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Potential applications of *Agrobacterium* virulence gene promoters in plant-protecting microbial inoculants

by

Mark Howard Levesley

A thesis submitted to the Department of Biological Sciences

University of Durham

In accordance with the requirements for the degree of

Doctor of Philosophy

April 1994

UNIV DURHAM

10 JUN 1994

For My Parents and the late Dr. Robbie Allen

Declaration

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Potential applications of *Agrobacterium* virulence gene promoters in plant-protecting microbial inoculants.

Mark Howard Levesley

PhD 1994

Abstract

The concept behind this project was to continue the development of strains of *Agrobacterium tumefaciens* that were capable of producing pesticidal proteins in response to plant wounding, thereby killing the invading organism.

To this end, *vir* induction was studied in *A. tumefaciens* and a protocol to elicit the maximum response was developed. In order for this concept to work, it was necessary to determine whether *vir* induction was occurring at plant wound sites and a method for showing this was developed, the results suggesting that indeed *vir* induction did occur. The stability of two types of plasmid was also analysed in this bacterium to ascertain how stable the proposed 'microbial inoculant' would be in the field. The results suggested that IncW plasmids should not be used in the final product.

The activities of two chitinases from *Serratia marcescens* were analysed and it was found that both chitinases were effective in controlling some types of fungus. In addition it was found that the expression of *chiB* in *Escherichia coli* led to the appearance of a filamentous phenotype at intermediate temperatures. A construct was made that linked the *virE* promoter to the *chiB* gene. This plasmid was introduced into *A. tumefaciens* but did not function as expected. However, other constructs were demonstrated to be inducible although they were only partially successful in controlling the fungi that the strains were assayed against.

The *Bacillus thuringiensis* δ-endotoxin gene, *cryIA(c)*, was cloned and various constructs were made to examine the effects of various regions of the native promoter. One construct was made that linked the *virB* promoter to *cryIA(c)* and this was introduced into *A. tumefaciens*. The resulting strain was capable of inducible δ-endotoxin expression and was also capable of controlling the larvae of the tobacco hornworm, *Manduca sexta*.

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I would also like to express my sincere thanks to my parents for their continued support throughout my education.

Abbreviations

Amp	=	ampicillin
bp	=	base pairs
BSA	=	bovine serum albumin
Cb	=	carbenicillin
CM-chitin-RBV	=	carboxymethyl-chitin-remazol blue violet
cpm	=	counts per minute
dH_2O	=	distilled water
DMSO	=	dimethylsulphoxide
DNAase	=	deoxyribonuclease
dNTP	=	deoxyribonucleoside triphosphate
DTT	=	dithiothreitol
EDTA	=	ethylenediaminetetraacetic acid
kb	=	kilobase pairs
kD	=	kilodaltons
MES	=	2-(*N*-morpholino)ethane sulphonic acid
PBS	=	phosphate buffered saline
PEG	=	polyethylene glycol
Rif	=	rifampicin
RNAase	=	ribonuclease
SDS	=	sodium dodecyl sulphate
Sm	=	streptomycin
SSC	=	saline sodium citrate
TBS	=	Tris buffered saline
T-DNA	=	transfer DNA
TEMED	=	*N,N,N',N'*,-tetramethylethylenediamine
Ti plasmid	=	tumour inducing plasmid
Tris	=	tris(hydroxymethyl)aminomethane
X-gal	=	5-bromo-4-chloro-3indolyl-β-D-galactopyranoside
UV	=	ultraviolet
wrt	=	with respect to
5'	=	5' terminal phosphate of DNA molecule
3'	=	3' terminal hydroxyl of DNA molecule

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I've been doing it for years,
My goal is moving near,
It says "Look I'm over here",
Then it up and disappears.

From *Sat in Your Lap* by Kate Bush

Chapter 1

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INTRODUCTION

1.1 *Agrobacterium tumefaciens*

The genus *Agrobacterium* , a member of the family Rhizobiaceae, consists of 3 - 5 species, *Agrobacterium tumefaciens* being considered as the type strain [66, 160, 282]. However, the division of the genus into various species is the subject of some controversy [172]. Traditionally the genus has been described as containing four species based on their pathogenic behaviour: *Agrobacterium tumefaciens* (which causes Crown Gall Tumour); *A. rhizogenes* (the causative agent of Hairy Root); *A. rubi* (which causes Cane Gall Tumour on Raspberry and Blackberry plants); *A. radiobacter* (which is avirulent) [218].

Despite the phytopathogenic differences between the species, these are not stable, since the behaviour of the species in question is determined by a type of large plasmid (Ti or Ri) that it may or may not contain [172, 217, 218, 314] which can be transferred from one strain to another [99, 170] or lost altogether [119, 172]. This loss or addition of a Ti or Ri plasmid can result in a strain that has a genetic background that is normally associated with one type of pathogenicity, behaving in an identical manner to another strain from a supposedly different species [307].

Thus, an alternative classification has been proposed [171, 172] which seeks to class the genus as three distinct biovars. These are based on various chromosomally encoded biochemical and physiological differences between various strains and take no account of pathogenicity. Despite this classification, the type strain remains the virulent *A. tumefaciens* [160] and thus the old nomenclature is the one that is still in common use and the one that will be used here. In addition, Ophel and Kerr [227] have proposed another small species of *Agrobacterium* (*A. vitis*) based on phenotype, DNA homology and serology.

Agrobacterium tumefaciens itself is a Gram-negative bacterium characterised by small 0.6 - 1.0 by 1.5 - 3.0 μm rods. The bacteria are non-sporulating and motile,

possessing between 1 and 6 peritrichously arranged flagella. The optimum temperature for growth is 25 - 28 °C and the colonies are convex, smooth and light beige in colour [169].

1.2 *Agrobacterium tumefaciens* infection - an overview

A. tumefaciens is an obligate pathogen of most dicotyledonous plants and some monocots (sections 1.3 and 1.4). It is found in soils around the world where its presence is indicated by tumours forming on the roots and sometimes the stems of infected plants.

The bacterium is able to detect wounded sites on plants, which are suitable targets for infection (section 1.5). By moving up a concentration gradient of various wound-specific chemicals in the soil, the bacterium is able to locate the wound site (section 1.6). Attachment then takes place and expression from *vir* (virulence) genes occurs upon stimulation by some of the wound-specific chemicals (sections 1.7, 1.8 and 1.9). The Vir proteins, thus produced, are able to copy and help to transfer a small piece of bacterial DNA (T-DNA) from bacterium to plant (section 1.10). The T-DNA then becomes incorporated into the plant genome and its genes become active (sections 1.11, 1.12 and 1.13). These genes encode plant growth regulators which cause a gall tumour to form, due to uncontrolled proliferation and expansion of the host cells (sections 1.2 and 1.13). The genes also encode enzymes responsible for the production and transport of derivatised amino acids and sugars, called opines (sections 1.8 and 1.13). These are useless to the plant, but the bacteria utilise them as a carbon and nitrogen source.

1.3 Habitat

Agrobacterium sp. are commonly isolated from both cultivated and uncultivated soils around the world and are routinely found in association with plant roots [33, 34, 168]. Estimations of the numbers of *Agrobacteria* present in soils range from 10^3 - 10^6 CFU.g^{-1} of soil [34] with the number of avirulent strains outnumbering virulent forms by around 10 -100 fold [26, 168]. The pathogenic forms of *Agrobacterium* are usually only detected around infected roots and in galls, since the Ti plasmid is easily lost without selective pressure, although exceptions have been found. Recently Bouzar *et al.* [34] isolated 72 strains from a fallow field which had not been cultivated for five years. The numbers of *Agrobacteria* were much higher than usual with 3 x 10^7 CFU.g^{-1} of soil, of which 33 % were virulent, a vastly higher than expected percentage. They postulated that the loamy soil of the

sample site could possibly support greater numbers of virulent *Agrobacteria* due to its capacity for water retention and acid pH. Another factor could have been the effect of the rhizospheres of the weeds particular to that sample site. Whatever the reasons, there appears to be a greater selective advantage for virulent strains in this soil than is usually the case.

Although this must be regarded as an exception, it is clear that the type of soil has a profound influence on the survival of any particular strain of *A. tumefaciens* (see section 3.6), since data obtained from other related species suggests that soil type affects survival of the bacterial strain, the stability of plasmids, motility, growth and pathogenicity [34, 140, 186, 193, 232].

The generally low ratio of tumourigenic to avirulent *Agrobacteria* in the soil does not seem to account for the regular occurrence of Crown Gall Tumour and Hairy Root [34]. However, when plant roots are wounded, various *Agrobacterium*-attracting phenolic compounds are released (see figure 1.3.1) [285, 289]. Virulent, Ti plasmid containing strains show a greater chemotactic response towards these compounds than those lacking the Ti plasmid [11, 12, 272, 273] and thus give the virulent strains an advantage in colonising the newly wounded root (see section 1.9). Once a wound site is infected by one species, another species is unable to invade [168].

Once a pathogenic strain has invaded the root, a small piece of DNA is transferred to the plant which encodes for the production of new compounds by the plant (see sections 1.12 and 1.13), including opines [55]. Opines are used as a source of nitrogen and carbon by the bacteria, but are not able to be used by the host plant or by the majority of other microorganisms [238], with the exception of some strains of *Pseudomonas* [257] and some fungi [21].

The opines have a secondary effect in that they stimulate the production of conjugating factor (CF) and its receptor TraR [239, 351, 352]. CF is produced by the invading *Agrobacteria* and its interaction with TraR is thought to induce the expression of the *tra* genes with the result that Ti/Ri plasmids are transferred to plasmidless *Agrobacteria* in the same vicinity (see section 1.8) [23, 112]. The presence of a greater number of virulent, Ti/Ri plasmid-containing bacteria could now be responsible for further infection of plants in the same locality.

In fact, in the absence of opines, bacteria carrying the Ti plasmid are at a selective disadvantage [116] and they are also targets for another strain of

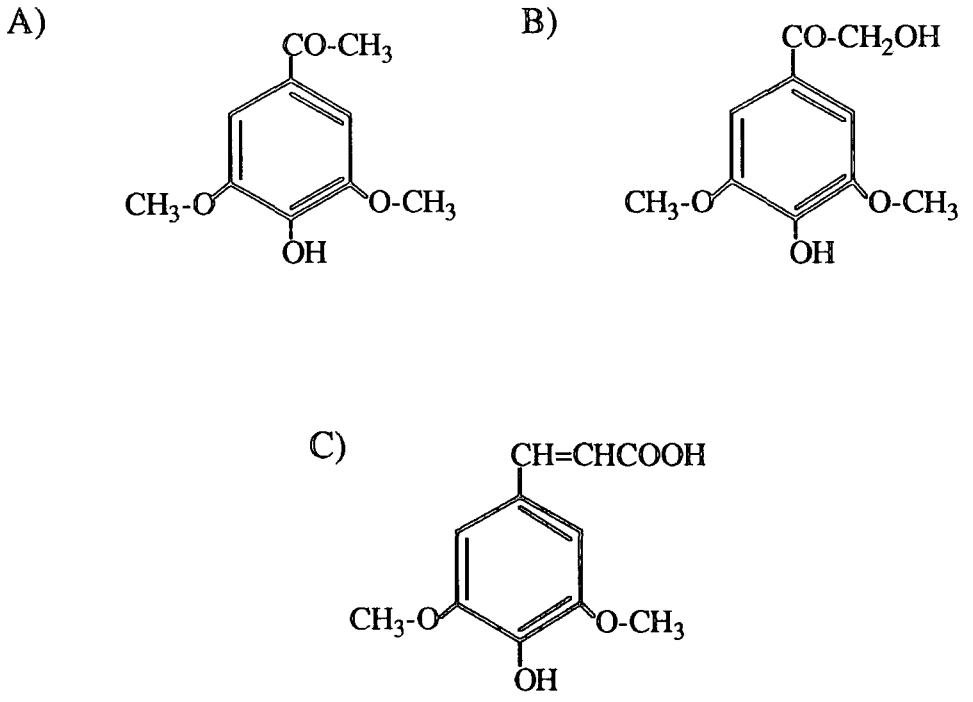


Figure 1.3.1 Structures of some wound-specific *vir*-inducing molecules. A) Acetosyringone, B) α-hydroxyacetosyringone and C) sinapinic acid.

Agrobacterium, A. radiobacter K84 [86, 127]. This produces agrocin 84, which is taken up and cleaved by strains able to utilise certain opines, thus releasing a modified nucleotide which is toxic to the cell [112].

This goes some way to account for the observation that Crown Gall Tumour can be prevalent in some areas and yet generally the numbers of virulent *Agrobacteria* in the soil are very low in comparison with non-pathogenic strains.

1.4 Crown Gall Tumour

The majority of dicotyledonous plants and some monocots are susceptible to attack by *A. tumefaciens* [67, 78] and the disease is practically universal [81].

The galls caused by the invading *A. tumefaciens* are neoplastic overgrowths of root or, less commonly, stem tissue [78, 217] and range from pea-size to over 23 kg [296]. The gall results from the uncontrolled division and an increase in size of the parenchyma cells, which can then develop into stunted shoots (teratoma) or a mass of undifferentiated cells [78, 217]. The bacteria are obligate aerobes and are generally found only on the outside of the gall and between the expeditiously growing epidermal cells [78]. The way in which a tumour develops in a particular species of plant is dependent on the type of Ti plasmid possessed by the invading *A. tumefaciens* strain [113]. So-called 'octopine-type' Ti plasmids (see section 1.8) generally result in undifferentiated and unorganised tumours and 'nopaline-type' Ti plasmids cause teratomas, although there are exceptions [217].

1.5 *Agrobacterium* chemotaxis

In order for the invading *Agrobacterium* to cause Crown Gall Tumour, it must come into contact with a susceptible plant [31]. *Agrobacterium* strains have been observed to accumulate in the rhizosphere of the root structure, at the root cap and at wounded or broken root hairs [268]. For this to occur the bacterium must have a way of detecting the roots and wound sites.

A. tumefaciens has a well-developed and selective chemotaxis system by which it is attracted to plants and this explains why it is so common in the rhizosphere [273]. An assortment of amino acids and sugars are present in plants [164] and these are released into the rhizosphere by the sloughing off of cells due to root growth. *A. tumefaciens* is strongly attracted to some of these compounds most notably sucrose [190]. The bacterium can detect this disaccharide down to a

concentration of less than 10^{-7} M although the greatest chemotactic response to it is at 10^{-6} M [190, 273]. Glucose and fructose also elicit a strong response from *A. tumefaciens*, and a response has been shown for many other sugars found in plants (including galactose, maltose and arabinose) and the amino acids arginine and valine [190, 273].

It is thought that these responses to sugars and amino acids are due to methyl-accepting chemotaxis proteins (MCPs), which have been studied most extensively in *Escherichia coli* and *Salmonella typhimurium* [166, 167, 287, 295]. There are four MCPs in *E. coli*, Tar, Tap, Tsr and Trg [84, 210] which are all inner membrane proteins of about 60 kD containing a periplasmic ligand-binding domain and a cytoplasmic signalling domain [210]. The periplasmic domain either binds the attractant/repellent ligand directly as in the case of serine or via an intermediate periplasmic binding-protein which is specific for the given ligand (e.g. galactose) [32].

The interaction of the ligand with the MCP causes a conformational change in the protein which generates a signal which is mediated through various other cytoplasmic proteins to the flagella apparatus. By the action of the flagella the net effect is to move the bacterium towards or away from a given MCP-binding ligand [32].

It has not been fully established whether this system operates in *A. tumefaciens* but methylation of MCP-like proteins has been detected [270, 273]. Antibodies to Trg (which is responsive to galactose [167]) have been shown to cross react with protein extracts from *Agrobacterium* [210] as have anti-Tar antibodies [38]. Both the proteins detected in these experiments were about 60 kD. Very recently Farrand and his co-workers have found an open-reading frame which could encode a protein with a 75 % amino acid similarity to MCPA from *Caulobacter crescentus* [87]. Thus it is almost certain that *Agrobacterium* uses MCPs to guide it to the rhizosphere of plants.

However, this system should work equally well for both virulent and avirulent forms of *Agrobacterium*. Given that the number of avirulent *Agrobacteria* in a given soil vastly outnumber the pathogenic bacteria [26, 168], the latter must have another chemotactic response system allowing it to be guided preferentially to susceptible sites on the plant. This is achieved by using proteins encoded by the *vir* region of the Ti plasmid.

1.6 Target site recognition

Only the wounded parts of plants are susceptible to attack by *A. tumefaciens* [36]. The wound sites themselves release many aromatic and aliphatic chemicals into the rhizosphere, some of which are specific to wound site exudates, such as acetosyringone and α-hydroxyacetosyringone (see figure 1.3.1) [289].

Acetosyringone and α-hydroxyacetosyringone are formed as a by-product of the β-oxidation of cinnamic acids [115]. These reactions, part of the shikimic acid pathway, provide the plant with many compounds including flavonoids and lignins [114, 117], both these sets of compounds being implicated in cell repair. Other precursors of the cell wall component lignin (coniferyl alcohol and sinapyl alcohol [114]) have been shown to elicit so-called *vir* induction in *A. tumefaciens* (see section 1.9) and so it may be that the organism has established a way of detecting and targeting those cells undergoing cell wall repair [285].

The chemoattractant ability of many wound exudate-specific phenolic compounds on *A. tumefaciens* were determined by Ashby *et al.* [9, 11]. These compounds could be divided into three classes - those requiring the bacterium to possess a Ti-plasmid to act as chemoattractants, those not requiring a Ti-plasmid and those that were non-chemoattractants. Of the first type, acetosyringone, sinapinic acid and syringic acid appeared to elicit the strongest response, with a peak at a concentration of 10^{-7} M and a threshold response of 10^{-8} M for acetosyringone (see figure 1.3.1) [9, 11, 12, 272]. The concentrations of the chemoattractants belonging to the second category had to be much higher to elicit a response, typically catechol and vanillyl alcohol gave chemotactic peaks at 10^{-2} M [9, 11]. Thus it would appear that there are two types of chemotaxis occurring in response to wound-specific phenolic compounds - one that is weak and chromosomally encoded, and another which is much more sensitive and is specified by the Ti-plasmid. For maximum chemotaxis in the latter system, the attractant molecule required a 4'-hydroxyl group, 3' and 5' O-methyl groups and a 1' polar side chain on the benzene ring [9, 11].

It is interesting to note that many of these wound exudate-specific compounds are also present in monocots, although monocots have been traditionally regarded as poor hosts for *Agrobacterium* [11, 67, 303]. Indeed Ashby *et al.* concluded that exudates from monocots and dicots had equal chemoattractant abilities, suggesting that the recognition and chemotaxis towards monocot wound sites occurs but a future step in the infection process is the one that is blocked [11]. Spencer and

Towers suggested that there might be 'phenolic inhibitors' present in monocotyledonous plants that might prevent the occurrence of subsequent events in the infection process [285]. Later work by Conner and Domisse has shown that many monocots can be infected by *Agrobacterium* but with certain provisos, such as the use of wide host range nopaline-type Ti-plasmid-carrying strains [67]. They also found that the site of inoculation on the plant and the stage of development of the site were both important [67].

This suggests that the 'phenolic inhibitors' are only produced in large amounts at certain times and in certain parts of the plant and possibly that the nopaline-type Ti-plasmid encodes a receptor that is more resistant to the action of the inhibitors at lower concentrations. Certainly it is not the attachment of the bacterium to the monocot (a necessary step for tumourigenisis [188]) that is the step that is blocked since *Agrobacterium* cells have been shown to bind to monocot plant cells in suspension culture [256].

The Ti-plasmid specified chemotaxis of *A. tumefaciens* to soluble factors produced by cells at a wound site would confer a selective advantage upon those *Agrobacterium* strains carrying a Ti-plasmid. However, the soil type also appears to play a crucial part in the infection process. When pea plants were grown in soil containing non-chemotactic *A. tumefaciens*, no tumour formation was found to occur [126]. Conversely, when sand was used instead of soil, tumours were formed [126]. It could therefore be argued that whilst chemotaxis is important in some soil types, it may only be of secondary significance in others [334]. Bouzar *et al.* noticed variations in the numbers of virulent *Agrobacteria* supported in different soil types and the effects that soil acidity may have on the tumour-forming ability of the *Agrobacterium* species present [34].

In general terms, however, it can be hypothesised that any *Agrobacterium* strain can be attracted towards the wound site of a plant but those possessing a Ti plasmid will be attracted more strongly given that the Ti-plasmid requiring attractants can be detected down to a concentration of 10^{-8} M by the relevant organisms. In a mixture of virulent and non-virulent strains in the soil, the Ti-plasmid bearing strains will be able to respond to wounding of a plant before the cured varieties are able to. Thus the number of tumours formed on plants in any given area is not reflective of the ratio of virulent to non-virulent strains in that soil.

1.7 Bacterial attachment

In order for *A. tumefaciens* to cause Crown Gall Tumour, attachment of the bacterium to a wound site is necessary [188]. The precise nature of this attachment has been open to a great deal of debate in recent years but the picture has recently become much clearer.

The facts that the binding of *Agrobacterium* cells to plant cells is saturable and that unrelated bacteria appear unable to compete for binding whereas related bacteria do, indicates the presence of specific binding sites [188].

Many chromosomal genes have been characterised that code for virulence functions - the *chv* (*ch*romosomal *v*irulence) genes. *chvA*, *chvB*, and *exoC* are all chromosomal genes whose products are responsible for the synthesis and export of β-1,2-glucan [43]. *chvA* codes for a 65 kD inner membrane protein that is required for transport of β-1,2-glucan into the periplasm and it may also play a role in processing the molecule [43]. *chvB* encodes a 235 kD inner membrane protein that is involved in the production of β-1,2-glucan [353]. The precise role of *exoC* has yet to be determined. A mutation in any one of these genes results in an avirulent Agrobacterium strain which is unable to attach to plant cells [242, 306]. This would suggest that β-1,2-glucan plays an essential role in attachment [242]. However, all these mutations also have indirect effects causing reduced conjugal transfer of some plasmids, reduced polysaccharide production and reduced motility [334].

Recently Swart *et al.* [299] have produced evidence suggesting that the active molecule involved in attachment is rhicadhesin. They found that although *chvB* mutants also produced this molecule, it was in an inactive form [299]. It has therefore been proposed that although rhicadhesin is the bacterial 'attachment molecule' it requires endogenous β-1,2-glucan either for correct processing or for anchoring of the molecule to the membrane. From these results, however, it is likely that another factor is involved since treatment of *chvB* mutants with rhicadhesin did not result in wild-type levels of attachment and this was unaffected by the presence or absence of β-1,2-glucan [299].

Mattysse [197] isolated three mutants (*att*) that were defective in attachment but appeared to have normal levels of polysaccharide production and β-1,2-glucan. These mutants failed to produce one or two outer membrane or periplasmic polypeptides of 33 kD, 34 kD and 38 kD. The precise nature of these proteins has

not been determined although it is possible that they too might be implicated in rhicadhesin production or anchorage.

There also appears to be a difference in binding between the different biotypes of *Agrobacterium*. It has been suggested that biotype 2 strain binding involves the interaction between negatively charged bacterial cells and glycoproteins with a positive charge on the plant cell surface [300]. Biotype 1 strains appear to require additional binding factors for the interaction [300].

It is not unlikely that a variety of these different interactions come into play at different times, depending on the biotype of the invading *Agrobacterium* and the precise environmental conditions present at any one particular wound site. However, these initial interactions between bacterium and plant are not strong enough to resist elemental forces and the invading bacteria is in danger of being washed away from the wound site. The next stage of the infection process involves the formation of cellulose fibrils [196].

In response to certain chemicals produced by the plant, the invading bacterium produces cellulose fibrils [196]. These fibrils serve two functions. First, they bind the bacterium firmly to the plant so that the organism is unlikely to be removed from the wound site [134, 195]. Secondly, the fibrils serve to entrap other *Agrobacteria* and thus form large aggregates which may serve to increase the chances of successful tumour formation [134, 195]. The genes responsible for the production of cellulose fibrils (*cel*), are all located close to one another on the bacterial chromosome in the same region that the *att* genes map to [249]. Robertson *et al.* have therefore proposed that there is a chromosomal region that is specifically responsible for bacterial attachment and cellulose synthesis [249].

The attachment of bacterium to plant cells at the wound site appears to involve specific saturable receptors [188, 189, 215, 256]. Current evidence regarding the plant cell receptor points to a cell surface protein with homology to the human serum factor, vitronectin, which has been identified in carrot [322]. Others have proposed that the receptor is pectic in nature [215]. This evidence does not however contradict the former in that it is possible that the vitronectin-like receptor is glycosylated or associated with pectic compounds [26].

1.8 The Ti plasmid

Hamilton and Fall were the first to report an avirulent form of *A. tumefaciens* that was stable and metabolically identical to known virulent strains [119]. This avirulence resulted from the incubation of *A. tumefaciens* at elevated temperatures and they thus postulated that this caused the loss of a 'virulence factor'. Van Larebeke *et al.* demonstrated that this loss of virulence was due to the loss of a large plasmid, which has come to be known as the Ti (*T*umour *i*nducing) plasmid [314].

Ti plasmids, of which there are four basic types, have sizes ranging from 190 - 240 kb [202, 334]. They each consist of 5 distinct regions: The T-DNA (*T*umour-DNA), the *vir* (*vir*ulence) genes, the opine metabolism region, a region for bacterial conjugation and one for replication incompatibility (see figure 1.8.1) [26].

The oncogenic functions of the bacterium are specified by the T-DNA [26, 217]. This region is transferred from the bacterium to the plant cell, whereupon it is stably inserted into the plant genome [26, 217]. The genes in this region encode proteins that are used for the manufacture of plant growth regulators (responsible for the tumour) and opines (see figure 1.8.2), which are small molecules used as a nitrogen and carbon source by the invading bacterium, but are of no use to the plant (see sections 1.10, 1.11, 1.12 and 1.13) [26, 217].

A Ti plasmid is classed as one of four types depending on the opine that is produced by the tumour that is caused as a result of the T-DNA integration into the plant genome. The classes are octopine-type, nopaline-type, agrocinopine-type and mannopine-type [334]. The grouping is, however, for convenience sake rather than a true reflection of the relatedness of the Ti plasmids to one another [202, 334]. The octopine-type Ti plasmids are a fairly homogenous group, but the nopaline-type plasmids are not a closely related group although some of them bear marked similarity to some octopine-type Ti plasmids [202, 334].

The transfer of the T-DNA to the plant is mediated by the action of the genes in the *vir* region and various chromosomal genes whose activity is determined by the presence of wound-specific phenolic compounds and sugars in the rhizosphere [26, 217]. The *vir* region is 35 - 40 kb and consists of the *vir* genes (*virA, virB, virG, virC, virD* and *virE* in that order) and a few other genes [26, 252]. Some Ti plasmids also contain another *vir* gene, *virF* (see figures 1.8.1 and 1.8.2) [144]. The products

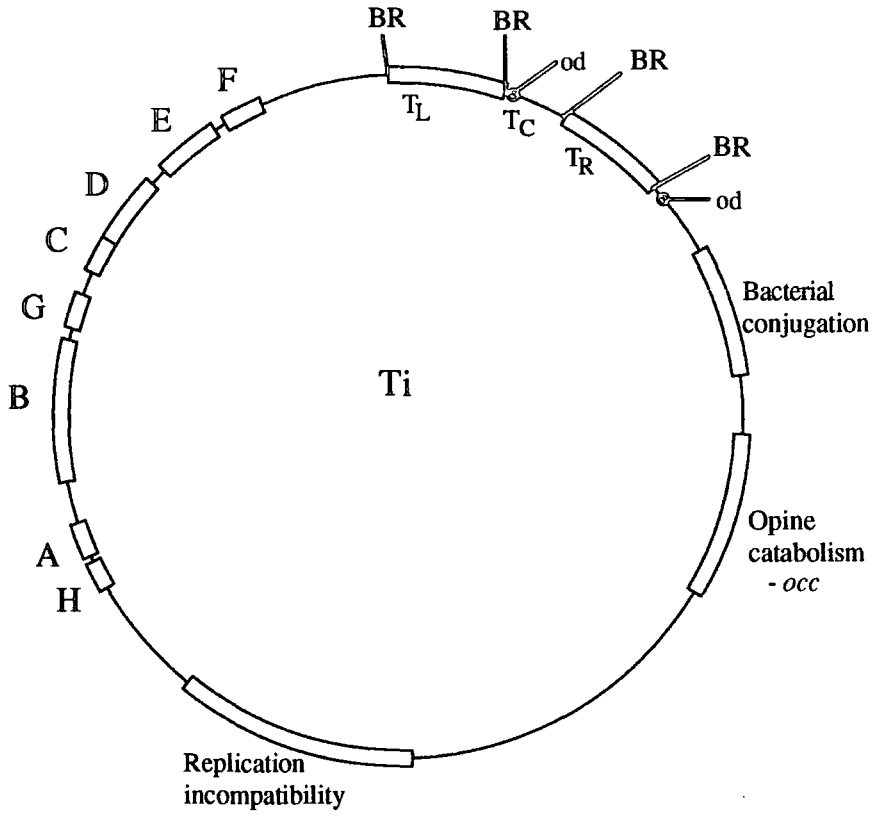


Figure 1.8.1 Octopine-type Ti plasmid showing approximate locations of the *vir* genes, the T-DNA and border repeats and the approximate locations of the loci for plasmid replication, bacterial conjugation and opine catabolism. Not to scale. BR = border repeat; od = overdrive sequence.

A)

B)

Figure 1.8.2 Organisation of the genes in the *vir* region. A) octopine-type, B) nopaline-type Ti plasmid.

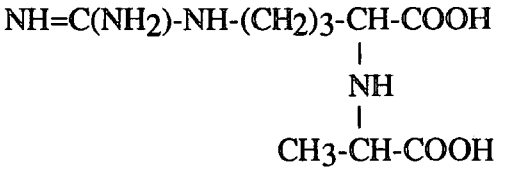
of the *vir* region are responsible for detection of some external stimuli and for the processing and transfer of the T-DNA.

The opine metabolism region encodes proteins that enable the invading *Agrobacterium* to take up and catabolise opines produced by the tumour [173, 217]. With very few exceptions, a strain which causes the production of a particular opine in the plant tumour also contains genes that are able to take up and catabolise that particular opine [305] as well as other related opines [350].

In octopine-type plasmids, part of the transferred T-DNA contains a gene (*ocs*) which encodes octopine synthase which catalyses the condensation of pyruvate and arginine to form octopine [161]. Another gene (*ons*) encodes a transport protein allowing octopine across the plant cell membrane [205]. Other genes (*mas1', mas2', ags'*) allow production of mannopine and agropine (see figure 1.13.1 and table 1.13.1) [26, 334]. On the corresponding octopine Ti plasmid in the bacterium, the *occ* region is responsible for opine catabolism and uptake, all of which are under the control of an opine-inducible promoter (see fig 1.8.4) [350]. The region consists of *ooxA* and *ooxB* which encode for octopine oxidase, *ocd* which encodes for ornithine cylodeaminase, *occQ* which may be involved in histidine transport, *occM* which may be involved in the transport of basic amino acids, *occP* which appears to be involved in some sort of active transport and *occT* which is likely to be involved in transport across the membrane [350]. Since all these genes are under the control of one octopine-inducible promoter (Pi1[occ]), it is likely that all the genes have a direct role in the transport and metabolism of octopine [334]. Octopine is thought to interact directly with the product of *occR,* which is the other gene in this region, functioning as a positive regulator which activates Pi1[occ] [334, 350]. A similar set of genes exists in nopaline type plasmids in the *noc* region [350].

The effect of opines in the environment, and the subsequent production of proteins involved in opine catalysis, has another effect - that of stimulating the production of conjugating factor (CF) and its receptor, TraR [239, 351, 352]. TraR is thought to act as a transcriptional activator which causes the expression of the *tra* genes, which are grouped into three loci (*traI, traII, traIII*) [22, 112]. In the nopaline Ti plasmid, pTiC58, the expression of TraR is thought to be mediated by the action of the repressor of the agrocinopine catabolism region (*acc*) - AccR [23]. Thus it would seem that agrocinopine catabolism and conjugal transfer are inextricably linked, although the genes required for CF production have not been characterised yet [112]. Although pTiC58 is usually classified as a nopaline plasmid, it is

A)



B)

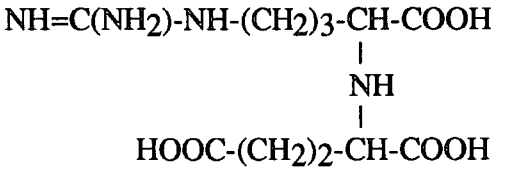


Figure 1.8.3 - Representative opines. A) octopine and B) nopaline

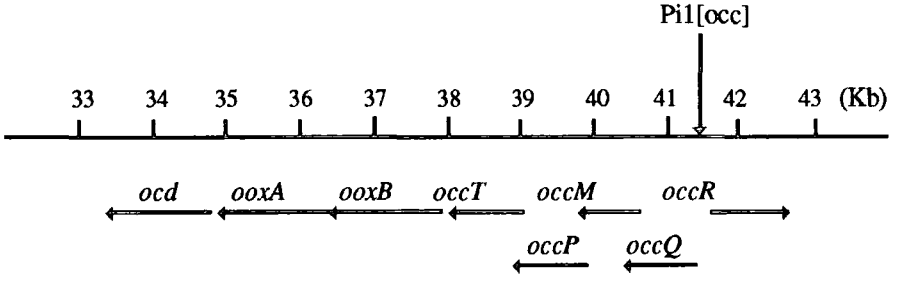


Figure 1.8.4 - The organsiation of genes encoding proteins required for opine transport and metabolism of the *occ* region in the octopine-type Ti plasmid, pTiAch5. After Zanker*et al.* [350].

agrocinopine A, and not nopaline, that induces conjugal transfer of this plasmid [23, 202]. Such an opine is termed a conjugal opine [23].

Octopine induces conjugal transfer of pTiAch5 [351], but whether this is via OccR (see figure 1.8.4) is not known at present [23]. However, NocR which regulates the nopaline catabolic functions on pTiC58 bears a close resemblance to OccR and yet nopaline does not induce conjugal transfer of pTiC58 [23, 350].

Another function encoded by some Ti plasmids is 'phage exclusion, by which replication of certain bacteriophages is inhibited [85]. Octopine plasmids inhibit the replication of 'phage Ψ and 'phage AP1 (a feature shared with some nopaline plasmids) [85, 137, 315].

Sensitivity to agrocin 84 is another function specified by the Ti plasmid (see section 1.2) [128].

Some Ti plasmids may also specify the production of certain plant growth regulators. Nopaline Ti plasmids contain a gene (*tzs*), whose product is involved in *trans*-zeatin production and also a locus that allows the production of indole acetic acid (IAA) [202].

Another region of the Ti plasmid is responsible for replication and incompatibility. Most Ti plasmids belong to one of two groups: *inc*Rh-1 and *inc*Rh-2 [202]. Another locus, associated with the replication locus, is *ein*, which is responsible for *e*ntry *in*hibition, an affect that is only apparent between non-homologous Ti plasmids [202]. Octopine plasmids all belong to the same *ein*-group, whereas nopaline plasmids belong to one of two different groups [202].

1.9 Molecular aspects of *vir* gene induction

The extent of induction of the *vir* genes is dependent on a number of environmental factors: pH, the presence of some low-molecular weight phenolic compounds, the presence of some monosaccharides, phosphate levels and the presence of some heavy metals. The levels of expression of the *vir* genes is determined by the pool size of VirG [194].

VirG is a transcriptional activator which positively controls the other *vir* gene promoters [292, 335]. The amount of VirG present in the cell is determined by the activity of two promoters, P1 and P2 [293, 333]. The upstream P1 promoter is

induced by phenolic compounds and low phosphate levels whereas the P2 promoter is induced by acidic pH [333]. The P2 promoter is also induced by other environmental stresses including heavy metals and certain mutagens and it bears a similarity to the consensus heat shock promoter of *E. coli* [194].

It is suggested, therefore, that low phosphate levels will induce the P1 promoter, thus increasing the VirG pool and so giving the bacterium a greater chance of successfully invading a plant which would provide this nutrient [333]. In the vicinity of a suitable infection site (a wound on the plant), the P2 promoter will also become activated since wound sites are acidic [161]. The P2 promoter may also be activated by phytoalexins, a group of antimicrobial compounds produced by some wounded plants [194]. Some phytoalexins have DNA-damaging properties and by acting through the P2 promoter, it may explain why some *Agrobacterium* strains appeared to be more pathogenic after treatment with UV light or mitomycin C [194].

With the activation of these two promoters, VirG should be present in high enough levels to allow the infection process to be successful when the main signal for the virulence process to begin is detected.

VirA is an environmental sensor whose function is to detect the presence of monosaccharides and plant released wound-specific phenolic compounds, and to pass this information on to the *vir* genes via VirG [48]. However, it appears that the VirA protein itself is not responsible for directly detecting the extracellular stimuli: Two small periplasmic proteins (p10 and p21) appear to bind the phenolic compounds and it is suggested that these then interact with the cytoplasmic domain of VirA [48, 184].

For the interaction with monosaccharides a chromosomally-encoded virulence gene is required, *ChvE* [42]. ChvE is a periplasmic binding protein that interacts with small sugars and then with the periplasmic region of VirA [42].

The putative interactions of the phenolic-bound p10 and p21 proteins with VirA causes the latter to become autophosphorylated [141, 153]. The interaction of sugar-bound ChvE with VirA appears to stimulate this autophosphorylation further [42]. The autophosphorylated VirA protein acts then as a protein kinase, phosphorylating VirG at aspartate 52 [141, 152, 153]. The phosphorylation of VirG either allows its interaction with RNA polymerase to be altered or alters its binding capabilities to the other *vir* gene promoters [334].

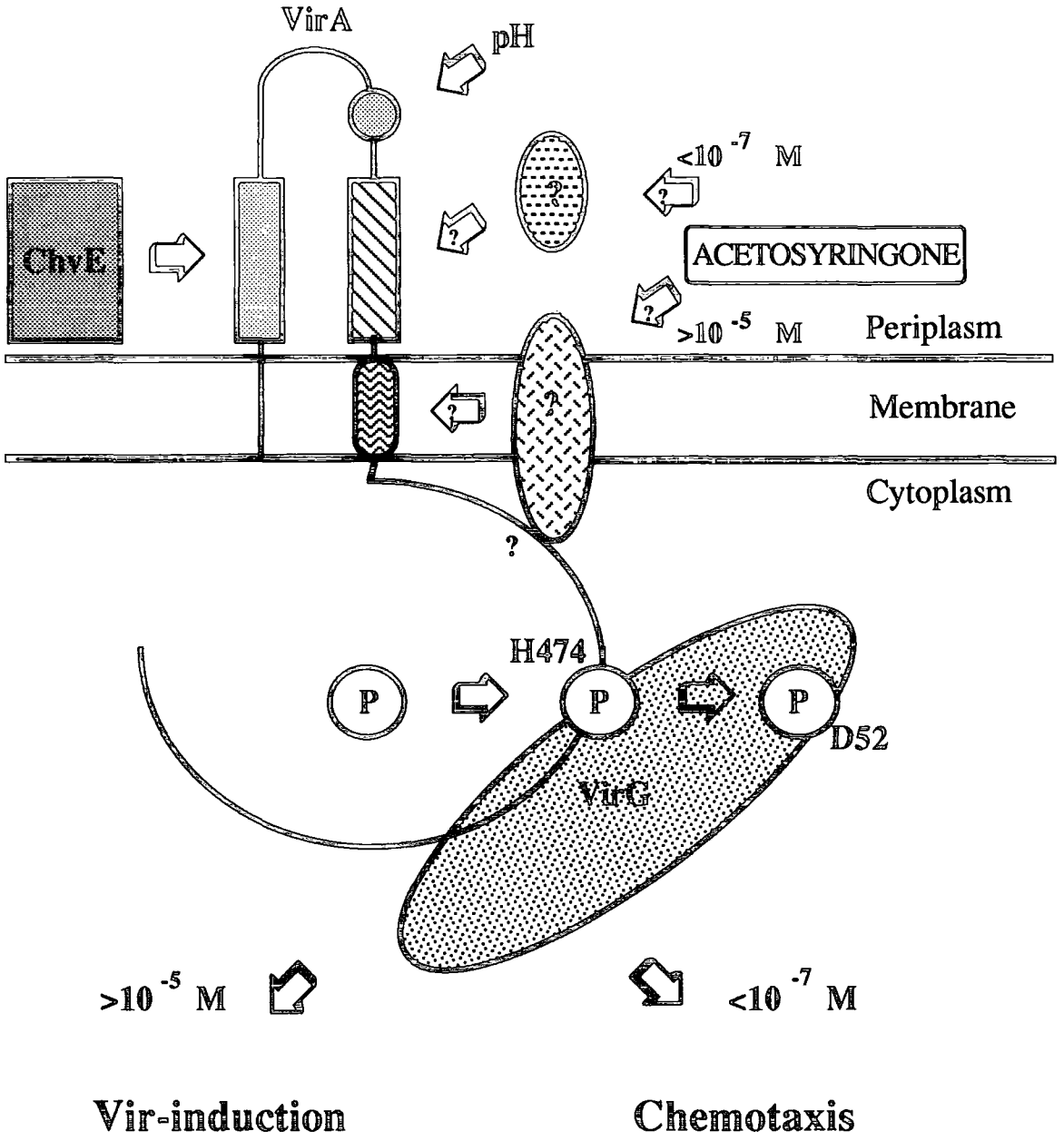


Figure 1.9.1 - Proposed mechanism for phenolic detection. Question marks represent hypothetical processes, whilst those within ovals represent putative phenolic binding proteins (one low affinity and one higher affinity), possibly p10 and p21 (see text). H474 and D52 are amino acid residues that are phosphorylated on VirA and VirG respectively. Redrawn from Shaw [271].

Interestingly, the C-terminus of VirA is homologous to the N-terminus of VirG - a so-called 'receiver domain' [48]. It is possible that these receiver domains compete with one another for the phosphorylation by the active VirA kinase, since the presence of the VirA receiver domain reduces the ability of VirA to phosphorylate VirG [48].

This functioning of VirA and VirG as a two-component regulatory system is complicated by the suggestion that VirA and VirG mediate the chemotactic response towards phenolics [231, 272]. It is hypothesised that the phosphorylated VirG component of this system has a greater affinity for part of the chemotaxis pathway and thus the small amounts of phosphorylated VirG produced at low concentrations of phenolics would be preferentially involved in chemotaxis signalling [231]. At higher concentrations, the chemotaxis component interaction becomes saturated and the phosphorylated VirG then activates the *vir* genes (see figure 1.9.1) [231]. However, it also apparent that chemotaxis towards sugars does not involve VirA. It is suggested instead that the sugar-bound ChvE protein interacts with a Trg-like protein [42].

Activation of the other *vir* genes appears to be by direct binding of the phosphorylated VirG to parts of the *vir* gene promoters called '*vir*' boxes [233, 234]. The consensus sequence for *vir* boxes is 5'dryTncAaTTGnAaY (R = A/G, Y = C/T) and each *vir* gene has up to five *vir* boxes, although not all of them are required for expression of the gene [233]. It is thought that VirG forms a dimer or tetramer and it is this that binds to the *vir* boxes and interacts with RNA polymerase [233]. A suggestion is that the phosphorylated form of VirG is able to convert the closed RNA polymerase-promoter complex to its open form, allowing transcription whereas the unphosphorylated form is unable to do this, although both forms can bind to *vir* boxes [152].

The interaction of phosphorylated VirG with the *vir* boxes serves to activate the expression of all the other *vir* genes, including *virA* and *virG*, forming a positive regulatory loop in the latter cases and committing the bacterium to the infection process [74].

virC and *virD* regulation is also negatively regulated by another factor, Ros, a chromosomally encoded protein [61]. This is a small (15.5 kD) protein that binds to a specific site in the *virC* and *virD* promoters, indicating that some form of competition between phosphorylated VirG and Ros might occur [69]. This might serve as a mechanism to control VirG-mediated expression of *virC* and *virD*, the

levels of which may determine how efficiently certain types of host plant are infected [69].

1.10 T-DNA transfer

The actions of the various Vir proteins allow the copying and transfer of small pieces of oncogenic DNA (T-DNA) to the plant [26].

The origin of the T-DNA is on the Ti plasmid and different types of Ti plasmid have different arrangements of the T-DNA region (see figures 1.8.1 and 1.13.1) [202]. T-DNA regions are delineated by imperfect 25 bp direct repeats [278, 340]. In the case of nopaline plasmids there is only one long region whereas in octopine-type Ti plasmids there are two regions, T_L and T_R, one or both of which can be transferred [202]. These two regions are separated by another region, T_C, which is not usually transferred [202].

Of the 25 bp direct repeats that border each T-DNA section, only the right border is essential for tumorigenesis, allowing DNA to its left to be transferred in a polar fashion [274]. The left border does not appear to be necessary for tumour formation [158]. In octopine-type plasmids, there is an additional DNA sequence (*overdrive*) found near to the right border that enhances the efficiency of tumour formation [236]. This is 24 bp in the case of pTiA6 and will function effectively in an inverted orientation and up to about 7 kb away from the right border repeat in either direction [236, 313]. There is no sequence homology between *overdrive* sequences from octopine plasmids and sequences to the right of the right border sequence in nopaline plasmids, but similar enhancing sequences may exist in these plasmids [150, 323].

The border repeats themselves function as 'nick sites' [324]. VirD1 is probably a topoisomerase involved in relaxing the DNA at the border repeats, thus allowing VirD2 to specifically nick the DNA between base pairs 3 and 4 from the left end of each border [100, 349]. It has also been suggested that VirD2 functions as a 'pilot protein' since it binds to the 5' end of the T-DNA strand, subsequent to nicking, and it also has two nuclear targeting signals [90, 129, 139]

The nicks in the T-DNA region allow the formation of a single stranded section of DNA (T-strand) from the lower strand that is unwound from the double stranded molecule in a 5' to 3' direction [291]. This process allows the synthesis of a new lower strand, in the T-DNA region, from the 3' end at the right border repeat,

and it is possible that this new synthesis helps the unwinding of the T-strand [291]. In addition to this VirC1 and VirC2 bind to *overdrive* in octopine plasmids and greatly facilitate the formation of the T-strand [334]. VirC2 may have a role to play in the unwinding of the DNA or in the new lower strand synthesis [334]. VirC1 appears to direct the T-strand processing proteins to the right border repeat [211]. VirD1 and VirD2 may also help in the unwinding process [349].

VirE2 is thought to bind to the T-strand and thus protect it from the actions of nucleases during its passage out of the bacterium and through the plant cell [59]. VirE1 may have a similar role [334]. These proteins may also serve to keep the T-strand in a linear unfolded form that is more easily transferable [59].

The eleven VirB proteins are thought to form a complex channel across both bacterial membranes [308]. Thorstenson *et al.* have suggested that where the inner and outer membranes are in close proximity to one another, the VirB proteins interact closely with each other forming a complex channel that opens in response to the presence of the T-strand [308]. The products of *virB4* and *virB11* may produce the energy required for translocation [271, 308]. Further to this, it has also been shown that VirD4 is associated with the inner membrane and it may also form part of the T-strand transfer apparatus [225]. It is certainly clear that VirD4 is essential for T-strand transfer [178].

As for the products of the other *vir* loci, the functions are unclear. VirD3 appears to have no role in the cell and it is not clear whether the fifth open reading frame (ORF5) in the *virD* operon is actually expressed [178]. *virF* (along with *virC* and *virE*) are host range genes that can affect the T-DNA transfer efficiency depending on the host plant species [211]. Its product may be responsible for the production of a secreted factor [211]. *virF* is however only present in octopine type plasmids [334]. *virH* (also known as *pinF*) is also only present in octopine-type plasmids, where it encodes two cytochrome P-450-type enzymes whose function may be to counteract the effects of certain bactericidal plant products encountered during the infection process [349].

1.11 Targeting of the T-strand

The T-strand consists of the T-DNA bound with one VirD2 pilot protein and hundreds of VirE2 molecules bound along its length, probably associated with other proteins [349]. The strand has a diameter of approximately 2 nm and a 20 kb T-DNA (the size of the nopaline-type T-DNA) would be about 3600 nm long [59,

349]. At one stage it was thought that the transferred molecule was circular but new evidence suggests that it is linear [17, 179, 349]. It is envisaged that the strand exits the cell, via the *virB* encoded transmembrane pore, in a polar fashion being led by the VirD2 pilot protein [138, 349]. Wagner and Mattysse have speculated that the binding of the bacterium to a vitronectin-like protein on the host cell surface affords the T-strand a direct channel to the centre of the cell [322]. They argue that the vitronectin-like protein is bound via integrin to the actin cytoskeleton and that the T-strand is transported into the centre of the cell via the 'actin tracks' already in place.

Once the T-strand is in the plant cell it needs to be directed to the nucleus, which is probably a function of the VirD2 and VirE2 proteins [349]. These both contain sequences that have high homology with nuclear localisation signals. The last 29 - 31 amino acids of the C-terminus of the VirD2 protein is thought to contain a very strong nuclear localisation signal (NLS) [349]. Zambryski speculates that the VirD2 pilot protein targets the T-strand to the nuclear pore in a polar fashion as a consequence of this NLS, however due to the length of the T-strand, this in itself is not sufficient to allow uninterrupted transfer into the nucleus. Therefore two separate NLS-homologous regions of the VirE2 protein are envisaged to allow this transfer to be facilitated [60, 348].

1.12 T-DNA integration into the plant genome

The target sites for T-DNA integration seem to share significant homology with the borders of the T-DNA and active transcriptional regions are preferred [177, 199]. A small deletion usually occurs at the target site and analysis of such integration sites has shown that the right border end of the T-DNA is usually preserved, but the left border end can lose up to 100 bp [199]. It is postulated that this is due to the right border end being protected, from the plant DNA repair/processing functions, by the tightly bound VirD2 molecule [349]. Integration is thought to occur in a manner analogous to 'illegitimate recombination', with the 5' end of the T-strand invading a nick in the plant DNA [199]. VirD2 itself may be involved in nicking the target site [178]. A portion of the VirD2 protein bears a strong identity to *E. coli* DNA ligase, suggesting that this protein may also have an active role in the integration of the T-DNA [334]. Some base pairing of the 3' end then takes place nearby and the DNA loop thus formed sets up stresses in the corresponding plant DNA which then nicks and gap repair and filling in produces a final product that includes the T-DNA as a double stranded region [349].

1.13 T-DNA expression *in planta*

The products of the T-DNA genes are involved in either opine synthesis and its subsequent transport or in altering the levels of plant growth regulators produced (see figure 1.13.1 and table 1.13.1) [334]. *iaaH* and *iaaM* are responsible for the production of indoleacetic acid [267, 316] and *ipt* provides a product that catalyses the first step towards the production of *trans*-zeatin and *trans*-ribosylzeatin [20, 40]. The other characterised genes are all involved in opine biosynthesis (see table 1.13.1). The precise nature of transcript 5 and *tml* have not been elucidated but they do have an effect on tumorigenicity in combination with the other genes [334].

Expression of the T-DNA genes is effected by the plant cell - the gene promoters possessing typical features of plant promoters, including CAAT and TATA boxes [334]. There may also be some functional enhancer regions in the T-DNA and it would appear that the mRNA contains eukaryotic poly-A-tails. Transcription is thought to involve RNA polymerase II [332].

However, the expression of these genes in the plant is not constitutive. Dymock *et al.* have shown that the *ipt* gene is not expressed in leaves [79].

1.14 *Agrobacterium tumefaciens* as a biocontrol agent

Over the last two decades there has been an increasing awareness of the environmental affects of large scale agriculture and the vast amounts of chemical pesticides that are used. Public concerns regarding the harmful nature of some of these pesticides has led to much research into finding alternatives.

The concept of the control of one organism by using another has been in existence since the beginning of the century, but it was generally considered too expensive and impractical and so only recently has 'biocontrol' started to be developed in any coherent fashion [16].

