term continuous flow experiments performed by Callow and colleagues (1971) using glycerol-limited cultures. Although less cell paste was produced by S10, the average yield of L-asparaginase II produced by this isolate in batch fermentation was 60.6 IU ml⁻¹, corresponding to a specific activity of 14.9 IU mg of protein⁻¹. This accounted for approximately 2% of the cell protein.

1.2.6.2 The purification process

A number of studies concerning the large-scale purification of L-asparaginase have been undertaken (Goward et al., 1989; Lee et al., 1986 a,b; Wade, 1972). The multistep procedure performed at C.A.M.R. is schematically represented in Figure 1.2, and has been greatly facilitated by the unusual stability of the Er. chrysanthemi NCPPB 1066 L-asparaginase II protein in the

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alkaline pH region, as well as its ability to be readily crystallized (Cammack, et al., 1972).

In brief, Er. chrysanthemi NCPPB 1066/S10 is initially recovered as a cell paste from mature cultures and quickly frozen at -20°C until required. Following resuspension of the bacterial cells, the crude enzyme is extracted at pH 12.5 and then adsorbed on CM-cellulose for 18 hours. Following elution, the enzyme is precipitated with 60% (w/v) saturated ammonium sulphate and re-adsorbed on DEAE-cellulose. The enzyme is crystallized with 40 to 50% (v/v) ethanol and subsequently treated with hydrated aluminium oxide gel to remove pyrogenic material. The final lyophilised product is greater than 95% homogenous by electrophoresis and has a specific activity of 650 to 700 IU mg of protein⁻¹. In contrast, the specific activity of E. coli preparations is generally lower, averaging 270 IU mg of protein⁻¹. Solutions of amorphous Er. chrysanthemi NCPPB 1066 L-asparaginase II protein (7 to 14 mg ml⁻¹) are sterilised by filtration and then adjusted to pH 7.4 with phosphoric acid prior to sealage in ampoules stored at -20°C , ready for clinical use.

1.2.7 Properties of clinically useful L-asparaginase enzymes

L-asparaginase is now known to be present within plant (Sodek et al., 1980) and animal cells (Broome, 1961), as well as fungi (Arst and Bailey, 1980), yeasts (Jones, 1977) and bacteria (Wade et al., 1971 a). Bacterial L-asparaginases are generally intracellular, but are freely accessible to exogenous substrate. Those of yeast and fungi are, on the other hand, usually exported to the extracellular environment (Wade, 1980).

Despite the widespread distribution of L-asparaginase, less than one half of the L-asparaginase enzymes examined to date have been found to be able

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to inhibit the growth of L-asparagine-requiring tumours. Indeed, only the L-asparaginase from E. coli, Er. chrysanthemi NCPPB 1066 and Serratia marcsecens has been shown to exhibit the desired properties for treatment of acute lymphoblastic leukaemia. In this regard, a number of fundamental criteria have been defined as necessary for antineoplastic activity (Holcenberg, 1982; Uren and Handschumacher, 1977). Firstly, an enzyme should be cleared from the circulation at a slow rate and have a high affinity for its substrate. Such a high affinity at physiological pH is an absolute prerequisite for antitumour activity, since the concentration of L-asparagine does not become rate-limiting in protein synthesis until it is reduced to 1.29×10^{-5} M, or less (Broome, 1968). Secondly, the enzyme should preferably display high activity in the presence of plasma or whole blood, a property which takes into account the question of naturally occurring inhibitors. Finally, it is desirable that the enzyme requires no cofactor, unless the latter is tightly bound. Moreover, it should display low antigenicity. Of these factors, two are particularly important, namely the affinity of the enzyme for substrate and the rate of clearance from the circulation. These aspects will now be discussed in relation to the physical and biochemical properties of both clinically effective Er. chrysanthemi NCPPB 1066 and E. coli L-asparaginase II enzymes. The characteristics of these enzymes are summarised in Table 1.2, alongside those of other microbial L-asparaginases.

1.2.7.1 Substrate affinity

Er. chrysanthemi NCPPB 1066 closely resembles E. coli and many other Gram-negative bacteria (Wade, 1980) in the fact that the antileukaemic L-asparaginase synthesised by this organism is a periplasmic enzyme with a high

Table 1.2 Physical and biochemical properties of L-asparaginase from different bacterial species.

SOURCE*	MOLECULAR WEIGHT [†]	pI VALUE [#]	CLEARANCE (hr)	Km VALUES (M)*	
				Asn	Gn
<u>Erwinia chrysanthemi</u> (+) NCPB 1066	135,000 ^a	8.6 ^e	4.1	1.50 X 10 ⁻⁵	1.4 X 10 ⁻³
Guinea pig serum (+)	133,000 ^b 138,000 ^a	3.6 - 4.5 ^f	26.0	7.20 X 10 ⁻⁵	0
<u>Escherichia coli</u> (+)	130,000 ^a	4.9 - 5.4 ^e	2.9-4.2	1.15 X 10 ⁻⁵	6.25 X 10 ⁻³
<u>Serratia marcescens</u> (+)	147,000 ^a 130,000 ^a 150,000 ^c	6.0 ^e	3.0-6.0	1.20 X 10 ⁻⁵	3.00 X 10 ⁻³
<u>Bacillus coagulans</u> (-)	85,000 ^c	-	< 0.5	4.00 X 10 ⁻³	-
<u>Acalligenes eutrophus</u> (+)	150,000 ^a	8.6 ^e	< <u>E.coli</u>	3.00 X 10 ⁻⁵	4.00 X 10 ⁻⁵
<u>Klebsiella aerogenes</u>	141,000 ^b	-	-	1.00 X 10 ⁻⁵	1.10 X 10 ⁻³
Yeast (-)	800,000 ^a	-	0.5	-	-
Chicken liver (+, weak)	306,000 ^b	-	0.5	6.00 X 10 ⁻⁵	-
<u>Pseudomonas</u> (ND)	122,000 ^b 118,000 ^a	7.6 ^d	0.6	-	-
<u>Fusarium tricinctum</u> (-)	160 - 171,000 ^c	5.18 ^e	0.5	5.00 X 10 ⁻⁴	-
<u>Acinetobacter</u> (+)	130,000 ^a	8.43 ^e	0.9	4.80 X 10 ⁻⁶	5.80 X 10 ⁻⁶
<u>Glutaminasaflicans</u>	97,000 ^b				
<u>Proteus vulgaris</u> (+)	-	5.08 ^e	0.9	2.60 X 10 ⁻⁵	4.3 X 10 ⁻⁴
<u>Mycobacterium bovis</u> (ND)	-	-	-	2.60 X 10 ⁻³	-
<u>Wollinella (Vibrio)</u> (+)	146,000	-	26-31	4.78 X 10 ⁻⁵	-

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affinity for its substrate (K_m value of 1×10^{-5} M). The periplasmic location of such an enzyme is no doubt important for the cell, because if situated in the cytoplasm, it would be in direct competition with vital anabolic enzymes such as aminoacyl tRNA synthetases, and hence lead to a disruption of protein synthesis.

In contrast to the above, the L-asparaginase enzymes synthesised by Gram-positive bacteria show relatively poor affinity for L-asparagine, and as a consequence, do not have much effect upon the growth of tumours. The affinities of L-asparaginases from yeasts and lower fungi are generally a little higher, but do not match those of Gram-negative bacteria (Wade, 1980). The possession of a high affinity L-asparaginase enzyme by the latter group of microorganisms may indeed have some relevance in terms of bacterial growth and survival. Under conditions of unrestricted growth, there is little evidence to suggest that a high affinity L-asparaginase serves a vital cellular role. However, in unfavourable circumstances, such an enzyme may adopt a scavenging role, possibly conferring upon the Gram-negative group of bacteria the advantage of competing successfully against other microorganisms for a valuable source of energy and ammonia.

1.2.7.2 Physical properties

Purification and characterisation of the antileukaemic L-asparaginase II from Er. chrysanthemi NCPPB 1066 has revealed it to be a tetrameric protein composed of four identical subunits of molecular mass 35,000 containing no detectable carbohydrate or phosphate groups (Cammack et al., 1972; Miller et al., 1971 a,b). In this respect it is very similar to the E. coli L-asparaginase II enzyme, which is also a tetrameric protein with a subunit molecular mass of 32,500 (Wriston and Yellin, 1973).

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The subunits of the Er. chrysanthemi NCPPB 1066 and E. coli L-asparaginase II molecules are held together by noncovalent bonding forces and may be reversibly dissociated in urea and sodium dodecyl sulphate (SDS). The greater difficulty experienced in dissociating the subunits of the Er. chrysanthemi NCPPB 1066 enzyme molecule, suggests that the interaction of the subunits in the tetramer involves stronger secondary bonding forces than those that maintain the integrity of the E. coli enzyme (Cammack et al., 1972; Marlborough et al., 1975; Shifrin et al., 1973). This feature may go some way to explaining why less pronounced penetration of tissue and a greater confinement to the vascular system is displayed by the Erwinia enzyme (Hall, 1970; Riccardi, 1981). To date, there is no evidence to suggest that the dissociated subunits of either the Erwinia or E. coli enzyme possess any catalytic activity (Cammack et al., 1972).

Optical rotary dispersion (ORD) data indicates that both the Er. chrysanthemi NCPPB 1066 (Cammack et al., 1972) and the E. coli L-asparaginase II enzymes (Wriston and Yellin, 1973) are globular proteins with a small amount of ordered secondary structure in the form of alpha helix (20% and 10%, respectively). The Er. chrysanthemi NCPPB 1066 L-asparaginase II is, however, a more basic protein than the E. coli enzyme, containing less aspartic acid and rather more arginine. Further differences are also seen in the isoleucine, leucine and histidine contents. One most notable feature of the Er. chrysanthemi L-asparaginase II protein is the absence of cysteine residues, thereby excluding the possibility of inter- or intra-molecular disulphide bond formation within the molecule. In contrast, the E. coli L-asparaginase II protein is reported to have a single intra-molecular disulphide bond per subunit (Frank et al., 1970). Tryptophan is also absent from the Er. chrysanthemi NCPPB 1066 enzyme, but the tyrosine content is the same as

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that of E. coli (Cammack et al., 1972).

A number of factors are recognised as influencing the plasma half-life of L-asparaginase, which in turn affect its ability to reduce and maintain a low concentration of plasma L-asparagine. One widely recognised property that governs enzyme persistence is the molecular mass of the protein. Sedimentation-equilibrium (Cammack et al., 1972; Miller et al., 1971a,b) and X-ray crystallography techniques (North et al., 1969), have indicated that L-asparaginase II from Er. chrysanthemi NCPPB 1066 has a molecular mass of 135,000. This is close to the values obtained for other antileukaemic enzymes from guinea pig serum, S. marcescens and E. coli, all of which remain in the circulation for a much longer period than Yeast L-asparaginase, which has been found to be ineffective against sensitive neoplastic cells. The high molecular mass of the latter enzyme (800,000) is believed to encourage its rapid elimination by phagocytosis (Broome, 1965). Likewise, the formation of aggregates during the preparation of a clinically effective L-asparaginase has to be avoided for the same reason. In the case of E. coli L-asparaginase II, several different aggregates may form depending on the method of preparation (Cammack et al., 1972; Laboureur et al., 1971), and have also been found to be rapidly engulfed by macrophages. This in turn stimulates the formation of circulating antibody and hastens the development of hypersensitivity. The Er. chrysanthemi NCPPB 1066 enzyme on the other hand, does not show the same tendency to aggregate after being subjected to freeze-drying or dialysis (Ho and Millkin, 1970).

Since the L-asparaginase enzymes from Bacillus coagulans and Fusarium tricinctum are found to be rapidly cleared from the circulation, even though they have a molecular mass of 85,000 and 165,000 respectively, it has been proposed that other factors may also influence the residence time. One such

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factor is the overall charge of the protein. The L-asparaginase II from Er. chrysanthemi NCPPB 1066 has an unusually high isoelectric point (pI) of 8.6, similar to that seen for the L-asparaginase of Er. aroideae (pI 8.2) and Alcaligenes eutrophus (pI 8.6) (Wriston and Yellin, 1973). However, this distinguishes it from the majority of other L-asparaginases that have been isolated, including that of E. coli which has a pI value of either 4.9 or 5.4 depending on the strain of origin and the mode of preparation (Wriston and Yellin, 1973).

It has been observed that alteration of the isoelectric point of the Er. chrysanthemi NCPPB 1066 (Rutter and Wade, 1971) and E. coli (Wagner et al., 1969) L-asparaginase II proteins by chemical deamination, acetylation and carbodimide reactions with free amino groups, can alter their persistence in the blood. In the case of the Er. chrysanthemi NCPPB 1066 enzyme, over a range of isoelectric points between pH 3 and pH 10, a value of 6 correlates with the maximum residence time (Rutter and Wade, 1971). At present, the physiological basis for this phenomenon remains unclear.

1.2.3 Deleterious effects of L-asparaginase treatment

Clinical trials carried out in the United Kingdom have indicated that the L-asparaginase II enzyme from Er. chrysanthemi NCPPB 1066 and E. coli, are roughly comparable in their clinical efficacy (Beard et al., 1970). Although treatment with either enzyme can cause a number of troublesome side effects, the serious toxicity displayed by conventional chemotherapeutic agents is fortunately uncommon, and adverse reactions are reversible and dose-dependent. This complication appears to be less pronounced for the Er. chrysanthemi NCPPB 1066 L-asparaginase II enzyme (Adamson, 1971; Evans et al., 1982; Peto et al., 1988; Sutow et al., 1976), which should now ideally be

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regarded as the primary choice for leukaemia treatment.

1.2.8.1 Toxic reactions

In approximately 40% of cases, L-asparaginase therapy is usually accompanied by pronounced toxicity reactions including liver and pancreatic complications (Crowther, 1971; Haskell *et al.*, 1969). Other adverse reactions include, nausea; anorexia; decreased levels of serum albumin, haemoglobin, lipoprotein, fibrinogen and cholesterol; bone marrow depression; decreased insulin synthesis; impaired renal function; and central nervous system dysfunction (Whitecar *et al.*, 1970). These findings have led to the realisation that L-asparaginase can limit protein synthesis even in normal tissues, particularly when it is administered in high dosage. In the case of organs such as the liver and the spleen, susceptibility to L-asparaginase treatment may be explained by the relatively low L-asparagine synthetase levels present within the cells (Prager and Derr, 1971).

1.2.8.2 Immunological reactions

The most common reason for terminating L-asparaginase treatment is the development of an allergic reaction by the patient to this foreign bacterial protein. As many as 25% of all patients develop hyper-sensitivity reactions, including fever, hypotension and agitation, 9% of which are severe enough to be classified as anaphylactic (Uren and Handschumacher, 1977). This low incidence of reaction reflects the fact that L-asparaginase is a mild immuno-suppressive agent (Durden and Distasio, 1977; Schwartz, 1969; Wade and Rutter, 1970). In certain instances, circulating antibodies, not only inhibit the catalytic activity of the enzyme but can facilitate removal from the

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circulation (Baechtel and Prager, 1973; Capizzi et al., 1969; Peterson et al., 1971). Fortunately, the L-asparaginase II enzymes from Er. chrysanthemi NCPPB 1066 and E. coli possess different immunological specificities (MacLennan et al., 1971), and as such offer an important alternative therapy if a patient develops hyper-sensitivity to one or other of the enzymes (Evans et al., 1982; King et al., 1974; Ohnuma et al., 1973).

1.2.8.3 Substrate specificity and L-asparaginase toxicity

Although L-asparagine is the preferred natural substrate for the Er. chrysanthemi NCPPB 1066 enzyme, activity is also observed with a variety of other amino acids, including L-glutamine (9%, relative to activity with L-asparagine), D-asparagine (5%) and L-B-cyanoalanine (5.7%); as well as acid analogues such as B-aspartohydroxamic acid (19%) and small L-asparaginyl peptides (Howard and Carpenter, 1972). The E. coli L-asparaginase II also has similar biochemical features. However, on the whole, it is a less active hydrolase with a relative L-glutaminase activity of only 3%, for example (Cooney and Rosenbluth, 1975).

It is difficult to assign responsibility for the side-effects described above solely on L-asparagine depletion, because in general they are seen only at dosage levels that have been shown to deplete L-glutamine as well (Uren and Handschumacher, 1977). Indeed, many investigators have implied that the intrinsic L-glutaminase activity displayed by the Er. chrysanthemi NCPPB 1066 and E. coli L-asparaginase preparations may, in fact, be the cause of immuno-suppression, although this has not been fully substantiated (Durden and Distasio, 1980). In this regard, it is interesting to note that both the L-asparaginase from guinea pig serum, which lacks detectable L-glutaminase activity, and the L-asparaginase from Wolinella (Vibrio) succinogenes, which

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has an L-glutaminase activity 130-600 fold less than E. coli enzyme (Distasio and Niederman, 1976), are non immuno-suppressive, and do not cause panereo- or hepatotoxicity (Gallagher et al., 1989).

Because the W. succinogenes enzyme is also immunologically distinct from E. coli L-asparaginase II, it may also prove extremely beneficial in the treatment of acute lymphoblastic leukaemia. Moreover, there is currently great interest at C.A.M.R. in cloning and sequencing the structural gene encoding the W. succinogenes L-asparaginase enzyme, since it is hoped that a comparison of the predicted W. succinogenes L-asparaginase polypeptide with the Er. chrysanthemi L-asparaginase II protein may lead to the identification of specific amino acid residues which account for the intrinsic glutaminase activity of the latter enzyme.

1.2.9 Overcoming L-asparaginase side-effects

Much effort has been concentrated on finding a means of reducing the antigenicity of L-asparaginase as well as extending the turnover time in the plasma. This has included chemical modification with the use of polyethylene glycol, attachment to dextran polymers and poly-D,L-alanine peptides (Uren and Ragin, 1979) or entrapment within nylon microcapsules (Siu Chong and Chang, 1974), acrylic microspheres (Edman and Sjöholm, 1981, 1983), liposomes (Neerunjun and Gregoriadis, 1976) and red blood cells (Urdike et al., 1976). Extracorporeal devices employing immobilised L-asparaginase have also been used (Allison et al., 1972; Horvath et al., 1973). Rather disappointingly, such techniques have resulted in an altered ability of the enzyme to deplete the plasma L-asparagine concentration rapidly. Insufficient positive data have therefore meant that they have not been used in a clinical context.

1.3 L-ASPARAGINASE REGULATION

In the majority of cases where it has been studied, the synthesis of a particular bacterial L-asparaginase appears to be influenced by conditions of growth. In general, in batch culture, these enzymes are produced at maximum levels towards the end of the logarithmic phase or in the stationary phase of growth. Physiological studies concerning the effect of growth conditions on L-asparaginase synthesis have yielded contrasting and sometimes contradictory data, strongly implying that many different mechanisms of control are involved in the regulation of enzyme expression. The following sections will therefore summarise the affects of differing physiological growth conditions on microbial L-asparaginase synthesis, whilst at the same time highlighting some of the molecular mechanisms which are thought to govern such physiological responses.

1.3.1 The effect of oxygen

When enteric bacteria such as E. coli and Salmonella typhimurium undergo the transition from aerobic to anaerobic growth, the synthesis of many proteins is repressed, whilst a specific class of approximately 50 proteins is induced (Clark, 1984; Strauch et al., 1985). In E. coli strains K12 (Cedar and Schwartz, 1968), B (Boeck and Ho, 1973), W (Resnick and Magasanik, 1976), ATCC 9637 (Svobodova and Serbanova-Necinova, 1973), 316/66 (Wade, 1980) and A1 (Barnes et al., 1977), anaerobiosis has been shown to induce L-asparaginase II synthesis upto 100-fold. Hence, this particular enzyme is a member of the latter class of proteins. In this instance, it has been suggested that L-asparaginase II could play a role in the anaerobic respiration of the cell by catalysing the formation of L-

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aspartic acid from L-asparagine, which in turn serves a precursor of the anaerobic electron acceptor, fumarate (Cedar and Schwartz, 1968; Svobodova and Serbanova-Necinova, 1973). Although it has been observed that L-asparagine and L-aspartate can replace fumarate in supporting anaerobic growth of *E. coli* on glycerol, this theory has not been fully substantiated.

More recent studies by Jerlstrom *et al.* (1987) have demonstrated that *E. coli fnr⁻* mutants grown anaerobically in a complex medium containing 0.2% (w/v) galactose, contain either 20% or less than 2% (depending on the mutant) of the L-asparaginase II activity compared to their *fnr⁺* counterparts. Furthermore, transformation of the *fnr⁻* derivatives with plasmid pGS24, harbouring the wild-type *E. coli fnr⁺* structural gene, resulted in high levels of anaerobically synthesised L-asparaginase II activity, several times higher than those seen in wild-type *fnr⁺* strains. From this evidence it can be concluded that the synthesis of L-asparaginase II in *E. coli* is positively controlled by the product of the *fnr* gene, Fnr.

The Fnr protein itself, has been extensively studied and is believed to be a sequence-specific DNA binding protein that functions as a global regulator, either activating or repressing transcription (reviewed by Spiro and Guest, 1990; Unden and Trageser, 1991). Indeed, Fnr displays striking homology at the level of amino acid sequence and predicted secondary structure to another global transcriptional regulator, namely the cAMP receptor protein, Crp (Shaw *et al.*, 1983). Further homology has also been noted between Fnr and the HylX protein of *Actino-bacillus pleuropneumoniae*, a proposed regulator of haemolytic activity, the product of the *orf20* gene of *Rhizobium leguminosarum*, involved in regulating microaerobic nitrogen fixation, and the FixK protein of *Rhizobium melliloti*, which is also involved in regulating nitrogen fixation (Unden and Trageser, 1991).

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Crp is a dimeric DNA binding protein composed of two identical subunits of 210 amino acids, each of which contains a structural and functional domain. The larger amino-terminal domain carries a site that interacts with cAMP and is involved in subunit-subunit interaction, whereas the smaller carboxy-terminal region contains a helix-turn-helix motif resembling a DNA binding domain (Busby, 1986; de Crombrughe *et al.*, 1984). The Fnr protein contains a homologous C-terminal domain (residues 196 to 219) which is presumed to interact with specific nucleotide sequences. The presence of a nucleotide-binding domain for Fnr has also been inferred (residues 54 to 104), although it differs from Crp in that cAMP binding residues are not conserved and cAMP is not bound.

Nucleotide sequence analysis of several Fnr regulated promoters has revealed the presence of a conserved region of dyad symmetry 5' AAANTGATNNNNATCAANTTT 3', occurring approximately 30 bp from the transcriptional start point, which is proposed to be the binding site for FNR (Bell *et al.*, 1989; Eiglmeier *et al.*, 1989). This Fnr consensus sequence is almost identical to the consensus sequence present in Crp regulated promoters (5' AANTGTGANNTNNNTCANATT 3'), which in contrast, occurs at varying distances from the transcriptional start site (Busby, 1986; Ebright *et al.*, 1984; Gaston *et al.*, 1990). This strongly suggests that like Crp, the Fnr protein binds as a dimer with each subunit recognising one arm of the repeat sequence. The similarity between Fnr and Crp is further illustrated by the fact that a single nucleotide substitution in the sequence TTGAT is sufficient to switch an Fnr-dependent promoter to Crp-dependence, and vice-versa for the TGTGA motif in the Crp binding site (Bell *et al.*, 1989). Furthermore, site-directed mutagenesis leading to the alteration of amino acid residues in the DNA-binding domain of Fnr and Crp can in turn change

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the binding specificity of the protein from one binding site to the other (Spiro et al., 1990).

The way in which Fnr senses anoxia and consequently activates transcription of genes such as the L-asparaginase II structural gene of E. coli, is not fully understood at present. However, site-directed mutagenesis data has indicated that a cysteine-rich amino terminal domain (residues 16 to 29) is essential for Fnr function (Spiro and Guest, 1988; Melville and Gunsalus, 1990; Sharrocks et al., 1990). Since this domain displays homology with metal binding domains of metal proteins (Cys-X₂₋₄-Cys-X₂₋₁₅-Cys-X₂₋₄-Cys; where X is any amino acid) (Berg, 1986), the interaction of a metal ion, such as Fe²⁺, has been suggested to be involved in regulating Fnr activity (Spiro et al., 1989; Uden and Trageser, 1991). This remains to be fully substantiated.

In addition to the aforementioned E. coli strains, the availability of oxygen appears to regulate the synthesis of L-asparaginase in a number of other microorganisms. For example, low oxygen conditions stimulate L-asparaginase synthesis by S. marcescens (Heinemann and Howard, 1969) and Bacillus cadaveris (Wade, 1980). Whether a transcriptional activator homologous to the Fnr protein is involved in this case, or some other mechanism, is not known. Indeed, not all anaerobically-inducible genes have been shown to be subject to the control of the Fnr protein. Studies by Birkmann and colleagues (1987) for example, have demonstrated that the sigma factor RpoN is required for the anaerobic induction of the Fnr-independent genes encoding formate dehydrogenase (fdhF) and hydrogenase isoenzyme 3 (hyd), both which contain functional -24/-12 promoter consensus sequences (see Section 1.4.2) characteristic of RpoN-regulated genes (Birkmann et al., 1987; Lutz et al., 1990). Moreover, a number of workers

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have presented experimental evidence clearly demonstrating the fact that environmental 'stresses' such as anaerobicity can alter the negative supercoiling of cellular DNA (Dorman et al., 1988; Higgins et al., 1988). This in turn appears to be responsible for regulating the expression of a number of specific genes which appear to be independent of activators such as Fnr (Ni Bhariain et al., 1989)

In contrast to the above, it is thought that the levels of available oxygen have no effect upon L-asparaginase production in Er. chrysanthemi NCPPB 1066 (Dr D. Cossar; personal communication). Similarly, L-asparaginase synthesis is unaffected by oxygen in E. coli strain HAP (Roberts et al., 1968); Er. aroideae NRL-B-138 (Liu and Zajic, 1973), Proteus vulgaris strain OUT8226 (Tosa et al., 1971), Citrobacter strain C6 (Bascomb et al., 1975), and Klebsiella aerogenes strain W-70 (Resnick and Magasanik, 1976).

1.3.2 The effect of carbon

As with other periplasmic hydrolytic enzymes such as acid hexose phosphatase and 2',3'-cyclic phosphodiesterase (Kler et al., 1977), the synthesis of the Er. chrysanthemi NCPPB 1066 L-asparaginase II enzyme has been shown to be subject to catabolite repression. Callow and colleagues (1971) have demonstrated that the synthesis of L-asparaginase II in Er. chrysanthemi NCPPB 1066 can be repressed to varying degrees by carbon substrates such as glucose, glycerol, mannitol, lactate and Kreb's cycle intermediates. Only L-glutamate has so far been found to be non-repressive (Callow et al., 1971). More recently, Gilbert and coworkers (1986) have reported that the expression of the cloned Er. chrysanthemi NCPPB 1066 ansB gene is also subject to catabolite repression in E. coli. However, in contrast to the regulatory control of L-asparaginase II synthesis in Erwinia, expression

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of the cloned gene was not repressed by glycerol in E. coli, thus inferring that the mechanisms of glycerol and glucose repression of the Erwinia L-asparaginase II are distinct.

Likewise, the production of L-asparaginase in E. coli strain K12 (Cedar and Schwartz, 1968) and strain W (Wade, 1980), as well as Proteus vulgaris (Wade, 1980), S. marcescens (Heinemann and Howard, 1969), Pseudomonas boreopolis (Wade, 1980) and Staphylococcus sp. (Wade, 1980) is also repressed in the presence of glucose.

In the case of E. coli, there have been a number of reports suggesting that, in addition to Fnr regulation, L-asparaginase II synthesis in E. coli is also subject to positive control by the cAMP receptor protein, Crp (Chesney, 1983; Russell and Yamazaki, 1978).

The activity of the Crp is modulated by the levels of cAMP within the cell. When cells experience carbon limitation, the intracellular levels of cAMP are raised, resulting in the formation of a cAMP-Crp complex. Upon binding of cAMP, the Crp dimer undergoes a conformational change which allows the cAMP-Crp complex to interact with specific DNA sequences at target promoters (Busby, 1986; de Crombrughe et al., 1984; Gronenborn et al., 1988). The effects of such cAMP-CRP binding are varied. For example, the cAMP-Crp complex may activate transcription at a number of different stages, depending on the promoter (Gaston et al., 1990). In contrast, the cAMP-Crp complex may also repress transcription. These two functions can be independent or, as is the case of the gal operon regulatory region, they can occur simultaneously (Busby and Buc, 1987). Moreover, the cAMP-Crp complex may act synergistically with other activators such as the AraC and MalT proteins to activate transcription of the araBAD and malK-lamB and malE,F and G operons, respectively (de Crombrughe et al., 1984).

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Unlike the examples listed above, the presence of glucose does not appear to influence L-asparaginase expression in K. aerogenes strain W-70 (Resnick and Magasanik, 1976), and in the case of E. coli B derivative C532.5, a natural phage-resistant derivative of ATCC 13706, the presence of glucose has actually been found to stimulate L-asparaginase expression (Boeck *et al.*, 1970; Boeck and Ho, 1973). Galactose also stimulates enzyme synthesis in C532.5, but to a lesser extent than glucose; whilst fructose, glycerol, arabinose and lactate produce a marginal positive response (Boeck and Ho, 1973)

1.3.3 The effect of nitrogen

The variable response of bacterial L-asparaginase synthesis to environmental factors such as oxygen level and carbon source, can also be seen with differing sources of nitrogen. Although it has been reported that ammonium ions do not affect the synthesis of L-asparaginase II in Er. chrysanthemi NCPPB 1066 (Minton *et al.*, 1986), this does not appear to be the case with P. vulgaris, which reduces the synthesis of L-asparaginase in the presence of ammonium ions (Tosa *et al.*, 1971). In the case of Azotobacter vinelandii, ammonium ions have actually been found to stimulate the synthesis of L-asparaginase in cells grown in a defined medium (Gaffar and Shetha, 1977). whilst in Wolinella succinogenes, even though L-asparaginase is synthesised constitutively throughout the growth phase, the presence of ammonium ions increases the overall level of enzyme within cells grown in complex medium (Albanese and Kafkewitz, 1978).

Further differences have been noted by Resnick and Magasanik, (1976), whilst directly comparing E. coli K12 and K. aerogenes grown in minimal glucose-glutamate medium with or without ammonium ions. For E. coli K12,

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addition of ammonium sulphate (0.2% w/v final concentration) stimulated L-asparaginase synthesis approximately two-fold, compared to cells grown in the absence of ammonium ions. In contrast, the level of L-asparaginase was reduced 10-fold when A. vinelandii was grown in the presence of ammonium sulphate. In this instance, evidence suggests that this particular enzyme is under control of the Ntr regulatory system (Section 1.4.2).

In certain instances, L-asparagine may induce L-asparaginase synthesis. For example, Svobodova and Strbanova-necinova, (1973) have reported that L-asparagine stimulates L-asparaginase synthesis approximately two-fold in E. coli strain AT9637, ML30 and B, cultured in minimal glycerol medium under anaerobic conditions. In contrast, L-asparagine does not act as an inducer for the synthesis of L-asparaginase in Er. chrysanthemi NCPPB 1066 (Minton et al., 1986), or E. coli A-1, a met⁻ derivative of strain HAP (Barnes et al., 1977). In E. coli K12, L-asparaginase II levels are low when cells were grown in defined medium with L-asparagine as the sole source of nitrogen (Cedar and Schwartz, 1968). At this point, growth becomes dependent on the low affinity cytoplasmic L-asparaginase (Willis and Woolfolk, 1974). On the other hand, rapid growth of K. aerogenes under these conditions is dependent on the high affinity periplasmic L-asparaginase (Resnick and Magasanik, 1976).

Aspartate has been reported to induce the synthesis of L-asparaginase in P. ovalis and E. coli strain 316/66 (Wade, 1980), but not in Er. chrysanthemi NCPPB 1066 (Gilbert et al., 1986).

Finally, workers have reported that glutamate has a stimulatory effect on L-asparaginase synthesis in a number of bacteria including Pseudomonas sp. (Wade, 1980), Er. chrysanthemi (Callow et al., 1971), E. coli strain HAP (Roberts et al., 1988), strain 316/66 (Wade, 1980) and strain A-1 (Barnes et al., 1977). However, no such effect is seen with P. vulgaris (Wade, 1980).

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1.4 THE Er. CHRYSANTHEMI NCPPB 1066 L-ASPARAGINASE II STRUCTURAL GENE

The structural gene encoding L-asparaginase II (ansB) has been isolated from the Er. chrysanthemi NCPPB 1066 genome on a 4.7 kb chromosomal EcoRI fragment (Gilbert et al., 1986), and the complete nucleotide sequence determined (Minton et al., 1986). Likewise, the ansB genes from Er. chrysanthemi 1125 (Filpula et al., 1986) and E. coli strains JM108 (Bonthon, 1990) and MC4100 (Jennings and Beacham, 1990) have also been cloned and sequenced. In the case of E. coli strain JM108, the ansB gene was mapped to approximately 63.8 minutes on the genetic map (Bonthon, 1990). Recently, Sun and Setlow (1991) have reported the cloning and sequencing of the L-asparaginase structural gene (designated ansA in this instance) from B. subtilis. This appears to be situated immediately upstream of, and is co-transcribed with, the L-aspartase structural gene.

The Er. chrysanthemi NCPPB 1066 ansB gene is now known to lie immediately upstream of the shikimate kinase structural gene (Minton et al., 1989), and consists of an open reading frame of 1044 bp, commencing with an ATG start codon and ending at a TGA stop codon. The latter lies immediately upstream of a GC-rich region of dyad symmetry (ΔG , -25 kcal) followed by a T-rich region of DNA, characteristic of an E. coli rho-independent transcriptional terminator (Figure 1.3).

1.4.1 The coding region

Translation of the derived nucleotide sequence indicates that the Er. chrysanthemi NCPPB 1066 ansB gene has a capacity to encode a predicted 332 amino acid protein of molecular mass 32 kDa. The codon utilisation of the Er. chrysanthemi NCPPB 1066 ansB gene shows no overall bias for codons

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Figure 1.3 The Er. chrysanthemi NCPPB 1066 L-asparaginase II (ansB) structural gene

Figure 1

The complete nucleotide sequence of the Er. chrysanthemi NCPPB 1066 L-asparaginase II (ansB) structural gene is shown opposite. The signal peptide sequence is highlighted by the heavy arrows. Three regions of dyad symmetry (I/I', II/II', III/III') are indicated by facing arrows. The position of the putative Crp and -24/-12 regions are indicated by alignment with the respective consensus sequences below and above the relevant region. Nucleotide homology to these sequences is indicated by the symbols : (for NTR) and (for Crp); while mismatch is highlighted by lower-case letters in the consensus sequence. N refers to any nucleotide, and W to either an A or T residue. Putative '-35' and '-10' regions are indicated by lines above and below the sequence. The putative ribosome-binding site (Shine-Dalgarno sequence) is marked S.D., whereas the stop codon is indicated by an asterisk.

Reproduced from Minton et al. (1986).

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Figure 1.3 The Er. chrysanthemi NCPPB 1066 L-asparaginase II (ansB) structural gene

The complete nucleotide sequence of the Er. chrysanthemi NCPPB 1066 L-asparaginase II (ansB) structural gene is shown opposite. The signal peptide sequence is highlighted by the heavy arrows. Three regions of dyad symmetry (I/I', II/II', III/III') are indicated by facing arrows. The position of the putative Crp and -24/-12 regions are indicated by alignment with the respective consensus sequences below and above the relevant region. Nucleotide homology to these sequences is indicated by the symbols : (for NTR) and (for Crp); while mismatch is highlighted by lower-case letters in the consensus sequence. N refers to any nucleotide, and W to either an A or T residue. Putative '-35' and '-10' regions are indicated by lines above and below the sequence. The putative ribosome-binding site (Shine-Dalgarno sequence) is marked S.D., whereas the stop codon is indicated by an asterisk.

Reproduced from Minton et al. (1986).

Figure 1.3

TC

GC
EH
I
GA

GC
P#

CT

F
TT

G
GG
45)

L
CT

G
GGK

S
AGK

G
GGC

P
CCA
75C

H
AAC

V
GTG

L
CTG

Y
TAC

L
CTG
110

Y
TAT

S
TCA

I
ATC

V
GTT

T
ACC
140C

TTCC

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which are indicative of high expression. Equally, it appears to make little use of the so-called 'modulator' codons that correspond to minor tRNAs in E. coli, and in this sense it is unlike weakly expressed regulatory genes, such as that encoding the AraC protein of Er. carotovora (Minton et al., 1986).

1.4.1.1 Primary structure

Residues 22 to 46 of the predicted Er. chrysanthemi NCPPB 1066 L-asparaginase II protein have been found to exhibit a perfect match to the first 25 amino acids of purified L-asparaginase from Er. chrysanthemi NCPPB 1066. This not only confirms the derived nucleotide sequence, but also suggests that the L-asparaginase II protein is processed post-translationally. In this respect, the 21 amino acids preceding the mature amino terminus are believed to represent the signal peptide, since they closely resemble sequences identified at the amino-terminus of proteins which are secreted (Perlman and Halvorson, 1983; von Heijne, 1984,1986). This is consistent with the fact that the Er. chrysanthemi NCPPB 1066 L-asparaginase II enzyme is located in the periplasmic space in both E. coli and Er. chrysanthemi NCPPB 1066 (Gilbert et al., 1986). Similarly, the homologous E. coli L-asparaginase II protein of E. coli (Bonthron, 1990; Jennings and Beacham, 1990) and the ASP3 gene product of Saccharomyces cerevisiae (Kim et al., 1988) have also been shown to possess a cleavable secretory signal peptide, whereas, as expected, the cytoplasmic L-asparaginase I of E. coli does not (Jerlstrom et al., 1989).

Alignment of the predicted primary amino acid sequence of the Er. chrysanthemi NCPPB 1125 and 1066 L-asparaginase II polypeptides has revealed them to be identical except for four conservative substitutions at position 156 (Ile ↔ Leu), position 178 (Arg ↔ Lys), position 267 (Leu ↔

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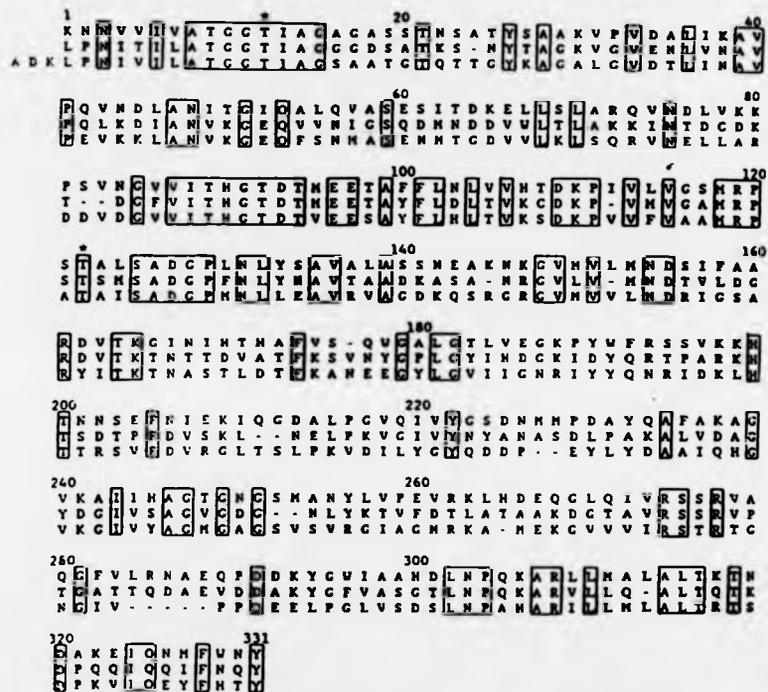
Met) and position 274 (Met ↔ Ile) (Filpula et al., 1988). A comparison of the predicted primary amino acid sequence of the mature Er. chrysanthemi NCPPB 1066 L-asparaginase II polypeptide, with the primary sequence of the E. coli A1-3 L-asparaginase II (Malta et al., 1974) and the Acinetobacter glutaminasificans L-asparaginase-glutaminase proteins (Tanaka et al., 1988) also reveals homology (Figure 1.4). In the case of the E. coli polypeptide, 143 amino acid residues are identical to those found in the Er. chrysanthemi NCPPB 1066 protein, and 43 similar. The A. glutaminasificans polypeptide is homologous to a slightly lesser degree, with 137 residues being identical and 32 being similar (Tanaka et al., 1988). Overall, the greatest proportion of homology for all three polypeptides is seen within the amino terminal region.

A number of regions appear to be perfectly conserved in all three of the aforementioned enzymes and may have some significance in terms of enzyme activity. This appears to be indeed the case with a conserved domain, situated close to the amino terminus which consists of eight amino acids (ATGGTIAG) (Figure 1.4). This motif can also be found within the amino terminus of the Pseudomonas 7A glutaminase-asparaginase and P. vulgaris enzymes (Tanaka et al., 1988), and, as such, has been described by Harms and colleagues (1991) as an L-asparaginase 'signature'. Holcenberg et al., (1978) have shown that the threonine residue, situated at position 12 within the conserved amino terminal domain of the A. glutaminasificans and P. vulgaris glutaminase-asparaginase enzymes, interacts with the glutamine analogue, DON (6-diazo-5-oxonorvaline), and have provided evidence to suggest that the DON binding site is also part of the catalytic site for L-asparagine and L-glutamine.

A slightly different situation has been noted for the corresponding threonine residue of the E. coli L-asparaginase II enzyme, which does not

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Figure 1.4 Alignment of the primary amino acid sequence of the Er. chrysanthemi NCPPB 1066 L-asparaginase II, E. coli L-asparaginase II and A. glutaminasificans L-asparaginase-glutaminase polypeptides.



The upper sequence represents the A. glutaminasificans L-asparaginase-glutaminase and is numbered. The middle and lower sequences represent the E. coli and the Er. chrysanthemi NCPPB 1066 L-asparaginase II polypeptides, respectively. Amino acids residues are denoted by the single letter code, and residues that are identical in all three proteins are boxed. Gaps have been introduced to maximise the alignment.

Reproduced from Tanaka et al. (1988).

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interact with the diazo ketone analogue of L-asparagine, DONV (5-diazo-4-oxo-L-norvaline) (Handschumacher, 1977). Instead, this compound is thought to bind to either Thr-118 or Ser-119 (Tanaka et al., 1988). More recent work carried out by Harms et al., (1991) has shown that the substitution of the Thr-12 residue within the E. coli L-asparaginase II polypeptide with alanine, using site-directed mutagenesis, results in a mutant polypeptide which displays 0.01% of the catalytic activity of the wild-type enzyme. Since the tertiary and quaternary structure of the mutant polypeptide was found to be unaffected by the substitution, and L-aspartic acid was found to bind normally, these workers have proposed that the E. coli threonine-12 is involved in catalysis but not substrate binding.

In addition to the above, a second perfectly conserved region of eight amino acid residues (VITHGTDI) occurs in all three of the polypeptides highlighted in Figure 1.4. However, the significance of this region has not been examined to date. Other identical residues can be found between positions 85 and 139 and 302 and 331, as illustrated in Figure 1.4.

1.4.1.2 Secondary structure

A comparison of the predicted secondary structures for the Er. chrysanthemi NCPPB 1066 L-asparaginase II, E. coli L-asparaginase II and the A. glutaminasificans L-asparaginase-glutaminase polypeptides also reveals a high degree of similarity; with 5 alpha-helices and 11 beta-strands of a minimum of 3 residues, predicted to be in similar positions (Tanaka et al., 1988).

1.4.2 The non-coding region

Promoters are regions of DNA to which RNA polymerase binds and

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initiates the transcription of DNA to RNA. Although it has been demonstrated that RNA polymerase binds asymmetrically to a region of DNA, stretching from a point approximately 44 to 50 bp upstream of the start of transcription to a point around 20 bp downstream, promoter recognition in bacteria is governed by two conserved hexanucleotide sequences, TTGACA and TATAAT, centered approximately 35 bp and 10 bp preceding the site of transcription initiation, respectively. These recognition sites are believed to interact directly with RNA polymerase and have been aptly designated the '-35' region and the '-10' region (or Pribnow box) (Lewin, 1983).

Analysis of the 5' non-coding DNA region upstream of the *Er. chrysanthemi* NCPPB 1066 *ansB* ATG start codon by Minton and colleagues (1986) has revealed no sequences displaying striking homology to the consensus '-10' and '-35' promoter sequences. These authors have, however, highlighted two regions of potential regulatory significance. The first of these is located at nucleotide position 142 of the published sequence and shows homology to the Crp consensus sequence. The second is located at position 162 and displays homology to the -24/-12 consensus sequence characteristic of promoters that are dependent on the minor form of RNA polymerase sigma factor, sigma 54 (also designated RpoN), encoded by the structural gene *rpoN* (also called *glnF* or *ntrA*) (Figure 1.3).

Bacterial sigma factors interact reversibly with the core RNA polymerase, consisting of four subunits in the stoichiometry $BB'\alpha_2$, to form a holoenzyme ($BB'\alpha_2\sigma$). Upon binding, the sigma factor reduces the capacity of the core RNA polymerase to interact with loose DNA-binding sites and catalyses its binding to promoter regions. The specificity of this interaction is controlled by the particular sigma factor involved, and most likely results from a sequence-specific contact between the sigma factor

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itself and the promoter (Doi and Wang, 1986; Helman and Chamberlain, 1988).

RpoN was first identified as a positive regulatory factor required for the expression of genes and operons which are positively controlled by the global nitrogen-regulatory (Ntr) system in enteric bacteria, under nitrogen-limiting conditions (Dixon, 1987; Kustu *et al.*, 1986; Magasanik, 1982; Merrick, 1987). This included the operon containing the glutamine synthetase gene (glnA) (Kustu *et al.*, 1986; Reitzer and Magasanik, 1987), the nitrogen fixation (nif) operons of Klebsiella pneumoniae (Dixon, 1984, 1987; Gussin *et al.*, 1986), and genes encoding amino acid transport components and degradative enzymes (Magasanik, 1982). Recently however, it has been demonstrated that RpoN is also required for the transcription of a set of genes which are not controlled by nitrogen availability, and whose products have diverse physiological functions. Examples include the dctA gene of rhizobia, encoding a transport component for dicarboxylic acids, genes on the P. putida TOL (toluene) plasmid involved in the catabolism of toluene and xylenes, genes encoding pilins of P. aeruginosa and Neisseria gonorrhoeae, and genes encoding hook and filament proteins of Caulobacter flagella (Kustu, 1989; Thony and Hennecke, 1989). Hence, it is clear that although RpoN is an essential component for transcription of Ntr-regulated promoters, it should by no means be considered a nitrogen-specific sigma factor. This is highlighted by the fact that studies with the rpoN structural gene of K. pneumoniae fused to 'reporter' genes encoding tetracycline-resistance (tet) and B-galactosidase (lacZ) (de Bruijn and Ausubel, 1983; Merrick and Stewart, 1985), as well as the E. coli rpoN structural gene fused with bacteriophage Mu dl (Ap lacZ). (Castano and Bastarrachea, 1984), have shown that the rpoN gene itself is transcribed constitutively at low level within the

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cell, and is not subject to regulation in response to nitrogen status.

It has been found that recognition complexes between RpoN-containing RNA polymerase and the promoter are closed and non-productive transcriptionally due to the fact that the DNA remains double-stranded. Initiation of transcription therefore requires an 'activator' protein, which catalyses the isomerisation of closed complexes to transcriptionally active open complexes where the DNA strands are locally denatured in the region of the transcriptional start site. All RpoN-dependent promoters described to date have an absolute requirement for an 'activator'. The transcription of the dct gene, xylCAB genes and the xylS gene for example, require the DctD and XylR 'activator' proteins, respectively. In the case of Ntr-regulated genes, initiation of transcription requires the bifunctional DNA binding protein NtrC (also designated NRI) encoded by the ntrC gene, which has the capacity to act as both an activator and repressor (Ames and Nikaido, 1985). Such 'activators' provide a common mechanism which allows the expression of RpoN-dependent promoters under diverse physiological conditions (Kustu, 1989).

All of the aforementioned activator proteins display strong homology in a 'central' domain spanning approximately 240 amino acids which is presumed to reflect a conserved mode of transcriptional activation. This domain, which is believed to interact directly with RpoN-containing RNA polymerase bound at the promoter, contains a predicted nucleotide-binding site (Drummond et al., 1986; Ronson et al., 1987) displaying homology to a glycine-rich phosphate binding loop characteristic of several ATP and GTP-binding proteins (consensus : G-X-X-G-X-G-K-E) (Saraste et al., 1990). Hence, it seems likely that the energy required for open complex formation is derived from the hydrolysis of ATP. Indeed, in vitro studies with a mutant form of S.

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typhimurium NtrC for example, have demonstrated an ATP-requirement for open complex formation (Popham et al., 1989). Moreover, a mutation in the proposed nucleotide-binding site of NtrC, replacing serine (170) with an alanine residue, has been shown to abolish the ability of this protein to form open complexes, even though it can still interact normally with bound RpoN-containing RNA polymerase (Austin et al., 1991).

The function of activator proteins, themselves, appears to be controlled by a variety of mechanisms. For example, the ability of the NtrC protein to activate transcription is regulated positively and negatively by phosphorylation and dephosphorylation, respectively. This modification is controlled by the action of a second protein NtrB (also designated NRII), encoded by the ntrB (or glnL) gene (Magasanik, 1988). Both proteins are now known to belong to a particular family of regulatory proteins which form so-called 'two component systems'. Such systems have been identified and characterised in many Gram-negative and Gram-positive species, and regulate a wide range of processes including outer membrane protein expression (EnvZ/OmpR), sporulation (SpoIIJ/SpoA,SpoF), phosphorous assimilation (PhoB/PhoR) and chemotaxis (CheA/CheY,CheB) (Stock, 1989,1990).

One component of the system functions as a histidine protein kinase (HPK) that acts as an environmental sensor transmitting a signal to a second component, the response regulator (RR). This in turn effects the response, usually by activating transcription. The process of signal transduction involves the sequential transfer of phosphoryl groups from ATP to histidine residues of the HPK protein, then from the HPK phosphohistidine side chains to aspartic acid residues in the RR, and ultimately from the RR phosphoaspartate side chains to water (Stock, 1990). The activator DctD is also a member of a two component system and is presumed to be phosphorylated by a second

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protein DctB (Kustu et al., 1989).

Proteins which comprise the histidine kinase family (eg. NtrB), are characterised by a conserved domain covering approximately 100 amino acids, generally situated near the carboxy terminus. There are additional sequence similarities surrounding a conserved tyrosine residue which precedes the C terminal domain by approximately 100 residues, and is presumed to be the site of autophosphorylation. The proteins belonging to the response regulator family on the other hand (eg. NtrC), are characterised by a conserved domain covering approximately 100 amino acid residues that usually extends from the amino terminus (Stock, 1989,1990).

NtrB-mediated phosphorylation of NtrC takes place under nitrogen-limiting conditions within the amino terminal domain and is presumed to result in a conformational change which stimulates the ability to bind DNA, and is required for the activation of transcription. Although phosphorylated NtrC has the capacity for auto-dephosphorylation (Keener and Kustu, 1988), it is also rapidly dephosphorylated by NtrB. This latter reaction requires ATP and an additional small protein, P_{II} encoded by the glnB gene, which interacts with NtrB. The ability of P_{II} to interact with NtrB is in turn determined by the action of a bifunctional enzyme encoded by the glnD gene, whose activity is controlled by the balance between 2-ketoglutarate and glutamine within the cell. Under conditions of nitrogen-limitation the ratio of ketoglutarate:glutamine is high and the glnD gene product functions as a uridylyltransferase (UT) that catalyses the uridylylation of tyrosine residues of P_{II}. This modified P_{II} protein is unable to interact with NtrB, and therefore NtrC remains phosphorylated and active. Conversely, under nitrogen excess conditions, the ketoglutarate : glutamine ratio is relatively low and the glnD product has uridylyl-removing (UR) activity. Unmodified P_{II}

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interacts with NtrB, leading to the dephosphorylation of NtrC, thereby halting transcription activation (Magasanik, 1988; Reitzer and Magasanik, 1987).

Other activators such as NifA and XylR appear to be controlled by a mechanism other than the phosphorylation seen for two component systems. This is reflected in the fact that NifA and XylR do not share homology in their amino-terminal domains to the activator proteins that have been shown to be members of the two component regulatory systems (Thony and Hennecke, 1989). Moreover, unlike NtrC, the amino-terminus of NifA is not absolutely required for transcriptional activation (Drummond et al., 1990), and the NifA protein of R. trifolii lacks the amino-terminal domain altogether (Iismaa and Watson, 1989).

In the case of XylR, direct binding of low molecular weight substrates of the xylene pathway is believed to lead to protein activation. Moreover, NifA from K. pneumoniae is synthesized in an active form which is inactivated by the protein NifL by an as yet unknown mechanism; whilst NifA from Rhizobium meliloti and Bradyrhizobium japonicum does not require NifL for inactivation (Kustu et al., 1989)

In many instances, but not all, optimal expression of RpoN-dependent promoters requires the binding of an activator to sites located at least 80 bp upstream of the -24/-12 consensus sequence. These have been termed upstream activator sequences (UAS) and are believed to function in a similar manner to eukaryotic enhancer elements. Hence, they are functional when placed up to 2 kb upstream of the transcription start site and can function independently of orientation (Dixon, 1987). For NtrC the consensus UAS motif is GCAC-N₆-GGTGCA; whilst for NifA it is TGT-N₁₀-ACA (Dixon, 1987). Evidence from in vivo and in vitro experimentation suggests that the process of NifA and NtrC activation may involve bending of the DNA

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between the upstream and downstream sites, thus increasing the local concentration of activator protein near to the RpoN-holoenzyme when it is at a low concentration (Dixon, 1987).

Where activation of transcription of an RpoN-dependent promoter is not strictly dependent on upstream DNA, as is the case with the fixA and fixR genes of B. japonicum and the argT gene of S. typhimurium (Thony and Hennecke, 1989), it has been proposed that the activator may interact directly with the RpoN-RNA polymerase complex.

All RpoN-dependent promoters so far studied at a molecular level, appear to lack the canonical '-10' and '-35' consensus sequences recognised by RNA polymerase holoenzyme containing the major sigma factor, sigma 70 (RpoD). Instead they are characterised by a conserved GGCACNNNNNTGCA motif, located between -26 and -11 with respect to the point of transcription initiation (Coppard and Merrick, 1991), and this constitutes the recognition site for RpoN-containing RNA polymerase.

Within the conserved motif itself, the GG and GC dinucleotides at -24 and -12 are separated by one turn of the helix and are invariant. The importance of such residues for promoter function has been confirmed by site-directed mutagenesis of the nifL and nifH promoters of K. pneumoniae (reviewed by Dixon, 1987). Moreover, Buck (1986) has reported that deletion of a single non-conserved nucleotide between the conserved dinucleotides of the rpoN-dependent nifH promoter of K. pneumoniae can eliminate transcriptional activation of the nifH promoter. This suggests that the spacing between the conserved dinucleotides is critical and that deletion of bases within the spacer region may disrupt a protein-DNA interaction which involves the simultaneous recognition of bases around positions -24 and -12.

The putative Er. chrysanthemi NCPPB 1066 ansB -24/-12 region contains

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the invariant -24 GG dinucleotide, but is atypical in the fact that the invariant -12 GC dinucleotide is replaced by a GA dinucleotide. This may be reconciled by the presence of an A residue at position -12 in the Rhizobium phaseoli (Quinto, et al., 1985) and Rhizobium trifolii (Scott et al., 1983) nifH -24/-12 promoter regions; therefore this situation is not unprecedented. Moreover, Buck et al. (1985) have demonstrated by mutational analysis of the Klebsiella pneumoniae nifH promoter, that a replacement of the C residue with an A residue unalters transcriptional activation of this gene by NifA.

The 5' region of the Er. chrysanthemi NCPPB 1125 ansB gene is almost identical to that of NCPPB 1066, with an identical putative -24/-12 region. It must be noted however, that the equivalent NCPPB 1125 Crp region has a T residue in place of a G residue at position 4 of the highly conserved TGTGA motif. This change occurs at a position known to be important for Crp binding, since site-directed mutagenesis to create a T residue instead of a G residue at position 4 has been shown to significantly reduce Crp binding (Jansen et al., 1987). A similar situation is seen with a putative Crp binding site identified in the promoter region of the E. coli ansB structural gene (Jennings and Beacham, 1990). It is a formal possibility that CRP may tolerate the aforementioned changes or, even though a putative binding site has been identified, Crp may act indirectly on the aforementioned promoter regions.

In addition to the above, Minton et al. (1986) also located two regions of dyad symmetry at positions 47 and 231, with the capacity to form a hairpin loop of G -16 and -14 kcal, respectively. Whether such structures play any significant role in the regulation of L-asparaginase expression is presently not known.

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Finally, it is of interest to note that nucleotide sequence analysis of the 5' non-coding region of the ansB structural gene isolated from the L-asparaginase II hyper-producing Er. chrysanthemi NCPPB 1066 industrial production strain, has revealed it to be identical to the 5' region of the ansB gene from the wild-type strain. This suggests that extragenic mutation(s) is responsible for increased L-asparaginase II synthesis in the former derivative (Dr. N.P. Minton; personal communication).

1.5 THE GENUS ERWINIA

The Erwinia genus belongs to the Enterobacteriaceae, and is composed of a diverse collection of Gram-negative, rod-shaped, non-spore-forming facultative anaerobic bacteria, which are motile by peritrichous flagella (Lelliott and Dickey, 1984). Many of the Erwinia species are pathogenic towards humans and animals, or are associated with plants as either opportunistic pathogens, saprophytes, or constituents of the epiphytic flora (Starr and Chatterjee, 1972; Lelliott and Dickey, 1984). Of all the Erwinia species, those belonging to the 'soft rot' group (which includes Er. chrysanthemi NCPPB 1066), have attracted the most interest.

1.5.1 The soft rot erwinias

The 'soft rot' group of erwinias are distinguished by their ability to cause a number of plant diseases, both in culture and in storage, such as soft rot, black leg and stem rot (Chatterjee and Starr, 1980; Perombelon, 1982), leading to crop losses estimated to total \$50-\$100 million annually on a worldwide basis (Perombelon and Kelman, 1980). As a consequence of their agricultural importance, this group has been the most extensively studied of

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all Erwinia species.

The soft rot erwinias essentially comprise four species; Er. carotovora, Er. chrysanthemi, Er. cyripedii and Er. rhapontici. However, as relatively little is known of the latter two species, the collective term "soft rot" erwinias is usually taken to represent Erwinia chrysanthemi and Erwinia carotovora. The latter species is further subdivided into the three subspecies, carotovora, atroseptica and betavasculorum. The taxonomy of the species named has been examined (Dickey, 1979) and their ecology reviewed (Perombelon, 1982; Perombelon and Kelman, 1980). Although these organisms are related both biochemically and serologically, they can nevertheless be differentiated in a number of ways as highlighted in Table 1.3.

The major pathogenicity determinants of the soft rot Erwinia group have been shown to be the extracellular pectinase (pectate lyase, pectin lyase and polygalacturonase), cellulase and protease enzymes synthesised by these bacteria, which cause maceration of plant cell walls (Chatterjee and Vidaver, 1986; Kotoujanski, 1987). A number of other extracellular enzymes including phospholipases, hemicellulases, arabinases and amylases have been reported to be synthesised, but have not been vigorously studied (Kotoujanski, 1987). As a consequence, Erwinia provides the geneticist with a doubly important model system with which to investigate both the molecular mechanisms of protein translocation and the fundamental principles of plant pathogenicity.

Aside from the academic challenges offered by Erwinia, there is also growing interest in this genus from industry. In addition to L-asparaginase, other enzymes and compounds produced by Erwinia species may prove to be industrially or medically useful. This is notably illustrated in the case of vitamin C (L-ascorbic acid) production. Until recently, most vitamin C was

Table 1.3 Biochemical differentiation of the 'soft rot' erwinias

Test	<u>Er. carotovora</u> subsp.		<u>Er.</u> <u>chrysanthem</u>
	<u>carotovora</u>	<u>atroseptica betavascularum</u>	
Pectate degradation	+	+	+
Gelatin liquifaction	+	+	V
Pigment production	-	-	V ^a
Sensitivity to erythromycin	-	-	+
Phosphatase production	-	-	+
Reducing substances from sucrose	-	V	V
Acid production from :			
glucose	-	-	+
maltose	-	V	-
trehalose	+	+	-
palatinose	-	+	-
alpha-methyl glucoside	-	V	-
cellobiose	+	+	V
Utilization of malonate	-	-	+
Utilization of tartarate	-	-	V
Growth at 36 - 37°C	+	-	+

Key : + indicates that 80% or more of tested strains were positive.
 - indicates that 20% or less of tested strains were positive.
 V indicates that 79 to 21% of tested strains were positive.

^a Blue pigment produced by certain strains of Er. chrysanthem when cultivated on YDC or PDA medium.

Data reproduced from Dickey and Kelman (1985). In "Laboratory guide for identification of plant pathogenic bacteria" (draft copy). Ed. N.W. Schaad.

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produced by the Reichstein-Grusser synthesis; a lengthy and capital intensive route that involves five complicated chemical steps and one fermentation. A number of Erwinia species are capable of oxidising D-glucose to 2,5 di-keto-D-gluconic acid (2,5-DKG). This compound is readily converted to 2-keto-L-gulonic acid (2-KLG), the last intermediate in the Reichstein-Grusser synthesis, by a number of organisms from the coryneform group of bacteria, via the enzyme 2,5-KDG reductase. Workers at Genentech and Biogen have introduced the 2,5-KDG reductase structural gene from Corynebacterium sp. into Er. herbicola (Anderson *et al.*, 1985) and Er. citreus (Grindley *et al.*, 1988) respectively, thereby achieving the synthesis of 2-KLG from D-glucose in a single fermentation. As this compound is easily converted to L-ascorbic acid by a simple acid or base-catalysed cyclisation, such recombinant Erwinia strains offer a more straightforward means of vitamin C production. The projected economics of this process are, however, only just competitive with the Reichstein-Grusser process. Consequently, such genetically engineered Erwinia strains are not yet in commercial use, although this situation may well change in the near future.

Since large amounts of pectinase, cellulase and protease enzymes are widely used in industry (Priest, 1984; Price and Stevens, 1989; Wiseman, 1983) the extracellular enzymes synthesised by Erwinia species may also prove to be of benefit. Mixtures of crude pectolytic enzymes from Aspergillus and Penicillium strains are currently used commercially in the food industry to clarify fruit juices and to increase the yield of apple and grape fruit (Rombouts and Pilnick, 1980). These enzymes have also found use in olive oil production, in the preparation of baby food puree and fruit nectar bases, and for the elimination of mucilage surrounding freshly picked coffee beans. Cellulases have a number of commercial applications,

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particularly in the breakdown of various cellulose-rich waste products. Proteases from microorganisms, such as Bacillus subtilis and Aspergillus niger, are widely used in the leather industry for tanning, in the textile industry to remove proteinaceous substances surrounding fibres such as silk, and in the photographic industry to hydrolyse the gelatin base of used photographic films in order to recover their silver content. Bacterial alkaline proteases are also included in biological detergents because of their tolerance to high pH and temperature. A thermotolerant Er. chrysanthemi protease has been discovered which may perhaps be put to a similar use.

Arai and Wantanabe (1986) have proposed a process for the freeze texturing of proteins using Er. ananas cells as ice-forming nuclei. This technique has been actively studied by many researchers and technologists to produce meat analogues from plant materials. Other industrial processes that could interest the food industry include the use of immobilised Er. carotovora cells for the production of N-acetyl-L-methionine (Nishida et al., 1984), and the use of immobilised Er. rhapontici cells to produce the artificial sweetener isomaltulose (palatinose) from sucrose (Cheetham, et al., 1985).

Finally, certain Erwinia strains could prove beneficial to the pharmaceutical industry. For example, Er. carotovora AJ2992 has been found to produce significant amounts of ribavirin from ortidine and tricarboxylic acid (TCA) (Shirae et al., 1988). This compound is recognised as a potent antiviral agent and has a broad antiviral spectrum. A number of other Erwinia species are known to synthesise a range of antimicrobial compounds which could perhaps be exploited. The production of broad and narrow host range antibiotics (Ishimaru et al., 1988; Parker et al., 1982; Winkelman et al., 1980) and bacteriocins (Stein and Beer, 1980) is common amongst strains

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of Er. herbicola, and has been exploited to achieve in planta protection against Fire Blight disease caused by Er. amylovora.

1.5.2 Erwinia genetics

The desire to understand the mechanisms of Erwinia pathogenicity, including a study of the synthesis, regulation and export of the pectinases, cellulases and proteases, has necessitated the development of a variety of genetic systems with which to analyse such processes at a molecular level. Clearly, the same genetic tools would also have to be employed in order to gain an understanding of the molecular mechanisms which regulate the synthesis of L-asparaginase II in Er. chrysanthemi NCPPB 1066.

The application of molecular genetic systems to Erwinia, has been fairly straightforward and relatively simpler than for phytopathogenic Pseudomonas and Xanthomonas species or plant-associated Agrobacterium and Rhizobium species. To date, it has proven possible to adapt a range of techniques, originally developed for E. coli, for various Erwinia strains due to the genetic relatedness between the two organisms.

The types of genetic tools/systems that are currently available have been described (Chatterjee and Starr, 1980; Van Gijsegem, 1987; Leary and Fulbright, 1982; Mills, 1985; Salmond, 1987; Starr and Chatterjee, 1972). The most significant developments, particularly in the context of classical in vivo genetic analysis are : 1) transpositional mutagenesis; 2) introduction of cloning vectors (including those containing the narrow-host-range Col E1 replicon), via transformation or conjugation; 3) sensitisation to bacteriophages such as lambda, Mu and P1; 4) marker-exchange mutagenesis; 5) in vivo cloning of large regions of the chromosome using, for example, the RP4::mini-Mu plasmid pULB113; and 6) the construction of gene fusions,

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suppressor mutants and recA strains. This, coupled with the more recent in vitro recombinant DNA technology, has brought Erwinia genetics into a very productive era. Apart from extracellular enzyme regulation and secretion, some of the details of cellulose and pectate degradation have also been defined (Mills, 1985), and biochemical and genetic aspects of lactose and melibiose metabolism studied in Er. chrysanthemi (Gray et al., 1986; Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1985 b).

It must, however, be noted that all the aforementioned genetic systems exhibit great strain dependence, particularly transposon mutagenesis (Zink et al., 1984). This fact is recognised amongst Erwinia researchers but has not been well documented in the literature. It must also be emphasised, that another great problem in Erwinia genetics lies in the variety of strains of Er. chrysanthemi and Er. carotovora that are used in different laboratories. It is known that strains of the same species can be fairly dissimilar genetically, as is the case with the genome organisation of Er. chrysanthemi strains B374, 3937j, and EC16 (Van Gijsegem, 1987). Hence, information that is derived from one particular strain may not relate directly to another.

The following sections will now outline the range of genetic systems that have been applied to a variety of soft rot Erwinia species.

1.5.3 Gene transfer systems

1.5.3.1 Conjugation

Conjugation involves the transfer of genetic material from one bacterium to another by direct physical contact (Willettts, 1988). To date, a number of studies dealing with the conjugal ability of soft rot Erwinia species have been performed. Chatterjee and Starr (1972 a) for example, were one of the first to demonstrate that F-plasmids, harbouring the lac⁺

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gene from E. coli (F'lac⁺), could be transferred inter-generically from E. coli to naturally occurring Lac⁻ derivatives of Er. herbicola, Er. chrysanthemi and Er. amylovora, and inter-specifically between Erwinia strains, by conjugation. Although the frequency of this transfer was relatively low compared to the transfer of lac⁺ from E. coli F' donor to E. coli F-recipient, in most cases (except for Er. chrysanthemi), such plasmids were stably maintained in Erwinia and rendered the heterogenotes sensitive to the F-specific bacteriophage M13.

Methods similar to those used in F-prime plasmid transfer have also been employed to introduce broad-host-range IncP plasmids (eg. RP1, R68.45, RK2, Rts1) into a number of Erwinia species (Chatterjee and Starr, 1972 b; Cho et al., 1975; Coplin, 1978; Gibbins et al., 1976; Lacy, 1978; Perombelon and Boucher, 1978). In this instance, plasmid genes conferring resistance to a variety of antibiotics have been used to select transconjugants.

On rare occasions, either spontaneously or in the presence of a plasmid curing agent such as acridine orange, F'lac⁺ plasmids have the capacity to integrate into the bacterial chromosome at sites of homology, and can subsequently promote the transfer of chromosomal DNA into recipient cells in a polarised fashion (ie. some genes are transferred at high frequency, whilst others are transferred at low frequency). This phenomenon has led to the isolation of so-called 'Hfr' donor strains (analogous to the E. coli system for high frequency of recombination) of Er. amylovora (Chatterjee and Starr, 1973; Pugashetti and Starr, 1975; Pugashetti et al., 1978) and Er. chrysanthemi (Chatterjee and Starr, 1977), and has enabled the mapping of genes by time of entry (interrupted matings), gradient of marker transmission and linkage analysis (two and three factor crosses). Moreover, these techniques have allowed the introduction of specific genes into new genetic

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backgrounds.

R plasmids also have the capacity to promote chromosome transfer, albeit at low frequency, and tend to transfer shorter regions of the genome compared to the Hfr system (Leary and Fulbright, 1972). However, despite this fact, they have nevertheless allowed the mapping of chromosomal genes by analysing co-inheritance data. (Chatterjee, 1980)

In order to achieve efficient mobilisation of chromosomal markers, bacteriophage Mu, antibiotic-resistant transposons and IS elements have been inserted into both F and R plasmids and into the genome, to serve as sites of homology for recombination. Using the thermo-sensitive episome F'(ts)::Tn10 lac⁺ for example, a series of Hfr strains of Er. chrysanthemi were isolated as temperature-resistant Lac⁺ derivatives (Kotoujansky et al., 1982). In this case, random integration of the plasmid occurred via replicon fusion mediated by the transposon; hence, each of the Hfr strains had a different origin of transfer and the capability to transfer chromosomal genes at frequencies similar to those observed for E. coli.

In addition, the IncP1 R plasmid R68.45, containing two copies of the active insertion sequence IS21, has been employed to readily transfer genes in Er. carotovora and Er. chrysanthemi, but not Er. amylovora (Chatterjee, 1980; Giumareas et al., 1978; Lacy, 1978; Perombelon and Boucher, 1979). Moreover, pULB113, an RP4-based plasmid harbouring a bacteriophage Mu derivative, Mu3A, has also been successfully used to mobilise a large number of chromosomal markers in Er. chrysanthemi, Er. amylovora, Er. carotovora subsp. carotovora and Er. carotovora subsp. atroseptica (Chatterjee et al., 1985; Schoonejans and Toussaint, 1983).

1.5.3.2 Transformation

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Transformation may be defined as the introduction of DNA into competent bacterial cells and its subsequent maintenance in the population (Smith and Danner, 1981). A transformation system is an essential tool for the introduction of non-conjugative plasmids into recipient strains. Furthermore, it allows the introduction of recombinant DNA molecules into the strain of interest, thus permitting its use as a host for subcloning and other in vitro manipulations without having to turn to E. coli as an intermediate.

Because of the aforementioned facts, there have been several attempts to develop efficient transformation systems for different Erwinia species. However, the success has been variable (Bauer and Beer, 1983; Berman et al., 1983; Hamamoto and Murooka, 1987; Lacy and Sparks, 1979; Lindow and Staskawicz, 1981; Reverchon and Robert-Baudouy, 1985). The basis for induction of competence in Gram-negative bacterial cells is poorly understood, and therefore the development of a transformation system in a new species requires an empirical approach.

A method yielding relatively efficient transformation of plasmids pAT153, pBR322 and pBR325 ranging from 1×10^2 to 4×10^4 colonies μg^{-1} of plasmid DNA, has been developed by Hinton et al. (1985 b) for both Er. carotovora subsp. carotovora SCRI 193 and Er. carotovora subsp. atroseptica SCRI 31. This system has been used for direct shotgun cloning of Erwinia DNA by complementation of transposon-induced mutants; however again, the efficiency of this process does display strain-dependence.

1.5.3.3 Electroporation

Electroporation (or electropermeabilisation) refers to the process of subjecting a suspension of living cells to a rapidly changing high strength

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electric field, thereby producing areas of reversible local disorganisation and transient breakdown ('pores') within the outer membrane. During the lifespan of the 'pores', diffusion and exchange of intracellular and extracellular components can take place (Chassy et al., 1988; Miller, 1988; Sowers and Lieber, 1986).

Electroporation has been used to render eukaryotic cells permeable to nucleic acids (Fromm et al., 1985; Potter et al., 1984; Toneguzzo and Keating, 1986) and to facilitate the fusion of protoplasts to create multinuclear eukaryotic cells (Zimmerman, 1983). More recently, this technique has been applied to the transformation of a wide range of Gram-negative and Gram-positive bacteria (Chassy et al., 1988; Fiedler and Wirth, 1988). Transformation frequencies reported for electroporation range from 10^4 to 10^5 transformants μg^{-1} of DNA for Gram-positive bacteria (Chassy and Flickinger, 1987, Powell et al., 1988) and up to 10^9 to 10^{10} μg^{-1} for Gram-negative bacteria (Dower et al., 1988; Miller, 1988).

In addition, electroporation has provided a means of extracting plasmid DNA from bacterial cells (Calvin and Hanawalt, 1988; Dower et al., 1988), as well as promoting the curing of plasmids (Heery et al., 1989) and allowing the inter and intraspecific transfer of plasmid DNA by a process termed 'electroduction' (Pfau and Youderian, 1990; Summers and Withers, 1990).

To date, electroporation has been used to transform a number of Erwinia species, including Er. stewartii and Er. herbicola (Chassy et al., 1988). Berman et al. (1987) successfully transformed Er. carotovora subspecies carotovora strain EC14 with plasmids pBR322 and pBR325 at a frequency of 1.3×10^3 transformants μg^{-1} using electroporation. Moreover, Ito and colleagues (1988) reported that plasmids pBR329 and pNN101 could be introduced into Er. carotovora strain Er by electroporation, at a frequency

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of approximately 2×10^7 transformants μg^{-1} of DNA. Unfortunately the effectiveness of this method was limited by the fact that it was applicable only to strain Er and not Er. carotovora strains T29 and Ar. These results indicate that electroporation may prove to be a valuable tool for the Erwinia geneticist, enabling the introduction of large non-conjugative plasmids that cannot be used to transform competent cells and those unable to be transferred with conjugative helper plasmids. However problems will undoubtedly arise in the future, due to the strain-dependence of this technique. The factors that will most likely determine the 'electrotransformability' of a particular strain of Erwinia are its cell size, chain length and degree of aggregation (Knight and Scrutton, 1986); the membrane composition, the presence of extracellular exonucleases; and strain-specific variables affecting plasmid establishment and maintenance.

1.5.3.4 Transduction

The process of bacteriophage-mediated transfer of bacterial DNA is known as transduction, and represents a powerful tool for bacterial strain construction and fine structure mapping of genes (Masters, 1985). A number of bacteriophages, including P1 and P22, are capable of performing so-called 'generalised' transduction, whereby any chromosomal gene can be transduced from donor to recipient. In contrast, other bacteriophages such as lambda, are able to carry out 'specialised' transduction, in which the only genes transduced are those adjacent to the chromosomal site of bacteriophage integration. These are occasionally incorporated in the bacteriophage genome during aberrant excision of the prophage (Stanisich, 1988).

Despite the occurrence of both virulent and temperate bacteriophages that are able to infect Erwinia species (Starr and Chatterjee, 1972), the

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transductional mode of gene transfer is only available for a limited number of Erwinia strains. Screening collections of naturally occurring bacterial isolates for the production of bacteriophages, following induction by either mitomycin C and UV irradiation, has led to the discovery of two generalised transducing bacteriophages for Er. chrysanthemi. The first, designated Echr-12, was originally isolated by Chatterjee and Brown (1980) and has been found to be capable of transducing an array of unlinked chromosomal markers between strains of Er chrysanthemi EC183 at frequencies ranging from 10^{-7} to 10^{-8} . This value can be increased approximately 50-fold by UV. irradiation of the Echr-12 lysate prior to infection (Chatterjee et al., 1981).

The second generalised transducing bacteriophage, designated phi EC-2, infects Er. chrysanthemi strains B374 and 3937j and is capable of transducing unlinked markers at a frequency ranging from 10^{-6} to 10^{-8} . In addition it has been used to demonstrate linkage between the thr and car genes in strain B374 (Resibois et al., 1984). A detailed analysis of the physical and genetic properties of phiEC2 has been performed (Resibois et al., 1984), and transposon carrying derivatives constructed (Hinton, 1986). Subsequently, phi EC-2 has been used for strain construction, plasmid transduction and analysis of mutants of strain B374 (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1985; Van Gijsegem et al., 1985 a). It has also been used to isolate bacteriophage resistant mutants which are currently being analysed for outer membrane alterations leading to enzyme export defects and loss of virulence (Schoonejans et al., 1987 a).

