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BIOLOGICAL CONTROL OF A PLANT PATHOGEN AND PEST BY EXPRESSION OF A CLONED *Serratia marcescens chiA* GENE AND *Bacillus thuringiensis cryIA(c)* GENE IN ENDOPHYTIC BACTERIA

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To my husband Roderick Westwood

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ABSTRACT

Protection of plants from pathogens and pests by introduced microorganisms provides an alternative to environmentally hazardous chemical pesticides and fungicides. Plant associated, free living, non-pathogenic bacteria found in the rhizosphere and phylloplane have been the emphasis of research on biological control agents. These regions however pose problems of competition between introduced microorganisms and native microflora and environmental extremes. Endophytic bacteria, present in the interior regions of healthy plants, offer a solution to these problems. Since little work has been done to date with endophytes, the work reported in this thesis comprises a novel approach to achieving biological control of a plant pest and pathogens.

An endophytic strain of *Pseudomonas fluorescens* was isolated from micropropagated apple plantlets and shown to be present in the roots of beans at a level of 1.2 x 10^5 CFU/g fresh weight 10 days after introduction. Generation of spontaneous rifampicin resistant mutants of this strain resulted in *P. fluorescens* Rif1. Isolates of *P. fluorescens* and *Aeromonas caviae* were recovered from the interior regions of surface-sterilized bean seeds. In addition, two sugarcane endophytes, *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*, which has also been isolated from rice, maize and sorghum, as well as an endophytic *Citrobacter* sp. of pine seeds and bean plants were used in this work.

The gene coding for the major chitinase of *S. marcescens*, *chiA*, was cloned under the control of the *tac* promoter by PCR amplification. The gene contained the endogenous Shine Dalgarno sequence 7 bases upstream of the ATG start codon in the plasmid pTC33. The plasmid p*tacchi*A had previously been constructed with a distance of 20 bases between the ribosome binding site of this vector and the ATG start codon of the gene. Gene expression of *chiA* carried on pTC33 was shown to be approximately 16-fold higher than that of p*tacchi*A. This clearly illustrated that the distance between the Shine Dalgarno sequence and ATG start codon was critical and that the shorter distance of 7 bases significantly increased the translation efficiency of *chiA*.

The first and second generation *tacchi*A cassettes from p*tacchi*A and pTC33 respectively were cloned into the broad host range plasmids pKT240, pDER405 and pML122 and into the integration vector pJFF350. These plasmids were introduced into the endophytes *P. fluorescens* Rif1, *H. seropedicae* and *Citrobacter* sp. AJ1 by conjugative transfer and electroporation and were shown to express the gene at varying levels. pKTC1 and pMTC33 carrying the 1st and 2nd generation *tacchi*A cassettes on pKT240 and pML122 respectively were not stably maintained in *P. fluorescens* Rif1 or *H. seropedicae*. The integration vector pJTC33 carrying the 2nd generation *tacchi*A cassette was shown to exist as a plasmid in *Citrobacter* AJ1. Chitinase assays showed that *P. fluorescens* Rif1 with the 1st generation Omegon-Km-*tacchi*A cassette integrated into the chromosome had considerably lower activity (0.2 units) than that of two strains carrying the integrated 2nd generation Omegon-Km-*tacchi*A cassette (3.27 and 2.93 units). Activity of *P. fluorescens*

Rif1(pKTC1) carrying the 1st generation cassette on a multicopy plasmid (2.64 units) was comparable with that of the latter strains.

P. fluorescens Rif1 carrying the 1st and 2nd generation *tacchi*A cassettes is an effective biocontrol agent of the phytopathogenic fungus *Rhizoctonia solani* under plant growth room conditions when it is present as an endophyte in beans. The new improved 2nd generation *tacchi*A cassette integrated as a single copy into the chromosome confers comparable biocontrol ability to the host as that of the 1st generation *tacchi*A cassette when present in greater copies on a plasmid. When bean seedlings were inoculated with *P. fluorescens* Rif1(pKTC1), carrying the 1st generation cassette and two strains of *P. fluorescens* Rif1::*tacchi*A33 carrying the 2nd generation cassette, disease incidence caused by *R. solani* was significantly reduced by 42%, 27% and 38% respectively but not by *P. fluorescens* Rif1 or *P. fluorescens* Rif1::*tacchiA*1, carrying the first generation *tacchiA* cassette. Disease incidence was not significantly different when strains were introduced into the plant as well as the soil compared to when strains were only introduced into the plant.

The *cryIA(c)* (new nomenclature *cry1Ac7*) gene of *B. thuringiensis* strain 234 under the control of the *tac* promoter was cloned into pKT240, pDER405 and pJFF350 and without the *tac* promoter into pML122 in both orientations with respect to the Nm^R promoter present on the vector. These plasmids were introduced by conjugation and electroporation into strain *P. fluorescens* 14 isolated from the phylloplane of sugarcane, *H. seropedicae* but not into *A. diazotrophicus*. High levels of the constitutively expressed gene on pKT240 were lethal in *P. fluorescens* 14. The *cryIA(c)* gene under the control of the Nm^R promoter on pML122 led to plasmid instability whereas the plasmid was stably maintained over 60 generations when the gene was cloned in the opposite orientation. Quantitative Western blot analysis showed that *P. fluorescens* 14 clones with the Omegon-Km-*tactox* cassette integrated into the chromosome had greater expression levels than those with the integrated Omegon-Km-*tox* cassette. In *H. seropedicae* more CryIA(c) toxin was produced when the gene was cloned under the control of the Nm^R promoter on pML122 than in the opposite orientation.

The effectiveness of the genetically modified *P. fluorescens* 14 and *H. seropedicae* as biological control agents of the sugarcane borer *Eldana saccharina* were evaluated. Bioassays showed that *P. fluorescens* 14::Omegon-Km-*tactox* resulted in 92% mortality of *E. saccharina* larvae after 192 h compared to 84% mortality caused by *P. fluorescens* 14::Omegon-Km-*tox*. *H. seropedicae* carrying the gene under the control of the Nm^R promoter on pML122 resulted in 80% mortality of larvae after the same period of time compared with 50% mortality when the gene was in the opposite orientation with respect to this promoter.

A synergistic toxic effect was observed when *P. fluorescens* 14::*tactox* was combined with the chitinase-producing strain *P. fluorescens* Rif1::*tacchiA* carrying the 2nd generation *tacchiA* cassette. This latter strain was not toxic on its own to larvae when included in the diet. In the absence of this strain 3 mg freeze-dried *P. fluorescens* 14::*tactox* per g artificial diet was required to cause 92% mortality after 168 h, while only 0.3 mg/g diet was necessary to obtain 96% mortality in the presence of the chitinase producer.

CHAPTER 1

GENERAL INTRODUCTION

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CHAPTER 1

GENERAL INTRODUCTION

Protection of plants from pathogens by introduced microorganisms provides an alternative to the use of environmentally hazardous chemical pesticides and fungicides. Biological control has been studied extensively for several years and is believed to have a beneficial role in agriculture and to be commercially feasible.

An important mechanism of biocontrol involves the degradation of the cell walls of pathogenic fungi. Of particular interest is the biocontrol of the phytopathogenic fungus *Rhizoctonia solani,* a widespread pathogen occurring in both cultivated and noncultivated soils. It is considered a genus of basidiomycetous imperfect fungi, as it lacks a sexual stage or telomorph which is known as *Thanatephorus cucumeris* (Ogoshi, 1987). *R. solani* are multinucleate with three or more nuclei per cell and have large hyphae. Isolates from diseased plants and soils differ in their pathogenicity and morphology. They have therefore been thought to have many intraspecific and anastomosis groups (AGs). If the mycelia of different isolates of *R. solani* on solid media grow together and overlap, and if attraction, fusion of hyphae and death of fused cells occurs, then these isolates belong to the same AG. If these events do not occur then isolates belong to different AGs (Ogoshi, 1987). Nine AGs have been identified for *R. solani* worldwide, although more groups may exist . They may be morphologically similar but each AG is a genetically isolated, noninterbreeding population. Different AGs also have different pathogenicity. For example, AG 1 isolates cause sheath and web blight; AG 2 are pathogens of sugar beet and vegetable crops; and AG 3 primarily infect potatoes (Anderson, 1982). AG 4 infects a wide variety of plant species, especially legumes. They cause seed and hypocotyl rot and form stem lesions near the soil of bean species, cotton and soybean resulting in damping off. They cause crown rot of alfalfa and sugar beet and aerial blight of sugar beet. This group is the most important group with respect to pathogenicity and potential disease reduction by biocontrol agents (Anderson, 1982).

A major cell wall component of fungi and other agronomically important pests is chitin. Chitin is an insoluble β-1,4 -linked, unbranched polymer of N-acetylglucosamine. Many organisms including bacteria, fungi and plants produce enzymes that degrade chitin. Chitinases hydrolyze the polymer to oligomers, mainly dimers, which are subsequently degraded by glucosaminidase to N-acetylglucosamine. Chitinases produced by higher plants may be part of a natural defense mechanism against fungal pathogens, since they do not have any apparent function in the metabolism of plants and can inhibit fungal growth. Development of fungal resistance in plants could therefore be achieved by either enhancement of the activity or availability of the chitinase of a plant or by the introduction of a foreign chitinase into plant cells. The

Gram negative, enteric, soilborne bacterium *Serratia marcescens* secretes several chitinolytic enzymes which retard fungal growth and is thus an effective agent for the biocontrol of widespread fungal pathogens of several economic crops. The gene coding for the major chitinase produced by *S. marcescens, chiA,* has been cloned (Shapira *et al.*, 1989) and supplied to us.

The sugarcane borer *Eldana saccharina* Walker causes extensive damage to sugarcane in South Africa. The natural hosts of this borer are the large Cyperaceae such as *Cyperus immensus* C.B.CI, which are found in wetlands. *E. saccharina* invaded sugarcane in the Umfolozi area of Natal (Kwazulu Natal) from 1939 until about 1950 and again in the Hluhluwe area in 1970. Since then it has become a widespread sugarcane pest in the cane growing areas of South Africa and Swaziland (Atkinson, 1979). It is believed that sugarcane has been invaded because *E. saccharina* prefers to lay its eggs in the abundant dead leaf material at the base of the plant. It attacks the middle and base of mature tillers and penetrates at the node or cracks, extending into the internode or throughout the segments. Older cane is infested more than younger cane and damage caused is usually neglible in cane younger than 9 - 10 months. This may be due to the presence of phenolic compounds in young cane (Atkinson and Nuss, 1989). Succeeding generations of larvae often penetrate the sugarcane through old borings. Infection by the borer fluctuates seasonally and is greater when the crop is stressed such as during drought (Carnegie and Leslie, 1990).

After the second outbreak of *E. saccharina* in 1970 a programme to combat the borer was established, which included biocontrol measures. Extensive field trials were conducted from the mid 1970's to test the toxicity of a wider range of insecticides to the borer. *E. saccharina* numbers were reduced by 85 to 100% when sugarcane was dipped into insecticides but this practice is not convenient in the field. Applications of insecticide to pretrashed and nontrashed cane reduced *E. saccharina* numbers by 75% but optimization of the frequency and rates of application was necessary (Heathcote, 1984). Biological control methods included the use of parasites, parasitoids and microorganisms as potential biocontrol agents. No natural parasites, besides ants which feed on the eggs and larvae, have been encountered which led to the testing of exotic parasites from other parts of Africa with limited success (Carnegie and Leslie, 1979). Seven natural larval parasitoids of the borer have been recognized but there is no known parasitoid in Africa that is particularly effective against *E. saccharina* in sugarcane (Conlong and Hastings, 1984). In West Africa egg and larval parasitoids of *E. saccharina* occur on maize. This natural parasitoid, along with other potential candidates, was imported from the Ivory Coast. Some of these appeared to be promising under laboratory conditions but when released in sugarcane fields the success was limited (Carnegie *et al.*, 1985). This could be due to the fact that *E. saccharina* lay their eggs in regions of the cane that are possibly inaccessible (Carnegie and Leslie, 1979).

Microorganisms are potential candidates for biocontrol of *E. saccharina*. The entomopathogenic fungus *Beauveria bassiana* was isolated in Natal from *E. saccharina* larvae and was used to infect larvae in the

laboratory by spraying with fungal spores. Since the spores are sensitive to dessication their effective use as a biocontrol agent in the field required the development of a suitable formulation. Fungal spores maintain viability and virulence when stored in coconut oil and this could potentially be used as a base in formulations (Jacobs, 1989). No baculovirus that infects *E. saccharina* has been observed in South Africa to date, so a screening programme for such a pathogen was undertaken. The larvae were also stressed in the laboratory in an attempt to activate any latent viral infection but no virus-like particles were isolated (Jacobs, 1989).

The biocontrol agent with the most potential is the Gram positive, aerobic, sporeforming bacterium *Bacillus thuringiensis*. It is a pathogen of insect larvae which produces highly specific crystal inclusions during sporulation. These parasporal crystals consist predominantly of protoxin molecules known as δ-endotoxins, Cry toxins or Cry proteins. The crystals are ingested by insect larva and dissolve in the alkaline midgut where the protoxin is converted to the active toxin by gut proteases. The activated toxin is highly toxic to the insect and very specific in its activity. However, *B. thuringiensis* does not survive well in the field and therefore cannot be used as an effective pest control agent. An alternative strategy has been to introduce insecticidal crystal protein genes into plant-associated bacteria or transgenic plants (Obukowicz *et al.*, 1986a nd b; Perlak *et al.*, 1991). In order to develop a biocontrol agent effective against *E. saccharina*, a δ-endotoxin gene was introduced into a bacterium capable of colonizing sugarcane (Herrera *et al.*, 1994). Several local *B. thuringiensis* isolates were obtained from infected *E. saccharina* cadavers. Some of these isolates were more toxic to *E. saccharina* larvae than the commercially available preparation of *B. thuringiensis* subsp. *kurstaki*, known as Thuricide and B401, a *B. thuringiensis* subsp. *aizawai* strain. One of the crystal protein genes, *cryIA(c)* was isolated from the local isolate *B. thuringiensis* strain 234 and introduced into *P. fluorescens* isolate 14 that effectively colonizes the phylloplane of sugarcane. This recombinant strain was toxic to *E. saccharina* larvae in bioassays and glasshouse trials. Although these results were promising, further improvement of this biocontrol strain by cloning the gene under the control of the strong *tac* promoter for increased expression was necessary and introduction of the gene into obligate sugarcane endophytes is required to address the problems of instability of this strain in the environment and inaccesibility to the interior regions where the larvae feed.

Research on biological control agents has emphasized free living, plant associated, nonpathogenic bacteria present in the rhizosphere and phylloplane. The rhizosphere is defined as the soil zone immediately surrounding the root which is influenced biologically and physically by the root (Kluepfel, 1993). It is an extremely complex environment which is inhabited by numerous microorganisms. Use of rhizosphere microorganisms as biocontrol agents provides frontline defence for the roots from invading pathogens (Weller, 1988). To be efficient biocontrol agents the bacteria must survive and become established in the soil and should be good root colonizers, including colonization of the internal or surface of the root as well as the rhizosphere soil (Weller, 1988). Factors such as temperature, soil moisture, pH and clay content will

influence the survival of introduced bacteria as biocontrol agents. They must also be able to compete and survive in the rhizosphere. The surface of aerial parts of the plant, known as the phylloplane, support the growth of microorganisms that can control diseases. The biocontrol activity of introduced organisms depends on both the microclimatic conditions at the plant surface as well as the chemical environment (Blakeman and Fokkema, 1982). Factors such as low and irregular provision of surface water, extreme temperatures and low nutrient availability on the surface of leaves influences the survival of microorganisms.

These problems could be circumvented by using endophytic bacteria that are present in the interior regions of healthy plants. They are found in the cortex, xylem of roots, stems and leaves and in seeds and ovules. There are additional advantages to using endophytic bacteria as biocontrol agents: they are likely to be shielded from the microbial competition and environmental extremes which exist in the rhizosphere and phylloplane and the chance of uncontrolled spreading of a genetically modified bacteria through the soil would be decreased. A potential limitation to the commercial use of endophytes would be the difficulty of reintroduction into the plant.

1.1 CHITINASES

1.1.1 CHITIN STRUCTURE

Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc), is one of the most abundant naturally occurring polysaccharides (Iliyina *et al.*, 1995). It is a major structural constituent of fungal cells walls, the exoskeletons of insects, organs of Molluscs and shells of crustaceans such as crabs, shrimps and krill (Flach *et al.*, 1992; Iliyina *et al.*, 1995). It is not found in plants or humans. Chitin which has been isolated from different species in nature and by different isolation methods differs in solubility, molecular weight, degree of deacetylation and specific rotations. This implies that it represents a family of closely related products (Austin *et al*, 1981). It is usually associated with protein although one type of chitin, called chitan, isolated from the spines of certain marine diatoms, occurs free of protein (Austin *et al.*, 1981; Blackwell, 1988).

The structure of chitin has been analysed by X-ray crystallography and infrared spectroscopy. X-ray data have shown that it is similar to cellulose in that both have the same 2_1 helical conformation. It has a fibrous morphology and is semicrystalline. As with many other crystalline polymers, chitin occurs in nature in more than one polymorphic form. Three have been identified from different sources, namely α, β and γ, which all have the same 2_1 helical conformation but differ in the mode of packing of adjacent chains. The α form is the most predominant occuring in most chitin of fungi, crustaceans and insects. The β form is rare and has been identified in only four sources. The γ form is not well characterized and is possibly not a true third

form (Blackwell, 1988). The unit cell of α chitin is orthorhombic with antiparallel packing of chains in sheets along the a axis. The intersheet hydrophobic bonds are responsible for the stability of the α chitin structure. The β chitin, unlike the α form, is unable to swell in water and has been shown to form a series of crystalline hydrate structures. The unit cell of β chitin is half the volume of that of α chitin and contains a dissaccharide unit of a single chain. The parallel chains of the structure are stacked along the a axis but there is no hydrogen bonding between adjacent stacks of chains. This allows the structure to swell in water which correlates well with the fact that β chitin is found exclusively in aquatic organisms (Blackwell, 1988). The unit of γ chitin has a three chain structure in which two " up" chains are followed by one "down" chain. Further analysis of this chitin is required to establish whether it is a separate form of chitin or a distorted, poorly crystalline α or β structure (Blackwell, 1988).

1.1.2 CHITINASE NOMENCLATURE

Most organisms that contain chitin produce chitinases, although they occur in a wide variety of bacteria, plants and humans which do not contain chitin. The first report of a chitinase was in 1911 by Bernard who isolated a thermostable and diffusible antifungal factor in orchid bulbs. In 1929 Karrer and Hoffman reported on a chitinase isolated from snail (cited in Flach *et al.,* 1992). Interest in chitinases has been renewed mainly due to the realisation of its potential roles in plant defence and in biological control.

Chitinases are defined as enzymes which cleave a bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitin (Flach *et al.*, 1992). The nomenclature of these enzymes is confusing and still under debate. In general two chitinolytic enzymes were recognised. According to the old nomenclature chitinases (EC 3.2.1.14) randomly cleave the chitin polymer to chitobiose which is then cleaved into units of N-acetylglucosamine by chitobiase (EC 3.2.1.29). The word chitinase may be used to refer to any enzyme with chitinolytic activity or only to endochitinases whose activity is described below (Tronsmo and Harman, 1993). The term chitobiase is no longer recognized (Harman *et al.*, 1993) and has been replaced by the term β-1,4 N-acetylglucosaminidase (EC 3.2.1.30) (Tronsmo and Harman, 1993; Sahai and Manocha, 1993).

These enzymes have endo and/or exochitinase activities. Endochitinases cleave the chitin polymer randomly at internal points resulting in chitotetraose, chitotriose and predominantly diacetylchitobiose which are tetramers, trimers and dimers of GlcNAc respectively (Sahai and Manocha, 1993). In the older literature exochitinases were defined as enzymes that release acetylglucosamine from the non-reducing ends of chitin (Robbins *et al.*, 1988); these were also known as chitobiases. A third type of enzyme nomenclature was necessary to describe the release of disaccharides from the chitin polymer. Exochitinases were then defined as enzymes that release diacetylchitobiose progressively from the non-reducing ends of chitin (Robbins *et al.*, 1988; Roberts and Selitrennikoff, 1988). Tronsmo and Harman (1993) have termed these

exochitinases 1,4-β-chitobiosidases or just chitobiosidases. Sahai and Manocha's (1993) definition of exochitinases is the same as that of Robbin *et al.* (1988), with the addition that no monosaccharides or oligosaccharides were formed by this enzyme. β-1,4 N-acetylglucosaminidase releases GlcNAc monomers from diacetylchitobiose as well as chitotriose and chitotetraose in an exo-type mechanism of action (Sahai and Manocha, 1993). Harman *et al.* (1993) refer to these enzymes simply as glucosaminidases.

In summary, it is now generally accepted that there are three types of chitinolytic enzyme activity (Brurberg *et al.*, 1996): a) N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) (or N-acetyl-β-glucosaminidase (EC 3.2.1.52)) abbreviated glucosaminidase that cleaves monomeric units of GlcNAc from the non-reducing end of chitin; b) Exo-N,N'-diacetylchitobiohydrolases (also called exochitinases or chitobiosidases) which catalyze the progressive release of the dimer, diacetylchitobiose in a stepwise fashion; and c) Endochitinases (EC 3.2.1.14) which randomly cleave the chitin polymer internally. In addition Exo-N,N',N''-acetylchitotriohydrolases cleave $(GlcNAc)_6$ to $(GlcNAc)_2$ and GlcNAc (Bruberg *et al.*, 1996). Some chitinases have transglycosylation activity with the ability to convert $(GlcNAc)_4$ to $(GlcNAc)_6$ (Takayanagi *et al.*, 1991).

1.1.3 CHITINASE ASSAYS

Several assays of chitinase activity have been reported. The viscosimetric method is based on the measurement of the viscosity of a glycol chitin solution which is reduced by chitinase, in an Oswald viscometer. Other compounds used in the assay have been chitosan acetate and carboxymethylchitin (Ohtakara, 1988). The colorimetric assay, used to assay both exo and endochitinases, is based on the determination of monomeric GlcNAc (exochitinase assay) released from colloidal chitin. In an endochitinase assay the predominant chitooligosaccharide products are enzymatically cleaved to monomeric GlcNAc before to the colorimetric measurement (Boller and Mauch, 1988). A radioactive assay based on the formation of soluble oligosaccharides from tritiated chitin is believed to be more sensitive than the previously mentioned assays (Cabib, 1988). The radioactive chitin is prepared by the reacetylation of chitosan, a colloidal chitin substitute, with tritiated acetic anhydride (Molano *et al.*, 1977). Wirth and Wolf (1990) developed the first commercially available soluble dye labelled and colloidal dye labelled substrates based on a carboxymethyl substituted soluble chitin and a reprecipitated colloidal chitin which were covalently linked to the dye Remazol brilliant violet. These dye labelled substrates can be used to quantitate activity in solution by colorimetric assays, in plate clearing assays for the detection of chitinolytic microorganisms (Wirth and Wolf, 1990) and as a substrate in polyacrylamide gels for detection of chitinolytic activity (Kalix and Hohenheim, 1995).

The chromogenic and fluorescent assays are based on the release of p-nitrophenol (pNp) and 4-methylumbelliferone (4-MU) respectively from synthetic substrates by chitinolytic enzymes. Two sets of

three highly sensitive substrates that produce a chromogenic or fluorescent product when enzymatically cleaved, are available commercially. The fluorescent substrates are, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide [4-MU-(GlcNAc]; 4-methylumbelliferyl-β-D-N,N'-diacetylchitobioside [4-MU-$(GlcNAc)_2$] and 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotriose [4-MU-$(GlcNAc)_3$]. The 4-methylumbelliferyl group is linked to the N-acetylglucosamine oligosaccharides (Haran *et al.*, 1995). The chromogenic compounds are similar to the fluorescent substrates. They are p-nitrophenyl-β-D-N-acetylglucosaminide; p-nitrophenyl-β-D-N,N'-diacetylchitobiose and p-nitrophenyl-β-D-N,N',N''-triacetylchitotriose (Tronsmo and Harman, 1993; Harman *et al.*, 1993). These two sets of compounds function as dimeric, trimeric and tetrameric substrates respectively with the p-nitrophenyl group or 4-MU group acting as one moiety (Fig 1.1 pg 16). Yellow colour formation or fluorescence is only produced by the hydrolysis of p-nitrophenol or 4-MU respectively. These compounds are used to identify glucosaminidases (dimeric substrate), exochitinases/chitobiosidases (trimeric substrate) and endochitinases (tetrameric substrate) (Roberts and Selitrennikoff, 1988; Harman *et al.*, 1993; Tronsmo and Harman, 1993 and Haran *et al.*, 1995).

1.1. 4 CLASSIFICATION OF CHITINASES

Based on amino acid sequence similarities the chitinases have been assigned two different families of the classification of glycosyl hydrolases, namely families 18 and 19, (Henrissat and Bairoch, 1993). Chitinases from bacteria, fungi, viruses, animals and the plant class III and V chitinases belong to family 18. Family 19 comprises only of the class I, II and IV plant chitinases. These families have different three dimensional structures and display no sequence homology (Ohno *et al.*, 1996).

1.1.5 BACTERIAL CHITINASES

Generally organisms that possess chitin as a structural constituent also produce chitinases. Exception to this general rule are provided by a wide variety of bacteria and actinomycetes which do not contain constituent chitin but do produce chitinases. These include species of the bacterial genera *Aeromonas* (Inbar and Chet, 1991), *Bacillus* (Watanabe *et al.*, 1990a; Pleban *et al.*, 1995), *Enterobacter* (Chernin *et al.*, 1995), *Serratia* (Monreal and Reese, 1969), *Streptomyces* (Blaak *et al.*, 1993) and *Vibrio* (Wortman *et al.*, 1986). Of 100 organisms tested for chitinolytic activity by Monreal and Reese (1969), the Gram negative enteric soil bacterium *Serratia marcescens* was found to be the best chitinase producer. This organism was shown to produce several chitinolytic proteins with subunit molecular masses of 21, 36, 48, 52/55.5, 58 and 95 kDa (Fuchs *et al.*, 1986; Kless *et al.*, 1989; Brurberg *et al.*, 1996). The most abundant chitinase produced by *S. marcescens* is the 58 kDa chitinase. Jones *et al.* (1986) isolated and characterized two chitinase genes, *chiA* and *chiB* from *S. marcescens* QMB1466 which encode two distinct chitinases of 58 kDa and 52 kDa respectively. These genes showed no detectable homology to each other, although Brurberg *et al.* (1996)

have reported 33% sequence identity between *chiA* and *chiB* of *S. marcescens* BJL200. The DNA sequence of *chi*A occurs within a 1686 bp open reading frame which encodes a 61 kDa protein with a 3 kDa signal peptide at the N terminus. This signal peptide is typical of genes encoding secreted bacterial proteins (Jones *et al.*, 1986). The nucleotide sequence surrounding the gene contains regulatory sequences and characteristic bacterial promoter sequences at -35 and -10 from the transcriptional start site. These are followed by a 158 base leader sequence containing a Shine Dalgarno sequence nine bases from the translational start site. A GC rich inverted repeat sequence indicative of a transcriptional termination signal occurs at the end of the chitinase gene. Similiar *chiA* genes have been isolated and sequenced from different strains of *S. marcescens* with variations in certain regions of their DNA sequences. (Harpster and Dunsmuir, 1989; Brurberg *et al.*, 1994; Brurberg *et al.*, 1995; Oppenheim and Chet, personal communication). The ones from QMB1466 and BJL200 which encode a periplasmic chitinolytic enzyme, are almost identical with 96.4% homology in the coding regions (Brurberg *et al.*, 1996). The *chiB* coding region is composed of 1497 bp which encodes a protein of 55.5 kDa, although Jones *et al.* (1986) reported it to be 52 kDa. The gene is preceded by a typical prokaryotic promoter and followed by a strong terminator. There is no characteristic signal peptide at the N terminus and no processing occurred in this region except for the removal of the N-terminal methionine residue. The enzyme, shown to be present in the periplasm of *S. marcescens* by fractionation and immunocytochemical studies, must therefore be exported by an unknown and as yet undefined mechanism (Brurberg *et al.,* 1995).

A chitobiase (glucosaminidase) gene encoding a protein of 95 kDa has also been isolated and characterized from *S. marcescens* (Kless *et al.*, 1989). This gene has been sequenced (Tews *et al.*, 1996).

Serratia liquefaciens contains five genes encoding proteins that are involved in the degradation of chitin (Joshi *et al.*, 1988). The *chiA* and *chiB* genes encode separate chitinases and the *chiC* gene encodes a chitobiase. The *chiA* and *chiB* genes showed no similarity to the chitinase genes from *S. marcescens*. The *chiD* and *chiE* genes are involved in the regulation of expression of the chitinolytic enzymes in this organism.

Aeromonas hydrophila contains a single excreted chitinase gene which encodes a protein of 85 kDa. The organism also contains a chitobiase (Chen *et al.*, 1991). Inbar and Chet (1991) partially purified a chitinase from *A. caviae* which yielded three bands on SDS PAGE of approximate molecular weights of 80, 48 and 59 kDa. This gene has been sequenced (Sitrit *et al.*, 1995). The 80-kDa protein band was the strongest.

Within the genus *Bacillus* most of the work on the characterization of chitinases and their corresponding genes has been done on those from *B. circulans* WL-12. The bacterium, which is lytic for yeast and fungal cell walls, produces at least six distinct chitinases which are secreted (Watanabe *et al.*, 1990a; 1994). The chitinases are named A1, A2, B1, B2, C and D with molecular weights of 74, 69, 38, 38, 39 and 52

respectively. It is believed that these are not coded for by six distinct genes but rather that some are derived from others. Chitinase A1 and A2, for example, have identical N-terminal amino acid sequences and the difference in size indicates that A2 was probably derived from A1 by proteolytic removal of the C-terminal region of chitinase A1. This conversion seems to occur after secretion of chitinase A1 into the growth medium. Similiarly chitinase B2 is a derivative of chitinase B1 since the two chitinases have identical N-terminal amino acid sequences. Although chitinase B1 and B2 do not differ in size, the isoelectric points of the two chitinases differ significantly (Watanabe *et al.*, 1990a). The key enzyme in the chitinase system of *B. circulans* is assumed to be chitinase A1 (and A2) as it is the most abundant in culture supernatant with the highest enzymatic activity on colloidal chitin (Watanabe *et al.*, 1990a). The *chiA* and *chiD* genes encoding chitinase A1 and D respectively have been cloned and sequenced. The calculated molecular mass of the mature protein of chitinase A1 of *B. circulans* WL-12 is 69.3 kDa which is approximately two thirds greater than the mature ChiA of *S. marcescens* (Watanabe *et al.*, 1990b). The deduced amino acid sequences of *chiA* genes of *S. marcescens* and *B. circulans* were compared. The N-terminal two thirds of the mature chitinase A1 was found to have 33% homology with almost the entire mature ChiA of *S. marcescens* indicating an evolutionary origin of the *chiA* genes of these organisms (Watanabe *et al.*, 1990b). The *chiD* gene is located upstream of the the *chiA* gene of *B. circulans* WL-12 with a calculated mass of the mature chitinase D of 48.2 kDa (Watanabe *et al.*, 1992). Four kinds of thermostable chitinases named chitinases I, II, III and IV with molecular weights of 89, 76, 66 and 59 kDa respectively were isolated from *B. licheniformis*. As with *B. circulans* WL-12 chitnases, it is believed that chitinases II, III and IV originated from the same enzyme and gene (probably chitinase II) and were processed differently during or after excretion (Takayanagi *et al.*, 1991).

In contrast to the relatively few chitinolytic bacterial species discussed above, nearly all species of *Streptomyces* have been shown to produce chitinases. Only a few of these or their corresponding genes have been analysed to date. The enzyme complex produced by *S. kurssanovii* consists of four chitinases of molecular masses 42, 40, 26 and 20 kDa (Ilyina *et al.*, 1995). A thermostable chitinase gene, *chi*40, was cloned from *S. thermoviolaceus* ORC-520 by Tsujibo *et al.* (1995). *S. lividans* secretes at least four chitinases, three of whose corresponding genes have been cloned and sequenced. DNA sequence analysis of the promoter region of the *chiC* gene showed two identical 12-bp direct repeat sequences. These direct repeats have also been reported in the promoter region of chitinase genes *chi*63 and *chi*35 of *S. plicatus* and *chiA* of *S. lividans* suggesting that these sequences are involved in the regulation of chitinase gene expression (Fujii and Miyashita, 1993). Chitinolytic activity in *S. abidoflavus* originates from seven different chitinases, the genes for which have still to be isolated (Broadway *et al.*, 1995). *S. griseus* secretes two major chitinases C-1 and C-2 into the culture medium which are suspected to be derived from a single gene, *chiC*. From the nucleotide sequence of this gene and the determined N terminus of both chitinases it is apparent that they are truncated forms of the initial product of this gene, chitinase C. As has been reported for many of the other bacterial chitinases, chitinases C-1 and C-2 are generated from initially secreted

chitinase C by proteases present in the culture supernatant. The presence of a second chitinase gene in *S. griseus* has been demonstrated (Ohno *et al.*, 1996). *S. olivaceovindis*, the most efficient chitin degrader, produces five chitinases (Blaak *et al.*, 1993). One of the corresponding genes, *exo-chi01* has been cloned and characterized. As is common in other species of *Streptomyces*, a mature 59-kDa protein encoded by *exo-chi01* is converted to a 47-kDa protein by proteolytic processing and found in the supernatant (Blaak and Schrempf, 1995).

1.1.6 THE STRUCTURE OF BACTERIAL CHITINASES

From the nucleotide sequences of the isolated chitinase genes the amino acid sequence and structure of bacterial chitinases could be determined. Bacterial chitinases consist of at least three functional domains, a chitin binding domain, a fibronectin type III-like module (domain) and a catalytic domain in addition to the signal sequence (Watanabe *et al.*, 1994). Most of the work done on identifying the structure and function of these domains has been carried out on *B. circulans* WL-12 chitinase A1. This consists of four functional domains, namely a C terminal domain, two fibronectin type III-like domains and a large N terminal domain (Watanabe *et al.*, 1994).

The C terminal domain of chitinase A1 has been shown to be involved in chitin binding and its loss results in a significantly reduced level of chitin binding and hydrolysis (Watanabe *et al.*, 1994). Although this has only been elucidated for chitinase A1 of *B. circulans,* evidence suggests that chitin binding domains are a general feature of many bacterial chitinases.

The deduced amino acid sequence of chitinase A1 revealed two 95-residue tandem repeats which linked the chitin binding domain to the catalytic domain. These tandem repeats were homologous to type III homology units of fibronectin (Watanabe *et al.*, 1990a). The fibronectin type III domain has been identified in at least 50 different animal proteins including human proteins but not in plant or fungal proteins (Bork and Doolittle, 1992). The occurrence of two type III domains in *B. circulans* chitinase A1 was the first report of such in prokaryotes (Bork and Doolittle, 1992). Subsequently this domain III has been identified in other bacterial enzymes such as an α-amylase, an α-amylase-pullanase, an endoglucanse, a galacturonidase and a depolymerase (Bork and Doolittle, 1992; Watanabe *et al.*, 1994). It is believed that the bacterial type III module originated from animal sources before the divergence of prokaryotes and eukaryotes (Bork and Doolittle, 1992). The fibronectin type III domain has been identified in all the bacterial chitinases discussed in section 1.1.5. The role of the two type III domains of chitinase A1 in chitin degradation was determined by site-directed mutagenesis. It was speculated that they were involved in chitin binding of chitinase A1 (Watanabe *et al.*, 1990a) but deletion of these regions did not alter chitin binding (Watanabe *et al.*, 1994). Results obtained indicated that these domains are involved in chitin hydrolysis by an unknown mechanism (Watanabe *et al.*, 1994).

The large N terminal domain of *B. circulans* WL-12 chitinase A1 contains the catalytic site of the enzyme which also has the ability to bind to chitin (Watanabe *et al.*, 1994). A region in the catalytic domain of chitinase A1, shown to be conserved in chitinases and related enzymes from evolutionary divergent organisms, was subjected to site-directed mutagenesis. From the results obtained it appeared that the two most conserved amino acid residues, Glu-204 and Asp-200, are directly involved in the catalytic activity of chitinase A1. This has also been confirmed from the 3 D structure of *S. marcescens* ChiA (Ohno *et al.*, 1996). This is analogous to the catalytic mechanism of lysozyme, which involves the Glu-35 and Asp-52 amino acid residues of the enzyme. The catalytic mechanism of chitinases has thus been suggested to be similar to that of lysozyme (Watanabe *et al.*, 1993). All prokaryotic chitinases sequenced so far share the amino acid sequences found in the catalytic region of chitinase A1 despite differences in their overall primary structure. A 73 amino acid segment located in this domain was found in the sequences of other bacterial chitinases, class III plant chitinases and a yeast killer toxin (Watanabe *et al.*, 1992).

Blaak *et al.* (1993) compared the deduced amino acid sequence of *Streptomyces olivaceoviridis* exo-Chi*01* with that of 25 other chitinolytic enzymes by hydrophobic cluster analysis. From this analysis the domain organization of the different bacterial chitinases could be compared as shown in Figure 1.2.

1.1.7 MODE OF ACTION OF CHITINASES

1.1.7.1 INDUCTION OF CHITINASES

Monreal and Reese (1969) showed that *S. marcescens* chitinase was induced by the presence of chitin in the culture medium. Very little induction of the enzyme occurred when the organism was grown on the monomeric GlcNAc or on non-chitinous substrates such as cellulose, lactose and sucrose. Chitinase production was repressed by glucose, presumably due to a drop in pH to 4.8 at which the enzyme is no longer produced. When the chitinase was cloned in *E. coli*, expression of the chitinase and chitobiase genes was constitutive (Shapira *et al.*, 1989; Kless *et al.*, 1989; Oppenheim *et al.*, 1990; Fuchs *et al.*, 1986; Jones *et al.*, 1986). In contrast although chitinase production by *Aeromonas hydophila* only occurred in medium containing chitin or colloidal chitin and was repressed by glucose, here expression of the cloned gene in *E. coli* was inducible by chitin, was not constitutively expressed and was strongly repressed by glucose. As in the case of chitinases cloned from *S. marcescens, A. hydrophila* chitinase is found mostly in the extracellular medium when cloned in *E. coli* (Chen *et al.*, 1991).

Bacterial chitinases discussed in section 1.1.5 are all induced by chitin or colloidal chitin and are secreted into the culture supernatant (Watanabe *et al.*, 1990a; Takayanagi *et al.*, 1991; Robbins *et al.*, 1988). An exception is ChiB of *S. marcescens* which is located in the cytoplasm in *E. coli*, whereas it is exported to the periplasm in *S. marcescens* without processing at the N terminus (Brurberg *et al.*, 1995).

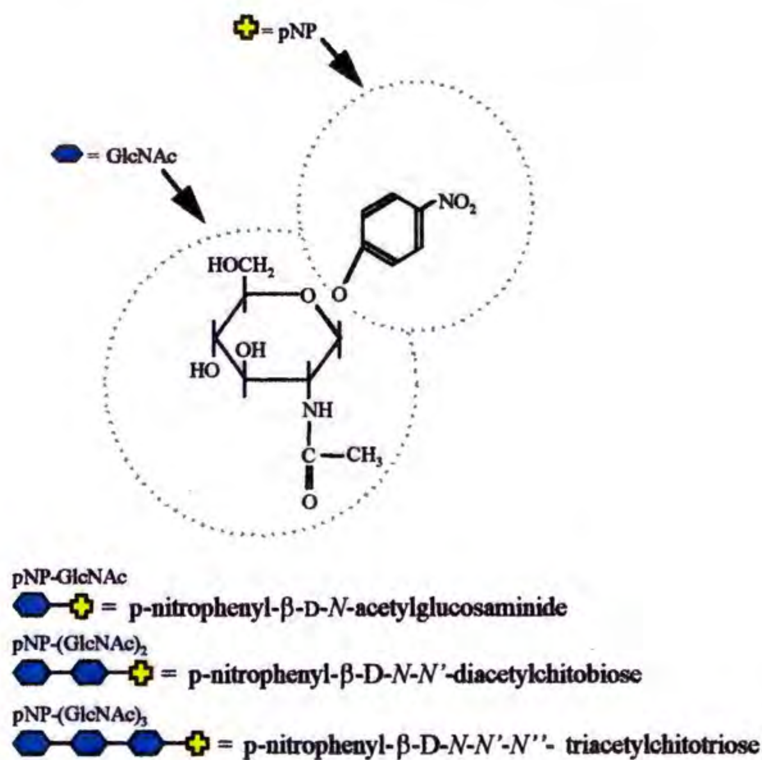

FIG. 1.1 Schematic representation of chromogenic N-acetylglucosamine oligosaccharide substrates for detection of chitinase activity. Adapted from Haran *et al.*(1995).

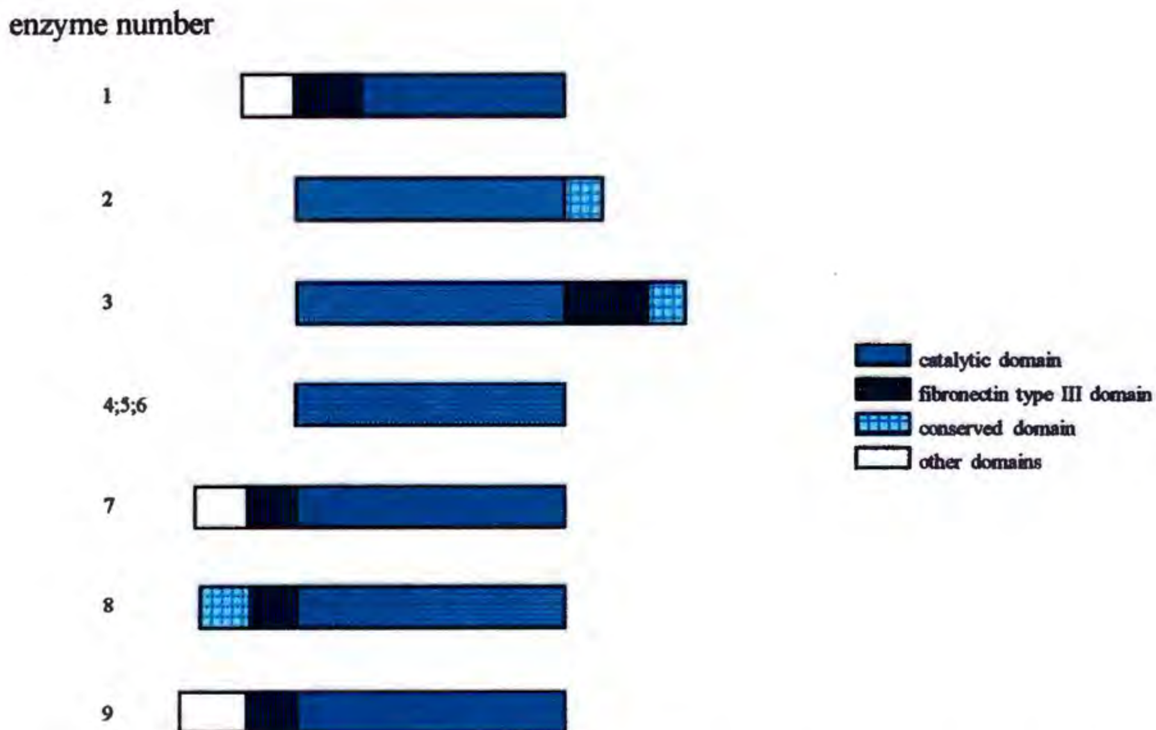

FIG. 1.2 Putative protein domains and their relative location within bacterial chitinases. 1, ChiA of *S. marcescens*; 2, ChiB of *S. marcescens*; 3, Chitinase A1 of *B. circulans*; 4, endoNAG of *Flavobacterium* sp.; 5, endoNAG F1 of *F. menigosepticum*; 6, EndoH of *S. olivaceoviridis*; 7, Chitinase-63 of *S. plicatus*; 8, Chitinase D of *B. circulans*; 9, exo-*chi01* of *S. olivaceoviridis*. Adapted from Blaak *et al.* 1993.