In general, the use of organisms as control agents has many possible advantages including the fact that they are generally safer than chemicals, they do not tend to accumulate in the food chain, resistance to them is generally slow in its development and they do not require repeated application [110]. However, the effectiveness of a biocontrol agent is very much dependent upon environmental

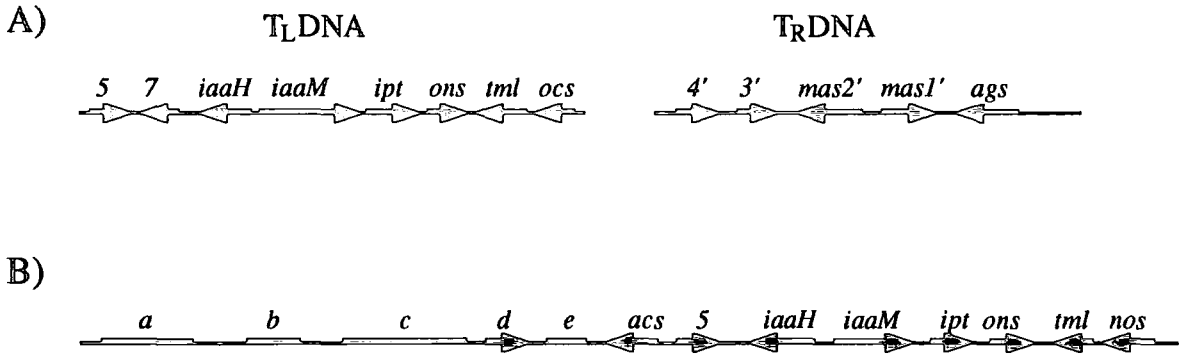


Figure 1.13.1 Organisation of the genes in the T-DNA regions of A) octopine-type plasmids and B) nopaline-type plasmids. Redrawn from Winans [334].

Locus	Protein Function	Role in tumorigenesis
iaaM	Tryptophan monooxygenase	Auxin synthesis
iaaH	Undoleacetamide hydrolase	Auxin synthesis
iptt	Isopentenyl transferase	Cytokinin synthesis
5	?	Tumorigenesis
tml	?	Tumorigenesis
ocs	Octopine synthase	Opine synthesis
nos	Nopaline synthase	Opine synthesis
acs	Agrocinopine synthase	Opine synthesis
mas2'	Mannopine synthesis	Opine synthesis
mas1'	Mannopine synthesis	Opine synthesis
ags	Agropine synthesis	Opine synthesis
ons	Permease	Opine secretion

Table 1.13.1 Genes present on the T-DNA region of Ti plasmids and their functions. From Winans [334].

conditions that it encounters and so its effectiveness may vary widely. It must also be ensured that the agent is being targeted properly [110].

Various microorganisms have been used as biocontrol agents against fungi and many more are under development [110]. *Bacillus subtilis* has been used to control *Fusarium roseum* infections in corn and fluorescent pseudomonads have been widely used to control many diseases affecting potatoes, wheat, tobacco and radish [110]. However, the most widely publicised research, in recent years, has been directed towards the δ-endotoxin produced by *Bacillus thuringiensis* to control insect pests [133]. Many commercial spray on 'Bt' products now exist which incorporate a crude preparation of *B. thuringiensis* spores, including Certan (Sandoz, Crop Protection Corp.), Skeetal (Novo Laboratories) and Dipel (Abbott Laboratories) [72]. However, there are problems associated with this method of dispersal since the preparations are liable to be washed off the plants and are inactivated by sunlight, requiring repeated applications [6, 241].

Many new approaches using *B. thuringiensis* (Bt) toxins involve the cloning of the toxin gene into the plant requiring protection [52, 91, 94, 237, 312]. However, this sort of approach has many problems associated with it, not least of which is the transformation of the plant and the continued expression of the gene in its progeny [72]. There is also the possibility that continuous expression of the toxin in plants could lead to a greater rate of the development of resistance in the target organism [72]. Consumer concerns may also play a part and a food substance from a plant producing a toxin may not gain consumer acceptance. The toxin genes themselves may also affect that growth and yield of particular plants. It is also worth noting that each different variety of crop plant needs to be genetically engineered with the toxin, which is far more costly than producing a biocontrol pesticide that can be used on a wide variety of crops. In the case of the δ-endotoxins, synergy is often observed between different toxins [301]. This synergy may be very important in the control of some insect pests, but to manipulate a crop plant to produce two or more toxins would be very difficult. It should also be remembered that the varieties of insects any particular crop is exposed to, varies from year to year and thus a more flexible system of applying the toxin to plants is required.

Rhizosphere microorganisms are good candidates for use as possible biocontrol agents since they can readily replicate (hopefully negating the need for repeated applications) and they are usually found in close proximity to the roots, the roots themselves providing a physical barrier to invading pathogens [325]. A non-

tumorigenic form of *A. tumefaciens* would therefore seem to be a good organism from which to develop a genetically engineered biocontrol agent.

Such a system has been proposed by Ashby *et al.* [9, 10]. It is envisaged that certain genes encoding toxins or lytic enzymes could be put under the control of a *vir* gene promoter, allowing expression of that gene only at a wound site. Thus an insect or fungal pest would cause localised wounding of a plant eliciting the release of wound specific phenolics. These would then attract the bacterial biocontrol agent to the wound site, and once there the higher concentration of phenolics would cause induction of the *vir* promoters and hence the bacterium would release the toxin/enzyme and destroy the pest [9, 10].

The main advantage of this system over other so-called 'microbial inoculant' systems, is that the pesticide is only produced in response to attack of the plant by the pest. It is hoped that this conservative process will ensure that large amounts of the pesticide will not amass in the rest of the surrounding area. This would ensure that only the harmful pests are targeted and that there is no build up of resistance.

Here, I show the construction or various plasmids containing a *vir* promoter linked to a toxin or a lytic enzyme gene and their expression in *A. tumefaciens* C58C1. The genes chosen for this work were *chiA* and *chiB* chitinase genes from *Serratia marcescens* and the δ-endotoxin from *Bacillus thuringiensis* var. *kurstaki* 4D4.

1.15 The chitinase genes of *Serratia marcescens*

Chitin is a major structural component of many organisms, being found in fungal cell walls (except Oomycetes), insect exoskeletons, nematode worms and the shells of crustaceans [209, 212]. The molecule itself consists of β-1,4-linked N-acetylglucosamine units forming an insoluble linear polymer [92].

The presence of chitin in a wide variety of pests makes chitinase a good candidate for a suitable biocontrol agent [53, 143, 229]. Chitinases exist in bacteria, fungi, higher plants and animals [30, 109, 209, 212]. Fungal growth has been shown to be inhibited by a variety of chitinases [229, 247, 248, 264]. It has been suggested that endogenous chitinases in higher plants are part of a defence mechanism against invading fungi [30]. Neuhaus *et al.* reasoned that the overexpression of a plant chitinase in the plant would produce greater resistance to fungal attack [221], however, certainly for the class of chitinase used, this did not appear to be the case.

Jach *et al.* suggested that the fungus had adapted to the plant defence mechanism and that to obtain protection from the fungus an unrelated chitinase was required [143]. This approach, using the *chiA* gene of *Serratia marcescens*, expressed in tobacco, proved more successful.

In a study, out of 203 different strains of bacteria, Ordentlich *et al.* showed that *Serratia marcescens* was the most effective as a biocontrol agent of *Sclerotium rolfsii* [228]. *S. marcescens* has also been shown to produce large amounts of chitinase [209, 246]. Subsequently the bacterium, a Gram-negative soil inhabitant, has been shown to produce five chitinases with molecular weights of 21, 36, 48, 52 and 57 kD [93]. The 57 kD protein being shown to be the most abundant, has been designated *chiA* , and it has been cloned by at least four groups [93, 157, 269, 297]. In all these studies, the *chiA* gene product was shown to have an inhibitory effect on fungal growth. Jones *et al.* also cloned another chitinase gene, producing a 52 kD protein, and this has been designated *chiB* [157].

Both these genes have been linked to *vir* promoters in previous studies and expressed in *A. tumefaciens* C58C1 [9, 187]. Two further constructs were made and examined in this study.

1.16 The δ-endotoxin of *Bacillus thuringiensis* var. *kurstaki* 4D4

Bacillus thuringiensis is a Gram-positive soil bacterium which produces characteristically shaped spore bound crystals during sporulation [180]. The crystals are composed of insecticidal proteins (δ-endotoxins) of which one or more are produced by a single *B. thuringiensis* subspecies [328].

Most of the crystal proteins (Cry) show specificity towards Lepidopteran insects but others are now being discovered with specificities to Coleopteran and Dipteran insects and even nematode worms and flatworms [89, 328, 354]. The Cry proteins were divided by Höfte and Whitely into four classes, which are related to their biological activity [133]. Toxins encoded by the *cryI* genes are Lepidopteran specific, those encoded by the *cryII* genes are active against Lepidopteran and Dipteran insects, *cryIII* genes encode Coleopteran specific toxins and *cryIV* gene products are specific for Dipterans [133]. Two novel δ-endotoxins have recently been reported which are toxic to both Coleoptera and Lepidoptera and these have been designated CryV [104, 302]. The *cytA* gene, present in some subspecies of *B. thuringiensis* also encodes a toxic protein which is Dipteran-specific but this is

unrelated to any of the *cry* genes, which probably share a common ancestral origin [133].

A *B. thuringiensis* preparation was first marketed in the early 1950s, but since then these preparations have grown in popularity, especially in recent years, as the cost has come down, the hazards of many chemical pesticides have become more clearly understood and insect resistance to the chemicals has increased [277]. In 1989 the market for *B. thuringiensis*-based pesticides accounted for about 95 % of the biocontrol pesticide market and was worth $107 M, this figure being expected to triple by the end of the millennium [89].

The preparations are usually made up of the spores and the crystals isolated from stationary phase cultures of the bacterium which are undergoing lysis. They are used at about 10 - 50 $g.acre^{-1}$, which is less than the concentration that conventional chemical pesticides are used at [89]. However, this method of dispersal has problems associated with it. The spores and crystals are easily washed off the plants and the preparations are inactivated by sunlight, requiring repeated application [6, 241]. Therefore, much current research interest has been directed towards developing a stable method of applying the pesticide.

There has been much work done on recombinant DNA *B. thuringiensis* products, but only two are currently licensed for use - Mycogen Corp.'s MVP and M-Trak products. These are *B. thuringiensis* δ-endotoxin-expressing strains of *Pseudomonas fluorescens* which are killed and fixed, forming capsules of toxin which persist longer in the field and have a greatly extended shelf-life [89]. Other recombinant microorganisms have also been used to try to deliver the toxin. Turner *et al.* have described a system using *Clavibacter xyli* which colonises the vascular system of some important crop plants, providing protection to plants possessing the recombinant toxin-producing bacterium [311]. Others have tried expressing the endotoxin in root-associating microorganisms such as *Pseudomonas fluorescens, Agrobacterium radiobacter* and *Rhizobium* strains [224, 281, 321].

Another approach to deliver the toxin to the target insects has been to develop a recombinant baculovirus which infects the insects [245].

Many groups have engineered *B. thuringiensis* toxins into plants, with varying degrees of success, but so far potato, cotton, tomato and tobacco plants have been successfully engineered with *cry* genes and shown to be more resistant to insect attack than their wild type counterparts [52, 91, 94, 237, 312].

At present, various companies have transgenic plants expressing *B. thuringiensis* toxins undergoing small-scale field trials, including Agrigenetics, Kubota, Monsanto and Plant Genetic Systems [89]. One, Crop Genetics International, is carrying out field trails using endophytic microorganisms expressing the toxin [89].

These biocontrol methods all have advantages and disadvantages. For transgenic plants the disadvantages include the difficulty of getting high levels of expression from the toxin genes; the fact that some useful toxin genes may not be able to be expressed at all in some plants; the length of time it takes to engineer a plant; it is unlikely that a broad range of toxins will be able to be expressed simultaneously in one plant; yields or the product quality may be adversely affected; consumers may not accept the products. For the bacterial systems, although more easily engineered, multiple applications may be needed - certainly for the 'dead systems'; the organisms may escape and deliver the toxin in an unwanted fashion in an unsuitable ecological niche; they are more likely to mutate and lose the toxin genes; they may only function effectively in certain environments [72, 110, 132]. Neither method is likely to prove ideal and thus a combination of both methods may be the realistic outcome of this sort of research.

One point to bear in mind, however, is the application of this new technology to the Developing World, where it could be of great benefit, since, in the end, it should be cheaper than chemical pesticide application. In tribal cultures, certain types of plants have various cultural significances and geographically close villages may grow different varieties of the same plant and be unwilling to change to a standard type. In this case, the 'microbial inoculant' system will be more advantageous especially if it can be incorporated into the soil on a more permanent basis.

1.17 The mode of action of *B. thuringiensis* δ-endotoxin

In this section, the mode of action and processing of CryIA(c) is briefly outlined. The general principles of these processes can also be applied to other Cry proteins.

CryIA(c) is a Lepidopteran-specific δ-endotoxin from *Bacillus thuringiensis* var. *kurstaki* 4D4, formerly *kurstaki* HD-73 [3]. The gene is found on a large 75 kb plasmid and is the only type of *cry* gene found in this subspecies, although many

subspecies possess more than one [133]. CryIA(c) is one of three CryIA δ-endotoxins all of which share over 80 % amino acid identity with one another [133].

Upon sporulation the bacterium produces characteristic bipyramidal crystals made up of the protoxin. This is a 133.3 kD protein consisting of 1 178 amino acids [3]. All Cry proteins appear to be composed of a highly conserved carboxy terminal half and a more variable amino terminal half, corresponding to a structural fragment and an active fragment (see figure 1.17.1) [133].

It has been suggested that the carboxyl half of the protoxin is important for crystal formation and it has been found that the protein crystal is stabilised by disulphide bonds formed in the carboxyl terminal half [133, 222].

Upon ingestion by the insect, gut proteases cleave the protoxin [6]. In the case of CryIA(c), it is proposed that this proteolysis occurs in a sequential fashion from the C terminus producing 80 - 90 amino acid fragments, which are then quickly degraded, thus leaving the 67 kD amino terminal half of the protoxin as a protease-resistant core [56]. It is likely that the first 28 amino acids from the amino terminus are also lost [6]. The activity of the δ-endotoxin is determined by the presence of other δ-endotoxins and also by the environment in the midgut; some insect midguts will dissolve and breakdown the protoxins in different ways to others [7, 118].

The active toxin, consisting of amino acids 29 - 695 consists of two domains - the toxic domain and the cell binding domain (see figure 1.17.1) [102]. The toxic domain contains five sequence homology blocks, common to most Cry toxins [133]. Also present are six α-helices which appear to be important for toxicity [102]. The binding domain is generally more conserved, having much β-sheet structure and determines the selectivity of the toxin, binding to the receptor in the membrane of midgut epithelial columnar cells [68, 136].

The proposed receptor for CryIA(c) is a 146 kD protein [176]. After binding it is proposed that the toxin undergoes a conformational change which allows the toxic domain to be inserted into the cell membrane [68]. It is proposed that the domains of six molecules then congregate forming a non-selective pore across the membrane [102]. The formation of the pore then upsets the K^+ driven uptake of nutrients and the pH balance resulting in cell lysis. Alternatively the influx of ions into the midgut cells results in the uptake of water, resulting in cell lysis [136]. The lysis of cells in the gut renders it useless and the consequent lack of nutrient uptake eventually

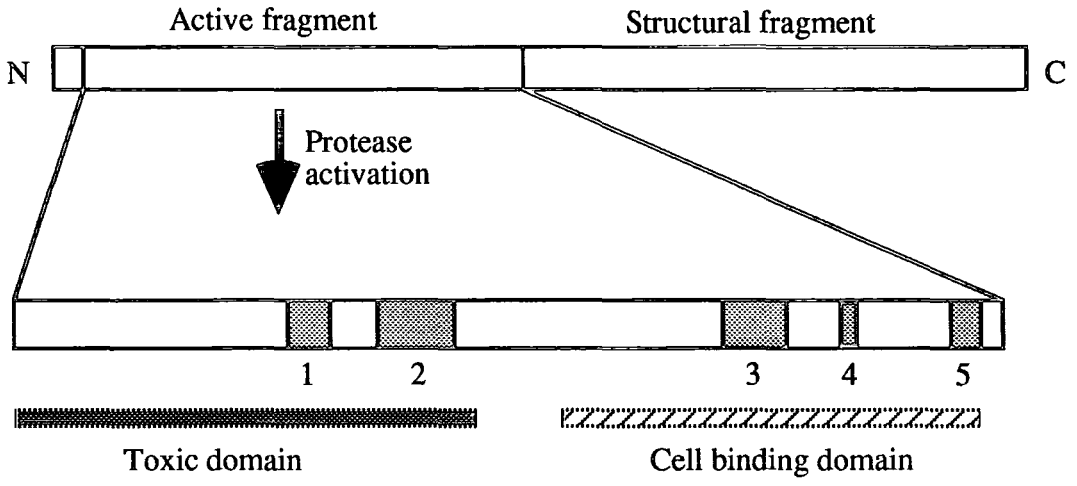


Figure 1.17.1 Diagram showing domains and activation of CryIA(c). Five conserved regions common to most Cry proteins are shaded. Redrawn from Gill *et al.* [102].

starves the insect. The insects are often seen to be paralysed after being exposed to the toxin and it is presumed that this is also caused by the changes in the midgut [136].

One of the major commercial problems associated with δ-endotoxins from *B. thuringiensis* is that the toxins are too specific and thus a range of different toxins are often applied to control more than one species of insect. 'Dipel' (Abbott Laboratories), which is a spray on formulation used for controlling caterpillars, contains CryIA(a), CryIA(b), CryIA(c) and CryIIA [89]. To engineer all of these into one plant would be very difficult if not impossible; a possible answer to this problem would be to engineer each one into the crop plant and mix the seeds whilst planting. A more practical approach might be to engineer each of these into some form of microbial inoculant. However, bearing in mind the current progress being made into the understanding of toxin-receptor binding a more practical approach may well be to broaden the host range of the toxin by altering the binding domain [118, 277].

Another commercial problem for *B. thuringiensis* based pesticides, is the potential for resistance development. Many workers have now shown resistance developing to these pesticides [102]. It has been predicted that if widespread use was made of toxin-expressing plants, the technology would become useless in 3 to 9 years [124]. Harris suggests that such problems could be curtailed by engineering the pesticide producing plants in such a way that the pesticide is only produced "in the susceptible tissues during the brief periods they are vulnerable to attack" [124].

This is the main advantage of the proposed *Agrobacterium*-based system. However, the real answer to controlling resistance must lie in the use of a range of biocontrol methods and in the case of the *B. thuringiensis*-based ones, using a wide variety of different δ-endotoxins. The Mycogen Corp. have a collection of over 3000 *Bacillus thuringiensis* strains and so many new endotoxins with differing specificities are bound to be found [89]. Using a broad range of *B. thuringiensis* toxins in a variety of delivery systems coupled with the use of other pesticidal proteins (including lectins and chitinases) and maybe selective application of chemicals, an effective but more environmentally responsible and healthier way of producing quality food products is within reach.

1.18 Aims of the project

In order for the proposed 'microbial inoculant' to function it was necessary to determine whether *vir* induction was actually capable of occurring at the wound sites of plants, since to our knowledge this had not previously been demonstrated. Lilley [187] had tried to show wound site induction using *lacZ* as a reporter gene, with some success, although this work needed to be confirmed. Attempts by Lilley to confirm this using *lux* as a reporter gene failed [187]. Thus in an attempt to show wound site *vir* induction, the work by Lilley using *lacZ* was to be repeated. This was to be confirmed using GUS or *lux* as a reporter gene. Two *vir*::GUS constructs were available for this work [97, 187] as were two *vir*::*lux* constructs [253].

It was also necessary to ascertain how stable the proposed plasmid containing the inducible pesticidal protein constructs were in soil. For this, plasmid stability assays were to be carried out, using soil, on *A. tumefaciens* carrying various plasmid types.

Since the work was to depend upon *vir* induction, a suitable assay procedure needed to be designed to elicit maximum responses from the various *vir* promoter constructs. To this end, four induction media, regularly used by other groups, were to be employed to determine which elicited the best response from the strains used in this study. A convenient method of setting up *vir* induction assays also needed to be devised.

Lilley [187] had previously made constructs containing the *virB* promoter linked to the *chiA* and *chiB* genes from *Serratia marcescens*, as well as one construct that contained *chiA* under *virB* promoter control. A further construct was to be produced in which the *chiB* gene was to be placed under *virE* promoter control. *A. tumefaciens* carrying these constructs were then to be assayed for their relative *vir* induction levels, allowing the construct that produced the most chitinase to be found. *A. tumefaciens* carrying these constructs were then to be assayed against various types of fungi to determine whether inhibition of fungal growth could be observed.

There is much confusion over the precise action of both ChiA and ChiB from *S. marcescens*. The chitinases were to be analysed by studying their effectiveness against a range of substrates, thus determining which parts of the chitin filament were susceptible to attack by the respective chitinases.

In order for the chitinases to be effective in controlling fungi, they need to be secreted by the 'microbial inoculant'. In order to study this, cellular fractionation studies were to be performed on *chi*$^+$ strains of *E. coli* to confirm that the chitinases were secreted. These studies were then to be extended to *chi*$^+$ strains of *A. tumefaciens* to examine the differences in expression levels and secretion of the two chitinases between the two bacteria. It was hoped that knowledge of the secretion and expression in *E. coli* might help elucidate any problems that might be encountered using *A. tumefaciens*.

The next part of the project was to try to express a *Bacillus thuringiensis* δ-endotoxin in *A. tumefaciens* to determine whether a functional toxin could be produced by this bacterium. Further to this, an inducible construct was to be made such that the gene was placed under the control of a *vir* promoter and the resulting plasmid was to be introduced into *A. tumefaciens* in order to determine whether the construct would function as expected. The toxicity of this strain, in both induced and uninduced states, to the larvae of a Lepidopteran insect was then to be determined.

It was thus hoped that at the end of the project the feasibility and effectiveness of an *A. tumefaciens*-based 'microbial inoculant' would have been determined.

Chapter 2

MATERIALS AND METHODS

2.1 Reagents

All inorganic chemicals were of AnalaR quality and from BDH Chemicals Ltd., Poole, Dorset, U. K. or Sigma Chemicals Plc., Poole, Dorset, U.K. unless otherwise specified.

Sodium chloride was from Riedel-de Haen, Seelze, Germany.

Anti-β-lactamase Ig was from 5 Prime → 3 Prime Inc., Boulder, Colorado, U.S.A.

Lab M Nutrient Broth (#2), Lab M Nutrient Agar, and nitrocellulose and nylon hybridisation transfer membranes were from Amersham Ltd., Bury, U.K..

Agar bacteriological (#1) and Yeast extract were from Oxoid Ltd., Basingstoke, Hants., U.K..

Trypticase Peptone was from BBL, Cockeysville, MD, U.S.A..

Bacto-peptone was from Difco Laboratories, Detroit, U. S. A..

Murashige and Skoog plant salt minimal organic mixture was from Flow Laboratories Ltd., Irvine, U.K..

Restriction endonucleases, corresponding buffers, T4 DNA Ligase and wild type λ DNA were from NBL, Cramlington, Northumberland, U. K., Boehringer Mannheim (UK) Ltd, Lewes, U. K., Gibco BRL Life Technologies Ltd., Paisley, U.K., United States Biochemical Corporation, Ohio, U.S.A., or New England Biolabs, CP Labs Ltd., Bishop's Stortford, Hertfordshire, U.K..

Caesium chloride, Klenow enzyme and Digoxygenin non-radioactive labelling kit were from Boehringer Mannheim Diagnostics and Biochemicals (England) Ltd., Lewes, East Sussex, U.K..

Agarose and RPMI i640 tissue culture medium was from GibcoBRL, Paisley, U.K..

Vacuum grease was from Dow Corning S. A., Seneffe, Belgium.

Reagent for the 'Bradford' protein estimation procedure was from Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, U.K..

Polaroid film was from Polaroid (UK) Ltd., St. Albans, Hertfordshire, U.K..

Filter paper (3MM) and laboratory sealing film was from Whatman International Ltd., Maidstone, U.K..

Nitrocellulose membrane filters for colony hybridisations were from Schleicher and Schuell, Dassel, Germany.

Wesson's salts and 'Vitamin mix' were from ICN Biomedicals Ltd., Cleveland, Ohio, U.S.A..

Wheatgerm was from Naturally, Spennymoor, U.K..

Isoton II was from Coulter Electronics Ltd., Luton, U.K..

Cucumber (var. Bedfordshire Prize) and mung bean seeds were from Kings Crown Ltd., Tyneside, U. K..

Minisart filters and 13 mm 8 μm pore filter discs were from Sartorius GmbH, Postfach 3243, D-3400 Göttingen, Germany.

Nitrocellulose discs (25 mm, 0.22 μm pore size) for tri-parental mating were from Millipore (UK) Ltd., Watford, U.K..

CM-Chitin-RBV (2 $mg.ml^{-1}$ solution) was from Loewe Biochemica, Otterfing, Germany.

Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) was from Janssen Chimica, Beerse, Belgium.

Vermiculite was from Silvaperl Ltd., Lincoln, U.K..

Compost was from Pan Britannica Industries Ltd., Waltham Cross, Hertfordshire, U.K..

2.2 Commonly Used Buffers, Media and Solutions

2.2.1 Inorganic Buffers and Solutions

5 X TBE Buffer [261]

54 g Tris
27.5 g Boric acid
20 ml 500 mM Na_2EDTA (pH 8.0)

Made up to 1000 ml in dH_2O (distilled water).

50 X TAE [261]

242 g Tris
100 ml 500 mM EDTA (pH 8.0)
57.1 ml glacial acetic acid

Made up to 1000 ml in dH_2O.

TE Buffer [261]

10 ml 1 M Tris.HCl (pH 8.0)
2 ml 500 mM EDTA (pH 8.0)

Made up to 1000 ml in dH_2O and autoclaved.

Phenol:chloroform [261]

50:50 mixture of phenol and 1:24 isoamyl alcohol:chloroform. Equilibrated with 100 ml TE (pH 8.0) three times. Stored under 25 ml TE (pH 8.0) at 4 °C in dark.

6 X DNA agarose gel loading buffer [261]

25 mg bromophenol blue
25 mg xylene cyanol
4 g sucrose

Made up to 10 ml with dH_2O. Stored at 4 °C.

20 x SSC [261]

175.3 g NaCl
88.2 g Na.citrate

Made up to 1000 ml with dH_2O. pH adjusted to pH 7.0 with HCl.

Denaturation Buffer [261]

87.7 g NaCl
20.0 g NaOH

Made up to 1000 ml with dH_2O.

Neutralisation buffer [261]

87.7 g NaCl
60.6 g Tris

Made up to 1000 ml with dH_2O. pH adjusted to 7.0 with HCl.

20 x SSPE [261]

174.0 g NaCl
27.6 g $NaH_2PO_4.H_2O$
7.4 g Na_2EDTA

Made up to 1000 ml with dH_2O. pH adjusted to 7.4 with NaOH.

50 x Denhardt's solution [261]

5 g Ficoll
5 g Polyvinylpyrrolidone
5 g BSA (pentax V fraction)

Made up to 500 ml in dH_2O and filter sterilised.

Benedict's solution [154]

173 g Na.citrate
100 g $NaCO_3$

The above were dissolved in 600 ml dH_2O by heating. 17.3 g $CuSO_4$ in 100 ml dH_2O was then added slowly with constant stirring. The solution was then allowed to cool and made up to 1 litre with dH_2O.

Sodium Iodide Solution [250]

98.5 g NaI
1.5 g Na_2CO_3

Made up to 100 ml in sterile dH_2O. Stored at 4 °C in the dark.

Silica fines [250]

250 ml of silica 325 mesh powder was resuspended in dH_2O to give a total volume of 500 ml. The suspension was stirred for 1 hour and left to settle for a further hour. The suspension was then centrifuged at 5000 rpm in a Beckman J2-HS centrifuge using a JA-14 rotor. The pellet was resuspended in 150 ml dH_2O and 150 ml nitric acid was added. The suspension was then heated to 98 °C and allowed to cool to room temperature. The silica fines were then washed with sterile dH_2O repeatedly until the pH was greater than 5.5. The fines were stored at 4 °C as a 50 % slurry in sterile dH_2O.

PBS [122]

8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4

Made up to 1000 ml in dH_2O. The pH was adjusted to 7.2 and the solution autoclaved.

TBS [122]

8.0 g NaCl
0.2 g KCl
3.0 g Tris

Made up to 1000 ml in dH_2O. The pH was adjusted to 8.0 with 1 M HCl and the solution autoclaved.

2.2.2 Antibiotic stock solutions

Ampicillin (Amp) - was dissolved in sterile Milli-Q water to give a stock solution of concentration 25 $mg.ml^{-1}$. It was used at a final concentration of 50 $\mu g.ml^{-1}$ for *E. coli*. Ampicillin affects bacterial cell wall synthesis. The resistance gene is *bla* and produces β-lactamase which cleaves the lactam ring of the antibiotic [223].

Carbenicillin (Cb) - was dissolved in sterile Milli-Q water to give a stock solution of concentration 50 $mg.ml^{-1}$. It was used at a final concentration of 200 $\mu g.ml^{-1}$ for *A. tumefaciens* instead of ampicillin [261].

Kanamycin (Km) - was dissolved in sterile Milli-Q water to give a stock solution of concentration 12.5 $mg.ml^{-1}$. For *A. tumefaciens* it was used at a final concentration of 25 $\mu g.ml^{-1}$ and for *E. coli* it was used at a final concentration of 50 $\mu g.ml^{-1}$. This is a bactericidal compound which binds to the 70S ribosomes and causes misreading of the mRNA. The *kan* gene product modifies the antibiotic preventing binding [223].

Rifampicin (Rif) - was dissolved in DMSO to give a stock solution of concentration 50 $mg.ml^{-1}$. It was used at a final concentration of 100 $\mu g.ml^{-1}$. Rifampicin inhibits the mRNA synthesis by binding to DNA-dependent RNA polymerase. Rifampicin resistant RNA polymerase is encoded chromosomally [120].

Streptomycin (Sm) - was dissolved in sterile Milli-Q water to give a stock solution of 20 $mg.ml^{-1}$. For *A. tumefaciens* it was used at a final concentration of 300 $\mu g.ml^{-1}$ and for *E. coli* at a final concentration of 25 $\mu g.ml^{-1}$. Streptomycin binds to the 50S ribosome subunit causing misreading of mRNA. The *str* gene product modifies the antibiotic and so prevents binding to the ribosome [223].

Tetracycline (Tc) - was dissolved in 50:50 ethanol:Milli-Q water at a concentration of 12.5 $mg.ml^{-1}$. For *A. tumefaciens* it was used at a final concentration of 10 $\mu g.ml^{-1}$ and for *E. coli* at 15 $\mu g.ml^{-1}$. The antibiotic binds to the 30S subunit of the ribosome, so preventing binding of the tRNA-amino acid complexes. The *tet* gene encodes a protein that modifies the bacterial membrane in such a way that tetracycline transport is prevented [120, 223].

2.2.3 Growth media

Lab M Nutrient Broth #2 (LM broth)

25 g made up to 1 litre with dH_2O gives final concentration of 10 $g.l^{-1}$ beef extract, 10 $g.l^{-1}$ balanced peptone #1, 5 $g.l^{-1}$ NaCl, pH 7.5 ± 0.2.

Lab M Nutrient agar (LM agar)

28 g made up to 1 litre with dH_2O gives final concentration of 5 $g.l^{-1}$ peptone, 3 $g.l^{-1}$ beef extract, 8 $g.l^{-1}$ NaCl, 12 $g.l^{-1}$ agar #2, pH 7.3 ± 0.2.

SCG agar [108]

1.5 g Bacto agar and 0.1 g casamino acids in 100 ml Spizizen's Medium [286] and 0.5 % glucose. Made up as follows:

0.2 g $(NH_4)_2SO_4$
1.4 g K_2HPO_4
0.6 g KH_2PO_4
0.1 g NA. citrate.$2H_2O$
0.02 g $MgSO_4.7H_2O$

in 50 ml of dH_2O and autoclaved. Once cooled, 1.5 g agar in 27.5 ml dH_2O (autoclaved and cooled) and 0.1 g casamino acids in 20 ml dH_2O (autoclaved and cooled) were added and mixed. Finally, 2.5 ml of 20 % glucose in dH_2O was added. 25 ml was used per plate.

SOC broth [261]

20 g tryptone
5 g yeast extract
0.5 g NaCl

The above were added to 950 ml dH_2O and stirred until the solutes had dissolved. Then 10 ml 250 mM KCl were added. The pH was adjusted to 7.0 with the addition of 10 N NaOH and the volume was made up to 975 ml. The medium was autoclaved, cooled and 20 ml sterile 1 M glucose and 5 ml sterile 2M $MgCl_2$ were added.

Manduca sexta diet [341]

3.83 g casein
8.35 g wheatgerm
1.09 g Wesson's salts
1.65 g yeast
3.39 g sucrose
0.11 g cholesterol
0.11 g methyl-4-hydroxybenzoate
0.17 g sorbic acid
0.43 g ascorbic acid
0.11 g choline chloride

The above were mixed together and 2.17 g of molten gum agar in 100 ml of dH_2O was added. The resulting mixture was mixed, allowed to cool and 1.07 ml of vitamin mix and 0.22 ml linseed oil were added.

Potato Dextrose Agar (PDA) [226]

200 g potatoes were boiled in 1 litre of distilled water for 1 hour and then blended in a food blender until the mixture was a thick paste. This was then pressed through a tea towel and autoclaved.

100 ml potato extract, as prepared above, was taken and 2 g dextrose and 2 g bacteriological agar were added. This was then autoclaved and poured into plates.

Potato Sucrose Agar (PSA) [226]

This was the same as PDA except that 2 g of sucrose was used instead of dextrose.

YM Agar [280]

5 g yeast extract
5 g peptone
10 g glucose
20 g agar

Made up to a total volume of 1 litre and autoclaved.

SPY [182, 286]

0.5 g yeast extract
1 g $(NH_4)_2SO_4$
7 g K_2HPO_4
3 g KH_2PO_4
0.5 g Na.citrate.$2H_2O$
0.1 g $MgSO_4.7H_2O$

The above were made up to 497.5 ml in dH_2O, autoclaved and cooled. 2.5 ml 20% glucose was added before use.

10 x Min A salts [207]

105 g K_2HPO_4
45 g KH_2PO_4
10 g $(NH_4)_2SO_4$
5 g Na.citrate.$2H_2O$

The above were made up to 1 litre in dH_2O and autoclaved.

Min A Medium [207]

10 ml 5 x Min A salts
1 ml 20 % D(+)galactose
0.1 ml 1 M $MgSO_4$

The above was made up to 100 ml in sterile dH_2O.

Min A agar

As for Min A medium but with the addition of 1 % bacteriological agar.

MinA swarm agar

As for MinA medium but with the addition of 0.16 % bacteriological agar.

M9 minimal medium [207]

6 g Na_2HPO_4
3 g KH_2PO_4
0.5 g NaCl
1 g NH_4Cl

Made up to 979 ml with dH_2O before autoclaving, cooling and the addition of 1 ml 1 M $MgSO_4.7H_2O$, 10 ml 20 % glucose (or glycerol where specified) and 10 ml 0.01 M $CaCl_2$.

M9 minimal medium agar

As for M9 medium but solidified with 1 % bacteriological agar.

Low phosphate medium [220]

14.54 g Tris
4.68 g NaCl
1.49 g KCl
1.07 g NH_4Cl
0.43 g Na_2SO_4
0.1 g $MgCl_2$
0.5 g Difco Bacto-peptone
22 mg $CaCl_2$
0.3 mg $ZnCl_2$

The solution was adjusted to pH 7.5, made up to 1000 ml with dH_2O and autoclaved.

2.2.4 Induction Media

Ghent [318]

12.5 g sorbitol and 2.5 g sucrose were dissolved in 400 ml dH_2O. 100 ml of 5 x stock salt solution (filter sterilised) was added and the solution was adjusted to pH 5.6.

5 x stock salt solution :

2.5 g $(NH_4)_2SO_4$
1.25 g Na.citrate
2.5 g KNO_3
4.4 g KH_2PO_4
0.8 g MES

The above were dissolved in 450 ml dH_2O followed by the addition of 6 ml 10 % $MgSO_4$ and 2.5 ml 1 % vitamin B1 (Thiamine) stock solution. The pH was adjusted to pH 5.45 with 1 M KOH and the volume was adjusted to 500 ml with dH_2O. The solution was filter sterilised.

Davis [253]

3.47 g of Murashige and Skoog plant salt minimal organic mixture was added to 98.75 ml of dH_2O, autoclaved and cooled. 81 µl of 1 M K_2HPO_4 and 1.169 ml of KH_2PO_4 were added. The resulting medium had a pH of 5.7.

Seattle [54, 336]

AB Salts:

3 g K_2HPO_4
1 g NaH_2PO_4
0.3 g NH_4Cl
0.15 g $MgSO_4.7H_2O$
0.15 g KCl
0.01 g $CaCl_2$
2.5 mg $FeSO_4.7H_2O$
30 g sucrose
4.27 g MES

The above were dissolved in dH_2O before the addition of 50 µl of 1 M K_2HPO_4 and 2.45 ml 1M KH_2PO_4. The resulting medium was adjusted to pH 5.5 if necessary, the volume made up to 1 litre and filter sterilised.

Rogowsky [254]

10 g sucrose
4 g yeast extract
0.2 g $MgSO_4.7H_2O$
0.57 g $K_2HPO_4.3H_2O$
3.9 g MES

The above were made up to 1 litre in dH_2O. The pH was adjusted if necessary to pH 5.5 and the medium was then autoclaved.

2.3 General Working Practices

All media and materials used in experimental work involving bacterial cultures were sterile. Solutions that were not able to be sterilised by autoclaving were filter sterilised by passing the solution through a Sartorius Minisart 0.2 µm nitrocellulose filter. Other solutions were sterilised by autoclaving for 15 minutes at 15 p.s.i, 123 °C.

All glassware or plasticware used in the maintenance of cultures or the manipulation of DNA was autoclaved before use.

All bottle necks were flamed in a roaring Bunsen flame before and after use and caps were replaced on all containers as soon as possible. Culture plates were sterilised after pouring by flaming the surface of the molten agar medium with a roaring Bunsen flame.

2.4 Strains and plasmids

2.4.1 Bacterial and fungal strains

The strains used in this study are listed below:

STRAIN	COMMENTS	REFERENCE
Escherichia coli DH5α	F^-, *recA1*, *endA1*, *hsdR17*($r_k^- m_k^+$), *supE44*, *thi-1*, λ^-, *gyrA96*, *relA1*, Δ(*lacZYA*-argF)U169, Ø80*lac*ZΔM15	[111]
Agrobacterium tumefaciens C58C1	Rif^R, Ti^-	[314]
Bacillus thuringiensis var. *kurstaki* 4D4	Donor: Dulmage, H. Genotype: cry(K73) Serotype: 3a3b	[75]
Serratia marcecens QMB1466	*chi*$^+$	[157]
Fusarium oxysporum Schlecht	IMI 329 662	[142]
Phycomyces blakesleeanus Burgeff	IMI 200 165	[142]
Thanatephorus cucumeris (Frank) Donk	IMI 323 817	[142]

Escherichia coli and *Agrobacterium tumefaciens* strains were cultured in LM - broth. Media used for bacteria containing plasmids contained the appropriate antibiotic selection. Cultures of *E. coli* were grown at 37 °C and cultures of *A. tumefaciens* were grown at 28 °C. These strains were maintained on LM-agar containing the appropriate antibiotics.

Serratia marcescens was cultured in LM-broth at 28 °C with the addition of 5 $mg.ml^{-1}$ crab shell chitin (Sigma) to induce the production of chitinase when required. The bacterium was maintaned on LM-agar.

Bacillus thuringiensis cultures were grown up at 37 °C or 28 °C in SPY. Again stocks were kept, produced in the same way as above. *Bacillus thuringiensis* was maintained on SCG agar.

Stocks of all bacterial cultures were kept in 15 % glycerol at - 80 °C. These were made by taking 0.5 ml of a 5 ml culture grown up overnight with shaking, and adding an equal volume of 30 % glycerol in a 1.5 ml screw-cap tube.

Fusarium oxysporum was maintained on PSA whereas *Thanatephorus cucumeris* and *Phycomyces blakesleeanus* were maintained on PDA. All fungus cultures were incubated at 28 °C unless otherwise stated.

2.4.2 Plasmids

The plasmids used in this study are listed below:

PLASMID	COMMENTS	REFERENCE
pVK 257	Km^R *virA, B, G, C* of pTiA6	[175]
pUCD1194	Cb^R *virE* promoter:lux	[253]
pUCD1187	Cb^R *virB* promoter:lux	[252, 253]
pKT230	Km^R, Sm^R Broad host range vector	[14, 15]
pUC18/19	Amp^R Multiple cloning site vector	[343]
pDUB2501	Amp^R, Km^R *Serratia* promoter, *chiA* gene	[9]
pDUB2502	Amp^R, Km^R *Serratia* promoter, *chiB* gene	[9]
pCHIT310	Amp^R *Serratia* promoter, *chiB* gene	[157]
pECA2	Amp^R, Tc^R *virE* promoter, *chiA* gene	[187]
pBCA2	Amp^R, Km^R *virB* promoter, *chiA* gene	[187]
pBCB2	Amp^R, Km^R, Tc^R *virB* promoter, *chiB* gene	[187]
pECB2	Amp^R, Sm^R *virE* promoter, *chiB* gene	This study
pCB1	Amp^R pUC19 containing *chiB* gene	[187]
pVB21	Amp^R pUC19 containing *virB* promoter	[187]
pUC1318::*virE*	Amp^R pUC1318 containing *virE* promoter	[187]

pBGB1	*cryIA(c)* in pUC19 native promoter and terminator	This study
pLEV1	*cryIA(c)* in pUC19 native promoter	This study
pLEV102	*cryIA(c)* in pUC19 partial promoter deletion	This study
pLEV103	*cryIA(c)* in pUC19 as pLEV103; P_{lac} control	This study
pLEV112	*cryIA(c)* in pUC19 complete promoter deletion	This study
pLEV113	*cryIA(c)* in pUC19 as pLEV112	This study
pLEV114	*cryIA(c)* in pUC19 as pLEV112; P_{lac} control	This study
pLEV115	*cryIA(c)* in pUC19 as pLEV112	This study
pLEV300	5' end of *cryIA(c)* containing native terminator in pUC19	This study
pLEV302	as pLEV102 with native terminator	This study
pLEV303	as pLEV103 with native terminator	This study
pLEV314	as pLEV114 with native terminator	This study
pLEV315	as pLEV315 with native terminator	This study

2.4.3 Measurement of Bacterial Growth

This was measured in a Beckman DU7500 spectrophotometer by measuring the OD_{600}, when the relationship between the number of bacteria and the optical density was known.

When this relationship was not known or a more precise count of bacterial numbers was required a Coulter Multisizer II with a 30 μm aperture was used to count the number of cells of the correct size in 30 μl. The cells were measured in Isoton II solution.

2.5 Methods

2.5.1 Plasmid DNA preparation

a) Standard 'mini-prep' method [261]

This was the preferred method of DNA isolation and it is to be assumed that this was the method used unless otherwise stated.

5 ml of LM broth was inoculated with a bacterial colony and grown overnight at 37 °C with shaking for *E. coli* or for 24 hours at 28 °C with shaking for *A. tumefaciens*. 1.5 ml of the culture was taken and pelleted in a microfuge tube (30 secs). The supernatant was removed and the pellet resuspended in 100 μl of ice cold Solution I (1% glucose, 10 mM EDTA, 25 mM Tris.HCl, pH 8.0) by vortexing. The suspension was stored at room temperature for 5 minutes. 200 μl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the contents of the tube were mixed by inverting the tube several times. The tube was stored on ice for 5 minutes. 150 μl of ice cold 'KAc' (pH 4.8, 3 M wrt potassium, 5 M wrt acetate) was added, the mixture was vortexed briefly and stored on ice for 5 minutes. It was then centrifuged in a bench microfuge for 5 minutes. The supernatant was transferred to a fresh tube (approximately 400 μl) and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added before the solution was vortexed and centrifuged for 2 minutes. The top aqueous layer was transferred to a fresh tube and 2 volumes of 100 % ethanol were added. This was left to stand for 2 minutes and centrifuged for 5 minutes. The supernatant was removed and 1 ml 70 % ethanol added to the pellet. This was vortexed briefly and centrifuged for a further 5 minutes. The supernatant was removed and the pellet dried under vacuum. It was resuspended in 50 μl TE (10 mM Tris. HCl, 1 mM EDTA, pH 8) and 1 μl of 10 $mg.ml^{-1}$ DNAase-free RNAase was added for plasmids extracted from *E. coli* and 10 μl of this solution for plasmids extracted from *A. tumefaciens*.

b) Quick 'mini-prep' method

This method was employed when large numbers of bacterial colonies needed to be examined for plasmids. It could only be used on *E. coli* .

1.5 ml of a 5 ml overnight culture was taken and spun down for 30 seconds in a microfuge. The pellet was resuspended in 100 µl of TE and 400 µl of phenol:chloroform was added. The suspension was vortexed for 5 seconds and spun at high speed for 3 minutes. The top layer was removed to a second microfuge tube and 400 µl of 1:24 isoamyl alcohol:chloroform was added. The suspension was vortexed for 5 seconds and spun for 3 minutes. The top layer, containing the plasmid, was removed to a fresh microfuge tube.

c) Standard 'maxi-prep' method [261]

The following procedure was used to prepare vector plasmids used in this study.

A 5 ml overnight culture was grown with the appropriate antibiotic selection and used to inoculate 500 ml of LM-broth. After 12 - 16 hours the cells were harvested by spinning at 4 000 g (6 000 rpm, MSE 18, 6 x 250 ml rotor) for 10 minutes using 2 centrifuge bottles. The pellets were washed in 20 ml STE (1 mM EDTA, 100 mM NaCl, 10 mM Tris.HCl, pH 8.0), transferred to 2 Oakridge tubes and spun at 4 000 g for 10 mins.

The pellets were resuspended in 10 ml Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl, pH 8.0) and left to stand at room temperature for 5 minutes. 20 ml freshly made Solution 2 (0.2 M NaOH, 1 % SDS) was added and the contents of the tubes mixed by inversion and left to stand on ice for 20 minutes.

15 ml ice-cold KAc (pH 4.8, 3 M wrt K, 5 M wrt Ac) was added and the tubes vortexed and left on ice for a further 10 minutes. The tubes were then centrifuged for 20 minutes at 2000 rpm (8 x 50 ml rotor, MSE 18) at 4 °C. The supernatants were then transferred to three 30 ml Corex tubes (12 ml per tube). 0.6 volumes of isopropanol were added, mixed and the tubes were left to stand at room temperature for 15 minutes.

The DNA was recovered by centrifugation at 12 000 g for 30 minutes at room temperature. The pellets were then washed with 70 % ethanol (1 ml), dried under vacuum and resuspended in 5 ml TE.

Caesium chloride gradients were made up as below.

Tube size	CsCl	Eth.Br 10 $mg.ml^{-1}$	Final Volume (TE)
Large	29.8 g	0.41 ml	39 ml
Medium	20.6 g	0.30 ml	27 ml

The DNA solution was added to Quickseal centrifuge tubes using a syringe, balanced, heat-sealed and centrifuged for 18 hours at 55 000 rpm at 15 °C , using a Sorvall 65 OTB ultracentrifuge and a VTi 50 rotor.

After centrifugation two bands appeared in the tube when viewed under UV light. The upper one was plasmid DNA in its open circular form and chromosomal DNA debris. The lower band contained the plasmid in its supercoiled form and it was this band that was removed using a syringe and wide-bore needle inserted through the tube wall just under the lower DNA band.

The DNA band was removed to a sterile plastic tube and extracted five times with H_2O/CsCl saturated butan-2-ol. The resulting solution was divided into 300 µl aliquots, to each of which was added 600 µl 0.45 M NaOAc and 540 µl isopropanol. The solutions were stored at -20 °C for 1 hour. The DNA was recovered by centrifugation at high speed in an MSE MicroCentaur microfuge for 10 minutes.

The pellets were washed in 70 % ethanol and recentrifuged. Finally the pellets were dried under vacuum and resuspended in TE.

d) 'Maxi-prep' method for large *Bacillus* plasmids [182]

One colony of *B. thuringiensis* was picked and cultured in 5 ml SPY at 37 °C for 24 hours. 1 ml was subcultured into 500 ml SPY and grown at 37 °C until an OD_{600} of 0.8 was reached.

The cells were harvested by centrifugation for 10 minutes at 8 000 rpm (6 x 250 ml rotor, MSE18) and 4 °C . The pellet was resuspended in 5 ml KTE (0.05 M Tris, 0.02 M EDTA, pH 7.9). Lysis was achieved by adding 95 ml KTE containing 1 % SDS and 0.085 M NaOH, pH 12.4. This was left for 30 minutes with occasional rocking. 10 ml of 10 % SDS was added, mixed in and then 10 ml 2 M Tris.HCl, pH 7.0 was added followed by 30 ml 5 M NaCl. The resulting mixture was stored overnight at 4 °C. It was then centrifuged at 11 000 g for 15 minutes. The supernatant was removed and added to 36 ml of 50 % PEG_{6000}. This was left on ice for at least 2 hours. The mixture was centrifuged and the pellet dried and resuspended in 4 ml TE.

2.5.2 Methods for introduction of plasmids into bacteria

a) Standard transformation method for *E.coli* [121]

5 ml of LM-broth (with appropriate selection) was inoculated with a bacterial colony from a plate using a sterile cocktail stick and incubated over night at 37 °C with shaking. This was added to 500 ml of LM-broth and incubated at 37 °C with shaking until the OD_{600} was 0.3 - 0.35. The suspension was chilled on ice for 5 minutes and spun down at 2 000 g for 7 minutes in 50 ml polypropylene tubes. The pellet was resuspended in 2/5 vol TFb1 (30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % glycerol, pH to 5.8 with acetic acid, filter sterilised). The suspension was then left on ice for 5 minutes before being recentrifuged (as above). The pellet was resuspended in 1/25 vol TFb2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15 % glycerol, pH 6.5 with 10 M KOH, filter sterilised) and left on ice for 15 minutes. 110 µl aliquots were then measured into prechilled microfuge tubes, frozen in liquid nitrogen and stored at - 80 °C.

Competent cells were thawed at room temperature and then left on ice for 10 minutes. Plasmid DNA was then added (up to 2/5 vol of cells, no more than 55 ng/110 µl cells).

Tubes were left on ice for 15 - 45 minutes, then heat shocked at 42 °C for 90 seconds and left on ice for a further 2 - 3 minutes. 890 µl of SOC was then added and the suspension incubated at 37 °C for 1 hour with occasional shaking. The cells were plated out on selective LM-agar.

b) Electroporation of *A. tumefaciens* [213, 231]

2 x 1.5 ml aliquots of bacteria from an overnight culture of *Agrobacterium tumefaciens* were taken and microfuged for 30 seconds at high speed. The pellets were resuspended in 0.5 ml of ice cold 1 mM HEPES/KOH, pH 7.0 and recentrifuged. This was repeated twice. The pellet was resuspended in 0.5 ml ice cold 10 % filter sterilised glycerol (autoclaving leads to the formation of aldehydes which inhibit electroporation [347]) and spun down. The pellets were resuspended in 20 µl 10 % glycerol and the contents of both tubes combined. DNA was added and the tube left on ice for 2 minutes. The mixture was pulsed in an ice cold 0.2 cm Bio-Rad electroporation cuvette in a Bio-Rad Gene Pulsar set at 25 µF capacitance, 400 Ω resistance and 2.5 kV charge. The time constant was typically about 9 ms in successful transformations. Immediately after the pulse, 1 ml SOC was added and the cells incubated at 28 °C for 4 to 6 hours before being plated out on selective media.

c) Tri-parental mating for *A. tumefaciens* [77]

These transconjugants were prepared using pRK2013 as the helper plasmid. 5 ml cultures of an *E. coli* donor strain, *E. coli* HB101(pRK2013) and the recipient *A. tumefaciens* strain were grown to exponential phase and then 100 µl of the donor, 100 µl of HB101(pRK2013) and 300 µl of the recipient strain were mixed together in a sterile plastic tube. 100 µl of this mixture was pipetted onto a nitrocellulose disc placed in the centre of an LM-agar plate. The bacteria were left on the plate overnight at 28 °C before the disc was removed and placed and shaken in 10 ml 10 mM $MgSO_4$. 100 µl samples of the suspension were plated out onto selective media and left to grow at 28 °C for 48 hours to allow for the appearance of transconjugant colonies.

