Heterologous expression from Agrobacterium virulence promoters

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A thesis submitted to the
University of Durham
for the degree of Doctor of Philosophy

Department of Biological Sciences
University of Durham

1991

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PhD 1991

ABSTRACT

The aim of this work was twofold: to construct plasmids with a gene encoding a pesticidal protein expressed from an *Agrobacterium tumefaciens* virulence promoter and to determine, *in planta*, the sites of *Agrobacterium vir*-induction.

A number of methods were employed to detect *in situ vir*-induction and, to this end, genes encoding β-glucuronidase (GUS) and bioluminescence (*lux*) were linked in plasmid constructs to *Agrobacterium vir*-promoters. In each case, expression of the gene was shown to be induced by the *vir*-inducing phenolic compound acetosyringone. An existing plasmid, in which the *lacZ* gene was under control of the *virB* promoter was utilised to demonstrate *vir*-induction occurring at sites of injury on the roots of mung bean seedlings.

Pesticidal genes expressed from *Agrobacterium* virulence promoters would form the basis of a biological control system. A microbial inoculant harbouring such a construct would produce the pesticidal protein only when in the presence of *vir*-inducing compounds in plant wound exudates. A chitinase gene, *chiB*, from *Serratia marcescens* was characterised and sequenced and, following removal of its promoter region, was linked to an *Agrobacterium virB* promoter. Plasmids were also constructed in which the *chiA* gene of *S. marcescens* was brought under the control of a *virB* or *virE* promoter. All the constructs specified acetosyringone-inducible production of chitinase. Chitinase is effective in the biological control of chitin containing organisms such as fungi.

DECLARATION

No part of this thesis has been previously submitted for a degree at this or any other university. All the material is my own original work except where otherwise stated.

ABBREVIATIONS

AS	acetosyringone
bp	base pairs
cpm	counts per minute
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate
DMF	dimethyl formamide
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropylthiogalactoside
kb	kilobase pairs
kDa	kilodaltons
MES	2-(*N*-morpholino)ethane sulphonic acid
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

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5.12 Acetosyringone-inducible chitinase production by *Agrobacterium* C58C^1 harbouring pVK257 and pBCA2.

CHAPTER 1

INTRODUCTION

1.1 The genus *Agrobacterium.*

The genus *Agrobacterium* is a member of the family Rhizobiaceae and is closely related to the genus *Rhizobium* (Kersters & DeLey, 1982). Agrobacteria are a common component of the soil microflora and are most usually found in association with plant roots, the number of bacteria around roots being roughly a thousand-fold greater than in nearby soil (New & Kerr, 1972). Morphologically, they are short, parallel-sided, Gram-negative rods with rounded ends. They are 0.6-1.0 μm wide and 1.5-3.0 μm long, with 1-6 peritrichous flagella which may be 4-5 times as long as the cell (Lippincott *et al.*, 1981).

The genus is traditionally considered to be divided into four species based on their phytopathogenicity. *Agrobacterium tumefaciens* causes crown galls on a wide variety of dicotyledonous and some monocotyledonous plants; *A. rhizogenes* induces root proliferation at the site it infects; *A. rubi* causes the disease cane gall; *A. radiobacter* is a non-pathogenic strain (Nester & Kosuge, 1981).

The pathogenicity in each case is determined by the presence or absence of a large plasmid termed either tumour-inducing (Ti) or root-inducing (Ri) (Van Larebeke *et al.*, 1974; Zaenen *et al.*, 1974; Watson *et al.*, 1975; Moore *et al.*, 1979) and since these plasmids are transferable from one strain of *Agrobacterium* to another (Kerr *et al.*, 1977; Genetello *et al.*, 1977) the above classification is controversial and subject to perpetual change. The transfer of plasmids between Agrobacteria confers pathogenic properties on the acceptor strain identical to those of the donor strain (Van Larebeke *et al.*, 1975; Thomashow *et al.*, 1980).

An alternative classification has been proposed which is based on a number of physiological and biochemical characteristics and DNA hybridisation studies (Kerr & Panagopoulos, 1977; Kersters & DeLey, 1982). As these characteristics are chromosomally encoded, they are more likely to be stable and thus legitimately form the basis for a true taxonomic classification. Under this classification, agrobacteria are subdivided into two major biotypes, 1 and 2, and a minor intermediate group, biotype 3, each group containing both pathogenic and non-pathogenic strains. There is also a corresponding regional specificity: biotype 1 predominant for Western Europe and the USA, biotype 2 for Australia and biotype 3 for Southeastern Europe.

For the purpose of this thesis, the traditional nomenclature will be used and henceforth *A. tumefaciens* will refer to a biotype of *Agrobacterium* which when isolated contained a Ti-plasmid and was thus capable of inciting the disease crown gall.

1.2 Crown Gall.

Crown gall is a neoplastic disease of plants induced by *A. tumefaciens* causing considerable losses in certain crops, notably grapes, stone fruits and ornamental plants. It is practically universal in its distribution, occurring in Africa, Asia, Australasia, Europe and North and South America (Elliott, 1951). The disease was first described by Aristotle, but it was in 1907 that Smith and Townsend identified *A. tumefaciens* as the causative agent. Since this time, crown gall and its inciting organism have been extensively and continuously studied, not only because of the economic impact of the disease, but also due to its possible relation to animal cancers and its value as a model system to study the control systems underlying cellular growth and differentiation.

In 1942 White and Braun showed that the presence of the bacteria was not necessary for tumour growth and maintenance. In fact, whereas normal plant tissue grows slowly in culture and then only when exogenous cytokinins and auxins are supplied, gall tissue grows rapidly in the absence of any added hormone. Braun proposed that the bacterium introduced a "tumour-inducing principle" into the plant cells so that they acquired a capacity for autonomous growth (Braun & Mandle, 1948).

In addition to the production of high levels of auxin and cytokinin, tumour cells have the peculiar characteristic of synthesising novel sugar and amino acid conjugates termed opines, which are not present in normal plant cells. In 1970, Petit *et al.* established a significant finding when they discovered that the type of opine synthesised in a tumour was specified by the inciting *Agrobacterium* strain and was independent of the host plant. It was further demonstrated that a secreted opine can be catabolised by the specific *Agrobacterium* strain that incited the tumour and used as an energy, nitrogen and carbon source, a trait which was shown to be Ti-plasmid determined (Bomhoff *et al.*, 1976). This may provide the inciting Agrobacteria with a competitive advantage over other soil microorganisms in the rhizosphere, a phenomenon which has been termed "genetic colonisation" (Schell *et al.*, 1979).

In 1975, there was a major breakthrough in the understanding of crown gall when it was demonstrated that the "tumour-inducing principle" of *Agrobacterium* was in fact carried on a large plasmid, the Ti-plasmid (Van Larebeke *et al.*, 1974; Watson *et al.*, 1975). The obvious way to explain the role played by Ti-plasmids in oncogenicity and opine synthesis was to

assume that part or all of the Ti-plasmid was somehow transferred to and stably maintained in, the transformed plant cells. By means of DNA hybridisation experiments it was indeed demonstrated that a small, discrete piece of DNA from the Ti-plasmid (the transfer, or T-DNA) was transferred to the plant genome (Chilton *et al.*, 1977; Chilton *et al.*, 1980; Willmitzer *et al.*, 1980). This T-DNA is responsible for causing the tumorous phenotype and for the synthesis of opines and it will be discussed in later sections, together with a detailed account of the Ti-plasmid and its functions.

1.3 *Agrobacterium* in the rhizosphere.

The interaction between *Agrobacterium* and plants which ultimately leads to plant cell transformation is often considered to start with the attachment of the bacteria to the plant cell surface. In fact, the plant:microbe interaction begins with the initial colonisation of the plant roots by the bacteria, a process which requires *Agrobacterium* to respond to plant chemical signals.

Agrobacterium sp. are commonly found in both cultivated and non-agricultural soils and are routinely isolated from the roots of plants. Although it was Kerr (1969 & 1974) who first made detailed studies of the occurrence of *Agrobacterium* species in the soil and demonstrated their abundance in the rhizosphere, the close association between *Agrobacterium* and plants was noted by Starkey as long ago as 1929. He investigated the rhizosphere effect in which plant exudates influence the microbial population of the soil and noted: "The development of higher plants exerts pronounced influences upon the soil population, but these influences are greater upon some organisms than upon others.....The greatest proportional increases appear in the *Bacterium radiobacter* (now *Agrobacterium*) group of organisms."

1.3.1 Motility and chemotaxis of rhizobacteria.

Motility is known to be a feature of many rhizosphere bacteria and the importance of flagella in the movement of bacteria in the soil has long been debated (Hamdi, 1971; Soby & Bergman, 1983; Howie *et al.*, 1987; Scher *et al.*, 1988). Howie *et al.* (1987) found that non-flagellated mutants of *Pseudomonas fluorescens* colonised wheat roots to the same extent as the wild types, even at a soil matric potential favourable for motility. Similarly, Scher *et al.* (1988), using strains of *Pseudomonas putida* and *Serratia* spp. showed no positive correlation between motility and root colonisation On the other hand, Soby and Bergman (1983) demonstrated that efficient spreading of *Rhizobium meliloti* in soil demanded active motility and chemotaxis and

experiments carried out by de Weger *et al.* (1987) indicated a requirement for flagella in the colonisation of potato roots by *P. fluorescens*.

Given soil conditions suitable for motility, chemotaxis toward seed or root exudates is likely to contribute to the ability of bacteria to colonise roots. Soil organisms which can detect and move towards a source of nutrients would obviously have a selective advantage over their non-chemotactic counterparts which rely on random rather than directed means of reaching their host organisms.

Chemotaxis has been observed in a number of rhizosphere bacteria (Chet *et al.*, 1973; Scher *et al.*, 1985; Heinrich & Hess, 1985; Bashan, 1986), although studies on the biological significance of the phenomenon have been limited. *Pseudomonas putida* can move 1cm in 12 hours towards a soybean seed in water saturated soil (Scher *et al.*, 1985) and *Azospirillum brasilense* has been shown to migrate distances of up to 160cm under field conditions, the movement being highly dependent on the presence of a plant source (Bashan & Levanony, 1987). The most detailed investigations have been undertaken with *Rhizobium* species and the general conclusion is that non-chemotactic mutants can nodulate host roots but their efficiency and competitiveness are reduced (Hunter & Fahring, 1980; Ames & Bergman, 1981; Bergman *et al.*, 1988; Caetano-Anolles *et al.*, 1988).

Rhizobia are known to be attracted towards substances secreted by plant roots (Currier & Strobel, 1976; Currier & Strobel, 1977; Gitte *et al.*, 1978; Gaworzewska & Carlile, 1982) and since these substances appear to be secreted chiefly by cells in the zone of elongation, then the bacteria will move towards root cells which are most susceptible to infection (Bhuvaneswari *et al.*, 1981). In addition to the chemotaxis exhibited towards nutrients such as sugars and amino acids (Goetz *et al.*, 1982), rhizobia also respond to diverse phenolic compounds by positive chemotaxis (Parke *et al.*, 1985). Most recently, chemoattraction has been demonstrated towards the *nod* gene inducing flavonoid compounds luteolin (Caetano-Anolles *et al.*, 1988) and naringenin (Aguilar *et al.*, 1988) and this highly potent and specific response requires functional nodulation genes. It has been postulated that motile cells in the rhizosphere, which can detect luteolin exuding from plant roots at concentrations between 10^{-10} and 10^{-9}M move towards the host until the luteolin concentration reaches 10^{-7}M (Caetano-Anolles *et al.*, 1988). At this point chemotaxis is diminished but the induction of *nod* gene expression is rapid (Peters *et al.*, 1986).

Over the past few years a highly comparable scenario has been demonstrated to exist for the *Agrobacterium*:plant interaction in the rhizosphere (Ashby *et al.*, 1987; Parke *et al.*, 1987; Ashby *et al.*, 1988; Shaw *et al.*, 1988a). The parallel effects of specific phenolic compounds on chemotaxis and *vir* gene induction suggest that this pattern of coordinated responses may be of general importance to plant:microbe interactions.

1.3.2 Chemotaxis in *Agrobacterium*.

A. tumefaciens strains are attracted to plant roots with broken root hairs in particular being major sites for bacterial accumulation (Schroth *et al.*, 1971). When Stachel and coworkers identified acetosyringone as a signal molecule produced by wounded plant cells which activates *vir* gene expression, they speculated that it might also serve to attract *Agrobacterium* to plants (Stachel *et al.*, 1985b). However, it is only recently that a detailed study of chemotaxis in *Agrobacterium* has been undertaken (Ashby *et al.*, 1987; Parke *et al.*, 1987; Ashby *et al.*, 1988; Loake *et al.*, 1988; Shaw *et al.*, 1988a). As a result of these investigations a scheme has been proposed for how *A. tumefaciens* recognizes host plants under natural conditions and becomes established in the rhizosphere, particularly around wounded plant tissue, in sufficiently high numbers to initiate infection (Shaw *et al.*, 1988b).

A. tumefaciens C58C^1 exhibits a highly sensitive chemotactic response towards a range of sugars, many of which are characteristic of plant exudates (Loake *et al.*, 1988). Sucrose, which is generally the most abundant translocated plant sugar, evokes by far the strongest and most sensitive response, with an optimum at 10^{-6}M. The responses towards glucose and fructose are equally as sensitive but of a much lower magnitude. In addition, the amino acids arginine and valine also act as chemoattractants for *Agrobacterium*. These responses are independent of the Ti-plasmid (Loake *et al.*, 1988). Since the sugars which act as attractants are components of plant exudates, these results suggest that the prevalence of *Agrobacterium* in the rhizosphere may be at least partly explained by its extremely sensitive chemotaxis towards plant saccharides. Both Ti-plasmid containing and non-pathogenic strains are attracted and, in fact, virulent strains often make up less than 1% of the rhizosphere agrobacteria (Kerr, 1969).

Once established in the rhizosphere, agrobacteria possessing a Ti-plasmid exhibit a second level of attraction which probably guides them specifically towards susceptible wounded plant cells releasing phenolic compounds (Ashby *et al.*, 1987; Ashby *et al.*, 1988). Ashby *et al.* (1987) demonstrated that acetosyringone, known to be a strong *vir*-inducer, elicits a slight, but sensitive, chemotactic response from *A. tumefaciens* C58C^1 harbouring a Ti-plasmid, with maximal attraction occurring at a concentration of 10^{-7}M. The otherwise isogenic, cured strain, exhibited no attraction towards acetosyringone. Alternatively, vanillyl alcohol, which does not induce *vir*-expression, evokes a much weaker chemotactic response which is independent of the Ti-plasmid.

Further studies, using a range of phenolic compounds, confirmed the correlation between *vir*-inducing ability and Ti-plasmid requirement for chemotaxis (Ashby *et al.*, 1988). Moreover, the Ti-dependent responses are significantly more sensitive than those evoked by non-inducers. For acetosyringone, the concentration giving peak attraction is some 100-fold lower than the

maximal *vir*-inducing concentration (Stachel *et al.*, 1985b). This suggests a scenario whereby virulent agrobacteria, which have already accumulated in the rhizosphere due to their strong attraction towards sugars in plant exudates, will be attracted towards wounded plant cells which are releasing *vir*-inducing phenolic compounds. The bacteria will migrate up the concentration gradient until they reach the cells, where the concentration of inducer will be high enough to effect *vir*-induction and thus initiate the chain of events leading to T-DNA transfer and plant transformation (see later sections).

Conflicting evidence has been provided by Parke *et al.* (1987) who reported that *A. tumefaciens* strain A348 is not attracted towards acetosyringone and chemotaxis towards other phenolic compounds does not require Ti-plasmid functions. A possible explanation for this discrepancy is that strain A348 is very poorly motile in comparison with C58C^1 (Ashby *et al.*, 1988).

Despite this controversy surrounding the specific action of phenolic compounds as chemoattractants for *Agrobacterium*, the importance of chemotaxis in the biology of *Agrobacterium* infection should not be overlooked. Ashby *et al.* (1988) showed that *A. tumefaciens* is attracted towards root and shoot extracts from both monocotyledonous and dicotyledonous plants, whilst Hawes and her coworkers have been using non-chemotactic mutants to assess the significance of chemotaxis in the pathogenicity of *A. tumefaciens* (Hawes *et al.*, 1988; Hawes & Smith, 1989). It was demonstrated that wild-type agrobacteria are attracted to isolated root cap cells of pea, then Tn5 mutants were selected which failed to show this response (Hawes *et al.*, 1988). The mutants still cause tumours when applied directly to wounded plants, but are completely avirulent when used in indirect soil assays (where wounded seedlings are planted in soil infested with bacteria). However, when sand is used for indirect inoculation assays, the mutant bacteria show only a slight reduction in virulence (Hawes & Smith, 1989). The authors conclude that in soil, chemotaxis is crucial to the pathogenicity of *A. tumefaciens* on pea plants, but in some environments it may be superfluous.

The same group also showed that the chemotactic response towards sugars appears to be more important than attraction to phenolic compounds in the establishment of *Agrobacterium* in the rhizosphere (Hawes & Smith, 1989; Hawes, 1990). They isolated a mutant which was not attracted to isolated root cap cells but retained an attraction to excised root tips. Such a phenotype implies that the attractants from live sloughed cells are distinct from those from wounded cells and this mutant is specifically attracted to those substances released by wounded tissue. However, this mutant is no more pathogenic in virulence assays than non-motile mutants. Loss of attraction to root cap cells always results in reduced virulence, whereas mutants which are only non-chemotactic towards excised root tips remain fully virulent. Hence, attraction to substances from non-wound exudates is apparently more important than the

chemotactic response to chemicals released from wounds. Attraction to wound sites is likely to be important only on a micrometre scale (Shaw *et al.*, 1989), and so it is possible that this response facilitates infection once the bacteria are in the rhizosphere.

1.4 The *Agrobacterium*: plant interaction.

1.4.1 Attachment of *Agrobacterium* to plant cells.

Once Agrobacteria are established in the rhizosphere the first stage in the actual transformation process is the attachment of the bacteria to the surface of susceptible plant cells. This binding has been observed with both light (Douglas *et al.*, 1985) and scanning electron microscopy (Matthysse *et al.*, 1981). It was Lippincott and Lippincott (1969) who first proposed the site-specific attachment of *A. tumefaciens* to plant cells and, although the existence of a plant cell receptor is not yet proven, it is supported by a number of pieces of evidence. Binding was found to be saturatable, with both tomato cells (Neff & Binns, 1985) and carrot cells (Gurlitz *et al.*, 1987) possessing about 200 attachment sites per cell. Neff and colleagues (1987) have put forward evidence for a pectin or pectin-associated receptor; a pectin-enriched soluble cell wall fraction inhibits *A. tumefaciens* binding and tumour formation. Alternatively, the plant receptor may be a protein as proposed by Gurlitz *et al.* (1987), or possibly a glycoprotein, since protease treatment partially abolishes the binding capacity of plant cells.

The binding itself appears to be a two-step process. The initial step is the attachment of single bacterial cells to the plant cell surface, a loose and reversible attachment, which is followed by a massive aggregation of bacteria. The aggregates result from the bacterial synthesis of cellulose fibrils which surround the bacteria and anchor them to the plant cell whilst also entrapping additional bacteria (Matthysse *et al.*, 1981) Cellulose-deficient mutants remain virulent and can still attach to plant cells but only singly and, since the bacteria can be easily washed off (Matthysse *et al.*, 1983), under natural conditions the production of cellulose appears to be important in firmly anchoring the bacteria.

1.4.2 Chromosomal virulence loci.

A number of constitutively expressed chromosomal loci have been identified which are necessary for *A. tumefaciens* virulence due to to their involvement in the attachment step. Mutants in these loci, *chvA*, *chvB* (Douglas *et al.*, 1985), *pscA* (Thomashow *et al.*, 1987) and *att* (Matthysse, 1987) are either avirulent or have severely attenuated virulence.

The *chvA* and *chvB* loci are two distinct transcriptional units of 1.5 and 5.0 kb respectively which are physically linked on the chromosome. *chvB* mutants lack flagella and do not synthesise a cyclic β-1,2-glucan which is found both extracellularly and in the periplasm of wild type cells (Puvanesarajah *et al.*, 1985). Zorreguieta and coworkers have established that the *chvB* region encodes a 235 kDa inner membrane bound protein which binds UDP-glucose and catalyses the synthesis of β-1,2-glucan (Zorreguieta *et al.*, 1988).

chvA mutants retain the ability to synthesise a β-1,2-glucan but it is not detectable extracellularly or in the periplasm (Cangelosi *et al.*, 1989). Additionally, most of the glucan in wild type cells is modified by the addition of anionic substituents (Miller *et al.*, 1987), whereas *chvA* mutants retain their cellular glucan in an unsubstituted form (Inon de Iannino & Ugalde, 1989). The *chvA* locus codes for a 75 kDa inner membrane protein which is highly homologous to bacterial and eukaryotic export proteins (Cangelosi *et al.*, 1989) and it has been suggested that the ChvA protein forms a complex in the membrane with ChvB (Inon de Iannino & Ugalde, 1989). The accumulation of non-substituted glucans in *chvA* mutants is probably due to a defect in the translocation of glucan into the periplasmic space, where the modifying reactions are likely to take place (Inon de Iannino & Ugalde, 1989).

Like *chvB* mutants, *pscA* mutants also lack β-1,2-glucan and are non-motile but in addition, they fail to synthesise exopolysaccharide (Thomashow *et al.*, 1987). Nevertheless it is again likely to be the absence of glucan which is the determining factor in causing avirulence, since *ros* mutants also lack polysaccharide but remain fully virulent (Close *et al.*, 1985). It has recently been discovered that the *pscA* mutant phenotype is caused by the lack of the enzyme phosphoglucomutase which carries out one step in the biosynthesis of UDP-glucose, a substrate in the biosynthesis of both β-1,2-glucan and succinoglycan (Uttaro *et al.*, 1990).

These three loci, *chvA*, *chvB* and *pscA* all have homologous loci in *Rhizobium* with which they are functionally interchangeable (Dylan *et al.*, 1986; Marks *et al.*, 1987) suggesting a general role of surface components in mediating bacterial-plant cell interactions. Despite the elucidation during recent years of the identity and function of these virulence loci and the recognition of the importance of β-1,2-glucan, the true role of this cyclic glucan in *A. tumefaciens* virulence has yet to be identified.

Matthysse (1987) has isolated a number of mutants, termed *att*, which are deficient in binding and tumorigenesis but retain an unchanged complement of polysaccharide and β-glucan. Closer investigation revealed that these mutants lacked one or two periplasmic or outer membrane proteins which presumably play a role in the bacterial attachment to plant cells, possibly in conjunction with β-1,2-glucan.

1.5 The Ti-plasmid.

All the other genetic components of *A. tumefaciens* which are required for plant transformation and tumorigenesis are located on a large, approximately 200 kb, tumour-inducing (Ti) plasmid (Figure 1.1). The regions of the plasmid which are essential for pathogenicity are the T-DNA, its flanking border sequences and the virulence (*vir*) region. The T-DNA is the DNA segment which is transferred to the plant cell and stably integrated into the genome, whereupon expression of the oncogenes results in tumorigenesis. Unlike transposons, the T-DNA does not mediate its own transfer. Border sequences and the *vir*-region are responsible for T-DNA processing and transit.

Not all Ti-plasmids are identical. They are classified according to the type of opine produced by the transformed cells and metabolised by the bacteria. The most commonly studied plasmids are of the octopine or nopaline type. Octopine type T-DNA genes encode the synthesis of octopine and agropine, eg. pTiA6, whilst nopaline and agrocinopines A and B are found in tumours incited by a nopaline Ti-plasmid, eg. pTiC58 (Bomhoff *et al.*, 1976). Other plasmids, such as pTi542, are responsible for the synthesis of leucinopine and succinamopine (Chang *et al.*, 1983; Chilton *et al.*, 1984).

Generally, each *Agrobacterium* strain catabolises only the opines synthesised by the tumour it induces. The genes required for the catabolism of opines are carried on a region of the Ti-plasmid distinct from the T-DNA and the *vir*-region and they are induced by the presence of the opine (Tempe & Petit, 1982). In addition, some opines induce conjugal transfer of Ti-plasmids between strains of *Agrobacterium* and thus confer virulence upon the non-pathogenic strains which predominate in the soil (Kerr & Ellis, 1982). However, the production and catabolism of opines may not provide *Agrobacterium* with the selective advantage one would expect, since *Agrobacterium* species comprise only a small proportion of the soil microorganisms capable of opine catabolism (Tremblay *et al.*, 1987; Rossignol & Dion, 1985; Beauchamp *et al.*, 1990). Add to this the fact that most *Agrobacterium* strains isolated from the soil do not contain a Ti-plasmid and it may be envisaged that opines could even invite competition from other species.

The restriction maps from each class of Ti-plasmid are different. Nevertheless, there are few known differences in *vir*-gene organisation, gene product functions and open reading frame sequences between the operons common to both nopaline and octopine Ti-plasmids. Within a class of Ti-plasmids the maps may be similar or even identical. The octopine Ti-plasmids form a very homogeneous group, although the nopaline plasmids are rather more heterogeneous (Melchers & Hooykaas, 1987).

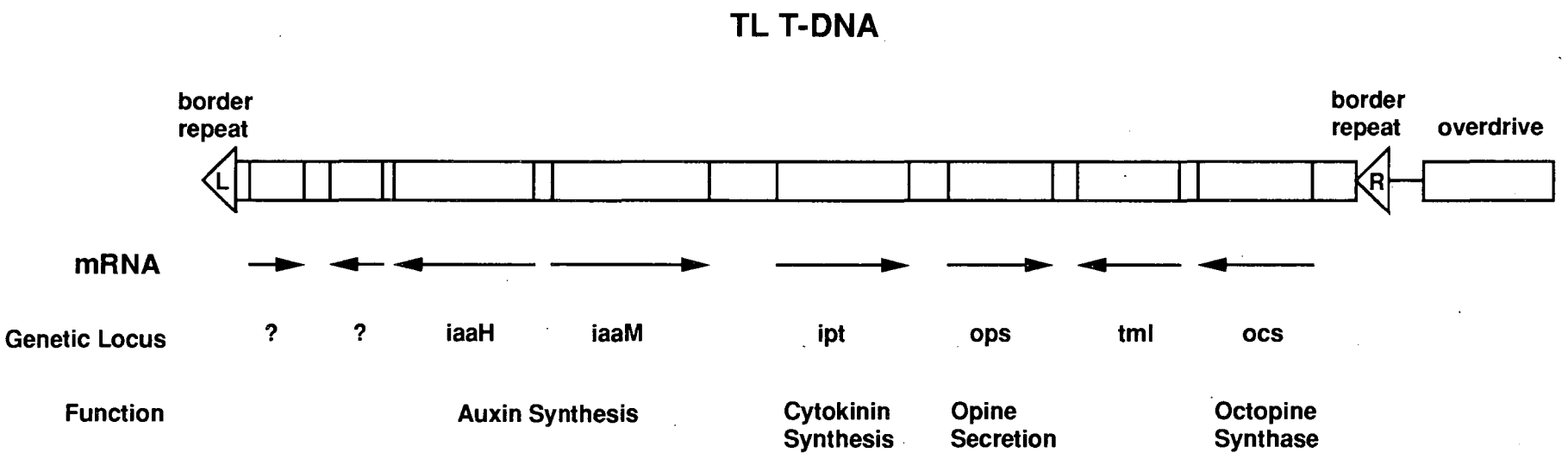

TL T-DNA
border repeat
border repeat
overdrive
L
R
mRNA
Genetic Locus
?
?
iaaH
iaaM
ipt
ops
tml
ocs
Function
Auxin Synthesis
Cytokinin Synthesis
Opine Secretion
Octopine Synthase

Figure 1.2 A genetic map of the TL T-DNA of an octopine-type Ti-plasmid.

The genetic loci are indoleacetamide hydrolase (*iaaH*), tryptophan monooxygenase (*iaaM*), isopentenyl transferase (*ipt*), opine secretion (*ops*), tumour morphology large (*tml*) and octopine synthase (*ocs*).

Adapted from Ream (1989).

1.6 T-DNA transfer.

The T-DNA region is defined as that part of the plasmid that is homologous to sequences present in transformed plants. Its size varies from one type of Ti-plasmid to another. The major difference between the two commonly studied Ti-plasmids is that the nopaline T-DNA is one large continuous segment of roughly 22kb, whilst the octopine Ti-plasmid contains three adjacent T-DNAs. The left T-DNA (TL) element is 13kb and encodes the oncogenes required for tumorous growth and also genes for octopine synthesis and secretion (Figure 1.2). The right T-DNA (TR) element of 7.8kb contains genes for the synthesis of agropine and mannopine, whilst the 1.5kb central T-DNA does not specify a phenotype in transformed cells.

Despite these differences in T-DNA structure most Ti-plasmids have a highly conserved set of oncogenes which are those loci responsible for hormone independence and thus tumorous growth of the transformed plant cells (Depicker *et al.*, 1978; Perry & Kado, 1982). For example, pTiC58 shows only about 15% overall homology with pTiA6 but encodes oncogenes that are very closely related to pTiA6 TL-DNA genes (Joos *et al.*, 1983; Willmitzer *et al.*, 1983).

Two of these genes, *iaaM* and *iaaH*, code for enzymes that establish a new auxin biosynthetic pathway in the transformed cells (Inze *et al.*, 1984; Schroeder *et al.*, 1984; Thomashow *et al.*, 1984/5; Van Onckelen *et al.*, 1986). *iaaM* encodes tryptophan monooxygenase which converts tryptophan to indole-3-acetamide. This is converted to indole acetic acid (IAA) by the action of indole-3-acetamide hydrolase, the product of the *iaaH* gene. The third oncogene is *iptZ*, coding for an isopentyl transferase which converts 5'AMP and isopentylpyrophosphate into isopentyl adenosine-5-monophosphate (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Buchmann *et al.*, 1985) The host cells then rapidly convert this to the cytokinins *trans*-zeatin and *trans*-ribosylzeatin.

Nopaline T-DNA also contains the *nos* gene encoding nopaline synthase, which lies to the right of the conserved oncogenic region and the *acs* gene encoding agrocinopine synthase which lies to the left.

All T-DNAs analysed to date are flanked by 25bp direct repeat sequences termed the border repeats (eg. Holsters *et al.*, 1983; Yadav *et al.*, 1982) and this is the only structural region of the T-DNA which is required in *cis* for its transfer. Transfer is unaffected by deletion of the internal T-DNA region and any DNA placed within the borders is transferred and integrated (Zambryski *et al.*, 1983). Thus the T-DNA element is not size restricted and is, in effect, simply a pair of borders and the DNA between them. The right border repeat is essential for transfer (Shaw *et al.*, 1984; Wang *et al.*, 1984) whilst deletion of the left border has no significant effect on transformation. Furthermore the data shows that T-DNA transfer is a polar

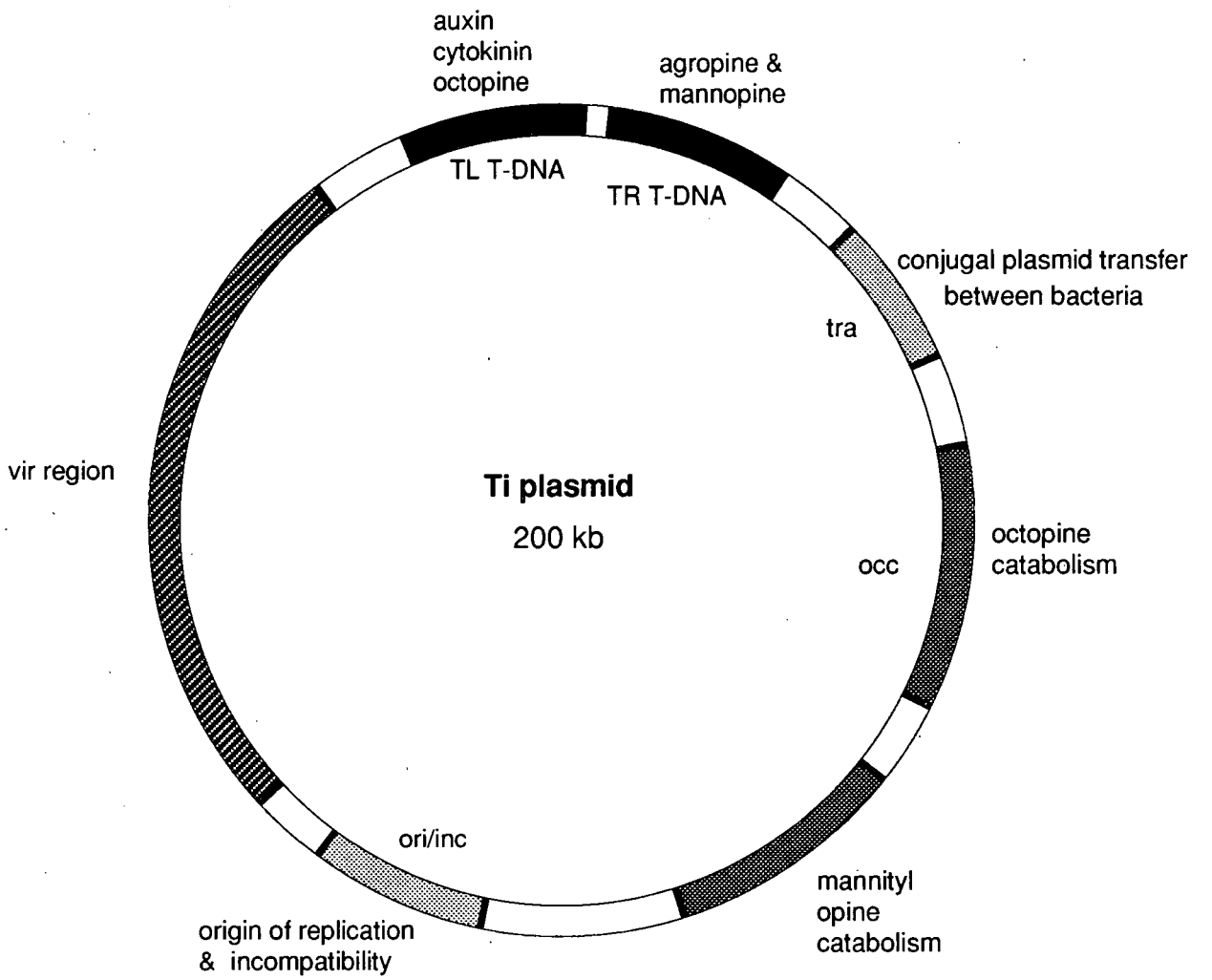

auxin
cytokinin
octopine
agropine &
mannopine
TL T-DNA
TR T-DNA
conjugal plasmid transfer
between bacteria
tra
vir region
Ti plasmid
200 kb
octopine
catabolism
occ
ori/inc
mannityl
opine
catabolism
origin of replication
& incompatibility

Figure 1.1 A genetic map of an octopine-type Ti-plasmid.

This map shows the main loci of the Ti-plasmid and their functions. The *vir*-region comprises six separate loci - *virA*, *virB*, *virG*, *virC*, *virD* and *virE* whose roles are described in the text.

Adapted from Ream (1989)

process since when the right border repeat is reversed with respect to its natural orientation transformation efficiency is greatly attenuated (Wang *et al.*, 1984; Peralta & Ream, 1985).

The sequence context of the border repeats is important. In nopaline Ti-plasmids sequences surrounding right borders enhance and sequences surrounding left borders attenuate polar DNA transfer (Jen & Chilton, 1986; Wang *et al.*, 1987). The situation with regard to the four border T-region of octopine Ti-plasmids is rather more complex. A specific 24bp sequence called *overdrive*, situated to the right of the right-hand border repeats of the TL and TR T-DNA elements has been shown to be essential for efficient T-DNA transfer (Peralta *et al.*, 1986). *Overdrive* can stimulate T-DNA transfer when placed in either orientation, on either side and at a variable distance from synthetic border repeat sequences (Peralta *et al.*, 1986; Van Haaren *et al.*, 1987). No sequences with homology to *overdrive* have been detected in nopaline Ti-plasmids (Wang *et al.*, 1987).

The role which the *vir* proteins play in T-DNA transfer will be discussed in the next section. For a comprehensive discussion of the possible mechanisms of T-DNA transfer and integration see Zambryski (1988) or Ream (1989).

1.7 The *vir* region.

The transfer of the T-DNA into the nuclei of infected plant cells and its integration into the plant genome is mediated by the *vir* regulon. This is a region of the Ti-plasmid, approximately 35kb in length, which lies to the left of the T-DNA and provides most of the *trans*-acting products for T-DNA transit (Figure 1.3). It is organised into six complementation groups (Rogowsky *et al.*, 1987; Stachel & Nester, 1986) four of which (*virA*, *virB*, *virD* and *virG*) are absolutely required for tumour formation. *virC* and *virE* enhance the efficiency of plant transformation and are necessary for the formation of tumours on certain host plants (Hirooka *et al.*, 1987; Yanofsky *et al.*, 1985).

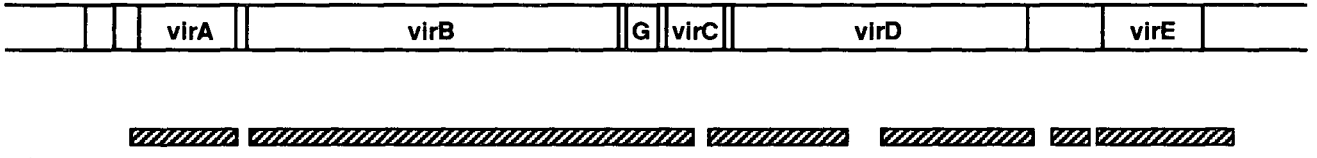
The six *vir* loci are highly conserved among different Ti plasmids and the physical organisation of octopine and nopaline *vir* regions is essentially identical. Furthermore, *vir* genes from a nopaline plasmid will complement mutations in an octopine plasmid and vice versa (Hooykaas *et al.*, 1984). Of the six loci only *virA* and *virG* are monocistronic whilst the other four encode multiple gene products. This reflects the differing roles the *vir* genes play in tumorigenesis: *virB*, *virC*, *virD* and *virE* encode proteins which are actively involved in the transfer of the T-DNA and these loci are transcriptionally regulated by the *virA* and *virG* gene products (Stachel & Nester, 1986; Stachel & Zambryski, 1986).

Figure 1.3 Genetic organisation of nopaline and octopine Ti-plasmid *vir* regions.

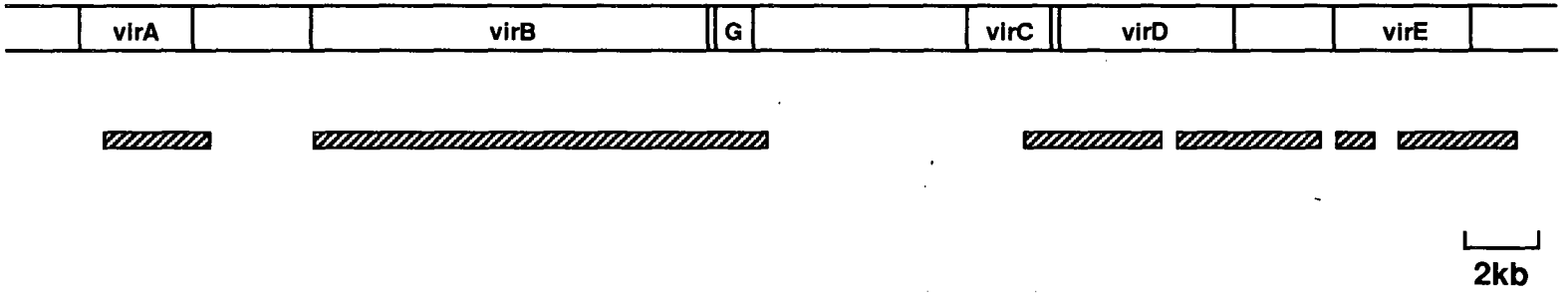
The locations of the six *vir* loci of a representative nopaline Ti-plasmid (pTiC58) and octopine Ti-plasmid (pTiAch5) are shown. Hatched boxes indicate regions of homology between pTiC58 and pTiAch5.

Adapted from Rogowsky *et al.* (1987).

pTiC58



pTiAch5



1.7.1 *virB*

This is the largest *vir* locus with a size of approximately 9.5kb. The complete nucleotide sequence of the operon from a number of Ti-plasmids has been deduced and this predicts eleven open reading frames (Ward *et al.*, 1988 & 1990; Thompson *et al.*, 1988; Kuldau *et al.*, 1990). The predicted polypeptides show no significant homology with any other known proteins although an increasing amount of evidence indicates that the *vir* genes are functionally analogous to the *tra* genes of conjugative plasmids (Stachel & Zambryski, 1986). Most of the *tra* proteins are located in the membrane and, similarly, hydrophobicity plots suggest that most of the *virB* proteins are likely to be membrane associated (Ward *et al.*, 1988; Thompson *et al.*, 1988). Cellular fractionation studies have confirmed a membrane localisation for several *virB* proteins (Engstrom *et al.*, 1987). More recently VirB10 has been shown to have a transmembrane topology and to be a component of an inner membrane protein complex (Ward *et al.*, 1990). It has also been demonstrated that VirB11 associates extrinsically with the inner membrane and is an autophosphorylating ATPase (Christie *et al.*, 1989) which has homology with *comG* ORF1, a protein required for DNA uptake by *Bacillus subtilis* (Albano *et al.*, 1989). The *virB5* gene product also has a consensus mononucleotide binding domain, suggesting that this too may couple energy to T-DNA transport by means of nucleotide triphosphate hydrolysis.

A likely scenario is that the VirB proteins are involved in the formation of a transmembrane complex capable of transferring the T-DNA into plant cells, in a manner analogous to that of the multicomponent membrane pore proposed to mediate DNA transfer in plasmid conjugation (Willetts & Skurray, 1987). VirB11 may function to regulate the conformation or activity of the pore via phosphorylation.

1.7.2 *virD*

Sequence analysis of the *virD* locus shows that it can encode at least four polypeptides of 16.2, 47.4, 21.3 and 75.8kDa (Yanofsky *et al.*, 1986; Porter *et al.*, 1987). The first two of these are required for endonucleolytic cleavage at the T-DNA borders (Jayaswal *et al.*, 1987; Yanofsky *et al.*, 1986). VirD1 contains a potential DNA-binding domain (Porter *et al.*, 1987) and exhibits a topoisomerase-like activity in its monomeric form, converting supercoiled DNA to its relaxed form (Ghai & Das, 1989). Together with VirD2 it participates directly in the generation of T-strand DNA molecules (linear, single-stranded DNA molecules corresponding to the bottom strand of the T-DNA region). VirD2 is a strand-specific and sequence-specific endonuclease which cleaves at the T-DNA border repeat sequences (Soliman & Das, 1990). In

addition it has been shown to become covalently attached to the 5' terminus of nicked T-DNA molecules after cleavage and is found associated with both double-stranded nicked DNA and single-stranded T-strands (Howard *et al.*, 1989; Young & Nester, 1988). This tight association probably prevents closure of the nick by ligase and has been demonstrated to protect the T-DNA from exonucleolytic degradation (Durrenberger *et al.*, 1989).

Only the first 203 amino acids of VirD2 are necessary for its participation in nicking border repeats (Stachel *et al.*, 1987; Yanofsky *et al.*, 1986) and binding the 5' end of the nicked DNA (Young & Nester, 1988). This N-terminal half of the protein shows a striking evolutionary conservation of amino acid sequence, with 90% homology of VirD2 from nopaline and octopine Ti-plasmids and Ri-plasmids (Wang *et al.*, 1990). Conversely, the C-terminal domain of the protein, which is necessary for virulence but is not required for T-DNA processing, is poorly conserved (Wang *et al.*, 1990; Steck *et al.*, 1990). However, all VirD2 proteins display a very similar hydropathy profile in this region which may perform other functions required for T-DNA transmission.

A number of conceivable roles have been attributed to the protein component of the T-DNA transfer complex. These include recognition of transmembrane routes from the bacterium to the plant cell, possibly involving interaction with VirB membrane proteins, targetting of the transfer complex to the plant nucleus and efficient integration of the T-DNA into the plant genome. Recently, Herrera-Estrella *et al.* (1990) provided confirmation of one of these functions when they showed that the first 292 amino acids of VirD2 are able to direct the cytoplasmic protein β-galactosidase to the plant nucleus. Thus the VirD2 protein linked to the T-DNA acts as a pilot protein and targets it to the plant nucleus. VirD2 contains three peptides which resemble a proposed consensus signal sequence for nuclear targetting (Wang *et al.*, 1990). Two of these are found in the C-terminal domain and whilst not essential for targetting the T-DNA they could act as auxiliary signals leading to a higher efficiency of transport through the nuclear envelope.

The VirD4 protein contains a transmembrane signal sequence and may interact with other *vir*-encoded proteins to promote T-DNA transfer (Porter *et al.*, 1987).

1.7.3 *virC*

The *virC* locus stimulates, but is not completely essential for, virulence. Its role seems to be host-species-dependent since the effect of a *virC* mutation is more pronounced on some plant hosts than on others (Close *et al.*, 1987). Moreover, the limited-host-range plasmid pTiAg162 does not have a functional *virC* locus but is nevertheless able to incite tumours on grapevines

(Yanofsky *et al.*, 1985). Two polypeptides of 26 and 23kDa are encoded by *virC* (Yanofsky & Nester, 1986). *virC* mutants transmit T-DNA inefficiently to plant cells suggesting that *virC* functions may help generate a transfer intermediate. Accordingly, VirC1 has been shown to bind specifically to the overdrive sequence and enhance the activity of the VirD endonuclease (Toro *et al.*, 1988 & 1989). VirC1 protein thus interacts directly with overdrive through specific DNA-protein binding and VirC2 may act indirectly, probably through interactions with VirD2 or VirC1 which bind overdrive or the border repeat.

1.7.4 *virE*

The *virE* locus contains at least two open reading frames, the largest of which, *virE2*, encodes a 65kDa non-specific single-stranded DNA-binding protein (Citovsky *et al.*, 1988; Das, 1988; Christie *et al.*, 1988). The octopine plasmid *virE* locus also encodes a second 7kDa polypeptide VirE1 (Winans *et al.*, 1987). Two small ORFs are found at the 5' end of the nopaline *virE* region (Hirooka *et al.*, 1987). The amino acid sequence of VirE2 (Hirooka *et al.*, 1987; Winans *et al.*, 1987) contains four potential single-stranded DNA-binding domains similar to those characteristic of other single-stranded binding proteins and it has been shown to bind cooperatively to T-strands (Christie *et al.*, 1988). By thus coating the T-strand DNA, VirE2 presumably protects it from degradation by either bacterial or plant enzymes during its transfer to the plant genome. The VirE1 protein is also required for full virulence and may increase the stability of VirE2 (McBride & Knauf, 1988). It is possible that the two proteins form a complex and that without the accompaniment of the 7kDa polypeptide VirE2 is rapidly turned over *in vivo*.

One interesting point is that the products of the *virE* locus seem to function outside the bacterial cells. Coinoculation of a *virE* mutant and a disarmed *virE*$^+$ helper strain leads to efficient tumour formation (Christie *et al.*, 1988) suggesting that the VirE2 protein can be exported form the cell. Localisation studies have assigned the majority of VirE2 to the cytoplasmic fraction but it is also present in membrane and periplasmic fractions. It is possible that the restoration of virulence in mixed infections results from the independent transfer of T-DNA and VirE2 from separate bacteria into the same plant cell. The virulence of *A. tumefaciens* is not completely abolished by a *virE2* mutation, so either the T-strands are transferred at low frequency in the absence of VirE2 functions, or another gene product can partially compensate for the lost functions.

1.8 Regulation of *vir*-gene expression.

One of the most interesting aspects of *Agrobacterium* tumorigenesis is the way in which the whole T-DNA transfer process is regulated. Transfer of the T-DNA is dependent upon the actions of the *vir* gene products and the *vir* loci are expressed only when *Agrobacterium* is in the presence of susceptible (ie. wounded) plant cells (Stachel *et al.*, 1986). Positive regulation involves extracellular recognition of plant signal molecules followed by an intracellular response leading to transcription initiation. These processes are mediated by *virA* and *virG* (Stachel & Zambryski, 1986) in a similar manner to that described for other two-component bacterial regulatory systems which respond to environmental stimuli (Ronson *et al.*, 1987). In addition, *virC* and *virD* have been shown to be regulated in a negative manner by a chromosomal locus, *ros* (Close *et al.*, 1985; Close *et al.*, 1987b).

The *vir*-induction system is of particular interest due to the possibility of *vir*-promoters being utilised in gene constructs, thus conferring upon a gene the property of plant inducibility. A number of *vir*-gene fusions have been made to the reporter genes *lacZ* (Stachel *et al.*, 1985), CAT and *lux* (Rogowsky *et al.*, 1987) coding for β-galactosidase, chloramphenicol acetyl transferase and luciferase respectively, in order to analyse *vir* induction and expression. However, it is envisaged that such gene fusions could have a wider application by linking a *vir* promoter to, for example, a gene encoding a pesticidal protein which only needs to be produced at a plant wound site (Ashby, 1988).

1.9 Positive regulation.

The available data indicate that the positive regulation of *vir*-gene expression is highly complex. Early reports recognised the role of plant phenolic compounds as *vir*-inducers (eg. Stachel *et al.*, 1985b) and later reports pointed out that acidic pH (Rogowsky *et al.*, 1987) and phosphate starvation (Winans *et al.*, 1988) both elevate *virG* expression thus leading to increased expression of the other *vir* loci. Most recently, another level of complexity in the induction process has been revealed; *vir*-expression can be induced by certain monosaccharides via a regulatory pathway which involves VirA and the chromosomally encoded ChvE protein (Cangelosi *et al.*, 1990; Shimoda *et al.*, 1990).

1.9.1 *Vir*-inducing molecules.

Early experiments with *Agrobacterium* indicated that plant wounding is required for tumour development. It was initially assumed that this was because wounded plant cells

presented less of a physical barrier to penetration, but the main reason is now clearly established: expression of the *vir* genes is induced by compounds produced in wounded plant tissues (Stachel *et al.*, 1985b; Stachel *et al.*, 1986). The first *vir*-inducing molecules to be identified, acetosyringone (AS) and hydroxyacetosyringone (OH-AS), were purified from the culture media of tobacco cells (Stachel *et al.*, 1985b). These low molecular weight phenolic compounds resemble products of phenylpropanoid metabolism, the pathway producing secondary metabolites, lignins and flavonoids which are important to plants under stress or injury (Darvill & Albersheim, 1984) (Figure 1.4a). They are present specifically in the exudates of wounded but actively metabolising plant tissue. Dead cells which would not be good targets for *Agrobacterium* transformation are therefore not capable of producing acetosyringone. It is likely that there is a continual low-level excretion of such wound related phenolics during growth of the plant due to abrasion from the soil. These levels may be high enough to help attract *Agrobacterium* to the root surface, but only at a wound site are the concentrations of inducers high enough for T-DNA transfer to occur.

Following this discovery, the same group assayed other related compounds eg. sinapinic acid and *p*-hydroxybenzoic acid for their *vir*-inducing activity. A correlation was established between structure and activity, providing limited information about the structural characteristics required by a *vir*-inducing molecule (Stachel *et al.*, 1985b). Spencer and Towers (1988) have since carried out a more extensive survey of a variety of plant-derived phenolic compounds with structural similarity to acetosyringone. The compounds tested could be classified into four groups: a) acetophenones and related structures, b) monolignols, c) cinnamic acid and related structures and d) chalcone derivatives (Figure 1.4b). From their results they conclude that in general two structural features are required for a compound to be a *vir*-inducer. These are a guaiacyl or syringyl substitution on a benzene ring and a carbonyl group on a substituent *para* to the hydroxy substituent on the ring. Furthermore, a guaiacyl substitution gives greater *vir*-induction than a syringyl one and the nature of the carbonyl group is an important factor in the level of *vir*-inducing activity possessed by a compound. In order to confer maximal activity there must be a double bond between the carbonyl carbon and the ring. The carbonyl group of an ester is more effective than that of the corresponding free acid.

Until recently, acetosyringone was the only native inducer to be isolated, but since it had never previously been reported from plants and is not anticipated to be of widespread occurrence, it seems unlikely that it is the major, universal *vir*-inducing compound. The immediate precursors of lignin, sinapyl alcohol and coniferyl alcohol, have now been found to be good *vir*-inducers with the activity of coniferyl alcohol approaching that of acetosyringone. *Agrobacterium* may therefore be capable of detecting plant cells which are undergoing lignin synthesis or cell wall repair as targets for transformation (Spencer & Towers, 1988). Recent

Figure 1.4 The structures of *vir*-inducing molecules.

a) From left to right:- acetosyringone, α-hydroxyacetosyringone and sinapinic acid.

Figure 1.4

b) Three of the four groups into which phenolic vir-inducing compounds may be divided.

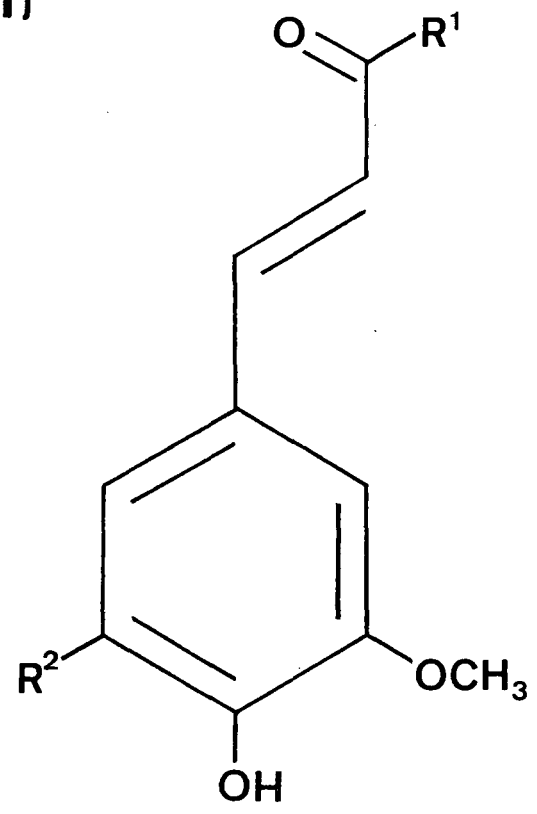
1 = cinnamic acid and related structures eg. ferulic acid R^1=OH R^2=H

2 = monolignols eg. coniferyl alcohol R=H

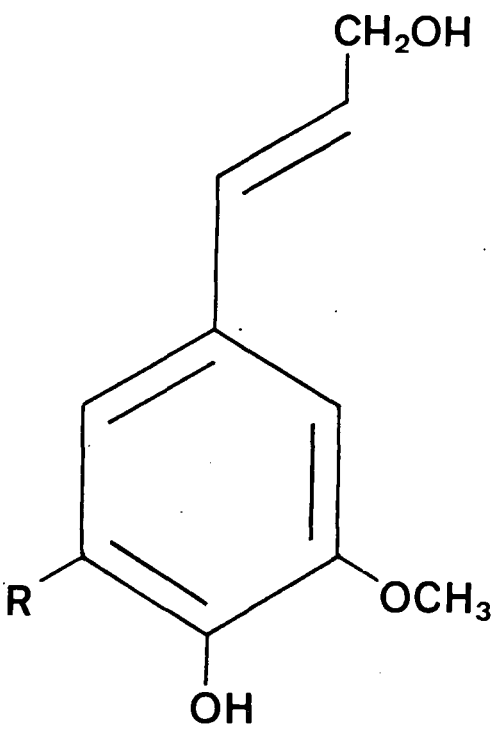
3 = chalcone derivatives eg. 2', 4', 4-trihydroxy-3-methoxychalcone R=H

The fourth group - acetophenones and related structures - is represented by acetosyringone in Figure 1.4a

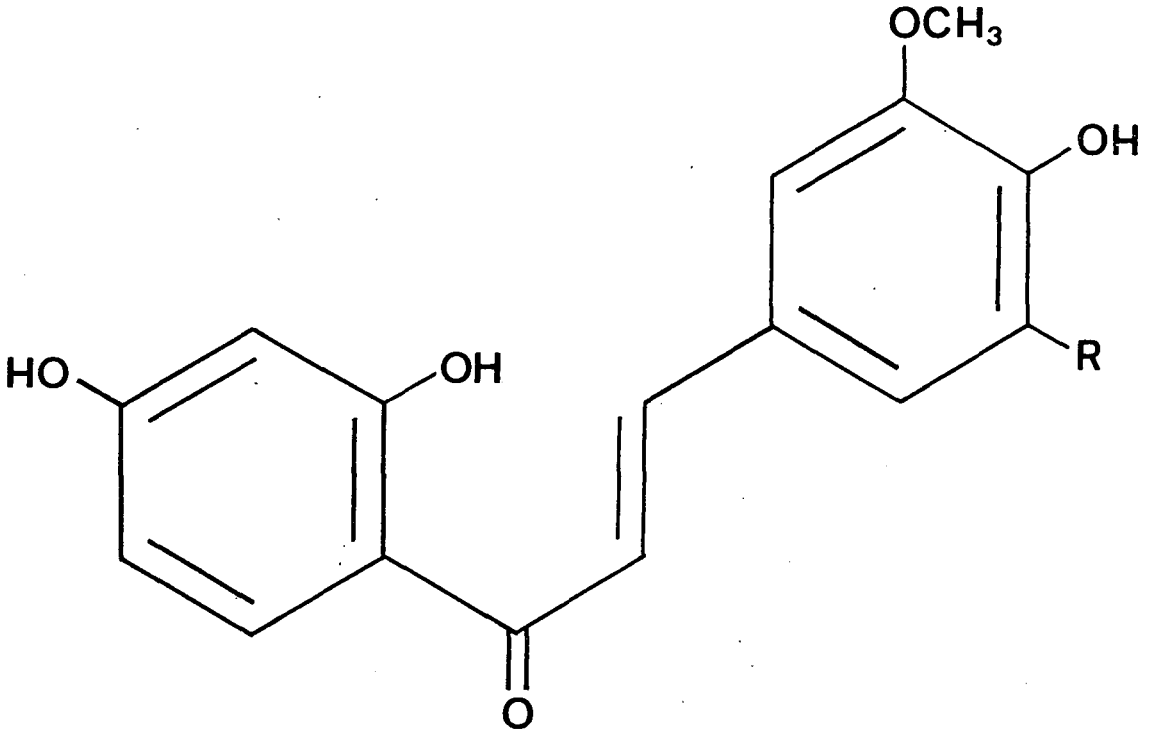
1]



2]



3]



reports have described the presence of a *vir*-inducing phenylpropanoid glucoside, coniferin, in the gymnosperm *Pseudotsuga menziesii* (Morris & Morris, 1990) and flavonoid compounds which elicit *vir*-induction released in high concentrations by the pollen of *Petunia hybrida* (Zerback *et al.*, 1989). It is possible that, together with other *vir*-inducing factors like cinnamic acids, such flavonoids which are known to be widespread in the plant kingdom, may play a more important general role in the natural infection of plants by *Agrobacterium* than acetosyringone.