1.1.7.2 ENZYMATIC ACTIVITY OF BACTERIAL CHITINASES

In general most bacterial chitinases are exochitinases which cleave diacetylchitobiose from the non-reducing ends of chitin. Other products such as GlcNAc and chitooligosaccharides larger than $(GlcNAc)_3$ are either produced in small amounts or not observed at all (Roberts and Selitrennikoff, 1988; Ohno *et al.*, 1996). According to these authors the ChiA of *S. marcescens* converts p-nitrophenyl-β-D,N,N'-diacetylchitobioside into p-nitrophenol and diacetylchitobiose, characteristic of an exo-N,N'-diacetylchitobiosidase. Brurberg *et al.* (1996) confirmed that ChiA converted $(GlcNAc)_4$ and $(GlcNAc)_6$ into $(GlcNAc)_2$ only but they also observed the presence of the monomer GlcNAc. This implied that ChiA possesses an endochitinase or an exo-N,N',N'' triacetylchitotriohydrolase in addition to the exo-N,N'-diacetylchitobiosidase activity previously reported but the reaction products of $(GlcNAc)_3$ and GlcNAc expected from the cleavage of GlcNAc by either of these enzymes was not detected. This could be explained if the extended chitin binding site of ChiA is designed to bind up to six GlcNAc moeities and if the catalytic centre is located between the fourth and fifth GlcNAc binding-site. Hexameric substrates or substrates with less than six GlcNAc moeities could therefore bind to the active site with all their moeities resulting in only the cleavage of $(GlcNAc)_2$ dimers from the non-reducing ends. In contrast to ChiA, the endochitinase or exo-N,N',N''-triacetylchitotriohydrolase activity of ChiB was confirmed by the detection of trimers and monomers resulting from the cleavage of $(GlcNAc)_6$. ChiB is believed to have a less extended chitin binding domain than ChiA and consequently ChiB may be optimized to cleave shorter oligosaccharides of chitin whereas ChiA is optimized for degradation of longer chito-oligosaccharides and chitin. From these observations, it is believed that ChiA has an endochitinase activity, which breaks down the larger substrate making it more available to ChiB which has an exo-N,N',N''-triacetylchitotriohydrolase activity (Brurberg *et al.*, 1996).

Chitinase A1 of *B. circulans* has been described as an exochitinase (Watanabe *et al.*, 1990a) but results show that it generates 4-MU from 4-MU-$(GlcNAc)_3$ at a 10 times greater rate than from 4-MU-$(GlcNAc)_2$. This is a similar situation to that observed with *S. marcescens* ChiA and implies that the enzyme is an endochitinase (Robbins *et al.*, 1988; Tronsmo and Harman, 1993; Haran *et al.*, 1995).

The thermostable chitinase I of *B. licheniformis* X-7U showed an exochitinase activity with the cleavage of $(GlcNAc)_2$ from the nonreducing end of colloidal chitin. The other three chitinases II, III and IV exhibited a transglycosylation activity with the ability to convert $(GlcNAc)_4$ into $(GlcNAc)_6$ and $(GlcNAc)_2$. These chitinases produced $(GlcNAc)_2$ and GlcNAc from colloidal chitin (Takayanagi *et al.*, 1991).

Chitinase 49 and 63 of *S. plicatus* are classified as endochitinases. On the other hand chitinases 47 and 61 are classified as exochitinases (Robbins *et al.*, 1988). The chitinases of *S. kurssanovii,* namely Chi 42 and Chi 26 hydrolyzed chitin, chitosan and carboxymethyl chitin by an endochitinse activity. Chi 26 also

exhibited transglycosylation as with *B. licheniformis* chitinase II, III and IV (Ilyina *et al.*, 1995). The chitinase encoded by *exo-chi01* of *S. olivaceoviridis* was classified as an exochitinase since it produced $(GlcNAc)_2$ exclusively from $(GlcNAc)_4$, $(GlcNAc)_6$ and colloidal chitin (Blaak *et al.*, 1993). The unique chitinase C-1 of *S. griseus* produced $(GlcNAc)_2$ and $(GlcNAc)_3$ as major reaction products of cleaved colloidal chitin. This indicated an endo-type action of this chitinase which is in agreement with the activity of plant chitinases, to which C-1 is related (Ohno *et al.*, 1996). The enzyme complex of *S. albidoflavus* consists of at least 7 chitinases. Two of these chitinases had chitobiosidase activity whilst the other five enzymes had endochitinase activity. N-glucosaminidase activity was also observed (Broadway *et al.*, 1995).

1.1.8 THE ROLE OF CHITINASES IN DEGRADATION OF CHITIN

The function of the multiple chitinase systems observed in bacteria is not well understood. Bacteria secrete multiple chitinases which work together to hydrolyze chitin for use as a carbon and/or nitrogen and an energy source (Watanabe *et al.*,1992; Fujii and Miyashita, 1993). *S. marcescens* ChiA and ChiB both possess exo-N,N'-diacetylchitobiohydrolase activity, combined with either an endochitinase (ChiA) or an exo-N',N'',N'''-triacetylchitotriohydrolase activity (ChiB) (Brurberg *et al.*, 1996). *S. marcescens* ChiA is secreted and located extracellualrly (Jones *et al.*, 1986). ChiB has been found in the supernatant of late stationary phase culture of *S. marcescens* (Roberts and Cabib, 1982) and has been shown to be exported to the periplasm, without being processed, in exponential and early stationary cultures of *S. marcescens* (Brurberg *et al.*, 1995). From the difference in enzyme activity, the cellular location of ChiA and ChiB, the presence of a fibronectin type III-like domain and an extended chitin binding domain of ChiA as well as having higher specific activity towards chitin than ChiB suggests that ChiA and ChiB act sequentially in that ChiA degrades chitin and the longer GlcNAc oligomers whereas ChiB digests the shorter GlcNAc oligomers that are capable of entering the periplasm (Brurberg *et al.*, 1996).

The roles of the other three chitinases produced by *S. marcescens* is not known. Joshi *et al.* (1988) were the first to report on the regulatory elements of chitin degradation. By transposon mutagenesis and deletion analysis, they showed that the *chiD* gene of *S. liquefaciens* encodes a trans-acting repressor of chitinase and glucosidase expression and the *chiE* gene from the same organism produces an inducer which activates the repressor. According to a model proposed by these authors in *S. liquefaciens*, a repressor is synthesized in the absence of chitin which blocks the expression of chitinase and glucosaminidase. When chitin is present an inducer is produced by modification of the degradation products present in a chitin preparation or provided by basal level expression of chitinase or glucosaminidase. This inducer inactivates the repressor and allows high levels of chitinolytic enzyme production.

The 6 chitinases of *B. circulans* have different activities and substrate specificities. Chitinases A1 and A2 hydrolyze colloidal chitin better than the other chitinases, producing mainly $(GlcNAc)_2$. Chitinase A1 has a

strong affinity for chitin whereas the other chitinases except D do not. Chitinase A1 is considered to be the key enzyme and chitinase D is believed to have an intimate relationship with chitinase A1 (Watanabe *et al.*, 1992). The roles of the other chitinases is not known but some of these may be involved in the induction of chitinase A1 (Watanabe *et al.*, 1990a).

1.1.9 THE ROLE OF CHITINASES IN BIOLOGICAL CONTROL

Chitinases from bacteria, fungi and plants can be used in a number of ways as biocontrol agents of phytopathogenic fungi. These control strategies include the treatment of plants with purified enzymes, the use of isolated soil microorganisms which are naturally chitinolytic, the introduction of genetically engineered rhizosphere or endophytic microorganisms expressing chitinase or the construction of transgenic plants which express chitinase.

1.1.9.1 GENETICALLY ENGINEERED BACTERIA EXPRESSING *S. marcescens* ChiA

Of the 203 different bacterial strains isolated from *S. rolfsii* infested soil, *S. marcescens* was found to be the best biocontrol agent of this pathogen (Ordentlich *et al.*, 1987). When this bacterium was applied to sterilized and unsterilized soils under greenhouse conditions, it caused a reduction in disease development by *S. rolfsii* (Ordentlich *et al.*, 1988). It also effectively controlled *R. solani,* resulting in a 50% reduction in fungal disease in beans. The *chiA* gene from this strain has been cloned and expressed in *E. coli* (Shapira *et al.*, 1989). Previously the *chiA* gene of *S. marcescens* QMB1466 (Jones *et al.*, 1986) was expressed in *E. coli* and shown to have high expression levels if the gene was expressed from the plasmid's *lacZ* promoter in addition to the endogenous promoter of *chiA*. Shapira *et al.* (1989) cloned their *chiA* gene under the control of the strong bacteriophage λ pL promoter into an *E. coli* strain which carries a thermosensitive cI repressor in order to obtain high levels of chitinase expression upon heat induction. The secreted enzyme was found to constitute over 10% of the total cellular protein (Oppenheim *et al.*, 1990).

A partially purified enzyme preparation was shown to be very effective in lysing hyphal tips of *S. rolfsii.* The number of diseased bean plants artificially infected with *S. rolfsii* was reduced when plants were watered daily with a diluted preparation of the purified chitinase. The recombinant *E. coli* strain was added to the irrigation water and was shown to be effective in reducing disease incidence in bean seedlings infected with *S. rolfsii*. Experiments were carried out at 40°C with beans artificially infected with the fungus and treated with whole, viable, recombinant *E. coli* cells added to the irrigation water. These cells were able to inhibit *S. rolfsii* but to a lower degree than the purified chitinase preparation. After 16 days disease incidence was 35% in water controls, 37% in soils treated with a control *E. coli* strain and 19% in soils treated with the engineered *E. coli* strain expressing chitinase. No protection was observed when the same experiment was carried out at 30°C, the temperature at which the pL promoter is repressed (Shapira *et*

al., 1989). The effectiveness of this engineered, non-soil bacterium served as a model, indicating the potential for engineering rhizosphere bacteria to express chitinase and to act as biocontrol agents. However, Fuchs *et al.* (1986) reported that expression levels of *chiA* in *E. coli* were greater than expression of the gene in *P. fluorescens.* This demonstrated the necessity for stable, constitutive, high level expression vectors for the production of chitinase in rhizosphere bacteria.

Sitrit *et al.* (1993) cloned the *S. marcescens chiA* gene under the control of the strong *tac* promoter, into a broad host range plasmid based on pRK290. This plasmid was transferred from *E. coli* to *P. putida* and *Rhizobium meliloti.* The *chiA* gene was constitutively expressed in both these bacteria as they do not encode a repressor of the *tac* promoter. Enzyme assays using the substrate p-nitrophenyl-β-D-N,N'-diacetylchitobiose showed high levels of chitinase activity, with 0.83 units (μmole of pNp/μg of protein/ hour released from pNp-chitobiose) for transformed *R. meliloti.* The introduced *chiA* gene in *P. putida* was not stably maintained with rapid loss of the plasmid after 20 generations without antibiotic selection. On the other hand the gene was stably maintained in *R. meliloti,* with 99% of cells still possessing the plasmid after 100 generations without selective pressure. After 45 days postinoculation, the plasmid carrying the *chiA* gene was detected in *R. meliloti* colonies present in the nodules (Chet *et al.*, 1993; Sitrit *et al.*, 1993). Hyphal tips of R. *solani* were rapidly lyzed by extracts from nodules containing the transformed *Rhizobium* verifying that the antifungal activity of the transformed bacteria during symbiosis on alfalfa plants. Bean plants were artificially infected with either *S. rolfsii* or *R. solani* and treated with 10^8 or 10^7 engineered *P. putida*/g soil added to the irrigation water for 18 or 9 days respectively. Reduction of disease was observed as compared to control plants (Chet *et al.*, 1993).

Due to the high instability of the plasmid-borne *chiA* gene in *Pseudomonas*, Koby *et al.* (1994) stably integrated the *chiA* gene, under the control of the *tac* promoter, into the chromosome of *P. fluorescens.* They constructed a mini Tn7 Km^R transposon carrying the *tacchiA* operon fusion on a plasmid, pUX-BF5, which was transferred into *P. fluorescens* isolated from the rhizosphere. They reported that chitinase activity under the control of the *tac* promoter in the pUC19 based integration vector was high in *E. coli.* Due to this high level of expression, mutants defective in expression of *chiA* accumulated rapidly in *E. coli* cell cultures. This necessitated the use of a *pcn*B mutant strain which reduced the plasmid copy number to 1 to 2 copies per cell (Liu and Parkinson, 1989). The expression levels of the chitinase in *P. fluorescens* carrying the integrated single copy Tn7-Km^R-P_{tac}*chiA* were similar to those obtained for the *E. coli pcn*B strain transformed with the integration vector. Chitinase activity in *E. coli* carrying the mini Tn7- Km^R-P_{tac}*chiA* plasmid was 1160 units/ml, in the *E. coli pcnB* strain activity was 262 units/ml and in two strains of *P. fluorescens* carrying the integrated *tacchiA* operon fusion activity was 186 and 210 units/ml. The engineered *P. fluorescens* strain showed effective biological control against *R. solani* infecting cotton seedlings. Cotton seeds artificially infected with *R. solani* were irrigated with the engineered *P. fluorescens* strain at a concentration of 10^7 cells/ml. The percentage of healthy plants resulting from treatment with

water only was 20%, with wild type *P. fluorescens* was 17% and with the engineered *P. fluorescens* was 85% (Koby *et al.*, 1994).

1.1.9.2 TRANSGENIC FUNGI EXPRESSING *S. marcescens* ChiA

The mycoparasite *T. harzianum,* which secretes lytic enzymes such as chitinase, is a known biocontrol agent of several phytopathogenic fungi. In an attempt to improve its effectiveness, the *S. marcescens chiA* gene was introduced into its genome through protoplast transformation (Haran *et al.*, 1993). It was cloned under the control of the cauliflower mosaic virus 35S (35S CaMV) promoter which allowed constitutive expression of the gene. Two approaches to transform *T. harzianum* with the *chiA* gene were followed. The first was transformation by a single plasmid carrying both the *chiA* gene under the control of its own promoter and a selectable marker gene *amds* from *Aspergillus nidulans* encoding an acetamidase which enables transformants to grow on media containing acrylamide (Chet *et al.*, 1993). The second approach was cotransformation using two plasmids, one of which carried the *chiA* gene under the control of the 35S CaMV promoter and the other contained the *amds* gene on plasmid p3SR2. The DNA of plasmid p3SR2 was shown to integrate into the fungal chromosome by recombination (Chet *et al.*, 1993). The single type of plasmid was transformed into *T. harzianum* (T-35) and was integrated in a non-site specific manner into the chromosome. No expression of the *chiA* gene was detected under the control of its own promoter (Chet *et al.*, 1993). In the cotransformation experiments, two transformants were obtained which had the *chiA* and *amds* genes integrated into their genomes. Both *Trichoderma* transformants, when grown on synthetic medium, produced and secreted a 58-kDa chitinase, the activity of which was significantly higher than that of the wild type *Trichoderma.* When grown in the presence of chitin however, both transformants had lower chitinase activity than the wild type. Western blot analysis showed that under non-inducing conditions the 58-kDa chitinase was cleaved into two fragments of 40 and 18 kDa. When induced one of the chitinases secreted by *T. harzianum* is 40 kDa (Haran *et al.,*1995). It is thought that the additional 40-kDa and 18-kDa fragments secreted by the transformed *T. harzianum* during induction could have interfered with the native 40-kDa chitinase. This could result in the decrease in the chitinase activity of the transformants under inducive conditions (Haran *et al.*, 1993). The constitutively expressed extracellular chitinase produced by the transformed *Trichoderma* protected seedlings from pathogens early on in germination and could therefore be useful as a biocontrol agent of chitin containing phytopathogenic fungi.

1.1.9.3 TRANSGENIC PLANTS EXPRESSING *S. marcescens chiA*

The *chiA* gene has also been used to construct transgenic plants. It was introduced into tobacco plants and detected, using ChiA specific antibodies, in the leaves, stems and roots of tobacco. Transgenic tobacco seedlings infected with *R. solani* showed reduced disease incidence as compared to control plants (Oppenheim and Chet, 1992).

1.1.9.4 ACTIVITY OF *S. marcescens* ChiA AGAINST INSECTS

The synergistic insecticidal effect of *B. thuringiensis* δ-endotoxins and bacterial chitinases has been reported in the literature. Smirnoff (1971) demonstrated that addition of both *B. thuringiensis* and chitinase increased the insecticidal effect on *Chonstoneura fumiferana* larvae significantly. Also co-application of *B. thuringiensis* subsp. *entomocidus* spore crystal preparations and chitinolytic bacteria resulted in a synergistic insecticidal effect against *Spodoptera littoralis* larvae (Sneh *et al.*, 1983, cited in Regev *et al.*, 1996). The synergistic insecticidal effect of a *B. thuringiensis* CryIC protein and the *S. marcescens* ChiA on the relatively Cry-insensitive larvae of *S. littoralis* has recently been demonstrated (Regev *et al.*, 1996). The *chiA* under the control of the pL promoter was expressed and secreted in *E. coli*. Even at low concentrations the chitinase was capable of perforating the chitin containing peritrophic membrane of the larval midgut,. A range of concentrations of CryIC and ChiA were combined and included in the larval diet. In control experiments, only one of the proteins was added to the diet. The lowest concentrations of CryIC (1.0 μg/ml) and ChiA (0.1 μg/ml) were within the expression range of single copy transgenes in plants. The combined toxic effect of CryIC and increasing concentrations of ChiA was a reduction in larval weight of 62.0 to 98.2%. CryIC (1 μg/ml) alone caused only 37% reduction in larval weight. Perforation of the peritrophic membrane by ChiA caused an increase in the toxicity of CryIC, possibly due to an increase in numbers of CryIC toxins binding to the membrane receptors present in the epithelium. A CryIC concentration of 20 μg/ml was required for a maximum toxic effect on larvae in the absence of chitinase, whereas only 3 μg of CryIC/ml was needed to have the same toxic effect in the presence of ChiA.

The introduction of both the CryIC and ChiA into bacteria or plants offers great potential for increasing the insecticidal activity in transgenic systems where the Cry toxins are expressed at low levels and/or in a crystalline form (Regev *et al.*, 1996).

1.1.9.5 NATURALLY OCCURING CHITINOLYTIC BACTERIA AS BIOCONTROL AGENTS

Various species of soil bacteria have been isolated and selected for their ability to produce chitinase and for their antagonism to phytopathogenic fungi in greenhouse and field trials. Fifteen chitinolytic bacteria were isolated from the rhizosphere of carnation and were shown to be capable of lyzing the hyphae of *F. oxysporum* (Sneh, 1981). A chitinolytic strain of *Aeromonas caviae* isolated from the roots of healthy bean plants grown in soil infested with *S. rolfsii* was shown to be an effective biocontrol agent against *R. solani* and *F. oxysporum* f.sp. *vasinfectum* infection of cotton and *S. rolfsii* infection of beans in greenhouse trials. Disease reduction was 78, 57 and 60% for the respective fungal infections (Inbar and Chet, 1991).

Pleban *et al.* (1995) reported on the antifungal activity of several endophytic bacteria, some of which had chitinolytic activity. These will be discussed in section 1.3.3.

The genus *Enterobacter* contains several species which are known to be efficient biocontrol agents of phytopathogenic fungi. Recently Chernin *et al.* (1995) provided evidence for the presence of a complex chitinolytic enzyme system in *E. agglomerans*. Three strains of this bacterium were isolated from the soil and shown to be antagonists of a variety of pathogenic fungi. In addition, the application of a suspension of the *E. agglomerans* strains to cotton, infested artificially with *R. solani*, resulted in significant disease reduction ranging from 64 to 86%. One of the three strains isolated, *E. agglomerans* IC 1270, was shown to also produce an antibiotic identified as pyrrolnitrin (Chernin *et al.*, 1996). This was the first report of the production of pyrrolnitrin by bacteria other than *Pseudomonas*.

1.1.9.6 CHITINOLYTIC FUNGI AS BIOCONTROL AGENTS

Trichoderma is a mycoparasite and antagonist of many fungi. Mycoparasitism involves lectin-sugar recognition and attachment to the target hypha either by coiling, hooks or appressoria, followed by penetration of the host cell walls (Elad *et al.*, 1983). This is achieved by the production and secretion of lytic enzymes including chitinases, β-1,3 glucanases, lipases and possibly proteases (Elad *et al.*, 1982; 1983; Ridout *et al.*, 1986). The induction of chitinolytic enzymes during mycoparasitism in *Trichoderma* is triggered by the recognition signal associated with lectins (Inbar and Chet, 1994). Several strains of *T. harzianum* have been shown to cause lysis of the cell walls of *S. rolfsii, R. solani* and *P. aphanidermatum* (Elad *et al.*, 1982; 1983).

The *chiA* gene from *S. marcescens* was shown by Southern blot analysis to share homology with one of the *Trichoderma* chitinase genes. Using *chiA* as a probe, the chitinase gene was isolated from a cDNA library of *T. harzianum* (T-35). This gene was cloned in the Bluescript expression vector in *E. coli* under the *lac* promoter. In greenhouse experiments, significant biocontrol activity was observed when bean seedlings infected with *S. rolfsii* were irrigated with the engineered *E. coli* (Chet *et al.*, 1993).

1.1.9.7 TRANSGENIC PLANTS EXPRESSING CHITINASE

Since the low basal levels of chitinase in many plant tissues and the relatively late chitinase induction after pathogen attack, are insufficient to protect plants against fungi (Vierheilig *et al.*, 1993) it was felt that constitutive expression of chitinases at high levels might prove beneficial in this regard. The first successful report using this approach was that of Broglie *et al.* (1991) who constitutively expressed a bean endochitinase gene, under the control of the 35S CaMV promoter, in transgenic tobacco and canola plants. These transgenic plants exhibited increased protection against attack by *R. solani*. Eighteen day old

transgenic tobacco progeny were grown in soil artificially infected with *R. solani* for 13 to 16 days. Seedling mortality of transformants ranged from 23 to 37% in comparison to 53% for nontransgenic controls. The control plants were stunted and the loss of root fresh weight was 46%, whereas the 35S chitinase plants were larger, hardier and had an average of 5 to 15% loss of root fresh weight. Inhibition of *R. solani* growth on 35S chitinase plants was proportional to the amount of bean chitinase expressed. Greater numbers of plants producing higher levels of bean chitinase survived infection by *R. solani*. Fungal resistance of these transgenic plants was shown to vary with the level of fungal inoculum. The transgenic tobacco plants were not protected from attack by the non-chitin containing fungus *Pythium aphanidermatum*. Introduction of the 35S chitinase gene into *Brassica napus* (canola or oilseed rape) also resulted in increased resistance of the transgenic plants to *R. solani*. Seedling survival of transgenic canola transplanted into *R. solani* infested soil was 53% after 15 days while only 24% of nontransgenic control plants survived.

Transgenic tobacco plants (*Nicotiana sylvestis)* constitutively expressing a vacuolar class I chitinase from *N. tabacum*, under the control of the 35S CaMV promoter showed increased resistance to *R. solani* as compared to control plants. All transgenic 35S chitinase tobacco plants tested were well colonized by the mycorrhizal symbiotic fungus *Glomus mosseae* indicating that the constitutively expressed chitinase conferred increased resistance to certain pathogenic fungi but did not interfer with the symbiosis with beneficial fungi, possibly because the chitin layer of the cell wall of *Glomus mosseae* is protected by a protein or polysaccharide cover (Vierheilig *et al.*, 1993).

Lin *et al.*(1995) have developed transgenic rice plants which constitutively express a rice class I chitinase gene. The level of chitinase expression in individual plants varied with the number of transgenes present and, as in the case of transgenic tobacco (Broglie *et al.*, 1991), correlated positively with resistance to *R. solani*

Increased fungal resistance by constitutively expressed chitinases in transgenic plants was demonstrated in the field for the first time by Grison *et al.* (1996). Previously such resistance had only been demonstrated for *R. solani* in greenhouse trials. A chimeric tomato endochitinase gene under the control of the 35S CaMV promoter was introduced into oilseed rape (*Brassica napus* var. *oleifera*) and third generation progeny were challenged with three different fungi in field trials in three different locations in Europe. Transgenic plants exhibited increased resistance to all three fungal pathogens as compared to controls, in all locations and under different climatic conditions. It was shown that only a two to five fold increase in plant chitinase activity in the transgenic plants was needed to cause increased fungal resistance.

The gene encoding the 42-kDa endochitinase of *T. harzianum* has been introduced into tobacco and potato. Constitutive expression of the chitinase was obtained at high levels in different parts of the plants. Detached

infected leaves from transgenic plants showed increased resistance to *Alternaria alternata* and *Botrytis cinera*. Entire plants have yet to be tested for resistance to the fungi (Lorita *et al.*, 1996).

1.2 *Bacillus thuringiensis* Cry PROTEINS

A large number of *B. thuringiensis* strains with different insect host ranges have been identified. The first report of *B. thuringiensis* was in 1901 where it devasted Japanese silk worm (*Bombyx mori*) colonies (Ishiwata, cited in Lambert and Perferoen, 1992). However, the organism was named by E. Berliner in 1915 after he isolated it from the Mediterranean flour moth (*Ephestia kuehniella*) and insect larvae in stored grains in the German city of Thuringen (Lambert and Perferoen, 1992). The earliest commercial *B. thuringiensis* product, Sporeine, was produced in France in 1938 (Lambert and Perferoen, 1992).

B. thuringiensis spores are found in large numbers within a variety of natural soils worldwide. The spores are able to survive for many years. Vegetative cells have not been detected in natural soils indicating that the organism does not germinate and multiply in this environment. It has been isolated from most leaf surfaces of trees grown in temperate climates but it is not known whether it multiplies on the leaf surface. How *B. thuringiensis* is able to persist in soils is not known but it is possible that it is able to infect and multiply in other soil organisms besides insects such as nematodes. On the other hand it is able to stably infect and multiply to high numbers in granaries and grain mills and other storage facilities which have high insect numbers. Within these contained environments, *B. thuringiensis* is relatively sheltered from UV light and adverse conditions (Lambert and Perferoen, 1992).

1.2.1 CLASSIFICATION OF CRYSTAL PROTEIN GENES

Initially *B. thuringiensis* isolates were classified according to morphological and biochemical characteristics which became inefficient when the numbers of different isolates increased. A classification scheme based on the presence of flagellar antigens or H-antigens on vegetative cells was introduced by de Barjac and Bonnefai in 1963 (de Barjac and Frachon, 1990). Serotyping has since been used to classify *B. thuringiensis* isolates into several serovars. An updated classification of *B. thuringiensis* was recently proposed which makes H serotyping the simplest and specific way to classify *B. thuringiensis* isolates (de Barjac and Frachon, 1990)

The genes coding for the proteins of *B. thuringiensis* are known as *cry* (crystal protein) genes. A large number of *cry* genes have been cloned since the first one in 1985 (Schnepf *et al.*, 1985). Höfte and Whiteley (1989) proposed the now commonly used scheme to classify Cry proteins according to their insect specificity and nucleotide sequence. The authors classified the 42 sequenced *B. thuringiensis* crystal protein genes into 14 distinct genes, 13 of which were grouped into 4 major classes and several subclasses based on

structural similarities and insect host range. The four major classes of genes are *cryI, II, III* and *IV* and are specifically active against Lepidoptera, Lepidoptera and Diptera, Coleoptera and Diptera larvae respectively. An additional class consists of a crystal protein gene of *B. thuringiensis* subsp. *israelensis* coding for a 27-kDa cytolytic protein which is structurally unrelated to the *cry* genes. Höfte and Whiteley's classification system, is widely used and has provided the basis for classification of the many new *cry* genes.

Eight different *cryI* genes have been recognised (*cryIA-G*) which code for Lepidopteran-specific Cry proteins. These 130-140 kDa protoxins accumulate in bipyramidal crystal inclusions and are proteolytically cleaved in the larval midgut into a toxic core of 60 to 70 kDa (Höfte and Whiteley, 1989). These proteins are closely related and all, except CryIB and CryIG have a common ancestor (Feitelson *et al.*, 1992). The CryI proteins, excluding the former two, have greater than 62% sequence homology (Feitelson *et al.*, 1992). The *cryIA* genes have been subdivided into three subgroups, *cryIA(a), cryIA(b)* and *cryIA(c)*, in which the Cry proteins that they encode have more than 80% amino acid homology (Höfte and Whiteley, 1989). The toxin-containing N terminal region of the CryI protoxins is variable whereas the C terminal region is highly conserved, is not essential for toxicity and is thought to be involved in crystallization (Höfte and Whiteley, 1989). The CryIB and CryIII proteins have greater than 53% sequence homology. The gene for an insecticidal crystal protein, designated CryIE cloned from *B. thuringiensis* subsp. *darmstadiensis* has 50% and 54% amino acid homology to CryIA(a) and CryIC crystal proteins.

Additional genes within the *cryI* family have recently been reported. These include the *cryIE* gene (Visser *et al.*, 1990) and the *cryIF* gene isolated from a novel grain dust isolate of *B. thuringiensis* subsp. *aizawai* (Chambers *et al.*, 1991). These genes differ significantly in sequences of the N terminal domain as well as in their insecticidal activities and host range.

The *cryII* genes encode crystal proteins which form cuboidal inclusions. They are active against both Lepidopteran and Dipteran larvae. The *cryIIA* and *cryIIB* genes cloned by Widner and Whiteley (1989) from *B. thuringiensis* subsp. *kurstaki* HD-1 both encode 71-kDa proteins. Although they share 87% amino acid homology, they have activity against different insects. CryIIA is toxic to both Lepidopteran and Dipteran insects whereas CryIIB is active against only against Lepidoptera (Widner and Whiteley, 1989). The *cryIIA* is part of an operon consisting of three open reading frames, ORF1, ORF2 and *cryIIA* (Widner and Whiteley, 1989). A third gene, *cryIIC*, has been isolated (Wu *et al.*, 1991) which is also part of an operon similiar to that of *cryIIA*. The *cryII* genes show very little homology to the other *cry* genes and the proteins they encode show significant amino acid homology to other Cry proteins only in the first of the five conserved domains of the N terminus (Höfte and Whiteley, 1989). The CryII proteins have more sequence homology to the CryIVD proteins than to the other Cry proteins (Feitelson *et al.*, 1992).

The *cryIII* genes encode Coleopteran-specific protoxins of approximately 73 kDa which produce rhomboidal crystals. The class is composed of 4 genes, *cryIIIA,B,C* and *D*.

The *cryIV* gene class is composed of 4 genes, *cryIVA-D*, which code for Dipteran-specific crystal proteins with molecular weights of 135, 128, 78 and 72 kDa respectively. All crystal proteins are toxic against larvae of certain mosquito species and form ovoid crystal complexes (Höfte and Whiteley, 1989). The CryIVA, B and C proteins have greater than 44% sequence homology, whereas CryIVD is more similiar to the CryII proteins (Freitelson *et al.*, 1992). The *cryIVA* and *cryIVB* genes are structurally more similiar to the *cryI* genes, with the 3' regions (corresponding to the conserved C terminal region of the protein) being almost identical to each other and to the 3' halves of the *cryI* genes. The *cryIVC* gene (ORF1) encodes a 78-kDa protein which is located 45 base pairs upstream of a second open reading frame, ORF2. The *cryIVC* is homologous to the 5' ends (corresponding to the variable N terminal end of the protein) of the other *cryIV* genes, whereas ORF2 is homologous to the 3' end. It is thought that these genes have evolved from either the *cryIVA* or *B* genes through an insertion (Höfte and Whiteley, 1989). The *cryIVD* gene encodes a 72-kDa protein, the amino acid sequence of which is unlike that of the other Cry proteins and the only homology is in a small region of the molecule in the first conserved region of the N terminal domain.

A novel 81.2-kDa CryV protein was isolated from a strain of *B. thuringiensis* subsp. *kurstaki* which differs in primary and secondary structure size and host range from other Cry proteins. It shares 62% amino acid identity with CryIB. This protein is toxic to a coleopteran species (Colorado potato beetle) and a lepidopteran species (European Corn borer) (Tailor *et al.*, 1992). This is the first example of a Cry protein which is toxic to the larvae of both insect types.

In addition Cry VI, VII, VIII and IX proteins have been isolated and classified.

It is believed that now that more than 90 *cry* genes have been cloned and sequenced, a simplified classification is no longer sufficient. The nomenclature for the *cry* genes has been revised and is based on amino acid sequence homologies rather than on insecticidal properties of the toxins (Crickmore *et al.*, 1995, cited in Baum and Malvar, 1995).

An additional class of toxin genes proposed by Höfte and Whiteley (1989) includes a 27-kDa protein isolated from *B. thuringiensis* subsp. *israelensis* encoded by *cytA*. It has no sequence homology to any of the other crystal protein genes. A second gene of the *cyt* family, *cytB*, was isolated from *B. thuringiensis* subsp. *kyushensis*. The CytB protein is not found with other Cry proteins and is specifically lethal to mosquito species. The Cyt crystal proteins have insecticidal activity against Dipteran insects including mosquito and blackfly. They are cytolytic to cells of these insects as well as to several other cell types including human erythrocytes *in vitro* (Höfte and Whiteley, 1989; Li *et al.*, 1996).

1.2.2 EXPRESSION OF THE *cry* GENES

The Cry proteins accumulate during stationary phase or sporulation to form crystal inclusions. In *Bacillus subtilis* sporulation is regulated by six transcription sigma factors which are activated sequentially during the different stages of sporulation. These various stages have also been observed in *B. thuringiensis* (Salamitou *et al.*, 1996). Transcription of *cryIA, cryIB, cryIIA* and *cytA* genes is dependent on the sporulation sigma factors σ35 and σ28 (Höfte and Whiteley, 1989) which are homologous to σE and σK sigma factors active during sporulation in *B. subtilis* (Salamitou *et al.*, 1996). Many of these genes also have a strong terminator (Höfte and Whiteley, 1989). Transcription of *cryIIIA* is different from that of the majority of *cry* genes in that the promoter is located further upstream from the translational start codon and the gene is expressed during late exponential/stationary phase rather than during sporulation. Also, transcription is not dependent on the major sporulation sigma factors (Salamitou *et al.*, 1996). *cytA* is regulated by a 20-kDa polypeptide which is required for efficient production of structural proteins (Höfte and Whiteley, 1989; Ben-Dov *et al.*, 1995). This product has been shown to raise the levels of *cytA, cryIVA* and *cryIVD* in *E.coli* and is thought to act as a chaperone (Ben-Dov *et al.*, 1995).

1.2.3 DIVERSITY OF *B. thuringiensis* STRAINS

Several *B. thuringiensis* strains and subspecies have been shown to contain *cry* genes that encode different Cry crystal proteins which occur in the same crystal inclusion. For example, *B. thuringiensis* subsp. *kurstaki* HD-1 contains genes for CryIA(a), CryIA(b), CryIA(c), CryIIA and CryIIB (Kyong Lee *et al.*, 1996a) and *B. thuringiensis* subsp. *israelensis* has been shown to contain the four CryIV crystal proteins (CryIVA-D) and CytA (Höfte and Whiteley, 1989; Ben-Dov *et al.*, 1995). Bioassays of individually isolated crystal proteins have shown that they have different insecticidal specificities and activities (Höfte and Whiteley, 1989; De Wald, 1995). The same protein isolated from different *B. thuringiensis* strains may vary only slightly in its amino acid sequence yet show very dramatic differences in insecticidal activity (De Wald, 1995).

The diversity of *B. thuringiensis* could be explained by the fact that the vast majority of toxin genes are located on self transmissible plasmids that range in size from 45 kb to greater than 225 kb (Kronstad and Whiteley, 1986, Ben-Dov *et al.*, 1995). *B. thuringiensis* subsp. *israelensis* carries several plasmids one of which, a 75-mDa plasmid, includes the mosquitocidal *cryIVA-D* and *cytA* genes as well as a gene encoding a 20-kDa regulatory protein (Ben-Dov *et al.*, 1995). The plasmids carrying the *cry* and *cyt* genes are capable of being transferred between *B. thuringiensis* strains by conjugation (Carlton, 1995); transduction (Lecadet *et al.*, 1980) or by transposon-like elements in the case of several *cryIA* and *cryIVB* genes which contain IS elements (Höfte and Whiteley, 1989).

1.2.4 STRUCTURE AND FUNCTION OF *B. thuringiensis* CRYSTAL PROTEINS

1.2.4.1 STRUCTURE OF *B. thuringiensis* CRYSTAL PROTEINS

The *cry* genes encode insecticidal protoxins of either 130 to 140 kDa, or approximately 70 kDa, which consist of two domains. The variable N terminal half contains the active toxin fragment of 50 to 70 kDa or 30 kDa in the case of *cryIVD*. The highly conserved C terminal half is not involved in toxicity but is thought to play a structural role in crystal formation (Höfte and Whiteley, 1989).

Evidence provided by Convents *et al.* (1990) showed that the active or mature toxin consists of at least two domains with specific functions. They predicted, using spectrophotometric techniques on a toxin from *B. thuringiensis* subsp. *berliner*, that the N terminal half of the toxin contained several α helices and the C terminal part contained β strand and coil structures in alternating fashion, indicative of a β sheet conformation. The N terminal domain consists of the toxin and the C terminal domain is thought to be involved in cell binding (Gill *et al.*, 1992, cited in Herrera, 1994). This is illustrated in Figure 1.3. The active toxin consists of five highly conserved amino acid sequence blocks, which are present in all Cry proteins except CryIII and CryIVD. These two proteins only have homology in Block 1. In different proteins each of the five blocks are separated by variable sequences of different lengths. A conserved region of hydrophobic amino acids is present in all proteins except CryII and CryIVD. This region corresponds to a predicted transmembrane sequence which is thought to play a role in toxin interaction with the midgut membrane epithelial cells of the insect host (Höfte and Whiteley, 1989).

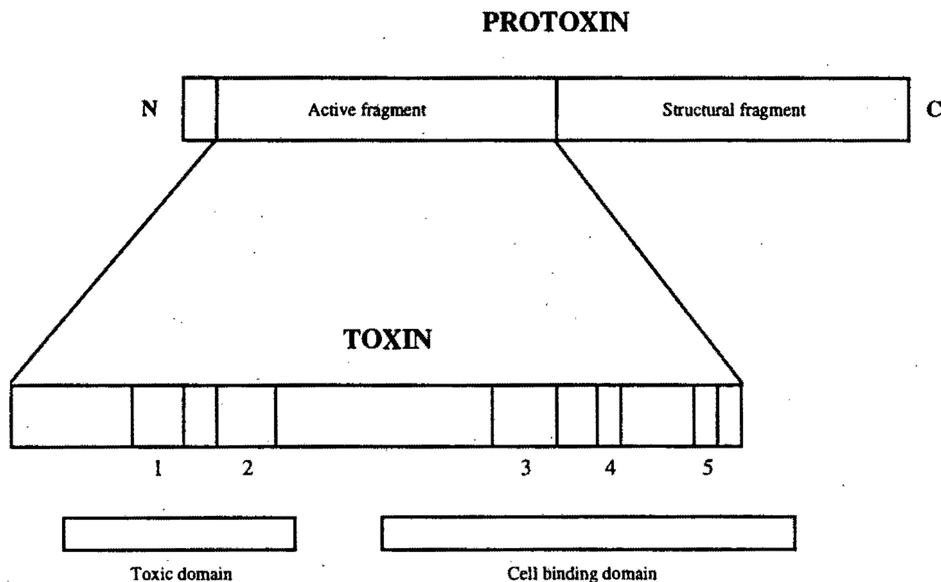

FIG. 1.3 Structure of a Cry protein. The protoxin molecule consists of a N-terminal active and C-terminal structural fragment which is lost when the molecule is proteolytically cleaved. The toxin (active fragment) comprises of the toxic, (within which are 5 conserved regions 1-5) and cell-binding domains. This domain consists of the variable and conserved C-terminal regions, which are believed to be involved in receptor binding and structural conformation or receptor interaction respectively. Adapted from Gill *et al.*,1992 cited in Herrera, 1994.

The 3D structure of the CryIIIA protein by X-ray crystallography (Li *et al.*, 1991), shows three structurally distinct domains. Domain I from the N terminus consists of seven α helices in a bundle. Domain II contains three β sheets and appears as a triangular column. Domain III at the C terminus is a sandwich of two antiparallel β sheets. The five highly conserved sequence blocks described by Höfte and Whiteley (1989) make up the core of the CryIIIA, which includes the interfaces of the three domains (Li *et al.*, 1991). Because of this high degree of conservation the authors proposed that this crystal sructure is a representative of homologous Cry toxins, and could be used as a model to propose the basis for the function of these proteins. Recently, the 3D crystal structure of CryIA(a) has been investigated and compared to that of CryIIIA (Grochulski *et al.*, 1995). Both structures show very high overall similarity, each with three domains that are folded in the same way. However variation occurs between domains. Domain III has the highest structural homology, followed by domain I. The largest differences occur in domain II in the position and length of connecting loops.

1.2.4.2 FUNCTION OF *B. thuringiensis* CRYSTAL PROTEINS

The parasporal crystal inclusions that contain the crystal protoxins are solubilized in the alkaline midgut of the susceptible insect. The large, 130-kDa protoxins are cleaved by larval gut proteases which remove the C-terminal halves of the protoxins and cleaves them at residue 28 or 29 from the N terminus. Activation of the smaller 70-kDa protoxins involves the removal of approximately 50 residues from the N terminus (Li *et al.*, 1991). This process results in the formation of active toxins of between 50 and 70 kDa (Höfte and Whiteley, 1989). There is a minimum size of the toxin below which toxicity is lost. This minimum size always contains the block 5 conserved amino acid sequence described by Höfte and Whiteley (Du and Nickelson, 1996). The activated or mature toxin binds to specific receptors on epithelial cells of the midgut and forms pores which result in cell lysis and death of the insect. Mature toxins bind specifically and with high affinity to receptors on the brush border membrane vesicles (BBMV) found in the larval midgut of susceptible insects (Hofmann *et al.*, 1988). These receptors have been identified in several insects including the tobacco hornworm (*Manduca sexta*), the cabbage butterfly (*Pieris brassicae*) and the tobacco budworm (*Heliothis virescens*) (Du and Nickleson, 1996). They are of extreme interest as they may account for the specificity of the endotoxins. For *M. sexta*, the receptors for CryIA(c) and CryIA(b) have been identified as glycoproteins of 120 kDa and 210 kDa respectively (Du and Nickelson, 1996). The 210-kDa protein is a cahedrin-like protein and the 120-kDa protein an aminopeptidase N (APN) (Kyong Lee *et al.*, 1996b). APN has also been purified from *Lymantria dispar* and shown to bind specifically a CryIA(c) protein (Kyong Lee *et al.*, 1996b). The APN's from *M. sexta* and *H. virescens* have 42% identity and 62% similarity (Gill *et al.*, 1995).

Binding studies of endotoxins to BBMVs have demonstrated that there is a correlation between the presence of specific receptors in the insects midgut and the insecticidal spectrum of the numerous endotoxins

(Hoffmann *et al.*, 1988). These authors used ^{125}I-labelled endotoxins to show that a 130-kDa toxin with insecticidal activity against the larvae of both *M. sexta* and *P. brassicae* showed saturable high affinity binding to the BBMVs from both insect species. The other 136-kDa toxin, which was highly toxic to *P. brassicae* but not to *M. sexta*, showed high affinity, saturable binding to the BBMVs of *P. brassicae* but not of *M. sexta*. Competition studies done between the two endotoxins showed that *P. brassicae* has two distinct binding sites for the different endotoxins since the toxins do not compete significantly for each other's receptor (Hoffmann *et al.*, 1988). Further evidence supporting the correlation between specific receptor binding and toxicity of different endotoxins was provided by Van Rie *et al.* (1990) who performed toxicity and binding assays with three toxins, CryIA(a), CryIC and CryIE and three insects from the lepidopteran family. The CryIE toxin, which had no activity against *H. virescens*, did not bind to membranes of this species whereas CryIA(a) exhibited strong binding and was toxic to *H. virescens*. The toxins CryIA(a), CryIA(c) and CryIB(b) lethal to *Chilo suppressalis* had shared binding sites but different affinities for the BBMV receptors in binding assays. They showed that the recognition of the binding site by a specific toxin varies from one species of insect to another (Fiuza *et al.*, 1996). Masson *et al.* (1995) presented evidence which supported the view taken by other researchers that toxin binding does not necessarily result in toxicity. They showed that CryIA(c) could specifically recognise binding sites on BBMV prepared from CryIA(c) resistant and sensitive *Plutella xylostella*. CryIC toxin also bound to a receptor on BBMV prepared from resistant and susceptible larvae which was different to that recognised by CryIA(c).

The three domains of the Cry toxin recognised by Li *et al.* (1991) and discussed in section 2.4.1 are believed to be involved in membrane penetration (domain I) and receptor recognition and binding (domain II). The function of domain III is not conclusively known but has been shown to be involved in binding of the toxin to gut epithelium membrane proteins and is thought to play an important role in the level of toxicity (de Maagd *et al.*, 1996a). Some elucidation of the functions of the three domains has come from studies of hybrid toxins in which DNA regions of closely related *cry* genes, encoding toxins with different insect specificity and activity, were exchanged. Construction of the hybrid CryIA(b)-CryIC, where domain III of CryIA(b) had been exchanged for that of CryIC, resulted in a greater level of toxicity to *Spodoptera exigo* than that observed for the moderately toxic CryIA(b) or the toxic CryIC (de Maagd *et al.*, 1996a). In a previous study conducted by Bosch *et al.* (1994) (cited in de Maagd *et al.*, 1996a), a CryIE-CryIC hybrid was toxic to *S. exiga* and *Mamestra brassicae* as was the unmodified CryIC, whereas the native CryIE was not toxic to either of the insects. These results strongly suggest that domain III is important in the determination of specific toxicity. Evidence for the involvement of domain III in binding to gut membrane proteins was provided by the exchange of domain III of CryIA(b) in hybrid toxins. This exchange abolished the binding of the hybrid to the receptor recognised by CryIA(b) on ligand blots, although this exchange did not affect the specificity of binding to intact BBMV of *S. exiga* (de Maagd *et al.*, 1996a). Further studies showed that binding of the endotoxins to binding sites of target insects may involve both domain II and III of

the toxin protein and this involvement is dependent, in ligand blots, on the type of toxin and insect species used (de Maagd *et al.*, 1996b).