2.5.3 DNA manipulations

a) Quantitation of DNA concentration [261]

DNA solutions were diluted 50 fold in TE and the absorbances were measured at 260 nm and 280 nm using a Beckman DU7500 spectrophotometer. A pure DNA sample has a 260 nm/280 nm ratio of 1.8 and a 260 nm reading of 1 was taken to be equivalent to a concentration of 50 $\mu g.ml^{-1}$ for double-stranded DNA.

b) Restriction enzyme digests

These were all performed according to the manufacturers' instructions unless phosphatase treatment of the DNA was subsequently required.

c) Phosphatase treatment of DNA

DNA was digested with the appropriate restriction enzyme in 1x or 2x Pharmacia 'One-Phor-All' buffer (according to the manufacturer's instructions) at 37 °C and 30 minutes before terminating the reaction 1 U of calf intestinal phosphatase was added. Upon completion of the reaction the enzymes were removed from the DNA by using the 'Magic DNA Clean-Up' system from Promega, according to the manufacturer's instructions.

d) Removal of 3' protruding termini [261]

DNA restriction was performed as above but after this 1 µl of a solution containing all of the dNTPs at a concentration of 2 mM was added as was 1 unit of T4 DNA polymerase. The DNA was then incubated at 15 °C for a further 15 minutes before the enzymes were removed using the Pharmacia 'Magic DNA Clean-Up' system or the DNA was subjected to electrophoresis.

e) Filling in 3' recessed termini [261]

DNA restriction was performed as above and then 1 µl of a solution containing all four dNTPs at a concentration of 1 mM and 1 unit of Klenow enzyme were added. The DNA was further incubated at room temperature for 15 minutes before the enzymes were removed using the Pharmacia 'Magic DNA Clean-Up' system or the DNA was subjected to electrophoresis.

f) Ligation of DNA fragments

DNA fragments with either cohesive or blunt termini were mixed together in the ratio of approximately 3:1 insert:vector (to a final concentration of approximately 50 $\mu g.ml^{-1}$) and 1 µl of 10 x T4 DNA ligase buffer was added. For blunt ended ligations 3 units of T4 DNA ligase was added to the reaction mixture and for cohesive termini 1 unit was used. The reaction mixture was made up to 10 µl and incubated at 4 °C overnight before being added directly to competent *E. coli* cells.

g) Agarose gel electrophoresis [261]

For minigels Pharmacia GNA-100 gel electrophoresis apparatus was employed. 50 ml of 1 x TAE was taken and an appropriate amount of agarose added (see table below). This was microwaved at 650 W for one minute in order to melt the agarose. Ethidium bromide was added to the cooled solution, to a final concentration of 0.2 $\mu g.ml^{-1}$, which was poured into a Pharmacia minigel mould, with the required comb in it. 350 µl of 1 x TAE was taken and ethidium bromide added to it (0.2 $\mu g.ml^{-1}$) to form the running buffer. The DNA samples to be run had DNA gel loading buffer added to them (1 µl for each 5 µl of sample). Once the gel had set, it was submerged in running buffer, so that it was just covered, in the Pharmacia minigel apparatus. The comb was removed and the DNA samples pipetted into the slots. Gels were normally electrophoresed at 75 V for 1 - 1.5 hours,

then viewed with long range UV light (302 nm) on a UV transilluminator (UVP Inc.) and photographed using a red filter with Polaroid 667 film in a Polaroid RP4 Land camera.

% agarose	DNA size range (kb)
0.3	60 - 5
0.6	20 - 1
0.7	10 - 0.8
0.9	7 - 0.5
1.2	6 - 0.4
1.5	4 - 0.2
2.0	3 - 0.1

For large gels, the same procedure was used, but with 200 ml of 1 x TAE and 2 litres of 1 x TAE for the running buffer. Gels were electrophoresed at 100 V for 2 - 3 hours or 20 V overnight.

h) DNA fragment isolation

Two methods were used.

The first method was from Robinson [250]. The band corresponding to the desired DNA fragment was excised from the gel using a scalpel blade. The gel slice was put in a large microfuge tube and 1 ml NaI solution (see pg 32) was added and the tube incubated at 70 °C for 10 minutes and then at 37 °C for 5 minutes. 5 µl of silica fines was added and the suspension left for 10 minutes. There followed a 30 second microfuge spin, washing of the pellet with 70 % ethanol and vacuum drying. The pellet was resuspended in TE, followed by a 10 minute incubation at 37 °C, a 30 second spin and the removal of the supernatant, containing the DNA, to a fresh tube.

Electroelution was the second method employed using a Scleicher and Schuell Biotrap according to the method of Göbel *et al.* [106]. The gel slice containing the DNA was removed from the agarose gel and placed in the elution chamber of the Biotrap in an electrophoresis tank. The tank was filled with 1 x TAE so that the level was about 5 mm below the top of the Biotrap and 500 µl of TAE was added into the collection trap. A voltage of 150 V was applied across the tank for a period of 4 hours, the current being directed solely through the Biotrap by positioning a perspex block across the rest of the tank. After electroelution the buffer in the collection trap

was removed, added to 1 ml of ethanol and left overnight at -20 °C . The tube was spun at high speed in a benchtop microfuge at high speed for 10 minutes. The DNA pellet was washed in 1 ml of 70 % ethanol, vacuum dried and dissolved in TE.

i) Colony hybridisation [261]

Using sterile toothpicks colonies were picked and streaked out on a gridded nitrocellulose filter on an agar plate and in an identical position on another agar plate (master plate). Positive and negative control colonies were also streaked out in this way. The plates were incubated at the appropriate temperature until bacterial streaks were 0.5 - 1.0 mm in width.

The filter was peeled from the agar and placed colony side up on a 10 % SDS saturated piece of Whatman 3MM paper for 3 minutes. The filter was placed on to 3MM paper saturated in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, followed by 5 minutes on paper soaked with neutralisation buffer (1.5 M NaCl, 0.5 M Tris.HCl, pH 8.0) and 5 minutes on paper saturated with 2 x SSC. The filter was allowed to air dry for 1 hour and was then sandwiched between two pieces of 3 MM paper and two sheets of glass and then baked under vacuum at 80 °C for 1.5 hours.

j) Southern blotting [261]

DNA samples were electrophoresed through agarose, the gel was photographed and soaked for 15 minutes in 1 % HCl. It was rinsed twice in dH_2O and soaked in denaturation buffer for 45 minutes. There followed two rinses in dH_2O, a 45 minute soak in neutralisation buffer and a rinse in 20 x SSC. The gel was then placed on a long piece of 3 MM paper, which was on a long glass plate, the ends of which were immersed in 10 x SSC. This was overlaid with a sheet of Hybond nylon (cut to size and pre wetted in dH_2O and then 10 x SSC) and then three 10 x SSC-soaked pieces of 3 MM paper cut to size. Over this were laid two layers of nappies cut to size, a glass plate and a mass of 1 kg. This was left overnight. Following this the nylon filter was removed and placed in clingfilm and placed on a UV transilluminator for 2 minutes. It was then air dried.

k) Radiolabelling of DNA fragments

The DNA to be labelled was boiled in a total volume of 28 µl for 5 minutes, and then held on ice for 2 minutes. 10 µl of labelling buffer (Amersham multiprime kit), 5 µl primers (Amersham multiprime kit), 5 µl ^{32}P-dCTP (400 Ci/mmol) were added, followed by 4 U Klenow enzyme. The mixture was then incubated at 37 °C for 2 - 3 hours. The probe was boiled for 5 minutes before use.

l) Hybridisation of radiolabelled probes to blots [261]

All the procedures outlined here were performed in Techne Hybridisation Tubes in a Techne Hybridiser HB-1.

For colony blots the filters were wetted in 6 x SSC and then washed in 25 ml prewash solution (50 mM Tris.HCl, 1 M NaCl, 1 mM EDTA, 0.1 % SDS, pH 8) per 100 cm^2 of filter, for 1 - 2 hours. The filters were incubated for 4 - 6 hours at 42 °C in 12.5 ml per 100 cm^2 prehybridisation solution (5 x Denhardt's, 5 x SSPE, 0.1 x SDS, 0.1 % pyrophosphate, 100 µg.ml^{-1} denatured salmon sperm DNA). The denatured ^{32}P probe was added directly to this solution and incubated at 42 °C overnight. The filters were washed twice in 150 ml 2 x SSC, 0.1 % SDS at room temperature for 10 minutes, and then washed twice in 250 ml 1 x SSC, 0.1 % SDS for 1 hour at 42 °C . If the background count was still high at this point a further wash in 0.2 x SSC, 0.1 % SDS was used.

For Southern blot hybridisation, the filters were wetted in 6 x SSC and soaked in prehybridisation solution at 68 °C for 2 - 4 hours, using 20 ml prehybridisation solution (6 x SSC, 5 x Denhardt's, 0.5 % SDS, 0.1 % sodium pyrophosphate, 100 µg.ml^{-1} denatured salmon sperm DNA) per 100 cm^2 of filter. The denatured probe was added directly to this and incubated at 68 °C overnight. The filters were washed in 2 x SSC for 15 minutes at 65 °C , in 2 x SSC, 0.1 % SDS for 30 minutes at 65 °C and in 0.1 x SSC for 10 minutes at 65 °C . If it was necessary to strip the blot in order to reprobe the filter, it was washed in 0.4 M NaOH for 30 minutes at 45 °C and then in 0.1 x SSC, 0.1 % SDS, 0.2 M Tris.HCl pH 7.5.

2.5.4 Cell extracts

a) Cell fractionation [220]

1.5 ml of a cell culture was taken and pelleted in a microfuge tube at low speed in a microfuge (MSE MicroCentaur) for 10 minutes. The supernatant was carefully removed and stored on ice as the extracellular fraction. The pellet was resuspended in 1.5 ml 20 % sucrose, 0.03 M Tris.HCl, pH 8 and then 3 µl of 500 mM EDTA, pH 8 was added. The cell suspension was shaken at room temperature for 10 minutes at low speed on a Scientific Industries Vortex Genie II mixer and then centrifuged for 10 minutes at low speed in a microfuge. The supernatant was saved as the wash fraction and the pellet was vortexed in 1.5 ml ice cold dH_2O and then shaken on ice at low speed for 10 minutes. The suspension was spun for 10 minutes at low speed and the supernatant was saved as the periplasmic fraction. The pellet was resuspended in 1.5 ml of dH_2O and sonicated for 1 minute at 14 µ amplitude using the narrow probe in an MSE Soniprep 150. The resulting suspension was spun for 5 minutes at high speed in a microfuge. The supernatant was saved as the cytoplasmic fraction. The pellet was resuspended in 1 % Triton X-100 and this was treated as the membrane fraction.

b) Total cell extracts

For determination of enzymatic activity or protein concentration 1.5 ml of a cell culture was sonicated for 1 minute at 14 µ amplitude using the narrow probe in an MSE Soniprep 150.

2.5.5 Protein detection and screening

a) SDS-PAGE

To run protein gels, Bio-Rad apparatus was used - either Mini Protean II or Protean II xi equipment.

Running gels were made using 10 % acrylamide, 0.1 % SDS, 0.07 % ammonium persulphate and 0.375 M Tris.HCl, pH 8.8. Stacker gels were made using 5 % acrylamide, 0.1 % SDS, 0.07 % ammonium persulphate and 0.125 M Tris.HCl, pH 6.8. The gels were set using TEMED - 1 $\mu l.ml^{-1}$ of gel. Protein samples containing the appropriate buffer (see below) were pipetted into the wells using Bio-Rad Prot/Elec Tips, as were the relevant standard protein markers. Gels

were run in reservoir buffer containing 0.025 M Tris.HCl pH 8.8, 0.19 M glycine, 0.1 % SDS, at 150 V until the blue marker was about 1 cm from the bottom.

The acrylamide used in the gels was diluted from a 30 % acrylamide stock which was made by the addition of 29.2 g acrylamide and 0.8 g bis-acrylamide to 500 ml dH_2O. This solution was stirred for 2 hours in the cold and filtered through Whatman #1 paper and stored at 4 °C.

Samples were diluted 2 fold in protein sample buffer (2 % SDS, 10 % glycerol, 0.01 % bromophenol blue, 5 % β-mercaptoethanol, 0.125 M Tris.HCl pH 6.8) and boiled for 5 minutes. *Bacillus thuringiensis* δ–endotoxin was not soluble in the standard sample buffer and δ-endotoxin expressing strains were boiled for 10 minutes in Bietlot buffer (8 M Urea, 2.5 % SDS, 5 % β-mercaptoethanol, 10 mM Tris.HCl, pH 8.3) instead [25].

b) Silver staining [80]

For large protein gels the gel was carefully removed from the glass plates of the electrophoresis apparatus and placed in a polythene box containing 600 ml of 5 % formaldehyde, 40 % ethanol solution for 30 minutes. This solution was replaced with 600 ml dH_2O and the gel left for 30 minutes before being placed in 50 % methanol overnight. The gel was soaked in 600 ml of dH_2O containing a small amount of DTT for 30 minutes followed by 200 ml of 0.1 % silver nitrate for 30 minutes. The gel was washed three times with dH_2O and once with 200 ml of developer (3 % $NaCO_3$, 0.0185 % formaldehyde) before being left to develop for 2 - 5 minutes in 400 ml of developer. Once the required intensity of the bands had been reached 20 ml of 50 % citric acid was added and the gel was washed in dH_2O.

c) Western blotting [122]

For this, Hoefer Semi-Phor TE70 semi-dry blotting apparatus was used. The bottom electrode (anode) was wetted with dH_2O and then three layers of 3MM paper soaked in transfer buffer (48 mM Tris, 39 mM glycine, 0.037 % SDS, 20 % methanol) were placed on top. With the addition of each successive sheet the stack was rolled with a glass Pasteur pipette to remove air bubbles. A nitrocellulose filter, pre-soaked in dH_2O was placed on the stack and an SDS-PAGE gel, run in the usual manner but incorporating prestained protein markers, was placed on the nitrocellulose followed by another three sheets of transfer buffer-soaked 3MM paper. The lid of the apparatus was put in place and a current of 0.8 $mA.cm^{-2}$ was applied for 1 hour. Transfer was deemed to be complete when no blue colouration

from the pre-stained protein markers was visible in the gel. The nitrocellulose filter was then removed and allowed to dry prior to subsequent processing. The positions of the pre-stained marker proteins were marked using a pencil.

d) Protein immunoscreening [122]

The filter was wetted in TBS and placed in a polythene box containing 200 ml of blocking solution (3 % BSA, 0.02 % NaN_3 in TBS). This was left shaking on a rotary shaker for 2 hours. The blocking solution was then replaced with 50 ml of TBS and the filter was agitated for 5 minutes. This last step was repeated once more. The filter was then placed in a Techne Hybridisation Tube and the primary antibody in blocking solution was added (50 $\mu l.cm^{-2}$). A 1/500 dilution was used of the anti-ChvE antibody [155] and a 1/1000 dilution was used of the anti-HD73 antibody [101]. The tube was placed in a Techne Hybridisation Oven and left at room temperature for 2 hours. The filter was washed four times with 30 ml TBS for 5 minutes. The secondary antibody (anti-rabbit Ig conjugated with alkaline phosphatase) was added at a concentration of 1/1000 in blocking solution (50 $\mu l.cm^{-2}$) and the filter was incubated for 2 hours. The blot was then washed four times with 30 ml of TBS for 5 minutes.

Developer was made by adding 66 µl of NBT stock and 33 µl BCIP stock to 10 ml phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5). This was added to the filter in a shallow polythene tray. The filter and the developer were agitated until the desired colour level of bands had appeared and then 100 ml of 20 mM EDTA in TBS was added to stop the reaction. (NBT stock was 50 $mg.ml^{-1}$ NBT in 70 % DMF; BCIP stock was 50 $mg.ml^{-1}$ BCIP in 100 % DMF).

e) ELISA [122]

50 µl of each of the protein samples was pipetted into the wells of a Falcon 'Pro-bind' 96 well assay plate and left for 2 hours. The samples were then shaken out of the plate and the wells were washed twice with PBS. The wells were filled with 300 µl blocking solution (3 % BSA in PBS) and left for 2 hours. The wells were washed with PBS four times before the addition of 50 µl of the primary antibody, diluted 1/1000 [101] in blocking solution. Plates were left for 2 hours and then the wells were washed four times with PBS before the addition of the secondary antibody (anti-rabbit Ig conjugated with alkaline phosphatase). The plates were left for two hours and the wells washed four times with PBS and twice with alkaline phosphatase buffer (0.5 mM $MgCl_2$, 10 mM diethanolamine, pH 9.5). 50 µl of substrate solution (1 $mg.ml^{-1}$ *p*-nitrophenyl phosphate in alkaline phosphatase

buffer) was added to each well and the colour allowed to develop for 30 minutes before addition of 50 µl stop solution (0.1 M EDTA, pH 8.0). The absorbance was then measured at 405 nm using a Titertek Multiscan MCC plate reader.

f) Protein concentration estimation

This was done using a modification of the method of Bradford [35] and using the kit supplied by Bio-Rad for that purpose.

800 µl of the test protein solution was taken and 200 µl of the Bio-Rad Bradford Assay Reagent was added in a microfuge tube. The contents of the tube were then mixed by inversion and the colour was allowed to develop for five minutes. 200 µl of the resulting solution was then taken and pipetted into a Greiner microtitre plate. The absorbances were read in a Titertek Multiscan MCC plate reader at 595 nm. The values obtained were compared to a standard curve prepared with various concentrations of BSA.

2.5.6 Enzyme assays

a) *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose assay for chitinase

p-nitrophenyl-β-D-N,N'-diacetylchitobiose (Sigma) was dissolved at a concentration of 300 $\mu g.ml^{-1}$ in 0.05 M sodium phosphate (pH 6.0). 100 µl aliquots of the samples were added to 100 µl of the substrate and incubated in a Greiner flat bottomed 96 well microtitre plate at 50 °C for 1 - 6 hours before the addition of 10 µl of 1 M NaOH. The absorbance was read at 405 nm using a Titertek Multiscan MCC plate reader. This method, based on that of Roberts and Selitrennikoff [248], was also used for the other chromogenic substrates, *p*-nitrophenyl-N-acetyl-glucosaminide and *p*-nitrophenyl-β-D-N,N',N''-triacetylchitotriose.

b) CM-chitin-RBV assay for chitinase

2 $mg.ml^{-1}$ CM-Chitin-RBV was diluted to give a 1 $mg.ml^{-1}$ solution in 0.05 M sodium phosphate (pH 6.0). 100 µl aliquots of the samples were added to 100 µl of the substrate in a microfuge tube and incubated at 50 °C for 1 - 6 hours before addition of 50 µl of 1 N HCl. The tubes were then put on ice for 10 minutes and then spun at high speed in a microfuge. 200 µl samples were then taken and added to a Greiner flat bottomed 96 well microtitre plate. The absorbance was measured at 540 nm. This procedure is based on that of Wirth and Wolf [337].

c) *p*-nitrophenyl phosphate assay for alkaline phosphatase [96]

The substrate solution was prepared by adding *p*-nitrophenyl phosphate (Sigma Substrate '104') to 1 M Tris, pH 8.0 to give a final concentration of 0.2 $mg.ml^{-1}$. 100 µl samples were added to 100 µl of the substrate solution in the wells of a microtitre plate and incubated at 37 °C for 30 minutes - 4 hours. The reaction was stopped by the addition of 100 µl of 0.2 M EDTA and the absorbance was measured at 405 nm.

d) Assay for β-lactamase [43, 63]

Solution A was 0.8 $mg.ml^{-1}$ neocuproine.HCl, 50 $mg.ml^{-1}$ SDS in 0.2 M sodium acetate pH 4.75. Solution B was 2 $mg.ml^{-1}$ $CuSO_4.5H_2O$, 50 $mg.ml^{-1}$ SDS in 0.2 M sodium acetate pH 4.75. Solution C was made by mixing equal volumes of Solutions A and B. 120 µl samples were added to 300 µl of 0.2 $mg.ml^{-1}$ penicillin G in 10 mM sodium phosphate buffer (pH 7.0) in a microfuge tube and incubated at room temperature for 30 minutes. 420 µl of Solution C was added and the colour left to develop for 8 minutes, before 200 µl of each sample was loaded into a microtitre plate and the absorbance measured at 450 nm.

e) Assay for invertase [107]

25 µl of 0.2 M sodium acetate (pH 4.9) was added to 10 µl of sample and the reaction was started by the addition of 12.5 µl 0.5 M sucrose. The reaction was left to proceed at 30 °C for 1.5 hours and then 50 µl of 0.5 M potassium phosphate buffer (pH 7.0) was added. The tube was further incubated for 3 minutes at 95 °C and then allowed to cool to 30 °C at which point 500 µl of Solution C was added. The tube was incubated at 30 °C for 20 minutes and then 750 µl of 6 N HCl was added and 200 µl of the resulting solution taken and added to a microtitre plate. The absorbance was read at 540 nm.

Solution C was prepared in the following way: 500 µl of a solution containing 0.8 $mg.ml^{-1}$ glucose oxidase (160 µmole U/mg) and 0.1 $mg.ml^{-1}$ peroxidase in 0.1 M potassium phosphate buffer (pH 7.0) was taken and added to 1 ml of 6 $mg.ml^{-1}$ *o*-dianisidine in dH_2O. The solution was made up to 10 ml with 45 % glycerol.

f) Assay for 5' nucleotidase [220]

100 µl of sample was taken and added to 100 µl of substrate solution which contained 8 mM 5'-AMP, 20 mM $CaCl_2$, 2 mM $CoCl_2$ in 200 mM sodium acetate buffer (pH 5.8). The mixture was incubated at 28 °C for 1 hour and then the amount of free phosphate liberated was determined.

This was performed using a Sigma Diagnostics kit. 100 µl of the above sample was taken and added to 500 µl of sterile dH_2O and 400 µl of 20 % trichloroacetic acid. The solution was mixed and left to stand for 10 minutes before being centrifuged in a microfuge at high speed for 5 minutes. 500 µl of the resulting solution was added to 750 µl sterile dH_2O and 250 µl molybdate solution (1.25 % ammonium molybdate.$4H_2O$ in 2.5 N sulphuric acid). The solution was mixed by inversion and 62.5 µl Fiske and Subbarow solution was added. The solution was mixed by inversion and left to stand for 10 minutes. 200 µl aliquots were loaded into a 96 well microtitre plate and the absorbance was measured at 620 nm.

g) Assay for acid phosphatase [220]

100 µl of sample was taken and added to 100 µl of substrate solution which contained 10 mM glucose-6-phosphate, 20 mM $CaCl_2$, 2 mM $CoCl_2$ in 180 mM sodium acetate buffer (pH 5.65). The mixture was incubated at 28 °C for 1 hour and then the amount of free phosphate liberated was determined as above.

h) Assay for 2'3' cyclic-AMP phosphodiesterase [220]

100 µl of sample was taken and added to 100 µl of substrate solution which contained 2 $mg.ml^{-1}$ bis(*p*-nitrophenylphosphate) in 10 mM $MgCl_2$, 2 mM $CoCl_2$, 100 mM sodium acetate (pH 6). After 1 hour at 28 °C the reaction was stopped by the addition of 10 µl 1 N NaOH and the absorbance measured at 405 nm.

i) Insertional inactivation of *lacZ* encoding β-galactosidase [261]

When cloning DNA fragments into pUC19, inactivation of *lacZ* α-complementation was used to distinguish between colonies containing plasmids with an insert and those containing pUC19. The multiple cloning site in pUC19 is located at the start of a shortened *lacZ* coding region, which is able to produce active β-galactosidase in bacterial strains containing a mutation in the N terminal end of the enzyme (*lacZ*ΔM15) - the shotened LacZ polypeptide from pUC19 is able to complement with the mutated host protein, forming an active enzyme. β-

galactosidase is able to cleave the substrate X-gal, forming a blue colouration. If DNA is inserted into the multiple cloning site of pUC19, the coding region is disrupted and cannot α-complement with the mutated host enzyme. Thus colonies of *E. coli* containing the *lac*ZΔM15 mutation containing recombinant plasmids can be identified as appearing 'white' when grown in the presence of X-gal.

40 μl of a 20 $mg.ml^{-1}$ solution of X-gal in dimethylformamide was evenly spread over the surface of selective agar plates and allowed to dry overnight at 37 °C. Transformed *E.coli* cells were then plated out onto these plates and incubated until distinct colonies could be detected.

2.5.7 *Agrobacterium* induction assay

The cells were grown for 48 hours in 5 ml of LM broth with antibiotic selection at 28 °C with shaking. Cells were then counted using a Coulter Multisizer II and diluted in Davis Induction Medium to a concentration of 1×10^7 $cells.ml^{-1}$. 2 ml of the resulting cell suspension was then added to sterile glass scintillation vials and incubated with shaking at 28 °C to allow the bacteria to adjust to the new medium. After 3 hours acetosyringone in 70 % methanol was added to a final concentration of 100 μM. Controls had the equivalent volume of 70 % methanol added. The vials were then sampled at various time intervals. The photons produced per minute per tube were counted using a Canberra Packard 2000 CA Tri-Carb Liquid Scintillation Analyser, by placing the vials directly into the counter. 50 μl of the cells were removed from the scintillation vial and placed in 20 ml of Isoton II in a Coulter vial. The number of bacteria was counted using a Coulter Multisizer II. Results were expressed as $photons.minute^{-1}.bacterium^{-1}$.

2.5.8 *Manduca sexta* bioassay

Manduca sexta diet [341] was made up and poured into Falcon flat-bottomed polystyrene 6 well culture plates so that the medium occupied about 1/3 rd of the well. The bacteria to be tested were diluted to 1×10^8 ml^{-1} in dH_2O and 50 μl of the suspension was spread evenly on the surface of the diet in the plate wells. One newly hatched 1st instar larva was added to each well and loosely fitting lids were placed on the plates to allow gaseous exchange but prevent the insects from escaping. They were kept under a 16 hour day at 26 °C. The development of the insects in each plate was monitored for a period of up to 5 days.

2.5.9 Fungal bioassays

A small sample of the fungus to be tested was scraped off a plate and placed in the centre of a petri dish containing Induction Medium solidified with 1 % bacteriological agar or YM agar. Around this 5 µl samples of bacterial cultures were placed and the plates incubated at 28 °C until the fungus had covered the bacterial colonies or zones of inhibition were observed.

2.5.10 Confirmation of the presence of *Agrobacterium*

This is a modified procedure of the one developed by Bernaerts and DeLay [24].

Agrobacterium colonies were grown for 48 hours at 28 °C on lactose agar (1 % lactose, 0.1 % yeast extract, 2 % agar). The plate was then covered in Benedict's solution and left for 5 minutes. A yellow colour was observed around the *Agrobacterium* colonies because of the bacterium's unique capability to produce 3-keto-lactose (a reducing sugar) from lactose.

2.5.11 Bacterial chemotaxis - the Blindwell Assay

This is a modified procedure of the one developed by Armitage *et al.* [5].

Motile cells of *A. tumefaciens* were obtained by inocluating a loopful of bacteria onto the centre of a MinA swarm agar plate. The plate was incubated at 28 °C for 48 hours before a loopful of motile cells from the leading outside edge of the bacterial swarm were transferred to the cetre of another MinA swarm agar plate. This process was repeated once more before cells from the outside of the edge were taken and streaked out onto a master plate with antibiotic selection, from which single colonies were taken and cultured when required.

Blindwell assay chambers (Bio-Rad) were greased with High Vacuum Grease and the lower chamber was filled with 200 µl of a bacterial suspension at about 1 x 10^7 cells.ml^{-1}. This was covered with a 13 mm nitrocellulose filter (pore size 8 µm) and the hollow Teflon plug was screwed down on top of this. 400 µl of attractant were pipetted into the plug, the top of which was then sealed with Whatman laboratory sealing film. The chambers were inverted for two hours and then 50 µl was removed from the top chamber and added to 20 ml of Isoton II. The number of bacteria in the resulting suspension was counted on a Coulter Multisizer II and the total number of bacteria in the upper chamber calculated.

Control assays were performed in parallel using medium as the attractant in the upper chamber. The Chemotactic Index [231] was calculated as follows:

$$CI = \frac{[\text{cells in upper chamber}] - [\text{cells in control upper chamber}]}{[\text{cells initially introduced in lower chamber}]} \times 100\%$$

2.5.12 Plasmid stability assay

Bio Multi Compost was sterilised and a bacterial suspension in sterile dH_2O was added to give a final concentration of between 1×10^5 and 1×10^6 bacteria.g^{-1} of soil. The soil was divided into plastic drainage tubing with a diameter of 4 cm and a height of 12 cm. The tubes had 1 cm diameter holes at 3, 7 and 10 cm from the base to allow sampling of the soil. These holes were sealed with masking tape during incubation of the tubes. The tubes were stood in a moist bed of Vermiperl vermiculite (which was kept moist with sterile dH_2O) and incubated at 28 °C in the dark. 0.1 g of soil was taken from each hole at regular intervals and added to 900 µl of sterile dH_2O giving a 10^{-2} dilution. 10^{-4} and 10^{-5} dilutions were plated out onto agar plates selective for *A. tumefaciens*. Colonies were allowed to grow for 48 hours before counting and then colonies from each were streaked out onto selective media to determine whether the plasmid was still present. Colonies that were of intermediate growth on the selective plates were grown up in selective liquid media and the plasmid content analysed by restriction analysis of 'minipreped' plasmid DNA. By comparing the total number of *A. tumefaciens* and the proportion of these which grew up on the selective plates it was possible to determine what percentage of the bacteria had lost the plasmids under study.

2.5.13 Antibiotic synergism assay

This is a modified version of the procedure outlined by Krogstad and Moellering [181]

Sterile strips of 3MM paper (35 mm by 10 mm) were soaked in 100 µl of the antibiotic solutions under test and placed on an LM-agar plate. To examine synergism or antagonism between two antibiotics, strips containing the two different compounds were placed perpendicular to one another with one strip overlapping the other. The strips on the agar were incubated at 28 °C for 1 hour before being removed. 100 µl of a stationary culture of the *Agrobacterium tumefaciens* strain under test was then spread evenly across the plate which was allowed to incubate at 28 °C for 48 hours. Synergism should have resulted in a greater than expected zone of clearing in the bacterial lawn whereas antagonism should have resulted in the opposite effect.

2.5.14 Electron microscopy

30 µl of the bacterial suspension were pippetted onto a Formvar-coated grid and left for 30 minutes. The excess medium was then blotted off and the grid was placed upside down in 50 µl 1 % uranyl acetate (w/v in 70 % ethanol) for 30 seconds. The grid was then washed by tranferring it five times into successive drops of distilled water and then air dried before viewing and photographing using a Philips EM400 microscope.

2.5.15 Thermotolerance assay [329]

Cells were cultured in M9 glycerol medium at 30 °C until an OD_{450} of 0.3 was reached at which stage the bacteria were diluted 10^4-fold in prewarmed (30 °C) M9 glycerol medium to give a final volume of 10 ml at which stage the bacteria were shifted to 50 °C. 100 µl samples were taken at regular intervals and plated out onto M9 glycerol plates and incubated at 30 °C until distinct colonies were apparent. The number of colonies on each plate were counted. Random colonies were picked, resuspended in a drop of LM-broth and examined under a light microscope (Nikon Optiphot) using phase contrast optics to confirm that there was no obvious contamination of the plates from other microorganisms.

Chapter 3

AGROBACTERIUM 'MICROBIAL INOCULANT' FEASIBILITY STUDIES

3.1 The *lux* reporter gene system

A general strategy for the use of *vir* gene promoters in *Agrobacterium tumefaciens* as a way of targeting pesticides in a conservative manner has been described in section 1.14 [9, 10]. In order to assess how well this system might operate, it was necessary to have a method for monitoring the amount of any particular pesticide that an induced *vir* gene promoter was capable of expressing.

For studies on the induction of the *vir* promoters by acetosyringone, the *lux* reporter system was used. The *lux* genes, from *Vibrio fischeri* (a bacterium that inhabits the 'light organ' of the fish *Monocentris japonicus* [135, 304]), encode enzymes that are responsible for light production [83].

There are three main benefits of using the *lux* system over more traditional reporter gene systems. First, the production of light does not destroy material or interfere with any other biochemical processes occurring in the bacterium. Second, the amount of light produced can be easily measured in a quantifiable manner. Third, the measurement of emitted light does not require sampling or the addition of extraneous substrates [275]. The detection of light is also easy to perform, extremely quick and involves no expensive substrates.

The enzyme responsible for light production is luciferase, a two subunit protein composed of α and β peptides encoded by *luxA* and *luxB* respectively each having a molecular weight of about 40 kD [83]. The functional enzyme oxidises a reduced flavin and a long-chain aldehyde to form an oxidised flavin and a long-chain fatty acid, a process which liberates a photon [82]. *luxC, luxD* and *luxE* code for the enzymes that provide the aldehyde substrate [82, 83]. Other *lux* genes (*luxF, luxG, luxH* and *luxY*) have also been found but their functions have not been elucidated [201].

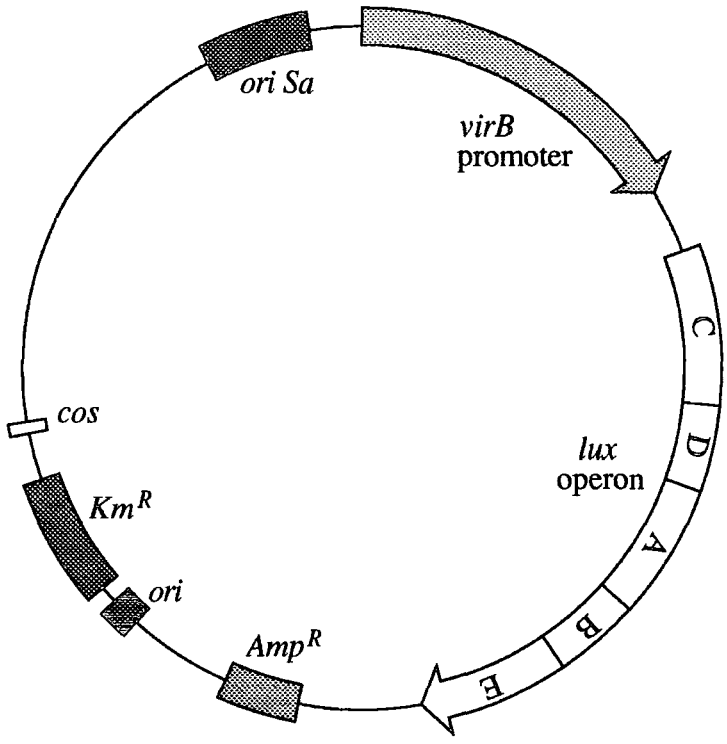


Figure 3.1.1 pUCD1187 [253], containing the *virB* promoter linked to the structural genes of the *lux* operon from *Vibrio fischeri*. *cos* = phage lambda packaging site, *ori Sa* = origin of replication from pSa, *ori* = origin of replication from pBR322. Not to scale.

Rogowsky *et al.* [253] constructed a promoterless *lux* operon, containing only the structural genes, in a broad-host range plasmid. This plasmid, pUCD615, also contained a multiple cloning site 600 bp upstream from the start codon of *luxC*, allowing promoters to be added and their effectiveness assayed [253]. Using this plasmid, Rogowsky *et al.* examined the acetosyringone-inducible nature of each of the *vir* promoters in pTiC58. They found that the *virB* promoter construct, pUCD1187 (see figure 3.1.1), was the most active followed by the *virE* promoter construct, pUCD1194 (see table 3.2.2).

These constructs were then used, in this study, to determine the optimum conditions for *vir* gene induction.

3.2 Monitoring *vir* induction using *lux*

A. tumefaciens LBA4301 (pVK257) [175] was transformed with pUCD1187 or with pUCD1194 [253] using electroporation (see section 2.5.2.b), both the resulting strains then being assayed for acetosyringone-inducibility in four different induction media in an attempt to find conditions most suitable for *vir* induction.

pVK257 [175] supplies the VirA and VirG proteins necessary for the detection of acetosyringone and subsequent expression from the *vir* gene promoters under test (section 1.9). A complicating factor, however, is the presence of dual promoters directing transcription of *virG*. All the induction media have low phosphate concentrations, which induce the P1 promoter and low pHs which induce the P2 promoter. When cells are moved from one medium with higher pH and phosphate concentration to another with lower pH and phosphate concentration, *vir* induction occurs, this phenomenon being known as 'media shift' [336].

In order to avoid 'media shift', the following procedure was used. One colony was taken from a selective LM agar plate and used to inoculate 5 ml LM broth, containing the relevant antibiotics. This culture was then left for 48 hours at 28 °C with shaking. After this period the pH of the culture was below pH 6 and although not determined, it seemed probable that at this stage the phosphate concentration would be very low. Using this approach no appreciable 'media shift' was found to occur.

The next problem encountered was the instability of light production. Although light was easily detected in the scintillation counter, initial experiments suggested that taking samples from induced cultures appeared to be detrimental. In

order to confirm this two cultures of *A. tumefaciens* C58C1 (pVK257, pUCD1187) were grown up in 3 ml of Seattle induction medium (one induced with 100 μM acetosyringone [289]) in scintillation vials for 15 hours at 28 °C with shaking. 1 ml was removed, the cells harvested by centrifugation and then resuspended and pipetted into a fresh scintillation vial. A further 1 ml was pipetted into a fresh scintillation vial and the photon production of the cells in the three vials was determined as was the number of cells in each vial, thereby determining the levels of induction for each treatment. The cells subjected to centrifugation and pipetting showed a 2 fold induction, those subjected to pipetting showed a 113 fold induction whilst those that were left undisturbed showed a 230 fold induction.

These results suggested that the cells should be disturbed as little as possible whilst taking photon measurements. Therefore, subsequent assays were performed by counting the photons liberated by whole cultures. Ideally, large cultures should have been grown up (to buffer temperature changes) in nephlo flasks and the light production measured in a luminometer [253]. However, no such apparatus was available and so a scintillation counter was used. This limited the size of culture that could be used so to minimise temperature changes in the culture the vials were taken out of the incubator for as little time as possible. A shift of even +/- 1 °C can affect the amount of light produced quite significantly [276]. Temperature changes, though, were inevitable and this probably accounts for the erratic nature of some of the readings of the induced cultures especially when using the *virE* promoter (see below).

The four induction media used were described as Davis [253], Ghent [318], Seattle [336] and Rogowsky [254] all of which were standard induction media used by other groups. Using *A. tumefaciens* C58C1(pVK257, pUCD1187) maximum induction occurred in Davis medium, 19 hours after addition of acetosyringone with a 8466 fold induction. Ghent medium produced a maximum induction level of 2756 fold after 15 hours, whereas for Seattle and Rogowsky medium maximum induction occurred between 10 and 11 hours with values of 4256 fold and 3168 fold respectively (see figure 3.1.1).

Rogowsky *et al.* [253] recorded a 1140 fold induction using pUCD1187 in Davis medium after 18 hours. This value is over 7 times less than the value reported here but there were differences between the two sets of experiments. Those performed by Rogowsky *et al.* were done at 25 °C using pTiC58 to provide the *virA* and *virG* functions. The experiments reported here were performed at 28 °C and used only *virA*, *virB*, *virC* and *virG* from pTiA6 [175]. It is possible that other

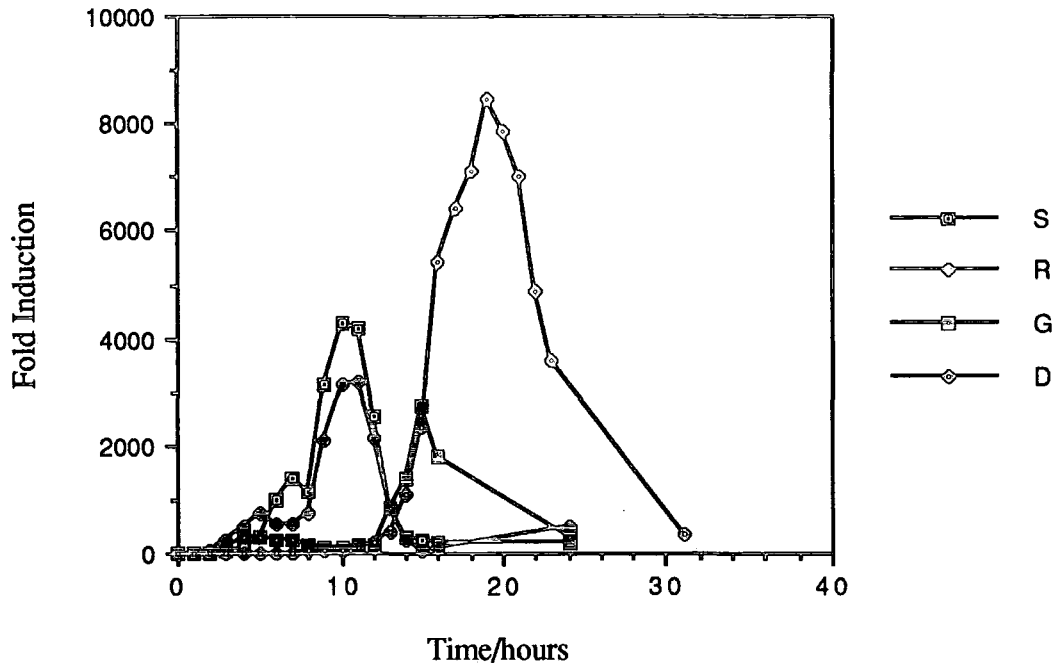


Figure 3.2.1 Showing induction of light production from *A. tumefaciens* C58C1(pVK257, pUCD1187) at various time intervals after addition of acetosyringone (time 0) in four different induction media. Fold Induction values are photons.min^{-1}.cell^{-1} values from an induced culture divided by the same value obtained at the same time point from a control culture induced with solvent. D = Davis medium, G = Ghent medium, S = Seattle medium, R = Rogowsky medium. Results shown are the means of two readings.

vir::*lux* plasmid	Gene fused	Fold induction
pUCD1186	*virA*	23
pUCD1187	*virB*	1140
pUCD1168	*virC*	>28
pUCD1173	*virD*	71
pUCD1194	*virE*	322
pUCD1195	*virG*	0.36
pUCD615	None	0.94

Table 3.2.2 Fold induction of various *vir* promoter::*lux* constructs 18 hours after addition of 100 μM acetosyringone in Davis medium. Data from Rogowsky *et al.* [253].

proteins encoded by pTiC58 down regulated the response from the *virB* promoter in the experiments by Rogowsky *et al.* The region upstream from the *virB* promoter contains two *vir* boxes, B1 and B2, B1 being sufficient for *virB* induction and B2 (downstream from B1) possibly altering the effect of VirG binding to B1 [233]. It may also be that VirG from pTiA6 is different in some important way from that of pTiC58. The temperature that the experiments were conducted at may also have been important.

Similar experiments were carried out using the *virE::lux* fusion, pUCD1194 [253]. However, these experiments produced consistently erratic results with low induction values, the highest of which was a 66 fold induction obtained after 14 hours in Seattle medium. This compares to a 322 fold induction obtained by Rogowsky *et al.* [253] after 18 hours in Davis medium. Again the temperature difference may have been a factor accounting for the variation in these results, as could the genetic differences as described above. Whilst the region upstream from *virB* contains two *vir* boxes, the similar region upstream of *virE* contains only one [233]. Possibly more importantly, Pazour and Das have previously reported that unmodified VirG will bind specifically to most *vir* promoter regions but not to the *virE* regulatory region [234]. It could be, therefore, that the induction of *virE* is a less stable event than the induction of *virB* due to poorer binding of VirG, the hourly measurements disrupting the induction process.

The experiments described here were performed to provide a standard method of conducting future *vir* induction assays involving *vir* promoters controlling the expression of chitinase or δ-endotoxin genes, allowing the production of large quantities of the relevant protein, although no attempt was made to elucidate which components of the various media were responsible for the differences in induction. For future induction assays using the *virE* promoter, the same conditions were used as for assays involving the *virB* promoter in order to make adequate comparisons between the two. *virE* promoter fusions have previously been shown to elicit high levels of induction [187, 253] and this was also seen to be the case in further studies involving the *virE* promoter (Chapter 4), indicating that for *virE* promoter::gene fusions the *lux* assay is not particularly suitable in its current form.

Davis medium was routinely used for liquid culture assays and Rogowsky medium was used for plate assays, since bacterial colonies did not grow well on solidified Davis medium. It should be noted that liquid Rogowsky medium allowed the production of most light (at its peak about 3 times as much as that obtained with

Davis medium at its peak), however, Rogowsky medium also gave the highest background values of all the media tested.

3.3 Demonstration of *vir* induction *in planta* using *lacZ*

Although it has long been assumed that phenolic compounds liberated by a wound site are responsible for causing *vir* induction in *A. tumefaciens*, to our knowledge this has never been conclusively demonstrated *in planta*. The *lux* system, used to monitor *vir* induction (section 3.2), was considered to be unwieldy in this respect since light is easily dispersed by media in which any seedling is growing and by interfaces. The alternative of taking plants out of the growth media and placing them against photographic film was not considered to be a viable approach, since removal of the seedlings was thought likely to cause wounding in itself.

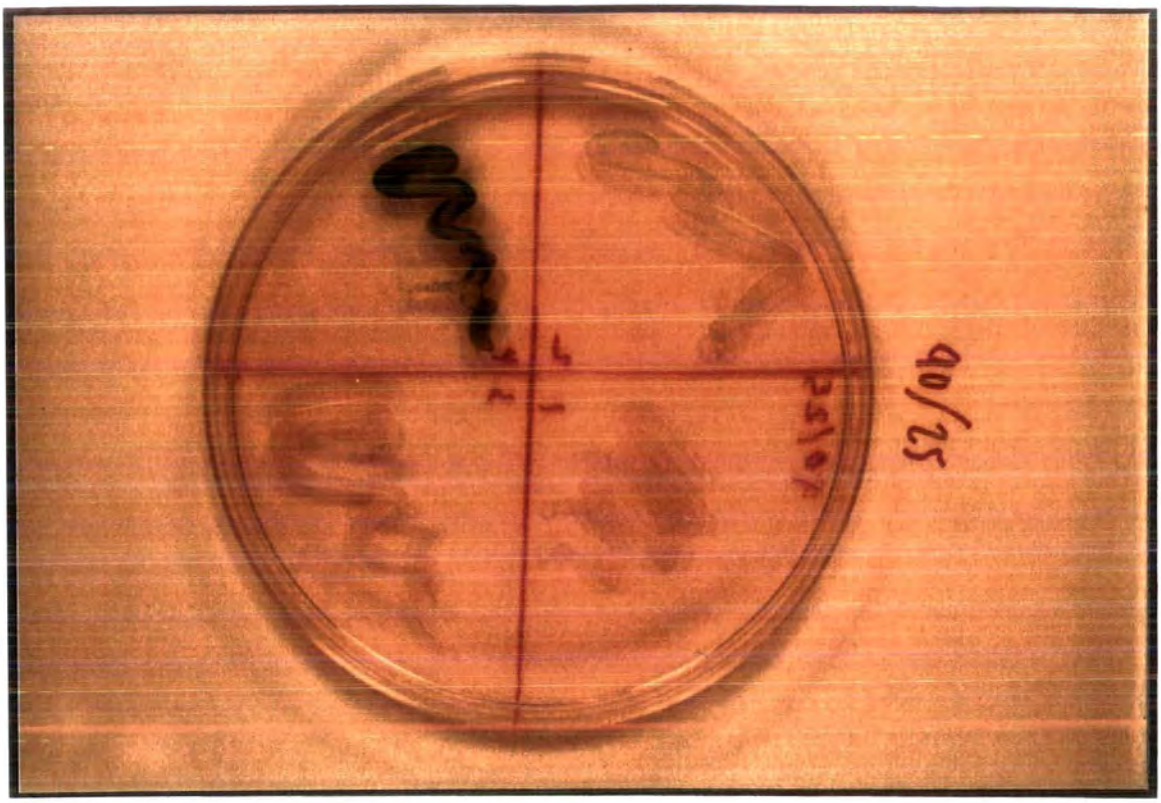
Various attempts were made to demonstrate *vir* induction occurring at the wound site on a plant root using the *E. coli lacZ* gene as a reporter [162]. *lacZ* encodes β-galactosidase which cleaves the artificial substrate X-gal producing a blue colour. This substrate was reasonably cheap, easy to use and was freely available in the lab.

vir promoter::*lacZ* fusions have been used successfully in the past to examine *vir* induction [4, 288] and *Agrobacterium* does not have any intrinsic β-galactosidase activity [331]. Thus, it was envisaged that seedlings grown in transparent plastic tubes in clear media would show a blue colouration at any wound site in the presence of an *A. tumefaciens* strain containing a *vir* promoter linked to the *lacZ* gene but not in the presence of control strains. Such a strain was *A. tumefaciens* C58C1 (pIB50, pIB100).

pIB50 is an IncP plasmid containing a *virB*::*lacZ* fusion and pIB100 contains the *virA* and *virG* functions necessary for *vir* induction [4]. *A. tumefaciens* C58C1 [314] was used since LBA4301 proved difficult to transform and for this reason C58C1 was used for all further investigations although Lilley found that it was not as effective a strain as LBA4301 for *vir* induction [187]. pIB50 and pIB100 have been used to demonstrate *vir* induction in a C58 chromosomal background in the past [4].