Until recently, no novel transducing bacteriophages had been isolated for either Er carotovora subsp. carotovora or Er. carotovora subsp. atroseptica. However, two bacteriophages, designated phiKP and phiM1, have now been demonstrated to transduce a range of chromosomal markers and

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plasmids between derivatives of Er. carotovora subsp. carotovora SCRI 193, ATCC 39048 and Er. carotovora subsp. atroseptica SCRI 1043, respectively (I. Toth; personal communication). The main drawback with all the Erwinia transducing bacteriophages is their limited host range; although this need not be a problem if all genetic analysis is restricted to a few sensitive strains.

Aside from the Erwinia bacteriophages described above, the bacteriophage Mu has also been shown to mediate gene transfer by generalised transduction, at a frequency of 10^{-7} (Bade et al., 1978; Howe, 1973). Moreover, certain strains of Er. carotovora subsp. carotovora and subsp. atroseptica have been found to possess a functional T4 receptor (the core structure of lipopolysaccharide). As such, they have been found to have the capacity to allow the adsorption of T4, and, in certain instances, sustaining lytic growth (Pirhonen and Palva, 1988). All of the aforementioned T4-sensitive strains were also found to be receptive to the generalised transducing derivative, T4GT7; thereby allowing the transduction of plasmids pBR322 and pBR325 from E. coli into the T4-sensitive Erwinia strains at a frequency ranging from 5×10^{-8} to 3×10^{-5} transductants per input bacteriophage particle.

1.5.4 Mutagenesis

Mutant isolation has proven extremely useful in the study of innumerable systems throughout biology. Conventional chemical and radiation mutagenesis techniques are relatively simple to perform and have been successfully applied to a number of Erwinia species, allowing the isolation of a range of missense and nonsense mutants (Andro et al., 1984; Chatterjee and Starr, 1977; Hinton et al., 1985 a; Hugouvieux-Cotte-Patatt et al., 1986;

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Kotoujanski et al., 1982; Rella et al., 1989). Coupled with this has been the generation of nonsense suppressor (sup⁻) mutants of Er. carotovora subsp. carotovora (Hinton et al., 1985 a) and Er. chrysanthemi (Schoonejans et al., 1987 b). Combination of the two will undoubtedly prove to be invaluable for the study of many different functions of Erwinia, as has been the case for E. coli and its bacteriophages (eg. for the study of operons; bacteriophage morphogenesis and the identification of genes encoding essential cellular functions).

Despite the advantages outlined above, one particular drawback of chemical or radiation mutagenesis is the possibility that multiple mutations may occur, thus complicating further genetic analysis. As a consequence of this fact, there has been an ongoing effort to develop insertion mutagenesis techniques for Erwinia, involving both transposons and bacteriophage Mu derivatives.

1.5.4.1 Transposon mutagenesis

Transposon mutagenesis is potentially one of the most powerful techniques available for investigating the structure and function of bacterial genes. (Kleckner, 1977). Bacterial transposable elements may be defined as mobile genetic entities which have the capacity to promote their own movement and integration into new target sites, independently of host cell recombination systems, and without the requirement for extensive areas of homology (for reviews see Craig and Kleckner, 1987; Kleckner, 1990).

Although bacterial transposable elements are diverse both in size and functional organisation, they can be essentially divided into three distinct classes : insertion sequences (or IS elements), transposons and transposing bacteriophages.

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Insertion elements are the simplest form of transposable element (700-1500 bp in size), and consist solely of a transposase gene(s) responsible for target-site selection, replication and transposition, flanked by perfect or near-perfect inverted repeat sequences of 10 - 40 bp. Transposons, on the other hand, are larger composite transposable elements which contain IS elements as an inverted or direct repeat at their termini. The intervening transposon DNA often encodes resistance to antibiotics or heavy metals; the ability to catabolise sugars, aromatics or hydrocarbons; the biosynthesis of amino acids; or the production of virulence factors (Craig and Kleckner, 1987; Duggleby *et al.*, 1990; Kleckner, 1990).

In addition to molecular structure, transposable elements can be further classified on the basis of their mode of transfer. Class I transposable elements include both the IS elements and the composite transposons, which transpose via a conservative (cut-and-paste) pathway. Class II elements are the Tn3-like family of transposons which transpose using the replicative pathway. Of the naturally-occurring transposable elements, the most commonly used for insertional mutagenesis have been the Class I composite transposons, Tn5, Tn9 and Tn10 and the class II transposons Tn3 and Tn1000 (gamma-delta).

The temperate bacteriophage Mu (Toussaint, 1985), represents a third class of transposable element that has been widely used by geneticists. This is due to its ability to integrate randomly and at high frequency within the host genome as an obligatory stage of both the lytic cycle and in initiating lysogeny (Mizuuchi and Craigie, 1986). Such transposition can result in insertion mutations as well as the inversion, deletion and duplication of DNA associated with other transposons.

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1.5.4.2 Advantages offered by transposon mutagenesis

Transposons offer a number of advantages over chemical or radiation mutagens, because of their capacity to insert randomly into a large number of sites at high frequency leading to the generation of stable mutations by insertional inactivation of a gene. This can cause a complete loss of function, whilst simultaneously providing a selectable marker (eg. antibiotic resistance) that can serve both as a genetic marker for mapping purposes and as a physical marker for subsequent manipulation of the target gene. It is then theoretically possible to clone the interrupted gene and to use this DNA to identify and to isolate the wild-type allele in the native strain. These properties are particularly important when trying to analyse genes encoding a non-selectable phenotype such as pathogenicity or virulence (Chatterjee *et al.*, 1983).

Further examples of the utility of transposons include the ability to generate both point mutations and genome rearrangements (duplications, deletions, inversions and translocations), and the characterisation of operon organisation and regulation (Berg and Berg, 1987). Moreover, numerous transposon derivatives carrying an array of genetic markers have been engineered to provide tools for specialised genetic analysis. These mobile markers include origins of conjugative transfer, different antibiotic resistance genes, fusion elements to monitor gene expression or allow the production of chimaeric proteins, polylinker restriction enzyme sites, portable regions of homology for *in vivo* recombination and origins of replication and DNA sequencing primers to enable the direct sequencing of the target region (Duggleby *et al.*, 1990).

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1.5.4.3 Suicide vehicles

Generalised transposon mutagenesis is frequently accomplished using a transposable element which resides on a so-called 'suicide vector', which can enter a recipient cell but fails to be maintained. During the transient period within the recipient cell, the transposon can transpose at a certain frequency to a site within the genome of the new host. Examples of suicide vehicles include bacteriophage P1 (see Section 1.5.4.3, b), P22 (Shanabruch et al., 1981), narrow-host-range plasmids (Ely, 1985; Simon et al., 1983 a) , plasmids containing the Mu prophage (see Section 1.5.4.3, a) and the lambda bacteriophage carrying nonsense mutations in the DNA replication genes (Simon et al., 1989). Alternative strategies have been to displace a resident plasmid by incompatibility (Elmerich, 1983) and to use plasmids which are temperature-sensitive for replication (Harayama et al., 1981; Laird and Young, 1980; O'Hoy and Krishnapillai, 1985).

1.5.4.3(a) The bacteriophage Mu

Despite the extensive use of Mu in the genetic analysis of sensitive Erwinia strains, an important limitation of this bacteriophage stems from its narrow-host-range. As a consequence of this fact, broad-host-range plasmids such as RP4, have served as a delivery vehicle for the interspecies transfer of this bacteriophage (Denarie et al., 1977). However, the presence of Mu on such plasmids has been found to greatly reduce their frequency of transfer compared to parental plasmids (Forbes and Perombelon, 1985; Zink et al., 1984), and cause instability in certain Gram-negative bacteria other than E. coli (Hirsch and Beringer, 1984). This basis for this phenomenon is not well understood, but is believed to be a consequence of either abortive replication of Mu, the action of host restriction systems or a Mu-encoded function that

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is lethal to the bacterial cells (Zink et al., 1985). Such instability of broad-host-range Mu containing plasmids has enabled them to serve as suicide vehicles for the introduction of transposons into the genome of a range of bacterial species including Erwinia. For example, the Mu-containing plasmid pJB4JI, which confers gentamycin-resistance and carries Tn₅ inserted in the Mu prophage (Beringer et al., 1978) has been widely used to generate insertional mutations in a variety of Erwinia species, including Er. chrysanthemi (Chatterjee et al., 1983), Er. carotovora subsp. carotovora (Zink et al., 1984, Hinton, 1986), Er. carotovora subsp. atroseptica (Zink et al., 1984), Er. amylovora (Steinberger and Beer, 1984), and Er. herbicola (Gantotti et al., 1981). These have been isolated mainly at loci that control biosynthetic and catabolic functions.

Unfortunately, in certain instances, the transposition events may be accompanied by insertion of Mu DNA, thus complicating genetic analysis and subsequent direct cloning experiments. Moreover, the applicability of pJB4JI varies dramatically between strains of Erwinia, (Hinton et al., 1987; Zink et al., 1984), however this may not be a problem if genetic analysis is confined to susceptible bacteria.

1.5.4.3(b) The bacteriophage P1

To some extent, the problem of Mu-resistance of Erwinia strains can be avoided by using a hybrid Mu bacteriophage which has the host range of a second bacteriophage, namely P1 (Csonka et al., 1981). The latter is able to infect E. coli and closely related enterics, however certain strains may be resistant. It is nevertheless possible to isolate P1-sensitive mutants of different bacterial strains, including those of Erwinia, by using P1-derivatives carrying antibiotic resistance genes (Goldberg et al., 1974; Jayasawal et al.,

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1984; Murooka and Harada, 1979; Quinto and Bender, 1984; Streicher et al., 1975). The sensitive bacteria allow adsorption of the P1-derivatives and the injection of DNA, but rarely provide the host functions necessary for replication. This therefore leads to the formation of antibiotic-resistant lysogens which can be readily detected on selective media.

Transposon mutagenesis may be achieved in P1-sensitive derivatives by superinfection of a P1 lysogen with a second incompatible P1 bacteriophage harbouring a transposable element. Upon infection, the transposon-carrying P1 bacteriophage is lost from the cell, leading to transposition of the transposon into the chromosome (Quinto and Bender, 1984). This super-infection protocol has been shown to be applicable to a range of Erwinia species (Hinton et al., 1987).

1.5.4.3(c) The bacteriophage lambda

The bacteriophage lambda has proven to be a powerful tool for molecular genetic analysis, and is commonly used as a vehicle for transposon mutagenesis, generation of in vivo gene fusions and cosmid cloning. Unfortunately, the power of lambda technology has been severely limited by the fact that its host range does not normally extend beyond that of E. coli K12. Although methods for the selection of lambda-sensitive mutants of other organisms are not available, this problem has been overcome by the introduction of the lamB structural gene of E. coli into certain species on multicopy vectors.

The lamB gene is part of the malK-lamB operon, situated in the malB region of E. coli (Raibaud et al., 1979; Silhavy et al., 1979) and encodes a single protein product which is a major constituent of the outer membrane. The LamB protein is generally classified as a porin (Hall and Silhavy, 1981),

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which serves as a receptor for several coliphages including lambda (Randall-Hazelbauer, 1973; Rao, 1979; Wandersman and Schwartz, 1978), and participates with maltose-binding protein (MalE) in the transport of maltose and maltodextrins across the outer membrane (Bavoll and Nikaido, 1981; Wandersman et al., 1979). Other species such as S. typhimurium possess LamB homologues, but these do not appear to be functional as lambda receptors (Palva, 1978).

Plasmids harbouring lamB have been used to create lambda-sensitive derivatives of a wide variety of bacteria, such as S. typhimurium (de Vries et al., 1984; Harkki and Palva, 1985; Palva et al., 1981), K. pneumoniae (de Vries et al., 1984; Kornacker, 1988), K. aerogenes (Bloom and Tyler, 1979); Vibrio cholerae (Harkki et al., 1986), and the non-enteric Agrobacterium tumefaciens, Rhizobium meliloti and P. aeruginosa (Ludwig, 1987). Such derivatives do not normally support the growth of lambda. However, the introduction of the nusA gene of E. coli (Holowachuk and Frieson, 1982; Kurihara and Nakamura, 1983) coding for a protein which affects the termination and anti-termination of transcription of lambda, to a lamB⁺ strain of S. typhimurium, allows absorption of lambda in this bacterium (Harkki and Palva, 1984; Harkki et al., 1987).

Plasmids carrying lamB have also been used successfully in a wide variety of Erwinia species (Ellard et al., 1989; Salmond et al., 1986; Steinberger and Beer, 1988). Despite the simplicity and wide-applicability of this technique for Erwinia, it must be noted that the success of this approach has been shown to be very strongly strain dependent (Ellard et al., 1989). In the instances where the construction of lambda-sensitive Erwinia strain has been possible, this bacteriophage has acted as a suicide vehicle for Tn5 and T_{phoA} mutagenesis, and allowed the subsequent isolation of

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auxotrophic and extracellular enzyme mutants of Er. carotovora subsp. carotovora SCRI 193 (Hinton and Salmond, 1987; Salmond et al., 1986), and reduced virulence mutants of Er. carotovora subsp. atroseptica (Hinton et al., 1989).

1.5.5 In vitro mutagenesis

In addition to the random insertion of transposons into Erwinia genes, in a number of cases, in vitro recombinant DNA technology has been used to mutate specific cloned Erwinia genes. Roeder and Collmer (1985) for example, have inactivated the pelC structural gene from Er. chrysanthemi CUCPB 1237 with a DNA fragment encoding a kanamycin-resistance determinant.

1.5.6 The cloning of Erwinia genes

To date, a large number of Erwinia genes have been cloned using both in vivo and in vitro techniques. In the majority of cases, E. coli has acted as a surrogate host for both the propagation and amplification of Erwinia genes, as well as the direct inter-generic complementation of mutants.

1.5.6.1(a) In vivo cloning

The in vivo cloning of Erwinia genes, has made use of RP4::mini-Mu plasmids, particularly pULB113. In addition to the capacity to mobilise the entire chromosome, pULB113 can resolve to form R-prime plasmids which carry part of the host genome. Whilst integrated in the chromosome, pULB113 is located between two mini-Mu prophages. In certain instances, according to Van Gijsegem and Toussaint (1982), one of the mini-Mu prophages can promote the deletion of an adjacent region of DNA, which in

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some instances, covers RP4, the second mini-Mu prophage, and a segment of adjacent chromosomal DNA. The DNA deleted from the chromosome is recovered as an R-prime plasmid carrying a single chromosomal DNA segment of up to 160 kb in size flanked by two mini-Mu copies in the same orientation.

The advantage of such large chromosomal inserts, is that only a small number of R-prime plasmids are required to represent the complete chromosome in the mapping and cloning of any new gene by complementation (Chatterjee et al., 1985). This has led to the isolation of Er. chrysanthemi genes involved in the catabolism of hexuronates (Van Gijsegem and Toussaint, 1983), as well as the clb genes (Barras et al., 1986 a) and the kdgT gene from Er. chrysanthemi (Condemine and Robert-Baudouy, 1987). Furthermore, due to the promiscuous nature of RP4, genes can be mobilised between Erwinia strains and species; as well as to other genera, including E. coli and Myxococcus (Schoonejans and Toussaint, 1983; Van Gijsegem et al., 1985 b). This latter ability has proven to be of benefit in the analysis of heterogenic gene expression because it has allowed a study of both the fidelity of synthesis of Erwinia proteins in foreign hosts and their cellular distribution.

1.5.6.2(b) In vitro cloning

A number of different approaches have been taken in the direct in vitro cloning of Erwinia genes. The maintenance of narrow-host-range ColE1 vectors in many Erwinia strains has proven important for the development of gene cloning technology in Erwinia. (Bauer and Beer, 1983; Hinton et al., 1985 b; Lacy and Sparks, 1979; Roeder and Collmer, 1985 b). Examples of the vectors that have been used for the in vitro cloning of genes from Erwinia

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include the cosmids pHc79 (Keen *et al.*, 1984), pSF6 (Murata *et al.*, 1990), pJB8 (Misawa *et al.*, 1990) and pMMB34 (Barras *et al.*, 1984); the lambda vector L47-1 (Kotoujanski *et al.*, 1985) and plasmids such as pBR322 (Collmer *et al.*, 1985), pBR329 (Keen *et al.*, 1984) and pUC8 (Plastow *et al.*, 1986).

1.5.7 Regulation and "reverse genetics"

In recent years much research effort has been concerned with elucidating the factors which regulate the expression of genes involved in the synthesis and secretion of the *Erwinia* extracellular enzymes and in cellulose and pectin degradation. Such studies have invariably involved the isolation of spontaneous and chemical-induced mutants affected in regulation, and the analysis of the role of various substrate compounds and cAMP.

In many instances, gene fusion technology has been employed to facilitate the monitoring of gene expression in *Erwinia*. Derivatives of bacteriophage Mu which behave as generalised mutagens in sensitive bacteria, have had the added advantage of facilitating the *in vivo* construction of gene fusions within a wide variety of *Erwinia* genes. The Mu derivative Mu dI (Casadaban and Cohen, 1979) and the mini-Mu derivatives Mu dII734 and Mu dIII734 (Castilho *et al.*, 1984) for example, have been extensively used by *Erwinia* geneticists to isolate both transcriptional and translational B-galactosidase (*lacZ*) gene fusions. Moreover, Mu dIIPR3 (Ratet and Richaud, 1986) has also been used. This derivative carries a promoterless neomycin phosphotransferase (*nptI*) gene which upon insertion, can generate translational fusions to promoters and confer kanamycin-resistance under conditions in which the promoters are active.

The aforementioned bacteriophage derivatives have been used to analyse the regulation of the cellulase (*celY* and *celZ*) genes (Aymeric *et al.*, 1986),

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the pectin methyl esterase (pem) gene (Boccaro and Chatain, 1989) of Er. chrysanthemi, and the pectin lyase (pnIA) gene of Er. carotovora subsp. carotovora (McEvoy et al., 1990). The transcriptional organisation and regulation of all the pel genes of Er. chrysanthemi (Diolez and Coleno, 1985; Diolez et al., 1986; Hugouvieux-Cotte-Pattat et al., 1986; Reverchon and Robert-Baudouy, 1987; Reverchon et al., 1986) has also been studied. Moreover, gene fusion technology has lead to the successful isolation of genes of Er. chrysanthemi 3937 which are inducible by Saintpaulia ionantha plant-extracts (Beaulieu and Van Gijsegem, 1990), and aided an analysis of the organisation and regulation of the Er. chrysanthemi arb genes which mediate the metabolism of the aromatic B-glucosides, arbutin and salicin (El Hassoum, 1990). It has also facilitated the selection of a regulatory mutation(s) allowing the constitutive expression of pelB and other pel genes (Diolez et al., 1986) and permitted the analysis of the regulation of the kdg genes involved in the uptake and metabolism of 2-keto-3-deoxygluconate, a common intermediate of the gluconate, galacturonate and pectin degradation pathways (Condemine and Robert-Baudouy, 1987; Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1985 a).

Although E. coli has been used as a surrogate host for Erwinia genes, in order to gain the most authentic picture of the regulation of erwinia genes, it has been ultimately desirable to perform regulatory studies in the parent Erwinia strain. This has been achieved by returning the mutated Erwinia gene back into the Erwinia chromosome, and replacing the wild-type allele via homologous recombination using a technique known as marker-exchange mutagenesis (Ruvkun and Ausubel, 1981).

Marker-exchange mutagenesis represents a so-called "reverse genetics" approach and can essentially be broken down into a number of distinct

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stages. The initial part of this strategy involves the cloning of the Erwinia gene of interest. Subsequently, an easily assayed fusion/insertion is constructed within the gene, which is then transferred back into an Erwinia strain on an unstable replicon (or one which can be rendered unstable). Finally, bacteria are screened for loss of the vector while retaining selection for the fusion/insertion in the genome. This technique has allowed the inactivation of individual pel genes within the Er. chrysanthemi genome, thereby facilitating a study of their role in pathogenicity and the factors that govern gene expression (Diolez and Coleno, 1985; Diolez et al., 1986; Payne et al., 1987; Roeder and Collmer, 1985, 1987; Reverchon and Robert-Baudouy, 1987). In addition, it has enabled an analysis of the expression of the Er. chrysanthemi kdgT gene encoding a transport system responsible for the uptake of ketodeoxyuronates in this organism (Condemine and Robert-Baudouy, 1987).

Despite the fact that this procedure has permitted the facile introduction of a directed mutation into the Erwinia genome, the construction of strains with multiple directed mutations has been complicated by the need for a corresponding number of antibiotic-resistant markers. To overcome this limitation, an extension of the marker-exchange mutagenesis technique has been devised by Ried and Collmer (1987) to allow the construction of an unlimited number of directed unmarked mutations within the Erwinia chromosome. In brief, this elegant technique firstly involves the introduction of a nptI-sacB-sacR cartridge, encoding kanamycin-resistance and sucrose sensitivity (due to the production of levansucrase by sacB), into a cloned Erwinia gene. This is subsequently introduced into the Erwinia genome by marker-exchange recombination. The resulting bacterial derivative is Kn^{r} and sucrose-sensitive. The second stage involves the removal of the cartridge

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from the cloned Erwinia gene to leave a 28 bp frame-shifting insertion. The mutated allele containing the frame-shifting insertion is then introduced into Erwinia and exchanged for the chromosomal allele by selecting for sucrose tolerance. This technique has provided a means to construct Er. chrysanthemi strains with mutations in all five pel genes (Ried and Collmer, 1988), allowing a further exploration of the role of the Pel isoenzymes in pathogenicity.

1.6 AIMS OF THIS STUDY

It is clear from the preceding sections of this chapter that the chemotherapeutic L-asparaginase II synthesised by Er. chrysanthemi NCPPB 1066, had been extensively studied both physically and biochemically prior to the onset of this project. In contrast however, limited work had been directed towards understanding the molecular mechanisms governing the expression of this medically and industrially important enzyme.

Following the isolation of the Er. chrysanthemi ansB structural gene and the nucleotide sequence determination, the present work was initiated in order to move towards unravelling the complexities of L-asparaginase II regulation. Such information would be of interest academically speaking, as well as hopefully being useful for enhancing industrial production yields. In this instance, molecular genetic studies were to be carried out ideally within Er. chrysanthemi NCPPB 1066 itself, rather than an artificial surrogate system such as E. coli, in order to gain an authentic picture of L-asparaginase regulation. As a pre-requisite for genetic analysis, attempts would be made to characterise Er. chrysanthemi NCPPB 1066 and to adapt a variety of genetic systems to this particular strain of Erwinia.

One immediate objective at C.A.M.R. Porton Down was to achieve

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over-expression of L-asparaginase II, therefore the aforementioned genetic techniques would also be employed to determine whether the ansB structural gene could be introduced into Er. chrysanthemi NCPPB 1066 in multicopy. If so, the levels of enzyme production in the resulting recombinant strains would be assessed in small-scale fermentation.

Within the project itself, two approaches were to be taken to study the molecular aspects of L-asparaginase II regulation. The first approach was non-targeted and would essentially involve the random generation of mutants altered in enzyme synthesis, followed by a characterisation of such mutants at a molecular level. To facilitate the monitoring of L-asparaginase II expression and allow the rapid screening and isolation of regulatory mutants, a series of gene fusions were to be generated between ansB and those encoding suitable 'reporter' proteins, such as alkaline phosphatase (phoA) or B-galactosidase (lacZ), using both in vivo and in vitro methodologies.

A second more targeted approach was to analyse the 5' non-coding region of the Er. chrysanthemi NCPPB 1066 ansB structural gene in more detail by mapping the transcriptional start-site using the technique of primer extension. Moreover, following the identification of a putative -24/-12 promoter consensus sequence, the significance of this region was also to be investigated.

CHAPTER TWO

MATERIALS AND METHODS

2. Materials and methods

2.1 MATERIALS

2.1.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1. Derivatives of strains carrying various plasmids were constructed by either transformation, electroporation or conjugation, and are defined in the text.

2.1.2 Bacteriophage strains

The bacteriophage strains used in this study are listed in Table 2.2.

2.1.3 Plasmids

All plasmids used in this study are listed in Table 2.3.

2.1.4 Media

Solutions, growth media, and assay media are listed in Table 2.4, and were prepared in double-distilled water. In order to prevent precipitate formation and to ensure reproducibility, all components of assay media were added in the order indicated. Sterilisation was achieved by autoclaving at 121°C (15 p.s.i.) for 20 mins, or by filtration through 0.22 μm Sterilin disposable filters.

Growth media were solidified with 1.5% (w/v) Difco Bacto-agar when necessary. Double Difco medium was used for bacteriophage work. "Top" agar and "sloppy" agar consisted of 0.6% (w/v) and 0.3% (w/v) Difco Bacto agar, respectively. Minimal medium and MacConkey agar were supplemented with amino acids and sugars at a final concentration of 20 $\mu\text{g ml}^{-1}$ or 0.2% (w/v), respectively, unless otherwise stated in the text.

2. Materials and methods

Table 2.1 Bacterial strains

Strain	Genotype	Source	Reference
<u>E. coli</u> derivatives			
CC118	<u>araD139</u> , Δ (<u>ara</u> , <u>leu</u>)7697, Δ <u>lacX74</u> , Δ <u>phoA20</u> , <u>galE</u> , <u>galK</u> , <u>thi</u> , <u>rpsE</u> , <u>rpoB</u> , <u>argE</u> _{am} , <u>recA1</u>	D. Gill	Manoil & Beckwith, 1985
LE392	F ⁻ , <u>hsdR514</u> , (<u>r_k</u> ⁻ , <u>m_k</u> ⁻), <u>supE44</u> , <u>supF58</u> , <u>lacY1</u> , <u>galK2</u> , <u>galT22</u> , <u>metB1</u> , <u>trpR55</u> , <u>lambda</u> ⁻	D. Gill	Salmond <u>et</u> <u>al.</u> , 1986
TG1	<u>supE</u> , <u>hsd</u> Δ 5, <u>thi</u> Δ (<u>lac-proAB</u>), F' (<u>traD36</u> , <u>proAB</u> , <u>lacI</u> ^q , <u>lacZ</u> Δ M15).	S. McGowan	Sambrook <u>et</u> <u>al.</u> , 1989
DH1	F ⁻ , <u>recA1</u> , <u>endA1</u> , <u>gyrA96</u> , <u>thi-1</u> , <u>hsdR17</u> (<u>r_k</u> ⁻ , <u>m_k</u> ⁻), <u>supE44</u> , <u>lambda</u> ⁻ .	D. Hodgson	Hanahan, 1983
ET8000	<u>rbs</u> , <u>lacZ</u> ::1S1, <u>gyrA</u> , <u>hutC</u> ^C _K .	M. Merrick	MacNeil <u>et</u> <u>al.</u> , 1982
ET8045	<u>rbs</u> , <u>lacZ</u> ::1S1, <u>gyrA</u> , <u>hutC</u> ^C _K , <u>rpoN208</u> ::Tn10.	M. Merrick	MacNeil <u>et</u> <u>al.</u> , 1982
ET8556	<u>rbs</u> , <u>lacZ</u> ::1S1, <u>gyrA</u> , <u>hutC</u> ^C _K , <u>ntnC1488</u> .	M. Merrick	Merrick, 1983
ET8894	<u>rbs</u> , <u>lacZ</u> ::1S1, <u>gyrA</u> , <u>hutC</u> ^C _K , Δ (<u>rha-ntnC</u>)1703::Mucts62.	M. Merrick	MacNeil <u>et</u> <u>al.</u> , 1982

2. Materials and methods

Table 2.1 continued....

Strain	Genotype	Source	Reference
C600	F^- , <u>thi-1</u> , <u>thr-1</u> , <u>leuB6</u> , <u>lacY1</u> , <u>tonA21</u> , <u>supE44</u> , λ <u>lambda</u> ⁻ .	T. Gibbs	Appleyard, 1954
CSH26 Δ F6	F' <u>ara</u> , Δ (<u>lac-pro</u>), <u>thi</u> , <u>rpsL</u> , Δ (<u>recA-srl</u>) F6, <u>sup</u> ^o , λ <u>lambda</u> ⁻ .	D. Gill	Jones & Holland, 1984
DW75	DH1 (pHPC2, pLVC9, pR64 <u>drd11</u>).	J. Hinton	Salmond <u>et al.</u> , 1986
S17-1	<u>thi</u> , <u>pro</u> , <u>hsdR</u> ⁻ , <u>hsdM</u> ⁺ , <u>recA</u> /RP4- Tc::Mu-Kn::Tn7, (Tp ^r , Sm ^r)	D. Hodgson	Simon <u>et al.</u> , 1983 b
HB101	<u>supE44</u> , <u>hsdS20</u> (<u>r_B</u> ⁻ , <u>m_B</u> ⁻), <u>recA13</u> , <u>ara-14</u> , <u>proA2</u> , <u>lacY1</u> , <u>galK2</u> , <u>rpsL20</u> , <u>xyl-5</u> , <u>mtl-1</u>	D. Gill	Bolivar & Backmann, 1979
Q358	<u>supE</u> , <u>hsdR</u> , phi 80 ^r	D. Hodgson	Karn <u>et al.</u> , 1980
ED8812	F^- , <u>thr</u> , <u>leu</u> , <u>thi</u> , <u>lacY</u> , <u>lacZ</u> Δ M15 <u>tonA</u> , <u>supE</u> , <u>hsdS</u> (<u>r_k</u> ⁻ , <u>m_k</u> ⁻), <u>recA</u> , λ <u>lambda</u> ⁻	D. Hodgson	pers. comm.
GJ342	JC2926 (pR64 <u>drd11</u> , pLVC9)	J. Hinton	van Haute <u>et al.</u> 1983

2. Materials and methods

Table 2.1 continued....

Strain	Genotype	Source	Reference
HE127	ECG18 (HB101 <u>lamB</u>) harbouring pHCP2	J. Hinton	pers. comm.
HE198	HB101 (pRK2013)	F. Ellard	Ellard <u>et al.</u> , 1989
HE168	ECG18 (pTROY9)	F. Ellard	Ellard <u>et al.</u> , 1989
<u>Er. carotovora subsp. carotovora</u>			
SCRI 193	wild-type (=Kelman's SR44 isolated from potato)	J. Hinton	Perombelon & Boucher, 1978
HC131	SCRI 193 (pHCP2)	J. Hinton	Salmond <u>et al.</u> , 1986
<u>Er. chrysanthemi</u>			
NCPB 1066	wild-type	N. Minton	Gilbert <u>et al.</u> , 1986
Industrial morphotype 1	morphological variant of L-asparaginase II hyper- producing NCPB 1066 strain S10	This work	-

2. Materials and methods

Table 2.1 continued....

Strain	Genotype	Source	Reference
Industrial morphotype 2	morphological variant of L-asparaginase II hyper-producing NCPPB 1066 strain S10	This work	-
Industrial morphotype 3	morphological variant of L-asparaginase II hyper-producing NCPPB 1066 strain S10	This work	-
Industrial morphotype 4	morphological variant of L-asparaginase II hyper-producing NCPPB 1066 strain S10	This work	-
Industrial morphotype 5	morphological variant of L-asparaginase II hyper-producing NCPPB 1066 strain S10	This work	-
CK1000	LacZ ⁻ derivative of wild-type NCPPB 1066 (EMS mutagenesis)	This work	-
CK1002	CK1000 (pCK11)	This work	-

2. Materials and methods

Table 2.1 continued....

Strain	Genotype	Source	Reference
CK1002	single copy <u>ansB::lacZ</u> fusion derivative of CK1000	This work	-
CK2000	LacZ ⁻ derivative of industrial morphotype 2 (EMS mutagenesis)	This work	-
CK2001	CK2000 (pCK11)	This work	-
CK3000	PhoA ⁻ derivative of wild-type NCPPB 1066 (EMS mutagenesis)	This work	-
CK4000	PhoA ⁻ derivative of industrial morphotype 2 (EMS mutagenesis)	This work	-
3937j	wild-type	J. Hinton	Kotoujanski <u>et al.</u> , 1982
<u>Er. uredovora</u>			
SCRI 423	wild-type (DNAase +ve)	F. Ellard	pers. comm.

EMS-generated mutants of wild-type Er. chrysanthemi NCPPB 1066 single copy ansB::lacZ fusion derivative CK1002 are listed in Table 7.5.

2. Materials and methods

Table 2.2 Bacteriophage

Bacteriophage	Characteristics	Source	Reference
lambda ⁺	wild-type	P. Reeves	lab stock
lambda <u>cl857</u>	lambda derivative with thermosensitive repressor	P. Reeves	lab stock
lambda <u>TnphoA</u>	<u>b221</u> · <u>cl857</u> · <u>P_{am3}</u> · (<u>rex</u>):: <u>TnphoA</u>	P. Reeves	Gutierrez <i>et al.</i> , 1987
lambda <u>TnlacZ-B20</u>	<u>b221</u> · <u>cl857</u> · <u>P_{am80}</u> · (<u>rex</u>):: <u>TnlacZ-B20</u>	C. Kennedy	Simon <i>et al.</i> , 1989
lambda ₄₆₇	<u>b221</u> · <u>cl857</u> · <u>P_{am80}</u> · <u>O_{am29}</u> · (<u>rex</u>):: <u>Tn5</u>	P. Reeves	de Bruijn & Lupski, 1984
lambda ₈₄₀	lambda <u>gt7-his</u> · <u>cl857</u> · <u>nin5,zzz</u> :: <u>Tn10-del4</u> - HH104	P. Reeves	Way <i>et al.</i> , 1984
<u>P1clr100CM</u>	thermo-inducible derivative of P1CM, encoding Cm ^r	This work	Rosner, 1972
phiEC-2	wild-type	F. Barras	Resibois <i>et al.</i> , 1984
<u>Mucts62</u>	thermo-inducible derivative of Mu	This work	Toussaint, 1985

2. Materials and methods

Table 2.2 continued....

Bacteriophage	Characteristics	Source	Reference
T4	wild-type	I. Toth	lab stock
phiCK-1	novel wild-type <u>Er.</u> <u>chrysanthem</u> i NCPPB 1066 bacteriophage	This work	-
phiCK-2	novel wild-type <u>Er.</u> <u>chrysanthem</u> i NCPPB 1066 bacteriophage	This work	-
phi CK-3	novel wild-type <u>Er.</u> <u>chrysanthem</u> i NCPPB 1066 bacteriophage	This work	-

2. Materials and methods

Table 2.3 Plasmids

Plasmid	Characteristics	Phenotype	Source	Reference
pBR322	multicopy cloning vector	Ap ^r , Tc ^r	Amersham	Bolivar <u>et al.</u> , 1977
pPHD7	<u>in vitro</u> <u>phoA</u> fusion vector	Ap ^r	C. Gutierrez	Gutierrez & Devedjian, 1989
pDAH276	<u>lacZ</u> promoter probe vector, P1 <u>inc</u>	Ap ^r , Kn ^r	D. Hodgson	-
pHP45 omega	pHP45::omega fragment	Ap ^r , Sm ^r , Sp ^r	D. Gill	Prentki & Krisch, 1984
pUC8	multicopy cloning vector	Ap ^r , <u>lacZ</u> ^r	D. Hodgson	Vieira & Messing, 1982
pUC9	multicopy cloning vector	Ap ^r , <u>lacZ</u> ^r	D. Hodgson	Vieira & Messing, 1982
pASN326	pUC9-based <u>ansB</u> expression vector	Ap ^r	N. Minton	Gilbert <u>et al.</u> , 1986

2. Materials and methods

Table 2.3 continued....

Plasmid	Characteristics	Phenotype	Source	Reference
pASN230	pKT230-based <u>ansB</u> expression vector	Kn ^r	N. Minton	Gilbert <i>et al.</i> , 1986
pMD114	pACYC177:: <u>ntrC_K</u>	Ap ^r	D. Cardy	Kennedy & Drummond, 1985
pMM17	pBR327:: <u>rpoN_K</u>	Ap ^r , Kn ^r	D. Cardy	Merrick & Stewart, 1985
pSM10	pBR322:: <u>ntrB_K</u>	Ap ^r	D. Cardy	MacFarlane & Merrick, 1987
pDAH331	pIC-19H-based cloning vector	Kn ^r , <u>lacZ'</u>	D. Hodgson	-
pSUP5011	pBR325:: <u>Tn5-Mob</u>	Ap ^r , Cm ^r , Kn ^r	D. Hodgson	Simon, 1984
pTROY9	pAFRB <u>malK</u> ::IS-3 <u>lamB^C</u>	Tc ^r	This study	De Vries <i>et al.</i> , 1984
pHCP2	pBR322:: <u>malK</u> , <u>lamB</u>	Ap ^r	This study	Clement <i>et al.</i> , 1982

2. Materials and methods

Table 2.3 continued....

Plasmid	Characteristics	Phenotype	Source	Reference
pHC79	cosmid cloning vector	Ap ^r , Tc ^r	Boehringer Mannheim	Hohn & Collins, 1988
pAT523	pBR325::glnA _{AV} , ntrC _{AV}	Ap ^r , Tc ^r	D. Cardy	Toukdarian & Kennedy, 1986
pAT705	pBR325::rpoN _{AV}	Ap ^r , Tc ^r	D. Cardy	Toukdarian & Kennedy, 1986
pME305	Temperature-sensitive RP1 derivative	Ap ^r , Tc ^r Cb ^r , Tra ⁺ , Rep(ts) IncP1	M. Rella	Rella <u>et al.</u> , 1985
pME9	pME305::Tn5-705	Ap ^r , Cb ^r Km ^r , Tc ^r Tp ^r	M. Rella	Rella <u>et al.</u> , 1985
pME461	pME305::Tn5 (inserted into Tn801)	Cb ^r , Tc ^r Km ^r , Tra ⁺ Rep(ts) IncP1	M. Rella	Rella <u>et al.</u> , 1985

2. Materials and methods

Table 2.3 continued.....

Plasmid	Characteristics	Phenotype	Source	Reference
pCHR81	pCHR71:: <u>Tn5</u>	Tp ^r Km ^r Rep(ts)	C. Sasakawa	Sasakawa & Yoshikawa, 1987
pCHR82	pCHR71:: <u>Tn5</u> -TC1	Tp ^r Tc ^r Rep(ts)	C. Sasakawa	Sasakawa & Yoshikawa, 1987
pCK1	pBR322-based <u>ansB</u> expression vector ⁽¹⁾	Ap ^r	This work	-
pCK2 Mob#1	pIC-19H-based <u>ansB</u> expression vector ⁽²⁾	Kn ^r	This work	-
pCK2 Mob#2	pIC-19H-based <u>ansB</u> expression vector ⁽³⁾	Kn ^r	This work	-
pCK3	pBR322:: <u>rpoN_E</u>	Ap ^r	This work	-
pCK4	pBR322:: <u>rpoN_E</u>	Ap ^r	This work	-
pCK5	pBR322:: <u>rpoN₂</u>	Ap ^r	This work	-
pCK6	pBR322:: <u>rpoN₂</u>	Ap ^r	This work	-
pCK7	pHC78:: <u>glnA</u> , <u>ntrB</u> , <u>ntrC_E</u>	Ap ^r	This work	-

2. Materials and methods

Table 2.3 continued....

Plasmid	Characteristics	Phenotype	Source	Reference
pCK8	pBR322-based <u>ansB</u> expression vector (4)	Ap ^r	This work	-
pCK9	pCK1::TnphoA	Ap ^r ,Kn ^r	This work	-
pCK10	pCK8::TnlacZ-B20	Ap ^r ,Kn ^r	This work	-
pCK11	<u>in vitro</u> constructed <u>ansB</u> :: <u>lacZ</u> fusion vector	Ap ^r ,Kn ^r	This work	-
pCK12 omega 1	<u>in vitro</u> constructed <u>ansB</u> :: <u>phoA</u> fusion vector containing omega fragment	Ap ^r ,Sp ^r , Sm ^r	This work	-
pCK12 omega 2	identical to pCK12 omega 1, except omega fragment cloned in opposite orientation	Ap ^r ,Sp ^r , Sm ^r	This work	-

Subscript K denotes that the cloned gene is of K. pneumoniae origin.

Subscript E denotes that the cloned gene is of wild-type Er. chrysanthemi NCPPB 1066 origin.

Subscript 2 denotes that the cloned gene is of Er. chrysanthemi NCPPB 1066 Industrial morphotype 2 origin.

Subscript AV denotes that the cloned gene is of A. vinelandii origin.

(1) A 2.8kb BamHI/EcoRI fragment from pASN326 harbouring the Er. chrysanthemi NCPPB 1066 structural gene, cloned into the BamHI/EcoRI sites of pBR322.

(2) A 1.8kb NarI/ClaI fragment from pASN326 harbouring the Er. chrysanthemi NCPPB 1066 ansB structural gene, cloned into the ClaI site of pDAH331. RP4 Mob region cloned into the BamHI site of pDAH331.

(3) Plasmid identical to (2) except the ansB structural gene is cloned in the opposite orientation.

(4) A 2.2kb BamHI/StuI fragment from pASN326 harbouring the Er. chrysanthemi NCPPB 1066 ansB structural gene, cloned into the BamHI/EcoRV sites of pBR322.

2. Materials and methods

Table 2.4 Media

Medium	Constituents per litre
Nutrient Broth (NB)	13g Oxoid nutrient broth
Luria Broth (LB)	10g Bacto tryptone 5g Bacto yeast extract 5g NaCl [pH 7.2]
LM	10g Bacto tryptone 5g Bacto yeast extract *10ml 1M NaCl *10ml 1M MgSO ₄
SOB	20g Bacto tryptone 5g Bacto yeast extract *10ml 1M NaCl *2.5ml 1M KCl *10ml 1M MgSO ₄ , 1 M MgCl ₂ (filter-sterile) [pH 6.9-7.0]
SOC	SOB plus *20ml 1M glucose
Stab medium	NB plus 7g Bacto agar

2. Materials and methods

Table 2.4 continued....

Medium	Constituents per litre
Phage buffer	10mM Tris base 10mM MgSO ₄ 0.01% (w/v) gelatin [pH 7.4]
Pectinase assay medium	16g Bacto agar *5ml 20%(w/v) Bacto yeast extract *10ml 10%(w/v) (NH ₄) ₂ SO ₄ *1ml 1M MgSO ₄ .7H ₂ O *10ml 50%(w/v) glycerol *250ml 2%(w/v) polygalacturonic acid *200ml Pel phosphate buffer
Pel phosphate buffer	15g Na ₂ HPO ₄ anhydrous 0.7g NaH ₂ PO ₄ .1H ₂ O [pH 8.0]
Cellulase assay medium	10g carboxymethyl cellulose 10g Bacto agar *25ml 20% (w/v) Bacto yeast extract *4ml 50% (w/v) glycerol *20ml 50 X phosphate solution *10ml 10% (w/v) (NH ₄) ₂ SO ₄ *10ml 1% (w/v) MgSO ₄
Protease assay medium (destructive)	16g Bacto agar 13g Oxoid nutrient broth 30g Oxoid gelatin

2. Materials and methods

Table 2.4 continued....

Medium	Constituents per litre
Double Difco medium (DD)	20g Bacto tryptone 8g NaCl (plus *10ml 20%(w/v) maltose and *10ml 1M MgSO ₄ for lambda work)
Motility broth	10g Bacto tryptone 5g NaCl
Motility agar	MB plus 3g Bacto agar
Terrific broth	12g Bacto tryptone 24g Bacto yeast extract 4ml glycerol *100ml (0.17M KH ₂ PO ₄ , 0.72M K ₂ HPO ₄)
DNAase detection medium	20g Bacto tryptose 2g DNA 5g NaCl 0.05g methyl green 15g Bacto agar
Minimal medium	*20ml 50 X phosphate solution *10ml 10%(w/v) (NH ₄) ₂ SO ₄ *10ml 1%(w/v) MgSO ₄
50 X Phosphate solution	350g K ₂ HPO ₄ 100g KH ₂ PO ₄ [pH 6.9-7.4]

2. Materials and methods

Table 2.4 continued....

Medium	Constituents per litre
Protease assay medium (non-destructive)	28g Oxoid Nutrient agar *500ml 2% (w/v) Difco skimmed milk
Freezing medium (2x)	12.6g K_2HPO_4 anhydrous 0.9g sodium citrate 0.18g $MgSO_4 \cdot 7H_2O$ 1.8g $(NH_4)_2SO_4$ 3.6g KH_2PO_4 anhydrous 88g glycerol
Low phosphate medium	0.1g $MgSO_4 \cdot 7H_2O$ 1.0g $(NH_4)_2SO_4$ 0.5g Na citrate 12.1g Trizma base *1ml 100mM KH_2PO_4 *10ml 20% (w/v) glucose

* solutions added from sterile stocks after autoclaving.

2. Materials and methods

Antibiotics were prepared as 100 X stocks and were added at the final concentration shown in Table 2.5, unless otherwise stated. Tetracycline and chloramphenicol were dissolved in 50% (v/v) ethanol and stored at -20°C . All other antibiotics were prepared in sterile double-distilled water, and stored at 4°C , with the exception of naladixic acid which was dissolved in 30mM NaOH, and trimethoprim which was dissolved in 1% (w/v) lactic acid.

TE buffer was used in all DNA manipulations and contained 10mM Tris.HCl, 1mM EDTA (pH8.0).

2.1.5 Chemicals

Media chemicals were generally obtained from either Fisons Scientific Apparatus (Loughborough, Leicestershire, UK.), BDH Chemicals Ltd. (Poole, Dorset, UK.), or Difco Laboratories (Detroit, Michigan, USA.), and were of Analar grade. Polygalacturonic acid, carboxymethyl cellulose, amino acids and vitamins were purchased from Sigma Chemical Company Ltd. (Poole, Dorset, UK.). All other chemicals, with the exception of those listed below, were obtained from Sigma.

Ethidium bromide, dimethyldichlorosilane, phenol, caesium chloride, liquid paraffin and B-mercaptoethanol came from BDH (Poole, Dorset, UK.). Organic acids, solvents and glycerol were obtained from May and Baker Ltd. (Dagenham, Kent, UK.). Ethanol and methanol were reagent grade. Glass-distilled dimethylsulphoxide (DMSO) was purchased from Fluka Chemicals Ltd. (Glossop, Derbyshire, UK.)

Acrylamide, developer D19 and Unifix fixer were obtained from Eastman Kodak (Rochester, New York, USA.). N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylene-ethylenediamine (TEMED), ammonium persulphate, urea, sodium dodecylsulphate (SDS), glycine and protein assay concentrate

2. Materials and methods

Table 2.5 Antibiotics

Antibiotic	Abbreviation	Final concentration ($\mu\text{g ml}^{-1}$)
Ampicillin (Na^+ salt)	Ap	50
Carbenicillin	Cb	100
Chloramphenicol	Cm	50
Kanamycin sulphate	Kn	50
Naladixic acid	Nm	50
Spectinomycin	Sp	50
Streptomycin sulphate	Sm	100
Tetracycline	Tc	10
Trimethoprim	Tp	10
Nitrofurantoin	Nft	10

2. Materials and methods

were purchased from Bio-Rad Laboratories Limited. (Watford, Hertfordshire, UK.).

Radiochemicals, T4 DNA ligase, T4 polynucleotide kinase, T4 DNA polymerase, E. coli polymerase I (Klenow fragment), pBR322 and bacteriophage lambda DNA were obtained from Amersham International PLC. (Amersham, Buckinghamshire, UK.). Calf intestinal alkaline phosphatase, deoxy- and dideoxy-nucleotides, Random Primed DNA labelling kit, DNAase I (RNAase-free), and RNAase A (DNAase-free) came from Boehringer Mannheim (BCL) (Lewes, East Sussex, UK.). Restriction endonucleases and corresponding "React" buffers were purchased from Gibco-BRL Ltd. (Uxbridge, Middlesex, UK.), as were 1kb DNA and 0.24 to 9.5kb RNA ladder markers.

Avian myeloblastosis virus reverse transcriptase came from Life Sciences Inc. (North Saint Petersburg, Florida, USA.). Gigapack Gold in vitro packaging kit (Cat. No. 200214,215,216) was obtained from Stratagene Cloning Systems (LA Jolla, California, USA.). Rabbit antibody to bacterial alkaline phosphatase (Cat. No. 5307-001360) and B-galactosidase (Cat. No. 5307-063100) were obtained from 5 Prime-3 Prime Incorporated (West Chester, Pennsylvania, USA.). The Sequenase sequencing kit was purchased from United States Biochemical Corporation (Cleveland, Ohio, USA.). Sephadex G25 and protein molecular weight standards were purchased from Pharmacia (GB.) Ltd. (London, UK.). X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside) and X-P (5-bromo-4-chloro-3-indolyl phosphate) was obtained from Bachem (UK.) Incorporated (Saffron Walden, Essex, UK.). API Biochemical test strips were purchased from API System S.A. (La Balme les Grottes, Montalleu-Vercieu, France).

2. Materials and methods

2.2 MICROBIOLOGICAL METHODS

2.2.1 Maintenance of bacterial strains and bacteriophage lysates

E. coli and Er. carotovora subsp. carotovora strains were maintained on either NB or LB agar containing appropriate antibiotics where required, for up to 6 weeks at 4°C. E. coli strain TG1 was stored on M9 minimal medium containing 0.1ml 0.1% (w/v) thiamine litre⁻¹, to ensure the presence of the F episome. In contrast, Er. chrysanthemi strains were found to lose viability rapidly if maintained at 4°C; hence, they were kept on solid medium at the higher temperature of 18°C, where viability remained for up to 4 weeks.

For long term storage, bacterial strains were either kept in stab agar at room temperature, or an overnight LB culture was mixed with an equal volume of 2 X freezing medium (Table 2.4), and placed at -70°C.

Bacteriophage lysates were stored at 4°C in the appropriate phage buffer over chloroform, for up to 6 months.

2.2.2 Growth of bacterial cultures

In general, E. coli and Erwinia strains were propagated at 37°C or 30°C, respectively, unless otherwise stated. Liquid cultures were routinely grown in 25ml Universal tubes in a Gallenkamp Orbital Shaker (150 rpm). When cells were required for transformation, cultivation was carried out in 250ml Ehrlenmeyer flasks in a New Brunswick Gyrorotary waterbath (275 rpm). In both instances, culture density was monitored at a wavelength of 550nm or 600nm with an LKB Ultrospec 4050 spectrophotometer.

2.2.3 Light microscopy

Cultures were viewed by phase contrast microscopy using an Olympus

2. Materials and methods

model stereoscopic microscope (X1000 magnification). Bacterial colonies and bacteriophage plaques were examined using a Carl Zeiss Jena Olympus plate microscope.

2.2.4 Antibiotic gradient plates

The formation of a gradient of antibiotic across an agar plate was achieved as follows. Molten agar (15ml) containing the appropriate antibiotic was poured into a Petri dish resting at an angle of approximately 15°, and allowed to set at room temperature. Following this period, the Petri dish was removed to a flat surface and the agar slant overlaid with a further 15ml of molten NB agar without antibiotic. This was again allowed to set at room temperature. Subsequent diffusion of the antibiotic from the primary layer into the upper layer of varying thickness leads to the creation of an antibiotic gradient.

2.2.5 Bacterial conjugation

Conjugal transfer of plasmids from one bacterium to another was routinely performed by mixing a loopful of donor and recipient cells on the surface of an NB agar plate. Following overnight incubation at 30°C, the patch-matings were streaked to single colonies on selective media.

When the frequency of plasmid transfer needed to be quantified, donor and recipient were grown in liquid culture overnight (80 rpm) at 30°C, with antibiotics if required, mixed in a 1.5ml Eppendorf tube, and concentrated by centrifugation in an MSE Microcentaur (6,500 rpm, 1 min). The mating mixture was then gently resuspended in 0.1ml of NB medium and spotted onto a dry NBA plate. Following overnight incubation at 30°C, cells were harvested into 1ml of phosphate buffered saline and serially-diluted on

2. Materials and methods

selective media. Transconjugants were streak-purified twice on selective media before further analysis. In every instance, donor and recipient alone controls were run in parallel. Furthermore, appropriate media were used in parallel to determine the viable count of the recipient strain. Mating frequency was expressed as the number of transconjugants per recipient cell (TPR), as described by Hinton and coworkers (1985 a).

2.2.6 Transformation

The following procedures were routinely used to transform E. coli and Erwinia strains. In both cases, pre-chilled pipettes, tubes and solutions were used throughout.

2.2.6.1 Preparation of competent E. coli cells

A single E. coli colony was inoculated into 5ml of SOB medium and incubated overnight at 37°C. Cells were then diluted 100-fold into pre-warmed SOB medium and grown with good aeration until an OD₅₅₀ value of 0.5 (ca. 3×10^8 cfu ml⁻¹) was reached. The culture was chilled on ice for 15 min and subsequently harvested in an MSE Chilspin (3000 rpm, 5 mins, 4°C). The resulting pellet was gently resuspended in an equal volume of ice-cold 0.1M MgCl₂ (Sigma No. M 9272), and kept on ice for 10 minutes. Cells were collected by centrifugation as described above, and carefully resuspended in 0.5 volume of 0.1M CaCl₂ (Grade 1, Sigma No. C 3881). Following a further 10 mins on ice, cells were re-pelleted as described, concentrated in 0.05 volume of 0.1M CaCl₂, and left on ice for a minimum of 2 hours to establish competence. In order to maximise transformation frequency, bacteria were left overnight at 4°C before use.

For long term storage of competent cells, suspensions were made up to

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15% (v/v) glycerol by the addition of an ice-cold sterile solution of 50% (v/v) glycerol/0.1M CaCl₂. Aliquots were frozen rapidly in a dry ice/ethanol bath for 5 mins and stored at -70°C.

2.2.6.2 Transformation of competent E. coli cells

Transformation of competent E. coli was initiated by incubating 200ul of cells with 1 to 10ul of DNA solution on ice for 30 mins, with occasional gentle mixing. After this period, the cell/DNA mixture was transferred to a 42°C waterbath for 2 mins and then returned to ice for a further 15 mins. Following this period, cells were incubated in 1ml of SOB medium at 37°C for 1 hour (200 rpm) to allow expression of plasmid-borne antibiotic resistance markers, and then spread-plated onto the appropriate selective medium.

2.2.6.3 Preparation of competent Erwinia cells

The preparation and transformation of competent Erwinia cells was carried out using the modified Hanahan method of Hinton et al. (1985 b). All solutions used in this procedure are listed in Table 2.6.

A single Erwinia colony was used to inoculate 5ml of SOB medium and incubated without shaking at 37°C for 16 hours. The culture was subsequently diluted 20-fold into 25ml of fresh SOB medium, and incubated at 37°C (275 rpm) until an OD₅₅₀ value of 0.3 (ca. 5×10^7 cfu ml⁻¹) was reached. At this point, bacteria were chilled on ice for 10 min; harvested in an MSE Chillspin (3000 rpm, 10 mins, 4°C), and then gently resuspended in 10ml of TFB solution. After 15 mins on ice, cells were re-pelleted as described, and carefully resuspended in 2ml of TFB solution on ice. Freshly thawed DMSO (70ul) was then added to the suspension and dispersed by gentle swirling.

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Table 2.6 Solutions used for Erwinia transformation

Solution	Constituents	Source
DTT stock	2.25M dithiothreitol	Sigma
	40mM potassium acetate (pH 6.0)	Sigma
TFB	10mM K-MES (pH 6.0)	Sigma
	100mM KCl	BDH
	45mM MnCl ₂ ·4H ₂ O	BDH
	10mM CaCl ₂ ·2H ₂ O	Sigma
	3mM hexamine cobalt (III) chloride (H ₆ CoCl ₃)	Fluka

The solutions listed in the table above were prepared in double-distilled water, exactly as described by Hanahan (1983, 1985). Dimethylsulphoxide (DMSO, Fluka No. 41645) was purchased freshly-distilled, and stored immediately in 0.5ml aliquots at -20°C. KCl was substituted for RbCl. DTT and DMSO stocks were discarded after single use. TFB was made as a filter sterile 5 X stock solution, and could be stored at 4°C for at least 2 years.

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After 5 mins, 70ul of DTT stock was added, and the mixture incubated for an additional 10 mins. Finally, 70ul of DMSO was again added, and cells incubated on ice for 5 mins prior to being dispersed in 210ul aliquots to pre-chilled 1.5ml Eppendorf tubes.

Competent cells prepared by the above method were unable to be stored for long periods, and had to be used within an hour of preparation.

2.2.6.4 Transformation of competent Erwinia cells

A sample of DNA solution (1-10ul) was added to each sample and dispersed by low speed vortexing. The cell/DNA mixture was incubated on ice for 30 mins before undergoing heat shock in a 30°C waterbath for 6 mins. SOC medium (800ul) was then added at room temperature and the culture incubated at 30°C for 70 mins (200 rpm) to allow antibiotic gene expression. Following this period, bacteria were pelleted in an MSE Microcentaur (6,500 rpm, 2 mins) and carefully resuspended in 100ul of SOC medium. Finally, transformants were spread-plated onto LM agar containing appropriate antibiotics and incubated at 30°C.

2.2.7 Electroporation

A fresh overnight bacterial culture was diluted 100-fold in LB medium and grown at 30°C with aeration (200 rpm) until an OD₅₅₀ value of 0.5 was reached. Cells were then collected by centrifugation in an MSE Chilspin (5,000 rpm, 10 mins, 4°C), washed once in an equal volume of ice-cold electroporation buffer (272mM sucrose, 7mM sodium phosphate buffer (pH 7.4), 1mM MgCl₂), and finally resuspended in 1/20 volume of the same buffer. A sample of the resulting cell suspension (0.8ml) was pipetted into a pre-chilled 1.5ml eppendorf tube containing an appropriate amount of DNA

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solution, and the contents mixed well. Following a 20 min incubation period on ice, the cell/DNA mixture was transferred to the bottom of a pre-chilled Bio-Rad electroporation cuvette (0.4cm inter-electrode distance), which was subsequently placed in the safety chamber slide of a Bio-Rad Gene Pulser apparatus, preset at 25uF and 2.5kV at room temperature. The sample was pulsed once and the cuvette immediately returned to ice for 10 mins. After this time, bacteria were diluted in 8 volumes of LB medium and incubated at 30°C with aeration (180rpm) for 1 hour. Aliquots (100ul) were then plated on the appropriate selective medium and incubation allowed to proceed at 30°C. On each occasion, a DNA-free cell suspension was run in parallel as an electroporation control.

2.2.8 Preparation of bacteriophage lysates

2.2.8.1 Bacteriophage lambda

Lambda lysates were prepared on the E. coli suppressing host LE392, by the method of de Bruijn and Lupski (1984). An overnight culture of LE392 was grown in LB medium supplemented with 0.2% (w/v) maltose and 10mM MgCl₂, and a sample of this (300ul) then infected with lambda bacteriophage (10^5 - 10^6 pfu) at 37°C for 20 mins. Following this period, 3ml of soft DDA agar was added, and the mixture overlaid upon a fresh wet DDA plate. Incubation was allowed to proceed for approximately 8 hours at 37°C until confluent lysis was observed (compared to a bacteriophage-free control lawn). The top agar was subsequently removed with a glass spreader and the plate washed with 1ml of phage buffer. Chloroform (500ul) was then added to the pooled slurry, and the mixture vortexed at room temperature for 15 min.

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Agar was removed by centrifugation in an MSE Chilspin (5000 rpm, 10 mins, 4°C) and the supernatant decanted and stored at 4°C.

2.2.8.2 Novel Er. chrysanthemi NCPPB 1066 bacteriophage

Lysates of novel Er. chrysanthemi NCPPB 1066 bacteriophage were prepared on the wild-type strain, essentially as described in section 2.2.8.1, except that in this instance soft LB agar was used and no cofactors were found to be required for bacteriophage adsorption. Moreover, infection was allowed to proceed at 30°C for 30 to 40 mins.

2.2.8.3 Bacteriophage P1 and Mu

High-titre lysates of bacteriophage P1 and Mu were prepared by thermal induction for a number of reasons. Firstly, P1 and Mu both contain an invertible region within their genome, the orientation of which determines the host range of the bacteriophage. In the case of Mu, the invertible region spans 3 kb and has been designated the G segment; whereas in bacteriophage P1, the invertible region covers 4.2 kb and is comprised of two large inverted repeats of 0.62 kb, flanking a 3 kb internal segment which displays homology to the G segment of Mu (Glasgow et al., 1989; Sternberg and Hoess, 1983). DNA isolated from Mu and P1 bacteriophage which have been produced by induction of a lysogen has been found to contain the G or C segment in both orientations (designated + and -). In contrast, bacteriophage prepared by infection contain the G or C segment in the + orientation only (Glasgow et al., 1989; Iida, 1984; Sternberg and Hoess, 1983). For this reason, induced lysates should be used to test an uncharacterised bacterial strain. Secondly, in the case of bacteriophage Mu, the mom function, which is responsible for the modification of approximately 15% of the adenine residues in the genome,

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thereby rendering the DNA resistant to many restriction endonucleases, is found to be expressed at a higher level following induction, rather than bacteriophage infection. Consequently, Mu bacteriophages prepared by induction are fully modified whilst lytically grown Mu bacteriophages are modified to a partial degree and are thus more sensitive to host-controlled restriction (Toussaint, 1985)

A lysogenic strain, carrying a P1 or Mu prophage with a thermo-sensitive repressor mutation was grown overnight at 30°C in LB medium, and then diluted 100-fold into fresh LB medium (supplemented with 20mM CaCl₂ for P1 work). The culture was placed at 30°C water bath and growth allowed to proceed until an OD₅₅₀ value of 0.3 was reached. At this point, bacteria were immediately transferred to a 42°C water bath and cultivated with vigorous shaking (250 rpm) for a further 30 mins to allow thermo-induction of the prophage. After this time, the culture was placed in a 37°C water bath and grown for 90 mins (250 rpm) to allow lysis to take place. Several drops of chloroform were added to the lysate to complete the process, and shaking continued for a further 15 mins. Cell debris was subsequently removed by centrifugation in an MSE Chilspin (5000 rpm, 10 mins, 4°C), and the supernatant stored at 4°C.

2.2.8.4 phi EC-2

Plate lysates of phi EC-2 were prepared on Er. chrysanthemi 3937j, essentially as described by Resibois et al. (1984).

2.2.9 Isolation of bacteriophage from sewage

Samples (500ml) of untreated sewage, activated sludge and effluent sewage were collected from Finham sewage works (Kenilworth, Warwickshire)

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and pooled before being centrifuged in an MSE Hi-Spin 21 centrifuge (9000 rpm, 15 mins, 4°C). The resulting supernatant was treated with 10ml of chloroform before being re-centrifuged, as described above. A 100ml sample of the aqueous supernatant was then added to 250ml Ehrlenmeyer flasks containing 1g Bacto tryptone, 0.5g yeast extract and 0.5g NaCl. To this solution was added MgSO₄, CaCl₂, tryptophan and casamino acids, each to a final concentration of 10mM. The constituents were then dissolved by gentle swirling.

A 100ul sample of a fresh overnight culture of wild-type Er. chrysanthemi NCPPB 1066 was inoculated into the aforementioned solution, and grown at 25°C with aeration (100rpm) until dense bacterial growth was achieved (the low temperature was chosen due to the possibility that potential bacteriophage isolated from the environment could have been sensitive to temperatures of 30°C and above). At this point, 1ml of chloroform was added and incubation continued at 25°C for 15 mins. Cells were subsequently pelleted in an MSE Hi-Spin 21 centrifuge (9000 rpm, 15 mins, 4°C) and the resulting supernatant decanted. This was then serially-diluted in phage buffer to 10⁻⁸, and 100ul of each dilution mixed with 200ul of a fresh overnight culture of wild-type Er. chrysanthemi NCPPB 1066, previously resuspended in LB medium containing MgSO₄, CaCl₂, casamino acids and tryptophan at the concentrations stated previously. Incubation was allowed to proceed at 25°C for 30 mins, after which time 3ml of top agar was added, and bacterial overlays made on LB agar plates. Following 24 hours incubation at 25°C, a suitable isolated bacteriophage plaque was cored with a Pasteur pipette and added to 0.3ml of phage buffer in a 1.5ml Eppendorf tube. This was vortexed for 3 min and left standing for 5 to 6 hours at 4°C to elute the bacteriophage from the agar. Following this period, the core

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lysate was serially-diluted in phage buffer and 100ul of each dilution used to infect 200ul of a fresh overnight culture of wild-type Er. chrysanthemi NCPPB 1066, as described previously. Top agar lawns were then prepared, and incubation allowed to proceed at 25°C overnight. Lawns were then inspected to determine whether the plaque purification had been successful.

2.2.10 Transduction assays

2.2.10.1 Bacteriophage lambda

An Erwinia recombinant, harbouring the lamB+ plasmid pHCP2 or pTROY9, was inoculated into 20ml of LB medium containing 10mM MgSO₄ and either Ap (50ug ml⁻¹) or Tc (10ug ml⁻¹), and grown overnight at 30°C with aeration (200 rpm). Following this period, cells were pelleted in a Wifug Labor 50M bench centrifuge (4,500 rpm, 10 mins), and resuspended in 2ml of fresh LB medium containing 10mM MgSO₄ alone. The cell suspension was then divided into two equal portions. The first acted as an uninfected control, whilst the second was infected with 100ul of a high-titre lambda₄₆₇ or lambda₈₄₀ lysate (10¹⁰ pfu). Following static incubation at 30°C for 60 mins, cells were collected by centrifugation in a Wifug Labor 50M bench centrifuge (4,500 rpm, 10 mins) and resuspended in 1ml of LB medium. Aliquots (100ul) were then spread-plated onto LB agar supplemented with the appropriate antibiotic, and growth allowed to proceed at 30°C for 36 hours.

2.2.10.2 Novel Er. chrysanthemi NCPPB 1066 bacteriophage

Transduction assays using novel Er. chrysanthemi NCPPB 1066 bacteriophage previously grown on the appropriate wild-type Er. chrysanthemi

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NCPPB 1066 derivative, were performed essentially as described in Section 2.2.10.1. Again, no cofactors were added to LB medium.

2.2.11 Isolation of bacterial strains lysogenic for bacteriophage P1

Attempts to isolate bacteria lysogenic for bacteriophage P1 were carried out essentially as described by Murooka and Harada (1979). Bacteria were grown in 10ml of LB medium containing 5mM CaCl_2 at 30°C for 24 hours and then centrifuged in a Wifug Labor 50M bench centrifuge (4,500 rpm, 15 mins). The resulting pellet was resuspended in 1ml of LB medium containing 10mM CaCl_2 , 10mM MgSO_4 , and a 200ul sample removed as an uninfected control. Further samples (200ul) of bacterial culture were then mixed with 250ul of undiluted P1_{clr}100CM bacteriophage lysate, and the mixtures incubated statically at 30°C for 30 min to allow adsorption and DNA injection. After this period, bacteria were collected by centrifugation in an MSE Microcentaur (13,500 rpm, 1 min) and the resulting supernatant discarded. The pellet was resuspended in 1ml of LB medium and cells incubated at 30°C (200rpm) for 30 min to allow expression of the chloramphenicol-resistance marker. The uninfected control was also run in parallel. Aliquots (100ul) were then spread-plated onto LB agar containing Cm (15ug ml^{-1}), and incubation allowed to proceed for up to 3 days at 30°C.

2.2.12 API 20 E biochemical tests

Rapid biochemical analysis of bacterial isolates was performed using the API 20 E microtube system. Cultures were grown in NB medium at 30°C for 24 hours, washed in sterile double-distilled water, and resuspended in 5ml of sterile double-distilled water at a density of approximately 10^8 cells ml^{-1} ,

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as recommended by Murray (1978). The API 20 E test strips were then handled according to the manufacturers instructions, and galleries incubated at 30°C or 37°C for 18 hours. The strips were read by referring to an interpretation table, and the results converted to a 7 digit profile number.

2.2.13 Antibiotic sensitivity tests

Bacterial cultures were grown to an OD₆₀₀ value of 1.0 in LB medium at 30°C and 300ul samples mixed with 3ml of Oxoid Iso-sensitest top agar. The mixture was then overlayed on top of a dry Iso-sensitest agar plate and allowed to set. At this point, Mastring-S antibiotic discs were aseptically placed onto the surface of the bacterial lawns and the plates incubated upright at 30°C for 18 hours.

2.2.14 Reversion analysis

Bacteria were inoculated into 10ml of LB medium containing no antibiotic, and grown with aeration (200 rpm) at 30°C to stationary phase. At this point, bacterial cells were collected by centrifugation in a Wifug Labor 50M bench centrifuge (4,500 rpm, 10 mins) and resuspended in 0.5ml of NB medium. Serial dilutions were then plated onto NB agar plates to determine viable counts, and onto minimal sucrose agar plates to select for spontaneous prototrophic revertants. Incubation was allowed to proceed at 30°C for 36 hours.

2.2.15 Low phosphate curing of plasmids

NB medium (5ml) supplemented with the appropriate antibiotic, was inoculated with the bacterial recombinant of interest and incubation allowed to proceed overnight at 30°C with aeration (200 rpm). A sample of this

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culture (10ul) was then added to 5ml of low phosphate medium (Table 2.4) containing the appropriate antibiotic, and the culture incubated at 30°C with aeration (200 rpm) for one round of 32 hours. Cells were then serially-diluted in phosphate buffered saline and spread-plated onto appropriate selective medium to assess plasmid loss. Successive rounds of treatment involved sub-culturing 10ul of the previous culture into 5ml fresh low phosphate medium with antibiotic, and the procedure repeated as described above.

2.2.16 Small-scale fermentation

2.2.16.1 Preparation of seed cultures

Seed cultures for 8 litre LH 2000 fermenter vessels were prepared by inoculation of 200ml of LB medium containing antibiotic if necessary, with a single colony from the surface of an agar plate. Cultures were grown in a 1 litre fluted Ehrlenmeyer flask at 30°C with aeration (180 rpm) for 12 hours.

2.2.16.2 Fermentation runs

LH Systems fermenters (2000 series) with a working volume of 8 litres were used for small-scale fermentation studies. Growth medium, consisting of yeast extract (40.9g l⁻¹) and glutamic acid (18.75g l⁻¹) was sterilised in situ and allowed to cool to the desired temperature for bacterial growth. Antibiotic was then added by filtration post-sterilisation, and medium inoculated with 200ml of seed culture. Growth was allowed to proceed batch-wise until the onset of stationary phase. Temperature (30°C) and pH (6.8) were monitored throughout by on-line computer and maintained at constant levels. In the case of the latter, 10M NaOH or 20% (v/v) phosphoric acid

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were added automatically, as required. Dissolved oxygen tension (DOT) was measured using a polarographic Ingold probe and maintained at 30 percent of saturation with air. Stirrer speed was initially set at 200 rpm, but was automatically increased by computer as growth ensued in order to maintain DOT levels. Head pressure was set at 0.2 bar and aeration rate at 4.0 l min^{-1} . Finally, exit gas in the fermenter space above the liquid phase, was analysed by an on-line Varion mass spectrophotometer.

2.2.16.3 Sampling

Culture samples (10ml) were periodically removed from reactor vessels aseptically for off-line analysis, by means of a sampling port. Optical density measurement of the fermentation broth was monitored at a wavelength of 600nm. Simultaneously, cells were washed and resuspended in 0.1M Boric acid/NaOH buffer, (pH8.5) to an OD_{600} value of 1.0, and stored at 4°C prior to automated L-asparaginase II and protein assays.

2.2.16.4 Plasmid stability

In order to gain an insight into the stability of plasmids in recombinant cultures, the proportion of cells bearing the plasmid-encoded antibiotic resistant phenotype was assessed by replica-plating, essentially as described by workers such as Bentley and Kompala (1990), Curless *et al.* (1989), Tolentino and San (1988) and Weber and San (1989).

A sample of fermentation culture was serially-diluted in LB medium and spread-plated onto LB agar plates. Following overnight incubation at 30°C , 300 colonies were replicated by toothpick onto LB agar supplemented with the appropriate antibiotic and onto LB agar alone, and incubation allowed to proceed for a further 24 hours at 30°C . The number of replicated colonies

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able to grow on the antibiotic medium compared to antibiotic-free medium was determined, and expressed as a percentage value.

2.2.17 Chemical mutagenesis

A fresh overnight bacterial culture (1ml) was inoculated into 30ml of pre-warmed LB medium in a 500ml Ehrlenmeyer flask, and propagated at 30°C (200 rpm) until an OD₆₀₀ value of 0.7 was reached. A 1ml sample was then removed at time zero as a control, and pelleted in an MSE Microcentaur (13,500 rpm, 2 mins). Cells were washed once in phosphate buffered saline and resuspended in 1ml of the same buffer. The remaining culture was treated with 500ul of ethylmethanesulphonate (EMS), shaken vigorously to dissolve the mutagen, and maintained at 30°C (200 rpm) for 2½ hours. Samples (1ml) were removed at various time points and treated as described above. Viable counts were determined by serially-diluting cells onto LB agar and incubating at 30°C for 24 hours. To allow segregation, the remaining mutagenised cells were added to 10ml of LB medium and incubated overnight at 30°C with aeration (200 rpm).

For long term storage of mutagenised cultures, aliquots were mixed with an equal volume of 2 X freezing medium and stored at -20°C.

2.2.18 UV. sensitivity test

Bacteria from a single colony were streaked in both directions on a NB plate (to overcome the dilution effects as the loop is moved across the surface of the agar), and allowed to dry in. A small glass sheet was placed between the cells and an ultraviolet lamp emitting light of 254nm (3200 erg sec⁻¹ cm⁻²) in the dark, and moved across the agar dish so that bacteria were exposed to UV. light for varying times. The lid was then replaced and

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the plate immediately wrapped in tin foil in order to prevent light-induced enzymatic photoreactivation occurring (Freidberg, 1985). Incubation was carried out at 30°C or 37°C for 24 hours before inspection.

2.2.19 Extracellular enzyme plate assays

2.2.19.1 Inoculation and incubation

Fresh colonies were either patched or streaked to single colonies onto pectin lyase, cellulase and protease assay media containing antibiotics if required, and incubated at 30°C for 24 to 48 hours.

2.2.19.2 Protease (Prt) assay

The non-destructive protease assay did not require development with chemical reagents, and was carried out using NB agar containing 1% (w/v) Difco Skim milk, according to Salmond *et al.* (1986). A positive reaction was observed as a zone of clearing against a turbid background.

The destructive protease assay was performed as described by Thurn and Chatterjee (1985), except that plates were flooded with saturated ammonium sulphate instead of the toxic mercuric chloride. Protease positive colonies were surrounded by a clear zone on an opaque white background.

2.2.19.3 Pectin lyase (Pel) assay

This assay was modified from Andro *et al.* (1984). Plates were flooded with 7.5% (w/v) copper acetate (Sigma No. C 5893) and left standing at room temperature for 1 to 2 hours. Pectin lyase positive colonies gave a white halo against a translucent light blue background.

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2.2.19.4 Cellulase (Cel) assay

The cellulase assay medium was modified from that of Gilkes *et al.* (1984). Cellulase activity was revealed by first staining carboxymethyl cellulose with 0.2% (w/v) Congo red (Sigma No. C 6767) for 20 mins, as described by Teacher and Wood (1982), followed by bleaching with 1M NaCl for 15 mins. Increased contrast was then obtained by flooding with 1M HCl for 5 mins. Cellulase positive colonies gave a red translucent halo upon a dark blue background.

2.2.20 Spectrophotometric assays

2.2.20.1 B-galactosidase

The method of Miller (1972) was used to assay B-galactosidase activity. A sample of bacterial culture to be assayed was mixed with Z buffer (60mM Na₂HPO₄ (anhydrous); 40mM NaH₂PO₄.2H₂O; 10mM KCl; 1mM MgSO₄.7H₂O; 0.27% (v/v) B-mercaptoethanol; pH 7.0) to a final volume of 1ml, and lysed by the addition of 2 drops of chloroform and 1 drop 0.1% (w/v) SDS with vortexing. Following incubation at 28°C for 5 mins, the reaction was started with the addition of 200ul of ONPG (*o*-nitrophenol-B-D-galactoside; 4 mg ml⁻¹ in Z buffer) and followed at 28°C. When sufficient colour had developed, the reaction was stopped with 0.5ml 1M Na₂SO₄, and the OD₄₂₀ and OD₅₅₀ measured. Units of B-galactosidase were calculated as follows :

$$\text{Units} = \frac{1000 \times \text{OD}_{420} - (1.75 \times \text{OD}_{550})}{\text{OD}_{600} \times v \times t}$$

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where OD_{600} reflects the cell density just before assay.

t is the time of reaction in min.

v is the volume of culture used in the assay in ml.

2.2.20.2 Alkaline phosphatase

The method of Brickman and Beckwith (1975) was used to assay alkaline phosphatase. Bacterial cultures (10ml) were spun in an MSE Chilspin (5000 rpm 10 mins, 4°C), and the supernatant removed and kept on ice. Pellets were washed once and resuspended in 8ml of 1M Tris.HCl (pH8.0). Cell-free extracts were prepared by sonication in a 150 Watt MSE ultrasonic disintegrater using a $\frac{1}{4}$ inch diameter probe. Disruption was performed on ice with four 30 sec 6 micron peak to peak bursts, with 30 sec cooling period between each burst. Cell debris was removed by centrifugation as described above, and sonicates stored on ice.

