CLONING AND SEQUENCE ANALYSIS OF A NOVEL *BACILLUS THURINGIENSIS* GENE ENCODING DELTA ENDOTOXIN ACTIVE AGAINST TSETSE (*GLOSSINA MORSITANS*) AND STEMBORER (*CHILO PARTELLUS*)

BY

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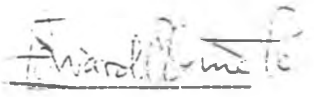
DECLARATION

I, Edward Enos Orony-Omolo, hereby declare that this thesis is my original work and the work described here has not been presented to any other University.



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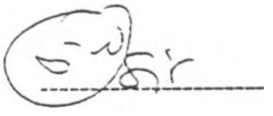

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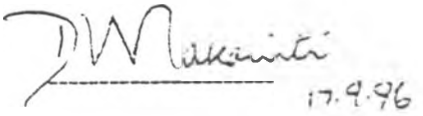

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DEDICATION

THIS THESIS IS DEDICATED TO MY LATE BROTHER AND MENTOR JUSTUS NGOJE OMOLO. MY PARENTS MAMA FLORA KORE OMOLO AND THE LATE JADUON'G ERASTO OMOLO; WIFE ROSE OWILI OMOLO; MY BROTHERS AND SISTERS FOR LOVE, PRAYERS, ENCOURAGEMENT AND SUPPORT THROUGHOUT MY WHOLE LIFE; TO MY SONS ELIJAH MATHEWS OMOLO AND EDWARD ORONY OMOLO JR. FOR THE MANY HOURS THEY HAD TO SPEND WITHOUT THEIR FATHER AROUND. ELIJAH AND EDWARD NEVER UNDERSTOOD WHY. BUT BORE IT SO COURAGEOUSLY.

ACKNOWLEDGMENTS

This research work was carried out at the International Center of Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya. I wish to extend my sincere gratitude to the ICIPE management and ARPPIS for providing me the opportunity and funding to pursue these studies. I wish to thank my first supervisor, Dr. Ellie Onyango Osir for intellectual guidance, constructive criticism, encouragement and patience. I am grateful to my second supervisor, Professor Dominic Makawiti for his constant encouragement and suggestions during the period of this study. Special recognition to Dr. Yuki Eshita and Dr. Phelix A. O Majiwa who first introduced me to molecular biology and encouraged me to pursue a Ph.D. work. I wish to express my gratitude to the following members of Molecular Biology and Biochemistry Research Unit - ICIPE for the training received in molecular biology and protein chemistry techniques: Dr. Mary Vundla, Dr. Mabel Imbuga, Dr. Dorrington Ogoyi, Dr. Ukaria Kenya, Dr. Edward Kinyua, Dr. Jedida Kongoro, Godwin Zimba and Mahamat Abakar. I am indebted to the staff of the Microbiology Department, University of Cape Town for their advice and support when I visited the Department, special thanks to Di James who helped in training me in subcloning and sequencing techniques, Dr. Andrew Hackland, Dr. Valerie Albright, Dr. Volker Mitterndorf, Dr. Qhobela Molapo, Brigid Campbell and Charles Hendrick for supplying the competent cells. Lastly, but not least, I thank my dear mother Mama Flora Kore Omolo, my dear wife Rose Owili Omolo, my sons Elijah Omolo and Edward Orony Omolo Jr, brothers and sisters for enduring the long years of separation during the period of my studies.

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ABBREVIATIONS

A	Adenosine
A_{562}	Absorbance at 562
ATP	Adenosine 5'-triphosphate
Amp	Ampicillin
bp	base pair
BSA	Bovine serum albumin
ca	Circa, about approximately
conc	concentration
cpm	counts per millicurie
C-terminal	carboxyl terminal
CsCl	caesium chloride
cry	crystal protein
DDT	dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1,4-dithio-L-threitol
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium bromide
et al.	and others
h	hour(s)
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kb	kilobase pairs
km	kilometers
l	Liter
LB	Luria-Bertani broth
LC_{50}	lethal concentration 50%

ABBREVIATIONS

min	minute(s)
MDa	Megadaltons
MNB	Modified nutrient broth
mol	molecular mass
M_r	Relative molecular mass
N-terminal	amino terminal
OD_{600}	Optical density at 600 nm
ORF	open reading frame
°C	degrees (Celsius)
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH	(negative logarithm of hydrogen ion concentration)
r	resistance (superscript)
RNA	ribonucleic acid
s	second(s)
SDS	sodium dodecyl sulfate
sp(p)	species
sq	square
TBBS	Tris-tween-buffered saline
TBS	Tris-buffered saline
TBE	Tris-borate-EDTA
TAE	Tris-acetate EDTA buffer
TTE	Tris-taurine-EDTA buffer
TEMED	N,N.N',N'-tetraethylethlenediamine
Tris	Tris (hydromethyl) aminomethane
U	units of enzyme activity

ABBREVIATIONS

UV	ultraviolet (light)
v/v	volume/volume
w/v	weight/volume
XGal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
α	Alpha
ß	Beta
µ	micro
λ	lambda

ABSTRACT

Local isolate of *B. thuringiensis*, designated L1-2 (*Bacillus thuringiensis L1-2)* was found to be toxic to adult tsetse, *Glossina morsitans morsitans* and fourth instar larvae of the stem-borer, *Chilo partellus* in bioassays. The δ-endotoxin crystals derived from *Bacillus thuringiensis L1-2* gave a major protein band of molecular weight of M_r ~ 130,000-140,000 on denaturing polyacrylamide gel electrophoresis. The cloning, expression in *E. coli* and the determination of the nucleotide sequence of a 3.5-kb DNA segment containing a cry gene from *B. thuringiensis L1-2* has been completed. The cloned gene from *B. thuringiensis L1-2* was found to be similar to that of the *B. thuringiensis* subsp. *kurstaki* HD-73 cryIA(c) gene having one amino acid difference at position 148 and 5 additional DNA differences. Bioassays using adult *G. m. morsitans* and larvae of *C. partellus* showed that the recombinant *B. thuringiensis L1-2* was as toxic to these insects as the original wild type. *B. thuringiensis L1-2* isolate is unique because, though the genes in this isolate belong to class I (lepidopteran-specific crystal proteins) which are synthesized as M_r 130-to 140,000 protoxins, the obvious advantage *B. thuringiensis L1-2 has over* the previously studied *B. thuringiensis,* is its wide range of insecticidal activity. Other previously reported *B. thuringiensis* of this class I, have shown only lepidopteran insecticidal activity but this isolate is also active against a adult dipteran. Also other classes which have shown insecticidal activities have so far shown that it is the larvae which suffered heavy mortalities, but with *B. thuringiensis L1-2* the insecticidal activity has been reported against adult *Glossina*. Amongst these dipteran *spp* studied so far (mosquitoes and black flies) both have aquatic larvae which offers a wide range of possibilities of infection by *B. thuringiensis*. *Glossina* spp the larvae does not feed and is restricted to a few minutes on the surface of soil before burrowing offering a narrow range of possibilities of infection by *B. thuringiensis*. So far no studies have shown cryIA gene specific to adult Diptera and show dual specificity and cross-reacts immunologically with dipteran and lepidopteran toxins.

This work describes the cloning, and expression of *Bacillus thuringiensis L1-2* gene as a first step to characterizing the gene.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Background

Bacillus thuringiensis (B. thuringiensis), is a gram-positive, rod shaped, spore forming soil bacterium that has a worldwide distribution (Martin and Travers, 1989). It is entomopathogenic to a wide variety of insect species. *B. thuringiensis* has shown great potential for use in the management of many lepidopteran and dipteran pests and disease vectors. A number of approaches have been employed for the use of δ-endotoxin in the biological control of pests. For example, *B. thuringiensis* formulations (Thuricide, Florobac XLC, Deflin, Dipel and Bactospeine XLV) have been applied for the control of *Pieris brassicae, Manduca sexta, Spodoptera littoris* and *Spodoptera excempta* larvae in the field (Bai *et al.*, 1993).

Similarly, control has been achieved for black flies by the World Health Organization's Onchocerciasis Control Program in West Africa. However, no *B. thuringiensis* has been reported to be active against tsetse. The major characteristic of *B. thuringiensis* is its ability to produce insecticidal crystalline proteins (ICPs) during the sporulation cycle (Höfte and Whiteley, 1989). These crystal proteins exhibit highly specific insecticidal activities against Lepidoptera, Diptera, or Coleoptera larvae (Dulmage, 1981; Whiteley and Schnepf, 1986; Federici *et al.*, 1988; Höfte and Whiteley, 1989). These inclusions consist of 1-5 protein subunits and are known as δ-endotoxin or insecticidal crystal proteins (ICPs) (Höfte and Whiteley, 1989). The subunits are held together by complex interactions including hydrophobic, hydrogen and disulphide bonds (Bulla *et al* 1979,1981). The disulphide bonds are important for holding the parasporal inclusions together (Aronson, 1986). The structure of *B. thuringiensis* endotoxin vary with the genes that encode the proteins and it has been demonstrated that *B. thuringiensis* strains can harbour different ICP genes (Kronstad *et al.*, 1983; Préfontaine *et al.*, 1987). It is now generally accepted that crystal protein genes are located on large (15- 300 MD) plasmids (Pang, 1994; Bhattacharya, 1993;

Aronson *et al.*, 1986; and Kronstad *et al.*, 1983) and the toxin activity appears to be mediated by binding of toxins to cell membrane phospholipids (Bhattacharya, 1993).

1.2 Literature review

1.2.1 Insects, pests, vectors and their control

1.2.1.1 Insects

Insects generally stimulate lots of interest in various studies due to their economic importance as pests of man, his crops and livestock. There are many insect pests and disease vectors in tropical Africa that cause major economic and public health problems. It has therefore, become all too easy to regard all insects as pests or disease vectors. Estimates of the number of insect species known vary considerably. There are many insect pests and disease vectors in tropical Africa that cause major economic and public health problems. Tropical Africa is one of the principal homes of insects in the world. It is estimated that area has over 3,000,000 insect species. Of that number, only about 0.3 % are major pests of man, his crops and his livestock (Odhiambo, 1975). Lepidoptera, Diptera and Coleoptera groups are the major pests and disease vectors due to the types of food they consume. The solution and management of the pest and vector problems of the continent must be investigated by studying the biology, ecology and behavior of the insects involved. Current control strategy for these pests rely heavily on the use of persistent synthetic pesticides. Odhiambo (1975) described the discovery of DDT as follows: "with its killing powers, its persistence, and its wide spectrum of insect targets, has put in the hand of man a new weapon of a bully-for clubbing to death all insects and sundry, friend and foe alike". Although DDT and other synthetic pesticides are generally very effective in the short term, their rigorous use invariably results in the rapid development of resistance. Moreover, the high cost of developing new pesticides coupled with the adverse effects of the pesticide residues on the biosphere renders this approach unacceptable as a long term solution. These and other concerns have spurred new interests in the search for alternative tactics, especially those that are affordable and non-pollutive to the environment.

Tsetse (*Glossina* species) are the insect vectors for trypanosomes, the causative agents for trypanosomiasis in both cattle and humans in Africa (Hoare, 1972). Cattle trypanosomiasis is a wasting disease responsible for massive losses in animal productivity in Sub-Saharan Africa. Similarly, the stem borer, *Chilo partellus*, is an important pest of maize and sorghum in many parts of Africa (Sheshu Reddy *et al.*, 1988)

1.2.3 Tsetse

Adult tsetse have adrenotrophic viviparity method of reproduction. *Glossina* spp feed only on bloodmeal. All life stages are dependent entirely on blood. The life span of an average adult *Glossina* spp is about 36 days (Jordon, 1986).

The *Trypanosoma*, a genus of parasitic tissue-dwelling Protozoa found in the blood is causative organism of African trypanosomiasis. *Trypanosoma* are transmitted from one host to another by tsetse, genus *Glossina* (Buxton, 1955; Jordan, 1974). *Trypanosomes* were first shown to be pathogenic to man and his livestock at the beginning of the century. Bruce (1902) found trypanosomes in the blood of a cow afflicted with the disease "Nagana", which was known to be associated with tsetse bite. "Nagana" is a wasting disease responsible for massive losses in animal productivity in tropical Africa. Trypanosomes were shortly afterwards found in the blood (Dutton, 1902) and then in the cerebrospinal fluid (Castellaani, 1903) of human beings suffering from sleeping sickness. The disease in man (sleeping sickness) is caused by *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *T. b. rhodesiense*. The more acute form of the disease (the *rhodesian* sleeping sickness) is caused by *T. b. rhodesiense* and classically occur in East and Central Africa. The milder and chronic form (the *gambian* sleeping sickness) of the disease is most commonly seen in West Africa and is caused by *T. b. gambiense*. In cattle, the disease, "Nagana" is caused by three species of trypanosomes, namely, *T. congolense, T vivax, and T. b. brucei*. Trypanosomiasis also occurs in pigs where it is caused by *T. simiae* (Stephen, 1966).

1.2.3.1 Tsetse distribution

Tsetse occur over 11 million square kilometers or about half of the non-desert area of Africa (Jordan, 1986; FAO, 1961; FAO/WHO/OIE, 1982). Trypanosomiasis is endemic in a vast region of Africa and its distribution is previously defined by the range of the tsetse distribution. There are 22 species and sub-species which are divided broadly into three sub-generic groups according to their habitat (Jordan, 1986). These are the forest (*fusca*), riverine (*palpalis*) and the savannah *(morsitans)* groups. Trypanosomiasis, however, also occurs outside the tsetse belt in Africa and in South America. The tsetse-infested areas cover an estimated 37% of the whole continent, involving 38 countries (Moloo,1993) and since the 1950's, tsetse have continued to spread (Maclennan, 1980) with approximately 50 million cattle being at risk. Such numbers represent about 30 % of the total cattle population on the African continent. Furthermore, approximately 25,000 cases of human sleeping sickness occur each year (ILRAD Reports, 1987). In their report on the impact of trypanosomiasis control and eradication of tsetse in several agro-ecological zones of africa, Jahnke *et al.* (1988) and Tacher *et al.* (1988) have assessed that within the 7 million Km^2 of tsetse-infested savannah, livestock numbers could be increased by approximately 120 million. Therefore, there is a need for proper tsetse trypanosomiasis control measures in order to realize the full socio-economic potential of livestock production on the continent. The benefits of tsetse and trypanosomiasis control are enormous especially when viewed, not only within the narrow context of animal production, but also, within the broader perspective that much of the tsetse infested land in the humid and sub-humid zones also has a relatively high agricultural potential, particularly with respect to growing staple foods. (FAO, 1961).

1.2.3.2 Current methods for Tsetse control

Current control of tsetse largely relies on:

1. Reduction of tsetse population by insecticides (Dieldrin and DDT).

   One such project was the extensive tsetse eradication project in Nigeria,

which aimed at the elimination of the tsetse from some 259,000 sq. km by 1986 (Jordan, 1978). Most of the project involved the application of residual insecticides both from the ground and the air. Similarly, the use of chemical sprays has led to the rapid development of resistance among the fly populations.

2. The destruction of tsetse habitats by clearing and elimination of wild animal reservoirs.

3. Biological approach, involving sterile insect technique (SIT) (Maclennan, 1968); in which artificially sterilized males compete with wild tsetse for mating. This has been employed for eradication or control of tsetse population (Politzar and Cuisance, 1982; Williamson *et al.*, 1983). However, not only is the method expensive, but sterile males of some species have been shown to be efficient vectors of pathogenic *Trypanosoma* species and could temporarily increase the disease risk (Moloo, 1982; Moloo and Kutuza, 1984).

4. Traps and screens have also been used successfully in different ecological zones to control tsetse. More recently, improvements in the design and colour of traps, such as the bi-conical trap (Challier and Laveissier, 1973), the beta trap (Vale, 1962) the F2 trap (Flint, 1982), the pyramidal trap (Lancien, 1981) and the NGU trap (Dransfield and Brightwell, 1988).

5. Use of traps and screens impregnated with insecticide and attractants, such as deltamethrin (Laveisser and Couret, 1981) and acetone (Vale, 1980). Insecticide impregnated screens offer relatively inexpensive and environmentally acceptable method of reducing tsetse population, which has made tsetse trapping an effective alternative method for tsetse control.