A plate assay was performed to demonstrate the production of β-galactosidase from pIB50 under conditions conducive to *vir* induction. Rogowsky medium was solidified with 0.5 % agar (Rogowsky Agar) and various concentrations of X-gal

4 2



3 1

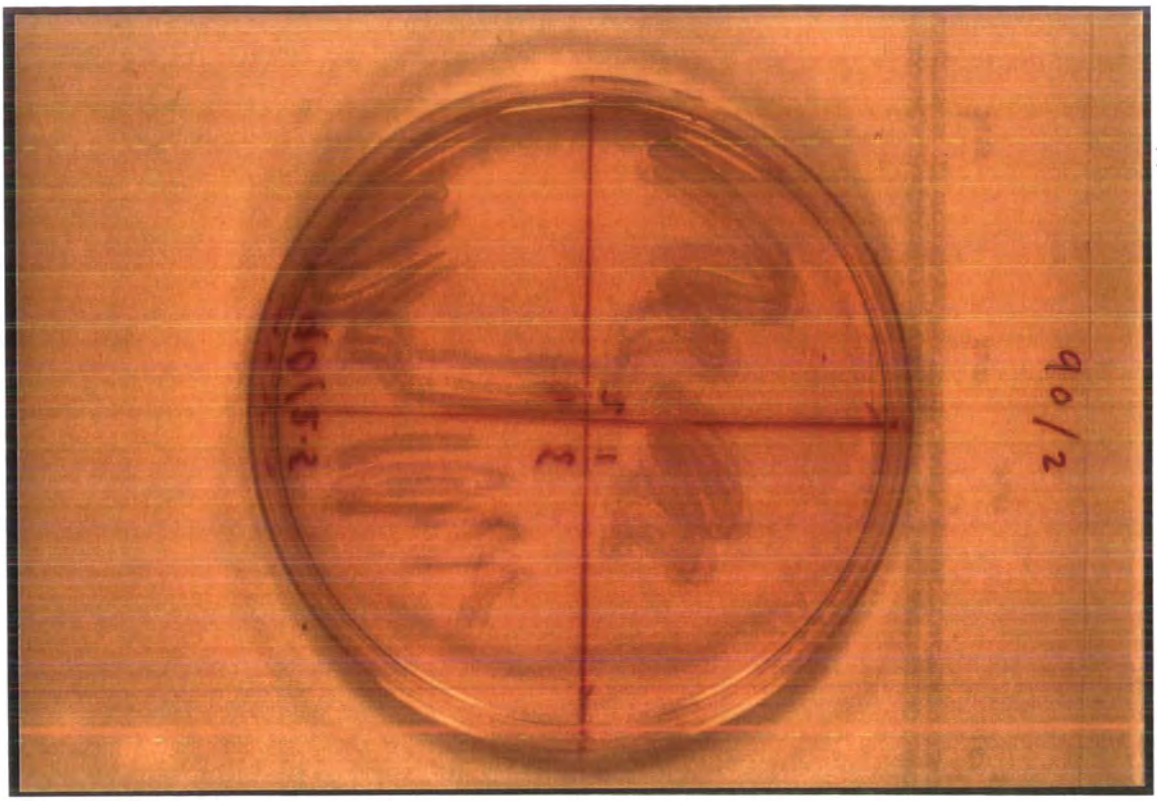
Figure 3.3.1 *vir* induced β-galactosidase induction of various *A. tumefaciens* strains after 48 hours growing on 0.5 % Rogowsky Agar containing 90 $\mu g.ml^{-1}$ X-gal and 100 μM acetosyringone. The plate is divided into four sections consisting of *A. tumefaciens* C58C1 containing the following: 1 - no plasmids; 2 - pIB50; 3 - pIB100; 4 - pIB50, pIB100.

and acetosyringone were added. The resulting medium was then poured into petri dishes and four strains of *A. tumefaciens* were streaked onto the surface of the agar and left to incubate at 28 °C.

Colour differences were detected at X-gal concentrations of 90 $\mu g.ml^{-1}$ and higher after about 16 hours, using 100 μM acetosyringone. After 48 hours, *A. tumefaciens* C58C1 (pIB50, pIB100) was an intense blue colour on these plates whilst strains, lacking pIB50 or both plasmids, remained beige. The strain containing pIB50 but lacking the *virA* and *virG* functions necessary for *vir* induction was a faint blue colour, indicating a low basal level of *lacZ* expression (see figure 3.3.1). On plates containing the same concentration of X-gal but only 10 μM acetosyringone, the intense blue colouration of *A. tumefaciens* C58C1 (pIB50, pIB100) was not observed indicating that low levels of phenolic compounds were not sufficient to cause *vir* induction in this set up (see figure 3.3.2). Using other concentrations of X-gal it was found that 90 $\mu g.ml^{-1}$ provided a quick assay that was not badly affected by the basal level of expression from *lacZ*. Below this concentration the vivid blue colour took longer to develop and above this concentration the background β-galactosidase caused a greater degree of blue colouration to develop in the control strain containing only pIB50, which might complicate the interpretation of results *in planta*.

From these observations it seemed likely that the use of the *virB::lacZ* fusion would provide an adequate means of detecting *vir* induction at wound sites inflicted on seedling roots. For this mung bean seedlings were used since they were quick and easy to germinate and produced a large tap root. The mung bean seeds were soaked in 5 % 'Chloros' (sodium hypochlorite) for 5 minutes and subsequently washed three times with sterile distilled water before being planted out in petri dishes containing 0.2 % Rogowsky Agar. This 'sloppy' agar allowed some support for the root structure but prevented too much diffusion of the blue colouration. It also allowed the bacteria to move in the medium and also allowed easy insertion of the seedlings into the medium without disrupting the root structure. The seeds were then germinated in a light cabinet at 28 °C for four days after which period of time they were removed and a proportion of the seedlings had the tap root wounded with a single gash made using a sterile scalpel blade. Unwounded and wounded seedlings were placed in clear plastic tubes containing 5 ml 0.2 % Rogowsky agar, 90 $\mu g.ml^{-1}$ X-gal and the strain of *A. tumefaciens* to be tested at a concentration of 1×10^7 $cells.ml^{-1}$. The tubes were then incubated at 28 °C in the dark. After 10 hours a blue colouration was observed at the wound sites of all the seedlings regardless of

4 2



3 1

Figure 3.3.2 *vir* induced β-galactosidase induction of various *A. tumefaciens* strains after 48 hours growing on 0.5 % Rogowsky Agar containing 90 $\mu g.ml^{-1}$ X-gal and 10 μM acetosyringone. The plate is divided into four sections consisting of *A. tumefaciens* C58C1 containing the following: 1 - no plasmids; 2 - pIB50; 3 - pIB100; 4 - pIB50, pIB100.

whether an *A. tumefaciens* strain was present or not. A blue colour was also observed at the root tips, lateral roots and also around the seed coat (see figure 3.3.3).

The tubes were observed for 48 hours but no difference in the intensity of the blue colouration was noticed between wounds surrounded by *A. tumefaciens* C58C1 (pIB50, pIB100) and those surrounded by control strains (see figure 3.3.3). The experiment was repeated using a range of X-gal concentrations, but again no differences were detected.

It was obvious that the mung bean seedlings were producing large amounts of endogenous β-galactosidase, or at least a functionally similar enzyme, a problem encountered in the past whilst using *lacZ* as a reporter gene in plants [149]. Similar problems were encountered when the experiment was repeated using cucumber seedlings.

However, it was not evident whether *vir* induction was capable of occurring in this set up, the conditions being fairly anaerobic. To test for this, strips of 3MM paper (30 x 10 mm) were soaked in an *A. tumefaciens* culture and placed in tubes containing 0.2 % Rogowsky Agar, 90 $\mu g.ml^{-1}$ X-gal and 100 μM acetosyringone. The tubes were incubated at 28 °C. After 16 hours a faint blue colouration was observed in tubes containing *A. tumefaciens* C58C1 (pIB50, pIB100). After 48 hours, the colour change was very marked but not as marked as that around the wound sites of the seedlings nor was the blue colour as deep as that observed during the plate assays (above). A faint blue colouration was noticed in the tubes containing *A. tumefaciens* C58C1 (pIB50) and no blue colouration was observed in the tubes containing bacteria lacking pIB50. It was concluded that the less aerobic conditions in the tube (as opposed to the agar surface in petri dishes) allowed less *vir* induction.

Lilley found that trying to do similar experiments using mung beans in petri dishes containing agar with a lawn of the indicator bacteria on it, resulted in further growth of the seedlings which lifted the roots up and so moved the wound sites away from the bacteria [187]. This was not, therefore, a satisfactory alternative.

However, given that some *vir* induction was observed in the tubes containing the paper strips soaked in *A. tumefaciens* C58C1 (pIB50, pIB100), it was decided to try the mung bean seedling experiments, as developed above, using an alternative reporter gene system. Any such reporter system would have to produce an enzyme

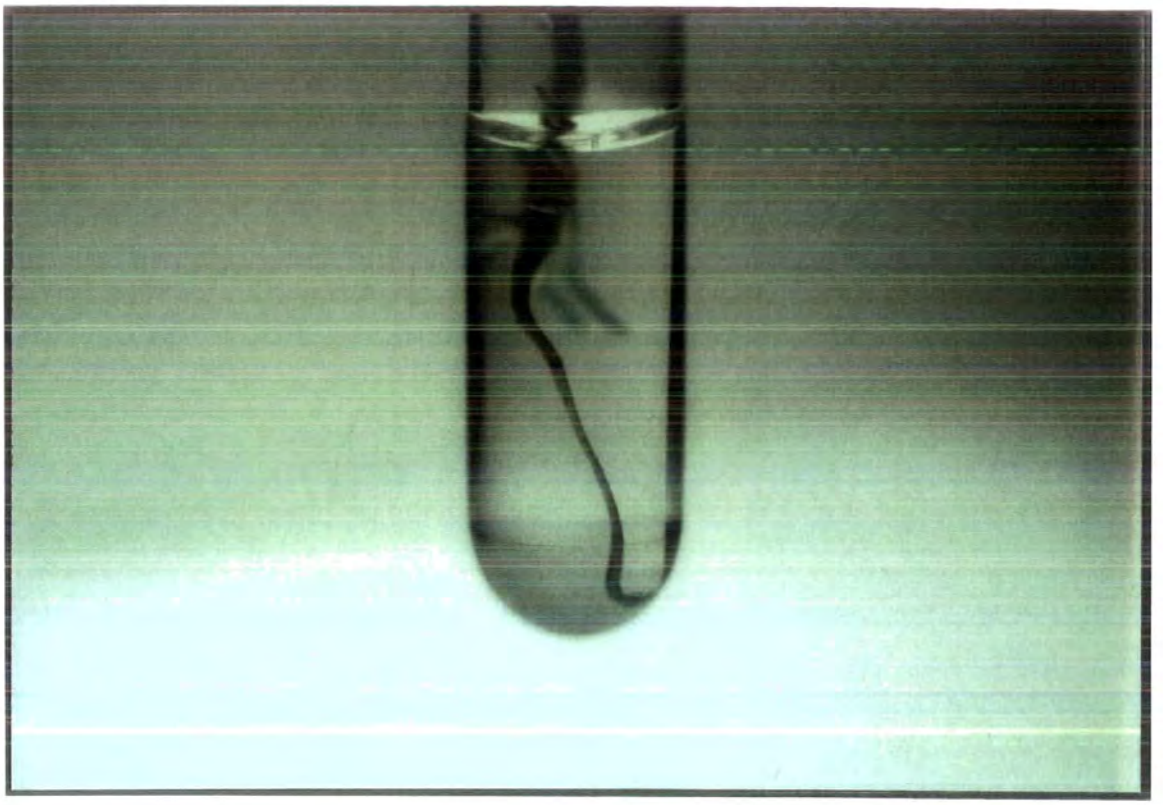


Figure 3.3.3 β-galactosidase production seen at various sites on the roots of a 4 day old mung bean seedling.after 9 hours incubation at 28 °C suspended in 0.2 % Rogowsky Agar containing 120 $\mu g.ml^{-1}$ X-gal. The wound site, a single gash made with a sterile scalpel blade, is clearly visible in the centre of the photograph.

capable of cleaving an available substrate to produce an observable colour change but would need to have no functionally similar analogues commonly found in plants.

3.4 Demonstration of *vir* induction *in planta* using GUS

The GUS reporter system consists of the *E. coli* genes *gusA* and *gusB* which are cocistronic and encode β-glucuronidase and glucuronide permease respectively [147]. The GUS system was developed to cope with some of the problems encountered using *lacZ*, including the intrinsic β-galactosidase activity associated with many plants, most plants having no detectable GUS activity [149].

β-glucuronidase is able to cleave a wide variety of substrates - virtually any aglycone conjugated to D-glucuronic acid via a β-o-glycosidic link (a glucuronide) [147]. The active enzyme is a tetramer, each subunit being about 68 kD [148]. Glucuronide permease has a molecular mass of about 49 kD and is responsible for transport of β-glucuronide substrates into the cell [147].

Lilley linked the *virE* promoter from pUCD1187 [253] to the *gusA/gusB* coding region. This construct was then transferred into the broad host range vector pMMB66, to allow replication in *Agrobacterium*, forming pEG2 [95, 187]. *A. tumefaciens* C58C1 (pVK257) [175, 314] was transformed with pEG2 by electroporation (section 2.5.2.b) and transformants were selected using colony hybridisation followed by probing with a radioactively labelled 0.8 kb *virE* promoter fragment from pUC1318::*virE* [187] (sections 2.5.3).

A plate assay was then performed using magenta-β-D-glcA (5-bromo-6-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt - Biosynth AG). The bacteria were streaked onto 0.5 % Rogowsky agar containing 100 μM acetosyringone (or the equivalent volume of 70 % methanol as a control) and varying concentrations of magenta-β-D-glcA. At a substrate concentration of 160 $\mu g.ml^{-1}$, the expected purple colouration of *A. tumefaciens* C58C1 (pVK257, pEG2) was observed after 24 hours. However the purple colouration was equally vivid on the control plates as it was on those plates containing acetosyringone. No colour development was observed for *A. tumefaciens* C58C1 (pVK257) growing on either type of plate.

This result was not unexpected. Using the same strain in liquid induction medium, Lilley had found that after 24 hours the GUS activity of the induced bacteria was 3.1 nmoles methylumbelliferone produced.min^{-1}.mg $protein^{-1}$ whilst

that of the uninduced culture was 1.4 nmoles.min^{-1}.mg protein^{-1} [187]. The high levels of background GUS activity meant that trying to distinguish between colour levels of the uninduced and induced bacteria on a plate assay was going to be difficult. The high basal level of GUS expression may have been the result of expression directly from the *tac* promoter found in pMMB66, the *virE::GUS* insert being in the correct orientation for this to occur [187]. No further trials were undertaken using pEG2.

Similar plate assays were performed using pVK257::*virB*GUS [97]. This plasmid contained the *virB* promoter region linked to the *gusA/gusB* region in pVK257 [175]. *A. tumefaciens* C58C1 (pVK257::*virB*GUS) was obtained using triparental mating (section 2.5.2.c) and confirmed as such using restriction digest patterns of 'minipreped' plasmid DNA (section 2.5.1.a). This strain was plated onto 0.5 % Rogowsky Agar containing a range of concentrations of magenta-β-D-glcA and 100 μM acetosyringone (or the equivalent volume of 70 % methanol as a control).

After 8 hours, *A. tumefaciens* C58C1 (pVK257::*virB*GUS) grown on the inducing medium containing 160 μg.ml^{-1} magenta-β-D-glcA had developed a clearly visible purple colour. *A. tumefaciens* C58C1 (pVK257::*virB*GUS) grown in the absence of acetosyringone but the same concentration of substrate, had remained beige in colour. After 24 hours, however, there was no difference in the colouration between the uninduced and the induced bacteria at 160 μg.ml^{-1} magenta-β-D-glcA nor at 80 μg.ml-1, the bacteria which had been grown on this lower concentration having become visibly purple by this stage. *A. tumefaciens* C58C1 (pVK257) was used as another control and this failed to produce a purple colour on any of the plates examined.

These results suggested that a clearly visible indication of *vir* induction might be possible at wound sites on mung bean seedlings using *A. tumefaciens* C58C1 (pVK257::*virB*GUS), providing that the length of the experiment was kept to a minimum. 4 - 5 day old mung bean seedlings (wounded or unwounded - see section 3.2) were added to tubes containing 0.2 % Rogowsky Agar, 240 μg.ml^{-1} magenta-β-D-glcA and *A. tumefaciens* C58C1 at a concentration of 1 x 10^7 cells.ml^{-1} (containing either the *virB*::GUS fusion plasmid or pVK257). The tubes were incubated in the dark at 28 °C and examined at regular intervals. No obvious change in colour occurred at the wound sites. Gradually, over a period of days, the agar medium became purple in all the tubes examined and this was probably due to background breakdown of the substrate [146]. The experiment was repeated using

Davis induction medium containing 0.2 % agar in an attempt to increase the induction levels of the *virB* promoter (see section 3.2) but this produced the same negative result. The anaerobic conditions in the tube may have been responsible for the lack of visible *vir* induction.

In hindsight, a series of experiments should have been carried out wounding the roots at the same level as the medium/air interface (or just below) in the tube to allow for a more aerobic response. All the experiments may have benefited from more air being let into the system. It would also have been interesting to add acetosyringone to some tubes instead of the seedling to see if there was a more rapid colour change than the background substrate degradation was producing, since at this stage it was still unclear as to whether wounded mung bean roots were capable of causing *vir* induction.

With the disappointing results using both the *lacZ* and the GUS reporter systems it was decided to have one more attempt to demonstrate *vir* induction at wound sites *in planta*, using the *lux* system.

3.5 Demonstration of *vir* induction *in planta* using *lux*

During the course of this work it was hoped that clearly identifiable *vir* induction could be shown to occur at wound sites using a non-invasive technique. The experiments using *lacZ* and GUS were not successful to this end and so experiments were undertaken using *lux* as a reporter system.

Despite the advantages of the *lux* system in monitoring *vir* induction in liquid media (see section 3.1), trying to detect light production *in planta* had many inherent difficulties that needed to be overcome. However, the *lux* system does have one main advantage over *lacZ* and GUS in this context, namely that the system is very sensitive, about 1000 times more so than the *lacZ* system [76].

Lilley had previously found that the light production from *A. tumefaciens* LBA4301 (pVK257, pUCD1187) (section 3.2) induced with acetosyringone was easily detectable using X-ray film [187]. It was therefore decided to use X-ray film in the experiments carried out in this work. However, light is easily scattered by media and interfaces between media and so a method had to be developed that would allow the film to be as close to the plant wound as possible whilst maintaining it and the bacteria in a nutrient medium.

Lilley had conducted experiments to try to demonstrate *vir* induction at wound sites *in planta* using *lux* but without success. In those experiments, tobacco seedlings were used, grown in compost treated with *A. tumefaciens* LBA4301 (pVK257, pUCD1187) or a control strain. After 5 days the seedlings were removed carefully from the compost, sandwiched between 3MM paper and cling film and then exposed to X-ray film. However, the presence of darkening on the developed film corresponded to neither the root structures of the seedlings nor to the treatments the plants had received [187].

In a second experiment Lilley used seedlings placed in tubes in a similar experiment to those described in sections 3.3 and 3.4. The tubes were taped to X-ray film and after an overnight exposure, the film was developed. Tubes containing wounded seedlings in the presence of *A. tumefaciens* LBA4301 (pVK257, pUCD1187) had caused more darkening of the film than those containing unwounded seedlings subjected to the same treatment. However, no specific darkening, corresponding to individual wound sites, was observed and it also appeared that the plastic of the tubes had caused some darkening of the film [187].

Plastic tubes were avoided in this study, but as work progressed it became clear that other substances were also capable of darkening the film, including soil and vacuum grease.

Mung bean seedlings were germinated in 0.2 % Rogowsky Agar for 4 days at 28 °C in a light cabinet after which period wounds were inflicted as a single gash on some of the tap roots using a sterile scalpel. The seedlings were then transferred to scintillation vials containing compost moistened with cultures of either *A. tumefaciens* LBA4301 (pVK257, pUCD1187) or *A. tumefaciens* LBA4301 (pVK257) as a control. An attempt was made to position the wound sites on the bottom of the vials which were then stood on X-ray film and sealed in a light-proof box for 48 hours. Upon development of the film, dark patches were observed where all the vials had been placed, although these areas were noticeably darker for the vials that contained wounded plants in the presence of *A. tumefaciens* LBA4301 (pVK257, pUCD1187). However, these darkened areas could not be correlated with the wound sites and this may have been due to the ridged bases of the scintillation vials having diffused or deflected any light emanating from bacteria at the wound site. Since all the vials had produced darkening it was not clear, however, that *vir* induction was really occurring, the compost itself could have been responsible by phosphorescing.

The experiment was repeated using Bio-Rad Protean II xi acrylamide gel electrophoresis equipment. Compost was placed between the two 4 mm glass plates separated by 3 mm spacers, held and sealed in the forming block. 4 day old mung bean seedlings (wounded and unwounded) were placed in the top of the set up between the plates. The compost was then soaked with Rogowsky medium containing 1 x 10^7 cells.ml^{-1} *A. tumefaciens* LBA4301 (pVK257, pUCD1187). The positions of the visible wound sites were marked on the glass and X-ray film was taped to this. The set up was left in a light-proof box for 48 hours.

Upon development of the film, various darkened patches appeared which bore no resemblance to the seedlings' root structure or more specifically to the wound sites. It was concluded that the compost itself was responsible for this, either by phosphorescence or by supplying *vir* inducing compounds to the bacteria in some areas. Due to this, subsequent experiments were performed in agar which had the added advantage that it produced a clear medium through which light could travel without being deflected so easily, whilst completely surrounding the root.

Small scintillation vials were filled with 2 ml 0.2 % Rogowsky Agar containing 1 x 10^7 cells.ml^{-1} *A. tumefaciens* LBA4301 (pVK257, pUCD1187). One 4 day old mung bean seedling was placed in each vial and wounds were then made in some roots, where they were touching the bottom of the vial, using a sterile spike. The positions of the wounds were marked and the vials were stood on X-ray film in a light-proof box for 24 hours. The film was then developed and showed clear *vir* induced light production in tubes containing the wounded plants. This light production was not evident in the vials containing unwounded plants. Despite this, the darkened areas were circular, corresponding to the bottom of the vial rather than any specific induction points at the marked wound sites. This was probably due to the slight convex nature of the glass forming the bottom of the small vials. Vials were thus considered unsuitable for future experiments.

The Bio-Rad Protean II xi equipment was again used, as above, but replacing the compost with 0.2 % Rogowsky Agar in the experiment. After 48 hours in the light tight box, the developed film showed no darkening. This was almost certainly due to the fact that by this time much of the agar had dried up and the roots and any associated bacteria had become dry.

The experiment was repeated using the smaller Bio-Rad Mini Protean II apparatus, the plates again being held in the forming block separated by 3 mm spacers. This set up had the added advantage that the glass plates used were 1 mm

thick, as opposed to 4 mm, allowing less chance of light scattering by the glass. In addition the gap at the top of the plates, between the seedlings, was sealed with vacuum grease in an attempt to limit drying of the agar. 3 seedlings (2 wounded, 1 unwounded) were placed between the plates at the top of the apparatus, the wound site positions were marked and X-ray film was taped to the glass and left for 24 hours.

The developed film showed one clear root structure on one of the wounded roots, although no definite single wound site was apparent. This result was marred by the fact that the vacuum grease had either reacted with the film or had itself caused *vir* induction in the bacteria. This reaction obscured the position of the other wounded root. Cling film was used in place of vacuum grease in all subsequent experiments, although this was less effective.

The experiment was repeated using three seedlings. One had a wound made at the air/agar interface, another had a wound completely surrounded by the medium and the other seedling was not wounded. After 24 hours, the developed film showed *vir* induction at a clearly defined wound site (see figure 3.5.1) at the air/agar interface. Below the wound site, the rest of the root was clearly visible, due possibly to wound exudate running down the outside of the wound under gravity. The wound site surrounded by medium had also caused a very faint darkening of the film (see figure 3.5.1). This result confirmed that *vir* induction does take place at the wounded parts of plants. It also confirmed earlier suspicions that the anaerobic conditions of the tube assays performed using *lacZ* and GUS as reporters, were not conducive to *vir* induction.

To try to increase the light produced at the wound sites, an attempt was made to repeat the experiment using vermiculite soaked in the bacterial suspension instead of agar, in order to allow more air into the system and thus create a more soil-like environment. However, the vermiculite quickly dried up and no light production was recorded.

It has been suggested by de Weger *et al.* [76] that low induction values may be due to the high energy demand placed on the cells to synthesise the aldehyde substrate. A future approach might involve linking the *vir* promoters only to the genes encoding luciferase (*luxA* and *luxB*) and then supplying the aldehyde substrate exogenously [76]. This high energy demand is likely to have greater consequences for the experiments performed in soil, since nutrient supply is much greater when

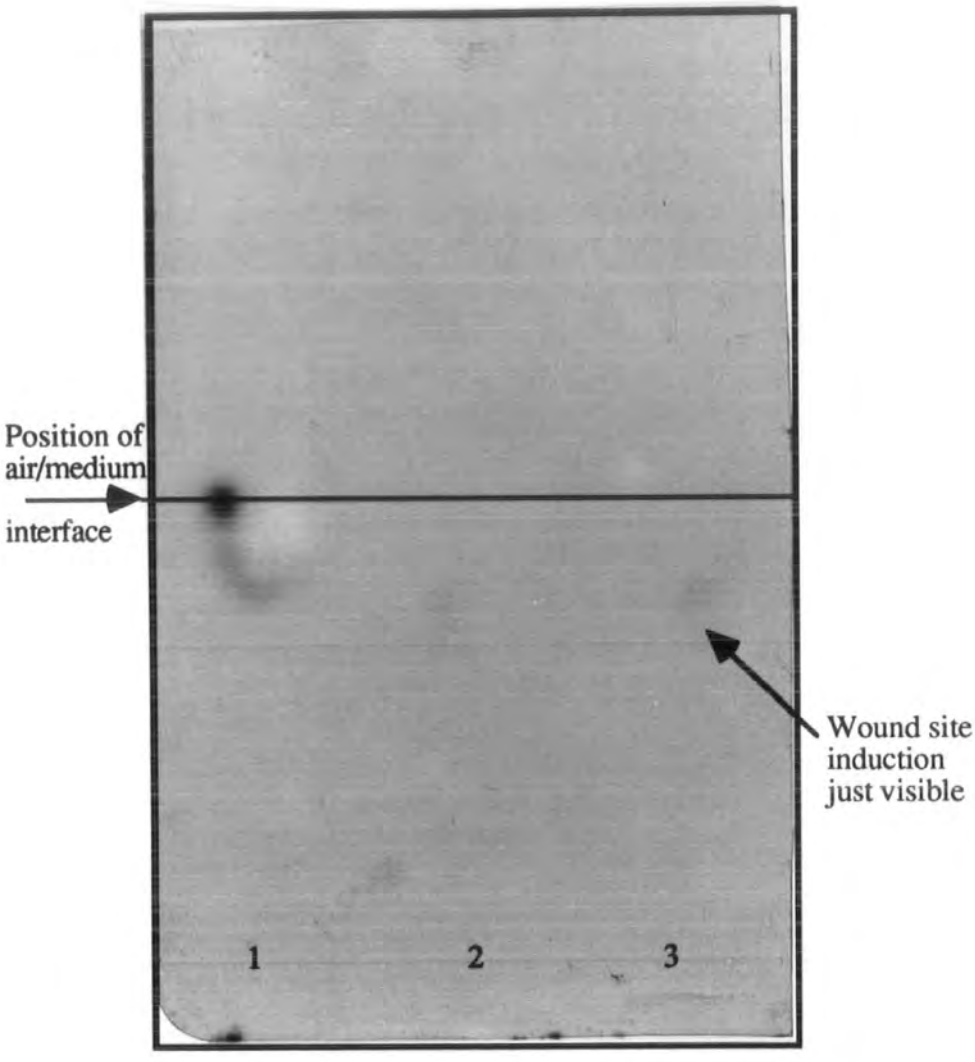


Figure 3.5.1 X-ray film, previously placed near three mung bean seedlings, showing darkening corresponding to light produced by *lux* genes under *virB* control. Numbers refer to positions of the plants: 1 - Seedling wounded on root at air/medium interface; 2 - Unwounded seedling; 3 - Seedling wounded lower on root. See text.

using artificial media. At some point, using only *luxA* and *luxB*, it may therefore be possible to demonstrate *in planta* wound induced *vir* induction occurring in soil.

These results suggested that a *vir* promoter-driven pesticidal gene could be expressed conservatively and specifically at a wound site, suggesting that the proposed 'microbial inoculant' system might be viable.

3.6 Plasmid stability

Much work has been carried out over the last decade to advance techniques to produce genetically engineered crops or crop protectants designed to allow the reduction of chemicals introduced into the environment. However, public mistrust and scientific concerns over the release of such organisms must be addressed.

The proposed outcome of this study is a genetically engineered microbe that will protect plants from fungal and insect attack. However, in order to qualify as a genuine alternative to existing agricultural practices, its use must be deemed to be safe and effective, and for this reason it was necessary to establish how stable the organism would be were it to be released [309].

It is known that differences in the composition of the soil into which any bacterial population is introduced have a profound impact on the survival of that species, the stability of any plasmids present, the rate of growth, motility and pathogenicity [34, 140, 186, 193, 232]. The viability of an *A. tumefaciens* 'microbial inoculant' as a commercial alternative to chemical pesticide application, will be greatly affected by these parameters in a range of different soils as will its ability to be applied and maintained in the soil in a controlled manner [309]. Some insight into the relative stabilities of various plasmids was required in order to judge how well the proposed microbial inoculant might perform in the field and to establish how successful experiments, involving fungal infected plants grown in soil containing the proposed microbial inoculants, might be.

In experiments conducted by Sundheim *et al.* [298], it was found that *Pseudomonas fluorescens* expressing a chitinase from *Serratia marcescens* in soil infected with *Gaeumannomyces graminis* had no effect on the fungus due to rapid loss of plasmids from the *Pseudomonas* strain. The vector used for construction of this plasmid, pLAFR3 [294], belongs to the IncP incompatibility group. This total loss of an IncP plasmid, in four weeks, from *Pseudomonas fluorescens* in soil, was not found to occur in *P. putida* carrying an IncP plasmid [193], nor in *A. tumefaciens*

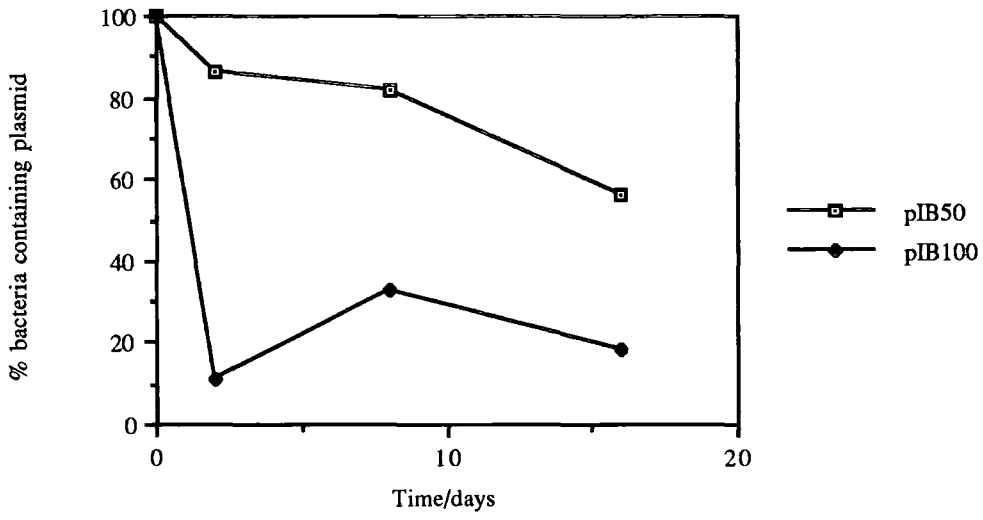


Figure 3.6.1 Graph showing relative rates of loss of two plasmids, pIB50 (IncP) and pIB100 (IncW) from an original strain of *A. tumefaciens* C58C1 containing both plasmids.

(results presented below). These results may suggest that the host strain carrying an IncP plasmid is very important in how stably these plasmids are maintained. Doubtless, there were also many differences between the soils used in each experiment and this will also have had an effect.

A preliminary study was performed using two plasmids, pIB50 and pIB100, which belonged to the incompatibility groups IncP and IncW respectively (see section 3.3). The genetic background used was *A. tumefaciens* C58C1 [314] which was resistant to rifampicin. This provided a useful marker for the strain since rifampicin has been shown to be a stable marker for related microorganisms in the field [103].

Since the results from the plasmid stability assay were to be determined by counting colonies on agar media with various selective properties, it was necessary to establish that none of the antibiotics used (kanamycin, carbenicillin, rifampicin) were either synergistic or antagonistic with one another in *A. tumefaciens*. There are many instances where different classes of antibiotic will interact with one another and therefore affect the growth of the bacteria in question [105, 181] making interpretation of the results difficult. Antibiotic synergism assays were performed (see section 2.5.13) but neither synergism nor antagonism between any of the antibiotics at any concentration was observed.

A preliminary plasmid stability assay was set up as described in section 2.5.12. The compost was originally moistened with a culture of *A. tumefaciens* C58C1 (pIB50, pIB100) at a concentration of approximately 3 x 10^5 cells.ml^{-1}. Sampling was done at 2, 8 and 16 days after this and dilutions were plated out on Rif plates. A proportion of these colonies were then taken and streaked out onto selective agar media, from which the percentage loss of plasmids from the parental strain was calculated (see figure 3.6.1). A certain number of colonies were also chosen at random from the original dilution plates and streaked out onto lactose plates and tested to confirm that they were *Agrobacterium* (section 2.5.10). All selected colonies proved positive.

From the data shown in figure 3.6.1, although only preliminary, it can be seen that the IncP plasmid is far more stable than the IncW plasmid in *A. tumefaciens* C58C1 in this compost. In experiments performed by de Weger *et al.* [76], 80 - 90 % of *Pseudomonas fluorescens* cells had lost an IncW plasmid after 6 days in soil. This is in broad agreement with the results presented here although it may be that *P. fluorescens* will not stably maintain either IncW or IncP plasmids in soil [76, 298].

Given that the IncW plasmid appears to be rather unstable in *A. tumefaciens* C58C1 in compost, this type of plasmid may have to be avoided when developing plasmids for use in a 'microbial inoculant' involving *A. tumefaciens*. IncW plasmids have also been implicated in interference with tumorigenicity in some *Agrobacterium* strains [88].

However, much work remains to be done on the stability of plasmids in soil in *Agrobacterium* species and with this in mind a much larger and more detailed experiment, based on the one above, is now planned using pVK257 (IncP) [175], pECA2 (IncW) [187] and pLEVB2114 (IncQ) (see section 5.3.2), individually and in combination with one another. These experiments will only measure the relative stabilities of these three plasmids in one type of compost but further experiments could be undertaken altering the particle size of the soil, the pH, nutrient and moisture contents, and adding plants and competing microorganisms.

3.7 Monitoring of *vir* induction in *A. tumefaciens*

In the following chapters, the development of an *A. tumefaciens* 'microbial inoculant' capable of conservative application of pesticidal proteins will be described.

A method for the monitoring of *vir* induction was developed (section 3.2) in order to determine a set of conditions that could be used to produce high levels of *vir* induction. This involved using Davis medium and measuring induction after 24 hours (see figure 3.2.1). However, using the *lux* system with the apparatus available only the *virB* promoter controlled genes appeared to produce consistent high level induction responses to the addition of acetosyringone. Further investigations into why the *virE* controlled system was not producing the expected results were not done, due to the limitations of time and because such an investigation would have been largely pointless since *virE* controlled genes had already been used to produce high levels of induction [42, 253, 290, 318]. Thus, for adequate comparisons to be made, *virE* fusion constructs were monitored in the same way.

It was assumed that the erratic results obtained using the *virE* constructs were probably due to the light measurement procedures used (resulting in temperature changes every hour) due to the less stable interaction with VirG. As will be seen (Chapter 4), this assumption appears to be correct, with undisturbed cultures of bacteria, containing *virE* promoter::gene fusions, showing high levels of *vir* induction.

Despite the successes of many other workers in using *lacZ* as a reporter gene for *vir* induction [4, 42, 288, 290, 318], this system did not prove useful in detecting *in planta vir* induction, due to the high levels of β-galactosidase (or a functional analogue) produced by the plants used.

The *vir* promoter::GUS fusions were not successful in demonstrating *vir* induction *in situ* either, although both the *virB* and *virE constructs* had previously been shown to be inducible [97, 187]. However, the *virE* construct produced a high background level of β-glucuronidase and despite a more encouraging plate assay, the *virB* construct was not sensitive enough to overcome the problems of an anaerobic environment.

The *vir*::*lux* constructs proved more successful in the detection of *vir* induction *in planta*, despite the more unwieldy methods that needed to be employed. This was probably due to the greater sensitivity of the system as opposed to *lacZ* and GUS. It may be possible to show an even greater induction response at wound sites by reducing the energy demand on the cell by using only the genes encoding luciferase and supplying the aldehyde substrate exogenously. This approach may even allow *vir* induction to be shown to be occurring at wound sites in soil.

3.8 Feasibility of an *A. tumefaciens* 'microbial inoculant'

This work has suggested that *vir* induction will occur specifically at the wound sites of mung bean seedlings and it is inferred that this will also happen at wound sites inflicted upon most other dicotyledonous and hopefully monocotyledonous plants.

The amount of induction also appears to be affected by the availability of air and it is therefore assumed that different soil types will allow differing levels of *vir* induction to occur. The soil will affect the growth of the 'microbial inoculant', its motility [34, 41, 284] and also the stability of the plasmids introduced into the bacteria (section 3.6).

It would therefore seem that to achieve a 'microbial inoculant' system that will work in most commonly found agricultural environments is rather ambitious. However, once a functioning *A. tumefaciens* 'microbial inoculant' has been shown to protect a crop in one set of conditions, the plasmids from that strain are easily transferable in the laboratory, to other *A. tumefaciens* strains that show different characteristics in other soil environments. This might allow a range of strains to be

produced, which, applied together in the field, would allow the different strains each to colonise their own particular niche in the field to optimum effect.

Whatever the outcome of these environmental problems, this work has suggested that *vir* induction does occur at wound sites and previous work has shown that *A. tumefaciens* strains carrying *virA* and *virG* will move towards a wound site [9, 11, 12]. If stable plasmids (most likely to be IncP) are used in genetically engineered *A. tumefaciens* strains, then this suggests that a 'microbial inoculant' system, based on *A. tumefaciens*, is feasible, certainly for some soil types. It is probable that this system would be used as part of a holistic approach to the biocontrol of pests and so guaranteed effectiveness in all soil types may not be required.

Chapter 4

USE OF CHITINASE FOR BIOCONTROL

4.1 The chitinolytic enzymes of *Serratia marcescens*

Serratia marcescens is a Gram-negative bacterium, found commonly in soil, belonging to the family Enterobacteriaceae [39]. The bacterium produces a high level of secreted chitinase activity [209, 246] which has been shown to be induced by the presence of chitin, laminarin and some fungal cell walls [209, 229].

This activity has been attributed to the presence of five chitinases with molecular weights of 21, 36, 48, 52 and 57 kD [93], the most abundant of which is the 57 kD protein, designated ChiA, the gene for which has been cloned by a number of groups [93, 157, 269, 297]. The 52 kD chitinase gene has also been cloned and designated *chiB* [157]. In addition, a chitobiase gene has been cloned from *S. marcescens* [174]. The degradation of chitin by the bacterium is thought to occur via a two-step process, with the substrate being degraded by chitinases to oligomers (mainly dimeric chitobiose) which are subsequently degraded by other enzymes including chitobiase [212].

4.2 Use of chitinases as biocontrol agents

Chitin, an insoluble linear polymer consisting of β-1,4-linked N-acetylglucosamine units, is a major structural element in many organisms, including fungi, insects and higher plants [92, 209, 212]. The widespread occurrence of chitin in pests makes the use of its degradative enzyme, as a biocontrol agent, an attractive proposition [53, 143, 229]. The growth of some fungi has been shown to be retarded by a variety of chitinases [53, 143, 157, 229, 247, 248, 264, 269, 297, 298]. ChiB has also been shown to be active against the larvae of the coleopteran insect *Diabrotica balteata* (the banded cucumber beetle) [187].

Ordentlich *et al.* examined 203 different bacteria from the rhizosphere of plants infested with *Sclerotium rolfsii* and found that *S. marcescens* was the most

effective of these in controlling the disease caused by the fungus [228]. It was subsequently shown that this biocontrol was mainly due to the activity of secreted chitinases from the bacterium, which, as well as degrading chitin, may cause the release of β-glucanase from the fungus [229]. Chitin and β-1,4-glucans form a large part of many fungal cell walls and therefore the presence of both these degradative enzymes is likely to enhance the destruction of the fungal hyphae. Certainly, the action of both these enzymes appeared to be responsible for the destruction of the fungal hyphae of *Sclerotium rolfsii* [229].

Serratia marcescens has also been shown to be effective in controlling *Thanatephorus cucumeris* (formerly *Rhizoctonia solani*), *Magnaporthe grisea* and *Fusarium oxysporum* [53, 298] and a partially purified preparation of *S. marcescens* ChiA, expressed in *E. coli*, has been shown to reduce the incidence of disease caused by both *Sclerotium rolfsii* and *Thanatephorus cucumeris* in cotton plants, when applied to the soil [53, 269].

In the light of these successful results, attempts have been made to clone chitinase genes from *S. marcescens* into other bacteria that are known to colonise plant roots. It is hoped that these bacterial biocontrol agents will therefore become associated with the roots and in so doing, protect the plants from fungal attack.

chiA, from *S. marcescens*, has been expressed in the plant-associated bacteria *Rhizobium meliloti* and *Pseudomonas fluorescens*; both the resulting bacterial strains being seen to be active against *Thanatephorus cucumeris* [279, 298]. In addition, the *Pseudomonas fluorescens* strain expressing *chiA* was shown to inhibit the growth of *Magnaporthe grisea* and *Fusarium oxysporum* [298].

Many chitinases are also found in plants where they are generally considered to be part of a response mounted against invading organisms (pathogenesis-related proteins), although some may also be involved in signal molecule production regulating plant development [65, 92]. Barley, bean, oat, pea, potato, tobacco, tomato and many other common plants are all known to produce chitinases [65, 92] many of which have been shown to have antifungal properties [30, 198, 247, 248, 264].

However, when Neuhaus *et al.* overexpressed a chitinase in *Nicotiana sylvestris* the resulting plant did not possess greater resistance to fungal attack by *Cercospora nicotiana* [221]. In this case, chitinase did not appear to be the limiting factor in the resistance of the plants. Mauch *et al.* found that in pea, the chitinase

produced had a synergistic activity with β-1,3-glucanase [198]. Many plants have now been shown to coexpress chitinases and β-1,3-glucanase in response to pathogen attack and it is likely that true plant resistance to some pests requires the action of both these enzymes [65, 92].

Although it may be that, in the experiments performed by Neuhaus *et al.*, the β-1,3-glucanase was the limiting factor [221], it could also be argued that the chitinase was located intracellularly in these plants and thus, despite obvious over-expression of the gene, the fungus did not come into contact with the enzyme [65]. Alternatively, it could also be argued that the fungus was resistant to that particular chitinase which is why it is a major pathogen of tobacco [65, 143, 221].

Jach *et al.* supported the latter argument and suggested that to produce a resistant tobacco plant, a chitinase from an unrelated species should be employed [143]. The *chiA* gene from *S. marcescens* was successfully expressed in tobacco, by this group, conferring resistance upon the plant to *Thanatephorus cucumeris* [143]. Similar resistant transgenic tobacco plants were produced by Broglie *et al.*, using a bean chitinase gene [37]. However, it must be mentioned that ChiA was also found to be secreted by the transgenic plants, which may partly account for the success of this approach over that of Neuhaus *et al.* [143, 192, 221].

It would therefore appear that for transgenic plants, a chitinase from an unrelated species is desirable to confer resistance to fungal pathogens. It may also be an advantage to be able to coexpress β-1,3-glucanase in these plants. However, the overexpression of two foreign genes in a plant is difficult to achieve.

Chitinases themselves appear to be good candidates for effective biocontrol systems, providing protection from many fungal pathogens. Results from Lilley [187] suggest that they may also be useful in controlling some insect pests. However, it is apparent that synergism between chitinases and β-1,3-glucanase (and possibly other plant pathogenesis-related proteins) does occur and for more effective biocontrol these may need to be expressed in combination with one another [65, 92, 198].

Bacteria, being simpler to transform, may prove to be a more viable alternative to transgenic plants, since more than one protein can be overexpressed at one time reasonably easily. However, new developments in plant biotechnology cannot be overlooked and it is more likely that any 'microbial inoculant' system will be used in conjunction with transgenic plants and possibly small amounts of chemicals.

In this chapter, the continued development of a bacterial biocontrol system involving chitinases expressed under the control of *A. tumefaciens vir* promoters, will be shown and discussed. It is our belief that such a system might eventually be used effectively against fungal, and possibly insect pathogens, in a manner that will be less deleterious to the environment than current methods involving chemical pesticides.

4.3 Cloned *S. marcescens* chitinase genes expressed in *E. coli*

4.3.1 Expression of chitinase in *E. coli*

The synthesis of both chitinases and chitobiase in *S. marcescens* is induced by the presence of chitin or related compounds [209, 229]. However, although induction occurs, the presence of chitin is not entirely required for chitinase production in *S. marcescens* QMB1466 [187]. This was confirmed in this study where chitinase activity in 5 ml cultures of *S. marcescens* grown for 48 hours at 28 °C was detected using the p-nitrophenyl-β-D-N,N'-diacetylchitobiose assay (section 2.5.6.a). Typically, cultures grown in the presence of chitin (5 $mg.ml^{-1}$ crab shell chitin - Sigma) contained twice as much chitinase activity as cultures grown in the absence of chitin, although activity in the latter case was still readily detectable. More discernible differences between the induced and uninduced cultures would probably have been noticed after longer periods of time, up to about 8 days [246].

When the structural genes for these enzymes have been expressed in *E. coli*, induction by chitin has not been observed [93, 174, 187]. The results of Fuchs *et al.* suggested that sequences some distance from the *chiA* gene were active in *E. coli* in repressing the expression of the gene [93]. Kless *et al.* suggested that the chitobiase gene is controlled by a repressor which was absent in their clone, leading to constitutive expression of the gene in *E. coli* [174].

Joshi *et al.* showed that control of chitinase production in *Serratia liquefaciens* is mediated by two other genes [159]. In this case, *chiD* produces a repressor protein that prevents expression from the *chiA*, *chiB* and *chiC* structural genes. However, in the presence of chitin, *chiE* is activated which produces an inducer which blocks the activity of the repressor [159]. These regulatory genes were also shown to function in *E. coli*. A similar regulation of the chitinase and chitobiase structural genes may well occur in *Serratia marcescens*.

The plasmids pCHIT1251 and pCHIT310 contain the genes *chiA* and *chiB* respectively, cloned into pUC8 [157]. These were both available in the laboratory. Given that the lengths of the inserts were 2.52 kb (*chiA*) and 3.28 kb (*chiB*) [157] and that only inserts of over 23.5 kb showed reduced chitinase activities in the case of *chiA* [93], it was unlikely that either of these plasmids contained the *S. marcescens* regulatory genes for the chitinase genes. Lilley found that indeed chitinase was constitutively expressed by both these plasmids in *E. coli* JM83 [187] and work done in this study confirmed that the same is true using *E. coli* DH5α.

4.3.2 Protein secretion in *E. coli*

In the following discussion, the term 'export' will be used to describe the translocation of proteins from the cytoplasm to the periplasm, whereas the term 'secretion' will be used to describe the translocation of proteins out of the cell and into the extracellular medium [240].

If chitinase is to be used as a biocontrol agent, more effective control of a pest will be achieved if the enzyme can be secreted into the surrounding medium rather than remaining within the bacterium. It was therefore necessary to determine whether *S. marcescens* chitinases can be secreted in *E. coli* expressing these genes as they are in *S. marcescens* [209]. However, the precise nature of extracellular release of chitinase in *S. marcescens* is not known at present [240]. What is evident is that both ChiA and ChiB possess signal peptides (see below) [123, 157] which must be involved, in some way, in the secretion of the enzymes. For this reason I shall confine the following brief description of secretion in Gram-negative bacteria, to the pathways used by proteins that possess a signal peptide.

Gram-negative bacteria possess two hydrophobic barriers, the inner and outer membranes, through which any secreted protein must pass. This usually involves other protein factors [240]. The secreted protein must first cross the cytoplasmic membrane to which it is directed by virtue of having an amino terminal 'signal peptide'. Signal peptides are composed of one or two positively charged amino acids in a short hydrophilic region (the N-domain), followed by a longer stretch of about 20 mainly hydrophobic amino acids (H-domain). This is followed by a short C-domain which contains a signal that is recognised by signal peptidase [240], allowing the 'signal peptide' to be cleaved from the rest of the protein.

In *E. coli* the Sec proteins are involved in the translocation of secretory proteins across the cytoplasmic membrane. A polypeptide that contains a signal peptide is unable to fold properly in the cytosol and therefore becomes bound with SecB [240]. It is thought that this guides the polypeptide to the inner membrane and, in association with SecA, aids the translocation of the polypeptide through a translocase apparatus composed of other Sec proteins inserted into the inner membrane [240]. Once in the periplasm, signal peptidase cleaves off the signal peptide and the protein is folded by other protein factors.

Many proteins that are released extracellularly in the host bacteria, are not secreted when the respective genes are expressed in *E. coli* [240]. Of those that possess a signal peptide, it would appear that although the signal peptide allows translocation through the inner membrane, the factors required for secretion are not present in *E. coli*, with the result that the protein becomes localised within the periplasm.

Secretion can sometimes occur if one other gene is present, linked to the structural gene, the product of which will allow translocation across the outer membrane, as is the case with the *S. marcescens* hemolysin [263]. It is apparent that secretion of this type requires a specific factor, apart from the secreted protein, and no such genes have been identified that are responsible for the secretion of chitinases in *S. marcescens*.

It is thought that the mechanisms by which proteins are translocated across the outer membrane are extremely selective and many secreted proteins have specific receptors and chaperones that allow them to be translocated [240]. One system that has been found to be common in Gram-negative bacteria is called the 'main terminal branch of the General Secretory Pathway'. Transport across the outer membrane using this pathway is highly selective and it is therefore proposed that proteins translocated in this manner possess a 'secretion signal'. However, no such signals have been identified and it may well be that only once the protein has been properly folded in the periplasm, are certain amino acids brought together, thus forming a 'signal patch' [240].

The chitobiase from *S. marcescens* appears to have a signal peptide and thus it has been suggested that the mature enzyme is located in the periplasm in *E. coli*, factors required for its secretion presumably being absent, since in *S. marcescens* it is thought to be secreted [174] (however, see section 4.4). A protease from *S. marcescens* also has a signal peptide, but in this case the proenzyme contains an

additional 637 amino acids at its C-terminus which are responsible for 'autosecretion' in which the C-terminal part of the proenzyme is inserted into the outer membrane which allows the N-terminal (that forms the mature enzyme) to be translocated through the membrane. The mature protein is then cleaved off, leaving it in the extracellular milieu [240, 342].

4.3.3 Secretion of chitinases in *E. coli*

The latter scenario is not likely in the case of ChiA and ChiB since processing of the enzymes only seems to occur at the N-terminal end to remove the signal peptide [123, 157]. If there are no extra factors present to enable chitinase secretion in *E. coli* and no evidence to support autosecretion, then it would appear that, like chitobiase (however, see section 4.4), the chitinases should be located in the periplasm, since very few proteins are secreted by *E. coli* [18, 174].

However, when isolating chitinase producing clones from a *S. marcescens* library, most researchers have used a plate-clearing assay in which colonies have been selected on the basis of their ability to degrade chitin extracellularly [93, 157, 298]. This has been used as evidence that chitinases are secreted by *E. coli* [9]. In addition to this, many workers have found ChiA and ChiB to be present in the culture medium, of *E. coli* expressing one or other of these genes [9, 53, 157, 187, 269]. It would therefore appear that these chitinases are, in some unknown way, secreted into the extracellular milieu by *E. coli*. Two groups have also demonstrated that *E. coli* cells expressing ChiA could themselves inhibit growth of some fungi [53, 157, 269]. However, none of the reports had examined marker enzymes known to be located in various parts of the *E. coli* cell (particularly the periplasm) in order to determine whether secretion was actually occurring or whether the supernatant-detection of chitinase activity was simply due to cell lysis in the stationary phase. It could also be that the presence of chitinase in the periplasm causes the cells to become more fragile and thus lyse, as was discovered to be the case when *S. marcescens* chitobiase was expressed in *E. coli* [174].

Experiments were carried out to examine the secretion of ChiA and ChiB in *E. coli*. The plasmids used for this study were constructed by Ashby [9]. pCHIT1251 and pCHIT310, containing *chiA* and *chiB* respectively, [157] were restricted with EcoRI and ligated with EcoRI-cut pGV1106 [185], a broad host range vector, forming pDUB2501 (*chiA*) and pDUB2502 (*chiB*) [9]. These double-replicon constructs allowed large copy numbers to be obtained in *E. coli* and replication in *A. tumefaciens* due to the presence of the *oriSa* origin of replication from pGV1106 [9].

The plasmids were transformed into *E. coli*DH5α (see section 2.5.2.a) and transformants were selected on plates containing ampicillin and kanamycin.