A particularly significant discovery was that the monocotyledon *Triticum monococcum* produces the potent *vir*-inducer ethyl ferulate which is more active at low concentrations than AS (Messens *et al.*, 1990). Monocotyledonous species, which comprise many important crop plants, have with a few exceptions proved to be resistant to *Agrobacterium* transformation and one explanation proposed for this was that monocotyledonous cells are not recognised as transformation targets since they fail to produce *vir*-inducing compounds. This latest finding refutes that hypothesis, demonstrating that the block to monocot transformation presumably lies in a step subsequent to *vir*-induction.

1.9.2 The role of VirA and VirG in *vir*-induction.

Following the discovery of *vir*-induction by plant exudates, Stachel and Zambryski (1986) conducted a series of experiments which showed that the response was mediated by the action of the *virA* and *virG* gene products. Sequence analysis of *virA* and *virG* revealed that the products of these genes have a striking homology to a large number of two-component regulatory systems including EnvZ-OmpR, NtrB-NtrC and CheA-CheY (Leroux *et al.*, 1987; Melchers *et al.*, 1986). In each case the first protein of the pair (sensor) detects an environmental signal and transmits the information to the activator protein which in turn functions as an effector of gene expression.

In the *Agrobacterium vir*-induction system, the VirA protein is the environmental sensor. It is an inner membrane protein with two membrane-spanning domains, an N-terminal periplasmic region and a C-terminal region which is in the cytoplasm (Winans *et al.*, 1989; Melchers *et al.*, 1989). Melchers *et al.* (1989) have shown that the second of the two transmembrane sequences is the important site for signal recognition, whilst the periplasmic portion is not required for *vir*-induction by phenolic compounds. Indeed, removal of the periplasmic domain has been found to increase AS-mediated *vir*-induction (Cangelosi *et al.*, 1990). The C-terminal cytoplasmic domain of VirA possesses autophosphorylating activity and upon sensing an inducer molecule it then phosphorylates VirG to commence the *vir*-induction process (Jin *et al.*, 1990b; Huang, Y. *et al.*, 1990). The phosphorylation site on VirA has been

identified as a specific histidine residue in a sequence which is highly conserved among all VirA homologues (Jin *et al.*, 1990b). Such autophosphorylation is also characteristic of other sensor molecules eg. CheA and EnvZ (Hess *et al.*, 1988; Igo & Silhavy, 1988).

The *virG* locus encodes a polypeptide of 241 amino acids, using an inefficient UUG translation initiation codon which probably down-regulates *virG* expression (Pazour & Das, 1990). The VirG protein is a sequence specific DNA-binding protein (Powell *et al.*, 1989) that specifically recognises and binds to a dodecamer DNA sequence called the *vir* box (Jin *et al.*, 1990c; Pazour & Das, 1990) which is present in upstream promoter regions of all genes that are regulated by VirA-VirG (Steck *et al.*, 1988). Protein sequence strongly suggests that it is the C-terminal half of VirG which possesses the DNA-binding property (Jin *et al.*, 1990c). The protein appears to have two functional domains, the C-terminal domain which binds to promoter sequences and the N-terminal domain which is directly phosphorylated by VirA at an aspartate residue (Jin *et al.*, 1990a) and is able to interact with other components.

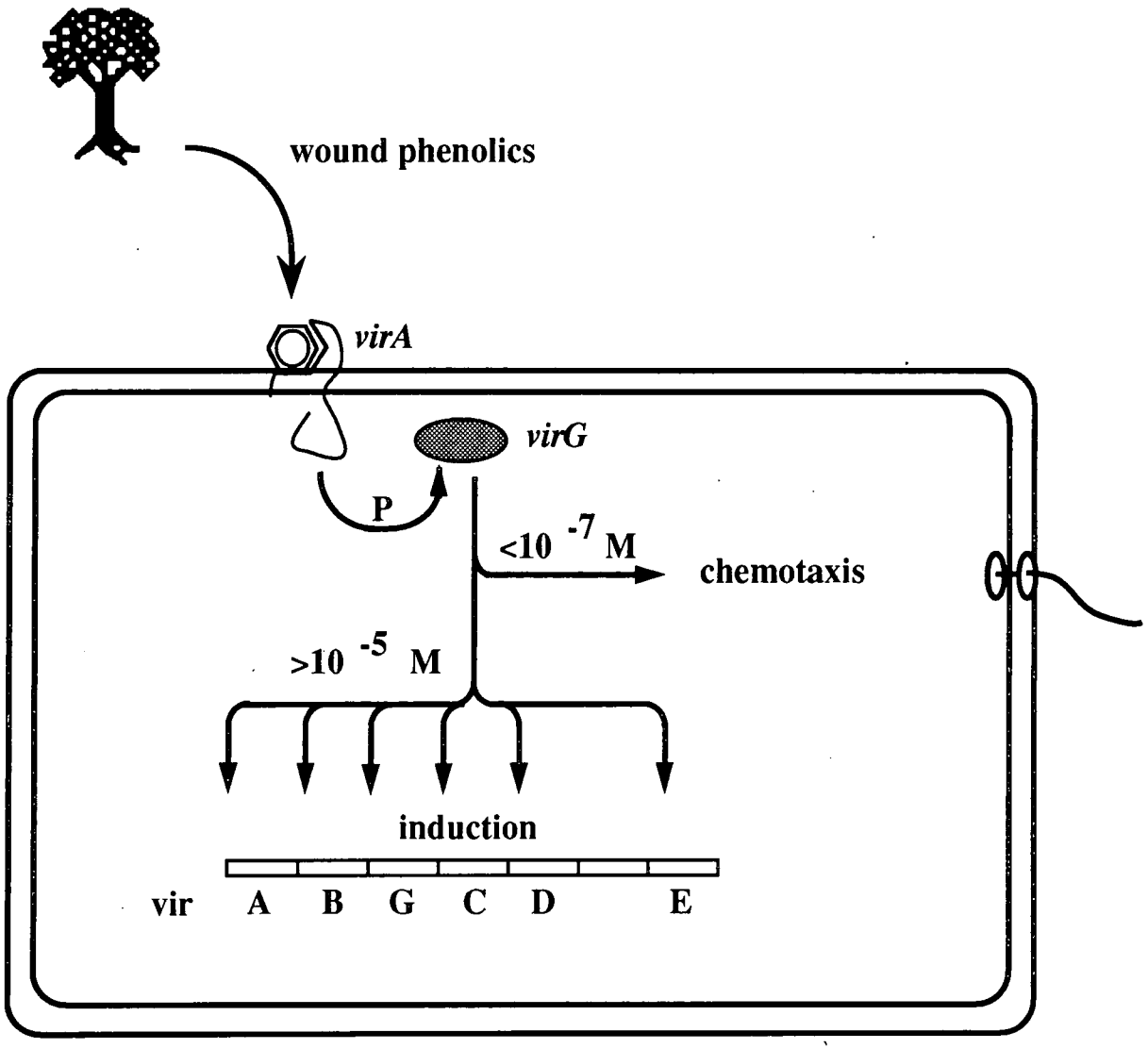
Since VirG is a transcriptional activator, it probably interacts with the transcriptional machinery, only the phosphorylated form being capable of such interaction. A similar scenario to the NtrB-NtrC system is envisaged, where both phosphorylated and unphosphorylated NtrC can bind DNA but only phosphorylation enables it to convert the closed RNA polymerase-promoter complex to an open complex (Popham *et al.*, 1989). In contrast to homologous systems, phospho-VirG was found to be very stable (Jin *et al.*, 1990a). This could be related to the fact that the *vir* genes are maximally induced in poor growth conditions such as low pH and phosphate starvation (Winans *et al.*, 1988; Winans, 1990), under which bacterial gene expression is slowed down. In this case it is necessary to have stable transcriptional and translational machinery in order that sufficient expression of *vir* genes continues.

1.9.2.1 The role of VirA and VirG in chemotaxis.

In addition to effecting *vir*-induction, acetosyringone and related phenolic compounds also serve as chemoattractants for *Agrobacterium* strains harbouring a Ti plasmid (section 1.3.2). Experiments have demonstrated that *virA* and *virG* are the Ti plasmid loci required to mediate this response (Shaw *et al.*, 1988a) suggesting a multifunctional role for the two proteins (Figure 1.5). Obviously the low constitutive level of *virA* and *virG* expression in the absence of *vir*-induction (Rogowsky *et al.*, 1987) is sufficient to mediate chemotaxis, since maximal attraction occurs at levels of acetosyringone which are sub-optimal for induction. *Agrobacterium* has a highly sensitive, chromosomally encoded chemotaxis system responding to a variety of sugars and amino acids (Loake *et al.*, 1988) and it is possible that VirA and VirG interact with this system in their role as mediators of chemotaxis towards *vir*-inducers. VirA has a secondary

Figure 1.5 A schematic diagram showing the dual role played by VirA and VirG in chemotaxis and *vir*-induction.

At concentrations of phenolic inducers below 10^{-7}M chemotaxis occurs but at concentrations greater than 10^{-5}M *vir*-induction is effected.


wound phenolics
virA
virG
P
<10 -7 M
chemotaxis
>10 -5 M
induction
vir
A
B
G
C
D
E

structure similar to that of methyl-accepting chemotaxis proteins (MCPs) which mediate chemotactic responses in *E. coli* (Springer *et al.*, 1979) and also has sequence conservation with CheA (Stock *et al.*, 1989), a protein kinase functioning in the signal transduction pathway of chemotaxis. Additionally, VirG possesses similarity to CheB, a methylesterase, and CheY, which is involved in the regulation of flagellar rotation (Winans *et al.*, 1986). However the exact functions of VirA and VirG in their role as mediators of the chemotactic response is unknown.

1.9.3 Monosaccharides as *vir*-inducers.

It has recently been discovered that certain sugars are capable of effecting *vir*-induction in *A. tumefaciens* when acetosyringone is absent or present in low concentrations (Cangelosi *et al.*, 1990; Shimoda *et al.*, 1990). This sugar-mediated induction requires VirA and the chromosomally encoded virulence protein ChvE (Cangelosi *et al.*, 1990).

Sequence analysis of ChvE shows that it is a periplasmic galactose-binding protein (GBP) with homology to similar sugar-binding proteins in *E. coli* (Huang, M. *et al.*, 1990). The *E. coli* proteins, upon binding sugars, interact with the periplasmic domain of the methyl-accepting transmembrane signal protein Trg resulting in chemotaxis towards the sugars, and also with separate transmembrane-bound uptake complexes. Similarly, ChvE mediates chemotaxis towards and uptake of galactose, glucose and several other sugars in *Agrobacterium* (Cangelosi *et al.*, 1990). In a manner analogous to the interaction of the *E. coli* GBP with Trg, sugar-bound ChvE probably interacts with periplasmic sites on VirA to stimulate *vir*-induction. Deletion of the VirA periplasmic domain, which is strongly homologous to Trg, abolishes *vir*-induction by sugars.

chvE mutants display a limited host-range, suggesting that on some plants the level of *vir*-induction by phenolic compounds alone may not be sufficient to bring about T-DNA transfer. Although AS is a strong *vir*-inducer, it is not known to be present in all plants, whereas a wide variety of lignin precursors which are generally present in higher plants are only weak inducers. However, coinduction with a weak *vir*-inducer such as syringic acid and an effective monosaccharide leads to high levels of *vir*-induction equivalent to those obtained with AS (Shimoda *et al.*, 1990). It is envisaged that plant cell wounding releases free sugars and oligosaccharides, generated by the action of cell wall glycosidases. These may then elicit the production of secondary metabolites including phenolic *vir*-inducers. Once *Agrobacterium* accumulates in the wound site, attracted by the sugars and phenolic compounds, the *vir*-genes are induced by a combination of these molecules acting synergistically (Ankenbauer & Nester, 1990).

1.9.4 Induction of *virG* by environmental stress.

The level of *virG* expression is a vital factor in *Agrobacterium* infection since the intracellular concentration of VirG is a rate limiting component of *vir*-gene induction (Jin *et al.*, 1988). Correspondingly, the regulation of transcription of *virG* is a complex process which appears to have at least two components, reflected by the fact that the *virG* gene utilises two distinct promoters (Stachel & Zambryski, 1986; Winans, 1990). The upstream promoter, designated P1, is required for the acetosyringone-mediated induction of *virG*. It contains three copies of the *vir* box sequence and, upon stimulation by AS, VirA phosphorylates VirG which binds to *vir* boxes 1 and 3 and induces transcription of its own gene (Winans, 1990).

virG is also induced by phosphate starvation and acidic growth media (Veluthambi *et al.*, 1987; Winans *et al.*, 1988). Such induction does not require any Ti plasmid functions, indicating that it must be chromosomally controlled. Indeed, a chromosomal mutant *chvD* has been identified which has severely attenuated virulence and only demonstrates partial induction by phosphate starvation and low pH (Winans *et al.*, 1988). The *chvD* gene shows homology to *nodI* from *Rhizobium* and others in a family of bacterial ATP-binding proteins with a role in active transport (Higgins *et al.*, 1986).

Induction by acidic media requires the downstream promoter P2. This has strong similarity to *E. coli* heat shock-inducible promoters (Cowing *et al.*, 1985). Genes under such control in *E. coli* are inducible by a wide range of environmental stresses including high temperature, alkaline pH and heavy metals (Van Bogelen *et al.*, 1987) and the P2 promoter of *virG* is also induced by these stimuli (Winans, 1990). Induction by phosphate starvation on the other hand requires P1 but does not involve interaction of VirG with the *vir* boxes. P1 contains a sequence highly similar to a so-called *pho* box found in *E. coli* promoters which are induced by phosphate starvation (Makino *et al.*, 1988; Winans, 1990).

Thus it appears that *virG* is controlled by three different global regulatory systems. Acidic growth conditions may serve as an additional signal of the presence of a susceptible wounded plant, whilst a variety of toxic compounds such as phytoalexins produced by wounded plant cells could act as inducers of the heat shock response and activate *virG* expression from P2. Scarcity of phosphate may signal to *Agrobacterium* that infection would be a pertinent survival strategy. All these stimuli would increase the intracellular pool of VirG to a level at which induction via VirG phosphorylation could occur. Therefore a number of signals indicating the likelihood of a successful infection need to be present before *Agrobacterium* becomes committed to *vir*-induction and T-DNA transfer.

1.10 Negative regulation by *ros*.

Spontaneous *Agrobacterium* mutants have been isolated which express *virC* and *virD* constitutively, at levels 10-100 times greater than their non-induced wild type counterparts (Close *et al.*, 1985; Close *et al.*, 1987b). However acetosyringone can still induce increased levels of expression of both loci in such mutants. The *ros* locus, which is responsible for this phenotype, is chromosomally encoded and has pleiotropic effects; *ros* mutants also produce colonies with a rough surface, do not form colonies at 12°C and do not produce the major extracellular polysaccharide, succinylglycan. Despite these effects virulence is not reduced.

Genetic and biochemical evidence suggests that the *ros* locus gene product is a negative regulator of *virC* and *virD*. Support for this comes from the finding that the *virD* locus is preceded by a pair of promoters: one responds to AS induction mediated by VirA and VirG whilst both are active in the presence of the chromosomal *ros* mutation. A *virC* promoter which responds to the *ros* mutation is transcriptionally divergent from and overlaps the tandem *virD* promoters (Tait & Kado, 1988). It has been postulated that these promoters may facilitate expression of *virC* and *virD* in the presence of sub-optimal levels of *vir*-inducers, thereby increasing the efficiency of the infection process (Tait & Kado, 1988).

1.11 Biological control using rhizosphere bacteria.

Recent public concern about the hazards associated with chemical pesticides and their accumulation in the environment has led to increased interest in the use of biological control methods (Baker, 1987). Biological control may be defined as the direct or indirect use of microorganisms both to reduce the effects of plant pests and pathogens and to improve plant survival and growth (Gould, 1990). Biological control of plant pests and pathogens is not a new practice. It has been studied for most of this century but until recently has not been considered commercially feasible. Now, however, several companies are developing biocontrol agents and a number have already appeared on the market (Currier & Gawron-Burke, 1990; Weller, 1988).

Microorganisms which colonise the rhizosphere are ideal candidates for biocontrol agents since the rhizosphere is the first line defence for roots against attack by pathogens, especially fungi (Weller, 1988). Many rhizosphere bacteria act as natural antagonists to pathogens both before and during infection. The three main forms of bacterial antagonism leading to pathogen suppression are competition, antibiosis and parasitism and the most successful biocontrol agents would probably suppress pathogens by a combination of these mechanisms.

1.11.1 Competition.

Microorganisms in the rhizosphere compete with each other for nutrients and essential minerals. Such competition between biocontrol bacteria and plant pathogens may result in displacement of the pathogen from the rhizosphere. Large populations of beneficial rhizobacteria which become established on roots act as a sink for nutrients so that reduced amounts of carbon and nitrogen are available for subsequent colonisation by pathogens (Elad & Chet, 1987). Bacteria which are aggressive colonisers of the rhizosphere, adhering to and multiplying on the root and able to utilise all available substrates are best suited to be biocontrol agents eg. the fluorescent pseudomonads (Weller, 1988). Competition for nutrients and sites on the root confers a low to moderate level of pathogen suppressiveness on many bacteria introduced onto seeds or planting material but it does not fully account for their efficacy as biocontrol agents.

One of the major ways in which biocontrol organisms exert their effects is by competing with pathogens for sources of iron (Neilands, 1981). This element is often unavailable in soil due to its low solubility at neutral and alkaline pH. To overcome the problem many bacteria possess high-affinity iron uptake systems involving siderophores and corresponding membrane receptors which are induced by low iron concentrations (Neilands, 1981). Siderophores are low molecular weight compounds which have a high affinity for ferric ions (Fe^{3+}) and serve to

transport Fe(III) into bacterial cells (Leong, 1986; Neilands, 1981). Siderophore-producing bacteria also possess specific membrane receptors and permeases that recognise the ferric-siderophore complex and transport it into the cell (de Weger *et al.*, 1986; Magazin *et al.*, 1986).

Although siderophores vary greatly in chemical structure, depending on the producer microorganism, most are either hydroxamate or catechol compounds (Neilands, 1981). Typical hydroxamate siderophores such as nocardamine (Meyer & Abdallah, 1980) and aerobactin (Gibson & Magrath, 1969) contain N^{δ}-hydroxyornithine as the ligand involved in the chelation of iron. Catechol based siderophores eg. agrobactin (Ong *et al.*, 1979) contain catechol functional groups as the iron-binding moieties and have higher affinity constants than the hydroxamate siderophores. The fluorescent pseudomonads, which have received much attention as proposed biocontrol organisms, are characterised by the production of yellow-green fluorescent pigments that function as siderophores. These are termed pyoverdines and are intermediate between catechol and hydroxamate-type siderophores (Cox, 1980; Teintze *et al.*, 1981)

The first demonstration of the importance of siderophores as a means of biological control was by Kloepper *et al.* (1980). Since then many reports have been published implicating siderophores in the suppression of a number of phytopathogenic fungi, eg. *Fusarium* spp. (Elad & Baker, 1985; Scher & Baker, 1982), *Pythium* spp. (Becker & Cook, 1988; Loper, 1988) and *Gaeumannomyces graminis* (Weller *et al.*, 1988). Siderophores exert their pathogen control by sequestering iron and denying it to other microorganisms. This is effective in suppressing pathogens which either do not produce siderophores of their own and are unable to use those produced by other organisms or produce smaller quantities of lower affinity siderophores (Weller, 1988). The most convincing evidence for the crucial role of siderophores in biological control has come from studies using siderophore-minus mutants (Elad & Baker, 1985; Loper, 1988; Marugg *et al.*, 1985). Such mutants lose the ability to suppress plant pathogens which is exhibited by the parental strains, whilst retaining their ability to colonise plant roots (Loper, 1988).

1.11.2 Antibiosis.

Another of the major mechanisms proposed for disease suppression in the soil is the production of antimicrobial compounds by the disease control agent (Gould, 1990). Antibiotics are low molecular weight organic compounds produced by microorganisms which at low concentrations are deleterious to the growth of other microorganisms (Fravel, 1988).

The fluorescent pseudomonads produce a large number of secondary metabolites with antimicrobial activity (Leisinger & Margraff, 1979). These fall into five major classes: phenazines, indoles, pyo compounds, phenylpyrroles and pterines (Gould, 1990). For example, *Pseudomonas fluorescens* 2-79, which is suppressive to take-all caused by *Gaeumannomyces graminis* var. *tritici*, produces a phenazine-type antibiotic with potent antifungal activity (Gurusiddaiah *et al.*, 1986). Howell and Stipanovich (1979 & 1980) demonstrated the production of two phenylpyrrole antibiotics by *P. fluorescens* (pf-5). The antibiotics, pyoluteorin and pyrrolnitrin, are active against *Pythium ultimum* and *Rhizoctonia solani* respectively.

Although antibiotics have been implicated in many instances of biological control most of the evidence for their involvement has until recently been indirect or circumstantial. Indeed, there are a number of reports of lack of correlation between *in vitro* antibiosis and biocontrol (Papavizas & Lewis, 1983; Utkhede & Gaunce, 1983). The fact that a biocontrol agent produces an antibiotic in culture does not indicate that it will perform likewise *in situ*. It is difficult to obtain experimental evidence for the direct involvement of antibiotics in disease control as there is no conclusive proof of their presence in natural soil (Williams & Vickers, 1986). Recently, however, the use of genetic approaches has produced firmer evidence for the role of antibiotics in biocontrol. Tn5 mutants of *P. fluorescens* 2-79 deficient in production of a phenazine antibiotic have been shown to be significantly less effective at protecting wheat from take-all disease, suggesting that the antibiotic plays a major role in the suppression of *G. graminis* (Thomashow & Weller, 1988). Howie and Suslow (1986) found that a mutant of *P. fluorescens* which does not produce an antifungal compound is less effective at suppressing *Pythium ultimum* on cotton than the antibiotic-producing parent strain.

1.11.3 Parasitism and lysis.

Many microorganisms produce potent extracellular enzymes which are capable of lysing other organisms and this has been suggested as a powerful disease control mechanism (Chet *et al.*, 1990; Henis & Chet, 1975; Mitchell & Alexander, 1969). The most effective of these hydrolytic enzymes in terms of biocontrol are the chitinases produced by various bacteria. A number of reports have proposed that chitinolytic activity plays a major role in plant protection (Barrows-Broaddus & Kerr, 1981; Morrissey *et al.*, 1976; Ordentlich *et al.*, 1988). This is due to the chitin content of the cell walls of many phytopathogenic fungi (Chet *et al.*, 1990). Mitchell and Alexander (1961) demonstrated that fungal cell wall-lytic bacteria added to soil controlled *Fusarium* spp. and *Pythium* sp. by destroying the fungal mycelium, whilst Mitchell and Hurwitz (1965) protected tomato plants against damping-off caused by *Pythium*

aphanidermatum with lytic *Arthrobacter*. Campbell and Ephgrave (1983) showed that the biological control of *G. graminis* by a *Bacillus* sp. in the soil was due to hyphal lysis.

In a survey of 100 microorganisms the enteric soil bacterium *Serratia marcescens* was found to be the most efficient producer of chitinase (Monreal & Reese, 1969). Consequently it has been the subject of investigations into its efficacy as a biocontrol agent (Ordentlich *et al.*, 1987; Ordentlich *et al.*, 1988). The genetic basis of chitinase production in this bacterium has also been investigated with the aim of using the information to produce improved biological control agents (Fuchs *et al.*, 1986; Jones *et al.*, 1986; Shapira *et al.*, 1989; Sundheim *et al.*, 1988). *S. marcescens* was found to be an effective biocontrol agent against *Sclerotium rolfsii* and *Rhizoctonia solani* under greenhouse conditions (Ordentlich *et al.*, 1987) and further investigations using chitinase preparations from the bacteria have shown that the lytic action of chitinase on fungal hyphae is responsible for the observed disease control (Ordentlich *et al.*, 1988; Shapira *et al.*, 1989).

Sundheim *et al.* (1988) transferred a plasmid carrying a chitinase gene from *S. marcescens* into *P. fluorescens* and the resulting strain inhibited growth of *Fusarium oxysporum* f. sp. *redolens* and reduced disease of radish caused by this pathogen. Similarly, a *S. marcescens* chitinase gene transferred to *E. coli* resulted in a strain that was inhibitory to *F. oxysporum* f. sp. *pisi* (Jones *et al.*, 1986).

1.12 *Agrobacterium* as a biological control agent.

One of the first commercial applications of biological control has been the use of *Agrobacterium radiobacter* K84 to control *A. tumefaciens* (New & Kerr, 1972). Strain K84 produces the bacteriocin agrocin 84 which is a disubstituted adenine nucleoside analogue (Tate *et al.*, 1979). Bacteriocins are antibiotic-like compounds bactericidal only to strains closely related to the producing organism (Vidaver, 1983). Agrocin 84 is classified as a bacteriocin because it exhibits specific toxicity toward *A. tumefaciens* strains able to utilise the agrocinopine-type opines (Engler *et al.*, 1975; Tate *et al.*, 1979). Sensitivity to agrocin 84 is related to agrocinopine uptake and it is likely that both molecules are transported by the same Ti-plasmid encoded permease (Murphy & Roberts, 1979). The synthesis of agrocin 84 is encoded by a 47.7kb plasmid pAgK84 (Ellis *et al.*, 1979) which also encodes immunity to agrocin 84 (Ryder *et al.*, 1987).

Although production of agrocin 84 by *A. radiobacter* is largely responsible for its control of crown gall there is a second element involved in suppression of the disease. A strain of *A. radiobacter* lacking pAgK84 was unable to prevent crown gall when coinoculated with *A.*

tumefaciens but was able to suppress the level of infection when inoculated 24 hours before the pathogen. Tumours which did occur under these circumstances were significantly smaller than tumours on untreated plants (Cooksey & Moore, 1982). This strongly suggests that prior establishment on the host and physical blockage of infection sites also contribute to biological control.

1.13 Future prospects for biological control.

Since biological control methods are considered less hazardous to the environment than chemical agents there is a promising future for the development of improved microbial inoculants. A considerable amount of research is being carried out in this area, although only a small number of products have reached the market and achieved commercial success. These include a *Pseudomonas*-based biofungicide called "Dagger-G" (Ecogen Inc.) currently being marketed for the prevention of damping-off of cotton seedlings and *Bacillus subtilis* A13 sold under the name of QUANTUM-4000 (Gustafson, Texas) as a treatment to suppress pathogens and improve the growth of peanut plants. These products are, in effect, natural biological control agents which have been marketed in an inoculant form that enables efficient application. The majority of research currently being undertaken has the aim of manipulating biocontrol bacteria to increase the level or consistency of their performance. Ultimately there exists the possibility of genetically engineering superior biocontrol agents by transferring genes from one bacterium to another and combining a number of desirable traits.

Genes controlling siderophore (Marugg *et al.*, 1985 & 1988; Moores *et al.*, 1984) and antibiotic (Gutterson *et al.*, 1986; Thomashow & Weller, 1990) synthesis have already been identified in several organisms. This opens the way for the possible use of molecular biology techniques both to modify the kinds and amounts of biocontrol molecules produced by existing strains and to express antibiotic or siderophore biosynthetic genes in nonproducer organisms with other desirable traits. In addition to the production of pathogen-antagonistic compounds, a successful biocontrol agent must also be an efficient and aggressive rhizosphere coloniser (Loper *et al.*, 1984; Weller, 1983). Since the characteristics which determine root-colonising ability are complex and poorly understood it would at present be impossible to engineer an existing biocontrol agent to create a more effective root coloniser. Therefore Juhnke *et al.* (1987) suggested that the way to achieve maximum proficiency from a biocontrol agent would be to first isolate an effective root coloniser and then introduce genes for antibiotic or siderophore production into this bacterial isolate. Thomashow and Weller (1990) have described the introduction of a 9.2kb fragment of DNA from *P. aureofaciens* 30-84 into *E. coli* and the resulting production by this strain of the two antibiotics phenazine-1-carboxylic acid

and 2-hydroxyphenazine-1-carboxylic acid. This result appears to demonstrate the feasibility of mobilising and expressing antibiotic genes in non-producer bacteria. Siderophore production and utilisation however is not determined by a simple, one-gene product and many antibiotics are secondary metabolites with complex biosynthetic pathways, therefore such an approach may not be entirely successful.

More suitable attributes for genetic manipulation are those determined by single, easily-characterised genes. Ideal candidates for transfer and manipulation are therefore genes encoding lytic enzymes such as chitinase which has a proven pathogen-suppressive action (Shapira *et al.*, 1989). Promising work has already been carried out using a chitinase gene from *S. marcescens* which has been transferred to *E. coli* (Jones *et al.*, 1986; Shapira *et al.*, 1989). The resulting strain in each case was effective in suppressing fungal pathogens. Perhaps more important were experiments where the chitinase gene was introduced into and expressed in root-colonising fluorescent pseudomonads (Fuchs *et al.*, 1986; Sundheim *et al.*, 1988). The chitinase-producing pseudomonads inhibited the growth of *Fusarium oxysporum, Rhizoctonia solani* and *Magnaporthe grisea in vitro* but were less effective in suppressing disease, since the vector carrying the chitinase gene was not maintained in the pseudomonad (Sundheim *et al.*, 1988). Further investigations using more stable extrachromosomal elements or integrating the chitinase gene into the chromosome of the biocontrol strain should be carried out.

Other studies may concentrate on improving the production of chitinase from a biocontrol agent by genetic manipulation techniques. One possible approach would be to bring the chitinase gene under the control of a stronger promoter to achieve higher levels of expression (Shapira *et al.*, 1989). A novel strategy has been proposed to control the expression of chitinase in a biological control agent such that maximal amounts of the enzyme are produced in response to plant wounding (Ashby *et al.*, 1986; Ashby, 1988). In this system a gene encoding chitinase, or any other lytic enzyme or toxin, is placed under the control of an *Agrobacterium* virulence promoter which is induced in the presence of plant wound exudates. Avirulent agrobacteria containing the modified gene and the necessary *vir*-functions would detect low levels of plant exudates in the rhizosphere and move chemotactically towards the wound site. Once there, the higher levels of plant exudate would initiate the *vir*-induction process and thus cause expression of the biocontrol product. The advantage of this system over existing bacterial pathogen-control systems is that the production of the enzyme or toxin is a conservative process, only occurring in the microrhizosphere around a wound site.

This delivery system is particularly suitable for the production of an insecticidal product such as the *Bacillus thuringiensis* (Bt) toxin (Hofte & Whiteley, 1989) since insect feeding would cause wounding and thus initiate expression of the toxin. Obukowicz *et al.*, (1986) have integrated the δ-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 into the

chromosome of root colonising *Pseudomonas fluorescens* and *Agrobacterium radiobacter* in an attempt to target the delivery of the insecticide to the rhizosphere. This approach would also reduce the current need for repeated applications of commercial Bt toxin preparations whose insecticidal activity declines rapidly in the environment (West, 1984). By employing the strategy of Ashby *et al.* the application process would be refined to an even greater extent and since production of the toxin is not constitutive there should be a lower risk of resistance developing in the insect population.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All chemicals and biological reagents were from Sigma Chemical Company Ltd or BDH Ltd with the exception of those listed below.

Restriction endonucleases, DNA modifying enzymes, X-gal and IPTG were from either Northumbria Biologicals Ltd or Boehringer Mannheim.

Agarose, LMP agarose and the M13 sequencing kit were from Bethesda Research Laboratories (UK) Ltd.

All radiochemicals and the oligonucleotide directed *in vitro* mutagenesis kit were from Amersham International plc.

Bacteriological agar and yeast extract were from Oxoid Ltd.

Trypticase peptone (tryptone) was from Becton Dickinson Microbiology Systems, Cockeysville USA.

The non-radioactive DNA labelling and detection kit was from Boehringer Mannheim.

Sodium chloride was from Reidel de Haen.

The reagent for the protein micro-assay was from Bio-Rad Laboratories, Herts., UK.

Nitrocellulose and nylon filters were from either Schleicher and Schuell or Amersham International plc.

2.2 Bacterial strains.

The bacterial strains used during the course of this work are outlined below.

E. coli.

JM83	*ara*, Δ(*lac-proAB*), *rpsL*, Φ80d*lacZ*ΔM15.
	Vieira and Messing (1982).
DH5α	*endA*1, *hsdR*17, *supE*44, *thi*-1, *recA*1, *gyrA*96, *relA*1, Δ(*lacZYA-argF*)
	U169, Φ80, d*lacZ*ΔM15.
	BRL (1986)
JM101	Δ(*lac-proAB*), *supE*, *thi*, F'(*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15).
	Yanisch-Peron *et al.* (1985).
TG2	Δ(*lac-proAB*), *supE*, *thi*, *hsdM*, *recA*1, *srl*::Tn10,
	F'(*traD*36, *proAB*$^+$, *lacI*^{q} *lacZ*ΔM15).
	Rec$^-$ derivative of TG1.
	Gibson (1984)

A. tumefaciens.

C58C[1]	Rif^R, Ti$^-$
	Van Larebeke *et al.* (1974).
LBA4301	Rec$^-$, Ach5 background
	Klapwijk *et al.* (1979).

2.3 Plasmids

Described below are the plasmids used during the course of this work.

Plasmid	Comments	Reference
pUC18/19	amp^R	Vieira & Messing (1982)
pUC1318	hybrid pUC vector	Kay & McPherson (1987)
pBluescript	amp^R, with T7 promoter	Stratagene
pCHIT310	amp^R, chitinase +ve	Jones *et al.* (1986)
pDUB2512	*virB* promoter:*chiA* coding region	Ashby (1988)
pVK257	*virA,B,G,C* of pTiA6	Knauf & Nester (1982)
pUCD607	constitutive *lux*, amp^R, kan^R	Shaw & Kado (1986)
pUCD615	*lux* promoter probe vector	Rogowsky *et al.* (1987)
pUCD1187	*virB* promoter from pTiC58 in pUCD615	"
pUCD1194	*virE* promoter from pTiC58 in pUCD615	"

2.4 Media and growth conditions.

Bacterial strains were routinely grown in L-broth or on L-agar at either 37°C (*E. coli*) or 28°C (*A. tumefaciens*).

L-broth:-	Tryptone	10g
	Yeast extract	5g
	NaCl	5g
	Distilled water	to 1 litre
	(For L-agar 10g of agar were added)	

The media was then autoclaved.

When growing bacteria in liquid culture for work with M13 bacteriophages, a richer media, 2YT, was used.

2YT:-	Tryptone	16g
	Yeast extract	10g
	NaCl	5g
	Distilled water	to 1 litre

If agar plates were required for blue/white colour selection when using pUC or pBluescript plasmids, X-gal was made up as a 2% solution in dimethylformamide and 200µl added for every 100ml of autoclaved agar.

Strains of *E. coli* harbouring F' plasmids were maintained on minimal agar plates lacking proline in order to select for the presence of the plasmid:-

Agar	10g
Distilled water	768ml

Autoclaved and cooled prior to the addition of:

200ml	5x M9 salts
2ml	1M $MgSO_4$
100µl	1M $CaCl_2$
20ml	1mg/ml thiamine

Chitin medium for the growth of *E. coli* harbouring chitinase constructs:-

Pure chitin	0.5g
Yeast extract	0.5g
Agar	1g
Distilled water	to 80ml

Autoclaved and cooled prior to the addition of :-

100µl	0.1M FeEDTA
100µl	1M N-acetylglucosamine
20ml	5x M9 salts

5x M9 salts contained:-

Na_2HPO_4	30g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5g
Distilled water	to 1 litre

Minimal medium for *A. tumefaciens* strains was:-

20ml	5x MinA salts
1ml	20% glucose
0.1ml	1M $MgSO_4$
76ml	sterile distilled water

5x MinA salts:-

K_2HPO_4	52.5g
KH_2PO_4	22.5g
$(NH_4)_2SO_4$	1g
Na citrate.$2H_2O$	2.5g
Distilled water	to 1 litre

When induction of *vir* genes was measured in *Agrobacterium*, the cells were grown in the following induction medium:

Sucrose	3g
Distilled water	85ml

Autoclaved and cooled prior to the addition of:

5ml	20x AB salts
10ml	0.2M MES, pH5.2
0.6ml	0.4M K_2HPO_4

20x AB salts contain:

NH_4Cl	20g
$MgSO_4.7H_2O$	6g
KCl	3g
$CaCl_2$	3g
$FeSO_4.7H_2O$	50mg
Distilled water	to 1 litre

When antibiotic selection was required, stock solutions were prepared, filter sterilised and the appropriate volume added to cooled, autoclaved media as detailed below.

Antibiotic	**Stock solution** (mg/ml)	**Solvent**	**Final conc.** (μg/ml)
Ampicillin (Amp)	50	H_2O	50
Kanamycin (Km)	25	H_2O	25
Carbenicillin (Cb)	50	H_2O	100
Tetracycline (Tc)	2.5	EtOH	10
Rifampicin (Rif)	50	DMSO	100

2.5 Isolation of DNA

2.5.1 Plasmid minipreps.

Small amounts of plasmid DNA were prepared essentially according to Maniatis *et al.* (1989).

A single bacterial colony was grown overnight in 5ml of L-broth with antibiotic selection, then a 1.5ml aliquot of the culture was transferred to a sterile eppendorf tube and centrifuged for 1-2 minutes in a microfuge (MSE microcentaur). The supernatant was removed and the bacterial pellet resuspended in 100µl of ice-cold Solution 1 (1% glucose, 10mM EDTA, 25mM Tris.HCl pH8.0). The bacterial suspension was allowed to stand at room temperature for 5 minutes, then 200µl of freshly made Solution 2 (0.2M NaOH, 1% SDS) was added, the tubes mixed gently by inversion and placed on ice for 5 minutes. 150µl of ice-cold Solution 3 (11.5ml of glacial acetic acid and 28.5ml of distilled water added to 60ml of 5M potassium acetate pH4.8) was added, the contents of the tube mixed by inversion then vortexed briefly and stored on ice for a further 5 minutes. Cell debris was pelleted by centrifugation for 5 minutes. The supernatant was removed to a fresh tube and extracted with an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was transferred to a clean tube and the DNA precipitated by the addition of 2 volumes of ethanol. After 5 minutes at room temperature, the plasmid DNA was pelleted by 5 minutes centrifugation, then washed with 70% ethanol, dried under vacuum and resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA pH8.0) plus RNase at a concentration of 20µg/ml.

2.5.2 Large scale plasmid preparation.

10ml of L-broth was inoculated with a single colony of a bacterial strain containing the desired plasmid and grown up overnight with the appropriate antibiotic selection. The resulting culture was added to 500ml of L-broth plus antibiotic and incubated with shaking overnight. The bacteria were harvested by centrifugation at 4000rpm for 10 minutes in an MSE High Speed 18 centrifuge. The supernatant was discarded and the pellets resuspended in 10ml of Solution 1 containing 2mg/ml lysozyme. The resulting suspension was transferred to two 50ml Oakridge tubes and left at room temperature for 30 minutes. 10ml of Solution 2 was added to each tube, the contents mixed by gentle inversion and kept on ice for 10 minutes. 7.5ml of ice-cold 3M sodium acetate pH4.8 was added to each tube, mixed well and left on ice for a further 10 minutes. The lysed cells were clarified by centrifugation at 19,000rpm for 20 minutes and the supernatant transferred to clean Corex tubes. An equal volume of isopropanol was added and the DNA left to precipitate at room temperature for 30 minutes. The DNA was pelleted by centrifugation at

12,000rpm for 30 minutes, then washed in 70% ethanol, dried under vacuum and resuspended in 5ml of TE buffer.

2.5.3 Caesium chloride/ethidium bromide density gradient centrifugation.

Plasmid DNA from large scale preparations was purified using the following method.

22.4g of caesium chloride and 0.33ml of a 10mg/ml ethidium bromide solution were added to the DNA sample and the volume made up to 30ml with TE buffer. The resulting solution was transferred to a Quickseal centrifuge tube using a needle and syringe. The tube was then balanced, heat-sealed and centrifuged for 17-20 hours at 55,000rpm and 15°C, using a Sorvall 65 OTB ultracentrifuge and a VTi 50 rotor.

Following centrifugation, the tube was carefully removed from the rotor and observed under UV light. Two fluorescent bands of DNA were usually visible, the upper band corresponding to the open circular form of the plasmid plus residual chromosomal DNA and the lower one to supercoiled plasmid DNA. A hole was made in the top of the tube and the supercoiled band collected using a syringe and wide-bore needle. The DNA solution was transferred to a sterile plastic tube and the ethidium bromide was removed by repeated extraction with an equal volume of sodium chloride-saturated isobutanol. The sample was then dialysed against TE buffer at 4°C for 24 hours with 3 changes of buffer.

2.6 Transformation of bacteria.

2.6.1 Calcium chloride method for preparing competent cells.

This was based on the method of Mandel and Higa (1970) and was used when a large number of transformations were to be carried out, or when competent cells were made for long term storage.

50ml of L-broth was inoculated with 1ml of an overnight culture of *E. coli* and incubated with shaking at 37°C until the absorbance at 550nm was 0.3-0.4 (2-3 hours). The culture was then placed on ice for 10 minutes before being transferred to a chilled, sterile Sorvall tube and centrifuged at 6,000rpm for 5 minutes at 4°C in an MSE 18 centrifuge. The supernatant was discarded and the cell pellet gently resuspended in 25ml of ice-cold 50mM $CaCl_2$, 10mM Tris.HCl (pH8.0). The cell suspension was maintained on ice for 15 minutes and then recentrifuged as before. The supernatant was removed and the pellet now resuspended in 2.5ml of the same

solution. For storage of the competent cells, 0.7ml of 80% glycerol was added and the cells dispensed into 200µl aliquots prior to flash freezing in liquid nitrogen and storage at -80°C.

2.6.2 Rubidium chloride method for preparation of competent cells.

This method was used when only a small number of transformations were to be performed and has been previously described by Rushner (1978).

100µl of an overnight culture of *E. coli* was used to inoculate 5ml of L-broth, which was shaken at 37°C for 2-3 hours until the density of the culture was approximately $2x10^8$ cells/ml. 1.5ml aliquots were transferred to eppendorf tubes and the cells pelleted by 30 seconds centrifugation. The supernatant was discarded and the pellet gently resuspended in 500µl of Solution A (10mM MOPS pH7.0; 10mM RbCl). The bacteria were pelleted by a 15 second centrifugation and the pellet now resuspended in 500µl of Solution B (100mM MOPS pH6.5; 10mM RbCl; 50mM $CaCl_2$). The suspension was held on ice for 90 minutes and then centrifuged for 10 seconds. The bacteria were finally resuspended in 150µl of Solution B and 3µl of DMSO was added.

2.6.3 Transformation of *E. coli* competent cells.

Competent cells prepared by either of the above methods were transformed as follows.

Pure DNA, or a ligation mixture was added to freshly made competent cells, or frozen cells which had been thawed on ice. The tube of cells was mixed gently and kept on ice for 30-60 minutes. The cells were then heat-shocked at either 42°C for 2 minutes, or 55°C for 30 seconds and placed back on ice for 5 minutes. 1ml of L-broth was added and the cells incubated at 37°C for 1 hour before appropriate aliquots, usually 100µl, were spread onto selective agar plates.

2.6.4 Transformation of *Agrobacterium.*

The method used for introducing plasmid DNA directly into strains of *Agrobacterium* was a slight modification of that used by Ebert et al. (1987).

100ml of L-broth was inoculated with 4ml of an overnight culture of *A. tumefaciens* and incubated with vigorous shaking at 28°C for approximately 4 hours, until the absorbance at 600nm reached 0.5-1.0. The cells were pelleted by centrifugation at 3,500 rpm for 10 minutes in a bench-top centrifuge (Wifug) and then resuspended in 2ml of L-broth. 100µl aliquots were transferred to

eppendorf tubes and held on ice for 30 minutes prior to the addition of up to 1μg of plasmid DNA. The cells were then subjected to two cycles of rapid freeze-thawing using liquid nitrogen and 5 minutes thawing in a 37°C water bath. 1ml of L-broth was added to the cells which were incubated at 28°C for 2 hours with gentle shaking. The cells were pelleted by a 1 minute centrifugation and then resusupended in 100μl of L-broth before being plated onto selective L-agar.

2.7 DNA manipulations.

2.7.1 Spectrophotometric quantitation of DNA solutions.

The DNA solution was diluted with an appropriate volume of TE buffer, usually a 1:50 or 1:100 dilution. The absorbance of the solution at 260nm and 280nm was measured using a UV spectrophotometer (LKB). A pure DNA sample has an $A_{260/280}$ ratio of 1.8. An A_{260} of 1.0 is equivalent to a double-stranded DNA concentration of 50μg/ml, or a single-stranded DNA concentration of 40 μg/ml.

2.7.2 Restriction enzyme digests.

Plasmid DNA was generally digested in a volume of 10-30μl, with 5 units of the desired restriction enzyme and 0.1 volumes of the appropriate 10X concentrated restriction enzyme buffer (REB). The volume was made up with sterile distilled water and the reaction incubated at the recommended temperature (usually 37°C) for 1-2 hours. Digestions which were to be analysed by gel electrophoresis were terminated by the addition of 0.1 volumes of stop-dye, which was made up as follows:-

1ml	10% SDS
2ml	250mM EDTA pH8.0
0.2ml	1M Tris.HCl pH8.0
5ml	glycerol
1.8ml	distilled water
10mg	bromophenol blue

If DNA was being digested for use in subcloning procedures, then the reaction was terminated by phenol/chloroform extraction (see section 2.7.3). Unless otherwise specified, restriction enzyme digests had a requirement for one of the following buffers.

	Buffer (X10)		
	low (µl)	medium (µl)	high (µl)
5M NaCl	0	100	200
1M Tris pH7.4	100	100	500
1M $MgSO_4$	100	100	100
1M DTT	10	10	0
distilled water	790	690	200

2.7.3 Phenol:chloroform extraction of DNA.

DNA samples were deproteinised by the addition of an equal volume of redistilled, water-saturated phenol equilibrated with 0.1M Tris.HCl pH8.0. The phases were mixed by vortexing and then separated by centrifugation for 3-4 minutes in a microfuge. The upper, aqueous layer was carefully removed and re-extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) saturated with the same buffer.

2.7.4 Ethanol precipitation of DNA.

DNA was precipitated from solution by the addition of 0.1 volumes of 3M sodium acetate pH4.8 and 2 volumes of ethanol, unless otherwise stated. The sample was mixed by vortexing and stored at -80°C for at least 30 minutes before centrifugation at 12,000g for 10 minutes in a microfuge. The DNA pellet was washed with 70% ethanol, dried under vacuum and resuspended in either TE buffer or sterile distilled water.

2.7.5 Phosphatase treatment of DNA.

In order to prevent recircularisation of digested vector DNA, calf intestinal phosphatase was used to remove the 5' phosphates from linear DNA.

30 minutes before the termination of a restriction digest using medium REB or Pharmacia "One-Phor-All" buffer, 1 unit of calf intestinal phosphatase was added to the reaction. Upon completion of the digest, 0.1 volumes of 0.1M nitrilotriacetic acid pH8.9 were added and the sample placed at 70°C for 15 minutes to denature the phosphatase. The solution was then

phenol:chloroform extracted, ethanol precipitated and resuspended in sterile water, ready for use in a ligation reaction.

2.7.6 Ligation of DNA.

DNA fragments with compatible cohesive or blunt termini generated by digestion with restriction enzymes were ligated together by the action of T4 DNA ligase. The fragments to be ligated were mixed together in the approximate ratio of 3:1 insert:vector moles of termini, with 0.1 volumes of 10X concentrated ligation buffer (0.66M Tris.HCl pH7.5; 50mM $MgCl_2$; 50mM DTT; 10mM ATP) and 1-2 units of T4 DNA ligase. For blunt-ended ligations the amount of enzyme was increased to 5 units. The ligation reaction was incubated at either 15°C overnight, or at room temperature for 2-3 hours before it was used to directly transform competent *E. coli* cells.

2.7.7 Agarose gel electrophoresis.

The correct amount of agarose required to give the desired concentration (usually 0.7-1.0% depending upon the sizes of the DNA fragments to be separated) was added to 200ml of TBE buffer (121.1g Tris-HCl, 51.35g boric acid, 3.72g EDTA/litre, for 10x) for a large gel, or 70ml for a minigel then heated in a microwave oven for 2-3 minutes. Ethidium bromide was added to a final concentration of 0.2µg/ml and the agarose cooled before being poured into the gel mould and allowed to set. The gel was then placed in a tank containing TBE buffer and ethidium bromide at 0.2µg/ml, the DNA samples containing stop-dye were loaded into the wells and electrophoresis was carried out at an appropriate voltage for the desired length of time. The gel was viewed on a UV transilluminator (UVP Inc.) and, if necessary, photographed using a red filter, Polaroid 667 film and a polaroid RP4 Land camera.

2.7.8 DNA fragment isolation.

Two different methods have been used to isolate fragments of DNA from agarose gels.

Method 1 employed low melting point (LMP) agarose. The DNA samples were separated through a LMP agarose gel and the fragments to be isolated were removed from the gel with a sterile scalpel blade and transferred to eppendorf tubes. The tubes were incubated at 65°C until the agarose was molten and 2 volumes of 50mM Tris.HCl; 0.5mM EDTA were added. The solution was mixed and incubated at 37°C for a few minutes, before being extracted twice with phenol and once with chloroform:isoamyl alcohol(24:1), then precipitated with ethanol.

Method 2 was a modified version of the freeze-squeeze method. The required DNA fragment was excised from an ordinary agarose gel and placed in a 0.8ml eppendorf tube which had been holed in the bottom and plugged with glass wool. The gel slice was frozen at -80°C for a minimum of 30 minutes and then the tube was placed into a 1.5ml eppendorf and centrifuged for 10 minutes whilst the agarose was still frozen. The DNA solution which collected in the large eppendorf was extracted once with chloroform:isoamyl alcohol (24:1) and precipitated with 0.1 volumes of 3M sodium acetate; 0.1M magnesium acetate pH5.2 and 2 volumes of ethanol.

2.7.9 Filling in 3' recessed ends.

If it was necessary to convert a DNA restriction fragment with 3' recessed ends to one with blunt ends, the following method was used.

The DNA fragment was resuspended in 13µl of sterile distilled water following isolation from an agarose gel or digestion with a restriction enzyme. To this was added 2µl of 10x polymerase reaction buffer (70mM Tris-HCl pH7.5; 70mM $MgCl_2$; 500mM NaCl), 1µl each of 0.5mM dATP, dCTP, dGTP and TTP and 1µl (1 unit) of Klenow enzyme. The reaction was incubated at room temperature for 15 minutes and was then phenol/chloroform extracted, ethanol precipitated and resuspended in sterile water.

2.7.10 Transfer of DNA to nitrocellulose membranes.

The method used was a modification of that of Southern (1975).

The DNA-containing agarose gel was photographed and then soaked for 45 minutes in denaturation buffer (1.5M NaCl, 0.5M NaOH) with occasional shaking. The gel was rinsed twice with distilled water and soaked for a further 45 minutes in neutralisation buffer (3M NaCl, 0.5M Tris-HCl pH7.0), then rinsed with 20x SSC (3M NaCl, 0.3M sodium citrate). It was then placed on a sheet of Whatman 3MM paper soaked in 10x SSC which was positioned over a glass plate "bridge", so that the ends dipped into a reservoir of 10x SSC and acted as a wick. A piece of nitrocellulose was cut to the same size as the gel, wetted with distilled water, immersed in 10x SSC and placed on top of the gel, taking care to remove any air bubbles. 3 pieces of Whatman 3MM paper, cut to the same size as the gel, were soaked in 10x SSC and laid on top of the nitrocellulose, again removing any air bubbles. Finally, 3 layers of absorbent nappies were positioned on top followed by a glass plate and the whole apparatus was compressed with a lead weight. DNA transfer was then allowed to proceed overnight.

The blotting apparatus was dismantled and the nitrocellulose filter removed, once the positions of the wells had been marked. The filter was baked between two pieces of 3MM paper in a vacuum oven at 80°C for 2 hours.

2.7.11 Preparation of digoxygenin labelled DNA probes.

In this method, DNA fragments were labelled by random primed incorporation (Feinberg & Vogelstein, 1983) of digoxygenin labelled dUTP, according to the protocol provided with the non-radioactive DNA labelling and detection kit (Boehringer Mannheim).

A fragment of DNA (10ng-3μg) which had been purified from an agarose gel (see 2.7.8) was denatured by heating at 95°C for 10 minutes and was then cooled quickly on ice. To the tube were added 2μl of a random hexanucleotide mixture, 2μl of dNTP labelling mix (1mM dATP. 1mM dCTP, 1mM dGTP, 0.65mM dTTP, 0.35mM Dig-UTP pH6.5) and distilled water to a final volume of 19μl. 1μl (2 units) of Klenow enzyme was added and the reaction incubated for 1-20 hours at 37°C. The reaction was stopped by the addition of 2μl of 0.2M EDTA pH8.0, and the labelled DNA was precipitated with 2μl of 4M LiCl and 60μl of prechilled ethanol, at -80°C for at least 30 minutes. The DNA was pelleted by centrifugation at 12,000g and washed with 70% ethanol, before being dried under vacuum and dissolved in 50μl TE buffer.

2.7.12 Hybridisation of labelled probes to Southern blots.

The baked nitrocellulose filter was placed inside a plastic bag and 50ml per 100cm^2 of prewarmed (65°C) hybridisation solution (5x SSC, 0.1% lauroylsarcosine, 0.02% SDS, 1% blocking reagent [Boehringer]) was added. The bag was heat sealed and the filter prehybridised for 2-4 hours at 65°C.

The prehybridisation solution was replaced with approximately 5ml per 100cm^2 filter of hybridisation solution containing freshly denatured probe DNA and the filter incubated overnight at 65°C. The filter was washed twice for 5 minutes with 50ml of 2x SSC, 0.1% SDS at room temperature and twice for 15 minutes with 0.1x SSC, 0.1% SDS at 65°C, then allowed to air dry.

2.7.13 Immunological detection of hybridised probe DNA.

The air-dried filter was washed briefly in Buffer 1 (0.1M Tris-HCl, 0.15M NaCl, pH7.5), and incubated for 30 minutes at room temperature with 100ml of Buffer 2 (Buffer 1 containing 1%

blocking reagent) with gentle shaking. It was then washed again briefly with Buffer 1 and incubated for 30 minutes with 20ml of antibody-conjugate solution (anti-digoxygenin AP-conjugate diluted to 150 mU/ml in Buffer 1). The unbound antibody-conjugate was removed by washing the filter twice for 15 minutes with 100ml of Buffer 1 and the filter was then equilibrated for 2 minutes with 20ml of Buffer 3 (0.1M Tris-HCl, 0.1M NaCl, 50mM $MgCl_2$, pH9.5). The bound antibody-conjugate was detected by the addition of 10ml of Buffer 3 containing 45µl of NBT solution (nitroblue tetrazolium salt; 75mg/ml in 70% DMF) and 35µl of X-phosphate solution (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt; 50mg/ml in DMF). The filter was incubated in the dark until the colour precipitate had formed and bands were visible. The reaction was stopped by washing the filter for 5 minutes with 50ml of TE buffer.

2.7.14 *In situ* hybridisation of bacterial colonies.

The method used was that described by Maniatis *et al.*, (1989).

A gridded nitrocellulose filter was placed onto a selective agar plate and individual colonies to be screened were transferred to it using sterile cocktail sticks. Each colony was also transferred to an identical position on a master plate. The plates were inverted and incubated at 37°C overnight and both the filter and the master plate were marked in three identical positions in order to identify positive colonies. Using forceps, the filter was peeled from the plate, placed colony side up on a piece of 3MM paper saturated with 10% SDS and left for 3 minutes. The filter was then transferred to 3MM paper soaked in denaturation solution (1.5M NaCl, 0.5M NaOH) for 5 minutes, followed by 5 minutes on 3MM paper saturated with neutralising solution (1.5M NaCl, 0.5M Tris.HCl pH8.0). The filter was laid on a sheet of dry 3MM paper and allowed to dry at room temperature for 30-60 minutes before being baked for 2 hours at 80°C in a vacuum oven.

To prepare the filter for hybridisation, it was first floated on the surface of a tray of 6x SSC until thoroughly wetted from beneath and then submerged for 5 minutes. A prewashing step followed, to remove any fragments of agar or loose bacterial debris. The filter was incubated at 65°C for 1-2 hours with shaking in 100ml of washing solution (1M NaCl, 1mM EDTA, 0.1% SDS, 50mM Tris.HCl pH8.0). Prehybridisation, hybridisation and detection of positive colonies were carried out as described in sections 2.7.12 and 2.7.13.

2.8 DNA sequencing.

DNA sequencing was carried out by the dideoxynucleotide chain termination method of Sanger *et al.* (1977), using a BRL M13 sequencing kit. DNA subclones for sequencing were constucted in the bacteriophage vectors M13mp18/19 (Yanisch-Peron *et al.*, 1985).

2.8.1 Transformation of recombinant M13 DNA.

Ligation reactions were transformed as follows into cells of *E. coli* strain JM101 or TG2 made competent by one of the methods in section 2.6.