A 0.1ml sample of sonicate or culture supernatant was mixed with 0.9ml 1M Tris.HCl (pH 8.0) and the reaction started with the addition of 0.1ml Sigma 104 solution (p-nitrophenyl phosphate, disodium salt; 4mg ml⁻¹ in double-distilled water). The mixture was incubated at 37°C until a faint yellow colour appeared, after which the reaction was terminated by the addition of 0.1ml of 1M KH₂PO₄. The absorbance at 420nm was measured for each sample and compared with a suitable blank. Units were expressed as OD₄₂₀ min⁻¹ ml⁻¹.

2.2.20.3 L-asparaginase

(1) Background

The L-asparaginase assay adopted in this study was based on the

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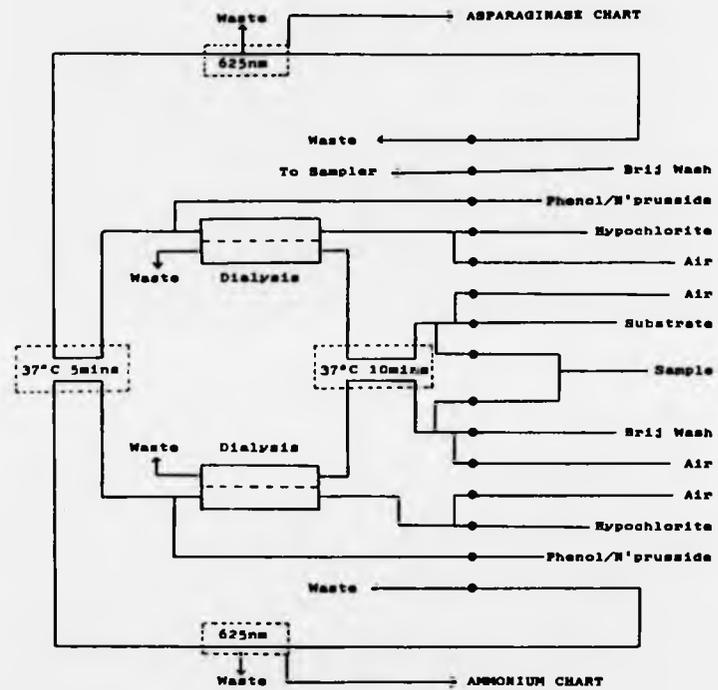
Berthelot reaction, in which the ammonia, enzymatically liberated from buffered L-asparagine substrate, is dialysed into a recipient solution containing sodium hypochlorite. Under alkaline conditions, the two components react to produce chloramine. Upon the addition of a solution containing phenol and sodium nitroprusside, chloramine reacts firstly with phenol to produce quinone chloramine, and then further to give indophenol. This process is catalysed by nitroprusside, and the indophenol product gives a blue colouration under the alkaline conditions used. This can then be measured spectrophotometrically at 650nm.

The level of L-asparaginase II activity within bacterial cultures of Er. chrysanthemi NCPPB 1066 was determined using a modified version of the continuous flow autoanalysis procedure, previously described by Wade and Phillips (1971). This system not only enables a large sample throughput, but also ensures reproducible incubation times. Moreover, studies by Wade et al. (1971 a) have demonstrated that suspensions of whole bacterial cells can be used for this assay, and provide a reliable measurement of L-asparaginase II activity in the cell. In this instance, the activity of the low affinity cytoplasmic L-asparaginase I is considered to be insignificant.

As can be seen from the flow diagram presented in Figure 2.1, the automated system essentially comprised of two identical systems. In one system, the sample came into contact with buffered L-asparagine substrate and the ammonium produced by the action of L-asparaginase plus any pre-formed ammonia was measured. In the other system, the sample was mixed with buffer alone, and hence only pre-formed ammonia was measured. The latter therefore acted as a sample blank, allowing correction for pre-formed ammonia and eliminating false positive readings. The proportions of sample to reagent was determined by flow-rated plastic tubing and a peristaltic pump.

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Figure 2.1 Continuous flow autoanalysis L-asparaginase II assay system



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A 1.2ml sample of whole bacterial cells diluted in 0.05M borate buffer, pH 8.5 (containing 0.005% (w/v) bovine serum albumin (BSA) to prevent cells binding to plasticware used in the analyser) was drawn into the system from a Technicon sampler at timed intervals, and shortly afterwards split into two identical portions of 0.6ml. These were then processed through the two systems previously described. In the case of the first system, the cell sample, flowing at a rate of 0.42 ml min^{-1} , entered into a segmented stream of L-asparagine substrate buffer (0.02% (w/v) L-asparagine in 0.05M borate buffer (pH 8.5), containing 0.15 % (w/v) EDTA), flowing at a rate of 0.8 ml min^{-1} . This mixture was then passed through a coil located in a water bath held at 37°C for 10 mins, after which it passed into one set of dialyzer plates. The enzymatically liberated ammonia then diffused into a segmented stream of alkaline hypochlorite solution (0.06% (v/v) hypochlorite in a solution of 1.25% (w/v) NaOH plus 0.015% (w/v) Brij 35 wetting agent), flowing at a rate of 1.00 ml min^{-1} . This stream was subsequently mixed with a segmented stream of phenol nitroprusside solution (0.4 % (w/v) phenol, 0.006 % (w/v) sodium nitroprusside) flowing at a rate of 1.2 ml min^{-1} and passed through a coil maintained in a water bath at 37°C for 5 mins. After a minimum delay, the product was passed through a flow cell of a colorimeter (650nm wavelength). The optical density was subsequently represented as a series of peaks on a chart recorder. The height of such peaks was proportional to the concentration of released ammonia. All assays were performed in duplicate, with the deviation between duplicate samples being less than 5% using this particular system (Dr. C. Goward; personal communication).

(2) Standards

A number of standards were employed in the automated assay in order

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to enable the amount of released ammonia in samples to be quantitated, and to ensure that the assay was functioning correctly. Firstly, a standard curve was constructed from known ammonium sulphate standard solutions (0.2, 0.4, 0.6, 0.8 and 1.0 mM NH_4^+). Ammonia concentrations from unknown samples were then interpolated from this calibration curve, and converted into enzyme activity. In this instance, a mid-range value of 0.5mM NH_4^+ was known to be equivalent to 0.05 IU ml^{-1} of L-asparaginase II enzyme. One International Unit (IU) of L-asparaginase activity is defined as the amount of enzyme which catalyses the liberation of of 1umol of ammonia $\text{min}^{-1} \text{ml}^{-1}$ of enzyme sample, at 37°C under the standard conditions of the assay. Calibration was also performed with a freeze-dried, purified L-asparaginase II standard, in order to check the enzyme incubation stage.

2.2.20.4 Protein

Protein concentration in cell-free extracts was estimated using the micro-assay protocol of the Bio-Rad single reagent method, as described by Bradford (1976). The protein sample was diluted to a concentration of 1 to 25 ug ml^{-1} in phosphate buffered saline and 0.8ml mixed with 0.2ml of Bio-Rad Coomassie Brilliant Blue G-250 dye concentrate. The OD_{595} versus a reagent blank was determined after 10 mins at room temperature, and the protein concentration estimated by reference to a calibration curve compiled with known amounts of BSA. Allowance was made for the fact that BSA behaves anomalously in this assay, in that for a given amount of this protein, the OD_{595} is twice that which would be obtained with an "average" protein.

Determination of protein was carried out in conjunction with the L-asparaginase assay using an automated Lowry procedure, the details of which have been described by Wade and Phillips (1971).

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2.3 DNA METHODS

2.3.1 Extraction of DNA with phenol/chloroform

Neutral phenol/chloroform mix was prepared by dissolving 100g of phenol and 100mg of 8-hydroxyquinoline in 100ml of chloroform and 4ml of Iso-amylalcohol. This was equilibrated by shaking the mixture with two changes of 0.2 volume 1M Tris.HCl (pH8.0) and two changes of 0.2 volume TE buffer, prior to storage at 4°C in the dark. Acid phenol/chloroform mix on the other hand, was made by dissolving 100g of phenol and 100mg 8-hydroxyquinoline in 100ml of chloroform and 20ml of double-distilled water, without equilibration with buffer.

Using gentle shaking, DNA samples were mixed with an equal volume of either neutral or acid phenol/chloroform to form an emulsion, and the phases separated by centrifugation in an MSE Microcentaur (13,500 rpm, 2 min). The upper aqueous phase was then recovered, avoiding the interface, and further extracted with an equal volume of chloroform/Iso-amylalcohol (24:1) to remove any traces of phenol. Finally, DNA was recovered by ethanol precipitation.

2.3.2 Precipitation of DNA

Unless otherwise stated, DNA in solution was routinely precipitated by the addition of 0.5 volume of 7.5M sodium acetate (pH 7.5; Sigma No. C 7262) and 3 volumes of 100% (v/v) ethanol, followed by incubation at -20°C for a minimum of 2 hours. In certain instances, 2ul of glycogen carrier (10mg ml⁻¹) was included if low amounts of DNA were present.

Precipitated DNA was recovered at room temperature by centrifugation in an

2. Materials and methods

MSE Microcentaur (13,500 rpm, 20 mins) and the supernatant carefully discarded. The resulting pellet was washed in 70% (v/v) ethanol and DNA subjected to further centrifugation in an MSE Microcentaur (13,500 rpm, 2 mins). The supernatant was discarded and the pellet dried under vacuum for 30 mins. Finally, the desiccated DNA pellet was resuspended in the appropriate amount of TE buffer.

2.3.3 Preparation of chromosomal DNA

The following protocol was developed for the rapid isolation of intact chromosomal DNA from Er. chrysanthemi NCPPB 1066. Bacterial cultures were grown to stationary phase in 50ml of LB medium (30°C, 150 rpm, 16 hours) and then harvested in an MSE Chilspin (5000 rpm, 15 mins, 4°C). Cells were resuspended in 2 X 8ml of 50mM Tris.HCl (pH 8.0)/50mM EDTA, and frozen at -20°C. Suspensions were subsequently thawed at room temperature and treated with 2.0ml of freshly-prepared lysozyme solution (5mg ml⁻¹ in 0.25 M Tris.HCl, pH 8.0). Following 5 mins on ice, 2.5ml of 0.5M EDTA (pH8.0) was added and incubation continued on ice for a further 5 mins. After this time, 0.25ml of 10% (w/v) SDS was then added and samples heated to 65°C for 10 mins to complete lysis. Cleared lysate (4.15ml) from each universal was pooled to form a third sample, and to each was added 2.0ml of 5M sodium perchlorate to aid DNA-membrane separation. Lysates were mixed by gentle inversion and appeared cloudy at this stage. Solutions were extracted twice with neutral phenol/chloroform and twice with chloroform/iso-amylalcohol (24:1). At each stage, the aqueous phase was separated in an MSE Chilspin (5000 rpm, 10 mins, 4°C) and removed using a wide-bore Pasteur pipette. Chromosomal DNA was precipitated with one volume of isopropanol in the presence of 0.3M sodium acetate (pH 4.9),

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spooled with a sealed bent Pasteur pipette, and resuspended in 1ml of TE buffer.

2.3.4 Large-scale preparation of plasmid DNA

2.3.4.1 E. coli

The basic procedure was that of Clewell and Helinski (1970). Strains were grown in 500ml of NB medium, containing antibiotics for plasmid selection, to an OD₆₀₀ value of 0.5 (ca. $2-4 \times 10^8$ cells ml⁻¹). In order to amplify plasmid, spectinomycin or chloramphenicol was then added to a final concentration of 300ug ml⁻¹ or 150ug ml⁻¹, respectively, and incubation continued for 18 hours. Cells were harvested in an MSE Hi-spin 21 centrifuge using a 6 X 300ml rotor (10,000 rpm, 10 mins, 4°C), resuspended in 16.5ml Tris-sucrose solution (0.05M Tris.HCl (pH 8.0), 25% (w/v) sucrose), and transferred to 50ml polycarbonate "Oakridge" tubes as 2 X 8.25ml aliquots. Samples were treated with 2.5ml of freshly prepared lysozyme solution (5mg ml⁻¹ in 0.25M Tris.HCl (pH 8.0), and stored on ice for 5 mins. Then, 2.25ml of 0.25M EDTA (pH 8.0) was added and the suspensions kept on ice for a further 5 mins. Cells were lysed by the addition of 9ml of lysis mix (0.05M Tris.HCl, 0.0625M EDTA, 2% (w/v) Brij 58, 0.4% (w/v) sodium deoxycholate, pH 8.0), prewarmed to 65°C, and the mixture inverted until it cleared. Incubation at 42°C aided this process. Unlysed cells, cell debris and intact chromosomal DNA were pelleted in an MSE Hi-spin 21 centrifuge using a 8 X 50ml rotor (18,000 rpm, 25 mins, 4°C). The supernatant was carefully decanted into a clean universal and stored at 4°C until required.

For a 5ml gradient, 4.1ml of cleared lysate, 4.28g of caesium chloride, and 0.2ml of ethidium bromide (10mg ml⁻¹) were gently mixed at room

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temperature, until all the salt had dissolved. The resulting solution (R_f ca. 1.393) was poured into 5ml Beckman heat seal polyallomer tubes and spun in a Beckman L8 ultracentrifuge using a Vti 65 rotor (45,000 rpm, 18 hours, 15°C). The plasmid DNA was visualised by long-wave UV. light and withdrawn from gradients with a size 21 needle and 2ml syringe.

In order to extract ethidium bromide, DNA samples were mixed with an equal volume of propan-2-ol equilibrated with caesium chloride and TE buffer (80ml propan-2-ol, 20g CsCl, 20ml TE), three times with vigorous shaking, followed by centrifugation in an MSE microcentaur (13,500 rpm, 1 min). On each occasion, the upper phase was carefully removed and discarded. The DNA solution was transferred to a 50ml 'Oakridge' tube and subsequently made up to 6ml with sterile double-distilled water. To this was added 12ml of 100% (v/v) ethanol and the mixture placed at -20°C for a minimum of 4 hours. Precipitated DNA was collected in an MSE Hi-spin centrifuge (16,000 rpm, 25 mins, 4°C), washed in 5ml 70% (v/v) ethanol, vacuum dried, and finally resuspended in 0.5ml of TE buffer.

2.3.4.2 Erwinia

Large-scale isolation of plasmid DNA from Erwinia was performed essentially as described above, except that cells were grown in Terrific broth (Table 2.4; Tartof and Hobbs, 1987) at 30°C for 18 hours without amplification. The addition of either spectinomycin or chloramphenicol was found to result in spontaneous cell lysis.

2.3.5 Rapid small-scale preparation of plasmid DNA

2. Materials and methods

2.3.5.1 E. coli

Rapid small-scale isolation of plasmid DNA was achieved using the alkaline lysis method of Birnboim and Doly (1979), as described by Maniatis et al. (1982).

2.3.5.2 Erwinia

Plasmid DNA was isolated on a small-scale from Er. carotovora subsp. carotovora and Er. chrysanthemi NCPPB 1066 strains, using a modified method of Kieser (1984). A sample of overnight culture (2.5ml) was pelleted in an MSE Microcentaur (13,500 rpm, 2 mins), washed in 2.5ml of phage buffer, and resuspended in 0.5ml of freshly-prepared lysozyme mix (2mg ml⁻¹ in 0.3M sucrose, 25mM Tris.HCl, 25mM EDTA, pH 8.0). Cells were stored on ice for 20 mins and then placed at 37°C for 10 mins, with gentle mixing. A 0.25ml sample of alkaline/SDS solution (0.3M NaOH, 0.2% (w/v) SDS) was added, and samples immediately vortexed until cell lysis occurred. Incubation at 65°C for 10 mins completed this process. Acid phenol/chloroform (80ul) was thoroughly mixed with cooled lysates and phases separated by centrifugation (13,500 rpm, 2 mins). The upper aqueous phase was transferred to a clean 1.5ml Eppendorf tube containing 70ul 3M unbuffered sodium acetate, and mixed by brief vortexing. Isopropanol (700ul) was then added and tubes stood at room temperature for 10 mins before being respun at 13,500 rpm, for 10 mins. The supernatant was discarded and the resulting pellet dissolved in 100ul of TE buffer. A 10ul sample of 3M unbuffered sodium acetate and 50ul of neutral phenol/chloroform were added and phases mixed by vortexing. The aqueous phase was separated by centrifugation as described previously, and transferred to a clean 1.5ml Eppendorf tube. Isopropanol (100ul) was added, and samples stood at room temperature for 10

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mins before being re-pelleted at 13,500 rpm for 10 mins. DNA was washed in 70% (v/v) ethanol, dried under vacuum, and resuspended in 50ul of TE buffer.

2.3.5.3 RNAase treatment

DNAase-free RNAase A was used for the selective removal of RNA from small-scale DNA samples. Stock solutions of RNAase A (10mg ml^{-1} in Tris.HCl (pH 7.5) were heated to 100°C for 15 mins and stored at -20°C . DNA samples prepared by the small-scale methods outlined in Sections 2.3.5.1 and 2.3.5.2, were typically treated with 1ul of stock RNAase solution at 37°C for 15 mins before being analysed by TAE agarose gel electrophoresis.

2.3.6 Concentration of DNA

The concentration of high molecular weight chromosomal DNA or plasmid DNA, prepared by caesium chloride gradients, was determined spectrophotometrically at 260nm. An OD_{260} value of 1.0 was taken to represent a double-stranded DNA concentration of 50ug ml^{-1} . The ratio obtained between readings at 260nm and 280nm provided an estimate of purity of the DNA sample. Pure DNA preparations had an $\text{OD}_{260}::\text{OD}_{280}$ ratio of 1.8. Values significantly less than 1.8 were an indication of either protein or phenol contamination and prevented an accurate quantitation of DNA.

When small amounts of DNA were available, the minigel method described by Manlatis *et al.* (1982) was used to estimate the concentration in a given sample. Lambda DNA restricted with HindIII at 50ng ul^{-1} acted as a standard.

2. Materials and methods

2.3.7 Synthetic oligodeoxyribonucleotides

The oligonucleotides used in this study are listed in Table 2.7.

2.3.7.1 Synthesis

Oligonucleotides were made by "solid phase oligonucleotide synthesis" (Gait *et al.*, 1982) using an Applied Biosystems Model 380A DNA synthesiser. The concentration and purity of de-salted preparations was determined using a Beckman DU-68 scanning spectrophotometer. An OD₂₆₀ value of 1.0 was taken to represent a concentration of 20ug ml⁻¹ (Maniatis *et al.*, 1982). Oligonucleotides were typically adjusted to 100ug ml⁻¹ in TE buffer and stored at -20°C.

2.3.7.2 Purification

An oligonucleotide solution (25ul) was added to an equal volume of denaturing gel loading buffer (90% (v/v) formamide, 10mM EDTA (pH8.0), 0.01% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol), and heated to 100°C for 5 mins. After cooling on ice, the sample was loaded onto a 20 X 20cm 10% (w/v) denaturing polyacrylamide gel and electrophoresed at 300 volts in 1X TBE buffer (0.89M Tris-base, 0.89M boric acid, 2mM EDTA, pH 8.0). Oligonucleotides were visualised by UV. shadowing at 254nm, and the band excised from the gel using a razor blade. The gel slice was cut into fine pieces, and the acrylamide transferred to a 1.5ml Eppendorf tube. To this was added 0.3ml of elution buffer (0.5M sodium acetate (pH 6.5), 1mM EDTA, 0.2% (w/v) SDS), and the mixture incubated overnight at 37°C with gentle shaking. The elution buffer was then removed and filtered through siliconised glass wool. A further 0.2ml of elution buffer was incubated with the acrylamide at 65°C for 5 mins, filtered through glass wool and combined

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Table 2.7 Synthetic oligonucleotides

Oligonucleotide	Length	5' to 3' sequence
Primer 1	19-mer	CAGGACGCTACTTGTGTAT
Primer 2	17-mer	ATCGTTATCCTGGCGAC
Primer 3	21-mer	AACAAAAAATAAAACCAGAAC
Primer 4	21-mer	GATAACGATATTGGGCAGTTT
Primer 5	21-mer	GCTCATAAOGTCTGGACCATG
-40 M13 sequencing primer	17-mer	GTTTTCCCGATCAGGAC

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with the previous sample. The resulting pool was extracted once with an equal volume of neutral phenol/chloroform and DNA precipitated in a dry ice/ethanol bath by the addition of 2 volumes of ethanol. Oligonucleotides were recovered in an MSE Microcentaur (13,500 rpm, 20 mins), washed in 70% (v/v) ethanol, vacuum dried and resuspended in TE buffer to 100ug ml⁻¹.

2.3.8 Agarose gel electrophoresis

Agarose gel electrophoresis was routinely used to examine and isolate individual DNA fragments. Horizontal agarose gel slabs were prepared by boiling agarose (Sigma type II medium EOO) in 1 X TAE electrophoresis buffer (40mM Tris-acetate, 1mM EDTA) and cooled to 45°C before pouring. DNA samples were prepared by adding 0.1 volume of loading buffer (0.25% (w/v) bromophenol blue, 15% (w/v) Ficoll Type 400), and loaded into the gel slots. Electrophoresis was carried out in a Bio-Rad minisub or subcell system, with the gel completely submerged in 1 X TAE electrophoresis buffer.

The DNA was stained within the agarose gels with ethidium bromide (0.5ug ml⁻¹) as described by Maniatis et al. (1982), and visualised by either long wave or short wave UV. trans-illumination. Gels were photographed when required using Polaroid Positive/Negative Land Pack film Type 665.

Appropriate DNA size markers were always run in parallel with samples and are listed in Table 2.8. Where necessary, restriction fragment sizes were calculated with the DNASIZE programme adapted from Schaffer and Sederoff (1981), and run on a BBC Model B micro-computer. Restriction mapping was performed essentially as described by Maniatis et al. (1982).

2.3.9 Preparation of DNA fragments from agarose gels

DNA was routinely purified from agarose gels essentially as described

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Table 2.8 DNA size markers

Marker.	Size of fragments in kb.
Lambda <u>Hind</u> III	23.131;9.418;6.557;4.361;2.322;2.028; 0.564;0.125.
Lambda <u>Pst</u> I	11.510;5.080;4.650;4.510;2.840;2.580; 2.450;2.440;2.140;1.990;1.770;1.160; 1.090;0.810;0.520;0.470;0.450;0.340; 0.270;0.250.
1kb ladder	12.216;11.198;10.180;9.162;8.144;7.126; 6.108;5.090;4.072;3.054;2.036;1.636; 1.018;0.517;0.396;0.344;0.298;0.222; 0.201;0.154;0.134;0.075.

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by McDonnell *et al.* (1977). Gel slices containing nucleic acid were dissected from agarose gels with a sharp scalpel blade and sealed with a minimal volume of 0.05 X TAE buffer in $\frac{1}{4}$ inch prepared dialysis tubing (Scientific Industries International Ltd.). Electro-elution was allowed to proceed at 200 volts for 90 mins in a shallow layer of 0.05 X TAE buffer in an electrophoresis tank. The polarity of the current was reversed for 2 mins to release DNA from the wall of the dialysis bag. The buffer from around the gel slice was removed, the bag washed with 0.5ml of fresh 0.05 X TBE, and samples pooled. DNA was extracted once with phenol/chloroform and twice with chloroform/Iso-amylalcohol (24:1), before being recovered by ethanol precipitation and resuspended in TE buffer.

On a number of occasions, DNA was extracted from agarose gels using a trough elution method. In this instance, the agarose gel was removed from the tank following electrophoresis and placed on top of a long-wave UV. light box, still resting on the gel plate. With the aid of a clean scalpel blade, a 1mm slot was cut into the gel immediately below the DNA band of interest and then filled with 1 X TAE buffer. The gel was subsequently returned to the tank, now containing sufficient 1 X TAE running buffer to make an electrical connection but leaving the top of the gel dry. Electrophoresis was carried out at 100 volts for 1 min after which the TAE buffer was removed from the slot and pipetted into a clean 1.5ml Eppendorf tube. The gel was removed from the tank, and examined on a long UV. transilluminator to ascertain the amount of DNA that had run into the trough. If further electrophoresis was required, the events described above were repeated until none of the desired DNA was visible within the gel. The eluted DNA samples were then pooled and extracted once with phenol/chloroform, and twice with

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chloroform/iso-amylalcohol (24:1), prior to recovery by ethanol precipitation. DNA was finally resuspended in the appropriate volume of TE buffer.

2.3.10 Restriction endonuclease digestion of DNA

In general, DNA was subject to restriction endonuclease digestion in the appropriate 1 X BRL "React" buffer in a 20ul volume. Five to ten units of enzyme were added for every 0.1 to 2.0ug of DNA, and never allowed to exceed 10% of the total reaction volume. Reactions were then carried out at 37°C for 90 min or up to 4 hours when digesting intact chromosomal and linear DNA fragments. In contrast restriction endonuclease reactions involving SmaI or BsmI were placed at either 25°C or 65°C (under liquid paraffin), respectively.

Following digestion, samples were either stored at -20°C for later use, prepared for gel electrophoresis, or phenol/chloroform-extracted if the DNA was required for ligation.

2.3.11 Ligation

Ligation of DNA fragments was routinely carried out in 0.5ml Eppendorf tubes containing 16ul of DNA solution, 4ul of 5 X ligase buffer (250mM Tris.HCl (pH 7.6), 50mM MgCl₂, 25% (w/v) PEG 8000, 5mM ATP, 5mM DTT), and 1ul of T4 polynucleotide ligase (5 to 10 units). The contents were mixed briefly, collected by centrifugation, and placed at 15°C for 16 hours. Samples were either stored at -20°C awaiting transformation or ethanol precipitated if required for electroporation.

For routine subcloning of DNA, a 4:1 insert to vector ratio was used with a DNA concentration less than 50ug ml⁻¹. In order to construct gene libraries and ligate blunt DNA fragments, an insert to vector ratio of 6:1

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was used, and the concentration of DNA increased to between 100 and 200ug ml⁻¹. DNA solutions were heated to 65°C for 5 mins and then placed on ice to allow slow re-annealing of fragments before ligation.

In certain instances, to determine whether in vitro ligation had taken place, a sample of the ligation reaction was analysed by TAE agarose gel electrophoresis for the presence of new molecular weight species.

2.3.12 Dephosphorylation of DNA

Terminal 5' phosphates were removed from DNA using Calf intestinal alkaline phosphatase (CIP). Following digestion of DNA with the appropriate restriction endonuclease, CIP was added to the reaction at a concentration of 1 to 5 units ug⁻¹ of DNA. Dephosphorylation was then allowed to proceed at 37°C for 30 to 60 mins. In certain instances, restricted DNA was ethanol precipitated and resuspended in the appropriate volume of 1 X CIP buffer (50mM Tris.HCl (pH 9.0), 1mM MgCl₂, 0.1mM ZnCl₂, 1mM spermidine). CIP was then added to a final concentration of 1 unit ug⁻¹ of DNA and the sample incubated at 37°C for 60 mins. The reaction was terminated by phenol/chloroform extraction and dephosphorylated DNA recovered by ethanol precipitation.

2.3.13 Generation of blunt-ended DNA fragments

T4 DNA polymerase was used in this study to generate 'blunt-ended' DNA fragments from 'sticky-ended' fragments resulting from restriction endonuclease digestion, as follows. The DNA sample was diluted in 20ul of 1 X T4 polymerase buffer (33mM Tris-acetate (pH 7.9), 66mM K acetate, 10mM Mg acetate, 0.5mM DTT, 0.1mg ml⁻¹ BSA) and incubated with 1ul T4 DNA polymerase (10 units) for 15 mins at 37°C. One-tenth volume of

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1.25mM dNTPs (dATP, dCTP, dGTP, dTTP) were then added and incubation continued at 37°C for an additional 15 mins. Treated DNA was extracted with phenol/chloroform and finally recovered by ethanol precipitation.

2.3.14 Amplification of DNA by the polymerase chain reaction

A specific nucleotide region was amplified from the bacterial genome using the polymerase chain reaction, as follows. A 10ul sample of intact chromosomal DNA solution (200ng) was pipetted into a clean sterile 0.5ml Eppendorf tube, along with 10ul of 10 X PCR buffer (500mM KCl, 100mM Tris.HCl (pH 8.3), 40mM MgCl₂), 16ul of 1.25mM dNTP mix (dATP, dCTP, dGTP, dTTP), 2ul of primer solution (100ug ml⁻¹), and 60ul of sterile double-distilled water. Taq polymerase (1 unit) was added last, and the reaction mixture gently mixed before being overlaid with 75ul of sterile liquid paraffin. The lid was tightly closed and the 0.5ml Eppendorf tube placed in a Techne Programmable Dri-block PHC-1 Thermal Cycler. Amplification was then performed using a "Step Cycle" programme set to denature at 94°C for 1.5 mins, anneal at 50°C for 1 min and extend at 72°C for 1.5 mins, for a total of 30 cycles. Following this period, the reaction mixture was allowed to cool to room temperature and then transferred to a clean 0.5ml Eppendorf tube, avoiding the liquid paraffin. DNA was then extracted once with phenol/chloroform and once with chloroform/IAA (24:1), prior to being ethanol precipitated overnight at -20°C. DNA was recovered by centrifugation in an MSE Microcentaur centrifuge (13,500 rpm, 15 mins) at room temperature and the resultant pellet allowed to air-dry on the bench for 30 mins. Finally, DNA was resuspended in 20ul of sterile double-distilled water.

2.3.15 In vitro packaging of cosmid DNA

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In vitro packaging of cosmid DNA was achieved using the Gigapack Gold in vitro packaging kit, as recommended by the manufacturers.

2.3.16 Radiolabelling DNA

2.3.16.1 Random primer labelling

The method of 'random-primed' DNA labelling was developed by Feinberg and Vogelstein (1983, 1984), and is based on the hybridisation of a mixture of hexanucleotides to the DNA to be labelled. Practically all sequence combinations are represented in the hexanucleotide primer mixture, leading to equal binding of primer along the entire length of the template. The complementary strand is synthesized from the 3' OH termini of the random hexanucleotide primer using labelling-grade Klenow enzyme. Radiolabelled deoxynucleoside triphosphates are incorporated in the process.

DNA probes were labelled to high activity using the Boehringer Mannheim Random Primed Labelling Kit, as recommended by the manufacturers. The DNA probe (100ng) was denatured in 10ul of sterile double-distilled water held at 95°C for 10 mins and then rapidly cooled on ice. The solution was then collected at the bottom of the tube by brief centrifugation in an MSE Microcentaur bench centrifuge (13,500 rpm, 30 sec) before being transferred to a 1.5ml Eppendorf tube on ice, containing 3ul of dATP, dGTP, dTTP (1:1:1), 2ul of hexanucleotide mixture in 10 X reaction buffer, and 2ul (10 Ci) of alpha-³²P dCTP (3000 Ci mmol⁻¹ aqueous solution). The volume was made up to 19ul with sterile double-distilled water and 1ul (2 units) of Klenow enzyme added. The contents were mixed by brief vortexing and spun in an MSE Microcentaur (13,500 rpm, 30 sec), before being placed in a 37°C water bath for 30 mins. After this time, the reaction

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was stopped by the addition of 2ul of 0.2M EDTA (pH 8.0) and stored on ice. Non-incorporated deoxyribonucleoside triphosphates were subsequently removed by passing the probe solution through a Sephadex G25 spin column, as outlined in Figure 2.2.

2.3.16.2 End-labelling with gamma-³²P-ATP and T4 polynucleotide kinase

Synthetic oligonucleotides are supplied dephosphorylated at the 5' end and can therefore be radiolabelled with gamma-³²P-ATP by T4 polynucleotide kinase. Single-stranded oligonucleotides for use in primer extension assays were labelled by the following method. A 15ul reaction was assembled in 1 X C buffer (66mM Tris.HCl (pH 7.6), 6.6mM MgCl₂, 10mM DTT) containing 4 to 10ng of oligonucleotide, 60 to 100uCi of gamma-³²P-ATP and 20 units of T4 polynucleotide kinase. The reaction was incubated at 37°C for 1 hour, after which the reaction volume was increased to 100ul with sterile double-distilled water and extracted once with neutral phenol/chloroform.

In order to remove unincorporated label, 10ul of 3M sodium acetate (pH 6.5), 200ul of 100% (v/v) ethanol and 2ul of glycogen (10mg ml⁻¹) were added, and the sample placed at -20°C for a minimum of 2 hours before being spun in an Eppendorf minifuge (10,000 rpm, 15 mins) at room temperature. The resultant pellet was resuspended in 100ul of sterile double-distilled water and the process repeated as described above. The oligonucleotide DNA pellet was air-dried at room temperature for 20 mins, and finally resuspended in 10ul of DEPC-treated water.

2.3.17 Southern blots

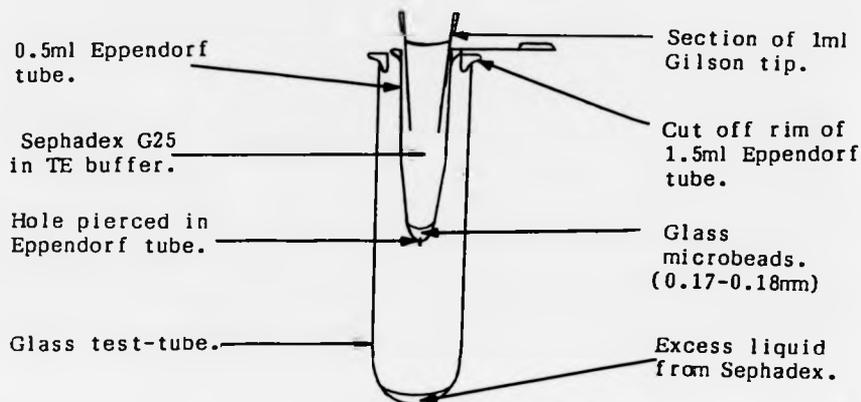
Transfer of DNA from agarose gels onto nylon membrane (Amersham 'Hybond-N' code RPN.303N) was performed using the capillary blotting

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Figure 2.2 Spin column technique for the purification of radiolabelled DNA probes

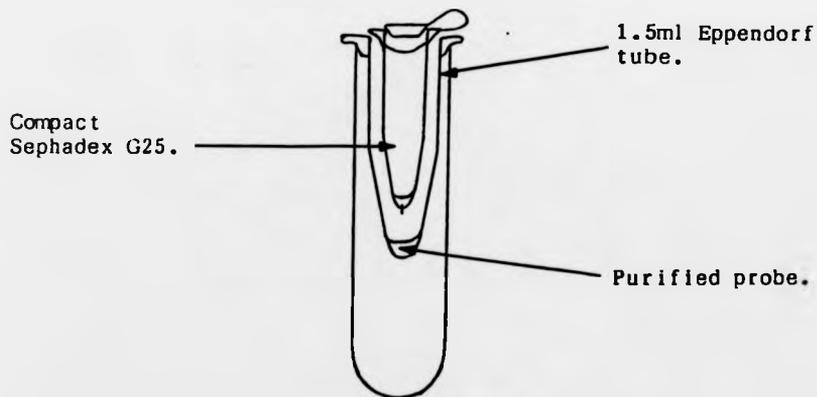
A) The method used to prepare a sephadex G25 spin column.

The assembly is spun with a swing out rotor in a Gallenkamp Labspin centrifuge at 3000 rpm for 4 min.



B) The arrangement for removing unincorporated nucleotides from the radiolabelled probe.

The 0.5ml Eppendorf tube now contains compact sephadex G25, and is placed in an intact 1.5ml Eppendorf tube. The labelling reaction is pipetted on the column and the assembly spun at 3000 rpm for 4 min, as described above.



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technique of Southern (1975). Chromosomal and plasmid DNA was digested to completion with the appropriate restriction endonucleases, and the products electrophoresed through an agarose gel at low voltage overnight. The gel was then trimmed to size and transferred to a tray containing denaturing solution (1.5M NaCl, 0.5M NaOH). After 30 mins, the gel was rinsed in double-distilled water and subsequently soaked for an additional 30 mins in neutralising solution (1.5M NaCl, 1M Tris.HCl, pH 8.0).

A folded piece of Whatman 3MM filter paper was supported over a tray containing 20 X SSC (3M NaCl, 0.3M Na₃citrate), with sides trailing in the buffer so that it remained saturated. The gel was carefully laid on this arrangement and covered with a nylon membrane (pre-wetted in 6 X SSC), excluding any air bubbles. Transfer was carried out by overlaying the nylon membrane with 2 pieces of Whatman 3MM filter paper cut to size, and a stack of paper towels pressed down with a weighted glass plate. After 16 hours, the nylon membrane was gently removed, washed in 2 X SSC for 10 sec, and air-dried. DNA was cross-linked by covering the membrane in Saran wrap, and irradiating on a short wave UV. transilluminator for 5 mins.

2.3.18 Dot blots

DNA solutions were denatured at 95°C for 5 mins and immediately placed on ice. After 5 mins, 2ul samples were spotted onto a nylon membrane (Amersham code RPN.303N) and air-dried. The membrane was then wetted with denaturing solution (1.5M NaCl, 0.5M NaOH) for 1 min, transferred to neutralising solution (1.5M NaCl, 0.5M Tris.HCl (pH 7.2), 1mM EDTA) for a further min and subsequently allowed to air-dry. To cross-link DNA, the membrane was covered in Saran wrap and irradiated DNA-side-down on a short wave UV. transilluminator for 5 mins.

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2.3.19 In situ colony blots

In situ colony blots were performed essentially as described by Grunstein and Hogness (1975). The recombinant bacterial colonies to be screened were patched (2 to 3mm in length) in duplicate onto gridded nylon membranes resting on the surface of a 20 X 20cm Petri dish containing LB medium supplemented with the appropriate antibiotic. A Petri dish without a membrane acted as a 'master'. Following overnight growth at 37°C, the 'master' plate was stored at 4°C, whilst membranes were carefully removed from the surface of the other plates and placed on Whatman 3MM paper saturated in 10% (w/v) SDS. After 3 mins, membranes were transferred to 3MM paper wetted with denaturing solution (1.5M NaCl, 0.5M NaOH), and left for 5 mins. Finally, membranes were rested on 3MM paper soaked with neutralising solution (1.5M NaCl, 1M Tris.HCl, pH 8.0) for 5 mins, and then air-dried for 20 mins at room temperature. In order to cross-link DNA released from the bacterial cells, membranes were covered in Saran wrap and irradiated colony-side-down on a short wave UV. transilluminator for 5 mins.

2.3.20 Hybridisation of Southern membranes, dot blots and colony blots

Dry Hybond-N membranes from either Southern, dot or colony blots were placed in heat sealed Pifco vacuum bags containing "Blotto" pre-hybridisation solution (0.5% (w/v) skimmed milk (Cadbury's Marvel), 1% (w/v) SDS, 6% (w/v) polyethylene glycol 8000, 0.27M NaCl, 1mM EDTA, 1.5mM sodium phosphate, pH 7.7), and incubated at 65°C for a minimum of 2 hours. Following pre-hybridisation, fresh "Blotto" supplemented with denatured ³²P-radiolabelled DNA probe was added, and hybridisation carried out at 65°C for an additional 18 hours. Radioactive probe was then discarded, and membranes

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washed twice in 2 X SSC at 55°C for 30 mins. Finally, membranes were blotted briefly on Whatman 3MM paper at room temperature for 2 to 3 mins, covered in Saran wrap and exposed to X-ray film as outlined in Section (2.3.24).

2.3.21 Sonication of DNA

Sonication of DNA was employed for the generation of a random population of DNA sub-fragments for sequencing. Initially, plasmid DNA was digested to completion with the appropriate restriction endonuclease, and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. The desired restriction fragment to be sequenced was purified from the gel by trough elution and then resuspended in double-distilled water to a final volume of 30ul.

Because maximum shear energy is experienced around the centre of a DNA molecule during sonication, circularised DNA will increase the random nature of this process. For this reason, the purified DNA fragment was self-ligated for 4 hours at room temperature in a total volume of 40ul, using 2 units of T4 polynucleotide ligase. After this period, the ligated DNA was transferred to a 1.5ml Eppendorf tube and placed in a Soniprep cuphorn sonicator filled with water to a depth of approximately 3cm, so that the tip of the tube was situated approximately 1mm above the surface of the probe. Sonication was then carried out at maximum power for 2 X 1 min bursts. After the initial burst, the water in the sonicator was changed so that the cool temperature of the probe was maintained, and the Eppendorf tube spun for a few seconds in an MSE Microcentaur at 13,500 rpm in order to concentrate the DNA solution for the next round of sonication.

Since the ends of the DNA molecules resulting from sonication, were

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not in a form which could be directly ligated into a suitable cloning vector, repair of the ends was carried out as follows. To the 40ul of DNA solution was added 2ul of a dNTP mix (1.5mM each of dATP, dCTP, dGTP, dTTP), 2ul of T4 DNA polymerase (4.45 U ml^{-1}), and 2ul of Klenow enzyme (5 U ml^{-1}). The reaction was then allowed to proceed at room temperature for 45 mins, after which time blunted DNA was loaded directly onto a 1.5% (w/v) TAE agarose gel, alongside size markers consisting of lambda DNA previously digested with either PstI or HindIII restriction endonucleases. Electrophoresis was then allowed to proceed until DNA had entered the gel to a distance of 1.5 to 2 cm. Fragments in the size-range 300 to 900bp were purified by trough elution and resuspended in 40ul of double-distilled water ready for ligation into the appropriate M13 cloning vector.

2.3.22 DNA sequencing

DNA sequence was determined using the dideoxynucleotide chain termination method of Sanger (1977, 1980).

2.3.22.1 Preparation of template

Single-stranded M13 template was prepared essentially as described by Sambrook et al. (1989). Competent cells of E. coli strain TG1 were transfected with recombinant M13 vector as described in Section 2.2.6.2, and overlaid on 2 X YT plates with 3ml of 2 X YT top agar containing 30ul isopropyl-B-D-thiogalactopyranoside (IPTG, 100mM in double-distilled water) and 50ul of X-gal solution (2% (w/v) in dimethyl-formamide). Following overnight incubation, colourless turbid recombinant plaques were cored using a Pasteur pipette and stored individually in 200ul of phage buffer at 4°C.

A 2ul sample of each bacteriophage cored lysate was spotted onto a

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top agar lawn containing 200ul of cells from a fresh overnight culture of E. coli TG1, and incubated overnight at 37°C. Freshly purified M13 plaques were then used to infect cells from a fresh overnight E. coli TG1 culture previously diluted 100-fold into 1.5ml of 2 X YT medium. Cultures were then propagated at 37°C with vigorous shaking (300 rpm) for 5 to 6 hours, and then pelleted in an MSE Microcentaur (13,500 rpm, 10 mins). The resulting supernatant was transferred to a clean 1.5ml Eppendorf tube containing 250ul of PEG/NaCl solution (20% (w/v) PEG 6000, 2.5M NaCl), and incubated at room temperature for 60 mins. M13 bacteriophage were subsequently harvested in an MSE Microcentaur (13,500, 10 mins) and all the PEG supernatant carefully removed by aspiration. The resultant pellet was gently resuspended in 100ul of TE buffer and extracted twice with an equal volume of phenol (equillbrated with 0.5M Tris.HCl, pH 7.8). On each occasion, phases were thoroughly mixed by vortexing and separated by centrifugation in an MSE Microcentaur (13,500 rpm, 5 mins). The upper aqueous phase was removed to a clean Eppendorf tube avoiding phenol, and mixed with 12ul of 2.5M sodium acetate (pH 6.0). Template DNA was precipitated by the addition of 300ul of 100% (v/v) ethanol and samples incubated at -70°C for 1 hour or -20°C overnight. DNA was recovered in an MSE Microcentaur (13,500 rpm, 15 mins) at room temperature, and the supernatant removed by aspiration. The pellet was washed in 100% (v/v) ethanol and liquid removed by aspiration again. Finally, DNA was dried under vacuum before being resuspended in 30ul of sterile double-distilled water and stored at -20°C.

2.3.22.2 T7 DNA polymerase 'Sequenase' sequencing

The DNA sequence of single-stranded templates was routinely determined using the Sequenase Version 2.0 kit, as recommended by the

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manufacturers. The Sequenase nucleotide triphosphate termination mixes are listed in Table 2.9.

2.3.22.3 Klenow sequencing

Control sequencing reactions used in primer extension experiments were performed using *E. coli* DNA polymerase 1 (Klenow fragment), essentially as described by Bankier *et al.* (1986). However, in this instance 5' ³²P end-labelled internal primers were used instead of M13 -40 sequencing primer, hence, no labelling step was required. The composition of nucleotide triphosphate termination mixes used in this procedure are listed in Table 2.9.

Annealing reactions were carried out in a 0.5ml Eppendorf tube containing 2ul 5 X sequencing buffer (200mM Tris.HCl (pH 7.5), 50mM MgCl₂, 250mM NaCl), 5ul template and 3ul end-labelled primer (0.5 pmol). The mixture was heated to 55°C for 1 hour, then collected by brief centrifugation and cooled on ice. A 1ul sample of 0.1M dithiothreitol and 5ul of ice-cold reaction solution (2 units of Klenow enzyme in 10ul of sterile doubl-distilled water) were added and briefly mixed. Aliquots (4ul) were then pipetted onto the sides of four wells of a Sterilin microtitre dish containing 2ul of NTP mix, and reactions initiated by spinning the aforementioned dish in an MSE Mistral 2000 plate centrifuge (2000 rpm, 3 sec). Following incubation at 37°C for 20 mins, 2ul of formamide dye was added to each well and the reactions heated to 80°C for 15 mins before being loaded on a denaturing polyacrylamide gel (Section 2.3.23). On a number of occasions, reactions were stored at -20°C without formamide dye until required. In this instance, the dish was stood at room temperature for 20 mins to allow the samples to thaw slowly, prior to denaturing.

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Table 2.9 The composition of nucleotide triphosphate termination mixes for Sequenase and Klenow sequencing reactions

Sequenase reaction

Nucleotide triphosphate	Concentration in μM			
	'A'mix*	'C'mix*	'G'mix*	'T'mix*
dATP	80	80	80	80
dCTP	80	80	80	80
dGTP	80	80	80	80
dTTP	80	80	80	80
ddATP	8	-	-	-
ddCTP	-	8	-	-
ddGTP	-	-	8	-
ddTTP	-	-	-	8

* All mixes were supplemented with 50mM NaCl

Klenow reaction

Nucleotide triphosphate	Concentration in μM			
	'A'mix	'C'mix	'G'mix	'T'mix
dATP	25	250	250	250
dCTP	250	25	250	250
dGTP	250	250	25	250
dTTP	250	250	250	25
ddATP	300	-	-	-
ddCTP	-	100	-	-
ddGTP	-	-	150	-
ddTTP	-	-	-	500

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2.3.22.4 Double-stranded sequencing

The following alkaline denaturing protocol was used to sequence double-stranded DNA template. Plasmid DNA was purified on a caesium chloride gradient, and 3 μ g denatured in 0.2M NaOH/ 0.2mM EDTA at room temperature for 5 mins. The mixture was then neutralised by adding 0.1 volume of 3M sodium acetate (pH 4.5), and DNA precipitated with 4 volumes of 100% (v/v) ethanol. Following incubation at -70°C for 30 mins, DNA was recovered in an MSE Microcentaur (13,500 rpm, 20 mins) at room temperature and washed in 70% (v/v) ethanol. The pellet was vacuum-dried, and finally resuspended in 7 μ l of sterile double-distilled water.

Sequenase buffer (2 μ l) and 1 μ l of primer (1.0 pmol) were mixed with the template solution, and annealing and sequencing reactions performed as described in section 2.3.22.2.

2.3.23 Denaturing polyacrylamide gels

Denaturing polyacrylamide gel electrophoresis was performed essentially as described by Bankier *et al.* (1986). The reagents used for this technique are listed in Table 2.10.

In general, 6% polyacrylamide buffer gradient gels (Biggin *et al.*, 1983; Hong, 1987) were poured between 50 X 20cm gel plates with 0.4mm spacers. A 45ml sample of 0.5 X TBE, 6% gel mix and a 7ml sample of 5 X TBE, 6% gel mix were each placed into two separate beakers. Polymerisation was initiated by the addition of a two thousandth volume of both 25% (w/v) ammonium persulphate and TEMED to each solution. The beakers were swirled, and 7ml of 0.5 X mix was pulled up in a 25ml pipette. The same pipette was then used to gently pull up 7ml of 5 X mix such that a clear interface between the two mixes was visible. A small number of air bubbles

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Table 2.10 Denaturing polyacrylamide gel reagents

Solution	Composition
40% (w/v) acrylamide	380g acrylamide 20g N,N-methylenebis-acrylamide Dissolved in double-distilled water to a final volume of 1 litre. De-ionized by stirring with 15g mixed bed resin for 15 min before filtration through Whatman 3MM paper. Stored at 4°C.
0.5 X TBE, 6% gel mix	75ml 40% (w/v) acrylamide 25ml 10 X TBE buffer 230g ultra-pure urea Dissolved in double-distilled water to a final volume of 500ml. Filtered through Whatman 3MM paper. Stored at 4°C.
5 X TBE, 6% gel mix	30ml 40% (w/v) acrylamide 100ml 10 X TBE buffer 92g ultra-pure urea 10mg bromophenol blue Dissolved in double-distilled water to a final volume of 200ml. Filtered through Whatman 3MM paper. Stored at 4°C.

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was then allowed to pass up the pipette to disrupt this interface. The solution was poured rapidly between gel plates previously taped and clamped, such that a 5cm wide stream remained down the length of one side. This was immediately followed by the rest of the 0.5 X mix. A gel comb (0.4mm thick) was placed to a depth of 5mm in order to form loading wells. The gel was then left at room temperature at an angle of 20° for 30 mins to fully polymerise. After this period, the gel was assembled into a Raven vertical slab gel electrophoresis chamber and upper and lower reservoirs filled with 1 X TBE buffer (89mM Tris-borate, 2mM EDTA, pH 8.3). The comb was carefully removed and wells flushed with buffer to remove any urea and unpolymerised acrylamide. Denatured samples were loaded in the order A, C, G, T, and electrophoresis carried out at constant 37 Watts for 2 to 3 hours. After running the gel, the siliconised glass plate was removed and the remaining plate, on which the exposed gel was resting, was immersed in 10% (v/v) acetic acid and 10% (v/v) methanol for 15 mins. The gel was then transferred to Whatman 3MM filter paper and covered with cling film, before being vacuum-dried in a Bio-Rad model 1125B gel drier at 80°C for 30 mins. Finally, the cling film was removed and the gel exposed to X-ray film as described in section 2.3.24.

In addition to the gradient gels described above, occasionally 'linear' gels were employed, where 0.5 X TBE, 6% gel mix was used throughout the length of the gel.

2.3.24 Autoradiography

Dried denaturing polyacrylamide gels or nylon membranes were rested in Harmer film cassettes and exposed to Fuji Nif RX X-ray film. For ³²P-radiolabelled material, autoradiography was carried out at -70°C with

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intensifying screens. For ^{35}S -radiolabelled material, autoradiography was carried out at room temperature without intensifying screens. In both cases, the resulting autoradiograms were developed in Kodak LX-24 developer and fixed in Kodak FX-40 fixer, according to the manufacturer's instructions.

2.3.25 Sequence analysis

DNA sequence data derived from this work was analysed on an IBM PCAT computer using the programmes supplied by DNASTAR Incorporated.

2.3.26 Siliconisation of glassware

Clean, grease-free glassware was rinsed thoroughly with 2% (v/v) dimethyldichlorosilane in 1,1,1-trichloroethane, and allowed to air dry at room temperature for 10 mins.

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2.4 RNA METHODS

2.4.1 Preparation of total cellular RNA

RNA was extracted from Er. chrysanthemi NCPPB 1066 using the hot phenol method of Aiba et al. (1981). To avoid contamination with ribonucleases, glassware used in the preparation of RNA was baked at 150°C overnight. Tubes, tips and reagents (except phenol and ethanol) were autoclaved at 121°C for 30 mins and handled with protective gloves. All solutions were made with double-distilled water previously treated with 0.1% (v/v) diethylpyrocarbonate (DEPC).

A 10ml overnight culture of wild-type Er. chrysanthemi NCPPB 1066 was diluted 100-fold in 10ml of suitable growth medium and propagated at 30°C (200 rpm) until an A_{600} value of 0.8 was reached. Cells were harvested in a Wifug Labor 50M centrifuge and resuspended in 450ul of SAE (0.5% (w/v) SDS, 1mM EDTA, 20mM sodium acetate, pH 5.5). An equal volume of BRL ultra-pure phenol (equilibrated with 20mM sodium acetate, pH 5.5) was then added, and an emulsion formed by vortexing. The solution was then incubated at 65°C for 5 mins with regular mixing, before being spun in an MSE microcentaur (13,500 rpm, 10 mins) at room temperature. The upper aqueous phase was carefully removed and the phenol extraction repeated. RNA was precipitated at -70°C for 30 mins by the addition of 1ml 100% (v/v) ethanol, and collected in an MSE Microcentaur (13,500 rpm, 10 mins, 4°C). The resulting pellet was resuspended in 333ul SAE on ice and re-precipitated as before. After harvesting a second time, RNA was dissolved in 500ul of DNAase buffer (100mM sodium acetate (pH 5.5), 10mM $MgCl_2$) and 2ul RNAase-free DNAase was added. The reaction was placed at 37°C for 30

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mins before being phenol extracted once. One-tenth volume of 3M sodium acetate (pH 5.5) and 1ml of 100% (v/v) ethanol was mixed with the aqueous phase and incubated at -70°C for 30 mins. Precipitated RNA was re-pelleted as described above, vacuum dried for 20 mins, and resuspended in 50ul of DEPC-treated water.

2.4.2 RNA formaldehyde gels

RNA was visualised using 1.5% (w/v) agarose/formaldehyde gels. These were prepared by boiling 0.75g of agarose (Sigma Type II medium EOO) in 31ml of double-distilled water and allowing the solution to cool to 60°C , before adding 10ml of 5 X MOPS buffer (0.2M MOPS (pH7.0), 50mM sodium acetate, 5mM EDTA, pH 8.0) and 9ml formaldehyde. The mixture was then poured into an RNA-free tank maintained within a fume hood. RNAase activity from previous gels was removed by washing the gel plate and gel tank with 1M NaOH.

A 5ul sample of RNA solution (ca. 2 - 3ug) was added to 15ul of Northern loading buffer (50% (v/v) deionised formamide, 6% (v/v) formaldehyde, 10% (w/v) glycerol, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue, 100ug ml^{-1} ethidium bromide), heated to 65°C for 10 mins and immediately loaded onto a formaldehyde gel completely submerged in 1 X MOPS buffer. Electrophoresis was then carried out with circulating buffer at 100V until the samples had entered the gel, and thereafter at 65V for 2 to 3 hours. After this period, RNA was examined by short wave UV shadowing in order to assess the concentration of the sample, and the level of degradation incurred during the preparation.

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2.4.3 Primer extension analysis of total cellular RNA

The single-stranded oligonucleotide to be used as the primer was end labelled with gamma-³²P-ATP as described in Section 2.3.16.2 and resuspended in 3 X PEB buffer (30mM PIPES (pH 6.4), 1.5mM EDTA (pH 8.0), 1.2M NaCl). Hybridisation reactions were set up by mixing the RNA sample (in 20ul of DEPC-treated water) and 10ul of the labelled primer (in 3 X PEB) in a 0.5ml Eppendorf tube. Samples were heated to 80°C for 10 mins, followed by incubation at 55°C for 3 hours. After hybridisation, 60ul of 100% (v/v) ethanol was added and the tubes placed at -20°C for 30 mins. Nucleic acids were recovered by centrifugation in an Eppendorf minifuge (10,000 rpm, 15 mins) at room temperature and dissolved in 20ul of reverse transcriptase reaction buffer (50mM Tris.HCl (pH 8.3), 6mM MgCl₂, 10mM DTT, 1mM each of dATP, dCTP, dGTP, dTTP), and Actinomycin D (25ug ml⁻¹). AMV reverse transcriptase (9 units) was then added and the reactions incubated at either 37°C for 30 mins, or 42°C for 60 mins. RNase A was then added to a final concentration of 2ug ml⁻¹ and tubes placed at 37°C for 10 mins. DNA was precipitated at -20°C overnight, following the addition of sodium acetate (pH 6.5) to a final concentration of 0.3M and 2 volumes of 100% (v/v) ethanol. Following centrifugation in an Eppendorf minifuge (10,000 rpm, 15 mins), the DNA pellet was resuspended in 4ul of denaturing gel loading dye (90% (v/v) deionised formamide, 10mM EDTA (pH 8.0), 0.01% (w/v) xylene cyanol, 0.01% (w/v) bromophenol blue), heated to 80°C for 15 mins, and then loaded alongside control sequencing reactions, on a 6% denaturing polyacrylamide linear wedge gel (Section 2.3.23). Electrophoresis was carried out at 55W (20mA) for 3½ hours, after which the gel was fixed, vacuum dried in a Bio-Rad model 1125B gel drier at 80°C for 3 hours and exposed to X-ray film as described in Section 2.3.24.

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2.5 PROTEIN METHODS

2.5.1 Preparation of protein samples

E. coli and Er. chrysanthemi NCPPB 1066 strains were propagated at 30°C in 10ml LB medium, supplemented with the appropriate antibiotics if required, until an OD₆₀₀ value of 1.0 was reached. Cultures were chilled on ice and harvested in a Wifug Labor 50M bench centrifuge (4,500 rpm, 10 mins). The cell pellet was then washed in 5ml phosphate buffered saline (PBS) and finally resuspended in 1ml of the same buffer. Cell-free extracts were prepared by sonication in a 150 Watt MSE ultrasonic disintegrator using the smallest probe. Disruption was carried out on ice with 4 x 30 sec 6 micron peak to peak bursts, with 30 sec cooling period between each burst. Unlysed cells and membranes were removed by centrifugation in an Eppendorf minifuge (10,000 rpm, 10 mins, 4°C) and supernatants stored in aliquots at -20°C until required. The concentration of soluble protein was estimated using the Bio-Rad micro-assay, as described in Section 2.2.20.4.

Alternatively, total cellular protein was prepared by growing strains in 5ml of LB medium with antibiotics if required, to an OD₆₀₀ value of 0.5. Cells were concentrated by centrifugation in a Wifug Labor 50M bench centrifuge (4,500 rpm, 10 mins), washed in 5ml of PBS, and resuspended in 100ul of Tris.HCl (pH 7.4). To this was added an equal volume of 2 X SDS sample buffer (Table 2.11), and the sample stored at -20°C until required.

2.5.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were routinely analysed on 11% (w/v) linear SDS polyacrylamide gels measuring 17cm x 16cm x 1mm, using the discontinuous

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buffer system of Laemmli (1970). The buffers and acrylamide solutions used are listed in Table 2.11 and 2.12, respectively. Both the separating gel and stacking gel mixtures were de-gassed for 15 mins prior to the addition of TEMED and ammonium persulphate for polymerisation.

A separating gel was cast first, and overlaid with water saturated iso-butanol until polymerisation was complete. After this time, the iso-butanol was removed by thorough washing with double-distilled water, and replaced with 10 ml of stacking gel. A Teflon comb was immediately inserted and the stacking gel allowed to set for 30 mins. Subsequently, the comb was removed and the wells flushed with electrophoresis buffer to remove any unpolymerised acrylamide.

Protein samples were thawed on ice, mixed with an equal volume of 2 X SDS sample buffer, and heated to 100°C for 5 mins prior to loading. Electrophoresis was carried out using a Bio-Rad Protean Dual Vertical Slab gel system at a constant 16mA for 12 hours, or 25mA for 3 hours. In all cases, protein molecular weight estimations were performed by running samples alongside the following Pharmacia protein standards : phosphorylase b (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20.1 kDa); and alpha lactalbumin (14.4 kDa).

2.5.3 Silver staining of SDS polyacrylamide gels

The rapid and sensitive procedure of Wray *et al.* (1981) was used to silver stain SDS polyacrylamide gels. Briefly, polyacrylamide gels were soaked in a 50% (v/v) methanol solution on the bench for a minimum of three hours with constant gentle agitation. After this period, they were transferred to a silver stain solution, previously prepared by adding solution A (0.8g of silver

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Table 2.11 Stock solutions for SDS-PAGE

Stock solution	Composition
Separating gel buffer	3M Tris.HCl (pH 8.8)
Stacking gel buffer	0.05M Tris.HCl (pH 6.8)
High bis-acrylamide	60% (w/v) acrylamide 1.6% (w/v) N,N'-methylene-bis-acrylamide
Stacking gel acrylamide	10% (w/v) acrylamide 0.5% (w/v) N,N'-methylene-bis-acrylamide
Electrophoresis buffer	0.025M Tris.HCl 0.192M glycine 0.1% (w/v) SDS
SDS-sample buffer (X 2)	0.05M Tris.HCl (pH 6.8) 10% (w/v) glycerol 2% (w/v) SDS 5% (w/v) B-mercaptoethanol 0.001% (w/v) bromophenol blue

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Table 2.12 **Composition of polyacrylamide separating and stacking gels**

Separating gel

Stock solution	Volume required for an 11% (w/v) gel (ml)
Lower gel buffer	3.76
High bis-acrylamide	5.45
10% (w/v) SDS	0.30
distilled water	20.35
10% (w/v) ammonium persulphate*	0.060
TEMED	0.006

Stacking gel

Stock solution	Volume required for an 11% (w/v) gel (ml)
Stacking gel buffer	2.40
Stacking acrylamide	3.00
10% (w/v) SDS	0.10
distilled water	4.40
10% (w/v) ammonium persulphate*	0.10
TEMED	0.005

* freshly prepared solution.

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nitrate dissolved in 4ml of double-distilled water) drop-wise into solution B (21ml of 0.365 (w/v) sodium hydroxide mixed with 1.4ml of 14.8 M ammonium hydroxide solution), and finally diluting to 100ml with double-distilled water. Following 20 mins gentle agitation at room temperature, gels were washed with copious amounts of double-distilled water for a period of 5 mins, and then developed in solution C (2.5ml of 1% (v/v) citric acid and 0.25ml of 38% (v/v) formaldehyde solution, made up to 500ml with double-distilled water) until bands appeared (usually between 10 and 20 mins). The development process was then arrested by washing gels briefly in double-distilled water and then placing them in a fixing solution (50% (v/v) methanol, 5% (v/v) acetic acid).

2.5.4 Western blots

Western analysis was performed using a modified method of Towbin et al. (1979). Following electrophoresis, SDS polyacrylamide gels were placed in Western transfer buffer (25mM Tris.HCl (pH 8.3), 193 mM glycine, 20% (v/v) methanol) for 10 mins, along with scouring pads, Whatman 3MM filter paper and Amersham 'Hybond-C' nitrocellulose membrane (Amersham code RPN.303C). Two sheets of 3MM paper were placed on a scouring pad and gently overlaid with the polyacrylamide gel. A nitrocellulose membrane previously cut to size, was then positioned on the gel, excluding any air bubbles, and covered with two sheets of 3MM paper. A second scouring pad was finally laid on top, and the arrangement clipped together before being lowered into a Bio-Rad Transblot cell filled with transfer buffer.

Electrophoresis was carried out with the gel facing the cathode, at 40 volts (300 mA) for 3 hours at 4°C. Following transfer, all manipulations were performed at room temperature with gentle agitation. The nitrocellulose

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membrane was stained with 0.5% (w/v) Ponceau S (dissolved in 5% (w/v) TCA) for 10 mins, and the position of the protein standards and lanes marked. The membrane was then de-stained for 10 mins in Tris buffered saline (TBS) (150mM NaCl, 50mM Tris.HCl, pH 8.0), and non-specific antibody binding sites blocked with TBS containing 2% (w/v) skimmed milk (Cadbury's Marvel) for a minimum of 1 hour. This was then replaced by fresh blocking solution containing 1/500 diluted Rabbit antibody raised to either B-galactosidase or alkaline phosphatase. After overnight shaking, the membrane was washed three times in TBS containing 0.1% (w/v) TWEEN 20, and incubated for two hours in TBS plus 0.1% (w/v) TWEEN 20 containing anti-Rabbit IgG (whole molecule) peroxidase conjugate (Sigma No. A 6154), diluted 300-fold. The membrane was then washed twice in TBS plus 0.1% (w/v) TWEEN 20 for 10 mins, and twice in TBS alone for 10 mins, before being developed.

A 50ul sample of hydrogen peroxide (100 volumes) was added to 50ml of solution A (0.51 M NaCl, 20mM Tris.HCl (pH 7.5), in double-distilled water), and combined with 50ml of solution B (3.4mM 4-chloro-1-naphthol (Sigma No. C 8890), 20% (v/v) methanol, in double-distilled water). The membrane was then covered in the A/B mix until a sufficient colour reaction was seen, and at this point, the membrane was washed three times in double-distilled water. Finally, the membrane was air-dried for 10 mins and stored in the dark.

CHAPTER THREE

A PHYSICAL, BIOCHEMICAL AND GENETIC ANALYSIS

OF ERWINIA CHRYSANTHEMI NCPPB 1066

3. Physical, biochemical and genetic analysis

3.1 INTRODUCTION

Prior to the onset of this project, limited genetic studies carried out with Er. chrysanthemi NCPPB 1066, both at C.A.M.R. Porton Down and in this laboratory, had met with no real success. Attempts had been made, for example, to introduce the promiscuous RP4::mini-Mu plasmid, pULB113, into a variety of Erwinia strains, including Er. chrysanthemi NCPPB 1066, via conjugal mobilisation (P. Reeves; personal communication). The frequency of transfer of pULB113 into the latter organism was found to be extremely low ($< 1 \times 10^{-8}$ transconjugants per recipient) compared to those obtained with Er. carotovora subsp. carotovora SCRI 193 (4.2×10^{-3} transconjugants per recipient) or subsp. atroseptica SCRI 1043 (9.5×10^{-3} transconjugants per recipient). The single Er. chrysanthemi NCPPB 1066 pULB113 transconjugant isolated from repeated patch matings was found to grow extremely poorly, hence, chromosomal mapping studies could not be performed.

Further experimentation had demonstrated that the Tn₅ suicide vector, pJB4JI, could be introduced into Er. chrysanthemi NCPPB 1066 by conjugal mobilisation, albeit at the low frequency of 5.8×10^{-8} transconjugants per recipient. Unfortunately this plasmid was found to be stably maintained and therefore was considered to be unsuitable as a suicide vehicle for transposon mutagenesis (D. Cardy; personal communication).

Finally, Gilbert and coworkers (1986) stated that repeated efforts to introduce the L-asparaginase II expression vectors pASN326 and pASN230 into Er. chrysanthemi NCPPB 1066, using a variety of techniques which had included the transformation protocol of Hinton et al. (1985 b), had failed. The same was also found to be true of plasmid pBR322 (Dr. N. Minton; personal communication). In control experiments, on the other hand, plasmids

3. Physical, biochemical and genetic analysis

pKT230 and pUC9 were successfully introduced into Er. carotovora subsp. carotovora SCRI 193 at frequencies of 6×10^4 and 1×10^3 transformants μg^{-1} DNA, respectively.

3.1.1 Aim

From the limited studies outlined above, it was clearly evident that Er. chrysanthemi NCPPB 1066 was a more difficult organism to work with in genetic terms compared to other Erwinia strains such as Er. carotovora subsp. carotovora SCRI 193. The aim of the work described in the following sections of this chapter was to direct further efforts towards adapting a number of genetic tools for Er. chrysanthemi NCPPB 1066, as a prerequisite for a study of L-asparaginase II regulation in this organism. To this end, both the wild-type Er. chrysanthemi NCPPB 1066 strain and a sample of the industrial production culture (batch 8) were obtained from C.A.M.R Porton Down for genetic analysis.

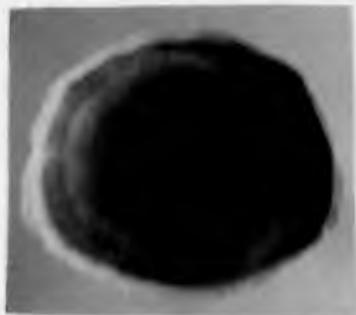
3.2 PHYSICAL AND BIOCHEMICAL CHARACTERISATION OF WILD-TYPE AND INDUSTRIAL ER. CHRYSANTHEMI NCPPB 1066 ISOLATES

3.2.1 Results and discussion

Wild-type Er. chrysanthemi NCPPB 1066 and the industrial production culture (batch 8) were initially streak-purified to isolated colonies on NB medium and incubated at 30°C for two days. Rather unexpectedly, the industrial production culture gave rise to five different colony morphologies which were clearly distinct from that of the wild-type strain (Figure 3.1). In most cases the industrial morphotypes were found to breed true, although industrial morphotype 1 displayed the additional feature of time-dependent

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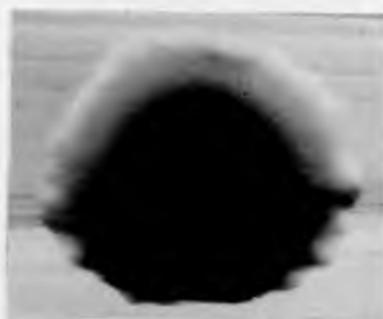
Figure 3.1 Colony morphology of wild-type *Er. chrysanthemi* NCPPB 1066 and isolates from the industrial L-asparaginase II production culture (batch 8)



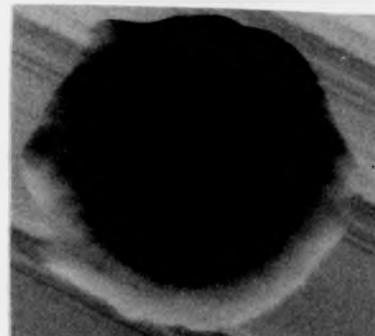
Wild-type



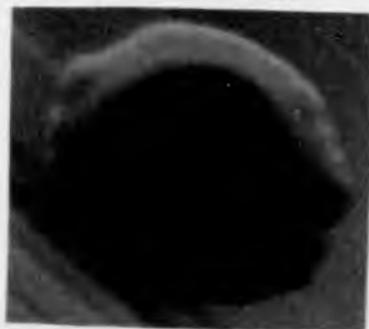
Industrial morphotype 1



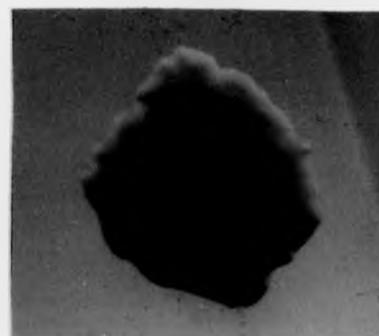
Industrial morphotype 2



Industrial morphotype 3



Industrial morphotype 4



Industrial morphotype 5

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sectoring, as illustrated in Figure 3.2. Following an 18 hour incubation on NB and LB medium at 30° C, approximately 7% of the colonies gave rise to this phenomenon. The exact basis for this is not understood at present. However, it does not appear to be linked to a change in bacterial motility, as assessed by viewing cells from sector and non-sector regions under the light microscope.

Based on these surprising morphological observations, it was not clear whether the industrial L-asparaginase II production culture was contaminated, or whether it could be viewed as axenic and, in fact, consisted of mutated forms of the Er. chrysanthemi NCPPB 1066 L-asparaginase II hyper-producing derivative S10. Further characterisation of the five morphological variants and the wild-type Er. chrysanthemi NCPPB 1066 strain was therefore performed in order to answer this question.

3.2.1.1 Physical studies

Phase contrast microscopy and Gram-staining revealed that both the wild-type Er. chrysanthemi NCPPB 1066 strain and the industrial Er. chrysanthemi NCPPB 1066 morphological variants were Gram-negative rods. On further examination, however, a number of physical differences between the industrial morphological variants and the wild-type strain became apparent. Firstly, the overall cell size of the industrial morphotypes appeared to be smaller than the wild-type strain. A second notable feature was the unique dramatic flocculating properties of industrial morphotype 1, which grew as visible clumps throughout the growth phase, rather than a uniform culture. Finally, variations in bacterial motility were also detected. In this respect, phase contrast microscopy revealed wild-type Er. chrysanthemi NCPPB 1066 cells to be highly motile, as were those of industrial morphotypes

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Figure 3.2 **Sectoring displayed by the industrial morphotype 1**



Bacteria were grown on NB medium at 30°C. Arrows highlight the areas of sectoring.

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2 and 4, but to a lesser extent. In contrast, industrial morphotypes 1, 3 and 5 were agitated by Brownian motion and were clearly non-motile.

These visual observations were corroborated by growing isolates to an OD₅₅₀ value of either 0.5 or 1.0 in motility broth, and spotting 2ul of the culture onto the surface of a motility agar plate. The results following an 8 hour incubation at 30°C are visually presented in Figure 3.3. Whether the non-motile industrial morphotypes 1, 3 and 5 represent paralysed mutants that have normal flagella which fail to rotate (Mot⁻ phenotype) even though the proton motive force is normal, or have in fact accumulated a number of mutation(s) within the fla (flb) genes and therefore lack flagella altogether (Fla⁻ phenotype), is not known at present.

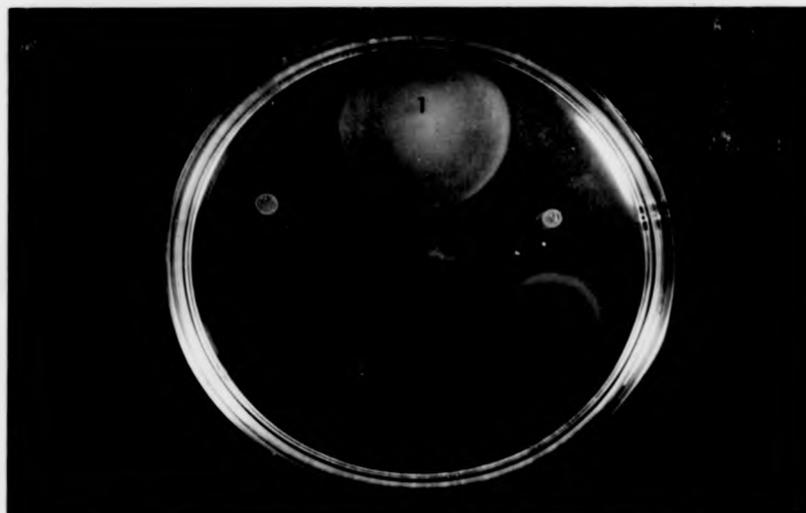
3.2.1.2 API 20E biochemical tests

Biochemical characterisation of wild-type and industrial Er. chrysanthemi NCPPB 1066 isolates was performed using the commercial API 20E system, as outlined in Section 2.2.12. This constitutes a rapid alternative to conventional phenotypical testing procedures, and has been used successfully by Mergaert and colleagues (1984) for the classification and identification of a range of Erwinia species where it was found to be highly reproducible and discriminatory.

The results of two independent tests performed in duplicate at 30°C are presented in Table 3.1. Overall, the API profiles recorded for both the wild-type Er. chrysanthemi NCPPB 1066 strain and the industrial morphotypes were found to be identical apart from two of the reactions. The first was melblose fermentation/oxidation, where the wild-type Er. chrysanthemi NCPPB 1066 strain gave a clear positive reaction and each of the industrial morphotypes did not. The second anomaly was seen for gelatin liquifaction,

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Figure 3.3 Motility agar test for wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes 1 - 5



Key : 1) wild-type Er. chrysanthemi NCPPB 1066; 2) morphotype 1; 3) morphotype 2; 4) morphotype 3; 5) morphotype 4; 6) morphotype 5

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Table 3.1 API biochemical profiles for wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotypes 1-5

Test	wild-type					industrial morphotype				
	1	2	3	4	5	1	2	3	4	5
B-galactosidase	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+
H ₂ S production	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-
Tryptophane desaminase	-	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+	+
Acetoin production	+	+	+	+	+	+	+	+	+	+
Proteolysis of gelatin	+	+	+	+	+	+	+	+	+	+
Glucose fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
Mannitol fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
Inositol fermentation/oxidation	-	-	-	-	-	-	-	-	-	-
Sorbitol fermentation/oxidation	-	-	-	-	-	-	-	-	-	-
Rhamnose fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
Saccharose fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
Melibiose fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
Amygdaline fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
L(+) arabinose fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-
Production of nitrites	+	+	+	+	+	+	+	+	+	+
Production of nitrogen	+	+	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+	+	+

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where again the wild-type gave a positive reaction in contrast to the industrial morphotypes. This latter result suggested that the wild-type strain had relatively greater proteolytic activity, compared to each of the industrial morphotypes. Subsequent analysis on extracellular protease detection medium reinforced this observation (see Figure 3.4).

The pattern of reactions obtained for both the wild-type and industrial morphotypes was translated into the seven digit profile number, 1247173, which was identical to the number recorded for Er. chrysanthemi NCPPB 1065 and several other Er. chrysanthemi strains, including the type strain Er. chrysanthemi NCPPB 402 (Mergaert et al., 1984). These results confirmed the re-classification of Er. chrysanthemi NCPPB 1066 and provided good evidence to suggest that the wild-type strain and the industrial morphotypes were fundamentally the same organism.

3.2.1.3 Sugar utilisation profiles

Both wild-type Er. chrysanthemi NCPPB 1066 and each of the industrial morphotypes were replicated from NB medium onto MacConkey medium containing a variety of sugars, and incubated at 30°C overnight. The results of two independent experiments are summarised in Table 3.2, where it can be seen that the profiles for wild-type and industrial isolates were very similar. However, slight differences were noted on both MacConkey cellobiose and raffinose medium, where wild-type Er. chrysanthemi NCPPB 1066 cells became pink and each of the industrial morphotypes remained white.