6. The use of trypanocidal drugs for prevention (prophylaxis) or curative treatment of infections, include treatment of the livestock with isometamidium, homidium chloride, (Novidium^{R}, May and Baker); (Ethidium^{R}, Boots) (Leach and Roberts, 1981) and iminazene aceturate (Berenil, Hoechst). Chemotherapeutic interventions to restore the health of the infected animal or human beings and to reduce indirectly the reservoir source of infection for other hosts is an important

element in the control of trypanosomiasis. Moreover, it has been observed that not only are the treated animals cured of the infection, but also they can develop a substantial degree of acquired immunity (Bevan, 1928; Wilson *et al.*, 1976). This approach has been used widely with considerable success, particularly in areas at the periphery of tsetse belt. However, in high fly challenge areas, the frequency of treatment required to control the disease may be economically unacceptable (Holmes, 1980). Nonetheless, the use of chemotherapeutic agents are plagued by problems such as drug resistance and toxicity. Homidium salts have been used widely, but a considerable number of cases of drug resistance to homidium have been reported and in many countries it has been necessary to suspend their use (Finelle, 1973).

7. Biological control agents using parasitoids. Three genera *Exhyalanthrax (Diptera: Bombyliddae); Chrestomutilla* and *Smicromyrme (Hymenoptera: Mutillidae)* and *Nesolynx (Hymenoptera: Eulophidae)* have been considered as natural enemies of *Glossina* spp but to date *Nesolynx,* is the only organism which has been used in an attempt to control puparia and adult tsetse (Jordan, 1986). But the failure of large release in Tanzania discouraged further attempts to use *Nesolynx* (Nash, 1933).

8. More than 30 entomopathogenic fungi have been tested as biological control preparations for different insects and although there is abundant literature describing the pathology of fungal infection in insects, little information is available on pathology of fungal infections in tsetse, despite their great importance as vectors of human and animal trypanosomiasis (Kaaya *et al.*, 1996). Biological control agents using entomogenous fungus *Metaharhizium anisopliae* and *Beauveria bassiana,* on tsetse have been shown to induce high mortalities in adult tsetse when applied topically as wet spores (Kaaya, 1989; Kaaya and Okech, 1990; Kaaya and Munyinyi, 1995).

In light of these limitations, there is an urgent need to develop alternative control measures for tsetse that are non-pollutive and for which resistance is not developed too rapidly.

1.2.4 Stem Borer

Stem borers, *Chilo partellus. Busseola fusca, Sesamia calamistis* and *Eldana saccharina* are major pests infesting maize, sorghum, millets, and other cereal grown in the region. They are responsible for decrease in cereal crop production. The borers cause losses of between 18 and 88% on unprotected crop (Odindo, 1991). Susceptibility of crops to infestation by stem borers depends directly on the phenological stage of the plants at the time of infestation. The pests feed on the leaves in the early instars and then burrow into the stem through the stalk. Therefore, they cause foliar damage, dead hearts, stem tunnelling, stem lodging and breakage of peduncle, all of which contribute to losses in yield. Direct loss may also be caused when larvae attack the grains of maize cob or sorghum head (Seshu Reddy, 1983; Ampofo, 1986; Unnithan, 1987).

Adult *Chilo partellus* are nocturnal insects and live for 2-3 days during which time each female lays a mean of 434 eggs (Delobel, 1975) in batches of 10 - 80 eggs on the under surface of leaves (Scheltes. 1978; Harris, 1990; Minja, 1990). The egg hatching is approximately 4-5 days at 26-28 °C. After hatching, larvae initially feed on the leaves and full larval development is completed in 2-4 weeks. Pupation is achieved by burrowing in the stem to facilitate eventual moth emergence. The pupal period last 4-8 days depending on environmental conditions, such as temperature, light, humidity, and the quality of the accessible food. The complete life cycle is, therefore, completed in 25-50 days depending environmental conditions such as the ones mentioned above (Ingram, 1958; Harris, 1990). Maize and sorghum rank fifth and fourth in acreage production, respectively, among the World's major crops (FAO, 1975). In tropical Africa, these two cereals account for some 90% of the grain crop production and are the staple food for the majority the population (Odindo, 1990).

An innovative approach to stem borer management is the application of entomopathogenic microorganisms. Investigations currently in progress show that certain microorganisms especially microsporidia, fungi and *B. thuringiensis* have a high pathogenicity to stem borers and as such may soon be developed for microbial control

of the borer (Odindo, 1991a.b). The bacterium, *B. thuringiensis,* has shown great potential for use in the management of many dipteran, lepidopteran and coleopteran pests (Brownbridge, 1991).

1.2.4.1 Current methods for stem borer control

Most of the present recommendations for cereal protection against pests are based on chemical insecticides including DDT, dieldrin, endrin, dimethoate and endosulfan sprays to the funnel of young plants, but the efficacy of the insecticides against these cereal stem borers are restricted by the feeding behavior of the larvae. Applications are only effective during the limited period before the larvae enter the concealed environment of the stems (Ingram, 1983; Scheltes, 1978; Warui *and* Kuria, 1983). Minja (1990) noted that all control strategies will have to be examined and incorporated into an integrated pest management programme if effective control of *C. partellus* and other stem borers is to be achieved. Current control measures for these pests in many parts of Africa rely on safer, faster acting, organophosphates and synthetic insecticides such as DDT, dieldrin, and aldrin which are available in a variety of formulations.

These organophosphates and synthetic insecticides are also preferred because they are highly effective, rapid in action and adaptable to most changing agronomic and ecological conditions. However, prolonged use of chemical pesticides is not ecologically sound, leading to such disadvantages as eradication of a wide range of useful insects and non-target organisms such as ants, spiders and parasitoids, as well as some predatory birds which are of great value in regulating pest populations. This results in unbalanced insect communities. This disruption of resident natural enemies allows the development of secondary pests many of which were present in insignificant numbers before the use of broad-spectrum pesticides. Some of these chemical pesticides (Dieldrin, DDT and Endrin) have been used and are known to be effective against these pests but are also not recommended on account of their high mammalian toxicity, objectionable build up of their toxic residues in the environment which are direct hazards to the user (Dixon, 1994). Furthermore, a period of time has to elapse

after plant treatment before crops can be harvested or animals allowed to graze.

Also the exorbitant cost of these synthetic chemical pesticides has led to their use being beyond the economic reach of the resource poor farmers of the developing countries who tend to rely instead on cultural practices such as early planting, rouging of infested plants and inter-cropping (Odindo, 1990).

1.3 Bacillus thuringiensis (B. thuringiensis)

1.3.1 General properties

Ishiwata (1901), a Japanese, bacteriologist isolated a *bacillus* from diseased Silkworm *(Bombyx mori)* larvae. In the early twentieth century, Berliner (1911) provided the first inkling that microbes could control insect pests when he isolated crystal-containing organism from diseased flour moth *(Anagasta kuehinella)* larvae species and named it *Bacillus thuringiensis*.

B. thuringiensis is distinguishable from the closely related species *B. cereus* and *B. anthracis* by the synthesis of several large crystalline cytoplasmic inclusion (δ-endotoxin) that appears during sporulation phase of development (Haider and Ellar, 1987). *B. thuringiensis* is a complex species spanning more than 30 varieties of flagellar serotypes based on serological and biochemical tests. The insecticidal crystalline proteins (ICPs) produced by *B. thuringiensis* are alpha-, beta-, and gamma-exotoxins and delta-endotoxins. Among these proteins, the delta-endotoxin and the beta-exotoxin are used in agriculture. The delta-endotoxin is the most extensively studied toxin; its larvicidal specificity includes members of lepidopteran, dipteran, and coleopteran insects. The pathological changes caused by *B. thuringiensis* depend on the host insect, dose, and strain involved. In general, δ-endotoxin destroys the integrity of the midgut epithelium. Some insects, especially those with high pH in the midgut, are rapidly paralysed by this toxin. These insects show pathological changes in the tissue and eventually die. Others, after ingestion of toxin, also show signs of midgut epithelium deterioration. This allows the toxin to enter the haemocoel and results in a lethal septicemia (Gill *et al.*, 1992).

Formulations of *B. thuringiensis (Berliner)* have been used for more than two

decades as biological insecticides (Rowe and Margaritis, 1987) to control agricultural pests, and more recently, insect vectors of a variety of human and animal diseases. Their increasing use and lack of mammalian toxicity have led to intensified efforts being directed at isolating new *B. thuringiensis* strains active against indigenous pests. In addition, the interest in finding novel strains has been heightened by an increasing interest in the preventive management of insect resistance to *B. thuringiensis*. Extensive screening programs are being carried out by respective groups to search for *B. thuringiensis* strains with different spectra of activities and best suited to the specific geographical localities in which they have been isolated. A recent survey carried out by ICIPE in different geographical locations in Kenya have led to the isolation of a large number of *B. thuringiensis* strains two of which are active against *C. partellus* and *Glossina* species. In addition to isolating these new strains, there is an urgent need to study and characterize the associated endotoxin in order to fully understand their biochemistry as well as the genes that encode their synthesis. These studies on the molecular basis for selective toxicity would allow for the development of improved strains through genetic manipulations of the endotoxin genes. The outcome would be an endotoxin with enhanced potency towards these pests.

1.3.2 B. thuringiensis growth phases

The growth cycle of *B. thuringiensis* can be divided into four phases whereby the first phase is spore germination. In this phase the spores lose heat resistance, when the growth conditions are favorable. The second phase of growth involves the biosynthesis of macromolecules, RNA, fatty acids and proteins necessary for growth in the vegetative phase. This stage is followed by the third phase of vegetative cells where cell division takes place after DNA replication. Excretion of ß and α-exotoxins occur at this phase in some varieties of *B. thuringiensis*. The fourth and last phase involves the formation of spores and δ-endotoxin. This stage occur due to exhaustion of nutrients in the media. The spores mature and there is lysis of the bacterial cell wall to release the spores and parasporal inclusions or delta endotoxin.

1.3.3 The δ-endotoxin structure

Held *et al.* (1982) first used the abbreviation "***cry***" (for crystal) to represent the insecticidal crystal producing genes of *B. thuringiensis* strains (the δ-endotoxins). The δ-endotoxin are glycoproteins (Pfannestiel *et al.*, 1986) and are normally referred to as protoxins according to the first reported studies (Hannay and Fitz-James, 1955). The crystalline inclusions may be of various morphologies. They are usually bipyramidal (rhombic) in shape. However, some *B. thuringiensis* strains are known to produce amorphous crystals. There is usually one crystal inclusion per cell but more than one inclusions have been known to occur (Luthy *et al.*, 1982).

These crystal inclusions have been reported to comprise up to 20 to 30% of the total dry weight of sporulated cells (Lilley *et al.*, 1980) and are toxic when ingested by susceptible insects. These parasporal crystals are synthesized within the sporangium as proteins of molecular weight between M_r ~70,000 to- 135,000 and are crystallized as parasporal inclusions also known as protoxins. The large endotoxin molecule requires processing to active toxin. This process is mediated by the alkaline pH and proteinaceous midgut of the insect, and involves the proteolytic cleavage of the C-terminal structural fragment, to yield a M_r ~ 65,000 to-70,000 proteinase-resistant core (Chungjatupornchai *et al.*, 1988). In the following proteolytic activation, approximately half of the protoxin molecule is removed from the C-terminal (about 500 amino acids), leaving the N-terminal half as the functional domain. Activation also appears to be accomplished by the removal of approximately 28-30 residues from the N-terminus. Crystallography studies of with *B. thuringiensis* cry IIIA protein indicate three structurally distinct domains (Li *et al.*, 1991) Domain I, a seven α-helix bundle, composed of the N-terminus 290 residues, is thought to play a role in forming a transmembrane spanning region, with the fifth α-helix potentially forming a key component of the pore; Domain II, a ß-sheet rich region, from residues 291 to 500, contains three anti-parallel ß-sheets each ending with a loop structure. Domain II apparently plays an essential role in toxin selectivity (Ge *et al.*, 1989), and is likely to be associated by the toxin receptor binding since it comprises most the hypervariable

region of the toxin. Domain III is a ß-sandwich containing the conserved C-terminus region of the crystal protein toxins. Although structural data suggest that Domain III is involved in structural stabilization (Li *et al.* 1991), recent mutagenesis data have indicated that this domain may also be involved in modulating toxin activity (Chen *et al.*, 1993) and is implicated in insect specificity and stability. The cryI, cryIVA and cryIVB *B. thuringiensis* protoxins, which are relatively large proteins of approximately M_r ~ 130,000, when ingested by insects are solubilized in the alkaline midgut (Gill *et al.*, 1992). The solubilized toxins are cleaved predominantly at the C-terminus by midgut proteases, for example trypsin and chymotrypsin. Additionally, there is N-terminal truncation; this, however, is relatively small usually of 7-30 amino acids (Gill *et al.*, 1992). The naturally truncated cryII, cryIII and cryIVD toxins can also undergo limited proteolysis in the insect midgut, with both truncated and the larger M_r ~ 130,000, the activated toxins are approximately M_r ~70,000 to- 135,000. These activated proteins retain apparently all the functional-domains I,II,III. Toxin processing is the key in the formation of an activated toxin and can affect the selectivity of a toxin. An example of this selectivity is the insecticidal activity of *B. thuringiensis* subsp. *kurstaki* cryIIA toxin to either mosquitoes or to lepidopteran insects if the toxin is first processed by either mosquitoes or lepidopteran midgut proteases, respectively (Widner and Whiteley. 1989). Similarly, Haider *et al*,1987 demonstrated that *B. thuringiensis subsp. aizawai* toxicity to mosquito or to lepidopteran larvae is dependent on the toxin C-terminal processing. If a dipteran enzyme is involved a toxin more active towards dipteran is obtained, similarly if processing is performed by lepidopteran proteases the toxins become active towards lepidopteran (Gill *et al.*, 1992). Toxin processing, therefore plays more important role in the selectivity of *B. thuringiensis*. Van Rie *et al.* (1990a,b) have reported that there is differential toxin processing in resistant *Plodia interpunctella*.

1.3.4 Mode of action of δ-endotoxin

Although, little is known about the site and mode of action of the *B. thuringiensis* endotoxin at the molecular level, the development of symptoms of *B. thuringiensis*

intoxication have been documented and the specificity of the *B. thuringiensis* endotoxin demonstrated but little information is available on the underlaying mechanisms. An understanding of insect midgut ion regulation is crucial for understanding *B. thuringiensis* toxin mode of action because a major consequence of toxicity is the disruption of the midgut cellular osmotic balance. The insect midgut plays a role in nutrition, and the enzymes involved in food digestion, such as trypsin, chymotrypsin, also play a role in *B. thuringiensis* toxin processing (Gill *et al.*, 1992). The insect midgut is made of number of different cell types; the columnar and the goblet cells predominate. The midgut lumen has a relatively high K^+ concentration compared to that in cell and/or in the hemolymph (Gill *et al.*, 1992). This K^+ concentration derives a number of secondary midgut cellular processes. As an example, amino acid and glucose transport into midgut cells is facilitated by high midgut lumen cation concentrations. The uptake of amino acids and other insect nutrients results in a relatively high insect hemolymph K^+ concentration (Gill *et al.*, 1992). However, for normal insect function it is essential that this K^+ hemolymph concentration be reduced. This reduction is achieved through the goblet cell. The goblet cell is known to exclude bicarbonate ions. This combination of bicarbonate and potassium ions excretion results in the formation of potassium carbonate/bicarbonate (Gill *et al.*, 1992). It is thought that it is this formation of carbonate/bicarbonate ions that leads to a relatively alkaline midgut (Ge *et al.*, 1991). Therefore, there is precise ionic regulation and osmotic balance in the insect gut. Insect ingestion of *B. thuringiensis* toxin therefore, affects the ionic regulation and osmotic balance and it has been observed that the columnar cell microvilli undergoes disruption resulting in insect death (Gill *et al.*, 1992; Singh *et al.*, 1986). Haider and Ellar (1988) contend that the active region of the toxin consists of a cell binding domain and a toxic domain and insect susceptibility to each toxin is believed to depend on both the differential proteolysis of the protoxin by the host proteinaceous and the presence of high affinity binding sites in the insect midgut epithelium, and probably differs between susceptible and non-susceptible insects (Haider and Ellar, 1987). However, the three dimensional conformation of the toxin is also essential for toxicity (Wu and Aronson, 1990). Differences in the solubilization

process, the enzyme profile and the presence of specific toxin binding sites in the insect midgut influences the host range of each crystal protein (Jaquet *et al.*, 1981), although susceptibility to certain crystal proteins is independent of activation (Jaquet *et al.*, 1987). This competitive displacement indicates the presence of selective receptors for the toxin (Gill *et al.*, 1992). The selectivity observed with a number of *B. thuringiensis* could in part explain the affinity of a toxin to a midgut brush border membrane receptors of a particular insect. Hence, the greater cryIA(c) toxicity to one lepidopteran insect is in part due to the higher affinity and receptor concentration of this toxin than for cryIA(a) toxin which could have lower toxicity. Consequently, when affinity is observed in an insect that is resistant to a particular toxin, a change in these binding constants is usually observed (Pietrantonio *et al.*, 1993). However, in resistant insects there is lack of identifiable affinity (Ferre *et al.*, 1991). Recent evidence suggest that an aminopeptidase N plays an important role in action of the cry protein toxicity (Sangadala *et al.*, 1994).