Ligation mix containing 10-20ng of DNA was added to an aliquot of competent cells and kept on ice for 30 minutes. The cells were heat shocked at 42°C for 2 minutes and placed back on ice for 5 minutes. 200µl of fresh log. phase cells were added and the transformation mix was transferred to a sterile tube containing 3ml of 0.6% L-agar which had been melted and held at 45°C. To this had previously been added 10µl of 100mM IPTG and 20µl of 10% X-gal. The contents of the tube were mixed gently and poured onto the surface of a prewarmed L-agar plate. Once the top agar had set, the plates were inverted and incubated at 37°C overnight. The presence of white plaques indicated recombinant phage.

2.8.2 Preparation of single-stranded M13 DNA.

Templates for sequencing were prepared by the following method.

50ml of 2YT broth was inoculated with 100µl of an overnight culture of JM101 or TG2 and 2ml aliquots were transferred to sterile McCartney bottles. Well spaced plaques were picked with sterile cocktail sticks and a single plaque was inoculated into each bottle. The cultures were incubated with shaking at 37°C for 8-10 hours. 1.5ml of each culture was transferred to a sterile eppendorf tube and centrifuged at 12,000g to pellet the bacterial cells. The supernatant was removed to a fresh tube and recentrifuged, whilst the bacterial pellet was saved for preparation of the RF (replicative form) DNA by the plasmid miniprep method, in order to carry out restriction analysis of the recombinant phage. 1ml of the supernatant was transferred to a clean tube and 200µl of 20% PEG/2.5M NaCl was added, the contents of the tube vortexed briefly and left to stand at room temperature for 45 minutes. The precipitated phage were pelleted by centrifugation at 12,000g for 10 minutes and the supernatant poured off. The tubes were respun for 10 seconds to collect any remaining supernatant in the bottom of the tube and this was removed with a drawn out glass pipette. The pellet was resuspended in 200µl of TE buffer and this was extracted once with

phenol, twice with phenol:chloroform:isoamyl alcohol, and finally, once with chloroform:isoamyl alcohol. To the aqueous phase was added 0.1 volumes of 3M sodium acetate pH6.0 and 3 volumes of ethanol and the DNA was allowed to precipitate at -20°C for 1 hour. Following centrifugation, the DNA was washed with 70% ethanol, dried and resuspended in 20µl of TE buffer. 2µl of the DNA was run on an agarose gel to estimate the concentration. Phage DNA which contained an insert showed a reduced mobility in comparison to control single-stranded M13 DNA.

2.8.3 Orientation test.

If a DNA fragment had been cloned into a single restriction site, then some of the template DNA could be used at this stage to carry out an orientation test, to determine whether inserts were in identical or opposite orientations and thus minimise repetitive sequencing.

1µl of single-stranded DNA of each of the pair of clones to be compared were mixed together with 11.5µl of TE buffer, 1.5µl of 5M NaCl and 5µl of formamide dye mix (3% SDS, 0.1% bromophenol blue, 60% formamide, 25mM EDTA pH8.0). The mixture was incubated at 65°C for 1 hour and samples then loaded on a 0.7% agarose gel, together with control samples. Electrophoresis was carried out at 100V for 2-3 hours and the gel then observed under UV light. If two templates contained the same insert in opposite orientations then they would anneal and show a reduced rate of migration in comparison with the control.

2.8.4 Dideoxysequencing reactions.

These were performed essentially as set out in the BRL M13 Cloning/DideoxySequencing Instruction Manual.

A sequencing primer (either the M13 universal 17bp primer, or an oligonucleotide complementary to a previously sequenced region of the cloned DNA) was first annealed to the template by mixing together 3µl of single-stranded DNA template, 7.4µl of distilled water, 1µl (4 nmoles) of primer and 1µl of 10x polymerase reaction buffer and heating to 95°C for 5 minutes. The mixture was then allowed to cool to room temperature. 10x polymerase reaction buffer was 70mM Tris.HCl, 70mM $MgCl_2$, 500mM NaCl pH7.5.

An aliquot of each of the 10mM dNTP stock solutions was diluted 1:20 in distilled water to give a final concentration of 0.5mM and these working solutions were used to prepare the nucleotide (N°) mixes, as set out below:-

	A°	C°	G°	T°
0.5mM dCTP	20µl	1µl	20µl	20µl
0.5mM dGTP	20µl	20µl	1µl	20µl
0.5mM dTTP	20µl	20µl	20µl	1µl
10x reaction buffer	20µl	20µl	20µl	20µl

10mM ddNTP stock solutions were diluted to give working concentrations of: 0.1mM ddATP; 0.3mM ddCTP; 0.5mM ddGTP; 1.0mM ddTTP.

Whilst the template and primer were annealing, 1µl of the appropriate N° mix and 1µl of the corresponding ddNTP solution were added to four tubes labelled A, C, G and T.

1µl of 0.1M DTT, 1µl of Klenow enzyme (1 unit) and 1µl of [^{35}]S-dATP (10 µCi) were added to the template/primer mix. A 3µl aliquot of this was added to each of the four tubes containing the nucleotides, mixed briefly and incubated at 37°C for 20 minutes. 1µl of a 0.5mM dNTP solution containing all four nucleotides was then added to each reaction tube, mixed and incubated for a further 20 minutes at 37°C. After this second incubation the reactions were terminated by the addition of 5µl of formamide dyes (0.1% bromophenol blue, 0.1% xylene cyanol, 10mM EDTA, 95% formamide) and the samples stored at -20°C until required.

2.8.5 Preparation and electrophoresis of sequencing gels.

Two glass sequencing plates were washed with detergent, rinsed with distilled water and then cleaned with ethanol. The inner surface of the smaller plate was siliconised with "Repelcote". Plastic side spacers were laid on the larger plate and the smaller plate placed on top. The plates were clamped together and taped around the edges with yellow plastic tape.

To prepare a 6% acrylamide gel, 7.25ml of a solution of 38% acrylamide:2% bis-acrylamide was mixed with 5ml of 10x TBE buffer, 21g of urea and distilled water to a total volume of 50ml. The solution was filtered and degassed, then 0.4ml of 10% ammonium persulphate and 10µl of TEMED were added. It was then poured between the glass plates, a well comb inserted and the gel allowed to set overnight covered in cling-film.

The yellow tape was removed from the bottom of the gel and the comb removed. The gel plates were then set up in the sequencing tank and the upper and lower reservoirs filled with 1x TBE buffer. An aluminium sheet was clamped across one plate to aid heat dissipation and the

wells were rinsed out with buffer. The gel was pre-electrophoresed at 35mA for 30-60 minutes, until the temperature of the gel reached approximately 50°C. The DNA samples were boiled for 3 minutes and 2µl samples were loaded onto the gel using a 10µl syringe. Immediately prior to loading the samples the wells were rinsed out using a syringe, to remove any diffused urea. The gel was electrophoresed at 25mA for at least 90 minutes and then it was removed from the tank and the two plates separated. A sheet of 3MM paper was laid on top of the gel and lifted off, taking the gel with it. The gel was covered with cling-film and dried under vacuum at 80°C for 3-4 hours. Once dry, the gel was taped into a metal cassette and exposed to preflashed X-ray film (Fuji RX) at room temperature for 24-72 hours, then the film was developed and the sequence read.

2.9 Oligonucleotide-directed *in vitro* mutagenesis.

In vitro mutagenesis was carried out using the Amersham system version 2, which is based on the method of Taylor *et al.* (1985).

2.9.1 5'-phosphorylation of the mutant oligonucleotide.

A 21-mer oligonucleotide, complementary to the single-stranded DNA template and carrying the desired base change was synthesised on an ABS oligonucleotide synthesiser. Before it could be used in the mutagenesis reaction it was then necessary to 5'-phosphorylate it as follows. 2.5µl of a 5 OD units/ml stock solution of the oligonucleotide was added to an eppendorf tube, together with 3µl of 10x kinase buffer (1M Tris-HCl pH8.0, 100mM $MgCl_2$, 70mM DTT, 10mM ATP), 25µl of distilled water and 2 units of T4 polynucleotide kinase. This was incubated at 37°C for 15 minutes and then heated at 70°C for a further 10 minutes. The oligonucleotide was now ready for use and was stored at -20°C until required.

2.9.2 Oligonucleotide directed mutagenesis reactions.

All buffers and nucleotide mixes specified in the following method were as supplied with the Amersham *in vitro* mutagenesis system.

The mutant oligonucleotide was first annealed to the DNA template (prepared as in section 2.8.2). 5µl of single-stranded DNA template (1µg/µl) was mixed with 2.5µl of phosphorylated mutant oligonucleotide, 3.5µl of Buffer 1 and 6µl of distilled water. This was placed at 70°C for 3 minutes, then at 37°C for 30 minutes before finally being put on ice. Synthesis and ligation of the mutant DNA strand was achieved by adding to the annealing reaction 5µl of 100mM $MgCl_2$, 19µl

of nucleotide mix 1, 6µl of water, 6 units of Klenow enzyme and 6 units of T4 DNA ligase, then incubating the reaction at 15°C overnight.

Any single-stranded, non-mutant DNA which remained was removed by centrifuging the reaction mixture through a pair of nitrocellulose filters. 170µl of water and 30µl of 5M NaCl were added to the reaction mixture and it was transferred to the upper chamber of the supplied filter unit. The unit was centrifuged at 1,500 rpm for 10 minutes in a bench top centrifuge. 100µl of 500mM NaCl were added to the upper chamber and the unit respun for 10 minutes to wash through any remaining double-stranded DNA.

The DNA sample was precipitated by the addition of 28µl of 3M sodium acetate pH6.0 and 700µl of cold ethanol. Once the DNA pellet was dry, it was resuspended in 10µl of Buffer 2 then 65µl of Buffer 3 and 5 units of *NciI* were added in order to nick the non-mutant strand. The reaction was incubated at 37°C for 90 minutes.

The next stage was to digest the non-mutant strand of DNA. The concentrated stock of exonuclease III was diluted to give a working stock of 25 units/µl. To the nicked reaction mix was added 10µl of Buffer 4, 12µl of 500mM NaCl and 50 units of exonuclease III. This was incubated at 37°C for 30 minutes then the enzymes were inactivated by heating at 70°C for 15 minutes. 13µl of nucleotide mix 2, 5µl of 100mM $MgCl_2$, 3 units of DNA polymerase I and 2 units of T4 DNA ligase were added and the reaction incubated at 15°C for 3 hours.

Upon completion of the mutagenesis reactions, 20µl aliquots of the final sample were used to transform competent TG2 cells as described in section 2.8.1. Mutant progeny were analysed either by sequencing or, if the mutagenesis had created a restriction site, by restriction analysis of the RF DNA.

2.10 *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose assay for chitinase production.

The assay used was a modified version of that of Roberts & Selitrennikoff (1985). The chromogenic substrate, *p*-nitrophenyl-β-D-N,N'-diacetylchitobioside, was dissolved at 300µg/ml in 0.05M phosphate buffer (pH6.0). 100µl aliquots were transferred to eppendorf tubes in an ice-bath. Samples of supernatants or cell extracts from chitinase producing cultures were added to the tubes and incubated at 50°C for 30 minutes to 24 hours depending upon the amount of chitinase in the sample. After incubation, 100µl aliquots of the assay samples were pipetted into a microtitre plate, mixed with 10µl of 1M NaOH to enhance colour formation and the absorbance read at 410nm using a Titertek Multiscan MCC plate reader. Duplicate aliquots of the same sample, when assayed, produced absorbance readings within approximately 5% of each other.

2.11 Assay for β-glucuronidase (GUS) activity.

Bacterial cultures were assayed for the production of β-glucuronidase (GUS) according to the method of Jefferson (1987).

1ml of the culture to be assayed was transferred to an Eppendorf tube and the cells pelleted by centrifugation for 2 minutes. The pellet was resuspended in 0.5ml of extraction buffer (50mM $NaPO_4$, pH7.0; 10mM β-mercaptoethanol; 10mM EDTA; 0.1% sodium lauryl sarcosine; 0.1% Triton X-100) then subjected to two cycles of rapid freeze-thawing using liquid nitrogen and a 37°C water bath. The resulting cell extract was assayed as follows. A 0.5ml aliquot of GUS assay buffer (1mM 4-methylumbelliferyl-β-glucuronide in extraction buffer) was prewarmed to 37°C. 20μl of cell extract was mixed thoroughly with the assay buffer and incubated at 37°C for 5 minutes before a 100μl aliquot was removed into 0.9ml of stop buffer (0.2M Na_2CO_3). Further 100μl aliquots were removed at regular time intervals in order to determine an accurate rate for the reaction. The concentration of methylumbelliferone present in the stopped reactions was determined using a Baird Atomic fluoripoint spectrofluorimeter, with excitation at 365nm and emission at 455nm. The fluorimeter was calibrated using 1μM and 100nM methylumbelliferone standards.

2.12 Conjugation of plasmids into *Agrobacterium.*

Plasmids were transferred into *Agrobacterium* strains by triparental mating using a method based on that of Ditta *et al.* (1980), with pRK2013 as a helper plasmid. Cultures of the *E. coli* donor strain, helper strain and recipient *Agrobacterium* were grown to exponential phase. 300μl of recipient were mixed with 100μl each of the donor and helper and 200μl of the mixture was carefully pipetted onto a nitrocellulose disc placed on the surface of an L-agar plate. This was incubated at 28°C overnight. The nitrocellulose disc was then transferred to a bottle containing 10ml of sterile 10mM $MgSO_4$ and shaken to resuspend the bacteria in the solution. Dilutions were made to 10^{-2} and 100μl aliquots of each dilution were plated onto selective agar plates. The plates were incubated at 28°C for 48 hours to allow for growth of transconjugant colonies.

2.13 Electroporation of *Agrobacterium.*

Once a Biorad GenePulser became available in the laboratory, electroporation was used as the preferred method of introducing plasmids into *Agrobacterium.* It had the advantages of being quicker and more efficient than the traditional triparental mating procedure. The method used for

the preparation of competent cells and the parameters for the electroporation process were those of Nagel *et al.* (1990).

250ml of L-broth was inoculated with a 5ml overnight culture of the recipient *Agrobacterium* strain and the culture grown with shaking at 28°C until the absorbance at 550nm was 0.5-1.0. The cells were harvested by centrifugation at 4000g for 15 minutes at 4°C. The pellets were resuspended on ice in a total of 250ml of 1mM HEPES pH7.0, then recentrifuged for a further 15 minutes. The resulting pellets were resuspended in 125ml of ice-cold HEPES buffer and centrifuged as before. The pellet was kept on ice and resuspended in 100ml of 10% glycerol before a final centrifugation step at 4000g for 15 minutes. The pellet was ultimately resuspended in 750µl of ice-cold 10% glycerol. The cells were either used immediately or divided into 80µl aliquots, frozen rapidly in liquid nitrogen and stored at -80°C.

For the electroporation process 80µl of competent cells were mixed with 1µl of plasmid DNA in TE buffer (100-200ng) in a precooled electroporation cuvette with a 2mm electrode gap (Biorad). The cells were subjected to a 2.5kV charge dissipated at 25µF and 200ohms. For a successful transformation, charge dissipation required at least 4 seconds. Following electroporation the cells were immediately diluted in 1ml of L-broth and incubated with shaking at 28°C for 6-8 hours prior to plating out on selective L-agar.

2.14 The 3-keto-lactose test for *Agrobacterium.*

The triparental mating procedure sometimes gave rise to spontaneous Rif^R *E. coli* cells, so the following test was used to confirm the identity of possible *Agrobacterium* transconjugants (Bernaerts & De Ley, 1963).

Presumptive *Agrobacterium* colonies were streaked onto $CaCO_3$ medium (2% $CaCO_3$, 2% glucose, 1% yeast extract, 2% agar) and incubated overnight at 28°C. A loopful of bacteria was transferred to a lactose agar plate (1% lactose, 0.1% yeast extract, 2% agar) to make a thick area of bacteria approximately 0.5cm in diameter. The plates were incubated at 28°C for 48 hours and were then flooded with Benedicts reagent (173g sodium citrate, 100g sodium carbonate, 17.3g copper sulphate per litre). A yellow zone could be seen around *Agrobacterium* colonies after 1-2 hours at room temperature.

2.15 Determination of protein concentration.

The protein concentration of cell extracts or culture supernatants was determined by a Bradford assay using the Bio-Rad micro assay reagent (Bradford, 1976). The dye reagent concentrate was diluted 1 in 5 with distilled water and 200µl of this was mixed with 100µl of sample, diluted if necessary. This was carried out in a microtitre plate (Falcon). The absorbance at 595nm was measured using a Titertek Multiscan MCC plate reader. The protein concentration was calculated from the absorbance readings using a calibration curve constructed using known quantities of IgG.

CHAPTER 3

THE *CHIB* GENE OF *SERRATIA MARCESCENS.*

3.1 Introduction.

Chitin, which is an insoluble, linear β-1,4-linked polymer of N-acetylglucosamine, is one of the most abundant polysaccharides in nature, being second only to cellulose in terms of biomass (Watanabe *et al.*, 1990). It is a major constituent of fungal cell walls, insect skeletons and the shells of crustaceans (Muzzarelli, 1977). Since many chitin-containing insects, fungi and nematodes are agronomically important pests it is not surprising that interest has been shown in the enzymatic degradation of the chitin component of these organisms as a method for their control (Fuchs *et al.*, 1986; Ordentlich *et al.*, 1988).

All organisms that contain chitin also contain the hydrolytic enzymes, chitinases, which are presumably required for morphogenesis of cell walls and exoskeletons (Gooday, 1977). However, many other organisms which do not contain chitin also produce chitinases. These include soil bacteria that secrete chitinases to degrade chitin in order to obtain nutrients (Roberts & Selitrennikoff, 1988) and a number of plants which produce chitinases in response to infection or injury (Boller, 1985). It has been suggested that the production of chitinase by plants is a defence mechanism to provide protection against fungal pathogens (Boller, 1985) and indeed a number of plant chitinases have been purified and shown to have antifungal activity (Roberts & Selitrennikoff, 1988; Schlumbaum *et al.*, 1986).

3.1.1 The chitinolytic enzymes of *Serratia marcescens.*

Serratia marcescens is a Gram-negative soil bacterium which secretes high levels of chitinase activity. In one survey it was found to be the most active chitinase producer of 100 organisms tested (Monreal & Reese, 1969). This has led to a number of investigations into the efficacy of *S. marcescens* as a biocontrol agent (Ordentlich *et al.*, 1988; Shapira *et al.*, 1989), the purification of its chitinases (Monreal & Reese, 1969; Roberts & Cabib, 1982) and cloning of the chitinase genes (Fuchs *et al.*, 1986; Jones *et al.*, 1986; Sundheim *et al.*, 1988).

The number of chitinolytic proteins reported to be produced by *S. marcescens* varies from 2 to 5. Fuchs *et al.* (1986) identified five individual proteins of 57, 52, 48, 36 and 21kDa, each possessing chitinase activity, although the 57kDa protein was preferentially synthesised. Using SDS PAGE, Roberts and Cabib (1982) located chitinase activity to two major protein bands at

58 and 52kDa and three weaker bands at approximately 21, 40 and 50kDa. However, under non-denaturing conditions only the 52 and 58kDa bands were present. A number of groups have cloned the gene encoding the 58kDa chitinase (Fuchs *et al.*, 1986; Jones *et al.*, 1986; Shapira *et al.*, 1989) which has been designated *chiA*, whilst Sundheim *et al.* (1988) isolated a 9.4kb fragment of DNA which is thought to contain the *chiA* gene. The failure of two of the groups to obtain more than one chitinase gene may reflect the abundance of the 57-58kDa protein (Fuchs *et al.*, 1986). Nevertheless, Jones *et al.* (1986) cloned a second chitinase gene, *chiB*, which encodes the 52kDa protein and Sundheim *et al.* (1988) cloned an 18kb *Eco*RI fragment which produced chitinase but they did not identify the protein product. In each case the cloned *chiA* gene product has been shown to have a suppressive effect on fungal pathogens.

Ashby (1988) elected to use the *chiA* protein as the active molecule in a novel biocontrol delivery system in which the chitinase gene is brought under the control of an inducible *Agrobacterium vir* promoter. The coding region and Shine-Dalgarno sequence of the *chiA* gene from pCHIT1251 (Jones *et al.*, 1986) was linked to a *virB* promoter cassette which utilised the promoter region of the *virB* operon from pSM30 (Stachel & Zambryski, 1986b). The resulting construct, when introduced into the *Agrobacterium* strain C58C^1 together with pVK257 (Knauf & Nester, 1982) to provide the necessary *vir* functions, produced chitinase upon induction with acetosyringone (Ashby, 1988).

3.2 *Serratia marcescens chiB* gene.

The second cloned chitinase of *S. marcescens*, designated *chiB*, is contained within a 3.28kb *Eco*RI-*Pst*I fragment of the plasmid pCHIT310 (Jones *et al.*, 1986). This plasmid, together with pCHIT1251, was kindly made available by John Bedbrook (AGS, Berkeley). pCHIT310 was obtained by first constructing a genomic library of *S. marcescens* QMB1466 DNA in the cosmid vector pLAFR1. Chitinase-producing clones were identified by clearance of chitin plates and nine such clones were found to contain an identical 30kb *Eco*RI fragment. The DNA from these was digested with *Eco*RI and partially with *Pst*I then ligated into pUC8. The resulting subclone, pCHIT310, was chitinase-positive and contained a 3.28kb insert (Jones *et al.*, 1986).

3.2.1 Decision to use the *chiB* gene in a biocontrol system.

The *chiB* gene was chosen as the second gene to be employed in the pesticide delivery system of Ashby *et al.* (1986) partly on the basis of the pesticidal activity its chitinase displayed in tests carried out at ICI's Jealotts Hill Research Station.

Two strains of *Agrobacterium* C58C[1] containing pDUB2501 and pDUB2502 which carry the *S. marcescens chiA* and *chiB* genes respectively (Ashby, 1988) and the corresponding *E. coli* JM83 containing pCHIT1251 or pCHIT310 were sent to Jealotts Hill for an assessment of their pesticidal effects. Each strain produced chitinase constitutively. A series of experiments were carried out as follows using *Diabrotica balteata* as the target organism. A loopful of culture was taken from a freshly grown plate and inoculated into 50ml of L-broth or chitin medium. This was shaken at 28°C (*Agrobacterium*) or 37°C (*E. coli*) for 48 hours prior to centrifugation and washing (twice) in sterile distilled water. The cells were finally resuspended in an equal volume of sterile distilled water and now formed the inoculum. Sprouted maize seeds were soaked in the bacterial inoculum for 1 hour and one seedling was placed in the bottom of each of ten 1.5" tall plastic containers. The seed was covered with 20g of compost which had been thoroughly mixed with 2ml of the corresponding bacterial suspension. Each container was infested with one healthy second stage *Diabrotica* larva and sealed with a clip-on polypropylene lid. The containers were incubated at 24-26°C for 6 days in a darkened environment. Control replicates were set up for each experiment using either an *Agrobacterium* or *E. coli* strain lacking a plasmid and using distilled water in place of the bacterial suspension.

In five separate sets of experiments using the *Agrobacterium* strains the strain containing the *chiB* gene consistently caused higher levels of larval mortality than either of the controls. A number of larvae which were still alive were either small or showed difficulty in moulting. However, in each case except one, the *Agrobacterium* control caused a low level of mortality suggesting that *Agrobacterium* itself may exert a toxic effect towards *Diabrotica.* In only one experiment did the strain containing the *chiA* gene cause higher mortality than the *Agrobacterium* control. One slightly disappointing result was that in each case no more than 20% of the larval mortality could be attributed to the effect of chitinase (see Table 1).

The mean results for the five sets of experiments were:-

Treatment	% mortality
Water control	2
C58C[1]	10
C58C[1] (pDUB2501)	12
C58C[1] (pDUB2502)	24

Table 1

A similar experiment was carried out using the *E. coli* strain JM83 containing the plasmids pCHIT1251 and pCHIT310. However in this case the inoculated maize seedling was placed on a filter paper in a petri dish which had been soaked in 1ml of bacterial suspension. This assay was considered to be less satisfactory since it continued for only 3 days and the conditions were not as suitable for the survival of the bacteria. Nevertheless the following results were obtained:-

Treatment	% mortality
JM83 control	0
JM83 (pCHIT1251)	0
JM83 (pCHIT310)	20

Table 2

Both sets of results appear to indicate that although the pesticidal effect is not great, the enzyme produced by the *chiB* gene has a greater effect against *Diabrotica balteata* than the *chiA* gene product.

Moreover it is known that the chitinases of *S. marcescens* are inhibitory to the growth of a number of plant pathogenic fungi and are responsible for the disease control shown by *S. marcescens* in a number of cases (Jones *et al.*, 1986; Ordentlich *et al.*, 1988; Sundheim *et al.*, 1988). Jones *et al.* (1986) showed that a *chiA* mutant of *S. marcescens* exhibited reduced inhibition of fungal spore germination and reduced control of *Fusarium oxysporum* f. sp. *pisi*. However a suppressive effect remained, suggesting that another factor such as *chiB* is necessary for efficient pathogen control. The two chitinases may act synergistically to degrade chitin and it was thought that a combination of the two enzymes in a biocontrol system would be more effective than a control agent producing just a single chitinase. Therefore it was essential to further characterise the *chiB* gene and determine its DNA sequence so that it could be utilised in the *vir*-inducible delivery system for biological control products (Ashby *et al.*, 1986).

3.3 Subcloning of the 3.28kb insert of pCHIT310.

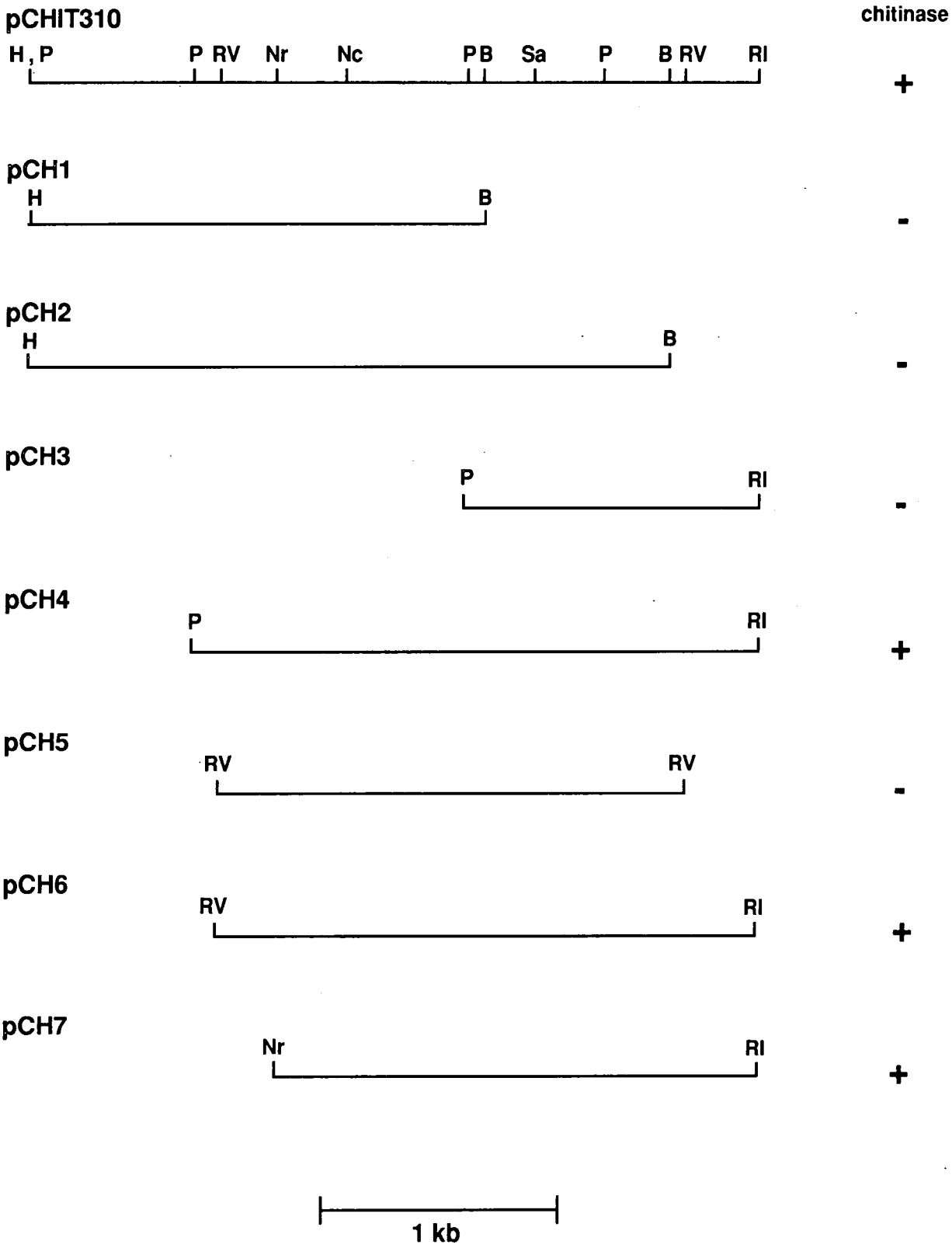
It was known that the *chiB* gene encoded a 52kDa protein and this implied that the gene was confined to only a part of the 3.28kb of *S. marcescens* DNA in pCHIT310. Functional deletion analysis was therefore carried out to define the minimal fragment required to express an active enzyme. A limited restriction map of pCHIT310 was available (Jones *et al.*, 1986)

Figure 3.1 pCHIT310 subclones and chitinase production.

A diagram showing the restriction map of the 3.28kb insert of pCHIT310 (Jones *et al.*, 1986) and the restriction fragments present in the subclones pCH1-pCH7.

The lefthand column indicates whether or not each subclone is capable of expressing an active chitinase.

H = *Hin*dIII; P = *Pst*I; B = *Bam*HI; Sa = *Sal*I; Nr = *Nru*I; Nc = *Nco*I; RV = *Eco*RV; RI = *Eco*RI.


pCHIT310
chitinase
H, P
P RV
Nr
Nc
P B
Sa
P
B RV
RI
+
pCH1
H
B
-
pCH2
H
B
-
pCH3
P
RI
-
pCH4
P
RI
+
pCH5
RV
RV
-
pCH6
RV
RI
+
pCH7
Nr
RI
+
1 kb

and this information was used to subclone smaller fragments from the original *S. marcescens* DNA fragment (Figure 3.1).

Plasmids pCH1 and pCH2 were constructed by first digesting 5µg of pCHIT310 DNA with *Hin*dIII at 37°C for 4-5 hours. The digest was terminated by carrying out a phenol/chloroform extraction to remove the restriction enzyme and the DNA was ethanol precipitated and resuspended in 50µl of TE buffer (see sections 2.73 & 2.74). The DNA was then partially digested with *Bam*HI, following optimisation of the digestion conditions in order to recover the required digestion products. 6µl of medium REB, 3µl of H_2O and 1µl (5 units) of BamHI were added to the resuspended DNA and the reaction allowed to proceed at room temperature. After 2 minutes a 5µl aliquot was removed into 2.4µl of 25mM EDTA pH8.0 and further 5µl aliquots were added every 2 minutes until the entire reaction had been terminated. The digested DNA was run on a 0.7% LMP agarose gel (section 2.7.7) and DNA fragments of approximately 2.9 and 2.1kb were recovered according to section 2.7.8. These DNA fragments were ligated into *Hin*dIII/*Bam*HI digested pUC19 (section 2.7.6) and the ligation mix used to transform competent JM83 cells (section 2.6.3). Resulting white colonies on X-gal/amp plates (section 2.4) were selected for analysis. DNA minipreps were made (section 2.5.1) and the plasmids digested with appropriate restriction enzymes and electrophoresed on an agarose gel to check for the presence of the correct inserts.

The construction of pCH3 and pCH4 followed a very similar procedure except that pCHIT310 was first digested to completion with *Eco*RI and then a partial *Pst*I digest was carried out on the resuspended *Eco*RI cut DNA. 10µl aliquots were removed every 2 minutes during the reaction into 2.4µl of 25mM EDTA. Again, the digestion products were separated on a 0.7% LMP agarose gel and DNA fragments of 2.6 and 1.3kb recovered according to section 2.7.8. These were ligated into pUC19 digested with *Eco*RI and *Pst*I and again transformed into JM83. White colonies on X-gal/amp plates were analysed as before.

To create pCH5, pCHIT310 DNA was digested with *Eco*RV at 37°C for 3 hours. The digested plasmid was electrophoresed on a 0.7% agarose gel and the 2.1kb *Eco*RV fragment was isolated (section 2.7.8) and ligated into pUC19 which had been digested with *Sma*I. This digest had been carried out in *Sma*I enzyme buffer (10mM Tris pH8.0, 20mM KCl, 10mM $MgSO_4$, 1mM DTT) at room temperature. Transformation and analysis of recombinants were carried out as for the other subclones.

For pCH6, pCHIT310 was first digested to completion with *Eco*RI and then a partial digest was carried out with *Eco*RV. Following a trial digest to optimise conditions it was discovered that by removing a 5µl aliquot of the digest every 4 minutes the required DNA fragment would

be obtained. The 2.4kb *Eco*RI-*Eco*RV fragment was isolated from a LMP agarose gel and ligated to pUC19 digested with *Eco*RI and *Sma*I.

pCH7 was created by digesting pCHIT310 with *Eco*RI and *Nru*I and ligating the gel isolated 2.2kb fragment to pUC19 digested with *Eco*RI and *Sma*I. *Nru*I digests DNA to give blunt ends which can be ligated to *Sma*I cut DNA.

3.4 Chitinase production by subclones of pCHIT310.

To determine which subclones were still capable of producing a functional chitinase, JM83 transformed with one of pCH1 to pCH7 or pCHIT310, *S. marcescens* as a positive control and JM83(pUC19) as a negative control, were streaked onto a minimal agar plate containing chitin (section 2.4). The plates were sealed with Nescofilm to prevent the agar drying out and incubated at 37°C (28°C for *S. marcescens*) for 7 days. The plates were examined and any clearing of the agar around the sites of bacterial growth was noted. This implied degradation of the chitin particles had taken place.

Clear areas were detected around *S. marcescens* and around bacteria containing pCHIT310, pCH4, pCH6 and pCH7 (see Figure 3.1) indicating that the DNA fragments in these plasmids specified the production of chitinase and therefore encompassed the complete *chiB* gene. Hence the *chiB* gene is located between the *Eco*RI site and the *Nru*I site of the *S. marcescens* DNA fragment. Further deletion from the *Eco*RI site to the *Eco*RV site as in pCH5 abolishes chitinase activity.

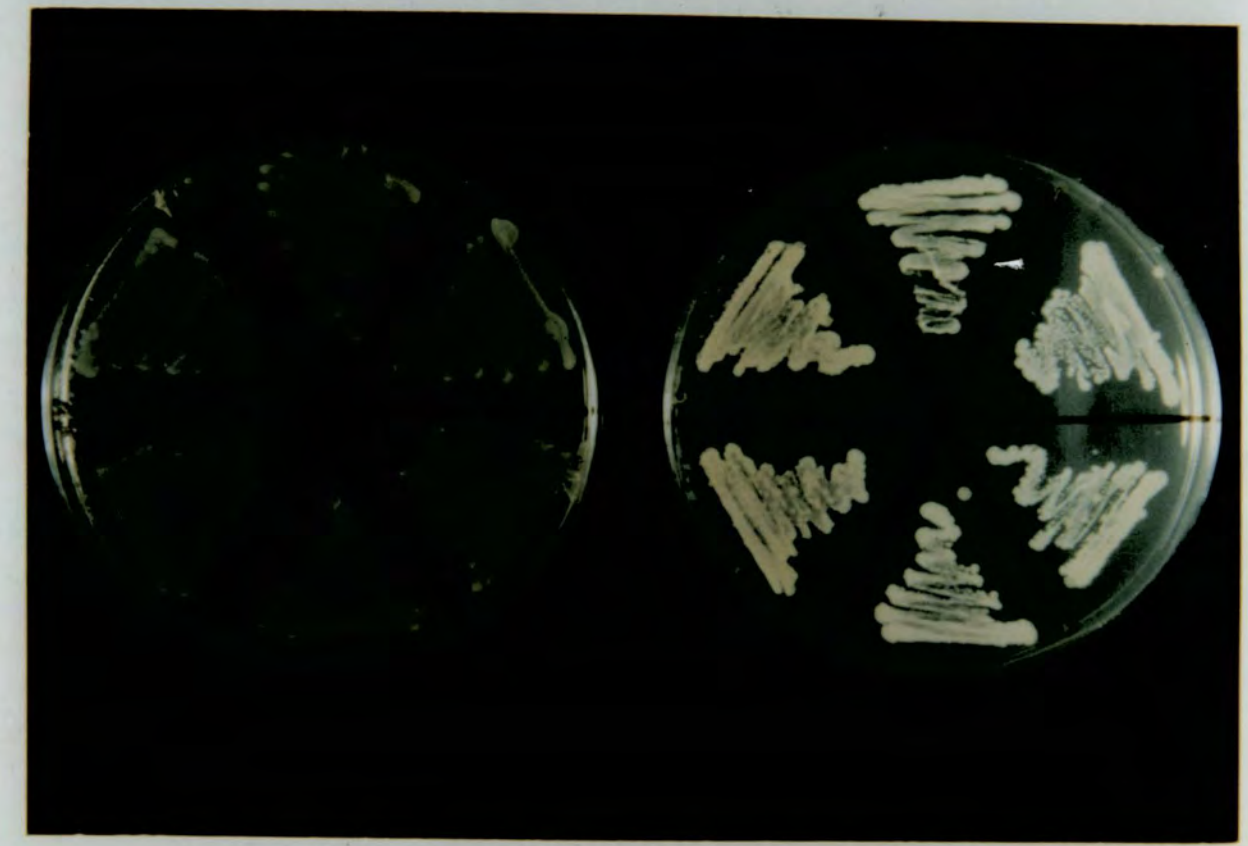
The area of clearing around *S. marcescens* was far greater and was detectable after a much shorter period of time than that around any of the *E. coli* clones. The clearing of the agar around *S. marcescens* was complete and the halo had a well defined outer edge whereas the halos around the *E. coli* clones still remained slightly opaque and had fuzzy, indistinct edges. Either the expression of the chitinase genes is less efficient in *E. coli* or a combination of enzymes are required for effective chitin digestion.

3.5 Distinct appearance of chitinase-producing colonies.

The plate-clearing assay was repeated a number of times since the results showed some inconsistency and the clearing was sometimes difficult to detect, even around the pCHIT310 positive control. It was noticed that chitinase producing colonies tended to have a different appearance when compared to non-chitinase producers. In contrast to the usual creamy,

Figure 3.2 Distinct appearance of chitinase producing *E. coli*.

E. coli JM83 containing either pCH6 (a) or pCH4 (b) was streaked onto an L-agar plate containing ampicillin. This photograph shows the difference in appearance of the two and is a typical result. The bacteria containing pCH6 retain the normal appearance of *E. coli* whilst those containing pCH4 have the unusual "white" appearance. Both are capable of producing an active chitinase.



a] pCH6

b] pCH4

somewhat shiny *E. coli* colonies, chitinase producing colonies were whiter and looked "dry" (Figure 3.2). When removing these chitinase producing bacteria from an agar plate with a bacteriological loop the cells did not have the usual tendency to adhere to each other or to the surface of the agar. This was not a constant phenotype dependent upon the presence of a particular plasmid, although some plasmids gave rise to a more variable phenotype than others. For instance, *E. coli* containing pCH7 have always appeared "whiter" whereas separate transformations with pCH6 have produced colonies with the two distinct appearances. It was speculated that colony appearance may be correlated to production of extracellular chitinase since it had been observed that bacteria which retained the normal colony appearance exhibited slower clearing of chitin agar plates and the cleared halos surrounding these bacteria were smaller).

3.6 An alternative method for assaying chitinase activity.

In order to investigate the production of the *chiB* enzyme in greater detail it was necessary to find a more reliable and quantitative assay for chitinase activity which was still simple and rapid to carry out. The principal drawbacks to the chitin plate assay were that it could not provide exact quantitative results, it measured only secreted chitinase and results were not available until after 7-10 days.

An alternative assay was employed, described by Roberts and Selitrennikoff (1988). This assay utilises the chromogenic substrate *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose which was originally synthesised as a potential substrate for lysozyme. Roberts and Selitrennikoff tested six different chitinases, three from bacteria and three from plants, for their ability to hydrolyse this substrate and found that only the bacterial enzymes were capable of doing so. This was reasoned to be due to the differing modes of action of the two classes of enzyme. Bacterial chitinases exhibit an exochitinolytic mechanism of chitin degradation, removing successive diacetylchitobiose units from the non-reducing end of the chitin molecule (Molano *et al.*, 1977; Robbins *et al.*, 1988; Roberts & Cabib, 1982) Obviously the action of such an exochitinase would liberate *p*-nitrophenol from this chromogenic substrate, resulting in assayable colour development. Conversely, plant-derived chitinases display an endochitinolytic mode of action, cleaving randomly at internal points within chitin chains and causing an accumulation of triacetylchitotriose (Boller *et al.*, 1983; Molano *et al.*, 1979). Therefore this chromogenic trisaccharide analogue would not be expected to act as a substrate for endochitinases.

Roberts and Selitrennikoff (1988) demonstrated that the purified 57kDa chitinase of *S. marcescens* was capable of hydrolysing *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose. Roberts and Cabib (1982) found that the major product of chitin digestion by a *S. marcescens* total

chitinase preparation was diacetylchitobiose, again signifying an exochitinolytic mechanism. Hence it was expected that this substrate could be used to assay the activity of the *chiB* enzyme, although it was necessary to check this. 0.5ml of overnight cultures of DH5α, DH5α(pCHIT1251), DH5α(pCHIT310) and *S. marcescens* were inoculated into 25ml of chitin medium and incubated at either 37°C (*E. coli*) or 28°C (*S. marcescens*) for 48 hours. After this time 1ml samples were removed from the cultures into Eppendorf tubes and centrifuged for 2 minutes in a microfuge. The supernatants were carefully removed into fresh tubes. For each assay, 100µl of supernatant was mixed in an Eppendorf tube with 100µl of *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose as described in section 2.10. The tubes were incubated at 50°C for 8 hours then 10µl of 1M NaOH was added to each tube to terminate the reaction and develop the colour. Each sample was transferred to a microtitre plate and the absorbance read at 410nm on a Titertek plate reader. Reaction blanks of supernatant plus buffer and substrate plus growth medium were included in the assay.

The results showed that hydrolysis of *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose could be used as an assay for ChiB activity, despite Jones *et al.* (1986) describing the *S. marcescens* enzymes as endochitinases. Positive results were also obtained with pCHIT1251 and *S. marcescens*, the latter showing the presence of much higher chitinase activity in the culture supernatant than either of the *E. coli* clones.

3.7 Is the *chiB* gene of pCHIT310 inducible?

It is known that the synthesis of chitinases in *S. marcescens* is inducible, with the yields of chitinase being much higher when the bacterium is grown in chitin-containing medium than when any other substrate is used (Monreal & Reese, 1969). However, it was probable that although the *chiB* gene carried on pCHIT310 was being expressed from its own promoter and not a vector promoter (Jones *et al.*, 1986) it lacked the *S. marcescens* regulatory genes and therefore chitinase expression could be constitutive.

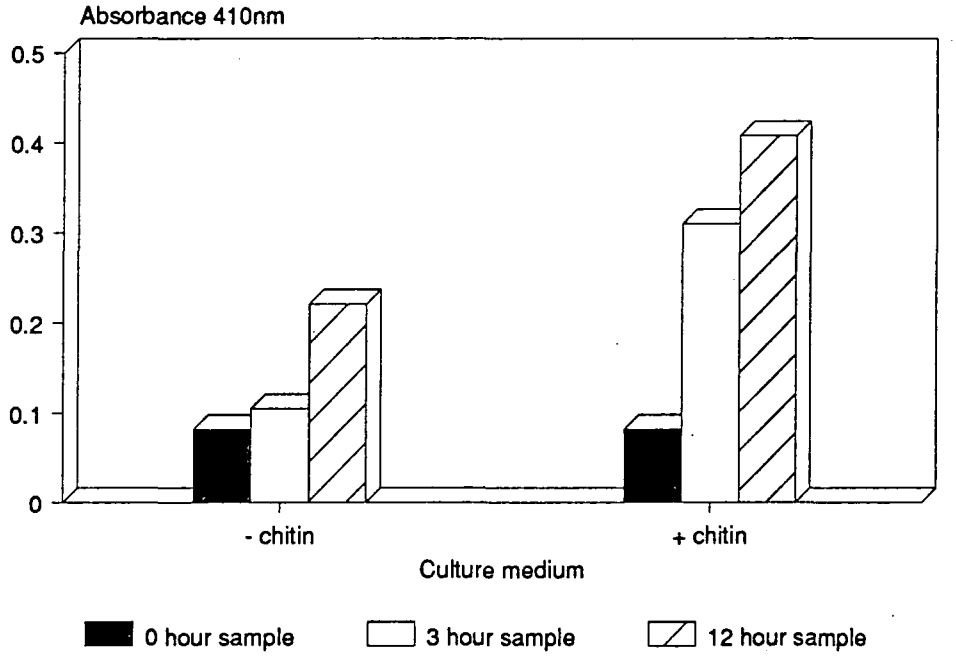
10ml aliquots of chitin medium lacking chitin were inoculated with 200µl of fresh overnight cultures of JM83(pCHIT310) or *S. marcescens*. The cultures were incubated with shaking at 37°C (*E. coli*) or 28°C (*S. marcescens*) for 3 hours when the OD_{550} was approximately 0.3. Each culture was then divided into two and 25mg of pure chitin added to one culture of each pair. A 0.5ml aliquot of each culture was taken for sampling. The cultures were then incubated for a further 3 hours and 0.5ml aliquots again removed. Samples were also taken after 12 hours. Each culture sample was centrifuged for 2 minutes in a microfuge and the supernatants transferred to fresh tubes. 100µl aliquots of these extracellular fractions were assayed for chitinase activity as described in section 3.6.

Figure 3.3 Effect of chitin supplementation on the production of chitinase.

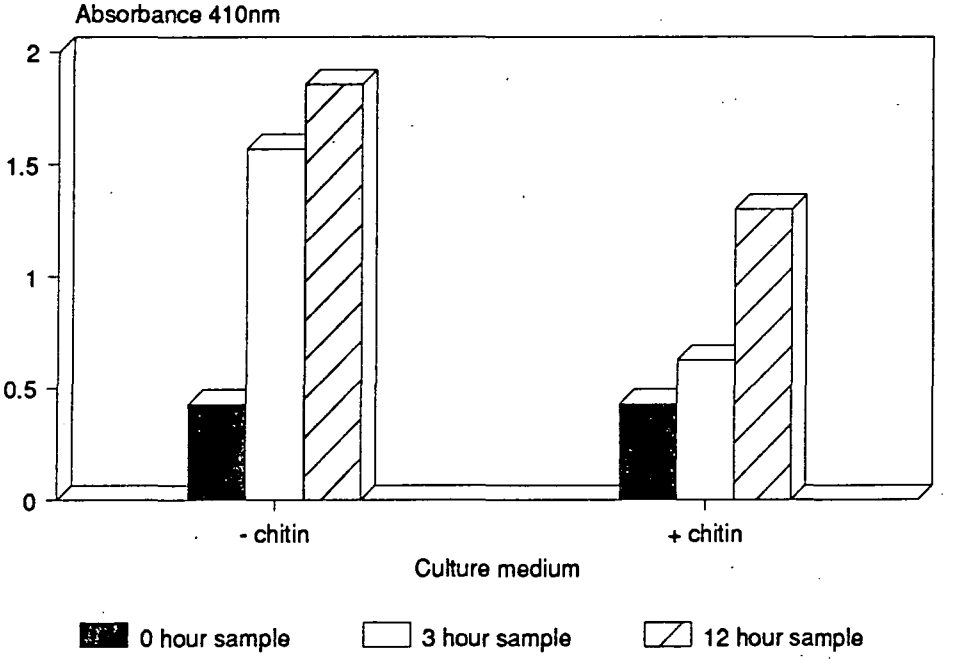
The histograms presented here show the chitinase activity present in the supernatants of cultures of *S. marcescens* and JM83(pCHIT310) grown either in the presence or absence of purified chitin.

The absorbance figures quoted are the total absorbance recorded for each assay. The experiment was repeated twice and the results each time showed the same trend. The results presented here are those obtained for a single representative experiment. Chitinase expression occurs in both *S. marcescens* and *E. coli* (pCHIT310) regardless of the presence of chitin, but the addition of chitin causes induction of expression in *S. marcescens*. No inducibility is seen for *E. coli* and the chitin in the medium appears to be inhibiting growth of the cells.

Induction of chitinase in S. marcescens.



Induction of chitinase from pCHIT310.



The results of the above experiment are presented in Figure 3.3. The supernatant of the culture containing pCHIT310 contained a small amount of chitinase at the point of addition of the chitin and this then increased in both cultures with or without added substrate. In fact, the culture lacking chitin exhibited higher levels of chitinase activity than the chitin supplemented culture. In contrast the supernatant of the *S. marcescens* culture contained a barely detectable level of chitinase activity prior to the addition of chitin. After 3 hours induction, there was a very low level of chitinase activity present in the supernatant of the chitin lacking culture and 2.95 times as much activity produced by the chitin supplemented culture. After 12 hours the amount of chitinase present in the supernatants of both chitin +/- cultures had risen with the chitin supplemented culture now containing only 1.8 times more chitinase. The *S. marcescens* culture containing chitin had also developed the red pigmentation typical of the strain whereas the absence of chitin led to a corresponding lack of pigment. This result suggested that a gene which causes repression of chitinase expression in the absence of chitin is not present on the cloned DNA fragment, resulting in constitutive expression from the *chiB* promoter.

An additional experiment demonstrated that a culture of JM83(pCHIT310) grown in L-broth also produced chitinase and so this was now routinely used as the culture medium for growth of all *E. coli* strains (result not shown).

3.8 Quantitative assay of chitinase production by pCHIT310 subclones.

Since an alternative method was available to assay chitinase activity quantitatively, it was decided to test all the pCHIT310 subclones which had been constructed, using the *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose assay. 100µl of fresh overnight cultures of pUC19, pCHIT310 and all the subclones in DH5α were inoculated into 5ml of L-broth and incubated with shaking at 37°C overnight. The OD_{550} of each culture was measured and 1ml aliquots of each culture were transferred to Eppendorf tubes and centrifuged for 2 minutes to pellet the cells. The supernatants were carefully transferred to fresh tubes and saved on ice as the extracellular fractions. Each cell pellet was then resuspended in 0.5ml of extraction buffer (50mM $NaPO_4$ pH7.0; 0.1% sodium lauryl sarcosine; 0.1% Triton X-100; 10mM β-mercaptoethanol) and subjected to two cycles of rapid freeze-thawing using liquid nitrogen and 50°C water baths. This was then stored as the cell extract. The extracellular fractions were assayed for chitinase activity by mixing 100µl of supernatant with 100µl of substrate and incubating at 50°C for 6 hours before stopping the reactions with 10µl of 1M NaOH. The cell extracts were assayed in the same way using 10µl of extract mixed with 90µl of H_2O and 100µl of substrate, but the reactions were only continued for 30 minutes. The protein concentration of the cell extracts was determined using the Bradford assay (section 2.15). Protein determinations

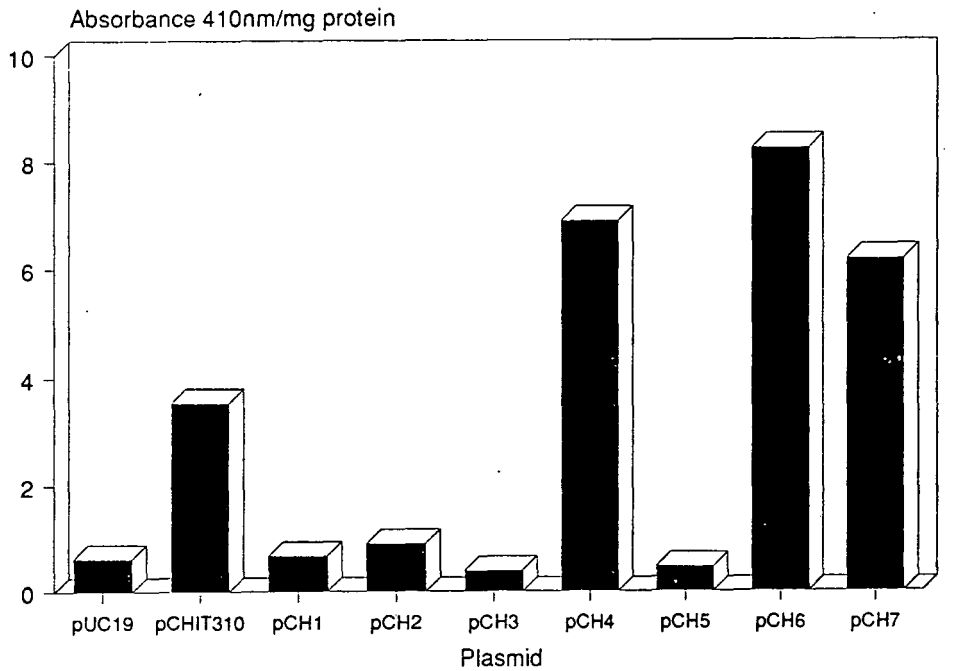
Figure 3.4 Results of chitinase assays carried out on cell extracts.

The histograms presented here show the chitinase activity present in the cell extracts of *E. coli* containing pUC19, pCHIT310 and the various subclones constructed.

In graph A the results are expressed as absorbance at 410nm per mg of protein whilst in graph B the same results are expressed as absorbance at 410nm per ml culture per OD_{550} unit.

The experiment was carried out three times although the results presented here are those obtained for a single representative experiment. Whilst the absolute values of absorbance varied slightly there was always a four-fold or greater difference in the values obtained for cultures containing pUC18. pCH1, pCH2, pCH3 and pCH5 and those containing pCHIT310, pCH4, pCH6 and pCH7.

Chitinase production by pCH1-pCH7.



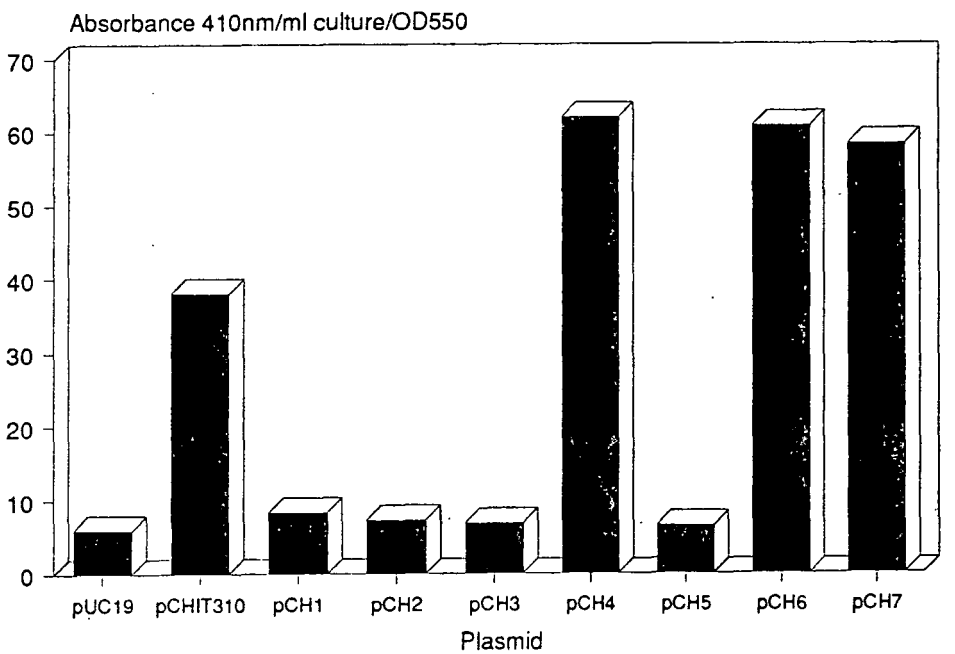
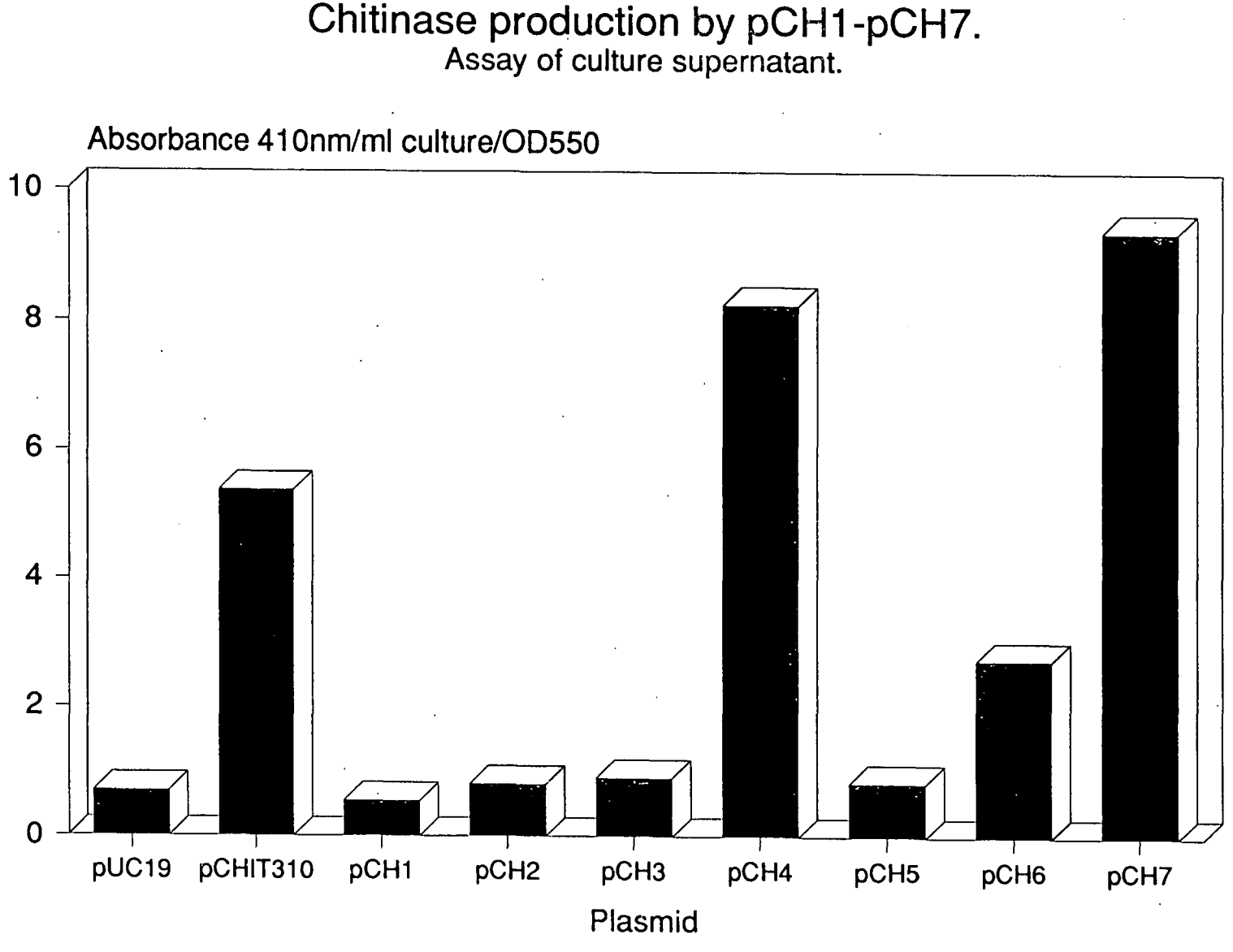


Figure 3.5 Result of chitinase assays carried out on culture supernatants.