The finding that both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes were positive for B-galactosidase activity (Section 3.3.1.1), but were unable to utilise lactose, is in accordance with what has been reported for many other Er. chrysanthemi strains which have either no

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Table 3.2 MacConkey sugar utilisation profiles for wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotypes 1 - 5

Sugar	wild-type	industrial morphotype				
		1	2	3	4	5
Mannitol	++	++	++	++	++	++
Trehalose	-	-	-	-	-	-
Cellobiose	+	-	-	-	-	-
Xylose	+	+	+	++	+	+
Sorbitol	-	-	-	-	-	-
Lactose	-	-	-	-	-	-
Maltose	-	-	-	-	-	-
Raffinose	+	-	-	-	-	-
Sucrose	+++	+++	+++	+++	+++	+++
Arabinose	+++	+++	+++	++	+++	++

Visual analogue scale of colony coloration :
 +++ dark red
 ++ red
 + pink
 - white

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ability, or a variable or delayed ability to ferment this sugar (Dickey, 1979; Lelliott and Dickey, 1984). Studies performed by Hugouvieux-Cotte-Pattat and Robert-Baudouy (1985 b) have indicated that wild-type strains of Er. chrysanthemi do not possess the lactose operon (comprised of the lacZ, lacY and lacA genes), which is found in E. coli (Miller and Reznikoff, 1978). Instead, Er. chrysanthemi is believed to contain two genes, designated lacZ and lacB. The first encodes B-galactosidase, whilst the second is a regulatory gene that controls expression of the lacZ gene itself. Moreover, Er. chrysanthemi does not appear to have a gene equivalent to lacA or lacY, and as such, lacks both galactoside transacetylase activity and a specific lactose permease. In this sense, the lactose system in Er. chrysanthemi NCPPB 1066 resembles that of Shigella dysenteriae, which possess only the lacI and lacZ genes (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1985 b). Hence, the absence of a lactose permease in Er. chrysanthemi NCPPB 1066 is taken to explain the negative results obtained on MacConkey lactose medium.

As a consequence of the above observations, MacConkey lactose medium was not used in further experiments aimed at isolating Er. chrysanthemi NCPPB 1066 lacZ⁻ mutants (Section 3.3.1.4), using ansB::lacZ gene fusions or isolating L-asparaginase II regulatory mutants (see Chapter 7). Instead, solid medium containing the chromogenic B-galactosidase substrate, X-gal was employed. This compound provides a sensitive assay for B-galactosidase activity, and is not totally dependent on a specific lactose permease for its transport into the cell (Silhavy and Beckwith, 1985).

Finally, wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes were also found to give a weak or negative reaction, respectively, on MacConkey raffinose medium, therefore suggesting that this sugar was not being utilised to a great extent, if at all. Similarly,

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Hugouvieux-Cotte-Pattat and Robert-Baudouy, (1985 b) have also noted that Er. chrysanthemi strain 3665 is unable to utilise raffinose, and in this respect, differs from Er. chrysanthemi strains 3937j and B374. On the other hand, both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes appeared to utilise sucrose to a much greater extent. Hence, it was decided that mating experiments would involve the use of this particular sugar in place of raffinose in order to perhaps achieve a more effective counterselection of E. coli.

3.2.1.4 Antibiotic sensitivity

The sensitivity of the wild-type Er. chrysanthemi NCPPB 1066 strain and the industrial morphotypes to a range of antibiotics, was investigated using Mastring-S antibiotic discs as described in Section 2.2.13. The results following overnight incubation at 30°C, are summarised in Table 3.3. Overall, the antibiogram profiles were very similar. However, the industrial morphotype 3 did appear to display slightly increased sensitivity to the antibiotics clindamycin, penicillin G, fusidic acid and erythromycin.

3.2.1.5 Extracellular enzyme production

Qualitative analysis of the synthesis of extracellular enzymes by wild-type Er. chrysanthemi NCPPB 1066 and all five of the industrial morphotypes was carried out by replicating colonies onto the appropriate extracellular enzyme detection medium, as described in Section 2.2.19. The results following a 36 hour incubation at 30°C are presented in Figure 3.4. In all cases, the size of the halos surrounding each of the industrial morphotypes was found to be significantly smaller than those produced by wild-type Er. chrysanthemi NCPPB 1066. It is believed that this reduction cannot be

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Table 3.3 Antibiogram profiles for wild-type Er. chrysanthemi
NCFPB 1066 and industrial morphotypes 1 - 5

Antibiotic	conc ⁿ . disc ⁻¹	wild-type	Industrial morphotype				
			1	2	3	4	5
Erythromycin	5ug	-	-	-	+R	-	-
Fusidic acid	10ug	-	-	-	+R	-	-
Gentamicin	10ug	+++	+++R	+++	+++	+++R	++++
Clindamycin	2ug	-	-	-	+R	-	-
Penicillin G	1 unit	-	-	-	++R	-	-
Tetracycline	10ug	+++	++	++	++	+++	++
Sulphamethoxazole	25ug	++	++R	++	++	++R	++R
Trimethoprim	1.25ul	++	++	++	+++	++	++

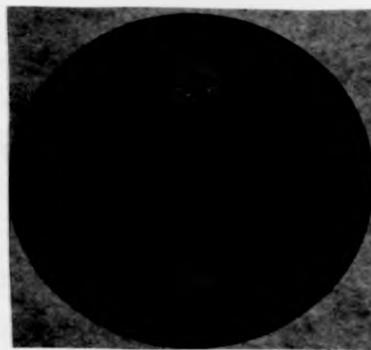
R denotes the presence of spontaneous antibiotic resistant colonies within the zone of clearing.

Key : ++++ zone of clearing of diameter 2.5 cm.
 +++ zone of clearing of diameter 1.9 - 2.1 cm.
 ++ zone of clearing of diameter 1.3 - 1.5 cm.
 + zone of clearing of diameter 1 cm.
 - no visible zone of clearing.

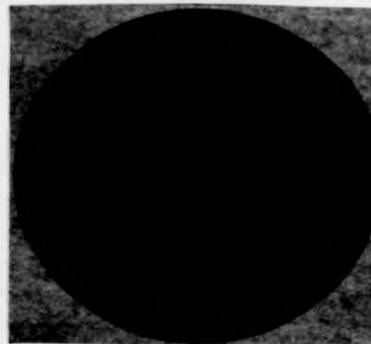
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Figure 3.4 Extracellular enzyme production by wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotypes 1 - 5

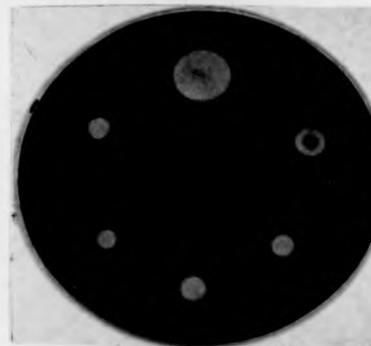
Protease detection
medium



Pectinase detection
medium



Cellulase detection
medium



Key : 1) Wild-type Er. chrysanthemi NCPPB 1066; 2) morphotype 1; 3) morphotype 2; 4) morphotype 3; 5) morphotype 4; 6) morphotype 5

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explained solely on the basis of differences in growth rate. Whether the industrial morphotypes are, in fact, reduced in the expression of the pel, cel and pri structural genes, or have suffered an alteration in the Out secretion system, or both, will require further investigation employing quantitative biochemical assay.

3.2.1.6 Comments

From the work described above, it was concluded that prolonged fermentation of Er. chrysanthemi NCPPB 1066 had resulted in an industrial culture which not only differed from the wild-type progenitor in terms of L-asparaginase II production (see Chapter 4), but also in a variety of physical and biochemical aspects. The five distinct colony types which were identified in this study all appeared to be Er. chrysanthemi NCPPB 1066 in nature and not contaminants of the production culture as originally thought. Such pleiomorphism is a well known side-effect of long-term fermentation (Prof. A. Atkinson; personal communication), and appears to be worsening with time in the case of the Er. chrysanthemi NCPPB 1066 process (Dr. R. Sharpe; personal communication). This no doubt reflects a steady accumulation of mutations arising from either spontaneous chromosomal re-arrangements, point mutations, or the action of transposable (IS) elements, some of which have affected either structural genes encoding a component of the outer surface of the cell, or genes involved in regulation of the former. Indeed, a number of genetic loci have now been identified in E. coli K12, for example, whose products can influence a number of cell surface properties, such as colony morphology and the ability to auto-aggregate. This includes a gene designated flu by Diderichsen (1980), which is located at 43' on the E. coli K12 chromosome, as well as a gene designated mor, which maps to 89'

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(Warne et al., 1990).

A spontaneous mutation affecting gene expression tends to be rare, usually occurring at a low frequency of less than one mutational event per 10^6 cells per generation. In contrast, programmed DNA rearrangements which control gene expression in bacterial and eukaryotic systems, arise in approximately one in 10^2 to 10^5 cells per generation (Glasgow et al., 1989). It is therefore tempting to speculate that the high frequency of sectoring displayed by the industrial morphotype 1 isolate may be a result of the latter genetic phenomenon. This could possibly involve a DNA inversion event, as has been found with flagellar variation in S. typhimurium, pilin variation in Moraxella bovis, and fimbrial variation in E. coli (Glasgow et al., 1989). Alternatively, it may involve duplicative transposition, as is the case with the switching of mating type in yeast and pilin variation in Neisseria gonorrhoeae, or a deletion/insertion event similar to that found in pigment variation in Streptomyces reticuli and opacity protein variation in Neisseria gonorrhoeae (Borst and Greaves, 1987; Glasgow et al., 1989).

3.3 GENETIC ANALYSIS OF ER. CHRYSANTHEMI NCPPB 1066

Although the Er. chrysanthemi NCPPB 1066 strains to be used in this study had been characterised to a certain extent, further work was required to adapt a number of genetic techniques to this hitherto genetically refractory organism.

3.3.1 Chemical mutagenesis

3.3.1.1 Approach

Alkylating agents form the largest class of mutagen and induce mutations both directly through mispairing, and indirectly through error-prone

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repair, giving rise to a plethora of genetic defects. Of this class, methylmethane sulphonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethylmethane sulphonate (EMS) are mutagens commonly applied to prokaryotic systems. Treatment with the latter compound primarily leads to G.C. to A.T. transitions of DNA, supporting the formation of O-6-alkyl guanidine as the primary mutagenic event in this case. In addition, a small fraction of A.T. to G.C. transitions, as well as some transversions, can also occur (Glass, 1982).

3.3.1.2 Aim

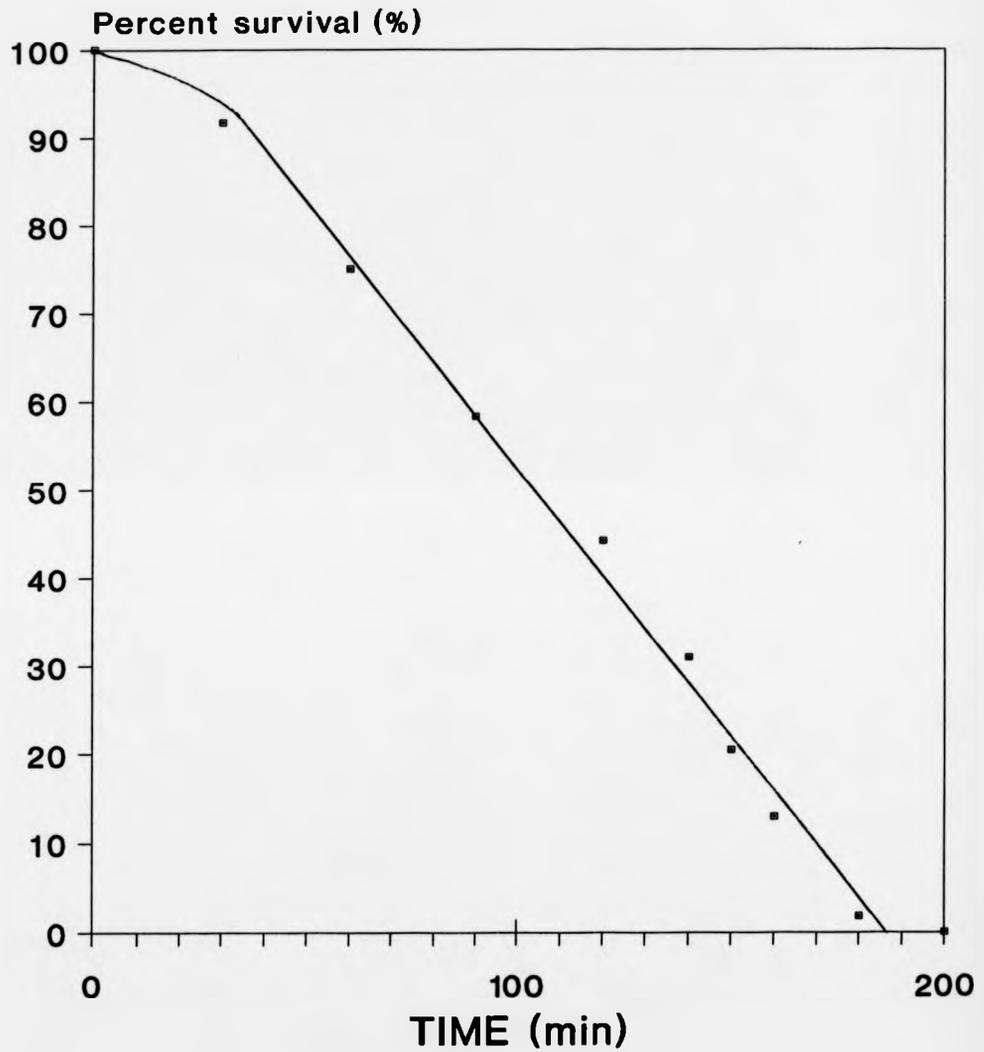
EMS mutagenesis has been successfully used in this laboratory to generate a range of nonsense, missense and temperature-sensitive mutations in a number of Erwinia strains, including Er. carotovora subsp. carotovora SCRI 193 (Hinton et al., 1985 a; P. Reeves; personal communication) and a carbapenem-producing strain, Er. carotovora subsp. carotovora ATCC 39048 (Dr. G.P.C. Salmond; personal communication). Therefore, the aim of this work was firstly to determine the susceptibility of Er. chrysanthemi NCPPB 1066 to EMS, and secondly to isolate both B-galactosidase (LacZ⁻) and alkaline phosphatase (PhoA⁻) mutants to act as recipients for L-asparaginase II fusion vectors (see Chapter 7).

3.3.1.3 Results and discussion

Preliminary EMS mutagenesis experiments were performed in which wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2 cells were exposed to EMS for varying times, as described in Section 2.2.17. The resulting survival curves are presented in Figure 3.5 and 3.6, respectively.

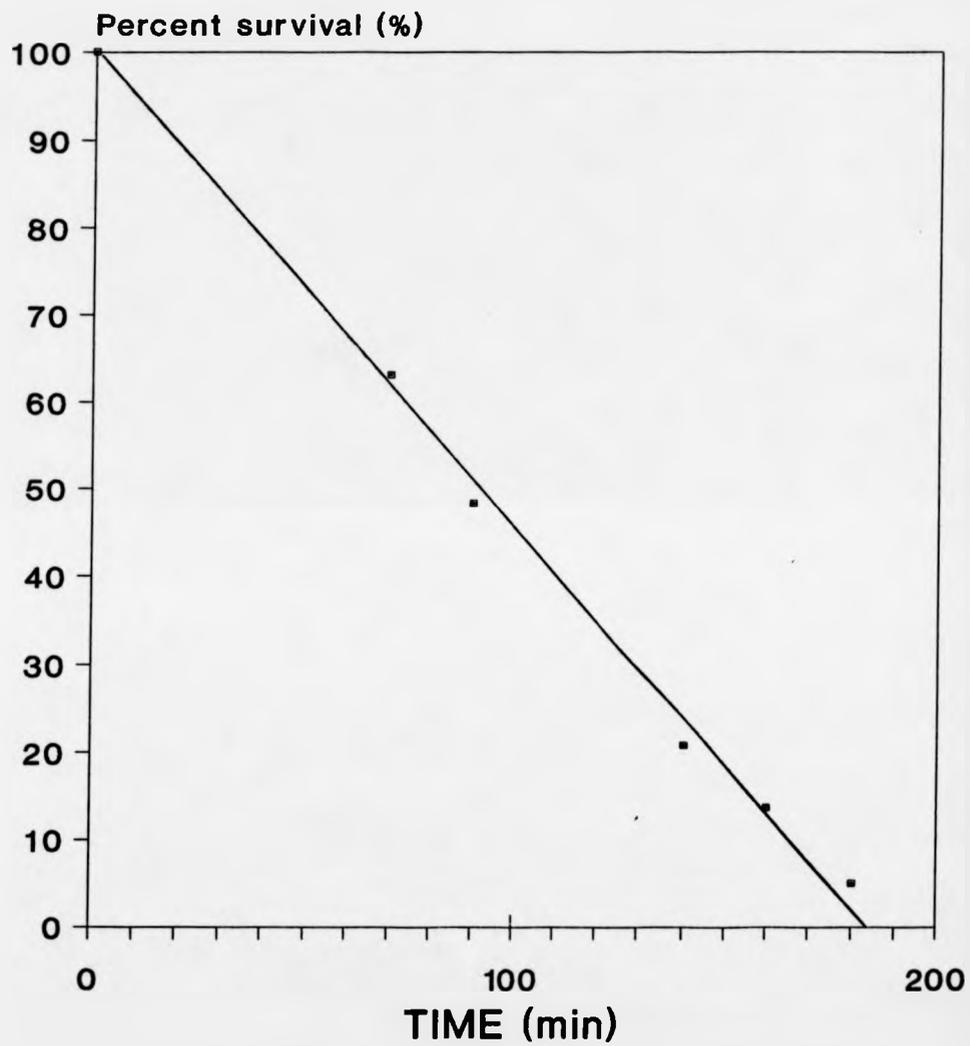
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Figure 3.5 EMS survival curve for wild-type *Er. chrysanthemi* NCPPB 1068



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Figure 3.6 EMS survival curve for industrial morphotype 2



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In order to gain an indication of the level of mutagenesis that had occurred during EMS treatment, the number of auxotrophic mutants arising in a non-segregated EMS-treated population of wild-type Er. chrysanthemi NCPPB 1066 was assessed. To this end, three hundred colonies from a number of time points were replicated from NB medium onto both sucrose minimal medium and NB medium, in that order. Following a 36 hour incubation at 30°C, the proportion of the replicated population unable to grow on minimal medium was determined. From the results of this experiment, a population survival of 2% and 13% was found to correspond with an auxotrophic frequency of 5.5% and 5%, respectively. At 20% survival, auxotrophic mutants arose at a frequency of 4%.

An auxotrophic frequency greater than 4% was taken to indicate that excess mutagenesis had taken place. Consequently, there was a potential risk of bacteria containing multiple mutations which could potentially complicate further genetic analysis. Hence, it was concluded that any mutants isolated from mutagenised cultures in which the survival was greater than 20%, were less likely to contain multiple mutations, compared with cells isolated from cultures in which cell death was greater than this value.

3.3.1.4 Isolation of Er. chrysanthemi NCPPB 1066 LacZ⁻ mutants CK1000 and CK2000

In order to isolate mutants of wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotype 2 which were devoid of B-galactosidase (LacZ⁻) activity, bacterial cultures were treated with EMS for varying times as described in Section 2.2.17. Non-segregated and segregated cells were subsequently serially-diluted in NB medium, and spread-plated onto NB agar plates containing X-gal (40ug ml⁻¹). Following a 2 day incubation at 30°C,

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white colonies displaying reduced β -galactosidase activity (as compared with an untreated time 0 control) were identified, and subsequently streak-purified onto the same medium. To reduce the risk of a LacZ^- derivative containing multiple mutations which could possibly complicate further genetic analysis, isolates that had been exposed to EMS for the shortest time were retained for further analysis.

The chosen wild-type Er. chrysanthemi NCPPB 1066 LacZ^- isolate was designated CK1000 (Figure 3.7), and originated from cells exposed to EMS for 90 mins, which had resulted in a population survival of 72%. The chosen industrial morphotype 2 LacZ^- isolate was designated CK2000 (Figure 3.7), and had arisen within a population of cells that had been exposed to EMS for 70 mins, corresponding to 63% survival.

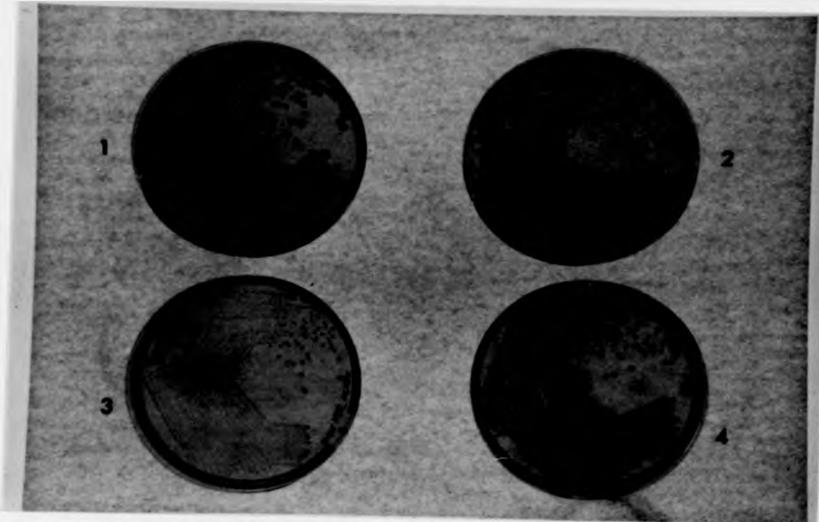
Both LacZ^- derivatives, CK1000 and CK2000, were found to be prototrophic, and displayed normal colony morphology on NB medium following mutagenesis. In addition, they did not appear to be noticeably affected in either growth in liquid NB medium or in extracellular Pel, Cel and Prt production on the appropriate enzyme detection medium, compared with un-mutagenised parental strains. Moreover, further experimentation revealed that the level of L-asparaginase II activity detectable within CK1000 and CK2000 was comparable to that of the parental strains, thus indicating that EMS mutagenesis had not adversely affected the synthesis of this particular enzyme. The level of β -galactosidase was, on the other hand, markedly reduced (Table 3.4).

3.3.1.5 Isolation of Er. chrysanthemi NCPPB 1066 PhoA^- mutants

The aforementioned EMS-treated cultures of wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotype 2 were also screened for mutants

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Figure 3.7 EMS-generated wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype 2 LacZ⁻ mutants, CK1000 and CK2000



Bacteria were cultivated on NB medium containing X-gal (40ug ml⁻¹) at 30°C for 24 hours. Key : 1) CK1000; 2) wild-type *Er. chrysanthemi* NCPPB 1066; 3) CK2000; 4) industrial morphotype 2

Table 3.4 Levels of B-galactosidase and L-asparaginase II in EMS-generated LacZ⁻ mutants CK1000 and CK2000

Strain	B-galactosidase activity [@]	L-asparaginase II activity [#]
Wild-type	31.6	2.5
Morphotype 2	36.7	7.3
CK1000	0.3	2.4
CK2000	ND	7.8

Prior to assay, bacterial cultures were grown to an OD₆₀₀ value of 2.0 in 20ml of LB medium at 30°C

[@] enzyme activity expressed as Miller units

[#] enzyme activity expressed as IU mg protein⁻¹

ND denotes that enzyme activity was not detected

Two independent cultures were sampled in duplicate. Values represent the mean

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reduced in alkaline phosphatase (PhoA⁻) activity. Again non-segregated and segregated cells were serially-diluted and spread-plated onto NB agar plates containing X-P (40ug ml⁻¹). Following a period of 36 hours at 30°C, two putative white wild-type Er. chrysanthemi NCPPB 1066 colonies (as compared with an untreated time 0 control) were identified. Upon streak-purification on the same selective medium, only one of the Pho⁻ candidates was found to remain white. This had arisen from a population of cells that had been treated with EMS for 120 mins corresponding to 41% survival, and was designated CK3000.

In contrast to wild-type Er. chrysanthemi NCPPB 1066, white colonies were more easily identified for the industrial morphotype 2 on the selective medium employed. Twelve such colonies were streak-purified onto NB medium containing X-P (40ug ml⁻¹) and were found to remain white. One of these, designated CK4000, originated from cells treated with EMS for 70 mins, corresponding to 63% survival, and was retained for further analysis.

Both CK3000 and CK4000 were found to be prototrophic and appeared to synthesise normal levels of Pel, Cel and Prt enzymes on the appropriate extracellular enzyme detection medium, compared to parental isolates. Alkaline phosphatase assay revealed that enzyme levels were reduced approximately three-fold in CK3000 and up to eight-fold in CK4000 (Table 3.5).

3.3.1.6 Comments

The preceding sections have described the successful mutagenesis of both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotype 2, using the alkylating agent EMS. In this instance, it has led to the isolation of LacZ⁻ (CK1000 and CK2000) and PhoA⁻ (CK3000 and CK4000) derivatives,

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Table 3.5 Alkaline phosphatase levels in EMS-generated wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype 2 PhoA^- mutants CK3000 and CK4000

strain	alkaline phosphatase activity [@]	
	sonicate	supernatant
Wild-type	0.013	ND
Morphotype 2	0.008	ND
CK3000	0.004	ND
CK4000	0.001	ND

Prior to sonication, cultures were grown to an OD_{600} value of 1.0 in 5ml of LB medium at 30°C.

@ enzyme activity expressed as units $\text{ml}^{-1} \text{min}^{-1}$.

ND denotes that enzyme activity was not detected.

Two independent cultures were sampled in duplicate. Values represent the mean.

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one of which (CK1000) was subsequently used for the construction of a single copy ansB::lacZ fusion derivative of Er. chrysanthemi NCPPB 1066 (see Section 7.5.2)

3.3.2 Transposon mutagenesis

3.3.2.1 Approach

As outlined in Chapter 1 of this thesis, there are many different types of suicide vehicle now available for the delivery of transposons into either the bacterial genome or plasmid DNA maintained within a bacterial cell. The use of plasmid vehicles which are temperature-sensitive for replication has been limited by the fact that many bacteria do not have the capacity to grow at the temperatures needed to prevent the establishment or maintenance of the thermo-sensitive vector (generally above 40°C).

In contrast to Er. carotovora subsp. carotovora SCRI 193, both wild-type Er. chrysanthemi NCPPB 1066 and each of the industrial morphotypes were found to grow well at 37°C, and to a slightly lesser extent at 42°C, both in liquid NB medium and on solid NB agar. Consequently, it was hoped that the introduction of a thermo-sensitive transposon delivery vehicle into Er. chrysanthemi NCPPB 1066, and the subsequent cultivation of the resulting recombinant culture at 42°C, would lead to loss of the plasmid and the concomitant generation of transposon insertions into the bacterial genome. Three such vectors were therefore chosen for study. Two of these were the plasmids pCHR81 and pCHR82, which had been originally isolated by Sasakawa and Yoshikawa (1987) following insertion of the transposon Tn5 and a hybrid Tn5 transposon, Tn5-TC1 (containing both the structural gene (tetA), encoding tetracycline-resistance and the structural gene for the tetA

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repressor (tetR), from the transposon Tn10), into plasmid pCHR71, respectively. The latter is a thermo-sensitive replication mutant of the 34 kb broad-host-range IncW plasmid R388 (Sasakawa and Yoshikawa, 1987) which encodes trimethoprim (Tp) and sulpha drug resistance (Ward and Grinsted, 1982), and is compatible with ColE1 derivatives and vectors derived from IncP and IncQ plasmids (Sasakawa and Yoshikawa, 1987).

Of the two plasmids, pCHR81 has been the most extensively used, and has facilitated the generation of Tn5 derivatives in a range of Gram-negative bacteria, including Er. carotovora strain EC7317 at 37°C and R. melliloti strain M103 at 40°C (Murata et al., 1988), as well as Shigella flexneri (Sasakawa et al., 1986) and S. typhi (Sasakawa and Yoshikawa, 1987) at 42°C. In Agrobacterium tumefaciens strain R66 and Agrobacterium radiobacter strain K84, plasmid pCHR81 has even been found to behave as a suicide vehicle at the lower temperature of 30°C (Murata et al., 1988).

The remaining thermo-sensitive transposon delivery vehicles chosen for study were plasmids pME461 and pME9. Both are based on the RP1 derivative, pME305, which encodes carbenicillin and tetracycline-resistance, and has undergone a 12 kb deletion leading to the removal of the primase genes, the kanamycin-resistance gene, and the insertion element, IS21. In addition, pME305 possesses a temperature-sensitive mutation in the trfA gene, leading to temperature-sensitive plasmid maintenance in E. coli and P. aeruginosa (Rella et al., 1985).

3.3.2.2 Results and discussion

3.3.2.2.1 Attempts to introduce plasmids pCHR81, pCHR82, pME9, pME461 and pME305 into wild-type Er. chrysanthemi NCPPB 1066

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In an attempt to introduce the thermo-sensitive transposon delivery vehicles into wild-type Er. chrysanthemi NCPPB 1066, patch-matings were carried out on NB medium between wild-type Er. chrysanthemi NCPPB 1066 and E. coli strain MC1061, harbouring either pCHR81 or pCHR82 (donor : recipient ratio of 1:1).

Additional patch-matings were set up on NB medium between E. coli strain ED8654 harbouring pME461 or pME9, and a Sm^r derivative of wild-type Er. chrysanthemi NCPPB 1066, which had spontaneously arisen at a frequency of 1×10^{-10} from a population of cells plated onto NB medium containing Sm (50ug ml⁻¹) (donor : recipient ratio of 1:1). Finally, E. coli strain JC3272 harbouring pME305 was mated with wild-type Er. chrysanthemi NCPPB 1066 (initial donor : recipient ratio of 1:1). In each instance, donor and recipient alone were run in parallel as conjugation controls.

Following a 24 hour incubation period at 30°C, cells were harvested, serially-diluted in phosphate buffered saline, and subsequently plated onto minimal sucrose medium containing Tp (10ug ml⁻¹) and Kn (25ug ml⁻¹) to select for pCHR81 transconjugants; Tp (10ug ml⁻¹) and Tc (10ug ml⁻¹) for pCHR82 transconjugants; Cb (100ug ml⁻¹) and Sm (50ug ml⁻¹) for pME461, and pME9 transconjugants, and Cb (100ug ml⁻¹) for pME305 transconjugants, and incubated at 30°C.

No transconjugant colonies were detected on the selective media for pCHR82, pME305, pME9, pME461 or the donor and recipient controls, after 36 hours at 30°C. In contrast, putative Tp^r Kn^r transconjugants were obtained from cells derived from the pCHR81 mating mix. One such colony was streak-purified on the same medium and analysed as described below. Disappointingly, further attempts to conjugally mobilise pCHR82, pME305,

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pME9 and pME461 into wild-type Er. chrysanthemi NCPPB 1066 with varying donor to recipient ratios, proved negative.

3.3.2.2.2 Analysis of a wild-type Er. chrysanthemi NCPPB 1066 pCHR81 transconjugant

The following lines of evidence suggested that pCHR81 had indeed been introduced into wild-type Er. chrysanthemi NCPPB 1066 from E. coli by conjugal mobilisation. Firstly, a Tp^f Kn^r colony was subjected to small-scale analysis and the presence of plasmid DNA verified by 0.4% (w/v) TAE agarose gel electrophoresis. DNA was then digested to completion with the restriction endonuclease BglII, and yielded four distinct DNA fragments of sizes 23.6, 9.0, 4.4 and 2.8 kb. Secondly, a patch-mating was set up on NB medium between putative wild-type Er. chrysanthemi NCPPB 1066 pCHR81 recombinant and E. coli strain CSH26 Δ F6 (Sm^r) cells. Donor and recipient alone were run in parallel as conjugation controls.

Following overnight incubation at 30°C, bacteria were harvested and then spread-plated onto NB medium containing Sm (50ug ml⁻¹) and Kn (50ug ml⁻¹). Further incubation at 30°C for 24 hours gave rise to confluent growth of cells derived from the Er. chrysanthemi NCPPB 1066/E. coli CSH26 Δ F6 mating. In contrast, no growth was seen for the donor and recipient alone controls.

3.3.2.2.3 Mutagenesis studies

Having gained physical and genetic evidence for the presence of pCHR81 within wild-type Er. chrysanthemi NCPPB 1066, further study was directed towards assessing the suitability of this vector to act as a suicide vehicle for transposon mutagenesis. To this end, recombinant wild-type Er.

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chrysanthemi NCPPB 1066 pCHR81 cells were grown with aeration (200 rpm) in minimal sucrose medium containing Tp ($10\mu\text{g ml}^{-1}$) at 30°C for 24 hours. Following this period, the culture was serially-diluted in phosphate buffered saline and a 100ul sample of each dilution spread-plated onto either LB medium alone, LB medium supplemented with Kn ($50\mu\text{g ml}^{-1}$), or minimal sucrose medium containing Tp ($10\mu\text{g ml}^{-1}$). Incubation was allowed to proceed at either 30°C or 42°C for 24 hours, after which time viable counts were determined on LB and minimal Tp medium.

At 30°C , no loss of the plasmid marker was observed. At 42°C , on the other hand, only 70% of the colony population was found to have retained the Tp^{r} plasmid marker, indicating that some plasmid loss had occurred at the non-permissive temperature. However, this was not as great as anticipated.

Incubation of recombinant Er. chrysanthemi NCPPB 1066 pCHR81 cells on NB Kn ($50\mu\text{g ml}^{-1}$) medium at 42°C gave rise to both small (1 mm in diameter) and large (2 - 3 mm in diameter) antibiotic-resistant colonies, with the latter occurring at a frequency of ca. 10^{-2} , compared to the former (Figure 3.8). Differences in colony size have also been noted by Rella et al. (1985) and Hinton (1986), for example, during mutagenesis studies with P. aeruginosa strain PAO 6049 and Er. carotovora subsp. carotovora SCRI 193, using plasmids pME9 and pJB4JI, respectively. In both cases, the large colonies were found to have lost vector antibiotic-resistance markers and retained only the transposon marker. In contrast, the smaller colonies had not. In the case of the latter, it has been suggested that the relatively small size of recombinant colonies perhaps reflects a metabolic load imposed upon the cell by the resident plasmid. Moreover, it has been speculated that the transposition of Tn_5 itself actually enhances the growth rate of the cell (Hinton, 1986), although the latter proposal remains to be substantiated.

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Figure 3.8 Colony morphology of wild-type *Er. chrysanthemi* NCPPB 1066 pCHR81 recombinants



Bacteria were cultivated at 42°C for 24 hours on LB Kn (50ug ml⁻¹) medium. Arrows indicate the 'large' colony type which was found to be Kn^r Tp^s.

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To test whether a similar situation was true in this study, both large and small colony types were replicated onto minimal sucrose Tp (10ug ml⁻¹) medium and NB medium containing Kn (50ug ml⁻¹), and incubated for a further 24 hours at 30°C. All of the large colonies studied were found to be Tp^S Kn^r, whereas each of the small colonies studied were Tp^r Kn^r. Hence, on the basis of antibiotic-resistance, the evidence suggested that the large colony type did indeed represent non-recombinant Er. chrysanthemi NCPPB 1066 cells that most likely contained Tn₅ insertions within the genome. Conversely, it was concluded that the small Kn^r colonies were derived from plasmid-containing cells.

Eighty-five Tp^S Kn^r isolates were replicated sequentially onto minimal glucose medium and NB medium containing Kn (50ug ml⁻¹), and incubated at 30°C for 24 hours. One such isolate was found to be auxotrophic, and subsequent analysis using the pool plate method of Clowes and Hayes (1968) revealed a requirement for cysteine. In order to determine whether the observed auxotrophy was due to Tn₅ insertion, reversion analysis was performed as described in Section 2.2.14. Prototrophic revertants were found to arise at a frequency of 3.5 X 10⁻⁸. A number of revertant colonies were then replicated onto NB medium containing Kn (50ug ml⁻¹) and onto Pel detection medium to verify their Erwinia origin. All (33/33) were subsequently found to be Pel⁺ and resistant to kanamycin.

This result is not unprecedented, since similar findings have been reported by Zink et al. (1985) and Hinton (1986), who studied the reversion of putative Tn₅-generated auxotrophs of Er. carotovora subsp. carotovora AC5001 and Er. carotovora subsp. carotovora SCRI193, respectively, following mutagenesis with the transposon delivery vehicle pJB4J1. In both cases, a large proportion of revertants still displayed kanamycin-resistance. Some

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possible reasons to account for this phenomenon include; 1) the presence of multiple copies of Tn5 within the bacterial genome; 2) extragenic suppression; 3) independent IS50 transposition; or 4) spontaneous re-insertion of Tn5 at another site within the genome, following precise excision.

3.3.2.2.4 Comments

The preceding sections have described the introduction of the thermo-sensitive transposon delivery vehicle, pCHR81, into wild-type Er. chrysanthemi NCPPB 1066 by conjugal mobilisation, and preliminary mutagenesis studies which lead to the identification of one putative Tn5-generated cysteine auxotroph. Although this system holds particular promise, it is clearly recognised that more work is required in the future. This would include Southern analysis of the Tp^S , Kn^r isolates to verify the existence of Tn5 within the genome, and reveal the number of insertions which had taken place. Furthermore, attempts should be made to optimise the conditions leading to the loss of the transposon delivery vehicle pCHR81.

3.3.3 Transformation studies

3.3.3.1 Aim

Although previous studies had demonstrated that the narrow-host-range ColE1 vector pBR322, could be introduced into and maintained within a number of Er. chrysanthemi strains, including 3937j (Reverchon and Robert-Baudouy, 1985) and SRI20A (Wang and Tseng, 1985); it was not known whether the same would be true of Er. chrysanthemi NCPPB 1066. Hence, the aim of the work described in the following sections was to introduce pBR322 DNA into wild-type Er. chrysanthemi NCPPB 1066 via transformation. In

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addition, efforts would also be made to introduce the high copy number L-asparaginase II expression vectors pASN326 and pASN230 (Gilbert *et al.*, 1986) into Er. chrysanthemi NCPPB 1066, using the same process.

3.3.3.2 Transformation of wild-type Er. chrysanthemi NCPPB 1066 with pBR322, pASN326 and pASN230

In preliminary experiments, attempts were made to transform wild-type Er. chrysanthemi NCPPB 1066 with 1 µg of CsCl-purified pBR322 (Amersham), using the published protocols of Wang and Tseng (1985) and Reverchon and Robert-Baudouy (1985). Unfortunately, on two separate occasions, no Ap^r transformants were detected, despite the high concentration of plasmid DNA used. Also investigated was the modified Hanahan protocol of Hinton *et al.* (1985 b). However, in this instance, the compound dimethylsulphoxide was substituted for dimethylformamide previously used by Gilbert and colleagues (1986) in their attempts at transforming Er. chrysanthemi NCPPB 1066, since the former is considered to be less toxic to bacterial cells compared with the latter (Dr. J. Hinton; personal communication).

Competent wild-type Er. chrysanthemi NCPPB 1066 cells were prepared as outlined in Section 2.2.6.3 and mixed with 1 µg of CsCl-purified pASN326 and pBR322 DNA (Amersham). Following transformation, cells were spread-plated onto LM medium containing Ap (35 µg ml⁻¹) and incubated at 30°C for 24 hours. After this time, one Ap^r colony was detected on the selective medium from the wild-type Er. chrysanthemi NCPPB 1066 transformation with pBR322; however, no transformants arose from the pASN326 transformation. As a control, competent Er. carotovora subsp. carotovora SCRI 193 cells were prepared in parallel and transformed with 1 µg of pBR322 DNA. In this instance, 1.08 X 10³ Ap^r transformants were obtained. No colonies were

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detected for untransformed controls.

In a second independent transformation experiment, employing the same amount of pBR322 and pASN326 DNA, four Ap^r colonies arose on LM Ap (35ug ml⁻¹) selective medium, following transformation of wild-type Er. chrysanthemi NCPPB 1066 cells with the former plasmid. Again, many more Ap^r colonies (5.6 X 10²) resulted from the control transformation of Er. carotovora subsp. carotovora SCRI 193 with 1ug of pBR322 DNA. In contrast, no Ap^r colonies were detected for the Er. chrysanthemi NCPPB 1066/pASN326 transformation, or from untransformed wild-type Er. chrysanthemi NCPPB 1066 and Er. carotovora subsp. carotovora SCRI 193 controls.

3.3.3.3 Analysis of putative wild-type Er. chrysanthemi NCPPB 1066 pBR322 recombinants

Following streak-purification of three wild-type Er. chrysanthemi NCPPB 1066 Ap^r colonies, confirmation that they were true pBR322 transformants came from the results outlined below. Firstly, although transformants were originally selected on the basis of ampicillin-resistance, all were found to have simultaneously attained resistance to tetracycline. Secondly, each candidate gave a distinct halo on pectinase, cellulase and protease detection medium. Finally, the presence of plasmid DNA was verified by small-scale analysis, as described in Section 2.3.5.2, followed by 0.6% (w/v) TAE agarose gel electrophoresis.

For a more detailed characterisation, plasmid DNA was extracted from one candidate on a large-scale, as outlined in Section 2.3.4.2, and subsequently digested to completion with a combination of BamHI and EcoRI restriction endonucleases. The products were then size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis, alongside Amersham pBR322 DNA

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restricted in a similar fashion. No differences in the patterns obtained for both sources of pBR322 DNA were seen.

3.3.3.4 Attempts to introduce plasmids pUC8, pUC9 and pASN230 into wild-type Er. chrysanthemi NCPPB 1066

In addition to the experiments described above, attempts were also made to introduce the high copy number cloning vehicles pUC8 and pUC9, as well as the L-asparaginase II expression vector pASN230, into wild-type Er. chrysanthemi NCPPB 1066, using the modified Hanahan procedure of Hinton *et al.* (1985 b). To this end, plasmid DNA (1 μ g) was mixed with competent wild-type Er. chrysanthemi NCPPB 1066 cells. Following transformation, bacteria were spread-plated onto LM Ap (35 μ g ml⁻¹) in the case of pUC8 and pUC9 selection, and LM Kn (25 μ g ml⁻¹) for pASN230 selection. Following a 24 hour incubation period at 30°C, no colonies were obtained for pUC8, pASN230, or for an untransformed wild-type Er. chrysanthemi NCPPB 1066 control. In contrast, one Ap^r colony arose on the selective medium for pUC9, which was subsequently found to be Pel⁺, Cel⁺ and Prt⁺ on extracellular enzyme detection medium. Plasmid DNA was isolated on a small-scale and digested to completion with the restriction endonuclease EcoR1. Subsequent analysis by 0.7% (w/v) TAE agarose gel electrophoresis revealed a single band of 2.8 kb.

3.3.3.5 Comments

From the above evidence, it was concluded that the multicopy cloning vectors pBR322 and pUC9 had been successfully introduced into wild-type Er. chrysanthemi NCPPB 1066 using the modified Hanahan procedure of Hinton *et al.*, (1985 b). In this instance however, the frequency of transformation was

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found to be two logs lower than that obtained for Er. carotovora subsp. carotovora SCRI 193, and therefore on the limits of detection.

3.3.3.6 DNAase production

In theory, one possible contributory factor leading to relatively poor transformation frequencies obtained with Er. chrysanthemi NCPPB 1066 was extracellular DNAase production. This question was addressed by using Difco DNAase detection medium containing DNA and the compound methyl green (Table 2.4). The latter combines with only highly polymerised DNA to give a green colouration. When DNA is degraded by the action of DNAase, combination with methyl green does not take place and the colour fades. This therefore leads to the formation of a clear halo around DNAase-producing bacterial colonies.

Both wild-type Er. chrysanthemi NCPPB 1066 and each of the industrial morphotypes were replicated onto DNAase detection medium, alongside E. coli strain CSH26 Δ F6 and Er. uredovora SCRI 423, which acted as negative and positive DNAase controls, respectively. Following a 3 day incubation at 30°C no zone of clearing was detected for either wild-type Er. chrysanthemi NCPPB 1066 or any of the industrial morphotypes, thus indicating that they were all DNAase-negative, and that this was unlikely to be the cause of the poor transformability.

3.3.3.7 Preliminary evidence for a restriction/modification system in

Er. chrysanthemi NCPPB 1066.

Transformation studies carried out by Reverchon and Robert-Baudouy (1985) demonstrated that pBR322 DNA obtained from Er. chrysanthemi 3937j could be used to transform Er. chrysanthemi 3937j at a frequency of 6 X

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10^3 transformants $\mu\text{g DNA}^{-1}$, compared to a frequency of 3.5×10^2 transformants $\mu\text{g DNA}^{-1}$ obtained with pBR322 DNA isolated from E. coli strain P4X. Similar results have also been reported by Hamamoto and Murooka (1987) who found that plasmid pBEH3-5 DNA prepared from Er. carotovora subsp. carotovora strain IFO 3380 transformed Er. carotovora subsp. carotovora strains IFO 3380 and IFO 3830, as well as Er. carotovora strain E7419, at frequencies of 1.6×10^2 , 2.1×10^2 and 1.7×10^2 transformants $\mu\text{g DNA}^{-1}$, respectively. In comparison, corresponding frequencies of 1, 2 and 0 transformants $\mu\text{g DNA}^{-1}$ were obtained with the equivalent DNA prepared from E. coli strain C600.

From the above observations, it is clear that in certain instances, host-controlled restriction can lower the efficiency of transformation of a particular strain of Erwinia. This phenomenon is widespread within the prokaryotic world and provides a means by which a particular bacterium can differentiate between 'self' DNA and 'foreign' DNA originating from another source. Upon entry into the bacterial cell, 'foreign' DNA is susceptible to the action of host-encoded site-specific restriction endonucleases and is degraded. The host DNA on the other hand is protected from the action of its own restriction endonucleases, due to the fact that specific residues within the recognition site for these enzymes are modified (Bickle, 1987). In E. coli, for example, such modification has been shown to involve the action of at least three distinct methylase enzymes, which add methyl groups to either adenine or cytosine residues following DNA replication (Marinus, 1987). The overall pattern of DNA methylation is characteristic of a particular bacterial species. Those 'foreign' DNA molecules which avoid the action of restriction endonucleases, will subsequently adopt the modification pattern of the host during subsequent rounds of DNA replication. This DNA will

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therefore transform the same host with higher efficiency compared to 'foreign' unmodified DNA due to its acquired resistance to the action of the host restriction endonucleases.

Recently, two further genetically distinct restriction activities, McrA and McrB, have been discovered in E. coli whose target is in fact methylated DNA itself, particularly DNA containing 5-methylcytosine. The best studied of the two restriction systems is the McrB system which recognises DNA methylated at the cytosine residue of the sequence 5'-GC-3'. The McrA system on the other hand, appears to have a different sequence specificity and is known to restrict DNA with the methylated sequence C^mCGG (Old and Primrose, 1989).

In order to determine whether Er. chrysanthemi NCPPB 1066-controlled restriction-modification could go some way to explaining the poor transformation efficiencies obtained previously, pBR322 DNA was isolated on a large-scale from wild-type Er. chrysanthemi NCPPB 1066 and E. coli HB101. This was subsequently used to transform competent wild-type Er. chrysanthemi cells prepared by the modified Hanahan method, as outlined in Section 2.2.6.4. Bacteria were subsequently spread-plated onto LM selective medium containing Ap (35ug ml⁻¹). Following a 24 hour incubation at 30°C, no transformant colonies were detected for pBR322 derived from E. coli HB101, whereas pBR322 isolated from Er. chrysanthemi NCPPB 1066 yielded 12 transformants ug DNA⁻¹. This was three-fold higher than had been obtained for the best transformation frequency recorded previously.

Further work carried out with plasmids pHCP2 (Section 3.3.5.3.4) and pCK 1 (Section 4.2.1), again showed that DNA isolated from Er. chrysanthemi NCPPB 1066 was transformed at a higher efficiency than equivalent DNA isolated from E. coli. It is therefore suggested that at least

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one of the factors leading to the poor transformability of Er. chrysanthemi NCPPB 1066 is restriction of unmodified 'foreign' DNA upon entry into this particular strain. Hence, it is envisaged that the isolation of a restriction-less mutant of Er. chrysanthemi NCPPB 1066 may go some way towards facilitating the transformation of this particular Erwinia strain, as has been found with strains of P. aeruginosa and P. putida, (Bagdasarian et al., 1981)

3.3.3.8 Transformation of industrial morphotypes 2 - 5 with pBR322

Using 'modified' pBR322 DNA that had been isolated from wild-type Er. chrysanthemi NCPPB 1066, competent industrial morphotype 2 - 5 cells prepared by the modified Hanahan procedure (Section 2.2.6.4) were transformed at frequencies ranging from 7.3×10^1 to 5.43×10^2 transformants $\mu\text{g DNA}^{-1}$ (Table 3.6).

3.3.4 Electroporation studies

Since efforts to introduce the L-asparaginase II expression vectors pASN326 and pASN230 into Er. chrysanthemi NCPPB 1066 via transformation had proven negative, attention was focused on electroporation to perhaps provide an alternative means of attaining this goal. At the time the following work was performed, the electroporation protocol outlined in Section 2.2.7 was the one which had been found to yield the highest electroporation efficiencies for E. coli; hence this was adopted for this particular study.

For bacteria such as E. coli, it has been demonstrated that cell survival declines steadily with increasing applied field strengths. In this instance, loss of viability has been attributed to irreversible membrane damage and/or the excessive loss of intracellular components (Chassy et al., 1988). Furthermore,

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Table 3.6 Transformation of industrial morphotypes 2 - 5 with pBR322 previously isolated from wild-type Er. chrysanthemi NCPPB 1066

Strain	No. of Ap ^r transformants ug DNA ⁻¹
Morphotype 2	7.30 X 10 ¹
Morphotype 3	3.35 X 10 ²
Morphotype 4	1.08 X 10 ²
Morphotype 5	5.43 X 10 ²

The industrial morphotype 1 was omitted from this work due to its poor growth characteristics in liquid medium.

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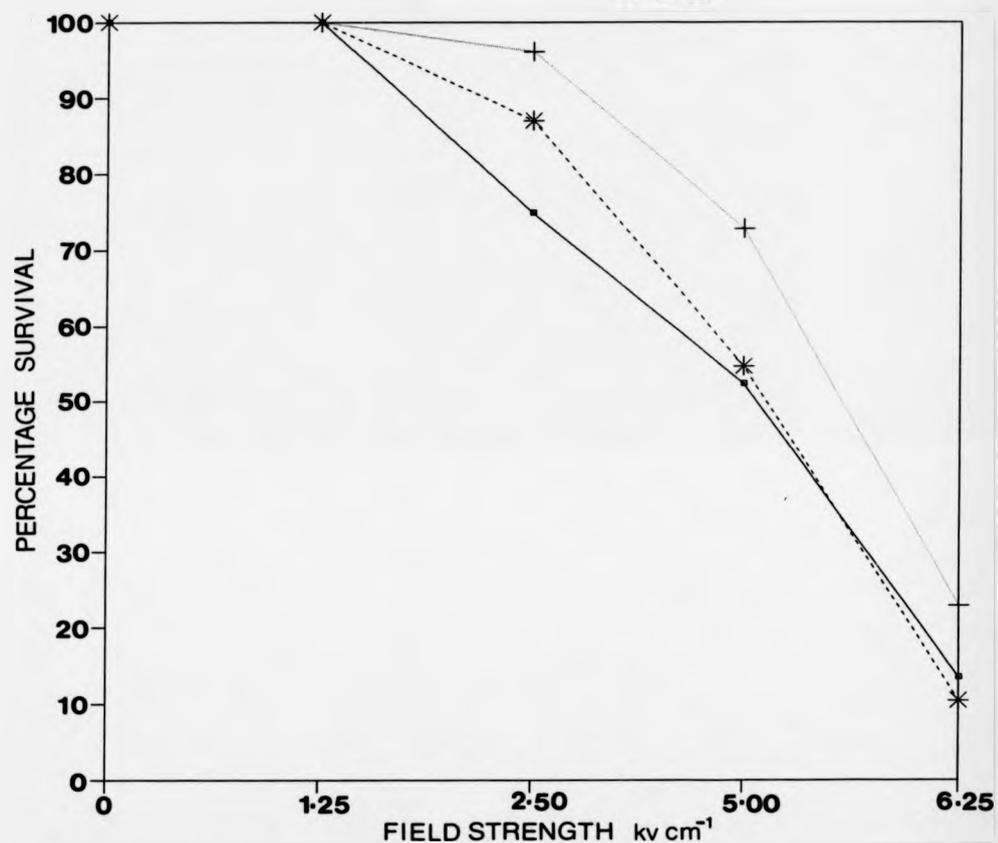
Calvin and Hanawalt (1988) have also noted the formation of filaments following electroporation. Therefore, these workers have proposed that the lack of macrocolony formation by cells subjected to electroporation may also be due to the onset of filamentous growth. This hypothesis has been reinforced by the fact that the presence of pantooyl lactone (a compound that inhibits filamentation) in solid medium, increases the transformation efficiency of E. coli strain JM109.

The extent to which a bacterial cell is susceptible to a particular amount of electric current varies from species to species, and from strain to strain. Hence, for this reason, a preliminary experiment was performed in order to determine the susceptibility of wild-type Er. chrysanthemi NCPPB 1066 to varying field strengths. At the same time, Er. carotovora subsp. carotovora SCRI 193 and E. coli strain CSH26 Δ F6 were run in parallel as a comparison.

Bacterial cells were prepared for electroporation as described in Section 2.2.7, and pulsed once in the absence of DNA, at a set capacitance of 25uF over a range of voltages up to 2,500 volts for a period of 5 - 6 msec. Following electroporation, cells were serially-diluted in LB medium and spread-plated onto LB agar plates. Viable counts were recorded following a 24 hour incubation at 30°C, and directly compared with a non-electroporated control. The results are presented in Figure 3.9, where it can be seen that for wild-type Er. chrysanthemi NCPPB 1066, Er. carotovora subsp. carotovora SCRI 193 and E. coli CSH26 Δ F6, a decline in cell viability began at field strengths greater than 1.25 kv cm⁻¹, and steadily declined thereafter to give a final value of 13%, 21% and 11% at the maximum field strength of 6.25 kv cm⁻¹, respectively.

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Figure 3.9 The effect of electrical field strength on the survival of wild-type Er. chrysanthemi NCPPB 1066, Er. carotovora subsp. carotovora SCRI 193 and E. coli CSH26 Δ F6



The percentage of the bacterial population surviving a single electrical pulse at a particular field strength is shown above. A range of 500 to 2,500 volts was applied at a set capacitance of 25 μ F. The inter-electrode distance of the cuvettes was 0.4cm.

Key : —•— Wild-type Er. chrysanthemi NCPPB 1066
—+— Er. carotovora subsp. carotovora SCRI 193
—*— E. coli CSH26 Δ F6

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3.3.4.1 Attempts to introduce plasmids pUC8, pUC9, pASN326 and pASN230 into Er. chrysanthemi NCPPB 1066 by electroporation

Having determined the effect of varying field strengths on the survival of wild-type Er. chrysanthemi NCPPB 1066, attempts were made to introduce the L-asparaginase II expression vector pASN326, as well as the high copy number plasmids pUC8, pUC9 into this organism. To this end, 1 μ g of plasmid DNA was mixed with wild-type Er. chrysanthemi NCPPB 1066 cells and electroporation allowed to proceed at a field strength of 6.25 kv cm⁻¹ for a period of 5.7 to 5.8 msec. Cells were subsequently spread-plated onto LB medium supplemented with Ap (50 μ g ml⁻¹) and incubated for 24 hours at 30°C. After this period, 574 Ap^r colonies were detected for pUC9, and only 1 such colony for pUC8. In contrast, no colonies were obtained for pASN326 DNA or with wild-type Er. chrysanthemi NCPPB 1066 cells electroporated in the absence of DNA.

Similar results to those described above were also obtained following electroporation of the industrial morphotypes 2, 3, 4 and 5 with 1 μ g of pUC8 and pUC9 DNA at a field strength of 6.25 kv cm⁻¹ for a period of between 5.6 and 6.2 msec (Table 3.7); and in parallel electroporation studies carried out at C.A.M.R. Porton Down, where pUC9 was transformed at a frequency of 165 transformants μ g DNA⁻¹ and no transformants were detected for pUC8 (Dr. N. Minton; personal communication).

The reason for the higher electroporation efficiencies seen for pUC9, compared with an equivalent amount of pUC8 DNA, cannot be explained at present; however they are curious considering the fact that the pUC8 and pUC9 DNA used in this particular study were originally prepared from the same E. coli strain (Dr. D. Hodgson; personal communication), and that the

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Table 3.7 Transformation of industrial morphotypes 2, 3, 4 and 5 with plasmids pUC8 and pUC9 by electroporation

Isolate	No. of Ap ^r transformant colonies ug DNA ⁻¹	
	pUC8	pUC9
Morphotype 2	3	375
Morphotype 3	ND	131
Morphotype 4	2	392
Morphotype 5	3	95

Industrial morphotype 1 was omitted from this work due to its poor growth characteristics in liquid medium.

ND denotes that transformants were not detected.

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only structural difference between the two plasmids is the orientation of a region containing multiple cloning sites.

3.3.4.2 Analysis of putative Er. chrysanthemi NCPPB 1066 pUC8 and pUC9 transformants

Putative wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2, 3, 4 and 5 pUC8 and pUC9 transformants were found to be Pel⁺, Cel⁺ and Prt⁺ on extracellular enzyme detection media. The presence of plasmid DNA was verified by small-scale purification followed by 0.7% (w/v) TAE agarose gel electrophoresis.

3.3.4.3 Comments

Although plasmids pUC8 and pUC9 were successfully introduced into both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes, no further work using electroporation was performed in this study. However, these results have been included in order to illustrate the potential of this particular technique. There is clearly a need for further optimisation of this technique for Er. chrysanthemi NCPPB 1066, since improvements to the electroporation protocol used in this study have now been developed (see Dower et al., 1988).

3.3.5 The sensitivity of Er. chrysanthemi NCPPB 1066 to coliphages and other Erwinia bacteriophages

Due to the fact that bacteriophages such as lambda, P1 and Mu have been successfully used for the genetic manipulation of a number of Erwinia strains (see Chapter 1), the aim of the work described in the following

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sections was to determine whether such bacteriophage systems could also be used to facilitate the genetic analysis of Er. chrysanthemi NCPPB 1066.

3.3.5.1 Sensitivity of Er. chrysanthemi NCPPB 1066 to bacteriophage

Mu, phi EC-2 and T4

High-titre lysates of bacteriophage Mu62 (1×10^9 pfu ml⁻¹) and phi EC-2 (3×10^8 pfu ml⁻¹) were prepared as described in Section 2.2.8.3 and 2.2.8.4. In addition, a high-titre lysate of bacteriophage T4 was kindly provided by I. Toth ($> 10^9$ pfu ml⁻¹). All of the aforementioned lysates were serially-diluted in ten-fold stages to 10^{-4} , and a 5ul sample of each dilution, as well as undiluted lysate (5ul), subsequently spot-tested along with a phage buffer alone control onto a top agar overlay containing log-phase cells of either wild-type Er. chrysanthemi NCPPB 1066 or the industrial morphotypes 1 - 5. The E. coli strain DH1 and Er. carotovora subsp. carotovora SCRI 193 were run in parallel as a positive and negative control, respectively, for bacteriophage T4 and Mu. In addition, Er. chrysanthemi strain 3937 and Er. carotovora subsp. carotovora SCRI 193 were included as a positive and negative control, respectively, for bacteriophage phi EC-2.

Following a 24 hour incubation at 30°C no plaques or zones of lysis could be detected on agar overlays for either wild-type Er. chrysanthemi NCPPB 1066 or any of the industrial morphotypes, thus indicating that they were resistant to the aforementioned bacteriophages.

3.3.5.2 Sensitivity to bacteriophage P1

As described in Chapter 1, the use of P1 derivatives harbouring antibiotic resistance genes has allowed the isolation of P1-sensitive variants from a population of resistant bacteria, by selecting for drug-resistant

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lysogens (Goldberg *et al.*, 1974). Since this approach has been successfully applied to a variety of Gram-negative genera, including E. coli, Klebsiella, Salmonella, Pseudomonas and Erwinia, the aim of this work was to attempt to isolate a P1-sensitive derivative of Er. chrysanthemi NCPPB 1066 in an identical manner.

A high-titre lysate (4×10^{10} pfu ml⁻¹) of the P1 derivative P1clr100CM was prepared by thermal induction of an E. coli lysogen as described in Section 2.2.8.3. A sample of lysate (250ul) was subsequently used to infect a fresh overnight culture of both E. coli C600 or wild-type Er. chrysanthemi, as described in Section 2.2.11. The former being run as a comparison. Following a 36 hour incubation at 30°C, infected E. coli C600 gave rise to confluent growth on LB agar supplemented with Cm (25ug ml⁻¹), indicating that P1clr100CM infection and lysogen formation had taken place. However, in contrast no Cm^r colonies were detected for infected wild-type Er. chrysanthemi NCPPB 1066 cells or for uninfected E. coli C600 and wild-type Er. chrysanthemi NCPPB 1066 controls. This experiment was repeated with E. coli C600, wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes 2 - 5, grown to an OD₆₀₀ value of 0.5 and 1.0 prior to infection. Unfortunately, similar results were obtained.

3.3.5.3 Attempts to create a lambda-sensitive derivative of Er. chrysanthemi NCPPB 1066

As spot-testing of wild-type lambda bacteriophage onto lawns of wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotypes 1 - 5 did not yield any plaques or zones of clearing, the aim of the following series of experiments was to determine whether lamB technology (see section 1.5.4.3 c) could be applied to Er. chrysanthemi NCPPB 1066, thereby leading to the

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construction of a Lambda-sensitive derivative. This would then allow an efficient means of introducing recombinant cosmids into this particular strain of Erwinia and, in addition, facilitate transposon mutagenesis.

3.3.5.3.1 Results and discussion

3.3.5.3.2 Conjugal transfer of lamB⁺ plasmids into wild-type Er. chrysanthemi NCPPB 1066

(a) pHCP2

The narrow-host-range replicon, pHCP2, is a pBR322-based derivative containing a 3kb EcoR1-BglII fragment harbouring the entire lamB⁺ structural gene of E. coli (Clement *et al.*, 1982), and has been used to render a variety of Erwinia strains sensitive to lambda infection (Ellard *et al.*, 1989; Salmond *et al.*, 1986). In order to introduce the aforementioned plasmid into wild-type Er. chrysanthemi NCPPB 1066, a patch-mating was set up with E. coli strain DW75 on NB medium at 30°C, as described in Section 2.2.5. In addition to pHCP2, DW75 harbours the conjugative 'helper' plasmids, pR64drd11 and pLVC9. The former is a 1.9 kb I α -type vector which encodes tetracycline and streptomycin-resistance, and provides tra mobilising functions (van Haute *et al.*, 1983). The latter is a 7.1 kb derivative of plasmid pGJ28 (van Haute *et al.*, 1983), which encodes chloramphenicol-resistance and provides the necessary mob mobilising functions (Salmond *et al.*, 1986).

Following a 24 hour incubation period, bacteria were harvested and serially-diluted in phosphate buffered saline, and 100ul samples of each dilution spread-plated on minimal agar containing Ap (50ug ml⁻¹) and 0.2% (w/v) sucrose for the selection of wild-type Er. chrysanthemi NCPPB 1066

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pHCP2 transconjugants. The results obtained after a 36 hour incubation at 30°C are presented in Table 3.8 (a).

(b) pTroy9

The broad-host-range IncP1 replicon, pTroy9, carries the E. coli lamB⁺ structural gene constitutively expressed from an IS3 promoter inserted upstream. It also contains the origin of transfer (oriT) of the parental plasmid pRK2 (pRP4), and transfers during conjugations between Gram-negative bacteria when a compatible 'helper' plasmid such as pRK2013 (Figurski et al., 1979) is present to supply in trans DNA transfer functions (Ditta et al., 1980).

A tripartite mating system was constructed using wild-type Er. chrysanthemi NCPPB 1066, E. coli strain HE198 (pRK2013) and E. coli strain HE168 (pTROY9) in varying ratios on NB medium at 30°C, as described in Section 2.2.5. Following a 24 hour incubation period, bacteria were harvested, serially-diluted in phosphate buffered saline, and 100ul samples of each dilution spread-plated onto minimal sucrose medium containing Tc (10ug ml⁻¹) to select for wild-type Er. chrysanthemi NCPPB 1066 pTROY9 transconjugants. The results following a 36 hour incubation period at 30°C are summarised in Table 3.8 (b)

3.3.5.3.3 Physical analysis of Er. chrysanthemi NCPPB 1066 lamB⁺ derivatives

Four putative Ap^r wild-type Er. chrysanthemi NCPPB 1066 pHCP2 transconjugant colonies, and five Tc^r wild-type Er. chrysanthemi NCPPB 1066 pTROY9 candidate transconjugants colonies were streak-purified twice on the appropriate selective medium, and subsequently replicated onto extracellular

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Table 3.8 (a) Conjugal transfer of the lamB⁺ plasmid pHCP2 into wild-type Er. chrysanthemi NCPPB 1066

Donor	Ratio of donor : recipient ⁺	Selection [#]	TPR [@]
DW75	1 : 1	Ap ^r	3.2 X 10 ⁻⁵

Table 3.8 (b) Conjugal transfer of the lamB⁺ plasmid pTBOY9 into wild-type Er. chrysanthemi NCPPB 1066

Ratio of HE168 : HE198 : recipient ⁺	Selection [#]	TPR [@]
1 : 1 : 1	Tc ^r	ND
1 : 10 : 1	Tc ^r	ND
10 : 10 : 1	Tc ^r	ND
1 : 0.1 : 1	Tc ^r	5 X 10 ⁻⁸
0.01 : 0.1 : 1	Tc ^r	ND

@ The frequency of conjugal transfer per Erwinia recipient. Total Erwinia viable count was determined by plating the mating culture onto minimal sucrose medium without antibiotic, to counterselect for the E. coli donor strains

Antibiotic selection for Erwinia transconjugants.

ND denotes that transconjugants were not detected.

+ Ratio of donor to recipient at the beginning of the mating

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enzyme detection medium. All were found to be positive for Pel^+ , Cel^+ and Prt^+ production. The absence of the corresponding conjugative 'helper' plasmids, pR64drd11, pLVC9 or pRK2013, was ascertained by replicating cells onto NB medium containing either Tc, Cm or Kn, respectively. Finally, small-scale analysis was performed on the each $lamB^+$ candidate, and the presence of plasmid DNA verified by 0.6% (w/v) TAE agarose gel electrophoresis. One wild-type Er. chrysanthemi NCPPB 1066 pHCP2 and pTROY9 recombinant derivative was retained for subsequent transductional analysis.

3.3.5.3.4 Construction of industrial morphotype $lamB^+$ derivatives

Transformation studies previously described in this chapter, indicated that modified plasmid DNA from Er. chrysanthemi NCPPB 1066 could be re-introduced into this strain at a higher frequency than equivalent DNA from E. coli. As a continuation of this theme, pHCP2 DNA was extracted from E. coli strain HE179 and wild-type Er. chrysanthemi NCPPB 1066 on a large-scale. Following cleavage with the restriction endonuclease EcoRI, 0.7% (w/v) TAE agarose gel electrophoresis revealed the presence of a 7.0 kb fragment in each case. Purified wild-type Er. chrysanthemi NCPPB 1066 and E. coli HE127 pHCP2 DNA (1 μ g) was subsequently used to transform competent cells of both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes 2 to 5, using the modified Hanahan procedure described in Section 2.2.6.3. Transformants were selected on LM agar containing Ap (35 μ g ml⁻¹) at 30°C. Following a 24 hour incubation period, transformation frequencies ranging between 8 and 196 transformants μ g⁻¹ were obtained using pHCP2 DNA derived from wild-type Er. chrysanthemi NCPPB 1066 (Table 3.9). In contrast, no transformants were obtained on selective medium

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Table 3.9 Transformation of wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotypes 1 - 5 with lanB⁺ plasmid pHCP2 prepared from Er. chrysanthemi NCPPB 1066

Strain	No. of Ap ^r transformants ug DNA ⁻¹ pHCP2 (NCPPB 1066)
Wild-type	1.96 X 10 ²
Morphotype 1	5.20 X 10 ¹
Morphotype 2	0.80 X 10 ¹
Morphotype 3	4.40 X 10 ¹
Morphotype 4	1.50 X 10 ¹
Morphotype 5	1.65 X 10 ²

Industrial morphotype 1 was omitted due to its poor growth characteristics in liquid medium

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from cells transformed with pHCP2 DNA that had originated from E. coli HE127, or from untransformed morphotype cells run in parallel as controls. One Ap^r colony was streak-purified from each transformation reaction and retained for further analysis. Each was shown to be Pel⁺, Cel⁺ and Prt⁺, and to contain plasmid DNA as determined by small-scale analysis followed by 0.6% (w/v) TAE agarose gel electrophoresis.

3.3.5.3.5 Transductional analysis

To determine whether the LamB protein was functional in both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes 2 - 5, thereby rendering them susceptible to lambda-mediated transduction, pHCP2 recombinant cultures were infected with either lambda₄₆₇ (Tn5) or lambda₈₄₀ (Tn10 high hopper) at titres of 10¹¹ pfu ml⁻¹; and transduction assays carried out as described in Section 2.2.10.1. For the wild-type Er. chrysanthemi NCPPB 1066 pTROY9 derivative, transduction experiments were performed with lambda₄₆₇ only. In all instances, Er. carotovora subsp. carotovora SCRI 193 and Er. carotovora subsp. carotovora HC131 were run in parallel as lamB⁺ negative and positive controls, respectively.

Transductants were selected on NB medium containing Kn (50ug ml⁻¹) for Tn5 selection, or Tc (10ug ml⁻¹) for Tn10 selection. Following a 36 hour incubation at 30°C, no Kn^r or Tc^r transductants were detected for any of the wild-type Er. chrysanthemi NCPPB 1066 or industrial lamB⁺ isolates tested, or the Er. carotovora subsp. carotovora SCRI 193 control. On the other hand, Er. carotovora HC131 yielded over 1000 Kn^r and Tc^r transductants following lambda₄₆₇ or lambda₈₄₀ infection. The above experiment was repeated on two further occasions and yielded similar results.

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One formal possibility to account for the apparent failure of Er. chrysanthemi lamB^+ derivatives to act as recipients for lambda-mediated transduction, was that structural alteration of the lamB^+ plasmid had taken place within the Erwinia background, leading to non-expression of the lamB structural gene, or production of a non-functional lamB gene product. Since this could have taken the form of a point mutation or small deletion not detectable by agarose gel electrophoresis, this particular question was addressed by isolating pHCP2 DNA on a large-scale from wild-type Er. chrysanthemi NCPPB 1066 recombinant pHCP2 cells, previously shown to be insensitive to lambda infection. This was subsequently introduced into Er. carotovora subsp. carotovora SCRI 193 using the modified Hanahan procedure, and transformants selected on LM medium supplemented with Ap (35ug ml^{-1}) at 30°C . Following a 36 hour incubation, representative Er. carotovora subsp. carotovora SCRI 193 pHCP2 recombinants were streak-purified on the same selective medium and then subjected to transductional analysis along with a non-recombinant control. Recombinant Er. carotovora subsp. carotovora cells were infected with λ_{467} and then spread-plated onto NB medium containing Kn (50ug ml^{-1}). Following a 36 hour incubation at 30°C , over 1000 Kn^r transductants were obtained. Hence, this evidence indicated that the lamB^+ plasmid pHCP2, originating from wild-type Er. chrysanthemi NCPPB 1066, still encoded a functional LamB protein, and that failure to transduce Er. chrysanthemi NCPPB 1066 was not due to deletion or mutation of the plasmid-borne lamB^+ gene. The same was likely to be true for pTROY9. At this point, a series of modifications to the basic transduction protocol were investigated :

(1) Growth phase

Cells were grown to varying densities corresponding to early and mid-

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log phase, instead of being allowed to enter stationary phase of growth before infection.

(2) Temperature and conditions of infection and transduction

Transductions were carried out at both 30°C and 37°C, where infection was performed statically or with shaking, ranging from 20 to 120 mins. In certain cases, 30 mins static infection followed by 90 mins shaking was employed.

(3) Treatment with NaCl

In an attempt to remove some of the extracellular polysaccharides from around Er. chrysanthemi NCPPB 1066 lamB derivatives, which could possibly occlude the lambda receptor protein, cells were washed for 0, 5, 10, 20 and 30 min with gentle agitation in 1M NaCl prior to infection. In order to assess the lethality of such a procedure, bacteria were serially-diluted in 1 X phosphate buffer (pH 7.0) before and after NaCl treatment, and subsequently spread-plated onto NB medium at 30°C. Viable counts were then determined following overnight incubation.

(4) Antibiotic concentration

At the time these particular experiments were performed, it was not known whether the Kn-resistance gene of Tn5 or the Tc-resistance gene of Tn10 could be efficiently expressed in Er. chrysanthemi NCPPB 1066. Therefore, the effect of altering the level of antibiotic selection was addressed by plating transductants onto NB agar containing a gradient of Kn or Tc up to 50ug ml⁻¹ and 10ug ml⁻¹, respectively, as described in Section 2.2.4.

3.3.5.3.6 Results

In summary, none of the alterations described above was found to lead

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to the isolation of Er. chrysanthemi NCPPB 1066 transductants. Treatment of Er. chrysanthemi NCPPB 1066 with 1M NaCl was found not to adversely affect cell viability, however as for the other procedures, this also did not yield transductants.

3.3.5.3.7 Cosmid transduction

Aside from transduction experiments using lambda₄₆₇ and lambda₈₄₀, an additional experiment was performed to assess whether an Er. chrysanthemi NCPPB 1066 lamB derivative would be sensitive to cosmid transduction. This has been found to occur at 100-fold higher frequency compared to transduction of transposons, due to the fact that one is not selecting for transposition following DNA injection (Dr. G.P.C. Salmond; personal communication). To this end, a 100ul sample of amplified Er. chrysanthemi NCPPB 1066 pHCT9 cosmid library (Section 5.3.2) was used to infect Er. chrysanthemi NCPPB 1066 cells harbouring pTROY9, as described in Section 2.2.10.1). Bacteria were subsequently selected on LB medium containing Ap (50ug ml⁻¹) at 30°C. Following 24 hours incubation, no Ap^r transductants were detected. This experiment was repeated a second time with similar results.

3.3.5.3.8 Comments

The preceding sections have described the introduction of the lamB⁺ plasmids pHCP2 and pTROY9 into wild-type Er. chrysanthemi NCPPB 1066 via conjugation, and the introduction of pHCP2 into the industrial morphotypes 2 - 5 using the modified Hanahan transformation procedure. The presence of multiple copies of the lamB⁺ structural gene within bacteria did not appear to affect cell growth or viability. Unfortunately, despite repeated and varied

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attempts all of the Er. chrysanthemi lamB⁺ derivatives appeared to remain resistant to lambda infection and as such, failed to transduce either lambda₄₆₇ or lambda₈₄₀ or in vivo packaged recombinant cosmids. In all cases, transduction experiments were carried out in the shortest possible time following the isolation of a lamB⁺ recombinant, since studies in this laboratory had found that certain Erwinia strains (eg. Er. rhapontici SCRI 423) harbouring pTROY9 lost the ability to transduce over a very short period of time. Evidence suggested that this was not due to structural alteration of the pTROY9 plasmid, but more likely resulted from mutation of the Erwinia host itself (Ellard et al., 1989).

Results from this study also implied that the failure of LamB technology was not due to an alteration in the structural or functional integrity of the relevant lamB⁺ plasmid. However, there are a number of possible alternative reasons to explain why Er. chrysanthemi NCPPB 1066 should fail to transduce. Firstly, the lamB gene may not be expressed or the LamB protein may be synthesised but not correctly processed and/or inserted into the outer membrane. Secondly, even though the lamB product may be targeted properly, it could be shielded by other membrane structures in such a way as to adversely affect lambda adsorption. Indeed, a number of studies have shown that many strains of E. coli and Shigella have a normal level of active lambda receptor, but are totally unable to adsorb the bacteriophage and cannot be labelled with antibody directed against the LamB protein. In this instance, it has been proposed that the O-specific side chains of the LPS molecules may mask the LamB protein (Gemski et al., 1972; Schwartz and Le Minor, 1975). An analogous situation is seen with the Salmonella bacteriophages, P221 and PH51, which use a 36 kDa and a 34 kDa outer membrane protein, respectively, as their receptor; and adsorb only to rough

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or semi-rough strains of S. typhimurium which have incomplete LPS structures (Schwartz, 1980). Alternatively, the capsule (or slime layer) surrounding the bacterial cell may block access to the receptor.