Single colonies of *E. coli*DH5α, DH5α(pDUB2501), DH5α(pDUB2502) and *S. marcescens*QMB1466 were inoculated into 5 ml LM-broth (containing 5 $mg.ml^{-1}$ chitin in the case of *S. marcescens*). The cultures were shaken for 12 hours at 37 °C (28 °C for *S. marcescens*) and then 1.5 ml aliquots were fractionated into the various cell compartments (see section 2.5.4.a). Each fraction (extracellular, wash, periplasmic and cytoplasmic) was then assayed for alkaline phosphatase activity, an enzyme commonly located in the periplasm of *E. coli* (see section 2.5.6.c) [220]. Protein concentration estimations were also performed (see sections 2.5.5.f) and the fractions were also assayed for chitinase activity (see section 2.5.6.b).

The OD readings (demonstrating relative amounts of the enzymes) from the two enzyme assays are displayed in figures 4.3.3.a and 4.3.3.b. From figure 4.3.3.a, it can clearly be seen that the majority of alkaline phosphatase is found in the periplasm in all cases except DH5α(pDUB2502). High alkaline phosphatase activities are seen in the cytoplasm due to the inefficient nature of the osmotic shock fractionation procedure. The release could be made more efficient by the addition of lysozyme, but this was found to be able to cleave CM-chitin-RBV. Further assays all confirmed these results, including the lack of apparent alkaline phosphatase activity in DH5α(pDUB2502). In this case ChiB appeared to be inhibiting the action of the enzyme, in some way, or to be blocking its translocation into the periplasm (the enzyme requires this translocation in order to become activated) [163] (see section 4.3.4).

To try to increase the amount of alkaline phosphatase produced by the strains (especially DH5α(pDUB2502)) trials were undertaken using minimal media (M9 - [207] and the low phosphate medium of Neu and Heppel [220]). Neither medium allowed production of alkaline phosphatase in DH5α, which was somewhat surprising, since the low phosphate medium was designed to enhance the production of this enzyme [220]. Whether this was simply an aspect of the molecular behaviour of DH5α was not determined. However, more importantly, DH5α(pDUB2502) showed no growth in these media at 37 °C.

In an attempt to find a marker for the periplasm that would be produced in DH5α(pDUB2502), β-lactamase activity was assayed, which should be located in the periplasm [29]. The gene that encodes this was present on both pDUB2501 and pDUB2502. The cloning vector, pUC19 [319], also contains this gene. Cultures of

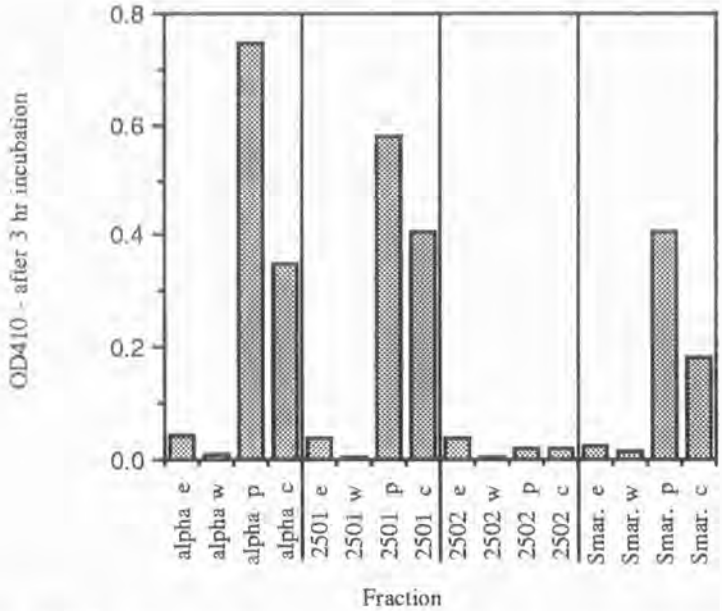


Figure 4.3.3.a Graph showing alkaline phosphatase activity of various fractions of four strains of bacteria: *E. coli*DH5α, DH5α(pDUB2501), DH5α(pDUB2502) and *S. marcescens* QMB1466. The activities are expressed as the optical density at 410 nm (the wavelength used in the assay) after 3 hours incubation at 37 °C. e = extracellular, w = wash, p = periplasmic, c = cytoplasmic.

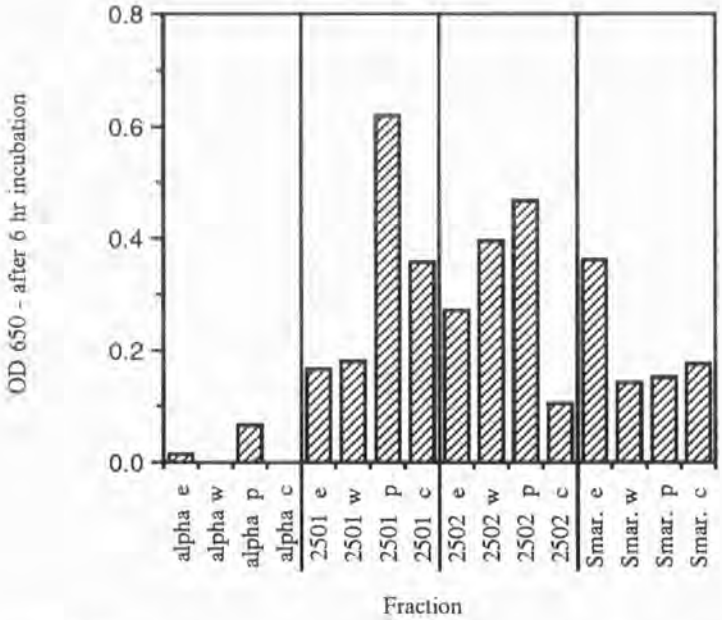


Figure 4.3.3.b Graph showing chitinase activity of various cell fractions of four strains of bacteria: *E. coli* DH5α, DH5α(pDUB2501), DH5α(pDUB2502), *S. marcescens* QMB1466. Chitinase activity is expressed as the optical density measured at 650 nm (the wavelength used for the assay) after 6 hours incubation at 50 °C. e = extracellular, w = wash, p = periplasmic, c = cytoplasmic.

DH5α(pDUB2501), DH5α(pDUB2502) and DH5α(pUC19) were grown and fractionated as described above. β-lactamase activities in the cell fractions were measured using the method described in section 2.5.6.d. However, this enzyme did not appear to be a useful marker since significant activity was found in all the fractions. This may have been due to the sensitivity of the assay method since it is likely that other β-lactamases are produced by *E. coli* [219].

Alternatively, overexpression of this gene in these strains may have led to some excretion of the protein. The fact that DH5α(pUC19) also had this activity in all cell fractions indicated that the presence of β-lactamase in the extracellular medium in DH5α(pDUB2501) and DH5α(pDUB2502) was not caused by the actions of ChiA and ChiB. DH5α also exhibited significant amounts of 'β-lactamase' activity, especially in the extracellular fraction.

Despite the drawbacks of the initial experiment, meaningful results were obtained. For DH5α(pDUB2501) 4 % of the alkaline phosphatase activity was located in the extracellular medium and the wash fractions. In the same fractions 26 % of the chitinase activity was found. For *S. marcescens* 6 % of the alkaline phosphatase was found in the extracellular and wash fractions, which also contained 61 % of the chitinase activity. On this basis, it is probable that ChiA is secreted in *E. coli,* but not as efficiently as it is in *S. marcescens*. By comparison of the chitinase activities of the different cell fractions of DH5α(pDUB2501) and DH5α(pDUB2502), it would seem that this is also true of ChiB (see figure 4.3.3.b). 52 % of the ChiB produced by this strain was found in the extracellular and wash fractions.

The fact that much of the chitinase activity appears to be present in the wash fractions of DH5α(pDUB2501) and DH5α(pDUB2502), indicates that after secretion a proportion of the enzyme remains loosely attached, in some way, to the outer membrane of the bacterium.

40 μl of each of the fractions, as prepared above, from DH5α, DH5α(pDUB2501) and DH5α(pDUB2502) were electrophoresed on a large protein gel (see section 2.5.5.a), shown in figure 4.3.3.c. What is clear is that cell breakdown did not occur to any appreciable extent, each fraction type remaining distinct from the others. ChiA and ChiB can be seen clearly in the periplasmic fractions at 58 kD and 52 kD respectively. What is also apparent is that the periplasmic fraction of DH5α(pDUB2502) bears little resemblance to the corresponding periplasmic fractions of the other bacteria. Therefore, ChiB had a severe affect on the molecular

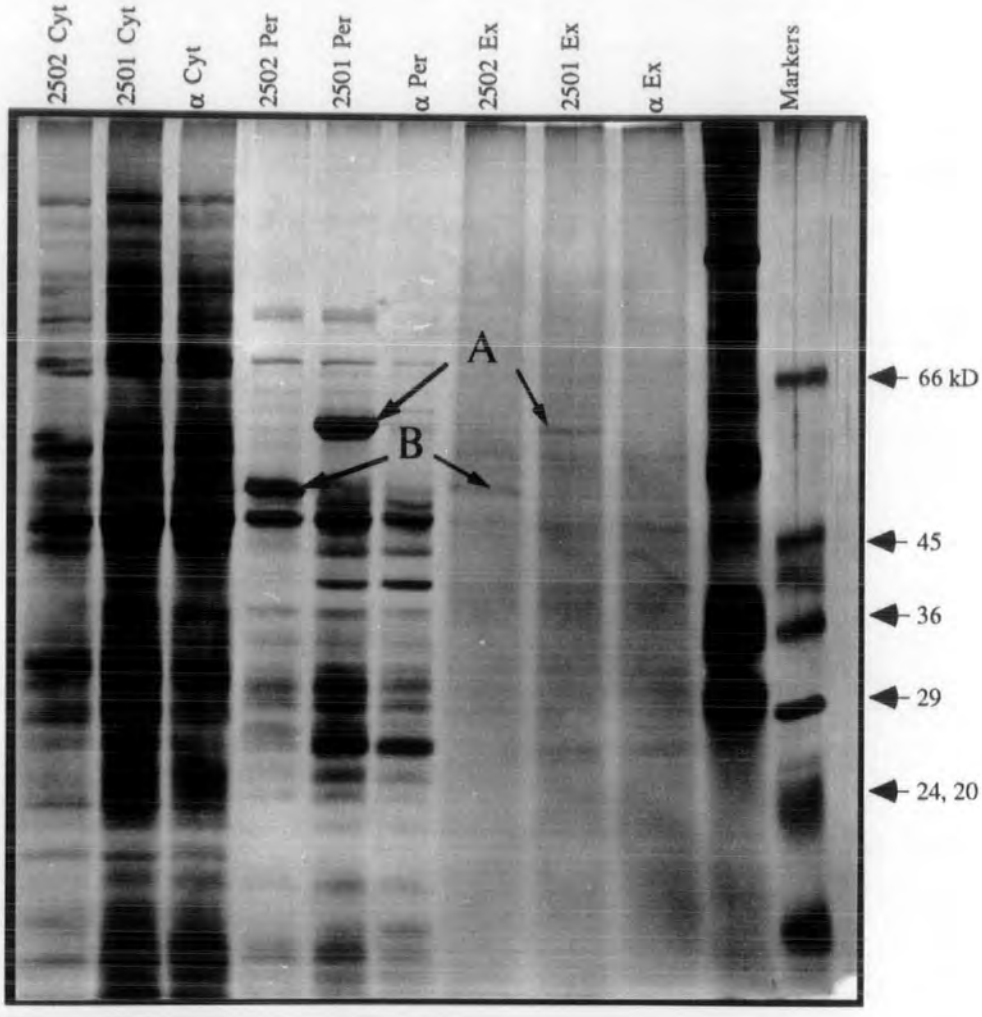


Figure 4.3.3.c Silver-stained SDS/PAGE gel loaded with 40 μl samples of various cell fractions of DH5α, DH5α(pDUB2501) and DH5α(pDUB2502). Bands corresponding to ChiA and ChiB are marked. The gel also shows clear differences between the periplasmic fractions of DH5α(pDUB2502) and the other two strains, which includes many missing proteins. Cyt = cytoplasm, Per = periplasm, Ex = extracellular medium. See text.

behaviour of DH5α resulting in a lack of active alkaline phosphatase and slow growth. Further investigations were therefore carried out on DH5α(pDUB2502) to try to elucidate the nature of the effect of ChiB (see section 4.3.4).

Quite how the secretion of ChiA and ChiB occurs in *E. coli* remains unclear. 'Autosecretion' does not appear to be an explanation, nor does the presence of other *S. marcescens* transcripts encoding factors that will allow secretion. Another method of secretion is the use of a pore that traverses both membranes [240]. The presence of large amounts of chitinase in the periplasmic fractions discounts this situation occurring in *chi*-expressing *E. coli* strains. One suggestion is that it might use the secretion pathway of another secreted protein, the chitinases mimicking a 'signal patch' allowing this to occur [18]. Ball *et al.* have suggested that the *S. marcescens* nuclease mimics an outer membrane protein which is directed to the outer membrane, becomes attached but fails to anchor in the membrane and can thus be lost from it to the extracellular medium [18]. Given that, in the case of DH5α(pDUB2501), 26 % of the chitinase appears to be secreted and in the case of DH5α(pDUB2502) 52 % of the chitinase appears to be secreted, it seems that this latter scenario is possible. If the chitinases were to mimic outer membrane proteins and be inserted into the outer membrane and then be displaced from it again, the displacement kinetics would suggest that at least half (and more likely, more than half) of the chitinase would remain in the periplasm, which is indeed the case. A more directional process would reverse this trend.

A further suggestion as to how this process might operate has been outlined by Jones *et al.* [156]. This group suggested that the incorporation of the *Salmonella typhimurium* flagella protein FlgH into the outer membrane was due to the high proportion of β-sheet secondary structure predicted for the protein using the algorithm of Chou and Fasman [57]. This amounted to 26 % of the protein and this compares well with ChiA and ChiB that have similarly high amounts of β-sheet using the same algorithm - 24 % and 33 % respectively. If the amount of β-sheet structure did have an effect on the secretion of the proteins through the outer membrane, it might be expected that the protein with more β-sheet would be secreted more efficiently (as suggested by Jones *et al.* [156]). This would appear to be the case, with 52 % of ChiB being secreted as compared with 26 % for ChiA. The same scenario might also be true for the *S. marcescens* nuclease which contains 28 % β-sheet.

In conclusion, it is apparent that *chiA* and *chiB* are efficiently expressed from their natural *S. marcescens* promoters, present on both the plasmids used, in *E coli*. On the basis of the experiments performed so far, it would appear that *chiA* is expressed slightly better than *chiB* (based on the activities of the enzymes produced as a function of the total protein present in the extracellular, wash, periplasmic and cytoplasmic fractions), although this may not actually be the case (see section 4.3.4). Both chitinases appear to be secreted in *E. coli* although the mechanism by which this occurs is unknown. ChiA appears to have no detrimental effect on the bacterial cells, whereas ChiB seems to inhibit the action of alkaline phosphatase and causes the bacteria to grow more slowly.

4.3.4 Effects of *chiB* expressed in *E. coli*

In the initial work carried out on *chiB*, Lilley described colonies of *E. coli* expressing the gene as 'white' in appearance rather than beige [187]. This observation was not followed up by any further investigations as to the causes of the altered bacterial appearance.

During the current work, it was noticed that DH5α(pDUB2502) was a slow growing strain when cultured in LM-broth at 37°C. Typically, after 12 hours cultures of DH5α and DH5α(pDUB2501) had OD_{600} values of between 2.1 and 2.3, whilst those of DH5α(pDUB2502) had values of only 0.7 - 0.8, as measured spectrophotometrically (see section 2.4.3). The *chiB*-expressing strain also showed no growth in either M9 minimal medium [207] or in low phosphate medium [220] at 37 °C. Moreover, DH5α(pDUB2502) cells were not able to pass efficiently through the 30 μm aperture of the Coulter Multisizer (see section 2.4.3). This, coupled with the previous observation that DH5α(pDUB2502) did not appear to possess active alkaline phosphatase, led to the conclusion that *chiB* had an adverse effect when expressed in *E. coli*.

Upon microscopic examination, it was discovered that these cells were filamentous in appearance. Closer examination using an electron microscope (see section 2.5.14) revealed the presence of darkly-staining, irregular hexagonal structures at asymmetric intervals along aseptate filaments (see figure 4.3.4.a).

ChiB was exerting an effect on the cell division of *E. coli*, but whether this was a direct or an indirect effect was unclear. DH5α(pDUB2502) cells were cultured in 10 ml LM-broth for 12 hours at 37 °C and the cells were removed by centrifugation. ChiB and any other possible 'growth factors' secreted by the cells

Scale : 10 μm

Figure 4.3.4.a Electron micrograph showing filamentous phenotype of DH5α(pDUB2502) photographed at a magnification of 2 800 X. Clear dark staining structures at asymmetric intervals along the filaments, which appear to be aseptate, can be seen. Some of these structures have a hexagonal geometry to them.

were now present in the culture medium. This was then divided into three and inoculated with single colonies of DH5α, DH5α(pDUB2501) and DH5α(pDUB2502). After a further 12 hours at 37 °C, the cells from each culture were examined microscopically. Neither the DH5α nor the DH5α(pDUB2501) strains had developed a filamentous phenotype although the DH5α(pDUB2502) strain was filamentous. This suggested that ChiB was not having a direct extracellular effect on the cells and nor was any 'growth factor', that may have been expressed in response to the presence of intracellular ChiB.

The blockage of an export pathway from cytosol to periplasm has been shown to result in the accumulation of protein precursors in the cytosol, which in turn elicits a heat shock response [329]. *E. coli* cells that contain a mutation in one of the 'heat shock genes' are known to form long filaments when undergoing a heat shock response [216]. It was therefore possible that ChiB was exerting a deleterious effect on the product of a heat shock gene whilst also eliciting a heat shock response by blocking an export pathway.

Single colonies of DH5α and DH5α(pDUB2502) were inoculated into LM-broth and cultured at 37 °C for 24 hours. The resulting bacterial suspensions were fractionated into various cellular compartments (see section 2.5.4.a) and 40 μl of each fraction was boiled for 2 minutes in an equal volume of standard SDS/PAGE buffer and electrophoresed through a SDS/PAGE gel (see section 2.5.5.a). Figure 4.3.4.b shows the silver stained gel (see section 2.5.5.b) clearly showing large amounts of ChiB present in the membrane fraction as compared with the other fractions. It seemed possible therefore that the jamming of the export machinery due to over expression of *chiB* led to a heat shock response and also to the lack of export of some proteins (including alkaline phosphatase) to the periplasm. Figure 4.3.4.b clearly shows certain proteins that are present in the periplasm in DH5α but are absent in the corresponding fraction from DH5α(pDUB2502).

At no time, though, was there a build up of possible precursors for these proteins (or indeed ChiB) seen to occur in the cytoplasm. This was puzzling but could be due to the cells having a fast rate of proteolysis of precursor proteins, something thought to occur in the heat shock response [216]. From figure 4.3.4.b, it is not clear whether alkaline phosphatase precursors are accumulating and being degraded. It can be seen that there are two periplasmic proteins that are absent in DH5α(pDUB2502), having molecular weights of 40 - 45 kD. The upper one of these could conceivably be alkaline phosphatase, the monomeric units of this enzyme having a molecular weight of 47 kD [64], although no antibody was available to test

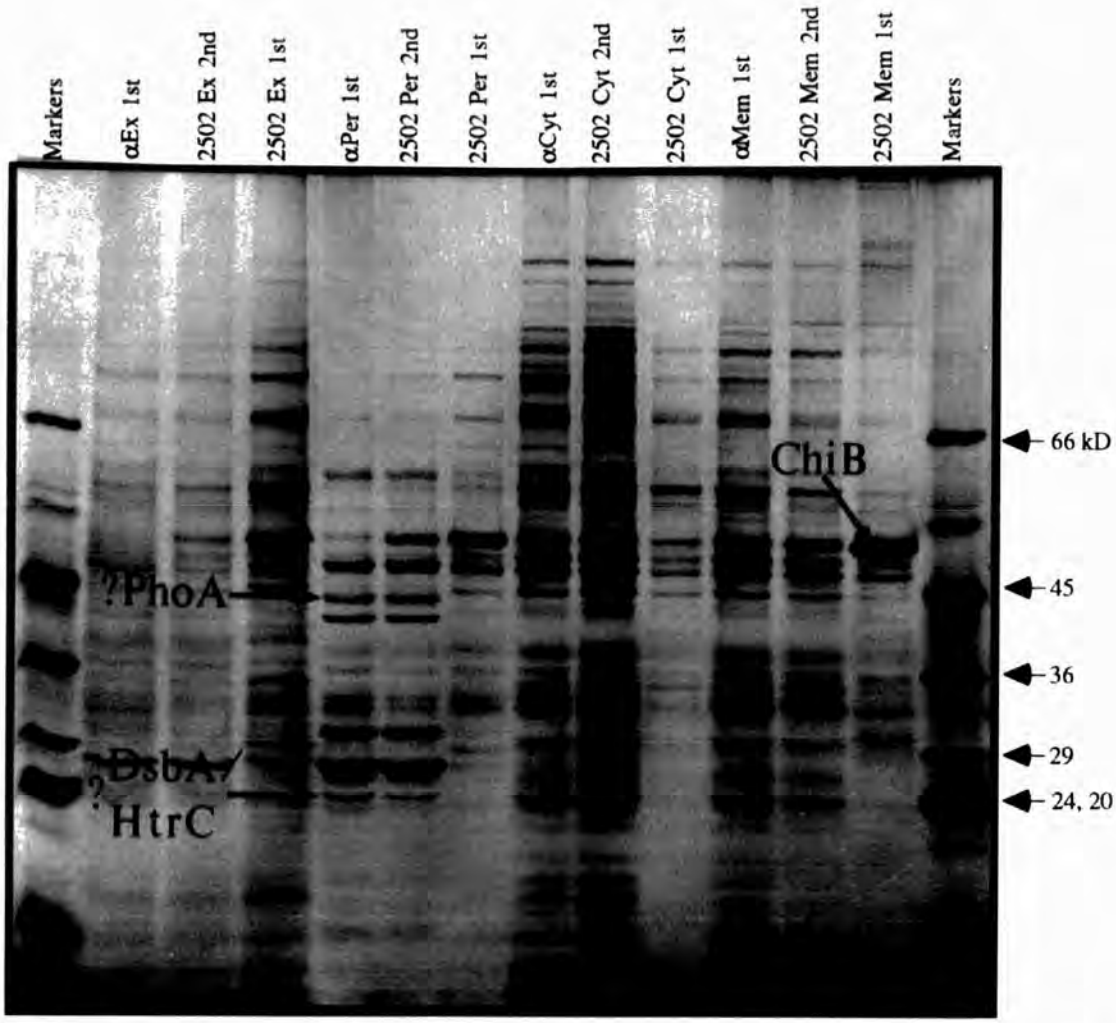


Figure 4.3.4.b Silver-stained SDS/PAGE gel loaded with 40 μl of various cell fractions of DH5α and DH5α(pDUB2502). 1st culture = original culture of bacterium at 37 °C for 24 hours, 2nd culture = subcultured cells grown for further 24 hours at 37 °C - see text for details. Mem = membrane fraction, Cyt = cytoplasmic fraction, Per = Periplasmic fraction, Ex = extracellular fraction.

A large amount of ChiB appears to be present in the membrane fraction of DH5α(pDUB2502) - 1st culture, as indicated. The periplasmic fraction of DH5α(pDUB2502) - 1st culture is clearly different from the corresponding fraction of DH5α.

The periplasmic fraction of the former has lost various proteins, one of which is approximately 21 kD (marked DsbA/HtrC?). Another is approximately 45 kD and could be alkaline phosphatase (marked PhoA?) - see section 4.3.4. However, all these periplasmic proteins appear to be regained in subcultured DH5α(pDUB2502) and less ChiB is seen to be present in the membrane fraction upon subculturing (see section 4.3.4)

this. However, there is no evidence to support accumulation of the alkaline phosphatase precursor in the cytoplasm. It seemed possible that the alkaline phosphatase was being exported but not folded correctly in the periplasm, resulting in its degradation.

The absence of certain proteins in the presence of ChiB suggested that the chitinase might have some specific protease activity. To test this, periplasmic fractions, as prepared above, from all three strains were mixed together in pairs. The mixtures were incubated at 37 °C for 30 minutes before electrophoresing 40 μl of each mixture through a SDS/PAGE gel and silver staining (see sections 2.5.5.a/.b). Proteins that were absent in the periplasm in DH5α(pDUB2502) but were present in the periplasm of the other strains did not undergo degradation in the presence of the periplasmic fraction from DH5α(pDUB2502). Thus, it was unlikely that ChiB was itself acting as a protease.

The heat shock response seemed a possible explanation for the observed phenotype of DH5α(pDUB2502), however SDS/PAGE gels of cellular fractions from this strain failed to show any overexpressed proteins, a feature indicative of a heat shock response [216]. However, overexpression of these proteins may only need to be short lived [58]. A thermotolerance assay was performed (see section 2.5.15) to find out whether DH5α(pDUB2502) was more thermotolerant than the other strains, which, if it is undergoing a heat shock response, it should be [329]. Figure 4.3.4.c suggests that the *chiB*-expressing strain is no more thermotolerant than DH5α or DH5α(pDUB2501). Thus it seemed probable that the filamentous phenotype was due, not to a heat shock response, but due to poor export of proteins important for cell division.

Two groups have isolated a periplasmic enzyme that is responsible for the folding and formation of disulphide bonds in alkaline phosphatase [19, 163]. The enzyme, encoded by *dsbA*, could therefore be responsible for the correct folding of proteins involved in cell division as well as alkaline phosphatase.

DsbA has a molecular weight of 21 kD, and is a periplasmic enzyme with a signal peptide [19, 163]. On figure 4.3.4.b, it can be seen that there is a protein missing in the periplasm of DH5α(pDUB2502) of about 21 kD. Bardwell *et al.* showed that mutations in *dsbA* rendered the bacterium unable to grow in minimal media [19] and the bacteria were deficient in active alkaline phosphatase (features also noted in DH5α(pDUB25020)). Kamitani *et al.* further indicated that a mutation in the gene led to "abnormal colony morphology" [163]. However, neither group

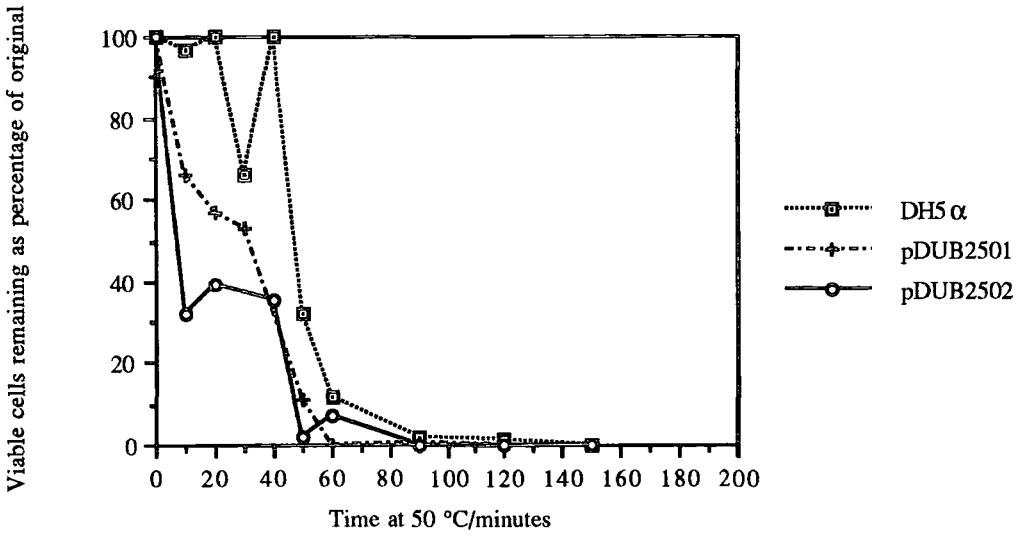


Figure 4.3.4.c Results of thermotolerance assay showing decrease in numbers of viable cells depending upon incubation time at 50 °C of various strains of *E. coli*:: DH5α, DH5α(pDUB2501) and DH5α(pDUB2502). It can be seen that DH5α(pDUB2502) is no more thermotolerant than DH5α. It could be suggested that both DH5α(pDUB2501) and DH5α(pDUB2502) were less thermotolerant than DH5α.

confirmed the presence of filamentous bacterial cells containing the mutation but it remained possible that the observed phenotype was not the result of a heat shock response.

To investigate this further, cultures of DH5α, DH5α(pDUB2501) and DH5α(pDUB2502) were grown in LM-broth at various temperatures. At 37 °C, DH5α and DH5α(pDUB2501) retained a normal phenotype, but DH5α(pDUB2502) showed the expected filamentous phenotype (see figure 4.3.4.a). However, DH5α(pDUB2502) exhibited a wild type phenotype when grown at 30 °C which strongly suggested that a heat-sensitive protein or one that is only required at higher growth temperatures was one of the proteins blocked in its export.

At 46 °C, both DH5α and DH5α(pDUB2501) showed evidence of a heat shock response, in that the cells were highly elongated and in some cases appeared filamentous. This filamentous phenotype however, did not appear to be the same as the one previously identified, in that the filaments were much shorter and no branch structures (see below) were observed. The darkly staining hexagonal structures were also less apparent, although no electron microscopic examination was carried out. At this temperature (46 °C) DH5α(pDUB2502) failed to grow. This suggested more strongly that the export of a heat shock protein, required for survival, at higher temperatures, was being blocked. This putative heat shock protein would also be necessary for cell division at intermediate temperatures.

What was more puzzling was the fact that at 42 °C and at 44 °C, the cells of DH5α(pDUB2502) appeared only slightly elongated and not filamentous. It may be that at these temperatures, other heat shock proteins are produced that can substitute for the putative heat shock protein, whereas at 46 °C, the latter is absolutely required or a further heat shock protein is required that is also blocked in its export.

Mutations in the heat shock gene *htrC* result in a similar phenotype to the one described above for DH5α(pDUB2502) grown at 37 °C [165, 243]. At intermediate temperatures *htrC*$^-$ bacteria were shown to be filamentous, the filaments being aseptate and containing asymmetrically distributed "nucleoid-like bodies" (cf. figure 4.3.4.a). These bacteria were also observed to grow in the same manner as wild type bacteria at 30 °C and they exhibited reduced production of FtsZ, a cell division protein [165, 243]. HtrC has a molecular weight of 21 kD [243] and thus the missing 21 kD band in the periplasmic fraction of DH5α(pDUB2502) may be this or both DsbA and HtrC.

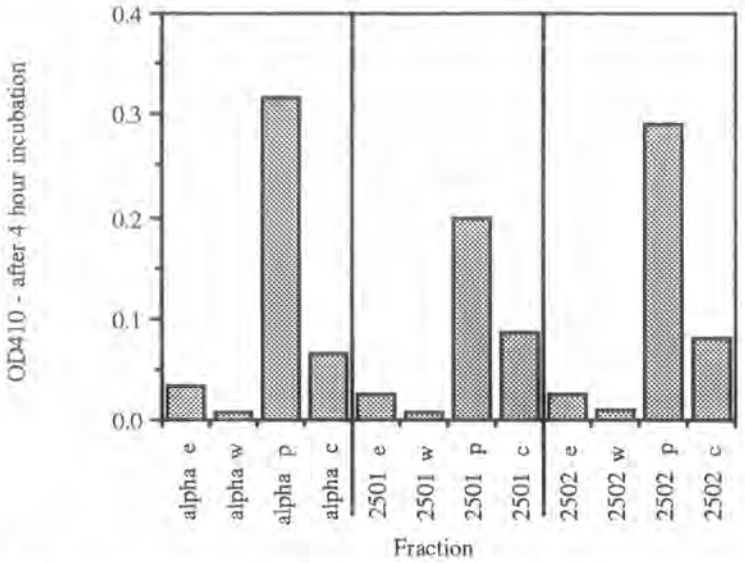


Figure 4.3.4.d Graph showing alkaline phosphatase activity of various fractions of four subcultured strains of bacteria: *E. coli*DH5α, DH5α(pDUB2501), DH5α(pDUB2502) and *S. marcescens* QMB1466. The activities are expressed as the optical density at 410 nm after 3 hours incubation at 37 °C. e = extracellular, w = wash, p = periplasmic, c = cytoplasmic.

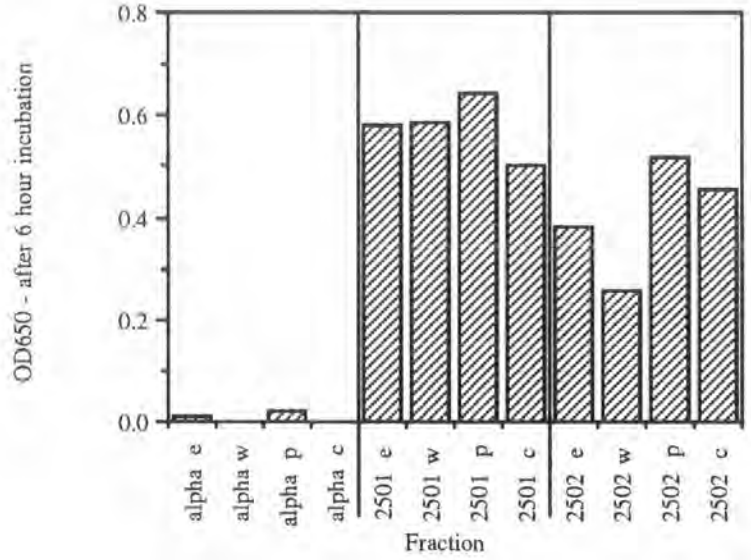


Figure 4.3.4.e Graph showing chitinase activity of various cell fractions of four subcultured strains of bacteria: *E. coli* DH5α, DH5α(pDUB2501), DH5α(pDUB2502), *S. marcescens* QMB1466. Chitinase activity is expressed as the optical denisty measured at 650 nm after 6 hours incubation at 50 °C. e = extracellular, w = wash, p = periplasmic, c = cytoplasmic.

However, HtrC, does not appear to have a signal peptide [28], suggesting that the protein might not be periplasmic, or if it is, that it uses a Sec-independent translocation pathway that proteins containing a signal peptide do not use.

It is unlikely that overproduction of ChiB will block two export pathways. Therefore, if HtrC is periplasmic, it is more likely that its action is inhibited due to the absence of DsbA, which might require the blocked export pathway to reach the periplasm. Alternatively the action of DsbA might be inhibited due to the absence of HtrC.

It is clear that the blockage of the translocation pathway has many effects, the results suggesting that this blockage does have effects on both DsbA and HtrC. DH5α(pDUB2502) may therefore prove useful in elucidating the precise functions of HtrC and DsbA, especially if the plasmid is used in genetic backgrounds containing mutations in these genes and the *sec* genes.

The reasons for the blockage are not immediately obvious, but it is likely that the signal peptide is not entirely complementary with the export pathway resulting in slow translocation. Alternatively, the ChiB molecule itself may not be able to be maintained in a translocation-competent form in the cytosol to allow efficient translocation. However, translocation does occur since ChiB is found in the extracellular medium and in the periplasm (see figure 4.3.3.b). What is intriguing is that upon subculturing filamentous DH5α(pDUB2502) into fresh LM-broth, the resulting culture exhibits a wild type phenotype. The periplasmic fraction of such subcultured cells are also seen to have regained proteins that the filamentous bacteria had lost (see figure 4.3.4.b) and alkaline phosphatase activity is restored (see figure 4.3.4.d). The membrane fraction can also be seen to contain much less ChiB, if any, and thus it would appear that the saturation of the export pathway is the result of overexpression. Figure 4.3.4.b suggests that the subcultured cells of DH5α(pDUB2502) are able to down regulate the expression of *chiB* in some manner, but chitinase activity assays do not support this conclusion (cf. figures 4.3.3.b and 4.3.4.e), although membrane fraction assays were not performed due to interference of Triton X-100 with the substrate. Once DH5α(pDUB2502) had adopted the more wild type phenotype, the filamentous phenotype never reappeared, suggesting that whatever the nature of the change, it was permanent.

The fact that subcultured DH5α(pDUB2502) was able to produce ChiB and possess a wild type phenotype, adds weight to the argument that the reason for the filamentous phenotype is as a result of export blockage. If ChiB were to have a

direct action on DsbA or HtrC in the cytoplasm, the filamentous phenotype would not be expected to change so completely to a wild type phenotype. However, an effect of ChiB could be that it just saturates out certain proteins at higher concentrations. It must also be pointed out that no experiments were performed to separate outer and inner membrane fractions, and the blockage (if there is one) may occur in the outer membrane, although this seems unlikely.

The appearance of a filamentous phenotype is not only specific to *E. coli* DH5α overexpressing *chiB*. *E. coli* JM83(pCHIT310) [157] were also observed to have a filamentous phenotype, although bacteria containing this plasmid were not reported as having a filamentous phenotype in the original paper by Jones *et al.*[157].

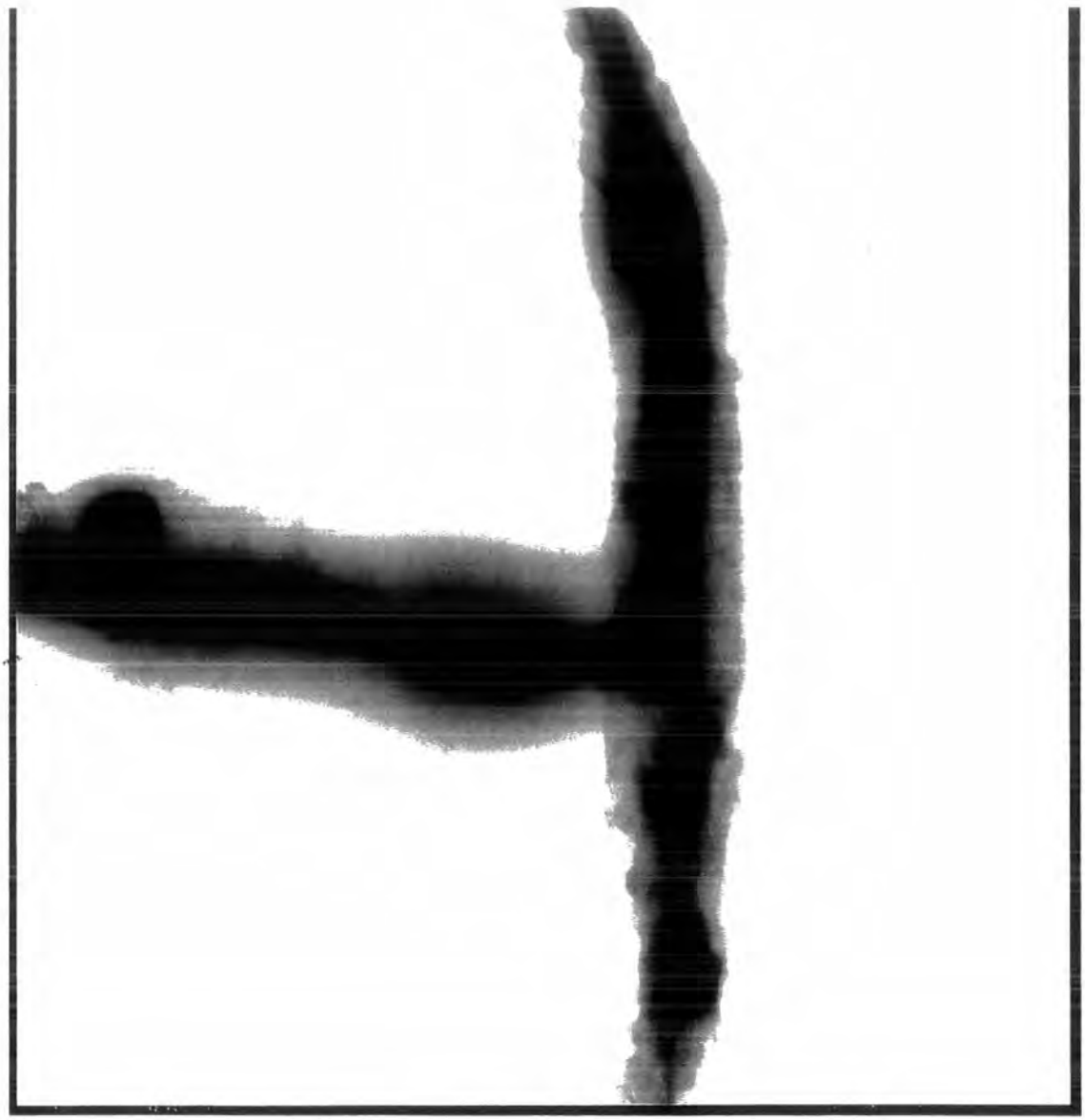
4.3.5 Further phenotypic analysis of filamentous DH5α(pDUB2502)

At various points along the filaments, branches were seen to occur (see figure 4.3.5.a) as were 'X'-shaped structures (4.3.5.b). The nature of the cause of these structures is unclear, although it is obviously a fault with some aspect of cell division.

One further point to note is that the darkly-staining structures appear to constrict the filament, or at least are found on the outside of the filament. The procedure used to stain the samples for the electron microscope (see section 2.5.14) causes disruption and swelling of the periplasm. From both figure 4.3.5.a and figure 4.3.5.b it can be seen that the periplasm appears to be constricted by the darkly-staining objects which might be forming some sort of 'collar' structure. However, again the nature of such a 'collar' and the mechanisms by which it might have been caused remain unclear.

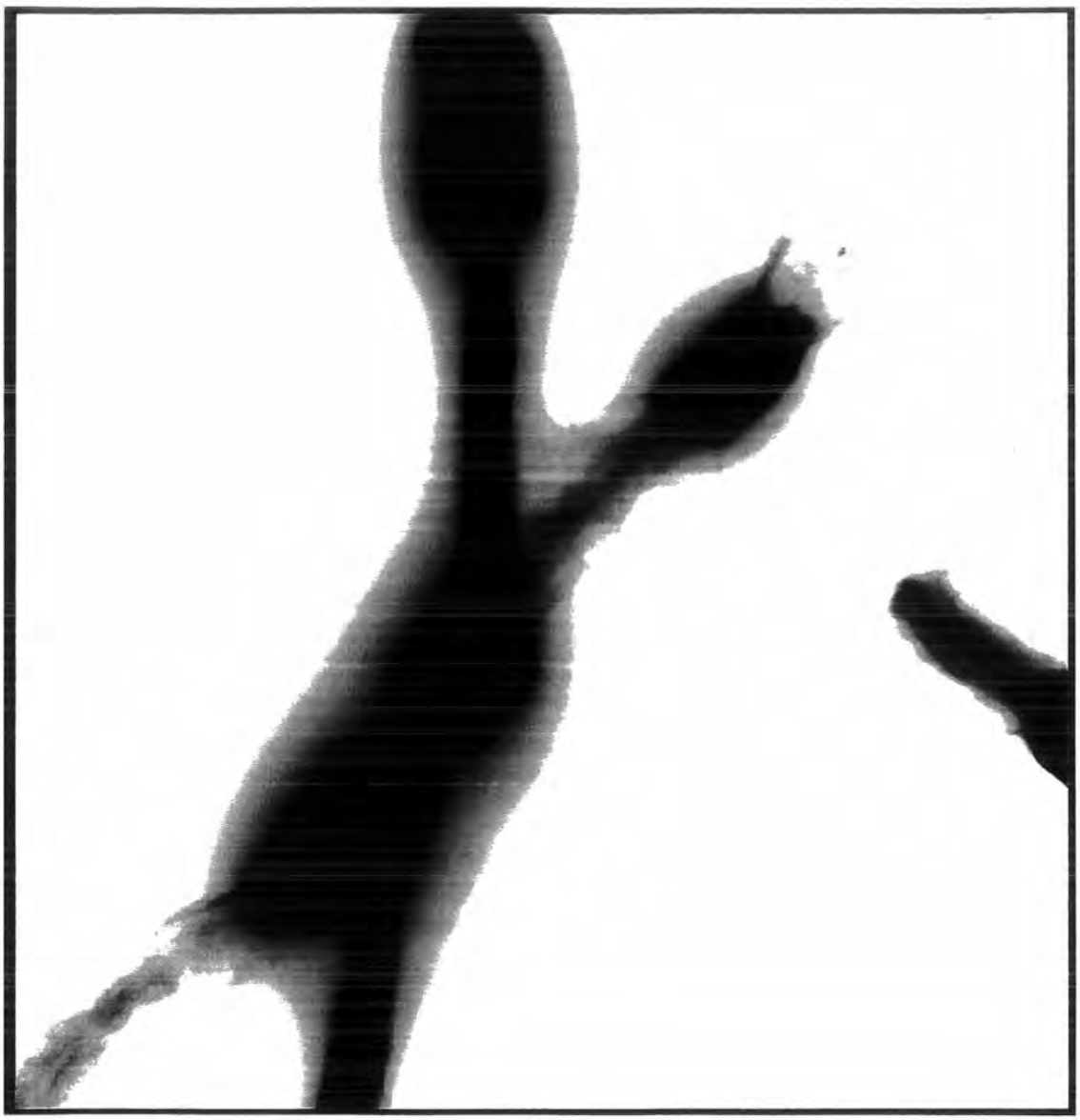
4.4 Mode of action of *Serratia marcescens* chitinases

There has been much confusion in the literature regarding the precise actions of the chitinases produced by *S. marcescens*, with some authors claiming that the chitinases were endo-acting [93, 157, 209] and others suggesting that the chitinases were exo-acting [187, 246, 248]. There is also much confusion over the definitions of the various enzyme activities and this may have led to some misnaming of the chitinases [92, 187, 310].



Scale : 1 μm

Figure 4.3.5.a Electron micrograph showing branch structure in the filamentous phenotype of DH5α(pDUB2502) photographed at a magnification of 13 000 X.



Scale : 1 μm

Figure 4.3.5.b Electron micrograph showing 'X'-shaped structure in the filamentous phenotype of *E. coli* DH5α(pDUB2502) photographed at a magnification of 13 000 X.

In the present study, a chitinase is described as an enzyme that cleaves a bond between carbon 1 and carbon 4 of two adjacent N-acetylglucosamine subunits. The term 'chitobiase' is used here to describe an enzyme capable of releasing single N-acetylglucosamine subunits from the non-reducing end of chitobiose (a molecule consisting of two N-acetylglucosamine subunits) and higher analogues. Other authors consider chitobiases to be β-N-glucosaminidases, but the former term is more widely used [92, 310]. An enzyme that releases chitobiose units from the non-reducing end of chitin is referred to here as an exochitinase, although Tronsmo and Harman consider this to be a chitin-1,4-β-chitobiosidase [310]. Again, the former terminology is more widely used. Enzymes capable of cleaving more than two residues from the non-reducing end of chitin are sometimes referred to as endochitinases [310]. However, if this is a consistent activity of the enzyme, they are better described as chitotriosidases, chitotetrasidases etc., reserving the term 'endochitinase' for enzymes that cleave randomly along the length of the chitin polymer.

Endochitinases will not cleave chitobiose or chitotriose substrates [248]. Lilley found that ChiA and ChiB were both able to cleave the artificial substrate p-nitrophenyl-β-D-N,N'-diacetylchitobiose (Sigma) [187] (see section 2.5.6.a). This substrate is equivalent to chitotriose, the nitrophenol group acting as one subunit [310]. On this basis, it was proposed that both ChiA and ChiB were exochitinases [187].

Studies were carried out in this work to further elucidate the activities of ChiA and ChiB. Overnight cultures of DH5α, DH5α(pDUB2501) and DH5α(pDUB2502) were fractionated (see section 2.5.4.a). Each fraction was then assayed using three different substrates: p-nitrophenyl-N-acetyl-β-D-glucosaminide (chitobiose equivalent), p-nitrophenyl-β-D-N,N'-diacetylchitobiose (chitotriose equivalent) and p-nitrophenyl-β-D-N,N',N''-triacetylchitotriose (see section 2.5.6.a). The different substrates allowed different chitinase activities to be assessed [310].

The activities for each fraction from the same strain were added together and the results are shown in figure 4.4.a. It can clearly be seen that neither ChiA nor ChiB have any chitobiase activity. Both chitinases appear to possess large amounts of exochitinase activity, ChiB being almost exclusive in this activity. ChiA, however, also possess much chitotriosidase activity. It was also noted that *E. coli* DH5α appeared to produce no enzymes with these activities.

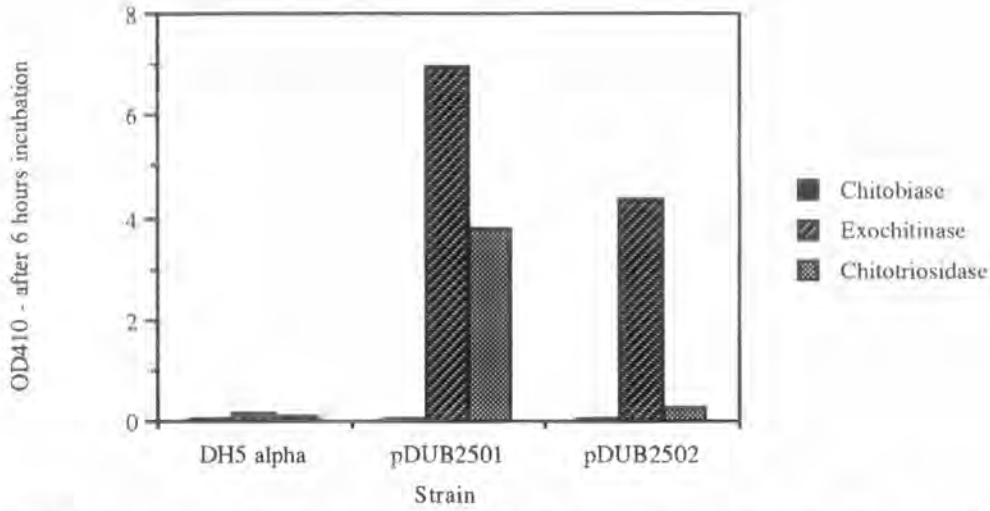


Figure 4.4.a Bar graph representation of different types of chitinase activity exhibited by DH5α, DH5α(pDUB2501) (*chiA*) and DH5α(pDUB2502) (*chiB*). Activity is expressed as the absorbance as measured at 410 nm of cell extracts after 6 hours incubation at 50 °C with the respective substrates.

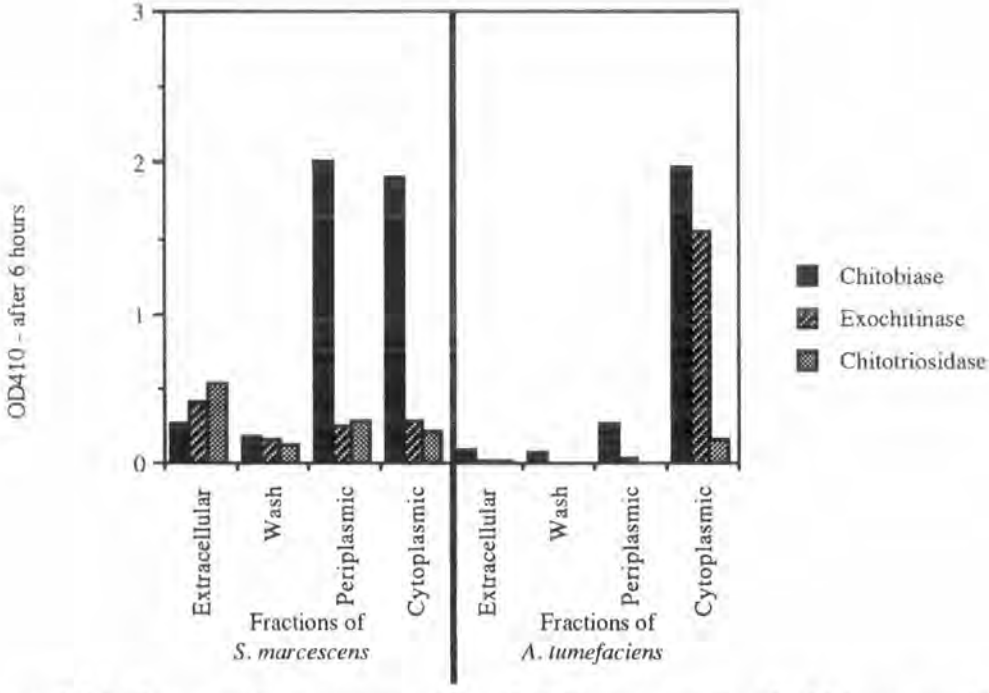


Figure 4.4.b Bar graph representation of different types of chitinase activity exhibited by cellular fractions of *S. marcescens* and *A. tumefaciens*. Activity is expressed as absorbance measured at 410 nm after 6 hours at 50 °C with the respective substrates.