This graph shows the chitinase activity present in the culture supernatants of *E. coli* containing pUC19, pCHIT310 or each of the subclones. Note the low level of chitinase activity present in the supernatant of the culture containing pCH6. This is correlated to the unusual colony morphology observed with this isolate.

The experiment was carried out three times and the results presented here are those obtained from a single representative experiment. With the exception of the unusual secretion observed with certain isolates (described in the text) the results were reproducible, with at least a five-fold greater activity in the supernatants of cultures containing pCHIT310, pCH4 and pCH7.


Chitinase production by pCH1-pCH7.
Assay of culture supernatant.
Absorbance 410nm/ml culture/OD550
10
8
6
4
2
0
pUC19
pCHIT310
pCH1
pCH2
pCH3
pCH4
pCH5
pCH6
pCH7
Plasmid

were also carried out on the supernatant fractions but these were misleading due to the protein content of the L-broth and the unreproducibility of the results obtained.

The results for the two fractions are presented in Figures 3.4 and 3.5. Those for the cell extracts are expressed as both absorbance at 410nm per mg of protein and absorbance at 410nm per ml culture per OD_{550}. The results for the extracellular fractions are merely expressed as absorbance per ml per OD_{550}. This should compensate for differences in the amount of chitinase due to differences in cell numbers.

The results correlate with those obtained from the plate clearing assay, confirming that pCH4, pCH6 and pCH7 each specify the production of an active chitinase. The results expressed as absorbance per mg protein show a very similar trend to those expressed as absorbance per ml culture per OD_{550} so either set of values could be used for analysis. For ease of comparison with the results of the assays carried out on the extracellular fractions the absorbances per ml culture per OD_{550} will be used. The relatively low level of chitinase activity detected in the supernatant of the culture containing pCH6 correlates with the fact that this isolate, uniquely among the chitinase producing cultures used in this experiment, did not have the unusual appearance described in section 3.5. The chitinase activity present in the cell extract of this culture was comparable to the levels in the other chitinase producing cultures.

3.9 Use of the *lacZ* promoter to enhance transcription of the *chiB* gene.

Now that the chiB gene had been assigned to the 2.2kb *Eco*RI-*Nru*I fragment of pCHIT310, further experiments were necessary to characterise the gene. The first step was to determine the direction of transcription.

During their characterisation of the *chiA* gene Jones *et al.* (1986) recloned the DNA insert of pCHIT1251, a pUC8 derivative, into pUC9 so that the fragment became reversed in orientation relative to the *lacZ* promoter contained on the vector. The resulting plasmid when transformed into JM83 caused the bacteria to produce a zone of clearing on chitin plates which was larger than that produced by the same strain containing pCHIT1251. This result led them to suggest that in the pUC9 derivative the chitinase gene was oriented such that the *lacZ* promoter was initiating transcription in the same direction as the *chiA* promoter and thus increasing expression. A similar experiment performed with pCHIT310 failed to show any detectable difference in the rates of clearing caused by the two plasmids. However the plate clearing assay has been observed to be a rather insensitive test (Fuchs *et al.*, 1986; Horwitz *et al.*, 1984) with variable rates of clearing produced by bacteria containing identical plasmids. Therefore it was considered worthwhile to repeat the experiment using the more sensitive and quantitative *p-*

nitrophenyl-β-D-N,N'-diacetylchitobiose assay to test both pCHIT310 and its equivalent pUC19 construct and also pUC18/19 pairs of the chitinase-encoding subclones.

It had already been observed that pCH4, pCH6 and pCH7, which have the DNA insert in the opposite orientation with respect to the *lacZ* promoter compared to pCHIT310, expressed higher levels of chitinase activity. It was possible that this was due to additional transcription from the *lacZ* promoter, but since the DNA inserts in each case were not identical a more controlled study was necessary. Therefore the 3.28kb *Hin*dIII/*Eco*RI fragment from pCHIT310 was transferred to pUC19 and the inserts from pCH4, pCH6 and pCH7 to pUC18.

3.9.1 Construction of pCHIT319.

1μg of pCHIT310 was digested with *Hin*dIII and *Eco*RI for 2 hours at 37°C. The digested DNA was electrophoresed on a 0.7% agarose gel, the 3.28kb band was excised and the DNA fragment was isolated using the freeze-squeeze method (section 2.7.8). The fragment was then ligated to pUC19 which had been digested with *Hin*dIII and *Eco*RI. Half the ligation mix was used to transform DH5α, with transformants being selected on plates containing X-gal and ampicillin. Resulting white colonies were chosen for analysis. Miniprep DNA from these transformants was digested with *Hin*dIII and *Eco*RI and the products electrophoresed on an agarose gel to check for the presence of the 3.28kb fragment. One clone which contained the correct insert was designated pCHIT319 and, together with pCHIT310, was digested with *Dra*I, an enzyme which cuts the 3.28kb insert once and also has sites in pUC19 outside the multiple cloning region. The products of these digestions were examined on an agarose gel to ensure that the correct vector was present in each case, since there was a possibility that some vector DNA had been included in the initial fragment isolation.

3.9.2 Construction of pCH48, pCH58, pCH68 and pCH78.

Each of the three subclones which specified the production of chitinase ie. pCH4, pCH6 and pCH7 was digested with *Hin*dIII and *Eco*RI which would excise the inserts intact. The insert DNA fragments were isolated from an agarose gel as above. These fragments were ligated to pUC18 and DH5α transformants were again selected on plates containing X-gal and ampicillin.

The insert of pCH5 was also excised as a *Hin*dIII-*Eco*RI fragment and recloned into pUC18 in the same way. In pCH5 the insert was in the same orientation with respect to the *lacZ* promoter as the other subclones. If the small *Eco*RI-*Eco*RV fragment, whose deletion in pCH5

Figure 3.6 Restriction digests of pUC18/19 subclone pairs.

The constructed plasmids pCH48, pCH58, pCH68 and pCH78 were digested with *Hin*dIII and *Dra*I to check for the presence of the correct vector as described in the text.

The digestion products were run on a 0.7% agarose gel and the result is presented here. Each plasmid was digested with *Hin*dIII and *Dra*I.

Lane 1 λ PstI
Lane 2 pUC19
Lane 3 pCH3
Lane 4 pCH48 (transformant 1)
Lane 5 pCH48 (transformant 2)
Lane 6 pCH48 (transformant 3)
Lane 7 pCH5
Lane 8 pCH58
Lane 9 pCH6
Lane 10 pCH68 (transformant 1)
Lane 11 Incorrect plasmid
Lane 12 pCH7
Lane 13 pCH78
Lane 14 λ PstI

The sizes of the λ *Pst*I markers, in kb, beginning with the top band are:-Band 1 - 14.05; Band 2 - 11.41; Band 3 - 5.08; Band 4 - 4.75; Band 5 - 4.51; Band 6 - 2.84; Band 7 - 2.56/2.45/2.44; Band 8 - 2.14; Band 9 - 1.97; Band 10 - 1.7.


14
1

was obviously responsible for the chitinase-minus phenotype, contained the vital promoter region of the *chiB* gene then it may be postulated that in pUC18, the *lacZ* promoter could substitute for the endogenous *chiB* promoter. If pCH5 still contained the complete coding region of the *chiB* gene, then recloning the *Eco*RV-*Eco*RV fragment in pUC18 could lead to expression of chitinase, if only at a low level.

Recloning of the inserts from pCH4, pCH5, pCH6 and pCH7 into pUC18 gave rise to plasmids which were designated pCH48, pCH58, pCH68 and pCH78 respectively. In all cases, the presence of the correct vector was checked by digesting the plasmids with *Hin*dIII and *Dra*I and analysing the products on an agarose gel. This is shown in Figure 3.6.

3.9.3 Comparison of chitinase production by pUC18/19 subclone pairs.

pCHIT310 and pCHIT319, together with the pairs of subclones described in section 3.9.2 were all transformed into DH5α and 100µl of a fresh overnight culture of each was used to inoculate 5ml of L-broth + ampicillin. The cultures were incubated with shaking at 37°C for approximately 16 hours. After this time the OD_{550} of each culture was measured and a 1ml aliquot was removed from each culture to an Eppendorf tube and centrifuged for 2 minutes to pellet the cells. The supernatants were removed to fresh tubes and stored on ice whilst the cell pellets were resuspended in 0.5ml of extraction buffer (section 3.8) and the cells frozen and thawed twice as described previously (section 3.8).

The resulting cell extracts were assayed for chitinase activity (section 2.10). The chitinase assay was carried out for 30 minutes using 50µl of extract. The results are presented in Figure 3.7. Chitinase assays were also carried out on the culture supernatants using 100µl of supernatant as before with an incubation time of 6 hours (Figure 3.7).

The results showed no consistent pattern of expression related to the direction of transcription from the *lacZ* promoter. The cell extracts for pCHIT319, pCH4 and pCH6 all displayed higher levels of chitinase than did the extracts of cells containing the corresponding pUC18 clones. However, the cell extract from DH5α(pCH78) exhibited greater chitinase activity than that from DH5α(pCH7). The supernatant assays followed a similar trend except that the supernatant from DH5α(pCHIT319) contained less chitinase than the supernatant from DH5α(pCHIT310). The low level of chitinase present in the supernatant of DH5α(pCH6) was most likely to be due to the fact that the bacteria used did not have the unusual "white" appearance and consequently released less enzyme. Neither pCH5 nor pCH58 caused significant chitinase production indicating that part of the coding region of the gene must have been deleted.

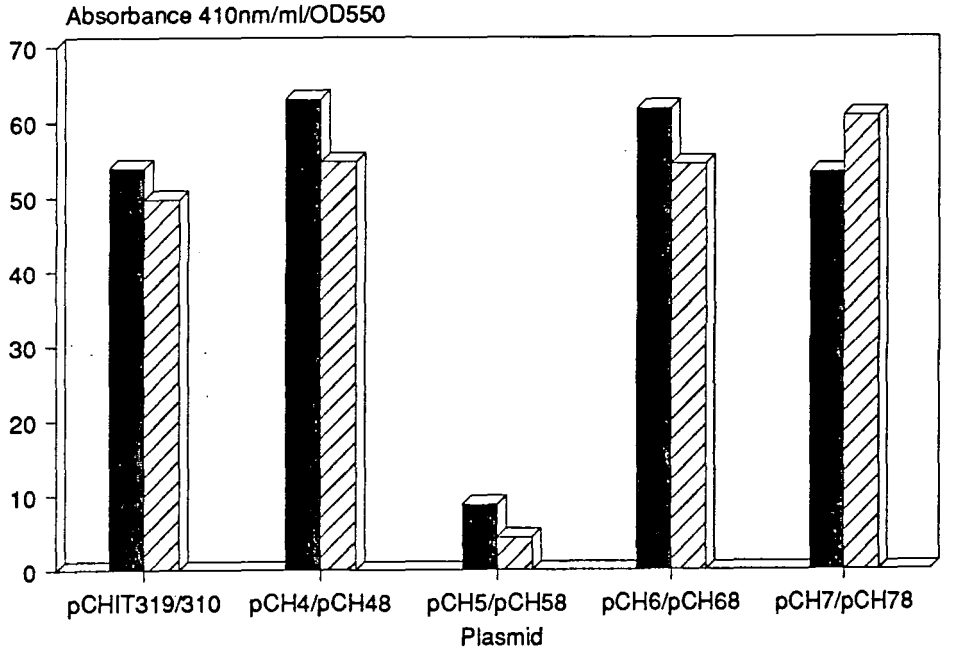
Figure 3.7 Chitinase production by pUC18/pUC19 subclones.

The chitinase activity present in both the cell extracts and culture supernatants was measured for DH5α containing each of the plasmids indicated. The results were expressed as absorbance at 410nm per ml of culture per OD_{550} and the values for pUC18/19 plasmid pairs were compared.

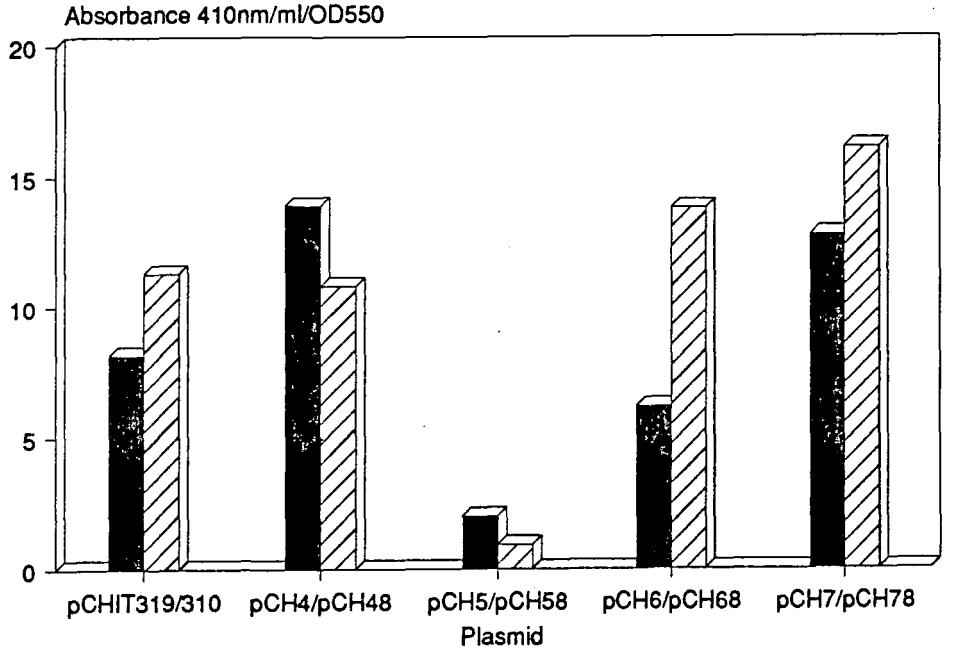
The solid bars represent plasmids constructed in pUC19 and the striped bars those plasmids constructed in pUC18. The experiment was carried out three times and the results presented here are for one representative experiment. No consistent difference was observed in the chitinase production of strains harbouring pUC18 or pUC19 derived plasmids. The low activity present in the supernatant of the culture containing pCH6 was again related to the unusual appearance of the colonies.

Chitinase production by pUC18/19 clones.

Assay of cell extract



Assay of culture supernatant



3.9.4 Induction of the *lacZ* promoter in a *lacI*^{q} strain.

At the same time, each of the plasmids used in the above experiment was transformed into *E. coli* TG2, a strain with the *lacI*^{q} mutation causing increased production of the lac repressor. In this bacterium transcription only occurs from the *lacZ* promoter on the pUC vector when the inducer IPTG is added to the system.

Each plasmid in TG2 was inoculated into 10ml of L-broth from a fresh overnight and the cultures were incubated with shaking at 37°C until an OD_{550} of 0.3 had been reached. At this point the cultures were divided into two and IPTG was added to half of each original culture to give a final concentration of 1mM. Incubation was continued for a further 12 hours then the OD_{550} of each culture was measured again and 1ml aliquots were transferred to Eppendorf tubes. Cell extracts were obtained as described previously (section 3.8) and 50µl aliquots were assayed for chitinase activity (section 2.10). The results are shown in Figure 3.8.

In each chitinase producing culture except one, the culture supplemented with IPTG produced slightly more chitinase. The highest increase caused by the addition of IPTG was approximately 12% for pCHIT310. However, if the *lacZ* promoter was enhancing transcription of the chitinase gene then the IPTG effect would not have been seen with every construct. As it was, these results gave no indication of the direction of transcription of the *chiB* gene.

3.10 Sequencing of the *chiB* gene.

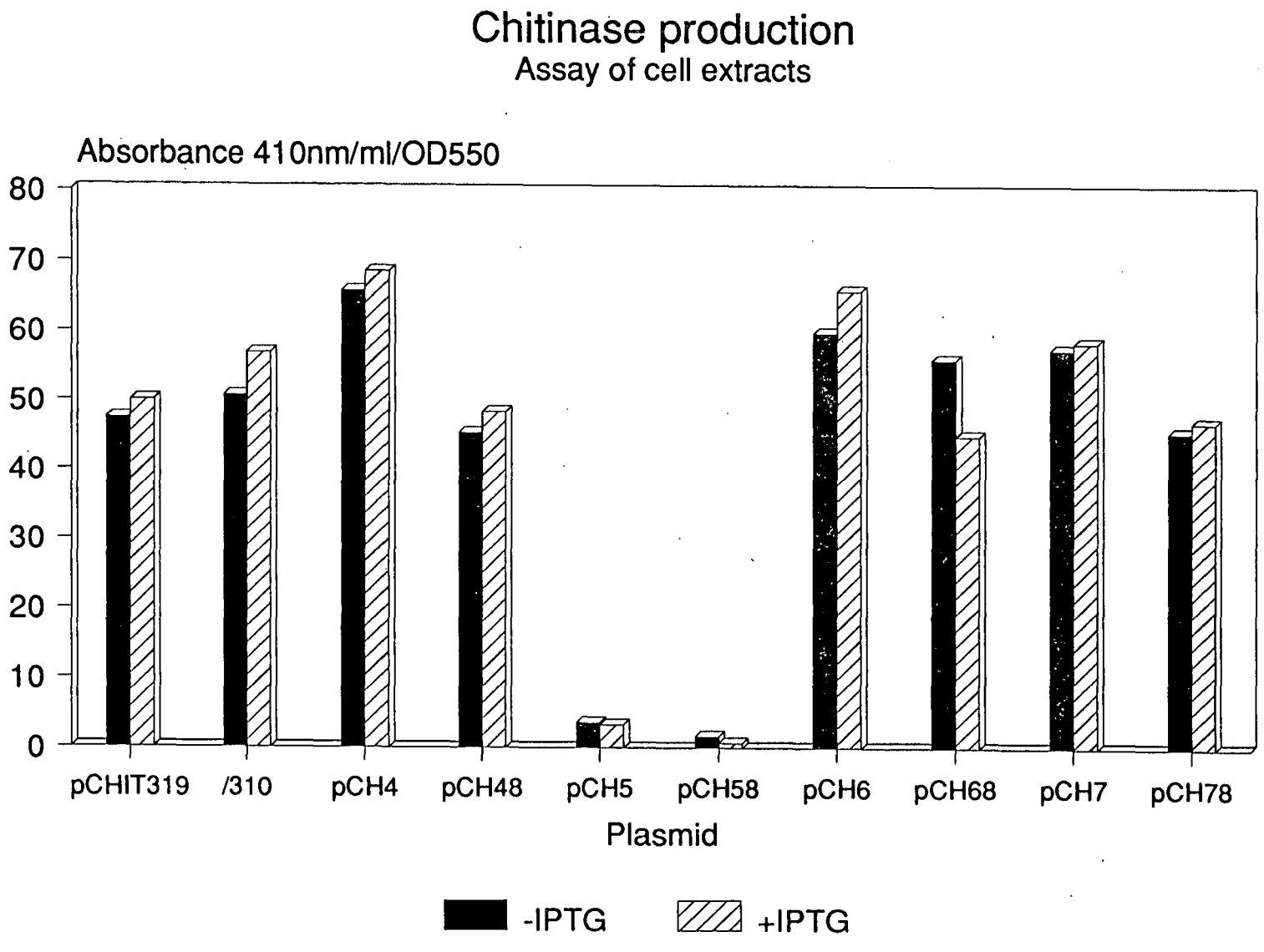
Neither of the experiments described in sections 3.9.3 and 3.9.4 successfully demonstrated the direction of transcription of the *chiB* gene. Therefore the sequencing of the pCHIT310 insert was carried out without this knowledge in the expectation that the position of the gene could be determined from the DNA sequence.

In order to sequence the entire *chiB* gene, numerous smaller fragments of the 3.28kb insert of pCHIT310 were subcloned into the M13 vectors mp18 and mp19. As far as possible, subclones were constructed with restriction fragments obtained using information from the restriction map of pCHIT310 (Figure 3.1).

In order to sequence from the *Nco*I site in each direction, existing M13mp18 subclones covering this region were taken and digested with both *Nco*I and *Hin*dIII which cut only in the M13 polylinker region between the insert DNA and the site of hybridisation of the sequencing primer. The digested DNA was electrophoresed on a 0.7% agarose gel and the large fragment which contained the vector DNA and part of the original insert was isolated. The purified DNA

Figure 3.8 Attempt to induce *chiB* expression using IPTG.

The histogram presented here shows the results of chitinase assays carried out on the cell extracts of *E. coli* cultures containing pUC18/pUC19 pairs of subclones from pCHIT310. Each culture was divided into two and one half was induced with IPTG. The values obtained for induced and non-induced cultures are compared.


Chitinase production
Assay of cell extracts
Absorbance 410nm/ml/OD550
80
70
60
50
40
30
20
10
0
pCHIT319
/310
pCH4
pCH48
pCH5
pCH58
pCH6
pCH68
pCH7
pCH78
Plasmid
-IPTG
+IPTG

fragment was then blunt-ended by filling in the 3' recessed ends as described in section 2.7.9. The blunt-ended DNA fragment consisting of vector and a reduced insert was religated and the ligation mix transformed into TG2. All transformants obviously produced clear plaques so a number were selected for restriction analysis. Miniprep DNA was used to identify clones which had lost the *Nco*I-*Hin*dIII fragment.

A number of clones for sequencing were obtained from a *Taq*I digest of pCHIT310. Previous work had shown the 3.28kb insert of pCHIT310 to contain at least 5 *Taq*I sites. Approximately 2μg of pCHIT310 was digested with *Taq*I in low REB at 65°C for 2 hours. Following phenol/chloroform extraction and ethanol precipitation, the entire digested DNA was ligated to M13mp18 cut with *Acc*I. Recombinant phage were analysed by restriction digests and those which contained a pUC8 vector fragment were discarded. Other recombinants were subjected to the orientation test (section 2.8.3) to identify pairs of clones with the same insert in opposite orientations and these were then sequenced.

All sequencing was carried out using the dideoxynucleotide chain termination method of Sanger *et al.* (1977) and the necessary procedures are described in section 2.8. The sequencing strategy is shown in Figure 3.9. In order to sequence one stretch of the gene (indicated in Figure 3.9) it was necessary to synthesise a 17-base oligonucleotide for use as a sequencing primer. The DNA sequence to which the primer was designed to hybridise had been obtained in a previous sequencing experiment.

Towards the end of the sequencing period a number of M13 subclones were resequenced using an Applied Biosystems automated DNA sequencer to obtain longer stretches of readable sequence. Both DNA strands were sequenced over the region containing the *chiB* gene. As the sequence data was being analysed, a report was published by Harpster and Dunsmuir (1989) presenting the sequence of the *chiB* gene of *S. marcescens* QMB1466. The published sequence demonstrated that the gene is transcribed away from the *Eco*RI site of pCHIT310 and matched the DNA sequence already obtained. This sequence, extending from the *Eco*RI site of pCHIT310 to the downstream region of the *chiB* gene is presented in Figure 3.11. In addition, the transcription start site had been determined and consensus promoter sequences and a 3' transcription termination region had been identified. Planned experiments to map the transcription start site using primer extension were now unnecessary due to the publication of this information. The transcription start site and regulatory sequences indicated in Figure 3.10 are those reported by Harpster and Dunsmuir (1989).

Figure 3.9 Sequencing strategy of the pCHIT310 insert.

This figure shows the sequencing strategy for the sequencing of the *chiB* gene. The region marked * was sequenced using an artificial oligonucleotide as a primer.

P = *Pst*I; B = *Bam*HI; Sa = *Sal*I; Nr = *Nru*I; Nc = *Nco*I; RV = *Eco*RV; RI = *Eco*RI.

pCHIT310

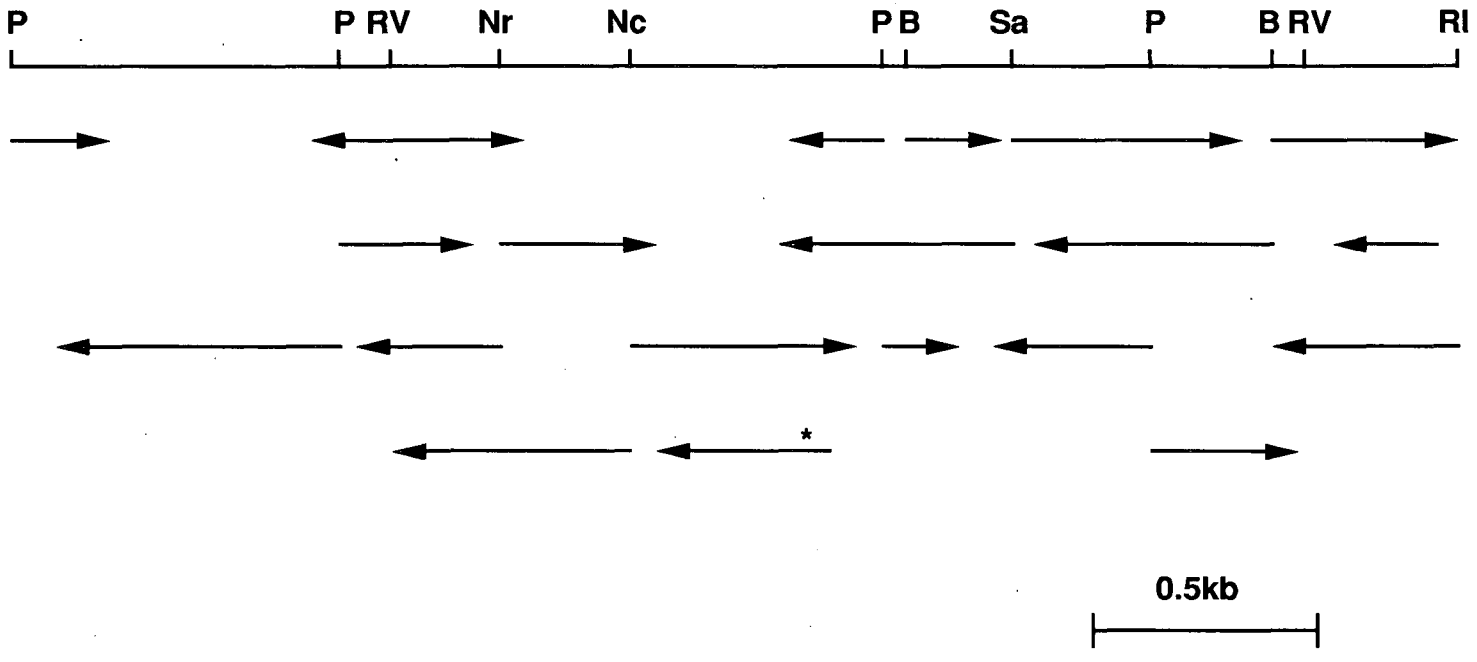


Figure 3.10 The sequence of the *S. marcescens chiB* gene.

The sequence presented here is the determined sequence. The published sequence of Harpster & Dunsmuir (1989) is identical to the sequence obtained.

The underlined bases in the upstream region of the gene are hexameric consensus sequences characteristic of bacterial promoters.

The underlined bases in the 3' flanking region form a near perfect inverse-repeat sequence which is characteristic of *rho*-dependent transcription termination.

The Shine-Dalgarno sequence is enclosed by a boxed area and the transcription start site is indicated by ★.

The encoded polypeptide is 55.5kDa and is thought to be processed at the carboxyl terminus of the alanine indicated (●) to yield a 51-52kDa mature protein (Harpster & Dunsmuir, 1989).

ATTCATTCACGCTGAACGTGGCACAACGCATAAAACGCCAATGCATGCGGCAGTTAATA

AAAATTCATTCTTATGGTGATTTATTTCGACTTTTATTCTCGAGGAAAATAAACATTAA

TGGCGACGGGGAATATTCCCCCATTGAAAAACATCCACTCTGGAGAAATGCCATG TCC
Met ser

ACA CGC AAA GCC GTT ATT GGG TAT TAT TTT ATT CCG ACC AAC CAA
thr arg lys ala val ile gly tyr tyr phe ile pro thr ile asn

ATC AAT AAT TAC ACC GAG ACG GAT ACG TCT GTC GTG CCG TTC CCG
asn gln asn tyr thr glu thr asp thr ser val val pro phe pro

GTT TCC AAC ATC ACG CCG GCC AAA GCC AAA CAG CTG ACG CAC ATT
val ser asn ile thr pro ala lys ala lys gln leu thr his ile

AAC TTC TCG TTC CTG GAT ATC AAC AGC AAC CTG GAA TGC GCC TGG
asn phe ser phe leu asp ile asn ser asn leu glu cys ala trp

GAT CCG GCC ACC AAC GAC GCC AAG GCG CGC GAT GTG GTC AAC CGT
asp pro ala thr asn asp ala lys ala arg asp val val asn arg

TTA ACC GCG CTC AAA GCG CAC AAC CCC AGC CTG CGC ATC ATG TTC
leu thr ala leu lys ala his asn pro ser leu arg ile met phe

TCC ATC GGC GGC TGG TAC TAC TCC AAC GAT CTG GGC GTG TCG CAC
ser ile gly gly trp tyr tyr ser asn asp leu gly val ser his

GCC AAC TAC GTC AAC GCG GTG AAA ACC CCG GCG GCG CGC ACC AAG
ala asn tyr val asn ala val lys thr pro ala ala arg thr lys

TTC GCC CAA TCC TGC GTG CGC ATC ATG AAG GAT TAC GGC TTC GAC
phe ala gln ser cys val arg ile met lys asp tyr gly phe asp

GGC GTG GAC ATC GAC TGG GAG TAT CCG CAG GCG GCG GAA GTG GAC
gly val asp ile asp trp glu tyr pro gln ala ala glu val asp

GGT TTC ATC GCC GCG CTG CAG GAG ATC CGC ACC TTG CTG AAC CAG
gly phe ile ala ala leu gln glu ile arg thr leu leu asn gln

CAA ACC ATC GCG GAC GGC CGC CAG GCG TTG CCG TAT CAG TTG ACC
gln thr ile ala asp gly arg gln ala leu pro tyr gln leu thr

ATC GCC GGC GCC GGC GGC GCC TTC TTC CTG TCG CGC TAT TAC AGC
ile ala gly ala gly gly ala phe phe leu ser arg tyr tyr ser

AAG CTG GCG CAA ATC GTC GCG CCA CTC GAT TAC ATC AAC CTG ATG
lys leu ala gln ile val ala pro leu asp tyr ile asn leu met

ACC TAC GAT CTG GCC GGC CCC TGG GAG AAG ATC ACC AAC CAC CAG
thr tyr asp leu ala gly pro trp glu lys ile thr asn his gln

GCG GCG CTG TTC GGC GAC GCG GCC GGG CCG ACC TTC TAC AAC GCA
ala ala leu phe gly asp ala ala gly pro thr phe tyr asn ala

CTG CGC GAA GCC AAT CTG GGC TGG AGC TGG GAA GAG CTG ACC CGC
leu arg glu ala asn leu gly trp ser trp glu glu leu thr arg

```
GCC TTC CCC AGC CCG TTC AGC CTG ACG GTC GAC GCC GCC GTG CAG
ala phe pro ser pro phe ser leu thr val asp ala ala val gln

CAG CAC CTG ATG ATG GAA GGC GTG CCG AGC GCC AAA ATC GTC ATG
gln his leu met met glu gly val pro ser ala lys ile val met

GGC GTG CCC TTC TAC GGC CGC GCC TTC AAG GGC GTC AGC GGC GGC
gly val pro phe tyr gly arg ala phe lys gly val ser gly gly

AAC GGC GGC CAG TAC AGC AGC CAC AGC ACG CCG GGC GAA GAT CCG
asn gly gly gln tyr ser ser his ser thr pro gly glu asp pro

TAT CCG AAC GCC GAT TAC TGG CTG GTG GGC TGC GAC GAG TGC GTG
tyr pro asn ala asp tyr trp leu val gly cys asp glu cys val

CGC GAC AAG GAT CCG CGC ATC GCC TCC TAT CGC CAG CTG GAG CAG
arg asp lys asp pro arg ile ala ser tyr arg gln leu glu gln

ATG CTG CAG GGC AAC TAC GGC TAT CAG CGG TTG TGG AAC GAT AAG
met leu gln gly asn tyr gly tyr gln arg leu trp asn asp lys

ACC AAA ACC CCG TAT CTG TAT CAT GCG CAG AAC GGG CTG TTT GTC
tre lys thr pro tyr leu tyr his ala gln asn gly leu phe val

ACC TAT GAC GAT GCC GAG AGC TTC AAA TAC AAA GCG AAG TAC ATC
thr tyr asp asp ala glu ser phe lys tyr lys ala lys tyr ile

AAG CAG CAG CAG CTG GGC GGC GTA ATG TTC TGG CAT TTG GGG CAA
lys gln gln gln leu gly gly val met phe trp his leu gly gln

GAC AAC CGC AAC GGC GAT CTG CTG GCC GCG CTG GAT CGC TAT TTC
asp asn arg asn gly asp leu leu ala ala leu asp arg tyr phe

AAC GCC GCA GAC TAC GAC GAC AGC CAG CTG GAT ATG GGG ACC GGC
asn ala ala asp tyr asp asp ser gln leu asp met gly thr gly

CTG CGA TAT ACC GGC GTC GGC CCC GGC AAC CTG CCG ATC ATG ACC
leu arg tyr thr gly val gly pro gly asn leu pro ile met thr

GCG CCG GCC TAT GTG CCG GGC ACC ACT TAC GCC CAG GGC GCG CTG
ala pro ala tyr val pro gly thr thr tyr ala gln gly ala leu

GTG TCC TAC CAA GGC TAC GTC TGG CAG ACC AAG TGG GGT TAT ATC
val ser tyr gln gly tyr val trp gln thr lys trp gly tyr ile

ACC TCG GCG CCC GGC TCA GAC AGC GCC TGG CTG AAG GTG GGC CGC
thr ser ala pro gly ser asp ser ala trp leu lys val gly arg

CTG GCG TAA GCCGTAAAAAAACCCCGTAGCCGAATGCTGCGGGGTTTTCATTGAGT
leu ala OCH

TAACCGTTTGATTTTCGCGTCCCTTCGTCTCTATT
```

3.11 DISCUSSION.

The *chiB* gene of *S. marcescens* was chosen for study so that it could then be utilised in a *vir* promoter-pesticide construct according to the scheme of Ashby *et al.* (1986). Chitinase was selected as the active molecule for this system for a number of reasons:- its antifungal activity is well documented, it is a single-gene product for which a number of genes have been cloned and there are straightforward assays available to evaluate chitinase production. Most published work has concentrated on either the complete spectrum of chitinase activity exhibited by *S. marcescens* or the *chiA* gene and its 58kDa protein. No investigations had been made into the *chiB* gene and its product following the initial cloning of the gene by Jones *et al.* (1986).

3.11.1 The effectiveness of the *chiB* enzyme as a biocontrol product.

Accordingly, there was no direct evidence for any antifungal or insecticidal activity associated with the *chiB* enzyme. Indirect evidence had come from studies on *chiA* in which a *chiA* mutant of *S. marcescens* retained a suppressive effect towards *Fusarium oxysporum* (Jones *et al.*, 1986). In order to assess the toxicity of the *chiB* enzyme towards *Diabrotica*, bacterial strains expressing the *chiB* gene were given to ICI Jealotts Hill. The results obtained in tests carried out there indicated that the *chiB* enzyme was actually more effective against this organism than the *chiA* enzyme. Earlier results, at the start of this project, appeared to be even more promising, with ChiB causing a 70% mortality rate amongst the *Diabrotica* larvae. It was on the basis of these results that the work on *chiB* was undertaken and although later experiments were never able to reproduce such high levels of mortality, their results were encouraging enough for the work to continue.

3.11.2 Mode of action of the *S. marcescens* chitinases.

Some reports have stated that the *S. marcescens* enzymes are endochitinases (Monreal & Reese, 1969; Jones *et al.*, 1986) whereas others have declared them to be exochitinases (Roberts & Cabib, 1982; Roberts & Selitrennikoff, 1988). Much of this confusion probably arises from the definition of the two terms. Monreal and Reese (1969) showed that the main product of chitin digestion by *S. marcescens* chitinase is the dimer, diacetylchitobiose. This activity they describe as involving "a random endoglycanase". Nevertheless, they reported that the trimer and tetramer were infrequently observed as reaction products. Convincing evidence comes from Roberts and Cabib (1982) who showed purified *S. marcescens* chitinase to degrade chitin by an exohydrolytic mechanism - removing diacetylchitobiose units from the non-reducing end

of the chitin chain in a stepwise fashion. This mode of action was confirmed by Roberts and Selitrennikoff (1988) using the chromogenic substrate described in section 3.6.

In order to clarify the situation concerning chitinase modes of action Robbins *et al.* (1988) proposed that exochitinase activity be defined as processive action starting at the non-reducing ends of chitin chains with release of diacetylchitobiose units and endochitinase activity be defined as random cleavage at internal points in chitin chains. According to this classification, both the ChiA and ChiB enzymes of *S. marcescens* are exochitinases.

One anomaly which still remains is the observation of Roberts and Selitrennikoff (1988) that the ChiA enzyme and bacterial chitinases in general do not possess antifungal activity. Plant chitinases on the other hand were found to inhibit fungal growth. This activity was correlated with the mode of action of the chitinases and the authors suggested that the bacterial enzymes, being exochitinases, are restricted to locating non-reducing termini of chitin chains as substrates, which may be difficult in intact fungal cell walls. However, the number of cited instances in which the *S. marcescens* chitinases have inhibited the growth of fungal pathogens provide sufficient evidence that they could be used successfully in a biological control system (Jones *et al.*, 1986; Ordentlich *et al.*, 1988; Shapira *et al.*, 1989).

The ChiA and ChiB enzymes of *S. marcescens* have no homology to each other and so it is possible that in a particular circumstance, one chitinase may be more effective than the other, hence the greater toxic effect of ChiB towards *Diabrotica.* Alternatively, a combination of the two enzymes acting together may prove to be better for suppressing fungal or insect growth.

3.11.3 Expression and secretion of *chiB* in *E. coli.*

The *chiB* gene could be expressed in *E. coli* to produce an active enzyme. Nevertheless, inconsistencies were observed in the secretion of the chitinase. The chitinases of *S. marcescens* are secreted enzymes (Monreal & Reese, 1969) and can be isolated from the culture supernatant. Apart from chitinases, *S. marcescens* is capable of secreting a number of other proteins including proteases, a nuclease and a lipase (Hines *et al.*, 1989).

3.11.3.1 Protein secretion by Gram-negative bacteria.

Gram-negative bacteria as a group are not generally renowned for their ability to secrete proteins, since the outer membrane provides an effective barrier, and secretion into the medium is restricted to a small number of proteins. This is in contrast to the greater number of proteins

exported to the cell membranes and periplasm. For instance, *E. coli* does not have an endogenous system for secreting proteins into the medium (Pugsley, 1989) and the few proteins which are secreted, eg. α-haemolysin, require the presence of specific gene products which are responsible for their release (Wagner *et al.*, 1983). Those proteins secreted to the cell envelope are mostly transported by the general export pathway which is composed of certain cytoplasmic and inner membrane proteins acting together to transfer periplasmic and outer membrane proteins across the inner membrane. All such exported proteins possess an N-terminal signal peptide of 12 or more hydrophobic or neutral residues which directs the protein to the inner membrane and facilitates translocation across it. The signal peptide is then removed by cleavage with a signal peptidase and remains embedded in the inner membrane (Pugsley *et al.*, 1990).

Some Gram-negative bacteria, including *S. marcescens*, selectively and efficiently secrete extracellular proteins such as hydrolases and toxins beyond the outer membrane. Over 30 genes for secreted proteins have now been cloned and characterised and sequence data has revealed that most of the proteins are synthesised as precursors with signal peptides. These display similarity to those signal sequences found in precursors of periplasmic or outer membrane proteins (Pugsley *et al.*, 1990). Thus it may be surmised that such proteins are secreted in a two-step pathway; initial transport to the periplasm is achieved via the the general export pathway for translocation through the inner membrane and a periplasmic intermediate is then secreted across the outer membrane with the aid of a specific secretion factor.

In all Gram-negative secretory systems characterised to date, a component has been found in the outer membrane which acts as a secretion factor. The extracellular proteins may be divided into three categories according to the nature of this component. Some proteins, for example the serine protease of *S. marcescens* (Miyazaki *et al.*, 1989; Yanagida *et al.*, 1986), and their specific secretion factors are synthesised as a single, large, precursor polypeptide. Following secretion through the inner cytoplasmic membrane the polypeptide inserts into the outer membrane with the N-terminal catalytic domain initially exposed on the cell surface and later released by proteolytic action. It is the C-terminal domain which acts as the secretion factor, facilitating translocation of the N-terminal domain through the outer membrane and remaining embedded within it.

In contrast, the α-haemolysin of *S. marcescens* requires a separate outer membrane protein in order to be secreted extracellularly. This membrane protein is encoded by the first gene in a two-gene operon - the haemolysin structural gene being the second of the two. Thus the membrane protein gene, which is coordinately regulated by iron, encodes a specfic secretion factor which may also be involved in activation of the haemolysin (Schiebel *et al.*, 1990)..

When bacteria have been screened for mutants which have lost the ability to secrete one or more proteins, a repeated observation is that different extracellular proteins are affected to different extents. It is possible that extracellular release of some proteins requires a number of secretion factors and although several proteins may share components of a common translocation system, other components are specific for one protein or group of proteins (Pugsley *et al.*, 1990). Certainly the production and secretion of the major metalloprotease of *S. marcescens* is a more complex process than that of the other *S. marcescens* extracellular proteins characterised so far (Hines *et al.*, 1988; Nakahama *et al.*, 1986).

3.11.3.2 Expression of foreign extracellular proteins in *E. coli.*

Many genes coding for secreted proteins from various Gram-negative bacteria have been expressed in *E. coli* and, with only a few exceptions, the proteins are localised in the periplasm rather than secreted into the medium. The signal peptides of these foreign protein precursors are obviously recognised by components of the general export pathway of *E. coli* and thus the proteins are transported across the inner membrane and into the periplasm. Once there however, they are unable to be secreted because *E. coli* lacks the secretion factors required for their translocation across the outer membrane. (Pugsley *et al.*, 1990).

Although there are several reports of proteins that are secreted extracellularly by both the natural producing bacterium and by *E. coli* expressing the cloned gene, the majority of these cases could be explained by one of two scenarios. Either there is spontaneous partial lysis of the cells leading to non-selective release of proteins, or the presence on the cloned DNA fragment of a second gene coding for a protein required for extracellular release.

Those proteins which are genuinely secreted in *E. coli* include the *S. marcescens* serine protease which contains its own secretion factor in the C-terminal portion of the precursor polypeptide (Yanagida *et al.*, 1986) and a *S. marcescens* nuclease which can be isolated from the growth medium (Ball *et al.*, 1987). The mechanism of its secretion is unknown, but non-selective release has been discounted since periplasmic and cytoplasmic enzymes remain localised and the cloned DNA fragment is not large enough to encompass a second gene.

Not all *S. marcescens* extracellular enzymes are secreted by *E. coli* however. The metalloprotease when expressed in *E. coli* is located in the cells and cannot be detected in the culture medium (Nakahama *et al.*, 1986). The chitobiase gene has been cloned and expressed in *E. coli* but rather than being secreted into the medium it accumulates in the periplasm (Kless *et al.*, 1989).

3.11.3.3 Is ChiB secreted by *E. coli*?

The fact that *E. coli* expressing the *chiB* gene is capable of producing cleared halos on chitin plates has been taken as evidence that the chitinase is secreted by *E. coli* (Ashby, 1988; Jones *et al.*, 1986). This is not necessarily the case. The plate clearing assay runs for a minimum of five to seven days and no clearing of the chitin is detectable around the *E. coli* clones until this time. This amount of extracellular activity could easily be accounted for by partial lysis of stationary cells and the subsequent non-specific release of cellular proteins. Likewise, the chitinase activity present in culture supernatants of stationary phase cultures could be derived from the same source.

It was noted that the amounts of extracellular chitinase produced by *E. coli* expressing the *chiB* gene were not consistent and in order to try to circumvent this problem, total cellular extracts were assayed for chitinase activity. This would include any active enzyme present in either the cytoplasm or the periplasm. It can be seen from the results that the combination of these two cell fractions contained a considerably greater level of chitinase activity than an equivalent sample of culture supernatant. At this stage it is not known for certain whether the greatest concentration of ChiB is to be found in the cytoplasm or the periplasm. Based on the behaviour of many other foreign proteins in *E. coli*, including the chitobiase of *S. marcescens*, it may be predicted that the ChiB protein accumulates in the periplasm. The nucleotide sequence of the *chiB* gene has shown the precursor polypeptide to possess a signal peptide at its N-terminal end and so this should direct the enzyme to the periplasmic space of *E. coli*, utilising the general export pathway.

Preliminary results of chitinase assays performed on the extracellular, periplasmic and cytoplasmic fractions of *E. coli* cells expressing the *chiB* gene indicate that the majority of the enzyme is indeed located in the periplasmic space, although chitinase activity is detected in the culture supernatant. In order to confirm and extend these observations it would be necessary to repeat the experiments and include assays for a typical periplasmic enzyme such as alkaline phosphatase or penicillinase and a cytoplasmic enzyme, eg. β-galactosidase. Such assays would demonstrate whether lysis of the cells was occurring, by measuring the amounts of known cytoplasmic and periplasmic proteins released into the growth medium. Assaying samples from cultures of different cell densities would indicate whether release of the chitinase was correlated to the growth stage of the cells.

If there was found to be no general release of cell proteins then the chitinase present in the culture medium may indeed be specifically secreted and the greater concentration of the enzyme in the periplasm could be due to saturation of the outer membrane transport system by the

artificially high levels of the cloned gene product. Such a situation has been proposed for when the *S. marcescens* nuclease gene is expressed in *E. coli* (Ball *et al.*, 1987).

On the other hand, if periplasmic and cytoplasmic enzymes were present in the culture supernatant, this would suggest that the ChiB protein alone does not possess a function allowing it to be secreted across the outer membrane, and thus release into the growth medium is due to spontaneous cell lysis. This would seem to be more likely, considering the fate of the *S. marcescens* chitobiase in *E. coli*. The gene encoding chitobiase, an enzyme involved in the final stage of chitin degradation, has been cloned and expressed in *E. coli* (Kless *et al.*, 1989). This enzyme was found to accumulate in the periplasmic space, where 90% of the enzyme activity was located, and was not actively secreted. However, an interesting point to note was that in stationary phase cultures of *E. coli* containing the chitobiase gene, 90% of the chitobiase activity and 60% of the β-galactosidase activity was found in the in the culture supernatant. This contrasted with only 1% of the β-galactosidase present in the growth medium of a stationary culture of cells containing merely the vector without the chitobiase gene. The result suggests that expression of the chitobiase gene leads to cell fragility and spontaneous lysis, causing release of the enzyme from the cells.

It seems feasible that the various extracellular enzymes of *S. marcescens* involved in chitin degradation may all utilise the same export system and thus no single protein has the ability to cross the outer membrane but requires another secretory factor (or factors) to be present. There may be a system comparable to that of the haemolysin gene where the structural gene and the gene encoding the outer membrane protein required for extracellular transport are linked in the same operon and are both induced by iron (Schiebel *et al.*, 1990). *S. marcescens* contains at least three structural genes involved in the degradation of chitin and these are induced by the presence of the substrate. Although the genes do not appear to form an operon, it is possible that they are clustered on the chromosome and the same regulatory system governs them all. A gene or genes coding for outer membrane transport proteins may be regulated in the same manner.

3.11.3.4 The effect of *chiB* expression on *E. coli*.

It was observed that the appearance of *E. coli* strains expressing the *chiB* gene was correlated to the amount of extracellular chitinase. When the bacteria possessed the unusual "white" appearance, higher levels of chitinase activity were found in the culture medium and such colonies gave rise to greater areas of clearing on chitin plates. The fact that the higher levels of extracellular chitinase were not accompanied by equally greater levels of cellular chitinase implies that these clones were not merely expressing the *chiB* gene more efficiently.

As with the cloned chitobiase gene of *S. marcescens*, the expression of *chiB* in *E. coli* may lead to cell fragility, with the altered appearance being a symptom of this. Obviously there would be more chitinase present in the growth medium of more fragile cells. Experiments should be performed to assay a number of marker enzymes in the various cell fractions to determine whether colony appearance is linked to cell fragility. Nevertheless, the question would still remain as to why this difference was not consistent.

3.11.4 Regulation of enzymes involved in chitin degradation.

In *S. marcescens*, all the enzymes involved in chitin degradation, ie. two or more chitinases and a chitobiase, are induced by chitin and only very low levels of the enzymes are present in the absence of the substrate (Monreal & Reese, 1969). The actual inducing molecules are likely to be soluble oligomers derived from chitin. It has been shown that the production of ChiB from a cloned DNA fragment in *E. coli* is constitutive and does not require the presence of chitin. A similar situation has been demonstrated for the chitobiase gene (Kless *et al.*, 1989). This suggests that regulation of the genes involves a repressor protein which is inactivated in the presence of inducing molecules. The gene encoding the repressor must not be present on the cloned DNA fragment.

Potential regulatory genes have been cloned from the related bacterium *Serratia liquefaciens* in addition to two chitinase genes and a chitobiase gene (Joshi *et al.*, 1988). When a region of DNA adjacent to one of the chitinase genes is deleted, or contains Tn5 insertions, there is a resulting increase in production of chitinase and chitobiase. The authors propose that this region encompasses a gene coding for a repressor protein. A second adjacent region may encode a gene product whose function is to catalyse a step in the synthesis of the active inducer. When Tn5 insertions are present in this region, no chitinase or chitobiase expression is observed.

The experiment described in section 3.7 demonstrated that the DNA fragment present in pCHIT310 did not contain any genes responsible for the regulation of enzyme production by chitin. High levels of chitinase activity were produced by *E. coli* cultures when grown in either the presence or absence of chitin. The observation that the chitin lacking culture produced more chitinase than the chitin supplemented culture could have been due to growth inhibition caused by the addition of chitin. It was not possible to take measurements of the culture density due to the particulate nature of the chitin in suspension. The lower levels of chitinase produced by *S. marcescens* were probably due to the fact that at the incubation temperature of 28°C, the *S. marcescens* culture was slower growing than the *E. coli* culture. Stringent regulation of chitinase production by *S. marcescens* was not observed, with chitinase detectable in the culture

supernatant when chitin was not present. However, twice as much chitinase activity was present in the induced culture than in the non-induced culture after 12 hours with a greater difference after only 3 hours.

CHAPTER 4

MONITORING *VIR*-INDUCTION IN *AGROBACTERIUM TUMEFACIENS*.

4.1 Introduction.

Phenolic compounds present in plant wound exudates play a dual role in the initial stages of *Agrobacterium* infection. At low concentrations they elicit an extremely sensitive chemotactic response from *Agrobacterium* harbouring a Ti-plasmid (Ashby *et al.*, 1987; Ashby *et al.*, 1988) and at higher concentrations they effect *vir*-induction (Stachel *et al.*, 1985b; Stachel *et al.*, 1986). Thus the initial interaction between the plant and the bacterium, involving signal recognition, is a vital first step in the infection process. Knowledge of the conditions which trigger chemotaxis and *vir*-induction in *A. tumefaciens* has led to the following scheme of events being proposed for the early stages of *Agrobacterium* infection (Shaw *et al.*, 1988b).

A chromosomally encoded recognition and chemotaxis system allows *Agrobacterium* to respond to sugars and amino acids in plant exudates (Loake *et al.*, 1988). The chemotactic response, particularly towards sucrose, the major translocated plant sugar, is strong and extremely sensitive, with a threshold at 10^{-7}M. This response is 1000-fold more sensitive than the equivalent response in *E. coli* (Shaw *et al.*, 1988b). It is known that bacteria are able to move great distances in the soil (Bashan & Levanony, 1987) and this capability, combined with such a strong chemotactic response ensures a prevalence of the bacterium in the rhizosphere. A further level of attraction then occurs for virulent *A. tumefaciens* cells harbouring a Ti-plasmid. Chemotaxis towards phenolic compounds released by wounded plant cells guides the bacteria specifically towards the site of wounding. This attraction probably only occurs on a micrometre scale and requires the presence of *virA* and *virG* (Shaw *et al.*, 1988a). At the wound site the concentrations of phenolic compounds reach the threshold level required for *vir*-induction.

The implication in this scheme is that *vir*-induction occurs specifically at wound sites on plants although, to our knowledge, this has not actually been demonstrated. However, it is the basic tenet upon which the concept of the *Agrobacterium* biocontrol system described in section 1.13 was established. Therefore it was of the utmost importance to attempt to identify the sites around a plant root where *vir*-induction really takes place. If it could be demonstrated that the *vir*-genes of *Agrobacterium* cells are specifically induced at wound sites, then this would show that the biocontrol system was likely to function as intended.

4.1.1 Reporter genes.

In order to ascertain the sites of *vir*-induction it was necessary to have an indicator which would show that induction was occurring. This could be achieved by linking one of the inducible *vir*-promoters to a reporter gene. Reporter genes express products which can be simply assayed, either by a colour reaction or by some other means. They can thus demonstrate the regulation of expression of a gene whose natural protein product has no known or easily assayable function, or of a gene that does not encode a protein (eg. t-RNA, r-RNA genes). Most reporter gene systems in current use involve genes that encode an enzymatically active protein.

A reporter gene commonly used in bacteria is the *E. coli lacZ* gene which encodes the enzyme β-galactosidase. This enzyme can be readily assayed by using either the artificial substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Miller, 1972) which liberates yellow nitrophenol, or X-gal, which develops a blue colour when subjected to the action of β-galactosidase. For *in situ* detection of enzyme activity, X-gal is the preferred indicator substrate. Another reporter gene, which tends to be used predominantly in transgenic plants, is that encoding β-glucuronidase, usually known as GUS. β-glucuronidase is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides. Many spectrophotometric, fluorometric and histochemical substrates are commercially available for this enzyme (Jefferson, 1987).

A reporter gene system which has proved useful in studying bacterial - plant interactions is the *lux* gene set derived from either *Vibrio harveyii* (Legocki *et al.*, 1986; Boivin *et al.*, 1988) or *Vibrio fischeri* (Shaw & Kado, 1986). The two sets of genes are similar, but the currently available *V. harveyii* clones suffer a disadvantage in that they do not contain functional fatty acid reductase genes. This means that exogenous aldehyde must be added as a substrate. In contrast, five genes, constituting a promoterless *lux* operon, have been cloned from *V. fischeri* and this *lux* cassette contains all the genes necessary for production of aldehyde by the fatty acid reductase pathway (Engebrecht *et al.*, 1985). Shaw & Kado (1986) have used the *V. fischeri lux* cassette in a number of different bacteria, causing them to produce detectable light.

4.2 Use of *lacZ* as a reporter gene for *vir*-induction.

LacZ was an obvious choice as the first reporter gene to be employed in an attempt to demonstrate, *in situ*, the sites of *vir*-induction. The plasmid pSM30 contains the *lacZ* gene under the control of a *virB* promoter (Stachel *et al.*, 1985a) and this plasmid was readily available. Additionally, detection of β-galactosidase required no specialised equipment and the indicator substrate, X-gal, was relatively cheap and routinely used in the laboratory.

As a preliminary experiment, *Agrobacterium* strain A348 harbouring both pTi6 and pSM30 was streaked onto induction medium agar (section 2.4) containing either 100μM acetosyringone, or an equivalent amount of the acetosyringone solvent, 70% methanol. The agar also contained 200μl of 2% X-gal in DMF per 100ml. Following 36 hours incubation at 28°C the bacteria streaked on the plate containing acetosyringone were mid - deep blue, whereas those bacteria growing on the plate lacking acetosyringone showed some very faint blue coloration which was only just detectable. After a further 24 hours incubation the colour of the bacteria on the plate lacking acetosyringone had become slightly more intense but was still only pale blue. Obviously there was a low basal level of *lacZ* expression in the non-induced state and the β-galactosidase produced was sufficient to cleave the substrate during extended incubation. This implied that any experiments to detect *vir*-induction in planta should not be allowed to run for prolonged periods of time.