There is increasing evidence that *B. thuringiensis* toxins insert into plasma membrane to form a pore that is permeable to small ions and molecules (Knowles and Ellar., 1986; Slatin *et al.*, 1990). Studies using a variety of techniques with or without insect membrane proteins show that pores formed are voltage independent cation pores (Gill *et al.*, 1992) and these studies have further demonstrated that like other proteins, the cry proteins can form cation channels, and the conductance of the channels formed are relatively large. Subcloning of the N- and C- terminal domains individually and together followed by analysis of their activity demonstrated that N-terminal retained most of the pore forming ability (Gill *et al.*, 1992). The N-terminal half by itself has the pore forming ability and is also likely that other parts of the toxin could contribute to pore forming characteristics.

A consequence of pore formation is a disruption of columnar cell osmotic balance. Since the midgut cells are very tightly regulated monolayer, any disruption of ion regulation in either the columnar cells or the goblet cells could result in loss of function, and ultimately that of midgut. With *B. thuringiensis* toxins midgut microvilli ultrastructural integrity is disrupted early in the poisoning syndrome (Gill *et al.*, 1992).

This loss of integrity could be due to loss of osmotic balance and subsequently resulting in loss of cell function and ultimately resulting in cell lysis and insect death.

1.3.5 Toxic fragments of the crystal proteins

The δ-endotoxin in the crystalline inclusions are generally protoxins which require activation by proteolytic processing in the larval midgut to yield toxic fragments which are highly alkaline in the case of Lepidoptera and Diptera and less so in the case of Coleoptera. For many of the toxins, proteolytic activation involves removal of the C-terminal half of the protoxin. This activation can be duplicated *in vitro* using alkaline buffers together with a combination of proteases. When the protoxin is activated *in vivo* in the larval midgut, it causes disruption of the gut epithelial cells which leads to larval mortality. Upon ingestion by a susceptible insect, the crystal endotoxin is processed and the resulting toxin interacts with cells of the insect midgut epithelium via the high affinity binding sites (Gill *et al.*, 1992). The activated toxins appear to generate pores in the midgut epithelial cells of susceptible insects. In some instances, specific high affinity binding sites have been shown to exist in the midgut epithelial cells of susceptible insects which may explain the specificity of the toxin (Höfte and Whiteley, 1989). The cytA is an exception as it is active in its native form, although a M_r ~25,000 proteolytically derived fragment can be obtained (Armstrong *et al.*, 1985; Chilcott and Ellar, 1988, Thomas and Ellar, 1983). The cryIVD polypeptide has been shown to be converted into an active fragment of M_r ~ 30,000 by protease (Chilcott and Ellar, 1988; Pfannensteil *et al.*, 1986), but it is not known where the active toxic fragment maps in the native protein. The toxic domain of the M_r ~ 130,000 protein has been localized in the amino-terminal half of the protoxins. This has been demonstrated for lepidopteran-active cryI protoxins including cryIA(a) (Schnepf and Whiteley 1981; Schnepf *et al* 1985), cryIA(b) (Wabiko *et al.*, 1986), cryIA(c) (Adang *et al.*, 1985), cryIC (Sanchis *et al.*, 1989) and cryIE (Botterman *et al.*, 1989) by deletion analysis of the corresponding genes. Similar experiments with the cryIVB gene also revealed that the carboxyl-terminal part of the cryIVB protein, active against dipteran larvae, is not essential for toxicity (Chungjatuporchai *et al.*, 1988; Delecluse *et al.*, 1988; Pao-intara *et al.*, 1988).

The determination of N-terminal amino acid sequence of the trypsin-activated toxin together with deletion analysis has shown that several amino acids from the N-terminus of the protoxin could be removed without affecting toxicity. The toxic fragment appears therefore to be about M_r ~ 60,000 to-65,000 and corresponds to the protoxin truncated both at its amino-acid and the carboxyl-termini. The minimal toxic core of cryI toxin is delineated by residues 29 and 607 (Höfte *et al.*, 1986; Nagamatsu *et al.*, 1984) and for cryIVB the toxic core is contained between residues 39 and 662 (Delecluse *et al.*, 1988; Pao-intara *et al.*, 1988). The cryIII toxin lacks a region corresponding to the C-terminal part of cryI proteins, cryIII is therefore considered as a naturally truncated version of M_r ~ 130,000 type protoxin. Sequence containing potential tryptic cleavage sites are found at positions approximately to the ends of active fragments. Compilation of results obtained by several groups shows that the sequence determined by the N-terminus of the toxic fragment is not found in all toxins and this suggests that N-terminal processing varies between toxins.

1.3.6 Receptor binding and membrane insertion

The specificity of a toxin is its ability to exhibit toxicity to limited insect species or other pests. Most *B. thuringiensis* strains simultaneously produce more than one crystal protein with diverse activities. These crystal proteins are known to exhibit highly specific insecticidal activity (Höfte and Whiteley, 1989). The differences between strains of the same subspecies toxic against a particular pest species have also been reported (Jaquet *et al.*, 1987). A number of reasons for this selective toxicity have been suggested. Both the bacterium and the insect species contribute to the specificity of the insecticidal activity. It has been demonstrated that the insecticidal crystal protein binds specifically to the brush border membrane vesicles (BBMV) prepared from the midgut columnar cells and that the genes encode protoxins that are cleaved in the insect gut to release a toxic fragment (Gringorten *et al.*, 1992; Gill *et al.*, 1992). Analysis of the insecticidal specificity of the products of several cloned genes or of proteins from strains of *B. thuringiensis* thought to carry a single toxin gene has shown that the individual gene products have differential activities toward specific insects

(Höfte *et al.*, 1989). Thus, the analysis of cloned gene products, has contributed an understanding of the diverse activity of *B. thuringiensis* However, few details are known about the mechanism involved in the intoxication of insect gut cells although it has been shown recently that the toxic proteins recognize specific high affinity receptors on the midgut brush border (Schnepf *et al.*, 1985). Although specific receptor binding is one determinant of species targeting, it is not known whether additional steps leading to intoxication are also species specific or whether different toxins may vary with regard to the region of the protein that determines specificity toward a given insect. However, the N-terminal sequences show less homology (Aronson *et al.*, 1986; Chunjatupornchai *et al.*, 1988). These differences may be partially responsible for the different activity spectra of the *B thuringiensis* strains (Chunjatupornchai *et al.*, 1988). Traditionally, the protein composition of *B. thuringiensis* crystals has been studied using polyacrylamide gel electrophoresis (Laemmli, 1970) and have been shown to contain one or more proteins with subunit M_r ~ 27,000 to- 160,000 (Höfte et al., 1989; Whiteley *et al.*, 1986; Wu *et al.*, 1985), and the M_r ~ 130 to- 160,000 protoxins are upon ingestion, proteolytically cleaved in the insect midgut into smaller, active forms (M_r ~ 60 to- 70,000) derived from the N-terminal half of the protein.

1.3.7 Selective toxicity of the δ-endotoxin

According to the current gene nomenclature, the first level of classification, the Roman numeral denotes host range; that is against lepidopterans, as that of the cryIII toxins against coleopterans. Even among toxins of the same classification, often there is significant differences in the degree of toxicity, for example, cryIA(a) is more active (400 times) against (*Bombyx mori*) than cryIA(c) and cryIA(c) is more active (10 times) against *heliothis virescens* or *Tricoplusia ni* than cryIA(a) (Ge *et al.*, 1989; Ge *et al.*, 1991).

B. thuringiensis strains contain different toxins, and it is this toxin diversity that determines the selective insecticidal activity of *B. thuringiensis* strain. The toxins are selective, not specific because although each toxin has its predominant activity towards a particular insect species, it usually also has very low insecticidal activity against other

insects. For example three toxins in the *B. thuringiensis* subsp. *kurstaki* strain HD-1, cryIA(a), cryIA(b) and cryIA(c) are toxic to *lepidopteran* insects, however, each of them have differing toxicity towards different *lepidopteran* species (Van Rie *et al.*, 1989). CryIA(b) protein, interestingly is toxic to both lepidopteran and dipteran larvae but despite this dual activity, the protein isolated from *B. thuringiensis IC1* has been classified as cryIA(b) because its amino acid sequence differs from that of a monospecific lepidopteran cryIA(b) polypeptide by only three amino acids (Haider and Ellar, 1988). This toxin, with dual specificity, appears to undergo differential conversion was shown to be related to the three amino acids that distinguish the dual-specific protein from the monospecific toxin (Haider and Ellar, 1989). One other toxin from this HD-1 strain, cryIIA, has toxicity towards both lepidopteran and *dipteran* insects.

The selective toxicity of the cryII type protein has been determined. The first cloned cryIIA was found to be toxic to lepidopteran larvae and the dipteran larvae *(Aedes aegypti)*. In contrast cryIIB and cryIIC toxins are active only against lepidopteran species, despite their relative similarity to cryIIA protein. A short region of 76 amino acids in cryIIA toxin has been identified as being important for mosquitocidal activity (Widner and Whiteley, 1990); this region differs in cryIIB and cryIIC at only 18 amino acid positions. It therefore, appears that a small number of changes can substantially alter the specificities of these toxins. The toxin determinants therefore could be located in a similar region, even for non-related toxins. Similarly, *B. thuringiensis* subsp. *israelensis* active against dipteran larvae are composite and has three different types of peptides and contain at least four different toxins;

(a) The M_r ~ 130,000 type cryIVA, cryIVB

(b) The M_r ~ 72,000 protein designated cryIVD

(c) A Cytolytic factor of M_r ~ 24,000 referred to as cytA.

Each of these toxins has differential toxicity to various mosquito species. The toxicity of each *B. thuringiensis* strain is dependent on the toxins that are present in that strain and selective toxicity of different *B. thuringiensis* proteins is therefore, determined by the receptors that are present in insect midgut. It is clear that there is a great diversity of toxin receptors, for example, with cryIA(c) toxin, a number of receptors have

been observed in some insects while others only one major protein appears to be involved (Sangadala *et al.*, 1994), while in other insects at least two proteins are involved (Garczynski *et al.*, 1991).

1.3.8 The δ-endotoxin synergism

A final factor that affects *B. thuringiensis* toxicity is synergism. Since a number of *B. thuringiensis* strains contain multiple toxins, these toxins at times do not act alone. *B. thuringiensis* subsp. *israelensis* illustrates interaction between toxins. This isolate contains four toxins, cryIVA, cryIVB, cryIVD and the cytA, all these toxins are essential for mosquitocidal toxicity and when combination of cryIVD and the cytA are expressed, there is 4-5 fold synergism. This suggests that the activity of the native crystal may be due to the presence of several polypeptides acting synergistically.

1.3.9 B. thuringiensis crystal protein genes and products

More than 20,000 *B. thuringiensis* isolates have been collected (Bai *et al.*, 1993). An updated classification of *B. thuringiensis* subspecies have been traditionally based upon flagellar (H) antigens. Twenty-seven antigenic groups and seven subgroups have been distinguished (34 serovers) but no relationship appears to exist between insecticidal specificity and serotypes (deBarjac and Frachon, 1990). Hofte and Whiteley (1989) have proposed a nomenclature and classification scheme for crystal proteins based on pathotypes and DNA sequence homology as well as protein structure. At the time of their publication, there were about 42 *B. thuringiensis* insecticidal crystal protein (ICP) genes cloned and sequenced. The determination of both the amino acid sequence similarities and the insecticidal activities of the encoded polypeptides has allowed the classification of these genes into five classes and 14 subclasses (Höfte and Whiteley, 1989; Lereclus et al., 1989a). These ICP genes specify a family of related insecticidal proteins (cry proteins) and have been referred as cry genes. These cry genes and their plasmids have been characterized on level of restriction maps (Boe *et al*, 1991; Lereclus *et al.*, 1988; Mahillon and Seurinck, 1988; Mahillon *et al.*, 1988; Miteva and Grigorova. 1986; Clark *et al.*, 1985; Herrera, 1994).

These genes have been further subdivided into 14 cry types, 13 of these cry genes have been divided into 4 major classes and several subclasses. Subsequently, these subclasses have been characterized by both the structural similarities and insecticidal spectra of the encoded proteins (Whiteley and Schnepf, 1986) and several sequences have been found to be identical or nearly identical.

1.3.10 Nomenclature of crystal protein genes

Currently known gene types encode toxin proteins that are specific to either Lepidoptera (cryI), Lepidoptera and Diptera (cryII), Coleoptera (cryIII), Diptera (cryIV), or Lepidoptera and Coleoptera (cryV) (Tailor *et al.*, 1992) and toxins with broad cytolytic activity (cyt) **(Table 0.1)**. The cryI gene class, the type mostly frequently identified contain 6 subclasses of genes (cryIA - F). The cryI encoded polypeptides with M_r ~ 130,000 to- 140,000 protoxins which are processed into toxic, protease-resistant fragments of M_r ~ 60,000 to- 70,000 and are active against lepidopteran larvae and are also related in amino acid sequences. More than 20 cryI gene sequences have been reported (Table 2), and 6 different cryI genes have been recognized. The sequence homology among cryI ICPs is 50% or higher (Bai *et al.*, 1993; Höfte and Whiteley, 1989) and because of this extensive homology have been divided into a minimum number of four major classes and several subclasses which are characterized by both structural similarities and the insecticidal spectra of encoded proteins (**Table 0. 1**); (Chambers *et al*, 1991).

Significant amino acid similarities have also been found to exist between the crystal proteins, with the carboxyl-terminal half of the crystal proteins highly conserved; the amino terminal part is more variable. However, five domains in the N-terminal part are conserved sequences in all of lepidopteran - active cryI toxins (Brown and Whiteley, 1992; Sanchis *et al.*, 1989) and this N-terminal variable region represents the active fragment. In contrast, the conserved C-terminal region is not required for toxicity but may be involved in crystallization. The cryIA are the most widely studied ICPs genes. On the basis of further refinement of sequence relationship a sub-ranking within the classification has been established on which a subfamily of three classes of

cryIA gene, originally differentiated according to the size of a characteristic HindIII fragment gene namely. The three classes are cryIA(a) designated **4.5 kb** gene, cryIA(b) designated **5.3 kb** gene and cryIA(c) designated **6.6 kb** gene. It is believed that differences within this variable region may account for the different host specificities exhibited by the three classes of cryIA genes (Höfte and Whiteley, 1989; Chambers *et al.* 1991). The amino acid sequences of the corresponding cry IA protein show more than 80% homology within each group with most of the sequence dissimilarity localized to start internal variable region. The cryII class, the encoded polypeptide with M_r ~ 71,000 protein are toxic for both lepidopteran and dipteran larvae. In the strain *B. thuringiensis kurstaki HD-1*, the gene encoding this polypeptide belongs to the cryII class, and is referred to as the cryIIA gene (Donovan *et al.*, 1988; Widner and Whiteley, 1989). The cryIIA gene is the distal gene of an operon which is comprised of 3 open reading frames (Widner and Whiteley, 1989). On the basis of sequence similarities the cryII has been divided into subgroups (cryIIA, cryIIB, cryIIC). The cryIIA, B, and C proteins share about 80-90% amino acid identity, but are dissimilar to the other cry proteins, except in the first N- terminal conserved domain (Hofte and Whiteley, 1989; Lereclus *et al.*, 1989; Sanchis *et al.*, 1989). The third class cryIII toxin genes encodes M_r ~ 73,000 coleopteran-specific proteins. Two genes belonging to this class have been characterized: cryIIIA (Donovan *et al.*, 1988) and cryIIIB (Sick *et al.*, 1990). The two genes are distantly related (only 67% DNA identity). The cryIIIA and cryIIIB proteins are homologous to the amino terminal half of cryI protoxins through the five conserved domains, but they lack the region corresponding to the carboxyl part of these molecules.