Thus ChiA and ChiB do have different spectra of activities, ChiA seeming to have a broader range of activity. It would have been interesting to use other longer p-nitrophenyl chitosides to further elucidate the activities, but the cost of these substrates was prohibitive.

The broader range of activity possessed by ChiA has probably caused some of the confusion over its mode of action. However, using the nomenclature set out above, neither chitinase can be considered to be an endochitinase, since they both cleave the smaller chitoside substrates [248]. Whether ChiA has further activities remains to be examined.

Further to these experiments, similar assays were performed using cell fractions from *S. marcescens* cultured in LM-broth containing 5 $mg.ml^{-1}$ chitin. Figure 4.4.b shows secreted chitinase activity with both exochitinase and chitotriosidase activities. There is relatively more activity in the latter case which was somewhat surprising, since both ChiA and ChiB have been shown to have substantial exochitinase activities (see above). However, *S. marcescens* produces at least 3 other chitinases apart from ChiA, ChiB and the cloned chitobiase [93, 174]. One or more of these other chitinases may have chitotriosidase activity, which combined with the activity of ChiA, may account for this result. Furthermore, most of the chitobiase activity was located in the periplasmic fraction. This may be accounted for by the activity of the chitobiase cloned by Kless *et al.*, suggesting that this enzyme is in fact located in the periplasm, and is not secreted, contrary to the surmises of Kless *et al* [174]. Being a large protein (95 kD) this would seem likely. However, there is some secreted chitobiase activity and it may be that either the 95 kD chitobiase is slowly secreted or that there is more than one enzyme with chitobiase activity secreted by *S. marcescens*.

Further assays of this type were performed using 24 hour *A. tumefaciens* cultures. Figure 4.4.b shows that *A. tumefaciens* might also possess at least one chitinolytic enzyme. Activity corresponding to a chitobiase was found in the cytoplasmic and periplasmic fractions. Exochitinase activity and some chitotriose activity was also evident. Surprisingly, though, when the same fractions were assayed using CM-chitin-RBV [337] (see section 2.5.6.b), no chitinase activity was observed. This indicates that the 'activity' found is probably not due to a chitinase, but to an enzyme that recognises and cleaves off the nitrophenol moiety. In the light of this, only CM-chitin-RBV was used as a substrate for subsequent assays for ChiA and ChiB expression in *A. tumefaciens*.

4.5 *chiA* and *chiB* expression in *A. tumefaciens*.

4.5.1 Expression and secretion of chitinases in *A. tumefaciens*.

pDUB2501 and pDUB2502 were conjugated into *A. tumefaciens* C58C1 using the triparental mating method (see section 2.5.2.c). Bacteria containing the plasmids were selected by plating out on selective agar. Plasmid DNA was prepared (see section 2.5.1.a) and then restricted and electrophoresed through a 0.7 % agarose gel (see sections 2.5.3.b/g) and the banding patterns were compared with known values.

To determine whether either ChiA or ChiB could be secreted in *A. tumefaciens*, strains C58C1, C58C1(pDUB2501), C58C1(pDUB2502) and *Serratia marcescens* QMB1466 were streaked out on LM-agar containing 5 mg.ml-1 CM-chitin-RBV [337]. The plates were incubated at 28 °C for 48 hours after which time clear zones were clearly visible around the *S. marcescens* and C58C1(pDUB2502) bacterial streaks. There was also a much smaller zone of clearing around C58C1(pDUB2501) but no zone of clearing was detected around the C58C1 control (see figure 4.5.1.a). This result suggested that, like DH5α(pDUB2502), the *A. tumefaciens* equivalent could also express *chiB* from its natural promoter and could secrete the protein. C58C1(pDUB2501), though, was either unable to secrete ChiA or was unable to express the gene efficiently. To investigate the relative expression levels of the two genes in *A. tumefaciens*, fractionation assays were performed, as described in sections 4.3 and 4.4.

24 hour cultures of C58C1(pDUB2501) and C58C1(pDUB2502) in MinA medium + 1 % mannitol were fractionated (see section 2.5.4.a) and the resulting cellular fractions were assayed for chitinase activity (see section 2.5.6.b). Typical results shown in figure 4.5.1.b, suggest that *chiB* is better expressed from its natural promoter in *A. tumefaciens* than *chiA*. It is further suggested that ChiB may well be secreted, albeit inefficiently, whereas ChiA is not. This latter conclusion is further supported by the banding patterns of 40 µl samples of the fractions electrophoresed through a SDS/PAGE gel (see section 2.5.5.a), shown in figure 4.5.1.c. The silver-stained gel suggests that ChiB release is due to secretion rather than cell lysis, due to the clear banding differences between the periplasmic and the extracellular fractions (see figure 4.5.1.c).

To confirm this, it was hoped to be able to use a marker enzyme for the periplasm (see section 4.3). Alkaline phosphatase was chosen, since it had been

Figure 4.5.1.a Agar plate containing 5 mg.ml^{-1} CM-chitin-RBV showing zones of clearing around various strains of bacteria: A = C58C1(pDUB2502), B= C58C1(pDUB2501), C = C58C1, D = *S. marcescens* QMB1466.

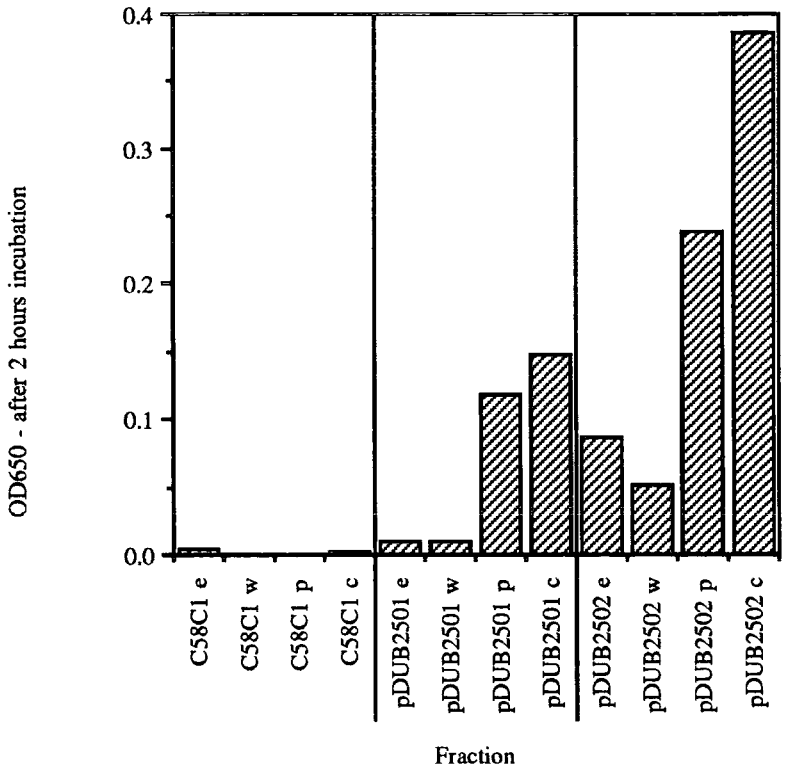


Figure 4.5.1.b Showing relative levels of chitinase activity present in cellular fractions of various bacterial strains: *A. tumefaciens* C58C1, C58C1(pDUB2501) and C58C1(pDUB2502). The activities are expressed as absorbance at 410 nm of the various fractions after 2 hours incubation with the substrate. e = extracellular, w = wash, p = periplasmic, c = cytoplasmic.

easily assayed during the work with *E. coli* (see section 4.3). However, no alkaline phosphatase activity, using the method as described in section 2.5.6.c, could be located in *A. tumefaciens*. This was found to be in common with the results of other researchers [43, 225].

Since both pDUB2501 and pDUB2502 contain the ampicillin resistance gene that encodes β-lactamase (*bla*), this enzyme was assayed (see section 2.5.6.d). This assay proved to produce very erratic results, typically indicating β-lactamase activity in all the cell fractions (as was also found to be the case in *E. coli*), including those of the control strain *A. tumefaciens* C58C1. A similar observation has been made previously [43] and it is likely that *A. tumefaciens* produces other β-lactamases [219] or enzymes that are able to cleave the substrate but are not true β-lactamases.

Assays for other enzymes known to be located in the *E. coli* periplasm were carried out on the cell fractions (see sections 2.5.6.e/f/g/h). No activities were found for 5' nucleotidase, acid phosphatase or invertase, whilst 2',3'cyclic AMP phosphodiesterase appeared to be located in the cytoplasm, although it is not known whether this result reflected true 2',3'cyclic AMP phosphodiesterase activity. It cannot be stated that *A. tumefaciens* does not posses 5' nucleotidase, acid phosphatase, invertase or indeed alkaline phosphatase activities, simply that under the conditions used, these enzymes did not appear to be active. It was not considered to be worthwhile pursuing the optimisation of these assays for *A. tumefaciens*.

One last attempt was made to find a periplasmic marker protein. An antibody to the glucose-binding protein ChvE (see section 1.9), was kindly donated by Colin Jones (University of Leicester, U.K.) [70, 71]. 40 μl samples of fractionated cultures of *A. tumefaciens* C58C1 grown for 24 hours in MinA medium + 0.5 % glucose were electrophoresed through a SDS/PAGE gel (see section 2.5.5.a) and the gel was subsequently western blotted and immunoscreened using the anti-ChvE antibody (see sections 2.5.5.c/d). However, no bands corresponding to ChvE could be detected. The antibody was tested once more on similar cell fractions using the ELISA technique (see section 2.5.5.e) and a range of dilutions (from 1/100 to 1/1000) of the anti-ChvE antibody. The secondary antibody did not bind, indicating a lack of ChvE in the fractions.

The reasons for this were not clear. It may have been that the antibody, which had been raised against ChvE from *A. radiobacter*, could not recognise the similar protein from *A. tumefaciens*. Alternatively, the antibody may have become denatured in some way.

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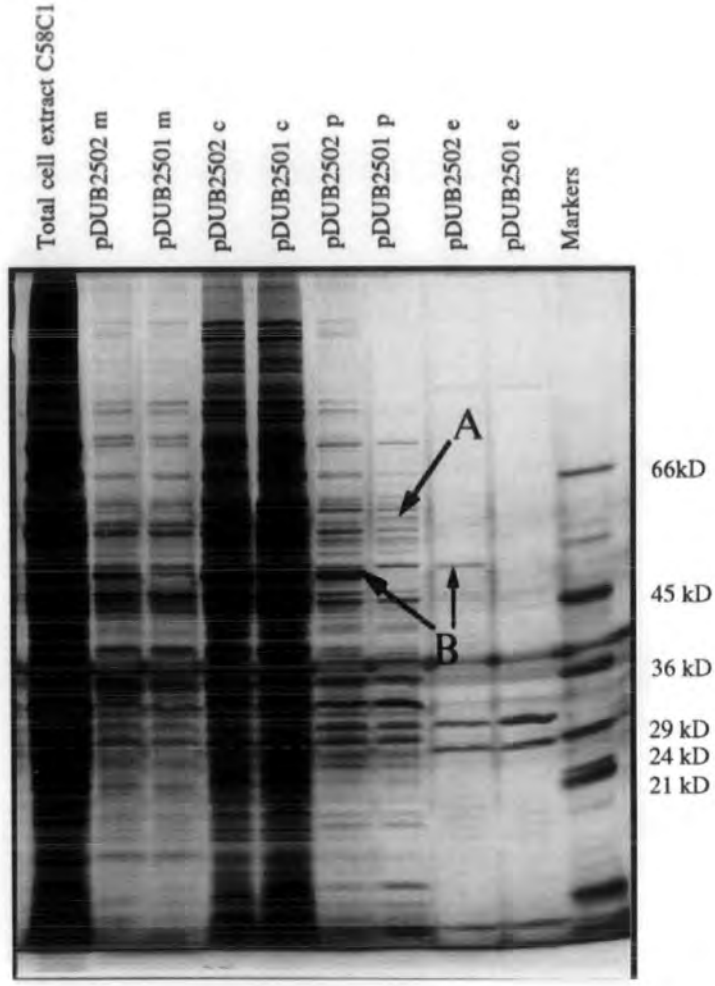


Figure 4.5.1.c Silver-stained SDS/PAGE gel showing various cellular fractions of *A. tumefaciens* C58C1(pDUB2501) and C58C1(pDUB2502). e = extracellular, p = periplasmic, c = cytoplasmic, m = membrane. ChiA and ChiB are labelled.

Despite the lack of a marker for the periplasm, on the basis of the plate assay, SDS/PAGE gels showing fractionation patterns and the presence or absence of ChiA or ChiB, and the CM-chitin-RBV assays on those fractions, it seemed likely that ChiB was secreted in *A. tumefaciens*, but ChiA was not. However, this latter point remains dubious since the levels of expression of the *chiA* gene were so low. The experiments did show that *chiB* was expressed more efficiently from its natural promoter than was *chiA*.

4.5.2 Construction of pECB2 and pECB2.2

Lilley had previously shown that *chiA* and *chiB* could be expressed under the control of the *virB* and *virE* promoters, in the presence of *virA* and *virG* provided by pVK257 [187]. The results of this work suggested that the *virE* promoter was more efficient in directing the transcription of *chiA* than the *virB* promoter was, although both these constructs were inducible by acetosyringone [187].

The suggestion that the *virE* promoter was more efficient than the *virB* promoter is in contradiction with the results of Rogowsky *et al.* [253] and the results obtained in the present work (see section 3.2). It was possible that the difference was a copy number effect, since pECA2 (containing the *virE* promoter linked to *chiA*) was based on the broad host range vector pUCD4 [62] and pBCBA2 (containing the *virB* promoter linked to *chiA*) was based on pGV1106 [185]. It is quite possible that pECA2 had a much greater copy number than that of pBCA2 and that this is responsible for the greater amounts of ChiA produced when using pECA2. This argument is supported to some extent by examining the data from Lilley [187], which shows a steeper curve of induction over 24 hours for pECA2, which may well be due to a greater initial concentration of VirG-binding sites as a result of a higher copy number (see section 1.9).

IncW plasmids (which both pUCD4 and pGV1106 are) have copy numbers of about 2 - 5 $cell^{-1}$ [185] and with this in mind, subsequent subcloning was carried out using an IncQ plasmid, pKT230 which, in *Pseudomonas,* has a copy number of between 15 - 20 $cell^{-1}$ [15].

However, it has been suggested by Hille and Schilperoort, that IncW plasmids themselves have an adverse effect on tumorigenicity [131] and this may thus be responsible for the low levels of induced chitinase reported by Lilley. IncQ plasmids appear to have no adverse effects on tumorigenicity [131] and this was a further reason for using pKT230 in subcloning in this work.

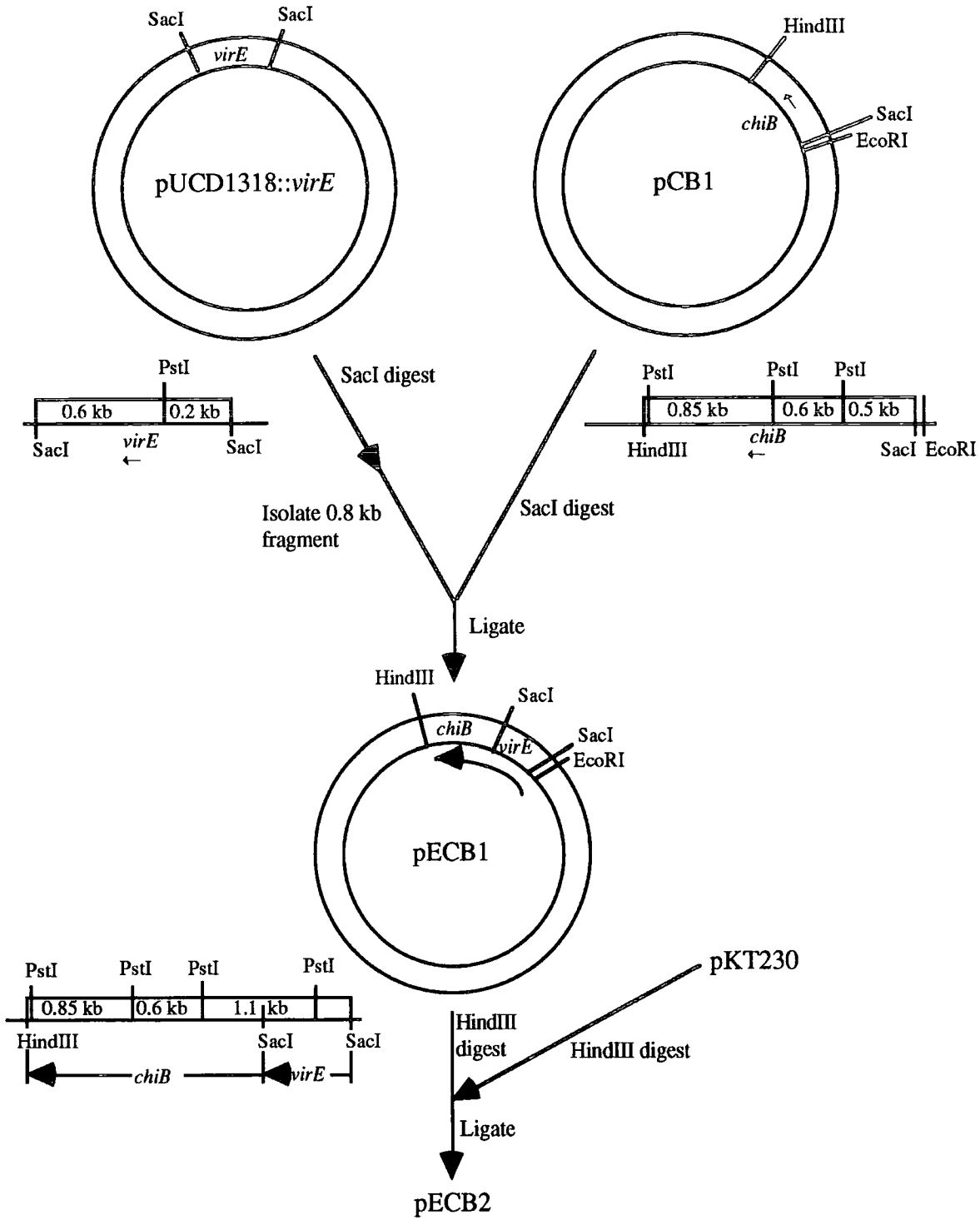


Figure 4.5.2.a Diagram showing construction of pECB2

In the experiments performed by Lilley, a culture of *A. tumefaciens* C58C1(pVK257, pECA2) when induced by acetosyringone produced four times as much ChiA as an uninduced culture after 24 hours. In a similar experiment using pBCA2, the amount of ChiA produced by an induced culture was only twice that of the uninduced culture. It was hoped that, in the light of experiments comparing different induction media, the amount of the chitinase produced by an induced culture might be increased using Davis medium and the procedure described in section 3.2, since the levels of induced chitinase recorded by Lilley appeared to be low in comparison with the induced *virB*::*lux* constructs (see section 3.2).

Lilley had also constructed a plasmid (pBCB2), based on pUCD4, in which the *virB* promoter from pUCD1187 [253] was linked to the ChiB gene. The construct made in this study was therefore one which contained the *virE* promoter (from pUCD1194 [253]) linked to the ChiB gene.

pCB1 contained the coding region of *chiB* only, in pUC19 [187]. This was digested with SacI and phosphatased to prevent religation of the cohesive termini (see sections 2.5.3.b/c). pUC1318::*virE* [187] was also restricted with SacI, and the digest was electrophoresed through a 0.7 % agarose gel after which the 0.8 kb *virE* promoter fragment was isolated using electroelution (see sections 2.5.3.b/g/h). A ligation was set up using this fragment and SacI-cut pCB1 (see section 2.5.3.f) and the mixture was subsequently transformed into *E. coli* DH5α using the standard technique (see section 2.5.2.a) and plated out on agar containing ampicillin. Random colonies were picked from the plates and cultured overnight in 5 ml LM-broth containing ampicillin. Plasmid DNA from the cultures was obtained using the quick 'miniprep' method (see section 2.5.1.b) and digested with SacI to confirm the presence of a 0.8 kb fragment (see figure 4.5.2.b). Plasmid preparations found to contain a 0.8 kb SacI fragment were taken and samples digested with PstI, to confirm the orientation of the insert. Those containing the *virE* promoter able to direct transcription of *chiB* contained a 1 kb PstI fragment, whereas those containing the *vir* promoter in the wrong orientation contained a 0.7 kb PstI fragment (see figure 4.5.2.a). The plasmid containing the correct insertion was designated pECB1. One plasmid preparation was found to contain two copies of the *virE* promoter both in the correct orientation relative to *chiB* and this was designated pECB1.2.

pECB1 was a pUC-based plasmid and could not therefore replicate in *A. tumefaciens*, thus a mixture of pKT230 and pCB1 was digested with HindIII in 1 x One-Phor-All Buffer (Pharmacia) for 4 hours. The mixture was then heated to 85 °C for 30 minutes to inactive the restriction enzyme and allowed to cool for 20 minutes

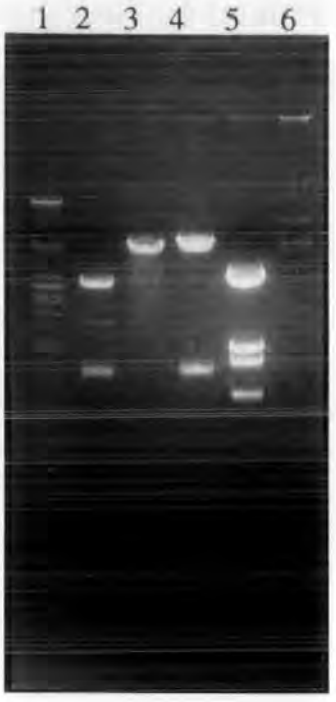


Figure 4.5.2.b Restriction patterns showing the construction of pECB2.

Lane 1 - λ - PstI markers (Bands at 14.05, 11.49, 5.07, 4.75, 4.51, 2.84, 2.5, 2.14, 1.99, 1.70, 1.16, 1.09, 0.81, 0.52 kb are visible)

Lane 2 - pUC1318::*virE* SacI digest

Lane 3 - pCB1 SacI digest

Lane 4 - pECB1 SacI digest

Lane 5 - pECB1 PstI digest

Lane 6 - pECB2 PstI digest, reflecting much lower copy number of this plasmid. Lower bands are not discernible.

prior to the addition of 5 mM ATP and 1 unit T4 DNA ligase. The ligation mixture was left overnight at 4 °C and then transformed directly into *E. coli* DH5α (see section 2.5.2), the resulting bacteria being plated out onto agar containing ampicillin and streptomycin. One colony grew on the plates and this was cultured overnight in 5 ml LM-broth containing antibiotics. The plasmid was obtained (see section 2.5.1.a) and then restricted once with HindIII and once with PstI, to confirm the presence of pECB2 (see figure 4.5.2.b). This process was repeated using pECB1.2 thus forming pECB2.2.

4.5.3 Relative chitinase expression levels of *vir::chi* constructs in *A. tumefaciens*

pBCA2, pECA2, pBCB2, pECB2 and pECB2.2 were conjugated into *A. tumefaciens* C58C1(pVK257) using the triparental mating method (see section 2.5.2.c). Bacteria containing the relevant plasmids were selected on LM-agar containing the appropriate antibiotics (see section 2.4). Random colonies of each strain were selected and cultured in 5 ml LM-broth containing antibiotics for 24 hours. The cultures were then minipreped (see section 2.5.1.a) and the resulting plasmid preparations were digested and electrophoresed in a 0.7 % agarose gel. The banding patterns were compared with known values for the plasmids and cultures containing the correct plasmids were plated out on selective agar.

48 hour cultures of the *chiA/B*-containing strains were set up in 5 ml LM-broth containing antibiotics. The number of bacteria in each culture was estimated (see section 2.4.3) and the bacteria were subcultured into 5 ml Davis medium (no antibiotics) at a concentration of approximately 1 x 10^7 cells.ml^{-1}. These cultures were allowed to grow for 3 hours prior to the addition of 100 μM acetosyringone (or the equivalent volume of the solvent, 70 % methanol, in the control cultures). After 24 hours, 100 μl samples of total cell extracts of the cultures (see section 2.5.4.b) were assayed for chitinase activity (see section 2.5.6.b) and the total amount of protein present in 100 μl was determined (see section 2.5.5.f). The results are displayed in figure 4.5.3.a as the total OD_{650} reading after 6 hours per μg of protein, corrected for the readings obtained using strain C58C1(pVK257).

Clear induction of chitinase production by acetosyringone can be seen. This is most evident in strain C58C1(pVK257, pECA2). Using Davis medium, the chitinase activities detected were approximately 100 fold greater than those reported by Lilley using the same strains [187]. However, the background values of these strains also increased in the same proportions and thus the overall fold induction values remained between 2.5 and 3 times. The reasons for this are unclear, although 'media

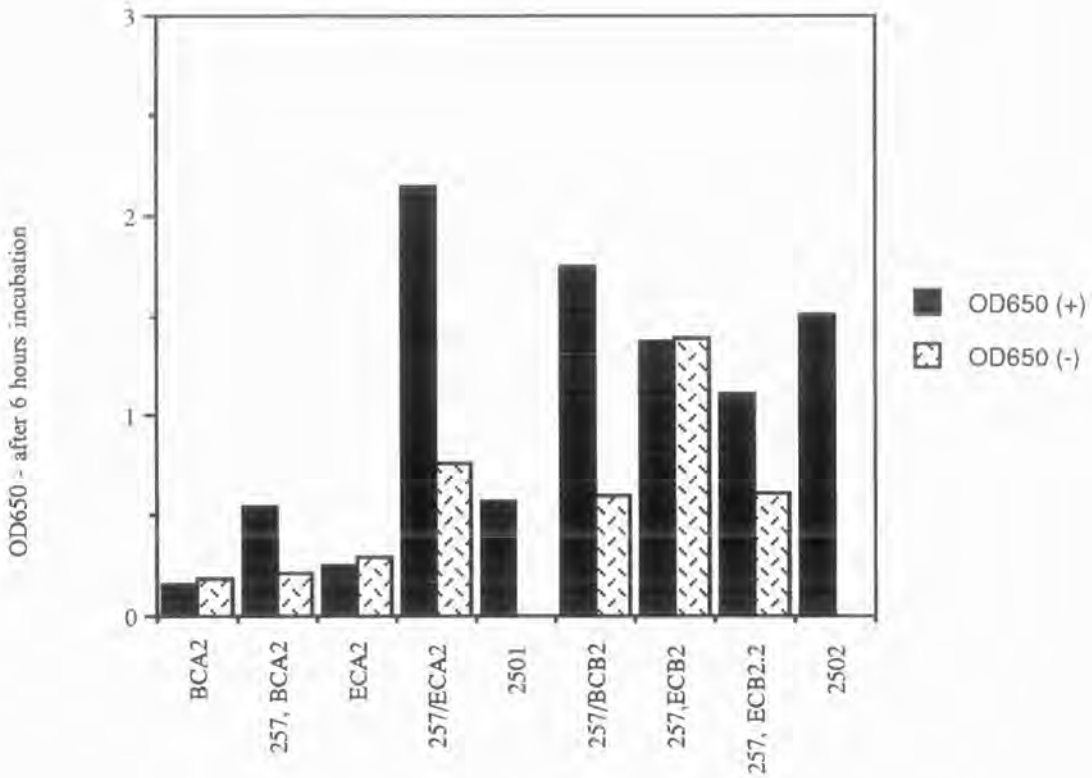


Figure 4.5.3.a Chitinase activities of *A. tumefaciens* C58C1, containing various plasmids: pVK257, pDUB2501, pDUB2502, pECA2, pBCA2, pBCB2, pECB2 and pECB2.2 as indicated, induced with acetosyringone (+) or uninduced (-). Activities are expressed as the absorbance at 650 nm per μg protein in each sample. No uninduced values were determined for C58C1(pDUB2501) or C58C1(pDUB2502).

shift' cannot be discounted (see section 3.2). The high backgrounds are probably not due to other promoters, in the broad host range plasmids, being able to transcribe the *chi* genes, since pECA2 in *A. tumefaciens* C58C1 in the absence of pVK257, produces much less background activity than when *virA* and *virG* are present. One explanation is that VirG could have binding-affinity with the *vir* boxes without being phosphorylated, something that has been observed previously [234].

The other feature of these results is that the plasmids based on pUCD4 (pECA2 and pBCB2) both seem to be capable of producing much more chitinase than the pGV1106 based plasmid, pBCA2. This might suggest a copy number difference between the two types of plasmid, as discussed above. Another explanation would be that the plasmid is not very stable in *A. tumefaciens* and thus is soon lost in the absence of selective pressure.

The results from strain C58C1(pVK257, pECB2) were not encouraging. It was apparent that another promoter was swamping the action of the *virE* promoter and thus inducibility was not observed. pECB2.2 was created in order to examine the effect of two *virE* promoters directing the transcription of the same gene. It was apparent that the addition of an extra 0.8 kb DNA fragment had attenuated expression of the gene from the putative 'promoter', and this plasmid was inducible. However, whether the addition of a second *virE* promoter increases expression is not evident from this experiment due to the action of the putative 'promoter'.

The nature of this 'promoter' is unclear. P_{lac}, on pUC19, transcribes in the opposite direction to the *chiB* gene in pCB1, and subsequent subcloning into pKT230 completely removes it from the vicinity of the *vir*::*chi* region. Nor is P_{bla}, responsible for directing the transcription of the ampicillin-resistance gene, a likely candidate, due to the action of terminators at the end of this gene. It could be that the cloning steps have generated a 'promoter' sequence 5' to *chiB* in pCB1. pBCB2 should exhibit a similar phenomenon. This is not the case (see figure 4.5.3.a) which might be explained due to the nature of the *virB promoter* fragment insert, which was 3 kb in size - much larger than the 0.8 kb *virE* promoter fragment - allowing a similar situation to occur as is proposed for pECB2.2, in which the second *virE* fragment attenuates the action of the putative 'promoter'.

The reasons for the lack of inducibility of pECB2 remain uncertain. Due to a lack of time, no attempt was made to remake the plasmid or alter pECB2 to try to allow it to express *chiB* in an inducible manner, as expected.

4.5.4 Secretion of ChiA by *A. tumefaciens*

Since there was much more ChiA production in strain C58C1(pVK257, pECA2) induced with acetosyringone, than in C58C1(pDUB2501), assays were performed on cellular fractions from these strains to determine whether ChiA was secreted when expressed at higher levels (see section 4.5.1). The results, displayed in figure 4.5.4.a showed that the periplasm was the major site for ChiA accumulation, although some activity appeared to be secreted from C58C1(pVK257, pECA2). Much chitinase activity was also present in the wash fraction, in this strain, suggesting that a secretion pathway is open to ChiA, but a final stage is being blocked.

In section 4.3.3, it was argued that ChiA and ChiB are secreted by *E. coli,* because they mimic outer membrane proteins, but fail to become anchored. The same may be true of *A. tumefaciens,* the exception being that in the case of ChiA, the protein becomes loosely anchored in the outer membrane, from which it is released by washing the cells.

4.5.5 Phenotypic analysis of *A. tumefaciens* expressing chitinase

A. tumefaciens strains expressing either ChiA or ChiB were examined using a Nikon Optiphot light microscope with phase contrast optics. All strains appeared to exhibit a wild type phenotype and neither chitinase appeared to have a detrimental affect on the bacterial cells when grown at 28 °C (all strains exhibited similar rates of growth). However, phenotypic investigations using different culture conditions (especially temperature) were not performed and it remains possible that an effect may be apparent at higher or lower temperatures.

4.6 Pesticidal action of *A. tumefaciens* expressing chitinase

4.6.1 Fungicidal nature of *S. marcescens*

There have been many reports of *S. marcescens* inhibiting the growth of fungi. Ordentlich *et al.* [228] found that *S. marcescens* was capable of controlling *Thanatephorus cucumeris* and *Sclerotium rolfsii* when applied to soils containing the fungi. The same group subsequently found that this biocontrol was due to the chitinases produced by *S. marcescens* [229]. Shapira *et al.* found that a crude preparation of ChiA, and *E. coli* expressing ChiA, were effective in reducing the incidence of disease caused by these two fungi [53, 269]. In addition, Jach *et al.*

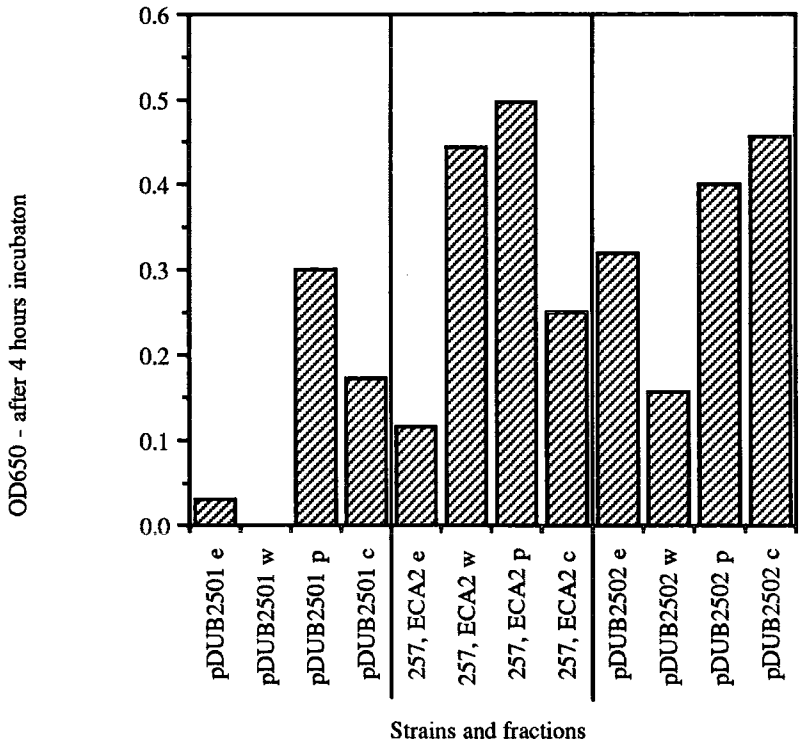


Figure 4.5.4.a Chitinase activities (expressed as absorbance at 650 nm), after 4 hour incubations with CM-chitin-RBV, of cell fractions of various strains of *A. tumefaciens*: C58C1(pDUB2501), C58C1(pDUB2502) and C58C1(pVK257, pECA2).

found that tobacco plants expressing ChiA were more resistant to *T. cucumeris* infection than wild type plants [143]. Sitrit *et al.* demonstrated that ChiA expressed by *Rhizobium meliloti* was able to lyse the hyphal tips of *T. cucumeris* [279]. Sundheim *et al.* have shown that *Pseudomonas* spp. expressing chitinases from *S. marcescens* were able to limit the hyphal growth of the same fungus and of *Magnaporthe grisea* and *Fusarium oxysporum* [298]. Jones *et al.* found that *E. coli* cells expressing ChiA were seen to inhibit the elongation of germ tubes formed by *F. oxysporum* spores [157]. However, Roberts and Selitrennikoff found that purified ChiA was not able to inhibit the growth of *Trichoderma reesi* or of *Phycomyces blakesleeanus* [248].

For the assays, three fungi were used: *Thanatephorus cucumeris,* a plant pathogen causing rice sheath blight [206]; *Fusarium oxysporum* which causes various types of wilt and damping-off [283]; and *Phycomyces blakesleeanus*, which causes certain rot diseases in plants like potato [47].

5 ml cultures of *S. marcescens* grown for 48 hours at 28 °C in LM-broth containing 5 $mg.ml^{-1}$ purified crab shell chitin (Sigma), were used in the assays. 5 µl aliquots were pipetted onto the surface of duplicate plates, each having a small sample of fungal mycelium placed in the centre (see section 2.5.9). One set of plates was placed in the dark at 28 °C, the other in a 16 hour day growth cabinet at 28 °C.

S. marcescens showed zones of inhibition towards dark grown *P. blakesleeanus* and light and dark grown *T. cucumeris*. *F. oxysporum* was seen to be inhibited better in the light grown example, although inhibition did occur in both experiments. The reasons for these differences are not known but are almost certainly due to the differing rates of growth and sporulation exhibited by the fungi in the different conditions.

The hyphae around the zones of inhibition of *P. blakesleeanus* (see figure 4.6.1.a) were examined microscopically. The 'inhibition zone' was seen to be mainly an absence of aerial hyphae, whereas the mycelial matrix within the agar was less affected. The experiments performed by Roberts and Selennitrokoff suggested that ChiA was ineffective in inhibiting the growth of this fungus [248]. However, their experiments were performed at 22 °C and on a richer medium (PDA - see section 2.2.3) which may account for this difference compared with *S. marcescens*. Alternatively, ChiA might be ineffective and other chitinases or degradative enzymes secreted by *S. marcescens* were responsible for the inhibition observed in these experiments.

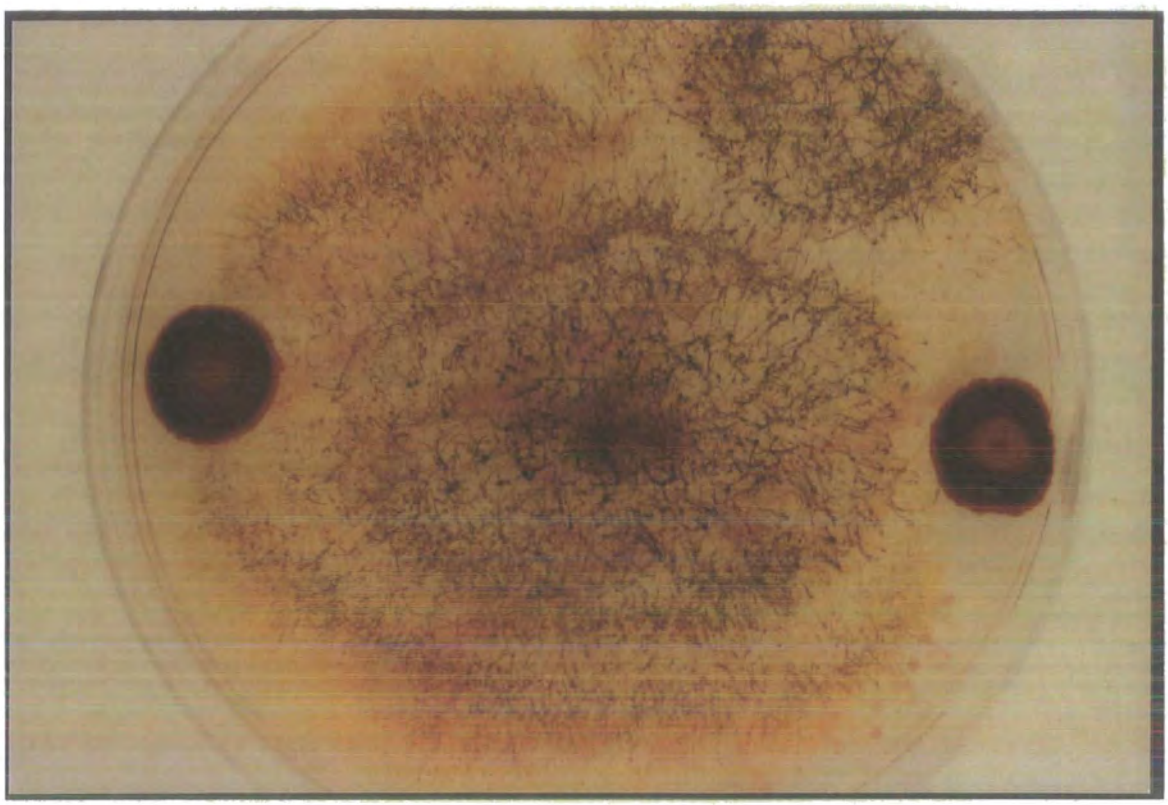


Figure 4.6.1.a Zones of inhibition of mycelial growth of *P. blakesleeanus* caused by *S. marcescens*.

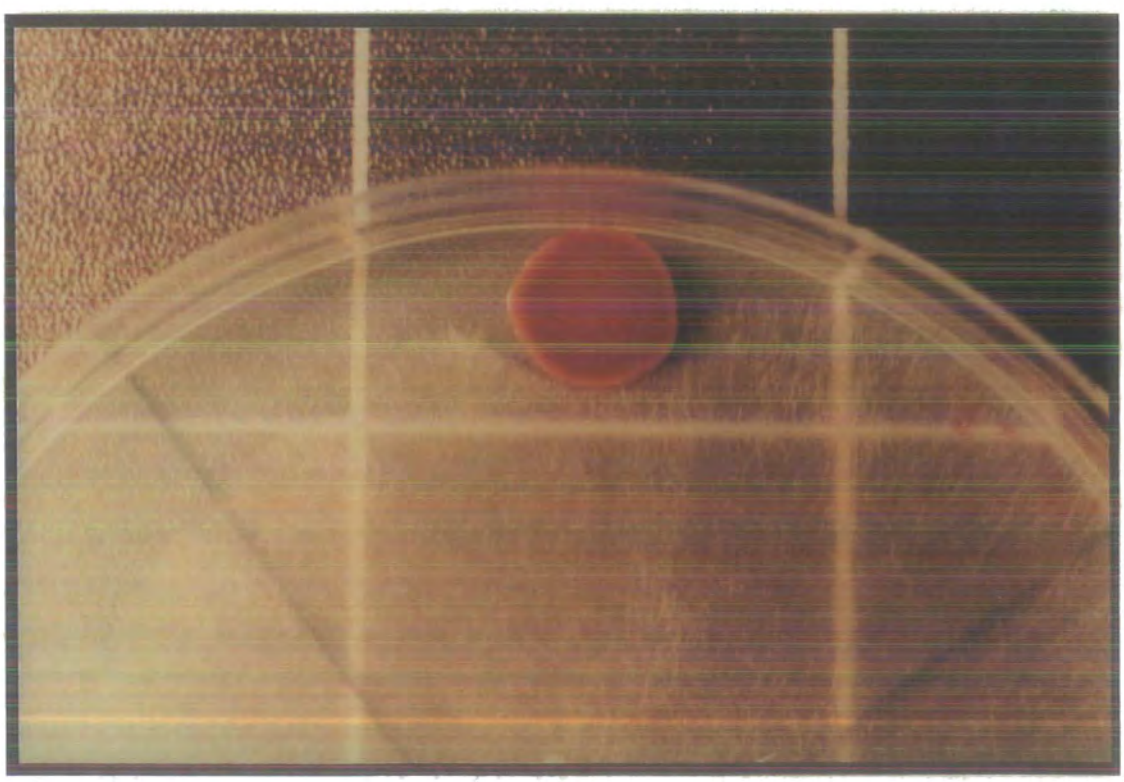


Figure 4.6.1.b Inhibition of *T. cucumeris* growth by *S. marcescens*.

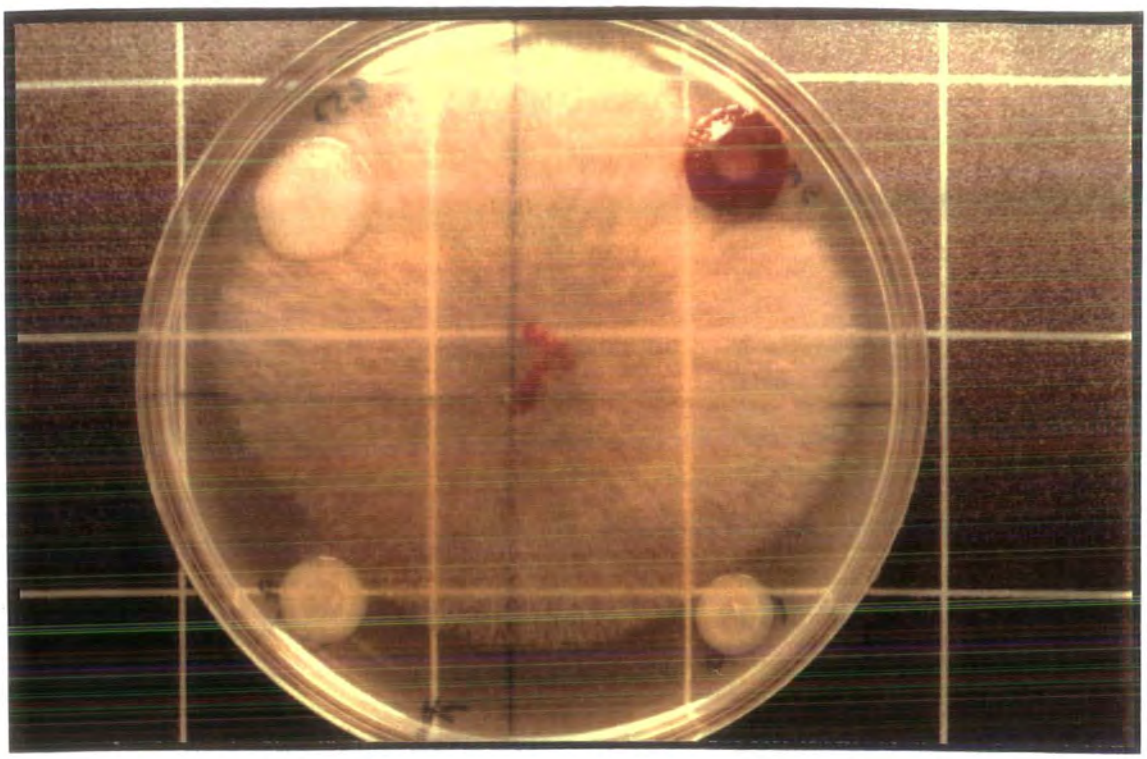
The cell wall of *P. blakesleeanus* is composed of three regions: an outer amorphous layer containing glycoprotein; a middle chitinous region; and a plasma membrane [47]. Of the three fungi assayed, the least inhibited was *P. blakesleeanus* and it is likely that the structure of the cell wall is responsible. The chitinases both appear to act at the termini of the chitin fibrils and if these are surrounded by a layer of glycoprotein, the target sites may not be exposed, making lysis of the hyphal tips difficult. Furthermore, the glycoprotein layer may also act to form a temporary barrier across any lesions that are inflicted upon the hyphae by the chitinases. It is only in the most actively growing part of the mycelium (i.e. the hyphal tips) that chitin will be exposed and the highly branched nature of the mycelium will soon allow for some hyphae to become mature before the actions of the chitinases can have an effect. The fungus might also be able to adapt to the presence of chitinases by producing more chitin synthase.

The action of chitinase on other fungal hyphae appears to be more direct in the absence of a glycoprotein layer. Holes have been shown to appear in the hyphae of *Sclerotium rolfsii* when incubated with *S. marcescens* [229].

Figure 4.6.1.b shows a zone of clearing in the mycelial mass of *Thanatephorus cucumeris*, surrounding a large colony of *S. marcescens*. Microscopic analysis of this zone revealed that the fungus was not present in this area and both aerial and mycelial hyphae were inhibited. However, after a few days the mycelium had overcome the inhibitory effect of the *S. marcescens* colony. This is again probably due to the highly branched nature of the mycelium eventually producing hyphae that reach a more mature stage before chitinase is able to produce holes that lead to hyphal swelling and lysis.

Figure 4.6.1.c shows a large inhibitory zone in the mycelium of *Fusarium oxysporum* in the vicinity of the *S. marcescens* colony. This zone of inhibition was the largest of the three fungi tested, although there was no definite cessation of fungal growth around the bacterial mass, as was observed with *T. cucumeris*. Thus it might be suggested that the hyphae of *T. cucumeris* are of a more homogenous type than those of *F. oxysporum*, which possess some differences in the cell wall structure between the aerial and the mycelial hyphae. Throughout the zone of inhibition in the latter case, there was a lack of aerial hyphae and the mycelial hyphae appeared to be much thinner and less branched.

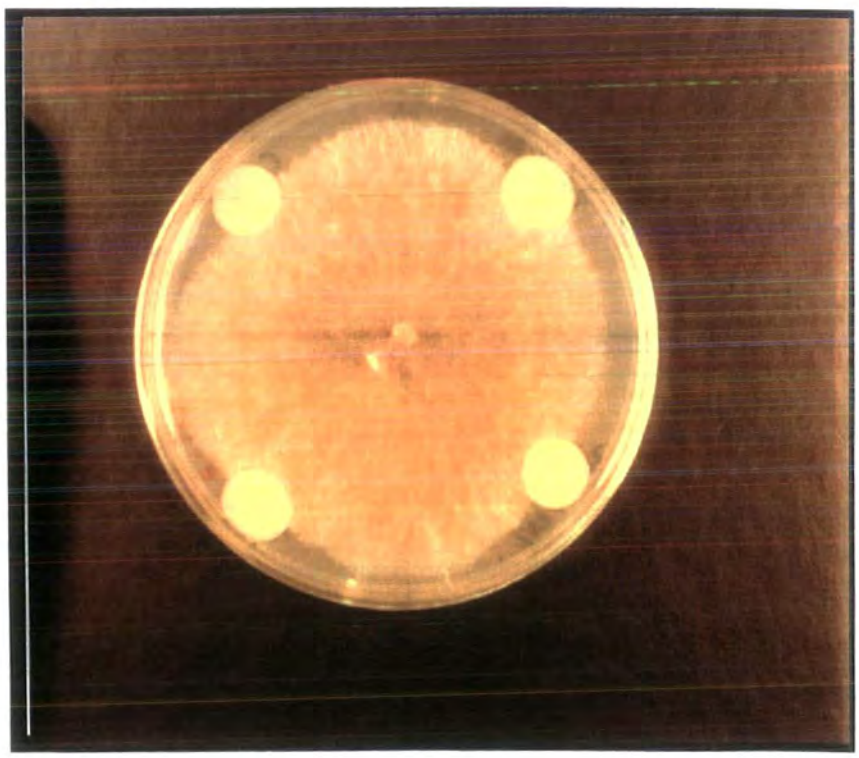
A D



B C

Figure 4.6.2.a Mycelial growth of *T. cucumeris* affected by various bacterial strains: *A. tumefaciens* C58C1(pVK257) (A), *E. coli* DH5α(pDUB2501) (B) and DH5α(pDUB2502) (C), *S. marcescens* (D).

A D



B C

Figure 4.6.2.b Mycelial growth of *T. cucumeris* affected by various *A. tumefaciens* strains: C58C1(pDUB2501) (A), C58C1(pECA2) (B), C58C1(pVK257, pECA2) (C), C58C1(pVK257, pBCA2) (D).

The greater inhibition observed with *F. oxysporum* relative to *T. cucumeris* is probably a function of the cell wall compositions of the two fungi. The cell walls of *F. oxysporum* are composed of up to 30 % chitin, whilst those of *T. cucumeris* consist of only about 5 % [53].

It was apparent that *S. marcescens* was able to inhibit the growth of these three fungi to different extents, probably reflecting the different compositions and constructions of the respective cell walls.