After binding to the receptor, the toxin is inserted into the midgut membrane forming pores by a process which is not yet fully understood. This pore formation leads to cell lysis and eventually death of the insect. The structure of the CryIIIA protein as determined by Li *et al.* (1991) contains a region in domain I which could penetrate the membrane and so form pores. These authors proposed that large conformational changes must occur between the toxin and the pore structure which is triggered by receptor binding and interaction of the toxin with the membrane bilayer. The toxin inserts into the membrane with the domain II region orientated towards the cytoplasm. One of the α helix pairs of domain I is thought to initiate membrane penetration. According to a model proposed by Knowles and Ellar (1987) (cited in Höfte and Whiteley, 1989), the formation of these pores in the membrane result in a net influx of ions and inflow of water causing the cells to swell and lyse. Biochemical studies suggest that a K^+ gradient is dissipated through the formation of the pores (Höfte and Whiteley, 1989).

1.2.5 THE USE OF *B. thuringiensis* CRY TOXINS IN PLANT PROTECTION

The chemical control of insect pests is one of the most costly aspects of crop protection and has been estimated to be $3-5 billion annually worldwide (Fischoff *et al.*, 1987; Bullock and Sollod, 1995). *B. thuringiensis* has been used as a safe alternative and supplement to chemical insecticides for over 20 years. These bioinsecticides, used as spore and crystalline inclusion preparations, account for 90-95% of the total biopesticide market (Feitelson *et al.*, 1992). Their successful application in agriculture and forestry and for the control of human and animal disease vectors is due to their high activity and specificity, features which make them harmless to the environment and humans.

Nonrecombinant *B. thuringiensis* products have traditionally been based on the single *B. thuringiensis* strain, *B. thuringiensis* subsp. *kurstaki* HD-1, which was first isolated in 1970 by Dr H. Dulmage (Carlton, 1995). This strain contains the genes for the crystal proteins CryIA(a), CryIA(b), CryIA(c), CryIIA and CryIIB and is active against lepidopteran insects (Kyong Lee *et al.*, 1996a). The bioinsecticide Dipel, produced by the American company Abbott Laboratories, is derived from this strain and contains all the crystal proteins except CryIIB (Feitelson *et al.*, 1992). Another *B. thuringiensis* based product, Vectobac, produced by Abbott Laboratories for mosquito control, contains CryIVA, CryIVB, CryIVD and CytA (Feitelson *et al.*, 1992). Table 1.1 lists other examples of *B. thuringiensis*-based products used commercially.

TABLE 1.1 Examples of commercially available *B. thuringiensis*-based products (Lambert and Perferoen, 1992).

B. *thuringiensis* SUBSPECIES	**INSECT TARGET**	**PRODUCT NAME**	**COMPANY**
kurstaki	Lepidoptera	Bactospeine	Duphar
		Biobit	Novo Labs
		Condor	Ecogen
		Cutlass	Ecogen
		DiPel	Abbott Labs
		Javelin	Sandoz
		Larvo Bt	Knoll Labs
		MVP*	Mycogen
		Thuricide	Sandoz
aizawai	Lepidoptera	Certan	Sandoz
israelensis	Diptera	Skeetal	Novo Labs
		Moskitocid	Radonja
		Teknar	Sandoz
		Vectobac	Abbott Labs
san diego	Coleoptera	M-One	Mycogen
		M-One Plus	Mycogen
tenebrionis	Coleoptera	Trident	Sandoz
tenebrionis/kurstaki conjugates	Coleoptera Lepidoptera	Foil*	Ecogen

*Genetically engineered, killed and encapsulated

These have several disadvantages as bioinsecticides. Although their high specificity is an advantage for the environment, the narrow host range of *B. thuringiensis* toxins is commercially problematic. *B. thuringiensis* being U.V sensitive is not stable in the environment resulting in the need for repeated applications of the bioinsecticide for effective biological control. *B. thuringiensis* is soil borne which makes its availability to insect pests found within or on plants difficult. The use of recombinant DNA technologies has provided solutions to these problems through the development of two new approaches, namely genetically modified microorganisms and transgenic plants.

The ability of *B. thuringiensis* to stably maintain several different types of crystal genes without gene rearrangements or loss has been exploited for the construction of recombinant *B. thuringiensis* strains which improved on the traditional wild type strains. Other advantages are that *B. thuringiensis* can express these genes to high levels and the natural *B. thuringiensis* plasmids, as well as a *B. thuringiensis* transposon that encodes a transposase and a site specific recombinase, can be used as cloning vectors. The new *B. thuringiensis* constructs will only contain *B. thuringiensis* DNA without any antibiotic resistance genes or other foreign genes, which would be an advantage when applying for regulatory approval for commercialization (Carlton, 1995).

The Biotechnology company Ecogen Inc. has constructed a *B. thuringiensis* strain known as Raven, which contains two different CryIII genes and a CryI gene, targeted at the Colorado potato beetle (CPB). The CryIII proteins expressed in the Raven strain have different binding characteristics on CPB midgut cell membranes. This is an important feature as development of resistance by CPB to this strain should be minimized. Laboratory studies conducted *in vitro* selected CPB that were resistant to one of the CryIII proteins and only slightly resistant to the other CryIII. Therefore in the field presumably the beetle would have to undergo two independent resistance mutations to become resistant to the Raven strain.

B. thuringiensis cry genes have been introduced into bacteria other than *B. thuringiensis*. Obukowicz *et al.* (1986a and b) reported the introduction of a *cry* gene from *B. thuringiensis* subsp. *kurstaki* HD-1 into the chromosome of 6 strains of *Pseudomonas fluorescens* and *Agrobacterium radiobacter* which colonize the roots of corn. The integration of the gene into the chromosome of these soil microorganisms ensured stability of the gene and minimized the risk of its horizontal transfer. These strains were toxic against the larvae of the tobacco hornworm (*Manduca sexta*).

Waalwijk *et al.* (1991) integrated a *cryIVB* gene under the control of the strong tac promoter into the chromosome of a root colonizing strain of *P. fluorescens*. Recombinant strains were toxic towards larvae of the malaria mosquito (*Anopheles stephensi*) and of leatherjacket (*Tipula oleracea*). The *cryIVB* gene was introduced into *Ancylobacter aquaticus*, a bacterium isolated from aquatic habitats. These recombinant bacteria were toxic to mosquito larvae and contain gas vacuoles which make them buoyant. This characteristic suggests that these recombinant organisms could be potential biocontrol agents of mosquito larvae which are found in the upper surfaces of water (Ho Yap *et al.*, 1994). These examples of microbial biopesticides overcame the drawbacks resulting from the instability of *B. thuringiensis* in the field and consequently the need for frequent reapplication.

Commercially available and Federally approved recombinant *B. thuringiensis* products MVP and M-Trak have been produced by Mycogen Corporation. The CryIA(c) and CryIIIA toxins respectively were encapsulated within *P. fluorescens* cells through a process called CellCap. These cells had been killed and

stabilized by a chemical fixative which strengthened the cell wall and inactivated proteolytic enzymes but left the toxins active. These products persisted longer in the field than traditional *B. thuringiensis*-based products (Feitelson *et al.*, 1992).

Endophytic bacteria engineered to express *cry* genes offer an alternative option to biological control of insect pests which penetrate the interior regions of plants. The *cryIA(c)* gene of *B. thuringiensis* subsp. *kurstaki* HD-73 was introduced into *Clavibacter xyli* subsp. *cynodontis*, which naturally colonizes the xylem of Bermuda grass. This recombinant endophyte was shown to colonize corn and was tested for its effectiveness against the European corn borer (*Ostrinia nubilalis*). Moderate control of this pest was achieved by expression of the toxin gene chromosomally integrated into the endophyte (Lampel *et al.*, 1994). However, integration of endotoxin gene sequences into the chromosome of *C. xyli* subsp. *cynodontis* was unstable and segregant colonies made up less than 15% of the colonies isolated from corn at the end of the growing season. The authors suggested that the loss of the integrated gene could serve as an environmental safety feature (Turner *et al.*, 1991).

Many transgenic plants protected from insect attack by expressed *B. thuringiensis* Cry proteins have been reported in the literature. These include tomato, potato, tobacco, maize, cotton and rice (cited in Kleiner *et al.*, 1996) as well as transgenic trees such as poplar (Kleiner *et al.*, 1996).

Although the expression of intact, unmodified *B. thuringiensis* protein in transgenic plants is poor, with levels of RNA and intact protein at about 0.001% of the total protein (Vaeck *et al.*,1987; Perlak *et al.*, 1991), truncated forms of insecticidal proteins result in increased levels of expression and insecticidal activity (Vaeck *et al.*, 1987; Perlak *et al.*, 1991; Fischhoff *et al.*, 1987). When tobacco plants were transformed with intact and modified *cryIA(b)* toxin genes from *B. thuringiensis* subsp. *berliner* 1715 under the control of the 2' promoter only transgenic tobacco plants which carried the truncated toxin genes gave rise to expression levels that were strongly insecticidal to *M. sexta* (Vaeck *et al.*, 1987). There was a correlation between the amount of toxin protein present in the plants and insecticidal activity and intact protein and RNA levels were 10-50 times lower than those for truncated or fusion *B. thuringiensis* proteins (Vaeck *et al.*, 1987). Similarly, transgenic tomato plants carrying truncated toxin genes from *B. thuringiensis* subsp. *kurstaki* under the control of the 35S CaMV promoter exhibited significantly greater levels of expression than those tomato plants transformed with the intact, full length gene under the control of the same promoter (Fischhoff *et al.*, 1987). Although significant insecticidal activity was detected the levels of mRNA from the truncated gene were much lower than expected for a gene expressed from the strong CaMV 35S promoter. Higher levels of expression, however, were required to control insects in field tests with tomato plants (Delannay *et al.*, 1989).

Perlak *et al.* (1991) having found that the use of different promoters and leader sequences had no significant effect upon the levels of *cry* gene mRNA and protein expression in plants came to the conclusion that the problem might be related to differences between the coding sequence in *B. thuringiensis* and plants which would preclude efficient expression of the bacterial genes in plants. Apart from differences in G+C content, the *cryIA(b)* DNA contained regions which resemble plant introns, potential polyadenylation signal sequences, rare plant codons and mRNA destabilizing ATTTA sequences. The DNA sequences of *cryIA(b)* and *cryIA(c)* from *B. thuringiensis* subsp. *kurstaki* HD-1 and HD-73 respectively were partially modified by site-directed mutagenesis to remove DNA sequences that were predicted to inhibit transcription and translation within plants. These genes retained less than 80% DNA homology to the wild type genes. The genes were also fully modified. These synthetic genes had almost 100% amino acid sequence homology to the wild type genes but were designed to have plant codon usage with the elimination of rare codons, all ATTTA sequences and potential polyadenylation signal sequences. Transgenic cotton plants carrying the partially modified *B. thuringiensis* genes produced significantly more of the toxin protein than those carrying the wild type, truncated genes (Perlak *et al.*, 1990). The expression of high levels of toxin protein correlated with an increased resistance of transgenic cotton to damage caused by lepidopteran insects.

The expression of the partially and fully modified genes was also determined in tobacco and tomato. Compared with plants expressing the wild type gene, those carrying the partially modified *cryIA(b)* genes had a 10-fold higher level of *cryIA(b)* protein and plants expressing the fully modified *cryIA(b)* had 100-fold. This was the rsult of improved translational efficiency in plants rather than improved transcription, since the increased levels of mRNA of the modified *cryIA(b)* were not directly proportional to the increased levels of protein (Perlak *et al.*, 1991).

Van der Salm *et al.* (1994) modified *cryIA(b)* and *cryIC* genes by removal of coding regions described by Perlak *et al.* (1991) which would negatively affect transcription and translation in plants. They too showed that limited modificatons resulted in increased expression of these genes in tobacco and tomato plants with increased resistance against *M. sexta*, *H. virescens* and *S. exigua*. A truncated *cryIA(b)* and modified gene was introduced into japonica rice. The plants were more resistant to two major insect pests of rice than untransformed control plants (Fujimoto *et al.*, 1993).

1.2.6 *B. thuringiensis* AND RESISTANCE MANAGEMENT

An important issue arising from the registration of increasingly more transgenic crops for pest control by *B. thuringiensis* toxins is that of insect resistance. To date only two species have evolved resistance to *B. thuringiensis* in the field (Gould, 1995), with the only documented instance being that of resistance to *B. thuringiensis* endotoxins in the diamondback moth (*Plutella xylestella*) (Stein and Lotstein, 1995). However, over 10 species of laboratory reared colonies of insects have been shown to be capable of

evolving resistance. An example is the tobacco budworm which has evolved a number of different types of resistance to *B. thuringiensis* in the laboratory. One strain developed resistance to the Cry1A(c) toxin by a mechanism that gave it broad resistance to many diverse *B. thuringiensis* toxins. Another strain developed a more specific mechanism for resistance to Cry1A(c) and now requires over 5000 times more toxin to kill this resistant strain than that required to kill normal strains. This strain has high resistance to other *B. thuringiensis* toxins including Cry1F. It has been estimated that one in a thousand tobacco budworms carries a gene for *B. thuringiensis* resistance, which is a higher frequency than that expected for conventional pesticides (Gould, 1995). It is of interest to note that several laboratory reared, endotoxin-tolerant colonies have been tested on transgenic plants expressing the same endotoxin or one which the insect has exhibited cross resistance to *in vitro* and in each case very few insects survived. This raises the question as to whether the mechanism(s) of *in vitro* resistance to *B. thuringiensis* endotoxins will be the same as the resistance mechanism(s) that may develop in the field (Stein and Lotstein, 1995).

With the high potential for resistance to *B. thuringiensis*, the Environmental Protection Agency (EPA) seriously considered pesticide resistance management strategies when registering the transgenic crops Newleaf Russet potato, Bollard cotton and Bt Corn expressing CryIIIA, CryIA(c) and CryIA(b) δ-endotoxins respectively. The EPA believes that if no resistance management plans are implemented widespread pest resistance could develop in less than five years after the uniform planting of transgenic crops over large areas (Matten and Lewis, 1995). In each of these three cases the registrants worked with the federal agencies in drawing up resistance management plans for their transgenic crops. Monsanto Co.'s strategies included the use of other pest management practices together with the planting of Newleaf potatoes, the monitoring of the susceptibility of the Colorado potato beetle (CPB) to the CryIIIA protein and the implementation of the "high dose" hypothesis. This hypothesis predicts that homozygous susceptible and heterozygous resistant insects will be killed by high dose expression of the *B. thuringiensis* toxin, if resistance to the protein is the result of a single gene that is inherited as a recessive or co-dominant trait. Therefore, only homozygous insects, which will be rare, will survive on the transgenic crop (Stone and Feldman, 1995). Strategies for the incorporation of non-toxic host plants as refugia for CryIIIA susceptible insects were part of Monsanto Co.'s resistance management plan as was the development of additional insect control proteins and other mechanisms of control different to that of the Newleaf insect control protein.

Ciba Seed's strategy for its transgenic corn producing the CryIA(b) protein against European corn borer (ECB) also included the adoption of the high dose strategy to ensure that all ECB are heterozygous for a resistant allele and the use of refugia to maintain sufficient susceptible populations. Similiarly, their strategy includes the establishment and monitoring of the insects population's baseline susceptibility to the toxin as well as mitigation measures should any confirmed findings of resistance be reported, such as the use of alternative ECB control measures. The company also intended to implement a grower education program and to continue with its resistnace management research programme (Stein and Lotstein, 1995).

1.2.6.1 MECHANISMS OF RESISTANCE DEVELOPMENT

Studies on the mechanism of resistance to *B. thuringiensis* toxins indicate that it is restricted to single groups of related toxins, is due to alterations in toxin membrane binding and is inherited as a partially or fully autosomal recessive trait (Gould *et al.* 1992, Tang *et al.*, 1996). The mechanism of resistance of the Indian meal moth, *Plodia interpunctella* to the commercial formulation of *B. thuringiensis* subsp *kurstaki*, known as Dipel, was due to alterations in toxin membrane binding (Van Rie *et al.*, 1989). Dipel consists of a mixture of CryIA(b) and CryII crystal proteins. Resistance to CryIA(b) toxin correlated to a reduced affinity of CryIA(b) to bind to BBMV from resistant larvae of *P. interpunctella*. Reduced binding of biotinylated CryIA(b) to tissue sections and BBMV from larvae of the Diamond moth resistant to *B. thuringiensis* subsp. *kurstaki* HD-1 was observed when compared with that of susceptible larvae (Tang *et al.*, 1996). Resistant larvae were still susceptible to CryIB, CryIC and CryID and binding studies showed that there was no difference in binding of CryIB or CryIC in resistant larvae (Tang *et al.*, 1996). This specificity of resistance has led to the suggestion that as insects become resistant to one toxin it could be replaced by a different one. Resistance of *H. virescens* to CryIA(c) differed in that it was not due to changes in toxin binding and was not inherited as a recessive trait at high doses of CryIA(c). This strain also exhibited cross resistance to *B. thuringiensis* toxins from different classes (for example between CryIA(c) and CryIIA; Gould *et al.*, 1992). These findings show that selection with a single type of toxin could lead to broad-spectrum resistance which has important implications for *B. thuringiensis* resistance management.

1.3 ENDOPHYTIC BACTERIA

1.3.1 ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA

The interior of healthy plants was initially believed to be free from microorganisms. This concept was fuelled by the observation made by Pasteur in 1876 that grape juice aseptically removed from the interior of healthy, intact fruit was sterile (Hollis, 1951; Mundt and Hinkle, 1976). However, Perotti in 1926 was chiefly responsible for changing this concept when he reported that non-pathogenic bacteria could be found within healthy root tissue (Philipson and Blair, 1957). The number of reports of similar findings of bacteria within leaves, stems and roots of healthy plants increased from 1935. Endophytic bacteria have been found in fruit (Samish *et al.*., 1963), vegetables (Hollis, 1951), stems (Whiteside and Spotts, 1991), roots (Philipson and Blair, 1957), seeds and ovules (Mundt and Hinkle, 1976). They have been observed in cells of the cortex, in the intercellular spaces between the cells of the cortex and endodermis, and in the cells of the xylem, parenchyma and pith. Examples of endophytic bacteria isolated from various plants and their population size, incidence, distribution, location and survival are summarized in Table 1.2.

TABLE 1.2 Examples of endophytic bacteria isolated from different plants, their population size, incidence and location.

PLANT SOURCE[a]	ENDOPHYTE[b]	POPULATION SIZE[c]	LOCATION[d]	REFERENCE
Various seeds and ovules (15 to 30%)	*Bacillus, Pseudomonas, Erwinia, Flavobacterium*	ND	interior	Mundt and and Hinkle (1976)
Florida citrus trees	*Pseudomonas* (40%), *Enterobacter* (18%), *Serratia* (16%), *Bacillus Corynebacterium*	10^2 to 2×10^4	xylem of roots	Gardner *et al.* (1982)
Cotton	*Erwinia* (69%), *Bacillus, Clavibacter, Xanthomonas*	0.4×10^3 to 11.6×10^3	radicles, roots, stems and bolls	Mishagi and Donndelinger (1990)
Sugar beet	*Bacillus, Erwinia, Pseudomonas, Corynebacterium, Lactobacillus, Xanthomonas*	ND	secondary roots	Jacobs *et al.* (1985)
Potato	*Micrococcus, Pseudomonas, Bacillus, Flavobacterium, Xathomonas, Agrobacterium*	ND	seed and tubers	De Boer and Copeman (1974)
Tomato, bean, peach, olive and cucumber	*Pseudomonas, Xanthomonas*	10^3	stem scar, core & fruit periphery	Samish *et al.* (1963)
Alfalfa	*Pseudomonas*	6×10^3 to 4×10^4	root, crown xylem	Gagné *et al.* (1987)
Apples (49%)	*Bacillus*	ND	ND	Sholberg *et al.* (1995)
Buttercup squash (70 %)	facultative anaerobes	10^2	fruit mesocarp	Sharrock *et al.* (1991)
Corn	*Clavibacter xyli* subsp. *cynodontis*	10^3 - 10^{10}	xylem of stem, husk and leaves	Tester (1992)
Pear (23.5%)	*Pseudomonas*	ND	roots and stems	Whiteside and Spotts (1991)
Corn	*Enterobacter*	ND	cortex, pericycle	Hinton and Bacon (1995)
Tomato	*Streptomyces*	ND	cortex	Sardi *et al.* (1992)

[a]Numbers in brackets represent the incidence of endophytic bacteria

[b]Numbers in brackets indicate the percentage representative of total numbers isolated. Most commonly isolated bacteria listed.

[c]CFU/g fresh weight of tissue [d]plant organ and tissue ND Not determined

1.3.2 NITROGEN-FIXING ENDOPHYTIC BACTERIA

Owing to the high price of nitrogen fertilizers in Brazil, plants such as sugarcane, cereals and forage grasses have been bred and selected for several decades with low nitrogen fertilizers. Nitrogen balance experiments carried out several years ago showed that high rates of biological nitrogen fixation (BNF) occurred in these plants. Since none of these plants had been artificially infected with nitrogen-fixing organisms, diazotrophic bacteria which were associated with these crops in a manner which differed from the classical rhizosphere associated bacteria (Döbreiner *et al.*, 1993) were sought. Several endophytic diazotrophs associated with these crops have subsequently been isolated and identified. These include bacteria from the genera *Azoarcus*, *Acetobacter* and *Herbaspirillum*. The latter two will be described in detail. Certain strains of *Azospirillum* have been isolated from surface sterilized roots and stems of cereal plants (Döbreiner, 1988; Döbreiner *et al.,* 1995). However they also occur in high numbers in the soil and are therefore rather referred to as rhizosphere associated (Döbreiner *et al.*, 1993).

1.3.2.1 *Acetobacter diazotrophicus*

A new N_2-fixing endophyte, originally named *Saccharobacter nitrocaptans,* was isolated from the roots and stems of sugarcane from four different regions in Brazil (Cavalcante and Döbreiner, 1988). Evidence obtained from DNA-RNA and DNA-DNA hybridizations as well as from phenotypic and chemotaxonomic analysis indicated that these isolates constituted a new species of *Acetobacter.* It was called *Acetobacter diazotrophicus*, the adjective *diazotrophicus* meaning one that feeds on nitrogen (Gillis *et al.*, 1989). These bacteria were isolated on a semisolid medium based on sugarcane juice inoculated with serial dilutions of macerated sugarcane roots and stems. The vials which showed nitrogenase activity (acetylene reduction) were replicated into semisolid acetic, N free, 10% sugar medium at pH 4.5. The bacteria showed characteristic growth in this medium forming a sub-surface pellicle initially which moved to the surface and became thick and dark orange-yellow while the medium below became colourless due to the assimilation of the bromothymol blue. Dark orange colonies formed on N poor (0.005%) acetic acid medium with 10% sucrose after 7-10 days and dark brown colonies on potato agar (Cavalcante and Döbreiner, 1988). An improved, more sensitive isolation medium for *A. diazotrophicus* that includes crystallized cane sugar at 100 g/l and cane juice at 5 ml/l at pH 5.5 was recently described has recently been described (Reis *et al.*, 1994).

The bacterium is a small, Gram negative, aerobic rod. It is a microaerobic nitrogen fixer which grows on N_2 as a sole nitrogen source in semisolid medium. It grows well in liquid media which has been supplemented with nitrogen. The nitrogenase is oxygen sensitive so growth occurs in N free semisolid medium at a point where the respiration rate is in equilibrium with the oxygen diffusion rate. The nitrogenase is only partially inhibited by NH_4 (Stephan *et al.*, 1991). It is also capable of producing H_2, a product of nitrogenase activity,

in the presence of high concentrations of acetylene at higher levels than expected for other N_2 fixing bacteria. Usually acetylene inhibits H_2 production due to the saturation of nitrogenase activity and suppression occurs at different degrees in different N_2 fixing bacteria (Dong *et al.*, 1995). It has no nitrate reductase and N_2 fixation occurs in the presence of 10mM NO_3.

Best growth occurs with high concentrations of sucrose (10%) and can occur up to 30% sucrose. Growth and N_2 fixation also occurs at high glucose concentrations (10 to 30%). Fructose and galactose can be used as carbon sources (Gillis *et al.*, 1989). Strong acid production results in a final pH of 3.0 or less at which growth and nitrogen fixation continues (Stephan *et al.*, 1991). Ethanol (1%), mannitol and glycerol are also carbon sources. Ethanol is oxidized to CO_2 and H_2O, as are acetic and lactic acids. Optimum growth is around 30°C (Cavalcante and Döbreiner, 1988).

A. diazotrophicus has only been isolated from sugarcane, sweet potatoes and cameroon grass, which are all vegetatively propagated plants with high sugar contents (Döbreiner *et al.*, 1995). It has been isolated from Brazilian sugarcane in high numbers of 10^3 to 10^7 bacteria/g fresh weight of roots and 10^3 to 10^4/g fresh weight of stems. It is not found in soil between rows of sugarcane nor in 12 species of weed growing in cane fields. It has also been isolated from sugarcane in Australia, South Africa, Uruguay, Cuba and Mexico (Döbreiner *et al.*, 1993). There is limited genetic diversity among strains of *A. diazotrophicus* isolated from sugarcane in diverse geographical locations in Mexico and Brazil (Caballero - Mellado and Martinez - Romero, 1994). Analysis of 11 metabolic enzyme loci by multilocus enzyme electrophoresis has shown that the chromosomal structural genes between these isolates are homogeneous. Dong *et al.* (1995) reported identical phenotypic and morphological characteristics of sugarcane isolates from field grown Cuban sugarcane and greenhouse propagated sugarcane in Canada. The levels of genetic diversity are the lowest reported for any bacterial species. This limited genetic diversity could be the result of the low levels of genetic diversity of commercial sugarcane varieties and the relatively constant internal environment of sugarcane stems. Several strains of *A. diazotrophicus* also contain highly conserved small (20-24kb) and large (170kb) plasmids (Caballero-Mellado and Martinez-Romero, 1994). *A. diazotrophicus* colonizes root, stems and leaves of Cameroon grass in numbers up to 10^6/g fresh weight and of sweet potato in numbers up to 10^5/g fresh weight of tubers (Döbreiner *et al.*, 1995).

The location of *A. diazotrophicus* within sugarcane was examined using light and electron microscopy and confirmed by immunogold labelling (James *et al.*, 1994). Aseptically propagated sugarcane plantlets were artificially infected with *A. diazotrophicus* and examined after 4, 7, 9 and 15 days. The bacteria externally colonized the roots and lower stems, particularly at cracks in lateral root junctions, and entered the root tissue via the loose cells of the root cap at the root tip. Bacteria were observed in enlarged, intact epidermal cells of the root and within the xylem vessels at the base of the stem. They appeared to be distributed

upward through these xylem vessels. These may also be possible sites of N_2 fixation as they provide the low pO_2 and energy necessary for nitrogenase activity.

A. diazotrophicus has not been isolated from seeds and does not survive in soil. The natural distribution of this bacterium seems to be via vegetative propagation of plants or by transfer directly from the sugarcane setts used for planting in the field to the emerging new plants (Döbreiner *et al.*, 1993). The most successful means of inoculating plantlets besides direct inoculation of micropropagated sugarcane plantlets is with the spores of vesicular-arbuscular mycorrhizal fungi (VAM) (Paula *et al.*, 1991). Spores of the VAM fungi *Glomus occultum* and *Acaulospora* sp. collected from field grown sweet potatoes naturally contained *A. diazotrophicus*. Spores of *G. clarum* were inoculated with the diazotrophs and used to infect sweet potatoes, sugarcane and sorghum. Results showed that these infected spores introduced as many bacteria into the roots of sweet potato as the inoculation with a pure culture. The bacteria were found only when inoculated with VAM spores containing the bacteria. Similar results were obtained in micropropagated sugarcane and sugar sorghum, except that the aerial parts of sweet sorgum did not contain bacteria. *A. diazotrophicus* enhanced VAM sporulation and colonization of the roots of these plants indicating the endophyte and VAM fungi mutually benefit from their interaction. The possibility of introducing N_2 fixing bacteria into sugarcane plants by VAM fungi could be important for the introduction of genetically engineered strains (Boddey, 1995).

A. diazotrophicus has been isolated from the pink sugarcane mealybug, *Saccharococcus sacchari* (Ashbolt and Inkerman, 1990). This mealybug is common to all sugarcane producing regions. Mealybugs can act as vectors for disease but this has not been demonstrated for the pink sugarcane mealybug. The honeydew secreted by the mealybugs is acidic (pH 3.0) and supports the growth of *A. diazotrophicus* as well as other acetic acid bacteria and acidophillic yeast species. There are strong implications that the pink sugarcane mealybug is a primary vector of these microorganisms and could be responsible for transmission of *A. diazotrophicus*.

In an attempt to demonstrate that N_2 fixing bacteria are responsible for the significant increase in biological nitrogen fixation observed, Cojho *et al.* (1993) set up a model system for plant - bacteria interactions. Mixed cultures of *A. diazotrophicus* and an amylolytic yeast, *Lipomyces kononenkoae*, were established in nitrogen-free batch culture. Results showed that approximately 50% of the total nitrogen fixed by the diazotroph was supplied to the yeast as early as the onset of the culture. This synergism indicated that, in sugarcane, substantial amounts of nitrogen produced by the bacteria could be transferred to the plant.

1.3.2.2 *Herbaspirillum* SPECIES

Herbaspirillum seropedicae (*Herbaspirillum* meaning small, spiral shaped bacteria from herbaceous seed bearing plants and *seropedicae* referring to the place where they were first isolated) was isolated originally from the roots of maize, sorghum and rice and from the soil (Baldani *et al.*, 1986). It has subsequently been shown that the organism does not survive well in the soil and the results obtained initially were probably due to the presence of root hairs or small pieces of root in the soil (Döbreiner *et al.*, 1993). *H. seropedicae* has been isolated from many samples of roots, stems, leaves and seeds of rice, sorghum and maize. It has been isolated from stems and leaves of Cameroon grass (*Pennisetum purpereum*), sugarcane, 13 different graminaceous weeds and from the roots of a pigeonpea plant (Döbreiner *et al.*, 1993). It has also been found in high numbers along with other diazotrophs in roots, stems and leaves of oil palms and pejibaye (Döbreiner, unpublished). It has never been found in dicotyledonous plants (Döbreiner *et al.*, 1995). The occurrence of *H. seropedicae* has been restricted to Brazil (Döbreiner *et al.*, 1993). These bacteria, originally thought to be *Azospirillum* species, are motile, viroid, Gram negative, rod shaped organisms which fix atmospheric N_2 under microaerobic conditions. In semisolid (NFb) medium and in a more specific medium (JNFb), they form a thin veil like surface pellicle. On solid media supplemented with nitrogen they form small white colonies with green-blue centres due to the assimilation of the bromothymol blue in the medium. Organic acids such as malate and fumarate are preferred carbon sources for growth but glucose, galactose, mannitol, sorbitol and glycerol are also used. The optimum pH range is between pH 5.3 and 8.0 and temperature is 34°C.

Herbaspirillum rubrisubalbicans, a mild pathogen known to cause mottled stripe disease of some sugarcane varieties in countries other than Brazil, was misclassified as *Pseudomonas rubrisubalbicans* (Gillis *et al.*, 1991). The inclusion of *P. rubrisubalbicans* within the genus *Herbaspirillum* has been proposed by Baldani *et al.* (Döbreiner *et al.*, 1995). DNA and rRNA analysis indicated a very close relationship with *H. seropedicae*, which was confirmed by auxotrophic tests (Gillis *et al.*, 1991; Döbreiner *et al.*, 1995). The physiological differentiation of these two strains is difficult. The only difference between these two species found so far is the use by *H. rubrisubalbicans* of mesoerythritol for growth with NH_4^+ but not N_2. *H. seropedicae* strains are unable to utilize mesoerythritol for growth but use N-acetylglucosamine as their sole carbon source. The optimum temperature differs between *H. rubrisubalbicans* (30°C) and *H. seropedicae* (34°C) (Boddey, 1995). *H. rubrisubalbicans* is also able to fix nitrogen at rates comparable to those of *H. seropedicae* (Döbreiner *et al.*, 1995). It has only been isolated from sugarcane stems and leaves from all over the world (Döbreiner *et al.*, 1993). The bacteria occur in high numbers in roots, stems and leaves of Brazilian sugarcane varieties which do not show symptoms of mottled stripe disease.

The mode of infection by *Herbaspirillum* spp. is not known. These bacteria were artificially inoculated into the leaves of sorghum and forage grasses and could be reisolated 60 days after infection (Pimentel *et al.*,

1991). *H. seropedicae,* artificially inoculated into sorghum, Napier grass and sugarcane caused mottled stripe disease symptoms on the former two plants but not on sugarcane (Pimentel *et al.*, 1991). *H. rubrisubalbicans* does not survive well in soil. Light and electron microscopy have shown that *Herbaspirillum* spp. are localized in the meta and protoxylem of sugarcane and sorghum plants. In sorghum, *H. rubrisubalbicans* formed globule like structures in the metaxylem vessels but these did not occur with *H. seropedicae* infection (Döbreiner *et al.*, 1993). In sugarcane varieties susceptible to mottle stripe disease, *H. rubrisubalbicans* completely blocked some of the xylem vessels, which could be the reason for the disease symptoms. In resistant varieties, the bacteria occurred in clusters of 10 to 20 cells encapsulated in membranes which were attached to the interior cell wall and therefore did not block the xylem vessels (Boddey, 1995).

1.3.3 POTENTIAL OF ENDOPHYTES AS BIOCONTROL AGENTS

Endophytic bacteria have several attributes which make them attractive as potential biocontrol agents. They colonize and form associations within plant tissues without causing disease and they have several advantages over bacteria found in the rhizosphere and phylloplane. In the interior of plants, endophytes are protected from harsh and extreme environmental conditions and from competition for limited nutrients (Chet *et al.*, 1993; Chen *et al.*, 1995). Despite these advantages there have only been a few reports in the literature on their use as biocontrol agents. Endophytes have been shown to provide effective control of the postharvest pathogen *Moninlinia laxa* and *Rhizopus stolonifer* in stonefruit (Pratella *et al.,* 1993 in Sholberg *et al.*, 1995). Fifteen endophytic bacteria isolated from apples stored at different temperatures reduced disease caused by *Pennicillium expansum.* Three isolates were also effective against *Botrytis cinerea* on stored fruits. Effectiveness against the fungi depended on the isolate and storage temperature. The majority of the endophytes were identified as *B. subtilis*, a recognized biocontrol agent (Sholberg *et al.,* 1995).

Enterobacter cloacae is a known biocontrol agent of postharvest diseases of fruits and vegetables and damping-off disease. However, it generally does not occur as an endophyte. Hinton and Bacon (1995) reported the first isolation of an endophytic strain of *E. cloacae* from the roots, stems and leaves of an Italian corn cultivar. This strain was capable of effectively controlling *Fusarium moniliforme, Aspergillus flavus* and *A. parasiticus* in inhibitory plate assays.

Of the 244 strains isolated from the internal tissues of cotton, 41 were potential biocontrol agents (Chen *et al.*, 1995). The strains were classified into 32 genera with the largest number belonging to the genera *Burkholderia* (27%), *Pseudomonas* (12%) and *Bacillus* (12%). They were introduced as endophytes into cotton and challenged by stem injection of the conidia of *F.oxysporum* f.sp. *vasinfectum*, which causes fusarium wilt. Symptom expression was evaluated 12 days after pathogen infection. Six strains caused disease reduction in two separate experiments. The bacteria survived in cotton stems for up to 28 days with

increase in the populations after 3 days indicating that these bacteria were able to survive and multiply after introduction into cotton (Chen *et al.*, 1995).

A variety of endophytic bacteria with the capacity for biocontrol have been isolated from seeds, plants and from micropropagated plants (Pleban *et al.*, 1995). The endophytic bacteria isolated from bean, *Sinapsis*, cauliflower, onion tissue culture, and sunflower were identified as *P. fluorescens*, *B. cereus*, *B. subtilis*, and *B. pumilus* respectively. These bacteria were able to inhibit some or all of the phytopathogenic fungi *R. solani*, *Sclerotium rolfsii* and *Pythium ultimum*. One *B. cereus* isolate exhibited chitinase activity and inhibited growth of all three fungi. When labelled radioactively it was found 72 days after introduction into cotton roots and stems at numbers of 2.8 x 10^5 and 5 x 10^4 CFU/g fresh weight respectively. The numbers of bacteria initially increased, then decreased and then, after day 30, again increased in numbers. They spread initially to all plant parts but were eventually found mainly in the stems and roots. The number of bacteria never decreased below 5 x 10^4 CFU/g fresh weight of stems and roots. Disease incidence caused by *R. solani* on cotton seedlings inoculated with this *B. cereus* isolate, *B. subtilis* and *B. pumilus* was reduced by 51, 46 and 56% respectively in greenhouse trials. Disease incidence caused by *S. rolfsii* in bean seedlings inoculated with , *B. subtilis*, *B. pumilus* and another *B. cereus* isolate was reduced by 72, 26 and 79% respectively. These endophytes were therefore efficient biocontrol agents which could survive and multiply within cotton and bean seedlings under greenhouse conditions.

The only report of a genetically engineered endophytic bacterium is that of *Clavibacter xyli* subsp. *cynodontis* isolated from bermuda grass, which was modified to produce a δ-endotoxin from *B. thuringiensis* subsp. *kurstaki* for the control of the European corn borer in corn. The engineered endophyte colonized the xylem of stems, leaves and husks of inoculated corn plants and was capable of reaching populations greater than 10^7 CFU/g plant tissue (Reeser and Kostka, 1988). The endophyte is not seed transmitted and does not survive well in the soil (Reeser and Sommerfeld, 1988; Turner and Tomasind, 1988). The recombinant strain that contained the toxin genes integrated into the chromosome, eventually lost these genes when it was grown *in vitro* and *in planta*. This organism has been discussed in section 1. 2.5.

Since little work has been done on endophytic bacteria to date, this project comprises a novel approach to achieving biological control. The objective of this work was to genetically engineer isolated endophytic bacteria from commercially important crops, including obligate sugarcane endophytes as well as the sugarcane phylloplane isolate, to overexpress the *S. marcescens chiA* and the *B. thuringiensis cryIA(c)* genes discussed above. These genes were cloned into broad host range plasmids and introduced into the endophytes. Integration vectors were also constructed to achieve stable integration of the overexpressed genes. The effectiveness of these bacteria as biocontrol agents was established.

CHAPTER 2

CLONING OF THE *Serratia marcescens chiA* AND *Bacillus thuringiensis cryIA(c)* GENE IN *Escherichia coli*

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CHAPTER 2

CLONING OF THE *Serratia marcescens chiA* and *Bacillus thuringiensis cryIA(c)* GENE IN *Escherichia coli*

2.1 INTRODUCTION

B. thuringiensis isolate 234 was obtained from dead *E. saccharina* Walker (Lepidoptera: Pyralidae) larvae at Mount Edgecombe, Natal, South Africa. This strain is toxic to the sugarcane borer *E. saccharina*, an endemic species in Africa which bores into the stalks of sugarcane and causes considerable crop loss. Isolate 234 was identified by H serotyping as belonging to the serovar *kurstaki*. It proved to be an atypical biovar in that it utilized citrate. Bt isolate 234 contains bipyrimadal crystals consisting of δ-endotoxin proteins with molecular weights of ca. 135 kDa and 61 kDa. Southern blot and PCR analysis showed that isolate 234 carries the *cryIA(a)* and *cryIA(c)* Lepidopteran specific genes. Just prior to submission of this thesis we were informed that the CryIA(c) δ-endotoxin from isolate 234 has now been incorporated into the nomenclature as Cry1Ac7 (accession number U87793; N. Crickmore, personal communication). The 61-kDa protein was thought to be a CryIIIA δ-endotoxin which is toxic to Dipteran species (Herrera, 1994).

The *cryIA(c)* gene of isolate 234 was cloned and sequenced and shown to be almost identical to the *cryIA(c)* gene of *B. thuringiensis* subsp. *kurstaki* HD-73 with only four different nucleotides, which did not result in changes in amino acids. A recombinant sugarcane colonizing strain of *Pseudomonas fluorescens* carrying this gene was toxic to *E. saccharina* larvae in bioassays and glasshouse tests (Herrera, 1994; Herrera *et al.*, 1994). Although this strain showed potential as a biocontrol agent, further improvement by cloning the *cryIA(c)* *(tox)* gene under the control of the strong *tac* promoter was necessary. This chapter describes the construction of broad host range vectors and an integration vector carrying the *tactox* cassette.

In order to obtain controlled expression of foreign genes at high levels in *E. coli*, De Boer *et al.* (1982) developed a "superpromoter" which they called *tac*. This hybrid promoter was derived from sequences of the commonly used *trp* and *lac*UV5 promoters. The *lac*UV5 promoter is induced when the *lac* operator is present which regulates the *lac* repressor. Expression from the *lac* promoter is repressed by overexpression of the *lac* repressor in *E coli* strains which possess a *lac*I^q gene. In these strains, the *lac* promoter can be induced by the addition of 1mM of isopropyl-β-D-thiogalactoside (IPTG). The *trp* promoter is repressed by the *trp* repressor which is activated and deactivated by levels of exogenous tryptophan present in the growth medium. Depletion of tryptophan in the medium deactivates the repressor. Control of the level of

repression and derepression is therefore difficult. The fully derepressed *trp* promoter is approximately five times as strong as the *lac*UV5 promoter. The *tac* promoter was shown to be at least 10 times stronger than the *lac*UV5 promoter, and at least 3 times as strong as the fully derepressed *trp*. The *tac* promoter is repressed in a *lac*I^q *E. coli* strain which prevents the detrimental effects on cell growth of constitutively expressed proteins from this promoter.

The consensus sequence of the Pribnow box is TATAATG and TTGACA for the -35 sequence. The *lac*UV5 promoter has a consensus Pribnow box but no consensus -35 sequence. Conversely the *trp* promoter has no consensus Pribnow box sequence but a consensus -35 sequence. Therefore the *tac* promoter has both a consensus Pribnow box and a consensus -35 sequence (De Boer *et al.*, 1982). This could explain the higher efficiency of *tac* compared to the *lac*UV5. The distance between these two sequences affects promoter strength. A distance of 17 bp occurs in the majority of the promoters sequenced to date. However, in the hybrid *tac* promoter the distance is 16 bp (the same as that found for strong ribosomal RNA promoters) and 18 bp in the *lac*UV5 promoter (De Boer *et al.*, 1982; 1983).

Although several strong promoters are efficiently used in *E. coli*, little is known about promoters that are useful for expression and regulation of cloned genes in other Gram negative bacteria. Bagdasarian *et al.* (1983) showed, using a broad host range plasmid, that the *tac* promoter and the promoter of the *E. coli lac*I^q gene is active in *Pseudomonas putida*. Expression of the catechol 2,3-oxygenase gene from the *tac* promoter in *P. putida* was similar to that in *E. coli*.

In this work the *tac*-carrying vector pKK223-3 (Brosius and Holy, 1984; Pharmacia Biotech) was used for the expression of the ChiA and Cry1A(c) proteins in *E. coli*. The *tac*-containing constructs were then subcloned in vectors suitable for other Gram negative bacteria. In pKK223-3, the multiple cloning site (MCS) of pUC8 is directly downstream of the *tac* promoter, followed by the *rrn*B 5S rRNA gene and strong ribosomal terminators T_1 and T_2 obtained from pKK10-2 (Brosius, 1984). These terminators help to stabilize the host vector system. If the foreign gene does not possess its own ribosome binding site (RBS) the RBS present on the vector can be used providing it is within the optimum 5-9 bp from the ATG start codon of the introduced gene (Kozak, 1983). If the cloned gene contains its own RBS and an ATG start codon it can be inserted anywhere within the MCS.