The cryIV class of protein genes encodes polypeptides with predicted M_r ~ 135,000, M_r ~ 28,000, M_r ~ 74,000 and M_r ~ 72,000, which are active against dipteran larvae. The class contains four genes (cryIVA, B, C and D). The amino acid sequence comparisons of cryIVA and cryIVB proteins revealed that these two M_r ~ 130,000 type toxins possess a common carboxyl-terminal part and differing amino halves (with 40% similarity). The M_r ~ 28,000 polypeptide, encoded by cytA gene is Diptera-specific. This cytA protein gene shows no sequence homology to the other crystal polypeptides

and has therefore been assigned to a class of proteins which does not belong to the cry IV endotoxin family.

The novel cryV gene encoding M_r ~ 81,000 protein was recently isolated (Blenk *et al.*, 1989). The encoded polypeptide presents a dual specificity, active against both lepidopteran and coleopteran (Tailor *et al.*, 1992).

1.3.10.1 Revised nomenclature of crystal protein genes

A revised Classification of *B. thuringiensis* toxins is listed on **Table 1**. However, there are inevitably some *B.thuringiensis* crystal proteins that do not fit into this classification due to attempts to accomodate genes that were highly homologous to known genes but didnot have a similar target specoficity. A revised nomenclature, based solely on amino acid acid idenity has been proposed to allow closely related genes to be ranked together (Crickmore *et al.*, 1995). In this classification Roman numerals have been replaced for Arabic numerals in the primary rank. The revised definition of a cry gene is rather broad: " a gene from Bacillus thuringiensis encoding parasporal inclusion protein that exhibits pesticide activity or is homologous to a known cry gene. To date, over 90 genes comprising 16 homology groups are systematically arranged.

Table 0.1: *B. thuringiensis* protein genes and product specificity (Entwistle *et al.*, 1993).

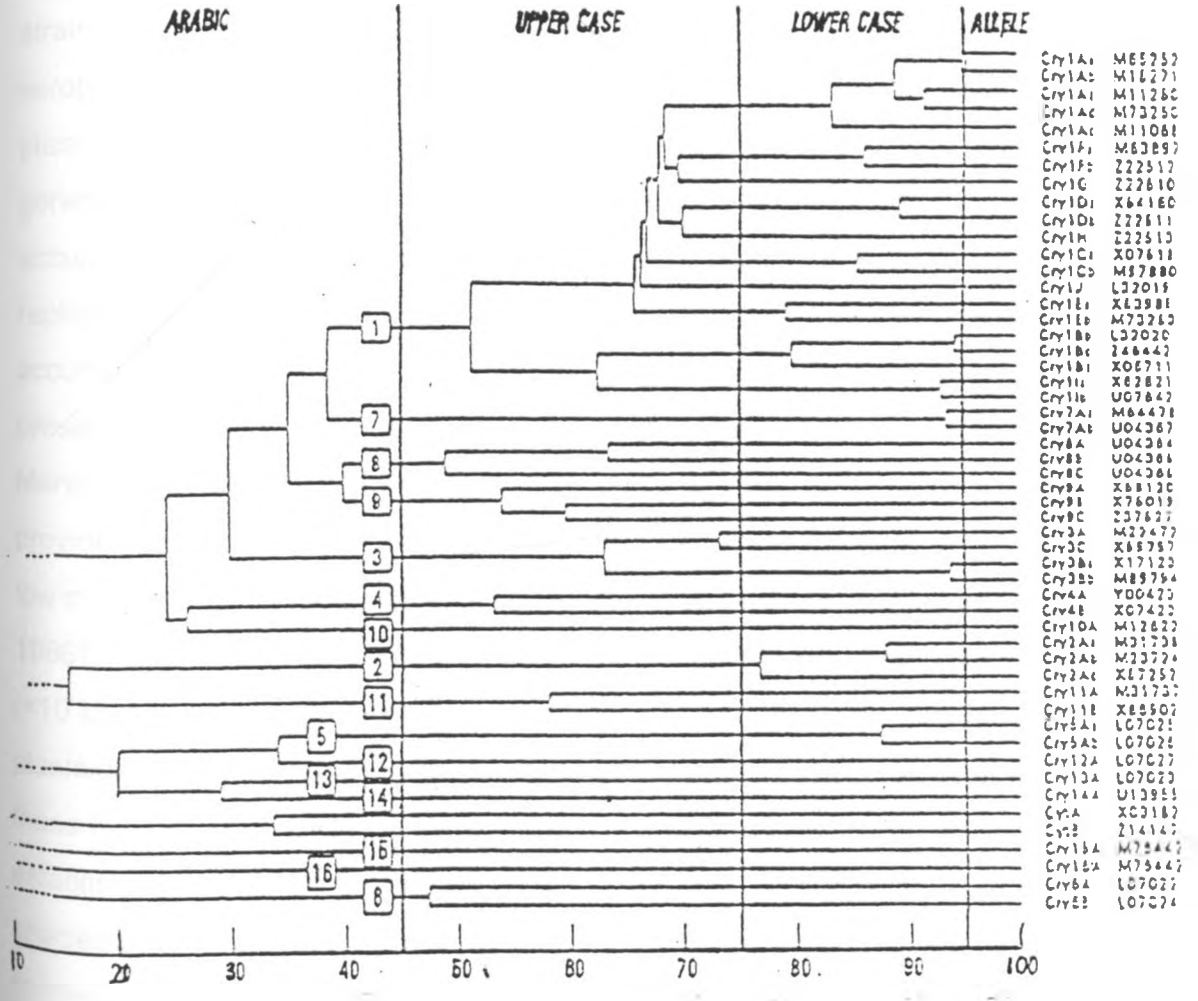
Gene type[a]	Class	Host range[b]	No. of amino acids	Predicted molecular mass (M_r)
cryIA(a)	I	L	1176	133500
cryIA(b)	I	L	1155	130600
cryIA(c)	I	L	1178	133300
cryIB	I	L	1207	139400
cryIC	I	L	1189	134700
cryID	I	L	1165	132500
cryIIA	II	L/D	663	70900
cryIIB cryIIC	II	L	633	70800
cryIIIA cryIIIB cryIIIB(b)	III	C	644	73100 74000
cryIVA	IV	D	1180	134500
cryIVB	IV	D	1136	127600
cryIVC	IV	D	675	77800
cryIVD	IV	D	643	72400
cryV cryIX	V	C/L VARIOUS	-	81200
cytA	-	D	248	27300

[a]According to Hofte and Whiteley (1989)

[b]Specified host ranges are L, Lepidoptera; D, Diptera; C, Coleoptera

Table 1: Dendogram showing sequence homology among the crystal proteins.

Created using the clustalV multiple alignment analysis. Gray vertical bars demarcate levels of sequence identity chosen in the proposed nomenclature to define the four levels of rank-Arabic numerals, upper and lower case letters, and allele numbers. Boxed numerals indicate the first level (Arabic numerals) grouping of proteins, example Cry1, Cry2, Cry3. Individual proteins are designed by their revised nomenclature assignment and database accession numbers (Crickmore *et al.*, 1995)



1.3.11 The expression of B. thuringiensis cry genes

The entomopathogenic functions of *B. thuringiensis* have stimulated the study of this organism. In the early 1980s, intensive research was directed toward the localization of the ICP genes among different *B. thuringiensis* subspecies. A number of early studies revealed direct correlation between the presence of plasmids and insecticidal crystal production. Stahly *et al.* (1978) first recognized that *B. thuringiensis* harbors plasmids and suggested they might be associated with crystal protein expression. Marin *et al.*, (1992) in their studies also discovered that nearly all the *B. thuringiensis* strains contained covalently closed circular (CCC) plasmid DNA. The number and size of these plasmids (1.4 MDa to 180 MDa) vary considerably between strains (Gonzalez and Carlton, 1980; Stahly *et al.*, 1978) but are independent of serotype and pathotype. Gruss and Ehrlich (1989) in their recent observation on plasmid replication attempted to relate the two sizes, the small size plasmids (<10 MDa, generally with a high copy-number) replicate by a rolling - circle mechanism involving accumulation of single stranded DNA (ss DNA) intermediates and a second group of replicons, including larger plasmids, replicate by a theta mechanism and do not accumulate single stranded DNA (ss DNA). These two sizes are structurally stable and present in low copy number (Bruand *et al.*, 1991; Janniere *et al.*, 1990; McDowell and Mann, 1991). Plasmid curing, conjugation (mating), and gene cloning experiments provided further evidence that the ICP genes are often located in larger (< 30 MDa), low copy-number cryptic plasmids (Gonzalez *et al.*, 1981; Whiteley and Schnepf, 1986). Other studies indicate that the plasmids fall into two size groups: small plasmids (<10 MDa) with some degree of relatedness and with no known function, and the large plasmids (<30 MDa) sharing homologous DNA sequences (Lereclus *et al.*, 1982) and these two groups appear unrelated. Carlton and Gonzalez (1985) in their work established that the genes coding for the insecticidal crystal proteins (ICPs) in different species of *B. thuringiensis* reside on large transmissible plasmids ranging in size from 5 to more than 30 MDa and that these large plasmids could be cured and were readily exchanged between strains by means of conjugation-like transfer. Carlton and Gonzalez (1985) made a survey of the plasmids present in most of the *B. thuringiensis*

strains. Their studies on 21 *B. thuringiensis* subspecies revealed that the number of plasmids varies from two to twelve between strains. Gene hybridization experiments provided additional evidence for the existence of multiple toxin-producing genes within a single bacterium. For example, the native plasmids of *B. thuringiensis* subspecies *aizawai* and *kurstaki* HD1 have as many as five separate ICP genes located at multiple sites (Sanchis *et al.*, 1988). In the case of *B. thuringiensis* subspecies *israelensis,* four dipteran-specific toxin genes and one cytolysin coding gene are located on a single 72 MDa plasmid (Aronson *et al.*, 1986; Lereclus *et al.*, 1989b). Their earlier studies have also shown that several lepidopteran-specific toxin genes, for example, *B. thuringiensis berliner* 1715 and *B. thuringiensis kurstaki* HD73 are flanked by two sets of insertion sequences and a transposable element (Kronstad and Whiteley., 1986; Bourgouin *et al.*, 1988). This structural organization of these toxin genes might give an evolutionary advantage to the bacteria by enabling them to adapt and proliferate among several insect species.

Despite their diversity and apparent absence of any essential functions the *B. thuringiensis* plasmids are generally inherited with remarkable stability suggesting efficient replication and maintenance mechanisms. Most *B. thuringiensis* plasmids are cryptic. The main function that has been assigned to them is the production of entomocidal toxins. Following the localization of toxin genes, many research groups began to clone ICP genes from several *B. thuringiensis* subspecies. Schnepf and Whiteley (1981), reported the first cloning and expression of a *cryI* type gene from *B. thuringiensis kurstaki* HD1 "Dipel" plasmid preparation. They expressed the ICP gene in *Escherichia coli* and showed activity to *Manduca sexta* larvae. The cloning of *B. thuringiensis kurstaki* crystal toxin gene in *Escherichia coli* has led to the cloning of additional toxin genes from other subspecies (Klier *et al.*, 1982, 1985; McLinden *et al.*, 1985; Whiteley and Schnepf, 1986). The availability of these cloned genes opened door to the sequencing of crystal protein genes, determination of their location in various subspecies (Kronstad *et al.*, 1983; Klier *et al.*, 1985), and investigations of the mechanisms regulating gene expression (Whiteley and Schnepf, 1986; Aronson *et al.*, 1986; Hofte and Whiteley, 1989; Lereclus *et al.*, 1989a). Based on these recombinant

DNA technology, in 1985 the first protein gene sequences were published (Adang *et al.*, 1985; McLinden *et al.*, 1985; Schnepf *et al.*, 1985; Shibano *et al.*, 1985), these sequences have provided the basis for the construction of gene specific probes to screen *B. thuringiensis* strains by hybridization analysis for the presence of known nucleotide sequences (Kronstad and Whiteley, 1986; Prefontaine *et al.*, 1987; Sanchis *et al.*, 1988; Visser 1989). *type, aizawai T.29, dendrolimus and wuhanensis)* has already been established (Aronson *et al.*, 1986; Lereclus *et al.*, 1989b) and the possibility that such genes could be carried by very large plasmids (> 150 MDa), which cannot be discriminated from the chromosome, the presence of one or several toxin genes on the same or different replicons, including very large plasmids or the chromosomes, is now an established feature of the crystal protein genes, for example the following strains: *kurstaki HD-1, israelensis* and *aizawai 7.29* harbour at least five separate crystal protein genes (Entwistle *et al.*, 1993).

The discovery that the δ-endotoxin genes are located on plasmids provided means of shuffling toxin genes between strains in an effort to unravel the relationship between the gene composition and phenotypic toxicity, and to construct strains with novel insecticidal activity spectra.

1.3.12 The Evaluation of B. thuringiensis as a commercial biopesticide

B. thuringiensis has emerged among the insect pathogenic bacteria as a possible candidate for pest control. *B. thuringiensis* was discovered in 1911 (Berliner,1911). This insect pathogen is simple, spore - forming, infectious bacterium, easy to grow on laboratory media and could be produced under large scale. *B. thuringiensis* has been the active ingredient in a wide array of biological insecticides for nearly a half a century. Bioassays of individual purified insecticidal crystal protein have spurred investigations into its use as a natural biological control agent in agriculture, forestry and human health for the elimination of disease vectors. The future prospects for the use of *B. thuringiensis* look favorable. It is now possible to identify *B. thuringiensis* that are particularly active on various target insects and to use cloning and expression of individual insecticidal crystal proteins to construct strains

carrying several ICPs selected could be used to construct new *B. thuringiensis* strains for different crops and insect applications. The choice of *B. thuringiensis* as expression host has several advantages. First, *B. thuringiensis* itself is is naturally capable of stably maintaining several different ICP genes without undergoing loss or gene rearrangement, second, *B. thuringiensis* can express these ICP genes to high level such that 25-30% of the *B. thuringiensis* total protein can be ICP proteins, third, natural *B. thuringiensis* plasmids can be used as cloning vectors for constructing new IC P combinations, as well as a *B. thuringiensis* transposon that encodes both transposase and a site-specific recombinase. These elements greatly facilitate the construction of new ICP combinations that do not contain antibiotic resistance genes or other undesired foreign genes. Thus, the new *B. thuringiensis* constructs consist only of *B. thuringiensis* DNA.

The first new *B. thuringiensis* product derived by this recombinant technology was developed as a superior product for control of Colorado potato beetle, as well as caterpillar pests of potato,tomato and eggplants by Ecogen. This new product contains two different ICP proteins of the active cryIII group, in addition to two caterpillar-active cryI genes.

In addition, these particular combination of genes are designed to minimize the development of resistance. First, the cryIII proteins expressed have different binding characteristics on midgut cell membranes . In laboratory selected insects that are resistant to one of the cryIII or CryI proteins showed only minimal resistance to the second cryIII or CryI. Thus, in practice, an individual insect would have to undergo two independent resistance mutations to become resistant to genes developed by this recombinat technique. Second, it has been found that insect strain selected for resistance to single cryIII protein when exposed to a mixture of the cryIII and the cryI protein contained in the recombinant gene, the cryIII resistance was strongly reduced. this efffect could be probably due to some protein-protein interaction that occurs bbetween the two ICPs at the level of midgut binding. Thus the recombinant strain incorporates two different strategies to minimize the likelihood that the principal insect target would develop resistance to the product. The combined approach will not only

allow for development of new and improved *B. thuringiensis* products, but also to effectively manage the potential for insect resistance development by continuing to exploit the ability to express multiple ICP genes having diverity of activities. The insects would have to overcome two or more insecticidal genes to become resistant, a less likely occurrence known as disruptional selection. Transgene combination also increases the toxic effect to insects.

The following advantages are in support of *B. thuringiensis*.