4.2.1 Devising a method for *in situ* detection of *vir*-induction.

All the following experiments were conducted using mung beans - the seeds germinated quickly and produced seedlings that were easy to handle and manipulate. Initial experiments were carried out in petri dishes. Induction medium containing 200μl of 2% X-gal per 100ml was solidified with 0.5% bacto-agar and used to fill petri dishes. The surface of each agar plate was spread with a lawn of *Agrobacterium* A348 containing pSM30. This was achieved by spreading 100μl of a fresh log. phase culture on each plate. Mung bean seedlings for the experiment were obtained as follows. Mung bean seeds were surface sterilised by immersion in 5% "Chloros" (sodium hypochlorite) for 2 minutes followed by three rinses in sterile distilled water. Using an ethanol-sterilised spatula each seed was pressed into 0.5% water agar in a petri dish. The seeds were then germinated at 25°C in a plant growth cabinet for 48 hours. 1cm root tip segments were excised from the seedlings and placed onto the surface of the agar sown with bacteria. The plates were incubated overnight at 25°C. Additionally, whole seedlings were taken and an excision made in the root, 1cm from the tip, with a scalpel blade. The seedlings were placed on the surface of the agar and the root pressed lightly into it. These were also incubated overnight at 25°C.

Upon examination of the plates it was seen that a lawn of *Agrobacterium* had grown and this had a pale blue colouration. Where the excised root tips were placed on the agar, a slightly deeper blue colour was observed around both the cut end of the root and at the root tip. The experiment involving the injured seedlings was not successful due to further growth of the seedling which had lifted the injured parts of the roots away from the surface of the agar. After a further 24 hours incubation the plates containing the root tips were reexamined. The blue

colour of the bacterial lawn had intensified and the root tips, whilst not causing significantly increased colour, had begun to turn brown and were obviously no longer fresh enough for the experiment to produce meaningful results.

The experiment was repeated a number of times with various minor modifications. Different dilutions of plating bacteria were used to create the lawn and the concentration of the agar was lowered so that the seedlings could be inserted into it more easily. A further modification was to include the bacteria in the semi-solid agar, rather than to spread them over the surface. However, none of these approaches were satisfactory, due mainly to the basic design of the experiments which used petri dishes. These were not ideal for positioning of whole seedlings. The excised root segments, although they appeared to indicate release of *vir*-inducing compounds from both the cut surface and the root tip, were poor substitutes for complete seedlings. The results obtained would need to be verified using an improved system which could accommodate growing seedlings.

Nevertheless, these trial attempts had demonstrated a number of important points which would prove useful in setting up further experiments, namely the most satisfactory concentration of agar to use and the need to ensure that the bacteria were actually in contact with the plant root within the agar.

4.2.2 Demonstration of *vir*-induction at sites on a root.

It was decided to repeat the experiments using sterile, clear plastic 10ml tubes in place of the petri dishes. 5ml of 0.2% water agar containing X-gal at the same concentration as before (section 4.2.1) was put into each tube. Initially it was attempted to actually germinate the mung bean seeds in the tubes, with the sterilised seeds pressed lightly into the surface of the agar. Prior to germination the seeds had been soaked for 10 minutes in an overnight culture of A348(pSM30) which had been centrifuged and resuspended in an equal volume of sterile water. The tubes were kept in a growth cabinet at 25°C for 48 hours and the germination was monitored over this period.

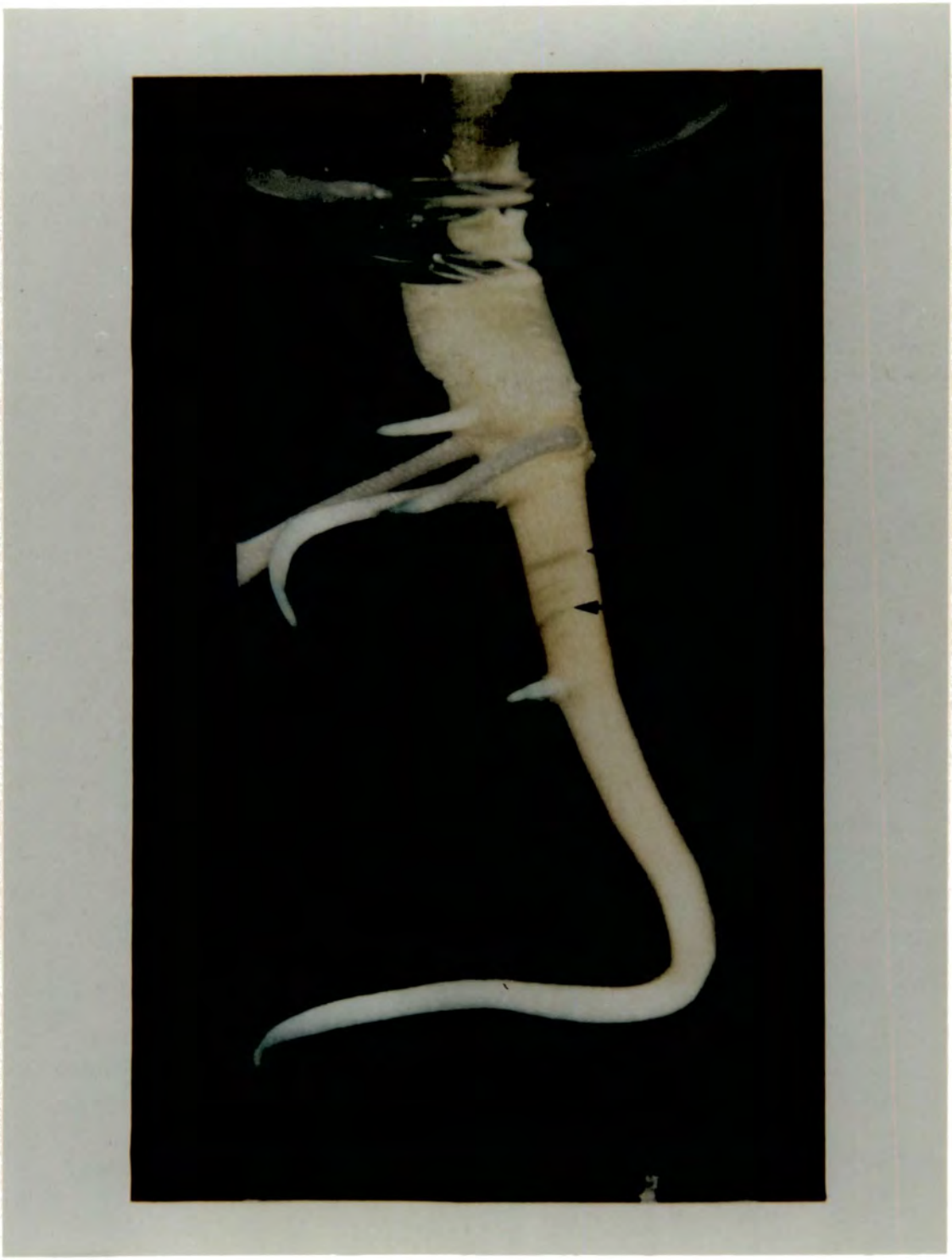
As germination occurred the seed coat was shed and this appeared to be a good source of *vir*-inducing compounds since a halo of bacteria and blue colouration surrounded it. Dark blue patches were also present on the seed coat itself. As the root tip emerged a narrow blue ring was evident around it, approximately 2mm behind the root tip. This observation was consistently made when seeds were germinated in this manner. Growth of the seedlings seemed to be inhibited by the presence of large numbers of bacteria surrounding the germinating seed and the roots did not grow longer than 1 - 1.5cm. Due to the excessive blue colour around the

Figure 4.1 The use of *lacZ* to show *vir*-induction at sites on a root.

These photographs show seedlings inoculated with A348(pSM30) and grown in agar containing X-gal. Injuries were made on the roots with a scalpel. Blue colour developed at the root tips, at the site of injury ⟶ and at a naturally occurring lesion on the root surface ►

Figure 4.2 A control experiment indicating endogenous β-galactosidase activity in the plant roots.

An injured seedling was grown in X-gal containing agar but had not been previously inoculated with the *Agrobacterium* culture. Blue colour developed around the root tips but was not evident at the site of wounding indicated by ⟵ .



seed coat which may have masked any sites of *vir*-induction on the root and the poor growth, this method was not continued.

Instead, sterilised seeds were germinated for 2 days in petri dishes of 0.5% water agar prior to immersion of the roots in a suspension of A348(pSM30). The seedlings were removed from the bacterial suspension after 5 minutes and carefully inserted into sterile plastic tubes containing 5ml of 0.2% water agar + X-gal. The tubes were incubated at 25°C in a plant growth cabinet overnight. Additional experiments were set up using either seedlings whose roots had been artificially injured with a scalpel blade before soaking in the bacterial suspension, or similarly injured but otherwise untreated seedlings.

Representative results of these experiments are shown in Figures 4.1 and 4.2. Those experiments which used wounded seedlings inoculated with *Agrobacterium* exhibited areas of blue colour following overnight incubation. The darkest blue areas were situated at the root tip and along the lines of the lesions caused by the scalpel blade. Intense blue colour had also developed at sites on the root surface where natural lesions had occurred as result of growth. Small, localised patches of lighter blue were additionally present on some areas of the root surface. Uninjured seedlings inoculated with the bacteria showed the same pattern of colouration, with the root tip having the most intense blue colouring and natural lesions also showing areas of dark blue. When no bacteria were present there was still some blue colouration but mainly at the root tip and not around the wound site. The root tips were a paler blue than when bacteria were present.

These results appeared to demonstrate that *vir*-induction, leading to expression of the *lacZ* gene, occurred chiefly at sites of natural or artificial wounding on the root surface and also at the root tip. However, the blue colour observed in the absence of bacteria suggested that a β-galactosidase occurring naturally in the plant was being released from the root tip zone and to a lesser extent from other areas of the root surface. Despite the success of these experiments, in which a system had finally been devised to monitor *vir*-induction *in situ*, improvements could be made. The main problems encountered when using the *virB-lacZ* fusion were the presence of an endogenous β-galactosidase in the seedlings and the basal level of *lacZ* expression from pSM30 when no inducing compounds were present. The wounded areas of the root would provide a more plentiful supply of nutrients for the growth of bacteria in addition to a suitable physical niche for colonisation. Thus more concentrated pockets of bacterial growth at these sites may have caused sufficient constitutive production of β-galactosidase to effect a colour change irrespective of whether *vir*-induction was taking place. Nevertheless it is unlikely, judging from the results of the experiment described in section 4.2, that constitutive expression could be responsible for the intensity of colour observed at the wound site after only an overnight incubation.

4.3 The *lux* system of *Vibrio fischeri.*

Seven genes constituting the *lux* operon were originally cloned from *Vibrio fischeri.* Two of these were found to be regulatory genes and not required for light production (Engebrecht & Silverman, 1984). These genes have been deleted and the remaining five genes form a promoterless *lux* cassette (Engebrecht *et al.*, 1985) which has been further modified for use in a number of plasmid constructs (Shaw *et al.*, 1987). *LuxA* and *luxB* encode the α and β subunits of the enzyme luciferase. This catalyses the oxidation of a reduced flavin mononucleotide and a long chain aldehyde, producing oxidised flavin, the corresponding long chain fatty acid and light. The other three genes of the cassette, *luxC*, *luxD* and *luxE* all provide functions necessary for the recycling of the aldehyde substrate (Engebrecht & Silverman, 1984).

The promoterless *lux* cassette has been cloned into the broad-host-range vector pUCD4, resulting in the recombinant plasmid pUCD607. In this construct the *lux* genes are constitutively expressed from the promoter of the tetracycline resistance gene (Shaw & Kado, 1986). The *lux* genes are also contained on the plasmid pUCD615 (Figure 4.3). This is a promoter probe vector which has a multiple cloning site at the 5' end of the *lux* cassette. When a fragment of DNA with promoter activity is inserted here in the correct orientation, transcription of the *lux* genes is initiated and light is generated. pUCD615 has two origins of replication; the broad-host-range *ori* of pSa allows replication in *A. tumefaciens* and many other Gram-negative bacteria, whilst the *ori* of pBR322 gives rise to a high copy number in *E. coli.* Mobilisation of pUCD615 can be achieved due to the presence of a *bom* site, therefore it can be used to study promoter activities in a variety of bacterial species (Rogowsky *et al.*, 1987).

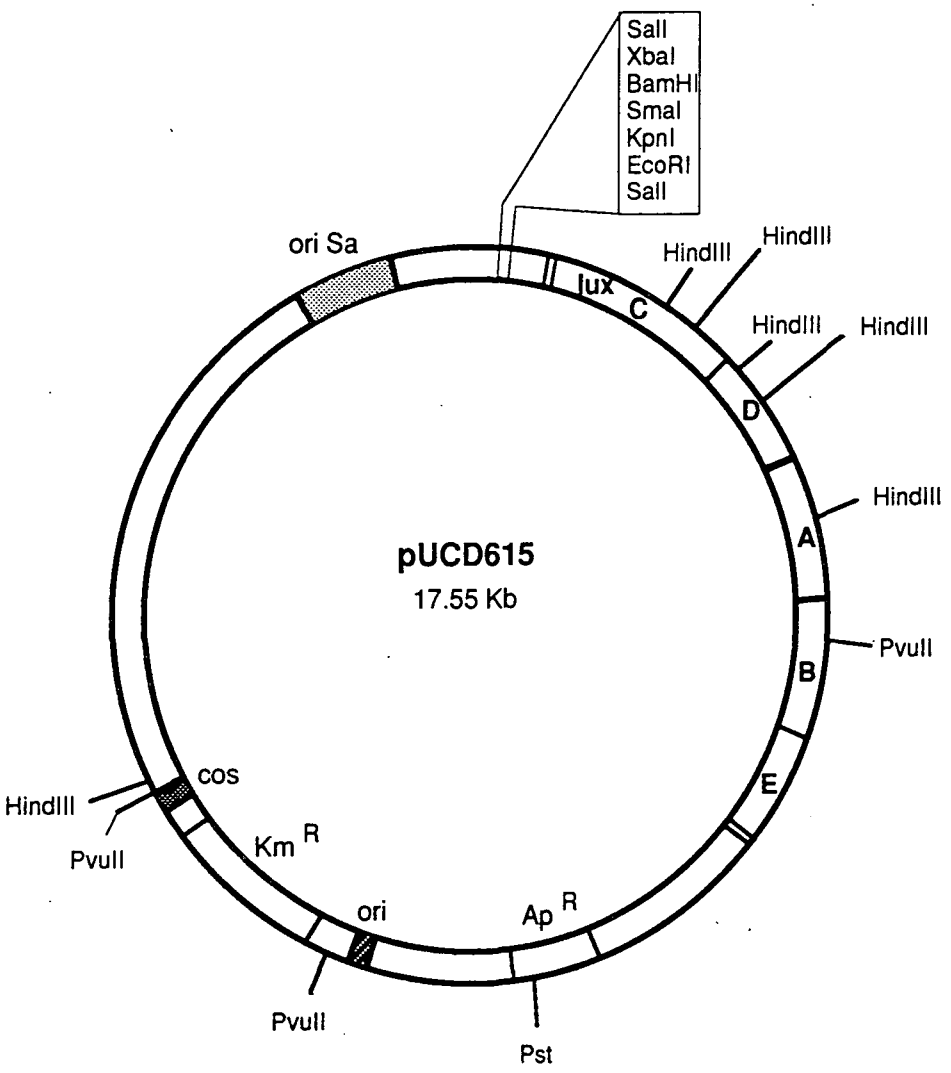
The *lux* bioluminescence genes had the potential to be a good reporter system for use in monitoring *vir*-induction *in situ.* Their use would overcome one of the problems encountered with *lacZ* since there would be no endogenous light production by the plant roots. Additionally it was hoped that bioluminescence would be a more sensitive reporter, able to be detected at low levels and maybe even in the soil rather than in an artificial situation. It was envisaged that *Agrobacterium* harbouring pUCD607 could be used to determine the total colonisation of the root by the bacteria. pUCD615 containing a cloned *vir*-promoter would provide a reporter system for *vir*-induction.

4.3.1 Bringing the *lux* operon under control of a *virB* promoter.

The promoter chosen for use was the *virB* promoter that was present in pSM30 (section 4.2). It had been removed from this plasmid, manipulated to delete any superfluous DNA sequence and then recloned in front of the *chiA* gene of *S. marcescens* to form pDUB2512

Figure 4.3 A diagram of the plasmid pUCD615.

This plasmid contains a promoterless *lux* operon preceded by a multiple cloning site.


SalI
XbaI
BamHI
SmaI
KpnI
EcoRI
SalI
ori Sa
lux C
HindIII
HindIII
HindIII
HindIII
D
HindIII
A
PvuII
B
E
pUCD615
17.55 Kb
cos
HindIII
PvuII
Km R
ori
Ap R
PvuII
Pst

(Ashby, 1988). In order to clone the *virB* promoter fragment into pUCD615 in the correct orientation it was first necessary to alter the restriction site at the 5' end of the fragment ie. the *Hin*dIII site (Figure 4.4).

The 0.5kb *virB* promoter fragment was excised from pDUB2512 with *Hin*dIII and *Bam*HI. Following isolation from an agarose gel (section 2.7.8) the promoter fragment was ligated to pUC1318 which had been digested with *Hin*dIII. pUC1318 is one of a pair of vectors which are primarily used as an alternative to linkers in order to add new restriction sites to the ends of DNA fragments (Kay & McPherson, 1987). It has a central *Hin*dIII site flanked by pairs of restriction sites in a cloning region which allows insertional activation of the *lacZ* gene. For recombinant plasmids to arise two promoter fragments would have to be inserted together, back to back, with their *Bam*HI sites linked together (Figure 4.4). The ligation mix was used to transform DH5α and transformants were selected on X-gal/amp plates. White colonies were screened by restriction analysis of miniprep DNA. The DNA samples were digested with both *Hin*dIII and *Hin*dIII/*Bam*HI and a clone which contained two copies of the *virB* promoter fragment was selected. pUC1318 contains two *Xba*I sites flanking the *Hin*dIII site. Thus by digesting the *virB* containing clone with both *Bam*HI and *Xba*I, the two *virB* promoter fragments could be excised individually (Figure 4.4). The *Bam*HI/*Xba*I fragments were isolated from an agarose gel and ligated to pUCD615 previously digested with *Bam*HI/*Xba*I and phosphatase treated. DH5α transformants were selected on amp/kan plates but there was no selection possible for recombinants. Twenty four transformants were chosen for restriction analysis of their miniprep DNA. DNA was digested with *Xba*I and *Bam*HI and one recombinant plasmid was identified which contained the *virB* promoter fragment. This was designated pVL13.

4.3.2 Transfer of pVL13 and pUCD607 to *Agrobacterium.*

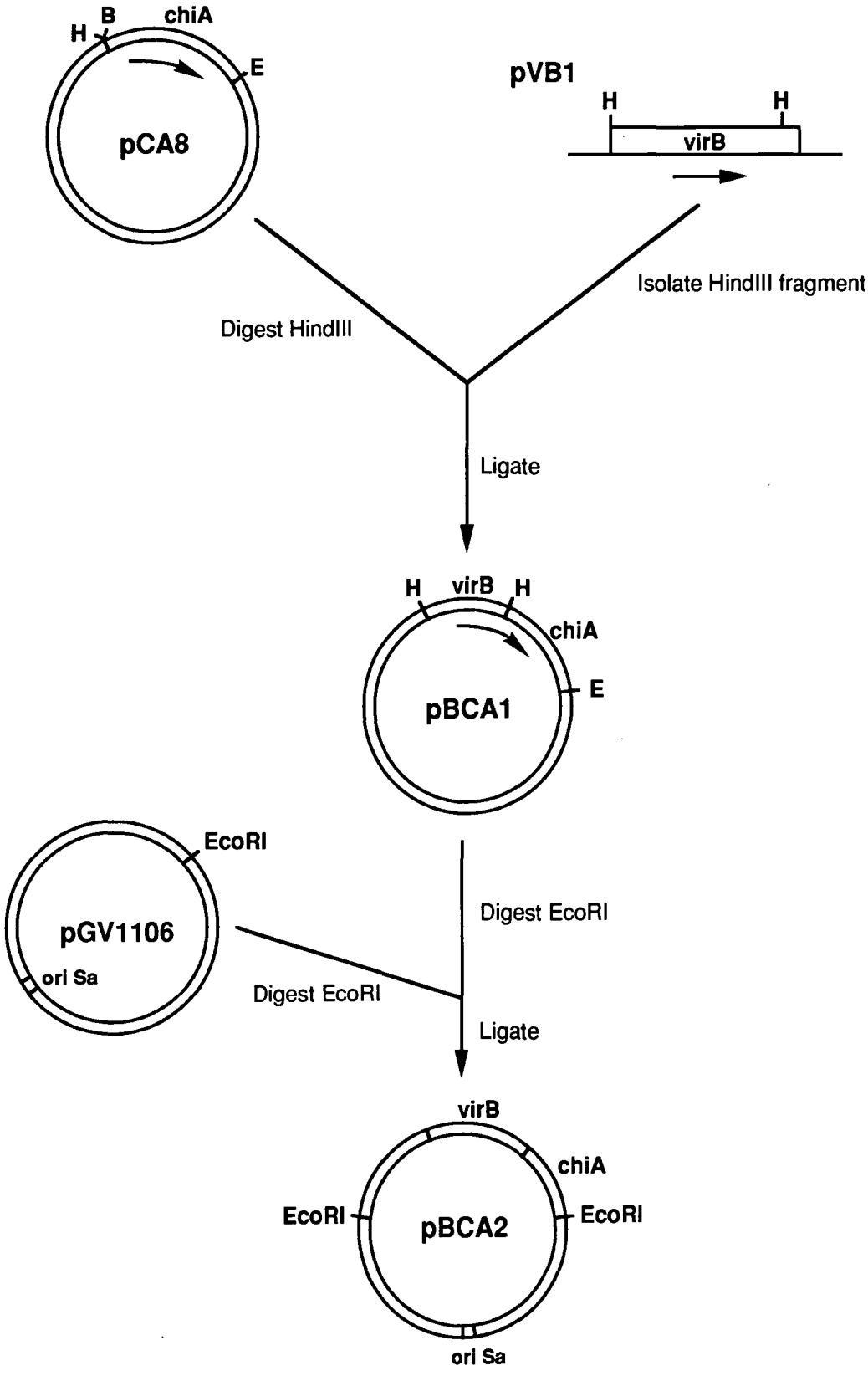
pVL13 was introduced into *Agrobacterium* C58C^1(pVK257) by means of a triparental mating as described in section 2.17. The donor strain was DH5α(pVL13), the helper strain was HB101(pRK2013) and the recipient strain was C58C^1(pVK257). The cosmid pVK257, containing *virA,B,G* and *C* had previously been introduced into C58C^1 by the same method. This was required to provide the necessary VirA and VirG functions of signal recognition and transcriptional activation since C58C^1 was cured of its Ti-plasmid. Transconjugants were selected on L-RifKmCb plates, with carbenicillin substituting for ampicillin as the selection involved *Agrobacterium.* pUCD607 was also introduced into C58C^1 in the same manner.

It had already been ascertained that when pUCD607 was present in *E. coli* HB101 or JM83, the light produced by individual colonies grown at 37°C and then removed to room

Figure 4.4 A schematic diagram showing the construction of pVL13.

This figure shows how the *virB* promoter fragment from pDUB2512 was manipulated for use in the *virB-lux* construct, pVL13.

Key:- H = *Hin*dIII; B = *Bam*HI; E = *Eco*RI; Sc = *Sac*I; Sm = *Sma*I; X = *Xba*I; Hc = *Hin*cII; P = *Pst*I; Sp = *Sph*I; K = *Kpn*I.



cointegrate plasmid

temperature for 5 hours could be visualised by observing the colonies in a dark room. The *lux* system is not active at temperatures above 33°C and lower temperatures give higher levels of light production (Shaw *et al.*, 1987). However, it had been reported that different species of bacteria display different levels of luminescence when harbouring the same *lux* plasmid, with enteric bacteria being the brightest (Shaw & Kado, 1986). Thus it was not known whether C58C^1 containing the *lux* constructs would produce sufficient light to be visible to the naked eye. C58C^1(pUCD607) was streaked thickly onto an L-agar plate containing kanamycin and carbenicillin and incubated for 36 hours at 25°C. No light was detectable when the plate was examined in a dark room. A more sensitive detection method was required. The agar plate of C58C^1(pUCD607) was taped to a piece of X-ray film (Fuji-RX) placed underneath the petri dish and this was wrapped first in foil and then in a black plastic bag. The film was developed after 6 hours kept at room temperature and darkened areas of the film corresponded to the streaks of bacterial growth.

Ten presumptive transconjugants expected to contain pVK257 and pVL13 were streaked in identical positions onto MinA plates (section 2.4) +/- 50μM acetosyringone. In all experiments involving acetosyringone, a 10^{-1}M stock solution in 70% methanol was used. The bacteria were grown at 25°C for 48 hours and the petri dishes were then exposed to X-ray film for 6 hours at room temperature. No darkening of the film was caused by the bacteria grown on the plate lacking acetosyringone. Seven of the ten colonies streaked onto the plate containing acetosyringone produced light which was detected by the X-ray film (Figure 4.5a) Thus in these cases the *virB-lux* construct was both functioning correctly and had been successfully introduced into *Agrobacterium*. The isolate which showed the greatest level of induction was selected for use in further experiments. When compared with C58C^1(pUCD607) streaked at an equal density, none of the *virB-lux* constructs produced such a high level of light.

4.4 The sensitivity of X-ray film for detecting bacterial bioluminescence.

It was important to try and determine what level of light could be detected by X-ray film. When using bioluminescence as an *in situ* indicator of *vir*-induction relatively low numbers of bacterial cells would be involved compared to the number of cells present during growth on an agar plate. Hence there would only be a low level of light production. It was a matter of concern that the level of light production in *Agrobacterium* may not be high enough to be used for *in situ* studies, even though the *lux* genes had previously been expressed in *Agrobacterium* and used with some success (Rogowsky *et al.*, 1987).

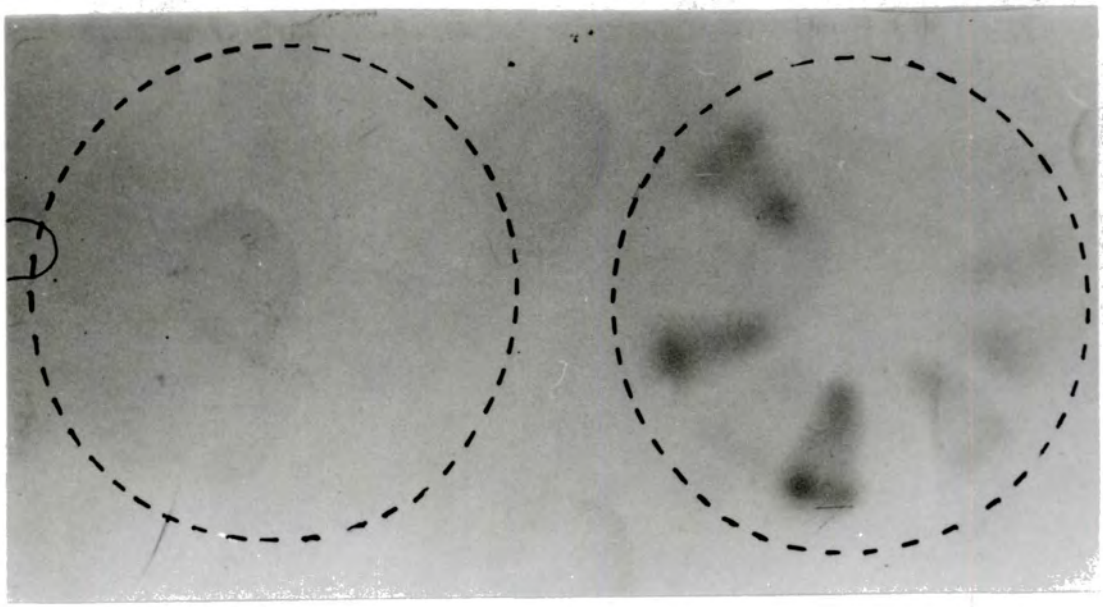
Dilutions of an overnight culture of C58C^1 containing pUCD607 were plated onto L-kan plates and incubated for 36 hours at 28°C. The dilution which gave well-spaced single colonies

Figure 4.5 Light production by *Agrobacterium* C58C1 harbouring *lux* plasmids.

a) Light production detected by X-ray film demonstrating which *Agrobacterium* transconjugants contained an acetosyringone-inducible *lux* plasmid. No light was detected from the bacteria when acetosyringone was not present in the agar.

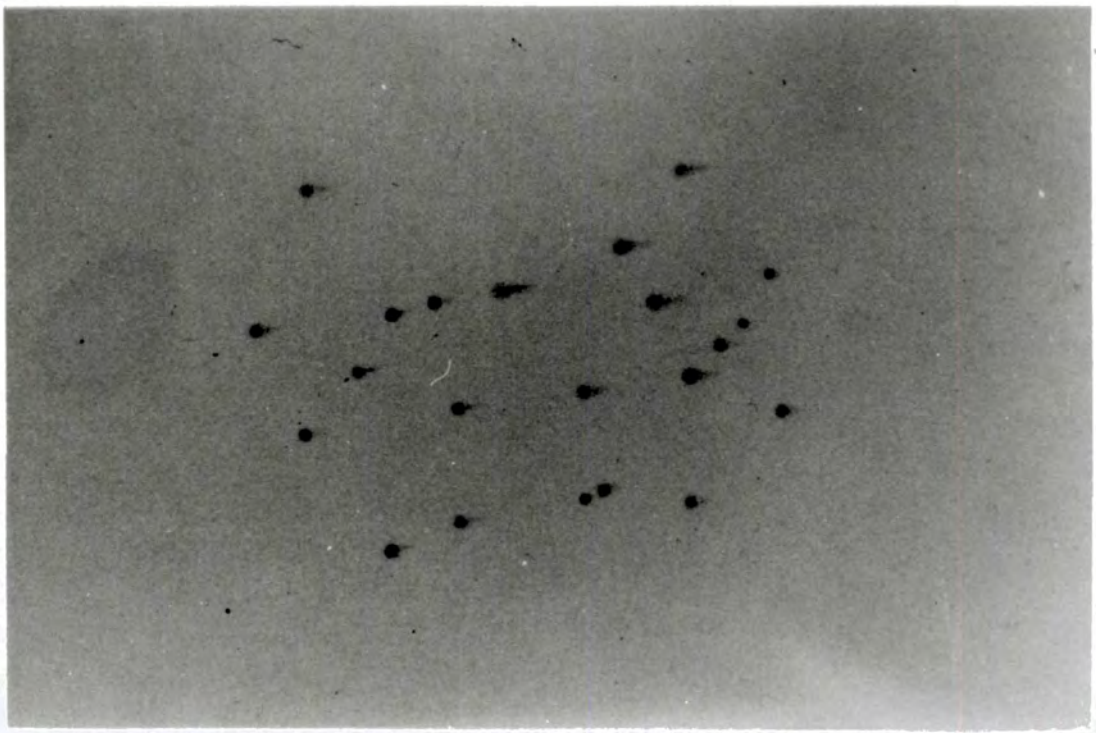
b) Light detected from individual colonies of *Agrobacterium* C58C[1] harbouring pUCD607. A "tail" can be seen extending from each colony due to lifting it from the plate on a nitrocellulose membrane.

A]



-AS +AS

B]



was chosen and the plating procedure was repeated with this dilution of bacteria. Incubation was this time carried out at 25°C. When the colonies were just visible (approximately 0.5mm diameter) the petri dish was exposed to X-ray film at room temperature for 6 hours. The film was then developed but light production could not be detected from any of the single colonies. The only darkening of the film occurred where three colonies were growing very close together, but the individual colonies could not be distinguished. It was likely that due to the thickness of the agar layer the light was being scattered so an alternative method was then employed.

Colonies (0.5mm diameter) were obtained as before then a nitrocellulose filter was placed on the surface of the agar and carefully removed. The filters with the replica colonies were immediately covered with cling film and then applied directly to X-ray film. This was sealed in a film cassette and left for 6 hours at room temperature. Upon developing the film, light could clearly be detected from each colony (Figure 4.5b). In order to obtain an estimate of the number of cells required to produce this amount of light, pieces of nitrocellulose carrying equal sized colony replicas had been cut out and vortexed thoroughly in 1ml of 10^{-2}M $MgSO_4$. Serial dilutions were made to 10^{-8} and duplicate 100µl aliquots were plated onto L-kan plates. The plates were incubated at 28°C for 36 hours and the numbers of colonies counted. A mean value was calculated of 4.87×10^5 bacteria present in each original colony. This was probably an overestimate of the number of cells producing the light detected since stationary cells emit very little light. Most of the luminescence would be coming from log. phase cells (Shaw *et al.*, 1987). The experiment still had not shown the minimum numbers of bacteria required to emit a detectable amount of light, but had given an idea of the sensitivity of the detection method. The experiments could have been repeated, attempting to monitor smaller and smaller colonies until light could no longer be detected. However, since there were no exact figures available for the numbers of Agrobacteria commonly colonising plant roots, then the results could not have been used to accurately predict whether bacteria could be detected in the rhizosphere. The only sure way of determining whether the *lux* system could be used to detect rhizosphere Agrobacteria was to actually try and use the system *in vivo*.

4.5 Measurement of bioluminescence using a scintillation counter.

The most sensitive method available for detecting bioluminescence is the use of a luminometer which incorporates a photomultiplier (Shaw *et al.*, 1987). This type of equipment was not available and so in order to obtain quantitative measurements of light production by the bacterial cultures, a programme was developed on a scintillation counter enabling single photons of light to be counted.

100µl of 36 hour cultures of C58C^1(pVK257) as a negative control, C58C^1(pUCD607) as a positive control and C58C^1(pVK257, pVL13) were each inoculated into 2 x 5ml of induction medium (section 2.4), containing either 5µl of 70% methanol or 5µl of 10^{-1}M acetosyringone. The cultures were incubated with shaking at 25°C for 24 hours. 100µl and 10µl samples were then removed and introduced into 15ml of fresh induction medium contained in glass scintillation vials. The contents of the vials were mixed gently by inversion and allowed to stand at room temperature for 5 minutes prior to measurement of light emission using the scintillation counter. A control blank of induction medium was also passed through the scintillation counter to monitor the background level of chemiluminescence.

Both the induced and non-induced cultures of the constitutive strain caused readings well above the background level of 10,000 counts per minute (cpm). A mean reading, calculated from 3 samples, of 108,624 cpm was achieved with 100µl of the C58C^1(pUCD607) culture lacking acetosyringone, with a similar reading obtained for the culture containing AS. 100µl of the C58C^1(pVL13) culture supplemented with AS gave a mean reading of 84,880 cpm. The equivalent non-induced culture gave a reading no higher than background. Serial dilutions were made of the two induced cultures and duplicate 100µl aliquots of each dilution were spread onto L-kan plates and incubated for 36 hours at 28°C. The number of colonies on each plate was counted and the number of colony forming units (cfus) present in each original culture was calculated.

The C58C^1(pUCD607) culture had contained 6.08 x 10^7 cfu in 100µl which gave rise to a photon count of 108,624 cpm. Therefore, the light production by this culture was 1.78 x 10^{-3} cpm per cell. The equivalent measurement for C58C^1(pVK257, pVL13) was 1.82 x 10^{-3} cpm/cell. This experiment was repeated twice and similar results were obtained each time. The level of light production from the *virB*:*lux* construct was disappointing and did not reach the levels reported by Rogowsky *et al.* (1987) although the expression of the *lux* genes was definitely inducible.

4.6 Comparison of light production by pVL13 and pUCD1187.

For *lux* to be used as an *in situ* reporter of *vir*-induction it was important to achieve maximum levels of expression from a *vir*-promoter:*lux* construct to enable the light to be detected. The results described above suggested that the *virB* promoter present in pVL13 may not have been functioning with maximum efficiency. The promoter fragment utilised in the construct was derived from pDUB2512 (Ashby, 1988). During the formation of this plasmid, part of the *virB* promoter, including the -10 region and the transcription start site, had been deleted. They had been replaced using synthetic oligonucleotides which, whilst maintaining the

correct spacing between the -10 and -35 regions, had altered some of these bases (Ashby, 1988). It was possible that this alteration had caused a loss of efficiency of the promoter. Obviously, since it was also intended to use a *vir*-promoter as part of an inducible pesticide system, it was important that the promoter was functioning correctly.

Agrobacterium strains were obtained from the University of California, Davis which contained the plasmids pUCD1187 and pUCD1194. These had been constructed by cloning a *virB* or *virE* promoter fragment respectively into the multiple cloning site of pUCD615 (Rogowsky *et al.*, 1987). The plasmids were present in the *recA*$^-$ *Agrobacterium* strain LBA4301 and the required *virA* and *virG* functions were provided by the Ti-plasmid pTiC58.

In order to compare the level of light produced by LBA4301(pUCD1187) and C58C^1(pVK257, pVL13), a culture of LBA4301(pUCD1187) was assayed for acetosyringone-inducible bioluminescence as described in section 4.5. LBA4301(pUCD607) was tested as a positive control and the cultures were allowed to incubate for 36 hours prior to the assay since LBA4301 was slower growing than C58C^1.

100µl of LBA4301(pUCD607) gave a mean reading of 374,482 cpm with a light production of 8×10^{-2} cpm/cell.

100µl of a 10^{-1} dilution of LBA4301(pUCD1187) induced with acetosyringone gave an average reading of 626,360 cpm with a light production of 3.5×10^{-1} cpm/cell.

Both of these values were higher than for the equivalent plasmids in C58C^1. It was known that different strains of bacteria containing the same *lux* plasmid produced differing amounts of light (Shaw & Kado, 1986) and so the possibility existed that LBA4301 merely provided a better physiological background for expression of the *lux* genes than C58C^1. However, although the light production by pUCD607 was better in LBA4301 than C58C^1, such an effect may not solely have accounted for the 100-fold increase in light production observed with pUCD1187 as compared to pVL13.

To make an accurate comparison of the efficiency of the two *virB* promoters it was necessary for both *virB*:*lux* constructs to be available in the same *Agrobacterium* strain. Therefore pUCD1187 plasmid DNA was prepared from LBA4301 (section 2.5.1) and transformed into *E. coli* DH5α (sections 2.6.1 & 2.6.3). A triparental mating was then carried out according to section 2.12 with C58C^1(pVK257) as the recipient strain. Transconjugants were selected on L-agar plates containing rifampicin, kanamycin and carbenicillin. Five presumptive transconjugants were subjected to the 3-keto-lactose plate test (section 2.14) and miniprep DNA was prepared from positive colonies. This was digested with *Sal*I and electrophoresed on an agarose gel to confirm the presence of both pVK257 and pUCD1187.

Cultures of C58C^1(pVK257) containing either pVL13 or pUCD1187 were then grown in induction medium +/- acetosyringone and light production was measured as described in section 4.5. 100µl of the cells harbouring pUCD1187 gave an average reading of 3.6 x 10^5 cpm when induced and 1.65 x 10^4 cpm uninduced. This was equivalent to an induced light production of 7.39 x 10^{-2} cpm/cell. The value for induced cells containing pVL13 was 1.74 x 10^{-3} cpm/cell. These results implied that although LBA4301 provided a better background for *lux* expression, the *virB* promoter in pUCD1187 was also functioning more efficiently than the promoter in pVL13. Attempts were made to introduce pVL13 into LBA4301 to assess its performance in this strain but both triparental mating procedures (section 2.14) and direct transformation (section 2.6.4) were unsuccessful. LBA4301 has been noted to be a poorly transformable strain (Farrand *et al.*, 1989).

4.7 Attempts to detect *lux* expression *in planta*.

In all further experiments employing *lux* as a reporter system, LBA4301 harbouring pUCD1187 was used, since this had been shown to produce the most light when in the acetosyringone-induced state. It was first attempted to detect light produced by bacteria surrounding the roots of seedlings grown in soil. This experiment was conducted initially to ascertain whether it would be feasible to use the *lux* system in an *in planta* situation rather than to determine the actual sites of *vir*-induction.

Tobacco seedlings were initially used since tobacco extracts are known to contain the potent *vir*-inducer acetosyringone. Seeds were germinated for six days in a tray of compost which was kept moist throughout and was maintained at 25°C in a plant growth cabinet. After this time the seedlings were large enough to be handled easily. Half of the seedlings (approximately 10) were immediately transplanted into larger pots of compost, whilst the other half were first dipped into a culture of either LBA4301(pUCD607) or LBA4301(pUCD1187) prior to replanting. The cultures used had been incubated in L-broth at 28°C for 48 hours then centrifuged and resuspended in an equal volume of sterile water. The seedlings were allowed to grow for a further 5 days and the compost was kept moist by standing the pots in a shallow tray containing wet vermiculite. The seedlings were then carefully removed from the compost and the rhizosphere soil was removed by gentle shaking. Three or four seedlings from each treatment were laid on pieces of Whatman 3MM paper, covered with a layer of cling film and then exposed to X-ray film (Fuji-RX) at room temperature overnight. The developed film was inspected for any areas of darkening which corresponded to the roots of the seedlings. It was expected that the handling of the seedlings would have caused sufficient damage to release *vir*-inducing compounds, since the root systems of the tobacco seedlings were somewhat fragile.

Darkening of the X-ray film was observed in the regions where the seedlings were located but this was not correlated to the treatment which the seedlings had undergone. Some of the seedlings which had not been in contact with the *Agrobacterium* cultures also caused darkening of the film. In addition, due to the nature of the root system of the tobacco seedlings, it was not possible to detect the outlines of individual roots since they were very fine and interwoven. The experiment had not been successful in demonstrating whether *lux* would be a viable reporter system and it was decided to attempt another method.

The second method used was similar to that described in section 4.2.2. Mung bean seedlings were again utilised due to their larger size and easily manipulated roots and these were germinated as before (section 4.2.1). Three of the seedlings had an injury made to their roots with a scalpel blade prior to being dipped in a suspension of LBA4301(pUCD1187) for 5 minutes. Three uninjured seedlings were treated in the same way and three seedlings underwent no treatment. All were then inserted into 0.2% water agar in sterile plastic tubes as in section 4.2.2. The seedlings were returned to a plant growth cabinet at 25°C for 12 hours and then the tubes were taped to X-ray film, covered with foil, wrapped in a black plastic bag and left overnight at room temperature. When the film was developed darkened areas were observed, corresponding to a line where the plastic tube had been in contact with the X-ray film. This line was slightly more intense for those seedlings which had been dipped in the bacteria. It appeared that either the plastic tube itself was causing the darkening of the film or any light being produced was being concentrated in one place due to the curvature of the tube. Lack of time prevented further investigations being carried out with these constructs. Suggestions for experiments which could valuably be undertaken will be discussed later.

4.8 Subcloning the *vir*-promoters from pUCD1187 and pUCD1194.

It had been observed that pUCD1187 caused a higher level of induced light production in *Agrobacterium* than pVL13. The plasmid pUCD1194 was known to cause an equally high level of light production upon induction with acetosyringone (Rogowsky *et al.*, 1987). Therefore it was decided to utilise one of the two pTiC58 *vir*-promoters from these plasmids in any future constructs involving a *vir*-promoter linked to a heterologous gene. For ease of manipulation the promoter fragments were transferred form their original broad host-range vectors to pUC19.

1.5µg of pUCD1187 DNA was digested with *Sal*I to excise the 3kb *virB* promoter fragment. Previous restriction digests had demonstrated that the fragment contained no internal *Sal*I sites. The gel-purified DNA fragment was ligated to pUC19 and the ligation mix used to transform DH5α. Transformants were selected on X-gal/amp plates and white colonies were taken and restreaked. Miniprep DNA from these colonies was digested with *Sal*I and

electrophoresed on an agarose gel. Those plasmids which contained the *virB* fragment were redigested with *Bam*HI. This was the only restriction enzyme known to cut within the *virB* promoter fragment (Rogowsky *et al.*, 1987) and was thus used to determine the orientations of the cloned fragments in pUC19. Two plasmids which contained the promoter in opposite orientations were designated pVB1 and pVB21 respectively. The promoter fragment in each plasmid was flanked by a range of restriction sites comprised of those present in the pUC19 multiple cloning site and those transferred with the promoter from pUCD1187 (Figure 4.6).

The 0.8kb *virE* promoter fragment was subcloned in a similar manner. It was excised from pUCD1194 as a *Bam*HI fragment and ligated to pUC19. Following transformation of DH5α, white colonies on X-gal/amp plates were analysed by restriction digests of miniprep DNA. Those plasmids which contained the correct fragment were redigested with *Pst*I. This enzyme was known to cut the promoter approximately 230bp from the 5' end (Hirooka *et al.*, 1987) and so the orientation of the fragment could be determined. All the plasmids obtained were found to contain the *virE* promoter in the same orientation (Figure 4.6) and the construct was designated pVE5.

4.9 Use of the GUS reporter gene system.

The enzyme β-glucuronidase catalyses the hydrolysis of a wide variety of substrates, each of which consist of D-glucuronic acid conjugated through a β-*o*-glycosidic linkage to virtually any aglycone (Jefferson, 1989). *E. coli* is unusual amongst bacteria because it is able to metabolise many β-glucuronides. It produces a β-glucuronidase enzyme which has been shown to be a stable homotetramer with a subunit relative molecular mass of 68,200 (Jefferson *et al.*, 1986).

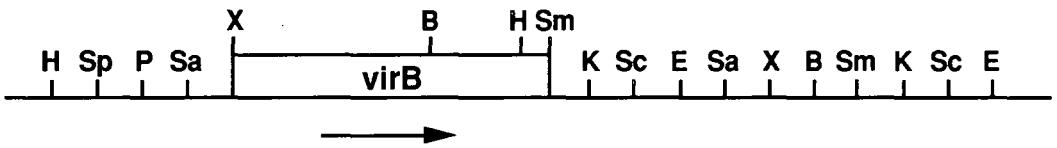
One of the main advantages of using GUS as a reporter is that GUS activity is largely absent from plants and most bacteria, fungi and insects that exist in the phyllo- and rhizosphere. Additionally, a variety of substrates are available for both sensitive histochemical analysis and for quantitative analysis. *E. coli* has evolved a mechanism for transmembrane transport of β-glucuronides involving a 49kDa permease which is encoded by *gusB*, a gene cocistronic with *gusA*. This allows accumulation of GUS substrates such as trifluoromethylumbelliferyl glucuronide within the cell.

Use of a *vir*:GUS construct in *Agrobacterium* would enable the sites of *vir*-induction to be assessed in a similar manner to that described in section 4.2.2. The sensitive histochemical substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) could be utilised in the GUS

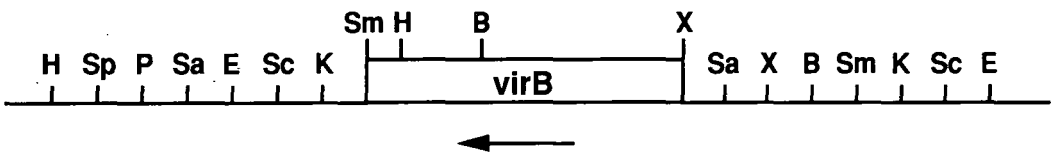
Figure 4.6 The subcloned *virB* and *virE* promoters from pUCD1187 and pUCD1194.

Schematic diagrams to show the restriction sites surrounding the pTiC58 *virB* and *virE* promoter fragments upon cloning into pUC19.

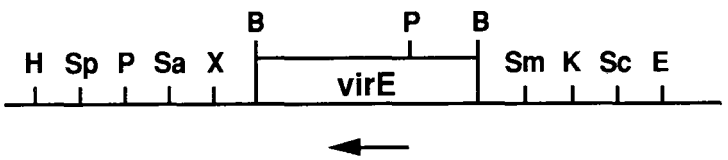
pVB1



pVB21



pVE5



→ = direction of transcription

system as X-gal was used with the *lacZ* reporter system. However, there would be an advantage over *lacZ* due to the absence of endogenous GUS activity in plants.

4.9.1 Linking the *virE* promoter to the GUS coding region.

The promoterless *gusA* gene, together with the *gusB* permease gene, was present on the plasmid pRAJ293 (Jefferson, R.A., personal communication). The two genes could be excised together as a single *Eco*RI/*Hin*dIII fragment. Approximately 1µg of pRAJ293 DNA was digested with *Hin*dIII/*Eco*RI and the 3.3kb GUS fragment was isolated from an agarose gel (section 2.7.8) and ligated to pBluescript SK$^+$. Transformed DH5α were selected on X-gal/amp plates and white colonies were chosen for analysis. A recombinant plasmid which contained the GUS fragment was identified from restriction digests of miniprep DNA and this became pSKG1.

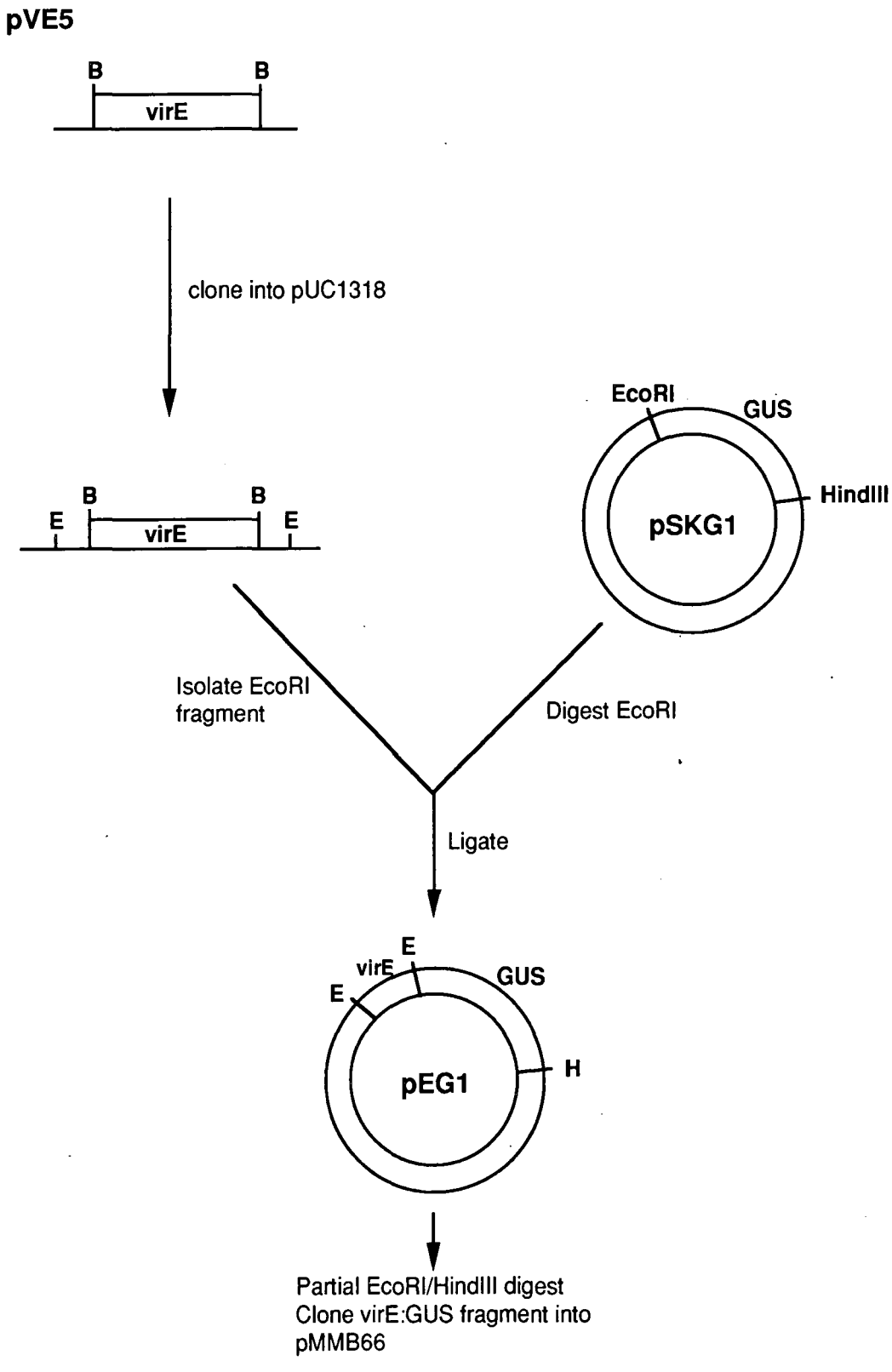
The *virE* promoter fragment from pUCD1194 was present as a *Bam*HI fragment in pVE5 (section 4.8). In order to clone this upstream of the GUS genes, it was first transferred to pUC1318 (section 4.3.1) and excised from there as an *Eco*RI fragment. This 0.8kb *virE* fragment was isolated from an agarose gel and ligated to *Eco*RI digested, phosphatase treated pSKG1. Transformed DH5α cells were selected on L-amp plates and since there was no selection for the insert, a colony hybridisation was carried out as described in section 2.7.14. The *virE* *Bam*HI fragment from pVE5 was labelled with digoxygenin according to section 2.7.11 and used to probe the filter. Miniprep DNA was obtained from positive colonies, digested with *Eco*RI and *Hin*dIII and electrophoresed on an agarose gel. Plasmids containing the correct fragments were then digested with *Pst*I, which cut the *virE* promoter fragment asymetrically, to determine the orientation of the promoter. One of the correct plasmids was selected for further use and was designated pEG1 (Figure 4.7).

4.9.2 Construction of pEG2.

The *virE*:GUS fusion was now present in a Bluescript vector. However, this vector, with a ColE1 origin of replication, was not capable of replicating in *Agrobacterium*. Therefore it was necessary to transfer the *virE*:GUS cassette to a broad host-range plasmid which did possess the requisite replication functions. The plasmid used was pMMB66, an 8.9kb broad host-range vector (Furste *et al.*, 1986). Approximately 3µg of pEG1 were digested to completion with *Hin*dIII. Following phenol/chloroform extraction and ethanol precipitation, the resuspended DNA was partially digested with *Eco*RI. This reaction was carried out at room temperature in a total volume of 30µl and 2µl aliquots were removed into stop dye every 2 minutes. The

Figure 4.7 A schematic diagram to show the construction of the *virE*:GUS plasmids.

Key:- B = *Bam*HI; E = *Eco*RI; H = *Hin*dIII.


pVE5
B
B
virE
clone into pUC1318
B
B
E
virE
E
EcoRI
GUS
HindIII
pSKG1
Isolate EcoRI fragment
Digest EcoRI
Ligate
E
virE
GUS
E
H
pEG1
Partial EcoRI/HindIII digest
Clone virE:GUS fragment into pMMB66

digestion products were separated on an agarose gel and the required 4.1kb *virE*:GUS fragment was isolated according to section 2.7.8. This was ligated to *Hin*dIII/*Eco*RI digested, phosphatase treated pMMB66 and an aliquot of the ligation mix was used to transform competent DH5α cells. Colonies selected on L-amp plates were analysed by restriction digests of miniprep DNA. A plasmid which contained the correct DNA fragments was chosen as pEG2.

pEG2 was introduced into *Agrobacterium* C58C^1 containing the cosmid pVK257 using the electroporation method described in section 2.13. Resulting cells were selected on L-kan,cb plates. Miniprep DNA was prepared from six colonies and this was digested with *Eco*RI and electrophoresed on an agarose gel. It was difficult to detect the presence of pEG2 and so the DNA was transferred to a nitrocellulose filter according to section 2.7.10. The digoxygenin-labelled *virE* fragment utilised in section 4.9.1 was used to probe the baked nitrocellulose filter (section 2.7.12) and the hybridised probe was detected as described in section 2.7.13. The probe was observed to hybridise to a 0.8kb fragment present in each miniprep digest and to the same fragment of control pEG2 DNA digested with *Eco*RI. No hybridisation was detected in the lane containing *Eco*RI digested pVK257. This confirmed that the electroporation procedure had been successful.

4.9.3 Acetosyringone-inducible GUS production from *Agrobacterium* harbouring pEG2.

If the *virE* promoter-GUS construct was to be used as a reporter for *vir*-induction then it was firstly important to demonstrate that it could specify acetosyringone-inducible production of β-glucuronidase. The level of GUS present in *Agrobacterium* cultures was measured over a period of 24 hours following induction with acetosyringone.

100ml of induction medium (section 2.4) was inoculated with 1ml of a fresh overnight culture of C58C^1 containing both pVK257 and pEG2. Incubation was carried out with shaking at 28°C for 9 hours by which time the cells were in log. phase. The culture was separated into two equal volumes of 50ml. To one flask was added 50μl of 10^{-1}M acetosyringone to give a final concentration of 100μM and to the other was added 50μl of 70% methanol to act as a control. A 1ml aliquot was immediately removed from each culture and these formed the zero time samples. Incubation of the cultures was then continued at 28°C and further 1ml samples were removed every 2 hours until 14 hours after induction. Final samples were taken after 24 hours. The GUS activity of each sample was determined using the method described in section 2.11 and the protein content of each cell extract was determined according to section 2.15.

During the incubation of the GUS assays, 100µl aliquots were removed from the reaction mixture into Stop Buffer after 5, 35, 65 and 125 minutes. The fluorescence of the resulting samples was measured (section 2.11) and the amount of methylumbelliferone (MU) present in each sample was determined. The rate of MU production was calculated for each original sample and GUS activity then expressed as nmoles MU produced/minute/mg protein (Figure 4.8).