The extensive array of phage and plasmid vectors developed for *E. coli* have very narrow host ranges. Since the majority are unable to replicate in other Gram negative organisms vectors were developed which could do so and could be transferred efficiently by conjugation. Several have been modified, including RSF1010 which has been used as the basic replicon for the construction of a number of vectors. It is a small, multicopy IncQ group plasmid which is not self transmissible but can be mobilized by co-existing conjugative plasmids (Priefer *et al.*, 1985). Other examples include the plasmids RK2 and RP4 and their

derivatives. One of these RSF1010 derived plasmids, pKT240, was constructed as a promoter probe vector containing the structural gene for aminoglycoside phosphotransferase (*aph*) without its promoter (Bagdasarian *et al.*, 1981; 1983). It contains the genes for Km^R and Ap^R from the plasmid pHSG415 but does not confer resistance to Sm. The *aph* gene is expressed if DNA carrying a promoter is inserted upstream of the gene resulting in Sm resistance of the host cell.

The plasmid pML122 is also based on RSF1010 (Labes *et al.*, 1990). It was constructed from the plasmid pML10 which contains the selectable Gm^R marker inserted into pSUP104. The promoter of the Nm^R gene (*npt*II) from Tn*5*, a MCS and a promoterless Nm^R gene downstream from the promoter and MCS were inserted into pML10. The Nm^R is expressed only if the DNA fragment inserted into the MCS does not have a transcriptional terminator.

The broad host range plasmid pDER405 originated as part of the development of genetic systems for *Thiobacillus* and was constructed by cloning a fragment of the *T. ferrooxidans* plasmid pTF-FC2 carrying its origin of replication into pBR325 (Rawlings *et al.*, 1986). It is capable of replicating in a wide variety of hosts including strains of *Pseudomonas*.

The Omegon (Ω) interposon was originally constructed to facilitate the genetic analysis of cloned genes in their natural hosts as well as in *E. coli* (Prentki and Krisch, 1984). This had previously been carried out successfully by transposition mutagenesis but with several disadvantages such as nonrandom insertion, transcription of adjacent DNA mediated by the transposon, genetic instability leading to DNA rearrangements and expression of transposon carried cryptic genes in certain bacteria. Use of the genetically engineered Ω interposon in mutagenesis was shown to avoid many of these problems (Fellay *et al.*, 1987). Based on the original Ω interposon, five new interposons were developed for insertion mutagenesis which had different selectable markers (Fellay *et al.*, 1987). One of these, Omegon-Km, was cloned into the plasmid pJFF350 which is capable of being conjugally mobilized into a broad range of Gram negative bacteria. This plasmid, derived from pBR322, was specifically designed as a transposon donor vector. The Omegon-Km module consists of the Ω interposon flanked by synthetic inverted 28 bp ends derived from the IS*1*. It carries the kanamycin-neomycin resistance gene which allows for selection of transconjugants. It also contains the *E. coli*-specific pBR322 derived origin of replication. Each end of Omegon-Km has the very strong transcription and translation terminators of the Ω interposon. The modified IS*1* element (IS*1**) although able to express the functional IS*1* transposase has mutations in the terminal two nucleotides and therefore both ends are no longer active in transposition. It is located on the plasmid, external to the Omegon-Km module, therefore insertions are very stable because they lack the capacity to transpose further. Plasmid pJFF350 also contains the origin of transfer (*ori*T) from RP4 which allows it to be conjugally transferred from *E. coli* to other Gram negative recipient strains in which it is unable to replicate.

Omegon-Km transposition has been observed at very high frequencies in *P. putida* and at lower frequencies in other Gram negative bacteria such as *R. leguminosarum* and *Agrobacterium tumefaciens*. Insertion of the Omegon-Km is random, stable and primarily occurs singly. I was interested in this plasmid because it provided the means to introduce the *chiA* and *cryIA(c)* genes under the control of the *tac* promoter, into the unique *Nde*I site of Omegon-Km. This enabled me to integrate these cassettes stably into the chromosomes of agriculturally important Gram negative bacteria for use as biocontrol agents.

2.2 MATERIALS AND METHODS

2.2.1 BACTERIAL STRAINS, PLASMIDS AND CULTURE CONDITIONS

Bacterial strains and plasmids used for cloning are listed in Appendix C. *E. coli* strain A5945 and plasmid p*tacchiA* were gifts from Professor Amos Oppenheim, Department of Molecular Genetics, Hadassar Medical School, The Hebrew University of Jerusalem, Israel. Plasmid pLCHIA in *E. coli* A2097 was a gift from Professor Ilan Chet, Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Israel. The plasmid pBS42 was donated by Dr Ivo Baldani of EMBRAPA, Brazil. *E. coli* strains JM105 and A5945 were cultured in Luria broth (Sambrook *et al.*, 1989) at 37°C unless stated, with vigorous shaking. Plasmids were maintained by selection in the presence of the appropriate antibiotic: ampicillin 100 μg/ml (Ap); kanamycin 100 μg/ml (Km); gentamycin 40 μg/ml (Gm); chloramphenicol 25 μg/ml (Cm).

Restriction maps of plasmids pLCHIA (Shapira *et al.*, 1989), p*tacchiA* (Koby *et al.*, 1994), pKK223-3 (Pharmacia Biotech), pKT240 (Bagdasarian *et al.*, 1983), pDER405 (Rawlings *et al.*, 1986), pML122 (Labes *et al.*, 1990) and pJFF350 (Fellay *et al.*, 1989), are shown in Appendix D. The λ *Pst*I molecular weight marker containing fragments of 11497, 5077, 4749, 4507, 2838, 2560, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339 bp was co-electrophoresed in all agarose gels to estimate the size of DNA fragments.

2.2.2 PREPARATION OF PLASMID DNA

Plasmid DNA was extracted from 400 ml of *E. coli* strain JM105 cultures containing the plasmids pKK223-3 and recombinant derivatives thereof, pGH37-D1 or pJFF350 and from 800 ml cultures of *E. coli* strain JM105 for all other plasmids. All plasmids in *E. coli* strain A5945 were isolated from 800 ml cultures. Large scale plasmid isolation was carried out according to the methods described by Sambrook *et al.* (1989) or using the Nucleobond® AX PC-kit 100 (Macherey-Nagel, Germany) according to the maufacturer's

instructions. Small scale plasmid isolation was carried out according to the method of Ish-Horowicz and Burke (1981).

2.2.3 MOLECULAR TECHNIQUES

Techniques, unless specified, were performed according to methods described by Sambrook *et al.* (1989). DNA was routinely digested with restriction endonucleases in a volume of 20 μl, examined by electrophoresis in 1% agarose gels in TBE or TAE buffer and purified from TAE agarose gels or low salt buffers with the GENECLEAN^R II kit (Bio 101 Inc.) according to the manufacturer's instructions. The 3' recessed ends of DNA were made blunt using Klenow and the 5' phosphate group was removed from linearized plasmid vector DNA with calf intestinal alkaline phosphatase (CIP). Ligation of cohesive ends was according to the method of Sambrook *et al.* (1989) and blunt ends using the Rapid DNA ligation kit (Boehringer Mannheim) following the manufacturer's instructions. PCR products were phosphorylated by treatment with T4 polynucleotide kinase prior to blunt end ligations. DNA was sequenced using the thermo-sequenase fluorescent labelled primer cycle sequencing kit (Amersham Life Science) and the automated ALFexpress™ DNA sequencer (Pharmacia Biotech AB, Sweden). Cells of *E. coli* A5945 and JM105 were made competent to take up DNA by the method of Chung and Miller (1988) and Armitage *et al.* (1988) respectively.

2.2.4 PLATE CLEARING ASSAY TO SCREEN *E. coli* STRAINS TRANSFORMED WITH PLASMIDS CARRYING THE *chiA* GENE.

Colonies to be screened were patched onto nutrient agar plates (NA; Biolabs) containing the substrate CM-CHITIN-RBV (Remazol Brilliant Violet) (2mg/ml; Loewe Biochemica) spread with 100 μl 1mM IPTG immediately prior to use and onto Luria agar plates (LA), supplemented with the appropriate antibiotic, which served as a master plate. The plates were grown at 37°C and 30°C respectively, the latter to prevent the possible accumulation of mutants defective in *chiA* gene expression.

2.2.5 SYNTHESIS OF THE *chiA* GENE BY THE POLYMERASE CHAIN REACTION (PCR) AND CLONING

The *chiA* gene was synthesized from plasmid pLCHIA. Samples were denatured at 94°C for 3 min, cycled for 30 cycles with a denaturation step at 94°C for 30 seconds, annealed at 58°C for 30 seconds and elongated at 72°C for 90 seconds.

2.2.6 CHITINASE ASSAY

Cultures were grown overnight at 30°C in LB media supplemented with the appropriate antibiotic, diluted 100 fold, grown to mid- exponential phase at 37°C and induced with 0.3 mM IPTG as described by Ausubel *et al.* (1994). Uninduced controls were prepared by dividing the mid-exponential phase (OD_{600} = 0.4) culture in two, before adding IPTG. Samples from both uninduced and induced cultures were removed at various time intervals after induction.

Two assays systems were used to measure chitinase activity of the bacteria. The first, as described by Koby *et al.*(1994) provided a convenient and rapid means of detecting and quantitating activity in the total culture. The second provided a more accurate measure of specific enzyme activity, in terms of units of enzyme per unit mass of protein in the cellular and extracellular fractions The first assay protocol was adopted from that used by Miller *et al.*(1972) to measure β-galactosidase activity. Here, 500 μl of cells of known OD_{600} were lysed by addition of 20 μl chloroform and 20 μl of 1% SDS. Two hundred microlitres (v) of this sample, comprising both lysed cells and culture medium, was then added to a standard assay mixture of buffer and the substrate p-nitrophenyl-β-D-N,N'-diacetylchitobiose prepared as described in Appendix A. This was incubated at 40°C and, when a yellow colour became visible, the time (t) in minutes was noted and 200 μl of 1M NaOH was added to terminate the reaction. The sample OD was then read at 410 nm (the E_{max} of liberated p-nitrophenol) and at 550 nm (to correct for light scattering). Enzyme activity in the culture was then calculated as Miller units (normalized for cell density) = $1000 \times (OD_{410} - 1.75(OD_{550})/(t \times v \times OD_{600})$.

Although this gave a useful measure of gene expression, a more precise protocol was required to measure enzyme activity as a function of cellular protein in both intracellular and extracellular compartments. The following method was therefore adopted. Measured samples of cultures, taken at given time periods, were centrifuged to separate cells and media. The cells were washed, resuspended and disrupted by sonication. The medium was clarified by a second centrifugation and the protein concentration of the sonicate and clarified medium was determined by the Bradford's assay as described in Appendix B. The solutions were assayed in the standard assay reaction mixture by incubating for a fixed period of 20 minutes. The assay was terminated as above and the OD_{410} measured and referred to a standard curve (Appendix B). Enzyme activity for the assay was expressed in units where 1 unit is equal to that amount of activity that will convert 1 μmol substrate/hour. Specific activity was expressed as units/mg protein.

2.2.7 WESTERN BLOT ANALYSIS

Cell extracts were prepared from 1 ml of stationary phase cultures by resuspending cell pellets in 100 μl denaturing loading buffer, boiling for 10 minutes, then centrifuging for 5 minutes at 14 000 rpm. Samples (20 μl) were loaded onto a denaturing gradient (10-5%) acrylamide gel and the proteins separated by SDS-PAGE according to the method of Laemmli (1970). For the quantitative analysis of δ-endotoxin production, cultures were induced as described in section 2.2.6. Samples (1 ml) of 24 h-induced cultures were sonicated and the protein concentration determined by the method of Bradford (1972). Volumes of denatured cell extracts representing 50 μg of protein were separated by SDS-PAGE.

Proteins were electrotransferred to nitrocellulose and analysed by Western blot according to Towbin *et al.* (1979) with modifications (Appendix B).

2.3 RESULTS AND DISCUSSION

2.3.1 CLONING OF THE *chiA* GENE INTO pKT240* *tac*

The plasmid pLCHIA (Appendix D) constructed by Shapira *et al.* (1989), carries the *S. marcescens chiA* gene under the control of the oLpL operator/promoter of bacteriophage lambda. This plasmid was introduced into *E. coli* strain A2097 which harbours a defective lambda prophage carrying a temperature sensitive repressor to yield strain A5745. However since this promoter is difficult to control in bacteria which do not carry a temperature sensitive repressor, the *chiA* gene was cloned under the control of the strong *tac* promoter for high level expression in Gram negative bacteria.

The plasmid pKT240**tac* constructed by R. Saunders (personal communication) contains part of the multiple cloning site of pUCBM20 and a 260-bp *Hind* III-*Eco*RI fragment carrying the *tac* promoter from pMMB24. Due to the difficulty of separating the 4.7- and 4.2-kb *Eco*RI fragments of pLCHIA it was decided to clone the entire pLCHIA plasmid into pKT240**tac*. The 8.936-kb pLCHIA linearized with *Bam*HI was ligated into the *Sal*I site of pKT240**tac*. Two transformants carrying the resulting plasmid pKT240**tac*pLCHIA (17* and 23*) produced clear halos on NA plates containing CM-CHITIN-RBV, supplemented with Ap (100 μg/ml) or Km (100 μg/ml). Restriction analysis showed that the *chiA* gene was in the same orientation as the *tac* promoter. The 4.2-kb *Eco*RI fragment from pLCHIA was excised from 17* and 23* generating the plasmid pKT240**tacchiA* (17** and 23**). However, a rearrangement had occurred in 23** resulting in the *chiA* gene being in the opposite orientation to the *tac* promoter and this was abandoned. Very low levels of chitinase activity were detected in an *E. coli* culture carrying 17**. As no difference in activity was observed between induced and uninduced cultures, it was concluded that the *chiA*

gene was not being transcribed from the *tac* promoter. This was probably due to the large distance of over 2 kb between the *tac* promoter and the *chiA* gene. Although this distance is the same as that between the λpL promoter and *chiA* in pLCHIA, the λpL promoter is much stronger than the *tac* promoter (cited in Kaderbhai *et al.*, 1992). The activity observed was assumed to be due to expression of the chitinase from its endogenous promoter. Shapira *et al.* (1989) reported very low chitinase activity in *E. coli* cultures carrying the *chiA* gene cloned into pBR322 (plasmid pCHIA) and attributed this to inefficient transcription of the *S. marcescens* gene in heterologous bacteria. When they cloned the *chiA* gene downstream of the thermosensitive operator/promoter of λ in pLCHIA and grew cells at 42°C high levels of expression of 0.228 units/ml of supernatant 3 hours after heat-induction were obtained. The supernatant of cells carrying plasmid pCHIA, which lacks the pL promoter, had considerably lower levels of chitinase activity of 0.0085 units/ml. In addition to the large distance between the *tac* promoter and the *chiA* gene I suspected from previous observations that the *tac* promoter in pKT240**tac* was not efficient. Therefore in order to improve the expression levels the *tacchiA* cassette of p*tacchiA* was subcloned into broad host range plasmids and an integration vector.

2.3.2 INTRODUCTION OF *tacchiA* INTO THE BROAD HOST RANGE PLASMID pKT240 AND THE INTEGRATION VECTOR pJFF350

Sitrit *et al.* (1993) cloned the *chiA* gene from pLCHIA into the expression vector pKK223-3 which carries the *tac* promoter, to give the plasmid p*tacchiA* (Appendix D). This plasmid was used to construct a broad host range plasmid pYZ291 (Sitrit *et al.*, 1993) and a transposon system based on Tn*7* (Koby *et al.*, 1994). The 2.1-kb *Bam*HI fragment of p*tacchiA* was cloned into the broad host range plasmid pKT240 and the integration vector pJFF350. The resulting plasmids were named pKTC1 (pKT240*tacchiA*) and pJTC1 (pJFF350*tacchiA*) respectively (Figs 2.1 and 2.2). Cultures of *E. coli* expressing the *chiA* gene at high levels have been reported to accumulate mutants defective in chitinase expression (Oppenheim, personal communication; Koby *et al.*, 1994). Therefore p*tacchiA*, pKTC1 and pJTC1 were maintained either in *E. coli* strain A5945 or JM105 grown at 30°C or for short periods at 37°C. A5945 carries a mutation in the *pcnB* locus which reduces the plasmid copy number to 1-2 per cell (Liu and Parkinson, 1989). This strain however is Km^R and as the only marker present on pJTC1 is Km, this plasmid was grown in JM105. As the frequency of transformation is reduced in A5945 (Oppenheim, personal communication), ligations wer initially transformed into JM105 which were then transferred into A5945.

2.3.3 CHITINASE EXPRESSION IN *E. coli* (p*tacchiA*), (pKTC1) AND (pJTC1)

Chitinase assays were carried out as described in section 2.2.6. The chitinase activity of *E. coli* JM105 (p*tacchiA*), (pKTC1) and (pJTC1) 4 h after induction was 406, 160 and 392 Miller units respectively. The specific activity of these clones will be discussed in section 2.3.8. Koby *et al.* (1994) reported high

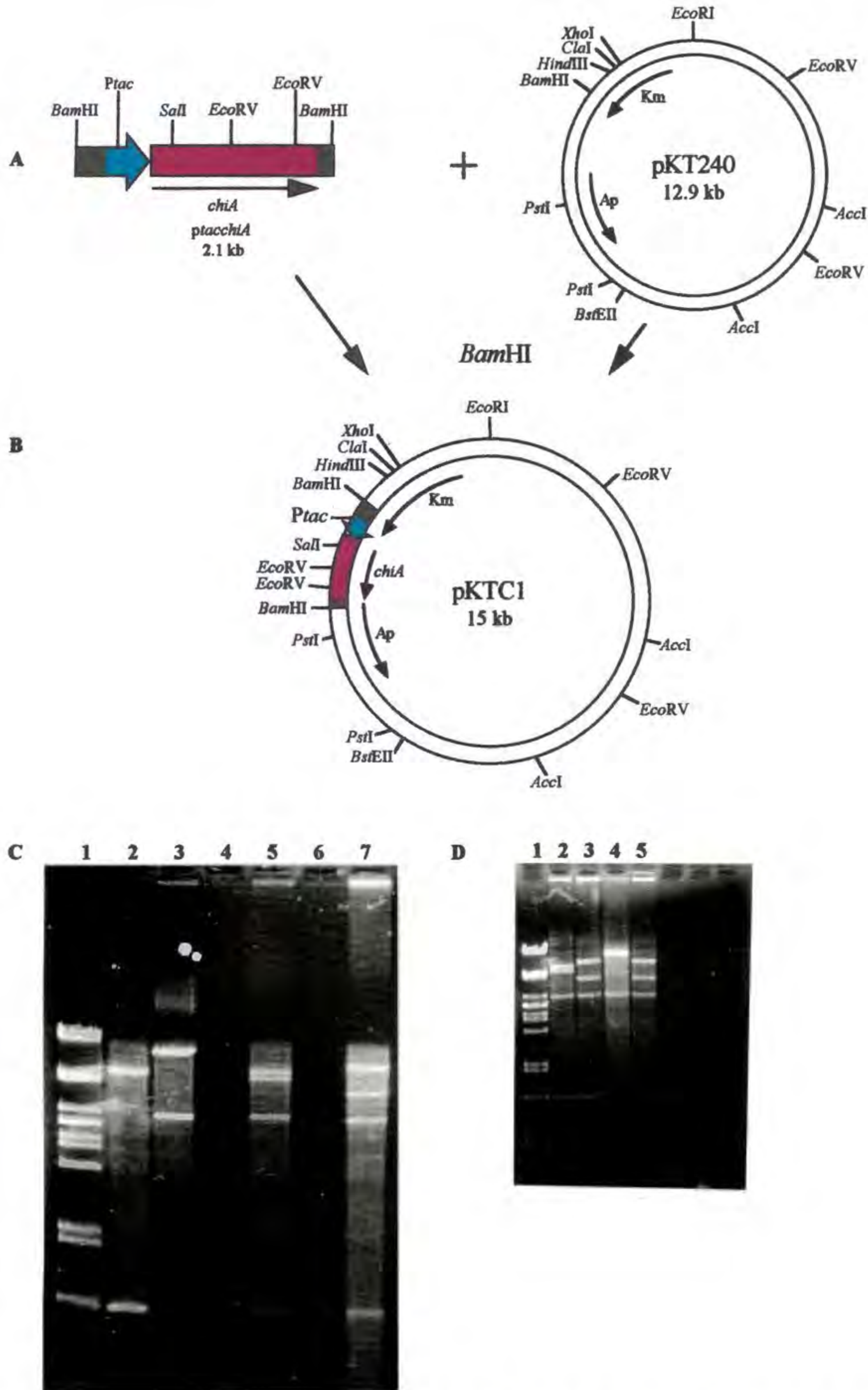

FIG. 2.1 Construction of pKTC1. Cloning of the 2.1-kb *Bam*HI fragment of p*tacchiA* into the *Bam*HI site of pKT240 resulted in pKTC1(A and B) in which the direction of transcription of the *tacchiA* cassette was in the same direction as that of the Km^R and Ap^R genes of the vector (lane 5, C), as was clone pKTC3 (lane 2, D). *Eco*RV cleaved the former to fragments of approximately 0.8 kb and 5.6 kb (lane 2, C) and the latter at two sites resulting in two fragments of ca. 2.5 kb and 10.4 kb (lane 3, C and 4, D). Restriction of pKTC1 (and pKTC3) with *Eco*RV resulted in 4 fragments of ca. 6.5, 5.5, 2.5 and 0.8 kb. Lanes 1 of both gels; λ *Pst*1 molecular weight markers.

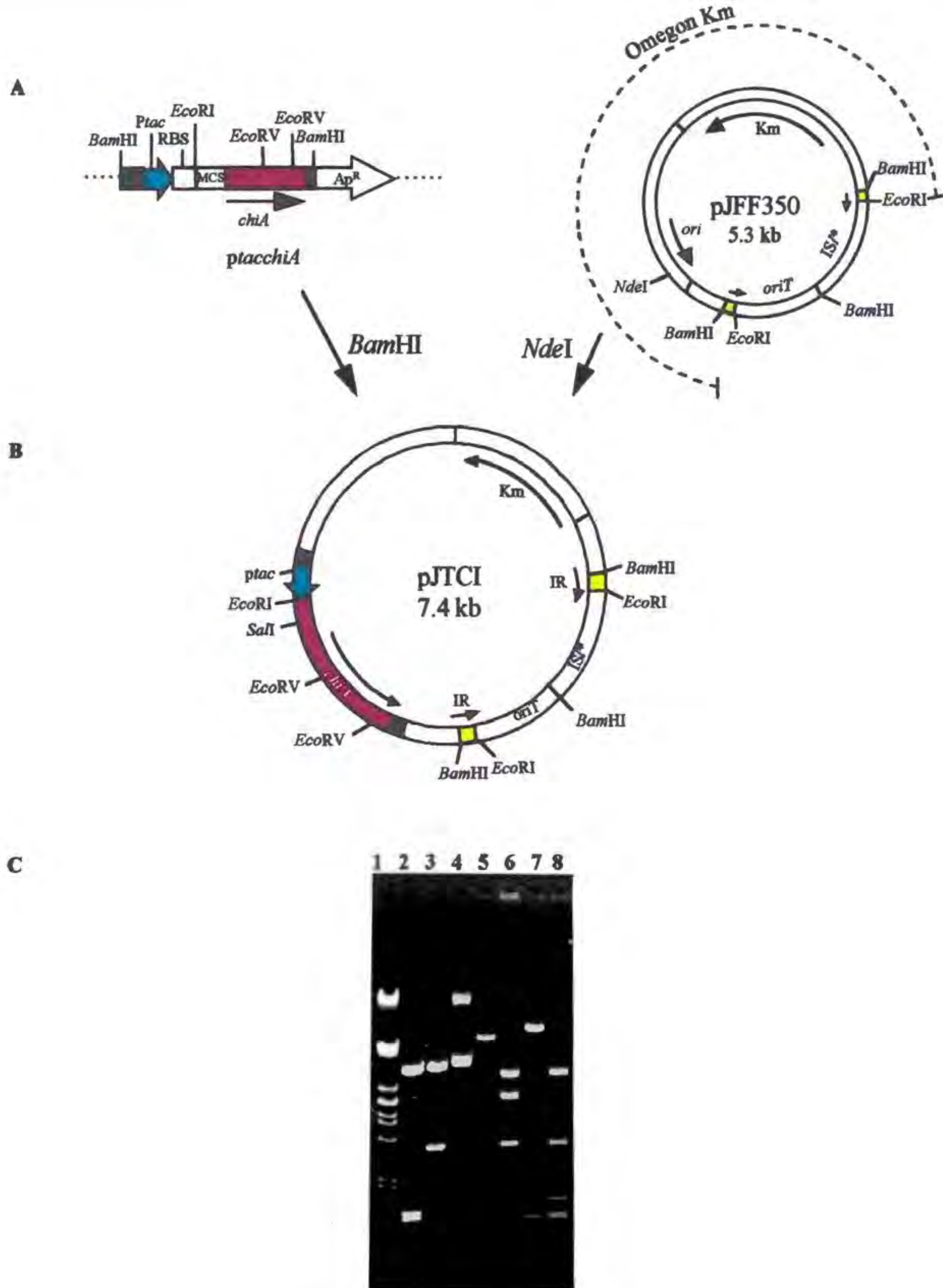

FIG. 2.2 Construction of pJTC1. The 2.1-kb *Bam*HI fragment of p*tacchiA* was cloned into the *Nde*I site of pJFF350 after the ends of both the insert and vector had been made blunt (A and B). The presence of the insert was confirmed by restriction analysis of pJTC1 with *Bam*HI (C) (lane 5). pJFF350 is cut three times resulting in fragments of ca. 3.7, 0.8 and 0.85 kb (lane 2). In pJTC1, a larger fragment of 5.8 replaced the 2.1-kb fragment. The *tacchiA* insert contains an *Eco*RI restriction site directly between the *tac* promoter and the *chiA* gene. Cutting pJTC1 with *Eco*RI resulted in three fragments of ca. 3.2, 2.5 and 1.6 kb (lane 6) in contrast to the two fragments of ca. 3.7 and 1.6 kb of pJFF350 (lane 3). Only the *chiA* gene is cleaved with *Eco*RV resulting in two fragments of pJTC1 of ca. 6.4 and 0.8 kb (lane 7). Restriction of pJTC1 with *Eco*RI and *Eco*RV, showed that the direction of transcription of the insert was the same as that of the Km^R gene (lane 8). Lane 1: λ *Pst*I molecular weight markers.

chitinase activity of 1160 units/ml for *E. coli* strain A7280 carrying the pUC19-derived plasmid pUXH2 carrying the *tacchiA* cassette from p*tacchiA* (500-700 copies per cell in *E. coli*; Sambrook *et al.*, 1989). When they introduced pUXH2 into *E. coli* strain A7279, which carries a *pcnB* mutation causing a reduction of plasmid copy number to 1-2 copies per cell, they reported chitinase activity of 262 units/ml. Comparison of the chitinase activity obtained in our assay (406 units/ml) with those obtained by Koby *et al.* (1994) (1160 and 262 units/ml) who had constructed p*tacchiA*, caused concern. This plasmid is pBR322-based, which has a copy number of 15-20 per cell in *E. coli.* In order to determine whether the differences in activity were due to a plasmid copy number effect, the 2.1-kb *Bam*HI fragment carrying the *tacchiA* cassette from p*tacchiA* was subcloned into the unique *Bam*HI site of plasmid pUC19. The resulting plasmid was called pUTC (pUC19*tacchiA*). The chitinase activity of *E. coli* JM105 (p*tacchiA*) and (pUTC) 24 hours after induction with IPTG was 392 and 406 Miller units respectively. The activity of *E. coli* (pUTC) assayed 4 hours after induction was 326 Miller units. This level of expression of the chitinase under our assay conditions was still lower than that reported for the pUC19-based plasmid pUXH2 of Koby *et al.* (1994). It was uncertain, therefore, whether chitinase expression of the original plasmid p*tacchiA* was optimal. This necessitated further investigation.

The level of expression of the *chiA* gene was further determined by SDS polyacrylamide gel electrophoresis. Samples of *E. coli* JM105 strains carrying the plasmids p*tacchi*A, pKTC1 and pJTC1 were prepared as described in section 2.2.6. Samples (1 ml) were removed prior to induction with IPTG and then 1, 2.5 and 4 hours after induction. As a control the plasmid pMAL-c (New England Biolabs; Ausubel *et al.*, 1994), a commercially available vector for the construction of fusion proteins, based on pKK223-3 was used. It has the maltose-binding protein (MBP), encoded by the *mal*E gene of *E. coli,* cloned downstream of the *tac* promoter from which this protein is overproduced resulting in a very abundant band visualized on an SDS polyacrylamide gel. The results (Fig. 2.3) showed the formation of a very intense protein band of 52 kDa for cultures carrying the pMAL-c plasmid 2.5 and 4 hours after induction (lanes 8 and 12) which is absent in the uninduced sample (lane 3). The *chiA* gene encodes a protein of 58 kDa (Shapira *et al.,* 1989) but no abundant protein band this size was observed in samples carrying the plasmids p*tacchiA* and pKTC1 1, 2.5 and 4 hours after induction (lanes 6, 7, 9, 10, 13, 14). Chet *et al.* (1993) had reported the formation of a new protein band of 58 kDa on a 10% SDS polyacrylamide gel produced by *E. coli* strains carrying the equivalent of p*tacchiA* (called pYZ223-3) and pYZ291. The latter plasmid is the broad host range plasmid pRK290 containing the *tacchiA* cassette from pYZ223-3 (Chet *et al.*, 1993; Sitrit *et al.*, 1993). Although the chitinase produced by induced cultures of *E. coli* carrying pYZ223-3 2.5 and 4 hours after growth was seen as a large band the difference in intensity, when compared with a protein band of the same size from the induced negative control (pKK223-3), was not as convincing as the difference in intensity between the 40-kDa protein of pMAL-c and the proteins of similar size produced by pKK223-3 that I observed. There did not appear to be any difference in the intensity of the 58-kDa protein band of induced and uninduced cultures of *E. coli* strains carrying the plasmid pYZ291 after 1, 2.5 and 4 hours growth nor between these

samples and the 58 kDa protein band produced by the negative control. These results confirmed my belief that *chiA* expression from the *tac* promoter in p*tacchiA* was not optimal which led to the construction of a new, improved *tacchiA* cassette by PCR amplification.

FIG. 2.3 Expression of the *chiA* gene from the *tac* promoter in *E. coli* strains. Cells were grown to midlog phase and induced with 0.3 mM IPTG. Samples (1 ml) were removed prior to induction and 1, 2.5 and 4 hours after induction, from which total protein was prepared and subjected to 10% SDS PAGE. The plasmids pKK223-3 and pMAL-c served as negative and positive controls respectively. Samples of uninduced cultures carrying pKK223-3, pMAL-c, p*tacchiA* and pKTC1 are represented in lanes 2-5 respectively. Lanes 6 and 7: *E. coli* (p*tacchiA*) and (pKTC1), 1h after induction. Lanes 8, 9, 10: *E. coli*(pMAL-c), (p*tacchiA*) and (pKTC1) respectively, 2.5h after induction. Lanes 11, 12, 13 and 14: *E. coli*(pKK223-3), (pMAL-c), (p*tacchiA*) and (pKTC1) respectively, 4h after induction.

2.3.4 PCR CLONING OF THE *chiA* GENE INTO THE EXPRESSION VECTOR pKK223-3

The results obtained with SDS-PAGE led to a review of the cloning strategy employed by Sitrit *et al.* (1993) and Koby *et al.* (1994). Analysis of the sequence of the *chiA* gene (Chet, personal communication) revealed that one of the *Hinf*I restriction sites that was used to isolate the 1.8-kb fragment from plasmid pLCHIA included part of the Shine Dalgarno (SD) sequence of the *chiA* gene. The sequence of the 5' region of the *chi*A gene upstream of the ATG start codon (dotted line) including the SD (solid line), and *Hinf*I site (bold, with arrow indicating cleavage point) is 5' AGG↓**AATC**AGTTATG 3'. Cleavage with this enzyme resulted in the destruction of the SD sequence of the gene. The 1.8-kb *Hinf*I fragment containing the *chiA* gene was cloned into the *Sma*I site of the plasmid pKK223-3. Although this plasmid contains a ribosome binding site (RBS), this can only be used for expression if the distance between the ATG start codon of the foreign DNA is within 5-9 bp of the *Eco*RI site (Kozak, 1983; Pharmacia Biotech). The efficiency of the SD interaction with the 16S rRNA is affected by the distance between the SD sequence and the ATG start codon, by the length of the complementarity between the SD sequence and the corresponding sequence of the 16S rRNA and by the extent to which the SD is masked by secondary structure. The spacing between the SD sequence and ATG codon is critical, with the average and optimal distance between the 3' end of the SD and the A of the ATG codon being seven nucleotides. A distance of more than nine or less than five nucleotides result in deficient translation. A deletion in the leader region of the tryptophan operon of *S. marcescens,* for example, that resulted in a distance of 11 bases, as opposed to 7 in the wild type, dropped *tpr*E expression to 53% (Stroynowski *et al.*, 1982).

In the plasmid p*tacchiA* the distance between the SD (solid line) sequence present on the plasmid and the ATG start codon (dotted line) of *chiA* (bold) was 20 bp, which was clearly too large for efficient translation (5' AGGAAACAGAATTCCC**AATCAGTTATG** 3'). The distance between the *chiA* gene's ATG start codon and its own SD sequence is 7 bp which is the optimal distance described by Kozak (1983). The decision was therefore made to reclone the *chiA* gene, with its own SD sequence but without its endogenous promoter, downstream of the *tac* promoter in plasmid pKK223-3 so that the requirements for efficient translation discussed above could be met. Since there were no suitable restriction endonuclease sites available, the gene had to be constructed by PCR from the plasmid pLCHIA. Primers of 28 nucleotides were designed from analysis of the sequence of the *chiA* gene (I. Chet, personal communication). The sequence of the forward primer was 5' **AAG↓AATTC**AAAGGAATCAGTTATGCGC 3'. This included the *Eco*RI restriction site (bold), the Shine Dalgarno sequence of *chiA* (solid line) and the ATG start codon of *chi*A (dotted line). The sequence of the reverse primer was 5' AAAG ↓**AATTC**TCGCCGTTAAAGACAGGAG 3' with the *Eco*RI restriction site in bold. The *Eco*RI restriction sites were included for the purpose of cloning the PCR product into the *Eco*R1 site of pKK223-3 by ligation of cohesive ends. The primers only had homology to each other at the 5' ends at the *Eco*RI sites. The forward primer ended with GC and the reverse primer with a G for increased efficiency of priming. The

predicted size of the PCR product was 1.87 kb. Using the intact plasmid pLCHIA as the template DNA and following the method described in section 2.2.5, a PCR product of ca. 1.8 kb as estimated on a 1% agarose gel was obtained. Initial attempts to clone the PCR product, restricted with *Eco*RI into the *Eco*RI site of pKK223-3 by ligation of compatible cohesive ends proved unsuccessful. It was suspected that the PCR product was not digested by *Eco*RI, despite the inclusion of a three base flanking spacer but this was not confirmed. A fault may have arisen during synthesis of the primers so that one or both of the *Eco*RI sites at the 5' termini were defective and thus would have prevented the cleavage of the product, but this was not confirmed by sequencing.

The cloning strategy of the second generation of *tacchiA*-carrying plasmids (pTC; p*tacchiA*) is outlined in Fig. 2.4 on pg 61. The PCR product purified from a 1% agarose TAE gel was polished with Klenow to remove the non-template nucleotide/s added to the ends by the terminal transferase activity of the *Taq* polymerase, generating blunt ends. It was successfully introduced into the *Eco*RI site of pKK223-3 by blunt end cloning. Screening of transformants on chitin containing plates yielded clones with clear halos. Restriction endonuclease mapping with *Bam*HI and *Sal*I showed that all contained inserts (Fig 2.4 c) and that the *chiA* gene was inserted in the correct orientation with respect to the direction of transcription of the *tac* promoter. This was expected as no activity would have been observed on the chitin containing plates if the gene had been in the opposite orientation.

2.3.5 CHITINASE EXPRESSION OF pTC CONSTRUCTS

The chitinase expression of the new pTC clones was determined 3 hours after induction. The level of expression of uninduced cultures carrying the original plasmid p*tacchiA* and the new second generation plasmids pTC17, pTC27 and pTC33 were 77, 608, 161 and 356 and of induced cultures 527, 2669, 1896 and 7345 Miller units respectively. The differences in the levels of expression of the three new clones could be explained by the fact that they grew to different cell densities after induction. The overproduced chitinase had a detrimental effect on the growth of induced *E. coli* (pTC27) and (pTC33) cells. This has been reported previously for constitutively expressed proteins from the *tac* promoter (De Boer *et al.*, 1982). Determination of chitinase expression based on the method of Miller *et al.* (1982) is highly variable. However, the data clearly illustrated that the distance between the SD sequence and ATG start codon was critical and that the shorter distance of 7 bases significantly increased the efficiency of translation of the *chiA* gene. The expression of the *chiA* gene from the *tac* promoter in *E. coli* JM105, which possesses a lacI^q gene, does not seem to be efficiently suppressed in this strain. Ge *et al.* (1990) also reported that the expression of a *cryIA(c)* gene from the *tac* promoter was not fully repressed in *E. coli* JM105.

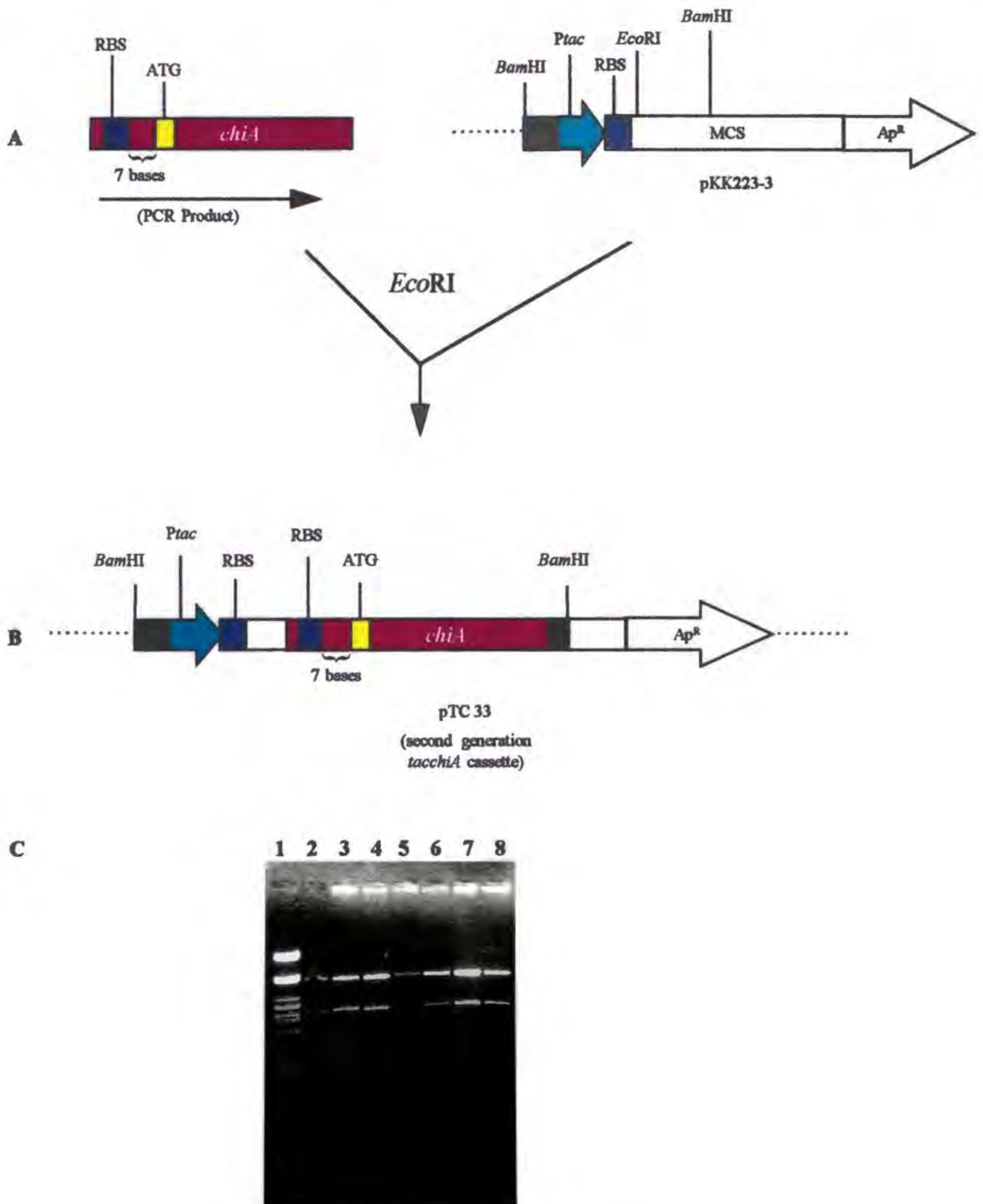

FIG. 2.4 Cloning of the PCR product of *chiA* under the control of the *tac* promoter in pKK223-3. The PCR product was cloned into the *Eco*RI site of pKK223-3 by blunt end cloning. In the second generation *tacchiA*-carrying vector (pTC) the distance between the gene's own SD sequence and the ATG codon is 7 bases (A and B). Clones were restricted with *Bam*HI resulting in two fragments of ca. 4.3 kb and 2.1 kb. The original plasmid p*tacchiA* restricted with *Bam*HI served as the positive control (lane 2) for the clones pTC11, 13, 17, 27, 29, 30 and 33 (lanes 3-8). Lane 1: λ *Pst*I molecular weight marker (C).

2.3.6 INTRODUCTION OF SECOND GENERATION *tacchiA* CASSETTE INTO pDER405, pML122 AND pJFF350

Since Koby *et al.* (1994) have reported the accumulation of mutants defective in *chiA* gene expression from the *tac* promoter during growth of *E. coli* cultures carrying the plasmid pUXH21, concerns that mutants might be generated during subsequent manipulations led to the decision to introduce the *tacchiA* cassette from pTC17, in addition to that from the apparently more highly expressed pTC33, into the vectors. No evidence of mutants defective in expression of the *chiA* gene was, however, observed.

Since it had been shown in stability assays that the plasmid pKTC1 was not stably maintained in *Pseudomonas fluorescens* and *Herbaspirillum seropedicae* (see section 3.3), it was decided not to introduce the improved second generation *tacchiA* cassette into the vector pKT240 and to consider the alternative broad host range plasmids pDER405 and pML122 that are capable of replicating in a number of Gram negative bacteria (Rawlings *et al.*, 1986; Labes *et al.*, 1990).

The plasmid pDER405 was shown to be stably maintained in *P. fluorescens* over at least 100 generations (Herrera, 1994). It originated from the broad host range, highly mobilizable plasmid pTF-FC2, isolated from a South African strain of *Thiobacillus ferrooxidans* FC (Rawlings *et al.*, 1986). Considering its uniqueness, broad host range and stability in *P. fluorescens*, this vector was selected for subsequent cloning experiments. The 2.1-kb *Bam*HI fragment containing the *tacchiA* cassette from the plasmids pTC17 and pTC33 were cloned separately into the unique *Bam*HI site of pDER405 (Appendix D). Transformants were screened on chitin containing plates supplemented with 25 µg/ml Cm and two, pDTC17 and pDTC33 (pDER405*tacchiA*), were selected for further work.

I did not have access to the plasmid pML122 (Appendix D) but to pBS42, which has the *B. thuringiensis cryIIIA* gene cloned in the MCS of pML122 with no other modifications (Baldani, personal communication). This gene was removed from pBS42 by cutting with *Eco*RI and gel-purifying the largest fragment representing pML122. The 2.1-kb *Bam*HI fragment of pTC33 was made blunt and cloned into this modified vector. The presence of an insert and its orientation were determined by restriction endonuclease analysis with *Sal*I. One of these clones, pMTC 33 (pML122*tacchiA*), was selected for further study.

The cloning strategy for the introduction of the improved second generation *tacchiA* cassette from pTC17 and pTC33 into the integration vector, pJFF350, is principally the same as outlined in Fig. 2.2 (A). Initial screening of transformants on chitin containing plates spread with 1 mM IPTG before patching of colonies resulted in very few (2 out of 100) colonies with clear zones whereas transformants on plates without IPTG gave many more colonies that expressed and secreted chitinase. The *lac*I^{q} gene of *E. coli* JM105 did not

fully repress the *tac* promoter as reported previously (Ge *et al.*, 1990). These results indicated that induction of the *tac* promoter with IPTG affected the expression of the *chiA* gene possibly due to a mutation(s) formed within the *tac* promoter and/or the *chiA* gene causing mutants defective in *chiA* gene expression as reported previously (Koby *et al.*, 1994). The growth of the colonies on these plates did not appear to be affected. Restriction enzyme analysis confirmed the presence and orientation of the *tacchiA* cassette as the same as the direction of transcription of the Km^R gene of pJTC17 and pJTC33 (pJFF350*tacchiA*).