1. The δ- endotoxin as well as the spores incorporated into the industrial products based *B. thuringiensis* have no mammalian toxicity. These microbial insecticides are specific to herbivorous insects and in aquatic environment, to filter feeders. It does not harm predators and non-target insect populations. Thus, *B. thuringiensis* are safe to man and for virtually all beneficial animals and plants. Besides being biodegradable, it has no phytoxicity. The safety record demonstrated with this insect pathogen is impeccable, there have been no reports of harm associated with its use for pest control. *B. thuringiensis* has proved totally safe even on food harvested the same day for human consumption, which is very important for plant protection in vegetable crops. The host specificity of *B. thuringiensis* is so restricted that it could never, pose a threat to human health and all beneficial animals and to plants, as well as being biodegradable. As expected, there are no conclusive indications that the organism is a conspicuous threat to human well-being, but there could be disquieting information that does exist.
2. The spores and crystals are readily produced by aerated liquid fermentation and are easily harvested and when properly formulated have a long shelf life and the resulting products are easy to apply with conventional machinery, both from ground and air.
3. *B. thuringiensis* possesses a considerable unexploited potential. The finding of new strains with increased activity or with an altered host spectrum are distinct possibilities. This has been shown, for example HD-1 strain, which has a multiple activity compared to other strains, and *B. thuringiensis* has been

demonstrated with discovery of the mosquito, black flies and now tsetse active variety, as a potent agent with which to attack these and other vectors of disease.

***B. thuringiensis* preparations have constraints:**

1. The narrow host spectrum, limited to lepidopteran and coleopteran in plant protection, in many cases precludes its use, since insect species belonging to other orders have to be controlled simultaneously, which can be achieved only with broad-spectrum chemical insecticides.
2. Use of *B. thuringiensis* requires more sophistication on the part of growers since proper timing is needed, where as chemicals are quite often applied on a prophylactic basis. Although the action of the δ-endotoxin is fast, insects die slowly, with chemical insecticides farmers are accustomed to seeing an immediate effect.
3. *B. thuringiensis* being a stomach poison, the toxin has to be ingested by target insects to take effect, so coverage of the plant has to be correspondingly better than for a contact insecticide. The toxin crystals are particulate, they are less easy to apply than soluble material, reaching only surface feeding pests. The feeding activity of the insect depends on environmental conditions, such as temperature, light, humidity, and the quality of the accessible food. Degradation in the environment is rather too rapid so that application often needs to be frequent. If not enough toxin is incorporated, the larvae or adult insect may recover from the gut lesions and resume feeding. In cases where the larvae are mainly found inside the stalk they are therefore, inaccessible to *B. thuringiensis* in case of stem borers. Also there is one obvious difference between *Glossina* spp and other *Diptera* spp (black flies and mosquitos) that have been controlled by *B. thuringiensis* is that tsetse do not have aquatic larvae offering a wide range of possibilities of infection by *B. thuringiensis*. In Lepidoptera spp it is the immature stages, eggs and larvae particularly, which suffer heavy mortality from a variety of causes; in *Glossina spp* each such stages are within the female parent or restricted to a few minutes on the surface of soil before burrowing and

pupariating

The advantages outweigh the constraints since the emphasis for insect control agents lies on safety aspects, delivery of the toxin and the development of resistance. Since each δ-endotoxin is the product of a single gene, the emergence of recombinant DNA technology and other genetic techniques have provided unprecedented opportunities for manipulating toxicity and toxin delivery systems. Recombinant DNA technology and other genetic techniques have enabled genes to be inserted into plants and expressed as toxin production in plants tissue, taking a prominent place among the growing number of factors that could be genetically engineered into plants to increase their protection against pests. Such systematic toxin will reach burrowing as well as surface-feeding pests. Filter feeding aquatic and blood feeding insects could be controlled by engineering toxin genes into some of their food microorganisms.

The major issue in the use of *B. thuringiensis* is the rather narrow range of hosts susceptible to any particular isolate but with the discovery of many agronomically important pests are not susceptible to any of the known *B. thuringiensis* isolates. Furthermore, restrictions are in force on the introduction of exotic microorganisms for pest control purposes Most countries, India being an example, require that only indigenous species of *B. thuringiensis* be used for insect control. There is no doubt whatsoever about the enormous benefits of *B. thuringiensis* as demonstrated in many different biological control projects in many different parts of the world.

The World Health Organization's (Geneva) Onchocerciasis Control Program in West Africa is arguably the most outstanding. Onchocerciasis (river blindness) is a chronic disease, caused by a nematode transmitted by black flies, which in the past has blinded over one million individuals. Over the past two decades, however, aerial spraying of *B. thuringiensis* to attack black fly larvae has interrupted transmission of the disease over some 60,000 square kilometers, with no cases of blindness reported among the nine million children within the area.

1.3.13 Source of B. thuringiensis used in the studies.

New *B. thuringiensis* strains have recently been isolated at ICIPE. Some of these have been shown to be active against the lepidopteran pests, *Chilo partellus Swinh (Pyralidae) and Spodoptera exempta Walker (Noctuidae)* (Brownbridge, 1991) and dipteran pests, *G. m. morsitans (Osir et al*, 1993). These species differ markedly in their relative responses to the *B. thuringiensis* strains which show little cross-genus activity. Thus, we have *B. thuringiensis* strains and pest species which have been little studied in relation to the nature of the subunits in the parasporal crystal, the nature of the protoxin and activated endotoxin and the types of binding sites involved in the intoxication process.

1.4 Rationale of the study

Insects have shown extraordinary ability to develop resistance to chemical insecticides. Over 500 insects are now resistant to all known classes of synthetic pesticides. The development of resistance is one of the most critical issues to be addressed by pest and disease vector managers at present. The large development and registration costs of chemical insecticides, increasingly stringent regulatory environment, pest resistance problems of new chemical pesticides have stimulated the critical need for new and existing insecticides to be used wisely. Insecticidal products based on *B. thuringiensis* are the most effective microbiological pesticides used in agriculture, forestry and public health today. Despite their success, *B. thuringiensis* usage in comparison to chemical insecticides has been small, but several developments have led to rapidly expanding usage of this insecticide products. These have increased environmental concerns leading to withdrawals of some chemical insecticides from the market, price competitive with chemical and improved product efficacy, and eventually use of *B. thuringiensis* as a resistance management tool.

B. thuringiensis use as a microbial insecticide have shown very few documented cases of *B. thuringiensis* resistance development in actual field use in the past 30 years. It was therefore, necessary to carry out *B. thuringiensis* research to manipulate

the *B. thuringiensis* genes with the aim of increasing the host range and identify possible mechanism by which both potency as well as activity spectrum could be enhanced leading to development of a vastly improved insect biological control products. Some microbial products under development are plant endophytes engineered to contain certain *B. thuringiensis* protein genes and *B. thuringiensis* genes engineered into *Pseudomonas* species have shown longer residual activity than conventional *B. thuringiensis* products and *B. thuringiensis* products based on recombinant DNA technology have so far shown the potential to improve efficacy and persistence, which will lead to wide use of *B. thuringiensis*-based biotechnology products with least resistance development. These, new *B. thuringiensis*-based biotechnology products, combined with established microbial techniques may provide answers to specific research problems like enhancing specificity or increasing the toxicity. Therefore, further genetic studies of the crystal toxin gene will allow for subsequent understanding of the biochemical mechanism of toxicity and the factors that determine the extreme specificity of the proteins towards insects and may ultimately lead to the design of proteins with improved toxicity and tailored specificity.

1.5 Aims and specific objectives.

The overall aim of the present study was to clone, screen, sequence and characterize *B. thuringiensis* gene encoding crystal protein genes active against *Glossina morsitans* and *Chilo partellus*. The *B. thuringiensis* gene was screened by hybridization analysis using known gene specific probes for the presence of known nucleotide sequences and characterization of the protein from this new isolate. Comparison of sequences of the proteins, therefore, offer unique opportunity to investigate the mode of action of the δ-endotoxin.

The specific objectives were as follows:

1. Isolation and Optimization of conditions for preparation of total cell and plasmid DNA from *B. thuringiensis*
2. Identification of delta endotoxin exhibiting toxicity to tsetse
3. N-terminal amino acid sequence analysis of the purified *B. thuringiensis* δ-

endotoxin

4. Cloning and sequence analysis of the genes responsible for production of the δ-endotoxin.
5. Characterization of crystal proteins from these new isolates.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Analytical grade chemicals were obtained from BDH (England); Sigma Chemical Co (USA); Pierce Chemical Co (Rockford, USA); Serva (Germany) and Bio-Rad (Richmond, USA). Restriction endonucleases, primers and radio-labelled compounds were obtained from Amersham (England), New England Biolabs (Beverly, MA, USA) and Boehringer Mannheim (Germany) respectively. pBluescript M13mp 18RFDNA, Sequenase version 2 sequencing kit and DNA maxi-prep plasmid Kit were purchased from Stratagene (USA); Lysozyme was obtained from Sigma Chemical Co. (USA); Nitrocellulose Hybond N+ membrane were obtained from Amersham (England). Dioxigenin labelling kit was obtained from Boehringer Mannheim (Germany); Nucleobond AX PC-100 Kit was obtained from Machery-Nagel (West Germany); S_1 nuclease, Klenow fragment of DNA polymerase and T_4 DNA ligase and dNTPs were obtained from Boehringer Mannheim (Germany).

2.2 Experimental insects

All insects were supplied by the Animal Rearing and Quarantine Unit of the ICIPE. Adult female tsetse, *Glossina morsitans morsitans* Westwood, were reared on a 12 h:12 h light:dark photoperiod at 70 % relative humidity and 27° C and fed on whole rabbit blood. Fourth instar *Chilo partellus* Swinhoe larvae were from a colony that had been reared on a natural diet of sorghum leaves and stems for more than 12 generations (Odindo *et al.*, 1992).

2.3 Bacterial strains and plasmids

The bacterial strains *Escherichia coli (E. coli)* LK 111 (Zabeau and Stanley, 1982) and JM 105 (Yanisch-Perron *et al.*, 1985) were used. Strain *B. thuringiensis L1-2* was obtained from the ICIPE Microbial Bank. The strain was originally isolated in Kenya and shown to be active against *C. partellus* (Brownbridge, 1989). The cloning

vector *pEcoR251* (Lin *et al.*, 1990) was the plasmid used to generate the gene library. The structure of *pEcoR251* is similar to other plasmids utilizing inactivation of the EcoRI endonuclease gene as a selective marker (Cheng and Modrich, 1983) and pBluescript vector (Stratagene, USA) was used for DNA sequencing. *pES1* (Schnepf and Whiteley, 1981) was the source of the cryIA(a) gene.

2.4 Media, buffers and storage of biological materials

All media, buffers and solutions not described in the text are in Appendix A. All biological study material were carefully stored in stab cultures containing glycerol in the dark at 4° C in the Microbial Bank at ICIPE (Sambrook *et al.*, 1989).

2.5 Growth conditions.

Escherichia coli (E. coli) LK 111 and *JM 105* were grown in Luria-Bertani (LB) medium (**Appendix A**) (Sambrook *et al.*, 1989) and ampicillin (100 µg/ml) was used to select for the transformants. *B. thuringiensis L1-2* were cultured using a single inoculum stage. During the inoculum stage, the seeding of culture was performed in 10 ml MNB or SPY broth (Appendix A) with bacteria from a slant culture which had been stored at 4° C. The broth was incubated (220 rev/min, 28° C, 16 h) on an orbital shaker (Labline, Melrose Park, USA). A 1/100 dilution of an overnight starter culture was used to inoculate 100 ml prewarmed (28° C) MNB or SPY media and incubated with shaking (220 rev/min, 28° C, 4 h) until the culture had reached early exponential phase (OD_{600} = 0.3 - 0.6). The *B thuringiensis* cultures used for the isolation of endotoxin crystals were incubated (220 rev/min, 28° C, 48-72 h) until autolysis of sporulating bacterial cells was completed (Krieg, 1981).

2.6 Preparation and transformation of competent E. coli cells

E. coli cells were made competent for DNA uptake according to the method of Sambrook *et al* 1989. A 1/100 dilution of an overnight *E. coli* cultures in LB plate was inoculated into 25 ml prewarmed LB medium and incubated at 37° C, with shaking, until the culture reached early exponential phase (OD_{600} = 0.3 - 0.6). The cell culture was

poured into a pre-cooled sterile SS-34 tube and the cells were harvested by centrifugation (5,000 x g, 10 min, 4° C). The cell pellet was resuspended in 2.5 ml (1/10) ice-cold transformation and storage buffer (TSB: 10 % (w/v) PEG M_r 4,000, 5 % (v/v) DMSO, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in LB medium) and incubated on ice for at least 10 min. Cells were then either used immediately for transformation or aliquoted and stored at -70° C. Frozen cells retained satisfactory competence for two weeks. For transformation, approximately 50 ng plasmid DNA was mixed with 100 µl *E. coli* competent cells and incubated on ice for 30 min. After the addition of 900 µl of preheated TSB, containing 20 mM glucose, cells were grown at 37° C for 60 min to allow expression of the ampicillin resistance gene. Aliquot of 100 µl transformed cells were plated on LB agar containing ampicillin (100 µg/ml) plates. In cases where the inactivation of the ß-galactosidase gene of the pBluescript SK+ vector was used as marker, 40 µl X-gal solution (20 mg/ml X-gal in N,N-dimethylformamide) was spread on LB agar plates containing ampicillin (100 µg/ml) before transformed cells were plated out.

2.7 Isolation of endotoxin crystals

The endotoxin crystals were harvested from the 48-72 h culture by a single step involving low speed centrifugation (10,000 x g, 10 min, 4° C) in a GSA rotor using a model RC-5C Sorvall centrifuge (Dupont, Delaware, USA). The sporangial debris were further removed by washing the crystal/spore pellet three times with distilled water, centrifuged (9,000 x g, 10 min, 4° C). The crystals were separated from the spores using a modification of a method described by Knowles and Ellar (1986). Briefly, the crystal/spore suspension (1 ml) were carefully layered onto 10 ml linear sucrose gradients (60-70 %). The gradients were centrifuged (8,000 x g, 30 min, 4° C) in a Beckman SW 41 Ti rotor using a model L8-M Beckman ultracentrifuge (Beckman, Palo Alto, Ca, USA). The interphase containing the crystals was carefully pipetted off and washed three times in phosphate buffered saline (PBS: 0.005 % $NaH_2PO_4.2H_2O$, 0.004 % $Na_2HPO_4.2H_2O$, 0.015 % NaCl) by centrifugation (10,000 x g, 10 min, 4° C) to remove the sucrose. The pellet was resuspended in PBS (1 ml) and layered onto a

second linear sucrose gradient. The pellet obtained from the above was washed in PBS as described above in order to remove sucrose. The crystals that settled at the bottom of the tube were washed three times with distilled water (each washing cycle included centrifugation at 10,000 x g, 10 min, 4° C). Purity of the crystals was ascertained by phase contrast microscopy or by compound microscopy after Smirnoff staining (Smirnoff, 1962). The purified crystals were resuspended in 1 ml PBS, lyophilized and stored at -20°C.

2.8 Solubilization of the endotoxin crystals

The purified endotoxin crystals (~0.5 mg) were suspended in carbonate buffer (50 mM Na_2CO_3.$NaHCO_3$, pH 9.5) containing 10 mM DTT, and incubated (37° C, 30 h) in a water-bath. After incubation, the suspension was centrifuged (10,000 x g, 10 min, 4° C) using an Eppendorf 5415C centrifuge (Eppendorf-Netheler-Hinz, Hamburg, Germany). The supernatant fraction (solubilized protoxin) was dialyzed for 6 h in PBS to remove Na_2CO_3.$NaHCO_3$ and pellet fractions were stored at -20° C.

2.9 Preparation of protein extracts

E. coli harboring plasmids encoding *B. thuringiensis* protein gene were grown in the 500 ml LB medium and ampicillin (100 µg/ml) for 24 h at 37° C and then harvested by centrifugation (10,000 x g, 10 min, 4° C) in a Sorvall centrifuge using an SS34 rotor (Sorvall, Dupont, Delaware, USA). The pellet was then frozen at -80° C overnight. After thawing in ice, the cells were resuspended in 25 ml lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, containing 0.1 mg/ml lysozyme). The cells were further disrupted by three rounds of sonication (30 sec each, with cooling in between). After centrifugation, the resulting pellet was resuspended in 25 ml lysis buffer, sonicated and centrifuged as described earlier, washed in 25 ml (0.5 M NaCl) and finally dissolved in 2 ml distilled water. Purity of protein was ascertained by Denaturing-Polyacrylamide gel electrophoresis (PAGE).

2.10 Protein determination

Protein was estimated by the Pierce Bicinchoninic acid (BCA) protein assay (Smith *et al.*, 1985) method according to instructions supplied by the manufacturer (Pierce Application Note 23225, 1984). Absorbance were measured at 562 nm using a model DU-50 Beckman spectrophotometer (Beckman, Palo Alto, CA, USA). BSA fraction V, (Serva, Heidelberg, Germany) was used as the protein standard.