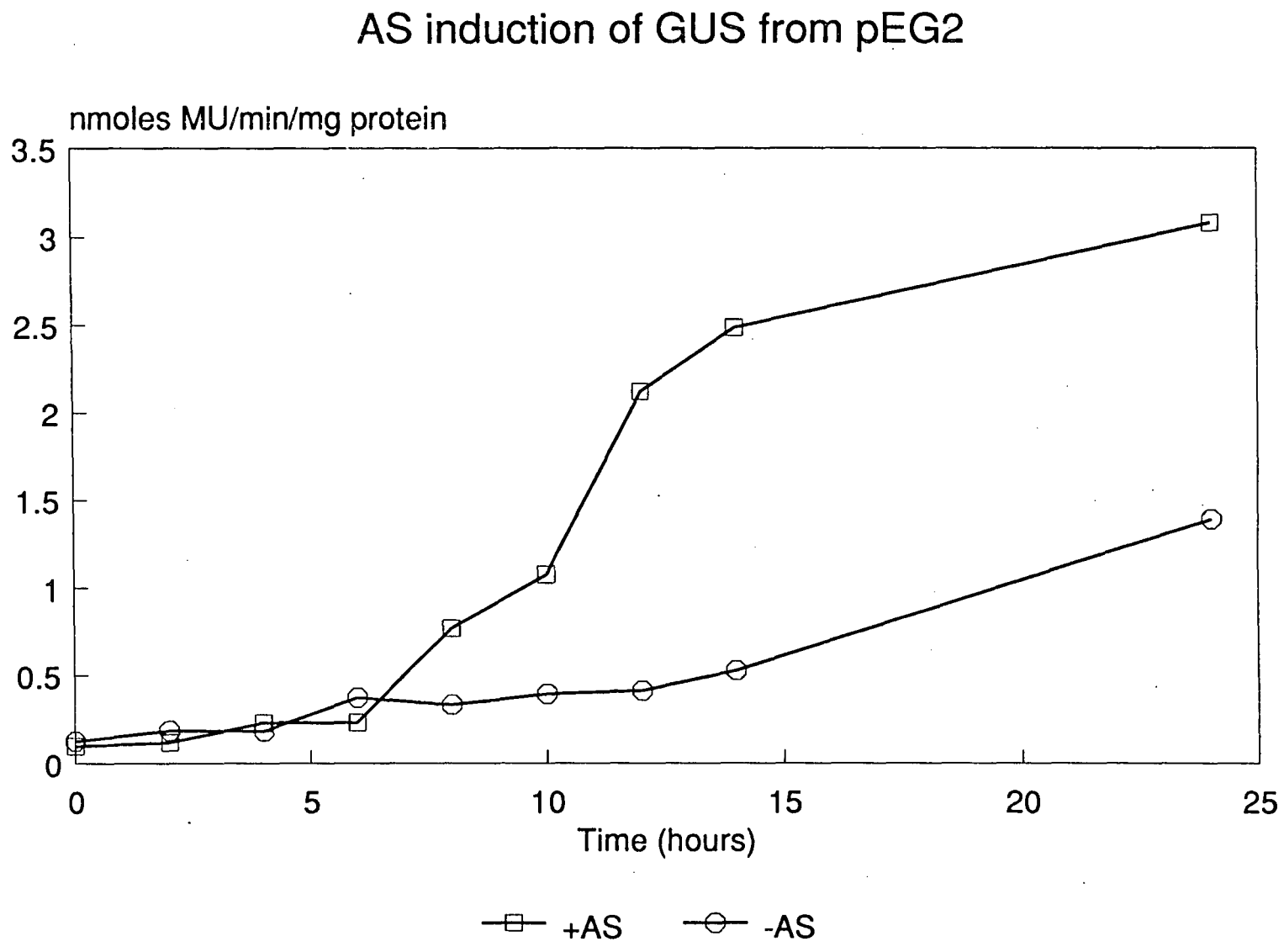
Both cultures initially contained a very low level of GUS activity and this continued until 8 hours after induction. At this point the GUS activity in the AS-induced cells increased 3-fold whereas the activity in the non-induced cells remained at the lower level. After 14 hours, the GUS activity in the non-induced culture had risen slightly to 0.52 nmoles MU/min/mg protein whereas that of the induced culture had reached a value of 2.48 nmoles MU/min/mg protein. Samples were not taken between 14 and 24 hours so it was not known exactly how the induction progressed throughout this period. However, after 24 hours, the GUS activity of the induced cells was 3.1 nmoles MU/min/mg protein and the activity present in the non-induced culture had risen to a value of 1.4 nmoles/min/mg protein. This experiment demonstrated that a *virE*:GUS construct had been successfully engineered and was capable of producing β-glucuronidase in response to induction with the strong *vir*-inducer acetosyringone. If time had allowed, the next step would have been to use *Agrobacterium* harbouring pEG2 in a system equivalent to that described in section 4.2.2. The chromogenic substrate X-gluc would have substituted for X-gal in the plant growth medium, with sites of *vir*-induction again being identified by the appearance of a blue colour.

4.10 DISCUSSION.

Three reporter genes were considered as a means of detecting the sites of *Agrobacterium vir*-induction *in planta*. One system, using the *lacZ* gene was readily available and some success was achieved when employing a construct in which a *virB* promoter was linked to the *lacZ* gene. Attempts to use the *lux* gene system to effect *vir*-inducible light production met with a number of problems at the *in planta* detection stage and thus far a set up has not been designed in which light emission by the bacteria surrounding plant roots can be accurately monitored. The third reporter system to be employed was GUS. A plasmid was successfully constructed in which a *virE* promoter was linked to the *gusA* and *gusB* genes and this was shown to specify acetosyringone-inducible production of β-glucuronidase. Further experiments may well prove this construct to be the most useful of the three since it should attain the same level of sensitivity as the *lacZ* system whilst overcoming the problems of background enzyme production encountered in this system.

Figure 4.8 Acetosyringone-inducible GUS expression by *Agrobacterium* C58C1(pVK257, pEG2).

This graph shows the induction of β-glucuronidase production by acetosyringone. The experiment was performed two times and the results shown are those obtained in one representative experiment. Both sets of results showed the same pattern of induction with equivalent levels of chitinase expression being achieved.


AS induction of GUS from pEG2
nmoles MU/min/mg protein
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Time (hours)
+AS
-AS

4.10.1 A comparison of the three reporter systems employed.

Of the three reporter systems described in this chapter, the one most commonly used in bacteria is the *E. coli lacZ* gene specifying the production of β-galactosidase. It has been employed many times to monitor the induction of *Agrobacterium* virulence genes, with various *vir*-promoters forming transcriptional and translational fusions to the *lacZ* coding region (Stachel *et al.*, 1985a; Spencer & Towers. 1988; Winans *et al.*, 1988; Zerback *et al.*, 1989; Cangelosi *et al.*, 1990; Winans, 1990). In these instances induction experiments were carried out using liquid cultures which were then assayed for β-galactosidase activity with the artificial substrate ONPG.

An alternative chromogenic substrate available for detecting β-galactosidase is X-gal, which is generally used for *in situ* demonstration of enzyme activity, particularly within tissue sections. One example of this, involving *Agrobacterium*, comes from experiments carried out by Herrera-Estrella *et al.* (1990). Using a gene fusion between *virD2* and *lacZ* it was demonstrated that the VirD2 polypeptide was able to direct the usually cytoplasmic β-galactosidase to the plant nucleus. Microscopy studies were able to detect the blue reaction product which arose when X-gal was cleaved by β-galactosidase. Generally, however, *lacZ* has not proved to be a useful reporter gene in plants. It is difficult to assay due to high endogenous β-galactosidase activity which is present in virtually all plants and in most tissues (Jefferson, 1987).

Despite this drawback, it was nevertheless decided to use a *virB:lacZ* fusion in an attempt to monitor *vir*-induction on the root surface. It was not known whether the endogenous cellular β-galactosidase would be released by the roots and, if so, whether it would be present in a sufficiently high concentration to cause a colour reaction with the X-gal present in the growth medium. The experiments undertaken demonstrated that β-galactosidase was indeed released from the roots of the seedlings, particularly at the root tips, as blue colour developed in these areas when no bacteria were present. However, the greater intensity of colour apparent when bacteria were colonising the roots was sufficient for *vir*-induction to be detected. In the experiments which have been carried out so far, the *lacZ* gene fusion has provided the best results with regard to identifying, *in planta*, the sites of *vir*-induction. However, the GUS sytem may prove to be superior for this purpose.

The greatest advantages of the *lacZ* system are the ease with which β-galactosidase activity can be detected using X-gal and the cheapness of this substrate. The disadvantages are the presence of an endogenous β-galactosidase in most plants and, at least with the construct used, a basal level of induced *lacZ* expression which may be high enough to produce a detectable

reaction. Additionally, it would be difficult to set up an experiment in which *vir*-induction was monitored *in situ* on plants growing in soil.

To overcome the problem of endogenous β-galactosidase activity in plants, a number of other reporter systems have been developed of which one of the most common is the *E. coli* β-glucuronidase (Jefferson, 1987). This has the same advantages as β-galactosidase in that there are simple assays and a variety of substrates available, for both histochemical and quantitative analysis. However, it has an additional advantage since GUS activity is absent from most organisms other than vertebrates and their microflora. The lack of GUS activity in plants and and soil organisms has meant that GUS fusions are now widely used when studying plant-pathogen and plant-symbiont interactions. They can be used to monitor populations of microorganisms in the rhizosphere and associated with plants and also to study the expression of particular genes (Jefferson, 1989).

In this study the *Agrobacterium virE* promoter was joined to the *gusA* and *gusB* genes which were present on a single fragment. The *gusB* permease gene would allow the accumulation of GUS substrates within the cell. The permease can transport a wide variety of β-glucuronide substrates ranging from simple aliphatic compounds to complex heterocyclic conjugates (Jefferson, 1989). The final *virE*:GUS construct caused *Agrobacterium* harbouring it to produce β-glucuronidase upon induction with the *vir*-inducer acetosyringone. It should therefore be possible to use this for monitoring *vir*-induction *in situ*. One slight problem with the construct was the increase in GUS activity in the uninduced culture after 24 hours. This may produce an unacceptable level of background enzyme activity if the experiments were to be carried for longer than an overnight incubation. A possible solution would be to transfer the *virE*:GUS cassette to an alternative broad host-range vector. With hindsight, pMMB66 was not the ideal plasmid to use since it carries the *tac* promoter. The orientation of the *virE*:GUS fragment in pEG2 was such that this promoter may have enhanced the uninduced levels of GUS expression. Once this problem has been resolved, the first experiments to be carried out would be identical to those described for the work with *lacZ* (section 4.2.2) but substituting X-gluc for X-gal.

The GUS system has the major advantage over *lacZ*, that there is no endogenous GUS activity present in plants, but it has the drawback that the chromogenic substrate, X-gluc, is prohibitively expensive for use in large scale experiments.

Monitoring bacterial:plant associations is a difficult procedure and although much work has been done to elucidate the infection process of plant pathogenic bacteria, most of the information has come from studies which caused disruption of the plant-bacterial interaction and interrupted the disease process. However, the *lux* reporter system has recently proved to be

a useful tool for the localisation and enumeration of plant associated bacteria and it can be used to directly monitor the ongoing interaction between plants and bacteria in a non-disruptive manner (Legocki *et al.*, 1986; Shaw & Kado, 1986). The broad host-range plasmid pUCD607, which constitutively expresses the *lux* operon, has been used to monitor the infection of cauliflower plants by *Xanthomonas campestris* pv. *campestris* which causes black rot (Shaw & Kado, 1986). Light production was detectable, using X-ray film, from leaves which had been inoculated with the plasmid-containing pathogen. The light was detected in areas of the leaves which later developed symptoms of the disease. Bioluminescence has also been previously used as a reporter of *vir*-induction (Rogowsky *et al.*, 1987). Light production by *Agrobacterium* strains harbouring *vir-lux* fusions was recorded by photographic film when the *lux* genes were induced in bacteria present on freshly sliced carrot discs.

Such reports indicate that bioluminescence is a sensitive reporter which has the potential to be used to directly monitor interactions between plants and bacteria. The failure to demonstrate *in situ vir*-induction using this system is likely to be due to shortcomings in the experimental design. An extended period of study would no doubt result in the development of an improved procedure to tackle the difficulties encountered with the detection methods.

4.10.2 Overcoming the problems of *in planta* detection.

It has become apparent throughout the course of this work that devising a method for *in planta* detection of bacteria and specifically of *vir*-induction is a difficult process. A set-up was achieved which gave limited success with the *lacZ* system and should give similar results with GUS, but no useful results have so far been obtained using bioluminescence as a reporter. Alternative strategies must be considered to improve the detection of the markers.

LacZ has been used as a marker in a study of rhizosphere colonisation by a *Pseudomonas fluorescens* strain and also as a reporter of gene expression in response to plant exudates (Lam *et al.*, 1990). Mutant strains of the bacterium, constitutively expressing β-galactosidase, were assessed for their root colonising ability when coinoculated onto seeds with the wild-type strain. Bacteria were recovered from seedling roots and plated onto indicator medium containing X-gal. The mutant cells could be distinguished from the wild-type cells by their blue colour and the numbers of cells of each strain present on the roots could be calculated. The same group also used *lacZ* as a reporter gene to obtain similar information to that required in the present study. Their aim was to test whether induction of *Pseudomonas* genes already found to be induced by root exudates was actually occurring on roots. To do this, bacteria containing the *lacZ* gene under the control of a plant inducible promoter were inoculated onto wheat seeds which were allowed to germinate and grow for 7 days. Bacteria were recovered from the roots

and immediately assayed for β-galactosidase activity using ONPG. The activity was compared to that present in overnight cultures of the same bacteria and was found to be higher in the bacteria isolated from the roots, indicating that the genes were indeed induced when the bacteria were colonising the roots.

It is possible that a comparable system could be employed with any of the three reporter systems described here. An advantage would be that the plants could be grown directly in soil rather than in an artificial medium. When the bacteria were recovered from the seedlings the roots could be divided into segments, enabling *vir*-induction at different points on the root to be monitored. Cutting the root in this way would release *vir*-inducing compounds but if the assays were carried out immediately and the bacteria washed after recovery from the root then the induction profiles obtained for the *lux* (Rogowsky *et al.*, 1987) and GUS (section 4.9.4) constructs indicate that this would not be a problem. GUS activity was not apparent until 7 hours after induction although increased light production was detectable after 2 hours. A further advantage is that differences in cell number at different parts of the root could be taken into account and the results of the assays calculated on a per cell basis. Nevertheless, the method reverts to a disruptive technique, whereas the main aim of this study was to devise a method to detect *vir*-induction in a non-disruptive manner as it was actually occurring.

The greatest improvement is necessary in the design of experiments to detect bioluminescence. One possibility is to grow seedlings as described in section 4.2.2 and then prior to detection, remove them from the agar and apply directly to X-ray film. This would require a suitable light-tight container to be organised to hold the seedling in place. An alternative method would be to employ photographic film in a manner such as that described by Rogowsky *et al.* (1987) when monitoring *vir*-induction on carrot discs. Hypersensitive astronomy film was used with an exposure time of 12 to 14 hours, but this would be difficult to organise with the available facilities.

Seedlings could be grown between glass plates held apart by 2-3mm spacers in a similar set-up to that used for acrylamide gels. The growth medium between the plates could be either agar or fine soil. Since it would be difficult to insert seedlings into such an apparatus, the seeds would need to be inoculated with the bacterial culture and then germinated directly in the soil or agar. Detection of the bioluminescence would be achieved by using X-ray film which could be easily attached to the glass plates. The thinness of the layer of agar or soil would ensure that the root surface was directly adjacent to the glass plate, thus minimising dispersal or interception of the light.

4.10.3 Comparison of the *virB* promoters in pVL13 and pUCD1187.

Results of assays using the two *virB:lux* plasmids pVL13 and pUCD1187 indicated that the promoter in pUCD1187 was functioning more efficiently (section 4.6). The two promoter fragments were derived from different sources - that in pUCD1187 is from the nopaline plasmid pTiC58 whereas the promoter used in pVL13 was originally derived from the octopine-type plasmid pTiA6. Rogowsky *et al.* (1987) reported similar results for the induction of pTiC58 *vir*-promoters and the equivalent promoters from pTiA6 and so it is unlikely that intrinsic differences in the origin of the two promoters could account for the observed differences in expression.

Investigation of the previous manipulations which the pTiA6 *virB* promoter had undergone showed that a part of the original sequence had been altered. In order to remove the coding sequence from the *virB* 1.3kb *Hin*dIII fragment present on plasmid pDUB2510, exonuclease deletions were carried out (Ashby, 1988). A deletion which resulted in a fragment of 0.47kb was sequenced and was found to have lost all the *virB* coding region and also part of the promoter sequence. The transcription initiation site and the -10 region had been deleted, although the -35 region was still intact. To correct the deletion, 32bp oligonucleotides corresponding to the deleted promoter region were synthesised and linked to the deleted fragment. The sequence of the oligonucleotides matched the published sequence (Das *et al.*, 1986; Ward *et al.*, 1988) as far as possible but to enable the small fragment to be cloned, a restriction site had to be created. This meant that whilst the spacing between the -10 and -35 sequences was maintained, 5 base changes were made in this intervening region (Figure 4.9).

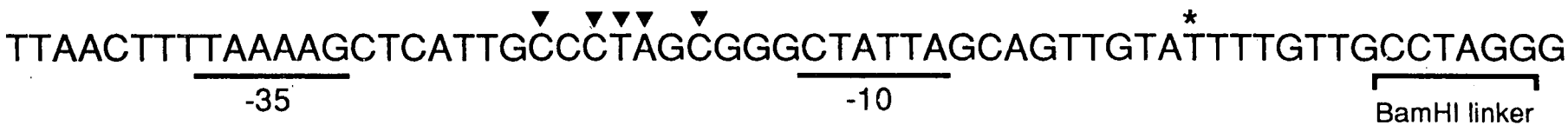
The -10 and -35 conserved regions have been identified from extensive sequence comparisons of bacterial promoters as being important for promoter activity (Reznikoff *et al.*, 1985; Harley & Reynolds, 1987). These regions show the greatest conservation between different promoters and are the sites of nearly all mutations which affect promoter strength. Variation in the spacing between the two regions also plays a role in promoter strength. However, other bases flanking these sequences occur at greater than random frequencies and sometimes affect promoter activity. Chemical protection experiments have shown that RNA polymerase interacts not only with the highly conserved -35 and -10 hexamers but also with regions immediately upstream of them (Siebenlist *et al.*, 1980). It is possible that alteration of the indicated bases (Figure 4.8) may have adversely affected the promoter activity and the ability of the RNA polymerase to initiate transcription, although it is the bases immediately flanking the -35 and -10 regions which have generally been found to be important (Harley & Reynolds, 1987).

Figure 4.9 A comparison of the original pTiA6 *virB* promoter and that present in pDUB2512.

This figure indicates the bases altered in the *virB* promoter during the construction of pDUB2512. The changed bases are marked by ▼ . ★ indicates the site of the start of transcription.

virB promoter sequence

altered promoter in pDUB2512



When RNA polymerase is bound to the *E. coli* promoter it covers approximately 70bp, including 20bp of the transcribed sequence and information encoded in this region may also participate in promoter function (Kammerer *et al.*, 1986). The *virB* promoter of pDUB2512 contains only 7bp of transcribed sequence, which could be affecting its activity. If the *virB* promoter utilised an alternative sigma factor then this might recognise bases other than those in the supposed -10 and -35 regions including some of those altered in replacement of the deleted region.

In all inducible *Agrobacterium vir*-genes, expression is also dependent on the presence of a conserved "vir-box" sequence (TNCAATTGAAAPy) in their 5' non-transcribed regions (Steck *et al.*, 1988). This sequence corresponds to the binding site of the transcriptional activator, VirG. The number and location of the vir-box sequence is highly variable in the various inducible *vir*-loci. The pTiA6 *virB* promoter has two vir-boxes - one is absolutely required for induction and the second contributes but is not absolutely required (Pazour & Das, 1990a). Both the vir-boxes are upstream of the -35 region and so the altered bases in the *virB* promoter fragment should not have affected their function.

CHAPTER 5

PLASMIDS FOR USE IN A PLANT PROTECTION SYSTEM INVOLVING *AGROBACTERIUM.*

5.1 Introduction.

A. tumefaciens was shown to exhibit positive chemotaxis towards compounds present in plant wound exudates (Ashby *et al.*, 1987). This led to the formulation of a novel idea for the use of *Agrobacterium* as a biological control agent (Ashby, 1988). The idea centres around the fact that at low concentrations of *vir*-inducing compounds released by wounded plant cells, *A. tumefaciens* responds chemotactically by moving towards the site of injury. The higher concentrations of *vir*-inducers present closer to the wound site then cause induction of the virulence operons. These responses require the presence of VirA and VirG which mediate both the chemotaxis and *vir*-induction processes (Shaw *et al.*, 1988a).

It was proposed to make use of these natural responses by placing a gene encoding a pesticidal protein under the control of one of the virulence promoters. *Agrobacterium* containing such a plasmid, together with the necessary *virA* and *virG* genes, would be attracted to wounded plant tissue. Once there the organism would produce the pesticide as a result of *vir*-induction by the high concentrations of phenolic inducing compounds. Thus pesticide production would be a conservative process, occurring only when a plant was wounded, at the time when it was most susceptible to attack by opportunist pathogens and then exclusively in the microrhizosphere around the wound site.

This chapter describes the construction of a number of plasmids which could be used in this control system. The finished constructs were then tested to demonstrate inducible production of the pesticide. The pesticide genes chosen for use were the chitinase genes of *S. marcescens*.

5.2 Manipulation of the *chiB* gene for use in a *vir* promoter - pesticide construct.

In order to use the *chiB* gene of *S. marcescens*, which had been previously characterised and sequenced (Chapter 3), in the plant protection system of Ashby *et al.* (1986) it was necessary to first delete the promoter region of the gene. By substituting a *vir*-promoter fragment for the upstream region of the *chiB* gene, including the transcription start site and any regulatory sequences 5' to this, the chitinase could be brought under inducible control. The

Shine-Dalgarno sequence and translation initiation codon of the *chiB* gene were to be left intact but the region of DNA between these and the upstream *Eco*RI site had to be removed to delete the promoter. The DNA sequence of this region was examined to determine whether any restriction sites which could be utilised lay between the transcription start site and the ribosome-binding site, but no suitable sites were found to be present.

One procedure which was considered utilised controlled deletions from the *Eco*RI site towards the coding region, using a combination of exonuclease III and mung bean nuclease. However, only approximately 150 base pairs needed to be deleted and there were only 20 base pairs between the transcription start site and the Shine-Dalgarno sequence within which the deletion could end. Previous experience of this technique had shown that it was difficult to control the exonuclease digestion with sufficient accuracy and so a large number of deletion products would need to be sequenced in order to select the one with the correct number of bases removed.

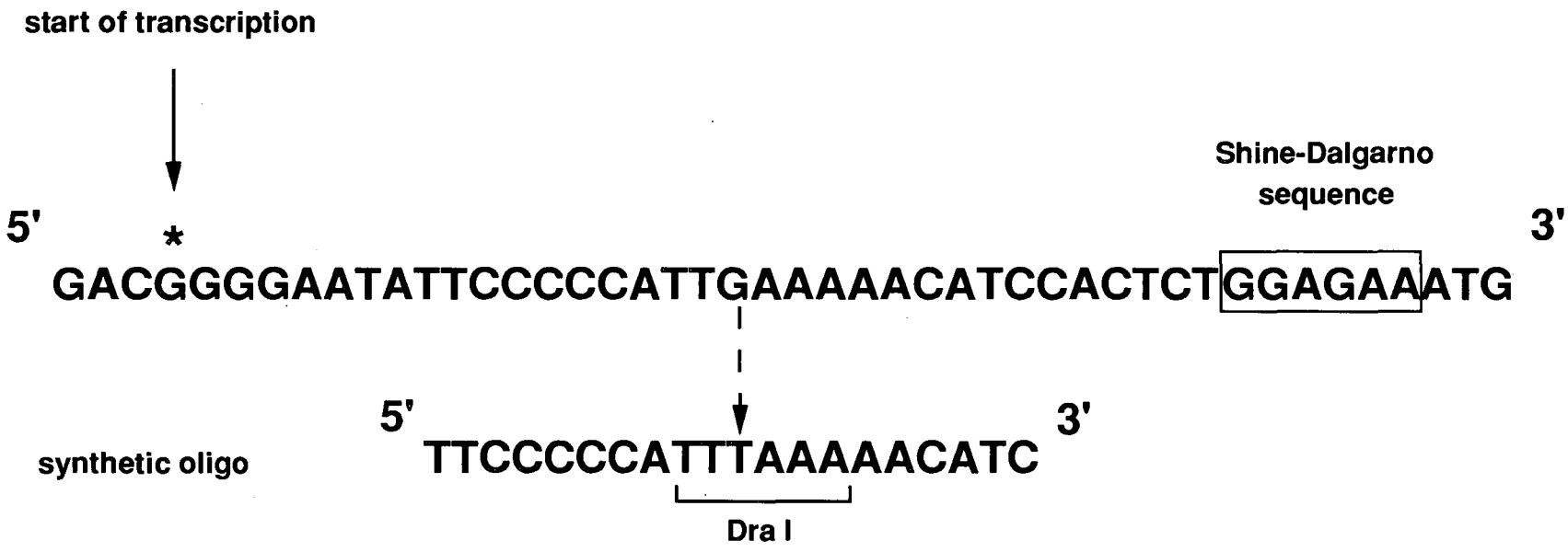
Therefore the technique of oligonucleotide-directed site-specific mutagenesis was used in order to create a restriction site in the stretch of DNA between the start of transcription and the ribosome binding site. This site could then be used, together with the *Hin*dIII site (see Figure 3.1), to excise the coding region of the *chiB* gene, whilst leaving behind its upstream regions. The nucleotide sequence was studied to find a position where a single base change would create a useful restriction site. Seventeen base pairs upstream of the translation initiation codon, a G to T change could create a *Dra*I site, which has the recognition sequence of TTTAAA (see Figure 5.1). This was an ideal restriction enzyme to use since there are no *Dra*I sites in the *chiB* gene.

The technique of oligonucleotide-directed site-specific mutagenesis utilises M13 bacteriophage vectors which have the ability to exist in both double-stranded/single-stranded forms and a complementary synthetic oligonucleotide containing the desired base change. The oligonucleotide is annealed to the single-stranded template DNA which has been cloned into an M13 vector. Second strand synthesis is initiated, with the oligonucleotide acting as a primer. The thionucleotide dCTPαS is included among the deoxynucleotides as a mutant heteroduplex is generated. The incorporation of the thionucleotide makes possible selective removal of the non-mutant strand, since the restriction enzyme *Nci*I cannot cleave phosphorothioate DNA. This enzyme merely nicks the non-mutant strand which is then digested away with exonuclease III. Using the mutant strand as a template, the double-stranded molecule is reformed to create a homoduplex mutated sequence.

Figure 5.1 Diagram showing the mutated sequence of the *chiB* gene.

This figure shows the sequence of the *chiB* gene immediately upstream of the translation initiation codon. The base which was altered by oligonucleotide-directed mutagenesis is indicated with an arrow and the sequence of the mutant oligonucleotide is shown.

5' region of the *chiB* gene



5.2.1 Preparation of the template for the mutagenesis reaction.

To be able to carry out the mutagenesis procedure, it was necessary to clone a DNA fragment containing the site to be altered into an M13 vector. The fragment chosen was the *Hin*dIII/*Eco*RI fragment of pCH7. This contained the entire *chiB* gene and yet was the smallest fragment to do so, hence it would be the most stable when cloned into M13. Additionally, this cloned fragment conferred consistently high levels of chitinase activity upon *E. coli.* (section 3.8).

The *Hin*dIII/*Eco*RI fragment was excised from pCH7 and isolated from an agarose gel by the freeze-squeeze method, according to section 2.7.8. It was then ligated into *Hin*dIII/*Eco*RI digested M13mp19 and the ligation mix used to transform competent TG2 cells as described in section 2.8.1. White plaques were picked for analysis and miniprep DNA digested with *Hin*dIII and *Eco*RI and analysed on an agarose gel. One of the clones containing the correct insert was chosen and single-stranded DNA prepared from the saved culture supernatant according to section 2.8.2.

5.2.2 Mutagenesis reactions.

A 21-mer oligonucleotide complementary to the template DNA and with the sequence shown in Figure 5.1 was synthesised on an ABI oligonucleotide synthesiser. Prior to use in the mutagenesis reaction it was phosphorylated at its 5' end as described in section 2.9.1. The mutagenesis reactions were then carried out using this phosphorylated oligonucleotide and the single-stranded DNA template from section 5.2.1 as detailed in section 2.9.2. Following transformation of 20µl of the final sample into TG2, 9 white plaques were picked and minipreps of the RF DNA were obtained. The miniprep DNA from each presumptive mutant was digested with *Hin*dIII and *Dra*I and electrophoresed on an agarose gel, together with the non-mutant template DNA. This gel is shown in Figure 5.2. If the mutagenesis reaction had been successful, then a mutant phage would possess an additional *Dra*I site to those contained in the template DNA. As the gel photograph shows, six of the nine DNA samples lost a fragment of approximately 2.6kb which was present when the template DNA was digested with *Hin*dIII and *Dra*I. This fragment was replaced in the mutants by two smaller fragments, one of approximately 650bp and another which ran together on the gel with one of the vector fragments of approximately 1.9kb. Thus these six clones appeared to be the products of successful mutagenesis reactions, each having acquired an extra *Dra*I site.

In order to confirm that the correct base change had been made, one of the mutant clones, designated MUT1, was sequenced using an automated DNA sequencer (ABI). The desired base

Figure 5.2 Restriction analysis of products of the mutagenesis reaction.

Double-stranded DNA from nine presumptive mutants was digested with *Hin*dIII and *Dra*I to look for the presence of the newly created *Dra*I site.

Lane 1	Template DNA	*Hin*dIII/*Dra*I
Lane 2	Mutant 1	*Hin*dIII/*Dra*I
Lane 3	Non-mutant	*Hin*dIII/*Dra*I
Lane 4	Mutant 2	*Hin*dIII/*Dra*I
Lane 5	Mutant 3	*Hin*dIII/*Dra*I
Lane 6	λ*Pst*I size markers	
Lane 7	Non-mutant	*Hin*dIII/*Dra*I
Lane 8	Mutant 4	*Hin*dIII/*Dra*I
Lane 9	Mutant 5	*Hin*dIII/*Dra*I
Lane 10	Mutant 6	*Hin*dIII/*Dra*I
Lane 11	Non-mutant	*Hin*dIII/*Dra*I

Six of the nine mutagenesis products tested had acquired an extra *Dra*I site.

Fragment a) is the fragment in which the *Dra*I site was created and is therefore absent in the mutants

Band b) contains two fragments from the mutants - the *Hin*dIII/*Dra*I fragment containing the *chiB* coding region runs together on the gel with a vector fragment.

Fragment c) is the second fragment derived in the mutants from the original fragment a).

The sizes of the λ*Pst*I size markers, starting at the top, in kb, are:- Band 1 - 14.09; Band 2 - 11.41; Band 3 - 5.08; Band 4 - 4.75; Band 5 - 4.51; Band 6 - 2.84; Band 7 - 2.56/2.45/2.44; Band 8 - 2.14; Band 9 - 1.97; Band 10 - 1.70; Band 11 - 1.16; Band 12 - 1.09; Band 13 - 0.8

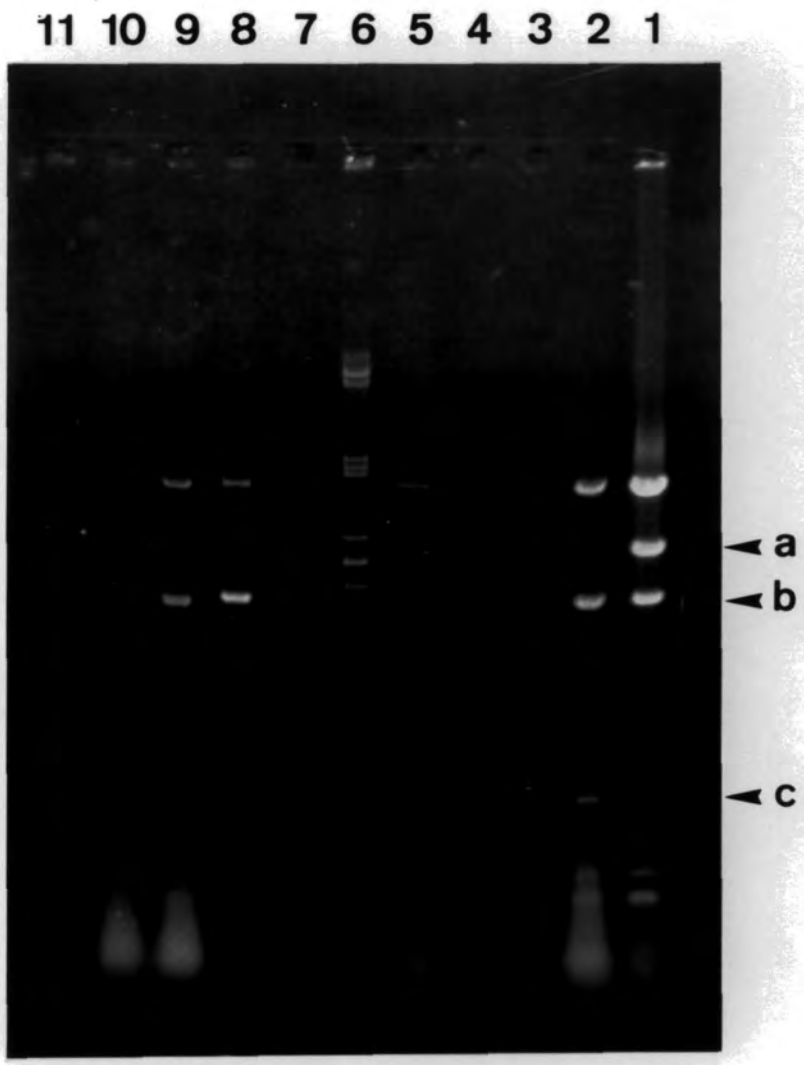
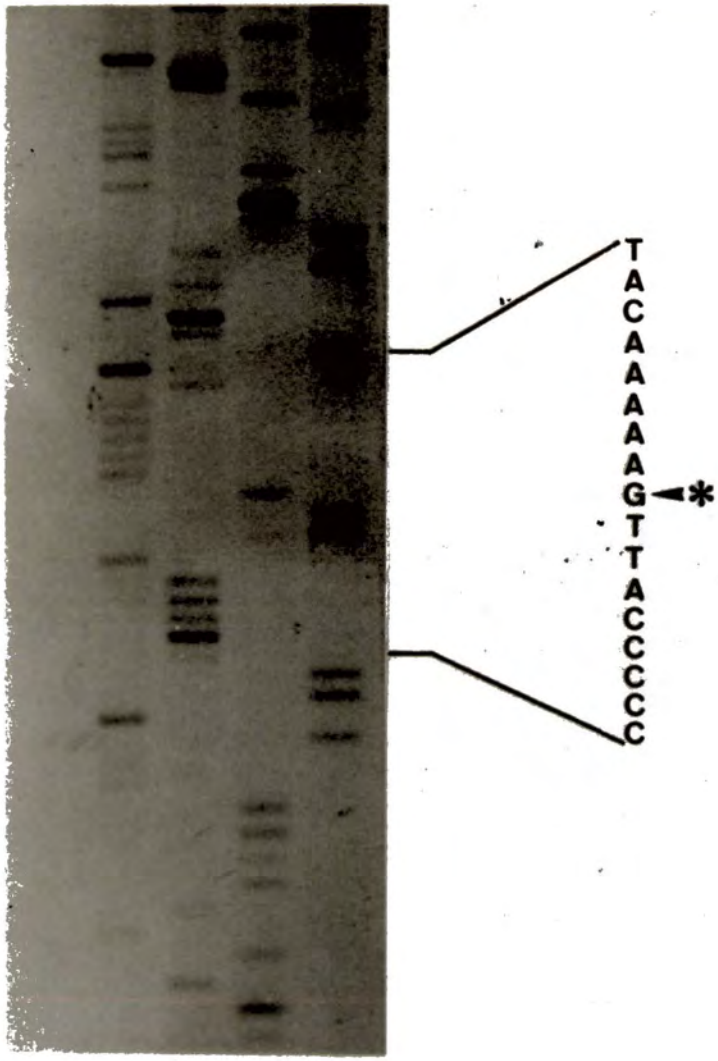

11 10 9 8 7 6 5 4 3 2 1
a
b
c

Figure 5.3 Comparison of the *chiB* DNA sequence before and after mutagenesis

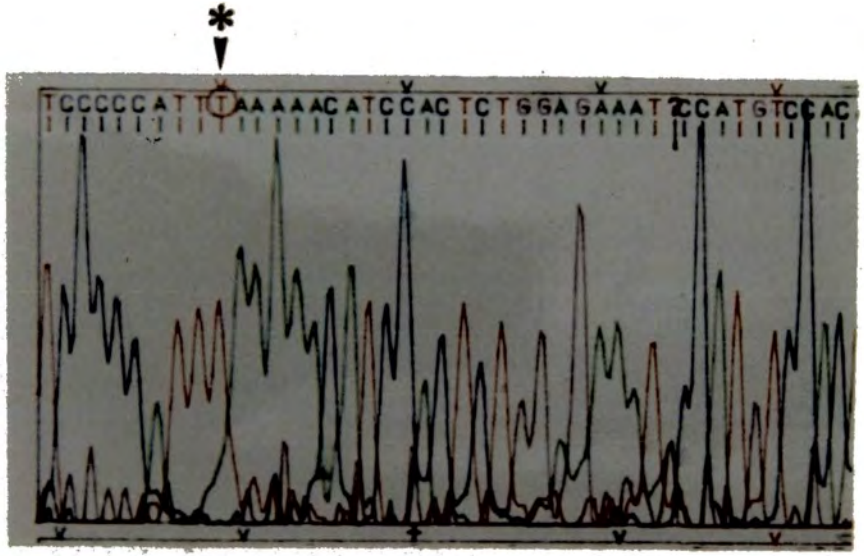
Figure 5.3a shows the original sequence of the *chiB* upstream region as obtained by manual sequencing.

Figure 5.3b shows the sequence of MUT1 over the same region as obtained using an automated sequencer.

The mutated base is indicated.



A



B

change had in fact occurred, as demonstrated in Figure 5.3 where the sequence of the original template (manually sequenced) is compared to the sequence of the mutant.

5.2.3 Recloning of the mutated *chiB* fragment.

Now that the *Dra*I site had been created, the *chiB* coding region was present within a *Hin*dIII/*Dra*I fragment which lacked the promoter sequences. The entire *Hin*dIII/*Eco*RI fragment was subcloned back to pUC19 prior to excision of the promoterless fragment. As can be seen in Figure 5.2, the *Hin*dIII/*Dra*I *chiB* fragment comigrates with a M13mp19 vector fragment when digested DNA is separated on an agarose gel. Also, DNA manipulation would be easier with a pUC-based plasmid since better DNA preparations can be obtained than from M13 constructs. Once in pUC19, chitinase production from the mutated fragment could be assayed to check that no extra mutations had occurred which affected the activity of the enzyme.

Therefore MUT1 miniprep DNA was digested with *Hin*dIII and *Eco*RI and the 2.2kb fragment was recovered from an agarose gel and ligated to pUC19. An aliquot of the ligation mix was used to transform competent DH5α and transformants were selected on X-gal/amp plates. Four white colonies were chosen for analysis and miniprep DNA from these was digested with *Hin*dIII and *Eco*RI and analysed on an agarose gel. The result is shown in Figure 5.4a and it can be seen that 3 of the 4 clones contained the correct 2.2kb insert. One of these was designated pMUT19 and was used in further DNA manipulations. To confirm the presence of the new *Dra*I site in this plasmid both pMUT19 and pCH7 (the equivalent unmutated plasmid) were digested with *Hin*dIII and *Dra*I and the DNA fragments separated on an agarose gel. The result is presented in Figure 5.4b and clearly demonstrates the presence of an extra restriction site in pMUT19. It also shows the *Hin*dIII/*Dra*I fragment which contains the *chiB* coding region and which will be used in the final *vir*-promoter:chitinase construct.

DH5α(pMUT19) and DH5α(pCH7) were inoculated into 5ml of L-broth and incubated overnight at 37°C. Cell extracts were prepared (section 3.8) and both they and the culture supernatants were assayed for chitinase activity (see section 2.16). DH5α(pMUT19) retained the ability to produce a high level of chitinase (9.1 OD_{410}/mg protein), comparable to that produced by DH5α(pCH7) (10.2 OD_{410}/mg protein).

Figure 5.4 Recloning of the mutated *chiB* fragment into pUC19.

A) Restriction digests of miniprep DNA from four transformants resulting from the ligation of the mutated chiB fragment to pUC19.

Lane 1 λ*Pst*I size markers
Lane 2 Empty
Lane 3 Transformant 1 *Hin*dIII/*Eco*RI
Lane 4 Transformant 2 *Hin*dIII/*Eco*RI
Lane 5 Transformant 3 *Hin*dIII/*Eco*RI
Lane 6 Transformant 4 *Hin*dIII/*Eco*RI
Lane 7 λ*Pst*I size markers

Transformants 1, 2 and 3 each contained the *chiB* fragment. Transformant 1 was chosen as pMUT19.

B) Confirmation of the new *Dra*I site in pMUT19.

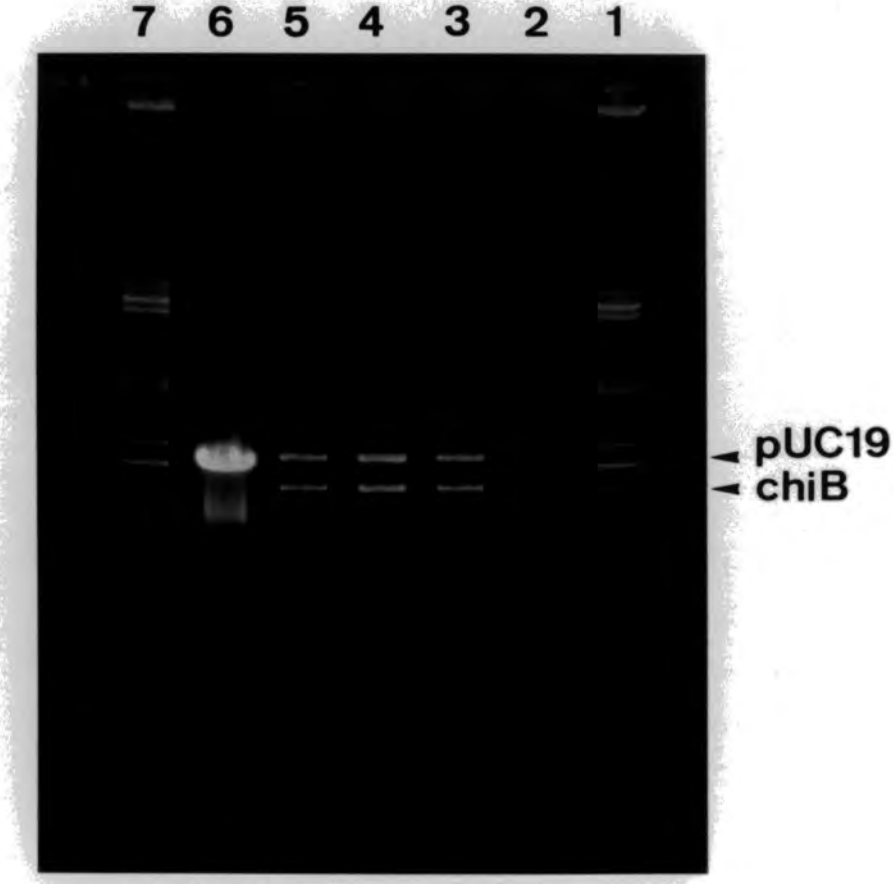
Lane 1 pCH7 *Hin*dIII/*Dra*I
Lane 2 pMUT19 *Hin*dIII/*Dra*I
Lane 3 λ*Pst*I size markers

Band a) is the fragment of pCH7 in which the *Dra*I site was created.

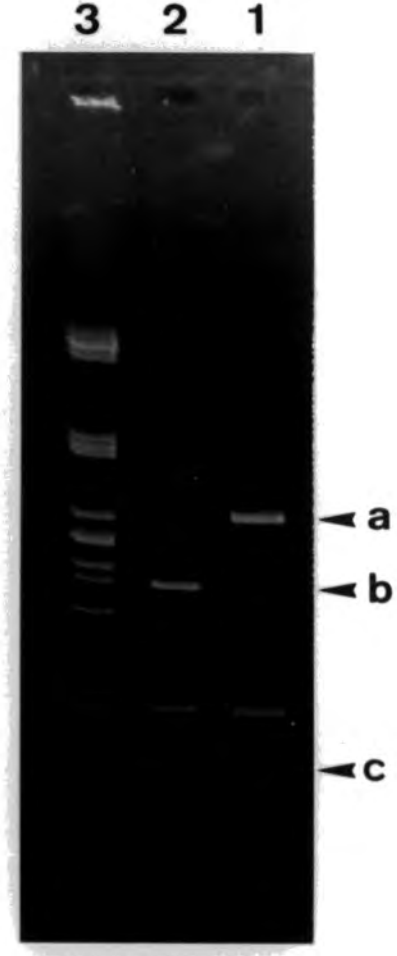
Band b) is the *Hin*dIII/*Dra*I fragment of pMUT19 which contains the *chiB* coding region.

Band c) is the second fragment formed by the creation of the *Dra*I site in pCH7.

A]



B]



5.2.4 Removal of the *chiB* promoter region.

This was achieved by digesting pMUT19 with *Hin*dIII and *Dra*I and isolating the required fragment (indicated in Figure 5.4b) from an agarose gel according to section 2.7.8. The fragment was ligated to pUC19 digested with *Hin*dIII and *Sma*I and the ligation mix used to transform competent DH5α cells. Transformants were again selected on X-gal/amp plates and 5 white colonies were chosen. Each miniprepped plasmid, upon digestion with *Hin*dIII and *Eco*RI, was found to contain the *chiB* gene and one was chosen as pCB1 (Figure 5.5). DH5α(pCB1) was inoculated into 5ml of L-broth and incubated at 37°C overnight. The cell extract was assayed for chitinase activity as previously described (section 3.8) and was not found to contain any enzyme activity (a figure of 0.885 OD*410*/mg protein). This demonstrated that all upstream regions of the *chiB* gene necessary for expression had been successfully removed. Hence the *chiB* gene was finally in a suitable form to be linked to either the *virB* or *virE* promoter fragments described in section 4.8.

5.3 Linking the *chiB* coding region to the *virB* promoter.

pCB1 was digested with *Eco*RI and phosphatased to prevent self-ligation of the plasmid (section 2.7.5). pVB21 (section 4.8) was also digested with *Eco*RI to excise the *virB* promoter fragment which was then isolated from an agarose gel following electrophoresis (section 2.7.8). The two were ligated and transformed into DH5α with transformants being selected on L-amp plates. Since blue/white selection on X-gal was not possible, transformants were streaked onto a master plate and a nitrocellulose filter in order to carry out a colony hybridisation as described in section 2.7.14. The digoxygenin-labelled DNA probe was prepared according to section 2.7.11 using the *virB Sal*I/*Hin*dIII fragment of pVB1 which had been purified from an agarose gel.

A number of colonies which were identified as being positive were inoculated into L-broth and incubated overnight at 37°C in order to prepare plasmid minipreps. Miniprep DNA was digested with *Eco*RI and analysed on an agarose gel to check for the presence of the *virB* promoter fragment. Those plasmids which contained the fragment (Figure 5.6b) were then digested with *Sal*I to determine the orientation of the *virB* promoter in each case. Figure 5.6a shows the result of electrophoresing the digested plasmids on an agarose gel. Of the five plasmids analysed, three contained the *virB* promoter in the correct orientation with respect to the *chiB* gene. The correct plasmid was designated pBCB1.

Figure 5.5 The construction of the *virB-chiB* plasmids.

A schematic diagram to show the construction of pCB1, pBCB1 and pBCB2.

Key:- H = *Hin*dIII; E = *Eco*RI; X = *Xba*I.

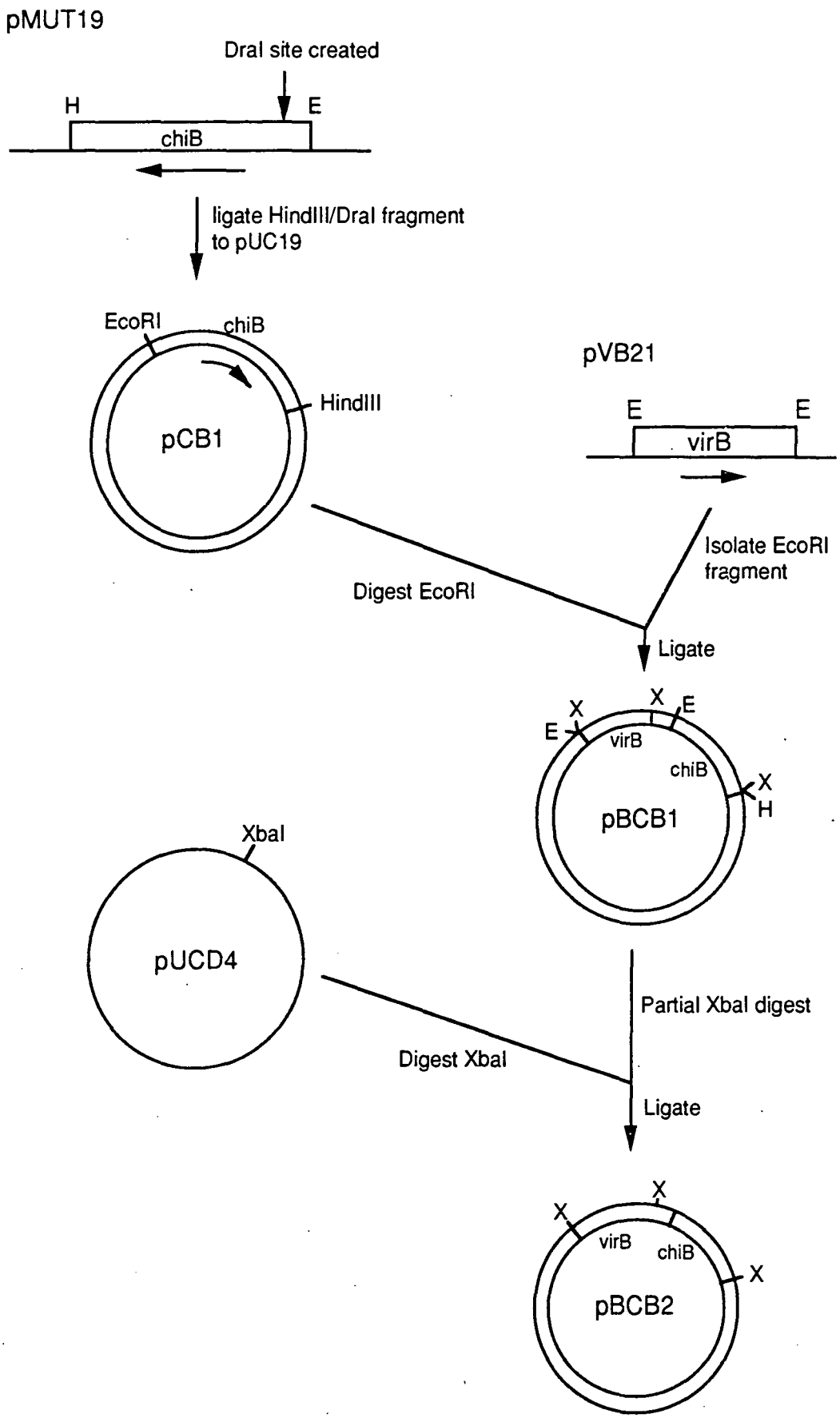

pMUT19
DraI site created
H
E
chiB
ligate HindIII/DraI fragment
to pUC19
EcoRI
chiB
HindIII
pCB1
pVB21
E
E
virB
Isolate EcoRI
fragment
Digest EcoRI
Ligate
X
X
E
E
virB
chiB
X
H
pBCB1
XbaI
pUCD4
Partial XbaI digest
Digest XbaI
Ligate
X
X
virB
chiB
X
pBCB2

Figure 5.6 Construction of plasmids carrying the *virB* promoter linked to the *chiB* coding region.

A) *Sal*I digests of miniprep DNA to determine which plasmid contained the *virB* promoter in the correct orientation.

Lane 1 λ*Pst*I
Lane 2 virB-chiB 1
Lane 3 virB-chiB 2
Lane 4 virB-chiB 3
Lane 5 virB-chiB 4
Lane 6 virB-chiB 5
Lane 7 -
Lane 8 λ*Pst*I

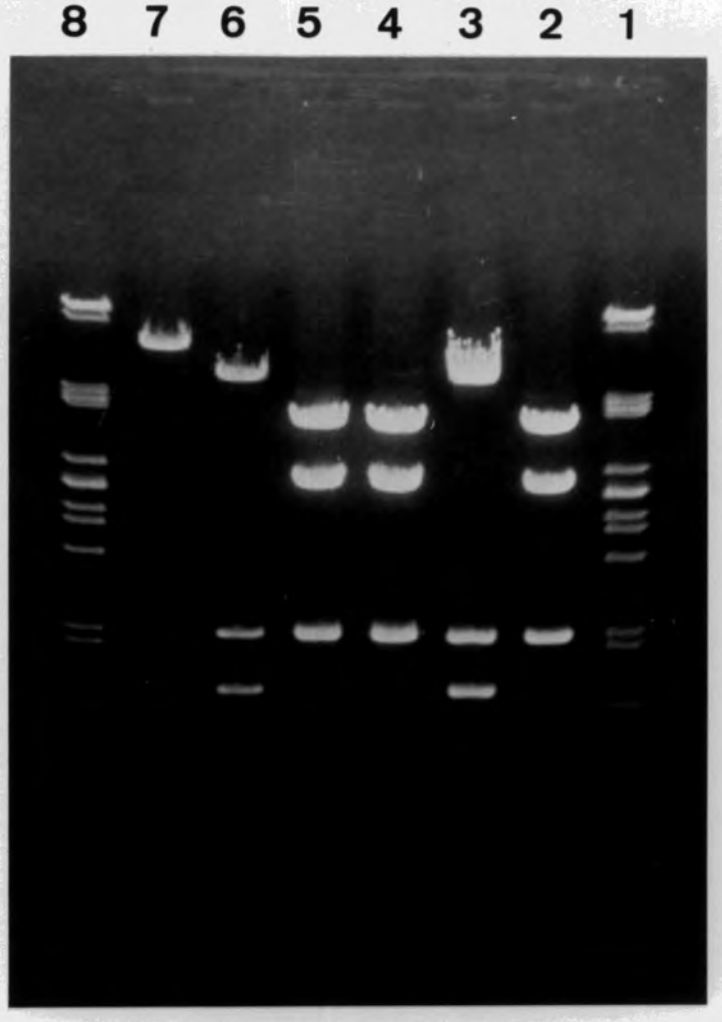
Lanes 2, 4 and 5 contained DNA from plasmids with the promoter in the correct orientation. *VirB-chiB* 1 was designated pBCB1.

B) Lane 1 λ*Pst*1 size markers
Lane 2 pBCB1 *Eco*RI
This shows the *virB* promoter *Eco*RI fragment cloned into pCB1.

C) Cloning of the *virB-chiB* cassette into pUCD4. Miniprep DNA from pBCB1 and pBCB2 was digested with *Xba*I to demonstrated the presence of the *Xba*I fragments containing the *virB-chiB* cassette.

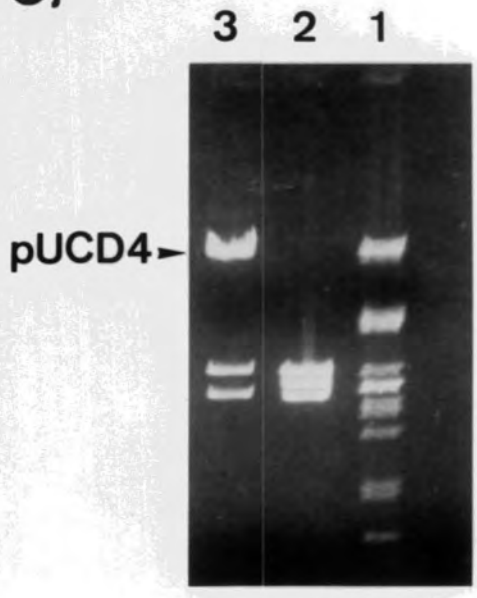
Lane 1 λPstI
Lane 2 pBCB1 XbaI
Lane 3 pBCB2 XbaI

A]



B]

C]



The promoterless *chiB* gene carried on pBC1 had now been linked to the *virB* promoter which had been taken from pUCD1187 and thus a *vir*-promoter-pesticide construct had been created (Figure 5.5).

5.4 Transfer of the *virB-chiB* cassette to a broad host-range vector.

Since pBCB1 was a pUC-based plasmid it did not possess the essential functions needed to replicate in *Agrobacterium*. Therefore it was necessary to transfer the *vir*-chitinase cassette to a broad host-range vector which was able to exist in both *E. coli* and *A. tumefaciens*. The vector chosen was pUCD4 which has a number of unique, screenable cloning sites. This plasmid was obtained from pUCD607 which is a derivative of pUCD4 containing a promoterless *lux* operon inserted as a single fragment into the *SalI* site (Shaw & Kado, 1986). In order to recreate pUCD4 for use as a cloning vector approximately 1µg of pUCD607 was digested with *SalI*. Following phenol/chloroform extraction and ethanol precipitation the DNA was resuspended in 10ml of distilled water and 2µl of this was self-ligated in a total reaction volume of 20µl. This dilution of the DNA should have ensured that the majority of the religated plasmids lacked the *SalI* fragment. The ligation mix was used to transform competent DH5α and transformants were selected on L-amp/tet plates since removal of the *lux* fragment should have restored tetracycline resistance. A plasmid corresponding to pUCD4 was identified from restriction analysis of miniprep DNA from a number of transformants.

The *virB-chiB* cassette was then removed from pBCB1 as an *XbaI* fragment and inserted into the *XbaI* site of pUCD4. To achieve this it was necessary to carry out a partial digest of pBCB1, digesting 2µg of pBCB1 in a total volume of 30µl at room temperature and removing 2µl from the digest every 4 minutes into 2.4µl of 50mM EDTA pH8.0 and 5µl of stop dye. The partial digest was electrophoresed on an agarose gel and the fragment isolated which corresponded to the entire *vir*-chitinase DNA. This was ligated to pUCD4 digested with *XbaI* and, since there was no selection for recombinant plasmids, phosphatase treated. Transformants were selected on L-amp plates and were streaked onto a master plate and nitrocellulose filter in order to carry out a colony hybridisation as described in section 2.7.14. The probe used was the same *virB* promoter fragment as that employed in section 5.3. Colonies which were identified as positive were grown up overnight in 5ml of L-broth and miniprep DNA was prepared. This was digested with *XbaI* and analysed on an agarose gel. A plasmid which showed the correct restriction pattern (see Figure 5.6c) was designated pBCB2.