2.3.7 *chiA* GENE EXPRESSION

The increased efficiency of the new second generation *tacchiA* cassettes was shown in chitinase assays (Table 2.3.1) and confirmed by SDS-PAGE analysis (results not shown).

TABLE 2.3.1 Chitinase activity of new *tacchiA* constructs in *E. coli* JM105

	INDUCED
	MILLER UNITS
pTC17	2669
pTC33	7345
pKTC1[a]	113
pDTC17	704
pDTC33	602
pMTC33	1772
pJTC1[a]	906
pJTC17	4190
pJTC33	6416

[a] original *tacchiA* cassette; all others contain the second generation cassette.

pTC: p*tacchiA* (new); pKTC: pKT240*tacchiA* (original); pDTC: pDER405*tacchiA*; pMTC: pML122*tacchiA*; pJTC1: pJFF350*tacchiA* (original); pJTC17/33: pJFF350*tacchiA* (new)

The low levels of expression of pDTC17 and pDTC33 were probably due to the large size of the plasmids (17.6 kb) and the lower copy numbers compared with pBR322-based vectors such as the pTC and pJTC plasmids (Rawlings *et al.*, 1986; Sambrook *et al.*, 1989). Nevertheless, these levels were greater than those seen with pKTC1 carrying the original *tacchiA* cassette, despite the fact that this plasmid probably has a greater copy number than the pDTC plasmids (Bagdasarian *et al.*, 1993; Rawlings *et al.*, 1986). This reflects the increased efficiency of the new cassette. The possibility of *chi*A expression from both the *tac*

and Nm[R] promoters in the pML122-derived constructs was considered. However chitinase expression levels were comparable for inserts in both orientations indicating that the gene was expressed from the *tac* promoter only.

The specific activity of cultures carrying the plasmids described above was determined (Table 2.3.2). The trend of these results is similar to that presented in Table 2.3.1. These confirmed the fact that the new *tacchiA* cassette resulted in higher expression than the original cassette. The highest activity was observed in the extracellular fractions which is in agreement with the results reported by Oppenheim *et al.* (1990) for chitinase expression from heat-induced cultures of *E. coli* carrying the plasmid pLCHIA. They reported a time-dependent increase with over 80% of activity found in the growth medium after 6 h. The authors suggested that either the chitinase is secreted from the cytoplasm via specific pores or via pores made during the induction step or alternatively, the leader sequence present at the amino-terminal end directs the chitinase across the periplasmic membrane and is subsequently removed.

The finding that uninduced cultures carrying pJTC33 produced higher level of enzyme was unexpected. The induced cultures appeared to grow more slowly than the uninduced cultures and had a considerably lower protein concentration as determined by the Bradford assay. This effect has been reported previously in the literature (Brosius *et al.*, 1984; Oeda *et al.*,1987). The possible reasons for the seemingly detrimental effect on induced cells carrying pJTC33 are discussed in section 2.3.10 with reference to the *cryIA(c)* constructs. The reason for the high level of expression in uninduced cultures carrying pTC33 is not known.

TABLE 2.3.2 Specific chitinase activity[†] of *chiA* constructs in *E. coli* JM105

	EXTRACELLULAR		CELLULAR		TOTAL	
	UNINDUCED	INDUCED	UNINDUCED	INDUCED	UNINDUCED	INDUCED
pKK223-3	0	0	0	0	0	0
p*tacchiA*[a]	0.35	91.3	9.2	101.6	9.55	192.9
pTC33	5.4	3000	60	8.8	65.4	3008.8
pKTC1[a]	0.133	12.56	0.3	1.5	0.433	14.06
pDTC	0.45	221.7	0.76	26	1.21	247.7
pMTC	1.47	360	1.36	26.6	2.83	386.6
pJTC1[a]	1.9	3667	37	2250	38.9	5917
pJTC33	5294	4200	2264	1935	7558	6135

[†]µmol/h/mg protein [a] original *tacchiA* cassette. All other constructs have the second generation cassette. See footnote to Table 2.3.1 for definition of constructs.

2.3.8 INTRODUCTION OF THE *cryIA(c)* GENE OF *B. thuringiensis* ISOLATE 234 INTO EXPRESSION, BROAD HOST RANGE AND INTEGRATION VECTORS

In order to improve the expression of the *cryIA(c)* gene, known as *tox*, from pGH37D-1 for subsequent use in biocontrol, it was cloned into the plasmid pKK223-3 for expression under the control of the *tac* promoter. The 3.7-kb *Nde*I fragment of pGH37D-1, carrying the *tox* gene under the control of its own promoter, was cloned into the *Sma*I site of pKK223-3. The resulting construct was called p*tactox* (Fig. 2.5, next pg). Difficulties with restriction of pGH37D-1 to completion with *Nde*I were encountered and as no convenient screening procedure was available, transformants had to be screened by restriction endonuclease mapping. *Xho*I was used to identify clones with inserts and *Pvu*II to determine the orientation of the gene with respect to the *tac* promoter. Out of a total of 58 colonies screened 7 were in the correct orientation. One of these was selected for further subcloning.

The 4-kb *Bam*HI fragment of p*tactox* was cloned into the BamH1 site of the broad host range plasmids pKT240 and pDER405 resulting in the plasmids pKTT (pKT240*tactox*) and pDTT(pDER405*tactox*) (Fig 2.5, next pg). The orientation of pKTT was not determined. In plasmid pDTT the orientation of the *tactox* cassette was the same as that of the Cm^R gene, as determined by restriction mapping with *Eco*RI, which cuts three times in the *tactox* cassette and twice in the vector.

For cloning into the integration vector, the *Bam*HI *tactox* fragment was made blunt and cloned into the blunted *Nde*I site of pJFF350 (Fig. 2.6, pg 66). The presence of the insert and orientation with respect to the Km^R gene was determined by cleavage with *Bam*HI and *Eco*RI. Out of 36 colonies, 27 contained inserts which occurred in both orientations with respect to the direction of transcription of the Km^R gene. One clone with the cassette in the same orientation as the Km^R gene was chosen for further work.

The plasmid pKTC1 carrying the original *tacchiA* cassette was shown to be highly unstable in *H. seropedicae* and *P. fluorescens* and the highly expressed *tox* gene on pKTT in the latter strain had a detrimental effect on these cells (see section 4.3). It was therefore decided to clone the *tox* gene and the *tactox* cassette separately into pML122 as this plasmid was shown to be stably maintained in *Herbaspirillum* spp. (Baldani, personal communication). The 3.7-kb blunted *Nde*I fragment of pGH37D-1 was cloned into the blunted *Eco*RI site of the vector and the resulting plasmid was named pMT7. Similarly, the blunt ended 4.0-kb fragment of p*tactox* was cloned into the blunt ended *Eco*RI site of the vector resulting in the plasmid pMTT11. This plasmid was sequenced using primers designed from the DNA sequence of the *tac* promoter (forward primer 5' GACAATTAATCATCGGCTCG 3') and the *tox* gene (reverse primer 5' GACAATTAATCATCGGCTCG 3') which were labelled using the Cy5™-dATP labelling mix kit (Pharmacia Biotech) according to the manufacturer's instructions. DNA sequence analysis revealed that

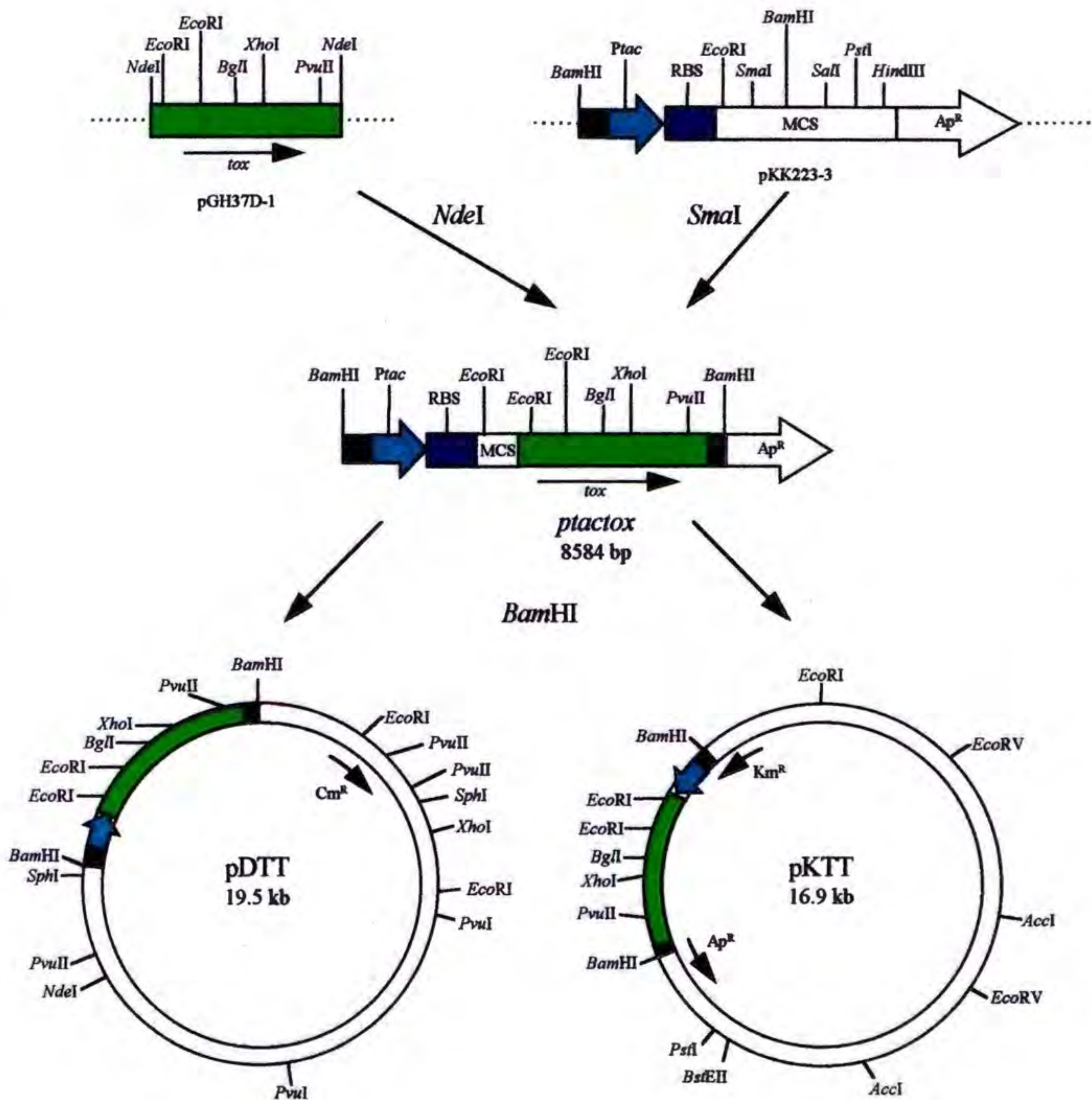

FIG. 2.5 Construction of p*tactox*, pDTT and pKTT. The 3.7-*Nde*I fragment of pGH37D-1 was made blunt and cloned into *Sma*I site of pKK223-3 to generate the plasmid p*tactox*. The 4.0-kb *Bam*HI fragment of p*tactox* was cloned independently into the *Bam*HI sites of pDER405 and pKT240, resulting in the plasmids pDTT and pKTT.

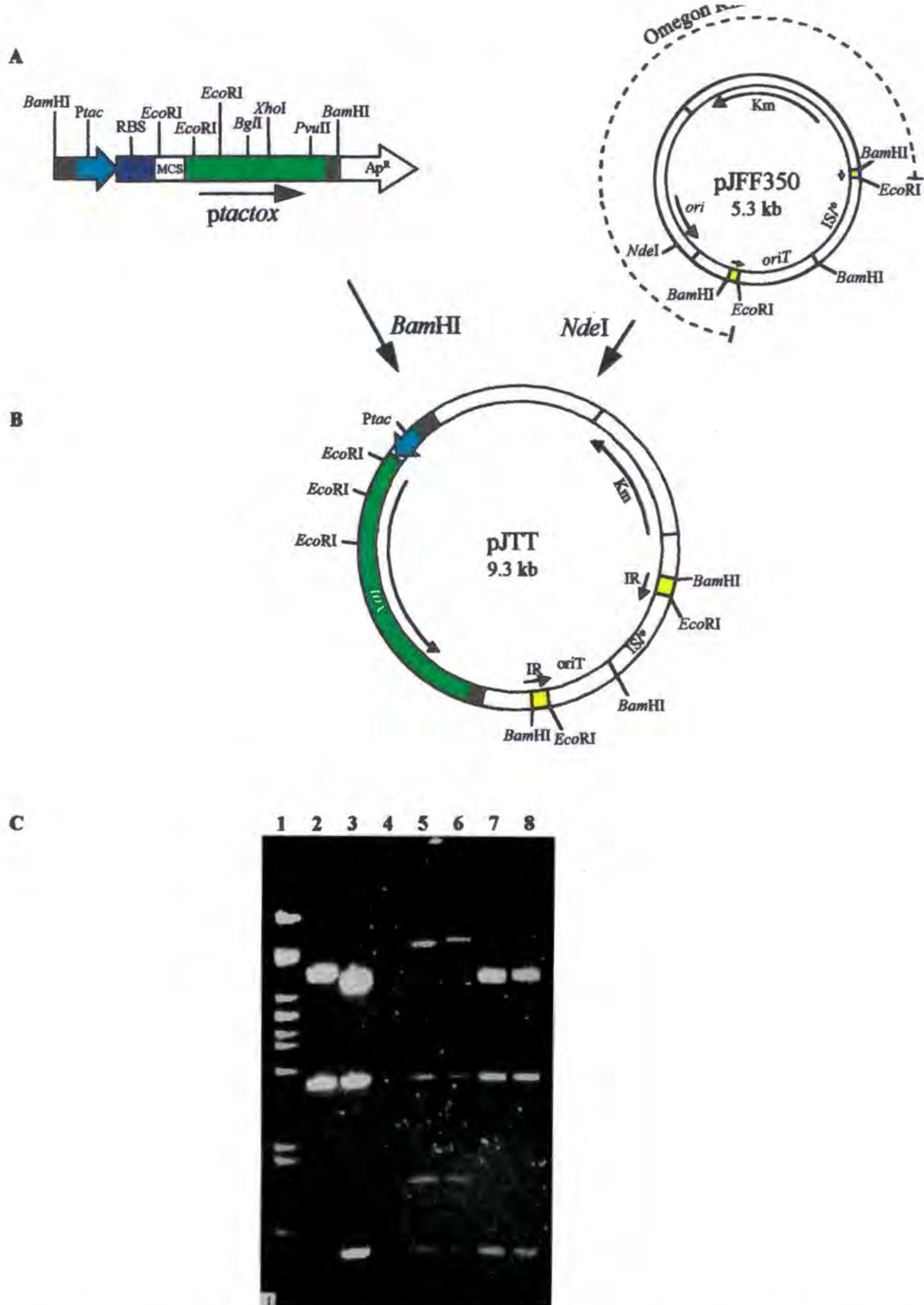

FIG. 2.6 Cloning of the *tactox* cassette from p*tactox* into the integration vector pJFF350. The 4-kb *Bam*HI fragment of p*tactox* was made blunt and cloned into the unique *Nde*I site, made blunt, of pJFF350 resulting in the plasmid pJTT. The orientation of the insert in this and other clones was determined by restriction of DNA with *Eco*RI, which cleaves the vector twice and the *tactox* cassette three times. The insert in the same orientation as the Km^R gene resulted in 5 fragments of ca. 3.5, 3.3, 1.6, 0.7 and 0.2 kb (last band not shown) as estimated from 1% agarose gel (lanes 5 and 6). The insert in pJTT was in this orientation (lane 5). The insert in the opposite orientation resulted in 5 fragments of 5.8, 1.6, 1.0, 0.7 and 0.2 kb (last band not shown) as estimated from 1% agarose gel (lanes 3, 7 and 8). Lanes 1, λ *Pst*I molecular weight marker; 2, pJFF350 cut with *Eco*RI ; 4, empty.

pMTT11 did not contain the *tac* promoter. Instead the *tox* gene, without any modifications, was present in the opposite orientation to the Nm promoter. A deletion of the entire *tac* promoter had occurred by an unknown mechanism. This plasmid was therefore renamed pMT11.

2.3.9 CRYIA(c) GENE EXPRESSION

The expression of the *tox* gene in *E. coli* JM105 was determined by Western blot analysis (Fig 2.7).

From these results it is clearly evident that the *cryIA(c)* gene is expressed from the *tac* promoter in p*tactox* at levels considerably greater than from its own promoter (lanes 3 and 4 compared with lanes 1 and 2). This apparent constitutive expression of *cry* genes from the *tac* promoter was also reported by Oeda *et al.* (1987) and Ge *et al.* (1990) in JM101, JM103 and JM105. It was suggested that the *lac*I^q gene or its product was not functional in these cases or that there were IPTG-like molecules present in the growth media which competitively bound to the repressor (Ge *et al.*, 1990). These authors also reported that the level of expression was best in JM103 and that long incubation of cultures, up to 60 h of growth, resulted in increased amount of toxin. This was due to the fact that *E. coli* cells do not lyse during stationary phase and therefore have more time to synthesize protein. Maximum expression levels in JM103 were obtained after 48 h of growth. Our results showed that the level of expression was greater after 24 and 48 h of *E. coli* JM105 (p*tactox)* as compared with that after 3 h of growth, but little difference could be detected between cultures grown for 24 and 48 h on SDS PAGE (results not shown). Cultures were therefore induced for 24 h and then examined by electrophoresis and immunoblotting. The constructs were not tested in JM103.

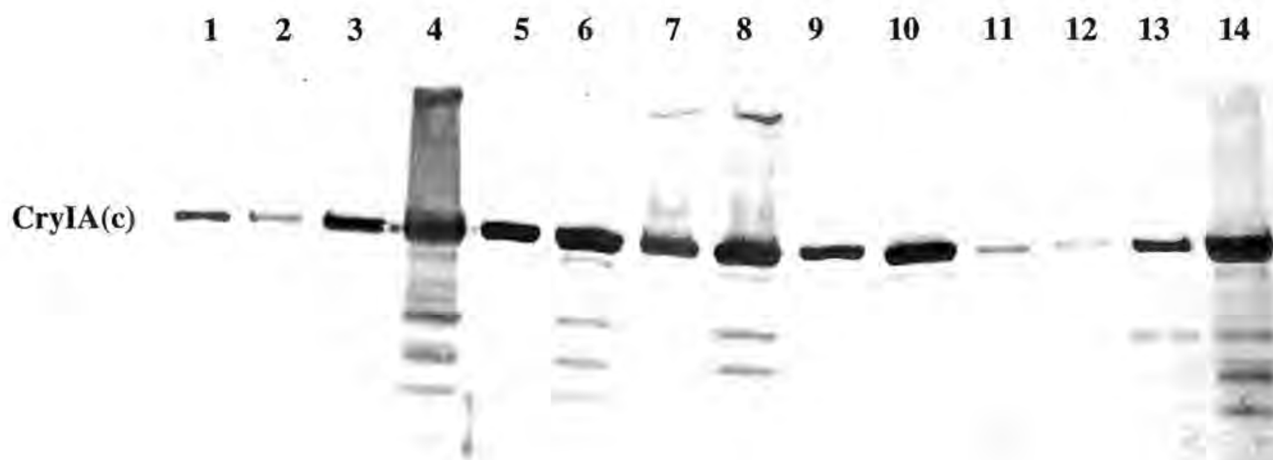

FIG. 2.7 Western blot analysis of *cryIA(c)* expression in *E. coli* JM105 from uninduced (U) and 24 h-induced (I) samples. Lanes 1 and 2, pGH37D-1 U, I; 3 and 4, p*tactox* U, I; 5 and 6, pKTT U, I; 7 and 8, pDTT U, I; 9 and 10, pMT7 U, I; 11 and 12, pMT11U, I; 13 and 14, pJTT I, U.

The ratio of Tox to total protein by ELISA was not determined. Others (Ge *et al.*, 1990) have reported that, while expression of the *cryIA(c)* 73 gene from its own promoter in *E. coli* amounted to 0.24% of total cellular protein, expression under the control of part of its own promoter plus the *tac* promoter in pKK223-3 led, after 48 h of induction, to about 50% of total cellular protein being present as CryIA(c) 73. In another system, expression of a *B. thuringiensis* subsp. *aizawai* 130-kDa protein gene from the *tac* promoter in *E. coli* was 38% of the total cellular protein after 20h induction, compared to 3% of total cellular protein from its own promoter (Oeda *et al.*, 1987).

The level of expression of the *cryIA(c)* under the control of the *tac* promoter in pJTT was higher under uninduced conditions than induced conditions (Fig 2.7, lane 14 compared to lane 13). This was not observed for any of the other constructs, where expression of induced cultures was always greater than uninduced cultures. The growth of induced pJTT cultures was retarded compared to that of uninduced cultures, regardless of the orientation of the *tactox* cassette with respect to that of the Km^R gene of Omegon-Km. Oeda *et al.* (1987) reported a similar observation for the 130-kDa protein gene in a plasmid based on pKK223-3, as did Brosius (1984) in induced cultures expressing the rat insulin gene from the *tac* promoter. Interestingly this was not observed with *E. coli* (p*tactox*) cultures (pKK223-3 carrying the *tactox* cassette). Both pJTT and p*tactox* are derivatives of pBR322, which has 5 major transcriptional units (Stüber and Bujard, 1981). A possible explanation for the above results could be the difference in the transcriptional terminators of pJFF350 and pKK223-3. Expression of genes from efficient promoters interferes with the plasmid's essential functions such as replication (Stüber and Bujard, 1981). This was observed by Gentz *et al.* (1981) when they attempted to clone bacteriophage T5 promoters in pBR322 without the presence of strong termination signals downstream. This destabilization is presumably due to the detrimental overexpression of one or more proteins on the plasmid, resulting in very slow growth of cultures (Brosius, 1984). In pKK223-3 (and p*tactox*), the strong *rrnB* transcription terminators T_1 and T_2 are distal to the *tac* promoter which stabilizes this vector host system by inhibiting read through from the *tac* promoter in the parent strain (Brosius, 1984). In pJFF350 (and pJTT) the transcription terminator from bacteriophage T4 gene 32 is carried in short inverted repeats which are symmetrically placed in the Ω fragment. It is possible that this terminator is inefficient for the termination of the *cryIA(c)* gene in pJTT so that slow growth of IPTG-inducted cultures results. The terminators of p*tactox* are more efficient and this effect is not seen.

No strong terminators are present in the broad host range plasmids pKTT and pDTT. Here the level of expression of *cryIA(c)* is lower than that of p*tactox* and pJTT, presumably due to the large size and low copy number of these plasmids, and this has a less detrimental effect on the cells.

CHAPTER 3

INTRODUCTION OF THE CLONED *Serratia marcescens chi*A GENE INTO ENDOPHYTIC BACTERIA FOR THE BIOCONTROL OF PHYTOPATHOGENIC FUNGI

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CHAPTER 3

INTRODUCTION OF THE CLONED *Serratia marcescens chi*A GENE INTO ENDOPHYTIC BACTERIA FOR THE BIOCONTROL OF PHYTOPATHOGENIC FUNGI

3.1 INTRODUCTION

Antagonism of fungal plant pathogens by microorganisms, especially fluorescent Pseudomonads, plays a major role in biological disease control (Schippers *et al.*,1987; Weller, 1988; Hill *et al.*, 1994). Species of *Pseudomonas,* especially *P. fluorescens* and *P. putida,* found in the plant rhizosphere have been most extensively studied as biocontrol agents (Davison, 1988; Jayaswal *et al.*, 1993). Antagonism by these bacteria may involve competition for nutrients or niches or the production of siderophores that chelate iron from the environment and make it unavailable to fungal and bacterial phytopathogens (Davison, 1988). In addition these microorganisms are capable of producing antibiotics, other secondary metabolites and enzymes, such as pyrrolnitrin, oomycin A, pyoluteorin, cyanide and chitinase which suppress disease (Gaffney *et al.*, 1994; Schnider *et al.*, 1995; Chernin *et al.*, 1996). The efficacy of chitinase - producing bacteria from the genera *Aeromonas* (Inbar and Chet, 1991b), *Serratia* (Ordentlich *et al.*, 1988) and *Enterobacter* (Chernin *et al.*, 1995) as biocontrol agents has been shown. A genetically modified strain of *P. fluorescens* carrying the *chiA* gene from *S. marcescens* suppressed disease caused by the plant pathogenic fungus *Rhizoctonia solani* (Koby *et al.*, 1994). Similarly, genetically modified *Rhizobium meliloti* and *P. putida* expressing the *chiA* gene under the control of the *tac* promoter had antifungal activity against *R. solani* and *Sclerotium rolfsii* (Chet *et al.*, 1993; Sitrit *et al.*, 1993). These examples were discussed in Chapter 1.

The use of endophytic bacteria for the control of plant pathogens and pests was discussed in Chapter 1. Many different endophytes have been isolated from the interior regions of healthy plants with *Pseudomonas* spp., specifically fluorescent Pseudomonads, being commonly encountered (Mundt and Hollis, 1976; Gardner *et al.*, 1982; Jacobs *et al.*, 1985; Gagné *et al.*, 1987; Mishagi and Donndlinger, 1990; Döbreiner *et al.*, 1993). Their population size, incidence, distribution and location within the interior of plants have been summarized in Chapter 1. The literature contains few reports on the use of endophytic bacteria as biocontrol agents with the only account of an endophytic bacterium being genetically engineered for this purpose being that of *Clavibacter xyli* subsp. *cynodontis* which carries a δ-endotoxin *cry1A(c)* gene for the control of the European corn borer (Turner *et al.*, 1991). This organism was discussed in Chapter 1.

The effectiveness of chitinases against phytopathogenic fungi, the advantages of endophytic bacteria as biocontrol agents and the lack of reports in the literature of endophytes genetically modified to express

chitinase genes, prompted the investigations described in this chapter. The aim of this work was to introduce the *S. marcescens chiA* gene under the control of the *tac* promoter into Gram negative endophytic bacteria from commercially important plants and to test their effectiveness as biocontrol agents against phytopathogenic fungi. These endophytes included strains of *P. fluorescens, Citrobacter* sp. and *H. seropedicae*. A model system using beans as the host plant and *R. solani* isolated from bean as the pathogen was used to determine whether *P. fluorescens* isolates expressing the *chiA* gene could reduce the severity of this fungal disease. This model was considered as one of several that could be used to analyse the effectiveness of genetically modified endophytic bacteria as biocontrol agents. The advantages of this system included the easy introduction of the representative endophyte, *P. fluorescens* Rif1, via the roots of germinated seeds and the short period of the assay due to the infectivity of *R. solani*. This fungus is the main cause of damping-off disease in bean which rapidly colonizes the plant and reaches a peak within the first 2-4 days of incubation (Sneh *et al.*, 1966). Initially broad host range plasmids were used to express the 1st and 2nd generation *tacchiA* cassettes in these endophytes. It had been reported that plasmids expressing *chiA* at high levels were unstable in *Pseudomonas* (Chet *et al.*, 1993; Koby *et al.*, 1994) and I also observed this. It was therefore decided to integrate the *tacchiA* cassette into the chromosome of these bacteria to achieve stability and to prevent horizontal transfer of the gene. The effectiveness of these modified microorganisms against the phytopathogenic fungi *R. solani, Fusarium moniliforme* and *F. nygamai* was tested.

3.2 MATERIALS AND METHODS

3.2.1 CULTURES, PLASMIDS AND GROWTH CONDITIONS

Bacterial and fungal strains and plasmids used are described in Appendices C and D. The *P. fluorescens* rhizosphere strains A7282 and A7283, containing the Tn7-Km^R-P_{tac}*chiA* transposon, were provided by Prof Amos Oppenheim of the Hebrew University-Hadassar Medical School, Israel and *H. seropedicae* HRC54 was a gift from Dr Johanna Döbreiner, EMBRAPA, Brazil. An endophytic strain isolated from pine pollen and tentatively classified by fatty acid analysis and API 20 E identification system (BioMerieux, France) as *Citrobacter* sp. (AJ1), was a gift from Dr Shlomo Pleban of the Otto Warburg Centre for Agricultural Biotechnology, the Hebrew University, Israel. The fungal isolates *Fusarium nyagamai*, MRC 5993, and *F. moniliforme* Mating Population F, MRC 6148, from sorghum were supplied by Prof Wally Marasas, the leader of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the South African Medical Research Council. The fungus *R. solani* was isolated from beans and supplied by the Plant Protection Research Institute of the Agricultural Research Council in Pretoria.

E. coli strains were cultured and maintained as described in section 2.2.1. Potential endophytes were isolated on nutrient agar (NA) and Kings B (KB) plates (King *et al.*, 1954) and grown in Luria broth (LB) (Sambrook *et al.*, 1989). *P. fluorescens* strains and AJ1 were grown on Luria agar (LA), LB or KB medium supplemented with Rif (100 μg/ml) and Km (100 μg/ml). *H. seropedicae* HRC54 was grown in JNFb solid, semisolid, or liquid media (Appendix A) supplemented with the appropriate antibiotic. Fungi were grown on Potato Dextrose agar (PDA) or in Potato Dextrose Broth (PDB; Difco Laboratories). All growth was at 30°C.

P. fluorescens strains were maintained in 0.1 M $MgSO_4$ at 4°C. *H. seropedicae* and all other bacterial strains were stored at -70°C in liquid JNFb or LB supplemented with 10% glycerol. Fungi were maintained at room temperature on PDA slants covered with mineral oil.

3.2.2 ISOLATION AND CLASSIFICATION OF ENDOPHYTIC BACTERIA

Endophytic bacteria were isolated from micropropagated plantlets obtained from plant tissue culture laboratories located in Cape Town and Stellenbosch. These included apricot, pear, apple, *Scadoxis* sp. and Black Stinkwood (*Ocotea bullata)*. Fluorescent bacteria where identified on KB medium using a 345 nm ultraviolet transilluminator. All Gram negative bacteria were further identified using the API 20 NE identification (BioMerieux, France) according to the manufacturers' instructions.

Endophytes were isolated from bean seeds obtained from a local supermarket and a cooperative specializing in the growth of kidney beans. Seeds which appeared to be in a healthy condition and without any visible lesions on the seed coat were selected. They were washed in tap water containing a few drops of detergent and rinsed well, immersed in 70% EtOH for 60 seconds, incubated in 1.5% sodium hypochlorite containing 0.01% Tween 20 for 15 minutes with shaking at room temperature, and then washed three times with sterile water. The seed coats were then aseptically removed, and the seeds surface sterilized in 70% EtOH and 1.5% sodium hypochlorite as above. The effectiveness of these procedures was determined by placing sterilized beans onto the surface of NA plates, incubating at 30°C for 48 hours and observing for bacterial growth. The seeds were further treated in one of the following ways. Whole seeds were placed in LB or NB and incubated with shaking at 30°C for 24-48 h until medium was turbid. Serial dilutions of these cultures were made and plated onto LA, NA or KB solid media and grown at 30°C. Single colonies were initially classified according to morphology, colour and ability to fluoresce on KB medium. A representative from each group was identified by Gram staining and all Gram negative bacteria were further identified using the API 20 NE kit. Alternatively seeds were macerated using a sterile pestle and mortar in 1 ml saline, plated undiluted onto LA, NA and KB medium and grown at 30°C. Resulting colonies were Gram stained and identified as before. Finally, interior sections of the cotyledons and the embryo were aseptically dissected

and placed onto NA solid media and grown at 30°C. Bacteria were streaked to single colonies from growth occuring on the surface of the NA plates around the edges of the plant tissue and classified as described.

3.2.3 SPONTANEOUS ANTIBIOTIC RESISTANT MUTANTS

Antibiotic gradient plates containing Rifampicin (Rif) or nalidixic acid (Nal; for *Herbaspirillum* species only) were used for the selection of spontaneous antibiotic resistant mutants. Cultures were grown from single colonies in LB overnight at 30°C with shaking and 100 µl plated onto gradient plates. These were then incubated at 30°C for 24-48 h. Colonies growing closest to the highest antibiotic concentration were streaked onto LA plates containing this concentration of antibiotic. In this way the antibiotic resistant mutants *P. fluorescens* Rif1, *H. seropediace* Nal1 and AJ1 Rif1 were generated.

3.2.4 INTRODUCTION OF ENDOPHYTES INTO BEAN SEEDS

Healthy been seeds without lesions of the seed coat were shaken in 70% ethanol for 60 seconds and washed 3 times with tap water to remove any contaminating fungi which would affect germination. They were then germinated in sterile vermiculite for 3-4 days. The root tips were removed and the seedlings placed into a petri dish as described by Pleban *et al.* (1995). Only the roots of the seedlings were in contact with the antibiotic resistant endophyte of interest at a concentration of 10^9 colony forming units (CFU)/ml in water for 24 h with gentle shaking. Seedlings dipped into water served as controls.

3.2.5 REISOLATION OF ENDOPHYTES FROM BEAN PLANTS

Bean seedlings, with or without introduced endophytes were planted in boxes (23 cm x 16 cm x 6 cm; 6 seedlings per box) containing baked soil. After 3-12; 21 and 30 days of incubation, plants were removed and washed thoroughly with tap water. Each plant was measured and dissected into root and stem, weighed and placed separately into sterile glass bottles. The plant sections were surface sterilized with 70% EtOH for 90 seconds, shaken in 1.5% sodium hypochlorite with 0.01% Tween 20 for 15 minutes and rinsed three times with sterile distilled water. They were then macerated individually in 2 ml saline or 0.1 M $MgSO_4$ in a sterile pestle and mortar and plated, undiluted, onto LA plates supplemented with Rif (100 µg/ml). Plates were incubated at 30°C and the number of CFU per gram fresh weight of plant material was calculated.

3.2.6 BACTERIAL TRANSFORMATION BY ELECTROPORATION

The integration vectors pJTC1 and pJTC33 (pJFF350*taccchiA*) carrying the 1st and 2nd generation *tacchiA* cassettes,) on pJFF350 were electroporated into *P. fluorescens* Rif1 using a modification of the method of Waalwijk *et al.* (1991). One ml of a stationary phase culture was inoculated into 100 ml LB and grown at

30°C with shaking until the cells had reached mid-exponential phase (OD_{650} between 0.7 and 0.9). The cells were harvested by centrifuging, the pellet washed three times in volumes of 100, 50 and 4 ml cold 300 mM sucrose, finally resuspended in 1 ml cold 300 mM sucrose and placed on ice. Electroporation was carried out using a Bio-Rad Gene Pulser set at 25 μF and 2.25 kV in conjunction with a Pulse Controller set at 200 ohm. In an ice-cold eppendorf tube, 40 μl of cells were mixed with 1-5 μl (1-3 μg) DNA immediately before electroporation and transferred to a cold 0.2 cm electroporation cuvette. Immediately after delivery of the high-voltage pulses, 1 ml LB was added for phenotypic expression at 30°C for 2 hours. The cells were plated undiluted onto LA plates supplemented with Rif (100 μg/ml) and Km (100 μg/ml) and grown at 30°C.

Electroporation of *H. seropedicae* HRC54 and Nal1 with the broad host range vectors pKT240, pDER405 and the integration vector pJFF350 and their derivatives pKTC1, pJTC1, pDTC33, pMTC33, and pJTC33 carrying the 1st and 2nd generation *tacchiA* cassettes was carried out as described in Chapter 2. In an attempt to increase electroporation efficiency, the method described by Wirth *et al.* (1989) was attempted. Transformants were selected on JNFb solid medium supplemented with Km (100 μg/ml).

Citrobacter sp. AJ1 cells were grown in LB to mid-exponential phase and washed as described above. Electroporation of 40 μl of cells with 1 or 2 μl DNA (1500 ng - 3500 ng) of the plasmids pKTC1, pDTC33, pMTC33 and pJTC33 was carried out in 0.2 cm cuvettes either at gene pulser and pulse controller settings of 25 μF, 2.5 kV and 200 ohms or 25 μF, 1.25 kV and 400 ohms

3.2.7 BACTERIAL CONJUGATION

Attempts to mobilize the broad host range plasmids and integration vectors and their recombinant *tacchiA* derivatives into *P. fluorescens* Rif1 and *Citrobacter* sp. AJ1 were carried out according to the method described by Simon *et al.* (1983) with modifications. Mobilization into *H. seropedicae* Nal1 was carried out according to the same method and with the modified procedure of Baldani (personal communication). For the former method, *E. coli* JM105 strains carrying the plasmids for mobilization (donor cells), *E. coli* HB101 carrying the helper plasmid pRK2013 for mobilization and the recipient bacteria *P. fluorescens* Rif1 or *Citrobacter* sp. AJ1, were grown overnight in 5 ml LB supplemented with the appropriate antibiotic at 30°C with shaking. An equal volume (1.5 ml) of each strain was collected by centrifugation in a microfuge and washed with 1.5 ml LB three times. Five hundred μl of each of the donor, recipient and helper strains were mixed, briefly microfuged and resuspended in 100 μl LB. The mixed culture was then spotted in 20 μl volumes onto LA plates without antibiotics and incubated at 30°C overnight. The resulting colonies were transferred and resuspended in 1 ml LB. Serial dilutions were made and plated onto LA supplemented with Km (100 μg/ml), Cm (25 μg/ml) or Rif (100 μg/ml) and LA supplemented with Rif (100 μg/ml) and Km

(100 μg/ml) or Cm (25 μg/ml). Donor, recipient and helper strains which had not been mixed were treated as described to serve as controls. Plates were incubated at 30°C.

For mobilization of pJTC33 (2nd generation *tacchiA* cassette in pJFF350) into *H. seropedicae* Nal1, the triparental method was used instead of using *E. coli* S17-1 as described by Baldani (personal communication) as pJTC33 was lethal in this strain since expression of the *chi*A from the *tac* promoter was constitutive. The plasmids pKT240, pKTC1 and pJTC1 carrying the 1st generation *tacchiA* cassette were successfully transformed into S17-1. Donor and recipient cells were grown as described, 500 μl of each mixed, placed on filter disks (Millipore) on plates containing a mixture of JNFb and LB and grown overnight at 30°C. Serial dilutions of the resulting colonies were plated onto JNFb supplemented with Nal (100 μg/ml) and Km (100 μg/ml). Controls were as described above.

3.2.8 SCREENING OF *P. fluorescens* Rif1, *H. seropedicae* HRC54, *H. seropedicae* Nal1 AND *Citrobacter* sp. AJ1 FOR EXPRESSION OF *chiA* AND CHITINASE ASSAYS

P. fluorescens, *H. seropedicae* and *Citrobacter* sp. AJ1 transformed with constructs carrying the *tacchiA* cassettes were screened for expression of the gene by means of the plate clearing assay described in section 2.2.4. In the case of *H. seropedicae*, electrotransformants and transconjugants were patched onto JNFb solid medium without the pH indicator (Appendix A) supplemented with the chitin substrate CM-CHITIN-RBV and the appropriate antibiotic.

Chitinase activity was determined in cultures of *P. fluorescens* Rif1 strains (carrying the *chiA* gene on pKT240 or integrated into the chromosome) and the positive control strains (*P. fluorescens* strains A7282 and A7283) that had been grown at 30°C in LB medium supplemented with Km (100 μg/ml) for 24 and 48 h. *Citrobacter* sp. AJ1 carrying pJTC33 was similarly grown. *H. seropedicae* Nal1 containing pKTC1 and HRC54 carrying pMTC33 were grown in liquid JNFb medium with Km (100 μg/ml) for 48 h at 30°C. Cultures were stored at -70°C with the addition of 20% glycerol prior to the assays. For *P. fluorescens, H. seropedicae* and their recombinant derivatives samples were prepared as described in section 2.2.6. The protein concentration was determined by the Bradford assay, using a standard curve as described in Appendix B. The chitinase enzyme assay was carried out as described in section 2.2.6.

3.2.9 SOUTHERN BLOT ANALYSIS

Southern blot analysis was used for to demonstrate random, single copy integration of Omegon-Km-*tacchiA* into the chromosome of the *P. fluorescens* Rif1 and *Citrobacter* sp. AJ1 electrotransformants. The 2.1-kb *Bam*HI fragment from *ptacchiA* and pTC33 containing the 1st and 2nd generation *tacchiA* cassettes was

labelled with Digoxigenin-11-dUTP (DIG; Boehringer Mannheim) by the random primed DNA labelling method according to the manufacturers' instructions and used as DNA probes. The efficiency of labelling was verified in a direct detection assay, as described in the DIG system users' guide for filter hybridization, and the probe was stored at - 20°C. Total DNA from several *P. fluorescens* and *Citrobacter* sp. AJ1 clones containing Omegon-Km-*tacchiA* cassettes was isolated as described by Ausubel *et al.* (1994). This DNA (10 µg), as well as pJTC1 and pJTC33 containing the 1st and 2nd generation *tacchiA* cassettes respectively, was restricted with *Eco*RI. Southern blot analysis was carried out according to the method described in the DIG sytem users' guide.

3.2.10 FUNGAL PLATE ASSAYS

The antifungal activity of the chitinase producing strains of *P. fluorescens* Rif1, *Citrobacter* sp. AJ1, *H. seropedicae* Nal1 and HRC54 was determined by fungal plate assays based on the method described by Chernin *et al.* (1995; personal communication with modifications). The antifungal activity of *E. coli* strains carrying pTC33 and pJTC33 was also determined. The bacterial cultures were grown in the appropriate antibiotic-supplemented media to stationary phase at 30°C with shaking. A 200 µl volume of the culture was spread across the centre of a PDA or LA plate in a straight line. Two 10 mm diameter agar disks taken from an actively growing PDA plate of *F. moniliforme* MRC 6148, *F. nygamai* MRC 5993 or *R. solani* were placed 3 cm away from the area of bacterial growth on opposite sides of the plate. The plates were incubated for 5 to 14 days at 30°C, until mycelia growing from the two disks on a control plate, had grown together. Zones of inhibition between fungi and bacteria were observed.

3.2.11 FUNGAL CHALLENGE OF BEANS CARRYING ENGINEERED *P. fluorescens* Rif1

The effectiveness of *P. fluorescens* Rif1 strains expressing the *chiA* gene as a biological control agent against the phytopathogenic fungus *R. solani* was tested *in vivo* according to the method of Pleban *et al.* (1995; personal communication). *P. fluorescens* Rif 1 and Rif 1 carrying either pKTC1 or the integrated *tacchiA* cassette were introduced into bean seedlings as described in section 3.2.4. They, together with uninoculated seedlings, were planted in baked soil (6 seedlings/tray; 3 replicates/ treatment) which had been artificially infested with *R. solani*. The fungus was grown in 100 ml PDB at 30°C without shaking for 3 - 4 days, weighed and 2 g macerated in 30 ml tapwater. This suspension was used to infest the top cover layer of the soil at 1 ml/kg of soil. The plants were grown under controlled conditions in a plant growth room. After 10 days, the percentage of seedlings with disease symptoms caused by *R. solani* were assessed. A disease index was used to describe the damping-off symptoms on a scale of 0 (no disease symptoms) to 5 (plant completely necrosed) as described by Sneh *et al.* 1966 (Fig. 3.1). The experiment was repeated 4 times. The presence of the endophyte was confirmed by reisolation of *P. fluorescens* from the plant on LA

supplemented with Rif (100 μg/ml) and Km (100 μg/ml) as described in section 3.2.5. Verification of chitinase expression was by the plate clearing assay described in section 2.2 6.

In a second set of experiments, endophytes were introduced into the plants as described above and by watering them into the soil. Plants were irrigated with freshly prepared cultures at a concentration of 10^7 cells /ml in a 15 ml volume 0, 3 and 6 days after planting. After 10 days of incubation the percentage of plants showing the symptoms of damping-off disease and the severity of the disease were determined. Each treatment was done in triplicate and the experiment repeated 4 times.

FIG. 3.1. Disease index of symptoms caused by *R. solani* in beans from left to right from 0 (no disease symptoms) to 5 (complete necrosis).

3.2.12 PLASMID STABILITY

The stability of pKTC1 in *P. fluorescens*, pKT240 in *H. seropedicae* Nal1, pMTC33 in *H. seropedicae* HRC54 and pJTC33 in *Citrobacter* sp. AJ1 was assessed. The strains were grown in the appropriate growth medium supplemented with Km (100 μg/ml) to stationary phase at 30°C, were diluted to 10^{-6} in 20 ml of appropriate medium without antibiotics and grown to stationary phase. This cycle of growth and dilution was repeated 5 times. The cells from a 10^{-6} dilution were plated onto LA plates after each cycle of growth. One hundred of the resulting CFU were patched onto LA plates supplemented with Km (100 μg/ml). The

percentage of patched colonies which grew on these plates was recorded. The experiment was repeated 3 times.