4.6.2 Fungicidal properties of *A. tumefaciens* expressing chitinase

Similar assays were performed using chitinase-expressing *A. tumefaciens* strains. 24 hour cultures were grown in LM-broth and 5 µl aliquots were placed on the surface of plates containing Rogowsky medium and 100 µM acetosyringone solidified with 1 % bacteriological agar (Davis medium solidified with agar was not able to support active growth of *A. tumefaciens* colonies). *E. coli* strains DH5α, DH5α(pDUB2501) and DH5α(pDUB2502) were also plated out on this medium. Samples of fungal mycelium were placed in the centre of the plates, which were then incubated at 28 °C - in a dark incubator for *T. cucumeris* and *P. blakesleeanus,* and in a 16 hour day growth cabinet for *F. oxysporum.*

Both DH5α(pDUB2501) and DH5α(pDUB2502) were clearly seen to inhibit growth of *T. cucumeris* (see figure 4.6.2.a). However, no inhibition of *P. blakesleeanus* was apparent. *F. oxysporum* was unable to grow on Rogowsky agar. DH5α showed no inhibition of growth towards either fungus.

The results from the experiments involving *A. tumefaciens* were less successful. Again no inhibition of *P. blakesleeanus* was observed. Some slight inhibition of *T. cucumeris* was observed using *chiA*-expressing strains. C58C1(pVK257, pBCA2) showed little or no inhibition of the growth of the fungus, whereas the leading edge of the mycelium was 'flattened' by the presence of C58C1(pVK257, pECA2), C58C1(pECA2) and C58C1(pDUB2501). The mycelial hyphae were also observed to be slightly less thick in the vicinity of C58C1(pDUB2501) and C58C1(pVK257, pECA2). This was surprising in the light of the previous studies suggesting that ChiA is not secreted well by *A. tumefaciens*, although the release of the protein from the cells may be different from colonies than cells cultured in liquid media.

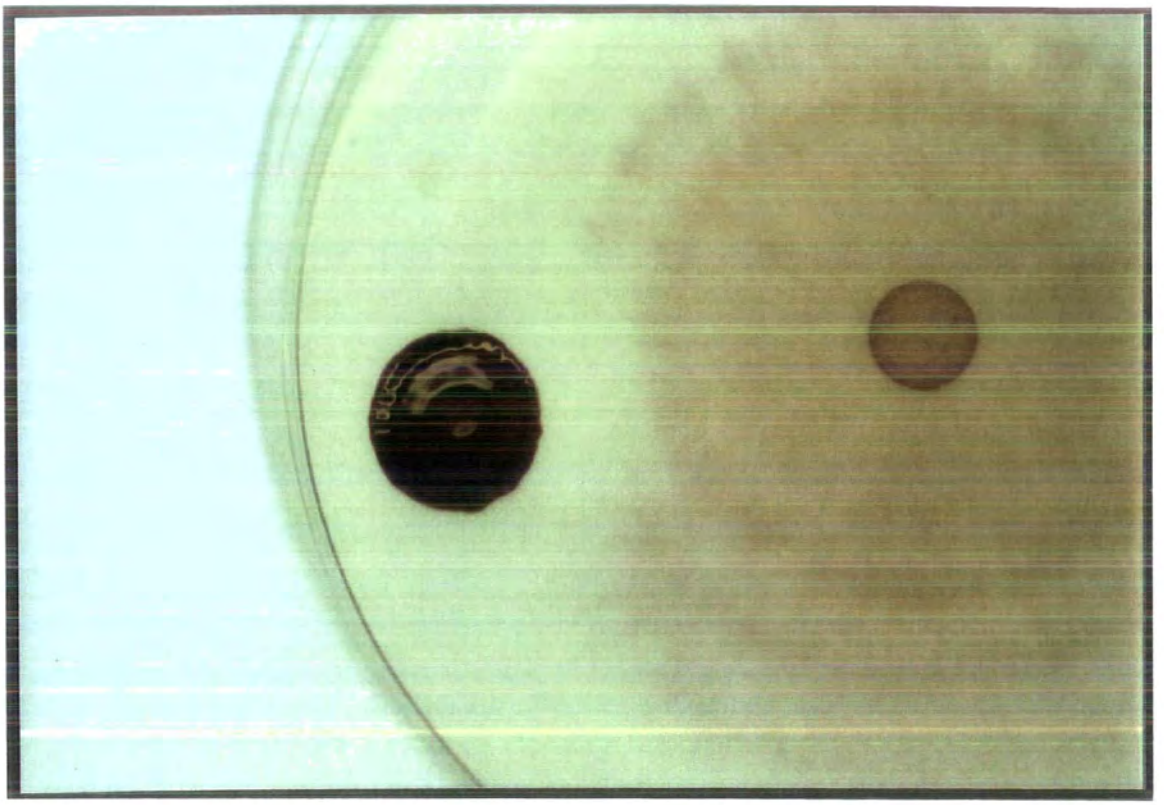


Figure 4.6.1.c Zone of growth inhibition of *F. oxysporum* by *S. marcecsens*.

Since DH5α(pDUB2502) was able to inhibit growth of *T. cucumeris*, while DH5α could not, it was expected that the *chiB*-expressing strains of *A. tumefaciens* would exhibit some inhibition of the fungus, especially considering that it seems to be secreted more effectively from *A. tumefaciens* than ChiA is. However, no inhibition was observed, the reasons for this being unclear. It may be that *vir* induction was not occurring in these strains, or had been 'switched off' in the outer cells of the colony. The secretion of ChiB may also differ between bacterial cells forming a colony and those grown in liquid media. Alternatively, plasmids may have been lost from the cells in the colonies.

These assays, although confirming growth inhibition by *chi*-expressing *E. coli* strains did not produce conclusive results when used with *A. tumefaciens*. The assays themselves are probably quite insensitive since secreted chitinases are required to have an observable action against a large area of actively growing hyphae. Secretion of the chitinases may also be different in the bacterial colonies, or plasmids may have been lost, or indeed *vir* induction may not be evident, although the plate assays performed using *vir*::*lacZ* constructs (see section 3.3) suggest that genes linked to *vir* promoters will be expressed consistently during the course of the experiment.

The assays are also far removed from the situation found in the soil, where the mycelia are more dispersed and the initial stages of mycelium development and germination of spores might also be countered by the presence of chitinase-expressing strains.

It is hoped that disease symptoms caused on plants by these fungi can be observed when grown in soil in the presence and absence of the *chi*-expressing bacteria, and plans are underway to conduct such experiments.

4.6.3 Insecticidal properties of *A. tumefaciens* expressing chitinase

Work conducted by ICI at Jealotts Hill using DH5α(pDUB2501), DH5α(pDUB2502), C58C1(pDUB2501) and C58C1(pDUB2502), suggested that ChiB was effective in controlling *Diabrotica balteata* [187]. Since chitin forms a major component of insect exo-skeletons, the mouth parts of the larval stages of some insects may well be sensitive to the action of chitinases [212]. Damage to the mouthparts could result in a lack of feeding and eventually larval death.

Thus trials were conducted using the lepidopteran insect pest *Manduca sexta* (the tobacco horn worm). Cultures of *chi*-expressing *A. tumefaciens* and *E. coli* were grown for 48 and 24 hours respectively in LM-broth containing antibiotic selection. Subcultures were grown up in 5 ml Davis medium (or LM-broth for *E. coli*) for 24 hours before an estimation of the bacterial cell numbers was made (see section 2.4.3). The bacteria were diluted with fresh Davis medium (or LM-broth) to a concentration of 1 x 10^8 ml^{-1} and 50 µl aliquots of these suspensions were spread across the surface of the insect diet (see section 2.5.8). Each strain was tested on 6 larvae which were observed for 7 days.

At no time during the experiment was any difference in growth or development noticed between the larvae feeding on chitinolytic bacteria and those growing on diet without the bacteria.

Lack of plasmid stability in these strains or lack of cell viability on the diet was unlikely to be the reason for the lack of activity against *M. sexta*, since the bacteria were readily reisolated from the plates and seen to grow on the appropriate selective media. A lack of expression of the *chi* genes under *vir* promoter control, when grown on the insect diet, is possible, but since strains that actively produce ChiA and ChiB (DH5α(pDUB2501) and DH5α(pDUB2502)) also failed to inhibit the growth of the larvae, this does not appear to be the reason that the *A. tumefaciens* strains failed to control the larvae.

It may be that the effectiveness of ChiB on insect larvae is specific to a few insects. *Manduca sexta* does not appear to be adversely affected by the presence of the chitinase-producing bacteria, whereas *Diabrotica balteata* does. The latter is a coleopteran insect whereas *Manduca sexta* is lepidopterous and herein may lie the reasons for the differences observed. Future trials should be conducted using other coleopteran insects to determine whether ChiB is effective against a range of these insects or simply just *Diabrotica balteata*.

4.7 Summary

Two chitinases from *S. marcescens*, ChiA and ChiB, have been shown to degrade chitin from the termini of chitin fibrils, ChiA having a broader range of activity than ChiB which must be considered to be a strict exochitinase. Both genes are expressed efficiently from their natural promoters in *E. coli* and both appear to be secreted. ChiB, however, has an adverse effect on *E. coli* cell division at

intermediate temperatures, probably by blocking the export pathway of one or two proteins (HtrC and DsbA). The cells of this strain appear filamentous at 37 °C.

The genes, *chiA* and *chiB*, can also be expressed from their natural promoters in *A. tumefaciens* although *chiA* is less well expressed than *chiB*. Replacement of the natural promoters with *vir* promoters leads to inducible production of both chitinases in response to acetosyringone, a compound commonly produced by plant wound sites [285, 289]. Both ChiA and ChiB appear to be secreted by *A. tumefaciens*, ChiA less well than ChiB, which may be due to an interaction with the outer membrane.

S. marcescens was seen to cause inhibition of growth of three fungi, *Phycomyces blakesleeanus, Thanatephorus cucumeris* and *Fusarium oxysporum* under certain conditions. ChiA and ChiB, expressed by *E. coli*, were seen to inhibit the growth of *T. cucumeris* but not of *P. blakesleeanus*. There appears to be slight inhibition of *T. cucumeris* mycelial growth in response to ChiA expressed by *A. tumefaciens*.

Neither ChiA nor ChiB was seen to be able to control the growth and development of the larvae of the lepidopteran insect, *Manduca sexta*, although ChiB has been shown previously to be insecticidal towards the coleopteran insect *Diabrotica balteata* [187].

These results suggest that the chitinases from *S. marcescens* may be useful in the biocontrol of fungi and some insect pests. The inducible high levels of expression (and some secretion) of these chitinases controlled by *vir* promoters in *A. tumefaciens*, suggest that this 'microbial inoculant' could be effective in the field.

4.8 Future work on this system

The disappointing results using the *A. tumefaciens* strains to try to control the growth of fungi may not be cause for concern, since the assay would appear to be insensitive. A better assay for their effectiveness would be to use the system against the fungi in infected soils containing plants. Such experiments are planned.

The fact that *Diabrotica balteata* has been shown to be controlled by ChiB, suggests that further experiments should also be carried out using other insects, particularly coleopterans.

One further avenue of research lies in the use of these chitinases to control nematode worms. The chitinases from *S. marcescens* have been shown to increase the hatching rate of embryonated nematode eggs [204]. This rate increase results in the premature hatching of the eggs causing greater juvenile mortality. Another group has suggested that *S. marcescens* chitinases kill younger eggs [145], whilst others have suggested that some juveniles can be killed by chitinases [208]. Using the soil-borne chitinase-expressing *Agrobacterium* 'microbial inoculant' system, described above, it may be possible to control nematodes to some extent.

Future work should also involve the coexpression of various pesticidal proteins, including chitinase, in *A. tumefaciens*. Proteins for consideration should include the δ-endotoxin from *Bacillus thuringiensis* (see Chapter 5) and β-1,4-glucanase (see section 4.2). Work should also be carried out to establish whether ChiA and ChiB are synergistic with one another.

The possible synergistic effects of such coexpressed proteins may lead to an effective biocontrol agent for use against fungi, nematodes and insects.

Chapter 5

'MICROBIAL INOCULANTS' USING A δ-ENDOTOXIN FROM *BACILLUS THURINGIENSIS*

5.1 *B. thuringiensis* var. *kurstaki* 4D4

B. thuringiensis var. *kurstaki* 4D4 contains the gene for one δ-endotoxin, *cryIA(c)* [3], which is present on a large 77 kb plasmid [108, 182] and probably also present on the chromosome [44]. The gene has been cloned previously and shown to be expressed in *E. coli* [3]. Since the sequence was available [3] and much research has been directed into the elucidation of the functioning and characteristics of this δ-endotoxin (see sections 1.16/17), *cryIA(c)* was chosen to be used as the active part of an *A. tumefaciens* 'microbial inoculant'.

Expression of *cryIA(c)* leads to the production of a 133 kD protein consisting of 1178 amino acids [3], which is converted to the active 67 kD toxin in the insect midgut [6, 56]. Toxin molecules recognise specific receptors in the insect midgut to which they bind. Pores are formed resulting in an ion imbalance in the cells which subsequently lyse [68, 136, 176] (see section 1.17), causing insect death.

CryIA(c) is lepidopteran specific, affecting only the larvae of certain moths and butterflies, notably *Manduca sexta*, the tobacco hornworm [3, 133]. In this study it was hoped to be able to produce a 'microbial inoculant' that was able to express this gene in response to a chemical often found in plant wounds, and that this toxin would then be present in high enough concentrations to kill the larvae of *Manduca sexta*.

5.2 Expression of a *B. thuringiensis* δ-endotoxin in *E. coli*

5.2.1 Cloning of *cryIA(c)* - construction of pBGB1

A plasmid, pIC4, containing the lepidopteran specific δ-endotoxin *cryIA(c)* gene, was obtained from ICI Seeds (Jealott's Hill, Berks., U.K.) [101]. However, total cell extracts (see section 2.4.5.b) of DH5α(pIC4) did not appear to contain the δ-endotoxin as judged by western blots using an anti-CryIA(c) antibody [101] (see sections 2.5.5.c/d). Furthermore, this strain appeared to be less toxic to the larvae of *Manduca sexta* than DH5α, using the insect bioassay (see section 2.5.8) with 24 hour cultures of the relevant strains in LM-broth, although *Manduca sexta* was the insect most commonly used in experiments of this type [2, 3, 6, 46, 224, 262, 265, 330].

It was possible that the lack of expression of *cryIA(c)* was due to its promoter being non-functional in *E. coli*. However, there were reports of the same or similar δ-endotoxin genes being cloned and expressed in *E. coli* using the natural *B. thuringiensis* promoters [3, 327].

pIC4 consists of a 17 kb fragment of a large plasmid from *B. thuringiensis* subsp. *kurstaki* HD-73 cloned into pUC19 [101]. Within this fragment there are three insertion sequences (see figure 5.2.1.a) [183, 203]. It had also been found that *B. thuringiensis* plasmids are commonly found to contain rearrangements and can become integrated into the chromosome [45, 108]. Kronstad and Whiteley suggested that these rearrangements and integrations were due to the insertion sequences found in close proximity to the δ-endotoxin genes [183] and it appears that this is the case [203]. It was subsequently found that both types of insertion sequence present in pIC4 (IS231 and IS232) were functional and able to transpose in *E. coli* [203]. It was therefore possible that a transposition event had occurred at some stage in this plasmid rendering the δ-endotoxin gene or its promoter non-functional. On this basis it was decided to reclone the HD-73 δ-endotoxin gene.

B. thuringiensis subsp. kurstaki 4D4 (formerly HD-73) was kindly provided by Dr. D. R. Zeigler from The Bacillus Genetic Stock Center (Ohio State University, Ohio, U.S.A.). Plasmid DNA was prepared (see section 2.5.1.d) and 10 μl of the preparation was electrophoresed through a 0.7 % agarose gel (see section 2.5.3.g) to check for the presence of plasmids corresponding to 7.5, 8.0, 8.3, 11.5 and 77 kb [108]. Figure 5.2.1.b clearly shows a range of plasmids corresponding

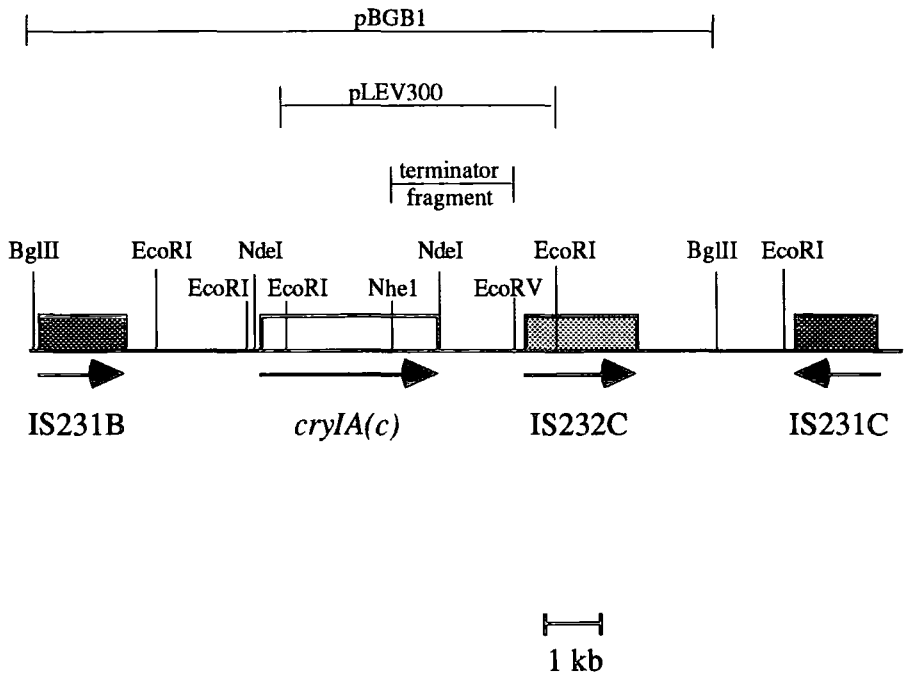


Figure 5.2.1.a - 17 kb fragment of a 77 kb plasmid from *B. thuringiensis* var. *kurstaki,* containing *cryIA(c)*, used in the construction of pIC4. Diagram shows the relative positions and orientations of three insertion sequences found in this piece of DNA. Positions of EcoRI, NdeI and BglII restriction sites also shown. After Menou *et al.* and Kronstad and Whiteley [Menou, 1990 #504; Kronstad, 1984 #178]. Also shown are the fragments used to create pBGB1, pLEV300 and the terminator fragment used to create the other pLEV3xx series plasmids.

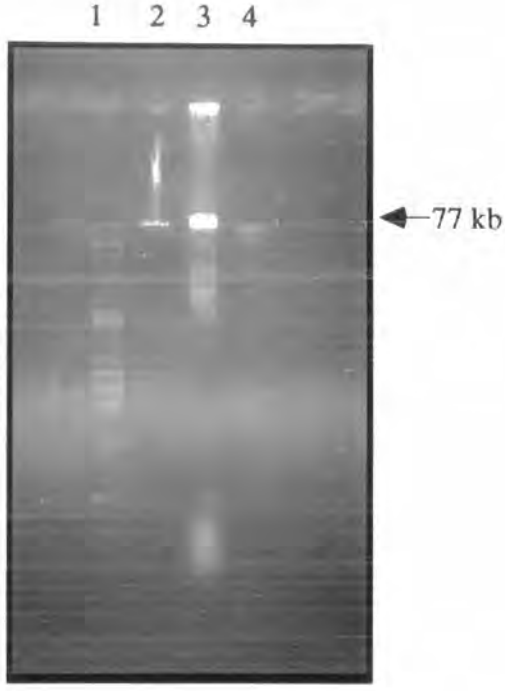


Figure 5.2.1.b - Photograph of a 0.7 % agarose gel, lanes containing DNA (from left) λ PstI (14.05, 11.49, 5.07, 4.75, 4.51, 2.84, 2.5 kb...); unrestricted wild type λ (50 kb); *B. thuringiensis* var. *kurstaki* 4D4 plasmid DNA; λ KpnI (29.95, 17.06, 1.5 kb). Bands roughly corresponding in size to 7.5, 8.0, 8.3, 11.5 and 77 kb can be seen in the lane containing *B. thuringiensis* plasmid DNA.

Figure 5.2.1.c - Showing restriction patterns of *B. thuringiensis* var. *kurstaki* 4D4 plasmids digested with various enzymes:

1 - λ PstI (14.05, 11.49, 5.07, 4.75, 4.51, 2.84, 2.5, 2.14, 1.99, 1.7, 1.16, 1.09, 0.81 kb ...)
2 - Uncut *B. thuringiensis* plasmids
3 - λ EcoRI (21.23, 7.42, 5.80, 5.64, 4.88, 3.53 kb)
4 - BglII-cut *B. thuringiensis* plasmids
5 - λ EcoRI
6 - EcoRI-cut *B. thuringiensis* plasmids
7 - λ EcoRI
8 - HindIII-cut *B. thuringiensis* plasmids
9 - λ PstI
10 - NdeI-cut *B. thuringiensis* plasmids
11 - λ EcoRI
12 - NdeI-cut pIC4
13 - λ EcoRI
14 - AccI-cut pBGH1 (see section 5.2.4)
15 - λ PstI

approximately to the sizes given by González *et al.* [108]. The plasmid profile suggested that no recombination or insertion events had occurred in this strain [45, 108].

The growth conditions used in the plasmid isolation method were optimised for the production of large plasmids [182]. As can be seen from figure 5.2.1.b, the 77 kb plasmid band (which actually contains two plasmids of similar size), appears to contain much more DNA than the other plasmid bands.

20 µl aliquots of the *B. thuringiensis* plasmid preparation were digested with a range of restriction enzymes and electrophoresed through a large 0.7 % agarose gel (see figure 5.2.1.c) and Southern blotted (see sections 2.5.3.b/c/g/j).

pIC4 was digested with NdeI and a 3.8 kb DNA fragment was isolated using electroelution (see sections 2.5.3.b/g/h). 50 ng of the resulting DNA was radiolabelled with ^{32}P (see sections 2.5.3.a/k) and hybridised to the Southern blot, as prepared above (see section 2.5.3.l). The 3.8 kb NdeI fragment should have contained the complete coding region from the δ-endotoxin gene (see figure 5.2.1.a) and it was hoped that even if the gene in pIC4 had somehow become non-functional, the labelled fragment would still be able to hybridise effectively to the natural gene fragments from *B. thuringiensis*. Figure 5.2.1.d shows that this was the case, although some 'extra bands' were detected above the 3.8 kb band in the lane containing NdeI-cut plasmids. This was thought to be due to partial digest products liberated by inefficient restriction by this enzyme.

Figure 5.2.1.d also shows that the gene was located in one of the large 77 kb plasmids, as was found in earlier studies [108, 182] and confirms that the entire coding region is located within a BglII fragment of about 13.5 kb (see figure 5.2.1.a).

This BglII fragment was used for the subsequent cloning of the gene since it contained not only the entire coding region but also the promoter and terminator sequences, which the 3.8 kb NdeI fragment did not (see figures 5.2.1.e) [183].

The *B. thuringiensis* plasmids were digested with BglII and electrophoresed through an agarose gel, the fragment corresponding to approximately 13.5 kb being isolated using silica fines (see sections 2.5.3.b/g/h). The DNA fragment was then ligated with BamHI-digested/phosphatased pUC19 (see sections 2.5.3.b/c/f). The ligation mixture was transformed into *E. coli* DH5α (see section 2.5.2.a) and

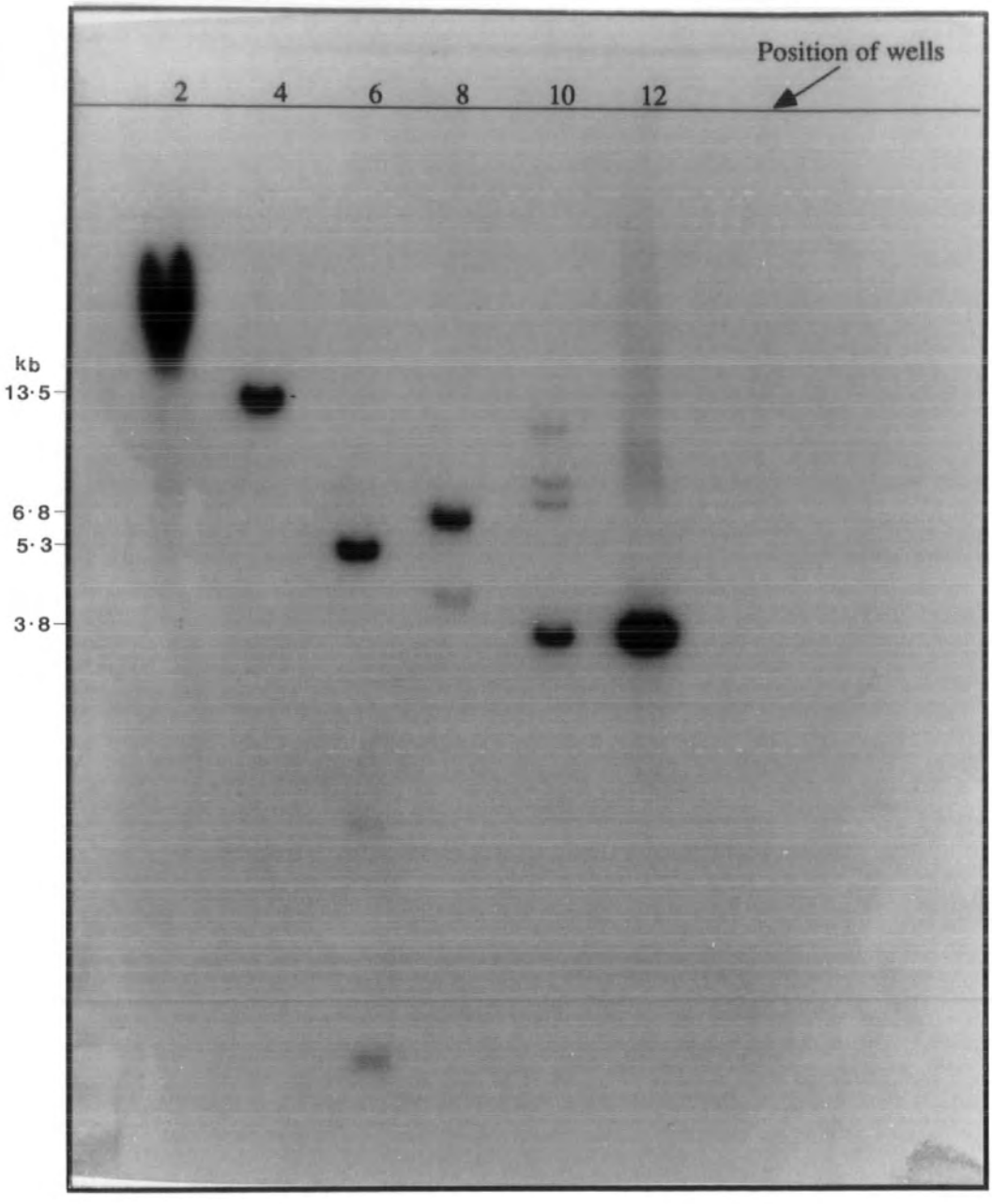


Figure 5.2.1.d - Southern blot of gel shown in figure 5.2.1.c. Bands of hybridisation found in lanes cut with (from left): uncut; BglII, EcoRI, HindIII, NdeI and pIC4 cut with NdeI. Shown full size.

DraI (-164)

AAAGTGGATTTTATATATAAGTATAAAAAGTAATAAGACTTTAAAATAA

HincII

GTTAACGGAATACAAACCCTTAATGCATTGGTTAAACATTGTAAAGTCT

HincII (-75)

AAAGCATGGATAATGGGCGAGAAGTAAGTAGATTGTTAACACCCTGGG

A/T rich region of promoter

TCAAAAATTGATATTTAGTAAAATTAGTTGCACTTTGTGCATTTTTTCAT

BtII NdeI EcoI BtI

AAGATGAGTCATATGTTTTAAATTGTAGTAATGAAAAACAGTATTATATC

S-D Start

ATAATGAATTGGTATCTTAATAAAAGAGATGGAGGTAACTTATGGATAA

CAATCCGAACAT.....3493 bp.....

Stop

.....GAATTACTCCTTATGGAGGAATAGTCTCATGCAAAC

DraI

TCAGGTTTAAATATCGTTTTCAAATCAATTGTCCAAGAGCAGCATTACAA

ATAGATAAGTAATTTGTTGTAATGAAAAACGGACATCACCTCCATTGAA

NdeI

ACGGAGTGATGTCCGTTTTACTATGTTATTTTCTAGTAATACATATGTAT

AGAGCAACTTAATCAAGCAGAGATATTTTCACCTATCGATGAAAATATCT

CTGCTTTTTCTTTTTTTATTTGGTATATGCTTTAC

Preferable transcription termination site

Figure 5.2.1.e - showing 5' and 3' ends of *cryIA(c)*. DraI, HincII and NdeI restriction sites are shown, the numbers in brackets corresponding to the distance away from the *E. coli* transcription start site in bp. BtI and BtII are the native *B. thuringiensis* transcription start sequences, EcoI is the *E. coli* transcription start sequence. S-D is the Shine-Dalgarno sequence. Start and stop sites for translation are boxed. Stem and loop transcription terminators are underlined [3, 338. 339].

Figure 5.2.1.f - Photograph of 0.7 % agarose gel: lanes from left: 1 - λ PstI; 2 - uncut pBGB1; 3 - EcoRI-cut pBGB1. pBGB1 is pUC19 containing a 13.5 kb BglII fragment (see figure 5.2.1.a).

ampicillin resistant colonies unable to produce a blue colouration were identified on selective agar plates containing ampicillin and X-gal (see section 2.5.6.i). Colonies selected in this way were cultured in 5 ml LM-broth overnight and plasmid preparations were obtained (see section 2.5.1.a). Restriction digests of these plasmid preparations revealed one containing the predicted banding patterns from the restriction map of Kronstad and Whiteley [183] and this was designated pBGB1 (see figures 5.2.1.a/f).

An ELISA was performed on total extracts of DH5α(pBGB1) and DH5α(pUC19) boiled for 10 minutes in Bietlot buffer (see sections 2.5.4.b and 2.5.5.a/e) using the anti-CryIA(c) antibody [101]. This showed that DH5α(pBGB1) produced a protein that was able to cross-react with the antibody whereas DH5α(pUC19) did not. To confirm that this was the δ-endotoxin, a western blot of the same cell extracts was immunoscreened with the same antibody (see sections 2.5.5.a/c/d). The result showed that the antibody was able to hybridise to a band present in the extract of DH5α(pBGB1), but not in DH5α(pUC19), which was approximately 130 kD (data not shown). The 130 kD band matched the reported size for the CryIA(c) δ-endotoxin [3, 327] and thus it appeared that the cloned gene and the natural *B. thuringiensis* promoter were functional in this plasmid.

The 13.5 kb insert in pBGB1 was also much shorter than the pIC4 insert and lacked one of the insertion sequences, which it was hoped would allow the plasmid to be more stable than pIC4. pBGB1 was used to subclone the gene and analyse the effects of the presence and absence of the natural promoter and terminator regions.

5.2.2 Subcloning *cryIA(c)*

The functioning of the promoter and terminator sequences was analysed, as was the effect of the *lacZ* promoter (P_{lac}) from pUC19, to determine what effect each would have on the overall expression of *cryIA(c)*.

pBGB1 was digested with DraI for a period of 20 minutes and electrophoresed through an agarose gel (see sections 2.5.3.b/g). Fragments with sizes in the region of 3.8 kb, corresponding to the complete coding and promoter regions of *cryIA(c)* but not the terminator sequence (see figure 5.2.1.e), were isolated using electroelution (see section 2.5.3.h). The resulting fragments were ligated with SmaI-cut/phosphatased pUC19 (see sections 2.5.3.b/c/f) and transformed into DH5α (see section 2.5.2.a) and plated out onto selective media coated with X-gal (see section 2.5.6.i). White colonies were picked, cultured in LM-broth containing ampicillin and

plasmid preparations were obtained (see section 2.5.1.b). These were digested with appropriate enzymes, electrophoresed through a 0.7 % agarose gel and the resulting banding patterns were compared with known values for the digestion of *cryIA(c)* [3] and pUC19 [343] with the same enzymes. One strain was discovered to possess a plasmid that contained the desired 3.8 kb fragment inserted into the multiple cloning site of pUC19 such that P_{lac} initiated transcription of *cryIA(c)* in the reverse direction. This plasmid was designated pLEV1.

The plasmids described below were created using the same method as described above. pLEV102 contains a 3.7 kb HincII/BamHI fragment from pLEV1 inserted into SmaI/BamHI-cut pUC19 such that 93 bp were removed from the 5' end of the pLEV1 insert - a deletion that might have affected the activity of the promoter (see figure 5.2.1.e). Again P_{lac} directed transcription from the 3' end of *cryIA(c)* and thus the same fragment was also subcloned into pUC18 (which is identical to pUC19 except that the orientation of the multiple cloning site is reversed). The resulting plasmid, pLEV103, allowed P_{lac} to direct transcription from upstream of the *cryIA(c)* promoter (see figure 5.2.2.a).

To remove the natural promoter completely, pLEV1 was digested with NdeI and the recessed 3' termini were 'filled in' (see section 2.5.3.e) producing a 'blunt end' (see figure 5.2.1.e). The plasmid was further cut with BamHI and the resulting fragment was cloned into HincII/BamHI-cut pUC19 and also into SmaI/BamHI-cut pUC19 producing pLEV114 and pLEV115 respectively. P_{lac} in pLEV114 initiates transcription of *cryIA(c)* in the correct direction, whereas in pLEV115 it initiates transcription of the gene in the reverse direction.

The same fragment that was used to create pLEV114 and pLEV115 was also used to create plasmids pLEV112 and pLEV113. In these cases, though, the NdeI-cut termini were not 'filled in', and the fragment was ligated into NdeI/BamHI cut pUC19 and pUC18 to create pLEV112 and pLEV113 respectively. In both cases P_{lac} initiates transcription of *cryIA(c)*, in the reverse direction both plasmids being almost identical except for different sets of restriction sites downstream of the gene.

The plasmids constructed above have no natural terminators and thus these were added. pBGB1 was restricted with EcoRI and a 5.25 kb fragment was ligated into EcoRI-cut pUC19 forming pLEV300 (see figure 5.2.1.a). Due to the cloning procedures used above and the large insert already contained in the pLEV plasmids, only restriction sites for enzymes liberating 'blunt ends' were available at the 3' end of *cryIA(c)* and thus pLEV300 was restricted with a number of these enzymes to try

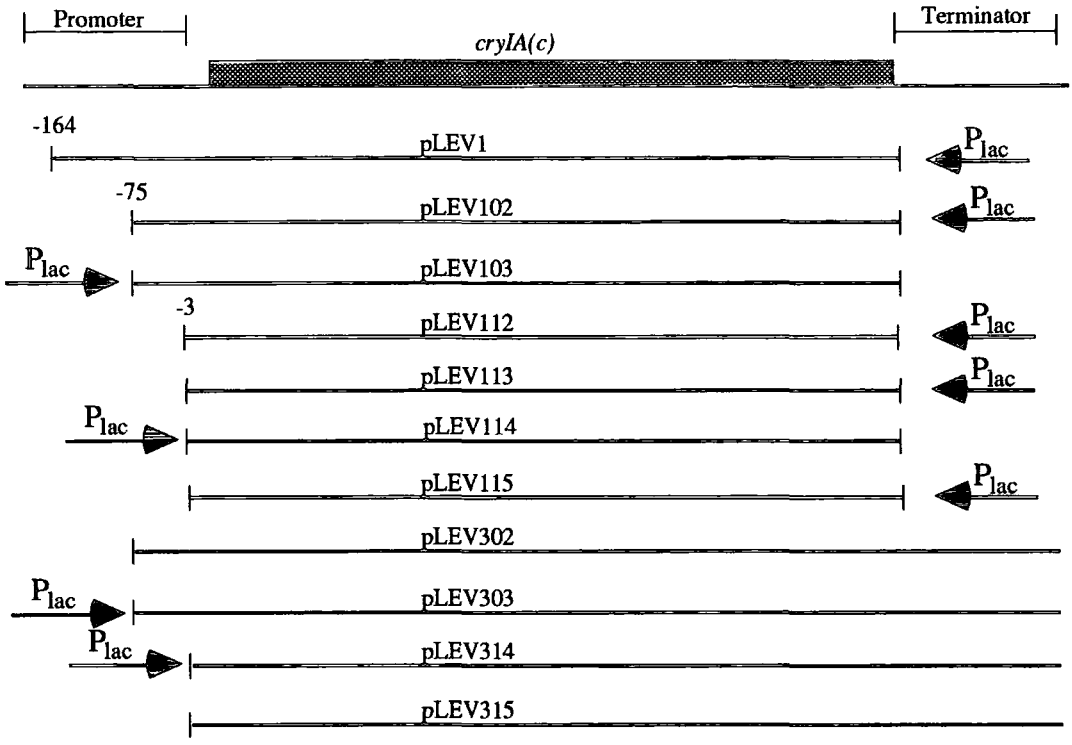


Figure 5.2.2.a - summary of the different pLEV plasmids constructed in this study. Digits refer to distance in bp from the *E. coli* transcription start site. Not to scale.

to discover a suitable site close to but downstream of the terminator region from which to add the terminator to the other pLEV plasmids. A restriction analysis of pLEV300 revealed very few enzyme sites that were suitable in this region of the DNA. Due to the absence of any unique site close to the terminator region, the fragment used to add the terminator, resulted in the addition of an extra 2.2 kb of DNA (see below).

pLEV102, pLEV103, pLEV114 and pLEV115 were all restricted with NheI and either SmaI (pLEV112 and pLEV114) or HincII. The larger fragment from each of these digests was isolated. pLEV300 was digested with NheI and EcoRV and a 3.2 kb fragment was isolated. This was then cloned into pLEV102, pLEV103, pLEV114 and pLEV115 (as restricted above) thus forming pLEV302, pLEV303, pLEV314 and pLEV315 respectively (see figure 5.2.2.a).

5.2.3 Expression analysis of pLEV plasmids in *E. coli*

Single colonies of each of the pLEV plasmids created thus far, were cultured in 5 ml LM-broth for 24 hours. Total cell extracts were made of each culture (see section 2.5.4.b) and two 40 µl aliquots of each were electrophoresed on two identical SDS/PAGE gels (see section 2.5.5.a). One gel was silver stained (see section 2.5.5.b) and the other was western blotted and immunoscreened using the anti-CryIA(c) antibody [101] (see sections 2.5.5.c/d). Figure 5.2.3.a clearly shows that all the pLEV plasmids are capable of producing full-length CryIA(c) in *E. coli*, with the possible exception of pLEV303.

Further assays were performed to determine the relative amounts of δ-endotoxin produced by each strain. 100 µl samples of each culture were centrifuged and the cells were resuspended in 100 µl of Bietlot buffer [25] (see section 2.5.5.a) in order to solublise the δ-endotoxin. The samples were allowed to cool to room temperature and then an ELISA was performed using them and the anti-CryIA(c) antibody (see section 2.5.5.e). The cells from 100 µl of each culture were also boiled for 10 minutes in 100 µl 100 mM Tris/50 mM EDTA in order to break open the cells, these samples being used to determine the amount of protein present in each sample used in the ELISA. A Tris/EDTA solution was used when measuring the protein concentrations since the urea in the Bietlot buffer interferes with the Bradford Protein Reagent [27]. The absorbance at 405 nm was measured and the values obtained were divided by the total amount of protein present in each sample used in the ELISA. The results are displayed in figure 5.2.3.b.

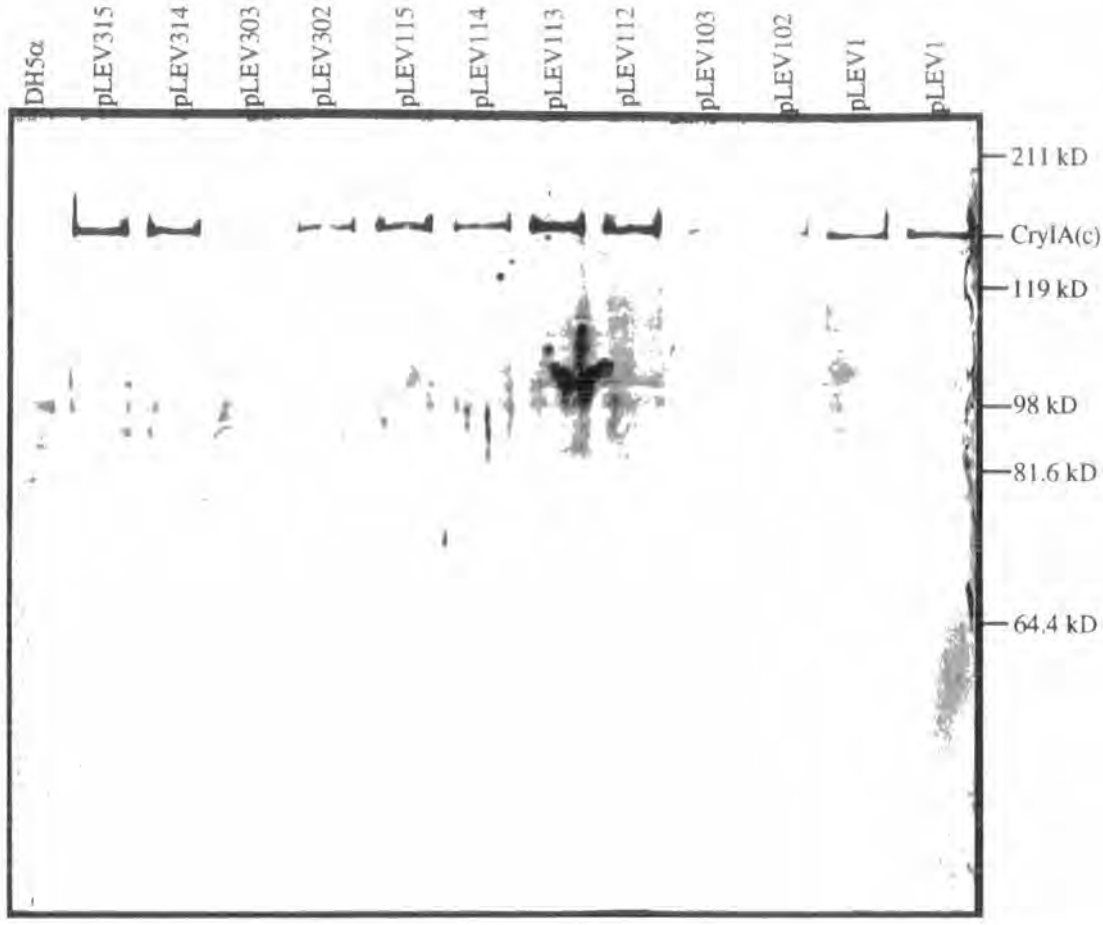


Figure 5.2.3.a - CryIA(c) production by various strains of *E. coli* containing pLEV plasmids. The bands correspond to a molecular weight of about 130 kD.

From figure 5.2.3.b it can be seen that pLEV300 produces very little detectable δ-endotoxin, as would be expected since much of the 5' coding region is absent and all of the promoter and terminator sequences are missing. pBGB1, also produces little δ-endotoxin despite the fact that the entire gene and all its regulatory sequences are present. This is in agreement with the data of Ge *et al.* who suggested that only about 0.24 % of the cellular protein is δ-endotoxin when expressed in this fashion [98]. This indicates that some sequences present on the insert of this plasmid repress the expression of the gene, or alternatively, the large insert size reduces the copy number in relation to the other pLEV plasmids or it reduces the stability of the plasmid.

Deletion of the regulatory sequences 3' of the coding region and up to -164 from the *E. coli* transcription start site [339] (see figure 5.2.1.e), greatly increased the amount of CryIA(c) produced, as shown by the expression levels in pLEV1. Further deleting the region from -164 to -75 from the *E. coli* transcription start site greatly reduces the amount of CryIA(c) produced (pLEV102). This is broadly in agreement with the analysis of Reznikoff *et al.* [244] who suggested that the deletion of sequences from -50 to -160 decreased the transcription from various promoters. However, the data presented here is in contrast to that of Schnepf *et al.* [266] who, using the same promoter as that of *cryIA(c)*, found that a deletion from -176 to -87 actually increased transcription. It is possible therefore that an enhancing region exists between -87 and -47, the effect of which is repressed by the region within -176 to -87. This putative enhancing region will have been disturbed or removed by a deletion to -75 (pLEV102). Indeed, when a further deletion removing the region to -47 was performed by Schnepf *et al.* the level of expression fell back to that detected using the promoter region which had been deleted to -176 (see figure 5.2.1.e) [266].

The effect of P_{lac} on the expression levels of pLEV102, was minimal. Only a slight increase was observed when P_{lac} was allowed to transcribe the gene in the same direction as the natural promoter (pLEV103), suggesting that in this context P_{lac} is not acting efficiently.

However, the results from the plasmids in which the entire *B. thuringiensis* DNA sequence upstream of -3 to the *E. coli* transcription start site had been deleted (pLEV112 - pLEV115), were more puzzling. Schnepf *et al.* found that a deletion of this type greatly reduced expression of the gene [266]. This is clearly not the case in the constructs created in this study. Only small decreases in expression were observed for pLEV114 as compared with pLEV103, and for pLEV115 as compared with pLEV102.

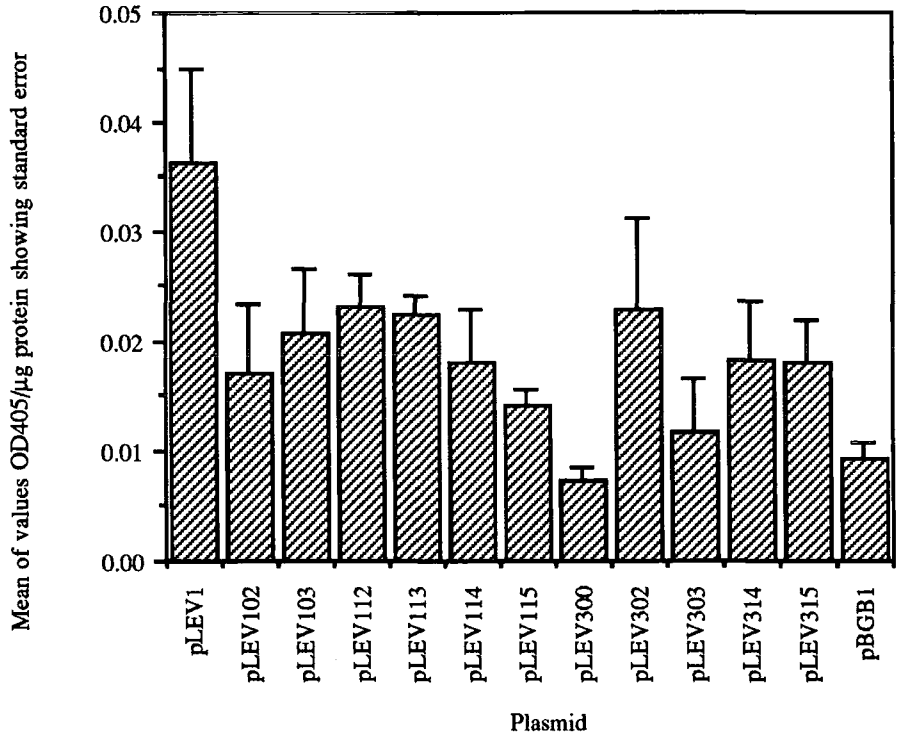


Figure 5.2.3.b - showing relative expression levels of *E. coli* DH5α containing a variety of plasmids which are subclones of *cryIA(c)* in pUC19/18. Values are the means of six experiments and are expressed as the absorbance at 405 nm as a function of the amount of protein present (µg).

pLEV112 and pLEV113 both exhibited the same levels of expression, as was expected since the plasmids are almost identical. However, the absence of any decrease in expression after the deletion of the promoter region, as compared with pLEV102 and pLEV103 remains unexplained, although it might be that the linking of the DNA fragments produced a sequence with promoter-like properties.

The addition of the native terminators to these constructs did not produce conclusive results either. The *cryIA(c)* terminator is extremely strong (ΔG = -30.4 kcal) [98] and has been identified as a positive retroregulator [338]. It is suggested that the transcribed retroregulator sequence forms a stem and loop structure at the 3' end of the mRNA and that this structure protects the mRNA from exonucleolytic degradation, thus increasing the half life of the molecule resulting in an increase in the amount of protein synthesised [338]. However, the addition of the native terminator did not increase the amount of protein produced and in most cases it actually decreased. It is possible that the extra DNA added in the 'terminator fragment' carried a repressor of protein synthesis, although it is more likely that the decrease in expression results from a larger amount of DNA causing a lower copy number or less stability of the plasmid.

It is apparent that the expression of δ-endotoxins in *E. coli* is dependent on a number of genetic factors [98]. One factor appears to be the strain of *E. coli* that is used. Ge *et al.* found large variations in the amount of δ-endotoxin produced by the same plasmid in different strains, some producing nearly 300 times as much cryIA(c) as others [98]. Although no further work was carried out using different strains (and Ge *et al.* did not use DH5α in their work), one report claimed that no CryIA(b) (a closely related δ-endotoxin) production was found using *cryIA(b)* under the control of P_{lac} in DH5α [258]. The reasons for this are unclear but it would appear that DH5α is not an effective host for these plasmids.

In conclusion, the regions flanking *cryIA(c)* appear to contain enhancer and repressor sequences although the exact nature of these was not elucidated. Expression results from the plasmids containing P_{lac} suggest that it is not an efficient promoter in these constructs and the results of other workers suggests that DH5α might not be a good host for the pLEV plasmids [98, 258].

5.2.4 Protein 'enhancers' of *cryIA(c)* expression in *E. coli*

A report by McClean and Whiteley suggested that for full expression of the mosquitocidal *B. thuringiensis* var. *israelensis* CytA peptide, a 0.8 kb DNA sequence 4 kb upstream of this gene was required [200]. Subsequently it was found that this DNA sequence encoded a 20 kD protein that protected CytA from proteolysis in *E. coli* [1, 320]. This 20 kD protein was also found to increase the amount of CryIVD and CryIVA produced by *E. coli*, the gene found to be just downstream from *cryIVD* [1, 320, 346]. Visick and Whiteley [320] suggested that the 20 kD protein did not have any effect on the production of CryIA(c). However, given that the 20 kD protein was produced naturally by *B. thuringiensis* var. *israelensis*, it was possible that var. *kurstaki* contained a similar protein.

pBGH1 was kindly donated by Dr. T. Komano (Kyoto University, Japan) (see figure 5.2.4.a). A 2.1 kb AccI fragment was isolated from this plasmid and used to make a radioactive probe (see sections 2.5.3.b/g/h/k). A Southern blot was made of *B. thuringiensis* var. *kurstaki* 4D4 plasmids cut with various restriction enzymes and the probe was hybridised to the blot at 68 °C overnight (see sections 2.5.3.j/l). Upon development of the photographic film, no hybridisation was observed to have occurred. The blot was reprobed at 45 °C, thus allowing sequences with a lower homology with one another to hybridise. In this case, hybridisation occurred but failed to identify any novel fragments of DNA - the probe bound to the fragments containing *cryIA(c)*.

The nucleotide sequence of the 20 kD protein, including much of the downstream flanking region, [1] was aligned with that of *cryIA(c)* using the UWGCG programme, GAP, which uses the algorithm of Needleman and Wunsch [214]. This showed that a 1.6 kb sequence (containing the 20 kD protein gene and flanking sequences of about 500 bp both 5' and 3' to it) had a 44 % identity with *cryIA(c)* overall, corresponding to the 3' region of the gene between 1583 and 3217 bp from the translation start site (ATG) (see figure 5.2.4.b).