In order to address the potential complication outlined above, both wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype lamB⁺ derivatives were soaked in NaCl prior to infection. This particular technique has been used to remove extracellular polysaccharide from the cell surface (Hartung et al., 1988) and has facilitated lambda transduction in strains of Er. amylovora (Steinberger and Beer, 1988) and Er. herbicola SCRI 463 (Ellard et al., 1989). In the latter instance, the number of transductants was reported to have been increased 10-fold. (Ellard et al., 1989)

Evidence from this laboratory (F. Ellard; personal communication), indicates that adsorption of the bacteriophage lambda does not occur in strains of Erwinia that do not transduce; although this was not tested for either the wild-type Er. chrysanthemi NCPPB 1066 or the industrial morphotype lamB⁺ derivatives. However, even if bacteriophage lambda does absorb correctly to Er. chrysanthemi NCPPB 1066, it may have failed to inject its DNA, as is the case with pei⁻ (for penetration of lambda) mutants of E. coli (Scandella and Arber, 1974). In this respect, a number of workers have shown that certain components of the E. coli mannose permease complex (encoded by the ptsL, P and M genes), present in the cytoplasmic membrane, are required for injection of lambda DNA into the cell (Erni and Zanolari, 1985; Erni et al., 1987). Certain components of this system may be absent or inactive in some Erwinia strains, including Er. chrysanthemi NCPPB 1066, although the latter wild-type strain was found to utilize mannose. Finally, host-controlled restriction may have prevented insertion of the transposon into the genome. Indeed, recent results obtained in this laboratory

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have suggested that restriction can play a big factor in allowing transduction to occur in Serratia λ MB⁺ recombinants (Dr. G.P.C. Salmond; personal communication.).

3.3.5.4 Sensitivity to novel Erwinia bacteriophage

Since a range of novel Erwinia bacteriophages had been isolated in this laboratory, for both Er. carotovora subsp. carotovora SCRI 193 (Phi DEL1, Phi KP) and Er. carotovora subsp. atroseptica SCRI 1043 (Phi MIK 1, 2, 3 and 4; Phi ART 1, 2, 3, 4, 5 and 6); their ability to infect wild-type Er. chrysanthemi NCPPB 1066 and each of the industrial morphotypes was investigated. To this end, high-titre lysates ($> 10^9$ pfu ml⁻¹) of the aforementioned bacteriophage were kindly provided by I. Toth, and a 5ul neat sample of each spot-tested onto overlays containing log-phase cells of both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes 1 - 5. Following a 36 hour incubation at 30°C, no plaques or zones of lysis were detected.

3.3.5.5 Isolation of novel bacteriophage for Er. chrysanthemi NCPPB 1066

Since it was clear that both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes were resistant to bacteriophages that have been commonly employed for genetic analysis in Erwinia, attempts were made to isolate novel bacteriophage for Er. chrysanthemi NCPPB 1066 from the environment. These would hopefully have the capacity to transfer DNA via the process of generalised transduction. In this instance, sewage was chosen as a potential source for novel Er. chrysanthemi NCPPB 1066 bacteriophages, due to the fact that a great variety of human-derived

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viruses, as well as those of higher plants, animals and the microflora (including bacteria), end up in this system from a wide range of environmental sources such as drainage from farms, slaughter houses and food processing factories, natural waterways and domestic homes (Slade, 1980). Since some of the aforementioned viruses are present in large numbers, equally some may be present in very small numbers. Consequently, enrichment must be performed (Adams, 1959).

3.3.5.5.1 Results

The bacteriophage isolation and enrichment procedure was carried out using wild-type Er. chrysanthemi NCPPB 1066 as outlined in Section 2.2.9, and was found to give rise to a number of bacteriophage plaques. Within this population, three distinct plaque morphologies were identified : (1) small turbid plaques approximately 1mm in diameter; (2) small clear plaques approximately 1mm in diameter and (3) larger clear plaques of diameter 2 - 3mm, surrounded by a turbid fringe. A single plaque of each type was subsequently purified and high-titre bacteriophage lysates prepared, as described in Section 2.2.8.2. These were subsequently designated phi CK 1, phi CK 2, and phi CK3, respectively. Further analysis revealed that none of the phi CK bacteriophage isolates required the addition of cofactors for infection, such as divalent cations Mg^{2+} or Ca^{2+} which were included in the original isolation procedure. Moreover, all were found to replicate at 25°C, 30°C, 37°C, but not at 42°C.

Rather unexpectedly, each of the bacteriophage originally isolated on the wild-type Er. chrysanthemi NCPPB 1066 strain did not give rise to plaques or zones of lysis when spot-tested on each of the industrial morphotypes, at either 25°C, 30°C or 37°C. This repeatable observation

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further highlighted the differences between wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotypes, and provided further evidence to suggest that the latter had suffered change(s) in an outer membrane component or components, which acted as a receptor for the novel bacteriophage isolated on wild-type Er. chrysanthemi NCPPB 1066. One prime candidate for such a receptor is the lipopolysaccharide (LPS) moiety of the outer membrane, since this is recognised as a cellular receptor unit by a wide variety of bacteriophages including T7, T3, P22 and P1, and may also be involved in subsequent stages of bacteriophage adsorption, including injection of DNA (Wright et al., 1980). Alternatively, for a number of bacteriophage receptors, an outer membrane protein may also be an essential component. In the case of the T5, T1 and phi80 for example, the outer membrane protein TonA, involved in the binding and transport of ferrichrome, forms part of the receptor. For bacteriophage T6, the presence of the Tsx protein, involved in the transport of nucleosides, is essential for absorption (Schwartz, 1980). Finally, the capsular slime layer may function as a bacteriophage receptor (Lindberg, 1973)

3.3.5.5.2 Generalised transduction assays

In an attempt to determine whether phi CK 1 - 3 isolates were capable of mediating generalised transduction, high-titre lysates (10^9 - 10^{10} pfu ml⁻¹) of each bacteriophage were prepared on wild-type Er. chrysanthemi NCPPB 1066 harbouring plasmid pBR322 (Section 3.3.3.3) and the wild-type Er. chrysanthemi ansB::lacZ single copy fusion derivative CK1002 (Section 7.5.2). Three independent transduction assays were subsequently performed at 30°C as described in Section 2.2.10.2. Unfortunately, on each occasion, no transductant colonies were detected either on NB medium containing Ap

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(50ug ml⁻¹) for pBR322 selection, or Kn (50ug ml⁻¹) for selection of the Kn-resistance gene situated adjacent to the ansB::lacZ fusion. Therefore, from the available evidence, it was concluded that, under the conditions of the assay, all of the bacteriophage isolates were incapable of transferring DNA between Er. chrysanthemi NCPPB 1066 derivatives via generalised transduction.

3.4 FINAL COMMENTS

This chapter has described a basic comparative physical and biochemical characterisation of both the wild-type Er. chrysanthemi NCPPB 1066 strain and five isolates from the industrial L-asparaginase II production culture (batch 8). Although the evidence indicated that the latter were Er. chrysanthemi NCPPB 1066 in nature, they clearly differed amongst themselves and from the wild-type strain in a number of respects. This included colony morphology, motility, extracellular enzyme production and sensitivity to novel bacteriophage isolated from sewage. It would now be of interest to determine whether changes in LPS structure or the protein composition of the outer membrane could explain some of these observations. This could possibly be addressed by purification of the aforementioned components, followed by their analysis using one or two-dimensional SDS polyacrylamide gel electrophoresis.

From the work that has been described in the latter sections of this chapter, it can be seen that both the wild-type Er. chrysanthemi NCPPB 1066 strain, as well as the industrial isolates were resistant to the commonly used bacteriophages Mu, phi EC-2, and T4. Moreover, isolation of a P1 sensitive derivative of Er. chrysanthemi NCPPB 1066 was unsuccessful. However, despite these setbacks, a number of techniques were successfully applied to

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wild-type Er. chrysanthemi NCPPB 1066 and some of the industrial morphotypes. For example, chemical mutagenesis using EMS provided a straightforward and effective means of generating mutations within the Er. chrysanthemi NCPPB 1066 genome.

Further success was attained using the modified transformation protocol of Hinton et al. (1985 b) leading to the introduction of the of the narrow-host-range medium copy cloning vector, pBR322, into wild-type strain and the industrial isolates. The high copy number cloning vehicles, pUC8 and pUC9 were also introduced into the wild-type strain and the industrial isolates, this time using electroporation. Unfortunately none of these techniques led to the construction of a recombinant Er. chrysanthemi NCPPB 1066 derivative harbouring the L-asparaginase II expression vectors pASN326 or pASN230.

Conjugal mobilisation was used to introduce the temperature-sensitive transposon delivery vehicle pCHR81, as well as the lamB⁺-containing plasmids pHCP2 and pTROY9 into the wild-type Er. chrysanthemi NCPPB 1066 strain. Although the E. coli lamB⁺ product failed to render Er. chrysanthemi NCPPB 1066 sensitive to lambda transduction, the data obtained from the pHCP2 mating experiment suggested that the pR64drd11/ pLVC9 mobilisation system had the potential of introducing a pBR322-based L-asparaginase II expression vector into this particular strain of Erwinia. Hence, attempts were made to determine whether this would, in fact, be the case. This will now be described in detail in the following chapter of this thesis.

In conclusion, the genetic data obtained in this study has highlighted the 'strain-dependent' applicability of genetic tools in Erwinia , and reinforced the findings of workers such as Gilbert et al. (1986) that Er. chrysanthemi NCPPB 1066 is not very amenable genetically speaking.

CHAPTER FOUR

OVER-EXPRESSION OF L-ASPARAGINASE II

4. L-asparaginase II over-expression

4.1 INTRODUCTION

4.1.1 Aim

Over recent years, one important objective at C.A.M.R. has been to increase the commercial production of the Er. chrysanthemi NCPPB 1066 L-asparaginase II enzyme, in order to meet the growing world demand for this product. In theory, the easiest way to achieve this goal without placing a further demand on fermentation facilities, is to construct a bacterial strain which will overproduce the protein. One of the most direct routes to achieve over-expression is to introduce the Er. chrysanthemi NCPPB 1066 ansB structural gene on a multicopy plasmid into the bacterial strain of interest, thereby achieving a gene dosage effect. Ideally, this genetic manipulation should be performed with Er. chrysanthemi NCPPB 1066, since all the fermentation technology at C.A.M.R. has been developed using this organism, and, more importantly, this strain is the only one licensed for L-asparaginase II production.

Following the isolation and cloning of the Er. chrysanthemi NCPPB 1066 ansB structural gene, Gilbert and colleagues (1986) had successfully introduced a number of recombinant ansB vectors into both E. coli JM83 and Er. carotovora subsp. carotovora SCRI 193 via transformation. In the case of the former bacterium, the presence of the ansB expression vectors pASN30, pASN32 and pASN42, was found to lead to the synthesis of the Er. chrysanthemi NCPPB 1066 AnsB protein up to levels of 32, 49 and 16 IU mg protein⁻¹, respectively. This was much higher than control E. coli JM83 cells harbouring pUC8 and pUC9, which were found to produce L-asparaginase to levels of 0.4 and 0.1 IU mg protein⁻¹, respectively. Similarly, recombinant Er. carotovora subsp. carotovora SCRI 193 cells harbouring the ansB vectors

4. L-asparaginase II over-expression

pASN326 and pASN230, were found to synthesise increased but variable levels the Er. chrysanthemi AnsB protein to levels of 26 and 31.8 IU mg protein⁻¹. Again, this was much higher than non-recombinant Er. carotovora subsp. carotovora SCRI 193 cells which only produced L-asparaginase to 1.1 IU mg protein⁻¹.

Although over-expression of the Er. chrysanthemi NCPPB 1066 L-asparaginase II protein had been in E. coli and Er. carotovora subsp. carotovora SCRI 193, attempts to transform Er. chrysanthemi NCPPB 1066 with the ansB expression vectors pASN326 and pASN230 DNA, using a number of techniques, including the modified Hanahan protocol of Hinton et al. (1985 b), proved negative. Consequently, the aim of the work described in the following sections of this chapter was to find a means of introducing the Er. chrysanthemi NCPPB 1066 ansB structural gene into the parent organism. This would not only solve an immediate industrial problem, but it would also be extremely useful for subsequent genetic studies concerning L-asparaginase II regulation in Er. chrysanthemi NCPPB 1066 itself.

4.1.2 Approach

From the preceding chapter of this thesis, one can see that additional efforts were made in this study to introduce both pASN326 and pASN230 into Er. chrysanthemi NCPPB 1066 by electroporation or transformation. Unfortunately, no recombinant colonies were detected, thereby echoing the difficulties experienced at C.A.M.R. by Gilbert and coworkers (1986). Hence, it was clear that an alternative genetic approach would have to be taken in order to introduce an ansB expression vector into Er. chrysanthemi NCPPB 1066. Moreover, it was possible that levels of expression of the ansB structural gene present on a high copy number vector may have been lethal

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for this particular strain of Erwinia, and could possibly have accounted for an inability to detect transformants. Therefore, to perhaps increase the chance of introducing the ansB structural gene into Er. chrysanthemi NCPPB 1066, a lower copy number ansB expression vector was to be constructed. One prime candidate to form the basis for such a vector was the medium copy number plasmid pBR322, since this had previously been shown to be able to be maintained within wild-type Er. chrysanthemi NCPPB 1066 (Section 3.3.3.3)

Theoretically, one alternative means of transferring a pBR322-based ansB expression vector into Er. chrysanthemi NCPPB 1066 would be to use conjugal mobilisation. However, pBR322 itself lacks the genes coding for proteins involved in mobilisation and is found to be inefficiently mobilised by large conjugative plasmids, such as the F episome or RP4 (Chambers et al., 1988 a; Simon et al., 1983 b). On the other hand, the presence of the site at which these proteins act, the nic/bom site, enables pBR322 to be readily mobilised if Mob proteins are supplied in trans from a co-resident plasmid such as ColK (Finnegan and Sherrat, 1982).

Studies described in Section 3.3.5.3.2 demonstrated that the pBR322-based lamB⁺ plasmid, pHCP2, could be conjugally mobilised into wild-type Er. chrysanthemi NCPPB 1066 relatively efficiently whilst in the presence of the trans-acting 'helper' plasmids pR64drd11 and pLVC9. This was similar to the findings of Roeder and Collmer (1985), who had demonstrated that a recombinant pBR322 plasmid containing the pelC structural gene of Er. chrysanthemi could be introduced by the same system into Er. chrysanthemi strain CUCPB 1237 at a frequency of 4×10^{-5} transconjugants per recipient. It was therefore reasonable to suppose that this system would also allow the introduction of a pBR322-based ansB vector into wild-type Er. chrysanthemi

4. L-asparaginase II over-expression

NCPPB 1066 and the industrial morphotypes in a similar manner. The following sections of this chapter now describe work which was aimed at testing this hypothesis.

4.2 RESULTS AND DISCUSSION

4.2.1 Construction of the pBR322-based ansB expression vector pCK1

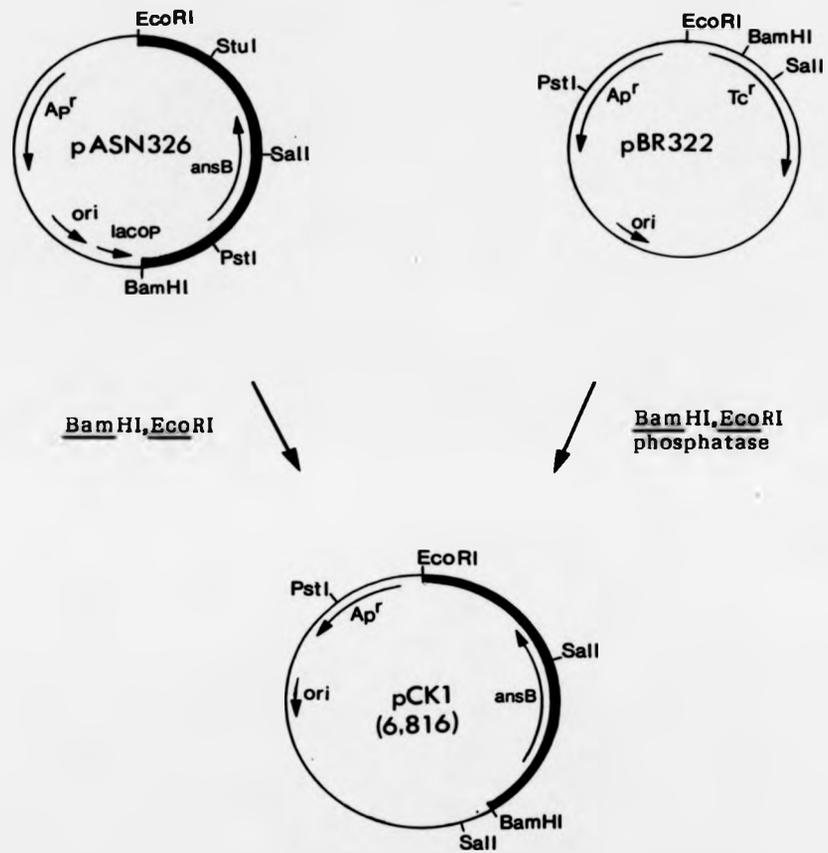
The construction of the pBR322-based ansB expression vector, pCK1, is schematically presented in Figure 4.1. Plasmid pASN326 was cleaved with a combination of BamH1 and EcoR1 restriction endonucleases, and the products size-fractionated on a preparative 0.7% (w/v) TAE agarose gel. A 2.83kb fragment containing the Er. chrysanthemi NCPPB 1066 ansB structural gene was purified by electro-elution and ligated with pBR322, previously restricted with BamH1/EcoR1 and dephosphorylated. Ligation products were then used to transform competent E. coli DH1 cells, and transformants selected on NB medium supplemented with Ap (50ug ml⁻¹). Plasmid DNA was extracted on a small-scale from six Ap^r Tc^s recombinants, and subjected to restriction endonuclease analysis with BamHI and EcoRI alone, or in combination, to verify the subcloning. One appropriate plasmid was subsequently retained for the large-scale isolation of DNA and designated pCK1.

4.2.2 Isolation of pCK1 recombinant derivatives

Plasmid pCK1 DNA was used to transform competent E. coli GJ342 cells harbouring the 'helper' plasmids pR64drd11 and pLVC9, and transformants selected on NB medium containing Ap (50ug ml⁻¹), Cm (50ug ml⁻¹) and Tc (10ug ml⁻¹). One representative Ap^rCm^rTc^r colony was then streak-purified on the same medium, prior to patch-mating, with wild-type

4. L-asparaginase II overexpression

Figure 4.1 Construction of the ansB expression vector pCK1



4. L-asparaginase II over-expression

Er. chrysanthemi NCPPB 1066 and each of the industrial morphotypes on NB agar at 30°C, as outlined in Section 2.2.5. Transconjugants were then selected on minimal sucrose medium containing Ap (50ug ml⁻¹) at 30°C. The results following a 2 day incubation are summarised in Table 4.1, where it can be seen that transfer of pCK1 into the aforementioned bacteria had occurred at a frequency ranging from 1.1 X 10⁻² to 2.4 X 10⁻⁵ transconjugants per recipient. In this instance, pCK1 appeared to be transferred to each of the industrial morphotypes with a higher efficiency than that seen for the wild-type strain, and could possibly correlate with the higher donor : recipient ratio recorded at the time mating mixes were harvested. Transconjugants from each mating were subsequently re-streaked twice on the same selective medium, before further analysis.

4.2.3 Verification of pCK1 transconjugants

Representative pCK1 transconjugant colonies from each mating were found to be Cm^S and Tc^S, indicating the absence of pR64drd11 and pLVC9, and were Pel⁺ Cel⁺ and Prt⁺ on extracellular enzyme detection medium. Furthermore, API biochemical profiles of transconjugants were identical to those seen for parent isolates. Verification of the presence of pCK1 DNA within cells was ascertained by small-scale analysis followed by 0.7% TAE agarose gel electrophoresis. DNA was subsequently cleaved with a combination of BamHI and PstI enzymes and the products were again analysed by 0.7% TAE agarose gel electrophoresis and shown to have the correct banding pattern.

4.2.4 Transformation studies with pCK1

Following from transformation experiments described in Section 3.3.3.7,

4. L-asparaginase II over-expression

Table 4.1 Conjugal transfer of plasmid pCK1 into wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotypes 1 - 5

Mating	Donor* (cfu ml ⁻¹)	Recipient ⁺ (cfu ml ⁻¹)	Ratio [#]	Transconjugants (cfu ml ⁻¹)	TPR [ⓐ]
wild-type	8.4 X 10 ⁸	1.1 X 10 ⁹	0.7:1	2.8 X 10 ⁴	2.5 X 10 ⁻⁵
IND1	2.3 X 10 ⁹	2.0 X 10 ⁶	1.2 X 10 ³ :1	2.2 X 10 ⁴	1.1 X 10 ⁻²
IND2	1.4 X 10 ⁹	6.4 X 10 ⁷	21.3:1	2.1 X 10 ⁵	3.3 X 10 ⁻³
IND3	1.3 X 10 ⁹	6.1 X 10 ⁷	20.8:1	1.3 X 10 ⁵	2.1 X 10 ⁻³
IND4	1.4 X 10 ⁹	2.5 X 10 ⁷	56.5:1	1.3 X 10 ⁵	5.2 X 10 ⁻³
IND5	1.7 X 10 ⁹	5.7 X 10 ⁷	29.4:1	2.0 X 10 ⁵	3.5 X 10 ⁻³

* Viable count for GJ342 donor cells was determined on NB agar plates supplemented with Ap (50ug ml⁻¹), Cm (50ug ml⁻¹) and Tc (10ug ml⁻¹) at 37°C.

Ratio of donor to recipient.

⁺ Erwinia viable count was determined on minimal sucrose medium at 30°C.

[ⓐ] Conjugation frequency expressed as number of transconjugants per recipient cell (TPR).

4. L-asparaginase II over-expression

it was of interest to determine whether pCK1 DNA isolated from wild-type Er. chrysanthemi NCPPB 1066 transconjugant cells would transform wild-type Er. chrysanthemi NCPPB 1066 at a higher frequency than pCK1 that had only resided in E. coli. To this end, DNA was prepared on a large-scale from both E. coli DH1 and wild-type Er. chrysanthemi NCPPB 1066 and used to transform the latter non-recombinant strain by the modified Hanahan procedure of Hinton et al. (1985 b) as outlined in Section 2.2.6.4.

In two independent experiments employing 0.5ug of plasmid DNA, no Ap^r colonies resulted from transformations involving E. coli pCK1 DNA. In contrast, Er. chrysanthemi NCPPB 1066-derived pCK1 DNA was found to transform competent wild-type Er. chrysanthemi NCPPB 1066 cells at a frequency of 11 and 14 transformants ug⁻¹ DNA, respectively.

4.2.5 Small-scale fermentation analysis of wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype pCK1 recombinants

From the aforementioned work, it was evident that introduction of the Er. chrysanthemi NCPPB 1066 ansB structural gene into both wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotypes had been successfully achieved by conjugal mobilisation, thereby solving an immediate industrial problem. The next stage of the work was to determine the levels of L-asparaginase II enzyme within recombinant derivatives, in order to ascertain whether over-expression had been achieved. To this end, pCK1 recombinant derivatives and their parental counterparts were cultured in the controlled environment of a small-scale (8 litre) LH 2000 bioreactor, with provision for accurate pH, temperature and dissolved oxygen control. Recombinant and parent strains were grown to stationary phase in complex yeast extract medium supplemented with Ap (50ug ml⁻¹), as described in Section 2.2.16.2.

4. L-asparaginase II over-expression

During fermentation runs, temperature (30°C), dissolved oxygen (30%), and pH (6.8) were kept constant by the aid of computer.

The results are presented in Figure 4.2 (a, b, c, d, e and f); and summarised in Table 4.2. Analysis of the non-recombinant isolates revealed that each industrial morphotype synthesised higher levels of L-asparaginase II enzyme at the end of the growth phase compared to the wild-type strain. In all cases, enzyme specific activity showed an initial fall over 3 hours post-inoculum, after which a subsequent rise was seen as the culture progressed. Finally, there was no indication of a significant accumulation of enzyme in the stationary phase, perhaps due to limiting nutrient concentration; although in general, maximal levels of specific enzyme activity were detected at the onset of stationary phase. In this respect, L-asparaginase synthesis in Er. chrysanthemi NCPPB 1066 follows a similar pattern to that seen for S. marcescens ATCC #60 (Novak and Phillips, 1974) and Citrobacter C6 (Bascomb et al., 1975) for example.

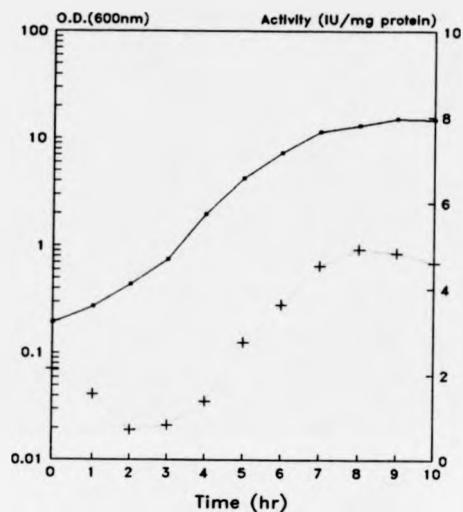
The initial drop in enzyme activity is a common feature of L-asparaginase II batch fermentation runs at C.A.M.R. (Dr. D. Cossar; personal communication); although the exact basis for this phenomenon is not understood. One possibility is that it reflects a transient inactivation of ansB expression due to the presence of some component of the growth medium. Alternatively, it may be a consequence of protein turnover (proteolysis), as cells are subjected to a rapid change in environment with a differing nutrient status. The latter suggestion could possibly be tested experimentally using anti-AnsB antibodies to see if pre-formed enzyme is degraded.

Similar fermentation profiles to those described above were also observed for the corresponding recombinant cultures, suggesting that multiple copies of the ansB gene did not significantly alter its regulation throughout

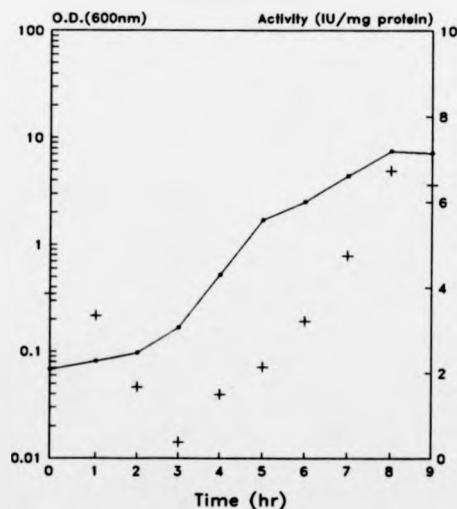
4. L-asparaginase II over-expression

Figure 4.2 (a) Fermentation profiles for wild-type *Es. chrysanthemi* NCPPB 1066 and industrial morphotype 1

WILD-TYPE



MORPHOTYPE 1

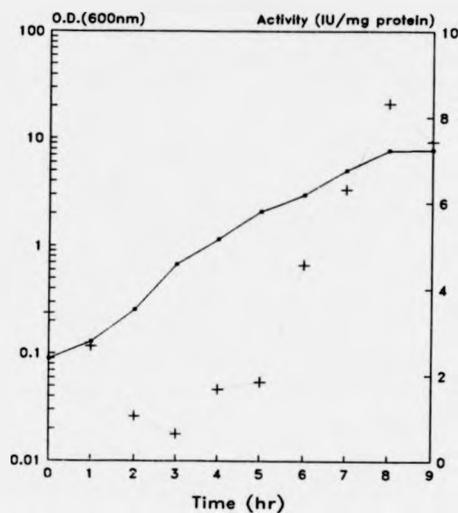


The optical density of the fermentation culture and the L-asparaginase II specific activity is represented by a solid and broken line, respectively.

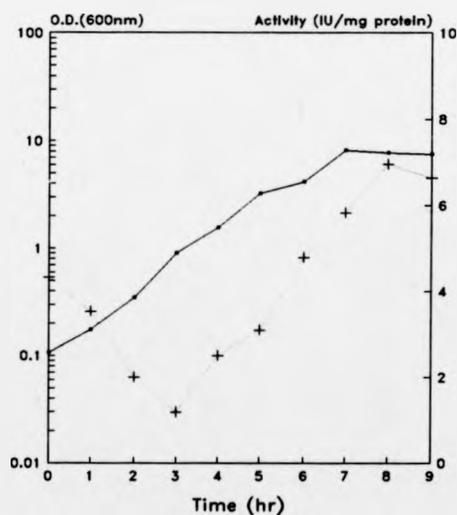
4. L-asparaginase II over-expression

Figure 4.2 (b) Fermentation profiles for industrial morphotype 2 and 3

MORPHOTYPE 2



MORPHOTYPE 3

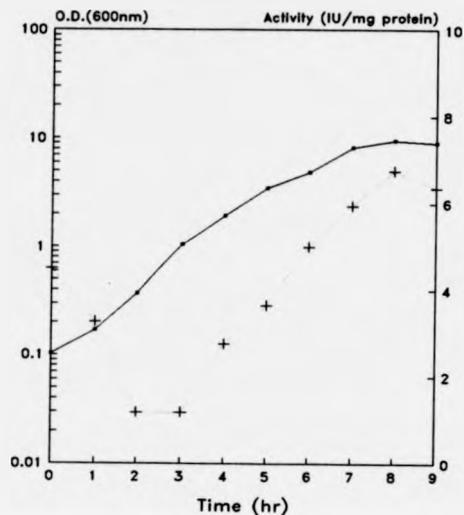


The optical density of the fermentation culture and the L-asparaginase II specific activity is represented by a solid and broken line, respectively.

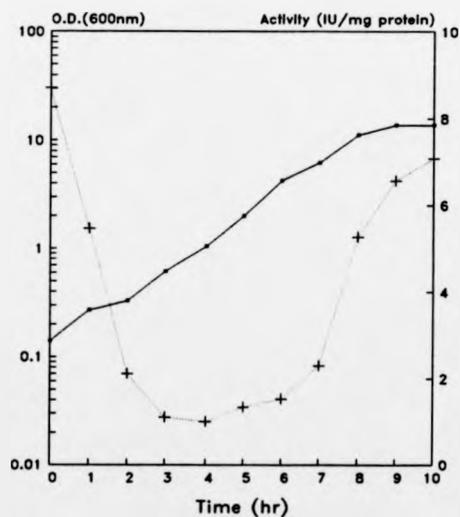
4. L-asparaginase II over-expression

Figure 4.2 (c) Fermentation profiles for industrial morphotype 4 and 5

MORPHOTYPE 4



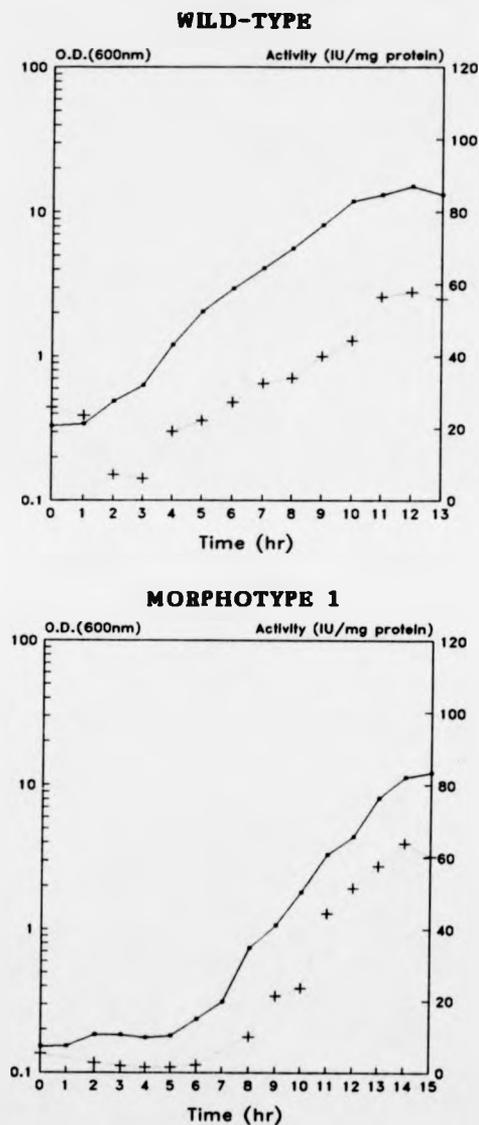
MORPHOTYPE 5



The optical density of the fermentation culture and the L-asparaginase II specific activity is represented by a solid and broken line, respectively.

4. L-asparaginase II over-expression

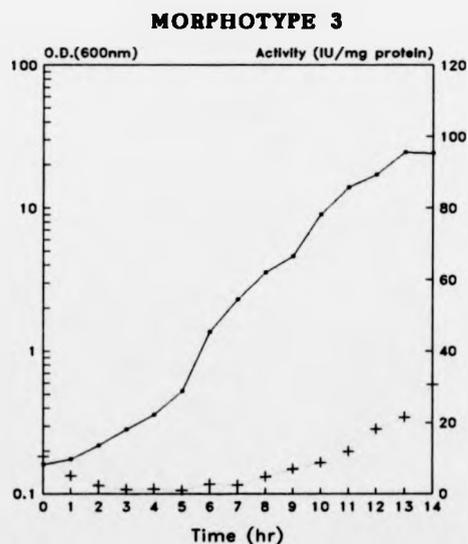
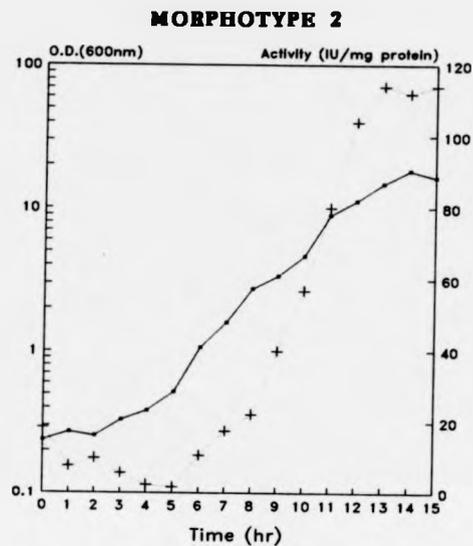
Figure 4.2 (d) Fermentation profiles for wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype 1 pGKI recombinants



The optical density of the fermentation culture and the L-asparaginase II specific activity is represented by a solid and broken line, respectively.

4. L-asparaginase II over-expression

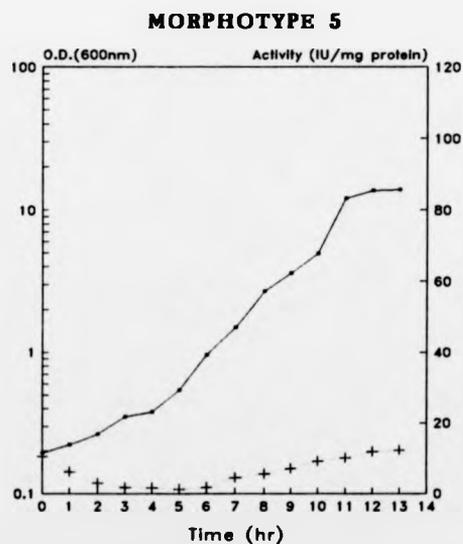
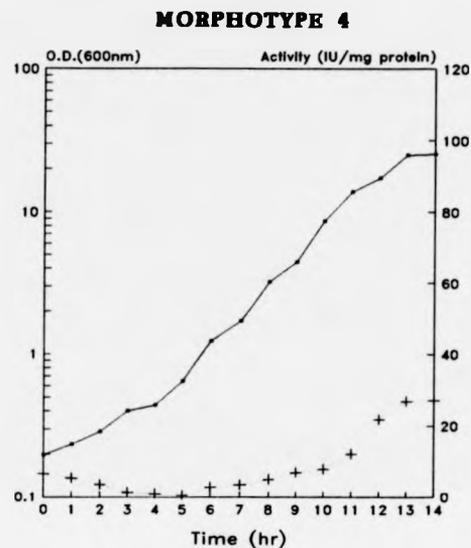
Figure 4.2 (e) Fermentation profiles for industrial morphotype 2 and 3 pCKI recombinants



The optical density of the fermentation culture and the L-asparaginase II specific activity is represented by a solid and broken line, respectively.

4. L-asparaginase II over-expression

Figure 4.2 (f) Fermentation profiles for industrial morphotype 4 and 5 pCKI recombinants



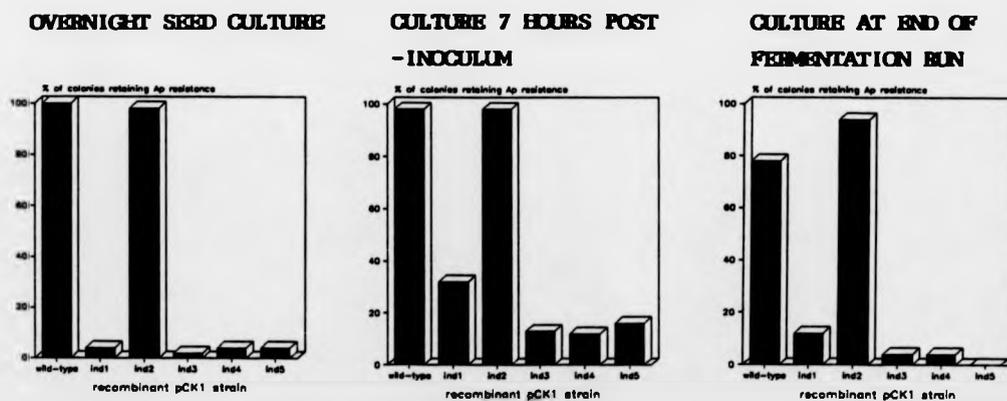
The optical density of the fermentation culture and the L-asparaginase II specific activity is represented by a solid and broken line, respectively.

4. L-asparaginase II over-expression

Table 4.2 Summary of the maximum levels of L-asparaginase II specific activity detected in wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype recombinant and parent isolates.

Isolate	Maximum levels of L-asparaginase II activity (IU mg protein ⁻¹)
Wild-type	4.9
Wild-type (pCK1)	57.8
Morphotype 1	6.7
Morphotype 1 (pCK1)	63.8
Morphotype 2	8.3
Morphotype 2 (pCK1)	114.0
Morphotype 3	7.0
Morphotype 3 (pCK1)	30.7
Morphotype 4	6.8
Morphotype 4 (pCK1)	27.1
Morphotype 5	7.1
Morphotype 5 (pCK1)	12.3

Figure 4.3 Maintenance of ampicillin-resistance in pCK1 recombinant isolates at various stages of fermentation



4. L-asparaginase II over-expression

the growth phase. In all cases, the maximum levels of L-asparaginase II enzyme produced by pCK1 recombinants, were found to be higher than those of parental strains, thus indicating that over-expression had been achieved. However, this was found to be highly variable from one pCK1 recombinant to another, and results presented in Figure 4.3 suggests that one factor may have been the difference in stability of pCK1 within recombinant derivatives. This was reflected by the fact that the proportion of the cell population retaining the plasmid-encoded Ap-resistance marker, varied from recombinant culture to recombinant culture (Section 2.2.16.4).

The occurrence of plasmid instability even when antibiotic has been added to growth medium has also been noted by Pierce and Gutteridge (1985) whilst studying an E. coli HB101 recombinant derivative containing a pBR322 clone which harboured the Rhodospirillum rubrum ribulosebisphosphate carboxylase structural gene. In this instance, plasmid-containing bacteria were found to produce and excrete high levels of B-lactamase, which rapidly degraded the available ampicillin in the culture medium. Consequently, antibiotic selection could only be maintained for a very short period of time, after which growth ensued under non-selective conditions. Plasmid-free cells quickly arose within the population and grew at an enhanced rate, eventually dominating the culture. In this instance, instability was found to be so severe, that even isolated colonies prepared on Ap-containing plates were impure and contained plasmid-free cells.

It is not known whether a similar situation occurred in recombinant pCK1 cultures. However, the particular example cited above illustrates the point that antibiotic selection involving Ap for example, may not be effective in a fermenter under certain circumstances.

Phenotypic plasmid instability itself, may be due to either the

4. L-asparaginase II over-expression

disappearance of the entire plasmid from host cells or structural alteration of the plasmid. The former can be influenced by a number of factors, including the copy number of the plasmid (Imanaka *et al.*, 1980), the degree of plasmid multimerisation (Summers and Sherratt, 1984), culture conditions (Shoham and Demain, 1990), and the population distribution of the recombinant plasmid (Georgiou, 1988). The latter may involve re-assortment and re-arrangement of DNA sequences, as well as recombination and DNA inversion of DNA, and may occur more frequently with chimaeric plasmids. This can depend on the genetic make-up of the host organism (Chang *et al.*, 1975; Kim and Ryu, 1984). In particular, the presence of transposable and IS1 genetic elements could cause insertional inactivation of plasmid encoded genes (Imanaka and Aiba, 1981; Rood *et al.*, 1980). Finally, plasmid instability may sometimes arise as a direct consequence of the metabolic effects of over-production of plasmid-encoded proteins (Adams and Hatfield, 1984).

It is not known which, if any of these explanations might account for the high intrinsic rates of plasmid loss that were observed for some pCK1 recombinants (attempts were made to determine the copy number of pCK1 within recombinant cells by scanning densitometry, but this was unsuccessful due to difficulty in resolving plasmid bands from chromosomal fragments in agarose gels).

Increased stabilisation of pCK1 in poor isolates, such as industrial morphotype 4 and 5, may perhaps be achieved using a highly dilute inocula to start cultures, as recommended by Pierce and Gutteridge (1985). This procedure is believed to allow the maintenance of antibiotic levels within the culture for longer periods and, as such, prolongs the selection of plasmid-containing organisms before the inevitable takeover by plasmid-free cells.

4. L-asparaginase II over-expression

Alternatively, genes such as par or cer can be cloned into a particular recombinant plasmid to potentially increase its stability. The former genetic determinant may increase the probability of equal distribution of plasmid molecules in the daughter cells (Gerdes and Molin, 1986; Martin et al., 1987; Ogura and Hiraga, 1983) and has been used to stabilise a number of expression vectors (Caulcott et al., 1985; Skogman et al., 1983; Zurita et al., 1984). The latter increases plasmid stability by reducing the level of plasmid multimerisation (Summers and Sherrat, 1984).

Several alternative strategies also exist to prevent plasmid-free segregants from dominating recombinant cultures, but at present none of these is generally accepted. For example, the use of plasmids containing genes for bacterial toxins that kill plasmid-free cells has been proposed (Lauffenburger, 1987). Furthermore, plasmid stability can be increased when a plasmid-encoded protein is essential for cellular metabolism and the host cell is unable to synthesise this protein based on its chromosomal DNA. This system has been employed successfully to confer 100% plasmid retention of an otherwise unstable plasmid for more than 150 generations (Nilsson and Skogman, 1986).

From Figure 4.3 it can be seen that pCK1 appeared to be maintained to the greatest degree in industrial morphotype 2, and therefore this recombinant derivative was chosen for further study. Unfortunately, following storage of morphotype 2 pCK1 ampoules in liquid nitrogen before seed inoculation, the levels of L-asparaginase II production which were initially recorded, could not be repeated (Dr. D. Cossar; personal communication). Instead, enzyme activity dropped to a steady value of between 55 and 70 IU mg protein⁻¹. Further parametric studies revealed that temperature had little effect on final enzyme yields (which were slightly lowered at 37°C, although

4. L-asparaginase II over-expression

the fermentation run time was increased), and the same could be said for oxygen tension (Dr. D. Cossar; personal communication). Additional studies also demonstrated that the presence of ampicillin in the culture medium was not essential for the maintenance of pCK1 (96% of the population retained plasmid marker (Ap^r) when grown in the absence of ampicillin). Moreover, the source of medium components did not influence growth or expression; neither did the age of the seed, nor the temperature at which the seed was grown (Dr. D. Cossar; personal communication).

4.3 Construction of a high copy number ansB expression vector

4.3.1 Aim

Having attained increased yields of L-asparaginase II with the ansB expression vector pCK1, an additional experiment was performed to determine whether the Er. chrysanthemi NCPPB 1066 ansB structural gene could be introduced into Er. chrysanthemi NCPPB 1066 on a higher copy number plasmid, and assess what effect this would have on cell viability, productivity and growth. On this occasion, both wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2 were included, since pCK1 had shown the greatest stability in these isolates.

4.3.2 Approach

The high copy number pUC cloning vectors have been widely used in genetic studies, but are recognised to be non-mobilisable, since they lack a functional oriT/bom site (Boyd *et al.*, 1989; Chambers *et al.*, 1988 a). One strategy for overcoming this limitation has come from the work of Simon and colleagues (1983 a,b; 1984), who have devised an RP4-specific

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conjugative system, applicable for a wide variety of Gram-negative organisms including Rhizobium, Agrobacterium, Alcaligenes and Rhodopseudomonas species. In brief, these particular workers constructed derivatives of E. coli (eg. S17-1) which contained a modified RP4 plasmid integrated into the chromosome. This provides trans acting tra gene products capable of efficiently mobilising modified narrow-host-range vectors containing a genetic locus necessary for mobilisation by the co-existing RP4 plasmid. This so-called cis acting Mob region, originates from RP4, and acts as a recognition site for the trans active RP4 transfer functions. It is believed to include the origin of transfer for replication (oriT), the site where a single-strand nick is introduced into the plasmid molecule as a prerequisite for its conjugative transfer into the recipient cell (Guiney and Helinski, 1979; Guiney and Yakobson, 1983; Simon et al., 1983 b).

Studies by Chambers et al. (1988 b) have indicated that problems encountered with cell viability and plasmid stability when a gene is cloned into an expression vector, are not just a consequence of the high expression of that gene. These workers noted that high unregulated expression of Pseudomonas carboxypeptidase G₂ (CPG₂), coupled with the synthesis of B-lactamase was detrimental to cell growth and viability. It was speculated that combined high expression of both proteins led to the overloading of the protein export machinery of the cell, resulting in the deleterious presence of CPG₂ in the cytoplasm. As protein export is generally believed to occur at specialised sites (Randall et al., 1987), the two proteins may have been in direct competition for translocation. Hence, it may be advantageous to utilise expression vectors carrying non-exported resistance products (for example, Tc^r, Cm^r and Kn^r) when the recombinant protein of interest is also being exported through the cytoplasmic membrane. In this respect, Cm^r and Kn^r

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genes may prove to be preferable to Tc^r , since the latter encodes a cytoplasmic membrane-associated protein (Tait and Boyer, 1978), that has been shown to effect the reproductive fitness of E. coli cells when expressed at high levels (Whan Lee and Edlin, 1985).

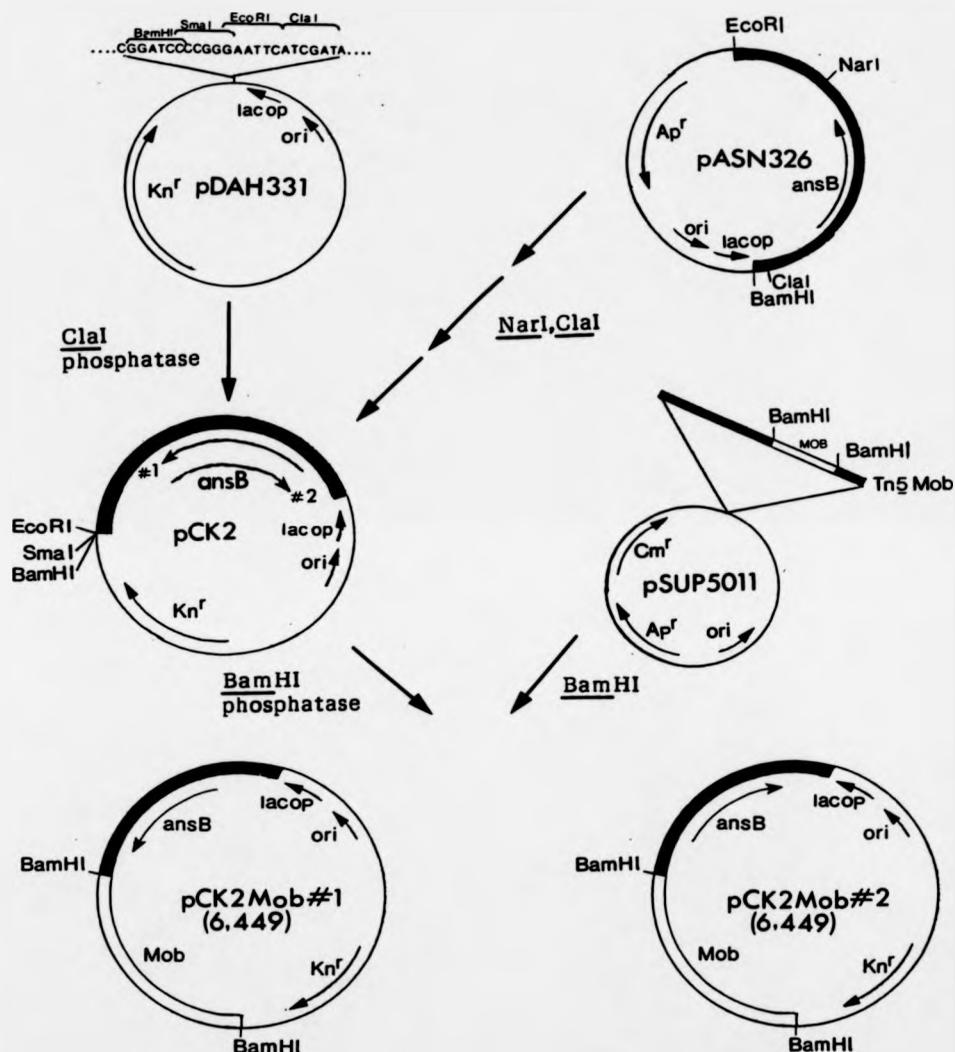
With the above in mind, work was carried out to construct a high copy number ansB expression vector encoding kanamycin-resistance, and to investigate whether the presence of the oriT/bom site of RP4 on such a vector would allow transfer into Er. chrysanthemi NCPPB 1066 using the E. coli mobilising strain S17-1.

4.3.3 Construction of the pIC-19H-based ansB expression vector, pCK2

The construction of a pIC-19H-based high copy number ansB expression vector encoding kanamycin-resistance, is schematically represented in Figure 4.4. Plasmid pASN326 was cleaved with both NarI and ClaI restriction endonucleases, and the products size-fractionated by 0.7% (w/v) agarose gel electrophoresis. A 1.6 kb fragment containing the Er. chrysanthemi NCPPB 1066 ansB structural gene was purified by electro-elution and ligated with the pIC-19H-based vector pDAH331 (kindly provided by Dr. D.A. Hodgson); previously linearised with ClaI and dephosphorylated. The ligation reaction was used to transform competent E. coli ED8812 cells and transformants selected on NB medium supplemented with Kn ($50\mu\text{g ml}^{-1}$), X-gal ($40\mu\text{g ml}^{-1}$) and IPTG (1mM). Four resulting white recombinant colonies were streak-purified on the same selective medium, and plasmid DNA extracted on a small-scale. Restriction endonuclease analysis with SmaI and PstI verified that the subcloning had been successful, and determined the orientation of the ansB gene. Two suitable plasmids containing the ansB gene in opposite

4. L-asparaginase II overexpression

Figure 4.4 Construction of the *ansB* expression vector pCK2 Mob#1 and pCK2 Mob#2



A portion of the polylinker region of pDAH331 containing the relevant restriction sites used for the construction of pCK2Mob#1 and pCK2Mob#2 is shown. The structure of the pIC-19H cloning vector is described by Marsh *et al.* (1984); (see addendum on page 431).

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orientations were retained for the large-scale preparation of DNA and designated pCK2 #1 and #2, respectively.

The final stage of the construction involved restricting plasmid pSUP5011 with BamHI to release a 1.8 kb fragment containing the Mob region from RP4. Following gel purification, the Mob fragment was ligated with pCK2 #1 and #2, previously digested with BamHI and dephosphorylated. Ligation products were used to transform competent E. coli ED8812 cells and transformants plated onto NB selective medium, as described above. Plasmid DNA was extracted on a small-scale from six streak-purified Kn^r candidates, and subjected to restriction endonuclease analysis to verify the insertion of the Mob region. One suitable #1 and #2 Mob chimaera was retained for the large-scale preparation of plasmid DNA and termed pCK2 Mob#1 and pCK2 Mob#2, respectively.

4.3.4 Isolation of Er. chrysanthemi NCPPB 1066 pCK2 Mob#1 and Mob#2 recombinant derivatives

Plasmids pCK2 Mob#1 and Mob#2 were used to transform competent E. coli S17-1 cells, and transformants selected onto NB medium containing Kn (50ug ml^{-1}). S17-1 bacteria, harbouring pCK2 Mob#1 and Mob#2 were streak-purified on the same selective medium, and then patch-mated with wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2 at 30°C , as described in Section 2.2.5. Transconjugants were selected on minimal sucrose medium plus Kn (50ug ml^{-1}). As a comparison, and to gain an indication of whether the transfer system was operative, a control reaction was run in parallel in which E. coli S17-1 (pCK2 Mob#1 and pCK2 Mob#2) was patch-mated with E. coli strain ET8000 (Nm^r) at 30°C . In this case, transconjugants were selected on NB medium plus Nm (50ug ml^{-1}) and Kn

4. L-asparaginase II over-expression

(50ug ml⁻¹).

Following a 2 day incubation, the ET8000 mating gave rise to confluent growth on selective medium, indicating that conjugal transfer of pCK2 Mob#1 and pCK2 Mob #2 had taken place efficiently in E. coli. In contrast, the transfer of pCK2 Mob#1 and pCK2 Mob#2 into wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2 was found to be drastically lower (1-2 X 10⁻⁸ per recipient), and in fact, no transconjugants were isolated from S17-1 (pCK2 Mob#1) and industrial morphotype 2 matings.

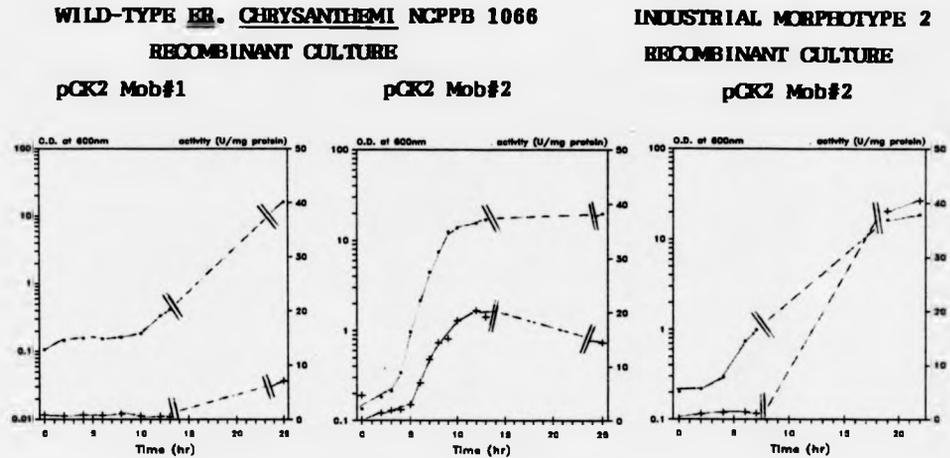
Attempts at improving the low transfer frequency met with no success. Matings were performed at both 30°C and 37°C with a range of donor to recipient ratios. In addition, cells were grown to differing stages of the growth cycle and patch-mated on nitrocellulose filters, as well as on the surface of solid medium. Despite this, the number of recombinant strains obtained, were subsequently studied in small-scale fermentation.

4.3.5 Small-scale fermentation analysis of pCK2 Mob#1 and pCK2 Mob#2 recombinants

Wild-type Er. chrysanthemi NCPPB 1066 pCK2 Mob#1 and pCK2 Mob#2 recombinants, along with the industrial morphotype 2 pCK2 Mob#2 recombinant, were cultivated batch-wise in LH 2000 series bioreactors under the same conditions as described in Section 4.2.5. In this case, however, the growth medium was supplemented with kanamycin at 50 ug ml⁻¹. The resulting small-scale fermentation profiles are shown in Figure 4.5, where it can be seen that for wild-type Er. chrysanthemi NCPPB 1066 pCK2 Mob#1 and morphotype 2 pCK2 Mob#2 recombinant cultures, growth was very slow compared to the wild-type Er. chrysanthemi NCPPB 1066 pCK2 Mob#2 culture. Despite this, reasonable biomass was attained at the end of the

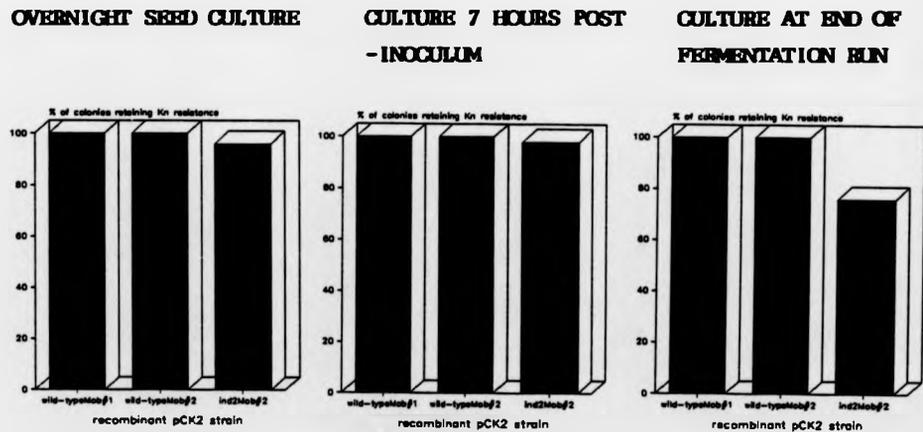
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Figure 4.5 Fermentation profiles for pCK2 Mob#1 and pCK2 Mob#2 recombinant strains



The optical density of the culture and L-asparaginase II activity are represented by dotted and crossed lines, respectively. These have been broken by parallel lines where sampling stopped and resumed.

Figure 4.6 Maintenance of kanamycin-resistance in pCK2 Mob#1 and pCK2 Mob#2 recombinant isolates at various stages of fermentation



4. L-asparaginase II over-expression

fermentation runs.

Although pCK2 Mob#1 and pCK2 Mob#2 recombinant derivatives showed good plasmid stability (Figure 4.6) on the basis of retention of the plasmid-encoded Kn-resistance marker, in general, they disappointingly produced lower levels of L-asparaginase II enzyme, compared with some of the pCK1 derivatives discussed earlier. In the case of wild-type Er. chrysanthemi NCPPB 1066 pCK2 Mob#2 recombinant culture, the maximum level of L-asparaginase II recorded was 20.4 IU mg protein⁻¹. Interestingly, drastically lower levels were seen for the wild-type Er. chrysanthemi NCPPB 1066 pCK2 Mob#1 culture (7.0 IU mg protein⁻¹). It is therefore a formal possibility that structural instability of pCK2 Mob#1 may have occurred, leading to reduced expression of the ansB structural gene.

On this occasion, the highest levels of L-asparaginase II were detected in cells of the morphotype 2 pCK2 Mob#2 culture, which displayed 2-fold higher levels (40.5 IU mg protein⁻¹) than the faster-growing wild-type equivalent. This compares well with the value of 26.0 IU mg protein⁻¹ recorded by Gilbert and coworkers (1986) for pASN326 maintained within Er. carotovora subsp. carotovora SCRI 193.

The basic trends seen in this study were repeatable in subsequent small-scale fermentation carried out at C.A.M.R. (Dr. D. Cossar; personal communication). On these occasions, morphotype 2 pCK2 Mob#2 recombinant cultures yielded maximum enzyme specific activities between 40 and 55 IU mg protein⁻¹, with an apparent plasmid stability of 70 to 80% at the end of the runs. The wild-type Er. chrysanthemi NCPPB 1066 pCK2 Mob#2 recombinant gave values of 17 to 20 IU mg protein⁻¹, and retained the Kn-resistance marker in 100% of the population tested. Finally, wild-type Er. chrysanthemi NCPPB 1066 pCK2 Mob#1 recombinant cultures grew at a

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slower rate, and yielded a maximum L-asparaginase II specific activity level of 9.0 IU mg protein⁻¹. Again, 100% of the colonies tested, retained the Kn-resistance marker.

4.4 FINAL COMMENTS

This study has shown that the problem of introducing the Er. chrysanthemi NCPPB 1066 ansB structural gene into Er. chrysanthemi NCPPB 1066 itself, can be solved using conjugal mobilisation. In this respect, two conjugative systems were analysed with Er. chrysanthemi NCPPB 1066. The first involved the conjugative 'helper' plasmids, pR64drd11 and pLVC9, and was found to elicit transfer of the ansB expression vector, pCK1, much more efficiently than the second system, which involved the interaction of trans-acting mobilisation functions encoded by a modified RP4 plasmid intergrated into the bacterial genome, with a cloned cis-acting Mob region of RP4.

Subsequent to the work described in this thesis, Parke (1990) constructed a series of mobilisable high copy number narrow-host-range plasmids, based on pUC18 and pUC19 (Yanisch-Perron et al., 1985), using a similar strategy to that described above. In this case, a segment of pSUP2021 (Simon et al., 1983 b) bearing the oriT/bom site of RP4 was used. Derivatives carrying both Ap and Kn-resistance were introduced efficiently into both Agrobacterium tumefaciens and Enterobacter aerogenes, and may prove useful for future studies with Er. chrysanthemi NCPPB 1066.

In this particular study, the presence of multiple copies of the ansB structural gene was found to increase the levels of L-asparaginase II enzyme within Er. chrysanthemi NCPPB 1066. Furthermore, results implied that, certainly under rich growth conditions, there was no marked titration of regulatory factors involved in ansB expression, since the pattern of enzyme

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activity detected throughout the growth phase followed a similar course in both recombinant derivatives and the non-recombinant parental isolates.

It is clear from this and other work, that a high copy number plasmid may not be beneficial for improving L-asparaginase II yields. One might envisage that a 'plateau' of activity is reached, resulting from the effect of gene dosage being counteracted by the increasing metabolic load on the cell. A lower copy number vector (<40 copies per cell), or a single chromosomal copy, where the ansB controlling regions have been replaced by a more amenable promoter system, would therefore be advisable. Results presented in Chapter 7 of this thesis have shown that the construction of the latter strain by exchange-recombination, is a real possibility. Today, a wide selection of strong, regulatable promoters now exists for the large-scale production of proteins (Georgiou, 1988; Glick and Whitney, 1987). Examples include the repressible trp (Somerville, 1988), and phoA (Oka et al., 1985) promoters; as well as the inducible lacUV5 (Fuller, 1982), tac (Amman et al., 1983; de Boer et al., 1983) and Lambda P_L (Mieschendahl et al., 1986; Remaut et al., 1981, 1987) promoters.

Finally, it must be stressed that the ansB expression vectors constructed in this work were simply designed to address fundamental questions. Clearly superior vectors can now be constructed and introduced into Er. chrysanthemi NCPPB 1066 by the methods described above. It was thought better to leave this task to the expertise at C.A.M.R., so that work of a more academic nature could be pursued.

CHAPTER FIVE

GENOMIC LIBRARY CONSTRUCTION AND AN INVESTIGATION
OF THE ROLE OF THE NTR SYSTEM IN THE REGULATION OF
L-ASPARAGINASE II SYNTHESIS IN ERWINIA CHRYSANTHEMI

NCPPB 1066

5. Library Construction and The Ntr System

5.1 Introduction

Following the identification of a putative -24/-12 sequence within the 5' non-coding region of the Er. chrysanthemi NCPPB 1066 ansB gene, preliminary experiments were carried out at C.A.M.R. in which the nifA structural gene from K. pneumoniae was supplied in trans to E. coli harbouring a plasmid carrying the Er. chrysanthemi NCPPB 1066 ansB structural gene. Minton and colleagues (1986) reported that the expression of the ansB structural gene was increased ten-fold by the presence of the nifA gene in multicopy. Moreover, the use of various E. coli mutant strains had apparently shown that the observed activation was rpoN-dependent.

The nifA plasmid used in the experiments described above was pMC71A (Dr. N. Minton; personal communication) which had been originally constructed by Buchanan-Wollaston et al. (1981) by subcloning a Sall restriction fragment into a Sall site of the cloning vector pACYC184 (Chang and Cohen, 1978), such that the nifA gene is constitutively expressed from the promoter of the tetracycline-resistance gene.

Studies by a number of workers have shown that the nifA product can substitute for the ntrC product in the activation of a number of Ntr-regulated genes including the nifLA promoter (Drummond et al., 1983; Ow and Ausubel, 1983), the glnA promoter (Ow and Ausubel, 1983) and the promoters of genes involved in the utilisation of histidine, proline and arginine (Merrick, 1983; Ow and Ausubel, 1983), when it is expressed constitutively in multicopy. Therefore, the observations reported by Minton et al. (1986) raised the possibility that the components of the Ntr regulatory system could act directly on the putative -24/-12 promoter of the Er. chrysanthemi ansB structural gene. However, they were somewhat surprising

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due to the fact that ammonia does not appear to regulate L-asparaginase II synthesis in Er. chrysanthemi NCPPB 1066. Consequently, Minton and colleagues (1986) put forward the proposal that this particular bacterium possibly did not contain the regulatory (ntr) genes necessary for the activation of such a promoter. These authors further speculated that Erwinia perhaps evolved from bacteria which did possess ntr genes, or possibly the ansB gene had been acquired from a member of the Enterobacteriaceae which could regulate -24/-12 promoters.

5.2 Aims

The work described in the latter sections of this chapter stemmed from the observations and proposals outlined above. The initial aim was to obtain direct physical evidence for the existence of ntr homologues within the Er. chrysanthemi NCPPB 1066 genome. If ntr homologues could be detected, then following their isolation, efforts would be directed towards determining whether they were functional. This would hopefully go some way to ruling out the suggestion of Minton et al. (1986) that this organism did not have the capacity to regulate -24/-12 promoters. Moreover, it was of interest to establish whether the introduction of such genes in trans in multicopy could influence the level of transcription from the Er. chrysanthemi NCPPB 1066 ansB promoter. Ideally these studies were to be performed in the wild-type Er. chrysanthemi NCPPB 1066 single copy ansB::lacZ transcriptional fusion derivative CK1002 (Section 7.5.3), rather than an E. coli host, since this would give a more authentic picture of the regulation of L-asparaginase II expression.

The initial sections of this chapter describe the construction of a total genomic library for wild-type Er. chrysanthemi NCPPB 1066 and experiments

5. Library Construction and The Ntr System

aimed at assessing its representation. This was subsequently to be used, not only for complementation studies, but also for the isolation of specific genes from this organism.

5.3 Construction of a wild-type Er. chrysanthemi NCPPB 1066 partial Sau3A total genomic library

Plasmids containing the cohesive end site (cos) of bacteriophage lambda are termed 'cosmids', and have been widely used as gene cloning vectors for the construction of both prokaryotic and eukaryotic total genomic libraries. The presence of the cos site enables recombinant cosmid molecules to be packaged into lambda particles in vitro, thereby allowing their introduction into a lambda-sensitive bacterium with high efficiency. Once inside the bacterium, the recombinant cosmid molecule behaves as an autonomous plasmid.

Because most cosmids are small (around 5 kb) and the maximum size of DNA that can be incorporated into lambda heads is 50 kb, it is possible to package recombinant cosmids containing upto 45 kb of chromosomal DNA. In contrast, plasmid vectors containing such large inserts are rarely isolated since their transformation efficiency is drastically reduced. Lambda cloning vectors themselves are relatively large and therefore can only accept DNA up to 20 kb in length (Grosveld and Dahl, 1983). As a consequence, the capacity to clone large chromosomal inserts into cosmids leads to the following advantages over plasmid and bacteriophage lambda cloning systems; firstly, fewer recombinants are required in order to cover the entire chromosome; secondly, there is a greater chance of isolating a number of closely linked genes, such as operons, on one recombinant molecule; and thirdly, it allows a more direct route to isolate overlapping clones containing

5. Library Construction and The Ntr System

the entire chromosomal region between linked genes. Furthermore, the size selectivity of the in vitro packaging process ensures that only cosmids containing inserts of greater than 30 kb are obtained and that the background of non-recombinant cosmids is low (Hohn and Collins, 1988).

With these points in mind, the 6 kb cosmid, pH79, was chosen as the vector for the construction of a genomic library of Er. chrysanthemi NCPPB 1066. This is a pBR322 derivative, harbouring genes that encode ampicillin and tetracycline-resistance, as well as several unique cloning sites for the introduction of 'foreign' chromosomal DNA. Furthermore, although it had been reported that pH79 cannot be mobilised (Pouwels et al., 1985), evidence from this laboratory suggested that this particular cosmid could, in fact be conjugally mobilised into Erwinia using the 'helper' plasmids pR64drd11 and pLVC9 (Dr. D. Whitcombe; personal communication).

5.3.1 Cloning strategy

Since the aim of this work was to construct a total genomic library, it was desirable that the starting collection of clonable Er. chrysanthemi NCPPB 1066 genomic fragments contained every sequence of the genome. This could be best achieved by fragmenting the chromosome in an entirely random manner. In order to obtain the most random genomic fragments, DNA would be partially digested with a restriction endonuclease that recognises a four base pair nucleotide sequence, rather than a six base pair sequence, since the former would occur more frequently within the genome (every 256 bp), compared with the latter (every 4096 bp). In this instance, genomic DNA would be digested with the restriction endonuclease, Sau3A, which recognises the nucleotide sequence GATC. The resulting restriction products would then be size-fractionated before being cloned into the compatible BamHI site of

5. Library Construction and The Ntr System

pHC79. Purification of DNA fragments in the size range 25 to 45 kb would hopefully increase the efficiency of the in vitro packaging reaction, whilst reducing the risk of cloning either several small non-contiguous fragments, or larger non-contiguous fragments caused by deletion events, into the same vector.

5.3.2 Results

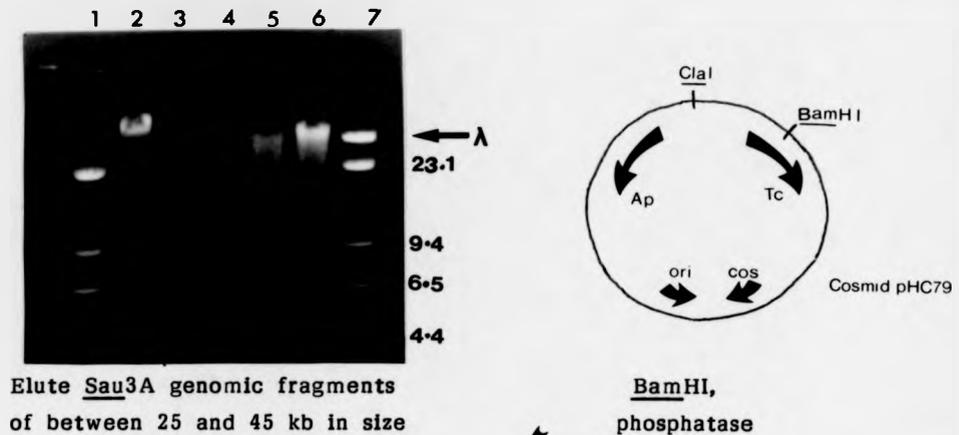
In order to determine the optimum conditions for creating random restriction fragments between 25 and 45 kb in size, chromosomal DNA was isolated from wild-type Er. chrysanthemi NCPPB 1066 as described in Section 2.3.3, and 1 μ g restricted with a range of Sau3A concentrations at 37°C for 1 hour. From the result of this preliminary experiment (Figure 5.1), a large-scale reaction employing 10 μ g of chromosomal DNA was performed with Sau3A at a concentration of 0.03 units μ g DNA⁻¹, and restriction products size-fractionated on a preparative 0.4% (w/v) TAE agarose gel. Fragments between 25 and 45 kb were purified by electroelution and ligated with cosmid pHC79 previously cut with BamHI and dephosphorylated, as outlined in Section 2.3.12. (Figure 5.1). The ligation mixture was in vitro packaged into bacteriophage lambda particles (Section 2.3.15) and 1/150th of the reaction used to transduce E. coli DH1 cells. Following a 36 hour incubation at 37°C, 198 transducant colonies were detected on NB medium supplemented with Ap (50 μ g ml⁻¹). In contrast, no colonies were detected with an uninfected or lysate alone control. It was therefore calculated that the in vitro packaged library represented close to 30,000 recombinants in total.

Cosmid DNA was extracted on a small-scale from 12 independent Ap^F colonies of varying size, and cleaved with the restriction endonuclease EcoR1. Agarose gel electrophoresis of the restriction products revealed that all

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Figure 5.1 Construction of an *Er. chrysanthemi* NCPPB 1066 partial Sau3A genomic library

A) TIME COURSE EXPERIMENT



Mix and ligate

In vitro package

Transduce *E. coli* DH1

Amplify

A) Intact chromosomal DNA was restricted with various concentrations of Sau3A as indicated in the text, and size-fractionated by 0.4% (w/v) TAE agarose gel electrophoresis. The running order is as follows : Track 1: lambda HindIII marker; Track 2: uncut chromosomal DNA; Track 3: 0.125 units of Sau3A $\mu\text{g DNA}^{-1}$; Track 4: 0.063 units of Sau3A $\mu\text{g DNA}^{-1}$; Track 5: 0.031 units of Sau3A $\mu\text{g DNA}^{-1}$; Track 6: 0.016 units of Sau3A $\mu\text{g DNA}^{-1}$; Track 7: lambda HindIII marker (linearised lambda DNA (49 kb) marker is indicated by an arrow).

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clones contained inserted DNA fragments averaging 35 kb in length; each possessing a unique restriction pattern. If one considers the size of the Er. chrysanthemi NCPPB 1066 genome to be approximately the same as that of E. coli, that is 4,700 kb (Bachman, 1990), then it was estimated that approximately 600 members of the gene bank would be needed to guarantee a 99% chance that a given sequence of DNA would be represented (Clarke and Carbon, 1976).

5.3.3 Amplification of the library

E. coli DH1 was infected with unamplified in vitro packaged cosmid library, and transductants selected on LB medium supplemented with Ap (50ug ml⁻¹). Following 24 hour incubation at 37°C, approximately 3000 Ap^r colonies were harvested into 25ml of LB medium, and cells concentrated five fold in a Labor 50M bench centrifuge (4,500rpm, 10 mins). A portion of this suspension (0.5ml) was sub-cultured into 25ml of prewarmed LB medium containing 0.2% (w/v) maltose and 10mM MgSO₄, and growth allowed to proceed at 30°C (250rpm) until an OD₅₅₀ value of 0.5 was reached. A 100ul sample of a high-titre lysate of lambda cl₈₅₇ was then added to the culture, which was then placed in a 42°C water bath for 20 mins, and then transferred to a second water bath held at 38°C. Growth was allowed to proceed until lysis was observed (around 4 hours), at which point, chloroform (0.5ml) was added and incubation continued for 15 mins to complete the process. Un-lysed cells and debris were removed by centrifugation in an MSE Chilspin (5000rpm, 15 mins, 4°C), and the resulting supernatant lysate decanted and stored at 4°C over chloroform.

5. Library Construction and The Ntr System

5.4 Library representation

In order to assess the representation of the constructed library, individual transductants were examined for the presence of specific genes of Er. chrysanthemi NCPPB 1066 encoding recA, cellulase, protease and pectolytic extracellular enzymes.

5.4.1 Isolation of the recA structural gene from wild-type Er. chrysanthemi NCPPB 1066

The construction of a recA⁻ derivative of Er. chrysanthemi NCPPB 1066 would undoubtedly be useful for facilitating the genetic analysis of genes and their protein products within this strain. For example, the inhibition of DNA repair in recA⁻ mutants (Little and Mount, 1983; Walker, 1984; Witkin, 1976) can be utilised in the development of the maxicell technique for identification of the protein products of cloned genes in E. coli (Sancar et al., 1979). Moreover, as the RecA protein also plays a central role in the cellular process of homologous recombination (Dressler and Potter, 1982; Radding, 1982), a recA⁻ deficient background allows the determination of the number and location of genes by complementation and F-prime analysis, and is essential for the stabilisation of repetitive foreign DNA molecules.

Clearly, a first stage towards obtaining a recA⁻ derivative would be the isolation of the Er. chrysanthemi NCPPB 1066 recA structural gene. A number of reports concerning the molecular cloning of the recA gene from a variety of Erwinia strains, have appeared in the literature (Faelen et al., 1985; Keener et al., 1984; Norelli et al., 1985; Zink et al., 1985). In these instances, recombinant recA clones were isolated which were able to complement a recA⁻ mutation of E. coli K12 by restoring resistance to DNA

5. Library Construction and The Ntr System

damaging agents such as UV. light and the mutagens, 4-nitroquinoline-1-oxide and methylmethanesulphonate (MMS). Erwinia recA⁻ derivatives were then constructed by exchange-recombination, whereby the wild-type chromosomal recA allele was replaced by the cloned recA structural gene that had been inactivated by insertional mutagenesis (Faelen et al., 1985; Zink et al., 1985).

5.4.1.1 Approach

Studies by Jenkins and Bennett (1976) have demonstrated that it is possible to distinguish between recA⁺ and recA⁻ bacterial strains using the observed sensitivity of the latter to nitrofurantoin (Nft). Members of the nitrofurantoin group of compounds are reduced in vivo to yield active antibiotics (McCalla et al., 1970) which have been shown to bind nucleic acid and are known to be mutagenic (Tazima et al., 1975). It was therefore anticipated that complementation of the recA⁻ mutation of E. coli strain DH1 by cosmids containing the Er. chrysanthemi NCPPB 1066 recA structural gene would lead to an increased resistance to nitrofurantoin.