3.3 RESULTS AND DISCUSSION

3.3.1 ISOLATION AND CLASSIFICATION OF ENDOPHYTIC BACTERIA

The presence of endophytic bacteria is a major problem experienced in plant tissue culture laboratories (Cassells, 1991 cited in Pleban *et al.*, 1995). Samples of contaminated plants were obtained from local commercial plant tissue culture laboratories and microorganisms cultured on nutrient medium. Preferential selection of fluorescent *Pseudomonas* spp. was the priority in isolation of potential endophytes. Gram negative, rod shaped bacteria were isolated from all tissue culture samples tested, except from apple cultivar MM109 which contained Gram variable rods (Table 3.1). Interestingly, the isolates of *P. fluorescens* from apple occurred in samples from two independent plant tissue culture laboratories. Both laboratories had obtained their explant from the quarantine unit at the Plant Protection Research Institute of the Agricultural Research Council, Pretoria, indicating that the mother plant was infected. The apple plantlets were micropropagated by aseptic organic cultures with the removal of the meristem from the shoot. The stage of the tissue culture process at which the endophyte was observed varied. Growth was detected either immediately after initiation of meristem cultures or after 8-12 weeks but was not observed in all cultures. These observations suggested that this organism was a true endophytic bacterium (S. Leivers, personal communication) and was selected for further work.

Several endophytes were isolated and characterized from bean seeds (Table 3.1). Endophytic bacteria have been isolated from the seeds of several plant species including cotton, squash, oats, cauliflower, sunflower, bean, cucumber, melon, *Sinapis* and *Mimosa* (Mundt and Hinkle, 1976; Mishagi and Donndelinger, 1990; Pleban *et al.*, 1995). Mundt and Hinkle (1976) found endophytes in 15% of the seeds of herbaceuos seeds, 16% of the seeds of woody plants, 13.5% of overwintered cereal seeds and 5.4% of overwintered noncereal seeds examined. Pleban *et al.*, (1995) isolated endophytes from 16.7% of surface-sterilized seeds. I isolated bacteria from 19% of bean seeds after sterilization. Two isolates, *A. caviae* and *P. fluorescens* known as X33, were selected for colonization assays.

The resistance of the *P. fluorescens* isolate from apple to some antibiotics commonly used as selection markers was determined to facilitate the choice of cloning vectors. The strain was resistant to Ap, moderately resistant to Nal and sensitive to Sm and Km at concentrations of 100 μg/ml. As Rif and Nal are commonly used as genetic markers for monitoring Gram negative bacteria in the environment (Kloepper and Scroth, 1981), Rif was selected for the generation of spontaneous antibiotic resistant mutants, one of which,

P. fluorescens Rif1, was selected for further work. Spontaneous Rif resistant mutants were also obtained for the bean isolates *A. caviae* and *P. fluorescens* known as X33.

TABLE 3.1. Endophytic bacteria isolated from micropropagated plants and seeds

CLASSIFICATION	SOURCE
Pseudomonas fluorescens	Apple M25 and Pear tissue culture, bean seeds
Pseudomonas paucimobilis	Apricot tissue culture
Pseudomonas pickettii	Black Stinkwood tissue culture
Pseudomonas chlororaphis	Bean seeds
Agrobacterium radiobacter	Bean seeds
Aeromonas caviae	Bean seeds
Flavobacterium spp.	Bean seeds
Chryseomonas luteola	Bean seeds

3.3.2 COLONIZATION OF BEANS

The numbers of *P. fluorescens* Rif1, *P. fluorescens* X33 and *A. caviae* isolates recovered from roots and stems of beans are given in Table 3.2.

TABLE 3.2. Numbers of endophytes recovered from roots and stems of infected bean seedlings

ISOLATE	NO.[a]	DAYS	ROOTS		STEMS	
			CFU/ml[b]	CFU/g[c]	CFU/ml[b]	CFU/g[c]
P. fluorescens Rif1	18	10	$2.5x10^5$	$1.2x10^5$	$1.5x10^2$	ND
	18	21	$1.95x10^2$	80	ND	ND
P. fluorescens X33	17	7	$3.6x10^3$	ND	ND	ND
	8	21	65	ND	ND	ND
A. caviae	17	12	$1.6x10^5$	$5x10^4$	$9x10^5$	$9x10^5$
	18	21	$1x10^2$	40	36	17

[a]Number of plants tested [b]Mean CFU/ml macerated sample

[c]Mean CFU/g fresh weight of tissue ND=Not determined

Kado (1992) defined endophytic bacteria as those living in plant tissues without doing substantial harm or gaining benefit other than residency. Based on this definition, Chen *et al.* (1994) claimed that their strains were endophytic and strains *P. fluorescens* Rif1, *P. fluorescens* X33 and *A. caviae* are also endophytic. All three isolates maintained detectable populations for 21 days. No Rif-resistant bacteria were detected in uninoculated plants.

Large variations in population numbers between samples were observed and some plant samples contained no detectable bacteria. These were included in the data as 0 CFU/ml macerated sample or /g fresh weight of tissue. Considerable variation was also observed between replicates within the colonization studies presented by Chen *et al.* (1994). These authors believed that populations of endophytic bacteria at low levels were probably underestimated. The population numbers of the three isolates obtained from beans were similar to those reported for some introduced endophyte populations and for naturally occurring endophytes present in low numbers of 10^2-10^4 CFU/g tissue in healthy tissue of a variety of plants (Gardner *et al.*, 1982; Streichan and Schink, 1986; Gagné *et al.*, 1987; Sharrock *et al.*,1991). The trend in my results is similar to that reported by Chen *et al.* (1994). All six model endophytic strains they chose for their ability to reduce disease severity of *Fusarium* wilt in cotton survived in cotton stems for 21 days post-infection. After an initial decrease in numbers of five of the strains by Day 7, the populations stabilized from Day 7 to 21 at $10^{3..5}$-$10^{5.5}$ CFU/g tissue. The population of one of the six strains decreased from $10^{3.36}$ CFU/g stem tissue at Day 14 to $10^{2.15}$ CFU/g stem tissue at Day 21. In my study bacterial numbers of all three isolates decreased from Day 7-12 to Day 21 from $2.5x10^5$-$1.6x10^5$ CFU/ml of macerated root sample to $1.95x10^2$-65 CFU/ml macerated root. After Day 30 bacteria were undetectable and their numbers were not determined after 30 days. Chen *et al.* (1994) did not report on the population levels after 28 days post-endophyte introduction. This decrease in numbers of introduced bacteria has also been reported by Mc Inroy and Kloepper (1990) (cited in Chen *et al.,* 1994) who found that populations of endophytes in cotton where initially 10^3 CFU/g tissue which increased to 10^5-10^6 during the growing season and then declined to 10^3 at maturity. Endophyte populations in arum lily decreased after 5 days from initial levels of 10^8-10^{10} CFU to populations of 10^5-10^7 CFU. These populations however were stable (Gunson and Spencer-Phillips, 1993 cited in Chen *et al.*, 1994) unlike our endophytes in beans. According to Chen *et al.* (1994) the reason for this reduction in population densities could have been due to actual decrease in numbers as the plant matured or the result of extraction techniques.

Pleban *et al.*, (1994) were able to recover the radioactively-labelled, introduced endophytic strain of *Bacillus cereus* from roots and stems of cotton after 72 days. Bacterial numbers increased initially to $6.4x10^6$/g fresh weight then decreased to $3.19x10^5$ and $1.93x10^5$ /g fresh weight stem and root respectively. However after 30 days the numbers increased again within the roots. The bacterial numbers never decreased below $5x10^4$/g fresh weight of stems and roots. These results showed that an endophyte isolated from *Sinapis* was capable of populating a different species. *P. fluorescens* Rif1 was similar in as much as it was capable of

colonizing bean, although originally isolated from micropropagated apple, but might reach higher, more stable populations in a plant species different from bean. In the bean model system used in this study *P. fluorescens* Rif1 is only required for a brief period as *R. solani* infects the seedlings within 2 to 4 days (Sneh *et al.*, 1966).

3.3.3 INTRODUCTION OF THE *tacchiA* CASSETTE INTO *P. fluorescens* Rif1

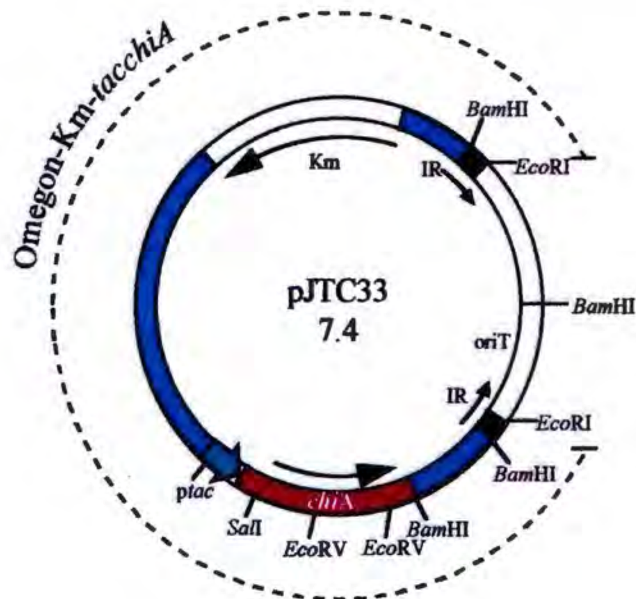
As pKT240 was known to be stable over 50 generations in *P. fluorescens* (Herrera, 1994; Herrera *et al.*, 1994), pKTC1 carrying the 1st generation *tacchiA* cassette was introduced into *P. fluorescens* Rif1 by triparental mating This was shown to be stable only for 20 generations and was lost from the majority of cells after 40 generations. Plasmids that express the *chiA* gene were found, by Chet *et al.* (1993) and Koby *et al.* (1994) to be unstable in *P. fluorescens* after overnight growth. The pJFF350-derived integration vectors, pJTC1 and pJTC33 for the integration of the 1st and 2nd generation *tacchiA* cassettes respectively into the chromosome of *P. fluorescens* Rif1 (section 2.3.2 and 2.3.6) were therefore constructed and introduced by electroporation. Selection of Km^R electrotransformants showed that the Omegon-Km-*tacchiA* cassette had been integrated into the chromosome as pJFF350 was unable to replicate in the host. Screening of electrotransformants on chitin-containing media resulted in production of cleared zones around the majority of colonies.

Integration of the two Omegon-Km-*tacchiA* cassettes was verified by Southern blot analysis (Fig. 3.2). Since the two *Eco*RI sites of pJFF350 are located outside of the 28-bp inverted repeats, they do not transpose. The *Bam*HI sites are internal to these repeats and therefore transpose with the Omegon-Km cassette. Chromosomal DNA of *P. fluorescens* Rif1::*tacchiA*1 (1st generation cassette from pJTC1) and *P. fluorescens* Rif1::*tacchiA*33 (2nd generation cassette from pJTC33) clones was cut with *Eco*RI and probed with the 2.1-kb *Bam*HI fragment from p*tacchiA* and pTC33 carrying the 1st and 2nd generation *tacchiA* cassette respectively. Hybridization of the probe to the *Eco*RI-restricted positive control pJTC1 resulted in two fragments of 3.2 and 2.5 kb. In *P. fluorescens* Rif1::*tacchiA*1 (1st generation cassette from pJTC1) clones two *Eco*RI fragments hybridized to the probe which had different sizes larger than 3.2 and 2.5 kb. The presence of only two larger *Eco*RI fragments indicated that a single copy of the Omegon-Km-*tacchiA* cassette had been integrated (results not shown). In the case of all but one of the *P. fluorescens* Rif1::*tacchiA*33 (2nd generation cassette from pJTC33) clones analysed only one *Eco*RI fragment hybridized to the probe since an *Eco*RI site had been lost in the construction of the new, improved *tacchiA* cassette. The presence of two bands in one of the clones (lane 4) indicated that the cassette had been integrated twice. The size of *Eco*RI fragments in these clones were different but all were larger than the 5.8-kb fragment of the *Eco*RI-digested positive control, pJTC33 (lane 1). The fragments larger than 5.8 kb in lane 1 are due to partial digestion of the plasmid DNA. The fact that all fragments from the clones analysed differed in size indicated that integration of the cassettes was random.

3.3.4 CHITINASE EXPRESSION OF *P. fluorescens* Rif1 CLONES CARRYING THE *tacchiA* CASSETTE

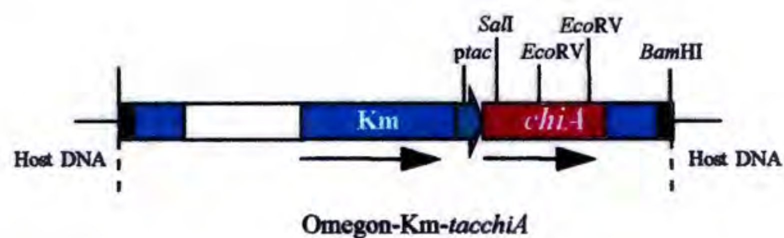
The expression of the *chiA* gene from the *tac* promoter in *P. fluorescens* Rif1 carrying pKTC1 (1st generation *tacchiA* cassette in pKT240) and from the integrated Omegon-Km-*tacchiA* cassettes (1st and 2nd generation) was observed initially on chitin-containing plates. Quantification of chitinase expression was carried out using the chitinase assays described in section 2.2.6 (Table 3.3). The strains A7282 and A7283 are two *P. fluorescens* exconjugants containing the Tn7-Km^R-P_{tac}*chiA* transposon constructed by Koby *et al.* (1994) with reported chitinase activity of 186 and 210 Miller units respectively. The *tacchiA* cassette integrated into the chromosome of these strains is the same as that cloned into pKTC1 and integrated into the chromosome of *P. fluorescens* Rif1::*tacchiA*1 (1st generation *tacchiA*), therefore they served as controls in our assay system. They were shown to have chitinase activity of 0.53 and 5 units/ml respectively compared with 20 and 266 units/ml for *P. fluorescens* Rif1::*tacchiA*1 and pKTC1 respectively. The higher chitinase activity of *P. fluorescens* Rif1(pKTC1) was due to the greater copy number of *tacchiA* compared with the single copy integrated into the chromosome of the host. The plasmid copy number of pKT240 carrying a *B. thuringiensis cryIA(c) gene* in *P. fluorescens* was shown to be 28 per cell (Herrera *et al.*, 1994). The chitinase activity of two *P. fluorescens* Rif1::*tacchiA*33 clones, namely 1 and 4, carrying the new, improved 2nd generation *tacchiA* cassette of 188 and 339 units/ml was comparable to that of

A



Transposition into *P. fluorescens* Rif1

B



C

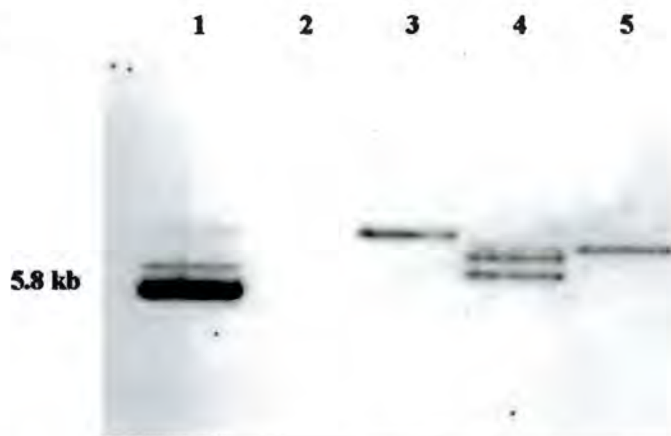


FIG. 3.2. Southern blot analysis of integration of the Omegon-Km-*tacchi*A cassette from pJTC33 into the chromosome of *P. fluorescens* Rif1. (A) The 2nd generation *tacchi*A cassette from pTC33 was cloned into the *Nde*I site of pJFF350 resulting in pJTC33. Electroporation of this plasmid into *P. fluorescens* Rif1 resulted in the transposition of the Omegon-Km-*tacchi*A cassette (B) which was verified by Southern blot analysis of *Eco*RI-digested chromosomal DNA (C). Lane 1, pJTC33; 2, *P. fluorescens* Rif1; 3,4 and 5, *P. fluorescens* Rif1::*tacchi*A33 (2nd generation cassette from pJTC33) clones 1, 3 and 4 respectively.

P. fluorescens Rif1(pKTC1) and considerably greater than that of the *P. fluorescens* strains carrying the integrated original 1st generation cassette. This was confirmed when the specific activity was determined with that of *P. fluorescens* Rif1::*tacchiA*33 clones 1 and 4 being 7 and 5 times greater than the activity of *P. fluorescens* Rif1::*tacchiA*1. In this assay *P. fluorescens* Rif1::*tacchiA*33 clone 1 had a higher activity than clone 4. The activity of both A7282 and A7283 was considerably lower than that of *P. fluorescens* Rif1::*tacchiA*1 however the standard deviation of the latter was large. After 48 h of growth majority of the chitinase had been secreted to the growth medium. These results demonstrated the increased efficiency of the new, improved second generation *tacchiA* cassette in *P. fluorescens* in addition to that observed in *E. coli* (section 2.3.7). Stable expression of the 1st generation *tacchiA* cassette in bacteria other than *Pseudomonas* spp. has only been reported for *Rhizobium meliloti* with a specific activity of 0.83 units (μmol/ h/μg protein; Sitrit *et al.*, 1993). It was therefore of interest to introduce this and the second generation cassette into the endophytes *H. seropedicae* and *Citrobacter* AJ1.

TABLE 3.3. Chitinase expression in *P. fluorescens* Rif1

STRAIN	Miller	SPECIFIC CHITINASE ACTIVITY[a]					
	Units	Extracellular	Cellular	Total	Extracellular	Cellular	Total
		24 h	24 h	24 h	48 h	48 h	48 h
A7282	0.53	0	0.036 (0.003)[b]	0.036 (0.003)[b]	ND	ND	ND
A7283	5	0	0.05 (0.008)[b]	0.05 (0.08)[b]	ND	ND	ND
Rif1(pKTC)	266	2.52 (0.33)[b]	1.24 (0.09)[b]	3.76 (0.3)[b]	2.5 (0.03)[b]	0.15 (0.09)[b]	2.64 (0.09)[b]
Rif1::*tacchiA*[c]	20	0.37 (0.34)[b]	0.05 (0.02)[b]	0.43 (0.32)[b]	0.20 (0.17)[b]	0	0.20 (0.17)[b]
Rif1::tc1[d]	188	1.9 (0.5)[b]	1.26 (0.23)[b]	3.16 (0.75)[b]	3.06 (0.55)[b]	0.2 (0.09)[b]	3.27 (0.5)[b]
Rif1::tc4[e]	339	1.19 (0.62)[b]	1.39 (0.62)[b]	2.12 (0.3)[b]	2.72 (0.45)[b]	0.22 (0.06)[b]	2.93 (0.43)[b]

[a]μmol/h/mg protein

[b]Numbers in brackets are the standard deviations of three replicates

[c]*P. fluorescens* Rif1::*tacchiA*1 (1st generation *tacchi*A cassette from pJTC1)

[d]*P. fluorescens* Rif1::*tacchiA*33 clone 1(2nd generation *tacchiA* cassette from pJTC33)

[e]*P. fluorescens* Rif1::*tacchiA*33 clone 4 (2nd generation *tacchiA* cassette from pJTC33)

ND = not determined

3.3.5 INTRODUCTION OF THE *tacchiA* CASSETTE INTO *H. seropedicae*

Few attempts have been made to introduce foreign genes into *H. seropedicae* and *H. rubrisubalbicans*, with the unpublished exception of *Bacillus thuringiensis cryIIIA* gene (J. Döbreiner and J.I Baldani, personal communications). Knowledge of suitable plasmid vectors and transformation systems in these species is limited. In this section the introduction of the pKT240-based pKTC1 (1st generation *tacchiA* cassette), the pML122-based pMTC33 (2nd generation), the pDER405-based pDTC33 (2nd generation) and the integration vectors pJTC1 and pJTC33 carrying the 1st and 2nd generation *tacchiA* cassettes respectively into *H. seropedicae* HRC54 and Nal1 will be reported.

Introduction of pKT240 and pKTC1 by electroporation was successful with efficiencies of $8x10^4$ and $1.2x10^3$ electrotransformants/µg DNA respectively, as was the triparental method described in section 3.2.7, although conjugation frequencies were not calculated. Since a recombinant derivative of pML122 was shown to be stably maintained in *Herbaspirillum* spp. (J.I Baldani, personal communication) I decided to introduce the 2nd generation *tacchiA* cassette into this plasmid for chitinase expression in *H. seropedicae*. Only 33 transformants/ug of pMTC33 DNA resulted from electroporation. This transformation efficiency was very low compared to that reported in *P. aeruginosa* of 10^4 transformants/picogram DNA, 10^9-10^{10} transformants/µg DNA in *E. coli* (Smith and Iglewski, 1989; Bio-Rad laboratories), and 10^2 to 10^5 transformants/µg DNA for various Gram negative bacteria using an electroporation method based on that optimized for *E. coli* K12 (Wirth *et al.*, 1989). These authors believed that efficiencies could be increased substantially if the method were optimized for each strain tested. Stability studies showed that pKT240 was unstable in *H. seropedicae* Nal1 with 54% of cells retaining the plasmid after 20 generations and only 4% of cells retaining the plasmid after 40 generations. However, pMTC33, carrying the 2nd generation *tacchiA* cassette in the opposite orientation to the direction of transcription of the Nm promoter, was shown to be very unstable with only 24% of cells containing the plasmid after overnight growth in the absence of Km selection. This instability confirmed the need to integrate the overexpressed *chiA* gene into the chromosome of *H. seropedicae*.

Introduction of pDTC33 based on the unique broad host range plasmid pDER405 (section 2.3.6) was not achieved as *H. seropedicae*, although reported to be susceptible to Cm (Baldani *et al.*, 1986), proved to be resistant to this antibiotic at the levels tested.

A biparental conjugation system for *Herbaspirillum* spp. resulting in the generation of transconjugants at high frequencies has been successful (J.I Baldani, personal communication). This technique was used to mobilize pKT240 and pKTC1 into *H. seropediacae* Nal1 but proved unsuitable for the introduction of pJTC33 as it uses the *E. coli* mobilization strain S17-1 (Simon *et al.*, 1983). Since this strain lacks the

repressor coded for by the *lacI*^{q} gene, expression of genes from the *tac* promoter is constitutive and overexpression of the *chiA* gene is lethal. pJTC1 was introduced, at very low transposition frequency, using the triparental method described in section 3.2.7.

Attempts to integrate the Omegon-Km-*tacchiA* cassettes by electroporation of pJTC1 and pJTC33 were unsuccessful due, supposedly, to the inefficient introduction of these vectors into *H. seropedicae*, supported by Fellay *et al.* (1989). They reported that the mobilization frequency of pJFF350 from S17-1 to a Rif ^{R} *E. coli* recipient strain was about 40% which was believed to be the same in non-*E. coli* strains. The number of Km^{R} exconjugants obtained is an indication of the frequency of Omegon-Km transposition which depends on the mobilization efficiency within the host. Low rates of occurrence of Km^{R} exconjugants reported for *Agrobacterium tumefaciens* and *Paracoccus denitrificans,* of $3x10^{-9}$ and $5x10^{-7}$ respectively, was speculated to be due to either poor mobilization of pJFF350 or to reduced transposition of Omegon-Km in these strains (Fellay *et al.*, 1989). The electroporation method described by Wirth *et al.* (1989) has been used in attempts to improve the frequency of transposition of the Omegon-Km-*tacchiA* cassettes in *H. seropedicae*.

The expression of the *chiA* gene in *H. seropedicae* transformants and exconjugants was determined (Table 3.4). The chitinase activity of H. *seropedicae* Nal1 carrying the Omegon-Km-*tacchiA* cassette (1st generation) was comparable to the level of expression of *P. fluorescens* Rif1::*tacchiA*1 (20 Miller units; Table 3.3). I assumed that the Omegon-Km-*tacchiA* cassette carried on pJTC1 had been integrated into the chromosome of *H. seropedicae* but this was not confirmed by Southern blot analysis, hence the reference to pJTC1 in Table 3.4. The level of expression of the improved 2nd generation *tacchiA* cassette in *H. seropedicae* HRC54 (pMTC33) was lower than that of the 1st generation pKTC1 which may have resulted from accumulation of mutants in the cell culture carrying pMTC33 defective in *chiA* expression as a result of the overexpression of this gene (Koby *et al.*, 1994). Alternatively, the instability of pMTC33 may have affected the level of expression.

TABLE 3.4. Chitinase expression in *H. seropedicae*

PLASMID	EXTRACELLULAR	CELLULAR	TOTAL
pKTC1[a]	1.6[c] (0.57)[e]	3.4[c] (1.7[e]	5.0[c] (2.08)[e]
pMTC33[b]	1.6[c] (0.16)[e]	0	1.6[c] (0.16)[e]
pJTC1[a]	ND	ND	22[d] (13.68)[e]

[a]1st generation *tacchiA* cassette [b]2nd generation *tacchiA* cassette

[c] Specific activity = µmol/h/mg protein [d]Miller units ND=not determined

[e]numbers in brackets are the standard deviations of three replicates

Future work includes the replacement of the Cm^R selection marker with the more suitable Km^R marker in pDTC33 and further optimization of the electroporation and/or conjugal transfer methods to achieve a greater transposition frequency of the integration vector pJTC33. If this fails alternative integration vectors such as the Tn7-based system described by Bao *et al.*, (1991) will be considered.

3.3.6 INTRODUCTION OF THE *tacchiA* CASSETTE INTO *Citrobacter* sp. AJ1

A strain identified as *Citrobacter* sp. AJ1 was isolated consistently from surface-sterilized pine pollen and believed to be endophytic (S. Pleban, personal communication). It is capable of producing high levels of the plant hormone IAA (indole acetic acid) which causes increases in root mass when introduced into beans, where it also occurs as an endophyte. Spontaneous Rif^R mutants of this endophyte were generated, introduced into beans and 51 CFU/g fresh weight bean root tissue were isolated 11 days after introduction although the root mass did not increase. In order to develop this strain as an agent for biocontrol, the effectiveness of which would be determined in bean in Israel, the 1st and 2nd generation *tacchiA* cassettes on pKTC1, pDTC33, pMTC33 and pJTC33 were electroporated into AJ1 but without success. This indicated that the electroporation protocol was not suitable, although efficiencies of $1x10^3$-$3.6x10^3$ transformants/μg DNA have been reported for *Citrobacter freundii* using this method (Wirth *et al.*, 1989). Although conjugal transfer of pDTC33 and pMTC33 was not successful, Km^R transconjugants resulting from the transfer of pKTC1 and pJTC33 were obtained but, considering previous plasmid instability results (see sections 3.3.3 and 3.3.4), it was decided not to continue with pKTC1.

In the case of pJTC33 Southern blot analysis showed that it still existed as a plasmid. This implied that the narrow host range, *E. coli* pBR322-based origin of replication within the Omegon-Km module of pJFF350, was recognised in *Citrobacter* sp. AJ1. The fact that the genera *Citrobacter* and *Escherichia* are both included in the family Enterobacteriaceae could be a possible explanation for this observation.

The chitinase activity of AJ1(pJTC33) clones was lower than that of *E. coli* strains carrying the same plasmid. Activity of six AJ1(pJTC33) clones varied between 224 and 1121 Miller units and the plasmid was stable over three cycles of growth to stationary phase.

3.3.7 BIOCONTROL ASSAYS

Fungal plate assays were performed to test the ability of *E. coli*, *P. fluorescens* Rif1 and *Citrobacter* sp. AJ1 expressing the *chiA* gene to inhibit the growth of *R. solani*. Since *H. seropedicae* occurs as an obligate endophyte of sorghum (Döbreiner *et al.*, 1983), it was decided to investigate the biocontrol ability of strains carrying the *chiA* gene against fungal pathogens of sorghum in addition to *R. solani*. The two *Fusarium* spp. used in this study were isolated from sorghum. *F. moniliforme* not only causes plant disease but produces

mycotoxins which are associated with several human and animal toxicoses (Hinton and Bacon, 1995). Although suppression of growth of *R. solani* was achieved with all strains (Fig 3.3), and with *H. seropedicae* clones against *Fusarium* spp. (results not shown), no significant difference in the antifungal activity of these strains compared with their unengineered controls was observed. *E. coli* strains were the exception. This implied that under these laboratory conditions the unmodified endophytes had the ability to inhibit fungal growth on agar media by a mechanism other than by the production of chitinase. Reports of biocontrol agents antagonistic to fungal pathogens abound in the literature. Various mechanisms, including the production of antifungal compounds such as antibiotics and siderophores are considered to be responsible for the biocontrol action of these strains (Chen *et al.*, 1994; Chernin *et al.*, 1996). It was assumed that one or more of these mechanisms was responsible for the antifungal activity seen in these preliminary studies. However, the use of *in vitro* antagonist screening to determine the biocontrol ability of microorganisms *in planta* is controversial, although widely used, and should be viewed with caution (Dickie and Bell, 1995). These authors reported that several factors including the type of growth medium, the pH of the medium, the temperature of growth and the medium of the assay plate influenced the results of *in vitro* antagonist screens.

The ability of *H. seropedicae* expressing the *chiA* gene to reduce disease incidence caused by pathogenic fungi *in planta*, specifically sorghum, needs to be investigated. The organism was isolated from 30 day old sorghum that had been planted in soil inoculated with the bacteria 78 days prior to planting implying that it could easily be delivered to plants by mixing in the soil (Boddey, 1995).

The ability of *P. fluorescens* Rif1 strains expressing the *chiA* gene to protect bean seedlings from *R. solani* was tested under plant growth room conditions. Variability in the disease control were observed between trials as a result of inconsistent growth of *R. solani* used to artificially infect seedlings. Standardization of the disease inoculum according to the method of Ko and Hora (1971) was also unreliable. Data were subjected to arcsine transformation prior to analysis of variance. Treatment means within the same trial were compared by analysis of variance followed by Duncan's multiple range test. *P. fluorescens* Rif1(pKTC1) carrying the first generation *tacchiA* cassette and two *P. fluorescens* Rif1:: *tacchiA*33 clones, 1 and 4, which have the second generation *tacchiA* cassette integrated into the chromosome, significantly reduced disease by 42%, 27% and 38% respectively when they were introduced into the plant during germination (Fig 3.4 A and B). Disease incidence was not significantly reduced by *P. fluorescens* Rif1 or *P. fluorescens* Rif1::*tacchiA*1, carrying the first generation *tacchiA* cassette. The results obtained for the former does not correlate with the observation of apparent biocontrol of this strain in fungal plate assays. Treatment means of the two trials were compared by Student's t test.

No significant disease reduction was observed when these strains were introduced into the plant and added to the soil compared to when strains were only introduced into the plant. Rif^R colonies were reisolated from

surface-sterilized root samples from one of the experiments after 10 days and shown to produce chitinase on chitin-containing plates. Koby *et al.* (1994) reported that 85% of cotton plants watered with a rhizosphere isolate of *P. fluorescens* carrying the integrated first generation *tacchiA* cassette (A7282 and A7283) and artificially infected with *R. solani* were disease free. This result is interesting as my equivalent, *P. fluorescens* Rif1::*tacchiA*1, did not significantly reduce disease incidence in my model system, despite having chitinase expression levels comparable to A7282 and A7283. Significant disease reduction was only obtained with the first generation *tacchiA* cassette when it was present on a plasmid, the copy number of which is around 28 copies per cell (Herrera, *et al.*, 1994). Pleban *et al.* (1995) reported that disease incidence caused by *R. solani* was reduced by 51%, 46% and 56% in cotton seedlings inoculated with three *Bacillus* endophytic strains. When bean seedlings were inoculated with these strains, disease incidence caused by *Sclerotium rolfsii* was reduced by 72%, 79% and 26%. They found that the disease index, which expresses the disease level (section 3.2.11), was also reduced but the index of our treatments was not significantly different from the disease controls as determined by Duncan's test. Chen *et al.* (1995) showed that the severity of disease caused by *Fusarium* wilt was reduced in cotton seedlings containing introduced endophytes.

Our results demonstrate that *P. fluorescens* Rif1 carrying the first and second generation *tacchiA* cassette on a plasmid and integrated into the chromosome respectively is an efficient biocontrol agent against *R. solani* under plant growth room conditions when it is present as an endophyte in beans.

FIG 3.3 Antifungal activity of endophytes against *R. solani*. Single plate on left: *R. solani*; Row 1: *Citrobacter* sp. AJ1; *Citrobacter* sp. AJ1 (pJTC33); Row 2: *P. fluorescens* Rif1; Rif1::*tacchiA*33 clone 4; Row 3: *H. seropedicae*; *H. seropedicae* (pMTC33); Row 4: *E. coli; E. coli* (pTC33); *E. coli* (pJTC33). All clones carry 2nd generation *tacchiA* cassettes. A 200 μl volume of each culture was spread across the centre of a PDA or LA plate in a straight line. Two 10 mm diameter agar disks of *R. solani* were placed 3 cm away from the area of bacterial growth on opposite sides of the plate.

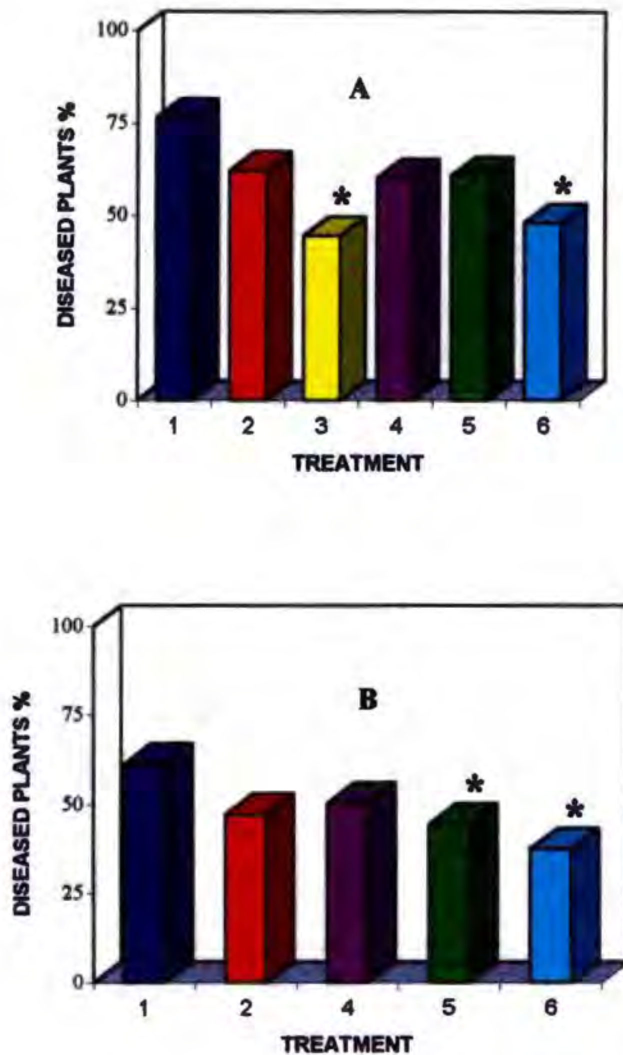


FIG. 3.4. The effect of application of *P. fluorescens* Rifl expressing the *chiA* gene on *R. solani* when the strains are (A) added to the roots and (B) added to the roots and soil. Data within the same trial topped with asterisks are significantly different from the disease control at the 5% level as determined by Duncan's test. Treatment means of the two trials were not significantly different from each other at the 5% level as determined by Student's t test. 1 = Disease control; 2 = *P. fluorescens* Rifl; 3 = *P. fluorescens* Rifl(pKTC1)(1st generation *tacchiA*); 4 = *P. fluorescens* Rifl:: *tacchiA*1 (1st generation); 5 = *P. fluorescens* Rifl:: *tacchiA*33 clone 1 (2nd generation *tacchiA*); 6 = *P. fluorescens* Rifl:: *tacchiA*33 clone 4 (2nd generation *tacchiA*). The standard deviations were 17.4; 24.6; 16.9; 37.7; 28.9; 21.6 for treatments 1-6 of Trial A and 25.9; 23.4; 15.3; 17.9; 20.2. for treatments 1, 2, 4, 5, 6 of Trial B.

CHAPTER 4

INTRODUCTION OF A CLONED *Bacillus thuringiensis cryIA(c)* GENE INTO *Pseudomonas fluorescens, Acetobacter diazotrophicus and Herbaspirillum seropedicae* FOR THE BIOCONTROL OF THE SUGARCANE BORER *Eldana saccharina*

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CHAPTER 4

INTRODUCTION OF A CLONED *Bacillus thuringiensis cryIA(c)* GENE INTO *Pseudomonas fluorescens, Acetobacter diazotrophicus and Herbaspirillum seropedicae* FOR THE BIOCONTROL OF THE SUGARCANE BORER *Eldana saccharina*

4.1 INTRODUCTION

Bacillus thuringiensis has been used as a safe alternative and supplement to chemical pesticides for over two decades. Despite the success of conventional *B. thuringiensis*-based products, they have several disadvantages as bioinsecticides. In the case of *E. saccharina* these include instability in the environment and on the surface of sugarcane as well as difficulty in reaching the internal regions where the larvae feed. The use of recombinant DNA technology has provided solutions to these problems through the development of two approaches, namely genetically modified microorganisms and transgenic plants. Examples of these were discussed in Chapter 1.

As part of an integrated pest management approach to the control of *E. saccharina* in South Africa, the *cryIA(c)* (*tox*) gene from *B. thuringiensis* strain 234 was previously introduced into *P. fluorescens* isolate 14 (Snyman *et al.*, 1993; Herrera *et al.*, 1994). This organism was isolated from the surface of sugarcane leaves, stems and borings and shown to be a good colonizer of the phylloplane of sugarcane. The gene was introduced into this strain on pKT240 and pDER405 and integrated into the chromosome using the Omegon-Km vector. Toxicity bioassays indicated that *P. fluorescens* 14 clones that expressed the gene were toxic to *E. saccharina* larvae and glasshouse trials showed that sugarcane inoculated with the strain carrying the integrated gene were more resistant to eldana damage than untreated controls.

Although these results were encouraging, it was felt that there was room for further improvement in the use of recombinant bacteria for the control of *E. saccharina*. The aim of the work presented in this chapter was to increase δ-endotoxin expression by cloning the *tox* gene under the control of the *tac* promoter with subsequent integration of the cassette into the chromosome of *P. fluorescens* 14. In addition, since recombinant *P. fluorescens* 14 populations are not stably maintained on sugarcane after 5 months (Snyman *et al.*, 1993), the potential of the obligate sugarcane endophytes *A. diazotrophicus* and *H. seropedicae* that expressed the gene as biocontrol agents was investigated. This was also seen as a possible solution to the problem of inaccessibility of conventional *B. thuringiensis*-based products to the interior regions of the plant. The advantages of using these recombinant endophytes, as discussed in Chapter 1, is their high stability in sugarcane, inability to survive outside the host plant, the ease of colonization and ability of *A.*

diazotrophicus to be transferred to subsequent generations via sugarcane setts. The introduction of the *cryIA(c)* gene from *B. thuringiensis* subsp. *kurstaki* into the chromosome of *Clavibacter xyli* subsp. *cynodontis* for the control of the European corn borer (ECB) is the only report of a genetically modified endophyte as a biocontrol agent. In these experiments a nonreplicating plasmid carrying a tetracycline resistance (Tc^R)gene, a sequence of *C. xyli* subsp. *cynodontis* DNA and two copies of a truncated form of the gene that included the first 1830 bp, fused with the entire kanamycin resistance gene as inverted repeats was inserted into the chromosome by homologous recombination. Integration was unstable *in vitro* and *in planta*, with the loss of the entire integrated plasmid, although the strain exhibited activity against ECB in diet assays (Turner *et al.*, 1991; Lampel *et al.*, 1994). A promoterless version of this gene was cloned into an integrative promoter-cloning plasmid carrying the Tc^R and a chromosomal fragment from the endophyte for homologous recombination gene. Introduction of this plasmid into the chromosome gave increased levels of the δ-endotoxin production, significant biological activity (with greater than 50% mortality of ECB larvae and reduction of damage to corn of about 60%) and higher stability in plants when compared with the previous construct (Lampel *et al.*, 1994).

A further strategy to improve the biocontrol of *E. saccharina* involved combining *P. fluorescens* strains producing the Cry (Tox) protein and the *S. marcescens* chitinase. Reports have shown that coapplication of *B. thuringiensis* and chitinase significantly increased the insecticidal effect of the former against insect larvae (Smirnoff, 1971, Regev *et al.*,1996). It is believed that the chitinase causes perforations in the chitin-containing peritrophic membrane of the larvae, thereby increasing the accessibility of the δ-endotoxin to the midgut membranes (Regev *et al.*, 1996).

4.2 MATERIALS AND METHODS

4.2.1 BACTERIAL STRAINS, PLASMIDS AND CULTURE CONDITIONS

Bacterial strains and plasmids used in this section are listed in Appendix C. The Rif-resistant *P. fluorescens* 14 was grown on LA or LB medium supplemented with Rif (100 μg/ml). The sugarcane endophytes *A. diazotrophicus* PAL 5 and *H. seropedicae* HRC54 were given to us by Dr Dobreiner of EMPRABA, Brazil. *A. diazotrophicus* PAL 5 was grown on semisolid, solid or liquid LGI as described by Cavalcante and Dobreiner (1988). For nitrogen dependent growth, yeast extract (20 mg/l) was added to solid and liquid LGI media. Cultures were also grown in DYGS liquid media (Appendix A) or in YPD media (Appendix A). *H. seropedicae* HRC54 was grown in semisolid, solid or liquid JNFb media (Appendix A).

All bacteria were grown at 30°C. Strains were maintained in 0.1 M $MgSO_4$ at 4°C for *P. fluorescens*, or the appropriate growth media supplemented with 10% glycerol at -70°C for *A. diazotrophicus* and *H. seropedicae* strains.

4.2.2 BACTERIAL TRANSFORMATION BY ELECTROPORATION AND CONJUGATION

Plasmids and the integration vector carrying the *taccryIA(c)* (*tactox*) cassette were electroporated into *P. fluorescens* 14, *A. diazotrophicus* PAL 5 and the Nal-resistant mutant of *H. seropedicae* (*H. seropedicae* Nal1) as described in section 3.2.6. The cultures were grown to mid-exponential phase in 100 ml LB, YPD or JNFb respectively. The cells were washed in 300 mM sucrose as described. The gene pulser and pulse controller were set at 2.5 kV, 25 µF and 200 ohms. The cells were plated onto appropriate growth media supplemented with Km (100 µg/ml). Plasmids were also mobilized into *H. seropedicae* Nal1 as described in section 3.2.7.

The plasmid pML122 (Appendix D) was shown to be stably maintained in *Herbaspirillum* spp. (Baldani, personal communication) and was therefore chosen for introduction of the *tox* gene into *H. seropedicae* Nal1. Plasmid stability of pMT7 and pMT11 carrying this gene on pML122 was determined as described in section 3.2.12.

4.2.3 WESTERN BLOT ANALYSIS

Determination of the expression of the *tox* gene in *P. fluorescens* 14 and *H. seropedicae* Nal1 was carried out by Western blot analysis as described in Appendix B.

4.2.4 SOUTHERN BLOT ANALYSIS

Total DNA from *P. fluorescens* 14 clones containing the Omegon-Km-*tactox* cassette was isolated as described by Ausubel *et al.* (1994). This DNA as well as pJTT carrying the *tactox* cassette on pJFF350 was cut to completion with *Eco*RI and analysed by Southern blot as described in section 3.2.9.

4.3 RESULTS AND DISCUSSION

4.3.1 INTRODUCTION OF THE *tactox* CASSETTE INTO *P. fluorescens* 14

The plasmids pKTT and pJTT, carrying the *tactox* cassette on pKT240 and the integration vector pJFF350 respectively were introduced into *P. fluorescens* 14 by electroporation. *P. fluorescens* 14 (pKTT)

electrotransformants grew poorly in liquid media indicating that constitutive expression of the *tox* gene at high levels had a lethal effect on this organism. This has been reported in the literature and was discussed in section 2.3.10. To circumvent this problem and to prevent the horizontal transfer of the gene to other bacterial species, the Omegon-Km-*tactox* cassette was inserted into the chromosome and integration was confirmed by Southern blot analysis. Total DNA of *P. fluorescens* 14::*tactox* was cut with *Eco*RI and probed with the 4-kb *Bam*HI fragment of p*tactox* carrying the *tactox* cassette (Fig 4.1). Four fragments of 3.5, 3.3, 0.7 and 0.2 kb hybridized to the *Eco*RI-restricted pJTT. In all clones analysed, two *Eco*RI fragments of 0.7 and 0.2 kb, corresponding to the fragments internal to the Omegon-Km-*tactox* cassette, and two of different sizes greater than 3.5 and kb, hybridized to the probe. Random, single integration of the cassette was indicated by the fact that the two larger *Eco*RI fragments were of different sizes and only two of the larger *Eco*RI fragments were detected in these clones.