5.5 Conjugation of pBCB2 into *Agrobacterium.*

As pUCD4 is a mobilisable broad host-range plasmid, pBCB2 could now be introduced into *Agrobacterium* by means of a triparental mating, using pRK2013 as a helper plasmid. This was carried out according to section 2.17 with DH5α(pBCB2) as the donor strain, HB101(pRK2013) as the helper strain and C58C^1(pVK257) as the recipient strain. Transconjugants were selected on L-RifKmCb plates. Carbenicillin was substituted for ampicillin when *Agrobacterium* was involved. Aliquots of donor and recipient strain cultures were also plated onto the same selective media to confirm that the selection was sufficiently stringent - neither single culture should have been able to grow under the selection. Presumptive transconjugants were subjected to the 3-keto-lactose test as described in section 2.14.

Two positive colonies were chosen for further analysis to check for the presence of both pVK257 and pBCB2, since C58C^1 which had acquired pBCB2 but had lost pVK257 would be resistant to the antibiotics used in the selection process. The two colonies were inoculated into L-broth containing carbenicillin and kanamycin and incubated with shaking at 28°C for 48 hours. Miniprep DNA was obtained from each culture, digested with *Sal*I and analysed on an agarose gel. In addition pVK257 and pBCB2 were each separately digested with *Sal*I and run on the same gel (Figure 5.7a). The second transconjugant did not contain pVK257 and so was discarded. The first transconjugant contained two plasmids. One of these was pVK257 whilst the other contained a *Sal*I fragment of approx. 11kb which was present in the pBCB2 digest. However, instead of two smaller fragments of 4.3 and 2.8kb there was a single fragment of approx. 7kb. Thus pBCB2 may have lost a *Sal*I site during its transfer to *Agrobacterium.*

The miniprep DNA from this transconjugant was digested again with each of *Sal*I, *Eco*RI and *Pst*I. Control digests were included and the DNA electrophoresed on an agarose gel (Figure 5.7b). The miniprep digests with *Eco*RI and *Pst*I show an identical fragment pattern to that displayed by similarly digested pVK257 and pBCB2. Only the fragments obtained from the *Sal*I digest differ in size and number. Upon examination of sequences present in pBCB2 it was noted that the following series of restriction sites existed:-*Bam*HI - *Xba*I - *Sal*I - *Xba*I followed immediately by the *virB* promoter fragment

During the construction of pBCB2 (see section 5.4) the partial *Xba*I digest gave rise to a fragment which included both *Xba*I sites 5' of the *virB* promoter. Upon conjugation of the plasmid into C58C^1 which is recombination proficient, the DNA between the two sites must have looped out causing the loss of the intervening *Sal*I site. This would explain the pattern of fragments observed following digestion of the transconjugant miniprep DNA with *Sal*I. A repeat conjugation of pBCB2 into C58C^1 gave rise to the same result (data not shown).

Figure 5.7 Restriction digests of miniprep DNA from *Agrobacterium* transconjugants possibly containing pBCB2.

A) Lane 1 size markers

Lane 2	pVK257	SalI
Lane 3	pBCB2	SalI
Lane 4	Transconjugant 1	SalI
Lane 5	Transconjugant 2	SalI

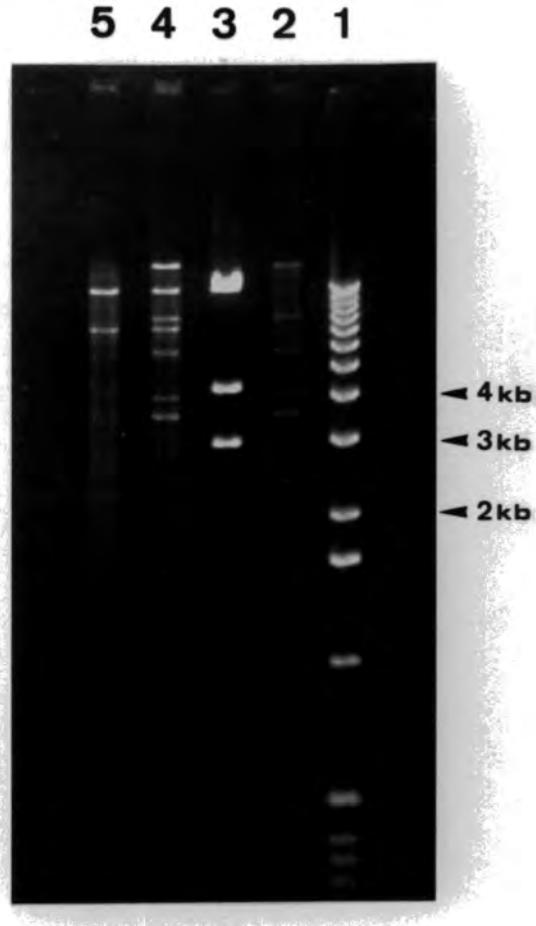
Transconjugant 2 did not contain pVK257.

Transconjugant 1 contained pBCB2 which had lost a SalI site.

B) Further restriction digests of miniprep DNA from transconjugant 1.

Lane 1	λ*Pst*I	
Lane 2	Transconjugant 1	*Eco*RI
Lane 3	pVK257	*Eco*RI
Lane 4	pBCB2	*Eco*RI
Lane 5	Transconjugant 1	*Sal*I
Lane 6	pVK257	*Sal*I
Lane 7	pBCB2	*Sal*I
Lane 8	Transconjugant 1	*Pst*I
Lane 9	pVK257	*Pst*I
Lane 10	pBCB2	*Pst*I
Lane 11	λ*Pst*I	

A)



B)

5.6 Acetosyringone-inducible chitinase production by *Agrobacterium* harbouring the *virB - chiB* construct.

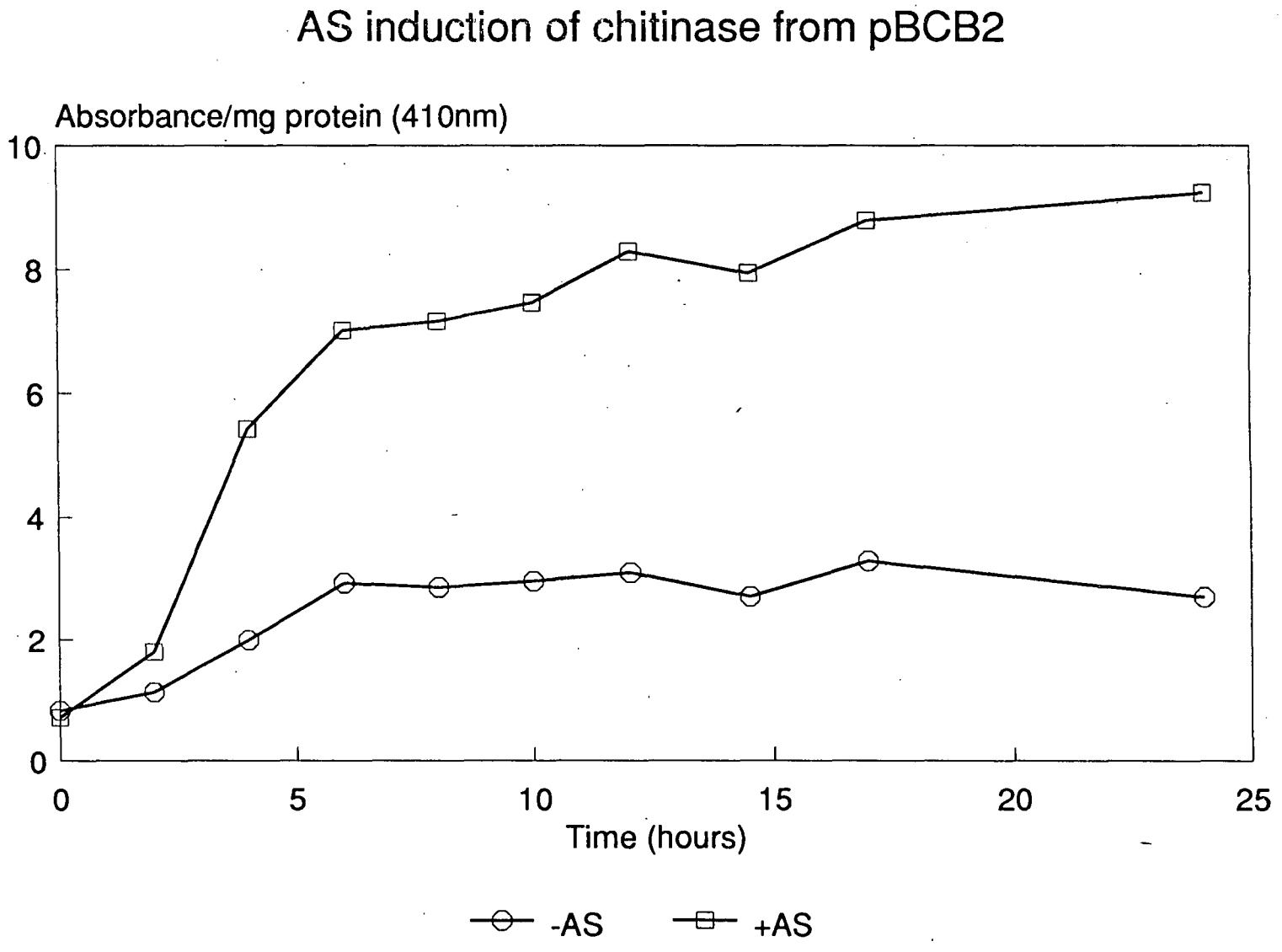
The aim of this project had been to construct plasmids which could be used as part of a plant protection system in *Agrobacterium*. A key feature of this system is that expression of a pesticidal protein is induced by phenolic compounds present in plant wound exudates. Therefore, as a last step, it was necessary to demonstrate that chitinase expression in C58C^1(pVK257, pBCB2) was inducible by acetosyringone, a potent *vir*-inducer. pVK257 provided the *virA* and *virG* recognition and transcriptional activation functions essential for the induction of the *virB* promoter. In order to investigate the expression of ChiB in this system chitinase activity was measured over a period of 24 hours following addition of acetosyringone to a culture.

100ml of induction medium (see section 2.4) was inoculated with 1ml of a fresh overnight culture of C58C^1 harbouring pVK257 and pBCB2. This was incubated with shaking at 28°C for 8 hours. At this point the cells were in early - mid log. phase and the culture was divided equally into two. To one half of the culture was added 50µl of 10^{-1}M acetosyringone in 70% methanol, to give a final concentration of 100µM. To the second half of the culture was added 50µl of 70% methanol as a control. A 1ml aliquot was immediately removed from each culture to form the zero time sample and the cultures were then incubated at 28°C with vigorous shaking. Further 1ml samples were removed every 2 hours until 14 hours after the addition of acetosyringone or solvent. After this time two further sets of samples were taken at 17 and 24 hours. Each sample, when taken, was centrifuged for 2 minutes to pellet the cells and the supernatant was removed and stored at 4°C. Cell extracts were prepared from the cell pellets as described previously (section 3.8) and 50µl aliquots were assayed for chitinase activity using *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose (section 2.10). The reactions were allowed to proceed at 50°C for 20 minutes before being terminated by the addition of 10µl of 1M NaOH. The absorbance of each sample was measured at 410nm as described in section 2.10. Protein determinations of each cell extract were also carried out using the Bradford assay (section 2.15). The results are shown in graphical form in Figure 5.8, expressed as absorbance at 410nm per mg of protein.

The level of chitinase in the cell extracts from the induced culture started to increase almost immediately following the addition of acetosyringone. In fact, the level had doubled after 2 hours incubation and it then increased rapidly up to 6 hours, after which time the rate of increase dropped. Between 17 and 24 hours following induction the level of chitinase in the cell extracts appeared to increase very slowly, but samples would need to be taken during the intervening hours to obtain an accurate view of the situation. The culture which did not contain

Figure 5.8 Acetosyringone-inducible chitinase production by *Agrobacterium* C58C[1] harbouring pVK257 and pBCB2.

Time zero is the point of addition of acetosyringone or control solvent to the log. phase cultures. The experiment was performed twice and the same pattern of induction was observed each time.


AS induction of chitinase from pBCB2
Absorbance/mg protein (410nm)
10
8
6
4
2
0
0
5
10
15
20
25
Time (hours)
-AS
+AS

acetosyringone also showed a small increase in the amount of chitinase in the cell extract. This increase continued up until 6 hours following addition of the control solvent but no further increase was observed after this time. Only a low level of chitinase was detected in this culture at any point however.

The supernatant from each sample was also assayed for chitinase (section 2.10) but no sample showed any detectable activity. This was a surprising result in view of a preliminary experiment which had been carried out (result not shown). In this, a 10ml culture of C58C^1(pVK257, pBCB2), grown to log. phase in L-broth was divided into two and one half was induced with acetosyringone. Following overnight incubation, chitinase activity was clearly present in the supernatant of the induced culture but was absent from the non-induced culture. Thinking that secretion of the chitinase may be linked to growth of the cells, further samples were taken from the timed experiment at 36 and 48 hours. No chitinase activity could be detected in the supernatant of the cultures at these times even though the cells would have been in stationary phase.

5.7 *Vir* - chitinase constructs utilising *chiA*.

Results obtained whilst constructing a *virB-lux* plasmid for use as a reporter system for *vir*-induction indicated that the *virB* promoter utilised in the initial construction (pDUB2512) was not functioning efficiently (see Chapter 4). Possible reasons for this have been discussed previously (section 4.10.3). Thus the decision was taken to use the *virB* and *virE* promoters described in section 4.8 to reconstruct *vir* promoter-pesticide plasmids containing *chiA*. This was in an attempt to increase the production of ChiA from an acetosyringone-induced culture of *Agrobacterium*, achieving better levels of expression than those previously reported (Ashby, 1988).

5.7.1 Cloning of the *chiA* region from pDUB2512.

The plasmid pDUB2512 contained the coding region of the *S. marcescens chiA* gene linked to a *virB* promoter fragment derived from pSM30 (Ashby, 1988). The promoter region of the *chiA* gene had been deleted prior to cloning, thus the gene was in a suitable form to be utilised in further plasmid constructions in conjunction with alternative promoters.

1μg of pDUB2512 was digested with *Eco*RI and *Bam*HI and the 2.2kb *chiA* fragment was isolated from an agarose gel by the freeze-squeeze method (section 2.7.8). The fragment was ligated to pUC19 and the ligation mix was used to transform competent DH5α cells to

ampicillin resistance. White colonies on X-gal/amp plates were selected and miniprep DNA was obtained. Restriction analysis of the miniprep DNA was carried out to identify a clone with the correct insert. This was designated pCA8.

5.7.2 Construction of pBCA1.

In order to link the *chiA* coding region to the *virB* promoter described in section 4.8 it was first necessary to excise the *virB* promoter fragment from pVB1. 1µg of pVB1 was digested with *Hin*dIII and the 3kb *virB* fragment was isolated following electrophoresis on an agarose gel (section 2.7.8). The fragment was ligated to pCA8 which had been digested with *Hin*dIII and phosphatased to prevent self-ligation. Transformants were selected on L-amp plates and a number were chosen for screening by restriction analysis of miniprep DNA. Those plasmids which were found to contain the correct *Hin*dIII fragment were then digested with *Bam*HI to identify one which contained the promoter in the desired orientation. pUC19 carrying the *virB-chiA* cassette was designated pBCA1 (Figure 5.9).

The *Hin*dIII fragment removed from pVB1 did not contain the entire fragment originally isolated from pUCD1187 but was approximately 200bp shorter at the 3' end. The limited sequence data available at the time indicated that this loss of such a small amount of DNA would not affect the promoter activity of the fragment. The sequence lost would only be a part of the coding region of one of the *virB* genes, whilst the promoter region of the operon was further upstream towards the 5' end of the DNA fragment.

5.7.3 Construction of the broad host-range plasmid pBCA2.

pBCA1, being a pUC-based plasmid, could not be maintained in *Agrobacterium*, so again it was necessary to employ a broad host-range vector. In this instance no suitable sites were present within pUCD4, so it was decided to employ an alternative strategy involving the formation of a cointegrate plasmid consisting of pBCA1 and pGV1106. pGV1106 is a broad host-range plasmid that carries the origin of replication of the W-type plasmid pSa thus allowing it to replicate in *Agrobacterium* (Leemans *et al.*, 1982). Both pBCA1 and pGV1106 possess a unique *Eco*RI site and so 500ng of each plasmid were combined in a total volume of 20µl and digested with *Eco*RI. The digest was terminated by phenol/chloroform extraction and following ethanol precipitation the DNA was resuspended in sterile distilled water. Ligation buffer (section 2.7.6) and 1 unit of T4 DNA ligase were added to the digested DNA and the ligation was allowed to proceed overnight at 15°C. *E. coli* DH5α transformants were selected on L-amp kan plates. Since pBCA1 possesses ampicillin resistance and pGV1106 is resistant to

Figure 5.9 Construction of *virB-chiA* plasmids.

A schematic diagram to show the construction of pBCA1 and pBCA2.

Key:- H = *Hin*dIII; B = *Bam*HI; E = *Eco*RI.

kanamycin and gentamycin, selection on L-amp kan plates would only allow the growth of cells containing a cointegrate plasmid, or possibly the two original plasmids coexisting in the same cell.

Five transformants were obtained under this selection and miniprep DNA was obtained from each. Undigested DNA was first electrophoresed on an agarose gel to ensure that only one plasmid was present in each case. Each plasmid was then digested with *Eco*RI and *Eco*RI/*Bam*HI and analysed by gel electrophoresis to verify that the correct fragments were present (Figure 5.10a). The required plasmid cointegrate was designated pBCA2 (Figure 5.9).

5.7.4 Transfer of pBCA2 to *Agrobacterium.*

pBCA2 was transferred to *Agrobacterium* C58C^1(pVK257) by means of a triparental mating as detailed in section 2.12. Resulting transconjugants, selected on L-RifKmCb plates, were subjected to the lactose plate test (section 2.14) to confirm that they were *Agrobacterium.* Four colonies which gave a positive result were inoculated into L-broth + ampicillin and kanamycin and grown overnight at 28°C so that miniprep DNA could be obtained. The miniprep DNA from each transconjugant was digested with *Eco*RI and analysed on an agarose gel together with pBCA2 and pVK257 each digested with *Eco*RI. The result is shown in Figure 5.10b. One transconjugant had lost pVK257 since there was no specific selection for this cosmid, but the other three each contained both pVK257 and pBCA2. One of the desired transconjugants was selected for studies on the expression of ChiA (see section 5.8).

5.7.5 Construction of pECA1.

To determine whether the *virE* or *virB* promoter would be the most effective one to use in further *vir* promoter-pesticide constructs a plasmid was also created in which the *virE* promoter of pTiC58 was linked to the *chiA* coding region. It was hoped to then compare the levels of chitinase expression generated by the two promoters.

The *virE* promoter fragment of pEV5 (see section 4.8) was excised as a *Bam*HI fragment and isolated from an agarose gel by the freeze-squeeze method (section 2.7.8). This was ligated to pCA81 digested with *Bam*HI and phosphatased. pCA81 had been constructed by transferring the *Bam*HI - *Eco*RI *chiA* fragment from pCA8 to the Bluescript vector SK$^+$. The ligation mix was used to transform competent *E. coli* DH5α and transformants were selected on L-amp plates. Resulting colonies were streaked onto a nitrocellulose filter and a corresponding master plate so that a colony hybridisation could be carried out to identify clones containing the *virE*

Figure 5.10 Construction of pBCA2 and its transfer to *Agrobacterium*.

A) Lane 1 pBCA2 *Eco*RI
Lane 2 pBCA2 *Eco*RI/*Bam*HI
Lane 3 λ*Pst*I size markers

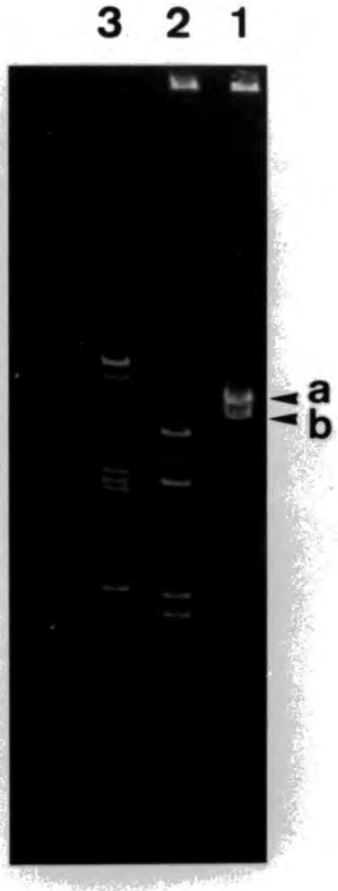
Fragment a) is the pGV1106 part of the cointegrate.
Fragment b) is the pBCA1 part of the cointegrate.

B) Confirmation that pBCA2 had been transferred to *Agrobacterium*.

Lane 1 λ*Pst*I size markers
Lane 2 Transconjugant 1 *Eco*RI
Lane 3 Transconjugant 2 *Eco*RI
Lane 4 Transconjugant 3 *Eco*RI
Lane 5 Transconjugant 4 *Eco*RI
Lane 6 pVK257 *Eco*RI
Lane 7 pBCA2 *Eco*RI
Lane 8 λ*Pst*I size markers

Transconjugant 4 did not contain pVK257.

A]



B]

promoter fragment (section 2.7.14). The probe used in this experiment was the *Bam*HI fragment of pVE5 containing the *virE* promoter. Colonies which were revealed as positive in the hybridisation screening were then chosen for restriction analysis of their miniprep DNA. Digestions were carried out first with *Bam*HI to confirm the presence of the correct insert and then with *Pst*I to identify a clone with the promoter in the correct orientation. This was nominated pECA1.

5.7.6 Construction of pECA2.

The broad host-range vector chosen to carry the *virE-chiA* cassette was again pUCD4. pECA1 was digested with *Xba*I and *Kpn*I which removed the entire *virE-chiA* cassette as a single fragment. This fragment of approximately 3kb was isolated from an agarose gel and ligated to pUCD4 which had been digested with *Xba*I and *Kpn*I. The ligation mix was used to transform competent DH5α and transformants were selected on L-amp plates. Resulting colonies were replica plated onto L-kan plates since insertion between the *Xba*I and *Kpn*I sites would have inactivated the kanamycin resistance gene of pUCD4. Miniprep DNA from colonies which were ampicillin resistant but kanamycin sensitive was digested with *Xba*I and *Kpn*I and analysed on an agarose gel. One plasmid which contained the correct *virE-chiA* insert was chosen as pECA2.

pECA2 was introduced into *Agrobacterium* C58C^1(pVK257) using the electroporation method described in section 2.13. Transformants were selected on L-RifKmCb plates and a number were chosen for restriction analysis of miniprep DNA to check that both pVK257 and pECA2 were present.

5.8 Acetosyringone-inducible chitinase expression from the *vir-chiA* constructs.

It was next essential to test the two *vir* promoter-*chiA* constructs for acetosyringone-inducible expression of chitinase. 100μl aliquots of an overnight culture of *Agrobacterium* harbouring one of the constructs were inoculated into 5ml of L-broth +/- acetosyringone. These were incubated for 24 hours at 28°C and the culture supernatants then assayed for chitinase as described in section 2.10. The supernatant of each culture lacking acetosyringone displayed negligible chitinase activity, whilst the supernatants from the induced cultures each contained an active chitinase. It appeared from this initial experiment that both constructs expressed inducible ChiA and the cells containing pECA2 produced more chitinase than those containing pBCA2. It was now necessary to carry out more rigorous assays to determine the comparative inducibility of the two constructs.

An identical procedure was followed for each construct. 100ml of induction medium (section 2.4) was inoculated with 1ml of a fresh overnight culture of C58C^1(pVK257) harbouring one of the constructs and incubated with shaking at 28°C for 8 hours. After this time the culture was divided equally into two. To one flask was added 50µl of 10^{-1}M acetosyringone and to the other was added 50µl of 70% methanol. A 1ml aliquot was immediately removed from each flask to act as a zero time sample and the cultures were then returned to incubate at 28°C. Further 1ml aliquots were removed at various time points. (Figures 5.11 and 5.12).

Cell extracts were prepared from each sample and the protein content determined using the Bradford assay (section 2.15). The protein content of the supernatant fraction of each sample was also determined. 50µl of each cell extract was assayed for chitinase activity according to section 2.10 and 100µl aliquots of the supernatant samples were also assayed. The cell extract assay was incubated at 50°C for 30 minutes, whilst the supernatant assay was allowed to proceed for 16 hours.

The results of the cell extract assays were calculated as absorbance at 410nm per mg of protein and are presented in Figure 5.11 (for C58C^1 containing pECA2) and Figure 5.12 (for C58C^1 harbouring pBCA2).

The results for pECA2 (Figure 5.11) indicated that induction of chitinase took place following addition of acetosyringone to the culture. The level of chitinase increased from the point of addition of acetosyringone up until 15 hours post-induction after which time it dropped slightly. The uninduced culture displayed only a small increase in the level of cellular chitinase and this remained well below the level present in the induced cells. This result demonstrated that the *virE-chiA* construct functioned correctly. The *virE* promoter responded to induction of the culture with acetosyringone, this induction being mediated by VirA and VirG expressed from pVK257.

Assays carried out on the corresponding extracellular fractions failed to detect any chitinase activity in the culture supernatants. This was a comparable result to that obtained with C58C^1 containing pBCB2: the cell extract but not the supernatant of induced cultures grown in induction medium contained active chitinase, whereas chitinase was present in the supernatant of induced cultures grown in L-broth.

A similar result was obtained with pBCA2. No chitinase activity was detectable in any of the supernatant samples but was present in the cell extracts (see Figure 5.12). Chitinase activity of the extract increased for both induced and non-induced cultures from the 2 hour time point.

Figure 5.11 Acetosyringone-inducible production of chitinase by *Agrobacterium* C58C1 harbouring pVK257 and pECA2.

The chitinase assays were carried out on cell extracts from induced and non-induced cultures. The experiment was performed twice and the same pattern of induction was observed each time.

AS induction of chitinase from pECA2

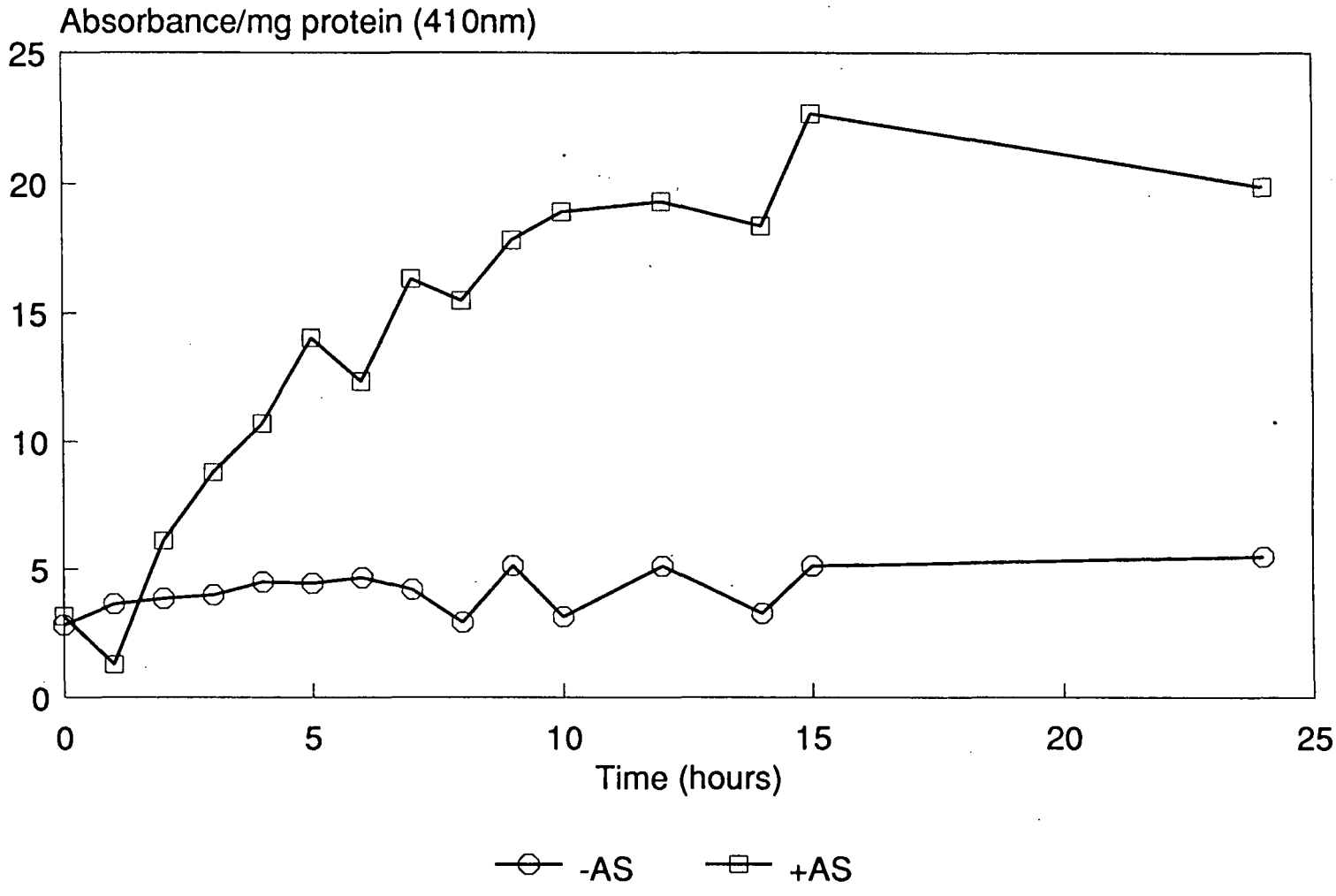
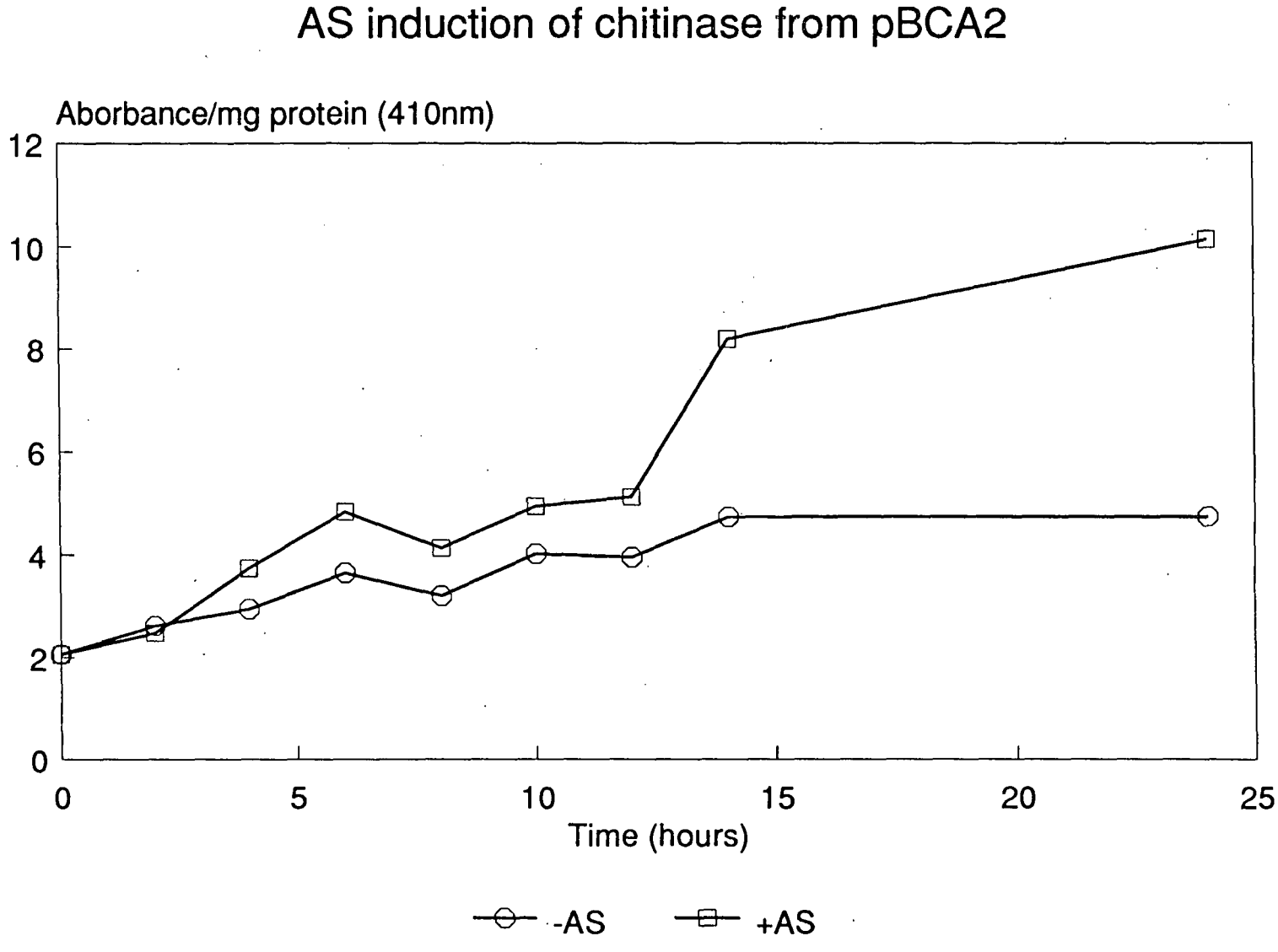


Figure 5.12 Acetosyringone-inducible chitinase production by *Agrobacterium* C58C1 harbouring pVK257 and pBCA2.

This graph shows the results of chitinase assays carried out on cell extracts as described in the text. The experiment was performed three times and neither time was there a large difference between the chitinase activity in the induced and non-induced cultures. In one experiment there was an equal amount of "induction" observed in each culture.


AS induction of chitinase from pBCA2
Aborbance/mg protein (410nm)
12
10
8
6
4
2
0
0
5
10
15
20
25
Time (hours)
-AS
+AS

Although the difference between the amounts of chitinase in the two cultures was relatively low, there was a greater increase in the induced culture.

5.9 DISCUSSION.

Three plasmids were constructed which could be utilised in a plant protection system involving *Agrobacterium* as described in section 5.1. Each plasmid was shown to confer upon *Agrobacterium* the ability to produce chitinase in the presence of the phenolic inducer acetosyringone. In each case, when cells were grown in induction medium, chitinase activity was detectable only in cell extracts and not in the culture supernatants. This corresponded to results obtained by Ashby (1988) and will be discussed later. The results also implied that the *virE* promoter, when used in such a construct, conferred greater inducibility and better control of expression of the pesticidal gene than did the *virB* promoter.

5.9.1 Relative efficiencies of the *virB* and *virE* promoters.

Earlier experiments had demonstrated that the *virB* promoter fragment from pDUB2512, when linked to the *lux* operon of pUCD615, only caused a low level of acetosyringone-inducible light production in *Agrobacterium* (see Chapter 4). This was in contrast to the results reported by Rogowsky *et al.* (1987) who achieved 100-fold greater induction using a *virB* promoter fragment from pTiC58. Thus it was decided that in all future constructs either the *virB* or *virE* promoter from pTiC58 should be used. These two promoters, when fused to the *lux* operon in pUCD1187 or pUCD1194 caused equally high levels of expression of the *lux* genes upon induction with acetosyringone, although pUCD1194 gave slightly higher levels of basal expression (Rogowsky *et al.*, 1987). Both promoter fragments had been excised from their respective *lux* plasmids and cloned into pUC19 for use in further plasmid constructs (section 4.8). The fragment containing the *virB* promoter was a large fragment of 3.4kb. Although no sequence data for the pTiC58 *virB* operon was available at the time it could be deduced that this DNA fragment contained extensive sequences both 5' to the promoter region, including the 3' end of *virA* and downstream of the promoter region encompassing the coding regions of one or more of the *virB* open reading frames. It was not therefore an ideal fragment to use although it had been successfully employed in pUCD1187. For this reason, the *virE* promoter fragment which was considerably smaller (0.7kb) and for which sequence data was available (Hirooka *et al.*, 1987) was selected as an alternative for use in *vir*-pesticide constructs.

In order to make an accurate comparison of the efficiencies of these two promoters in such constructs, each promoter was linked to the coding region of the *chiA* gene of *S. marcescens*.

This gene had been successfully utilised in a *virB-chiA* construct by Ashby (1988). The pTiA6 *virB* promoter fragment included in the construct was less efficient than either the pTiC58 *virB* or *virE* promoter (Chapter 4). It was therefore appropriate to recreate a *vir*-inducible *chiA* gene to achieve higher levels of expression.

Agrobacterium containing pECA2 produced the greatest amount of chitinase with an absorbance/mg protein of 22.67 being recorded at one point. The maximum value obtained with either *virB* construct was 10.14. However, the *virE* construct also caused a slightly higher level of basal chitinase expression. This reflected the result obtained by Rogowsky *et al.* (1987) who found the *virE-lux* plasmid, pUCD1194, caused higher basal expression than the *virB-lux* fusion.

The results obtained did not show a large difference between the induced and non-induced levels of chitinase activity. In particular, the culture containing pBCA2 displayed an increase in chitinase production when no acetosyringone was added. After 24 hours the induced culture contained only twice the chitinase activity of the non-induced culture.. A possible explanation for this is the growth conditions used for the experiment. When 5ml cultures were grown in L-broth +/- acetosyringone and samples taken after 16 hours, the basal expression in the non-induced culture was barely detectable with much higher levels of chitinase activity present in the induced cultures. L-broth, having a pH of approximately 7.0 is recognised as being a poor medium for *vir*-induction (Alt-Moerbe *et al.*, 1989). In contrast, the induction medium used in the time course experiments was optimised to provide the best conditions for *vir*-induction. These conditions included a low pH of 5.5 and a low phosphate concentration. Both of these enhance *vir*-induction by initiating transcription of *virG* from its P1 and P2 promoters in the absence of acetosyringone, thus increasing the pool of VirG available for phosphorylation by VirA (Winans, 1990). It has been reported that an increase in the copy number of *virG* led to considerably elevated basal expression of *virB* and *virE* in the absence of acetosyringone. It was suggested that since increases in VirG alone were sufficient to increase *vir*-gene expression, then the VirG protein in its unactivated (ie. unphosphorylated) form possessed some transcriptional activation capability (Rogowsky *et al.*, 1987).

The increase of VirG protein caused by the low pH and low phosphate content of the induction medium may have been responsible for the high basal level of chitinase found in all cultures. Any attempts to initiate *vir*-induction of an *Agrobacterium* culture under optimum conditions in the laboratory are obviously far removed from the *in situ* soil environment of *Agrobacterium*. The experiments described in this chapter merely demonstrated that inducible expression of chitinase genes under *vir*-control was possible. The implication from this result is that the system will perform similarly in a natural environment where *vir*-induction would

normally occur. In such conditions it is possible that the high constitutive chitinase expression observed *in vitro* would not be present.

Overall, the results of the induction studies indicated that the *virE* promoter was probably a better choice for use in such constructs than the *virB* promoter. The result obtained with pEG2 (section 4.9.3) provided further evidence that high levels of inducibility were possible using the *virE* promoter. It is difficult to compare the levels of chitinase produced by the three constructs described here with that of the *virB-chiA* construct of Ashby (1988). The experiment carried out with pDUB2513 measured only the chitinase activity present in the culture supernatant and the results were presented merely as absorbance values with no indication of either protein content or cell numbers. Additionally, the length of incubation of the chitinase assay was not stated but can be assumed to be overnight. Nevertheless, by comparing these results with readings taken during the course of the experiments described in this chapter, it would appear that pECA2 gives much higher expression of chitinase than pDUB2513 although the level of constitutive expression is also slightly higher (possibly for reasons described above). The *virB-chiA* constructs also produce a greater amount of chitinase although the difference is not as marked. The maximum absorbance value recorded by Ashby during the chitinase assays was 0.7 whereas a value of 2.5 was recorded when assaying extracts of an induced culture of pECA2. By making a comparison with the expression of chitinase in *E. coli*, the level of enzyme in the culture supernatant would be expected to be lower than that found in the cell extract. However, since the cell extract assays were only allowed to proceed for 30 minutes whereas the assays carried out by Ashby on the culture supernatants were incubated for at least 16 hours it appears that the constructs described in this chapter cause greater amounts of chitinase to be produced.

5.9.2 The effect of pH on chitinase activity.

No chitinase activity could be detected in the culture supernatant of either construct, even when incubation with acetosyringone was continued for 48 hours, ensuring the cells would be in stationary phase. A similar situation was reported by Ashby (1988) when assaying chitinase production by C58C[1](pDUB2513). In this case, chitinase assays were only carried out on culture supernatants. No activity was evident upon induction at pH5.7 in either MinA or MS medium. Activity was detected however at pH7.0. Two hypotheses have been proposed to account for this observation (Ashby, 1988). Either modifications made to the *virB* promoter sequence had caused a "pH mutation" leading to a shift in the optimum pH for *vir*-induction, or the chitinase enzyme was not stable at the lower pH.

The results presented here challenge the hypothesis which suggests that the pH optimum for *vir*-induction had been altered. Using a different *vir*-promoter which had not been modified in any way, chitinase activity was also found to be absent from the supernatant of a culture grown in induction medium at pH5.5. However, it was present in the supernatant of a culture grown in L-broth at pH7.0. The cell extracts of cultures grown in both media contained chitinase. Since chitinase was produced in media of both pH5.5 and 7.0, then the pH was not affecting the *vir*-induction process or the expression of the chitinase genes. It is suggested that if Ashby had assayed cell extracts from cultures grown at pH5.7 then chitinase activity would have been present. Thus the lack of chitinase activity in the supernatants must have been due to either the non-release of the enzyme from the cells or the detrimental effect of low pH on the activity of the enzyme. Raising the pH of the induction medium to 6.0 did not alter the level of chitinase activity detectable in the supernatants (results not shown).

Monreal and Reese (1969) reported that the optimal pH for chitinase production by *S. marcescens* was 7.5, whilst Ashby (1988) found that *S. marcescens* grown on agar plates containing chitin produced an active chitinase at pH7.0 but not at pH5.7. These figures do not reflect the conditions for activity of the enzymes however. The crude chitinase of *S. marcescens*, ie. the enzyme present in the culture supernatant, was found to have maximum activity at pH6.4. Relative activity dropped to approximately 10% at a pH of 5.0, although at pH5.5 the activity was still 80% of the maximum. Enzyme stability was only slightly reduced at pH5.5 and the pH had to fall to 4.8 before 50% of the enzyme activity was lost in one hour (Monreal & Reese, 1969).

If the pH of the growth media used in the experiments described in sections 5.6 and 5.8 had remained constant then the activity of the chitinase enzymes should not have been adversely affected. However if, despite the buffering capacity of the medium, the pH had dropped during incubation of the culture to pH5.0 or below, then this could account for the lack of chitinase activity observed in the culture medium. Only a relatively small drop in pH would be necessary to cause a large drop in activity. To clarify this situation the experiments would need to be repeated with the pH of the medium measured at intervals throughout the incubation.

5.9.3 Advantages of using an inducible microbial inoculant as a biocontrol agent.

Conventional disease and pest control has relied heavily on chemical fungicides and pesticides. Precise application of these chemicals to the desired location on the plant foliage or roots is impractical and therefore general spraying of crops and soil is widespread. This results in more pesticides being introduced into the environment than is necessary. Recently, the hazards associated with the use of chemicals in this manner, including the accumulation of

pesticides in water supplies and contamination of food products, have led to general public concern. This has been partly responsible for a renewed interest in biological control with several companies operating programmes to develop biocontrol agents as commercial products (Weller,1988). The use of microbial inoculants as biological disease-control organisms has several advantages over the use of conventional chemical control. Microbial inoculants are considered safer than many of the chemicals currently in use since if properly developed they are not harmful to the environment and will not accumulate in the food chain. As they are self-replicating this eliminates the need for repeated application throughout the growing season. Finally, resistance of the target organism is less likely to occur than when chemical products are used. This is aided by the fact that delivery of the active biocontrol product is more precise and so excessive levels are not widespread in the environment.

The use of a system such as the one described in section 5.1 has a further advantage in that expression of the active biocontrol product by the microbial inoculant is regulated. By using the *vir*-gene system of *A. tumefaciens* it has been possible to engineer *Agrobacterium* to express a pesticidal product only when in the presence of inducing compounds released from wounded plant cells. The bacteria can detect and respond to very low concentrations of compounds in plant wound exudates by moving chemotactically towards the site of the injury. Once at the wound site the higher concentrations of inducing compounds trigger the induction of the *vir*-gene system and thus the expression of the linked gene and the production of its pesticidal protein. Hence the pesticide is only produced when it is required and at the site where it will be most effective.

This mirrors a situation found in some plants where expression of pathogenesis-related defence proteins is induced by stress or pathogen attack. Proteins induced by this response include enzymes involved in the synthesis of phytoalexins (Darvill & Albersheim, 1984) and lytic enzymes (eg. chitinases and glucanases) which are capable of degrading fungal cell walls (Boller *et al.*, 1983). Such a system is obviously of benefit to the plant since it is not using valuable resources to constitutively express proteins which are only required at specific times. Equally, a regulated system for the production of a pesticide will afford similar benefits to a bacterium.

The development of successful plant transformation systems has led to the production of transgenic plants which will express additional pesticidal proteins to enhance their natural defence strategies. The *chiA* gene of *S. marcescens* has been introduced into tobacco and is constitutively expressed from two photosynthetic gene promoters (Jones *et al.*, 1988). The *Agrobacterium* system may offer advantages over transgenic plants. Each plant type to be protected by introduction of foreign genes must be individually transformed, whereas a single transformed *Agrobacterium* strain will be able to be used with a wide range of plant species.

Vir-inducing compounds have been identified in a large number of plants so the biocontrol system should function in each of these cases. Additionally, successful transformation of economically important monocotyledonous crop plants has not yet been achieved. Such plants, however, produce *vir*-inducing compounds (Messens *et al.*, 1990; Usami *et al.*, 1988) and chemotaxis has been observed towards monocot root and shoot extracts (Ashby *et al.*, 1988). Thus the *Agrobacterium* biocontrol system has the added benefit that it can be used to protect both monocot and dicot species.

5.9.4 Possible improvements to the *Agrobacterium* biocontrol system.

The plasmids whose construction has been described in this chapter form the most basic elements of an inducible biocontrol system involving *Agrobacterium*. A number of refinements could be made to improve the system. Obviously, *Agrobacterium* containing a plasmid with either an inducible *chiA* or *chiB* gene should be tested to assess its plant protecting ability. The constructs of choice would be pECA2 and a plasmid combining the *virE* promoter with the *chiB* gene, since this promoter gave better production of chitinase. However, one of these chitinase genes acting on its own may not be the most effective pesticidal protein. It may be that a new construct combining the two chitinase genes under the control of the same *vir*-promoter would create a more effective biocontrol agent since the two enzymes acting together may enhance the degradation of chitin. Alternatively, a chitinase gene could be linked in the same plasmid with a gene encoding a β-1,3-glucanase. In many plants this enzyme is coordinately induced with chitinase upon infection (Shinshi *et al.*, 1987). A combination of the two enzymes may afford better protection against fungal infection.

In the area of biological control, much interest has centered around the δ-endotoxin of *Bacillus thuringiensis*. During sporulation, *B. thuringiensis* produces insecticidal, bipyramidal inclusion bodies that are composed of one or more polypeptides called crystal proteins. There are many distinct proteins produced by various *B. thuringiensis* isolates, each with a specific toxic effect directed against insects belonging to a certain order. The majority of *Bt* toxins are effective against Lepidoptera or Diptera (Hofte & Whitely, 1989). Thus a *Bt* toxin gene is a major contender for inclusion in a *vir*-promoter-pesticide construct. This would extend the usefulness of the system - chitinase producing microbial inoculants would primarily control fungi whereas bacteria producing *Bt* toxin would be effective in controlling insects. The δ-endotoxin gene from *B. thuringiensis* subsp. *kurstaki* HD-1 has been integrated into the chromosome of root-colonising *Pseudomonas fluorescens* and *Agrobacterium radiobacter* by Obukowicz *et al.* (1986) in an attempt to target the delivery of the insecticide to the rhizosphere. Utilising such a gene in the *Agrobacterium* biocontrol system described previously would refine

the approach further by adding a degree of inducibility to the delivery of the insecticide. As part of this project, work has already commenced on linking the δ-endotoxin gene from *B. thuringiensis* subsp. *kurstaki* HD-73 to a *vir*-promoter. The promoter region has been removed from the gene and the *virE* promoter fragment described in section 4.8 has been cloned upstream of the *Bt* coding region. Further work needs to be carried out with this construct to transfer the *virE*-toxin cassette to a broad host-range vector and test for inducibility of the toxin in *Agrobacterium*.

A vital part of the inducible *Agrobacterium* biocontrol system is the presence of both the *virA* and *virG* genes. These provide the necessary functions to ensure induction of the *vir*-promoter linked to the pesticide gene. Currently, these genes are located on the cosmid pVK257 which also carries the *virB* and *virC* operons of pTiA6. This is not an ideal situation since it requires the maintenance of two separate plasmids in the *Agrobacterium* cell. In the cases described in this chapter there is no individual antibiotic selection for pVK257 and so maintenance of this cosmid cannot be guaranteed. In a natural environment there would be no selection for either plasmid and the need for two plasmids to be present to enable the system to work would make it particularly inefficient. Initially it may be possible to combine both the *vir*-promoter-pesticide cassette and the *virA* and *G* genes on the same broad host-range vector. Ultimately, the ideal situation may be to integrate both these components into the bacterial chromosome to ensure maximum stability.

SUMMARY

SUMMARY

Agrobacterium tumefaciens, the causative organism of the disease Crown Gall, is a common component of the soil microflora and is found predominantly in the rhizosphere (Kerr, 1969 & 1974). The prevalence of *Agrobacterium* in the rhizosphere is likely to be due in part to the sensitive chemotactic response which it exhibits towards many sugars characteristic of plant exudates (Loake *et al.*, 1988). This response is mediated by chromosomal genes and so occurs for both virulent and avirulent bacteria. A futher level of attraction is then displayed as virulent Agrobacteria harbouring Ti-plasmids show positive chemotaxis towards phenolic compounds in plant wound exudates (Ashby *et al.*, 1987). This response requires the presence of two of the *Agrobacterium* virulence gene products, VirA and VirG (Shaw *et al.*, 1988). It is possible that VirA and VirG interact with components of the chromosomally encoded chemotaxis system. The attraction towards phenolic *vir*-inducing compounds such as acetosyringone is extremely sensitive with maximal levels of attraction occurring at a concentration of 10^{-7}M. This is 100-fold lower than the concentraion of acetosyringone which gives maximum *vir*-induction. Thus the bacteria are first attracted towards wounded plant cells by the low concentrations of phenolic compounds which they detect, then as they reach the plant the concentration of wound exudates increases until it is high enough to effect *vir*-induction.

These two responses of *Agrobacterium* combine to make it an effective root coloniser and disease causing organism, since *vir*-induction leads to transfer of the T-DNA and ultimately to the formation of a tumour at the site of infection. However, it has been proposed to make use of these responses in a different way and employ *Agrobacterium* as a disease control organism (Ashby, 1988). The idea was to place a gene encoding a pesticidal protein under the control of a *vir*-promoter so that its expression would be induced by compounds released from wounded plant cells. An avirulent *Agrobacterium* containing this construct and introduced into the rhizosphere would be attracted to wounded plant tissue provided the required *virA* and *virG* genes were also present in the cell. Once the bacterium reached the wound site the higher concentrations of *vir*-inducers would elicit expression of the pesticide.

The aim of this project was two-fold - to engineer constructs consisting of a *vir*-promoter and a pesticide gene which could be used in such a system and to devise a method of monitoring the actual sites where *vir*-induction was occurring in the rhizosphere. This was important because wherever *vir*-induction took place, then the pesticidal product would be produced. It was assumed that since *vir*-inducing compounds are present in the exudate from wounded plant

cells, *vir*-induction occurs specifically at sites of injury on the plant root. When a plant is injured it is particularly susceptible to invasion by opportunist pathogens. A biocontrol organism which can deliver a pesticidally active protein at this site would therefore be especially effective.

Biological control is currently enjoying renewed interest, although it has been practised for many years. Concern has increased recently about the hazards associated with the chemical fungicides and pesticides relied on for conventional forms of control and several companies are researching the possibility of introducing biocontrol agents onto the market. Biological control agents offer several advantages over chemical pesticides. Any active molecules they produce are generally harmless to the environment and will not accumulate to toxic levels in the food chain. Delivery of the active biocontrol product occurs at the site where it is needed and since the biocontrol agent is a living organism, its self-replication eliminates the need for repeated application. This ensures that excessive amounts of pesticides are not introduced into the environment unnecessarily. The inducible system described here refines the process even further as the active control product is expressed only at the precise time and place that it is required ie. when the bacterium is in the presence of wounded plant cells.

Chitinase was chosen as the first active molecule to be used in the inducible biocontrol system. Many agronomically important disease-causing organisms and pests such as fungi and nematodes contain chitin therefore chitinase may be useful in the control of such organisms. The Gram-negative bacterium *Serratia marcescens* produces a number of proteins with chitinolytic activity (Fuchs *et al.*, 1986) and the genes for two of these enzymes have been cloned (Jones *et al.*, 1986).

As part of this project, the *chiB* gene of *S. marcescens* was characterised and sequenced so that it could be utilised in a construct where it was under the control of an *Agrobacterium* virulence promoter. Its own promoter region was removed and replaced by a DNA fragment containing the *virB* promoter from pTiC58. The final construct when mobilised into *Agrobacterium* was demonstrated to produce chitinase upon induction with acetosyringone. Two other plasmids were also constructed using the *chiA* gene from *S. marcescens* linked to either the *virB* or *virE* promoter from pTiC58. Any of these plasmids could now be used in the biocontrol system and experiments need to be carried out to determine their efficacy against a variety of plant pathogenic fungi and nematodes. It is possible that a single chitinase enzyme acting alone will not be sufficient to fully protect a plant from pathogen attack and either a combination of chitinases with differing modes of action or a chitinase acting together with a glucanase will be more effective. Alternative genes could also be considered, in particular a gene encoding the δ-endotoxin from *Bacillus thuringiensis* as the Bt toxins have well

documented insecticidal activity (Hofte & Whiteley, 1989). This would extend the range of the biocontrol system allowing it to be used to control insect pests as well.

Experiments to demonstrate *in situ* the sites of *vir*-induction were only partially successful. By using *lacZ* as a reporter gene it was possible to observe *vir*-induction at artificially created wound sites on the roots of seedlings, but the experiment was hampered by the endogenous β-galactosidase produced by the plant. A reporter construct was created in which the *gus* gene was brought under the control of a *vir*-promoter. A lack of time precluded further experiments from being undertaken with this construct and also with the *lux* reporter plasmid. As detailed in Chapter 4 there are a number of possibilities which could valuably be explored for the utilisation of both of these plasmids.

The work carried out during this project has gone some way towards achieving a biocontrol agent such as that envisaged previously (Ashby, 1988). An efficient *vir*-promoter has been selected for use and plasmids have been constructed using two different chitinase genes. These are now at the stage where *in planta* trials are crucial to determine what plant protecting capabilities they possess. Any further work should concentrate on refining the system. This may be achieved by removing the need to maintain two separate plasmids in the cell or even by integrating all components of the system into the bacterial chromosome. Incompatibility functions may be incorporated into the system to prevent the avirulent *Agrobacterium* used as the microbial inoculant from acquiring a Ti-plasmid.

It is likely that concern would be expressed over releasing a genetically engineered known plant pathogen into the environment. Therefore it would be of interest to determine whether the inducible *vir*-system could be transferred to other, non-pathogenic bacterial species which are also good rhizosphere colonisers. It is already known that the Ti plasmid genes function in *Rhizobium* since expression of a Ti plasmid in this bacterium makes it tumourigenic (Hooykaas *et al.*, 1977). However, it is not known whether the VirA and VirG mediated chemotaxis to phenolic compounds would still occur .

REFERENCES

REFERENCES

Aguilar, J.M.M., Ashby, A.M., Richards, A.J.M., Loake, G.J., Watson, M.D. and Shaw, C.H. (1988) Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes. *J. Gen. Microbiol.* **134:** 2741-2746.

Akiyoshi, D.E., Klee, H., Amasino, R.M., Nester, E.W. and Gordon, M.P. (1984) T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA* **81:** 5994-5998.

Albano, M., Breitling, R. and Dubnau, D.A. (1989) Nucleotide sequence and genetic organisation of the *Bacillus subtilis comG* operon. *J. Bacteriol.* **171:** 5386-5404.

Alt - Moerbe, J., Kuhlmann, H. and Schroeder, J. (1989) Differences in induction of Ti-plasmid virulence genes *virG* and *virD* and continued control of *virD* expression by four external factors. *Mol. Plant-Microbe Interact.* **2:** 301-308.

Ames, P. and Bergman, K. (1981) Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. *J. Bacteriol.* **148:** 728-729.

Ankenbauer, R.G. and Nester, E.W. (1990) Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. *J.Bacteriol.* **172:** 6442-6446.

Ashby, A.M. (1988) *Agrobacterium tumefaciens*: Chemotaxis and crop protection. PhD thesis, University of Durham.

Ashby, A.M., Watson, M.D., Shaw, C.H. and Richards, A.J.M. (1986) European Patent Application EPO 256682 A2.

Ashby, A.M., Watson, M.D. and Shaw, C.H. (1987) A Ti-plasmid determined function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. *FEMS Microbiol. Letts.* **41:** 189-192.

Ashby, A.M., Watson, M.D., Loake, G.J. and Shaw, C.H. (1988) Ti-plasmid specified chemotaxis of *Agrobacterium tumefaciens* C58C^1 toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. *J. Bacteriol.* **170:** 4181-4187.

Baker, K.F. (1987) Evolving concepts of biological control of plant pathogens. *Annu. Rev. Phytopathol.* **25:** 67-85.