2.11 Protein electrophoresis

Electrophoresis on denaturing polyacrylamide gels was carried out by the method of Laemmli (Laemmli, 1970). Gradients (4 - 15 %) were cast using a gradient maker (BRL, Gaithersburg USA). Samples were dissociated by heating at 95° C for 5 min with an equal volume of sample buffer (125 mM Tris-Cl pH 6.8; 10 % (w/v) SDS; 1 % (v/v) ß-mercaptoethanol; 20 % (v/v) Glycerol; 0.002 % (w/v) Bromophenol blue). High and low molecular weight standards were from Bio-Rad. Gels were stained for protein with Coomassie Brilliant Blue R250 (0.25 % (w/v) in staining solution (Appendix A) and destained in destain solution (25 % (v/v) methanol, 10 % (v/v) acetic acid, 65 % (v/v) water. Molecular weights were determined from plots of log of molecular weight versus the relative migration of the standards.

2.12 Bioassays

2.12.1 Chilo partellus

In all experiments, the larvae were maintained on artificial diet (4 ml). Petri-dishes (8.5 cm, i.d.). The toxicity of the δ-endotoxin against *Chilo partellus*, was tested using artificial diet (4 ml) maintained at 28° C with a photoperiod of 12:12 (Light:dark) and relative humidity was of 70%. For the first screening for mortality, samples containing known δ-endotoxin concentration were added to the artificial diet (4 ml) and thoroughly mixed to give a final concentration of 100 µg/ml. For the LC_{50} values on the larvae, dilutions of δ-endotoxin were made in PBS solution. The control were treated in the same way but without the δ-endotoxin added to the diet. These diets were allowed

to solidify and cool to 27° C. Ten fourth instar *C. partellus* larvae, previously starved for 24 h, were introduced into each petri-dish, including the control. The experiment was carried out at 27° C, and the mortalities scored at fixed time intervals for up to 72 h The assay were replicated three times. The data were fed onto a computer and slopes of the lines calculated and analyzed with the Probit analysis (SAS, USA). The LC_{50} values were obtained from the Probit analysis. The Probit analysis calculates the index of significance for toxicity estimation (g), if $g > 0.5$, then confidence levels are not calculated. Therefore, only confidence limits fitting the given parameter were included in the results.

2.12.2 Glossina morsitans morsitans

In all experiments, the adult *G. m. morsitans* were maintained on whole rabbit blood. In the toxicity assays for the δ-endotoxin against *G. m. morsitans*, δ-endotoxin samples prepared in PBS buffer were thoroughly mixed with the blood to give concentrations of 25, 50, 100, 150 or 200 μg/ml respectively. The control did not contain the δ-endotoxin. Twenty five teneral (24 h post-emergence) female *G. m. morsitans* were starved for 24 h. The δ-endotoxin preparations were added to whole rabbit blood, and equal volume of PBS buffer was added to each control sample. The tsetse were allowed to feed on the blood through an artificial silicone membrane (Mews and Ruhm, 1971) maintained at 37° C. Mortality was scored at fixed intervals for up to 54 h. The assay was replicated three times. The LC_{50} values were calculated from the slopes of the lines and analyzed with the Probit analysis.

2.12.3 Production of antibodies

Antibodies against the *B. thuringiensis* protoxin and *E. coli* LK111 containing recombinant *B. thuringiensis L1-2* was produced in New Zealand White rabbits using a previously described protocol (Osir *et al.*, 1986). The animals were bled through the main ear artery and the blood allowed to clot in capped plastic tubes for 3 h at 27° C and then stored at 4° C overnight. Serum was separated from the clots by centrifugation (7,000 x g. 15 min). 0.04 % sodium azide was added to the serum and

stored at -70° C. In order to test the binding properties of the antibodies, protein samples were first separated by SDS-PAGE and then electrophoretically transferred onto Immun-Lite nitrocellulose membranes (Towbin *et al.*, 1979) using the LKB-NOVA BLOT (Broma, Sweden) transfer system (Osir *et al.*, 1995). The transfer was performed for 2 h at a constant current of 0.8 mA per cm^2. Immuno detection was carried out by the Immuno-Lite chemiluminiscent assay kit (Bio-Rad, USA) as follows. After the transfer, the Immuno-Lite membrane was immersed into 5 ml Tris-buffered saline (5 % Non-fat dry milk in TBS; TBS: 20 mM Tris, 500 mM NaCl, pH 7.5). The membrane was gently agitated using an orbital shaker platform and incubated (27° C, 30 min) followed by washing in 5 ml Tris-Tween buffered saline (TBBS: 20 mM Tris, 0.05 % Tween-20, 500 mM NaCl, pH 7.5) solution for 5 min. The primary antibodies anti-*B. thuringiensis L1-2* was used at a dilution of 1:100 in TTBS (1 % non-fat dry milk in TBBS) for 60 min with gentle agitation. Unbound primary antibodies were removed by washing the membrane three times in 5 ml TBBS (5 min each wash) at 27° C. Conjugate binding step was performed by incubating the membrane in second antibody (goat anti-rabbit IgG (H+L) AP conjugate at a dilution of 1:330 in antibody buffer (1 % non-fat dry milk in TBBS) for 30 min with gentle agitation. The conjugate solution was removed and the membrane rinsed in TBBS for 5 min. The solution was decanted and membrane washed twice in 5 ml TBBS and once in TBS. Blot development was achieved by immersing the membrane in the chemiluminscent substrate solution (0.1 M Diethanolamine, 1 mM $MgCl_2$, pH 10) at a dilution of 1:1880 and incubated by agitation for 5 min. Excess solution were drained and membrane sealed in heat sealable bag. Autoradiography carried out for 0.5 - 1.5 h at 27° C.

2.13 Miscellaneous DNA techniques

Standard recombinant DNA techniques unless otherwise described, were performed as described by Ausubel *et al.*, (1991) and Sambrook *et al.*, 1991.

2.13.1 Small scale preparations of plasmid DNA

Cell cultures were grown overnight at 37° C in 3 ml LB medium and 100 μg/ml ampicillin, with vigorous shaking. Cells were harvested by centrifugation in a eppendorf 5415C centrifuge, and the cell pellet drained and resuspended in 300 μl of solution I (25 mM Tris-HCl, pH 8.0; 10 mM EDTA; 50 mM glucose). To this, 600 μl freshly prepared solution II (0.2 M NaOH; 1 % SDS) was added, the contents gently mixed by inverting the tube, and then incubated on ice for exactly 5 min. 450 μl of ice cold solution III (3 M potassium acetate, pH 4 8) was added, the tube contents was gently mixed again, and incubated on ice for 5 min. The sample was then centrifuged for 10 min, the supernatant solution removed to a clean tube, RNase A was added to a final concentration of 50 mg/ml, and the sample incubated (27° C, 30 min). The sample was extracted with phenol/chloroform and the DNA precipitated with an equal volume of isopropanol. After centrifugation, the DNA pellet was washed with 70 % ethanol, lyophilized, and resuspended in TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA).

2.13.2 Large scale preparations of plasmid DNA

Cells cultures were grown overnight at 37° C in 100 ml LB medium and ampicillin (100 μg/ml). 1/100 volume of the overnight culture was inoculated 500 ml LB medium and ampicillin 100 μg/ml (prewarmed to 37° C). The culture was incubated at 37° C with vigorous shaking (200 rev/min) on a rotary shaker to late log phase (OD_{600} of ~ 0.4). The culture was harvested by centrifugation (5000 x g, 5 min). The cells were resuspended in 10 ml solution I (solutions I, II and III were the same as used in the small scale preparations), 20 ml solution II was added and gently mixed with the resuspended cells. The sample was then incubated on ice for 5 min, 15 ml of ice-cold solution III added, again gently mixed and left on ice for 10 min. The bacterial lysate were centrifuged (5.000 x g, 10 min) and the supernatant solution transferred to a clean tube. DNA was precipitated with 0 6 volume of isopropanol and collected by centrifugation (5, 000 x g, 15 min). The supernatant solution was decanted carefully and the pellet rinsed with 70 % ethanol. DNA pellet was resuspended in 3 ml of TE

buffer. Caesium chloride and ethidium bromide were added to final concentrations of 1 μg/ml and 500 μg/ml, respectively. The samples were transferred into Beckman QuickSeal tubes sealed and centrifuged (55,000 x g. 20° C, 12 h) in a Beckman VTi 65 rotor. After centrifugation, DNA bands were visualized by fluorescence under UV illumination (365 nm). The DNA band containing closed circular plasmid DNA were collected through the side of the tube using an 18 gauge hypodermic needle. Residual ethidium bromide was removed from the DNA by adding an equal volume of water 1-butanol saturated with water and the CsCl removed by diluting with 3 volumes of water. The DNA was precipitated with 0.6 volume isopropanol and centrifuged (10,000 x g, 15 min, 4° C). The DNA pellet was resuspended in 1 ml TE buffer (pH 8.0), RNase A was added to a concentration of 50 μg/ml, and the sample incubated (27° C, 30 min). OD_{260} of the final solution of DNA was measured and concentration of plasmid DNA calculated. DNA was stored in aliquots at -20° C.

2.13.3 Mini-preparation of Chromosomal DNA

Small quantities of chromosomal DNA could be isolated in less than four hours by this method. Cells from transformed colonies were inoculated in 750 μl medium (LB or MNB or SPY) and ampicillin (100 μg/ml). The culture were incubated at 37° C with vigorous shaking (200 cycles/minute on a rotary shaker) with tubes in a horizontal position for 2-3 h, cells were harvested by centrifugation (12,000 x g, 60 sec) in Eppendorf 5415C centrifuge. The cells were resuspended in 40 μl STE (10 mM Tris-HCl pH 8.0; 100 mM NaCl; 1 mM EDTA). An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added, the mixture was thoroughly vortexed, and the phases separated by centrifugation (12,000 x g, 3 min) in a microcentrifuge. The top aqueous phase was transferred to a new Microfuge tube. 5 μl of the aqueous phase was loaded directly onto a 0.8 % agarose gel to check for the chromosomal DNA. This technique was used extensively for sizing and selection of inserts with right sizes especially during library screening and for searching for suitably-sized shortened inserts after Henikoff shortening to which will be used for sequencing purposes.

2.13.4 Large scale preparation for Chromosomal DNA

Chromosomal DNA was obtained by the method of Kronstad *et al.*, (1983) with the following modifications. An overnight culture was started from a fresh plate in LB-medium at 30° C, with shaking at 200 rev/min. A 1/100 dilution of the overnight culture in LB medium was inoculated into 200 ml prewarmed (37° C) MNB or SPY culture and incubated at 37° C with shaking (200 rev/min), until the culture had reached early exponential phase (OD_{600} = 0.3 - 0.7). The cells culture was harvested by centrifugation (5,000 x g. 10 min, 4° C). Cell pellet was washed in 2 ml of 1M NaCl and incubated at 37° C with shaking (200 rev/min), pelleted by centrifugation (5,000 x g, 10 min, 4° C). Cells were washed twice in 5 ml STE buffer. The cell pellet was resuspended in 5 ml of 50 % sterilized glycerol before freezing at -80° C overnight. Cells were thawed and then resuspended in 5 ml of lysis buffer (150 mM NaCl, 100 mM EDTA, pH 7.9 containing 0.25 mg/ml lysozyme) and the cells were incubated (37° C, 20 min). Further lysis was effected by addition of 6.25 ml of lysis buffer (100 mM Tris, pH 7.9, 100 mM NaCl, 2 % SDS) followed by incubation (65° C, 1 h). The lysate was first extracted three times with 15 ml 50:50 mixture of phenol-chloroform, followed by two times extraction with 5 ml of chloroform, then 5 ml of water-saturated ether. After the final extraction, DNA was precipitated with 1/10 volume of 7.5 M ammonium acetate and 2.5 volumes ethanol (absolute) and placed at -20° C for 1 h, then DNA was spooled onto a thin glass rod, rinsed with 70 % ethanol and dissolved in 3 ml TE buffer (pH 8.0).

2.13.5 Restriction endonuclease digests

Both small and large scale plasmid DNA preparations were used in restriction endonuclease digests. Restriction endonuclease digests were carried out using the manufacturer's restriction endonuclease buffers. For digestion with KpnI, Bovine serum albumin (BSA) was added to the low salt incubation buffer to a concentration of 100 mg/ml. All digestions were carried out by incubation (37° C, 10-120 min) in water-bath, except for SmaI, which was incubated at 25° C. Typically, DNA (1-5 μg) was digested

using, 2 units of restriction enzyme per μg DNA in a 20 μl total reaction volume. Multiple digests were done where necessary, or simultaneously in cases of buffer and temperature compatibility, or sequentially (starting with the enzyme with the lowest salt requirement) if buffers salt concentration varied. In cases of buffer incompatibility DNA samples were phenol/chloroform extracted and ethanol precipitated between digestions. Small aliquot of digested DNA were routinely checked for complete digestion by agarose gel electrophoresis in a small "slide" gel.

2.13.6 Preparation of DNA templates for sequencing

Double stranded DNA templates used in sequencing reactions were purified by caesium chloride density gradient and digested (30 min, 20° C) with DNase-free RNase A (1 μl of a 10 mg/ml aqueous stock solution), followed by one phenol/chloroform extraction and ethanol precipitation. 5 μg of template DNA was then diluted to 18 μl with H_2O, mixed with 2 μl of 2 N NaOH, and left at 27° C for 5 min. The alkaline denatured DNA was then precipitated by addition of 120 μl neutralizing/precipitating solution (5% 2 M ammonium acetate pH 7.5, 95 % ethanol). After 10 min incubation on ice, denatured DNA templates were centrifuged (12,000 x g, 20 min, 4° C). The pellet was washed gently with ice cold 70 % ethanol and vacuum-dried.

2.13.7 Phenol/Chloroform extractions

Nucleic acid preparations were phenol/chloroform extracted to remove contaminating proteins during purification, or for the removal of enzymes after enzymatic manipulations of DNA. An equal volume of buffered phenol/chloroform/isoamylalcohol (25:24:1) was added to the DNA preparation, the mixture emulsified by vortexing, and the nucleic acid-containing aqueous phase separated by centrifugation. The extraction was repeated until no precipitate could be seen on the aqueous/organic phase interface (normally three times). Trace amounts of phenol were removed from the preparations by extraction with water-saturated diethyl-ether. Nucleic acids were then precipitated from solution by the addition of

sodium acetate (pH 5.2) to 120 mM and either 2.5 volumes of absolute ethanol or 0.6 volume of isopropanol, followed by centrifugation (12,000 x g, 5 min, 4° C). DNA pellets were washed with ice-cold 70 % ethanol, dried, and resuspended in an appropriate buffer.

2.13.8 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a horizontal GIBCO-BRL-Submarine agarose gel units (BRL Scientific Instruments, San Francisco). Electrophoresis of large (20 cm long x 15 cm wide x 0.7 cm thick) gels were carried out at 50 V for 16 h, or 100 V for approximately 5 h. To obtain results more quickly, mini submarine agarose gel units (10 x 6.5 x 0.7 cm) or small (5 x 7.5 x 0.3 cm) gels were run (100 V. 60 or 15 min respectively). Agarose (Sigma Type 1) or low gelling temperature agarose (SeaPlaque, FMC Bio Products, ME, USA) were routinely used with TBE or TAE buffers, respectively (Appendix A). Agarose (0.6 % to 0.8 %, depending on sizes of fragments being analyzed) was dissolved in TBE or TAE buffer by melting in a Microwave oven, and cooled to approximately 50° C before pouring. The amount of DNA loaded per lane also varied with the sizes and number of fragments but under normal circumstances about 330 ng of plasmid DNA was used. Gels were run either with ethidium bromide (0.5 μg/ml) in the running buffer or were stained afterwards for 15-30 min. DNA bands were visualized on a 254 nm UV transilluminator. Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3,000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f4.7 was used. Fragment sizes were calculated by extrapolation from a standard curve of the mobility of Lambda DNA fragments, digested with either Hind III or PstI, plotted against the $\log_{10}$ of their molecular weights.

2.14 Construction and screening of B. thuringiensis L1-2 library

Plasmid DNA were digested with *Bgl*II endonuclease and ligated to *Bgl*II-digested pEcoR251 vector. The ligated DNA were used to transform competent *E. coli* LK111 cells (Appendix C). The transformants were selected on LB agar and ampicillin (100 μg/ml). Plasmids were analyzed by standard procedures (Sambrook *et al.*, 1989).