The stability of the integrated cassette was not definitively established, but there was no evidence of decreased CryIA(c) (Tox) expression in SDS-PAGE after 48 h (results not shown) and the growth rate of the recombinant strain did not appear to be different from that of *P. fluorescens* 14. Herrera (1994) showed that the Omegon-Km-*tox* cassette in *P. fluorescens* 14 was stably integrated for at least 100 generations and stable integration of *cry* genes into root colonizing *P. fluorescens* strains using a transposon Tn*5*-mediated system or suicide vectors for integration by homologous recombination have been described in the literature (Obukowicz *et al.*, 1986a and b; Waalwijk *et al.*, 1991).

4.3.2 ATTEMPTS TO INTRODUCE THE *tactox* CASSETTE INTO *A. diazotrophicus* PAL 5 AND *H. seropedicae* Nal1

The plasmids pKT240, pJFF350 (Appendix D), their recombinant *tactox* derivatives, and the pML122-derived plasmids carrying the *tox* gene were electroporated into *A. diazotrophicus* and *H. seropedicae*. The pML122-based plasmid pBS42, containing a *cryIIIA* gene, served as a transformation control as it has been successfully introduced into these sugarcane endophytes (Baldani, personal communication). pDER405 and its derivative were not used due to the inherent resistance of *H. seropedicae* to Cm. Transformation efficiencies of pKT240 in these endophytes were $6x10^3$ and respectively.

Although Km^R electrotransformants were obtained for all plasmids introduced into *A. diazotrophicus,* growth in liquid media was only possible for clones transformed with pKT240 without the *tactox* cassette, the pML122-based pMT7 and pMT11 carrying the *tox* gene alone and pBS42. Accumulation of spontaneous Km^R mutants resulted in all cultures. This indicated that the *tox* gene, under the control of *tac* promoter and expressed constitutively in this strain, had a lethal effect on the growth of clones in liquid culture although apparently not on solid media. Since pJFF350 is unable to replicate in the majority of Gram negative bacteria and clones did not grow in liquid culture containing Km it was assumed that this

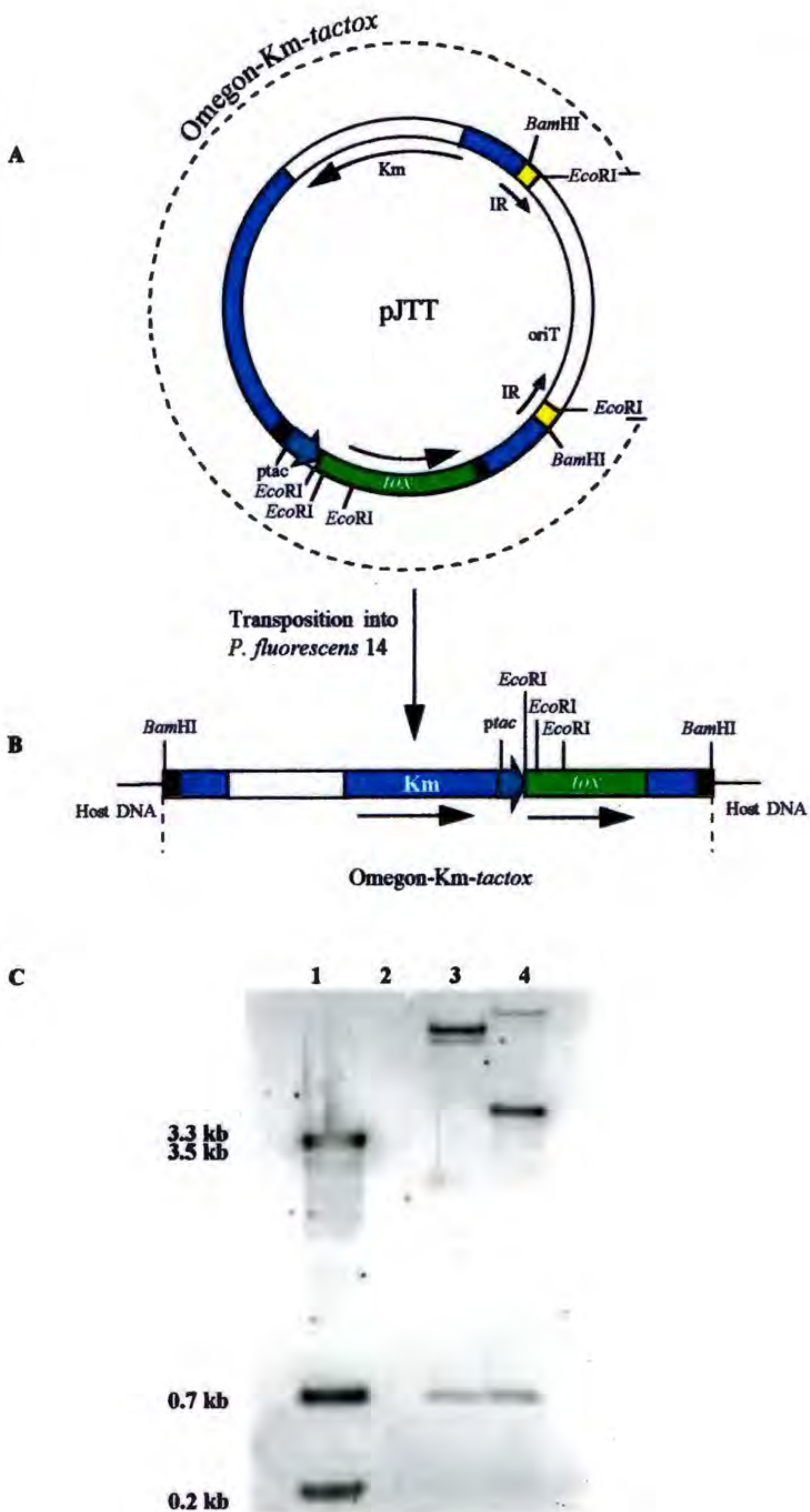


FIG. 4.1 Integration of Omegon-Km-*tactox* into the chromosome of *P. fluorescens* 14. The *tactox* cassette from p*tactox* was cloned into the *Nde*I site of pJFF350 resulting in pJTT (A). The plasmid was electroporated into *P. fluorescens* 14 with the transposition of the Omegon-Km-*tactox* cassette into the chromosome (B). (C) Southern blot analysis of DNA probed with the 4-kb *Bam*HI fragment carrying the *tactox* cassette. Lanes 1, pJTT; 2, *P. fluorescens* 14; 3, 4 and 5, *P. fluorescens* 14:: *tactox* clones 1, 2 and 3 respectively.

Although Km^R electrotransformants were obtained for all plasmids introduced into *A. diazotrophicus,* growth in liquid media was only possible for clones transformed with pKT240 without the *tactox* cassette, the pML122-based pMT7 and pMT11 carrying the *tox* gene alone and pBS42. Accumulation of spontaneous Km^R mutants resulted in all cultures. This indicated that the *tox* gene, under the control of *tac* promoter and expressed constitutively in this strain, had a lethal effect on the growth of clones in liquid culture although apparently not on solid media. Since pJFF350 is unable to replicate in the majority of Gram negative bacteria and clones did not grow in liquid culture containing Km it was assumed that this vector with and without the *tactox* cassette had not been integrated into the chromosome, possibly as a result of low transposition frequency due to inefficient electroporation (see section 3.3.5).

pKT240 and the pML122-based plasmids carrying the *tox* gene were successfully introduced into *H. seropedicae* with an efficiency of $8x10^4$ transformants/µg DNA for the former. Electroporation of the pKT240-derived pKTT carrying the *tactox* cassette resulted in only a few Km^R colonies. As described in section 2.3.8 cloning of the *tactox* cassette into pML122 resulted in the deletion of the entire *tac* promoter. However, plasmid stability studies showed that pMT7 carrying the *tox* gene inserted downstream of the strong Nm^R promoter on pML122 was extremely unstable in *H. seropedicae* Nal1 after overnight growth whereas pMT11 with the gene in the opposite orientation with respect to this promoter was stable over 60 generations. As will be seen in section 4.3.3 this is likely to be due to the intolerance of high levels of constitutively expressed *tox* in *H. seropedicae* cells. Efforts to introduce pJTT carrying the *tactox* cassette on pJFF350 into the Nal-resistant strain *H. seropedicae* by electroporation and conjugative transfer resulted in very low numbers of Km^R colonies, indicative of low transposition frequencies believed to be due to inefficient transformation as discussed in section 3.3.5.

4.3.3 EXPRESSION OF THE δ-ENDOTOXIN GENE

Expression of the *tox* gene in *P. fluorescens* 14, *A. diazotrophicus* PAL 5 and *H. seropedicae* Nal1 was determined by quantitative Western blot analysis. The 134-kDa Tox protein was not detected immunologically in *P. fluorescens* 14 (pKTT) clones carrying the *tactox* cassette on pKT240 (Fig 4.2A, lane 4). However, this gene under the control of its endogenous promoter on pKT240 and pDER405 was expressed in *P. fluorescens* 14 at toxin protein levels of 3.5 and 2.2% respectively of their total protein respectively (Herrera, 1994). This implied that constitutive expression of *tox* at high levels in *P. fluorescens* 14 (pKTT) must have resulted in the accumulation of mutants defective in *tox* expression after overnight growth.

All the analysed *P. fluorescens* 14::*tactox* clones, carrying the integrated Omegon-Km-*tactox* cassette, produced the 134-kDa protein at levels considerably greater than that of the previously constructed *P. fluorescens* 14::Omegon-Km-*cry*, referred to in this work as *P. fluorescens* 14::*tox* (Fig 4.2A, lanes 6-10

compared with lane 5). These results proved that the *tac* promoter is capable of operating efficiently in *Pseudomonas* and is responsible for the increased levels of expression of the gene. I am unaware of any reports of a *cryIA(c)* gene under the control of the *tac* promoter having been integrated into the chromosome of a *Pseudomonas* sp. Quantitative analysis of the δ-endotoxin by ELISA in *P. fluorescens* 14::*tactox* clones was not determined but Herrera *et al.* (1994) showed that *P. fluorescens* 14:: *tox* clones produced high levels of Tox protein similar to those produced by pKT240-*cry* clones representing 3.7 and 3.5 % of the total protein respectively. These levels were comparable to those of 0.5-1% reported by Obuckowickz (1986) for a similar *tox* gene in root-colonizing pseudomonads. Ge *et al.* (1990) reported that expression of *cryIA(c)* 73 gene from its own promoter in *E. coli* was 0.24% of total cellular protein and from the *tac* promoter after induction was about 50% of total cellular protein. In another system, expression of a *B. thuringiensis* subsp. *aizawai* 130-kDa protein gene from the *tac* promoter in *E. coli* was 38% of the total cellular protein compared to 3% of total cellular protein from its own promoter (Oeda *et al.*, 1987). Therefore it is believed that the levels of the Tox protein expressed from the *tac* promoter in *P. fluorescens* would be considerably greater than 3.7 %. This would only result if the *tactox* cassette was present in the cell as a single integrated copy as it is clear from the non-expressing mutants of *P. fluorescens* carrying the cassette on the multicopy plasmid pKTT that only a certain level of expression is tolerated by these cells.

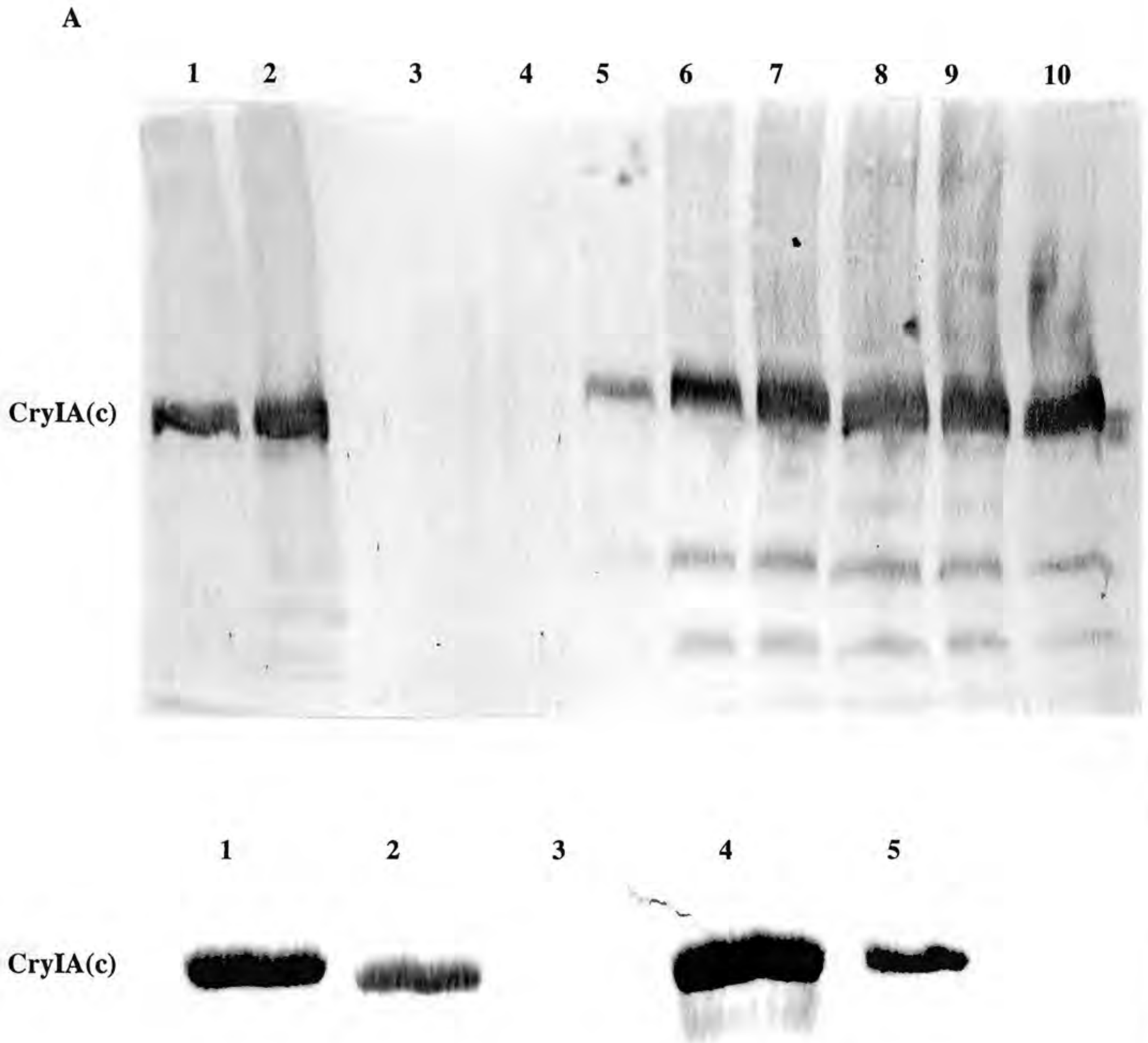
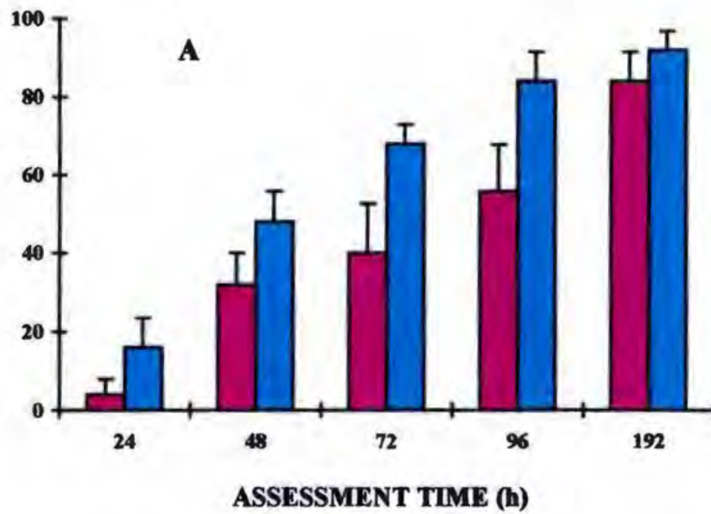


FIG. 4.2 Western blot analysis of *tox* expression in recombinant clones. (A) Lanes 1-2, *E. coli* (pKTT) and *E. coli*(pJTT) carrying the *tactox* cassette on pKT240 and pJFF350 respectively; 3, *P. fluorescens* 14; 4, *P. fluorescens* 14 (pKTT); 5, *P. fluorescens* 14::*tox* clone 2; 6-10, *P. fluorescens* 14::*tactox* clones 1 and 2. (B) Lanes 1, *E. coli* (pMT7); 2, *E. coli* (pMT11); 3, *H. seropedicae* Nal1; 4, *H. seropedicae* Nal1 (pMT7); 5, *H. seropedicae* Nal1 (pMT11).

The Tox protein was not detected in *A. diazotrophicus* PAL 5 carrying the *tox* gene on pML122-based pMT7 and pMT11. Similarly, the *cryIIIA* gene carried on pML122 (pBS42) was not expressed in this organism (Baldani, personal communication). The explanation for this is not known since there have been no other attempts to manipulate this organism genetically. It could be related to its unusual growth characteristics since growth is best in 10% sugar at pH 5.5 or less with the final pH of the medium reaching below 3.0 and is possible that this would lead to the denaturation of any expressed Tox protein present in the samples. Alternatively, it is possible that pML122 and its derivatives are not stably maintained, but this was not established and work with this organism was discontinued.

The Tox protein was not detected immunologically in *H. seropedicae*(pKTT) carrying the *tactox* cassette on pKT240 (results not shown). As in *P. fluorescens* 14(pKTT) clones this is possibly due to the accumulation of Tox$^-$ mutants resulting from high levels of the constitutively expressed *tox* gene in *H. seropedicae*(pKTT). In contrast, *H. seropedicae* (pMT7) clones with the *tox* gene downstream of the Nm^R promoter on pML122, produced higher levels of the Tox protein than *H. seropedicae* (pMT11) clones with the gene in the opposite orientation with respect to this promoter (Fig 4.2B). This indicated that expression of the gene in the former clones was under the control of the Nm^R promoter which could explaine the high levels of instability of this plasmid (see section 4.3.2). Labes *et al.* (1990) reported that the Nm^R promoter was an efficient and more effective promoter than the *tac* promoter for overexpression of foreign genes in soil bacteria, including *Pseudomonas* spp.

The biological activity of the above strains was determined in toxicity bioassays performed at the South African Sugarcane Experiment Station (SASEX) at Mt Edgecombe, Kwazulu Natal. The results showed that *P. fluorescens* 14::*tactox* was more toxic than *P. fluorescens* 14::*tox* (Fig 4.3A). Similarly, *H. seropedicae* (pMT7) caused greater mortality of larvae than *H. seropedicae* (pMT11) (Fig 4.3B). The results obtained support our hypothesis that increased expression of the *tox* gene in both *P. fluorescens* 14 and *H. seropedicae* improves the control of *E. saccharina* larvae and corroborates the usefulness of genetically modified obligate sugarcane endophytes as biocontrol agents of this insect pest. However, it is important to consider that although increased expression leads to increased toxicity, it can also be a burden on bacterial cells resulting in instability, as in the case of *H. seropedicae* (pMT7) clones the accumulation of non-expressing mutants or in lethality. All these factors need to be taken into account when planning future strategies for biological control of *E. saccharina* in sugarcane.



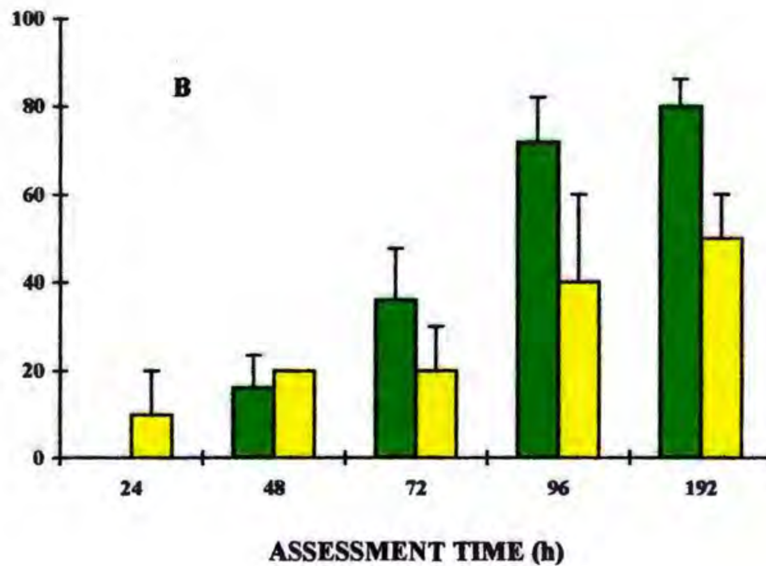


FIG. 4.3 Toxicity to *E. saccharina* neonate larvae of *P. fluorescens* 14 and *H. seropedicae* Nal1 strains expressing the *tox* gene. (A) *P. fluorescens* 14::*tox* (pink) and *P. fluorescens* 14::*tactox* (blue). (B) *H. seropedicae* (pMT7; green) and (pMT11; yellow). Neonate larvae were fed on an artificial diet to which 3 mg of freeze-dried recombinant strains were added per g diet. Mortality of larvae were determined after 24, 48, 72, 96 and 192 h. Standard error of means are indicated by the error bars above each column.

Herrera *et al.* (1994) showed, in glasshouse trials of sugarcane, that *P. fluorescens* 14::*tox* caused a decrease in the presence of larvae and consequent damage of approximately 60% after 4 weeks. Preliminary *in planta* assays carried out in growth chambers at the University of Cape Town were unsuccessful due to a malfunction in the temperature control in one of the chambers where control sugarcane inoculated with *P. fluorescens* 14 were grown.

Future investigations include the optimization of the conjugal transfer method using the mobilization strain *E. coli* S17-1 for improved transposition of the integration vector pJTT necessary to increase the chance of integration into the chromosome of *H. seropedicae*. The possibility that overexpression of the *tox* gene from the *tac* promoter in pJTT had a detrimental effect on *H. seropedicae* cells was also considered. To investigate this, the *tox* gene will be cloned under the control of its own promoter, into a pDER405-derived vector carrying a Km^R gene instead of the Cm^R gene and pJFF350. If an increased transposition frequency and stable integration of the Omegon-Km-*tox* cassette into the chromosome of *H. seropedicae* is achieved, insertion of the Omegon-Km-*tactox* cassette will be reinvestigated. The effectiveness of these strains as biocontrol agents of *E. saccharina* larvae will be determined in bioassays. The ability of the recombinant phylloplane and endophytic strains to survive in the environment and protect sugarcane from *E. saccharina* larvae must be determined in glasshouse trials in Kwazulu Natal .

A synergistic toxic effect was observed when *P. fluorescens* 14::*tactox* was combined with the chitinase-producing strain *P. fluorescens* Rif1::*tacchiA* carrying the 2nd generation *tacchiA* cassette (Table 4.1). This latter strain was not toxic on its own to larvae when included in the diet. In the absence of this strain, 3 mg freeze-dried *P. fluorescens* 14::*tactox* per g artificial diet was required to cause 92% mortality after 168 h, while only 0.3 mg/g diet was necessary to obtain 96% mortality in the presence of the chitinase producer. Synergistic insecticidal effects with combined *B. thuringiensis* suspensions and chitinase, or chitinase-producing bacteria as well as the combined effects of a Cry1C protein and the *S. marcescens* ChiA have previously been demonstrated (Smirnoff, 1971; Regev *et al.*, 1996). In this last report, the chitinase alone was capable of perforating the chitin-containing peritrophic membranes of insect larvae, indicating the mechanism involved when combined with the Cry protein. My results, although preliminary, demonstrate that by cointroduction of these genes, increased biocontrol of insect pests could be achieved by using lower levels of the Cry protein in recombinant bacteria. This is advantageous since these may be able to compete better in the environment with a diminished risk of generating resistant larval populations resulting from exposure to high levels of Cry protein. Future experiments include the determination of optimum, effective concentrations of the recombinant strains as well as the synergistic toxic effect of *H. seropedicae* strains producing the Tox protein and chitinase.

TABLE 4.1. Synergistic effect of *P. fluorescens* 14::*tactox* and *P. fluorescens* Rif1::*tacchiA*

TREATMENT	CONC.	MEAN % MORTALITY OF LARVAE		
	mg/g[a]	24 h	72 h	168 h
Control	0	8 (8.0)[d]	4 (4.0)[d]	36 (18.3)[d]
14[b]	3.0	0	8 (4.9)[d]	32 (13.5)[d]
14[b]::*tox*	3.0	20 (8.94)[d]	40 (6.3)[d]	88 (4.9)[d]
14[b]::*tactox*	3.0	36 (11.66)[d]	52 (10.20)[d]	92 (4.9)[d]
Rif1[c]::*tacchiA*	3.0	0	0	12 (4.9)[d]
14[b]::*tactox* + **Rif1[c]::*tacchiA***	0.3 3.0	24 (7.48)[d]	60	96 (4.0)[d]

[a]Concentration of freeze dried bacteria in mg added per g artificial diet

[b]*P. fluorescens* 14

[c]*P. fluorescens* Rif1

[d]Values in brackets give the standard error of means

APPENDIX A

MEDIA AND SOLUTIONS

A.1 MEDIA

A.1.1 JNFb SOLID MEDIUM

Media Component	Stock solution	/1000ml
Malic acid		5 g
K_2HPO_4	10%	6 ml
KH_2PO_4	10%	18 ml
$MgSO_4.7H_2O$	10%	2 ml
NaCl	10%	1 ml
$CaCl_2.H_2O$	1%	2 ml
Bromothymol blue	0.5%	2 ml
Micronutrients*		2 ml
FeEDTA	1.64%	4 ml
KOH		4.5 g
Yeast extract	1%	2 ml
Agar		17 g

pH 5.8

***Micronutrients**

$Na_2MoO_4.2H_2O$	0.2 g
$MnSO_4H_2O$	0.235 g
H_3BO_3	0.28 g
$CuSO_4.5H_2O$	0.008 g
$ZnSO_4.7H_2O$	0.024 g
Distilled water	to 200 ml

A.1.1.1 SEMISOLID AND LIQUID JNFb

As above but the yeast extract is omitted and 1.9 g/l agar added in semisolid medium. In liquid media 1 g/l NH4Cl is added in addition to Yeast extract. The pH of both is 5.8.

A.1.2 DYGS

Glucose	2 g
Yeast Extract	2 g
Peptone	1.5 g
$MgSO_4.7H_2O$	0.5 g
Glutamic acid	1.5 g
K_2HPO_4	0.5 g
Distilled water	to 1000 ml
pH 6.0	

A.1.3 YPD

Yeast Extract	10 g
Polypeptone	10 g
Glucose (20% stock)	3 %
Distilled water	to 1000 ml

All components except glucose were added autoclaved together with 15 g/l agar for solid media. Glucose stock was autoclaved separately and added to rest of autoclaved components.

A.2 p-nitrophenyl-β-D-N.N'-diacetylchitobiose STOCK SOLUTION

A 2 mg/ml stock solution was prepared by adding O.5 ml N,N-Dimethylformamide and 4.5 ml H2O to 10 mg p-nitrophenyl-β-D-N.N'-diacetylchitobiose (Sigma) and stored in a light-tight container at 4°C.

APPENDIX B

STANDARD TECHNIQUES

B.1 DETERMINATION OF PROTEIN CONCENTRATION

Total protein concentration of cultures containing Cry1A(c) and ChiA was determined independently according to the instructions in the Bio-Rad Laboratories manual based on the method of Bradford (1976). Two standard curves were prepared separately in duplicate or triplicate for the respective cultures using BSA fraction V (Boehringer Mannheim) as the protein standard. A stock of BSA (14 mg/ml for Cry1A(c) containing cultures or 25 mg/ml for ChiA containing cultures) was prepared in H_2O and diluted to 1.4 mg/ml or 2.5 mg/ml in H_2O, from which the standard curves were prepared. The dilutions of BSA in µg were made up to a total volume of 800 µl with H_2O were placed in test tubes together with 10 µl sample buffer (or growth medium for extracellular samples) and 200 µl Bio-Rad dye reagent concentrate. The tubes were mixed by vortexing and incubated at room temperature for 2 (for Cry1A(c) samples) and 20 minutes (for ChiA samples). After this time period, the OD_{595} was read against the reagent blank. The OD_{595} versus concentration of the standards was plotted and the regression curve (y=mx+c) determined manually or using the computer software package Microsoft Excel for Windows 95 for determination of unknowns.

B.2 WESTERN BLOTS

Proteins were transferred from SDS polyacrylamide gels onto nitrocellulose membranes according to the method of Towbin *et al.* (1979). Electrotransfer was carried out using a Hoeffer Transphor (TE 42) unit in transfer buffer at 18 V for 8 hours or overnight at 15 V at 4°C, after which the blot was airdried overnight before placing in 4.5% skim milk powder in 1xPBS as blocking for 1 hour with shaking. The anti-Cry1A(c) antibody supplied by SASEX was added at a dilution of 1:4000 in blocking buffer for 1-1.5 hours. The blot was rinsed and washed 3 times in wash solution (1x PBS + 0.5 ml/l Tween 20) before incubating with the second antibody, Goat-anti-rabbit-IgG conjugated to alkaline phosphatase (Sigma) diluted 1:2000 or 1:500 if laboratory prepared second antibody was used for 1-1.5 hours. It was rinsed, washed in wash buffer then in 1x PBS three times. Freshly prepared staining solution (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-Cl pH 9.5, 50 µl BCIP/10 ml, 50 µl NBT/10 ml) was added for 30-60 minutes.

B.3 p-NITROPHENOL STANDARD CURVES

For the determination of the amount of p-nitrophenol released in the chitinase enzyme assays, two p-nitrophenol standard curves were prepared, ranging from 0.009 to 0.09 mM and 0.009 to 0.001 mM. A 100 mM stock of p-nitrophenol (Sigma) was dissolved in 9.5 ml $H_2 0$ and 0.5 ml N,N' dimethylformamide with vortexing. The stock was diluted to 1 mM in $H_2 0$ from which the standard curves were prepared. The dilutions were added to test tubes together with 200 µl sample buffer or growth medium and 50 µl 0.1 M phosphate buffer pH 8.0. The volume was made up to 500 µl with $H_2 0$ to which 200 µl 1 M NaOH was added. The tubes were mixed by vortexing and the OD_{410} was read against the reagent blank. The assay was carried out in triplicate. The OD_{410} versus concentration of the standards was plotted and the regression curve (y = mx+c) determined using the computer software package Microsoft Excel for Windows 95 for determination of unknowns.

APPENDIX C

BACTERIAL AND FUNGAL STRAINS AND PLASMIDS

Bacterial Species	Strain	Relevant genotype/phenotype	Ref/Source
E. coli	JM105	F' *tra*D36 *lacI*^{q}Δ(*lac*Z)M15 *pro*AB/*thi rps*L (Str^r) *end*A *sbc*B15 (*sbc*C) *hsd*R4 ($r_K^- m_K^+$) Δ(*lac-pro*AB)	Yanish-Perron *et al.*, 1985
E. coli	S17-1	*rec*A *thi pro hs*R$^-$M$^+$ <RP4:2-tc:Mu:Km:Tn7> Tp^R Sm^R	Simon *et al.*, 1983
E. coli	A5945	K37 *lac*Z::Tn*5* *lacI*^{q} *pcnB*::Tn*10*	Oppenheim, Israel
P. fluorescens	Rif1	Rif^R	This work
P. fluorescens	14	Rif^R, Nal^R	Jacobs, 1989
P. fluorescens	14::*tox*	Rif^R, Nal^R, Omegon-Km-*cryIA(c)*	Herrera *et al.*, 1994
P. fluorescens	A7282; A7283	Tn7; Km^R, P*tac*, *chiA*	Koby *et al.*, 1994
H. seropedicae	HRC54	Ap^R	Baldani *et al.*, 1986
H. seropedicae	Nal1	Nal^R	This work
A. diazotrophicus	PAL 5	Ap^R	Cavalcante and Döbreiner, 1988
Citrobacter sp.	AJ1	Rif^R	Pleban, Israel, this work

Fungal species	Strain	Source
F. nyagamai	MRC 5993	W. Marasas, PROMEC
F. moniliforme	MRC 6148	W. Marasas, PROMEC
R. solani	from beans	ARC

Plasmid	Relevant genotype/phenotype	Ref/Source
pLCHIA	*chiA*, pL, Ap^R	Shapira *et al.*, 1986
pKT240	Km^R, Ap^R	Bagdasarian *et al*, 1983
pKT240**tac*	Km^R, Ap^R, P*tac*	Saunders, UCT
p*tacchiA*	Ap^R, *chiA*, P*tac*	Oppenheim, Israel
pUC19	Ap^R	Vieira and Messing, 1982
pKK223-3	Ap^R, P*tac*, *rrnB* T_1, T_2	Pharmacia Biotech
pML122	Gm^R, 'Nm, PNm	Labes *et al*, 1990
pDER405	Cm^R, Tc^R	Rawlings *et al*, 1986
pJFF350	Km^R	Fellay *et al*, 1989
pMAL-c	Ap^R, P*tac*, *mal*E-*lacZ*, *lacI*^{q}	New England Biolabs
pBS42	Km^R, *cryIIIA*	Baldani, Brazil

APPENDIX D

CLONING VECTORS

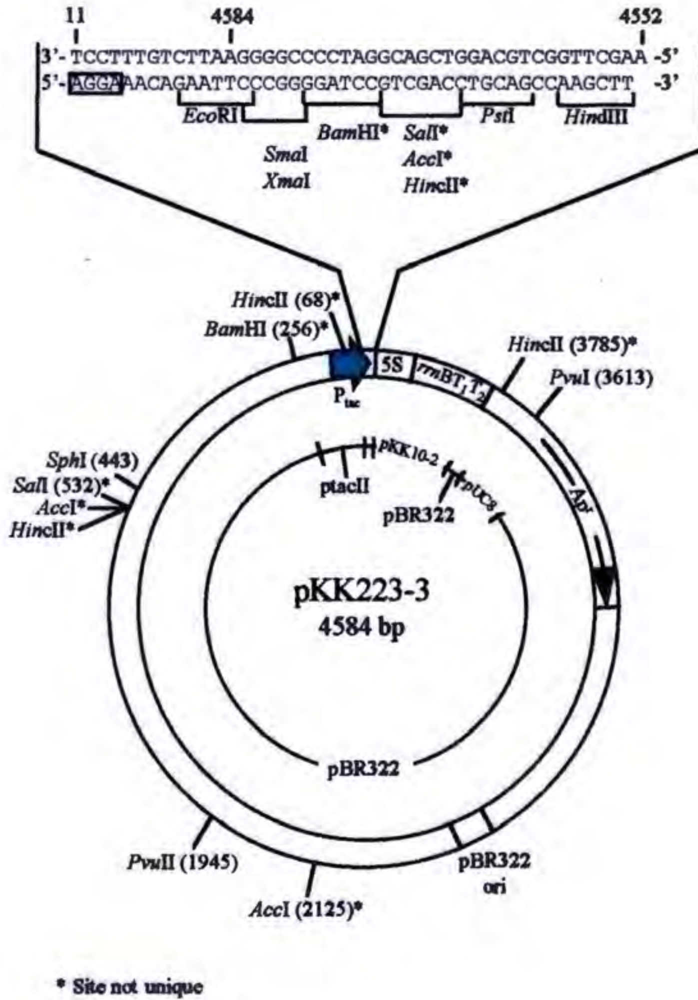


FIG. D.1 Physical map of the expression vector pKK223-3 (Pharmacia Biotech; Brosius and Holy, 1984) carrying the strong *tac* promoter, the pUC8 MCS, and the strong *rrn*B ribosomal terminator. Genes containing a ribosome binding site (RBS) and an ATG codon may be expressed by insertion into any unique site in the MCS. The RBS of the vector can be utilized if the start codon of the insert is within 5-9 bp from it.

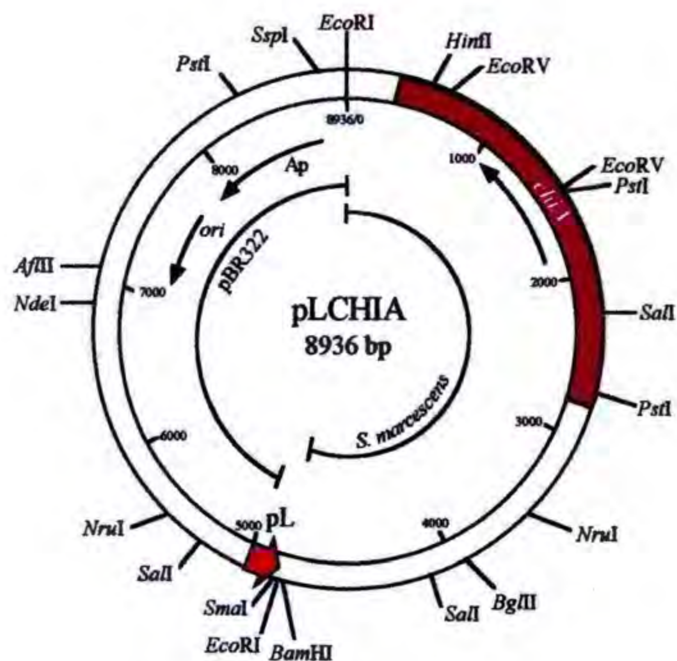


FIG. D.2 Physical map of the plasmid expressing the *S. marcescens chiA* gene under the control of the bacteriophage λ pL promoter. A 4.7-kb *Bam*HI/*Eco*RI fragment carrying the *chiA* gene was subcloned into pBR322 containing the promoter on a 250-bp *Bgl*II/BamHI fragment (Shapira *et al.*, 1989).

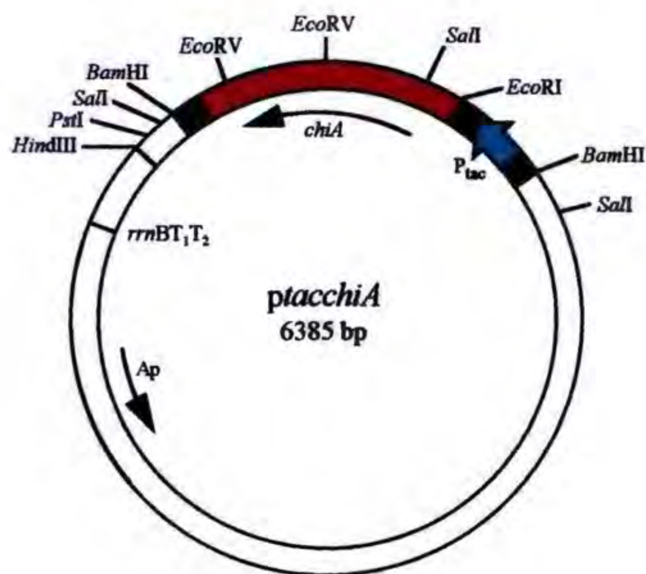


FIG. D.3 Physical map of the plasmid carrying the *chiA* gene under the control of the *tac* promoter. A 1.8-kb *Hinf*I fragment from pLCHIA was introduced into the *Sma*I site of pKK223-3 (Koby *et al.*, 1994; A. Oppenheim, personal communication).

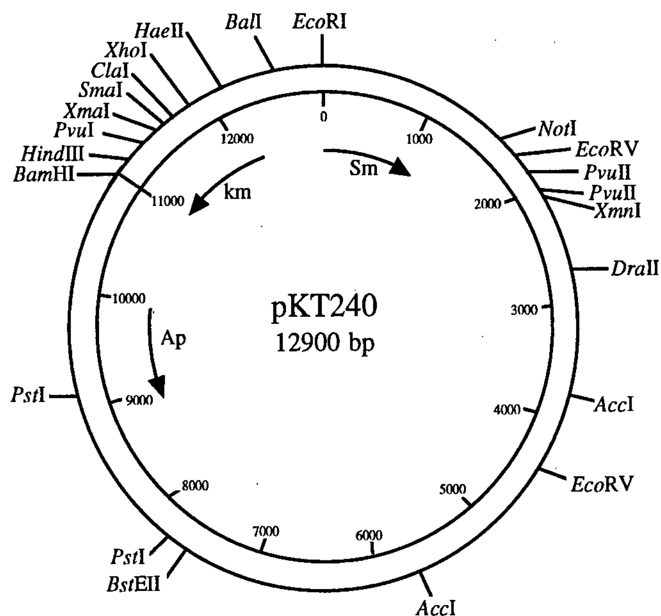


FIG. D.4 Physical map of the broad-host-range vector pKT240 containing the Km^R, Ap^R and promoterless Sm^R genes on RSF1010. (Bagdasarian *et al.*, 1983).

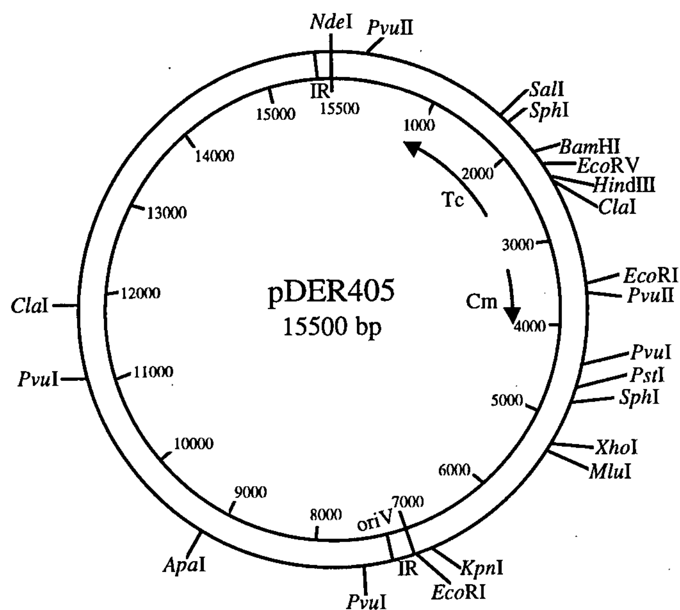


FIG. D.5 Physical map of the broad-host-range plasmid pDER405 constructed by cloning a 4.2-kb *PstI/NdeI* fragment, carrying the mobilization region from the *Thiobacillus ferrooxidans* plasmid pTF-FC2, into pBR325 (Rawlings *et al.*, 1986).

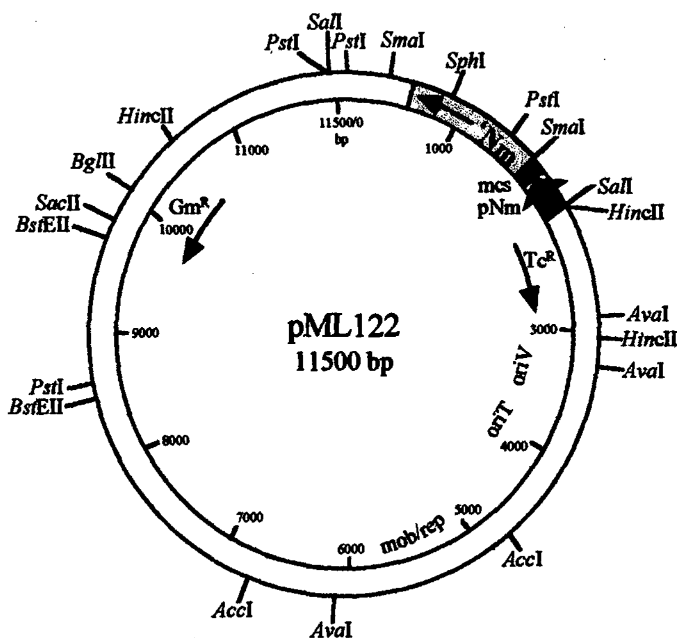


FIG. D.6 Physical map of pML122 consisting of the pSUP104-derived pML10 carrying the Gm^R gene, the promoter of the Nm^R gene (pNm), a MCS and promoterless Nm^R gene ('Nm) which serves to monitor expression as it will only be expressed if the cloned gene does not carry a strong terminator. The Tc^R gene is inactive (Labes *et al.*, 1990).