Closer inspection revealed a region with much greater sequence identity (59 %) corresponding to 2902 - 3055 bp after the initiation codon. This region was 239 bp downstream of the translation stop codon of the 20 kD protein gene and contained a small open reading frame (designated ORFQ) encoding a putative protein of 51 amino acids. It is likely that this region of DNA was responsible for the hybridisation observed (see figure 5.2.4.c). A translation of ORFQ was also analysed for homology with other δ-endotoxin genes using the ALIEN programme by

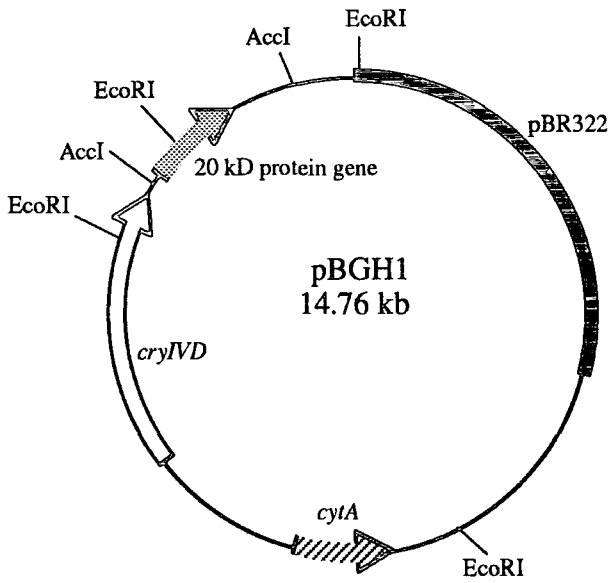


Figure 5.2.4.a - plasmid map of pBGH1, [346]. All EcoRI restriction sites and selected AccI sites shown. Not to scale.

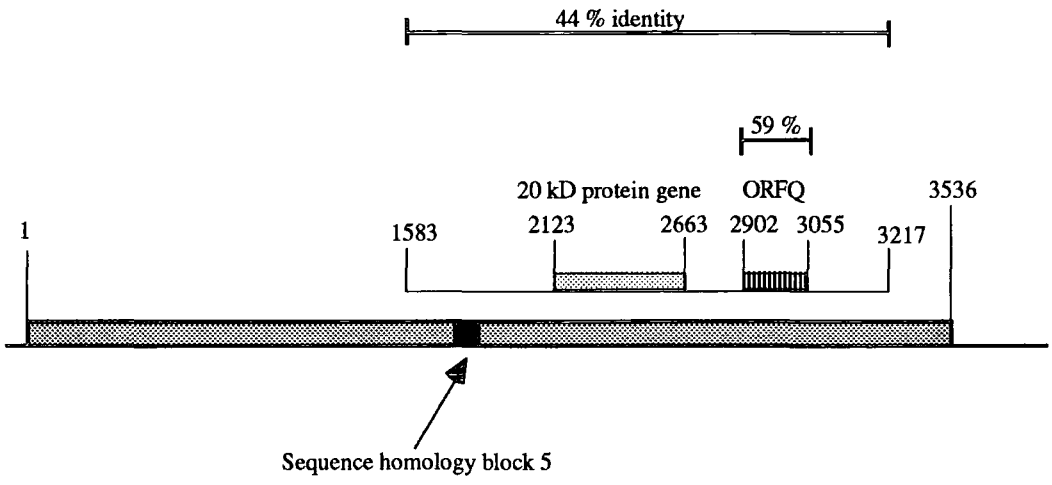


Figure 5.2.4.b - showing region of homology of *cryIA(C)* with a 1.6 kb DNA fragment from *Bacillus thuringiensis* var. *israelensis* containing the 20 kD protein gene. Numbers refer to distance in bp from the start of translation (ATG). The sequence homology block 5 is the last of the sequence blocks, from the 5' end of the gene, that are conserved in many *cry* genes (see section 1.17). Percentage figure refer to the identity of the two sequences with one another. Not to scale.

Bleasby which shares similar searching parameters with CLUSTAL [130]. It was found that the putative protein corresponded well with a 30 amino acid stretch found in CryIA(c), CryIVA and CryIVB (see figure 5.2.4.d) but not in CryIVD. This region was not one of the homology blocks designated by Höfte *et al.* (see figure 1.17.1) as being common to most δ-endotoxin genes, but is closer to the C terminus [133].

It seemed possible that since most of the δ-endotoxin genes are more than 25 % similar to one another [89], in the evolution of these genes, the functions of smaller stretches of DNA found downstream of the structural toxin genes, might be incorporated into the 5' end of these genes. Thus the scaffolding or proteolytic protection functions of the 20 kD [320] protein might be incorporated into the C terminal half of the CryIA(c) protein. This hypothesis is supported by the fact that the 133 kD CryIA(c) protein is much longer than CryIVD (72 kD) [3, 73].

CryIVA and CryIVB are about 35 % similar to CryIA(c) [89], but all contain a region that is highly homologous with ORFQ, a region not present in CryIVD. These δ-endotoxins are produced as protoxins of 125 kD (CryIVB) and 135 kD (CryIVA). If the C terminal ends of these proteins were to provide the same functions as the 20 kD protein then it would be expected that increased production of CryIA(c), CryIVA and CryIVB would not be observed when supplied with the 20 kD protein *in trans*, whereas increased production of CryIVD would. This holds true for all the proteins except CryIVA which appears to be increased in its production under these circumstances [320, 346]. Therefore there are probably other factors that determine whether a δ-endotoxin requires other protein factors for efficient production in *E. coli*.

It is not known whether ORFQ is expressed in *B. thuringiensis* but its small size suggests that it is not. It is clear that the presence of this region has no effect on the production of either CytA or CryIVD in *B. thuringiensis* [49]. A search of the GenBank database using the FastA programme (which performs a Pearson and Lipman search [235]) with the nucleotide sequence of ORFQ revealed that a similar sequence was found in a wide variety of δ-endotoxin genes which suggests that the analysis of regions downstream from the structural toxin genes may provide a good insight into the evolution of the various δ-endotoxins. Whether the functions of the 20 kD protein are retained by the C terminus of the larger δ-endotoxin genes is unclear, but further research into the evolution of these genes and analysis of the products formed by truncated genes may be able to clarify this.

```
         Gap Weight:  5.000      Average Match:  1.000
      Length Weight:  0.300   Average Mismatch:  0.000

            Quality:   96.4             Length:   3538
              Ratio:  0.533               Gaps:      2
 Percent Similarity: 59.444   Percent Identity: 59.444
```

ORFQ x *cryIA(c)*

```
   1 ....GCATTTTATCTATATGCACGTTCGAAATTTTATTAAAAATGGTGAC 46
         ||||| |  ||||||| | |   ||||| | ||||||||||||||
2900 CACTGCATTCTCCCTATATG.ATGCGAGAAATGTCATTAAAAATGGTGAT 2949

  47 TTTAAATATGGCCTAGAGGAGTGGCATGTCAAAGGTGATGCAAACGTACA 96
     |||||  ||||| ||      ||| | || |||||  ||| | | ||| |
2950 TTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGA 2999

  97 ACAAATAAATGGTACACCTGTGTTCAGTAAT.TCCCTAATTGGAGTGCTC 145
     | ||  |||      || |  | ||  |  | | ||  | |||   ||
3000 AGAACAAAACAACCAACGTTCGGTCCTTGTTGTTCCGGAATGGGAAGCAG 3049

 146 AAGTATCACAAAATATATGCTTAAACACGATCATGG............... 181
     |||| |||||| |  |  |  |     ||      |
3050 AAGTGTCACAAGAAGTTCGTGTCTGTCCGGGTCGTGGCTATATCCTTCGT 3099
```

Figure 5.2.4.c - Alignment of small open reading frame (ORFQ), located 239 bp downstream from the translation stop site of the 20 kD protein gene, with *cryIA(c)*. Alignment performed using the UWGCG programme GAP with the default parameters as shown at the top of the figure. Numbers of ORFQ are arbitrary whereas numbers of *cryIA(c)* refer to bp after the *E. coli* start of transcription site. Putative start and stop of translation signals are underlined and in bold.

```
ORFQ ......1..M H V R N F I K N G D F K Y G L E E W H V K
CryIA(c).844..Y D A R N V I K N G D F N N G L S C W N V K
CryIVA..1061..Y D I R N I I K N G D F T Q G V M G W H V T
CRYIVB..1007..Y D T R N I I K N G D F T Q G V M G W H V T
                    * *   * * * * * *     * ^     * ^ *

      G D A N V Q - Q I N G T P V         F S N S L I G V
      G H V D V E E Q N N Q R S V..61 aa..Y P N N T V T C
      G N A D V Q - Q I D G V S V.56 aa.E
      G N A D V Q - Q I D G V S V.56 aa.E
      * ^   ^ * ^   *   ^     ^ *         ^ ^ * ^   ^

      L K Y H K I Y....51
      N D Y T V N Q...953
          *
```

Figure 5.2.4.d - Alignment of the putative protein translation of ORFQ against CryIA(c), CryIVA and CryIVB using the programme ALIEN. * = identity, ^ = conservative change of amino acids. Numbers refer to amino acids in protein.

5.2.5 Insecticidal nature of *E. coli* expressing *cryIA(c)*

Bioassays were conducted using the tobacco hornworm (*Manduca sexta*) and 24 hour cultures of DH5α strains containing pLEV plasmids or pBGB1 (see section 2.5.8). *B. thuringiensis* var. *kurstaki* 4D4 cultured for 48 hours in SPY was also used. The insects were monitored for a period of 4 days, 4 1st instar larvae being tested against each strain. Larvae that exhibited no reaction to being prodded using forceps were described as being dead.

After 24 hours all the insects feeding on the diet containing *B. thuringiensis* had died. After 48 hours those feeding on DH5α containing pLEV100, pLEV112, pLEV113, pLEV114, pLEV115, pLEV302, pLEV314, pLEV315, pBGB1 or pLEV2114 (see section 5.3.1) had all died. At 72 hours only insects feeding on diet spread with DH5α , DH5α (pLEV102), DH5α (pLEV103), DH5α (pLEV300) or diet alone were alive and at the end of the assay (96 hours) the same was true.

At the end of the experiment the masses of the insects on the diet alone, all of which had survived to 3rd instar, were measured, the mean being 42.5 mg. For those feeding on diet spread with DH5α (pLEV102), two larvae remained alive, both in 2nd instar with masses of 15.7 and 12.5 mg. One 2nd instar larva remained, of those fed on DH5α (pLEV103) having a mass of 15.5 mg. Two remained alive of those fed on DH5α (pLEV300), one being in 3rd instar of 41.2 mg in mass and the other a 2nd instar larva of mass 41 mg. Of those fed on DH5α all had survived, two were in 3rd instar (37.3 mg, 46 mg), one was in 2nd instar (43.2 mg) and the other was a 1st instar larva of mass 6.9 mg.

From these results it can be seen that DH5α probably has little effect on the growth of these larvae - the one remaining in 1st instar may have been abnormal in some respect, may have become stuck in the medium, may have been damaged during handling or may have started to drown. Occasionally small pools of condensation were observed in the medium which were the result of poor ventilation in some of the wells.

It is quite clear that *B. thuringiensis* had the most insecticidal activity of all the strains assayed, but all the *cryIA(c)* expressing DH5α strains were highly effective in killing the larvae. It is doubtful, though, that any meaningful conclusions can be drawn concerning the relative insecticidal activities of the different strains containing pLEV plasmids or pBGB1, since the sample sizes were too small. If a study was to be conducted to try to elucidate differences between the activities of the

strains, much greater numbers of larvae would have to be used. A slight change in protocol design would also probably help. It is possible that due to the particulate nature of the diet, some areas of the diet surface allowed greater numbers of bacteria to accumulate (due to spreading the bacterial solution on an uneven surface), it being chance that a larva would feed on one of these areas rather than an area containing less bacteria. Thus a better way of delivering each larva with the same amount of bacteria would be to incorporate the microbes directly into the diet. However, for practical reasons, this was not attempted.

In conclusion, it can be said that *E. coli* DH5α is able to express *cryIA(c)* sufficiently well, to allow it to be highly effective in controlling the larvae of the lepidopteran insect, *Manduca sexta.*

5.3 Expression of a δ-endotoxin in *Agrobacterium tumefaciens*

5.3.1 Constitutive expression of *cryIA(c)* in *A. tumefaciens*

pLEV114 was digested with EheI which cut once 200 bp downstream from the insertion containing *cryIA(c)* (see sections 5.2.2, 2.5.3.b). The digest was heated to 80 °C for 30 minutes to inactivate the enzyme and allowed to cool before the addition of pKT230 [14, 15] cut with SmaI and phosphatased (see sections 2.5.3.b/c). The resulting mixture was ligated overnight at 4 °C (see section 2.5.3.f), before being directly transformed into *E. coli* DH5α (see section 2.5.2.a) and plated out on selective agar containing ampicillin and streptomycin. Single colonies were cultured in LM-broth containing the antibiotics and plasmid DNA was obtained (see section 2.5.1.a).

The plasmid DNA was restricted and electrophoresed in a 0.7 % agarose gel and the banding patterns were compared with predicted values. In this way, pLEV2114 was obtained, which contained *cryIA(c)* without the *B. thuringiensis* promoter or terminator regions, pUC19 (allowing high copy number in *E. coli*) and pKT230 (allowing replication in *A. tumefaciens*). The δ-endotoxin gene was under P_{lac} control. Using the same method as described above, but using pLEV1, pLEV2001 was produced containing the *B. thuringiensis* promoter region but not the native terminator region.

These plasmids were introduced into *A. tumefaciens* C58C1 using triparental mating (see section 2.5.2.c). Confirmation that the strains contained the appropriate plasmids was performed by culturing the strains in LM-broth with antibiotic

selection and then digesting plasmid DNA 'minipreps', followed electrophoresis through an agarose gel and comparing the banding patterns obtained on an agarose gel with known values. The resulting strains, and DH5α strains containing the two plasmids, were cultured for 48 and 24 hours respectively in LM-broth, and the cultures were then assayed for the production of CryIA(c) using the ELISA technique described above (see section 5.2.3).

The DH5α strains both produced CryIA(c) but neither of the *A. tumefaciens* strains showed any detectable expression of the gene. This was disappointing especially considering that *virG* had been shown to be expressed well in *A. tumefaciens* under the control of P_{lac} [51] and that the almost identical δ-endotoxin gene, *cryIA(a)* was able to be expressed in *A. radiobacter* [224]. However, in the latter case, the expression of *cryIA(a)* was extremely low and it may have been that the amount of δ-endotoxin produced by both C58C1(pLEV2001) and C58C1(pLEV2114) was below the limits of detection of the ELISA technique used. It was therefore decided to construct a plasmid containing *cryIA(c)* under the control of a *vir* gene promoter.

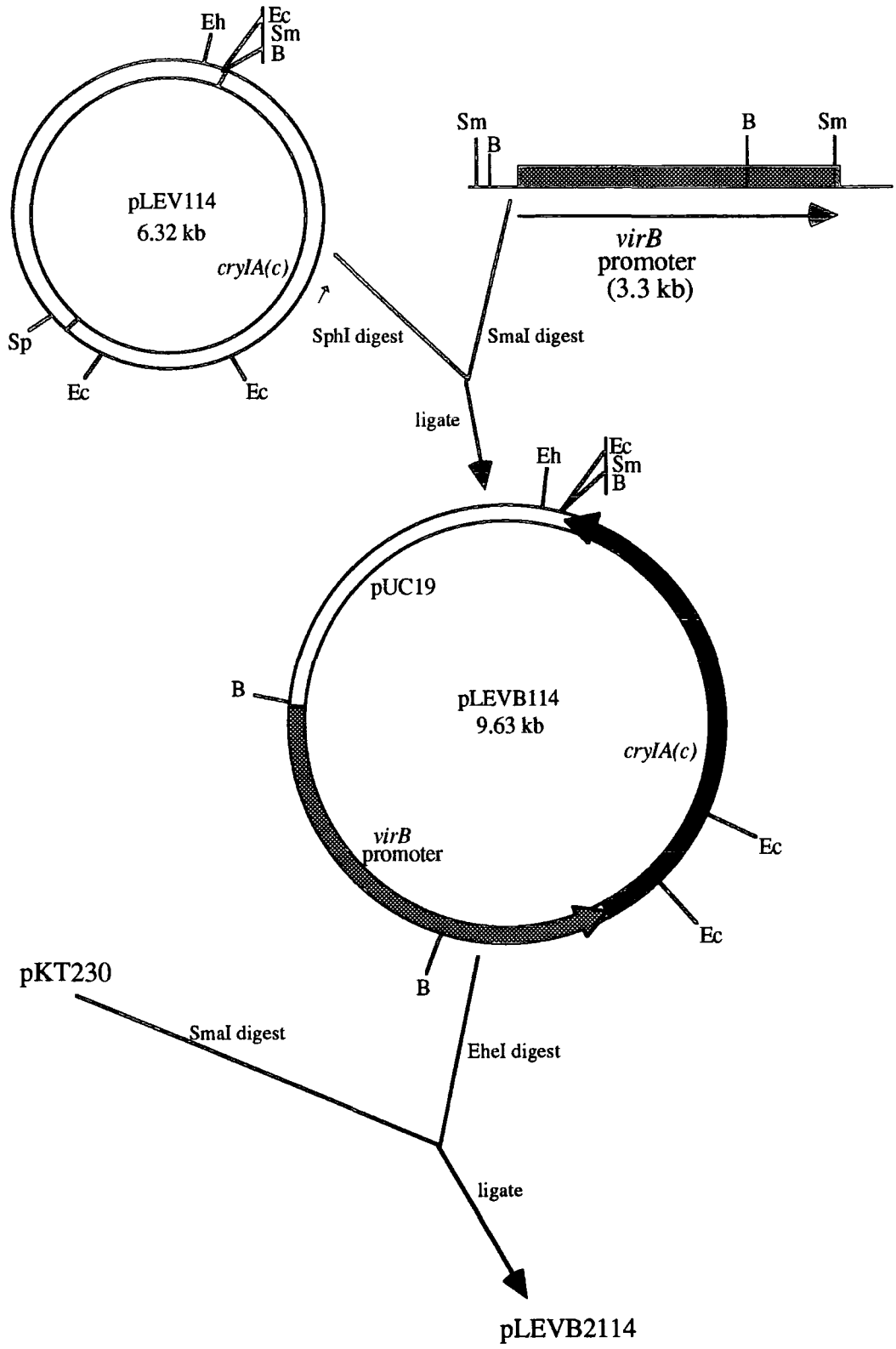


Figure 5.3.2.a - showing construction of the broad host range plasmid, pLEVB2114, containing *vir*-inducible *cryIA(c)*. Restriction sites shown - B = BamHI, Ec = EcoRI, Eh = EheI, Sm = SmaI, Sp = SphI.

5.3.2 Inducible expression of *cryIA(c)* in *A. tumefaciens*

To create an inducible construct, the *virB* promoter was chosen since this had previously been demonstrated to be more easily induced and to higher levels than the *virE* promoter [253] (see section 3.2). It was hoped to be able to produce a *virE* promoter::*cryIA(c)* construct as well, since the results obtained when working with the chitinase constructs had suggested that the *virE* promoter was working more efficiently.

pLEV114 was linearised with SphI and the 3' protruding termini were removed (see sections 2.5.3.b/d). pVB21 [187] was restricted with SmaI and a 3.3 kb fragment, corresponding to the *virB* promoter region was isolated (see sections 2.5.3.b/g/h). This fragment was ligated to the linearised pLEV114 (see section 2.5.3.f) and then transformed into DH5α (see section 2.5.2.a). Transformants were selected on agar containing ampicillin and single colonies were picked at random and cultured overnight in LM-broth. Plasmid preparations were obtained from the cultures (see section 2.5.1.b) and restricted with various enzymes to determine whether any contained the appropriate insert. Plasmids containing the insert were further restricted with BamHI to determine the orientation of the insert (see figure 5.3.2.a).

The correct plasmid was designated pLEVB114 and this was further linearised with EheI, ligated into SmaI-cut/phosphatased pKT230 and transformed into DH5α (see sections 2.5.3.b/c/f and 2.5.2.a). Transformants were selected on agar containing ampicillin and streptomycin and plasmid preparations were restricted with EcoRI to determine whether the desired plasmid had been constructed. The plasmid, once created, was designated pLEVB2114 and contained the *virB* promoter directing transcription of *cryIA(c)* on a broad host range plasmid. pLEVB2114 was introduced into *A. tumefaciens* C58C1(pVK257) using triparental mating (see section 2.5.2.c). Colonies containing the plasmid were selected using rifampicin, streptomycin and carbenicillin and plasmid preparations from the resulting colonies were restricted with EcoRI and electrophoresed through a 0.7 % agarose gel. The banding patterns were compared with known values for both pLEVB2114 and pVK257 and the desired strain was thus isolated.

C58C1(pVK257, pLEVB2114) and C58C1(pVK257) were both cultured in 5 ml LM-broth for 48 hours before 100 μl aliquots were subcultured into culture tubes containing 5 ml Davis medium. The bacteria were incubated for 3 hours prior to the addition of 100 μM acetosyringone (or the equivalent volume of 70 % methanol).

The cultures were further incubated for 24 hours before an ELISA was performed and the total amount of protein estimated, as described above (see section 5.2.3).

The results of the ELISA were very encouraging. The amounts of CryIA(c) produced by both C58C1(pVK257) and uninduced C58C1(pVK257, pLEVB2114) were below the detection limit of the assay. However, there was clear induction and expression of the *cryIA(c)* in the induced cultures of C58C1(pVK257, pLEVB2114). The OD_{405} readings, as a function of the amount of protein, averaged 0.019 OD units/μg protein which compared favourably with the results of the expression studies in *E. coli* (see figure 5.2.3.b).

These experiments suggested that by using a δ-endotoxin gene under the control of the *virB* promoter, *A. tumefaciens* was able to express the gene to approximately the same levels as *E. coli.*

5.3.3 Insecticidal nature of C58C1(pVK257, pLEVB2114)

24 hour cultures of C58C1(pVK257, pLEVB2114), C58C1(pLEV2001), C58C1(pLEV2114) and C58C1(pVK257) in Davis medium were used in an insect bioassay against *Manduca sexta*, as described above (see sections 5.2.5). The bacteria had all been subcultured from 48 hour cultures in LM-broth, C58C1(pVK257, pLEVB2114) being subcultured in two, one of the resulting cultures being induced with 100 μM acetosyringone and the other with the equivalent volume of 70 % methanol. 4 insects were used against each strain and were monitored for 5 days.

After 24 hours, 2 of the insects feeding on the induced culture of C58C1(pVK257, pLEVB2114) had died although it took a further 72 hours before all 4 larvae had died. Of those fed on the uninduced culture of C58C1(pVK257, pLEVB2114), one had died after 96 hours, the others remained alive and had developed to 2nd instar after 5 days, the masses being 41.3, 37.2 and 29.8 mg.

All of those grown on diet alone had developed into 3rd instar larvae by the end of the experiment, the mean mass being 54.7 mg. Those fed on C58C1 had all survived and had a mean mass of 56.6 mg, although only one of these had reached 3rd instar. Of those fed on C58C1(pLEV2001), all bar one had reached 3rd instar and a mean mass of 45.3 mg was achieved. On C58C1(pLEV2114), one larvae had died after 96 hours, the others reaching 2nd instar and having a mean mass of 28.5 mg at the end on the experiment (see table 5.3.3.a).

A. tumefaciens strain	Larvae surviving after 5 days	Mean mass of surviving larvae (mg)
Diet only	4	54.7
C58C1	4	56.6
C58C1(pLEV2001)	3	45.3
C58C1(pLEV2114)	3	28.5
C58C1(pVK257, pLEVB2114) induced	0	-
C58C1(pVK257, pLEVB2114) uninduced	3	36.1

Table 5.3.3.a - showing survival of *Manduca sexta* larvae when assayed against various strains of *A. tumefaciens* C58C1 over a period of 5 days.

From these results it would appear that *A. tumefaciens* alone has little effect on the growth of *Manduca sexta* larvae. What is apparent is that *A. tumefaciens* containing either pLEV2001 or pLEV2114 was somewhat effective in slowing the growth of these larvae, those feeding on C58C1(pLEV2114) having a mean mass, after 5 days, nearly 50 % less than those fed on C58C1. This implies that contrary to the results of the ELISA performed (see section 5.3.1), some CryIA(c) is produced by these strains, C58C1(pLEV2114) producing more than C58C1(pLEV2001) suggesting that P_{lac} was more effective in directing transcription of the gene than the native *B. thuringiensis* promoter.

What is clear is that, when induced, C58C1(pVK257, pLEVB2114) is capable of controlling the larvae of *Manduca sexta.* In the uninduced state, this strain appears to be capable of some background expression of *cryIA(c)* since the mass of those larvae surviving this treatment was 36.1 mg.

These results were very encouraging since it was now evident that a strain of *A. tumefaciens* C58C1 was capable of producing enough δ-endotoxin to kill the larvae of *Manduca sexta* when induced by a compound produced by plant wounds, acetosyringone (see sections 1.6 - 1.9).

5.4 Summary

A plasmid, pIC4, obtained from ICI Seeds, that contained the lepidopteran-specific *B. thuringiensis* δ-endotoxin gene *cryIA(c),* appeared not to be able to produce the protein. This plasmid had only been used by Gibb and his co-workers as a molecular probe for other δ-endotoxin genes and so it was not known whether the gene on the plasmid had ever been capable of being expressed [101], or whether, as suspected, an insertion sequence had disrupted the promoter or coding region of the gene. Thus *cryIA(c)* was recloned as a 13.5 kb BglII fragment which was ligated into pUC19, forming pBGB1.

E. coli cells containing pBGB1 showed expression of the δ-endotoxin from its native promoter and it was confirmed that the cells were toxic to the larvae of the lepidopteran insect pest, *Manduca sexta.* Various subclones of pBGB1 were made, which lacked either the natural *B. thuringiensis* promoter or terminator sequences or both. In *E. coli* these plasmids all showed expression of *cryIA(c)* although P_{lac} did not appear to be an efficient promoter in these constructs and the strain of *E. coli* used, DH5α, may not have been a good host for the plasmids.

In an attempt to find a transacting protein 'enhancer' of *cryIA(c)* expression, it was discovered that part of the region of the δ-endotoxin gene bore significant sequence homology to a small open reading frame (designated ORFQ) downstream of the gene encoding a 20 kD protein 'enhancer' of *cryIVD* expression from *B. thuringiensis* var. *israelensis*. It was speculated that ORFs downstream of a δ-endotoxin gene might become incorporated into the gene itself during the evolution of a particular subspecies of *B. thuringiensis* . The functions of these ORFs, if they were normally expressed, might thus be incorporated into the mature protoxin. It was also suggested that analysis of regions of DNA downstream of δ-endotoxin genes might help to elucidate the evolution of these genes.

E. coli DH5α containing subcloned *cryIA(c)* plasmids, were all found to be toxic to *Manduca sexta* larvae. Two of these plasmids were ligated into pKT230 and introduced into *A. tumefaciens*. However, no expression of *cryIA(c)* could be detected using an ELISA in these strains.

One broad host range plasmid was constructed such that it contained *cryIA(c)* under the control of the *virB* promoter from *A. tumefaciens*. When pLEVB2114 was introduced into *A. tumefaciens* C58C1(pVK257) and was induced by the addition of acetosyringone, CryIA(c) was detected using ELISAs, at levels comparable to those produced using *cryIA(c)*-expressing strains of *E. coli* DH5α. This strain was also found to be toxic to the larvae of *Manduca sexta*, when induced, but markedly less toxic when uninduced with the plant wound-specific chemical acetosyringone.

These results strongly suggest that C58C1(pVK257, pLEVB2114) could be used as a 'microbial inoculant'. It is hoped that such a microorganism would be able to detect the wounding of plants by hatching larvae and thus migrate to the area in which the larvae were to be found. Once there, the bacterium would produce the δ-endotoxin and therefore kill the larvae.

5.5 Future work on δ-endotoxin-based 'microbial inoculants'

Unfortunately a construct containing the *virE* promoter controlling *cryIA(c)* was not made due to time limitations. It would be interesting to determine how well the *virE* promoter directs the transcription of the gene, since results in previous parts of this study were conflicting - In section 3.2 the *virB* promoter appeared to be stronger and more reliable but in section 4.5.3 the *virE* promoter appeared more efficient in directing the transcription of the *chi* genes.

The *virB* promoter fragment used in this study was very large and thus various deletions of it should be made to see if acetosyringone induced expression can be optimised in this way.

Despite the good results obtained during this work, it is unlikely that *cryIA(c)* would be used successfully in a 'microbial inoculant' system of this nature, due to its activity. Lepidopteran insects lay their eggs mainly on leaves or stems and it is unlikely that an aerial application of *A. tumefaciens* would be able to control newly hatched larvae on these surfaces since the bacterium is usually soil borne and is unlikely to be maintained on the aerial parts of plants in sufficient numbers to effect the desired control.

A more likely scenario is one in which *cryIA(c)* is replaced with a coleopteran-specific δ-endotoxin gene (*cryIII* or *cryV*) [104, 133]. Many coleopteran larvae attack the roots of plants, such as *Diabrotica undecimpunctata howardii* which attacks maize roots and *Leptinotarsa decemlineata* which attacks potato roots. These pests are therefore ideal as targets for the proposed *A. tumefaciens* 'microbial inoculant' which would primarily be soil-borne. δ-endotoxins active against these pests have already been identified [259] and it is probable that these genes can be successfully expressed in *A. tumefaciens* based on the results above.

The *A. tumefaciens* 'microbial inoculant' system may also be able to control nematode worms. There have recently been reports of δ-endotoxins that are able to control these parasites [89] and again it should be possible to express these δ-endotoxins in *A. tumefaciens* .

The future for the 'microbial inoculant' system, as described above looks interesting and the encouraging results obtained so far suggest that this might be one method of controlling some insect pests in a more environmentally responsible manner.

Chapter 6

FURTHER APPLICATIONS

6.1 'Microbial inoculants' for cancer treatment in humans

During the course of this work two papers were published that suggested that the δ-endotoxin from *B. thuringiensis* var. *israelensis* (CryIVD) was able to potentiate the effects of bleomycin, an anticancer drug, towards several mouse cancers [344, 345]. It was possible that a 'microbial inoculant' based on the *A. tumefaciens* system, might be used to deliver bleomycin (or other anticancer agents), in association with a δ-endotoxin, to tumours.

Neural crest tumours, such as neuroblastoma, show a marked increase in their production of phenolic compounds such as vanillylmandelic acid (VMA) and homovanillic acid (HVA) [251, 260]. These compounds bare a marked structural homology to the *vir*-inducing plant-wound specific compounds like acetosyringone (see figure 6.1.a).

It was therefore possible that cancers that secrete these compounds would be able to attract an *A. tumefaciens*-based 'microbial inoculant' which would then produce the anti-cancer agents under *vir* promoter control.

6.2 *A. tumefaciens* chemotaxis towards colonic cancer cell lines

The obvious cancer types to choose for the initial investigations were gut derived in nature, since bacteria would be able to gain access to these tumours *in vivo*. Whether these were able to secrete large amounts of chemoattractant/*vir*-inducing phenolics was not known and thus the initial experiments concentrated on chemoattraction to the various cancer types chosen.

Four cell lines were tested. HRT18 was a human rectum adenocarcinoma cell line obtained from the European Collection of Animal Cell Cultures (Salisbury, U. K.) [317]. HT115 was a human colon carcinoma again obtained from the European

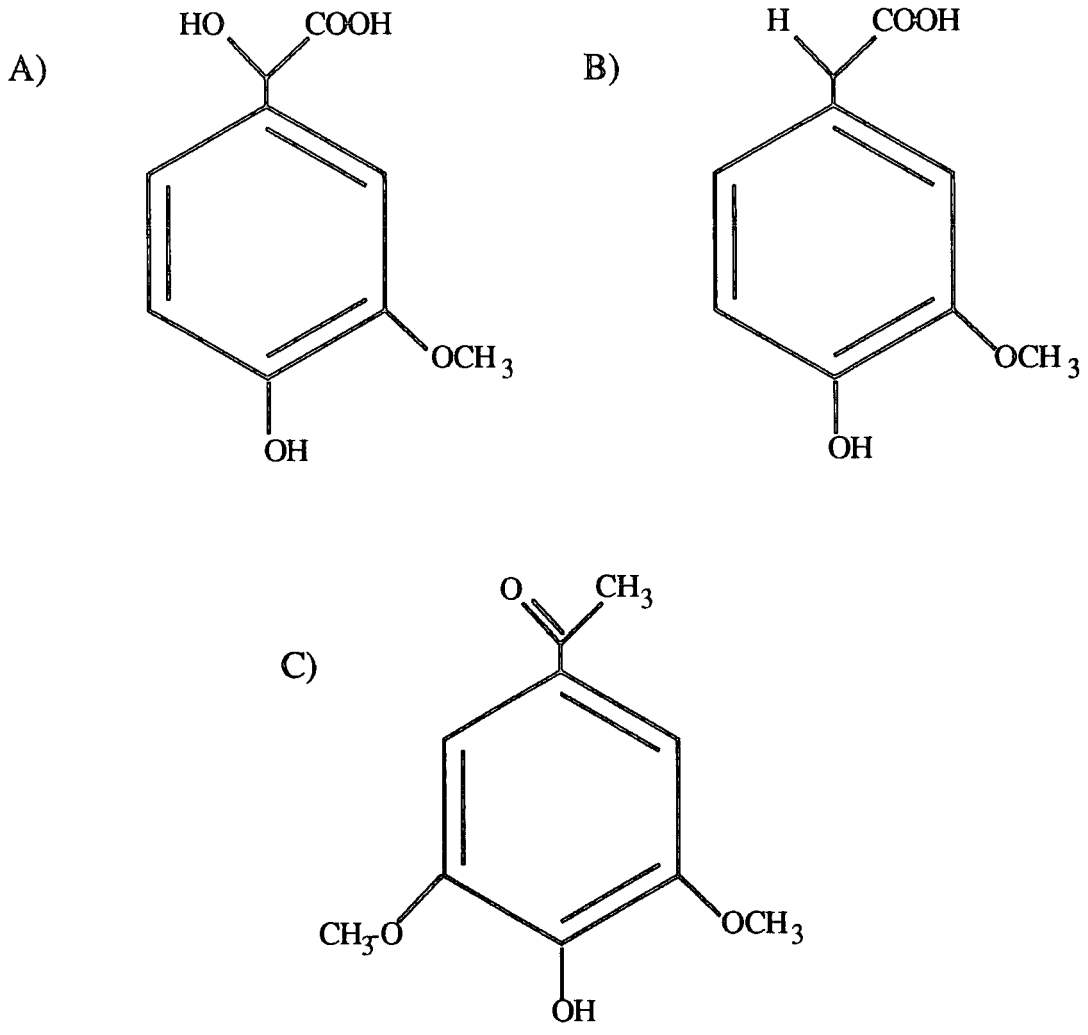


Figure 6.1.a Structures of A) vanillylmandelic acid (VMA); B) homovanillic acid (HVA); C) acetosyringone.

Collection of Animal Cell Cultures [8]. SW837 was another human rectum adenocarcinoma, obtained from Dr. C. Dixon (ICRF, Lincoln's Inn Fields, London, U. K.). SK-CO-1 was also obtained from Dr. Dixon and was a human colon adenocarcinoma.

For comparison to the above, a neuroblastoma cell line (SK-N-BE2C) was obtained from Dr. J. Kemshead (Institute of Child Health, Bristol, U. K.). All of the cell lines were cultured as monolayers to confluence at 37 °C under 5 % CO_2 in 'Tissue Culture Medium' - RPMI 1640 medium (GibcoBRL) supplemented with 10 % heat inactivated foetal calf serum and 2 mM L-glutamine. The tissue culturing of the cell lines was kindly performed by Dr. I. Still (University of Durham, Durham, U. K.).

Once the cells had reached confluence they were removed by centrifugation and the resulting medium was used in chemotaxis assays as described in section 2.5.11. In all the experiments, actively motile bacteria were selected and cultured from swarm plates (see section 2.5.11). C58C1 and C58C1(pVK257) were cultured for 24 hours in LM-broth before 100 µl of each suspension was subcultured into 5 ml of tissue culture medium and cultured for a further 24 hours after which time the cells were diluted in fresh culture medium to a concentration of 1×10^7 cells.ml^{-1}. This suspension was used in the lower chamber for the blindwell assays. The upper chamber was filled with the supernatant tissue culture medium from the growing cell lines, any secreted phenolics (e.g. VMA or HVA) being now present in this (see section 2.5.11).

After 2 hours the numbers of bacteria that had moved into the upper chambers were counted and compared to control assays in which the bacteria had been exposed only to fresh tissue culture medium. The results were expressed using the Chemotactic Index (CI) [231] (see section 2.5.11).

Palmer and Shaw had shown that the CI of C58C1(pVK257), using 10^{-7} M acetosyringone in this set up, was 64 %, whereas strains of C58C1 lacking *virA* and *virG* showed little or no response [231]. In the current study, a comparison of chemotaxis by C58C1 and C58C1(pVK257) towards the culture supernatant of SW837 showed CI values of 13 % and 28 % respectively. This strongly suggested that pVK257 (which contains *virABGC*) was able to respond to a compound produced by the SW837 cell line. On the basis of the results of Ashby *et al.* [9, 11, 12, 273] it appeared that this compound (or compounds) was phenolic in nature

bearing a resemblance to acetosyringone, since the *virA* and *virG* functions were required for chemotaxis.

Taking the culture supernatant from one flask of SW837 cells and diluting it with fresh tissue culture medium and then carrying out the blindwell assays, showed that the response was due to a diffusable compound produced by the cells themselves - the more dilute the supernatant, the less the chemotactic response (see figure 6.2.a).

Four further experiments were carried out on the cell lines and the CI values obtained for C58C1(pVK257) are displayed in table 6.2.a. Although the C58C1(pVK257) cultures used should have all contained bacteria at the log phase of growth, confluence in the cancer cell line cultures was more difficult to judge and thus it might be invalid to compare the experiments with one another. However, it is clear that cells of SW837 elicited the strongest response and HRT18 cells also seemed to produce a good chemoattractant. Supernatants from cultures of SK-CO-1 consistently showed a weak response, whereas the supernatant from HT115 cultures varied. This variation might be the result of the cells being in different phases of growth in the different experiments, producing different amounts of chemoattractant compounds. It is, though, interesting to note that the supernatants from the two cultures containing rectal cancer cells produced the two strongest responses, whereas those from the colonic cancer cell line cultures produced much weaker responses. This might suggest that epithelial tumours of the rectum produce different compounds from their colonic counterparts.

Results obtained using the neuroblastoma cell line, SK-N-BE2C, known to produce VMA and HVA also produced quite a strong response (CI = 18 % - mean of two separate assays). However, this is much less than the response obtained when using the two rectal cell lines, suggesting that the latter might produce more of the chemoattractant molecule(s) or chemicals that are stronger chemoattractants than either VMA or HVA.

These results were very encouraging, suggesting that an *A. tumefaciens* 'microbial inoculant' might be attracted to a rectal tumour *in vivo*. This could be delivered by using a simple suppository. Unlike many chemicals applied in this way, the bacteria should remain in the vicinity of the cancer, due to the chemotactic response, providing a localised application of the anti-tumour agents. However, this idea does not take into account the effects that amino acids and sugars within the

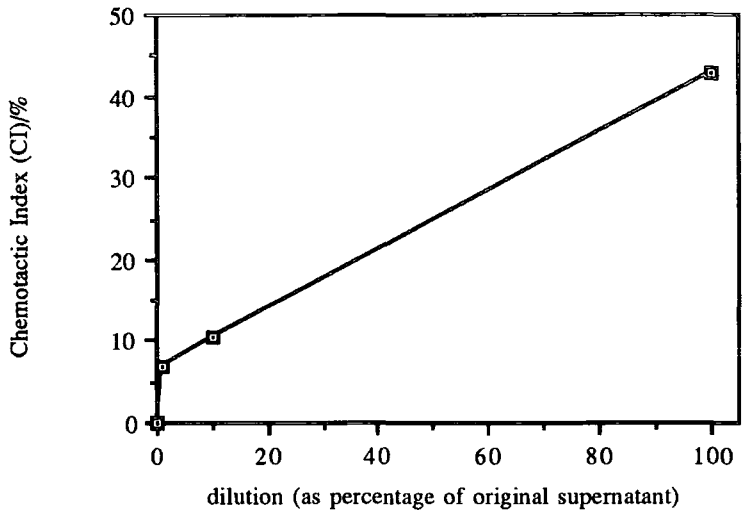


Figure 6.2.a - showing linear decrease of CI with decreasing concentration of original SW837 cell supernatant when diluted with fresh tissue culture medium.

Cell type	1	2	3	4	Mean
HRT18	24 %	31 %	29 %	23 %	27 %
SW837	34 %	40 %	35 %	31 %	35 %
HT115	3 %	3 %	19 %	16 %	10 %
SK-CO-1	1 %	7 %	2 %	2 %	3 %

Table 6.2.a - Showing CI (Chemotactic Index) values for C58C1(pVK257) against cell culture supernatants of various cancer cell types obtained in four separate experiments.

rectum might have on the bacterium, and the proposed system relies on the phenolic compounds having a stronger chemotactic affect on the bacteria than other compounds. Whether this is the case was not investigated. The next aspect of the idea was to see whether the culture supernatants could elicit *vir* induction.

6.3 *vir* induction in response to secreted factors from cancer cells

For these experiments strains C58C1(pVK257, pUCD1187) (*lux*) and C58C1(pIB50, pIB100) (*lacZ*) were employed (see sections 3.1/2/3). Chemotaxis assays confirmed that the introduction of pUCD1187 into C58C1(pVK257) made no difference to the CI values obtained for the cells in response to the supernatants from the different cell lines. In the first assay, X-ray film was simply placed over the open ends of the upper blindwell assay chambers once the cell count readings had been taken. This set up was placed in a light-proof box for 12 hours but upon development it was found that all the chambers had darkened the film. It was suspected that some chemical interaction may have occurred between the film and the medium in the chambers.

Therefore the *lux* induction assay was performed as described in section 2.5.7 using Rogowsky medium and supplementing this with 0.5 ml of culture supernatant from a confluent culture of SW837 cells in tissue culture medium. 0.5 ml of fresh tissue culture medium was used as a control. Readings of the light produced by the cultures and the total number of cells present were taken over a period of 22 hours. However, no *vir* induction was evident.

Further assays involved soaking small strips (30 mm x 10 mm) of Whatman 3MM paper in the culture supernatants and leaving these on the surface of LM-agar plates for 3 hours to allow chemicals from the supernatants to diffuse into the agar. The strips were then removed and 100 µl aliquots of a culture of C58C1(pIB50, pIB100) were spread across the plates to produce a bacterial lawn. Signs of a blue colouration were looked for in the vicinity of where the paper strips had been, but at no time was this observed.

It was concluded therefore that the compounds produced by SW837 and HRT18 cells were able to elicit chemotaxis but not *vir*-induction. This is in agreement with the results of Palmer who found the same phenomenon occurring in chemotaxis/*vir*-induction assays using VMA and HVA [230]. Shaw suggested that these responses might be due to the interaction of VirA with two different phenolic binding proteins [271]. It was hypothesised that there might be a high affinity

binding protein and one with a lower affinity. The high affinity protein would bind the periplasmic region of VirA, triggering low levels of phosphorylation resulting in small amounts of phospho-VirG which in turn would have a higher affinity for the chemotaxis pathway. The lower affinity binding protein would only bind the phenolic compound when present in much higher concentrations. This would then interact with the cytoplasmic domain of VirA resulting in phosphorylation of much more VirG which would then saturate the chemotaxis pathway and thus enable the phospho-VirG to interact with the *vir* boxes, causing *vir* induction. This is only a hypothesis at present, but the discovery of two phenolic binding proteins (p10 and p21) by Lee *et al.* [184] does lend some weight to this argument.

It is therefore possible that the structures of HVA and VMA and related compounds are sufficiently different from acetosyringone and other wound-specific phenolic compounds that they are not able to be bound by the low affinity binding protein.

Shaw *et al.* suggested three classes of phenolic compound depending on the response elicited by *A. tumefaciens*: *vir*-inducers, requiring Ti plasmid for chemotaxis; weak/non-*vir*-inducers, chemoattractant for cured strains; and non-*vir*-inducing, non-chemoattractants [273]. From the results presented here, and from those of Palmer [230], it would appear that an extra group needs to be added - non-*vir*-inducers, requiring Ti plasmid for chemotaxis.

6.4 Future developments of this system

No further experimental work was performed on developing this system, although the results obtained so far suggest that a bacterial method of delivering anti-tumour agents to intestinal cancers might be possible. However, Prof. B. Westley (Prof. Molecular Pathology, University of Newcastle-Upon-Tyne, U. K.) was of the opinion that such a system would not be effective in the treatment of epithelial colonic and rectal cancers of the type tested above since the tumours that develop do no protrude into the gut but rather invaginate into the body cavity [326]. It was suggested that a cancer that caused polyps to form in the intestine might be more suitable [326]. Such a cancer is familial adenomatous polyposis (FAP) which can cause the formation of over a thousand polyps protruding from the wall of the colon into the gut [13]. It is not known whether this cancer can produce the compounds required to elicit chemotaxis, or indeed *vir*-induction . Thus if a cell line can be obtained, the experiments described above will be repeated to test for these compounds. Following any successful outcome of these experiments faecal/urine

samples should be taken from patients suffering from FAP and those who do not and chemotaxis/*vir*-induction assays repeated to see whether any of the compounds normally present in such materials will mask the presence of cancer-specific phenolics.

If a difference in chemotactic responses is observed (but no difference in *vir* induction is noticed) between the two sets of samples then a system of delivery of anti-tumour agents constitutively expressed in the bacterium might be appropriate.

Whatever the outcome of these experiments, it is unlikely that any clinical product would use *A. tumefaciens* since it has been found to cause disease in humans [125, 255]. Thus the system would have to be engineered into a more suitable, harmless host, probably a natural gut inhabitant such as *E. coli*. This process would obviously be difficult and take many years to achieve.

However, through better understanding of the interactions of the phenolic binding proteins, it might be possible to develop a diagnostic 'test' for certain types of cancer based on *A. tumefaciens*. If the hypothesis of Shaw [271] is correct, then if the interaction between phospho-VirG and the chemotaxis pathway can be elucidated, a system could be developed whereby the phospho-VirG 'switches on' the production of an enzyme able to cleave a chromogenic substrate, rather than the chemotaxis pathway. This, though, depends on the absence of interfering compounds that might be present in urine or faecal samples and no experiments have been performed as yet to make this suggestion of an *A. tumefaciens* diagnostic test for some cancers any more than an idea.

Through further work in this area it might be possible to develop a delivery method for certain types of anti-tumour agent. This would be particularly appropriate for agents that have a deleterious effect on other non-cancerous cells, making a method for targeting desirable. It might be possible to develop a diagnostic test for some forms of cancer using an *A. tumefaciens* system. Whatever the outcome of these ideas, it is apparent that work done in this area will also help the further understanding of the mechanisms of chemotaxis and *vir*-induction in *Agrobacterium tumefaciens* and also to identify novel compounds produced by some tumours.

Chapter 7

SUMMARY

7.1 Summary and future work on this system

Ashby designed a theoretical system whereby pesticidal proteins expressed under the control of a *vir* promoter in *A. tumefaciens* could be selectively applied to crops undergoing insect or fungal attack [9, 10].

A. tumefaciens is a common rhizosphere bacterium causing a neoplastic disease of dicotyledonous plants, crown gall tumour [168]. It is postulated that the bacterium is able to chemotactically respond to wound-specific phenolic compounds, such as acetosyringone, and thus migrate towards the wounded sites of plants by virtue of an interaction of the phenolic compounds with the *virA/virG* sensing/control mechanism [12, 272, 273]. Once at the wound site, the higher concentration of the phenolic compounds is able to cause *vir*-induction which in turn results in the transfer of a piece of DNA to the plant genome which causes a neoplastic overgrowth to form [271].

Thus by removing the tumorigenic functions of the bacterium and placing pesticidal proteins under *vir*-promoter control, it should be possible to engineer the bacterium in such a way that it will migrate to the plant wound sites and subsequently produce the pesticides necessary to control the pest causing the damage [9, 10].

Results from this study suggested that *vir*-induction does occur at the wound sites - a necessary prerequisite for the proposed biocontrol agent to function properly. Two chitinase genes [157] were placed under *vir* promoter control in the work by Lilley [187] and in this study, and inducible expression of the genes was found to occur (in all but one case) in response to the presence of the wound-specific compound, acetosyringone. The chitinases were also demonstrated to be effective in the control of some species of fungus, although the results using the inducible

chitinase-producing strains of *A. tumefaciens* were not convincing. It thus remains to be seen whether these strains are able to control the fungi in the field.

A second pesticidal protein gene, for a lepidopteran-specific δ-endotoxin from *B. thuringiensis* var. *kurstaki* (*cryIA(c)*), was placed under *vir* promoter control in *A. tumefaciens*. In the presence of acetosyringone the protein was seen to be produced and to be effective in controlling the larvae of *Manduca sexta*.

It is unlikely that this strain would be able to control lepidopteran insects in the field since the larvae are normally associated with the aerial parts of the plants and it is thought unlikely that, being a soil-borne bacterium, the *A. tumefaciens* strain could be maintained at high enough concentrations to effect the desired level of control. However, many δ-endotoxins exist that are toxic to coleopteran larvae (CryIII and CryV-type) such as *Diabrotica spp.*, and use of one or more of these genes might provide a fully functional 'microbial inoculant' when inserted into the system described. Since it has also been found that *Diabrotica balteata* is susceptible to ChiB, a more effective biocontrol agent might be obtained through expressing both *chiB* and a *cryIII* or *cryV* gene in the same bacterium.

For effective control of fungi, two genes might also be used in the same *A. tumefaciens* strain including a chitinase and maybe β-1,3-glucanase.

This system of applying pesticides has some advantages over transgenic plants in that bacteria are easier to engineer, more than one foreign gene can easily be expressed and the pesticide is only applied when and where it is necessary which it is hoped will go some way towards combating resistance build-up in the pests. 'Microbial inoculants' are also able to replicate in the soil, thus negating the need for reapplications of the pesticide which is a current problem with *B. thuringiensis* δ-endotoxin preparations currently employed. Transgenic plants also suffer from consumer concerns regarding the ingestion of genetically altered material. However, it is envisaged that both 'microbial inoculants' and transgenic plants would be used together to effect the desired level of control of certain pests in a more environmentally responsible manner.

Further improvements to this system can still be made. A plasmid stability assay carried out in this study suggested that some types of plasmid would not be stable in the field and thus efforts should be made to engineer the functions of the 'microbial inoculant' into one plasmid that would be stably inherited or possibly into the chromosome. Use of incompatibility functions in the bacterium might be able to

prevent the 'microbial inoculant' reacquiring a Ti plasmid [99, 170] and thus becoming pathogenic.

It is clear that part of the success of an *A. tumefaciens*-based 'microbial inoculant' would depend on the production of sufficient amounts of pesticide to allow effective control. Further increases in the expression of the genes used might be achieved by using certain 'supervirulent' forms of *virG* that have been identified and shown to increase the pathogenicity of the host strains by making more VirG available after *vir* induction [50, 151]. The action of more efficient terminators might also be examined and codon usage of the genes might be optimised for expression in *A. tumefaciens*. The replacement of the second codon could also be examined, substituting it for the strongest reported codon for *A. tumefaciens* . Looman *et al.* [191] found that gene expression was enhanced in *E. coli* when a weak second codon was replaced with a stronger one and this effect was also seen when the weak codon (GAT) of *cryIA(c)* was replaced with the much stronger AAA and expressed in *E. coli* [98]. This effect was unrelated to codon preference [191].

There is public concern over the release of genetically-engineered microorganisms and it is unlikely that any form of known plant pathogen would be released into the environment. Thus it should be determined whether the *vir* system could operate effectively in related non-pathogenic bacteria such as *Rhizobium spp.*. It is known that tumorigenic forms of *Rhizobacterium spp.* can be developed by the introduction of a Ti plasmid although this does not appear to occur in the field [137]. It appears that the genes used in this study can also be expressed in the related *Rhizobium* genus [224, 279].

It is therefore hoped that a safe system of delivering pesticidal proteins to wounded plants in a conservative manner can be developed and that this system, based on the *vir* induction pathway of *A. tumefaciens*, will allow the cultivation of quality crops with less requirement for dangerous chemicals.

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