2.14.1 Ligation reaction

The methods of Sambrook *et al.*, (1989) were generally used. Recircularization reactions for isolating deletion plasmids contained DNA concentrations of 1 pmole/ml or less. A DNA concentration of 5 pmole/ml was used for recombination reactions. Ligation reactions containing DNA, ligation buffer (Appendix A) and water to the required volume, were performed in sterile Microfuge tubes. Sticky-end ligations were performed at 27° C for 3 h or at 15° C overnight using 0.1-0.25 units of ligase, whereas blunt-end ligations were performed at 27° C for 2-20 h using 20-200 times more ligase.

2.14.2 Subcloning protocol

The rapid subcloning protocol of Struhl (1985) was used. The DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (1 %) (SeaPlaque, FMC Bio Products, ME, USA) in TAE buffer (Appendix A). The gel was stained with ethidium bromide after electrophoresis and the DNA bands viewed under UV light (310 nm), as briefly as possible. The desired bands were excised using sterile scalpel blades, in as small a volume as possible. The gel slices were melted at 70° C for 5 min in a Microfuge tube and the required amounts (2 μl vector DNA, 8 μl insert DNA) were added hot to the prepared ligation mixture containing ligation buffer, ligase and water (10 μl). The ligation was incubated at 27° C for 3 h, before transformation into *E. coli* competent cells.

2.15 Nucleic acid labelling

2.15.1 Southern blotting of DNA to nylon membranes

The procedures for Southern blot analysis were carried out as described by Sambrook *et al.*, (1989). Chromosomal and plasmid DNA from *B. thuringiensis L1-2* were digested with restriction endonucleases, and resolved by electrophoresis in an agarose gel (0.8 %, w/v) in TBE or TAE buffer. The DNA was transferred to Hybond-N membranes (Amersham, U.K) (Smith and Summers, 1980). The use of a nylon membrane allowed the capillary transfer of DNA restriction fragments in alkali rather than in neutral, high ionic strength solvents (used in conventional Southern transfer), and eliminated the need for post-transfer fixation. After electrophoresis, the agarose gel was incubated (15 min, 27° C) in two volumes of 0.25 M HCl with gentle agitation, followed by a brief rinse in distilled water to partially hydrolyse the DNA by acid depurination. The gel was then placed on top of three layers of Whatman 3 MM filter paper (wetted with 0.4 N NaOH, and placed on top of an inverted gel-casting tray in a plastic box such that the filter papers touched the base of the box, forming a wick), and overlaid with a nylon membrane and three layers of filter paper, all which had been pre-wetted in 0.4 N NaOH. A 5 cm-thick wad of dry tissue paper was placed on the gel/membrane/filter paper sandwich, followed by another glass plate and a 5 kg weight. Blotting was continued by capillary transfer to the membrane, using 0.4 N NaOH buffer. For efficient transfer, blotting was carried overnight. DNA was fixed onto the membrane by either UV-cross-linking the membrane (254 nm) for 5 min or by baking in an oven at 120° C for 30 min. Membranes were used for hybridization immediately, or stored at 4° C in a sealed plastic bag. After DNA fixing by UV illumination, nylon membranes filter was placed in a thick plastic bag with pre-hybridization solution (5X SSC; 5X Denhardt's solution, 0.5 % SDS, 50 ml/cm^2 filter). DNA probe was either radioactively labelled using random priming method (Feinberg and Vogelstein, 1983), or by incorporation of dioxigenin (DIG) nucleotides (Boehringer-Manneheim, Germany), denatured by boiling for 5 min, snap-cooled on ice, and added to the pre-hybridization solution. The bag was sealed and incubated (68 °C, 4-16 h) with shaking (200

rev/min). For radioactive blots, the filter were washed at 65°C for 10 min in low stringency washing buffer (2.0X SSC; 0.1 % SDS). Moderate (0.5X SSC; 0.1 % SDS) and high stringency (0.1X SSC; 0.1 % SDS) washes were done when necessary (as judged using a hand-held Geiger counter). Finally the filter was wrapped in Saran wrap and subjected to autoradiography.

2.15.2 Colony hybridization assays

Colony hybridization assays were carried out according to the method of Sambrook *et al.*, (1989). Bacterial colonies were transferred to nylon filter membranes (Hybond N, Amersham, UK) by placing dry filters on plates, and peeling them off gently. Filters were placed, colonies facing up, on filter paper disks soaked in 2X SSC, 5 % SDS and then transferred to filter paper disks soaked in 5X SSC; 0.1 % SDS for 5 min; then sandwiched between clean filter paper disks, and sealed in plastic bags for hybridization.

2.15.3 Radioactive labelled Nucleic Acids

Hybridization and washing conditions were essentially those described by Sambrook *et al.*, (1982). The membrane were gently shaken in pre-hybridization solution (Appendix A) for 4 h at 68° C, while the probe was being prepared DNA probes were random primed by [α -^{32} P]dCTP (Amersham, U.K), denatured by boiling for 10 min and was added to the prehybridization solution. Hybridization was carried out at 68° C for 1 h. The washing conditions were those described by Sambrook *et al.*, (1989) in low stringency washing buffer (10 min, 27° C followed by high stringency, 30 min, 27° C) and sealed in a plastic bag. The membrane were exposed to autoradiographic film (XAR-5) film using a Cronex Lightning Plus intensifying screen (Dupont, Delaware, U.S.A) overnight at -70° C.

2.15.4 Sephadex column chromatography for purification of radiolabelled DNA

Sephadex G50 (Pharmacia Fine Chemicals, Sweden) was soaked in TE buffer for a few hours and then autoclaved. A column was poured in a sterile 1 ml syringe blocked with sterile siliconized glass wool. The sample was loaded and washed through with 150 µl aliquot of TE buffer. Radiolabelled DNA fractions (as determined with a hand-held Geiger counter) were pooled, a 2 µl aliquot was removed and the cpm determined by Cerenkov counting in a Beckman liquid scintillation counter.

2.15.5 Digoxigenin (DIG)-labelling of Nucleic Acids

The blot was placed in a hybridization bag containing 20 ml standard hybridization solution (5X SSC; 0.1 % Sodium-lauroylsarcosine; 0.02 % SDS; 1 % blocking reagent). The bag was sealed and prehybridized at 68° C for 1 h. Digoxigenin (DIG) (Boehringer Mannenheim, Germany) labelled probe was heated in a boiling water bath for 10 min to denature the DNA and chilled immediately on ice. The prehybridization solution was discarded and the hybridization solution containing the Digoxigenin-labelled probe was added and the probe left to hybridize overnight at 68° C. Hybridization solution was poured into a capped polypropylene tube and stored at -20° C to be reused for future hybridizations. The membrane was washed twice, 5 min per wash, in low stringency washing solution (2X SSC; 0.1 % SDS) at 27° C to remove unbound probe. The membrane was washed twice, 15 min per wash, in high stringency washing solution (0.1X SSC; 0.1 % SDS) at 68° C. The hybridization signals can be detected directly, or the filters can be air-dried and stored for later detection.

2.15.6 Detection of Digoxigenin-labelled Nucleic Acids

After hybridization and post-hybridization washes, the membrane was equilibrated in Buffer 1 (150 mM NaCl; 100 mM Maleic acid, pH 7.5) for 1 min. The membrane was transferred to fresh bag and blocked by gentle agitation in buffer 2 (blocking reagent is dissolved in Buffer 1 to a final concentration of 10 % (w/v)) for 30-

60 min. The antibody solution was prepared by diluting the anti-DIG-alkaline phosphate 1:10,000 in buffer 2. The diluted antibody solution was mixed by inverting the tube a few times. The membrane was transferred to fresh bag containing the antibody solution, the bag was sealed and incubated (30 min, 200 rev/min). The antibody solution was discarded and the membrane transferred to a fresh bag and was washed twice, 15 min per wash, in buffer 1 (Buffer 1 + 0.3 % Tween 20), and membrane was equilibrated by washing in buffer 3 (100 mM Tris-HCl pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$) for 2 min. If Lumigen™ PPD was to be used for detection, Lumigen™ PPD had to be diluted 1:100 in buffer 3. Lumi-phos™530 was used without dilutions. The equilibrated membrane was placed in a fresh bag and 0.5 ml of Lumi-phos ™ 530 or diluted Lumigen™PPD was added and the detection reagents distributed evenly over the surface.

2.15.7 Autoradiography

Radioactive blots were exposed to Fuji 1 X-ray film (Fuji, Japan) at -70° C in X-ray cassettes with intensifying screens (Dupont, Delaware, USA). In cases of a weaker signal, as judged using a hand-held Geiger counter, the blots were exposed for longer periods on Kodak XAR 5 X-ray film (Eastman Kodak Company, NY, USA). Digoxigenin (DIG) labelled membranes were placed in the detection reagents and incubated (15 min, 37° C), exposed to X-ray film (Kodak XAR, Fuji or Chronex 8) for 7-8 h.

2.15.8 Reprobing the nylon membranes

2.15.8.1 Stripping colour-detected (NBT/Phosphate) membrane.

The colour precipitate was removed by washing the membrane with ethanol (red colour: 27° C; blue and green: + 50° - (+) 65° C). After the colour had been removed, the membrane was rinsed thoroughly in distilled water. Incubated twice for 20 min in 0.2 N NaOH, 0.1 % SDS solution at +37° C and rinsed thoroughly in 2X SSC. Then reprobing was recommenced with the prehybridization steps or stored wet in 2X SSC in a sealed plastic bag

2.15.8.2 Reprobing colour-detected (NBT/Phosphate) membrane.

The membrane to be reprobed was kept wet and washed in distilled water twice for 15 min in 50 mM EDTA, pH 8.0; containing 2X SSC at +85° C, washed twice for 15 min in 0.1X SDS, 0.2 N NaOH buffer at +37° C. Finally the reprobed membrane was rinsed briefly in 2X SSC and then reprobing was recommenced with the prehybridization steps.

2.16 Sequencing of the B. thuringiensis L1-2

2.16.1 N-terminal amino acid sequencing

The N-terminal amino acid sequence of *B. thuringiensis L1-2* was carried out by automated Edman degradation (Edman and Begg, 1967) using an Applied BioSystems model 477 A protein/peptide sequencer. The amino acid derivatives (phenylthiohydrantoin-amino acids, PTH-AA) were analyzed by an interfaced model 120 A HPLC using a C-18 PTH reversed-phase column. Amino acid sequence alignment comparision to the already sequenced cry1(A) gene classification were carried out by Osir, *et al.*, (1993).

2.16.2 Nucleotide sequencing

Plasmid DNA templates were prepared from large plasmid preparations. The DNA fragments to be sequenced were subcloned in pBluescript vectors (Stratagene, Ca, USA) and *Exonuclase*III/*S*I were used to generate two sets of overlapping deletions of opposite polarity (Henikoff, 1984). Nucleotide sequencing was performed by a modification of the dideoxy chain termination method of Sanger *et al.*, (1977) using Sequenase 2TM (a modified T7 DNA polymerase) from a Sequenase version 2.0 kit (US Biochemicals, Ohio, USA) (Tabor & Richardson, 1987).

2.16.3 Exonuclease III/S1 nuclease shortening

Shortening of DNA to obtain deletion mutants for sequence analysis was performed according to the method of Henikoff (1984). This method involved the

digestion of the recombinant clone DNA with two appropriate unique restriction endonucleases to obtain cleavage at two sites in the polylinker region on the same side of the insert DNA. Enzymes were chosen such that the one closest to the insert produced a 5' overhang (from where shortening proceeds), while the site more distal from the insert produced a 3' overhang (protected site resisting exonuclease attack). By exposing such DNA to differential time-intervals of exonuclease attack, a set of nested deletion mutants capable of self-religated were created. A recombinant plasmid *pL12.7-4* with insert of approximately 16-kb was selected for sequence analysis. Those plasmids were subjected to unidirectional shortening from each end of the insert, using exonucleaseIII and S1 nuclease (Boehringer Mannheim, Germany) to produce two sets of overlapping deletion mutants for sequence analysis of each strand.

For the reverse sequencing a primer (5' CAGGAAACAGCTATGAC 3') was obtained from Amersham (UK), 10 μg purified *B. thuringiensis L1-2* DNA were digested to completion with PstI (protection site) and BamHI (shortening site), extracted by phenol/chloroform, and precipitated with ethanol. For sequencing the complementary strand with the "forward" sequencing primer (5' GTTTTCCCAGTCACGAC 3') was obtained from Amersham (UK), the same was done using EcoRI (protected site) and KpnI (shortening site). The lyophilized DNA pellet was resuspended in 70 μl Exonuclease III buffer and equilibrated at 37°C for 5 min. An 8 μl fraction was transferred into another tube (time T=0) containing 25 μl S1 Nuclease reaction mix, and placed on ice. ExonucleaseIII (Boehringer Mannheim, 175 units/μl) was added to the remaining DNA to a concentration of 150 units per pmol of DNA 3' termini. Aliquot of 8 μl were removed at 30 sec intervals to tubes (T=1 to T=12) on ice containing 25 μl S_1 nuclease reaction mix each. The tubes were incubated at 27° C for 30 min before reactions were stopped by addition of 3.5 μl S1 nuclease stop buffer and incubated (70° C, 10 min). Fractions of 5 μl were removed from each time interval and separated on a 0.8 % agarose gel. 4 μl of a Klenow reaction mix was added to each tube (to "polish" ends), and the tubes incubated (3 min, 27° C); then 1 μl dNTP mix was added and tubes incubated for a further (5 min, 27° C). Ligation was carried out (16 h, 15°C)

after which 115 μl ligase reaction mix was added to each tube. The integrity of the "Reverse Primer" binding site in each deletion mutant was verified by HindIII restriction. Since HindIII recognition site is located between the PstI site of pBluescriptSK+ and the "reverse primer" binding site, thus the presence of the HindIII site served as an indirect indication that the site was still present. No restriction sites between EcoRI and the "forward primer binding site were available, and the presence of these priming sites had to be confirmed by probing deletion mutant transformant colonies with [α-^{32}P]-dATP (Amersham, U.K) endlabelled "forward primer" DNA

2.16.4 Priming, labelling and termination

For sequencing of the "reverse strand", (5' CAGGAAACAGCTATGAC 3') was used as a primer, while 5' GTTTTCCCAGTCACGAC 3' used as the "forward" sequencing primer. Vacuum-dried DNA templates were resuspended in 7 μl H_2O, 2 μl Sequenase buffer (supplied in kit) and 1 μl of appropriate primer (1 pmol/ml). Primers were annealed to the template by heating (42° C, 5 min) followed by slow cooling (15 min) to 27° C. To each 10 μl primer/template complex were added: 1 μl 0 1 M DTT, 2 μl labelling mix (1.5 mM each of dCTP, dGTP and dTTP), 1.75 μl DMSO, 0.5 ml [α-35S]dATP (100 Ci/mmol; 10 Ci mg/ml) and 2 μl Sequenase 2^{TM} (1/8 dilution in cold 10 mM Tris-HCl pH 7.5; 5 mM DTT; 0.5 mg/ml BSA). Labelling reactions were mixed thoroughly and incubated (20° C. 3 min). Four aliquot of 4.3 μl each were then transferred, and mixed well with four respective prewarmed termination mixes, each containing a different dNTP/ddNTP Tubes containing termination mix (2.75 μl) were prepared before starting the labelling reactions. Each termination mix consisted of a particular dNTP/ddNTP in a 80 mM/8 mM ratio, 50 mM NaCl and 10 % (v/v) DMSO. For each template there were four tubes: dATP/ddATP, dCTP/ddCTP, dGTP/ddGTP and dTTP/ddTTP. These tubes were pre-warmed (45° C, 1 min) before addition of the labelling mix. Termination reactions were incubated (45° C, 5 min) before being stopped by the addition of 5 μl stop buffer (95 % Formamide; 20 mM EDTA: 0.05 % bromophenol blue; 0.05 % xylene cyanol). Reactions were either electrophoresed

immediately on a denaturing sequencing gel, or stored at -20° C for up to two weeks.

2.17 Preparation of sequencing gels

The gel apparatus used for DNA sequencing was obtained from GIBCO-BRL Scientific, (USA). Glass plates were washed and rinsed thoroughly with deionized water. Denaturing gels of 4.8 % polyacrylamide (for extended runs) or 6 % for normal runs, each containing 7 M urea (ICN Biomedicals, Inc., OH, USA) were prepared by mixing 60 ml of gel mix with 60 µl of freshly prepared 50 % ammonium persulphate (Sigma) and 60 µl TEMED just before pouring. The gel mix was poured smoothly, avoiding bubbles, by using two 60 ml syringes, after which the gel was allowed to polymerize for 30 min before use.