5.4.1.2 Results

A 100ul sample of the amplified wild-type Er. chrysanthemi NCPPB 1066 cosmid library was used to infect E. coli DH1 cells and transductants selected on NB medium containing Ap (50ug ml⁻¹). Following a 24 hour incubation at 37°C, two hundred Ap^r colonies were replicated onto NB medium containing both Ap (50ug ml⁻¹) and Nft (10ug ml⁻¹). The concentration of the latter compound was used because it has been found to be effective at overcoming the artificial resistance that sometimes arises due to the high cell density within the patches. E. coli DH1 and Q358 were run

5. Library Construction and The Ntr System

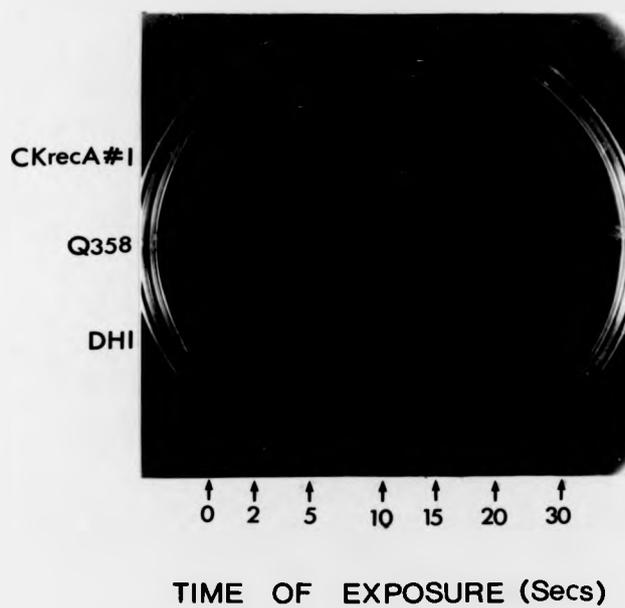
in parallel on NB medium supplemented with Nft (10ug ml^{-1}), as negative and positive recA controls, respectively. Following a 36 hour incubation at 37°C , one Ap^{r} candidate displaying resistance to Nft was detected. Cosmid DNA was subsequently recovered on a small-scale from this isolate and used to transform competent E. coli DH1 cells. Transformants were spread-plated onto NB medium containing Ap (50ug ml^{-1}) and Nft (5ug ml^{-1}) and incubated for 36 hours at 37°C . Again, Ap^{r} and Nft^{r} cells were detected. In contrast, no Nft^{r} colonies arose from untransformed control E. coli DH1 cells plated onto NB medium containing Nft (5ug ml^{-1}) alone. One of the resulting E. coli DH1 Ap^{r} Nft^{r} transformants, designated CK recA#1, was streak-purified on the same selective medium and subjected to a UV. plate sensitivity test on two separate occasions, as described in Section 2.2.18. In each case, CK recA#1 was found to be more UV. resistant than E. coli DH1, but less compared with the wild-type E. coli recA⁺ strain Q358, as illustrated in Figure 5.2.

5.4.1.3 Comments

Although CK recA#1 was not studied further, the above evidence suggested that some degree of complementation of the E. coli DH1 recA⁻ mutation had been achieved. Hence, it is likely that the cosmid clone maintained within this isolate contains part, or all, of the Er. chrysanthemi NCPPB 1066 recA structural gene, and will perhaps prove useful for future studies aimed at constructing a recA⁻ derivative of Er. chrysanthemi NCPPB 1066.

5. Library construction and the Ntr system

Figure 5.2 UV. plate sensitivity test for CKrecA#1



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5.4.2 Isolation of extracellular enzyme structural genes from wild-type Er. chrysanthemi NCPPB 1066

To date, structural genes encoding extracellular pectinase (Pel), cellulase (Cel) and protease (Prt) enzymes have been cloned from a wide variety of Erwinia strains. This has included pel genes from Er. chrysanthemi (Barras and Chatterjee, 1987; Barras et al., 1987; Collmer et al., 1985; Keen et al., 1984; Kotoujanski et al., 1985; Reverchon et al., 1985,1986; Van Gijsegem et al., 1985 b) and Er. carotovora subsp. carotovora (Lei et al., 1985; Nikaido et al., 1985; Plastow et al., 1986; Roberts et al., 1986; Zink et al., 1985); cel genes from Er. chrysanthemi strains 3665 (Barras et al., 1984; Boyer, 1987 a,b); 3937 (Kotoujanski et al., 1985), and Er. carotovora subsp. carotovora EC14 (Allen et al., 1985); and prt genes from Er. chrysanthemi strains B374 (Wandersman et al., 1987), and EC16 (Barras et al., 1986 b).

5.4.2.1 Approach

In many instances, it has been possible to isolate plasmids or cosmids harbouring Erwinia extracellular enzyme structural genes directly from E. coli, by either immunoblotting, or plating recombinant cells directly onto enzyme detection medium. Er. chrysanthemi is known to synthesise and secrete three antigenically-related metalloproteases, designated PrtA, B and C (Delepelaire and Wandersman, 1989) by a Sec-independent pathway (He et al., 1991 a). The corresponding structural genes (prtA, B and C) reside within a 40kb genomic region, with prtB and C being clustered together on a 8.8 kb EcoRI fragment. When present in E. coli, the latter two genes are found to be expressed; however, their products are not secreted (Wandersman et al., 1988). This process only takes place when the structural genes that encode the specific Prt secretion functions are also present within the same cell.

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Such functions are encoded by three co-transcribed genes, namely, prtD, E and F, present on a 5.5 kb region which lies adjacent to the prtB structural gene. The gene products of prtD, E and F have been found to share significant homology to the E. coli proteins HylB, HylD and TolC, respectively (Letoffe et al., 1990; Wandersman and Delepelaire, 1990). Therefore, evidence suggests that the secretion system in Er. chrysanthemi is homologous to the E. coli alpha-haemolysin secretion pathway, and recent studies by Guzzo et al. (1991) have reinforced this view with the finding that PrtD, E and F can mediate the secretion of alkaline metalloprotease of P. aeruginosa, as can HylB, HylD and TolC.

In contrast to the Prt secretory pathway described above, plant cell wall degrading enzymes such as cellulase, exo-poly α -D-galacturonidase, pectin methylesterase and at least four of the pectate lyase isoenzymes are secreted into the extracellular environment by Er. chrysanthemi by what now appears to be an extension of the Sec pathway for general protein export. This begins with Sec-mediated translocation across the inner membrane, with subsequent export requiring specific secretory functions which comprise the so-called OUT secretory system (He et al., 1991 a,b). Because E. coli lacks an analogous OUT system, Pel and Cel enzymes can be expressed, but their products are only exported to the periplasmic space by the Sec pathway and no further. However, even in the absence of the OUT system functions in E. coli, Pel and Cel activity may still be detected on the appropriate assay medium, since enzyme can be released into the extracellular environment due to non-specific leakage or autolysis of E. coli cells.

5.4.2.2 Results

A 100ul sample of the amplified wild-type Er. chrysanthemi NCPPB

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1066 cosmid library was used to infect E. coli DH1 cells, and transductants plated onto non-destructive Prt assay medium containing Ap (50ug ml⁻¹). Following a 2 day incubation at 30°C, four Ap^r, Prt⁺ colonies displaying opaque halos were detected. These were streak-purified to single colonies and re-tested on destructive protease medium containing Ap (50ug ml⁻¹), where halos were clearer and thus more distinct. Transductants were also replicated onto Pel and Cel assay media at the same time. E. coli DH1 and Er. chrysanthemi NCPPB 1066 were run in parallel on extracellular enzyme detection media lacking antibiotic, as negative and positive enzyme controls, respectively. In all cases, the Prt⁺ candidates gave clear halos on destructive Prt medium, but did not display Pel or Cel activity, thereby ruling out the possibility of Erwinia contamination.

In addition, 100 transductants from the non-destructive protease plates were replicated onto Pel, Cel, destructive Prt, and NB medium supplemented with Ap (50ug ml⁻¹) at 30°C. Development of the assay medium after 2 days clearly revealed two recombinants displaying Pel activity and a further two which were positive for Cel activity. A further Prt⁺ clone was also discovered. All isolates were re-checked on Pel, Cel and destructive Prt medium, alongside the aforementioned controls. The Pel⁺ candidates were found to be Cel⁻ and Prt⁻, whilst the Cel⁺ candidates were Pel⁻ and Prt⁻. The fifth Prt⁺ candidate again displayed no pel or cel activity.

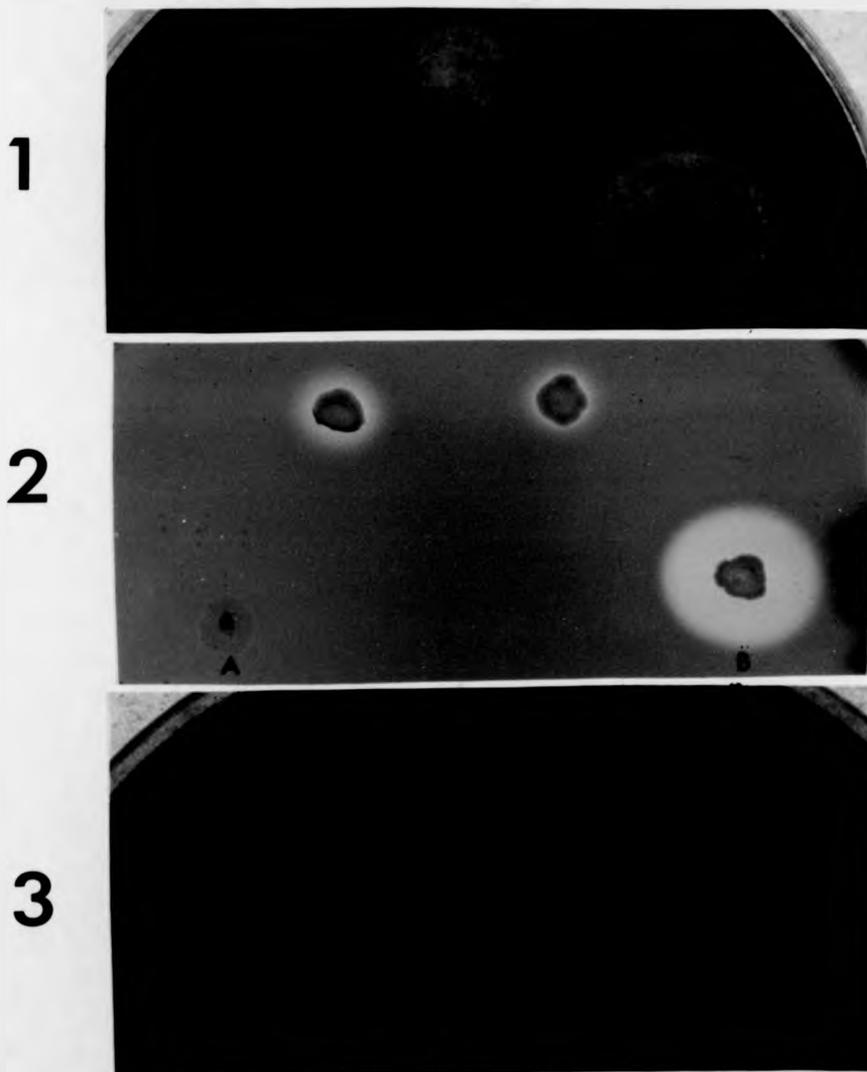
These results strongly implied that cosmid clones harbouring Er. chrysanthemi NCPPB 1066 extracellular pel, cel and prt structural genes had been isolated and were capable of being expressed in E. coli. Representative E. coli DH1 recombinant Pel⁺, Cel⁺ and Prt⁺ colonies are shown in Figure 5.3.

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Figure 5.3 *E. coli* DH1 cells harbouring cloned *Er. chrysanthemi* NCPPB 1066 extracellular enzyme structural genes.

Recombinant bacteria were cultivated on the appropriate extracellular enzyme detection medium at 30°C. Plates were developed as outlined in Section 2.2.19, except for the cellulase medium which was stained with Congo red only. Plate 1, cellulase medium; Plate 2, protease medium; Plate 3, pectinase medium.

Controls are: A) *E. coli* DH1 B) wild-type *Er. chrysanthemi* NCPPB 1066.



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5.4.2.3 Comments

From the experiments described above, it was concluded that the constructed total genomic library was representative of the Er. chrysanthemi NCPPB 1066 chromosome. This would now be used for complementation studies and for the isolation of additional genes from this organism.

5.5 CLONING AND CHARACTERISATION OF THE ER. CHRYSANTHEMI NCPPB 1066 rpoN STRUCTURAL GENE

5.5.1 Approach

The structural gene encoding sigma 54 (rpoN) has been cloned from a variety of bacterial genera, including E. coli (Hunt and Magasanik, 1985), K. pneumoniae (Merrick and Gibbins, 1985), A. vinelandii (Merrick et al., 1987), Rhizobium meliloti (Ronson et al., 1987), Rhizobium sp. strain NGR234 (Stanley et al., 1989), Rhodobacter capsulatus (Alias et al., 1989; Jones and Haselkorn, 1989), P. putida (Inouye et al., 1989; Kohler et al., 1989), P. facilis (Romermann et al., 1989), Alcaligenes eutrophus (Romermann et al., 1989), and Thiobacillus ferrooxidans (Berger et al., 1990). Due to the availability of these cloned genes for use as probes, a heterologous hybridisation approach was employed in order to attempt to identify and isolate the rpoN structural gene from Er. chrysanthemi NCPPB 1066.

5.5.2 Heterologous hybridisation studies using cloned K. pneumoniae and A. vinelandii rpoN gene probes

The first stage of this work was to ascertain whether the chromosome of Er. chrysanthemi NCPPB 1066 contained any sequences that displayed homology with either the rpoN structural gene of K. pneumoniae or A.

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vinelandii. To this end, Southern analysis of genomic DNA isolated from both wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2 was carried out, using the 1.9 kb ClaI K. pneumoniae rpoN fragment from plasmid pMM17, as a probe. In this instance, K. pneumoniae genomic DNA and cosmid pH79 were run in parallel as positive and negative hybridisation controls, respectively.

The results are presented in Figure 5.4, where it can be seen that the resulting autoradiograph revealed the presence of a number of chromosomal restriction fragments that hybridised with the Klebsiella rpoN probe. Moreover, positive fragments were identical for both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotype 2. The most notable of these from a cloning standpoint, was a single hybridising ClaI fragment of approximately 2.4 kb in size.

In addition to the above, Southern analysis was also carried out on wild-type Er. chrysanthemi NCPPB 1066 genomic DNA, using a 2.0 kb EcoRI/HindIII A. vinelandii rpoN fragment isolated from plasmid pAT705, as a probe. The result of this experiment is shown in Figure 5.5, where it can be seen that restriction fragments which hybridised to the K. pneumoniae rpoN probe, also hybridised to the A. vinelandii probe. Again, a single ClaI fragment of 2.4 kb was detected amongst these.

5.5.3 Isolation of the Er. chrysanthemi NCPPB 1066 rpoN structural gene

5.5.3.1 Approach

Following Southern analysis of chromosomal DNA from wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2, attempts were made to isolate the 2.4 kb ClaI chromosomal fragment which hybridised with both

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Figure 5.4 Identification of wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype 2 genomic sequences exhibiting homology with a *K. pneumoniae* *ropN* gene probe

Intact chromosomal DNA isolated from both wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype 2 was digested to completion with a range of restriction endonucleases and the products size-fractionated by 0.7 % (w/v) TAE agarose gel electrophoresis. DNA was transferred to a nylon membrane and probed with a ^{32}P -radiolabelled 1.9 kb *Cla*I fragment from plasmid pMM17 overnight at 65°C in 1 X 'Blotto'. The membrane was washed at 55°C in 2 X SSC for 1 hour and subjected to autoradiography at -70°C.

The running order is as follows :

- Track 1 : lambda DNA restricted with *Hind*III
- Track 2 : wild-type genomic DNA restricted with *Eco*RI
- Track 3 : wild-type genomic DNA restricted with *Sal*I
- Track 4 : wild-type genomic DNA restricted with *Bam*HI
- Track 5 : wild-type genomic DNA restricted with *Cla*I
- Track 6 : wild-type genomic DNA restricted with *Pst*I
- Track 7 : morphotype 2 genomic DNA restricted with *Eco*RI
- Track 8 : morphotype 2 genomic DNA restricted with *Sal*I
- Track 9 : morphotype 2 genomic DNA restricted with *Bam*HI
- Track 10 : morphotype 2 genomic DNA restricted with *Cla*I
- Track 11 : morphotype 2 genomic DNA restricted with *Pst*I
- Track 12 : *K. pneumoniae* genomic DNA restricted with *Cla*I
- Track 13 : no DNA
- Track 14 : pMM17 supercoil DNA
- Track 15 : lambda DNA restricted with *Hind*III

Tracks 16 to 29 are autoradiographs of corresponding tracks 1 to 14 probed with the 1.9 kb *ropN* *Cla*I fragment from pMM17.

Figure 5.4

23-1

9-4

6-5

4-4

2-3

2-0

16

23-1

9-4

6-5

4-4

2-3

2-0

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Figure 5.5 Identification of wild-type Er. chrysanthemi NCPPB 1066 genomic sequences exhibiting homology with an A. vinelandii rpcN gene probe

Intact chromosomal DNA isolated from wild-type Er. chrysanthemi NCPPB 1066 was digested to completion with a variety of restriction endonucleases and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. DNA was transferred to a nylon membrane and probed with a ³²P-radiolabelled 2.0 kb EcoRI/HindIII fragment from plasmid pAT705 overnight at 65°C in 1 X 'Blotto'. The membrane was washed at 55°C in 2 X SSC for 1 hour and then subjected to autoradiography at -70°C.

The running order is as follows :

- Track 1 : lambda DNA digested with HindIII
- Track 2 : wild-type Er. chrysanthemi NCPPB 1066 genomic DNA digested with EcoRI
- Track 3 : wild-type Er. chrysanthemi NCPPB 1066 genomic DNA digested with Sall
- Track 4 : wild-type Er. chrysanthemi NCPPB 1066 genomic DNA digested with GlaI
- Track 5 : wild-type Er. chrysanthemi NCPPB 1066 genomic DNA digested with KpnI
- Track 6 : wild-type Er. chrysanthemi NCPPB 1066 genomic DNA digested with PstI
- Track 7 : wild-type Er. chrysanthemi NCPPB 1066 genomic DNA digested with BamHI

Tracks 8 to 14 are autoradiographs of corresponding tracks 1 to 7 probed with the 2.0 kb EcoRI/HindIII fragment from pAT705.

Figure 5.5

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the K. pneumoniae and A. vinelandii rpoN gene probes. The cloning strategy involved the construction of a restricted gene library in the vector pBR322, and screening of the recombinants by heterologous hybridisation. This procedure would not only enrich for a region of interest, but in addition would permit its direct subcloning as a small fragment onto a multicopy vector.

5.5.3.2 Construction and screening of a restricted ClaI library

Intact chromosomal DNA from wild-type and industrial isolate 2 Er. chrysanthemi NCPPB 1066 was digested to completion with ClaI and size-fractionated through a 0.8 % (w/v) TAE agarose gel. Fragments between 2.0 and 3.0 kb were purified by electro-elution and ligated with pBR322, previously restricted with ClaI and dephosphorylated. Ligation products were subsequently introduced into competent E. coli DH1 cells by transformation and transformants selected on NB medium supplemented with Ap (50ug ml⁻¹). Following overnight incubation at 37°C, Grunstein analysis was performed in duplicate on 900 colonies resulting from each cloning reaction, as described in Section 2.3.19. E. coli DH1 alone was run in parallel as a control for antibiotic selection. Moreover, E. coli DH1 cells harbouring either pBR322 or pMM17 were included as negative and positive hybridisation controls, respectively.

DNA from a total of 9 recombinant E. coli DH1 colonies representing part of the Er. chrysanthemi NCPPB 1066 restricted library, and 5 recombinant E. coli colonies representing part of the industrial isolate 2 Er. chrysanthemi NCPPB 1066 restricted library, were found to clearly hybridise to the K. pneumoniae rpoN gene probe. One such positive clone is illustrated

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in Figure 5.6. All the positive candidates were subsequently streak-purified to single colonies on NB medium containing Ap (50ug ml⁻¹) for further analysis.

5.5.3.3 Physical analysis of positive clones

To verify the colony hybridisation results, plasmid DNA was isolated on a large-scale from all of the positive Ap^r *E. coli* DH1 recombinants, and subjected to dot-blot analysis, as described in Section 2.3.18, using the 1.9 kb *Clal* *K. pneumoniae* *rpoN* gene probe from pMM17. Supercoil pBR322 and pMM17 DNA was run in parallel as a negative and positive hybridisation control, respectively. As can be seen from Figure 5.7, all candidates were found to re-hybridise with the *Klebsiella* *rpoN* gene probe, thus reconfirming that the cloned *Erwinia* DNA did indeed have sequences displaying homology with the aforementioned gene.

Restriction endonuclease digestion of purified plasmid DNA with *Clal*, followed by size-fractionation of the products by 0.7% (w/v) TAE agarose gel electrophoresis, revealed that in all cases the pBR322 vector contained a single *Clal* insert of 2.4 kb. Further digests with *EcoRI*, *BamHI* and *Sall* restriction endonucleases indicated that the 2.4 kb *Clal* fragment isolated from both wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype 2 recombinant plasmids were identical, and had been cloned in both orientations. Two representative wild-type *Er. chrysanthemi* NCPPB 1066 and industrial morphotype 2 clones were retained for further analysis, and designated pCK3 and pCK4, or pCK5 and pCK6, respectively (Figure 5.8).

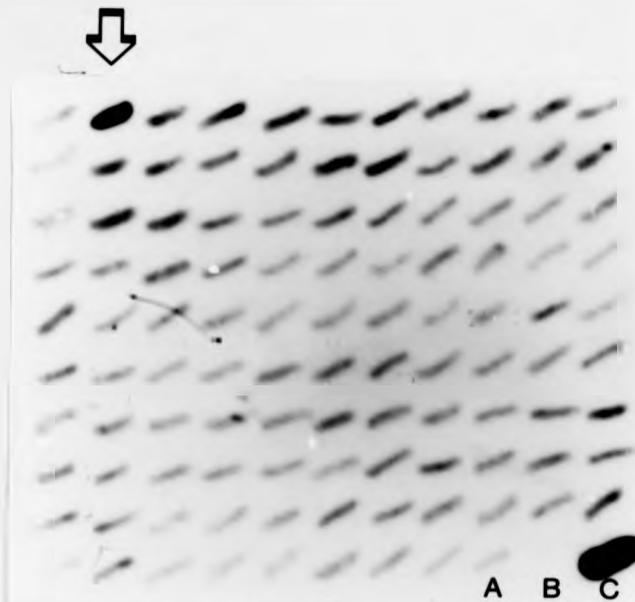
5.5.3.4 Complementation analysis of positive clones

Following on from the physical studies described above, the next stage of the work was aimed at ascertaining whether pCK3, pCK4, pCK5 and pCK6

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Figure 5.6 Grunstein analysis of E. coli DH1 recombinants harbouring a restricted ClaI genomic library of Er. chrysanthemi NCPPB 1066

Recombinant E. coli DH1 cells were transferred to nylon membrane resting on NB medium containing Ap ($50\mu\text{g ml}^{-1}$), and grown overnight at 37°C . DNA was released from bacteria, fixed, and then probed with a ^{32}P -radiolabelled 1.9 kb ClaI fragment from plasmid pMM17 at 65°C in 1 X 'Blotto' overnight. Membranes were then washed at 55°C in 2 X SSC for 1 hour and then subjected to autoradiography at -70°C . The result obtained from one of the membranes is shown below. The arrow highlights a clear positive detectable above background.



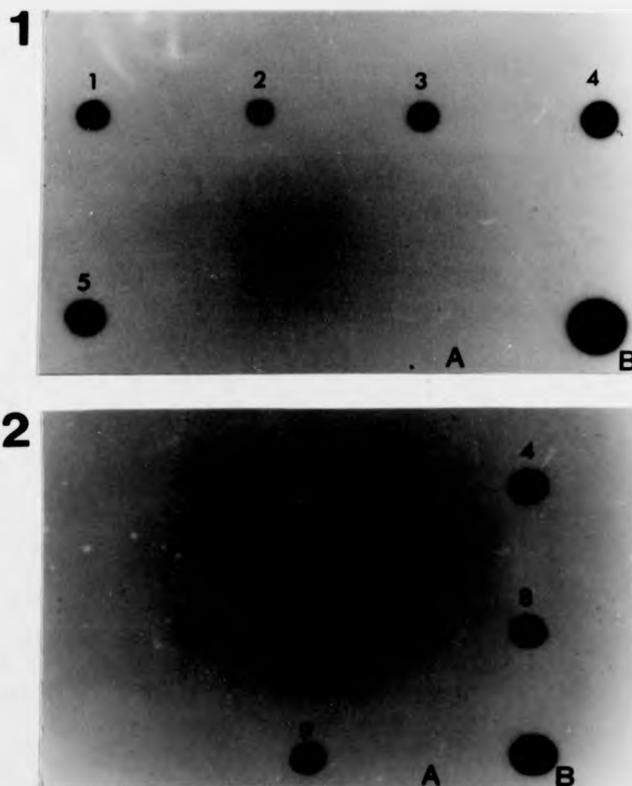
Key to controls:

- A) E. coli DH1 harbouring pBR322
- B) E. coli DH1 alone
- C) E. coli DH1 harbouring pMM17

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Figure 5.7 Dot blot analysis of wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2 pBR322::rpoN candidates

CsCl-purified supercoil plasmid DNA (2ul) was bound to a nylon membrane and probed with a ^{32}P -radiolabelled 1.9 kb ClaI fragment from plasmid pMM17 overnight at 65°C in 1 X 'Blotto'. Membranes were washed at 55°C in 2 X SSC for 1 hour and then subjected to autoradiography at -70°C. The results are shown below : Plate 1 represents wild-type Er. chrysanthemi NCPPB 1066 pBR322::rpoN candidates. Plate 2 represents industrial morphotype 2 Er. chrysanthemi NCPPB 1066 pBR322::rpoN candidates.



Key to controls: A) pBR322 supercoil DNA B) pMM17 supercoil DNA

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Figure 5.8 Restriction endonuclease analysis of rpoN complementing recombinant pBR322 clones pCK3, pCK4, pCK5 and pCK6

A) Plasmid DNA was digested to completion with a number of restriction endonucleases and the resulting DNA fragments size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis.

The running order is as follows :

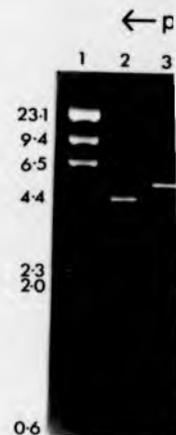
- Track 1 : lambda DNA digested with HindIII
- Track 2 : plasmid DNA digested with ClaI
- Track 3 : plasmid DNA digested with BamHI
- Track 4 : plasmid DNA digested with Sall
- Track 5 : plasmid DNA digested with EcoRI
- Track 6 : plasmid DNA digested with ClaI
- Track 7 : plasmid DNA digested with BamHI
- Track 8 : plasmid DNA digested with Sall
- Track 9 : plasmid DNA digested with EcoRI
- Track 10 : lambda DNA digested with HindIII

B) Restriction endonuclease map of the rpoN-complementing recombinant pBR322 clones pCK3, pCK4, pCK5 and pCK6.

Key to restriction sites : EI = EcoRI
CI = ClaI
BI = BamHI
SI = Sall

Figure 5.8

A)



B)

Recombinant p

Recombinant pl

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had the ability to complement the rpoN mutation of E. coli strain ET8045. Since a functional RpoN protein is required for the assimilation of the amino acids arginine (Aut⁺ phenotype), proline (Put⁺ phenotype) and histidine (Hut⁺ phenotype) by E. coli, complementation would be assessed by the ability of the recombinant clones to allow the growth of E. coli strain ET8045 on minimal medium containing one of the aforementioned amino acids as a sole source of nitrogen.

To this end, pCK3, pCK4, pCK5, and pCK6 DNA was introduced into competent E. coli ET8045 cells by transformation. Plasmids pBR322 and pMM17 were also included in this experiment as negative and positive rpoN controls, respectively. Transformants were washed in 1 X phosphate buffer to prevent the carry-over of nutrients, before being selected on minimal glucose medium supplemented with Ap (50ug ml⁻¹), Nm (50ug ml⁻¹) and 0.2% (w/v) arginine as sole nitrogen source. Untransformed ET8000 and ET8045 controls were plated in parallel on the same selective medium containing Nm (50ug ml⁻¹) alone. The results obtained after 2 days of incubation at 37°C are summarised in Table 5.1, and provided strong evidence to suggest that a functional Er. chrysanthemi NCPPB 1066 rpoN homologue had been expressed in E. coli. Moreover, the fact that complementation occurred with recombinant pBR322 plasmids with the ClaI insert in opposite orientations, strongly implied that the Er. chrysanthemi NCPPB 1066 rpoN homologue was being expressed from a regulatory region contained within the cloned DNA fragment and was not due to read-through from a foreign (vector) promoter.

A small number of transformant colonies were subsequently replicated from the minimal arginine plates onto minimal glucose medium containing Ap (50ug ml⁻¹), and either 0.2% (w/v) proline or 0.2% (w/v) histidine, as a sole nitrogen source. In all cases (12/12), transformants were able to grow, thus

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Table 5.1 Complementation analysis of recombinant pBR322 clones pCK3, pCK4, pCK5 and pCK6

<u>E. coli</u> strain	Sole nitrogen source for growth* 0.2% (w/v) arginine	Phenotype
ET8000	++	RpoN ⁺
ET8045	-	RpoN ⁻
ET8045(pBR322)	-	RpoN ⁻
ET8045(pMM17)	++	RpoN ⁺
ET8045(pCK3)	+	RpoN ⁺
ET8045(pCK4)	++	RpoN ⁺
ET8045(pCK5)	++	RpoN ⁺
ET8045(pCK6)	+	RpoN ⁺

* Growth was scored after 2 days incubation at 37°C.

Key : ++ Good growth (colonies 2 mm in diameter)

+ Growth (colonies 1 mm in diameter)

- No growth

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indicating that the Put⁻ and Hut⁻ phenotype of E. coli ET8045 had also been corrected. A repeated observation was that recombinant ET8045 colonies harbouring pCK4 and pCK5 were larger in diameter than corresponding pCK3 and pCK6 derivatives on all selective media. Such a size difference may have reflected an altered level of expression of the Er. chrysanthemi NCPPB 1066 rpoN gene, possibly due transcriptional read-through from other promoters such as the anti-tet promoter of pBR322. This phenomenon has also been reported for the K. pneumoniae rpoN structural gene cloned into the plasmid pACYC184 (Birkmann et al., 1987), as well as other genes inserted into pBR322 (Barry et al., 1979; Brosius et al., 1982; Emtage et al., 1989).

5.5.3.5 Comments

From the results of the work described in the preceding sections of this chapter, it was likely that the rpoN structural gene from both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotype 2 had been isolated by heterologous hybridisation. Although expression of the cloned gene was not relied upon for the initial isolation, further experiments demonstrated that a functional RpoN homologue was expressed in E. coli ET8045, and was capable of complementing the Aut⁻, Hut⁻, and Put⁻ phenotype of this particular strain. In order to determine whether the entire rpoN structural gene had been cloned, the nucleotide sequence of the 2.4 kb ClaI insert from pCK4 was determined.

5.6 NUCLEOTIDE SEQUENCE DETERMINATION OF THE 2.4 KB ClaI INSERT FROM pCK4

Approximately 120ug of supercoil pCK4 DNA was digested to completion with ClaI and the 2.4 kb insert isolated by electro-elution. A random

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population of blunt ended sub-fragments (300-900bp) were generated by the sonication procedure, as outlined in Section 2.3.21. Samples of sonicated DNA (0.5, 1 and 3ul) were then ligated with 20ng of M13mp8 DNA previously restricted with SmaI and dephosphorylated. Control ligation reactions were also performed in parallel and comprised of either SmaI dephosphorylated M13mp8 DNA alone, or SmaI M13mp8 DNA and bacteriophage lambda DNA previously digested with AluI. These were then used to transfect competent E. coli TG1 cells, along with 200pg of intact M13 DNA which acted as a transfection control.

Following overnight incubation at 37°C, approximately 160 colourless recombinant M13 plaques resulting from sonicated DNA ligation reactions were cored and single-stranded template prepared, as described in section 2.3.22.1. The dideoxy 'chain-termination' procedure was then used to sequence 96 templates as outlined in Section 2.3.22.2. The resulting sequence data, determined on both complementary strands, was subsequently compiled into one complete sequence of 2,388 bp using the programs of DNASTAR Incorporated. This is illustrated in Figure 5.9.

5.6.1 Features of the derived nucleotide sequence

It is evident from Figure 5.9 that the nucleotide sequence of the 2.4 kb chromosomal ClaI fragment from pCK4 displays extensive homology with the genomic region surrounding and including the rpoN structural gene of K. pneumoniae (Merrick and Gibbins, 1985; Merrick and Coppard, 1989). Translation of the derived nucleotide sequence in all possible reading frames, identified the presence of four putative open reading frames (ORFs) which were subsequently designated ORF A, B, C and D, respectively. ORFs A and D are considered to be incomplete, and are predicted to extend beyond the

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Clal site at either end of the sequence into the genome.

ORF A extends 204 bp from the most 5' nucleotide of the Er. chrysanthemi NCPPB 1066 sequence up to a TGA termination codon at nucleotide positions 205 to 207. ORF B is located immediately downstream of ORF A and constitutes the largest open reading frame. This extends from a typical ATG methionine start codon at nucleotides 256 to 258, and terminates 1430 bp downstream at a TGA stop codon at nucleotides 1686 to 1688. Analysis of the 48 bp intergenic region between ORF A and ORF B did not reveal any sequences that were characteristic of a rho-independent transcription terminator (GC-rich stem loop followed by several T residues), or any sequences that resembled '-10' or '-35' promoter consensus regions. A putative Shine-Dalgarno ribosome binding site (5' AAAGGAAG 3') was, however, identified 12 bp upstream of the ORF B start codon.

ORF C begins at an ATG initiation codon at positions 1714 to 1716 and terminates 284 bp downstream at an TGA stop codon at nucleotides 1999 to 2001. The non-coding region between ORF B and ORF C is 24 bp in length. Again, no rho-independent terminator or '-10' or '-35' promoter consensus sequence could be located within the non-coding region between ORF B and ORF C, although a stretch of nucleotides constituting a putative Shine-Dalgarno sequence (5' AAGGAAG 3') precede the ORF C start codon by 5 bp. Finally, ORF D covers 293 bp from an ATG start codon at nucleotides 2095 to 2097, and ends at the most 3' nucleotide of the derived sequence. The intergenic region between ORF C and ORF D is 93 bp in length. No rho-independent terminator, or a '-10' or '-35' promoter consensus sequence was apparent within this region. However, a sequence (5' AAGTGAA 3') showing some homology to a Shine-Dalgarno ribosome binding site is present 6 bp upstream of the translational start codon of ORF D.

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The % G+C content of the Er. chrysanthemi chromosome has been calculated to be between 55.7 and 57.1% (Lelliott and Dickey, 1984). In comparison, the predicted % G+C values for ORFs A, B, C and D are 48.5%, 54.9%, 47.4% and 56.8%, respectively.

5.6.2 ORFB is the rpoN structural gene of Er. chrysanthemi NCPPB 1066

The predicted polypeptide encoded by ORF B comprises of 477 amino acids with a calculated molecular weight of 53,776 Daltons. Alignment of the primary amino acid sequence of the ORFB polypeptide with the rpoN product of K. pneumoniae revealed extensive homology (in terms of identical amino acid residues), along the entire length of both proteins (84.7% identity over 477 amino acids; see Appendix, Figure A1). This is also the case for the RpoN protein of E. coli (84.3% identity over 477 amino acids; see Appendix, Figure A1), and to a lesser extent with the RpoN protein of A. vinelandii (54.7% identity over 503 amino acids; see Appendix, Figure A2), P. putida (52.6% identity over 498 amino acids, see Appendix, Figure A2) and R. meliloti (37.4% identity over 508 amino acids; see Appendix, Figure A3). It was therefore concluded that ORF B encodes the RpoN homologue of Er. chrysanthemi NCPPB 1066.

As is the case with the RpoN proteins from other bacterial genera, a significant proportion of the amino acid residues of the predicted ORFB polypeptide are aspartate and glutamate. Therefore, this protein is predicted to be acidic overall with a pI value calculated to be 4.6.

5.6.2.1 Features of the ORF B polypeptide

Even though the RpoN protein is known to function as an RNA

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polymerase sigma factor, it does not share extensive homology with other prokaryotic RNA polymerase sigma factors such as RpoD, RpoH, SpoIIG, SpoIIAC and SigB (Helmann and Chamberlin, 1988; Merrick and Gibbins, 1985; Merrick et al., 1987). Despite this fact, alignment of the predicted amino acid sequences of K. pneumoniae, E. coli, P. putida, A. vinelandii, and R. melloti RpoN proteins for example, has led to the identification of a number of conserved regions within the RpoN protein itself that are considered to be significant in terms of sigma function (Merrick et al., 1987; Sasse-Dwight and Gralla, 1990; van Slooten et al., 1990). Interestingly, some of these regions are more characteristic of eukaryotic transcription factors, suggesting that RpoN represents a type of hybrid sigma factor (Sasse-Dwight and Gralla, 1990). On the basis of these studies, the RpoN protein may be divided into essentially three regions, which will now be briefly discussed in relation to the ORF B polypeptide.

Region I :

The first area of conservation found amongst bacterial RpoN proteins, typically spans the first 48 amino acids at the amino-terminus. This region is particularly rich in leucine and glutamine residues, and is not characteristic of other bacterial sigma factors. Such a region is also to be found within the predicted ORF B polypeptide, where leucine and glutamine account for 54% of the total number of residues.

Region II :

Region II of the RpoN protein appears to be much more variable than region I in terms of amino acid similarity and is therefore considered to be less essential for RpoN function. For the ORF B polypeptide, region II is taken to cover the amino acid residues between glutamine (48) and leucine (120).

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Region III :

Finally, region III comprises the remaining two thirds of the carboxy-terminus of the RpoN polypeptide, and is quite highly conserved throughout. Four specific subregions have been identified within this domain, which are believed to play an important role in the protein-protein and protein-DNA interactions between RpoN, core RNA polymerase and the -24/-12 promoter. According to van Slooten and colleagues, (1990) these specific subregions may be regarded as the 'fingerprint' of the RpoN family of proteins.

The first C-terminal subregion of RpoN covers twenty amino acids which have been found to display significant homology with conserved residues located within other bacterial sigma factors such as RpoD of *E. coli* and SpoDIIAC of *B. subtilis*, which may be involved in the interaction of the sigma factor with core RNA polymerase (Gribskov and Burgess, 1986). This proposal has been substantiated by the fact that a point mutation, leading to the modification of an amino acid residue within the conserved region of SpoIIAC (Ser 73; see Figure 5.10), prevents the toxicity normally observed when the SpoIIAC protein is over-produced in *E. coli*. Such toxicity is believed to be a consequence of SpoIIAC binding to *E. coli* core RNA polymerase, and that the aforementioned mutations prevent such binding (Yudkin, 1987). For the ORF B polypeptide, an amino acid sequence resembling subregion I extends from amino acid residue 188 to 187, as highlighted in Figure 5.10.

Subregion II spans 30 amino acid residues that are homologous to a sequence situated close to the amino-terminus of the B' subunit of core RNA polymerase (RpoC). It is likely that conservation of such residues has some functional significance, but the question of why such a conserved sequence is present in both RpoN and RpoC proteins has not been answered to date. In

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this regard, Merrick *et al.* (1987) have proposed that binding of RpoN to core RNA polymerase results in an alteration of the structure of the enzyme, and that the conserved RpoN region can thus take over the function normally performed by the homologous region of RpoC. For the ORFB polypeptide, subregion II appears to be located between residues 328 and 357, inclusive (Figure 5.11).

Subregion III and subregion IV are found to be highly conserved in the RpoN protein and are therefore likely to play an intrinsic role in RpoN function. Subregion III is believed to be involved in promoter recognition and has been identified as a potential DNA-binding domain. Such domains are typically characterised by a 20 amino acid motif that consists of two short alpha-helices connected by a short turn having the consensus: glycine, valine or isoleucine, followed by serine. The first helix is believed to interact with the bound core RNA polymerase, and help to correctly orientate the second helix within the major groove of the DNA molecule. It is the latter that is believed to primarily determine the specificity of the protein-DNA interaction (Harrison and Aggarwal, 1990; Helmann and Chamberlin, 1988; Pabo and Sauer, 1984).

Recently, Coppard and Merrick (1991) have performed a detailed molecular analysis of the region containing helix 2 of the proposed helix-turn-helix (HTH) motif of the *K. pneumoniae* RpoN polypeptide, and have obtained genetic evidence consistent with the view that this region is involved in the binding of RpoN to the -24/-12 promoter region. In this instance, cassette mutagenesis was used to alter a number of amino acid residues within helix 2, subsequently leading to the generation of mutant RpoN proteins that had either an altered promoter specificity, caused an increase in the levels of expression from the RpoN-dependent *nifLA*

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promoter, or were inactive. Analysis of the latter class, using the technique of in vivo footprinting, indicated that the RpoN protein was unable to interact with the -24/-12 region of the glnAp2 promoter.

The amino acid sequence corresponding to subregion III of the ORF B polypeptide is illustrated in Figure 5.12. As is the case with other helix-turn-helix motifs that have been studied, an alanine residue is conserved at position five and hydrophobic amino acids are located at positions 4, 8, 10, 15, 18 and 19. The latter are thought to act as a 'hydrophobic brace' which contributes to the stabilisation and correct orientation of the HTH motif structure. The turn between the two helices is usually occupied by a glycine residue at position 9 because this amino acid residue allows conformational flexibility within this region. In the ORF B polypeptide however, the favoured glycine residue is replaced by aspartate. Although this differs from other RpoN proteins that have been studied, such a residue is in fact found at position 9 in the helix-turn-helix motif of the B. subtilis sigma factor SpoII_G, SigB and RpoD, and is therefore not unprecedented.

Subregion IV is located close to the carboxy-terminal end of the RpoN protein and is represented by a region of 13 conserved amino acids, containing a core of 9 perfectly conserved residues (ARRTVAKYR). This has been termed the 'RpoN box' by van Slooten and colleagues (1990) by analogy to a conserved sequence of 13 amino acids (the so-called 'RpoD box'), located within the RpoD protein of B. subtilis, E. coli and Streptomyces coelicolor (Tanaka et al., 1988). A sequence resembling the 'RpoN box' can be found within the ORFB polypeptide between residues 454 and 462, inclusive.

Leucine zipper motifs and an acid domain :

Finally, Sasse-Dwight and Gralla (1990) have identified two conserved

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Figure 5.12 Alignment of a proposed helix-turn-helix motif within the predicted RpoN polypeptide of a number of bacterial species

		1		10		20																
ORF B	(366)	V	L	A	D	I	A	Q	A	V	D	M	H	E	S	T	I	S	R	V	T	T
<u>K. pneumoniae</u>	(366)	V	L	A	D	I	A	Q	A	V	E	M	H	E	S	T	I	S	R	V	T	T
<u>E. coli</u>	(366)	V	L	A	D	I	A	Q	A	V	E	M	H	E	S	T	I	S	R	V	T	T
<u>A. vinelandii</u>	(391)	V	L	H	D	I	A	E	A	V	G	M	H	E	S	T	I	S	R	V	T	T
<u>P. putida</u>	(386)	V	L	H	D	I	A	E	A	V	G	M	H	E	S	T	I	S	R	V	T	T
<u>R. meliloti</u>	(393)	N	L	R	I	V	A	D	A	I	K	M	H	E	S	T	V	S	R	V	T	S
<u>R. capsulatus</u>	(312)	T	L	E	D	V	A	S	E	L	G	L	H	A	S	T	I	S	R	A	V	S
		*		*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		←	HELIX 1	→	←	TURN	→	←	HELIX 2	→												

The numbers in brackets denote the position of the amino acid residue at the beginning of each of the above sequences, within the RpoN polypeptide. Vertical lines highlight residues that are perfectly conserved. The position of significantly conserved residues is indicated by an asterisk.

All data, except for the ORF B polypeptide, is reproduced from Coppard and Merrick (1991).

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heptad hydrophobic repeats within the RpoN protein which display homology to so-called leucine zipper motifs (Landshulz *et al.*, 1988; Vinson *et al.*, 1989) found in eukaryotic transcription factors. Such structures have been shown to be required for transcription, and are believed to interact to form a coiled coil helical structure which can act as a DNA binding domain (O'Shea *et al.*, 1989 a,b). Deletion of residues within either of the aforementioned motifs within *E. coli* RpoN protein using Bal 31 nuclease has been shown to prevent binding of the protein to the -12 region but not the -24 region of the *glnAp2* promoter and does not lead to the formation of an active open complex. Instead, the DNA remains unmelted (Sasse-Dwight and Gralla, 1990). It has therefore been suggested that the DNA leucine zipper binding motifs within the RpoN protein come together to form an intramolecular DNA binding domain which is required for RpoN to contact the -12 region of the promoter. This would in turn correctly position the activator domain to allow melting of the DNA strands. A domain poorly conserved in terms of sequence, but much more so in terms of overall acidity appears to be involved in the melting process. This spans region II and part of region III up to and including most of the second leucine zipper motif. Indeed, deletion of residues 51 to 77 within the acidic region of the *E. coli* RpoN polypeptide has been shown not to affect binding to the *glnAp2* -24/-12 promoter region but to reduce DNA melting (Sasse-Dwight and Gralla, 1990).

For the ORF B polypeptide, the first leucine zipper motif occurs within region I between residues 19 and 44, respectively; whereas the second motif is situated between residues 158 and 183, respectively (Figure 5.13).

5.6.3 ORF A

Previous analysis of the chromosomal region immediately upstream of

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Figure 5.13 Alignment of two conserved heptad hydrophobic repeat sequences within the RpoN polypeptides of various bacterial species and ORF B

REGION I

ORF B	(19)	L	Q	Q	A	I	R	L	L	Q	L	S	T	L	E	L	Q	Q	E	I	Q	Q	A	L	E	S	N
<u>E. coli</u>	(19)	L	Q	Q	A	I	R	L	L	Q	L	S	T	L	E	L	Q	Q	E	L	Q	Q	A	L	E	S	N
<u>K. pneumoniae</u>	(19)	L	Q	Q	A	I	R	L	L	Q	L	S	T	L	E	L	Q	Q	E	L	Q	Q	A	L	D	S	N
<u>A. vinelandii</u>	(19)	L	Q	Q	A	I	R	L	L	Q	L	S	T	L	D	L	Q	Q	E	I	Q	E	A	L	D	S	N
<u>R. meliloti</u>	(21)	L	M	Q	S	I	Q	L	L	Q	M	N	H	L	E	L	S	H	F	I	A	Q	E	V	E	R	N
<u>P. putida</u>	(19)	L	Q	Q	A	I	R	L	L	Q	L	S	T	L	D	L	Q	Q	E	I	Q	E	A	L	E	S	N

REGION III

ORF B	(158)	L	D	E	I	R	E	S	I	G	N	E	-	E	V	T	L	E	E	V	E	A	V	L	K	R	V	Q
<u>E. coli</u>	(158)	L	E	D	I	L	E	S	I	G	D	E	-	E	I	D	I	D	E	V	E	A	V	L	K	R	I	Q
<u>K. pneumoniae</u>	(158)	V	E	D	I	V	E	S	I	G	D	D	-	E	I	G	L	E	E	V	E	A	V	L	K	R	I	Q
<u>A. vinelandii</u>	(186)	L	A	S	L	D	P	E	L	G	V	E	-	L	D	E	V	E	M	V	L	R	R	I	Q	Q	F	E
<u>R. meliloti</u>	(189)	L	A	E	T	A	E	T	L	G	A	A	-	G	E	D	V	A	R	V	L	H	V	L	Q	Q	F	D
<u>P. putida</u>	(177)	L	E	E	I	C	A	G	F	D	P	E	L	D	I	E	L	D	E	V	E	A	V	L	H	R	I	Q

Residues which are involved in the hydrophobic heptad repeat are boxed. Numbers in brackets denote the position of the amino acid residue at the beginning of each of the above sequences within the RpoN polypeptide. A gap has been introduced in the lower set of sequences to maximise the alignment.

All data except for ORF B (this work) and P. putida (Inouye *et al.*, 1989), is reproduced from Sasse-Dwight and Gralla (1990).

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the rpoN structural gene of K. pneumoniae, S. typhimurium, R. meliloti, Rhizobium strain NGR234, P. putida, and T. ferrooxidans, has revealed the presence of a closely linked conserved open reading frame, designated ORF 1. Similarly, a small incomplete open reading frame, ORF A, was found to be located immediately upstream of ORF B (the rpoN homologue of Er. chrysanthemi), encoding a predicted 68 amino acid polypeptide with a calculated molecular weight of 7,658 Daltons. As can be seen from Figure A4 (see Appendix), subsequent alignment of the primary amino acid sequence of the ORF A polypeptide with the ORF 1 product of K. pneumoniae, P. putida and R. meliloti, revealed homology in each case.

The reason for the presence of a conserved open reading frame situated immediately upstream of rpoN in a number of diverse bacterial species including Er. chrysanthemi NCPPB 1066, is presently not understood since experimental evidence gathered from a number of sources indicates that the product of the conserved upstream ORF does not directly influence RpoN function. Work performed by Berger and colleagues (1990) for example, has demonstrated that subclones of the T. ferrooxidans rpoN region which have been deleted for most of the upstream ORF 1, retain the capacity to complement an E. coli rpoN mutant, regardless of orientation. Similar results have also been obtained by Ronson et al. (1987) following deletion analysis of a 3.5 kb HindIII restriction fragment harbouring both ORF 1 and rpoN structural genes of R. meliloti; and also from the present study.

The functional independence of RpoN and the polypeptide encoded by the conserved upstream ORF is further highlighted by the fact that transcription of the two genes appears to be uncoupled. RNAase protection studies and primer extension analysis, have clearly shown that in R. meliloti, transcription of rpoN originates within the non-coding region between rpoN

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and the conserved upstream ORF, 66bp upstream of the translational start (Albright *et al.*, 1989). Furthermore, insertion of a strong transcriptional terminator (ω S1), within the upstream ORF of *Rhizobium* sp. strain NGR234, does not appear to affect expression of RpoN (van Slooten *et al.*, 1990). Finally, *lacZ* gene fusion analysis and complementation data have together indicated that the *A. vinelandii* and *K. pneumoniae* *rpoN* promoter is located between nucleotides 1-145 and 1-279, upstream of the structural gene, respectively. In the former, no ORF has been detected within the available 168 bp of nucleotide sequence upstream of *rpoN*. Berger *et al.* (1990) have proposed that the promoter of the *T. ferrooxidans* *rpoN* structural gene actually lies within the carboxy-terminus of the conserved upstream ORF, since the non-coding region between the two genes is only 12 bp in length. It is likely that the same is true of *K. pneumoniae* and *Er. chrysanthemi* NCPPB 1066 because both contain relatively small intergenic regions also. Indeed, Merrick and Gibbins (1985) have identified putative '-10' and '-35' regions at nucleotide positions 206 and 180 upstream of the *rpoN* structural gene. Analysis of the nucleotide sequence at the carboxy terminus of the *Er. chrysanthemi* NCPPB 1066 ORF A revealed two sequences (TATACT and TTGCCC), separated by 16 bp, which show good homology to the '-10' and '-35' promoter consensus sequences, respectively (see Figure 5.9).

A clue as to the biological role of the polypeptide encoded by the conserved upstream ORF has come from recent work by Albright and colleagues (1989), which has highlighted significant homology between the predicted polypeptide of *R. melloti* ORF 1, and members of a superfamily of ATP-binding proteins involved in a wide range of cellular processes. This has also been found to be true of the polypeptide encoded by ORF 1 of *T.*

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ferrooxidans, P. putida and K. pneumoniae. Members of the ATP-binding superfamily include HisP, MalK, OppD and PstB, all of which form part of a multi-component, periplasmic binding protein-dependent transport system, consisting of a periplasmic substrate binding protein and two highly hydrophobic integral membrane proteins, in addition to the conserved ATP-binding protein. Several other members of the ATP-binding protein superfamily function in transport processes that are distinct from the periplasmic protein-dependent systems previously described. These include HylB, which is involved in export of haemolysin in E. coli, and the product of the ndvV gene of R. melliloti, believed to be involved in the export of B-(1,2) glucan. Furthermore, some members do not appear to be involved in transport processes at all (eg UvrA, an ATP-dependent DNA repair enzyme), or have unknown functions (eg. FtsE, required for cell division in E. coli and NodI of Rhizobium leguminosarum, involved in the nodulation process). The conservation between the aforementioned group of proteins spans a region of approximately 250 amino acids, and includes two highly conserved regions that are thought to form a nucleotide binding pocket. In certain members of the superfamily (eg. FtsE) the ATP binding domain covers the entire protein, whereas in others (eg. HylB) it forms part of a much larger polypeptide.

Higgins and colleagues (1986) have suggested that the role of the superfamily of ATP binding proteins is to provide energy for such diverse biological processes, via ATP hydrolysis. The exact nature of the cellular process involving the conserved ORF upstream of rpoN is not known. Attempts by Albright et al. (1989) to generate an ORF 1 mutant of R. melliloti by replacing the wild-type ORF 1 chromosomal allele with ORF 1::Tn5 via marker-exchange mutagenesis, were unsuccessful. Failure in this case did not appear to be a consequence of re-arrangement of the cosmid

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harbouring ORF 1::Tn5, or limited recombination within the ORF 1 region of the genome, since rpoN::Tn5 was successfully homogenotised with much less flanking homology. It was therefore concluded that ORF 1 encodes a protein that is involved in an essential cellular process.

5.6.4 ORF C and ORF D

In addition to an analysis of the conserved ORF upstream of the rpoN structural gene of K. pneumoniae, Merrick and Coppard, (1989) have carried out a detailed study of the chromosomal region immediately downstream of the rpoN structural gene of K. pneumoniae, and identified three additional ORFs (two complete and one incomplete), transcribed in the same direction as rpoN. The first complete open reading frame, ORF 95, was situated 22 bp from the 3' terminus of rpoN and encoded a predicted protein of 10,759 daltons. The second complete open reading frame, ORF 162, was positioned 64bp downstream of ORF 95, and encoded a predicted polypeptide of 17,721 Daltons. Finally, 45 bp beyond ORF 162 was an incomplete open reading frame, ORF193', encoding a polypeptide of 21,778 Daltons. Analysis of the derived nucleotide sequence between the coding regions of rpoN, ORF 95 and ORF 162 could not identify any sequence resembling a rho-independent terminator and, therefore, suggested to Merrick and Coppard, (1989) that the latter two ORFs were co-transcribed with rpoN. In contrast, two regions of dyad symmetry characteristic of a rho-independent transcriptional terminator were identified within the non-coding region between ORF 162 and ORF 193', and implies that this ORF does not form part of the rpoN operon.

An open reading frame homologous to ORF 95 has also been located immediately downstream of all of the remaining rpoN structural genes studied to date, except that of R. capsulatus which is flanked by the genes

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nifK and nifA. Alignment of the primary amino acid sequence of the predicted ORF C polypeptide of Er. chrysanthemi NCPPB 1066 with the ORF 95 product of K. pneumoniae, and homologous proteins from A. vinelandii, P. putida and R. meliloti, revealed homology in each case (see Appendix, Figure A5). Likewise, homology was apparent between the primary amino acid sequences of the predicted ORF D polypeptide of Er. chrysanthemi NCPPB 1066 and the ORF 162 polypeptide of K. pneumoniae and its P. putida counterpart, as illustrated in Figure A6 (see Appendix).

Conservation of two open reading frames downstream of rpoN in a wide range of bacteria (including Er. chrysanthemi NCPPB 1066) implies that they either perform an essential cellular function (as is the case with the conserved ORF upstream of rpoN), or in fact play a significant role in terms of RpoN function. In this regard, Merrick and Coppard, (1989) have reported that for K. pneumoniae at least, mutation of either ORF 95 and ORF 162 leads to a significant rise in the transcription from the sigma 54-dependent promoters of the nifH, nifL or fdhF genes, for example. Hence, it seems as if the products of these particular ORFs have the capacity to modulate the activity of sigma 54. However, the mechanism by which this is achieved is not understood at present. In this regard, it is interesting to note that the K. pneumoniae ORF 95 product displays 38% homology with the polypeptide encoded by the open reading frame situated adjacent to the pheA gene of E. coli (URF 1). The role of the URF 1 protein in the latter bacterium and the significance of the homology with ORF 95 is also not understood to date (Merrick and Coppard, 1989).

5.7 CLONING OF THE glnA, ntrB.C REGION FROM ER. CHRYSANTHEMI NCPPB 1066

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5.7.1 Approach

In all the enteric bacteria so far studied, the ntrB and ntrC structural genes have been found to lie immediately downstream of the structural gene encoding glutamine synthetase (glnA), and form a complex operon. The rpoN gene is unlinked and maps close to argG (Leonardo and Goldberg, 1980). The cloning strategy for the isolation of the glnA, ntrB,C region from a number of organisms has relied solely upon a 'shotgun' approach, in which gene libraries are either conjugated or used to transform corresponding E. coli or K. pneumoniae mutants. The desired recombinant is thus isolated by complementation of the lesions of these bacteria, thereby allowing growth on minimal glucose medium containing ammonium but no glutamine (GlnA^+ phenotype), or arginine, proline or histidine as sole nitrogen source (Ntr^+ phenotype). The following section describes attempts aimed at identifying and isolating the glnA, ntrB and ntrC homologues of Er. chrysanthemi NCPPB 1066 using the constructed partial Sau3A genomic library described in Section 5.3.2.

5.7.2 Heterologous hybridisation studies using a cloned K. pneumoniae ntrB gene probe

Prior to complementation studies, a preliminary experiment was carried out to determine whether an ntrB homologue was present within the genome of Er. chrysanthemi NCPPB 1066; since this would provide some physical evidence to suggest that an ntrB,C regulatory system existed in this particular organism. To this end, Southern blot analysis was performed on wild-type Er. chrysanthemi NCPPB 1066 genomic DNA, using a 1.0 kb fragment from plasmid pSM10, which contains the ntrB structural gene of K. pneumoniae, as a probe.

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The results are presented in Figure 5.14, where it is evident that a number of chromosomal restriction fragments hybridised to the K. pneumoniae ntrB gene probe, strongly suggesting the existence of a Er. chrysanthemi NCPPB 1066 ntrB homologue. Further experimentation was thus aimed at isolating this gene and surrounding regions (hopefully including the ntrC and glnA structural genes).

5.7.3 Screening the wild-type Er. chrysanthemi NCPPB 1066 partial Sau3A cosmid library

E. coli strain ET8894 [(glnA, ntrB,C)⁻] was grown overnight at 30°C in 10ml of LB medium supplemented with 15mM glutamine, 0.2% (w/v) maltose, 10mM MgSO₄ and Nm (50ug ml⁻¹). The culture was concentrated ten-fold and statically incubated with 100ul of amplified pHC79 cosmid library lysate at 30°C for 30 min. Following infection, bacteria were sub-cultured 1 in 10 into 10ml of fresh LB glutamine medium and aerated at 30°C for 60 min. Cells were subsequently washed once in 1 X phosphate buffer and resuspended in 1ml of the same medium to prevent carry over of nutrients. Transductants were then selected on minimal glucose medium containing 0.1% (w/v) (NH₄)₂SO₄ as sole nitrogen source, Ap (50ug ml⁻¹) and Nm (50ug ml⁻¹) at 30°C. Uninfected E. coli ET8894 and ET8000 were run in parallel as negative and positive GlnA⁺ controls, respectively.

Following a 2 day incubation, one Ap^r, Nm^r transductant was isolated and re-streaked on the same medium. Cosmid DNA was isolated on a small-scale and designated pCK7. This was used to transform competent E. coli ET8894 and ET8556 (ntrC⁻) cells, along with plasmids pHC79 and pAT523. The latter contains the glnA, ntrB,C region from A. vinelandii, and thereby acted as a positive complementation control. Transformants were selected on

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Figure 5.14 Identification of *Er. chrysanthemi* NCPPB 1066 genomic sequences exhibiting homology with a *K. pneumoniae ntrB* gene probe

Intact wild-type *Er. chrysanthemi* NCPPB 1066 chromosomal DNA was digested to completion with a range of restriction endonucleases and the products size-fractionated by 0.7 % (w/v) TAE agarose gel electrophoresis. DNA was transferred to a nylon membrane and probed with a ³²P-radiolabelled 1.0 kb *KpnI* fragment from plasmid pSM10 overnight at 65°C in 1 X 'Blotto'. The membrane was washed at 55°C in 2 X SSC and subjected to autoradiography at -70°C.

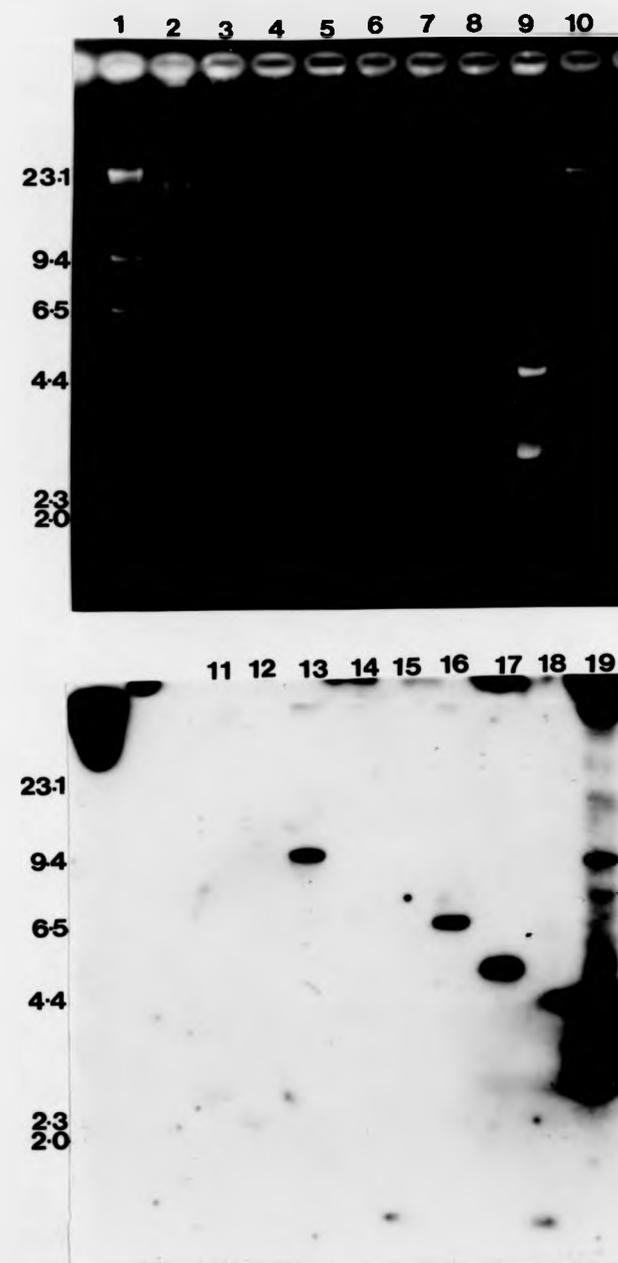
The running order is as follows :

- Track 1 : lambda DNA restricted with *HindIII*
- Track 2 : wild-type genomic DNA restricted with *EcoRI*
- Track 3 : wild-type genomic DNA restricted with *SalI*
- Track 4 : wild-type genomic DNA restricted with *KpnI*
- Track 5 : wild-type genomic DNA restricted with *Clal*
- Track 6 : wild-type genomic DNA restricted with *PstI*
- Track 7 : wild-type genomic DNA restricted with *BamHI*
- Track 8 : *K. pneumoniae* genomic DNA restricted with *KpnI*
- Track 9 : pSM10 supercoil DNA
- Track 10 : lambda DNA restricted with *HindIII*

Tracks 11 to 19 are autoradiographs of corresponding tracks 1 to 9 probed with the 1.0 kb *KpnI* fragment from pSM10.

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Figure 5.14



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minimal glucose medium containing Ap (50ug ml⁻¹), Nm (50ug ml⁻¹) and either 0.1% (w/v) (NH₄)₂SO₄ (for GlnA⁺ phenotype) or 0.2% (w/v) arginine (for Ntr⁺ phenotype), as sole nitrogen source. The results obtained from this experiment (Table 5.2) indicated that sequences contained within pCK7 were able to correct the Ntr⁻ phenotype of both ET8894 and ET8556, and the glutamine auxotrophy of the former strain. Hence, this strongly suggested that the Erwinia glnA and ntrB,C homologues had been cloned on one recombinant pHC79 cosmid, and were therefore likely to be situated in close proximity on the chromosome of this organism.

5.7.4 Hybridisation studies with pCK7

In order to verify that the cloned fragment in pCK7 contained ntr homologues, and at the same time check that it was derived from the Er. chrysanthemi NCPPB 1066 genome (ie. that no re-arrangements had occurred during the isolation process), the following control experiment was performed. Both wild-type Er. chrysanthemi NCPPB 1066 chromosomal and pCK7 DNA was digested to completion with an identical set of restriction endonucleases and run on an agarose gel in parallel. DNA was then subjected to Southern blot analysis using the K. pneumoniae ntrB gene probe. From Figure 5.15, it can be seen that the restriction fragments of pCK7 which hybridised to the probe were identical in size to the hybridising fragments derived from the wild-type Er. chrysanthemi NCPPB 1066 genome. It was therefore concluded that the cloned DNA maintained in pCK7 originated from the Er. chrysanthemi NCPPB 1066 genome and had been cloned intact.

The physical presence of an ntrC homologue on the cloned fragment was determined by digesting pCK7 to completion with a range of restriction endonucleases, and performing Southern blot analysis on the restriction

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Table 5.2 Complementation analysis of pCK7

<u>E. coli</u> strain	Sole nitrogen source for growth*		Phenotype
	0.1% (w/v) (NH ₄) ₂ SO ₄	0.2% (w/v) arginine	
ET8000	+	+	GlnA ⁺ , RpoN ⁺
ET8894	-	-	GlnA ⁻ , RpoN ⁻
ET8894(pHC79)	-	-	GlnA ⁻ , RpoN ⁻
ET8894(pCK7)	+	+	GlnA ⁺ , RpoN ⁺
ET8894(pAT523)	+	+	GlnA ⁺ , RpoN ⁺
ET8556	+	-	GlnA ⁺ , RpoN ⁻
ET8556(pHC79)	+	-	GlnA ⁺ , RpoN ⁻
ET8556(pCK7)	+	+	GlnA ⁺ , RpoN ⁺
ET8556(pAT523)	+	+	GlnA ⁺ , RpoN ⁺

* Plates inspected following a 2 day incubation at 30°C.

Key : + Growth (colonies 2-3 mm in diameter)
 - No growth

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Figure 5.15 Verification of the origin and continuity of the chromosomal insert of the recombinant cosmid pCK7

Recombinant cosmid clone pCK7 and wild-type *Er. chrysanthemi* NCPPB 1066 chromosomal DNA was digested to completion with a range of restriction endonucleases and the products size-fractionated by 0.7 % (w/v) TAE agarose gel electrophoresis. DNA was transferred to a nylon membrane and probed with a ³²P-radiolabelled 1.0 kb *Kpn*I fragment from plasmid pSM10 overnight at 65°C in 1 X 'Blotto'. The membrane was washed at 55°C in 2 X SSC for 1 hour and then subjected to autoradiography at -70°C.

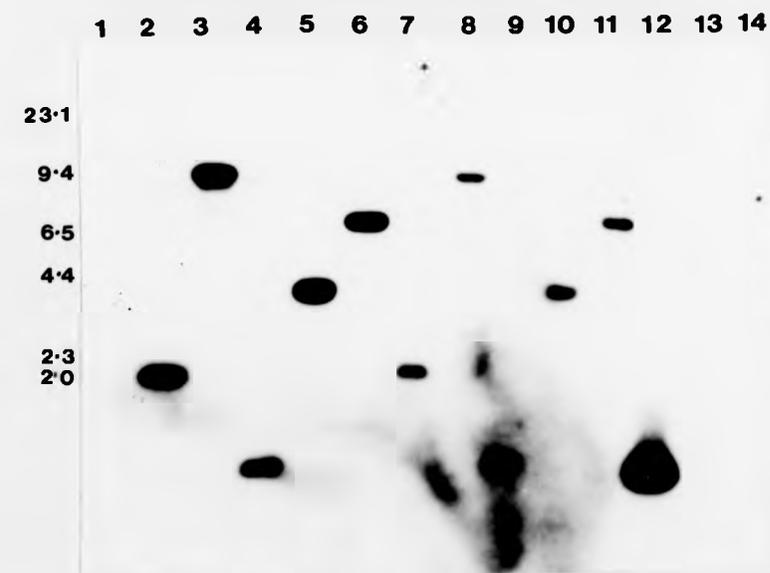
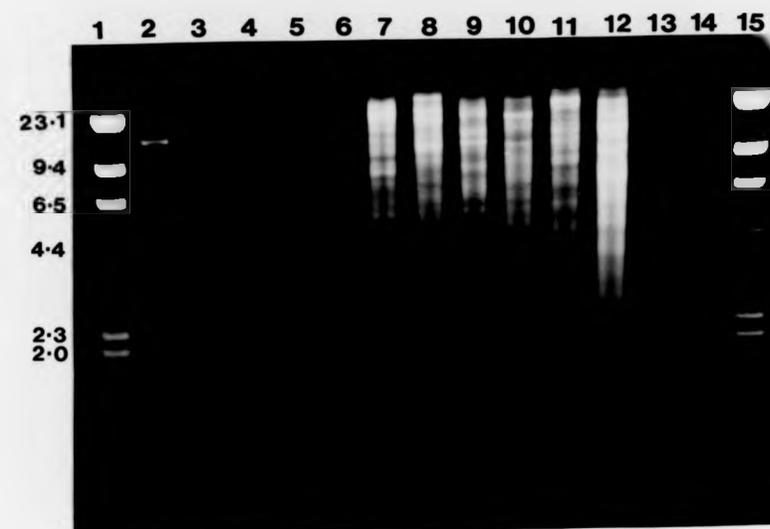
The running order is as follows :

- Track 1 : lambda DNA restricted with *Hind*III
- Track 2 : pCK7 DNA restricted with *Eco*RI
- Track 3 : pCK7 DNA restricted with *Sal*I
- Track 4 : pCK7 DNA restricted with *Cl*aI
- Track 5 : pCK7 DNA restricted with *Kpn*I
- Track 6 : pCK7 DNA restricted with *Pst*I
- Track 7 : wild-type genomic DNA restricted with *Eco*RI
- Track 8 : wild-type genomic DNA restricted with *Sal*I
- Track 9 : wild-type genomic DNA restricted with *Cl*aI
- Track 10 : wild-type genomic DNA restricted with *Kpn*I
- Track 11 : wild-type genomic DNA restricted with *Pst*I
- Track 12 : *K. pneumoniae* genomic DNA restricted with *Kpn*I
- Track 13 : no DNA
- Track 14 : pHC79 supercoil DNA
- Track 15 : lambda DNA restricted with *Hind*III

Tracks 1 to 14 of the autoradiograph shown opposite, correspond to the DNA shown in tracks 1 to 14 of the agarose gel, probed with the 1.0 kb *Kpn*I fragment from pSM10.

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Figure 5.15



5. Library Construction and The Ntr System

products using a *K. pneumoniae* ntrC gene probe. Wild-type *Er. chrysanthemi* NCPPB 1066 chromosomal DNA, restricted in an identical fashion, was run in parallel. As can be seen from Figure 5.16, a number of restriction fragments derived from pCK7 hybridised to the ntrC probe. Again, positive pCK7 fragments co-migrated with hybridising fragments derived from the genome. Moreover, restriction fragments which hybridised to the ntrC probe were the same fragments which had hybridised to the ntrB gene probe discussed earlier. This provided additional evidence that the ntrB and ntrC homologues of *Er. chrysanthemi* were closely linked on the chromosome.

5.8 Comments

From the work described above, it appeared that the glnA, ntrB and ntrC homologues from wild-type *Er. chrysanthemi* NCPPB 1066, had been isolated on a single chromosomal fragment, from a partial Sau3A genomic library of this organism. From hybridisation studies, it is likely that the ntrB and ntrC homologues are closely linked in an operon arrangement as has been found with all other Enterobacteria so far studied. Furthermore, this region was found to complement both glnA and ntrB,C mutations of *E. coli*, suggesting that the *Er. chrysanthemi* NCPPB 1066 genes are functional.

5.9 Preliminary evidence for an rpoN-like gene in *Er. chrysanthemi* NCPPB 1066

During the course of this work, overlapping cosmid clones were isolated from the amplified *Er. chrysanthemi* NCPPB 1066 cosmid library on two separate occasions, which appeared to complement the rpoN⁻ phenotype of *E. coli* strain ET8045, by allowing growth on minimal glucose medium containing 0.2% (w/v) arginine as sole nitrogen source. These are shown in

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Figure 5.16 Physical evidence for the presence of the Er. chrysanthemi NCPPB 1066 ntrC homologue within the recombinant cosmid pCK7 and the bacterial genome

Recombinant cosmid pCK7 and wild-type Er. chrysanthemi NCPPB 1066 intact chromosomal DNA was digested to completion with a variety of restriction endonucleases and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. DNA was transferred to a nylon membrane, and probed with a ^{32}P -radiolabelled 1.5 kb EcoRI/HindIII fragment from plasmid pMD114 overnight at 65°C in 1 X 'Blotto'.

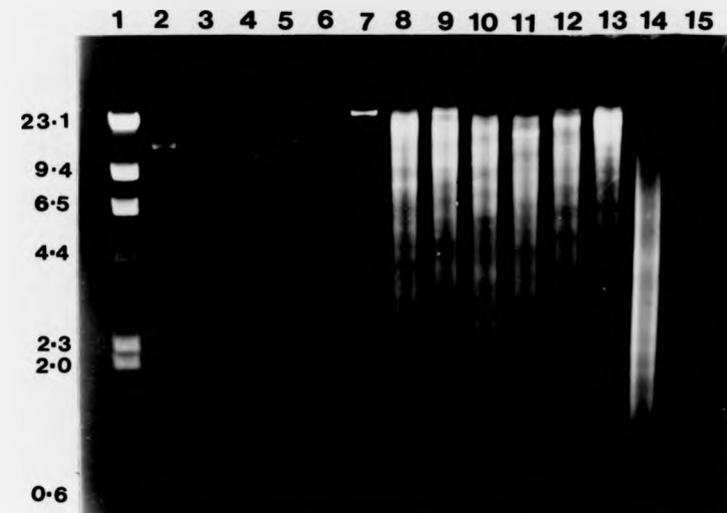
The running order is as follows :

- Track 1 : lambda DNA digested with HindIII
- Track 2 : plasmid pCK7 DNA digested with EcoRI
- Track 3 : plasmid pCK7 DNA digested with Sall
- Track 4 : plasmid pCK7 DNA digested with Clal
- Track 5 : plasmid pCK7 DNA digested with KpnI
- Track 6 : plasmid pCK7 DNA digested with PstI
- Track 7 : plasmid pCK7 DNA digested with BamHI
- Track 8 : wild-type genomic DNA digested with EcoRI
- Track 9 : wild-type genomic DNA digested with Sall
- Track 10 : wild-type genomic DNA digested with Clal
- Track 11 : wild-type genomic DNA digested with KpnI
- Track 12 : wild-type genomic DNA digested with PstI
- Track 13 : wild-type genomic DNA digested with BamHI
- Track 14 : K. pneumoniae chromosomal DNA digested with EcoRI and HindIII
- Track 15 : pHC79 supercoil DNA

Tracks 16 to 30 are corresponding autoradiographs of tracks 1 to 15 probed with the 1.5 kb EcoRI/HindIII fragment from pMD114.

5. Library construction and the Ntr system

Figure 5.16



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Figure 5.17 (a). Interestingly, Southern analysis revealed that the inserted Er. chrysanthemi NCPPB 1066 chromosomal DNA did not contain sequences with homology to the K. pneumoniae rpoN structural gene (Figure 5.17, b), and therefore did not contain the Er. chrysanthemi NCPPB 1066 rpoN homologue described in previous sections. The fact that E. coli ET8045 transductants were capable of growth on minimal glucose medium containing Ap (50ug ml⁻¹), and either 0.2% (w/v) proline or 0.2% (w/v) histidine as a sole source of nitrogen, excluded the possibility that an arginase had been cloned. This therefore highlights the potential danger that one can encounter when attempting to clone a gene on the basis of complementation.