Ball, T.K., Saurugger, P.N. and Benedik, M.J. (1987) The extracellular nuclease gene of *Serratia marcescens* and its secretion from *Escherichia coli*. *Gene* **57:** 183-192.

Barrows-Broaddus, J. and Kerr, T.K. (1981) Inhibition of *Fusarium moniliforme* var. *subglutinans*, the causal agent of pitch canker, by the soil bacterium *Arthrobacter* sp. *Can. J. Microbiol.* **27:** 20-27.

Barry, G.F., Rogers, D.A., Fraley, R.T. and Brand, L. (1984) Identification of a cloned cytokinin biosynthetic gene. *Proc. Natl. Acad. Sci. USA* **81:** 4776-4780.

Bashan, Y. (1986) Migration of the rhizosphere bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* toward wheat roots in soil. *J. Gen. Microbiol.* **132:** 3407-3414.

Bashan, Y. and Levanony, H. (1987) Horizontal and vertical movement of *Azospirillum brasilense* Cd in the soil and along the rhizosphere of wheat and weeds in controlled and field environments. *J. Gen. Microbiol.* **133:** 3473-3480.

Beauchamp, C.J., Chilton W.S., Dion, P. and Antoun, H. (1990) Fungal catabolism of crown gall opines. *Appl. Env. Microbiol.* **56:** 150-155.

Becker, O. and Cook, R.J. (1988) Role of siderophores in suppression of *Pythium* species and production of increased growth response of wheat by fluorescent pseudomonads. *Phytopathology* **78:** 778-782.

Bergman, K., Gulash-Hoffee, M., Hovestadt, R.E., Larosiliere, R.C., Ronco, P.G. and Su, L. (1988) Physiology of behavioural mutants of *Rhizobium meliloti*: Evidence for a dual chemotaxis pathway. *J. Bacteriol.* **170:** 3249-3254.

Bernaerts, M.J. and DeLey, J. (1963) A biochemical test for crown-gall bacteria. *Nature (London)* **197:** 406-407.

Bhuvaneswari, T.V., Bhagwat, A. and Bauer, W.D. (1981) Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. *Plant Physiol.* **68:** 1114-1149.

Boivin, R., Chalifour, F.P. and Dion, P. (1988) Construction of a Tn5 derivative encoding bioluminescence and its introduction into *Pseudomonas*, *Agrobacterium* and *Rhizobium*. *Mol. Gen. Genet.* **213:** 50-55.

Bomhoff, G., Klapwijk, P.M., Kester, H.C.M., Schilperoort, R.A., Hernalsteens, J.P. and Schell, J. (1976) Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* **145:** 177-181.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgramme quantities of protein utilising the principle of protein dye binding. *Anal. Biochem.* **72:** 248-254.

Braun, A.C. and Mandle, R.J. (1948) Studies on the inactivation of the tumour-inducing principle in crown gall. *Growth* **12:** 255-269.

Buchmann, I., Marner, F.J., Schroeder, G., Waffenschmidt, S. and Schroeder, J. (1985) Tumor genes in plants: T-DNA encoded cytokinin biosynthesis. *EMBO J.* **4:** 853-859.

Caetano-Anolles, G., Christ-Estes, D.K. and Bauer, W.D. (1988) Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. *J. Bacteriol.* **170:** 3164-3169.

Caetano-Anolles, G., Wall, L.H., De Micheli, A.T., Macchi, E.M., Bauer, W.D. and Favelukes, G. (1988) Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meliloti. Plant Physiol.* **86:** 1228-1235.

Cangelosi, G.A., Ankenbauer,R.G. and Nester, E.W. (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc. Natl. Acad. Sci. USA* **87:** 6708-6712.

Cangelosi, G.A., Martinetti, G., Leigh, J.A, Lee, C., Theines, C. and Nester, E.W. (1989) Role of *Agrobacterium tumefaciens* ChvA protein in export of β-1,2-glucan. *J. Bacteriol.* **171:** 1609-1615.

Chang, C.-C., Chen, C.-M., Adams, D.R. and Trost, B.M. (1983) Leucinopine, a characteristic compound of some crown gall tumours. *Proc. Natl. Acad. Sci. USA* **80:** 3573-3576.

Chet, I., Ordentlich, A., Shapira, R. and Oppenheim, A. (1990) Mechanisms of biocontrol of soil-borne plant pathogens by Rhizobacteria. *Plant Soil* **129:** 85-92.

Chet, I., Zilberstein, Y. and Henis, Y. (1973) Chemotaxis of *Pseudomonas lachrymans* to plant extracts and to water droplets collected from the leaf surfaces of resistant and susceptible plants. *Physiol. Plant Pathol.* **3:** 473-479.

Chilton, M.-D., Saiki, R.K., Yadav, N., Gordon, M.P. and Quetier, F. (1980) T-DNA from *Agrobacterium* Ti-plasmid is in the nuclear DNA fraction of crown gall tumour cells. *Proc. Natl. Acad. Sci. USA* **77:** 4060-4064.

Chilton, W.S., Tempe, J., Matzke, M. and Chilton, M.D. (1984) Succinamopine: a new crown gall opine. *J. Bacteriol.* **157:** 357-362.

Chilton. M.-D., Drummond, M.H., Merlo, D.J., Sciaky, D. and Montoya, A.L. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11:** 263-271.

Christie, P.J., Ward, J.E., Gordon, M.P. and Nester, E.W. (1989) A gene required for the transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity. *Proc. Natl. Acad. Sci. USA* **86:** 9677-9681.

Christie, P.J., Ward, J.E., Winans, S.C. and Nester, E.W. (1988) The *Agrobacterium tumefaciens virE2* gene product is a single-stranded DNA-binding protein that associates with T-DNA. *J. Bacteriol.* **170:** 2659-2667.

Citovsky, V., De Vos, G. and Zambryski, P. (1988) Single-stranded DNA-binding protein encoded by the *virE* locus of *Agrobacterium tumefaciens*. *Science* **240:** 501-504.

Close, T.J., Rogowsky, P.M., Kado, C.I., Winans, S.C., Yanofsky, M.F. and Nester, E.W. (1987b) Dual control of *Agrobacterium tumefaciens* Ti plasmid virulence genes. *J. Bacteriol.* **169:** 5113-5118.

Close, T.J., Tait, R.C. and Kado, C.I. (1985) Regulation of Ti-plasmid virulence genes by a chromosomal locus of *Agrobacterium tumefaciens*. *J. Bacteriol.* **164:** 774-781.

Close, T.J., Tait, R.C., Rempel, H.C., Hirooka, T., Kim, L. and Kado, C.I. (1987a) Molecular characterisation of the *virC* genes of the Ti-plasmid. *J. Bacteriol.* **169:** 2336-2344.

Cooksey, D.A. and Moore, L.W. (1982) Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. *Phytopathology* **72:** 919-921.

Cowing, D.E., Bardwell, J.C.A., Craig, E.A., Woolford, C., Hendrix, R.W. and Gross, C.A. (1985) Consensus sequence for *Escherichia coli* heat shock promoters. *Proc. Natl. Acad. Sci. USA* **82:** 2909-2913.

Cox, C.D. (1980) Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. *J. Bacteriol.* **142:** 581-587.

Currier, T.C. and Gawron-Burke, C. (1990) Commercial development of *Bacillus thuringiensis* bioinsecticide products. In: *Biotechnology of Plant-Microbe Interactions*. J.P. Nakas and C. Hagedorn (Eds.)

Currier, W.W. and Strobel, G.A. (1976) Chemotaxis of *Rhizobium* spp. to plant root exudates. *Plant Physiol.* **57:** 820-823.

Currier, W.W. and Strobel, G.A. (1977) Chemotaxis of *Rhizobium* spp. to a glycoprotein produced by Birdsfoot trefoil roots. *Science* **196:** 434-436.

Darvill, A. and Albersheim, P. (1984) Phytoalexins and their elicitors- a defence against microbial infection in plants. *Annu. Rev. Plant Physiol.* **35:** 243-275.

Das, A. (1988) *Agrobacterium tumefaciens virE* operon encodes a single-stranded DNA-binding protein. *Proc. Natl. Acad. Sci. USA* **85:** 2909-2913.

Das, A., Stachel, S.E., Ebert, P., Allenza, P., Montoya, A. and Nester, E.W. (1986) Promoters of *A. tumefaciens* Ti plasmid virulence genes. *Nucl. Acids Res.* **14:** 1355-1364.

De Weger, L.A., Van Boxtel, R., Van Der Burg, B., Gruters, R.A., Geels, F.P., Schippers, B. and Lugtenberg, B. (1986) Siderophores and outer membrane proteins of antagonistic, plant growth-stimulating, root-colonising *Pseudomonas* spp. *J. Bacteriol.* **165:** 585-594.

De Weger, L.A., Van Der Vlugt, C.I.M., Wijfjes, A.H.M., Bakker, P.A.H.M., Schippers, B. and Lugtenberg, B. (1987) Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonisation of potato roots. *J. Bacteriol.* **169:** 2769-2773.

Depicker, A., Van Montagu, M. and Schell, J. (1978) Homologous DNA sequences in different Ti plasmids are essential for oncogenicity. *Nature* **275:** 150-153.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980) Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA* **77:** 7347-7351.

Douglas, C.J., Staneloni, R.J., Rubin, R.A. and Nester, E.W. (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. *J. Bacteriol.* **161:** 850-860.

Durrenberger, F., Crameri, A., Hohn, B. and Koukolokova-Nicola, Z. (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-strand from exonucleolytic degradation. *Proc. Natl. Acad. Sci. USA* **86:** 9154-9158.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E.W., Helinski, D.R. and Ditta, G. (1986) *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *A. tumefaciens*. *Proc. Natl. Acad. Sci. USA* **83:** 4403-4407.

Ebert, P.R., Ha, S.B. and An, G. (1987) Identification of an essential upstream element in the nopaline synthase promoter by stable and transient assays. *Proc. Natl. Acad. Sci. USA* **84:** 5745-5749.

Elad, Y. and Chet, I. (1987) Possible role of competition for nutrients in biocontrol of *Pythium* damping off by bacteria. *Phytopathology* **77:** 190-195.

Elliott, C. (1951) *Manual of bacterial plant pathogens*, 2nd edition. Chronica Botanica, Waltham, Mass.

Ellis, J.G., Kerr, A., Van Montagu, M. and Schell, J. (1979) *Agrobacterium*: Genetic studies on agrocin 84 production and the biological control of crown gall. *Physiol. Plant Pathol.* **15:** 311-319.

Engebrecht, J. and Silverman, M. (1984) Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **81:** 4154-4158.

Engebrecht, J., Simon, M. and Silverman, M. (1985) Measuring gene expression with light. *Science* **227:** 1345-1347.

Engler, G., Holsters, M., Van Montagu, M., Schell, J., Hernalsteens, J.P. and Schilperoort, R.A. (1975) Agrocin 84 sensitivity: a plasmid determined property in *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* **138:** 345-349.

Engstrom, P. Zambryski, P., Van Montagu, M. and Stachel, S. (1987) Characterisation of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. *J. Mol. Biol.* **197:** 635-645.

Farrand, S.K., O'Morchoe, S.P. and McCutchan, J. (1989) Construction of an *Agrobacterium tumefaciens* C58 *recA* mutant. *J. Bacteriol.* **171:** 5314-5321.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling restriction endonuclease fragments to a high specific activity. *Anal. Biochem.* **132:** 6-13.

Fuchs, R.L., McPherson, S.A. and Drahos, D.J. (1986) Cloning of a *Serratia marcescens* gene encoding chitinase. *Appl. Env. Microbiol.* **51:** 504-509.

Furste, J.P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M. and Lanka, E. (1986) Molecular cloning of the RP4 DNA primase region in a multirange *tacP* expression vector. *Gene* **48:** 119-131.

Fravel, D.R. (1988) Role of antibiosis in the biocontrol of plant diseases. *Annu. Rev. Phytopathol.* **26:** 75-91.

Gaworzewska, E.T. and Carlile, M.J. (1982) Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J. Gen. Microbiol.* **128:** 1179-1188.

Genetello, C., Van Larebeke, N., Holsters, M., Depicker, A., Van Montagu, M. and Schell, J. (1977) Ti-plasmids of *Agrobacterium* as conjugative plasmids. *Nature* **265:** 561-563.

Ghai, J. and Das, A. (1989) The *virD* operon of *Agrobacterium tumefaciens* Ti plasmid encodes a DNA-relaxing enzyme. *Proc. Natl. Acad. Sci. USA* **86:** 3109-3113.

Gibson, F. and Magrath, D.I. (1969) The isolation and characterisation of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. *Biochim. Biophys. Acta* **192:** 175-184.

Gitte, R.R., Rai, P.V. and Patil, R.B. (1978) Chemotaxis of *Rhizobium* sp. towards root exudate of *Cicer arietinum L. Plant Soil* **50:** 553-566.

Goetz, R., Limmer, N., Ober, K. and Schmidt, R. (1982) Motility and chemotaxis in two strains of *Rhizobium* with complex flagella. *J. Gen. Microbiol.* **128:** 789-798.

Gooday, G.W. (1977) Biosynthesis of the fungal wall - mechanisms and implications. *J. Gen. Microbiol.* **99:** 1-11.

Gould, W.G. (1990) Biological control of plant root diseases by bacteria. In: *Biotechnology of Plant-Microbe Interactions.* J.P. Nakas and C. Hagedorn (Eds.)

Gurlitz, R.H.G., Lamb, P.W. and Matthysse, A.G. (1987) Involvement of carrot cell surface proteins in attachment of *Agrobacterium tumefaciens. Plant Physiol.* **83:** 564-568.

Gurusiddaiah, S., Weller, D.M., Sarkar, A. and Cook, R.J. (1986) Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. *Antimicrob. Agents Chemother.* **29:** 488-495.

Gutterson, N.I., Layton, T.J., Ziegle, J.S. and Warren, G.J. (1986) Molecular cloning of genetic determinants for inhibition of fungal growth by a fluorescent pseudomonad. *J. Bacteriol.* **165:** 696-703.

Hamdi, Y.A. (1971) Soil-water tension and the movement of Rhizobia. *Soil Biol. Biochem.* **3:** 121-126.

Harley, C.B. and Reynolds, R.P. (1987) Analysis of *E. coli* promoter sequences. *Nucl. Acids Res.* **15:** 2343-2361.

Harpster, M.H. and Dunsmuir, P. (1989) Nucleotide sequence of the chitinase B gene of *Serratia marcescens* QMB1466. *Nucl. Acids Res.* **17:**?

Hawes, M.C. (1990) Living plant cells released from the root cap: A regulator of microbial populations in the rhizosphere? *Plant Soil* **129**: 19-27.

Hawes, M.C. and Smith, L.Y. (1989) Requirement for chemotaxis in pathogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea plants. *J. Bacteriol.* **171:** 5668-5671.

Hawes, M.C., Smith, L.Y. and Howarth, A.J. (1988) *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Molec. Plant-Microbe Interac.* **1:** 182-186.

Heinrich, D. and Hess, D. (1985) Chemotactic attraction of *Azospirillum lipoferum* by wheat roots and characterisation of some attractants. *Can. J. Microbiol.* **31:** 26-31.

Henis, Y. and Chet, I. (1975) Microbial control of plant pathogens. *Adv. Appl. Microbiol.* **19:** 85-111.

Herrera-Estrella, A., Van Montagu, M. and Wang, K. (1990) A bacterial peptide acting as a plant nuclear targeting signal: The amino-terminal portion of *Agrobacterium* VirD2 protein directs a β-galactosidase fusion protein into tobacco nuclei. *Proc. Natl. Acad. Sci. USA* **87:** 9534-9537.

Hess, J.F., Bourret, R.B. and Simon, M.I. (1988) Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. *Nature* **336:** 139-143.

Higgins, C.F., Hiles, I.D., Salmond, G.P.C., Gill, D.R., Downie, J.A., Evans, I.J., Holland, I.B., Gray, L., Buckel, S.D., Bell, A.W. and Hermondson, M.A. (1986) A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature* **323:** 448-450.

Hines, D.A., Saurugger, P.N., Ihler, G.M. and Benedik, M.J. (1988) Genetic analysis of extracellular proteins of *Serratia marcescens*. *J. Bacteriol.* **170:** 4141-4146.

Hirooka, T., Rogowsky, P.M. and Kado, C.I. (1987) Characterisation of the *virE* locus of *Agrobacterium tumefaciens* plasmid pTiC58. *J. Bacteriol.* **169:** 1529-1536.

Hoefte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53:** 242-255.

Holsters, M., Villarroel, R., Gielen, J., Seurinck, J., De Greve, H., Van Montagu, M. and Schell, J. (1983) An analysis of the boundaries of the octopine TL-DNA in tumours induced by *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* **190:** 35-41.

Hooykaas, P.J.J., Hoftker, M., den Dulk-Ras, H. and Schilperoort, R.A. (1984) A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens*. *Plasmid* **11:** 195-205.

Hooykaas, P.J.J., Klapwijk, P.M., Nuti, M.P., Schilperoort, R.A. and Rorsch, A. (1977) Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to *Rhizobium ex planta*. *J. Gen. Microbiol.* **98:** 477-484.

Horwitz, M., Reid, J. and Ogrydziak, D. (1984) Genetic improvement of chitinase production by *Serratia marcescens*. In: *Chitin, chitosan and related enzymes*. pp.191-208. J.P.Zikakis (Ed.) Academic Press Inc., Orlando, Florida.

Howard, E.A., Winsor, B.A., De Vos, G. and Zambryski, P. (1989) Activation of the T-DNA transfer process in *Agrobacterium* results in the generation of a T-strand-protein complex: Tight association of VirD2 with the 5' ends of T-strands. *Proc. Natl. Acad. Sci. USA* **86:** 4017-4021.

Howell, C.R. and Stipanovich, R.D. (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* **69:** 480-482.

Howell, C.R. and Stipanovich, R.D. (1980) Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* **70:** 712-715.

Howie, W.J. and Suslow, T.V. (1986) Effect of antifungal compound biosynthesis on cotton root colonization and *Pythium* suppression by a strain of *Pseudomonas fluorescens* and its antifungal minus isogenic mutant. *Phytopathology* **76:** 1069.

Howie, W.J., Cook, R.J. and Weller, D.M. (1987) Effects of soil matric potential and cell motility on wheat root colonisation by fluorescent Pseudomonads suppressive to take-all.. *Phytopathol.* **77:** 286-292.

Huang, M-L.W., Cangelosi, G.A., Halperin, W. and Nester, E.W. (1990) A chromosomal *Agrobacterium tumefaciens* gene required for effective plant signal transduction. *J. Bacteriol.* **172:** 1814-1822.

Huang, Y., Morel, P., Powell, B. and Kado, C.I. (1990) VirA, a coregulator of Ti-specified virulence genes, is phosphorylated *in vitro*. *J. Bacteriol.* **172:** 1142-1144.

Hunter, W.J. and Fahring, C.J. (1980) Movement by *Rhizobium* and nodulation of legumes. *Soil Biol. Biochem.* **12:** 537-542.

Igo, M.M. and Silhavy, T.J. (1988) EnvZ, a transmembrane environmental sensor of *Escherichia coli* K12, is phosphorylated *in vitro*. *J. Bacteriol.* **170:** 5971-5973.

Inon de Iannino, N. Ugalde, R.A. (1989) Biochemical characterisation of avirulent *Agrobacterium tumefaciens chvA* mutants: Synthesis and excretion of β-(1,2)-glucan. *J. Bacteriol.* **171:** 2842-2849.

Inze, D., Follin, A., Van Lijsebettens, M., Simoens, C., Genetello, C., Van Montagu, M. and Schell, J. (1984) Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*: further evidence that two genes are involved in indole-3-acetic acid synthesis. *Mol. Gen. Genet.* **194:** 265-274.

Jayaswal, R.K., Veluthambi, K., Gelvin, S.B. and Slightom, J.L. (1987) Double-stranded cleavage of T-DNA molecules in *Escherichia coli* by a *virD*-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. *J. Bacteriol.* **169:** 5035-5045.

Jefferson R.A. (1987) Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* **5:** 387-405.

Jefferson, R.A. (1989) The GUS reporter gene system. *Nature (London)* **342:** 837-838.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. (1986) β-glucuronidase from *Escherichia coli* as a gene fusion marker. *Proc. Natl. Acad. Sci. USA* **86:** 8447-8451.

Jen, G. and Chilton, M.-D. (1986) The right border region of pTiT37 T-DNA is intrinsically more active than the left border region in promoting T-DNA transformation. *Proc. Natl. Acad. Sci. USA* **83:** 3895-3899.

Jin, S., Komari, T., Gordon, M.P. and Nester, E.W. (1988) Genes responsible for the supervirulent phenotype of *Agrobacterium tumefaciens* strain A281. *J. Bacteriol.* **169:** 4417-4425.

Jin, S., Prusti, R.K., Roitsch, T., Ankenbauer, R.G. and Nester, E.W. (1990a) Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: Essential role in biological activity of VirG. *J. Bacteriol.* **172:** 4945-4950.

Jin, S., Roitsch, T., Ankenbauer, R.G., Gordon, M.P. and Nester, E.W. (1990b) The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene regulation. *J. Bacteriol.* **172:** 525-530.

Jin, S., Roitsch, T., Christie, P. and Nester, E.W. (1990c) The regulatory VirG protein specifically binds to a *cis*-acting regulatory sequence involved in transcriptional activation of *Agrobacterium tumefaciens* virulence genes. *J. Bacteriol.* **172:** 531-537.

Jones, J.D.G., Dean, C., Gidoni, D., Gilbert, D., Bond-Nutter, D., Lee, R., Bedbrook, J. and Dunsmuir, P. (1988) Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. *Mol. Gen. Genet.* **212:** 536-542.

Jones, J.D.G., Grady, K.L., Suslow, T.V. and Bedbrook, J.R. (1986) Isolation and characterisation of genes encoding two chitinase enzymes from *Serratia marcescens*. *EMBO J.* **5:** 467-473.

Joos, H., Inze, D., Caplan, A., Sormann, M., Van Montagu, M. and Schell, J. (1983) Genetic analysis of T-DNA transcripts in nopaline crown galls. *Cell* **32:** 1057-1067.

Joshi, S., Kozlowski, M., Selvaraj, G., Iyer, V.N. and Davies, R.W. (1988) Cloning of the genes of the chitin utilization regulon of *Serratia liquefaciens*. *J. Bacteriol.* **170:** 2984-2988.

Juhnke, M.E., Mathre, D.E. and Sands, D.C. (1987) Identification and characterisation of rhizosphere-competent bacteria of wheat. *Appl. Environ. Microbiol.* **53:** 2793-2799.

Kammerer, W., Deuschle, U., Gentz, R. and Bujard, H. (1986) Functional dissection of *Escherichia coli* promoters: information in the transcribed region is involved in late steps of the overall process. *EMBO J.* **5:** 2995-3000.

Kay, R. and McPherson, J. (1987) Hybrid pUC vectors for addition of new restriction enzyme sites to the ends of DNA fragments. *Nucl. Acids Res.* **15:** 2778.

Kerr, A. (1969) Crown gall of stone fruit I: Isolation of *Agrobacterium tumefaciens* and related species. *Aust. J. Biol. Sci.* **22:** 111-116.

Kerr, A. (1974) Soil microbiological studies on *Agrobacterium radiobacter* and biological control of crown gall. *Soil Sci.* **118:** 168-172.

Kerr, A. and Ellis, J.G. (1982) Conjugation and transfer of Ti-plasmids in *Agrobacterium tumefaciens*. In: "*Molecular Biology of Plant Tumours*" pp. 321-344 G. Kahl and J. Schell (Eds.) New York: Academic.

Kerr, A., Manigault, P. and Tempe, J. (1977) Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium. Nature (London)* **265:** 560-561.

Kerr, A. and Panagopoulos, C.G. (1977) Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathol. Z.* **90:** 172-179.

Kersters, K. and DeLey, J. (1982) Family Rhizobiaceae - genus *Agrobacterium*. In: "*Bergey's Manual of Systematic Bacteriology.*" Vol. 1, 9th edition. N.R. Krieg (Ed.) Williams & Wilkins, Baltimore.

Klapwijk, P.M., Van Beelen, P. and Schilperoort, R.A. (1979) Isolation of a recombination-deficient *Agrobacterium tumefaciens* mutant. *Mol. Gen. Genet.* **173:** 171-175.

Kless, H., Sitrit, Y., Chet, I. and Oppenheim, A.B. (1989) Cloning of the gene coding for chitobiase of *Serratia marcescens. Mol. Gen. Genet.* **217:** 471-473.

Kloepper, J.W., Leong, J., Teintze, M. and Schroth, M.N. (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature (London)* **286:** 835-836.

Knauf, V.C. and Nester, E.W. (1982) *Plasmid* 8: 45-54.

Kuldau, G.A., De Vos, G., Owen, J., McCaffrey, G. and Zambryski, P. (1990) The *virB* operon of *Agrobacterium tumefaciens* pTiC58 encodes 11 open reading frames. *Mol. Gen. Genet.* **221:** 256-266.

Lam, S.T., Ellis, D.M. and Ligon, J.M. (1990) Genetic approaches for studying rhizosphere colonisation. *Plant Soil* **129:**11-18.

Legocki, R.P., Legocki, M., Baldwin, T.O. and Szalay, A.A. (1986) Bioluminescence in soybean root nodules: Demonstration of a general approach to assay gene expression *in vivo* by using bacterial luciferase. *Proc. Natl. Acad. Sci. USA* **83:** 9080-9084.

Leisinger, T. and Margraff, R. (1979) Secondary metabolites of the fluorescent pseudomonads. *Microbiol. Rev.* **43:** 422-442.

Leong, J. (1986) Siderophores; their biochemistry and possible role in the biocontrol of plant pathogens. *Annu. Rev. Phytopathol.* **24:** 187-209.

Leroux, B., Yanofsky, M.F., Winans, S.C., Ward, J.E., Ziegler, S.F. and Nester, E.W. (1987) Characterisation of the VirA locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host-range determinant. *EMBO J.* **6:** 849-856.

Lippincott, B.B. and Lippincott, J.A. (1969) Bacterial attachment to a specific wound site as an essential stage in tumour initiation by *Agrobacterium tumefaciens*. *J. Bacteriol.* **97:** 620-628.

Lippincott, J.A., Lippincott, B.B. and Starr, M.P. (1981) The genus *Agrobacterium*. In: "*The Prokaryotes*" Chapter **68:** 842-855. M.P. Starr, H. Stolp, H.G. Trueper, A. Balows and H.G. Schlegel (Eds.) Springer - Verlag.

Loake, G.J., Ashby, A.M. and Shaw C.H. (1988) Attraction of *Agrobacterium tumefaciens* $C58C^1$ towards sugars involves a highly sensitive chemotaxis system. *J. Gen. Micro.* **134:** 1427-1432.

Loper, J.E. (1988) Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. *Phytopathology* **78:** 166-172.

Loper, J.E., Suslow, T.V. and Schroth, M.N. (1984) Log-normal distribution of bacterial populations in the rhizosphere. *Phytopathology* **74:** 1454-1460.

Magazin, M.D., Moores, J.C. and Leong, J. (1986) Cloning of the gene coding for the outer membrane receptor protein for ferric pseudobactin, a siderophore from a plant growth-promoting *Pseudomonas* strain. *J. Biol. Chem.* **261:** 791-795.

Makino, K., Shinagawa, H., Amemura, M., Kimura, S., Nakata, A. and Ishihama, A. (1988) Regulation of the phosphate regulon of *Escherichia coli*. Activation of *pstS* transcription by PhoB protein *in vitro*. *J. Mol. Biol.* **203:** 85-95.

Mandel, M. and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection of *E. coli*. *J. Mol. Biol.* **98:** 503-517.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) *Molecular cloning. A laboratory manual.* 2nd edition Cold Spring Harbour, Laboratory Press, New York.

Marks, J.R., Lynch, T.J., Karlinsey, J.E. and Thomashow, M.F. (1987) *A. tumefaciens* virulence locus *pscA* is related to the *Rhizobium meliloti exoC* locus. *J. Bacteriol.* **169:** 5835-5837.

Marugg, J.D., Nielander, H.B., Horrevoets, A.J.G., Van Megen, I., Van Genderen, I. and Weisbeek, P.J. (1988) Genetic organisation and transcriptional analysis of a major gene cluster involved in siderophore biosynthesis in *Pseudomonas putida* WCS358. *J. Bacteriol.* **170:** 1812-1819.

Marugg, J.D., Van Spanje, M., Hoekstra, W.P.M., Schippers, B. and Weisbeck, P.J. (1985) Isolation and analysis of genes involved in siderophore biosynthesis in plant growth-stimulating *Pseudomonas putida*, WCS358. *J. Bacteriol.* **164:** 563-570.

Matthysse, A.G. (1983) Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. *J. Bacteriol.* **154:** 906-915.

Matthysse, A.G. (1987) Characterisation of non-attaching mutants of *Agrobacterium tumefaciens*. *J. Bacteriol.* **169:** 313-323.

Matthysse, A.G., Holmes, K.V. and Gurlitz, R.H.G. (1981) Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. *J. Bacteriol.* **145:** 583-595.

McBride, K.E. and Knauf, V.C. (1988) Genetic analysis of the *virE* operon of the *Agrobacterium* Ti-plasmid pTiA6. *J. Bacteriol.* **170:** 1430-1437.

Melchers, L.S. and Hooykaas, P.J.J. (1987) Virulence of *Agrobacterium*. In:"*Oxford Surveys of Plant Molecular and Cellular Biology.*" **4:** 167-220. B.J. Miflin (Ed.) Oxford University Press.

Melchers, L.S., Regensburg-Tuink, T.J.G., Bourret, R.B., Sedee, N.J.A., Schilperoort, R.A. and Hooykaas, P.J.J. (1989) Membrane topology and functional analysis of the sensory protein VirA of *Agrobacterium tumefaciens*. *EMBO J.* **8:** 1919-1925.

Melchers, L.S., Thompson, D.V., Idler, K.B., Schilperoort, R.A. and Hooykaas, P.J.J. (1986) Nucleotide sequence of the virulence gene *virG* of the *Agrobacterium tumefaciens* octopine Ti-plasmid: significant homology between *virG* and the regulatory genes *ompR*, *phoB* and *dye* of *E. coli*. *Nucl. Acids Res.* **14:** 9933-9942.

Messens, E., Dekeyser, R. and Stachel, S.E. (1990) A non-transformable *Triticum monococcum* monocotyledonous culture produces the potent *Agrobacterium vir*-inducing compound ethyl ferulate. *Proc. Natl. Acad. Sci. USA* **87:** 4368-4372.

Meyer, J.-M. and Abdallah, M.A. (1980) The siderochromes of non-fluorescent pseudomonads: production of nocardamine by *Pseudomonas stutzeri*. *J. Gen. Microbiol.* **118:** 125-129.

Miller, K.J., Reinhold, V.N., Weissborn, A.C. and Kennedy, E.P. (1987) Cyclic glucans produced by *Agrobacterium tumefaciens* are substituted with *sn*-1-phosphoglycerol residues. *Biochim. Biophys. Acta* **901:** 112-118.

Mitchell, R. and Alexander, M. (1961) The mycolytic phenomenon and biological control of *Fusarium* in soil. *Nature (London)* **190:** 109-110.

Miyazaki, H., Yanagida, H., Hirinouchi, S. and Beppu, T. (1989) Characterisation of the precursor of *Serratia marcescens* serine protease and COOH-terminal processing during secretion through the outer membrane of *Escherichia coli*. *J. Bacteriol.* **171:** 6566-6572.

Molano, J., Duran, A. and Cabib, E. (1977) A rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* **83:** 648-656.

Molano,J., Polacheck, I., Duran, A. and Cabib, E. (1979) An endochitinase from wheatgerm. *J. Biol. Chem.* **254:** 4901-4907.

Monreal, J. and Reese, E.T. (1969) The chitinase of *Serratia marcescens*. *Can. J. Microbiol.* **15:** 689-696.

Moore, L., Warren, G. and Strobel, G. (1979) Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. *Plasmid* **2:** 617-626.

Moores, J.C., Magazin, M., Ditta, G.S. and Leong, J. (1984) Cloning of genes involved in the biosynthesis of pseudobactin, a high-affinity iron transport agent of a plant growth-promoting *Pseudomonas* strain. *J. Bacteriol.* **157:** 53-58.

Morris, J.W. and Morris, R.O. (1990) Identification of an *Agrobacterium tumefaciens* virulence gene inducer from the pinaceous gymnosperm *Pseudotsuga menziesii*. *Proc. Natl. Acad. Sci. USA* **87:** 3614-3618.

Morrissey, R.F., Dugan, E.P. and Koths, J.S. (1976) Chitinase production by an *Arthrobacter* sp. lysing cells of *Fusarium roseum*. *Soil Biol. Biochem.* **8:** 23-28.

Murphy, P.J. and Roberts, W.P. (1979) A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. *J. Gen. Microbiol.* **114:** 207-213.

Muzzarelli, R.A. (1977) *Chitin* Pergamon Press, New York.

Nagel, R., Elliott, A., Masel, A., Bioch, R.G. and Manners, J.M. (1990) Electroporation of binary Ti-plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *FEMS Microbiol. Letts.* **67:** 325-328.

Nakahama, K., Yoshimura, K., Marumoto, R., Kikuchi, M., Lee, I.S., Hase, T. and Matsubara, H. (1986) Cloning and sequencing of *Serratia* protease gene. *Nucl. Acids Res.* **14:** 5843-5855.

Neff, N.T. and Binns, A.N. (1985) *Agrobacterium tumefaciens* interaction with suspension cultured tomato cells. *Plant Physiol.* **77:** 35-42.

Neff, N.T., Binns, A.N. and Brandt, C. (1987) Inhibitory effects of a pectin-enriched tomato cell wall fraction on *Agrobacterium tumefaciens* binding and tumour formation. *Plant Physiol.* **83:** 525-528.

Neilands, J.B. (1981) Microbial iron compounds. *Annu. Rev. Biochem.* **50:** 715-731.

Nester, E.W. and Kosuge, T. (1981) Plasmids specifying plant hyperplasias. *Annu. Rev. Microbiol.* **35:** 531-565.

New, P.B. and Kerr, A. (1972) Biological control of crown gall: Field measurements and glasshouse experiments. *J. Appl. Bacteriol.* **35:** 279-287.

Obukowicz, M.G., Perlak, F.J., Kusano-Kretzmer, K., Mayer, E.J. and Watrud, L.S. (1986) Integration of the δ-endotoxin gene of *Bacillus thuringiensis* into the chromosome of root-colonising strains of pseudomonads using Tn5. *Gene* **45:** 327-331.

Ong, S.A., Peterson, T. and Neilands, J.M. (1979) Agrobactin, a siderophore from *Agrobacterium tumefaciens*. *J. Biol. Chem.* **254:** 1860-1865.

Ordentlich, A., Elad, Y. and Chet, I. (1987) Rhizosphere colonisation by *Serratia marcescens* for the control of *Sclerotium rolfsii*. *Soil Biol. Biochem.* **19:** 747 - 751.

Ordentlich, A. Elad, Y. and Chet, I. (1988) The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* **78:** 84-88.

Papavizas, G.C. and Lewis, J.A. (1983) Physiological and biocontrol characteristics of stable mutants of *Trichoderma viride* resistant to MBC fungicides. *Phytopathology* **73:** 407-411.

Parke, D., Ornston, L.N. and Nester, E.W. (1987) Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti-plasmid in *Agrobacterium tumefaciens*. *J. Bacteriol.* **169:** 5336-5338.

Parke, D., Rivelli, M. and Ornston, L.N. (1985) Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. *J. Bacteriol.* **163:** 417-422.

Pazour, G.J. and Das, A. (1990) *virG*, an *Agrobacterium tumefaciens* transcriptional activator, initiates translation at a UUG codon and is a sequence-specific DNA-binding protein. *J. Bacteriol.* **172:** 1241-1249.

Peralta, E.G., Hellmiss, R. and Ream, W. (1986) *Overdrive*, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid. *EMBO J.* **5:** 1137-1142.

Peralta, E.G. and Ream, W. (1985) T-DNA border sequences required for crown gall tumorigenesis. *Proc. Natl. Acad. Sci. USA* **82:** 5112-5116.

Perry, K.L. and Kado, C.I. (1982) Characteristics of Ti-plasmids from broad host-range and ecologically specific biotype 2 and 3 strains of *Agrobacterium tumefaciens*. *J. Bacteriol.* **151:** 343-350.

Petit, A., Delhaye, S., Tempe, J. and Morel, G. (1970) Recherches sur les guanidines des tissues de crown gall. Mise en evidence d'une relation biochemique specifique entre les souches d'*Agrobacterium* et les tumeurs qu'elles induisent. *Physiol. Veg.* **8:** 205-213.

Popham, D.L., Szeto, D., Keener, J. and Kustu, S. (1989) Function of a bacterial activator protein that binds to transcriptional enhancers. *Science* **243:** 629-635.

Porter, S.G., Yanofsky, M.F. and Nester, E.W. (1987) Molecular characterisation of the *virD* operon from *Agrobacterium tumefaciens*. *Nucl. Acids Res.* **15:** 7503-7517.

Powell, B.S., Rogowsky, P.M. and Kado, C.I. (1989) *virG* of *Agrobacterium tumefaciens* plasmid pTiC58 encodes a DNA-binding protein. *Mol. Microbiol.* **3:** 411-419.

Pugsley, A.P. (1989) *Protein Targetting* Academic Press, Inc. San Diego, CA.

Pugsley, A.P., d'Enfert, C., Reyss, I. and Kornacker, M.G. (1990) Genetics of extracellular protein secretion by Gram-negative bacteria. *Annu. Rev. Genet.* **24:** 67-90.

Puvanesarajah, V., Schell, F.M., Stacey, G., Douglas, C.J. and Nester, E.W. (1985) Role for 2-linked-β-D-glucan in the virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* **164:** 102-106.

Ream, W. (1989) *Agrobacterium tumefaciens* and interkingdom genetic exchange. *Annu. Rev. Phytopathol.* **27:** 583-618.

Reznikoff, W.S., Siegele, D.A., Cowing, D.W. and Gross, C.A. (1985) The regulation of transcription initiation in bacteria. *Ann. Rev. Genet.* **19:** 355-387.

Robbins, P.W., Albright, C. and Benfield, B. (1988) Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. *J. Biol. Chem.* **263:**443-

Roberts, R.L. and Cabib, E. (1982) *Serratia marcescens* chitinase: One-step purification and use for the determination of chitin. *Anal. Biochem.* **127:** 402-412.

Roberts, W.K. and Selitrennikoff, C.P. (1988) Plant and bacterial chitinases differ in antifungal activity. *J. Gen. Microbiol.* **134:** 169-176.

Rogowsky, P.M., Close, T.J., Chimera, J.A., Shaw, J.J. and Kado, C.I. (1987) Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. *J. Bacteriol.* **169:** 5101-5112.

Ronson, C.W., Nixon, B.T. and Ausubel, F.M. (1987) Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. *Cell* **49:** 579-581.

Rossignol, G. and Dion, P. (1985) Octopine, nopaline and octopinic acid utilisation in *Pseudomonas*. *Can. J. Microbiol.* **31:** 68-74.

Rushner, S.R. (1978) An improved method for transformation of *E. coli* with Col-E1 derived plasmids. In: *Genetic Engineering*. H.W. Boyer and S. Nicosia (Eds.) pp. 17-23.

Ryder, M.H., Slota, J.E., Scarim, A. and Farrand, S.K. (1987) Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. *J. Bacteriol.* **169:** 4184-4189.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463-5467.

Schell, J., Van Montagu, M., De Beuckeleer, M., De Block, M., Depicker, A., De Wilde, M., Engler, G., Genetello, C., Hernalsteens, J.P., Holsters, M., Seurinck, J., Silva, B., Van Vuet, F. and Villarroel, R. (1979) Interactions and DNA transfer between *Agrobacterium tumefaciens*, the Ti-plasmid and the plant host. *Proc. R. Soc. Lond. B* **204:** 251-266.

Scher, F.M. and Baker, R. (1982) Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Phytopathology* **72:** 1567-1573.

Scher, F.M., Kloepper, J.W. and Singleton, C.A. (1985) Chemotaxis of fluorescent *Pseudomonas* spp. to soybean seed exudates *in vitro* and in soil. *Can. J. Microbiol.* **31:** 570-574.

Scher, F.M., Kloepper, J.W., Singleton, C., Zaleska, I. and Laliberte, M. (1988) Colonization of soybean roots by *Pseudomonas* and *Serratia* species: Relationship to bacterial motility, chemotaxis and generation time. *Phytopathology* **78:** 1055-1059.

Schiebel, E., Schwartz, H. and Braun, V. (1990) Subcellular location and unique secretion of the hemolysin of *Serratia marcescens*. *J. Biol. Chem.* **264:** 16311-16320.

Schlumbaum, A., Mauch, F., Voegeli, U. and Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature (London)* **324:** 365-367.

Schroeder, G., Waffenschmidt, S., Weiler, E.W. and Schroeder, J. (1984) The T-region of Ti plasmids codes for an enzyme synthesising indole-3-acetic acid. *Eur. J. Biochem.* **138:** 387-391.

Schroth, M.N., Weinhold, A.R., McCain, A.H., Hildebrand, D.C. and Ross, N. (1971) Biology and control of *Agrobacterium tumefaciens*. *Hilgardia* **40:** 537-552.

Shapira, R., Ordentlich, A., Chet, I. and Oppenheim, A.B. (1989) Control of plant diseases by chitinase expressed from cloned DNA in *E. coli*. *Phytopathology* **79:** 1246-1249.

Shaw, C.H., Watson, M.D., Carter, G.H. and Shaw, C.H. (1984) The right-hand copy of the nopaline Ti plasmid 25bp repeat is required for tumour formation. *Nucl. Acids Res.* **12:** 6031-6041.

Shaw, C.H., Ashby, A.M., Brown, A.B., Royal, C., Loake, G.J. and Shaw, C.H. (1988a) *virA* and *virG* are the Ti-plasmid functions required for chemotaxis of *Agrobacterium tumefaciens* towards acetosyringone. *Molec. Microbiol.* **2:** 413-417.

Shaw, C.H., Ashby, A.M., Loake, G.J. and Watson, M.D. (1988b) One small step: The missing link in crown gall. In: *Oxford Surveys of Plant Molecular and Cell Biology* Vol. **5:** 177-183. B.J. Miflin (Ed.) Oxford University Press.

Shaw, C.H., Loake, G.J., Brown, A.P. and Garrett, C.S. (1989) Molecular biology of chemotaxis in *Agrobacterium.* In: *Molecular Signals in Microbe-Plant Symbiotic and Pathogenic Systems.* B. Lugtenberg (Ed.) Springer-Verlag KG. Berlin.

Shaw, J.J. and Kado, C.I. (1986) Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner. *Biotechnology* **4:** 560-565.

Shaw, J.J., Rogowsky, P., Close, T.J. and Kado, C.I. (1987) Working with bacterial bioluminescence. *Plant Mol. Biol. Rep.* **5:** 225-236.

Shimoda, N., Toyoda-Yamamoto, A., Nagamine, J., Usami, S., Katayama, M., Sakagami, Y. and Machida, Y. (1990) Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. *Proc. Natl. Acad. Sci. USA* **87:** 6684-6688.

Shinshi, H., Mohnen, D. and Meins, F. (1987) Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *Proc. Natl. Acad. Sci. USA* **84:** 89-93.

Siebenlist, U., Simpson, R.B. and Gilbert, W. (1980) *E. coli* RNA polymerase interacts homologously with two promoters. *Cell* **20:** 269-281.

Smith, E.F. and Townsend, C.O. (1907) A plant tumour of bacterial origin. *Science* **25:** 671-673.

Soby, S. and Bergman, K. (1983) Motility and chemotaxis of *Rhizobium meliloti* in soil. *Appl. Env. Microbiol.* **46:** 995-998.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503-517.

Spencer, P.A. and Towers, G.H.N. (1988) Specificity of signal compounds detected by *Agrobacterium tumefaciens*. *Phytochemistry* **27:** 2781-2785.

Springer, M.S., Goy, M.F. and Adler, J. (1979) Protein methylation in behavioural control mechanisms and in signal transduction. *Nature (London)* **280:** 279-284.

Stachel, S.E., An, G., Flores, C. and Nester, E.W. (1985a) A Tn3 *lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression of *Agrobacterium*. *EMBO J.* **4:** 891-898.

Stachel, S.E., Messens, E., Van Montagu, M. and Zambryski, P. (1985b) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature (London)* **318:** 624-629.

Stachel, S.E. and Nester, E.W. (1986) The genetic and transcriptional organisation of the *vir*-region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J.* **5:** 1445-1454.

Stachel, S.E., Nester, E.W. and Zambryski, P.C. (1986) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proc. Natl. Acad. Sci. USA* **83:** 379-383.

Stachel, S.E., Timmerman, B. and Zambryski, P. (1987) Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement of the 5' *virD* gene products. *EMBO J.* **6:** 857-863.

Stachel, S.E. and Zambryski, P. (1986a) *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. *Cell* **47:** 155-157.

Stachel, S.E. and Zambryski, P.C. (1986b) *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *Agrobacterium tumefaciens*. *Cell* **46:** 325-333.

Starkey, R.L. (1929) Some influences of the development of higher plants upon the microorganisms in the soil. *Soil Sci.* **27:** 355-378.

Steck, T.R., Lin, T-S. and Kado, C.I. (1990) *VirD2* gene product from the nopaline plasmid pTiC58 has at least two activities required for virulence. *Nucl. Acids Res.* **18:** 6953-6958.

Steck, T.R., Morel, P. and Kado, C.I. (1988) *Vir* box sequences in *Agrobacterium tumefaciens* pTiC58 and A6. *Nucl. Acids Res.* **16:** 8736.

Stock, J.B., Ninfa, A.J. and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Revs.* **53:** 450-490.

Sundheim, L., Poplawsky, A.R. and Ellingboe, A.H. (1988) Molecular cloning of two chitinase genes from *Serratia marcescens* and their expression in *Pseudomonas* species. *Physiol. Mol. Plant Pathol.* **33:** 483-491.

Tait, R.C. and Kado, C.I. (1988) Regulation of the *virC* and *virD* promoters of pTiC58 by the *ros* chromosomal mutation of *Agrobacterium tumefaciens*. *Mol. Microbiol.* **2:** 385-392.

Tate, M.E., Murphy, P.J., Roberts, W.P. and Kerr, A. (1979) Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. *Nature (London)* **280:** 697-699.

Taylor, J., Ott, J. and Eckstein, F. (1985) The rapid generation of oligonucleotide-directed mutagenesis at high frequency using phosphothioate-modified DNA. *Nucl. Acids Res.* **13:** 8765-8785.

Teintze, M., Hossain, M.B., Barnes, C.L., Leong, J. and Van der Helm, D. (1981) Structure of ferric pseudobactin, a siderophore from a plant growth promoting *Pseudomonas*. *Biochemistry* **20:** 6446-6457.

Tempe, J. and Petit, A. (1982) Opine utilisation by *Agrobacterium*. In: *Molecular Biology of Plant Tumours*. pp.451-459. G. Kahl & J. Schell (Eds.) Academic Press, New York.

Thomashow, M.F., Panagopoulos, C.G., Gordon, M.P. and Nester, E.W. (1980) Host range of *Agrobacterium tumefaciens* is determined by the Ti-plasmid. *Nature (London)* **283:** 794-796.

Thomashow, M.F., Karlinsey, J.E., Marks, J.R. and Hurlbert, R.E. (1987) Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. *J. Bacteriol.* **169:** 3209-3216.

Thomashow, L.S., Reeves, S. and Thomashow, M.F. (1984) Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyses synthesis of indoleacetic acid. *Proc. Natl. Acad. Sci. USA* **81:** 5071-5075.

Thomashow, L.S. and Weller, D.M. (1988) Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J. Bacteriol.* **170:** 3499-3508.

Thomashow, L.S. and Weller, D.M. (1990) Role of antibiotics and siderophores in biocontrol of take-all disease of wheat. *Plant Soil* **129:** 93-99.

Thompson, D.V., Melchers, L.S., Idler, K.B., Schilperoort, R.A. and Hooykaas, P.J.J. (1988) Analysis of the complete nucleotide sequence of the *Agrobacterium tumefaciens virB* operon. *Nucl. Acids Res.* **16:** 4621-4636.

Toro, N., Datta, A., Carmi, O.A., Young, C., Prusti, R.K. and Nester, E.W. (1989) The *Agrobacterium tumefaciens virC1* gene product binds to overdrive, a T-DNA transfer enhancer. *J. Bacteriol.* **171:** 6845-6849.

Toro, N., Datta, A., Yanofsky, M. and Nester, E. (1988) Role of the overdrive sequence in T-DNA border cleavage in *Agrobacterium. Proc. Natl. Acad. Sci. USA* **85:** 8558-8562.

Tremblay, G., Gagliardo, R., Chilton, W.S. and Dion, P. (1987) Diversity among opine-utilising bacteria: identification of coryneform isolates. *Appl. Environ. Microbiol.* **53:** 1519-1524.

Usami, S., Okamoto, S., Takebe, I. and Machida, Y. (1988) Factor inducing *Agrobacterium tumefaciens vir* gene expression is present in monocotyledonous plants. *Proc. Natl. Acad. Sci. USA* **85:**3748-3752.

Utkhede, R.S. and Gaunce, A.P. (1983) Inhibition of *Phytophthora cactorum* by a bacterial antagonist. *Can. J. Bot.* **61:** 3343-3348.

Uttaro, A.D., Cangelosi, G.A., Geremia, R.A., Nester, E.W. and Ugalde, R.A. (1990) Biochemical characterisation of avirulent *exoC* mutants of *Agrobacterium tumefaciens. J. Bacteriol.* **172:** 1640-1646.

Van Bogelen, R.A., Kelley, P.M. and Neidhart, F.C. (1987) Differential induction of heat shock, SOS and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli. J. Bacteriol.* **169:** 26-32.

Van Haaren, M.J.J., Sedee, N.J.A., Schilperoort, R.A. and Hooykaas, P.J.J. (1987) *Overdrive* is a T-region transfer enhancer which stimulates T-strand production in *A. tumefaciens. Nucl. Acids Res.* **15:** 8983-8997.

Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I., Schilperoort, R.A. and Schell, J. (1974) Large plasmid in *Agrobacterium tumefaciens* essential for crown-gall inducing ability. *Nature (London)* **252:** 167-170.

Van Larebeke, N., Genetello, C., Schell, J., Schilperoort, R.A., Hermans, A.K., Hernalsteens, J.P. and Van Montagu, M. (1975) Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. *Nature (London)* **255:** 742-743.

Van Onckelen, H., Prinsen, E., Inze, D., Rudelsheim, P., Van Lijsebettens, M., Follin, A., Schell, J., Van Montagu, M. and De Greef, J. (1986) *Agrobacterium* T-DNA gene 1 codes for tryptophan 2-monooxygenase activity in tobacco crown gall cells. *FEBS Lett.* **198:** 357-360.

Veluthambi, K., Jayaswal, R.K. and Gelvin, S.B. (1987) Virulence genes A, G and D mediate the double stranded border cleavage of T-DNA from the *Agrobacterium* Ti plasmid. *Proc. Natl. Acad. Sci. USA* **84:** 1881-1885.

Veluthambi, K., Ream, W. and Gelvin, S.B. (1988) Virulence genes, borders and overdrive generate single-stranded T-DNA molecules from the A6 Ti-plasmid of *Agrobacterium tumefaciens*. *J. Bacteriol.* **170:** 1523-1532.

Vidaver, A.K. (1983) Bacteriocins: The lure and the reality. *Plant Dis.* **67:** 471-475.

Vieira, J. and Messing, J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19:** 259-268.

Wagner, W., Vogel, M. and Goebel, W. (1983) Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. *J. Bacteriol.* **154:** 200-210.

Wang, K., Genetello, C., Van Montagu, M. and Zambryski, P. (1987) Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. *Mol. Gen. Genet.* **210:** 338-346.

Wang, K., Herrera-Estrella, L., Van Montagu, M. and Zambryski, P. (1984) Right 25bp terminus sequences of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell* **38:** 35-41.

Wang, K., Herrera-Estrella, A. and Van Montagu, M. (1990) Overexpression of *virD1* and *virD2* genes in *Agrobacterium tumefaciens* enhances T-complex formation and plant transformation. *J. Bacteriol.* **172:** 4432-4440.

Ward, J.E., Akiyoshi, D.E., Regier, D., Datta, A., Gordon, M.P. and Nester, E.W. (1988) Characterisation of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. *J. Biol. Chem.* **263:** 5804-5814.

Ward, J.E., Akiyoshi, D.E., Regier, D., Datta, A., Gordon, M.P. and Nester, E.W. (1990) Characterisation of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. *J. Biol. Chem.* **265:** 4768.

Ward, E.J., Dale, E.M., Nester, E.W. and Binns, A.N. (1990) Identification of a *virB* 10 protein aggregate in the inner membrane of *Agrobacterium tumefaciens*. *J. Bacteriol.* **172:** 5200-5210.

Watanabe, T., Oyanagi, W., Suzuki, K. and Tanaka, H. (1990) Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol.* **172:** 4017-4022.

Watson, B., Currier, T.C., Gordon, M.P., Chilton, M-D. and Nester, E.W. (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* **123:** 255-264.

Weller, D.M. (1983) Colonisation of wheat roots by a fluorescent pseudomonad suppressive to take-all. *Phytopathology* **73:** 1548-1553.

Weller, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* **26:** 379-407.

Weller, D.M., Howie, W.J. and Cook, R. (1988) Relationships between *in vitro* inhibition of *Gaeumannomyces graminis* var. *tritici* and suppression of take-all of wheat by fluorescent pseudomonads. *Phytopathology* **78:** 1094-1100.

West, A.W. (1984) Fate of the insecticidal, proteinaceous parasporal crystal of *Bacillus thuringiensis* in soil. *Soil Biol. Biochem.* **16:** 357-360.

White, P.R. and Braun, A.C. (1942) A cancerous neoplasm of plants - autonomous bacteria-free crown gall tissue. *Cancer Res.* **2:** 597-617.

Willets, N. and Skurry, R. (1987) Structure and function of the F factor and mechanism of conjugation. In: *Escherichia coli and Salmonella typhimuriam: cellular and molecular biology, Vol. 2.* F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter and H.E. Umbrager (Eds.) American Society for Microbiology, Washington, D. C.

Williams, S.T. and Vickers, J.C. (1986) The ecology of antibiotic production. *Microb. Ecol.* **12:** 43-52.

Willmitzer, L., De Beuckeleer, M., Lemmers, M., Van Montagu, M. and Schell, J. (1980) DNA from Ti-plasmid is present in the nucleus and absent from plastids of plant crown-gall cells. *Nature (London)* **287:** 359-361.

Willmitzer, L., Dhaese, P., Schreier, P.H., Schmalenbach, W., Van Montagu, M. and Schell, J. (1983) Size, location and polarity of T-DNA encoded transcripts in nopaline crown gall tumours: common transcripts in octopine and nopaline tumours. *Cell* **32:** 1045-1056.

Winans, S.C. (1990) Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promoters by plant-released phenolic compounds, phosphate starvation and acidic growth media. *J. Bacteriol.* **172:** 2433-2438.

Winans, S.C., Allenza, P., Stachel, S.E., McBride, K.E. and Nester, E.W. (1987) Characterisation of the *virE* operon of the *Agrobacterium* Ti plasmid pTiA6. *Nucl. Acids Res.* **15:** 825-838.

Winans, S.C., Ebert, P.R., Stachel, S.E., Gordon, M.P. and Nester, E.W. (1986) A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. *Proc. Natl. Acad. Sci. USA* **83:** 8278-8282.

Winans, S.C., Kerstetter, R.A. and Nester, E.W. (1988) Transcriptional regulation of the *virA* and *virG* genes of *Agrobacterium tumefaciens. J. Bacteriol.* **170:** 4047-4054.

Winans, S.C., Kerstetter, R.A., Ward, J.E. and Nester, E.W. (1989) A protein required for transcriptional regulation of *Agrobacterium* virulence genes spans the cytoplasmic membrane. *J. Bacteriol.* **171:** 1616-1622.

Yadav, N.S., Vanderleyden, J., Bennett, D.R., Barnes, W.M. and Chilton, M-D. (1982) Short direct repeats flank the T-DNA on a nopaline Ti plasmid. *Proc. Natl. Acad. Sci. USA* **79:** 6322-6326.

Yanagida, N., Uozumi, T. and Beppu, T. (1986) Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli. J. Bacteriol.* **166:** 937-944.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC18 vectors. *Gene* **33:** 103-119.

Yanofsky, M., Lowe, B., Montoya, A., Rubin, R., Krul, W., Gordon, M.P. and Nester, E.W. (1985) Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens. Mol. Gen. Genet.* **201:** 237-246.

Yanofsky, M.F. and Nester, E.W. (1986) Molecular characterisation of a host-range determining locus from *Agrobacterium tumefaciens*. *J. Bacteriol.* **168:** 244-250.

Yanofsky, M.F., Porter, S.G., Young, C., Albright, L.M., Gordon, M.P. and Nester, E.W. (1986) The *virD* operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. *Cell* **47:** 471-477.

Young, C. and Nester, E.W. (1988) Association of the VirD2 protein with the 5' end of T-strands in *Agrobacterium tumefaciens*. *J. Bacteriol.* **170:** 3367-3374.

Zaenen, I., Van Larebeke, N., Teuchy, H., Van Montagu, M. and Schell, J. (1974) Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. *J. Mol. Biol.* **86:** 109-127.

Zambryski, P. (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annu. Rev. Genet.* **22:** 1-30.

Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M. and Schell, J. (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* **2:** 2143-2150.

Zerback, R., Dressler, K. and Hess, D. (1989) Flavonoid compounds from pollen and stigma of *Petunia hybrida*: Inducers of the *vir* region of the *Agrobacterium tumefaciens* Ti plasmid. *Plant Sci.* **62:**83-91.

Zorreguieta, A., Geremia, R.A., Cavaignac, S., Cangelosi, G.A., Nester, E.W. and Ugalde, R.A. (1988) Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. *Mol. Plant-Microbe Interact.* **1:** 121-127.