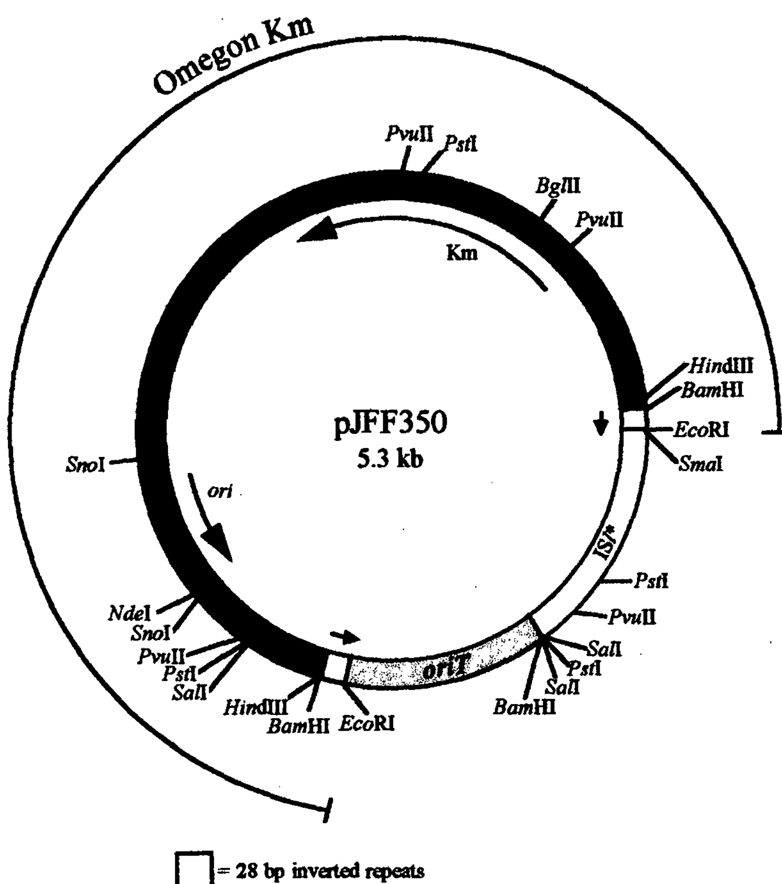


FIG. D.7 Physical map of the integration vector pJFF350 which carries the pBR322 origin of replication, the Omegon-Km (dark blue and red respectively) interposon flanked by synthetic inverted repeats (yellow and small arrows), a disabled IS*1* element (IS*1**) and the *oriT* of RP4 (light blue). The IS*1** element is still able to contribute functional transposition products, but both ends have mutations in the terminal two nucleotides and are no longer active in transposition because both ends are no longer active in transposition (Fellay *et al.*, 1989).

ABBREVIATIONS

Ap	ampicillin
ARC	Agricultural Research Council
BCIP	5-bromo-4-chloro-3-indol phosphate toluidine salt
bp	base pair
BSA	bovine serum albumin
C-	carboxy terminal (end of protein)
CFU	colony forming units
Cm	chloramphenicol
CM-CHITIN-RBV	carboxymethylated chitin attached to remazol brilliant violet
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetra-acetic acid
ELISA	Enzyme-Linked-Immunosorbent Assay
Gm	gentamycin
h	hour (s)
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
KB	Kings B medium
kDa	kilodalton
Km	Kanamycin
LB	Luria-Bertani broth
min	minute (s)
MRC	Medical Research Council
N-	amino terminal (end of protein)
NBT	p-nitro blue tetrazolium chloride
ND	not determined
Nm	neomycin
OD	optical density

p	plasmid
P	promoter
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
pNP	p-Nitrophenol
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
R	(superscript) resistance
RBS	Ribosome binding site
Rif	Rifampicin
SASEX	South African Sugarcane Experiment Station
SD	Shine Dalgarno sequence
SDS	sodium dodecyl sulphate
Sm	Streptomycin
TAE	tris-acetate EDTA buffer
TBE	tris-borate EDTA buffer
Tc	teracycline
Tn	transposon
UV	ultraviolet light
::	novel joint (fusion)

REFERENCES

Anderson, N.A. 1982. The genetics and pathology of *Rhizoctonia solani*. Annu. Rev. Phytopathol. **20**:329-347.

Armitage, P., R. Walden, and J. Draper. 1988. Vectors for the transformation of plant cells using Agrobacterium. *In* Draper. D., R. Scott, P. Armitage, and W. Walden (eds). Plant genetic transformation and gene expression, A laboratory manual, Blackwell Scientific Publications Ltd, Oxford.

Ashbolt, N.J., and Inkerman, P.A. 1990. Acetic acid bacterial biota of the pink sugar cane Mealybug, *Saccharococcus sacchari,* and its environs. Appl. Environ. Microbiol. **56:**707-712.

Atkinson, P.R. 1979. Distribution and natural hosts of *Eldana saccharina* Walker in Natal, its oviposition sites and feeding patterns. Proc. S. Afr. Sug. Technol. Ass. **53:**111-115.

Atkinson, P.R., and K.J. Nuss. 1989. Associations between host-plant nitrogen and infestations of the sugarcane borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Bull. Ent. Res. **79:**489-506.

Austin, P.R., C.J. Brine, J.E. Castle, and J.P. Zikakis. 1981. Chitin: New facets of research. Science **212:** 749-753.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J. Smith, and K. Struhl. 1992. Current Protocols in Molecular Biology. Greene Publishing Associates and John Wiley & Sons, New York.

Bagdasarian, M., R. Lurz, B. Rückert, F.C.H. Franklin, M.M. Bagdasarian, J. Frey, and K.N. Timmis. 1981. Specific-purpose plasmid cloning vectors II. Broad host range, high copy numbers, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene **16:**237-247.

Bagdasarian, M. M, E. Amann, R. Lurz, B. Rückert, and M. Bagdasarian. 1983. Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida.* Construction of broad-host-range, controlled-expression vectors. Gene **26:**273-282.

Bao, Y., D. P. Lies, H. fu, and G.P. Roberts. 1991. An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene **109:**167-168.

Baldani, J.I., V.L.D Baldani, L. Seldin, and J. Döbreiner. 1986. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. Int. J. Syst. Bacteriol. **36:**86-93.

Baum, J.A., and T. Malvar. 1995. Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. Mol. Microbiol. **18:**1-12.

Ben-Dov, E., S. Boussiba, and A. Zaritsky. 1995. Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **177:**2851-2857.

Blaak, H., J. Schnellmann, S. Walter, B. Henrissat, and H. Schrempf. 1993. Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. Eur. J. Biochem. **214:**659-669.

Blaak, H., and H. Schrempf. 1995. Binding and substrate specificities of a *Streptomyces olivaceoviridis* chitinase in comparison with its proteolytic processed form. Eur. J. Biochem. **229:** 132-139.

Blackwell, J. 1988. Physical methods for the determination of chitin structure and conformation. Methods in Enzymology **161:** 435-442.

Blakeman, J.P., and N.J. Fokkema. 1982. Potential for biological control of plant diseases on the phylloplane. Annu. Rev. Phytopathol. **20:**167-192.

Boller, T, and F. Mauch. 1988. Colorimetric assay for chitinase. Methods in Enzymology **161:**430-435.

Boddey, R. M. 1995. Biological nitrogen fixation in sugar cane: A key to energetically viable biofuel production. Critical Reviews in Plant Science **14:**263-279.

Bork, P., and R. F. Doolittle. 1992. Proposed acquisition of an animal protein domain by bacteria. Proc. Natl. Acad. Sci. USA **89:**8990-8994.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.

Broadway, R. A., D. L. Williams, W. C. Kain, G. E. Harman, M. Lorito, and D. P. Labeda. 1995. Partial characterization of chitinolytic enzymes from *Streptomyces albidoflavus.* Lett. Appl. Microbiol. **20:**271-276.

Broglie, K., I. Chet, M. Holliday, R. Cressman, P. Biddle, S. Knowlton, C. J. Mauvals, and R. Broglie. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani.* Science **254:**1194-1197.

Brosius, J. 1984. Toxicity of an overproduced foreign product in *Escherichia coli* and its use in plasmid vectors for the selection of transcription terminators. Gene **27:**161-172.

Brosius, J., and A. Holy. 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. **81:**6929-6933.

Brurberg, M. B., V. G. H. Eijsink and I. F. Nes. 1994. Characterization of a chitinase gene (*chiA*) from *Serratia marcescens* BJL200 and one-step purification of the gene product. FEMS Microbiol. Lett. **124:**399-404.

Brurberg, M. B., V. G. H. Eijsink, A. J. Haandrikman, G. Venema, and I. F. Nes. 1995. Chitinase B from *Serratia marcescens* BJL200 is exported to the periplasm without processing. Microbiology **141:**123-131.

Brurberg, M. B., I. F. Nes, and V. G. H. Eijsink. 1996. Comparative studies of chitinases A and B from *Serratia marcescens*. Microbiology **142:**1581-1589.

Bullock, W. O., and C. Sollod. 1995. The business side of Bt products: A market analysis. Information Systems for Biotechnology News Report - December p15-17.

Caballero-Mellado, J., and E. Martinez-Romero. 1994. Limited genetic diversity in the endophytic sugarcane bacterium *Acetobacter diazotrophicus*. Appl. Environ. Microbiol. **60:**1532-1537.

Cabib, E. 1988. Assay for chitinase using tritiated chitin. Methods in Enzymology. **161:**424-426.

Carlton, B. C. 1995. Developing new recombinant strains of Bt. Information Systems for Biotechnology News Report - December p12-14.

Carnegie, A. J. M., and G. W. Leslie. 1979. Attempts at the biological control of *Eldana saccharina* Walker (Lepidoptera: Pyralididae). Proc. S. Afr. Sug. Technol. Ass. **53:**116-119.

Carnegie, A. J. M., and G. W. Leslie. 1990. *Eldana saccharina* (Lepidoptera: Pyralidae): Ten years of light trapping. Proc. S. Afr. Sug. Technol. Ass. **64:**107-110.

Carnegie, A. J. M., D. E. Conlong, and D. Y. Graham. 1985. Recent introductions of parasitoids against *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Proc. S. Afr. Sug. Technol. Ass. **59:**160-163.

Cavalcante, V. A., and J. Döbreiner. 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant and Soil **108:**23-31.

Chambers, J. A., A. Jelen, M. P. Gilbert, C. S. Jany, T. B. Johnson, and C. Gawron-Burke. 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. J. Bacteriol. **173:**3966-3976.

Chen, C., E.M. Bauske, G. Musson, R. Roderíguez-Kábana, and J.W. Kloepper. 1995. Biological control of Fusarium wilt on cotton by use of endophytic bacteria. Biological Control **5:**83-91.

Chen, J. P., F. Nagayama, and M. C. Chang. 1991. Cloning and expression of a chitinase gene from *Aeromonas hydrophila* in *Escherichia coli*. Appl. Environ. Microbiol. **57:**2426-2428.

Chernin, L., A. Brandis, Z. Ismailov, and I. Chet. 1996. Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. Curr. Microbiol. **32:**208-212.

Chernin, L., Z. Ismailov, S. Haran, and I. Chet. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl. Environ. Microbiol. **61:**1720-1726.

Chet, I., Z. Barak, and A. Oppenheim. 1993. Genetic engineering of microorganisms for improved biocontrol activity, p 211-235. *In* Chet, I (ed.), Biotechnology in plant disease control. Wiley-Liss, Inc., New York.

Chung, C. T., and H. Miller. 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucl. Acid Res. **16:**3580.

Cojho, E. H., V. M. Reis, A. C. G. Schenberg and J. Döbreiner. 1993. Interactions of *Acetobacter diazotrophicus* with an amylolytic yeast in nitrogen-free batch culture. FEMS Microbiol. Lett. **106:** 341-346.

Conlong, D. E., and H. Hastings. 1984. Evaluation of egg parasitoids in the biological control of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Proc. S. Afr. Sug. Technol. Ass. **58:**168-172.

Convents, D., C. Houssier, I. Lasters, and M. Lauwereys. 1990. The *Bacillus thuringiensis* δ-endotoxin. J. Biol. Chem. **265:**1369-1375.

Davison, J. 1988. Plant beneficial bacteria. Bio/Technology **6:**282-286.

De Barjac, H., and E. Frachon. 1990. Classification of *Bacillus thuringiensis* strains. Entomophaga **35:**233-240.

De Boer, H.A, L.J. Comstock, D.G. Yansura, and H.L. Heyneker. 1982. Construction of a tandem *trp-lac* promoter and a hybrid *trp-lac* promoter for efficient and controlled expression of the Human Growth Hormone gene in *Escherichia coli* p 462-481. *In* Rodriguez, R.L, and M.J. Chamberlin (eds.), Promoters: Structure and function. Praeger.

De Boer, H.A., L.J. Comstock, and M. Vasser. 1983. The *tac* promoter: A functional hybrid derived from the *trp* and *lac* promoters. Proc. Natl. Acad. Sci. **80:**21-25.

De Boer, S.H., and R.J. Copeman. 1974. Endophytic bacterial flora in *Solanum tuberosum* and its significance in bacterial ring rot diagnosis. Can. J. Plant Sci. **54:**115-122.

Delannay, X., B. J. LaVallee, R. K. Proksch, R. L. Fuchs, S. R. Sims, J. T. Greenplate, P. G. Marrone, R. B. Dodson, J. J. Augustine, J. G. Layton, and D. A Fischhoff. 1989. Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. *kurstaki* insect control protein. Bio/Technology **7:**1265-1269.

de Maagd, R.A., M.S.G. Kwa, H. van der Klei, T. Yamamoto, B. Schipper, J.M. Vlak, W.J. Stiekema, and D. Bosch. 1996a. Domain III substitutions in *Bacillus thuringiensis* delta-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Appl. Environ. Microbiol. **62:**1537-1543.

de Maagd, R.A., H. van der Klei, P. Bakker, W.J. Stiekema, and D. Bosch. 1996b. Different domains of *Bacillus thuringiensis* δ-endotoxins can bind to insect midgut membrane proteins on ligand blots. Appl. Environ. Microbiol. **62:**2753-2757.

DeWald, S. 1995. Bt insecticidal crystal proteins and their mode of action. Information Systems for Biotechnology News Report - December p 2-3.

Dickie, G.A, and C.R. Bell. 1995. A full factorial analysis of nine factors influencing *in vitro* antagonistic screens for potential biocontrol agents. Can. J. Microbiol. **41:**284-293.

Döbreiner, J. 1988. Biotechnology: Recent advances in biological nitrogen fixation. *In* VI Japan-Brazil symposium on science and technology, Vol IV, Academia de Ciência do Estado de São Paulo Publicação ACIESP.

Döbreiner, J., V. M. Reis, M. A. Paula, and F. Olivares. 1993. Endophytic diazotrophs in sugar cane, cereals and tuber plants p 671-676. *In* Palacios, R. *et al.* (eds.), New horizons in Nitrogen Fixation. Kluwer Academic Publishers, Netherlands.

Döbreiner, J., V. L. D. Baldani, and V. M. Reis. 1995. Endophytic occurrence of diazotrophic bacteria in non-leguminous crops p 3-14. *In* Fendrik, I. *Et al.* (eds.), *Azospirillum* VI and related microrganisms. Springer-Verlag, Berlin, Heidelberg.

Dong, Z., M. Heydrich, K. Bernard, and M. E. McCully. 1995. Further evidence that the N_2-fixing endophytic bacterium from the intercellular spaces of sugarcane stems is *Acetobacter diazotrophicus*. Appl. Environ. Microbiol. **61:**1843-1846.

Du, C., and K. W. Nickerson. 1996. The *Bacillus thuringiensis* insecticidal toxin binds biotin-containing proteins. Appl. Environ. Microbiol. **62:**2932-2939.

Elad,Y., I. Chet, and Y. Henis. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*

Elad,Y., I. Chet, P. Boyle, and Y. Henis. 1983. Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii*- Scanning electron microscopy and fluorescence microscopy. Phytopathology. **73:**85-88.

Feitelson, J. S., J. Payne, and L. Kim. 1992. *Bacillus thuringiensis*: insects and beyond. Bio/Technology **10:**271-275.

Fellay, R., J. Frey and H. Krisch. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. Gene **52:**147-154.

Fellay, R., H.M. Krisch, P.Prentki, and J. Frey. 1989. Omegon-Km: a transposable element designed for *in vivo* insertional mutagenesis and cloning of genes in Gram-negative bacteria. Gene **76:**215-226.

Fischhoff, D. A., K. S. Bowdish, F. J. Perlak, P. G. Marrone, S. M. McCormick, J. G. Niedermeyer, D. A. Dean, K. Kunsano-Kretzmer, E. J. Mayer, D. E. Rochester, S. G. Rogers, and R.T. Fraley. 1987. Insect tolerant transgenic tomato plants. Bio/Technology **5:**807-813.

Fiuza, L., C. Nielsen-Leroux, E. Gozé, R. Frutos, and J. Charles. 1996. Binding of *Bacillus thuringiensis* CryI toxins to the midgut brush border membrane vesicles of *Chilo suppressalis* (Lepidoptera: Pyralidae): Evidence of shared binding sites. Appl. Environ. Microbiol. **62:**1544-1549.

Flach, J., P.-E. Pilet, and P. Jollès. 1992. What's new in chitinase research?. Experientia **48:**701-716.

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

Fujii, T., and K. Miyashita. 1993. Multiple domain structure in a chitinase gene (*chiC*) of *Streptomyces lividans*. J. Gen. Microbiol. **139:** 677-686.

Fujimoto, H., K. Itoh, M. Yamamoto, J. Kyozuka, and K. Shimamoto. 1993. Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Bio/Technology **11:**1151-1155.

Gaffney, T.D., S.T. Lam, J. Ligon, K. Gates, A. Frazelle, J.D. Malo, S. Hill, S. Goodwin, N. Torkewitz, A.M. Allshouse, H.-J. Kempf, and J.O. Becker. 1994. Global regulation of expression of antifungal factors by a *Pseudomonas fluorescens* biological control strain. Molec. Plant Microbe Interactions **7:**455-463.

Gagné, S., C. Richard, H. Rousseau, and H. Antoun. 1987. Xylem-residing bacteria in alfalfa roots. Can. J. Microbiol. **33:**996-1000.

Gardner, J.M., A.W. Feldman, and R.M. Zablotowicz. 1982. Identity and behavior of Xylem-resisding bacteria in rough lemon roots of Florida citrus trees. Appl. Environ. Microbiol. **43:**1335-1342.

Gardner, J.M., J.L. Chandler, and A.W. Feldman. 1985. Growth responses and vascular plugging of citrus inoculated with rhizobacteria and xylem-resident bacteria. Plant and Soil **86:**333-345.

Ge, A. Z., R. M. Pfister, and D. H. Dean. 1990. Hyperexpression of a *Bacillus thuringiensis* delta-endotoxin-encoding gene in *Escherichia coli*: properties of the product. Gene **93:**49-54.

Gentz, R., A. Langner, A. C. Y. Chang, S. N. Cohen, and H. Bujard. 1981. Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. Proc. Natl. Acad. Sci. **78:**4936-4940.

Gill, S. S, E. A. Cowles, and V. Francis. 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIA(c) toxin-binding protein from the midgut of the Lepidopteran insect *Heliothis virescens*. J. Biol. Chem. **270:**27277-27282.

Gillis, M., K. Kersters, B. Hoste, D. Janssens, R. M. Kroppenstedt, M. P. Stephan, K. R. S. Teixeira, and J. De Ley. 1989. *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. Int. J. Syst. Bacteriol. **39:**361-364.

Gould, F. 1995. The empirical and theoretical basis for Bt resistance management. Information Systems for Biotechnology News Report - December p3-5.

Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. J. Silva, and W. Moar. 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. **89:**7986-7990.

Grochulski,P., L. Masson, S. Borisova, M. Pusztai-Carey, J-L. Schwartz, R. Brousseau, and M. Cygler. 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. **254**:447-464.

Grison, R., B. Grezes-Besset, M. Schneider, N. Lucante, L. Olsen, J-J. Leguay, and A. Toppan. 1996. Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nature Biotechnology. **14**:643-646.

Haran, S., H. Schickler, S. Pe'er, S. Logemann, A. Oppenheim, and I. Chet. 1993. Increased constitutive chitinase activity in transformed *Trichoderma harzianum.* Biol. Control **3**:101-108.

Haran, S., H. Schickler, A. Oppenheim, and I. Chet. 1995. New components of the chitinolytic system of *Trichoderma harzianum.* Mycol. Res. **4**:441-446.

Harman, G. E., C. K. Hayes, M. Lorita, A. Di Pietro, C. Peterbauer, and A. Tronsmo. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: Purification of chitobiosidase and endochitinase. Phytopathology **83**: 313-318.

Harpster, M. H., and P. Dunsmuir. 1989. Nucleotide sequence of the chitinase B gene of *Serratia marcescens* QMB1466. Nucl. Acids Res. **17**:5395.

Heathcote, R.J. 1984. Insecticide testing against *Eldana saccharina* Walker. Proc. S. Afr. Sug. Technol. Ass. **58**:154-164.

Henrissat, B., and A. Bairoch. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J. **293**:781-788.

Herrera, G. 1994. Biological control of *Eldana saccharina* Walker using a cloned *Bacillus thuringiensis cry* gene. PhD thesis. University of Cape Town.

Herrera, G., S. J. Snyman and J. A. Thomson. 1994. Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugarcane borer, *Eldana saccharina.* Appl. Environ. Microbiol. **60**:682-690.

Hill, D.S., J.I. Stein, N. R. Torkewitz, A.M. Morse, C.R. Horwell, J. P. Pachlatko, J.O. Becker, and J.M. Ligon. 1994. Cloning of genes involved in the synthesis of pyrrolnitrin from *Pseudomonas fluorescens* and role of pyrrolnitrin synthesis in biological control of plant disease. Appl. Environ. Microbiol. **60**:78-85.

Hinton, D. M., and C. W. Bacon. 1995. *Enterobacter cloacae* is an endophytic symbiont of corn. Mycopathologia **129**:117-125.

Ho Yap, W., T. Thanabalu, and A. G. Porter. 1994. Expression of mosquitocidal toxin genes in a gas-vacuolated strain of *Ancylobacter aquaticus*. Appl. Environ. Microbiol. **60**:4199-4202.

Hofmann, C., H. Vanderbruggen, H. Höfte, J. Van Rie, S. Jansens, and H. Van Mellaert. 1988. Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. **85**:7844-7848.

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

Hollis, J.P. 1951. Bacteria in healthy potato tissue. Phytopathology **41**:350-366.

Ilyina, A., V. E. Tikhonov, V. P. Varlamov, L. A. Radigina, N. Y. Tatarinova, and I. A. Yamskov. 1995. Preparation of affinity sorbent and isolation of individual chitinases from a crude supernatant produced by *Streptomyces kurssanovii* by a one-step affinity-chromatographic system. Biotechnol. Appl. Biochem. **21:**139-148.

Inbar, J., and I. Chet. 1991. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. Soil Biol. Biochem. **23:**973-978.

Inbar, J., and I. Chet. 1994. A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii:* purification, characterization and its role in mycoparasitism. Microbiol. **140:**651-657.

Ish-Horowicz, D., and J.F. Burke. 1981. Rapid and efficient cosmid cloning. Nucl. Acids Res. **9:**2989-2998.

Jacobs, S. J. 1989. Micro-organisms as potential biological control agents of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Proc. S. Afr. Sug. Technol. Ass. **63:**186-188.

Jacobs, M.J., W.M. Bugbee, and D.A. Gabrielson. 1985. Enumeration, location, and characterization of endophytic bacteria within sugar beet roots. Can. J. Bot. **63:**1262-1265.

James, E. K., V. M. Reis, F. L. Olivares, J. I. Baldani, and J. Döbreiner. 1994. Infection of sugarcane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. J. Exp. Bot. **45:**757-766.

Jayaswal, R.K., M. Fernandez, R.S. Upadhyay, L. Visintin, M. Kurz, J. Webb, and K. Rinehart. 1993. Antagonism of *Pseudomonas cepacia* against phytopathogenic fungi. Curr. Microbiol. **26:**17-22.

Jones, J. D.G., K. L. Grady, T. V. Suslow, and J. R. bedbrook. 1986. Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO J. **5:**467-473.

Joshi, S., M. Kozlowski, G. Selvaraj, V. N. Iyer, and R. W. Davies. 1988. Cloning of the genes of the chitin utilization regulon of *Serratia liquefaciens*. J. Bacteriol. **170:**2984-2988.

Kaderbhai, N., J. Gallagher, M. He, and M. A. Kaderbhai. 1992. A pink bacterium as a receptor system signaling expression of a recombinant protein. DNA and Cell Biology **11:**567-577.

Kado, C.I. 1992. The Prokaryotes. Vol1, 2nd Ed. Springer-Verlag, New York.

Kalix, S., and H. Buchenauer. 1995. Direct detection of β-1,3-glucanase in plant extracts by polyacrylamide gel electrophoresis. Electrophoresis **16:** 1016-1018

King, E.O., M.K. Ward, D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301-307.

Kleiner, K.W, D.D. Ellis, B.H. McCown, and K.F. Raffa. 1996. Field evaluation of transgenic poplar expressing a *Bacillus thuringiensis cryIA(a)* d-endotoxin gene against forest tent caterpillar (Lepidoptera: Lasiocampidae) and gypsy moth (Lepidoptera: Lymantriidae) following winter dormancy. Biological control **24:**1358-1364.

Kless, H., Y. Sitrit, I. Chet, and A. Oppenheim. 1989. Cloning of the gene coding for chitobiase of *Serratia marcescens*. Mol. Gen. Genet. **217:**471-473.

Kloepper, J.W., and M.N. Scroth. 1981. Relationship of *in vitro* antibiosis of plant growth-promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology **71:**1020-1024.

Kluepfel, D.A. 1993. The behavior and tracking of bacteria in the rhizosphere. Annu. Rev. Phytopathol. **31**:441-472.

Ko, W., and F.K. Hora. 1971. A selective medium for the quantitative determination of *Rhizoctonia solani* in soil. Phytopathology **61**:707-710.

Koby, S., H. Schickler, I. Chet and A. Oppenheim. 1994. The chitinase encoding Tn7-based *chiA* gene endows *Pseudomonas fluorescens* with the capacity to control plant pathogens in soil. Gene **147**:81-83.

Kozak, M. 1985. Comparison of initiation of protein synthesis in Procaryotes, Eukaryotes and organelles. Microbiol. Rev. **47**:1-45

Kronstad, J. W., and H. R. Whiteley. 1986. Three classes of homologous *Bacillus thuringiensis* crystal-protein genes. Gene **43**:29-40.

Kyong Lee, M. I., A. Curtiss, E. Alcantara, and D. H. Dean. 1996. Synergistic effect of the *Bacillus thuringiensis* toxins CryIAa and CryIAc on the gypsy moth, *Lymantria dispar*. Appl. Environ. Microbiol. **62**:583-586.

Kyong Lee, M.I., T. H. You, B. A. Young, J. A. Cotrill, A. P. Valaitis and D. H. Dean. 1996. Aminopeptidase N purified from gypsi moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* CryIAc toxin. Appl. Environ. Microbiol. **62**:2845-2849.

Labes, M., A. Pühler, and R. Simon. 1990. A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for gram-negative bacteria. Gene **89**:37-46.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

Lam, S. T., and T. D. Gaffney. 1993. Biological activities of bacteria used in plant pathogen control p 291-320. *In* Chet, I (ed.), Biotechnology in plant disease control. Wiley-Liss, New York.

Lampel, J. S., G. L. Canter, M. B. Dimock, J. L. Kelley, J. J. Anderson, B. B. Uratani, J. S. Foulke JR., and J. T. Turner. 1994. Integrative cloning, expression and stability of the *cryIA(c)* gene from *Bacillus thuringiensis* subsp. *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp. *cynodontis*. Appl. Environ. Microbiol. **60**:501-508.

Lambert, B., and M. Perferoen. 1992. Insecticidal promise of *Bacillus thuringiensis*. BioScience **42**:112-122.

Lecadet, M-M., M-O. Blondel, and J. Ribier. 1980. Generalized transduction in *Bacillus thuringiensis* var. *berliner* 1715 using bacteriophage CP-54Ber. J. Gen. Microbiol. **121**:203-212.

Li, J., J. Carroll, and D.J. Ellar. 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature **353**:815-821.

Li, J., P.A. Koni, and D.J. Ellar. 1996. Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J. Mol. Biol. **257**:129-152.

Lin, W., C. S. Anuratha, K. Datta, I. Potrykus, S. Muthukrishnan, and S. K. Datta. 1995. Genetic engineering of rice for resistance to sheath blight. Bio/Technology **13**:686-691.

Liu, J., and J.S. Parkinson. 1989. Genetics and sequence analysis of the *pcnB* locus, an *Escherichia coli* gene involved in plasmid copy number control. J. Bacteriol. **171**:1254-1261.

Masson, L., A. Mazza, R. Brousseau, and B. Tabashnik. 1995. Kinetics of *Bacillus thuringiensis* toxin binding with brush border membrane vesicles from susceptible and resistant larvae of *Plutella xylostella*. J. Biol. Chem. **270:**11887-11896.

Matten, S. R., and P. I. Lewis. 1995. EPA and Bt plant-pesticide resistance management. Information Systems for Biotechnology News Report - December p5-7.

Miller, J. W. 1972. Experiments in molecular genetics. Cold Spring harbour Laboratory, Cold Spring harbour, N.Y.

Mishagi, I.J., and C.R. Donndelinger. 1990. Endophytic bacteria in symptom-free cotton plants. Phytopathology **80:**808-811.

Molano, J., A. Durán, and E. Cabib. 1977. A rapid and sensitive assay for chitinase using tritiated chitin. Analytical Biochemistry **83:**638-656.

Monreal, J., and E. T. Reese. 1969. The chitinase of *Serratia marcescens*. Can. J. Microbiol. **15:** 689-696.

Mundt, J.O., and N.F. Hinkle. 1976. Bacteria within ovules and seeds. Appl. Environ. Microbiol. **32:**694-698.

Obukowicz, M.G., F.J. Perlak, K. Kusano-Kretzmer, E.J. Mayer, and L.S. Watrud. 1986. Integration of the delta-endotoxin gene from *Bacillus thuringiensis* into the chromosome of root colonizing pseudomonads using Tn*5*. Gene **45:**327-331.

Obukowicz, M.G., F.J. Perlak, K. Kusano-Kretzmer, E.J. Mayer, S.L. Bolten, and L.S. Watrud. 1986. Tn*5*-mediated integration of the delta-endotoxin gene from *Bacillus thuringiensis* into the chromosome of root colonizing Pseudomonads. J. Bacteriol. **168:**982-989.

Oeda, K., K. Oshie, M. Shimizu, K. Nakamura, H. Yamamoto, I. Nakayama, and H. Ohkawa. 1987. Nucleotide sequence of the insecticidal protein gene of *Bacillus thuringiensis* strain *aizawai* IPL7 and its high-level expression in *Escherichia coli*. Gene **53:**113-119.

Ogoshi, A. 1987. Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annu. Rev. Phytopathol. **25:**125-143.

Ohno, T., S. Armand, T. Hata, N. Nikaidou, B. Henrissat, M. Mitsutomi, and T. Watanabe. 1996. A modular family 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT 6037. J. Bacteriol. **178:**5065-5070.

Ohtakara, A. 1988. Viscosimetric assay for chitinase. Methods in Enzymology **161:** 426-430.

Oppenheim, A.B., Y. Hirsch, S. Koby, and I. Chet. 1990. Protein secretion in biotechnology p 63-70. *In* White, M. D., S. Reuveny, and A. Shafferman (eds), Biologicals from recombinant microorganisms and animal cells, Production and recovery, Proceedings of the 34th Oholo Conference Eilat, Israel. Balaban Publishers, Weinheim, New York, Basel and Cambridge.

Oppenheim, A.B., and I. Chet. 1992. Cloned chitinases in fungal plant-pathogen control strategies. TIBTECH **10:**392-394.

Ordentlich, A., Y. Elad, and I. Chet. 1987. Rhizosphere colonization by *Serratia marcescens* for the control of *Sclerotium rolfsii*. Soil Biol. Biochem. **19:**747-751.

Ordentlich, A., Y. Elad, and I. Chet. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology. **78:**84-87.

Paula, M.A., V.M. Reis, and J. Döbreiner. 1991. Interactions of *Glomus clarum* with *Acetobacter diazotrophicus* in infection of sweet potato (*Ipomoea batatas*), sugarcane (*Saccharum* spp.), and sweet sorghum (*Sorghum vulgare*). Biol. Fertil. Soils **11:**111-115.

Perlak, F.J., R.W. Deaton, T.A. Armstrong, R.L. Fuchs, S.R. Sims, J.T. Greenplate, and D.A. Fischhoff. 1990. Insect resistant cotton plants. Bio/Technology **8:**941-943.

Perlak, F.J., R.L. Fuchs, D.A. Dean, S.L. McPherson, and D.A. Fischhoff. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. USA **88:**3324-3328.

Philipson, M.N., and I.D. Blair. 1957. Bacteria in clover root tissue. Can. J. Microbiol. **3:**125-129.

Pimentel, J. P., F. Olivares, R. M. Pitard, S. Urquiaga, F. Akiba, and J. Döbreiner. 1991. Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum seropedicae.* Plant and Soil **137:**61-65.

Pleban, S., F. Ingel, and I. Chet. 1995. Control of *Rhizoctonia solani* and *Sclerotium rolfsii* in greenhouse using endophytic *Bacillus* spp. Eur. J. Plant Pathol. **101:**665-672.

Prentki, P., and H.M. Krisch. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303-313.

Priefer, U.B., R. Simon, and A. Pühler. 1985. Extension of the host range of *Escherichia coli* vectors by incorporation of RSF1010 replication and mobilization functions. J. Bacteriol. **163:**324-330.

Rawlings, D.E., I-M. Pretorius, and D.R. Woods. 1986. Expression of *Thiobacillus ferrooxidans* plasmid functions and the development of genetic systems for the Thiobacilli. *In* Ehrlich, H.L., and D.S. Holmes (eds.), Biotechnology and Bioengineering Symp. **16:**281-287.

Reeser, P.W., and S.J. Kostka. 1988. Population dynamics of *Clavibacter xyli* subsp. *cynodontis* (CXC) and a CXC/*Bacillus thuringiensis* subsp. *kurstaki* (BT) recombinant in corn (*Zea Mays*). Phytopathology **78:**1540.

Reeser, P.W., and M.L. Sommerfeld. 1988. Seed transmission of *Clavibacter xyli* subsp. *cynodontis* (CXC) in corn. Phytopathology **78:**1540.

Regev, A., M. Keller, N. Strizhov, B. Sneh, E. Prudovsky, I. Chet, I. Ginzberg, Z. Koncz-Kalman, C. Koncz, J. Schell and A. Zilberstein. 1996. Synergistic activity of a *Bacillus thuringiensis* δ-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Appl. Environ. Microbiol. **62:**3581-3586.

Reis, V. M., F. L. Olivares, and J. Döbreiner. 1994. Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. World J. Microbiol. Biotech. **10:**401-405.

Ridout, C.J., J.R. Coley-Smith, and J.M. Lynch. 1986. Enzyme activity and electrophoretic profile of extracellular protein induced in *Trichoderma* spp. by cell walls of *Rhizoctonia solani*. J. Gen. Microbiol. **132:**2345-2352.

Robbins, P. W., C. Albright, and B. Benfield. 1988. Cloning and expression of a *Streptomyces plicatus* chitinase (Chitinase-63) in *Escherichia coli.* J. Biol. Chem. **263:** 443-447.

Roberts, R. L, and E. Cabib. 1982. *Serratia marcescens* chitinase: One-step purification and use for the determination of chitin. Anal. Biochem. **127:**402-412.

Roberts, W. K., and C. P. Selitrennikoff. 1988. Plant and bacterial chitinases differ in antifungal activity. J. Gen. Microbiol. **134:**169-176.

Sahai, A. S., and M. S. Manocha. 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol. Rev. **11:**317-338.

Salamitou, S., H. Agaisse, A. Bravo, and D. Lereclus. 1996. Genetic analysis of *cryIIIA* gene expression in *Bacillus thuringiensis*. Microbiology **142:**2049-2055.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbour, NY.

Samish, Z., R. Etinger-Tulczynska, and M. Bick. 1962. The microflora within the tissue of fruits and vegetables. J. Food Sci. **28:**259-266.

Sardi, P., M. Saracchi, S. Quaroni, B. Petrolini, G.E. Borgonovi, and S. Merli. 1992. Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. Appl. Environ. Microbiol. **58:**2691-2693.

Schippers, B., A.W. Bakker, and P.A.H.M. Bakker. 1987. Interactions of deleterious and beneficial microorganisms and the effect of cropping practises. Annu. Rev. Phytopathol. **25:**339-358.

Schnepf, H.E., H.C. Wong, and H.R. Whiteley. 1985. The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. J. Biol. Chem. **260:**6264-6272.

Schnider, U., C. Keel, C. Blumer, J. Troxler, G. Défago, and D. Haas. 1995. Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J. Bacteriol. **177:**5387-5392.

Shapira, R., A. Ordentlich, I. Chet, and A. Oppenheim. 1989. Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. Phytopathology **79:**1246-1249.

Sharrock, K.R., S.L. Parkes, H.K. Jack, J. Rees-George, B.T. Hawthorne. 1991. Involvement of bacterial endophytes in storage rots of buttercup squash (*Cucurbita maxima* D. hybrid 'Delica'). New Z. J. Crop Hort. Sci. **19:**157-165.

Sholberg, P.L., A. Marchi, and J. Bechard. 1995. Biocontrol of postharvest diseases of apple using *Bacillus* spp. isolated from stored apples. Can. J. Microbiol. **41:**247-252.

Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784-791.

Sitrit, Y., Z. Barak, Y. Kapulnik, A. B. Oppenheim, and I. Chet. 1993. Expression of *Serratia marcescens* chitinase gene in *Rhizobium meliloti* during symbiosis on alfalfa roots. MPMI **6:**293-298.

Sitrit, Y., C. E. Vorgias, I. Chet, and A. B. Oppenheim. 1995. Cloning and primary structure of the *chiA* gene from *Aeromonas caviae*. J. Bacteriol. **177:**4187-4189.

Smirnoff, W.A. 1971. Effect of chitinase on the action of *Bacillus thuringiensis*. Can. Entomol. **103:**1829-1831.

Sneh, B. 1981. Use of rhizosphere chitinolytic bacteria for biological control of *Fusarium oxysporum* f. sp. *dianthi* in Carnation. Phytopath. Z. **100:**251-256.

Sneh, B., J. Katan, Y. Henis, and I. Wahl. 1966. Methods for evaluating inoculum density of Rhizoctonia in naturally infested soil. Phytopathology **56:**74-78.

Snyman, S. J., K.G. Black, G. Herrera, and J. A. Thomson. 1993. *Pseudomonas fluorescens* genetically engineered to produce an insect toxin: A culmination of five years of collaborative research. Proc. S. Afr. Sug. Technol. Ass. **67:**78-81.

Stein, J., and R. Lotstein. 1995. Resistance management strategy for CIBA Seeds' transgenic Bt corn. Information Systems for Biotechnology News Report - December p 9-12.

Stephan, M. P., M. Oliveira, K. R. S. Teixeira, G. Martinez-Drets, and J. Döbreiner. 1991. Physiology and dinitrogen fixation of *Acetobacter diazotrophicus*. FEMS Microbiol. Lett. **77:**67-72.

Stone, T., and J. Feldman. 1995. Development of a comprehensive resistance management plan for Newleaf potatoes. Information Systems for Biotechnology News Report - December p 7-9.

Stüber, D., and H. Bujard. 1981. Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. **78:**167-171.

Streichan, M., and B. Schink. 1986. Microbial populations in wetwood of European white fir (*Abies alba* Mill.). FEMS Microbiol. Ecol. **38:**141-150.

Stroynowski, I., M. van Cleemput, and C. Yanofsky. 1982. Superattenuation in the tryptophan operon of *Serratia marcescens*. Nature **298:**38-41.

Tailor, R., J. Tippett, G. Gibb, S. Pells, D. Pike, L. Jordan, and S. Ely. 1992. Identification and characterization of a novel *Bacillus thuringiensis* δ-endotoxin entomocidal to coleopteran and lepidopteran larvae. Mol. Microbiol. **6:**1211-1217.

Takayanagi,T., K. Ajisaka, Y. Takiguchi, and K. Shimahara. 1991. Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. Biochim. Biophys. Acta **1078:**404-410.

Tang, J.D., A.M. Shelton, J. Van Rie, S. De Roeck, W.J. Moar, R.T. Roush, and M. Peferoen. 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella).* Appl. Environ. Microbiol. **62:**564-569.

Tester, C.F. 1992. Influence of a genetically modified endophytic bacterium on composition and decomposition of corn resisdue. Soil Biol. Biochem. **24:**1107-1112.

Tews, I., R. Vincentelli, and C. E. Vorgias. 1996. N-acetylglucosaminidase (chitobiase) from *Serratia marcescens* gene sequence and protein production and purification in *Escherichia coli*. Gene **170**:63-67.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Proc. Natl. Acad. Sci. USA. **76:**4350-4354.

Tronsmo, A., and G. E. Harman. 1993. Detection and quantification of N-acetyl-β-D-glucosaminidase, chitobiosidase, and endochitinase in solutions and on gels. Anal. Biochem. **208:**74-79.

Takayanagi, T., K. Ajisaka, Y. Takiguchi, and K. Shimahara. 1991. Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. Biochim. Biophys. Acta **1078:**404-410.

Tsujibo, H., H. endo, K. Miyamoto, and Y. Inamori. 1995. Expression in *Escherichia coli* of a gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520.

Turner, J.T., and S.F. Tomasino. 1988. Persistence of *Clavibacter xyli* subsp. *cynodontis* (CXC) and a (CXC)/*bacillus thuringiensis* recombinant (CXC/BT) in soil, water and plant debris. Phytopathology **78:**1541.

Turner, J.T., J.S. Lampel, R.S. Stearman, G.W. Sundin, P. Gunyuzlu, and J.J. Anderson. 1991. Stability of the δ-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp. *cynodontis*. Appl. Environ. Microbiol. **57:**3522-3528.

Vaeck, M., A. Reynaerts, H. Höfte, S. Jansens, M. De Beuckeleer, C. Dean, M. Zabeau, M. Van Montagu and J. Leemans. 1987. Transgenic plants protected from insect attack. Nature **328:**33-37.

Van der Salm, T., D. Bosch, G. Honée, L. Feng, E. Munsterman, P. Bakker, W.J. Stiekema, and B. Visser. 1994. Insect resistance of transgenic plants that express modified *Bacillus thuringiensis cryIA(b)* and *cryIC* genes: a resistance management strategy. Plant Mol. Biol. **26:**51-59.

Van Rie, J., S. Jansens, H. Höfte, D. Degheele, and H. Van Mellaert. 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Appl. Environ. Microbiol. **56:**1378-1385.

Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259-268.

Vierheilig, H., M. Alt, J-M. Neuhaus, T. Boller, and A. Wiemken. 1993. Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. MPMI **6:**261-264.

Visser, B., E. Munsterman, A. Stoker, and W.G. Dirkse. 1990. A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua*-specific crystal protein. J. Bacteriol. **172:**6783-6788.

Waalwijk, C., A. Dullemans, and C. Maat. 1991. Construction of a bioinsecticidal rhizosphere isolate of *Pseudomonas fluorescens*. FEMS Microbiol. Lett. **77:**257-264.

Watanabe, T., W. Oyanagi, K. Suzuki, and H. Tanaka. 1990a. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. J. Bacteriol. **172:**4017-4022.

Watanabe, T., K. Suzuki, W. Oyanagi, K. Ohnishi, and H. Tanaka. 1990b. Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the Type III homology units of fibronectin. J. Biol. Chem. **265:**15659-15665.

Watanabe, T., W. Oyanagi, K. Suzuki, K. Ohnishi, and H. Tanaka. 1992. Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. J. Bacteriol. **174:**408-414.

Watanabe, T., K. Kobori, K. Miyashita, T. Fujii, H. Sakai, M, Uchida and H. Tanaka. 1993. Identification of Glutamic acid 204 and Aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. J. Biol. Chem. **268:**18567-18572.

Watanabe, T., Y. Ito, T. Yamada, M. Hashimoto, S. Sekine and H. Tanaka. 1994. The roles of the C-terminal domain and type III domains if chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. J. Bacteriol. **176:**4465-4472.

Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379-407.

Whitesides, S. K., and R.A. Spotts. 1991. Frequency, distribution, and characteristics of endophytic *Pseudomonas syringae* in pear trees. Phytopathology **81:**453-457.

Wider, W.R., and H.R. Whitely. 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol. **171:**965-974

Wirth, R., A. Friesenegger, and S. Fiedler. 1989. Transformation of various species of Gram-negative bacteria belonging to 11 different genera by electroporation. Mol. Gen. Genet. **216:**175-177.

Wirth, S.J., and G.A. Wolf. 1990. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. J. Microbiol. Meth. **12:**197-205.

Wortman, A.T., C.C. Somerville, and R.R. Colwell. 1986. Chitinase determinants of *Vibrio vulnificus:* Gene cloning and applications of a chitinase probe. Appl. Environ. Microbiol. **52:**142-145.

Wu, D., X.L. Cao, Y.Y. Bai, and A.I. Aronson. 1991. Sequence of an operon containing a novel δ-endotoxin gene from *Bacillus thuringiensis*. FEMS Microbiol. Lett. **81:**31-36.

Yanish-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of M13mp18 and pUC19 vectors. Gene **33:**103-119.