It is a formal possibility that the aforementioned cosmid clones harbour additional gene(s) involved in the regulation of nitrogen metabolism in Erwinia. Indeed, pleiotropic mutations affecting nitrogen metabolism, that are located outside the ntr and glnA genes have been identified in a number of enteric bacteria (Bender et al., 1983; Broach et al., 1976; Close and Shanmugan, 1980). Moreover, studies performed by Allibert and colleagues (1987) have resulted in the isolation and characterisation of an E. coli gene able to restore a ntr⁺ phenotype to ntr⁻ strains of R. capsulatus. Although this gene appeared to be involved in the utilisation of nitrogen sources, it displayed no homology with known sequences of E. coli genes, nor the ntr genes from K. pneumoniae. Recently however, a Rhodobacter gene designated adgA has been isolated by Willison and coworkers, (1990, cited in Albertini and Galizzi, 1990), whose predicted translated product displays 25% homology with the predicted translated product of the aforementioned E. coli ntr-like gene. Similarity values have also been detected with the Bacillus subtilis outB gene, whose product is involved in the process of spore outgrowth (Albertini and Galizzi, 1990), implying that nitrogen regulatory elements

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Figure 17 (a) Restriction endonuclease analysis of *rpoN*-complementing, non-hybridising recombinant cosmid pHC79 clones

Plasmid DNA was digested to completion with *Cla*I or *Eco*RI restriction endonucleases and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. The running order is as follows :

- Track 1 : lambda DNA digested with *Hind*III
- Track 2 : clone 1 DNA digested with *Cla*I
- Track 3 : clone 3 DNA digested with *Cla*I
- Track 4 : clone 4 DNA digested with *Cla*I
- Track 5 : clone 6 DNA digested with *Cla*I
- Track 6 : clone 11 DNA digested with *Cla*I
- Track 7 : lambda DNA digested with *Hind*III
- Track 8 : lambda DNA digested with *Hind*III
- Track 9 : clone 1 DNA digested with *Eco*RI
- Track 10 : clone 3 DNA digested with *Eco*RI
- Track 11 : clone 4 DNA digested with *Eco*RI
- Track 12 : clone 6 DNA digested with *Eco*RI
- Track 13 : clone 11 DNA digested with *Eco*RI
- Track 14 : lambda DNA digested with *Hind*III

Figure 17 (b) Southern analysis of *rpoN*-complementing, non-hybridising recombinant cosmid pHC79 clones

Plasmid DNA (1 µg) and chromosomal DNA was digested with a variety of restriction endonucleases and size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. DNA was then transferred to a nylon membrane and probed with a ³²P-radiolabelled 1.0 kb *Cla*I fragment from plasmid pMM17 overnight at 65°C in 1 X 'Blotto'. The membrane was then washed in 2 X SSC at 55°C for 1 hour and then subjected to autoradiography at -70°C. The running order is as follows :

- Track 1 : lambda DNA digested with *Hind*III
- Track 2 : clone 1 DNA digested with *Cla*I
- Track 3 : clone 3 DNA digested with *Cla*I
- Track 4 : clone 4 DNA digested with *Cla*I
- Track 5 : clone 6 DNA digested with *Cla*I
- Track 6 : clone 11 DNA digested with *Cla*I
- Track 7 : no DNA sample
- Track 8 : no DNA sample
- Track 9 : wild-type *Er. chrysanthemi* NCPPB 1066 chromosomal DNA digested with *Cla*I
- Track 10 : no DNA
- Track 11 : *K. pneumoniae* chromosomal DNA digested with *Kpn*I
- Track 12 : no DNA sample
- Track 13 : plasmid pMM17 supercoil DNA
- Track 14 : cosmid pHC79 supercoil DNA

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Figure 5.17 (a)

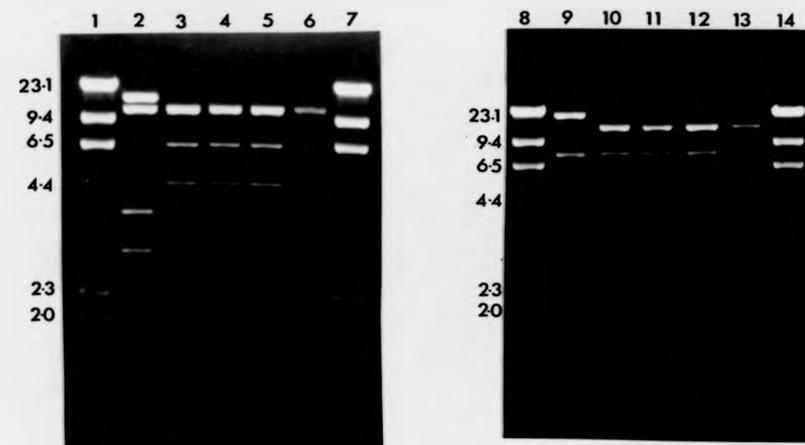
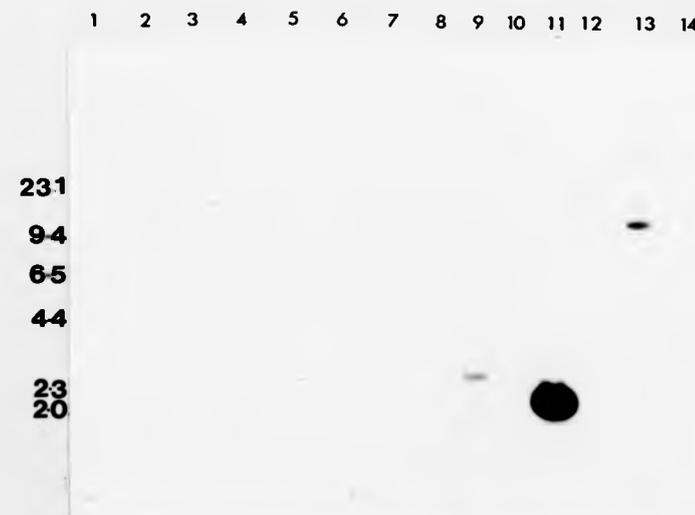


Figure 5.17 (b)



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distinct from the Ntr system could be common to a wide range of microorganisms.

5.10 The effect of multicopies of the Er. chrysanthemi NCPPB 1066 rpoN structural gene on the expression of the Er. chrysanthemi ansB structural gene

Studies carried out by a number of workers have indicated that the level of the RpoN protein within the bacterial cell may limit transcription from a -24/-12 promoter, not only under conditions of nitrogen-limitation, but also under conditions of nitrogen-excess. Merrick and Stewart (1985), for example, introduced plasmid pFB71 (pACYC184 harbouring the K. pneumoniae rpoN structural gene) in trans to the nifL::lacZ fusion plasmid, pRD514, maintained within K. pneumoniae strain UNF931. The presence of multiple copies of the rpoN structural gene was found to cause a 2-fold increase in nifL expression under nitrogen-limiting conditions, and a 5-fold increase under nitrogen-rich growth conditions, compared to levels detected within a K. pneumoniae UNF931 pFB71-free control. Moreover, pFB71 was also found to stimulate nifL expression to a similar extent in E. coli strain ET8000, under conditions of nitrogen-limitation.

A similar phenomenon had also been noted in earlier studies performed by de Bruijn and Ausubel (1983) in which the introduction of pFB71 into K. pneumoniae strain UNF1748, harbouring plasmid pVSA2 (nifH::lacZ), led to an increase in the level of transcription from the K. pneumoniae nifH promoter under both nitrogen-limiting and nitrogen-rich conditions.

Interestingly, in contrast to the above, Merrick and Coppard (1985) introduced pFB71 into E. coli strain ET8000 harbouring plasmid pAM125,

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which contains an RpoN-independent promoter ntrBC_p (Alvarez-Morales *et al.*, 1984) fused to lacZ, and observed no increase in transcription from the aforementioned promoter. In this instance, a 1.4-fold stimulation of transcription was only seen when the rpoD structural gene was supplied in trans in multicopy.

One theory to explain the observed effects of multicopy rpoN on transcription of RpoN-dependent promoters is that the RpoN protein is in direct competition with other sigma factors, such as RpoD, for core RNA polymerase. By increasing the levels of RpoN within the cell one possibly increases the formation of RpoN-core RNA polymerase complexes at -24/-12 promoters, which in turn, increases the levels of transcription. Such an increase could also take place under nitrogen rich conditions, since a certain amount of phosphorylated NtrC protein is also present within the cell under these conditions (Dr. R. Dixon; personal communication).

Having isolated and characterised the rpoN structural gene from both wild-type Er. chrysanthemi NCPPB 1066 and the industrial morphotype 2, the following experiment was carried out in order to investigate whether multiple copies of this particular gene could influence the level of transcription from the Er. chrysanthemi NCPPB 1066 ansB promoter region.

5.10.1 Introduction of the Er. chrysanthemi NCPPB 1066 rpoN structural gene into the Er. chrysanthemi NCPPB 1066 single copy ansB::lacZ fusion derivative CK1002 and the effects on ansB expression

Because the rpoN structural gene had been cloned into pBR322, attempts were made to introduce such a recombinant plasmid into the wild-type Er. chrysanthemi NCPPB 1066 ansB::lacZ fusion derivative CK1002

5. Library construction and the Ntr system

(Section 7.5.4) using the pR64~~drd11~~/pLVC9 mobilisation system. To this end, plasmid pCK3, pCK4, pCK5 and pCK6 DNA was used to transform competent cells of E. coli GJ342, and transformants selected on NB medium containing Ap (50ug ml⁻¹), Cm (50ug ml⁻¹) and Tc (10ug ml⁻¹), at 30°C. This was also performed for pBR322 DNA, as this plasmid was to act as a control in subsequent expression studies.

Following overnight incubation, a single Ap^r, Cm^r, Tc^r colony from each transformation was streak-purified on the same selective medium, prior to patch-mating with wild-type Er. chrysanthemi NCPPB 1066 CK1002 on NB medium at 30°C. After 18 hours, each mating mix, as well as donor and recipient alone controls, were streaked onto minimal sucrose medium supplemented with Ap (50ug ml⁻¹) and Kn (50ug ml⁻¹) and incubated at 30°C. After this time, Ap^r Kn^r transconjugants arose from each of the mating mixes, but not for the donor and recipient controls. One such colony from each mating was subsequently streak-purified twice on selective medium prior to further analysis. Putative CK1002 recombinants were shown to be Pel⁺, Cel⁺ and Prt⁺, and to be sensitive to Cm and Tc. The presence of plasmid DNA was verified by small-scale analysis followed by 0.7% (w/v) TAE agarose gel electrophoresis.

Er. chrysanthemi CK1002 cells harbouring either pCK3, pCK4, pCK5, pCK6 or pBR322, were inoculated into 20ml of minimal medium containing 0.1% (w/v) glycerol as carbon source, Ap (50ug ml⁻¹) and Kn (50ug ml⁻¹). In addition, ammonium sulphate (15mM) or proline (15mM) were included as a sole nitrogen source (representing nitrogen-rich or nitrogen-limiting growth conditions, respectively). Cultures were then grown with aeration at 200rpm at 30°C in 250ml Erlenmeyer flasks until an OD₆₀₀ value of between 1.25 and 1.36 was reached. At this point, 4ml samples were removed and cells

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harvested by centrifugation. The resulting pellets were then resuspended in 1ml of phosphate buffered saline and cells disrupted by sonication, as described in Section 2.5.1. Cell-free extracts were subsequently assayed for B-galactosidase activity (Section 2.2.20.1) and protein (Section 2.2.20.4). In order to determine the proportion of the population retaining the Ap resistance plasmid markers at the time of sampling, a 100ul sample of each culture was removed and serially-diluted in NB medium. A sample (100ul) of each dilution was spread-plated onto NB agar alone, or on NB agar supplemented with either Ap (50ug ml⁻¹) or Kn (50ug ml⁻¹). Viable cell counts were then determined following a 36 hour incubation at 30°C.

The results obtained from the aforementioned work are summarised in Table 5.3., where it can be seen that only a small proportion of the pCK3 and pCK6 recombinant cultures displayed Ap resistance when cells had been cultivated under conditions of both nitrogen-excess and nitrogen-limitation. Whether this was a result of plasmid-loss or plasmid re-arrangement, leading to the inactivation of the Ap resistance gene, is not known at present. In contrast, recombinant pBR322, pCK4 and pCK5 cultures had maintained the plasmid Ap^r marker to a much greater extent. It was therefore concluded that pCK3 and pCK6 were much more unstable compared with pCK4 and pCK5, and that this may go some way to account for the smaller size of colony noted during complementation studies (see section 5.5.3.4). This, in turn, may be related to the level of expression of the Er. chrysanthemi NCPPB 1066 rpoN gene. From restriction mapping and the nucleotide sequence data which is now available, it is clear that for both pCK3 and pCK6, the rpoN gene is being transcribed in the same direction as the anti-tet promoter. Hence, read-through from the latter may possibly have increased the level of rpoN expression to a point which is detrimental to the

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Table 5.3 The effect of multiple copies of the *Er. chrysanthemi* NCPPB 1066 *rpoN* structural gene on *ansB* expression

Recombinant	B-galactosidase activity [@]		Plasmid stability (%) [*]	
	+NH ₄	-NH ₄	+NH ₄	-NH ₄
CK1002 (pBR322)	7.6	1.0	96.8	83.2
CK1002 (pCK3)	7.0	0.9	7.4	<1.0
CK1002 (pCK4)	6.1	0.6	80.2	77.0
CK1002 (pCK5)	5.8	0.7	89.5	79.1
CK1002 (pCK6)	6.9	1.1	8.9	<1.0

+NH₄ denotes that cells were cultivated in the presence of 15mM (NH₄)₂SO₄ as sole nitrogen source.

-NH₄ denotes that cells were cultivated in the presence of 15mM proline as sole nitrogen source.

@ B-galactosidase activity expressed as umoles of ONP released min⁻¹ mg protein⁻¹

* Plasmid stability is expressed as the % of the population retaining the plasmid-encoded Ap-resistance marker

Samples were assayed in quadruplicate for B-galactosidase and triplicate for protein. Values represent the mean.

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cell.

Overall, no stimulation in the level of transcription from the Er. chrysanthemi ansB promoter region was detected under nitrogen-limiting growth conditions compared with nitrogen-excess within the CK1002 pBR322 recombinant. Moreover, the presence of multiple copies of the Er. chrysanthemi NCPPB 1066 rpoN structural gene did not appear to increase ansB expression under nitrogen-limitation or nitrogen-excess conditions, as compared with the aforementioned control.

5.11 FINAL COMMENTS

From the work described in the latter sections of this chapter, it is clear that Er. chrysanthemi NCPPB 1066 does contain rpoN, ntrB and ntrC homologues, and that these particular genes appear to be functional. This conclusion is based on the finding that recombinant clones had the capacity to complement the corresponding mutations within the E. coli genome. Furthermore, nucleotide sequence analysis of the Er. chrysanthemi NCPPB 1066 rpoN locus and Southern analysis of pCK7, has indicated that the chromosomal organisation of the rpoN, ntrB and ntrC homologues is very similar, if not identical, to what has been found for K. pneumoniae and E. coli. Hence, it is likely that a functional Ntr system is operative within Er. chrysanthemi NCPPB 1066.

With the above knowledge in mind, the proposals put forward by Minton and coworkers (1986) to explain the paradox between the effect of multiple copies of the K. pneumoniae nifA gene on ansB expression and the fact that expression of the latter is not influenced by the levels of ammonia, can now be ruled out. Consequently, it is very difficult to imagine the nifA gene product interacting directly with the ansB promoter itself. One formal

5. Library construction and the Ntr system

possibility is that the presence of multiple copies of the K. pneumoniae nifA structural gene indirectly effected ansB expression in some way.

The question of the potential significance of the putative -24/-12 consensus sequence identified within the 5' non-coding region of the ansB structural gene was further addressed by introducing the Er. chrysanthemi NCPPB 1066 rpoN structural gene and surrounding regions in trans to a single copy ansB::lacZ fusion present within the chromosome of the cell. The availability of an rpoN⁻ derivative of Er. chrysanthemi NCPPB 1066 for such studies was not considered critical since, as stated earlier, the experiments performed by Merrick and Coppard (1985) involved the use of K. pneumoniae strain UNF931 and E. coli strain ET8000, both of which contain a functional rpoN structural gene within the chromosome. If the ansB promoter was rpoN-dependent, one might have expected to detect an increase in the level of expression of this gene in the presence of multiple copies of the rpoN structural gene, assuming that sufficient levels of chromosomally-encoded ntrC product were also present. No such increase was seen.

To summarise, this study has obtained direct physical and genetic evidence to suggest that a functional Ntr system is present within Er. chrysanthemi NCPPB 1066. However, in contrast to the scenario proposed by Minton et al. (1986), the expression of the Er. chrysanthemi NCPPB 1066 ansB structural gene does not appear to be regulated by such a system. The results obtained in this study implied that the ansB gene is not, in fact RpoN-dependent and that the putative -24/-12 consensus sequence within the 5' non-coding region is not significant in terms of ansB regulation.

To investigate this area further, the ansB promoter had to be accurately mapped. To achieve this, the 5' non-coding region of the Er.

5. Library construction and the Ntr system

chrysanthemi NCPPB 1066 ansB gene was analysed by primer extension analysis, as described in the following chapter of this thesis.

CHAPTER SIX

ansB TRANSCRIPT MAPPING

6.1 INTRODUCTION

6.1.1 Aim

The fact that Gilbert and coworkers (1986) demonstrated that the ansB structural gene from Er. chrysanthemi NCPPB 1066 could be highly expressed in E. coli when introduced on a multicopy plasmid, suggests that sequences within the ansB promoter region can be recognised and efficiently utilised by the E. coli RNA polymerase. Subsequent nucleotide sequence analysis of the 5' non-coding region by Minton et al. (1986) revealed no regions which closely resembled the E. coli '-10' and '-35' consensus sequences. However, two areas of potential regulatory importance were identified, one of which was a putative -24/-12 consensus sequence (see Chapter 1.0).

Following on from the work described above, and in conjunction with studies described in the preceding chapter, transcript mapping of total cellular RNA from wild-type Er. chrysanthemi NCPPB 1066 was to be carried out by primer extension to accurately determine the transcriptional start site(s) within the 5' non-coding region, and thereby allow a more detailed structural analysis of this region. This would be performed under both nitrogen-rich and nitrogen-poor growth conditions in a further attempt to ascertain the significance of the -24/-12 region.

6.1.2 Approach

Primer extension provides a rapid and straightforward means of mapping and quantifying the 5' termini of mRNA, although it relies upon a prior knowledge of at least part of the sequence of the mRNA of interest. The sensitivity and precision of this technique results from the ability to use

6. Primer extension

large amounts of RNA and to accurately determine the size of short extended products. In theory, the test RNA is hybridised with an excess of a single-stranded DNA primer radiolabelled at its 5' terminus. Reverse transcriptase is then used to extend this primer to produce DNA complementary to the RNA template. The length of the resulting end-labelled DNA, as measured by electrophoresis through a polyacrylamide gel under denaturing conditions, reflects the distance between the end-labelled nucleotide of the primer and the 5' terminus of the RNA.

6.2 METHODOLOGY

In designing transcript mapping experiments, the following points were considered :

6.2.1 Primer selection

Oligonucleotides offer several advantages over restriction fragments as primers. Technically speaking, oligonucleotides can be efficiently radiolabelled to a higher specific activity than restriction fragments, because they possess free 5'-hydroxyl groups. Moreover, the short single-stranded nature of oligonucleotides allows the primer to hybridise to RNA without competition from a complementary DNA strand. Consequently, this greatly reduces the risk of self-priming. Finally, choosing an oligonucleotide primer that has a unique sequence greatly reduces the potential problem of non-specific hybridisation or cross-hybridisation to related genes.

6.2.2 Primer location

When mapping the position of RNA 5' termini without any prior

6. Primer extension

information regarding the initiation site, it is advantageous to choose a primer with a 3' terminus just within the amino terminal end of the coding region. Mapping resolution is maximised with small extended products up to around 150 bp. With this in mind, two 21-mer oligonucleotides (primer 3 and 4; see Table 2.7) were synthesised, which were complementary to the DNA template strand and would anneal at different distances from the putative promoter. Primer 3 was complementary to nucleotides between positions 372 and 392, whereas primer 4 was complementary to nucleotides between positions 414 and 434, of the published ansB nucleotide sequence (Minton et al., 1986), as illustrated in Figure 6.3. These staggered primers were used in separate experiments to enable confirmation of any result obtained with just one primer.

6.2.3 Control and sequencing reactions

It is important to include controls whilst performing primer extension analysis to help identify contaminating labelled fragments, self-priming, or utilisation of contaminating nucleic acids as templates. These background signals can be recognised by performing a hybridisation-extension reaction with primer or RNA template alone.

In order to pinpoint the transcriptional start point to a single nucleotide, the extension products are run alongside control sequencing reactions (Section 2.3.22.3) which are derived from the hybridisation of the end-labelled primer used in primer extension reactions, to an M13 clone containing DNA which spans the region of interest. This ensures that the nucleotide base within the sequence which co-migrates with the extension product represents the start position of the mRNA, and therefore no counting

6. Primer extension

of base pairs is necessary. Such precision cannot be obtained if any other sequence is used as the control, since it is impossible to tell whether secondary structure affects the position of particular DNA fragments within the gel.

The template used for sequencing was the 1.2 kb Clal fragment from pCK9 cloned into the AccI site in M13mp19.

6.3 RESULTS

6.3.1 Transcript mapping under nitrogen-rich non-defined growth conditions

Preliminary experiments were aimed at defining the mRNA start point within the Er. chrysanthemi NCPPB 1066 ansB 5' non-coding region under non-defined nitrogen-rich growth conditions. To this end, primers 3 and 4 were annealed to total cellular RNA isolated from wild-type Er. chrysanthemi NCPPB 1066 grown with aeration (200 rpm) in LB medium at 30°C. Reverse transcription was then allowed to proceed at both 37°C and 42°C in the presence of Actinomycin D, as described in Section 2.4.3. Reactions were carried out at the higher temperature to reduce the risk of RNA secondary structure causing premature termination of the extension product.

The result of this experiment was found to be identical at both 37°C and 42°C for both primers. In each instance, the extension reaction yielded two distinct radiolabelled DNA fragments which differed by one base pair. The more 5' band originated at position 228 of the published sequence (Minton et al., 1986), corresponding to a cytosine residue 117 nucleotide bases upstream from the translational startpoint, and was taken to represent

6. Primer extension

the Er. chrysanthemi NCPPB 1066 ansB mRNA start site. Figure 6.1 shows an autoradiograph of the reaction performed at 42°C.

6.3.2 Transcript mapping under nitrogen-poor and nitrogen-rich defined growth conditions

To investigate whether the transcriptional start varied under differing nitrogen regimes, wild-type Er. chrysanthemi NCPPB 1066 was grown with aeration (200 rpm) at 30°C, in defined minimal medium supplemented with 0.1% (w/v) glycerol and a number of different nitrogen sources. In this instance, only primer 3 was annealed to the total cellular RNA isolated.

Bacteria were cultured under nitrogen-rich conditions by using 15mM $(\text{NH}_4)_2\text{SO}_4$ or 15mM glutamine as sole sources of nitrogen. In contrast, nitrogen-limited cultures were cultivated in minimal medium supplemented with 15mM proline or 0.15mM glutamine as sole sources of nitrogen. The latter growth conditions were designed to lead to an activation of the Ntr regulatory system within Er. chrysanthemi NCPPB 1066. Medium containing low and high levels of glutamine was also supplemented with 15mM glutamate, as recommended by Cardy and Murrell (1990). In addition, Er. chrysanthemi NCPPB 1066 was grown in minimal medium containing 15mM L-asparagine, since this was the preferred substrate of the L-asparaginase II enzyme, and it was of interest to see whether the ansB mRNA start site would be altered in any way when it was present as a sole source of nitrogen.

It was anticipated that some repression of ansB expression may occur due to the presence of glycerol in the culture medium, albeit at a fairly low concentration. Hence, all of the total cellular RNA sample was annealed

6. Primer extension

Figure 6.1 Primer extension analysis of total cellular RNA isolated from wild-type *Er. chrysanthemi* NCPPB 1066 cultivated in nitrogen-rich non-defined growth conditions

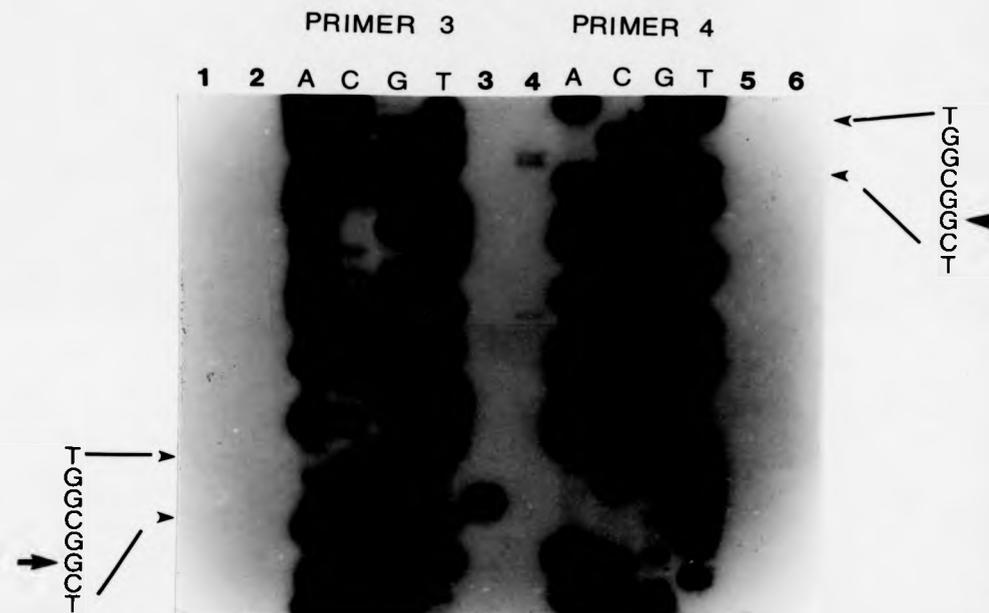
Reverse transcripts were prepared at 42°C and electrophoresed on a 6% (w/v) denaturing polyacrylamide linear wedge gel alongside control sequencing reactions. The resulting autoradiograph is shown opposite. Lanes A, C, G and T represent nucleotide sequencing reactions. The nucleotide base in the sequencing reaction which co-migrates with the most 5' transcription product is indicated by an arrow.

The running order is as follows :

- Track 1 : primer 3, no RNA template
- Track 2 : RNA template, no primer 3
- Track 3 : primer 3, RNA template
- Track 4 : primer 4, RNA template
- Track 5 : primer 4, no RNA template
- Track 6 : RNA template, no primer 4

6. Primer extension

Figure 6.1



6. Primer extension

with primer 3 alone. Primer extension reactions were performed at 42°C only and are the results shown in Figure 6.2. In all cases the transcriptional start was found to be identical to that detected previously for cells grown in LB medium.

6.4 FINAL COMMENTS

Two independent primer extension reactions performed at 37°C and 42°C with total cellular RNA isolated from wild-type Er. chrysanthemi NCPPB 1066 cells grown in complex LB medium, yielded two distinct products separated by one base pair. This phenomenon is very often seen following primer extension and perhaps reflects an inability of the reverse transcriptase enzyme to fully extend to the last 5' base (Dr. R. Old; personal communication). The upper band was found to correspond with a cytosine residue at position 228 of the published sequence (Minton et al., 1986), and was taken to represent the ansB mRNA start site.

Assuming that no post-transcriptional processing of the RNA has taken place, it is proposed that this position also represents the transcriptional initiation site. This result is consistent with the observation made by Gilbert and coworkers (1986) that promoter activity was destroyed when the non-coding region was cleaved with PstI. This restriction endonuclease cuts within a region of dyad symmetry capable of forming a stem loop of -14 kcal, previously identified by Minton et al. (1986), which lies immediately downstream of the identified transcriptional start. The significance of this stem loop structure with respect to ansB regulation awaits further investigation. However, it is interesting to note that an inverted repeat sequence capable of forming a stem loop is also situated immediately

6. Primer extension

Figure 6.2 Primer extension analysis of total cellular RNA isolated from wild-type *Er. chrysanthemi* NCPPB 1066 cultivated in nitrogen-rich and nitrogen-poor defined growth conditions

Reverse transcripts were prepared at 42°C and electrophoresed on a 6% (w/v) denaturing polyacrylamide linear wedge gel alongside a control sequencing reaction. Lanes A, C, G and T represent the nucleotide sequencing reaction. The nucleotide base in the sequencing reaction which co-migrates with the most 5' transcription product is indicated by an arrow.

The running order is as follows :

Track 1 : primer 3, no RNA template

Track 2 : primer 3, RNA template from NH_4^+ grown cells

Track 3 : primer 3, RNA template from proline grown cells

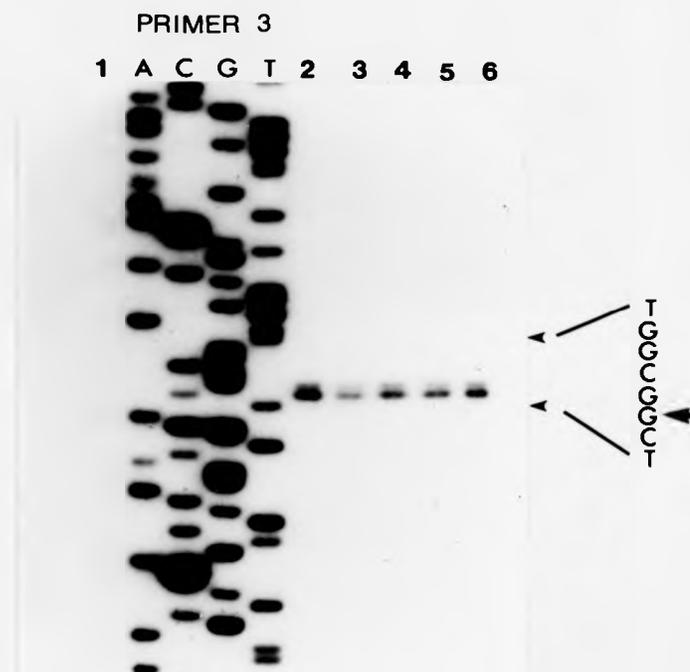
Track 4 : primer 3, RNA template from asparagine grown cells

Track 5 : primer 3, RNA template from 0.15mM glutamine grown cells

Track 6 : primer 3, RNA template from 15mM glutamine grown cells

6. Primer extension

Figure 6.2



6. Primer extension

downstream of the transcriptional start site of the *E. coli* MC4100 ansB structural gene (Jennings and Beacham, 1990). Indeed, Jennings and Beacham (1990) have speculated that this structure, if significant, could be involved in some form of ansB regulation by interacting with a regulatory protein, thereby occluding RNA polymerase binding.

The transcriptional start position of the wild-type *Er. chrysanthemi* NCPPB 1066 ansB structural gene was found to be invariant in bacteria grown under either nitrogen-rich or nitrogen-limiting growth conditions. These preliminary results therefore imply that the putative -24/-12 consensus sequence CTGGCTCTCCTCTTGAT identified by Minton *et al.* (1986), does not in fact represent the promoter region of the ansB gene. The nitrogen-limiting conditions used in this study have previously been shown to activate the Ntr regulatory system in both *E. coli* and *K. pneumoniae*, and it is reasonable to suppose that the same is true for the Ntr system in *Er. chrysanthemi* NCPPB 1066. If the components of this system had interacted with the ansB promoter, one would have expected to have detected transcription initiation around the thymine residue at position 191, eleven nucleotides downstream of the aforementioned sequence. This, however, was found not to be the case.

Aside from the aforementioned promoter consensus sequence, the theoretical '-10' and '-35' regions proposed by Minton *et al.* (1986) also do not appear to correspond to the experimental data obtained. Hence, these results illustrate the potential danger of over-interpreting nucleotide sequence homology, and clearly without direct physical evidence (ie transcript mapping), any interpretation must be done with caution. This has been illustrated in the case of the calY structural gene of *Er. chrysanthemi* 3937j, encoding the endoglucanase isoenzyme Y. Analysis of the 5' non-coding region of this

6. Primer extension

particular gene led to the identification of nucleotide regions which resembled '-10' and '-35' promoter consensus sequences; as well as a putative -24/-12 consensus sequence (Guiseppi et al., 1988). However, S1 mapping failed to identify a cognate -24/-12 transcript, but instead revealed a mRNA transcript which did not correspond to any of the identified promoter sequences (Guiseppi et al., 1988).

The newly proposed '-10' and '-35' regions based on transcript mapping data obtained in this study are highlighted in Figure 6.3, and have been aligned with the transcriptional start site according to a number of general observations which have arisen from the analysis of a large number of promoters (Hawley and McClure, 1983; Rosenberg and Court, 1979; Siebenlist et al., 1980). In E. coli, as well as Gram-negative organisms, initiation of transcription takes place preferentially at a purine residue situated 5 to 9 base pairs downstream of the '-10' region (Aoyama and Takanami, 1985; Murray and Rabinowitz, 1982). In this instance, the Er. chrysanthemi NCPPB 1066 ansB initiation point appears to be a pyrimidine residue.

Generally, the distance between the '-35' and the '-10' region is between 16 to 19 base pairs in length. The TTG in the '-35' region and the TA---T in the '-10' region, have been found to to be the most highly conserved base pairs within the promoter region. Of all the known E. coli promoters that have been sequenced, each shows a match of at least two out of the three conserved base pairs within the '-10' region and one out of three in the '-35' region (McClure, 1985). The T residue at -7 been referred to as "invariant" because it occurs in virtually all of the promoter regions studied, although the A residue at -11 appears to be nearly as conserved as the -7 T.

6. Primer extension

On the basis of this, the nucleotides CAACAT, displaying 50% identity with the '-10' consensus sequence, have been chosen to represent the '-10' region of the Er. chrysanthemi NCPPB 1066 ansB gene. The nucleotides TATACA, displaying 67% identity with the '-35' consensus sequence, are situated 19 bp upstream of the proposed '-10' region, and could possibly represent the '-35' region.

Overall, the Er. chrysanthemi NCPPB 1066 ansB structural gene shows rather poor homology to the promoter consensus sequences. However, one can imagine two reasons why functional promoters might display this feature. Firstly, the sequence of the promoter could overlap with other control sites. Secondly, the interaction of a positive control element could convert a weak promoter showing little homology into one of greater strength, thereby providing some essential adaptability to changes in cellular conditions. (Reznikoff and McClure, 1986). Indeed, it has been noted in the past that many promoters which are subject to positive control in vivo have relatively poor homology to the consensus sequence in the -35 region (Raibaud and Schwartz, 1984). Moreover, in a number of cases, a 'TG' motif preceding the -10 region has been shown to compensate for the absence of a -35 sequence (Ponnambalan et al., 1986). Interestingly, a 'TG' sequence directly precedes the proposed '-10' region in the 5' non-coding region of the Er. chrysanthemi NCPPB 1066 ansB gene, and may be of some significance. Such a motif also precedes the '-10' region of the E. coli ansB gene (Jennings and Beacham, 1990) and the E. coli gyrA gene (Hussain et al., 1987), both of which lack an identifiable -35 region.

CHAPTER SEVEN

L-ASPARAGINASE II GENE FUSIONS

7.1 INTRODUCTION

7.1.1 Aim

Although a number of sensitive and rapid assay systems have been devised for detecting L-asparaginase activity (for examples see; Drainas and Drainas, 1985; Frohwein *et al.*, 1971; Jayaram *et al.*, 1974; Rekharsky *et al.*, 1988; Takenaka *et al.*, 1971; Wade and Phillips, 1971), none has lead to the innovation of a direct plate visualisation technique for detecting enzyme activity. Indeed, for many years it has been recognised that until such a technique became available, genetic studies concerning L-asparaginase II regulation would be drastically hindered by the necessity of performing innumerable time consuming biochemical assays (Jeffries, 1976).

To date, the fundamental problem outlined above remains. However, the advent of gene fusion technology has now provided a means by which it can be overcome. Consequently, the aim of the work described in the following sections of this chapter was to use ansB gene fusions to facilitate the study of L-asparaginase II regulation. The long-term goal was to use such fusions to isolate mutants of Er. chrysanthemi NCPPB 1066 altered in ansB gene expression, and to analyse these at a molecular level. Such a 'black box' approach would hopefully lead to the identification of regulatory genes, and help decipher some of the regulatory mechanisms governing the expression of the Er. chrysanthemi NCPPB 1066 ansB structural gene.

7.1.2 Approach

Gene fusion technology has proven to be an invaluable tool, not only for the genetic analysis of biological regulatory mechanisms, but also for

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protein localisation and cellular differentiation studies in both prokaryotic and eukaryotic systems, as well as protein purification (for reviews see; Benson *et al.*, 1985; Guarente, 1984; Manoil *et al.*, 1990; Silhavy and Beckwith, 1985). Conventionally this approach involves the fusion of an assayable, selectable 'reporter' gene to the promoter and controlling regions of the particular gene under study. As a result, the expression of the 'reporter' gene is placed under regulatory control of the gene of interest. Hence, by assaying the activity of the 'reporter' gene, the expression of the hybrid gene can be monitored, which in turn reflects the activity of the exogenous promoter.

The 'reporter' gene may be used in either transcriptional (operon) fusions, where it retains its own translational start but is dependent on the attached DNA for transcription or in translational (protein) fusions, where both transcription and translation are dependent on signals in the attached upstream DNA. Although a variety of 'reporter' genes have been used in the construction of gene fusions, the most commonly used have been the lacZ gene of E. coli, encoding the cytoplasmic enzyme B-galactosidase (B-D-galactoside galactohydrolase, EC 3.2.1.23), and the phoA gene of E. coli, encoding for the periplasmic enzyme alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1). This popularity has been mainly due to that fact that both gene products are stable in many cellular backgrounds and can be precisely quantitated by a very simple and sensitive colorimetric assay. Moreover, one can extend all the experimental convenience of the lacZ or phoA genetic system to the gene under study. In particular, a variety of sophisticated genetic techniques are available, including indicator and selective media, which can be employed for the identification and/or selection of mutations that alter expression of the fused 'reporter' gene.

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Aside from the similarities outlined above, one important fundamental difference between alkaline phosphatase and B-galactosidase is the fact that the former enzyme is inactive in the cytoplasm. This is believed to be a result of the fact that it must dimerise to be active, and the environment in the cytoplasm of the bacterial cell is too reducing for the formation of the disulphide bond necessary for such a process to occur. Therefore, active phoA fusions are only obtained with genes encoding membrane-spanning or periplasmic proteins (Reuber et al., 1991). In contrast, B-galactosidase is only active in the cytoplasm, and attempts by the cell to export a hybrid LacZ protein leads to a loss of B-galactosidase activity and can be lethal to the cell. This phenotype is known as 'over-production lethality', and is thought to be a consequence of the cell's abortive attempts to export the B-galactosidase portion of the hybrid protein. The hybrid protein is thought to contain sequences that effectively 'jam' the cell export machinery, thus hindering normal protein export. When the hybrid protein is highly expressed, such inhibition of normal export is lethal (Palva and Silhavy, 1984).

Gene fusions may be constructed in vivo by spontaneous non-homologous recombination or semi site-specific transposon recombination; or in vitro with recombinant DNA technology. In particular, derivatives of bacteriophage Mu (Bremer et al., 1984,1985; Casadaban and Cohen, 1979; Casadaban and Chou, 1984), and the transposons Tn917 (Youngman et al., 1985) and Tn5 (Kroos and Kieser, 1984; Manoil, 1990; Manoil and Beckwith, 1985) have been widely used in this approach. In certain instances, there is a possibility that in vivo generated fusions may be rendered unstable, due to transposase functions of both Mu and the transposon derivatives remaining intact upon insertion. This could potentially lead to secondary transposition events which would complicate subsequent studies. Alternatively, potentially more stable fusions

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can be created in vitro from defined restriction fragments with the aid of recombinant DNA technology.

The initial sections of this chapter describe the construction of a series of ansB fusion vectors using both in vivo and in vitro techniques. In the present study, the Tn5 derivatives TnphoA (Manoil and Beckwith, 1985) and TnlacZ-B20 (Simon et al., 1989), were chosen to create random insertions within the Er. chrysanthemi NCPPB 1066 ansB structural gene (Figure 7.1). When TnphoA integrates within a gene in the correct orientation and reading frame, a hybrid protein is generated. In contrast, TnlacZ-B20 forms transcriptional fusions upon insertion, and therefore need not be inserted in the correct reading frame to produce B-galactosidase activity.

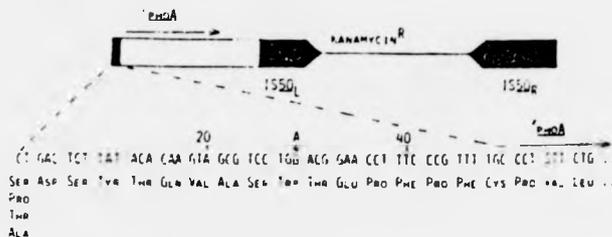
Both of the aforementioned transposon derivatives may be efficiently delivered into E. coli on a defective lambda suicide vehicle, which contains a nonsense mutation within a gene essential for bacteriophage replication. Such a bacteriophage can propagate in a suppressing (Su⁺) host, but is unable to establish itself in a non-suppressing (Su⁰) host (Gutierrez et al., 1987).

7.2 ISOLATION OF IN VIVO-GENERATED ansB GENE FUSIONS

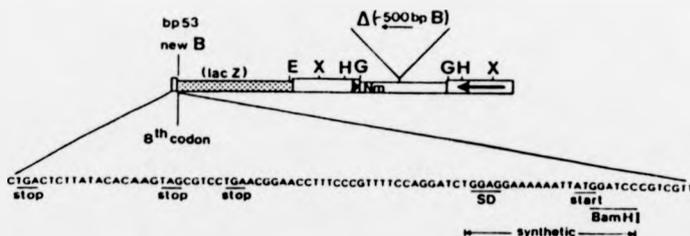
7.2.1 Methods

7.2.1.1 TnphoA insertion into the ansB expression vector pCK1

A 10ml culture of E. coli CC118, harbouring the plasmid pCK1, was grown in LB medium supplemented with 0.2% (w/v) maltose, 10mM MgSO₄ and Ap (50ug ml⁻¹), to an OD₆₀₀ value of 0.6. Cells were centrifuged in a Wifug Labor 50M bench centrifuge (4,500 rpm, 10 mins) and resuspended in 1ml of the same medium. Lambda TnphoA was then added at an m.o.i. of

Figure 7.1 Structure of *TnphoA* and *TnlacZ-B20*

Detailed structure of *TnphoA* : There are 50bp of DNA at the beginning of the *phoA* coding region. Of this sequence, 48bp derive from *IS50L* of transposon *Tn5* and 2 bp originate from the *PstI* linker of plasmid pCH39 (Hoffman and Wright, 1985). The amino acid residues that are shown, are present at the fusion joint of every hybrid protein generated. The DNA sequence differs from that of the *IS50L* region of *Tn5* by an A-to-G change at position 29 (Auerswald *et al.*, 1980). This substitution eliminates the opal nonsense codon in-frame with the *phoA* coding sequence. All of *phoA* is encoded except the signal sequence and 5 amino acid residues of the mature protein (Inouye *et al.*, 1982).



Detailed structure of *TnlacZ-B20* : The internal *BamHI* site of the transposon *Tn5* (encompassing approximately 500bp to its left end and including the central *XhoI* and *PstI* site) is deleted (Δ). The *BamHI* site at nucleotide position 53, results from the insertion of a *BamHI* fragment containing a promoterless *lacZ* gene into to the former *Sau3A* site at this position. A synthetic 21 bp DNA fragment has been cloned into the aforementioned *BamHI* site which contains the translational initiation sites (SD) (Shine-Dalgarno region) and ATG initiation codon. Stop codons are present in all three reading frames within the remaining *IS50L* region upstream of *lacZ* gene (Auerswald *et al.*, 1980).

7. Gene fusions

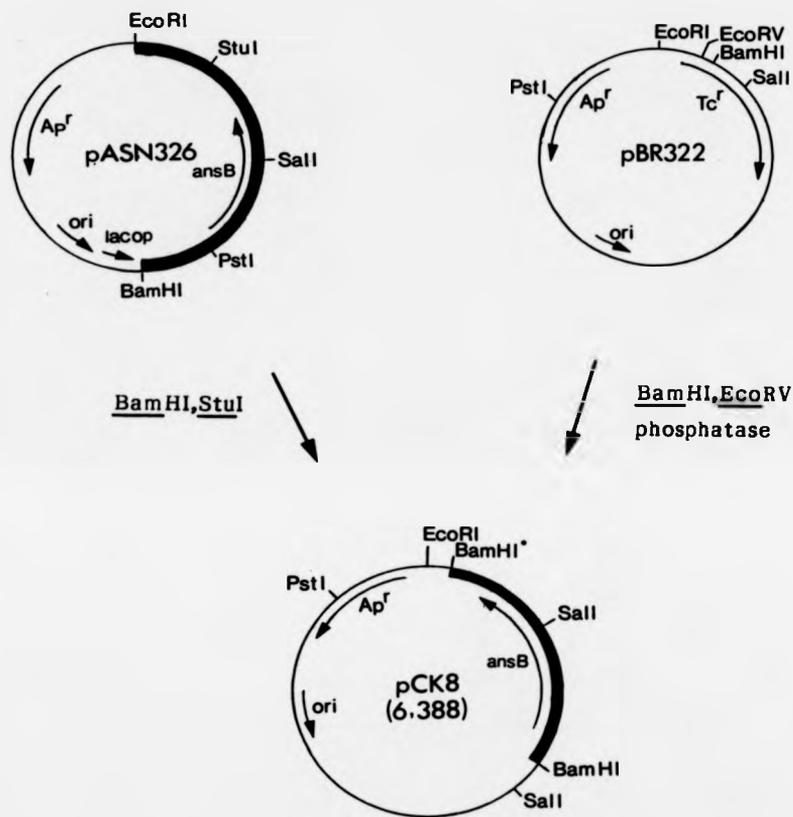
1.0, and the culture incubated statically at 30°C for 30 mins to allow bacteriophage absorption and DNA injection. Following this period, cells were diluted 10-fold with fresh LB medium and incubated with shaking at 30°C for 1 hour. The culture was subsequently concentrated 10-fold as described above, and 100ul aliquots spread-plated onto LB agar containing Ap (50ug ml⁻¹), Kn (300ug ml⁻¹) and X-P (40ug ml⁻¹); along with uninfected and lysate alone controls. The high concentration of kanamycin in this medium enriches for cells carrying insertions of the transposon into the multicopy plasmid (Berg *et al.*, 1983).

Following 2 days of incubation at 37°C, blue colonies displaying alkaline phosphatase activity were detected and streak-purified onto the same medium. Plasmid DNA was then prepared from 8 recombinants on a small-scale, following an additional day of growth at 37°C.

7.2.1.2 TnlacZ-B20 insertions into the ansB expression vector pCK8

Since lacZ does not need to be integrated into a gene coding for an exported protein to generate an hybrid protein with B-galactosidase activity; theoretically, the transposon TnlacZ-B20 could create active fusions at more sites within an ansB expression vector. Therefore, to increase the chance of transposon insertion into the Er. chrysanthemi NCPPB 1066 ansB structural gene, as well as simplify restriction mapping, plasmid pCK8 was constructed as outlined in Figure 7.2. Essentially, this plasmid was identical to pCK1, except that the majority of the shikimate kinase structural gene, situated immediately downstream from the Er. chrysanthemi NCPPB 1066 ansB gene, was deleted.

A 2.2kb BamHI/StuI fragment from pASN326 was subcloned into pBR322, which had been previously cleaved with a combination of BamHI/EcoRV

Figure 7.2 In vitro construction of plasmid pCK8

The asterisk indicates a new BamHI site created by the fusion of the EcoRV and StuI termini.

7. Gene fusions

restriction endonucleases and dephosphorylated. Restriction mapping revealed that the fusion of the EcoRV and StuI termini resulted in the formation of a new BamHI site.

E. coli CC118 cells harbouring pCK8, were infected with lambda TnlacZ-B20 at a m.o.i. of 1.0, as described in Section 7.2.1.1. Cells were then spread-plated onto LB medium supplemented with Ap (50ug ml⁻¹), Kn (300ug ml⁻¹) and X-gal (40ug ml⁻¹), along with uninfected and lysate alone controls. Following incubation at 37°C for 2 days, blue colonies displaying B-galactosidase activity were streak-purified on the same medium. In this instance, plasmid DNA was extracted from 32 recombinants on a small-scale after an additional day of growth at 37°C.

7.3 RESULTS AND DISCUSSION

7.3.1 Restriction mapping of TnphoA and TnlacZ insertions

The presence of a transposon within plasmids pCK1 and pCK8 was initially confirmed by restriction endonuclease analysis using BamHI. To ascertain the position and orientation of the transposon insertion, further digests were performed using BamHI, EcoRI, Sall, PstI and HindIII restriction endonucleases, alone or in combination. On the basis of this, plasmid pCK9 was identified as containing a single TnphoA insertion, approximately 500 bp within the Er. chrysanthemi NCPPB 1066 ansB structural gene. A second plasmid, pCK10, was identified as having a single TnlacZ insertion around 700 bp within the ansB structural gene (Figure 7.3). Both plasmids were subsequently retained for further study.

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Figure 7.3 Restriction endonuclease analysis of the *in vivo* - generated ansB fusion plasmids pCK9 and pCK10

Plasmid DNA was digested to completion with a variety of restriction endonucleases and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis.

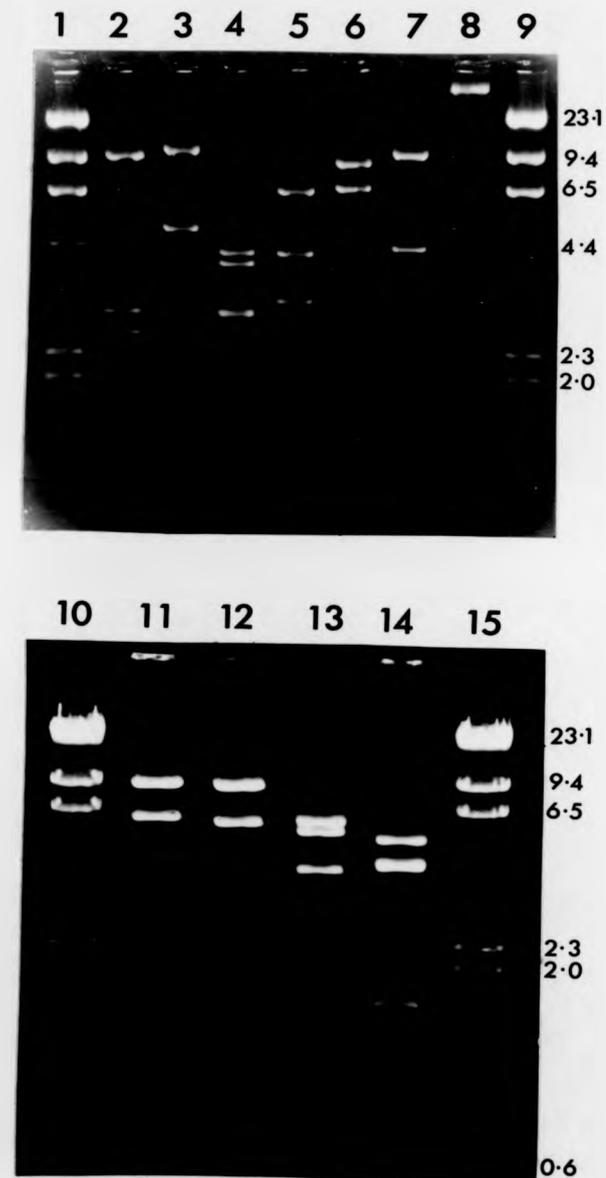
The running order is as follows :

- Track 1 : lambda DNA digested with HindIII
- Track 2 : plasmid pCK10 DNA digested with HindIII
- Track 3 : plasmid pCK10 DNA digested with SalI
- Track 4 : plasmid pCK10 DNA digested with PstI
- Track 5 : plasmid pCK10 DNA digested with BamHI and EcoRI
- Track 6 : plasmid pCK10 DNA digested with EcoRI
- Track 7 : plasmid pCK10 DNA digested with BamHI
- Track 8 : supercoil pCK10 DNA
- Track 9 : lambda DNA digested with HindIII

- Track 10 : lambda DNA digested with HindIII
- Track 11 : plasmid pCK9 DNA digested with BamHI
- Track 11 : plasmid pCK9 DNA digested with EcoRI
- Track 12 : plasmid pCK9 DNA digested with SalI
- Track 13 : plasmid pCK9 DNA digested with BamHI and EcoRI
- Track 14 : lambda DNA digested with HindIII

7. Gene fusions

Figure 7.3



7. Gene fusions

7.3.2 Sequence analysis of pCK9 and pCK10

As stated previously, TnphoA must insert itself in the correct orientation and reading frame to create an active fusion. Recently, however, Gott and Boos (1988) have shown TnphoA to be capable of generating active fusions, even when inserted out of frame by +1. These authors suggested that this is due to so-called 'in vivo frameshifting' (Craigien and Caskey, 1987; Roth, 1981), where the translational machinery corrects the wrong frame by reading a sequence of four nucleotides as a sense codon.

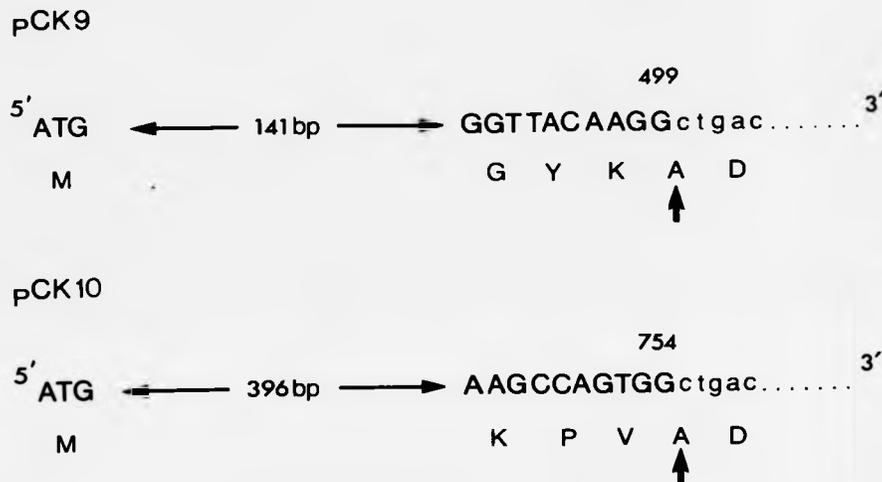
In order to locate the exact position of TnphoA in pCK9 and to determine whether an in-frame fusion had been created, a 1.2 kb Clal fragment spanning the fusion junction was cloned into M13 mp19, previously digested with AccI and dephosphorylated. Likewise, the fusion junction in pCK10 was cloned into BamHI dephosphorylated M13 mp19 on a 1.1 kb BamHI fragment. Sequencing of single-stranded DNA across the fusion joint was primed by a synthetic 19-mer oligonucleotide (Primer 1; Table 2.7) which was complimentary to nucleotides 10 to 28 in the intragenic IS50L region between the ansB gene and either phoA or lacZ. The results are summarised in Figure 7.4. Transposon TnphoA was found to have inserted in-frame adjacent to nucleotide 499 of the published Er. chrysanthemi NCPPB 1066 ansB sequence, whilst transposon TnlacZ was positioned adjacent to nucleotide 754.

7.3.3 Western blot analysis of pCK9

From the sequence data derived in Section 7.3.2, it was predicted that plasmid pCK9 would encode for a chimaeric protein of 54 kDa, assuming that that (1) the average molecular weight of one amino acid residue is 110 (Boquet et al., 1985), (2) the phoA gene in TnphoA encodes a protein of 450

7. Gene fusions

Figure 7.4 The nucleotide sequence across the fusion junction of plasmid pCK9 and pCK10



A portion of the amino terminal region of the *Er. chrysanthemi* NCPPB 1066 *ansB* structural gene and a portion of the IS50 L region of the Tn5 derivative TnphoA or TnlacZ-B20, is shown. Capital letters represent the *ansB* coding region, whereas lower case letters represent the IS50 L region. The predicted translated polypeptide product is represented by the single letter amino acid code, with each amino acid symbol positioned below the middle nucleotide of the relevant codon. The amino acid found at the fusion junction is indicated by an arrow. The number above the nucleotide sequence denotes the position within the published *ansB* sequence (Minton *et al.*, 1986).

7. Gene fusions

amino acids in length, and (3) correct processing of the AnsB signal peptide takes place when the hybrid protein is exported to the periplasm of the bacterial cell. Western blot analysis of pCK9 (Figure 7.5) revealed two over-expressed protein species which cross-reacted with anti-alkaline phosphatase antibody. The larger protein migrated at 57 kDa, whilst the smaller protein had an apparent molecular mass of 54 kDa. Since the molecular mass between these 2 species was approximately 2 kDa, the size of the AnsB 21 amino acid signal peptide, it is possible that they represent pro AnsB-PhoA and mature AnsB-PhoA fusion proteins, respectively. A minor band of approximately 47 kDa was also detected. Bla-PhoA hybrid proteins have been reported to be relatively unstable and slowly degraded to fragments of around 47 kDa, the size of mature alkaline phosphatase (Manoil and Beckwith, 1985), as have App-PhoA chimaeras (Boquet *et al.*, 1987).

7.4 IN VITRO CONSTRUCTION OF ansB GENE FUSIONS

The following sections of this chapter describe the in vitro construction of ansB::phoA and ansB::lacZ gene fusion vectors. In this instance, such plasmids were designed so that the 'reporter' gene was 'tagged' by an antibiotic-resistance marker, and that both genes were flanked by at least 500 bp of Erwinia DNA.

7.4.1 The in vitro constructed ansB::lacZ fusion vector pCK11

The in vitro construction of the ansB::lacZ transcriptional fusion vector pCK11, is schematically represented in Figure 7.6. The first phase involved the cleavage of pCK8 with the restriction endonucleases AvaI and SphI, in order to delete the Sall site situated in the pBR322 backbone of the vector. This would then leave a unique Sall site within the ansB structural gene.

7. Gene fusions

Figure 7.5 Identification of the fusion protein from the *in vivo*-generated ansB::phoA fusion plasmid pCK9

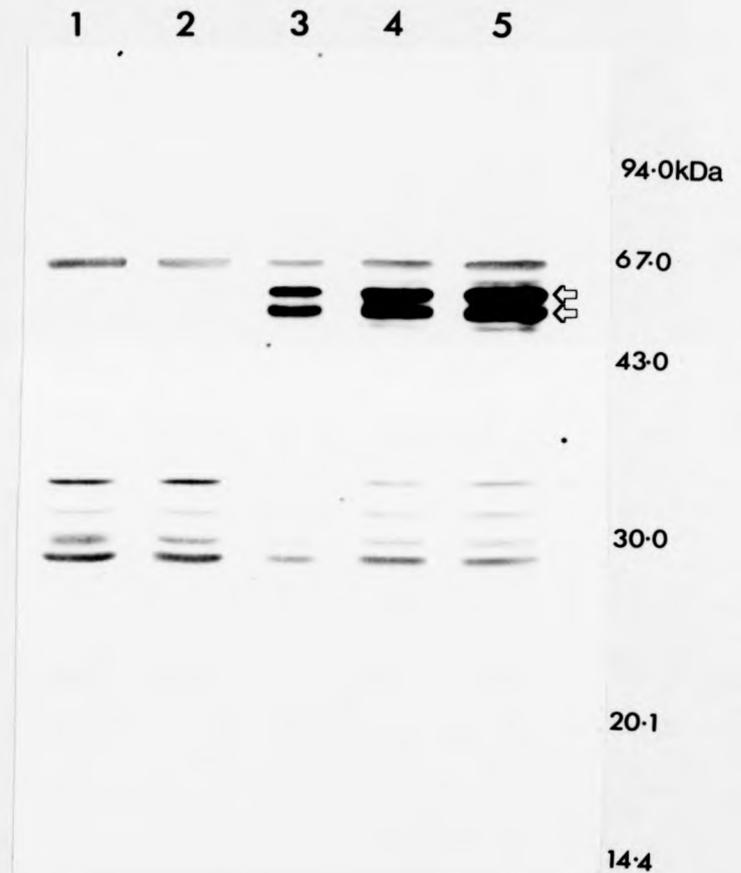
Total cell protein (Section 2.5.1) was separated by electrophoresis through an 11% (w/v) linear SDS polyacrylamide gel and immunoblotted with Rabbit anti-alkaline phosphatase antibody, as described in Section 2.5.4.

The running order is as follows :

- Track 1 : E. coli CC118 alone (10ul sample)
- Track 2 : E. coli CC118 harbouring pCK1 (10ul sample)
- Track 3 : E. coli CC118 harbouring pCK9 (10ul sample)
- Track 4 : E. coli CC118 harbouring pCK9 (20ul sample)
- Track 5 : E. coli CC118 harbouring pCK9 (30ul sample)

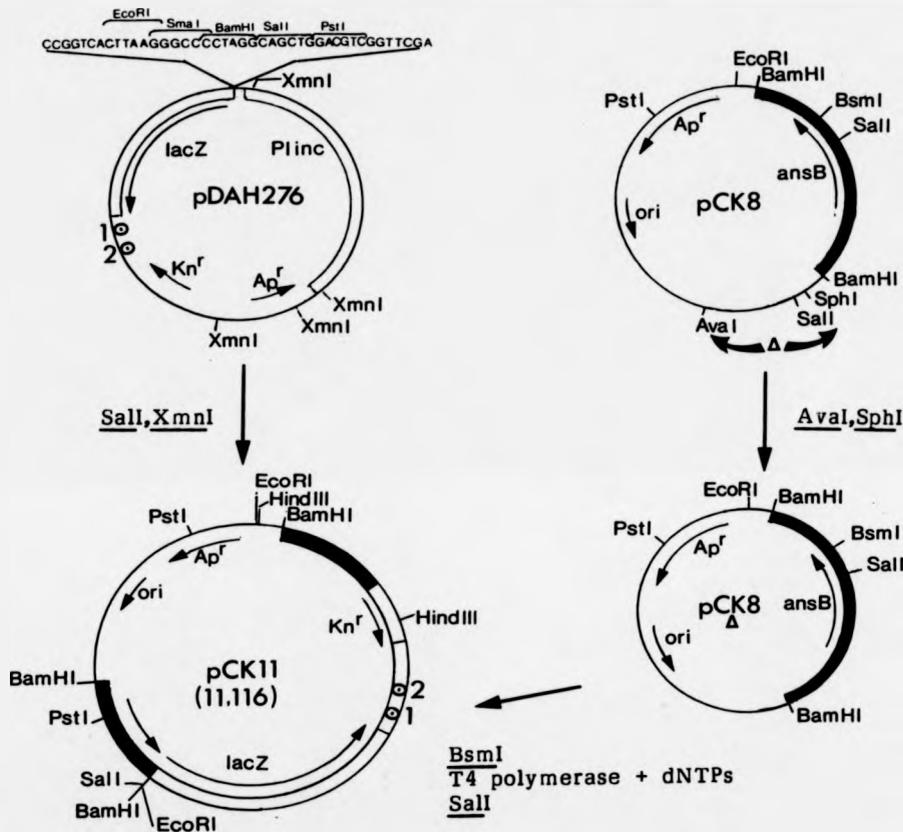
7. Gene fusions

Figure 7.5



7. Gene fusions

Figure 7.6 In vitro construction of the ansB::lacZ fusion vector, pCK11



The lacZ promoter probe vector pDAH276 was chosen as the source of the promoterless lacZ structural gene and Kn^r-resistance gene (originating from transposon Tn903). This plasmid was originally constructed by removing the promoterless lacZ structural gene on a blunted HindIII fragment from plasmid pDAH216 (Scanlan *et al.*, 1990), and subsequently cloning this into plasmid pDAH211 (Scanlan *et al.*, 1990), previously restricted with BamHI and EcoRI (ends filled-in). The region between the lacZ gene and the Kn^r gene contains translational terminators, as well as two transcriptional terminators that are represented by the symbol \odot . The terminator labelled 1 is a B. subtilis alpha-amylase transcriptional terminator, whereas the terminator labelled 2 is a bacteriophage Fd transcriptional terminator. The Kn^r-resistance gene, Ap^r-resistance gene and the ori region originate from the cloning vector pACYC177 (Chang and Cohen, 1978) (Dr. D. Hodgson; personal communication).

7. Gene fusions

Restricted DNA was size-fractionated by agarose gel electrophoresis, and the 5.54 kb vector fragment purified by electro-elution. Termini were blunted using T4 polymerase and then self-ligated. The ligation products were used to transform competent *E. coli* DH1 cells, and transformants selected on LB medium supplemented with Ap (50ug ml⁻¹). Plasmid DNA was subsequently isolated on a small-scale from four Ap^r clones, and found to be linearised after *Sall* digestion. One of the appropriate plasmids was kept for large-scale isolation of DNA and designated pCK8 delete.

To complete the cloning strategy, the promoter probe plasmid pDAH276 (kindly donated by Dr. D.A. Hodgson) was restricted with a combination of *XmnI/Sall* restriction endonucleases, whilst plasmid pCK8 delete was linearised with *BsmI*, blunted using T4 polymerase, and then cleaved with *Sall*. Restriction products from each reaction were subsequently size-fractionated by agarose gel electrophoresis. The 5.84 kb *XmnI/Sall* product from pDAH276 containing the *lacZ* and Kn^r genes, and the 5.29 kb blunt/*Sall* fragment from pCK8 delete were isolated by electro-elution and ligated. The ligation products were introduced into *E. coli* CC118 cells by transformation and transformants subsequently selected on LB medium supplemented with Ap (50ug ml⁻¹), Kn (50ug ml⁻¹) and X-gal (40ug ml⁻¹). Plasmid DNA was extracted on a small-scale from 6 blue Ap^rKn^r colonies, and subjected to restriction endonuclease analysis to confirm the fusion of *lacZ* to the amino terminus of the *Er. chrysanthemi ansB* structural gene. An appropriate plasmid was subsequently retained for the large scale isolation of DNA, and designated pCK11 (Figure 7.7).

7.4.2 The *in vitro* constructed *ansB::phoA* fusion vector pCK12

The *in vitro* constructed *ansB::phoA* translational fusion vector pCK12,

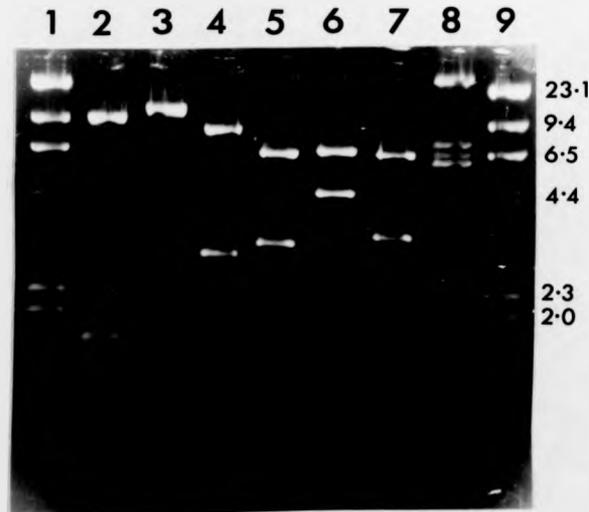
7. Gene fusions

Figure 7.7 Restriction endonuclease analysis of in vitro-generated ansB::lacZ fusion plasmid pCK11

Plasmid DNA was digested to completion with a variety of restriction endonucleases and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis.

The running order is as follows :

- Track 1 : lambda DNA digested with HindIII
- Track 2 : plasmid pCK11 DNA digested with HindIII
- Track 3 : plasmid pCK11 DNA digested with Sall
- Track 4 : plasmid pCK11 DNA digested with PstI
- Track 5 : plasmid pCK11 DNA digested with BamHI and EcoRI
- Track 6 : plasmid pCK11 DNA digested with EcoRI
- Track 7 : plasmid pCK11 DNA digested with BamHI
- Track 8 : supercoil plasmid pCK11 DNA
- Track 9 : lambda DNA digested with HindIII



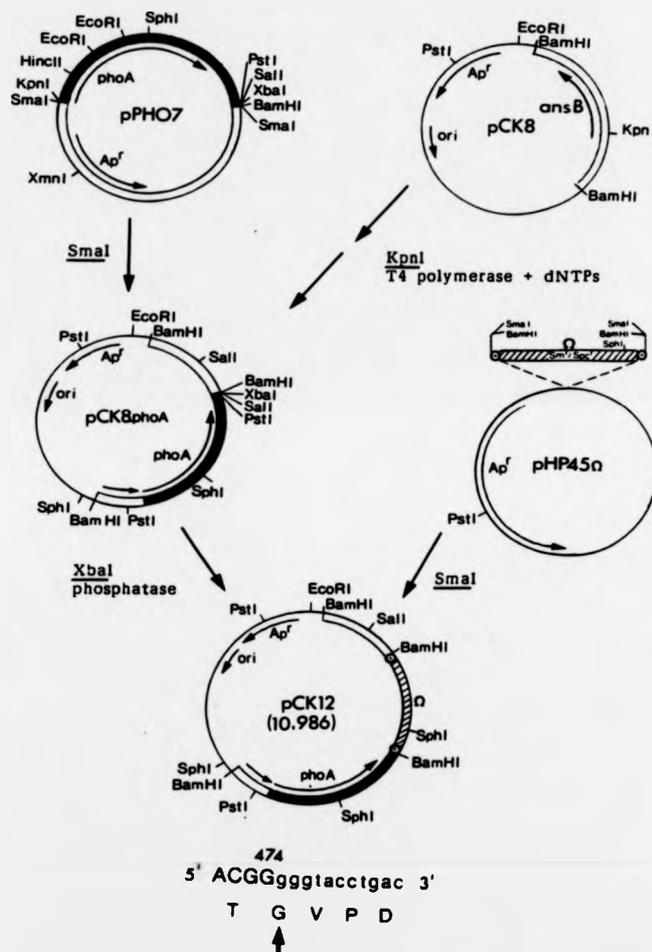
7. Gene fusions

was constructed in two stages following the strategy shown in Figure 7.8. Plasmid pPHO7 (Gutierrez and Devedjian, 1989) was cleaved with SmaI to release a fragment of 2.6 kb containing the promoterless phoA structural gene. Simultaneous treatment with XmnI reduced the size of the 2.9 kb pTZ18R vector region and therefore aided the separation of DNA for purification. The phoA region was then ligated to plasmid pCK8, previously linearised with KpnI and blunted with T4 polymerase. The ligation products were used to transform competent E. coli CC118 cells, and transformants selected on LB medium containing Ap (50ug ml⁻¹) and X-P (40ug ml⁻¹). Plasmid DNA was isolated on a small-scale from six blue Ap^r clones and digested with KpnI. Restriction products were then analysed by agarose gel electrophoresis, to corroborate the joining of phoA to the amino terminal region of the ansB structural gene. One of the appropriate plasmids was retained and designated pCK8 phoA. Double-stranded sequencing across the fusion junction was achieved as described in Section 2.3.22.4, using primer 2 which annealed 32 bp upstream of the blunted KpnI site. The resulting sequence is shown in Figure 7.8

The omega fragment from plasmid pHP45 omega (Prentki and Krisch, 1984) was chosen to 'tag' the in vitro fusion. This 2.0kb DNA segment has been widely used as an interposon for the in vitro insertional mutagenesis of genes. It consists of the Sm^R/Sp^R aadA gene of the R100.1 plasmid, flanked by short inverted repeats carrying transcriptional/translational terminators and synthetic restriction site polylinkers. Plasmid pHP45 omega was digested with SmaI and the products resolved on a preparative agarose gel. The 2.0 kb omega fragment was extracted from the gel by electro-elution and then ligated with pCK8 phoA, which had been linearised with XbaI and dephosphorylated. The ligation products were used to transform competent E.

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Figure 7.8 In vitro construction of the ansB::phoA fusion vector, pCK12



The nucleotide sequence across the ansB::phoA fusion junction in plasmid pCK8 phoA. Capital letters represent the ansB gene and lower case letters represent the phoA fragment from plasmid pPHO7. The number above the nucleotide sequence indicates the position within the published sequence (Minton *et al.*, 1986). Amino acid residues are represented by the single letter code and are beneath the middle nucleotide of the relevant codon. The residue at the fusion point is highlighted by an arrow.

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coli CC118 cells and subsequent selection on LB agar supplemented with Ap(50ug ml⁻¹), Sm (20ug ml⁻¹), and X-P (40ug ml⁻¹) yielded blue transformants, six of which were employed for the small-scale isolation of plasmid DNA. Restriction endonuclease analysis with HindIII, followed by size-fractionation of the products by agarose gel electrophoresis confirmed that the desired construct had been obtained. SphI digestion revealed that the omega fragment had been cloned in both orientations. Two appropriate plasmids were retained for the large-scale isolation of DNA, and designated pCK12 omega1 and pCK12 omega2, respectively (Figure 7.9).

7.5 CREATION OF A SINGLE COPY FUSION DERIVATIVE OF ER.

CHRYSANTHEMI NCPPB 1066

Since the ultimate aim of the present study was to carry out work concerning the regulation of L-asparaginase II synthesis within Er. chrysanthemi NCPPB 1066 itself, attempts were to be made to introduce fusion plasmids into Er. chrysanthemi NCPPB 1066. Although many regulatory studies have involved the use of multicopy fusion plasmids, a single copy derivative of Er. chrysanthemi NCPPB 1066 was desirable for a number of reasons. Firstly, fusions carried on multicopy plasmids can be subject to several intrinsic problems which cause misleading or erroneous results that no longer accurately reflect the normal level of transcription of the test sequence. For example, plasmid copy number can vary with growth conditions, size of DNA insert, the strength of the cloned promoter (Adams and Hatfield, 1984), as well as in the presence of other plasmids. Secondly, certain complete or truncated gene products can be detrimental to the cell when expressed at high levels from multicopy plasmids, thereby providing a selective pressure for reduced expression of the gene or reduction in plasmid

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Figure 7.9 Restriction endonuclease analysis of the *in vitro*-generated *angB::phoA* fusion vector pCK12 omega 1 and pCK12 omega 2

Plasmid DNA was digested to completion with a variety of restriction endonucleases and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis.

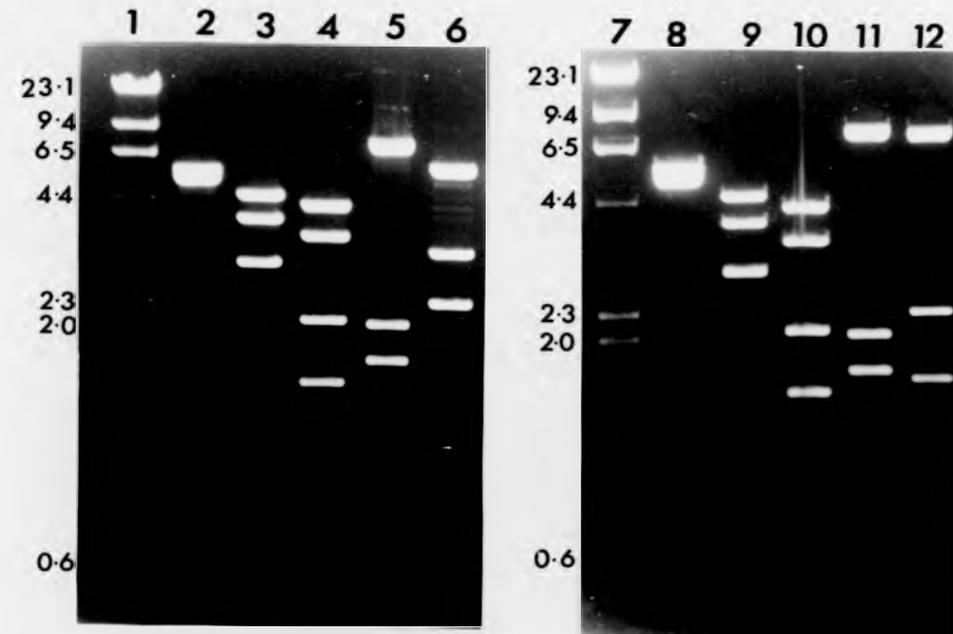
The running order is as follows :

- Track 1 : lambda DNA digested with HindIII
- Track 2 : plasmid pCK12 omega 1 DNA digested with EcoRI
- Track 3 : plasmid pCK12 omega 1 DNA digested with PstI
- Track 4 : plasmid pCK12 omega 1 DNA digested with BamHI
- Track 5 : plasmid pCK12 omega 1 DNA digested with HindIII
- Track 6 : plasmid pCK12 omega 1 DNA digested with SphI

- Track 7 : lambda DNA digested with HindIII
- Track 8 : plasmid pCK12 omega 2 DNA digested with EcoRI
- Track 9 : plasmid pCK12 omega 2 DNA digested with PstI
- Track 10 : plasmid pCK12 omega 2 DNA digested with BamHI
- Track 11 : plasmid pCK12 omega 2 DNA digested with HindIII
- Track 12 : plasmid pCK12 omega 2 DNA digested with SphI

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Figure 7.9



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copy number. Finally, multiple copies of some genes, or over-expression of their products, could potentially titrate regulatory components present in low numbers in the cell, and thus lead to abnormal expression.

7.5.1 Approach

The strategy for creating a single copy derivative of Er. chrysanthemi NCPPB 1066 was based on de-stabilising the fusion vector so that it is lost from the cell. During the process, homologous recombination would hopefully occur between the Erwinia sequences on the plasmid and the equivalent regions on the Er. chrysanthemi NCPPB 1066 chromosome at a defined frequency. This so-called 'exchange-recombination' would thus result in disruption of the chromosomal ansB structural gene, leaving a single copy of the fusion within the bacterial cell.

Jones and colleagues (1980) have previously reported that the plasmid pBR322 is not stably maintained in chemostat cultures of E. coli under phosphate-limited growth conditions, and postulated that the cause was due to a lack of an efficient segregation mechanism in pBR322. Under normal growth conditions, the high plasmid copy number ensures continued distribution among the daughter cells. Under limited growth conditions, however, the plasmid copy number is reduced, thus diminishing the probability that the plasmid will be distributed to both daughter cells during cell division. Subsequent work carried out by Roeder and Collmer (1985) demonstrated that pBR322 could be readily lost from Er. chrysanthemi CUCPB 1237 when small batch cultures were grown in phosphate-limited medium, and, by taking advantage of this effect, these workers obtained high frequencies of exchange-recombination without introduction of an incompatible plasmid (Ruvkun et al., 1981), or prior subcloning into a plasmid

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unable to replicate in the host organism (Comai et al., 1983; Van Haute et al., 1983).

As a consequence of the above, a similar approach was to be used in an attempt to construct a single copy fusion derivative of Er. chrysanthemi NCPPB 1066. In this instance, attempts would be made to introduce the ansB::lacZ fusion plasmids pCK10 and pCK11 into the EMS-generated LacZ⁻ derivatives, CK1000 and CK2000 (see Section 3.3.1.4), since it was anticipated that at a later stage, such hosts would facilitate the selection of ansB 'down' mutants on solid media.

7.5.2 Introduction of pCK10 and pCK11 into Er. chrysanthemi NCPPB 1066 LacZ⁻ derivatives CK1000 and CK2000

Plasmid pCK10 and pCK11 DNA was used to transform competent E. coli GJ342 cells, and transformants selected on NB agar containing Ap (50ug ml⁻¹), Cm (50ug ml⁻¹) and Kn (300ug ml⁻¹ for pCK10 or 50ug ml⁻¹ for pCK11 selection). Cells displaying Ap, Cm, Tc and Kn-resistance were isolated and subsequently patch-mated with wild-type Er. chrysanthemi NCPPB 1066 LacZ⁻ derivative CK1000 and industrial morphotype 2 LacZ⁻ derivative CK2000 at both 30°C and 37°C, as described in Section 2.2.4. Bacteria were then harvested into 1ml of phosphate buffered saline, and serial dilutions spread-plated onto minimal sucrose selection medium supplemented with Ap (50ug ml⁻¹), Kn (300 or 50ug ml⁻¹) and X-gal (40ug ml⁻¹). Growth was then allowed to proceed at 30°C and 37°C.

Following 3 days of incubation at both temperatures, small pale blue Ap^r Kn^r colonies were detected on selective medium. Representative colonies were streak-purified onto the same medium, and all were subsequently found to be Tc^s and Cm^s, indicating the absence of the 'helper' plasmids

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pR64drd11 and pLVC9, and gave distinct halos on Pel, Cel and Prt extracellular enzyme detection medium. Small-scale analysis followed by agarose gel electrophoresis, revealed the presence of plasmid DNA within isolates. This was subsequently verified by the fact that DNA preparations were able to transform E. coli DH1 cells to Ap^r and Kn^r. DNA was extracted from E. coli DH1 transformants and restricted with EcoRI, HindIII and PstI restriction endonucleases. The products were then analysed by 0.7% (w/v) TAE agarose gel electrophoresis to verify plasmid structure and origin.

Representative clones of CK1000 and CK2000, containing pCK10 and pCK11 were grown in LB medium with Ap (50ug ml⁻¹) and Kn (300ug ml⁻¹ or 50ug ml⁻¹) to an OD₆₀₀ value of between 0.7 and 0.8 (cultures were found not to grow to higher cell densities), and assayed for B-galactosidase activity, as described in Section 2.2.20.1. The results are shown in Table 7.1, where it can be seen that slightly higher levels were detected within CK2000 recombinants. These formed noticeably smaller colonies on solid medium and appeared to grow at a slower rate in liquid medium compared with CK1000 recombinants, although this latter feature was not quantified.

From the above evidence, it was concluded that ansB::lacZ fusion plasmids pCK10 and pCK11 had been successfully introduced into CK1000 and CK2000. Since plasmid pCK11 was potentially more structurally stable than pCK10, due to the fact that TnlacZ-B20 could potentially transpose randomly onto the chromosome upon plasmid loss; CK1000 and CK2000 derivatives harbouring the former plasmid were initially chosen for low phosphate treatment. These were designated CK1001 and CK2001, respectively.

7.5.3 Low phosphate treatment of CK1001 and CK2001

Strains CK1001 and CK2001 were grown in low phosphate medium

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Table 7.1 B-galactosidase activity of pCK10 and pCK11 in Er. chrysanthemi NCPPB 1066 LacZ⁻ derivatives CK1000 and CK2000.

Strain	Plasmid	B-galactosidase activity*
CK1000	pCK10	5,135
CK1000	pCK11	4,667
CK2000	pCK10	6,831
CK2000	pCK11	6,915

Cultures were grown in LB medium containing Ap (50ug ml⁻¹) and Kn (300 or 50ug ml⁻¹) until an OD₆₀₀ value of 0.7 - 0.8 was reached.

* B-galactosidase activity is expressed as Miller units. Assays were performed in duplicate on two separate cultures. Values represent the mean.

Table 7.2 Low phosphate treatment of recombinant derivatives CK1001 and CK2001.

Strain	Viable count (cfu ml ⁻¹)		
	LB	LB Ap	LB Kn
CK1001	5.0 x 10 ⁶	1.8 x 10 ²	5.4 x 10 ⁶
CK2001	3.2 x 10 ⁶	3.0 x 10 ⁶	3.4 x 10 ⁶

Following 32 hours growth in low phosphate Kn medium, cultures were serially-diluted and spread-plated in duplicate onto selective media. Ampicillin-resistant colonies were taken to represent cells that had retained the plasmid, pCK11.

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containing Kn (50ug ml^{-1}) for 32 hours, as described in Section 2.2.15. Cells were then serially-diluted in phosphate buffered saline, and 100ul samples of each dilution spread-plated onto LB agar alone, or onto LB agar containing either Ap (50ug ml^{-1}) or Kn (50ug ml^{-1}). The results are summarised in Table 7.2, where it can be seen that for the CK1001 culture, the number of Ap^{r} cells within the population had dropped four logs whilst 100% still retained the Kn^{r} marker. In contrast, 100% of the CK2001 culture still possessed both antibiotic-resistance markers. This suggested that exchange-recombination had taken place for CK1001 but not for CK2001. Repeated rounds of low phosphate treatment failed to cure CK2001 of plasmid. The reason for the difference in plasmid stability between the two strains is unknown at present (additional experimentation showed that pCK11 was also retained 100% in industrial morphotypes 3, 4 and 5 when grown for 14 days under low phosphate conditions); however, at this point it was decided to concentrate on the $\text{Ap}^{\text{s}} \text{Kn}^{\text{r}}$ derivatives of CK1001 to prove that exchange-recombination had indeed taken place.

7.5.4 Evidence for exchange-recombination in CK1001

Colonies from the LB Ap serial dilution plates of the experiment described above, were patched onto LB Kn medium and all (50/50) were found to display Kn-resistance. Similarly, colonies from the LB Kn serial dilution plates were patched onto LB Ap medium. However, in contrast none were found to be Ap^{r} . The following tests were subsequently performed on a number of $\text{Ap}^{\text{s}} \text{Kn}^{\text{r}}$ colonies.

7.5.4.1 Resistance to B-aspartylhydroxamate

L-asparaginase is able to catalyse the conversion of aspartic

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hydroxymate to asparagine and hydroxylamine (Ehrman *et al.*, 1971). Because the latter product is extremely toxic to the cell (Drainas *et al.*, 1977), it was possible that disruption of the Er. chrysanthemi *ansB* gene following exchange-recombination would confer resistance to the lethal analogue B-aspartylhydroximate, even though the low affinity cytoplasmic L-asparaginase I still existed. To test this hypothesis, 40 Ap^S Kn^r isolates were streaked to single colonies on minimal sucrose medium containing 4mM B-aspartylhydroximate, along with wild-type Er. chrysanthemi NCPPB 1066 and CK1000 controls. Following 48 hours incubation at 30°C, good growth leading to single colonies of 2 - 3mm diameter was detected for all single copy candidates. No growth was seen on control plates even after 7 days, indicating that the above hypothesis was correct.

7.5.4.2 Presence of plasmid DNA

To verify that Ap-sensitivity was in fact a result of plasmid loss, rather than deletion or mutation of the plasmid-borne Ap-resistance gene, small-scale analysis of 12 Ap^S Kn^r candidates was performed. CK1000 and CK1001 were studied in parallel as negative and positive pCK11 controls, respectively. DNA preparations were used to transform competent E. coli DH1 cells, and simultaneously analysed by 0.7% (w/v) TAE agarose gel electrophoresis. No plasmid DNA could be visually detected for the single copy candidate samples. Moreover, only the CK1001 sample was found to transform E. coli DH1 to Ap and Kn-resistance.

7.5.4.3 B-galactosidase activity

B-galactosidase assays were performed on 5 of the single copy candidates, along with CK1000 as a control. In this instance, cultures were

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grown to an OD₆₀₀ value of between 1.8 and 2.0 in LB medium supplemented with Kn (50ug ml⁻¹) if required, and sampled in duplicate. From Table 7.3, it can be seen that B-galactosidase was synthesised at significantly higher levels compared with the LacZ⁻ control parent strain. Indeed, this was also much higher than wild-type levels (Table 3.4).

7.5.4.4 Growth and maintenance of the Kn-resistance marker

Another line of evidence in favour of exchange-recombination was gleaned from the fact that the single copy candidates under study grew to a much higher cell density than the recombinant derivative CK1001, and that even in the absence of antibiotic selection, 100% of the population maintained Kn^r. This was in contrast to the observation that CK1001 readily lost Kn^r and Ap^r when cultured in the absence of antibiotics (Table 7.4).

7.5.4.5 Southern and Western blot analysis

Chromosomal DNA was extracted from four Ap^s Kn^r candidates as described in Section 2.3.3, and digested with the restriction endonuclease EcoRI. The products were then separated by agarose gel electrophoresis alongside wild-type Er. chrysanthemi NCPPB 1066 chromosomal DNA, also cleaved with the same enzyme. Intact pBR322 and EcoRI digested pCK10 DNA was included as hybridisation controls. DNA was probed with a ³²P-radiolabelled 2.2kb BamHI fragment from pCK8, harbouring the entire Er. chrysanthemi NCPPB 1066 ansB structural gene. The results are shown in Figure 7.10, where it is evident that a single hybridising chromosomal restriction fragment was detected for wild-type Er. chrysanthemi NCPPB 1066; whereas two such fragments were visible for the single copy fusion candidates. Figure 7.11 summarises the interpretation of this data, which

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Table 7.3 B-galactosidase activity within single copy candidates

Strain	B-galactosidase activity*
Candidate 1	1,396
Candidate 2	1,095
Candidate 3	1,398
Candidate 4	1,433
Candidate 5	1,494
CK1000	ND

* B-galactosidase activity expressed as Miller units
 ND denotes that enzyme activity was not detected

Table 7.4 Retention of the Kn-resistance marker in the single copy candidate 1 and derivative CK1001 when grown in the absence of antibiotic selection.

Strain	Viable count (cfu ml ⁻¹)		
	LB	LB Kn	LB Ap
Single-copy candidate 1	1.05 x 10 ⁸	1.02 x 10 ⁸	ND
CK1001	2.90 x 10 ⁷	3.63 x 10 ⁴	3.38 x 10 ⁴

Cultures were grown in LB medium overnight without antibiotic selection, and serially-diluted. Samples (100ul) were then spread-plated onto LB and LB Kn (50ug ml⁻¹) medium in duplicate. CK1001 was also plated onto LB Ap (50ug ml⁻¹)
 ND indicates that spread-plating was not performed

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Figure 7.10 Southern analysis of four *Er. chrysanthemi* NCPPB 1066 single copy *ansB::lacZ* fusion candidates

Intact chromosomal DNA was digested to completion with the restriction endonuclease *EcoRI* and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. DNA was then transferred to a nylon membrane and probed with a ³²P-radiolabelled 2.2kb *Bam*HI fragment from plasmid pCK8, harbouring the *Er. chrysanthemi* NCPPB 1066 *ansB* structural gene, overnight at 65°C in 1 X 'Blotto'. The membrane was subsequently washed at 55°C in 2 X SSC and subjected to autoradiography at -70°C.

The running order is as follows :

Track 1 : wild-type *Er. chrysanthemi* NCPPB 1066 chromosomal DNA digested with *EcoRI*

Track 2 : single copy fusion candidate 1 chromosomal DNA digested with *EcoRI*

Track 3 : single copy fusion candidate 2 chromosomal DNA digested with *EcoRI*

Track 4 : single copy fusion candidate 3 chromosomal DNA digested with *EcoRI*

Track 5 : single copy fusion candidate 4 chromosomal DNA digested with *EcoRI*

Track 6 : no DNA sample

Track 7 : plasmid pBR322 supercoil DNA

Track 8 : plasmid pCK11 DNA digested with *EcoRI*

Track 9 : lambda DNA digested with *Hind*III

Tracks 10 to 18 are autoradiograms of corresponding tracks 1 to 9 probed with the 2.2kb *Bam*HI *ansB* fragment from pCK8.

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Figure 7.10

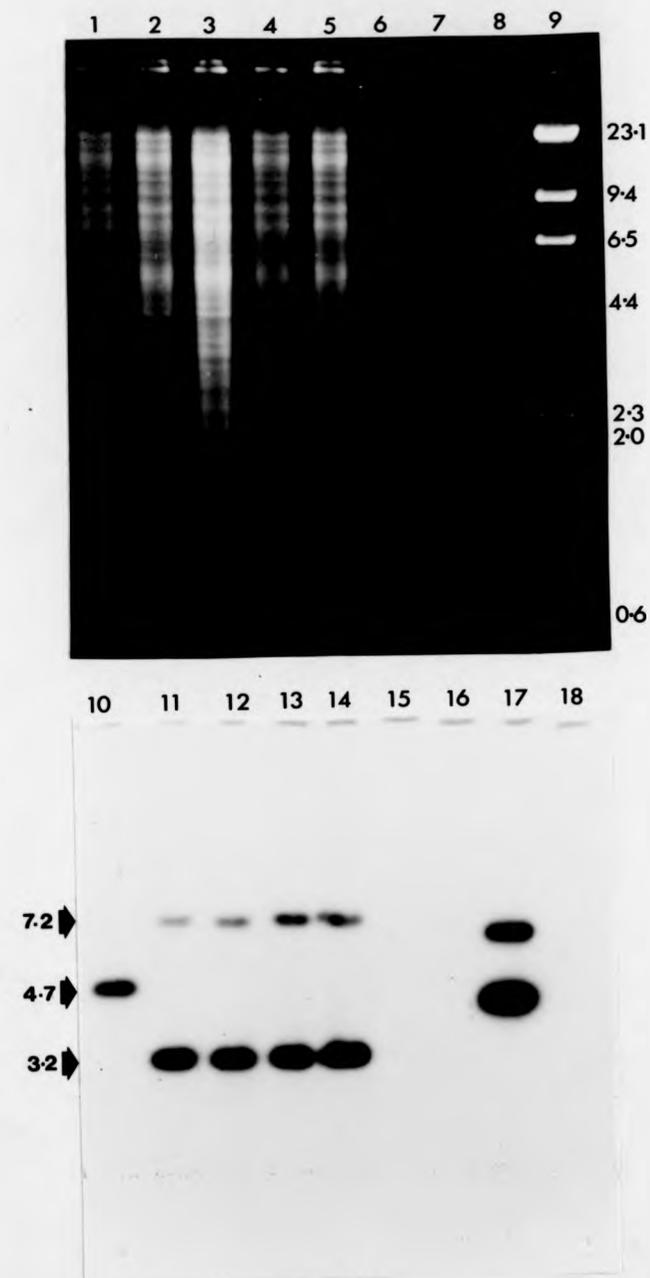
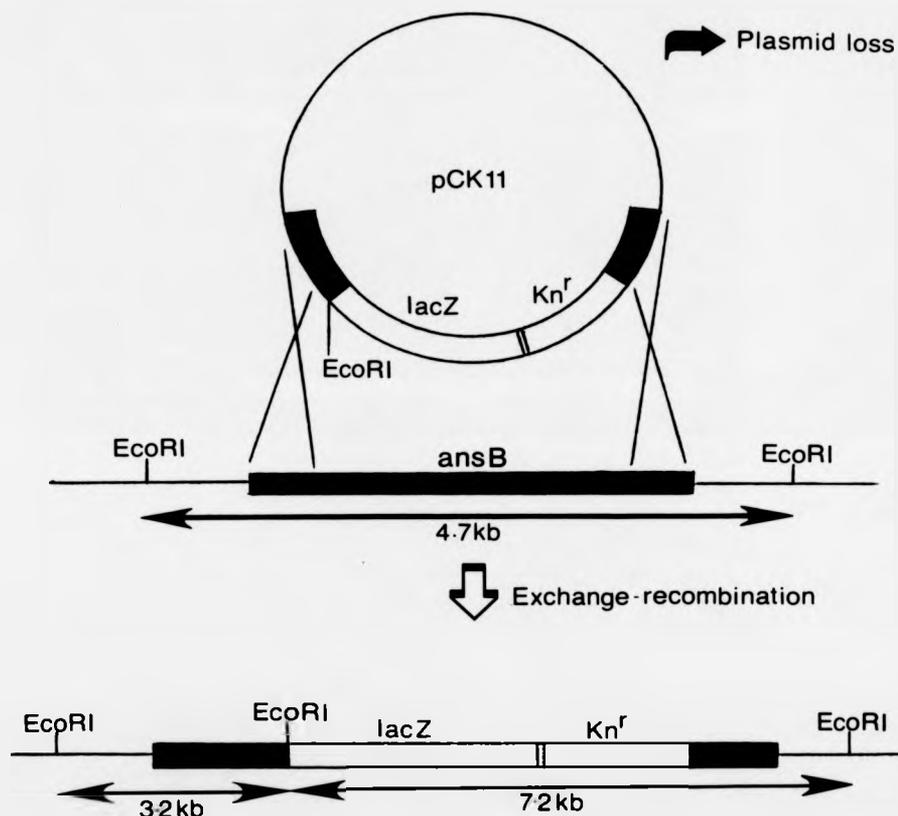


Figure 7.11 Interpretation of the data obtained from Southern analysis of the *Er. chrysanthemi* NCPPB 1066 ansB::lacZ candidates 1 - 4



Integration of the ansB::lacZ transcriptional fusion and the *Kn^r* gene from plasmid pCK11 into the wild-type *Er. chrysanthemi* NCPPB 1066 ansB structural gene within the genome introduces a single *EcoRI* site into this region. Restriction of chromosomal DNA with *EcoRI* will therefore result in the generation of two ansB containing fragments, rather than a single ansB fragment of 4.7 kb (Gilbert *et al.*, 1986).

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provided direct physical evidence to suggest that exchange-recombination had taken place.

Candidate 1 was retained for further experimentation and was designated CK1002. Western blot analysis of this derivative was performed as outlined in Section 2.5.4 and the results are presented in Figure 7.12. An over-expressed cross-reacting protein, which co-migrated with purified E. coli B-galactosidase (lane 1), was clearly visible in both the Western blot and silver-stained SDS polyacrylamide gel. This was consistent with the fact that the constructed ansB::lacZ fusion from pCK11 was of a transcriptional nature.

7.5.4.6 L-asparaginase II activity

Finally, CK1002 was subjected to automated L-asparaginase II assay in parallel with the isolates listed in Table 3.4, and was found not to contain detectable levels of enzyme activity.

7.6 COMMENTS

The above lines of evidence (i.e. loss of plasmid marker and retention of Kn-resistance, loss of B-aspartylhydroxymate sensitivity, disruption of the ansB structural gene, loss of L-asparaginase II activity and over-production of B-galactosidase) strongly suggested that a single copy fusion derivative of Er. chrysanthemi NCPPB 1066 had been constructed. This strain was to form the basis for the following studies aimed at isolating and characterising mutants affected in ansB expression.

7.7 ISOLATION AND CHARACTERISATION OF EXPRESSION MUTANTS OF ER. CHRYSANTHEMI NCPPB 1066 derivative CK1002

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Figure 7.12 Western analysis of wild-type *Er. chrysanthemi* NCPPB 1066 derivative CK1002

Cell-free extracts (15ug) were separated by electrophoresis through an 11 % (w/v) linear SDS polyacrylamide gel and immunoblotted with Rabbit anti-B-galactosidase antibody, as described in Section 2.5.4.

The running order is as follows :

Track 1 : *E. coli* B-galactosidase standard (Sigma Grade X)

Track 2 : CK1002 extract

Track 3 : CK1000 extract

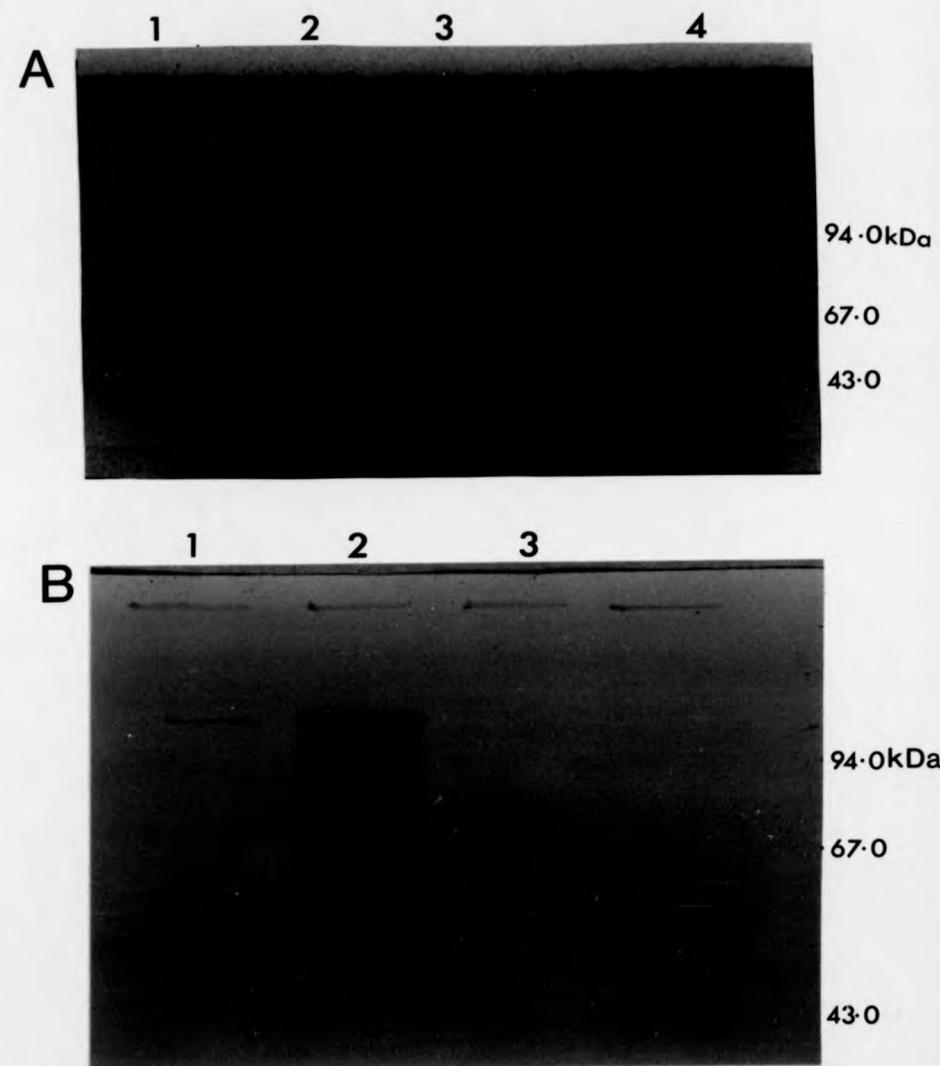
Track 4 : Pharmacia protein standards

Plate A shows a silver stain of the polyacrylamide gel following the electro-transfer of protein onto nitrocellulose filter.

Plate B shows the membrane following immuno-staining. The multiple bands visible below the B-galactosidase protein band in lane 2, are taken to represent proteolysis or degradation products that have arisen during sample preparation.

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Figure 7.12



7. Gene fusions

7.7.1 EMS mutagenesis of derivative CK1002

Because transposon mutagenesis systems were still at a rudimentary stage, it was decided to treat derivative CK1002 with EMS and to screen mutagenised cultures on solid medium containing Kn and X-gal. Clearly, it was recognised that as a result, characterisation of any mutants would be more complicated. However, EMS does have the advantage of generating subtle mutations, such as temperature-sensitive mutations, that cannot be attained with transposons. It was hoped that mutations would be generated in the ansB promoter region, or in genes governing ansB expression.

EMS mutagenesis was performed on 8 separate occasions, as described in Section 2.17, except that the procedure was scaled down so that 100ul of EMS was added to 6ml of culture maintained within a 100ml conical flask. Following mutagenesis, cells from segregated and non-segregated cultures were plated onto NB agar supplemented with Kn (50ug ml⁻¹) and X-gal (40ug ml⁻¹), followed by a 2 day incubation at 30°C. Twenty-four white colonies displaying a marked 'down' phenotype were isolated and streak-purified on the same medium. The isolates are listed in Table 7.5 and corresponded to population survivals ranging between 24 and 55%. All mutants were found to be prototrophic, and were not markedly affected in growth, as compared with the CK1002 parental derivative.

The lacZ 'down' expression mutations may have arisen for a number of reasons. Firstly, mutations may have occurred within the catalytic site of the B-galactosidase polypeptide, resulting in the synthesis of a full length but non-active protein. Secondly, nonsense mutations may have occurred causing non-functional truncated gene products, or thirdly, insertions or deletions may have taken place to inactivate enzyme activity (the lacZ gene is prone to

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Table 7.5 EMS-generated LacZ⁻ 'down' expression mutants of the Er. chrysanthemi NCPPB 1066 single copy ansB::lacZ fusion derivative CK1002

Mutant	Mutagenesis no.	Segregated [@]	B-galactosidase activity
CK1004	1	-	ND
CK1041	1	-	2.4
CK1042	1	-	ND
CK1044	1	+	16.1
CK1054	1	+	9.6
CK1063	1	+	1.7
CK1077	4	-	10.1
CK1080	2	+	1.5
CK1082	2	+	5.8
CK1084	3	+	ND
CK1085	3	+	ND
CK1091	2	+	ND
CK1092	8	-	1.8
CK1093	8	-	9.6
CK1094	8	-	7.2
CK1095	8	-	3.2
CK1096	8	-	2.5
CK1101	7	-	3.0
CK1108	7	+	4.4
CK1112	6	+	1.3
CK1113	7	+	2.1
CK1116	5	+	2.3
CK1117	8	+	ND
CK1119	5	+	5.0

* Cultures were grown in LB Kn (50ug ml⁻¹) to an OD₆₀₀ value of 1.11 - 1.35 and sampled in triplicate. Activity is expressed as Miller units. Values represent the mean.

@ A + symbol denotes that mutants were derived from cells segregated overnight following mutagenesis. The symbol - denotes that mutants were isolated from non-segregated cells plated directly onto the selective medium following mutagenesis.

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insertion by insertion (IS) elements; Dr. D. Hodgson, personal communication). More interestingly, some mutants may represent promoter or regulatory gene mutations that had the effect of reducing the level of transcription from the ansB promoter. In order to characterise the aforementioned mutants further, Southern and Western blot analysis was carried out, as described below.

7.7.2 Characterisation of the lacZ expression mutants of CK1002

Southern analysis was performed, as described in Section 7.5.4.5 and the results shown in Figure 7.13 (a and b). In all cases, the restricted chromosomal DNA isolated from the LacZ⁻ mutants, gave rise to hybridising bands which were indistinguishable in size compared to the hybridising bands seen for CK1002 chromosomal DNA. This implied that no gross insertion or deletion event had taken place.

Cell-free protein extracts were prepared as described in Section 2.5.1, and size-fractionated by SDS polyacrylamide gel electrophoresis. Blots were reacted with anti- B-galactosidase antibody and immuno-stained, as outlined in Section 2.5.4. In all cases, samples from the derivatives CK1000 and CK1002 were included as negative and positive LacZ controls, respectively.

The results are highlighted in Figure 7.14 (a and b), where it can be seen that mutants CK1112, CK1044, CK1063, CK1093, and CK1101 produced similar levels of B-galactosidase polypeptide, which co-migrated with the LacZ protein synthesised by the CK1001 control. This strongly implies that these particular isolates had undergone mutations within the lacZ structural gene leading to a loss of catalytic activity of the LacZ enzyme. Mutants CK1092, CK1095, CK1119, CK1004, CK1042, CK1054, CK1085 and CK1116 on the other hand, clearly synthesised truncated forms of the LacZ protein,

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Figure 7.13 (a) Southern analysis of EMS-generated *lacZ*⁻ 'down' expression mutants of *Er. chrysanthemi* NCPPB 1066 derivative CK1002

Intact chromosomal DNA was digested to completion with *EcoRI* and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. DNA was then transferred to nylon membrane and probed with a ³²-P radiolabelled 2.2 kb *Bam*HI fragment from plasmid pCK8, harbouring the *Er. chrysanthemi ansB* structural gene, overnight at 65°C in 1 X 'Blotto'. The membrane was subsequently washed at 55°C in 2 X SSC and subjected to autoradiography at -70°C.

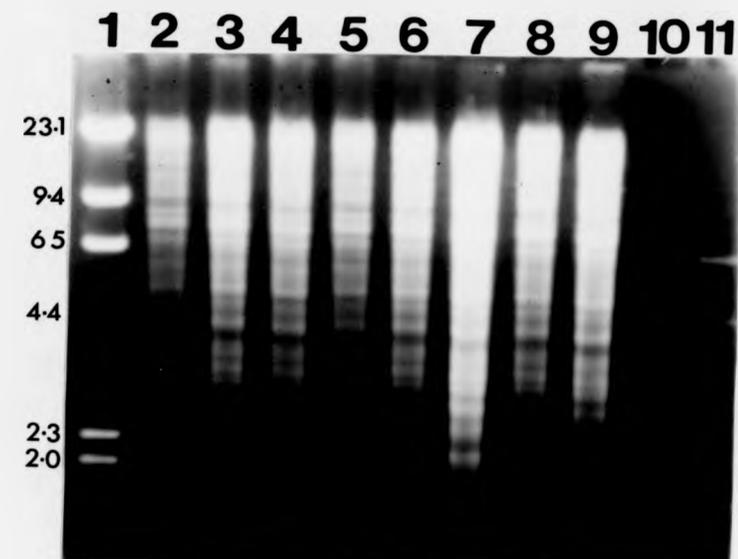
The running order is as follows :

- Track 1 : lambda DNA digested with *Hind*III
- Track 2 : wild-type *Er. chrysanthemi* NCPPB 1066 DNA digested with *EcoRI*
- Track 3 : CK1002 DNA digested with *EcoRI*
- Track 4 : CK1091 DNA digested with *EcoRI*
- Track 5 : CK1084 DNA digested with *EcoRI*
- Track 6 : CK1096 DNA digested with *EcoRI*
- Track 7 : CK1082 DNA digested with *EcoRI*
- Track 8 : CK1113 DNA digested with *EcoRI*
- Track 9 : CK1108 DNA digested with *EcoRI*
- Track 10 : plasmid pBR322 supercoil DNA
- Track 11 : plasmid pCK11 DNA digested with *EcoRI*

Tracks 12 to 21 are autoradiograms of corresponding tracks 2 to 11 probed with the 2.2 kb *Bam*HI *ansB* fragment from pCK8.

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Figure 7.13 (a)



12 13 14 15 16 17 18 19 20 21

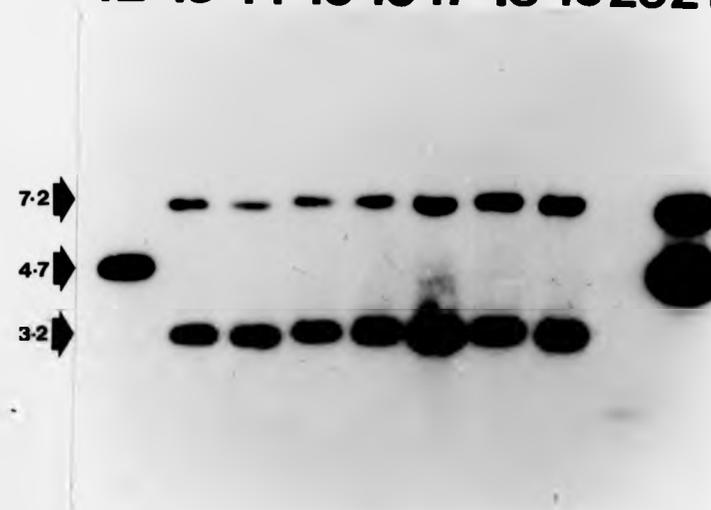


Figure 7.13 (b) Southern analysis of EMS-generated *lacZ*⁻ 'down' expression mutants of *Er. chrysanthemi* NCPPB 1066 derivative CK1002

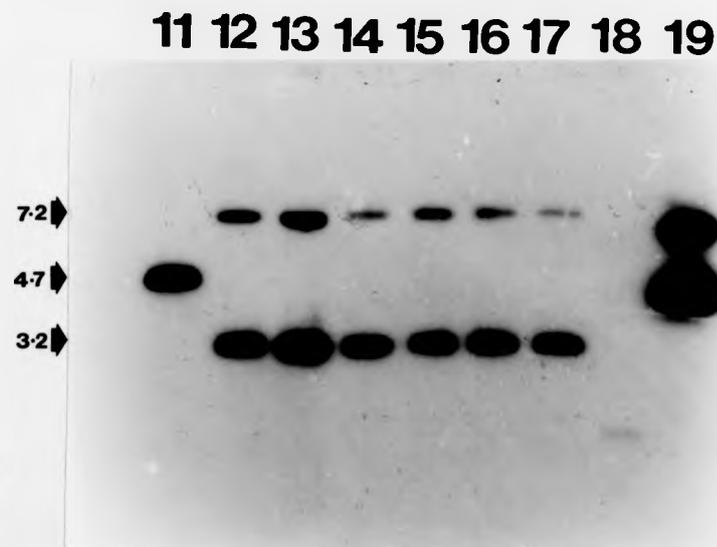
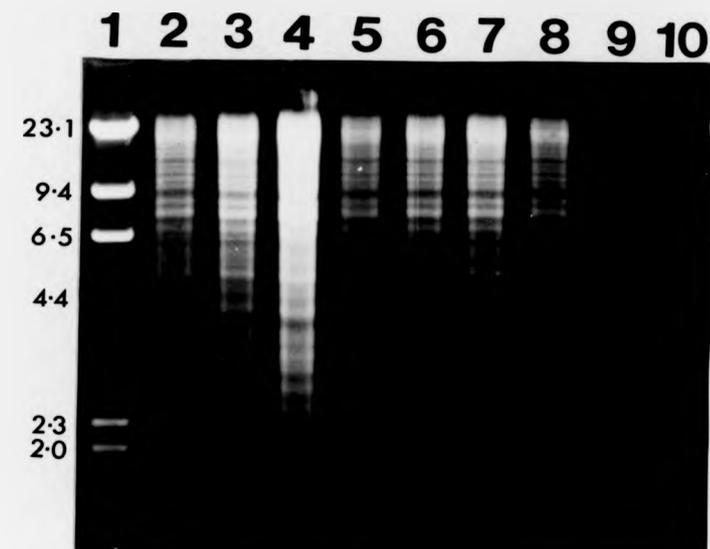
Intact chromosomal DNA was digested to completion with *Eco*RI and the products size-fractionated by 0.7% (w/v) TAE agarose gel electrophoresis. DNA was then transferred to nylon membrane and probed with a ³²-P radiolabelled 2.2 kb *Bam*HI fragment from plasmid pCK8, harbouring the *Er. chrysanthemi ansB* structural gene, overnight at 65°C in 1 X 'Blotto'. The membrane was subsequently washed at 55°C in 2 X SSC and subjected to autoradiography at -70°C.

The running order is as follows :

- Track 1 : lambda DNA digested with *Hind*III
- Track 2 : wild-type *Er. chrysanthemi* NCPPB 1066 DNA digested with *Eco*RI
- Track 3 : CK1002 DNA digested with *Eco*RI
- Track 4 : CK1094 DNA digested with *Eco*RI
- Track 5 : CK1077 DNA digested with *Eco*RI
- Track 6 : CK1041 DNA digested with *Eco*RI
- Track 7 : CK1117 DNA digested with *Eco*RI
- Track 8 : CK1080 DNA digested with *Eco*RI
- Track 9 : plasmid pBR322 supercoil DNA
- Track 10 : plasmid pCK11 DNA digested with *Eco*RI

Tracks 11 to 19 are autoradiograms of corresponding tracks 2 to 10 probed with the 2.2 kb *Bam*HI *ansB* fragment from pCK8.

Figure 7.13 (b)



7. Gene Fusions

Figure 7.14 (a) Western analysis of EMS-generated *lacZ*⁻ 'down' expression mutants of *Er. chrysanthemi* NCPPB 1066 derivative CK1002

Cell-free extract (30ug) was electrophoresed through an 11 % (w/v) linear SDS polyacrylamide gel and immunoblotted with Rabbit anti-B-galactosidase antibody, as described in Section 2.5.4.

The running order is as follows :

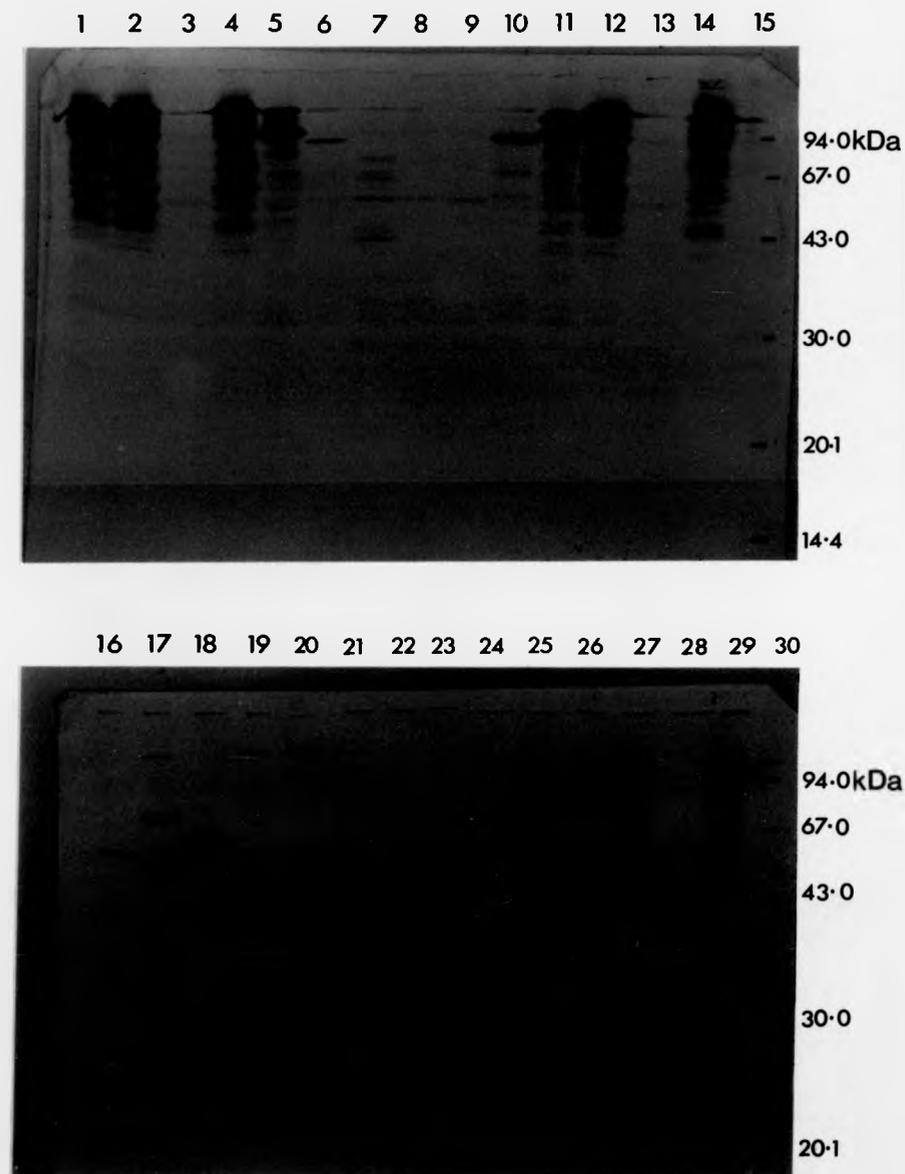
Track 1 : CK1112 extract
Track 2 : CK1044 extract
Track 3 : CK1094 extract
Track 4 : CK1063 extract
Track 5 : CK1054 extract
Track 6 : CK1077 extract
Track 7 : CK1041 extract
Track 8 : CK1117 extract
Track 9 : CK1080 extract
Track 10 : CK1085 extract
Track 11 : CK1116 extract
Track 12 : CK1002 extract
Track 13 : CK1000 extract
Track 14 : *E. coli* B-galactosidase standard (Sigma Grade X)
Track 15 : Pharmacia protein standards

Track 16 : CK1084 extract
Track 17 : CK1092 extract
Track 18 : CK1095 extract
Track 19 : CK1096 extract
Track 20 : CK1082 extract
Track 21 : CK1113 extract
Track 22 : CK1108 extract
Track 23 : CK1091 extract
Track 24 : CK1119 extract
Track 25 : CK1004 extract
Track 26 : CK1042 extract
Track 27 : CK1002 extract
Track 28 : CK1000 extract
Track 29 : *E. coli* B-galactosidase standard (Sigma Grade X)
Track 30 : Pharmacia protein standards

The multiple bands visible below the B-galactosidase protein band in a number of tracks, is taken to represent proteolysis or degradation products that have arisen during sample preparation.

7. Gene fusions

Figure 7.14 (a)



7. Gene Fusions

Figure 7.14 (b) Western analysis of EMS-generated *lacZ*⁻ 'down' expression mutants of *Er. chrysanthemi* NCPPB 1066 derivative CK1002

Cell-free extract (40ug) was electrophoresed through an 11 % (w/v) linear SDS polyacrylamide gel and immunoblotted with Rabbit anti-B-galactosidase antibody, as described in Section 2.5.4.

The running order is as follows :

Track 1 : Pharmacia protein standards

Track 2 : *E. coli* B-galactosidase standard (Sigma Grade X)

Track 3 : CK1000 extract

Track 4 : CK1002 extract

Track 5 : CK1093 extract

Track 6 : CK1101 extract

Track 7 : no protein sample

Track 8 : Pharmacia protein standards

Multiple bands seen below the B-galactosidase protein band are taken to represent degradation or proteolysis products which have arisen during sample preparation.

7. Gene fusions

Figure 7.14 (b)



7. Gene fusions

which may have arisen from nonsense mutations. Indeed, it can be seen for isolates CK1004 and CK1054 that a small but significant amount of full length protein is produced. This is a common phenomenon with weak nonsense suppression (Dr. G.P.C. Salmond; personal communication). Interestingly, for mutant CK1116, two bands of similar intensity are seen. The upper band is not full length, and therefore two mutations could have arisen. It is possible that the first mutation is being suppressed; however, read-through is arrested at the second mutation further downstream, that cannot be suppressed.

The aforementioned isolates were not studied further, but the remaining 11 isolates, CK1084, CK1094, CK1096, CK1082, CK1113, CK1108, CK1091, CK1077, CK1041, CK1117 and CK1080 were potentially more interesting because of the vastly reduced levels of B-galactosidase protein. This could have been due to extreme amino terminal lacZ mutations, or mutations in the ansB gene itself. Of the latter, some could possibly occur in the promoter or controlling regions, thereby reducing the levels of transcription. Alternatively, these isolates may represent mutations which have reduced the half-life of the lacZ messenger RNA.

A closer examination of the ansB promoter region of the aforementioned 11 lacZ 'down' mutants, was performed using the polymerase chain reaction (PCR) (Ehrlich, 1989; Innis *et al.*, 1990). A comparison of the size of the amplified PCR products from each of the mutants with the size of the amplified PCR product from wild-type *Er. chrysanthemi* NCPPB 1066 control DNA, would perhaps give a better indication of whether smaller deletions not detectable by Southern analysis, had taken place. Moreover, this technique would provide the most direct route to obtaining DNA for sequence analysis.

In this instance, primer 3 and primer 5 (see Table 2.7) were chosen to

7. Gene fusions

anneal to intact chromosomal DNA, separated by a distance of 344bp which covered the promoter region of the ansB structural gene. The polymerase chain reaction was carried out as described in Section 2.3.14, and the results are shown in Figure 7.15. In all cases, a PCR product of the predicted size was generated. Hence, it was concluded that a deletion of greater than 50bp within the promoter region of the ansB gene could not account for the 'down' phenotype of the aforementioned EMS mutants.

7.7.3 Complementation studies

In addition to the work described above, efforts were also made to determine whether the 'down' phenotype of the remaining 11 EMS-generated lacZ⁻ mutants was a result of a mutation at an extragenic site. If this was the case, then theoretically such a mutation would be complemented in trans by the cosmid library constructed as described in Section 5.3.2.

The amplified cosmid library of wild-type Er. chrysanthemi NCPPB 1066 genomic DNA was transduced into E. coli GJ342 as described in Section 2.2.10.1, and cells selected on LB medium containing Ap (50ug ml⁻¹), Tc (10ug ml⁻¹), and Cm (50ug ml⁻¹). Over 10,000 Ap^r Tc^r Cm^r colonies were pooled into 10ml of LB medium, and 100ul of the resulting suspension mixed with 1ml of fresh overnight culture of each of the 11 aforementioned 'down' mutants. Patch-matings were then set up on LB agar and incubation allowed to proceed at 30°C overnight. Transconjugants were harvested into 1ml of phosphate buffered saline and then serially-diluted onto LB medium containing Ap (50ug ml⁻¹), Kn (50ug ml⁻¹) and X-gal (40ug ml⁻¹). CK1002 alone was run in parallel as a positive LacZ control, and was plated onto LB medium supplemented with Kn (50ug ml⁻¹) and X-gal (40ug ml⁻¹) at 30°C. Over 700

7. Gene Fusions

Figure 7.15 Amplification of the ansB promoter region from the genome of wild-type Er. chrysanthemi NCPPB 1066 and the EMS-generated lacZ⁻ 'down' expression mutants CK1084, CK1096, CK1082, CK1113, CK1108, CK1091, CK1094, CK1077, CK1041, CK1117 and CK1080, using the polymerase chain reaction

A sample of the polymerase chain reaction (1/30) was electrophoresed through a 1.5% (w/v) TAE agarose gel.

The running order is as follows :

Track 1 : 1kb ladder markers

Track 2 : CK1091

Track 3 : CK1113

Track 4 : CK1096

Track 5 : CK1077

Track 6 : CK1080

Track 7 : wild-type Er. chrysanthemi NCPPB 1066

Track 8 : 1kb ladder markers

Track 9 : 1kb ladder markers

Track 10 : CK1084

Track 11 : CK1108

Track 12 : CK1094

Track 13 : CK1041

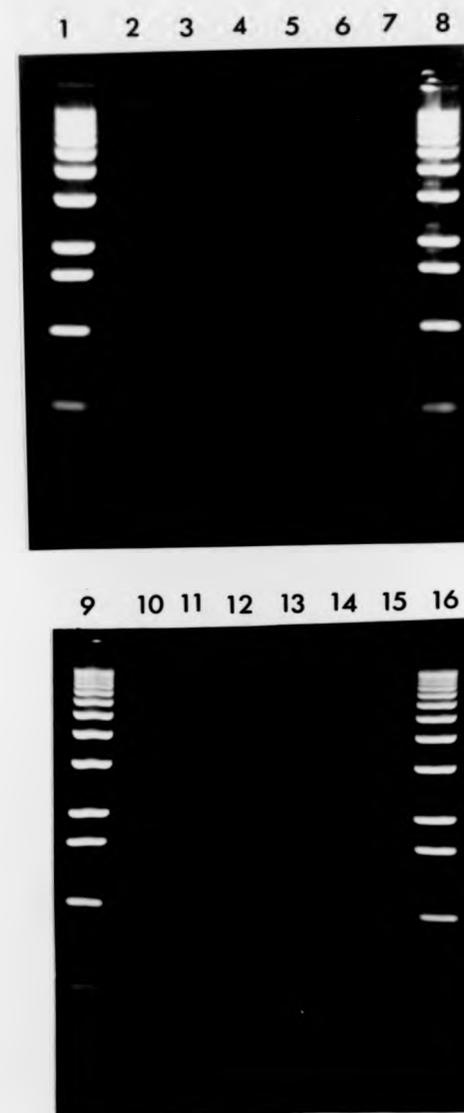
Track 14 : CK1082

Track 15 : CK1117

Track 16 : 1kb ladder markers

7. Gene fusions

Figure 7.15



7. Gene fusions

transconjugants were screened for each mutant, unfortunately no evidence of complementation (ie. restoration of blue coloration) was seen.

7.8 FINAL COMMENTS

The initial sections of this chapter have described the successful fusion of both phoA and lacZ 'reporter' genes to the Er. chrysanthemi NCPPB 1066 ansB structural gene, so that expression of the former is brought under the control of the ansB regulatory regions. Following from this work, the ansB::lacZ transcriptional fusion vector pCK11 was introduced into the wild-type Er. chrysanthemi NCPPB 1066 and industrial morphotype 2 LacZ⁻ derivatives, CK1000 and CK2000, respectively, by conjugal mobilisation. The ultimate aim was to construct a single copy fusion derivative of Er. chrysanthemi NCPPB 1066, and this goal was achieved by replacing the chromosomal ansB allele of the wild-type Er. chrysanthemi NCPPB 1066 LacZ⁻ mutant CK1000 with the ansB::lacZ region from pCK11 via exchange-recombination. The absence of the L-asparaginase II enzyme was found to bestow resistance to the lethal analogue B-aspartylhydroxymate, but did not appear to severely effect the growth or viability of the resulting single copy fusion derivative, CK1002, under rich growth conditions; although a slight degree of cell clumping was evident.

In contrast to recombinant derivative CK1001, growth under low phosphate conditions was found not to lead to exchange-recombination in CK2001. The reason(s) for the increased stability of pCK11 in the latter derivative (or industrial morphotypes 3 and 4, for that matter) are not fully understood at present. Clearly, an alternative means of plasmid curing will have to be adopted in the future, in order to create a single copy fusion derivative of an industrial Er. chrysanthemi NCPPB 1066 morphotype. This

7. Gene fusions

would perhaps involve using a commercially available compound, such as alpha-santonin, which has been reported to be specific for the curing of plasmids belonging to the ColE 1 group, including pBR322 (Bharathi and Polasa, 1990).

Mutagenesis of derivative CK1002 using EMS, led to the isolation of 24 mutants which displayed lowered levels of B-galactosidase activity. In this instance, however, bacterial colonies displaying noticeably increased levels of B-galactosidase were not identified.

Despite the fact that the availability of a generalised transducing bacteriophage for Er. chrysanthemi NCPPB 1066 would have greatly facilitated the genetic analysis of the aforementioned isolates, Southern and Western blotting techniques nevertheless enabled characterisation to be carried out at a molecular level. This led to the identification of 11 mutants which did not appear to have arisen as a consequence of either a gross insertion or deletion event within the ansB or lacZ gene. Moreover, evidence suggested that their 'down' phenotype did not result from mutations within the catalytic site of the LacZ polypeptide, or within the carboxy-terminus of the lacZ gene, leading to the synthesis of a truncated inactive form of LacZ. Furthermore, since complementation of the 11 mutants proved negative, the evidence to date rules out the existence of an extragenic mutation. However, further attempts at complementation should be made in the future.

In conclusion, a number of possible explanations still remain to account for the 'down' phenotype of the 11 partially characterised mutants. The first, and most interesting, is the possibility that a mutation has arisen within the promoter or regulatory region of the Er. chrysanthemi NCPPB 1066 ansB gene. The second is that an extreme amino terminal mutation within the lacZ gene may have arisen, thereby leading to the synthesis of a

7. Gene fusions

truncated LacZ protein, not detectable by SDS-polyacrylamide gel electrophoresis. Thirdly, a mutation may be present within the coding region of the ansB gene itself. It is therefore clear, that further work is now required in order to characterise the 11 aforementioned 'down' mutants more fully. This would include nucleotide sequence analysis of the ansB promoter of each particular mutant. To this end, PCR fragments covering this region (Section 7.7.2) were digested with HinPI and DraI restriction endonucleases, and attempts were made to subclone the resulting products into M13 mp8, previously digested with SmaI and AccI. Unfortunately, this proved beyond the scope of this study.

CHAPTER EIGHT
GENERAL DISCUSSION

8. General discussion

The plant pathogen Er. chrysanthemi NCPPB 1066 has been found to synthesise relatively high levels of the periplasmic L-asparaginase II enzyme, compared with other Erwinia species and strains. Consequently, this bacterium has been employed over many years for the commercial production of this particular anti-leukaemic enzyme. Despite the fact that knowledge has been gained from studies concerning the effect of growth conditions on L-asparaginase II production by Er. chrysanthemi NCPPB 1066, relatively little work has been involved with understanding the molecular mechanisms which govern the synthesis of this enzyme.

This particular study was initiated soon after the cloning and sequencing of the Er. chrysanthemi NCPPB 1066 L-asparaginase II structural gene (ansB), with the aim of studying L-asparaginase II regulation in Er. chrysanthemi NCPPB 1066 itself, and not in a surrogate host such as E. coli. However, at the outset of the work described in this thesis, no genetic systems were available to allow such an analysis to be carried out in this strain of Erwinia. Indeed, evidence from a number of sources suggested that Er. chrysanthemi NCPPB 1066 was not as genetically amenable as some of the other strains that are currently under study in this laboratory.

8.1 Summary of major results

This project began with attempts to apply a number of genetic techniques to the wild-type Er. chrysanthemi NCPPB 1066 strain and the five distinct morphotypes that comprised the L-asparaginase II hyper-producing industrial production culture (Chapter 3). During the course of these genetic studies, several plasmids, including the narrow-host-range multicopy cloning vector pBR322, were successfully introduced into both wild-type Er.

8. General discussion

chrysanthemi NCPPB 1066 and the industrial morphotypes via transformation, electroporation or conjugal mobilisation. However, despite this success, a range of other genetic techniques that had proven useful for the genetic analysis of a variety of other Erwinia strains, were found to be inapplicable for Er. chrysanthemi NCPPB 1066. This perhaps comes as no surprise considering the fact that many other Erwinia geneticists have reported such strain-dependence. Probably the most disappointing result, in this respect, was the failure of λ B⁺ technology to render Er. chrysanthemi NCPPB 1066 sensitive to the bacteriophage lambda, thereby preventing a straightforward means for allowing transposon mutagenesis and cosmid complementation.

Despite the aforementioned setback, one important positive aspect did emerge from this particular set of experiments; namely, the finding that a pBR322-based plasmid (in this case, pHCP2) could be introduced into Er. chrysanthemi NCPPB 1066 at a relatively high frequency via conjugal mobilisation, using the 'helper' plasmids, pR64drd11 and pLVC9. Capitalising on this information, both the L-asparaginase II expression vectors and gene fusion vectors constructed during the remaining course of this work were all based on pBR322, with the anticipation that this particular gene transfer system would allow straightforward transfer into Er. chrysanthemi NCPPB 1066. This was indeed found to be true of the pBR322-based L-asparaginase II expression vector pCK1, which was successfully transferred into both wild-type Er. chrysanthemi NCPPB 1066 and each of the industrial morphotypes at a relatively high frequency, thereby solving a fundamental industrial problem experienced at C.A.M.R. following the cloning of the Er. chrysanthemi NCPPB 1066 ansB structural gene (Chapter 4).

Analysis of the recombinant Er. chrysanthemi NCPPB 1066 pCK1 derivatives in the controlled environment of an 8 litre small-scale fermenter,

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subsequently revealed that multiple copies of the Er. chrysanthemi NCPPB 1066 ansB gene led to an increase in the production of L-asparaginase II, compared with non-recombinant parental isolates. In this instance, the highest level of L-asparaginase II enzyme and the greatest stability of the pCK1 vector was recorded for industrial morphotype 2.

In contrast to the above, Jennings and Beacham (1990) have reported that no such gene dosage effect is seen under anaerobic conditions when multiple copies of the E. coli ansB gene are maintained in E. coli. In this case, lack of over-expression of E. coli L-asparaginase II is believed to be due to the titration of the positive activator proteins, Crp and Fnr.

Following on from the pCK1 work, further experimentation was aimed at increasing L-asparaginase II production yields in Er. chrysanthemi NCPPB 1066, whilst at the same time investigating the applicability of another conjugal mobilisation system for the introduction of plasmid DNA into this bacterium. This involved the construction of the high copy number pIC-19H-based L-asparaginase II expression vectors pCK2 Mob#1 and pCK2 Mob#2, encoding kanamycin-resistance and harbouring the RP4 Mob (oriT) region from plasmid pSUP5011. Results indicated that the presence of the Mob region provided a further means by which recombinant plasmids could be introduced into Er. chrysanthemi NCPPB 1066 from an E. coli derivative providing RP4 mobilising functions in trans, albeit at low frequency. Unfortunately, the presence of pCK2 Mob#1 and pCK2 Mob#2 within Er. chrysanthemi NCPPB 1066 was found not to lead to an improvement in the L-asparaginase II levels previously attained with the industrial morphotype 2 pCK1 recombinant derivative. More recently, an identical approach has been successfully employed by workers at C.A.M.R. to introduce the cloning vectors, pMTL30 and pMTL31 (Williams et al., 1990), into the industrial

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morphotype 2 isolate, at frequencies similar to those reported in this study (Dr. N. Minton; personal communication).

Moving away from the genetic analysis of Er. chrysanthemi NCPPB 1066, further work was concerned with studying L-asparaginase II regulation at a molecular level. One area of study stemmed from experiments performed at C.A.M.R. Porton Down, which implied that the positive activator protein, NifA, in combination with the alternative sigma factor RpoN, could influence L-asparaginase II expression in E. coli, by possibly interacting with the putative -24/-12 consensus sequence identified within the 5' non-coding region of the Er. chrysanthemi NCPPB 1066. This was contradictory to the reports that L-asparaginase II expression was unaffected by the level of available ammonia. Hence, it had been proposed that perhaps Er. chrysanthemi NCPPB 1066 perhaps did not contain a functional Ntr regulatory system. However, during the course of this study, heterologous hybridisation was used to demonstrate the presence of rpoN, ntrB and ntrC homologues within the genome of Er. chrysanthemi NCPPB 1066 (Chapter 5). This was subsequently verified by isolating the appropriate genomic regions and demonstrating that they had the capacity to complement the corresponding E. coli rpoN and ntrB,C mutants (Chapter 5).

The Er. chrysanthemi NCPPB 1066 rpoN homologue was studied in further detail, with the complete nucleotide sequence of the 2.4 kb Clal chromosomal fragment harbouring this gene being determined (Chapter 5). Analysis of the derived sequence verified the cloning of the entire Er. chrysanthemi NCPPB 1066 rpoN structural gene, and revealed a high degree of homology throughout to the rpoN region of K. pneumoniae. Introduction of multiple copies of the Er. chrysanthemi NCPPB 1066 rpoN structural gene and surrounding regions into the wild-type Er. chrysanthemi NCPPB 1066 single

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copy ansB::lacZ transcriptional fusion derivative, CK1002 (Chapter 7), was found not to lead to a stimulation in the level of transcription from the ansB promoter, under either nitrogen-rich or nitrogen-poor growth conditions (Chapter 5).

These latter findings were reinforced by the results gained from transcript mapping of the wild-type Er. chrysanthemi NCPPB 1066 ansB structural gene using primer extension (Chapter 6). Under the rich and poor nitrogen growth conditions employed in this study, transcription of the ansB gene in Er. chrysanthemi NCPPB 1066 was found to initiate from a cytosine residue, 117 nucleotides from the translational start site. This did not correspond to the putative -24/-12 consensus sequence. Hence, no evidence was found in this study to suggest that expression of the ansB structural gene was RpoN-dependent in Er. chrysanthemi NCPPB 1066.

A less targetted approach to analysing L-asparaginase II regulation involved the random generation of L-asparaginase II expression mutants and their subsequent molecular characterisation. To facilitate the screening for expression mutants, a series of ansB::phoA and ansB::lacZ fusion vectors were constructed both in vitro by recombinant DNA technology, and in vivo using the transposons TnphoA and TnlacZ-B20, respectively (Chapter 7). Again, using the pR64drd11, pLVC9 mobilisation system mentioned above, one such vector, pCK11 (harbouring a transcriptional ansB::lacZ fusion), was introduced into the EMS-generated wild-type Er. chrysanthemi NCPPB 1066 lacZ⁻ mutant CK1000 (Chapter 3), eventually leading to the construction of the single copy fusion derivative, CK1002. This was mutagenised with EMS and a total of 24 mutants lowered in B-galactosidase activity were isolated. Southern analysis indicated that reduced enzyme levels were not due to the insertion of a transposable element into the lacZ structural gene, or a gross deletion event.

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Western blotting subsequently allowed a more detailed characterisation of the LacZ⁻ isolates, and revealed that 5 were LacZ catalytic mutants, 8 synthesised a truncated form of the LacZ polypeptide, and that 11 were due to some other cause, possibly a 'down' promoter mutation. Further analysis using the polymerase chain reaction indicated that a deletion of greater than 50 bp had not occurred within the promoter region of the latter mutants.

8.2 Suggestions for future work

Due to the broad nature of this project, there still remains a number of areas that are worthy of further investigation, and these will now be outlined below :

(1) The genetic analysis of Er. chrysanthemi NCPPB 1066 is clearly an area that will require further study. In particular, electroporation and transposon mutagenesis using the thermo-sensitive transposon delivery vehicle pCHR81, are two areas that should be concentrated upon, since it is believed that both these techniques hold particular promise. Moreover, from the experience gained in this laboratory with Er. carotovora subsp. carotovora SCRI 193 and Er. carotovora subsp. atroseptica SCRI 1043, it is anticipated that further screening of sewage samples will eventually lead to the isolation of a novel transducing bacteriophage for Er. chrysanthemi NCPPB 1066. Moreover, because of the surprising observation that each of the novel bacteriophage isolated from sewage in this study failed to plaque on the industrial morphotypes, one or all of the latter should be included in future isolation experiments.

(2) With regard to L-asparaginase II over-expression in Er. chrysanthemi NCPPB 1066, a further enhancement in enzyme production yields could possibly be attained by replacing the promoter and controlling regions of the

8. General discussion

Er. chrysanthemi NCPPB 1066 ansB structural gene with an alternative strong regulatable promoter of the type mentioned in Section 4.4. This construct could then be 'tagged' with a suitable transposon and then used to replace the wild-type Er. chrysanthemi NCPPB 1066 ansB chromosomal allele via exchange-recombination, thereby avoiding the potential problems of plasmid instability. Loss of the transposon could then be achieved under non-selective conditions by allowing for precise excision of the transposon.

(3) Following on from primer extension mapping of the mRNA transcript originating from the 5' non-coding region of the Er. chrysanthemi NCPPB 1066 ansB structural gene, detailed site-directed mutagenesis and deletion analysis of the newly proposed '-10' and '-35' promoter consensus regions should be carried out. Moreover, another potentially interesting target for future mutagenesis would be the predicted stem loop structure (II/II') identified by Minton et al. (1986), situated immediately downstream of the experimentally determined transcriptional start site.

(4) Both single copy and multiple copies of the Er. chrysanthemi NCPPB 1066 ansB structural gene have been shown to be subject to catabolite repression in the presence of glucose for example, in both Er. chrysanthemi NCPPB 1066 (Callow et al., 1971) and E. coli (Gilbert et al., 1986). The possible role of cAMP and the positive activator Crp in the catabolite repression of L-asparaginase II synthesis was an area not touched upon in the present study, but clearly this should be a subject for future work. This could possibly involve the introduction of an appropriate L-asparaginase II fusion vector into an E. coli derivative deficient in either cAMP synthesis (cya⁻) or Crp production (crp⁻), and monitoring ansB expression in the presence and absence of glucose. In conjunction with this, site-directed mutagenesis of the putative Crp binding site, previously identified by Minton

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et al. (1986), should also been performed, and the effect on ansB expression assessed.

(5) Finally, the analysis of the EMS generated lacZ⁻ expression mutants of the single copy fusion derivative, CK1002, has been left at an interesting stage. Clearly, further characterisation of the 11 isolates which did not appear to result from the synthesis of a truncated LacZ polypeptide or from the synthesis of a LacZ polypeptide which was devoid of catalytic activity, is required. This would involve nucleotide sequence analysis in order to determine whether mutation within the promoter region of the Er. chrysanthemi NCPPB 1066 ansB structural gene is the cause of their 'down' phenotype. This could involve further attempts to subclone PCR products into M13 for single-stranded sequencing, or directly sequencing the products themselves. In addition to this, further screening of EMS-generated mutants should be carried out, perhaps on different media and at different temperatures.

8.3 Concluding remarks

In conclusion, many of the basic obstacles that have prevented the genetic analysis of L-asparaginase II regulation in Er. chrysanthemi NCPPB 1066 in the past, can now be overcome. The availability of a single copy ansB::lacZ fusion derivative of Er. chrysanthemi NCPPB 1066 should simplify the isolation of L-asparaginase II expression mutants. Moreover, it should greatly facilitate the monitoring of L-asparaginase II activity in a variety of genetic backgrounds and/or under different physiological conditions, using either Western blot analysis or the relatively straightforward B-galactosidase biochemical assay. It is therefore hoped that the results obtained in this study will now pave the way towards a greater understanding of the

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mechanisms governing the synthesis of L-asparaginase II by Er. chrysanthemi
NCPPB 1066.

APPENDIX

Figure A1 Alignment of the predicted primary amino acid sequence of the wild-type *Er. chrysanthemi* NCPPB 1066 ORFB polypeptide (Erwin) with the RpoN polypeptide of *K. pneumoniae* (Klebs) and *E. coli* (Eco)

84.7% identity in 477 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	90v
Klebs	MEGGLQLRLSQAANTPOLQQAIRLLQLSTLELOEIQDALESNPLLEQDINDEVEYTKAEADRESLDTVDALKEKPELPLDAADIEI								
Erwin	MEGGLQLRLSQAANTPOLQQAIRLLQLSTLELOEIQDALESNPLLEQDINDEVEYTKAEADRESLDTVDALKEKPELPLDAADIEI								
	10"	20"	30"	40"	50"	60"	70"	80"	90"
	100v	110v	120v	130v	140v	150v	160v	170v	180v
	YTACTPSGCVGVDDELPTVYGETTQSLQDYLMQVVELTPFDTORAIATSIQVAVDVTGYLTVSVDIRESIQHEEVTLEEVAVERVDRFDPVGVAAARDLRECLLVQ								
	100"	110"	120"	130"	140"	150"	160"	170"	180"
	190v	200v	210v	220v	230v	240v	250v	260v	270v
	QRFDPVGVAAARDLRECLLVQSLQDYLMQVVELTPFDTORAIATSIQVAVDVTGYLTVSVDIRESIQHEEVTLEEVAVERVDRFDPVGVAAARDLRECLLVQ								
	190"	200"	210"	220"	230"	240"	250"	260"	270"
	280v	290v	300v	310v	320v	330v	340v	350v	360v
	PDVLRVYVQGMUTVELNDSVRLQINQOYAAAGSARMSDGGFIRSHLQEARMLKESLESNDTLLVRCIVEQDGAFFEGEEFKMPVGLADIAADVHNESTISRVITQKFLNSP								
	280"	290"	300"	310"	320"	330"	340"	350"	360"
	370v	380v	390v	400v	410v	420v	430v	440v	450v
	HWLADIAADVHNESTISRVITQKFLNSPQIFELKYFFSNNVTGCGEASSTAIRALVKLLIAAENPAKPLSDSKLITLLSEGGIIVARRVAKYRESLISPPSNORKQLV								
	370"	380"	390"	400"	410"	420"	430"	440"	450"
	460v	470v							
	RTVAKYRESLISPPSNORKQLV								
	RTVAKYRESLISPPSNORKQLV								
	RTVAKYRESLISPPSNORKQLV								
	460"	470"							

84.3% identity in 477 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	90v	100v
Erwin	MEGGLQLRLSQAANTPOLQQAIRLLQLSTLELOEIQDALESNPLLEQDINDEVEYTKAEADRESLDTVDALKEKPELPLDAADIEI									
Eco	MEGGLQLRLSQAANTPOLQQAIRLLQLSTLELOEIQDALESNPLLEQDINDEVEYTKAEADRESLDTVDALKEKPELPLDAADIEI									
	10"	20"	30"	40"	50"	60"	70"	80"	90"	100"
	110v	120v	130v	140v	150v	160v	170v	180v	190v	200v
	TDYDEELPTVYGETTQSLQDYLMQVVELTPFDTORAIATSIQVAVDVTGYLTVSVDIRESIQHEEVTLEEVAVERVDRFDPVGVAAARDLRECLLVQ									
	110"	120"	130"	140"	150"	160"	170"	180"	190"	200"
	210v	220v	230v	240v	250v	260v	270v	280v	290v	300v
	LSQFNSDIPRLSEARLIVSDYLDLQAMDFRLLRSHLQEARMLKESLESNDTLLVRCIVEQDGAFFEGEEFKMPVGLADIAADVHNESTISRVITQKFLNSP									
	210"	220"	230"	240"	250"	260"	270"	280"	290"	300"
	310v	320v	330v	340v	350v	360v	370v	380v	390v	400v
	AALGHSARMSDGGFIRSHLQEARMLKESLESNDTLLVRCIVEQDGAFFEGEEFKMPVGLADIAADVHNESTISRVITQKFLNSPQIFELKYFFS									
	310"	320"	330"	340"	350"	360"	370"	380"	390"	400"
	410v	420v	430v	440v	450v	460v	470v			
	SNVITGCGEASSTAIRALVKLLIAAENPAKPLSDSKLITLLSEGGIIVARRVAKYRESLISPPSNORKQLV									
	410"	420"	430"	440"	450"	460"	470"			

The middle amino acid sequence highlights the residues which are common to both of the polypeptides being compared.

Figure A2 Alignment of the predicted primary amino acid sequence of the wild-type *Er. chrysanthemi* NCPPB 1066 ORFB polypeptide (Erwin) with the RpoN polypeptide of *A. vinelandii* (Azoto) and *P. putida* (Pseud)

54.7% identity in 503 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	90v		
Azoto	MKPSLVKNGQGLTHTPOLGQAI	RLLOLSTL	LDLQGEIQEALDSNPHLE	EGERAEQD	SPDMLG	EGWGDOS	TLDTT	PGSTGEGY	SCAA	SEDC	
Erwin	MK L L QGL	HTPOLGQAI	RLLOLSTL	LDLQGEIQEALDSNPHLE	EGERAEQD	SPDMLG	EGWGDOS	TLDTT	PGSTGEGY	SCAA	SEDC
	10°	20°	30°	40°	50°	60°	70°	80°	90°	70°	
	100v	110v	120v	130v	140v	150v	160v	170v	180v		
	100°	110°	120°	130°	140°	150°	160°	170°	180°		
	190v	200v	210v	220v	230v	240v	250v	260v	270v		
	170°	180°	190°	200°	210°	220°	230°	240°	250°		
	280v	290v	300v	310v	320v	330v	340v	350v	360v		
	260°	270°	280°	290°	300°	310°	320°	330°	340°		
	370v	380v	390v	400v	410v	420v	430v	440v	450v		
	350°	360°	370°	380°	390°	400°	410°	420°	430°		
	460v	470v	480v	490v	500v						
	440°	450°	460°	470°							

52.6% identity in 498 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	90v		
Pseud	MKPSLVKNGQGLTHTPOLGQAI	RLLOLSTL	LDLQGEIQEALDSNPHLE	EGERAEQD	SPDMLG	EGWGDOS	TLDTT	PGSTGEGY	SCAA	SEDC	
Erwin	MK L L QGL	HTPOLGQAI	RLLOLSTL	LDLQGEIQEALDSNPHLE	EGERAEQD	SPDMLG	EGWGDOS	TLDTT	PGSTGEGY	SCAA	SEDC
	10°	20°	30°	40°	50°	60°	70°	80°	90°	70°	
	100v	110v	120v	130v	140v	150v	160v	170v	180v		
	100°	110°	120°	130°	140°	150°	160°	170°	180°		
	190v	200v	210v	220v	230v	240v	250v	260v	270v		
	170°	180°	190°	200°	210°	220°	230°	240°	250°		
	280v	290v	300v	310v	320v	330v	340v	350v	360v		
	260°	270°	280°	290°	300°	310°	320°	330°	340°		
	370v	380v	390v	400v	410v	420v	430v	440v	450v		
	350°	360°	370°	380°	390°	400°	410°	420°	430°		
	460v	470v	480v	490v							
	440°	450°	460°	470°							

The middle amino acid sequence highlights the residues which are common to both of polypeptides being compared. Gaps have been introduced to maximise the alignment.

Figure A5 Alignment of the predicted primary amino acid sequence of the wild-type Er. chrysanthemi NCPPB 1066 ORFC polypeptide (Erwin) with the amino terminal residues of the K. pneumoniae ORF95 (Klebs), A. vinelandii ORF107 (Azoto), P. putida (Pseud) ORF3 and R. meliloti (Rhizo) ORF3 polypeptides

	10v	20v	30v	40v	50v	60v	70v	80v	90v
Azoto	MOVNI	SGHLDVTDALBDYVEEKISPLE	ENFRITTSVQVIVIVKELKQKIEATLNV	SGAEVWANAENEDHYAAIDDLADKLRDL	IKNEKQIER				
Erwin	MO	WJ	GH	TDA	R	V	K	LE	FDR
	10*	20*	30*	40*	50*	60*	70*	80*	90*

54.9% identity in 91 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	90v
Klebs	MOLNIT	GHVVEITP	ANDVYAKFSKLE	DFDRINOV	TIKVEKVTQIDANL	VWGGELN	ASAE	GDHYAAIDDLADKLRDL	IKNEKQIER
Erwin	MOLNIT	GHVVEITP	ANDVYAKFSKLE	DFDRINOV	TIKVEKVTQIDANL	VWGGELN	ASAE	GDHYAAIDDLADKLRDL	IKNEKQIER
	10*	20*	30*	40*	50*	60*	70*	80*	90*

80.0% identity in 95 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	90v
Pseud	MOVNI	SGHVEVTP	LRDYLVEKLRVESH	DFDKITNVQVIVIVKELKQKIEATL	NVSGAEVWANAENEDHYAAIDDLADKLRDL	IKNEKQIER			
Erwin	MO	WJ	GH	RVE	I	R	V	K	LE
	10*	20*	30*	40*	50*	60*	70*	80*	90*

52.7% identity in 91 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	90v
Rhizo	MSVRS	SGHNEIGDS	FRVIEG	IEGADVTEYFDGGYS	SDVIVKSGSRFSADCKL	MLDTGVVLRGANGA	NEPOSADFAASERIER	KLRYKLEKDH	
Erwin	MOLNIT	GHVVEITP	ANDVYAKFSKLE	DFDRINOV	TIKVEKVTQIDANL	VWGGELN	ASAE	GDHYAAIDDLADKLRDL	IKNEKQIER
	10*	20*	30*	40*	50*	60*	70*	80*	90*

26.8% identity in 97 aa overlap

Figure A6 Alignment of the predicted primary amino acid sequence of the wild-type Er. chrysanthemi NCPPB 1066 ORFD polypeptide (Erwin) with the amino acid residues of the K. pneumoniae ORF162 (Klebs) and homologous P. putida (Pseud) polypeptides

	10v	20v	30v	40v	50v	60v	70v	80v	90v
Klebs	MIND	SALQ	LSHVLVQEC	TRSOVH	CSKRALEI	ISELA	AKGLSSOIV	FEAILTR	ENKSTGICGIAIPNCKLEED
Erwin	MM	QLS	VL	ECTRS	VHCSKRALEI	ISELAA	DL	L	G
	10*	20*	30*	40*	50*	60*	70*	80*	90*

78.6% identity in 98 aa overlap

	10v	20v	30v	40v	50v	60v	70v	80v	
Pseud	MIRLET	ITPGRSL	VHVP	GGSKRALEK	QVATV	ADQVPE	LEMDV	FFKLVAREK	LSGICGICGIAIPNCKLEED
Erwin	MNEP	WMLSTVLR	KECTRS	VAHCSKRALEI	ISELA	AKGLSSOIV	FEAILTR	ENKSTGICGIAIPNCKLEED	NTLQAVGV
	10*	20*	30*	40*	50*	60*	70*	80*	90*

42.2% identity in 90 aa overlap

The middle amino acid sequence highlights the residues which are common to both of the polypeptides which are being compared. Gaps have been introduced to maximise the alignment.

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ADDENDUM

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