2.17.1 Electrophoresis (polyacrylamide denaturing gels)

After assembly of the gel apparatus, the buffer tank was filled with TBE had been pre-heated to 65° C, (preheating eliminated the need for pre-running the gel to heat it up to the necessary running temperature (40 - 45° C)). Templates (prepared as described above), were heat denatured in a 80° C water bath for 2 min immediately prior to loading, and snap cooled on ice. The wells were cleared from crystallizing urea by blowing out with a buffer filled syringe, and samples were run at 96 W for approximately 60 min in 6 % gels (normal runs) or 92 W for 2.5-4 hr in 4.8 % gels (extended runs). After running, the top glass plate was lifted and a sheet of Whatman filter paper was layered over the gel. The gel was "dry-lifted" from the glass plate, and dried onto the filter paper (80°C, 30 min) using a vacuum dryer coupled to a water trap and a vacuum pump, before being exposed to Fuji X-ray film.

2.18 Computer software

The computer software used to compile and analyze the nucleotide and deduced amino acid sequence data was an IBM XT PC computer and DNA tools using the Genetics Computer Group programme (GCG) of the University of Wisconsin, USA or Genepro (version 3.1).

CHAPTER 3

RESULTS

3.1 Cloning, sequencing and analysis of expression of B. thuringiensis (L1-2) gene

3.2 *B. thuringiensis L1-2* had previously been shown to be active against *C. partellus* (Brownbridge, 1989). Further bioassays showed that this isolate was also toxic to adult tsetse. Effective dose estimates were obtained by Probit analysis (SAS Institute, Inc. 1988) for 50 % lethal concentration estimates (LC_{50} to clone and sequence the gene involved.

3.2 Identification of a crylA-like gene in B. thuringiensis L1-2

The N-terminal amino acid sequence of *B. thuringiensis L1-2* previously carried out by Osir et al.,1993 (**Table 2**), *had shown* high sequence homology between the 5' ends of crylA gene. This sequence homology facilitated the use of a 1.9 kb PvuII fragment from pES1 (Schnepf and Whiteley, 1981) encoding terminal amino acids of the crylA(a) protein from *B. thuringiensis var. kurstaki* as a DNA probe for the identification for the possible presence of a similar crylA gene in *B. thuringiensis L1-2*.

B. thuringiensis L1-2 chromosomal DNA and plasmid were digested with different restriction endonuclease whith known sites present in pES1. Some of these restriction endonuclease were HpaII, KpnI, AluI, HaeIII, NcoI, ApaI, BglII, PstI, XhoI, SfiI, and NruI (**Fig. 3.1**). These restriction digests were probed with 1.9 kb PvuII fragment from pES1. The probe hybridized to various restriction fragments **(Fig. 3. 1, Panel B).** In the ApaI, BglII, SfiI, PstI, XhoI and NruI digests the probe hybridized to one large fragment (ca 12-24 kb). These results demonstrated the presence of a crylA-type gene in *B. thuringiensis L1-2*. It was of interest that digestion with PstI also gave only one band. As PstI cuts crylA(a) twice, yielding a fragment of 360 bp and a fragment larger than 2.4 kb, it would appear that these sites are either absent or are not being cut in the *B. thuringiensis L1-2* gene. Chromosomal and plasmid DNA preparations from *B. thuringiensis L1-2* were digested with PstI, BglII, and XhoI (**Fig. 3.2**) and probed as above using conditions of low stringency DNA - DNA hybridization. These data further

confirmed the presence of the single PstI band and showed that the δ-endotoxin gene was present on both the chromosomal and the plasmid(s) DNA in *B thuringiensis L1-2* (Fig. 3.2, Panel B).

Table 2: N-terminal amino acid sequence of *B. thuringiensis L1-2*

lleu--glu--thr--met--gln--thr--prp--ileu--asp--ileu--ser--leu--ser--phe-

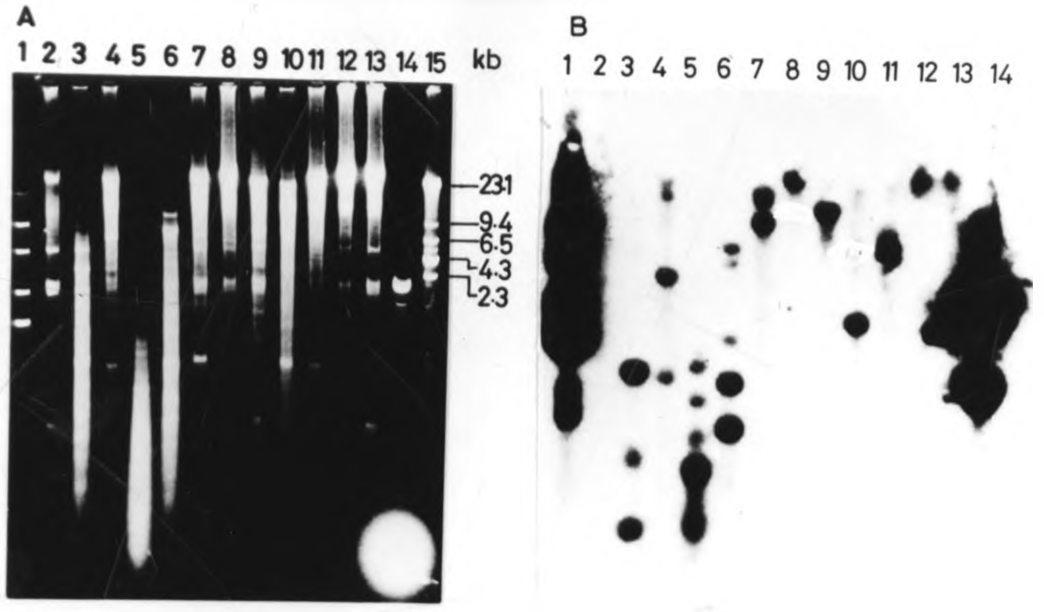


Fig. 3.1 **Restriction digests and Southern blot Analysis of Chromosomal DNA from *B. thuringiensis L1-2*.**

Panel A: **1**, crylA(a); **2**, undigested L1-2; **3** HpaII; **4**, KpnI; **5**, AluI; **6**, HaeIII; **7**, NcoI; **8**, ApaI; **9**, BglII; **10**, PstI; **11**, XhoI; **12**, SfiI; **13**, NruI; lane **14**, crylA(c) DNA; **15**, molecular size marker (Ms) are λ DNA digested with restriction endonuclease HindIII.

Panel B: **Autoradiogram of Panel A** blotted onto Hybond N and probed with Digoxigenin-labelled 1.9-kb PvuII pES1 fragment.

3.3 *Cloning and sequence analysis of the δ-endotoxin gene of B. thuringiensis L1-2*

In order to clone the *B. thuringiensis L1-2* gene, plasmid DNA sample was digested with BglII, shot gun cloned by ligating to BglII-digested pEcoR251 vector and transformed into *E. coli* LK111 cells. The special advantage over using pEcoR251 over other cloning vectors is that the structure of pEcoR251 is similar to other plasmids utilizing inactivation of the EcoRI endonuclease gene as selective marker, pEcoR251 has a positive selection vector containing the *E. coli* gene, as well as a derivative which contain large deletion within the gene for methylase. The EcoRI gene product expressed at high levels by the lambda promoter on pEcoR251 is lethal unless insertionally inactivated or regulated by plasmid which contains a repressor gene (Remault *et al.*, 1983). The EcoRI gene has a single BglII cloning site and EcoRI endonuclease gene downsteam from Lambda p_L It does not yield transformants upon introduction into E. coli unless as a positive-selection cloning vehicle which has been employed for regulated overproduction of hybrid proteins. Failure to obtain transformants is due to lethal effects of endonuclease production upon cell entry. A plasmid library consisting of 2258 clones was screened by colony hybridization using a digoxigenin-labelled 1.9 kb PvuII fragment from pES1 as a probe. One positive *E. coli LK 111* transformant was isolated on LB agar plates containing ampicillin. The *E. coli* cells contained a recombinant plasmid which was designated pL12-7.4. pL12-7.4 when digested with BglII and XhoI displayed an insert estimated to 16-kb **(Fig. 3.3)**.

Recombinant plasmid pL12-7.4 was digested with different restriction endonuclease, Southern blotted and probed with Digoxigenin (DIG) labelled 1.9 kb PvuII fragment from pES1 in order to determine the origin and orientation of the cloned fragment on pL12-7 4. **(Fig. 3.4)**

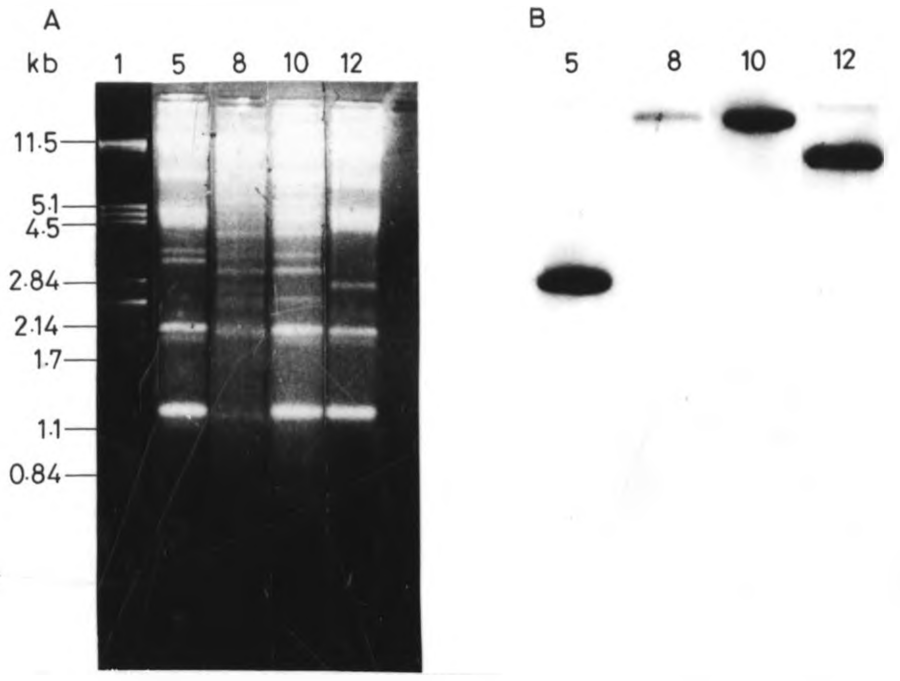


Fig. 3.2 **Comparison of *B. thuringiensis L1-2* plasmid and chromosomal DNA.**

Panel A: *B. thuringiensis L1-2* **Plasmid DNA** digested with restriction endonuclease;**1**, molecular size marker (Ms) are λ DNA digested with PstI; **5**, PstI; **12**, XhoI

B. thuringiensis L1-2 **Chromosomal DNA** digested with restriction endonuclease. **8** and **10**, BglII.

Panel B: **Autoradiogram of Panel A** blotted onto Hybond N and probed with Digoxigenin-labelled 1.9-kb PvuII pES1 fragment.

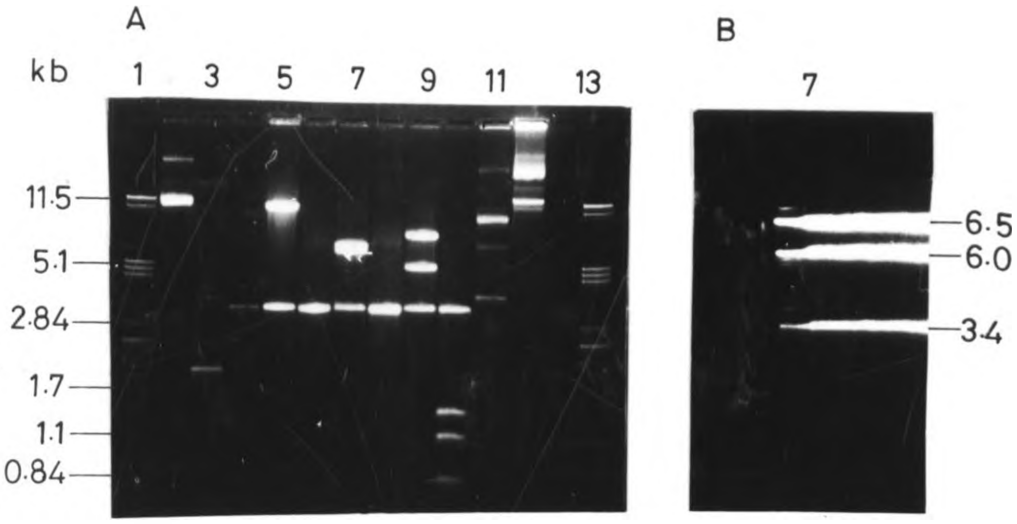


Fig. 3.3 **Confirmation of insert size of Putative clone pL12-7.4 derived from *B. thuringiensis L1-2*.**

Panel A: *B. thuringiensis L1-2* **plasmid DNA** digested with restriction endonuclease

1 and **13**, molecular size marker (Ms) are λ DNA digested with PstI

2, undigested pEcoR251; **3**, pL12-7.4 with PstI/XhoI

4, pEcoR251, BglII; **5**, pL12-7.4, BglII; **6**, pEcoR251, BglII

7, pL12-7.4, BglII-XhoI; **8**, pEcoR251 PstI ; **9**, pL12-7.4, BglII-PvuII, lane

10, pL12-7.4, PvuII/XhoI; **11**, pL12-7.4 PstI/BglII; **12**, undigested pL12-7.4

Panel B: **Preparative agarose gel electrophoresis.**

7, pL12-7.4, BglII-XhoI

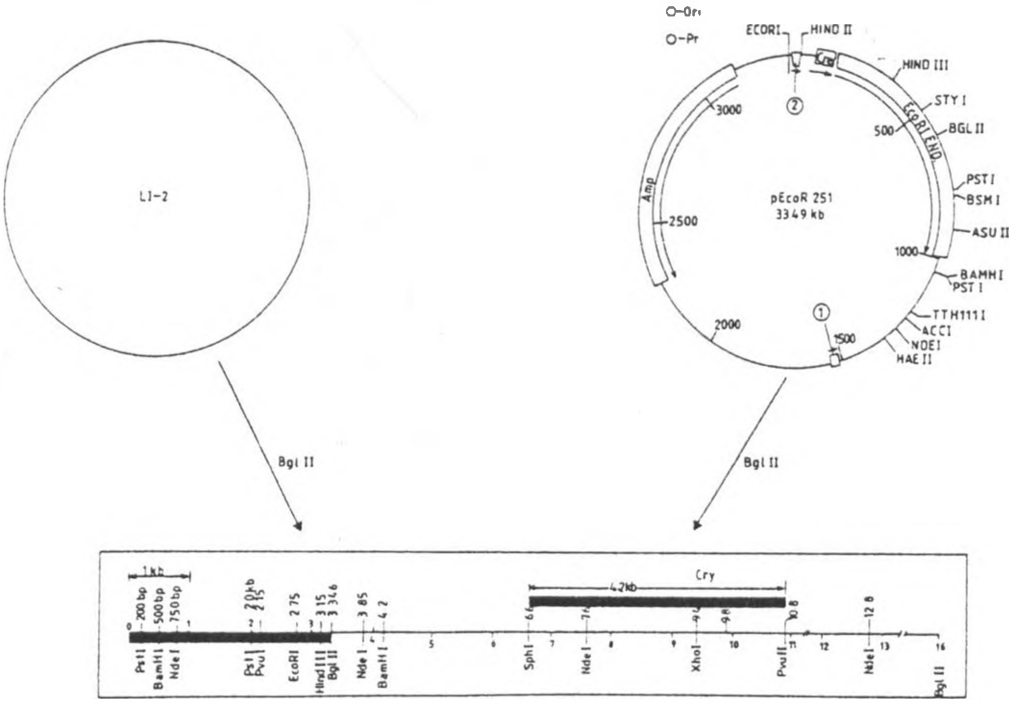


Fig 3.4 Cloning strategy of *B. thuringiensis L1-2* gene.

The circles represent unlinearized plasmids and the boxed segement illustrate the putative restriction endonuclease map of recombinant plasmid pL12-7.4. The location of the δ-endotoxin gene is shown in bold as cry between restriction endonuclease SphI and PvuII. The other bold line between PstI and BglII show the location of pEcoR251 vector.

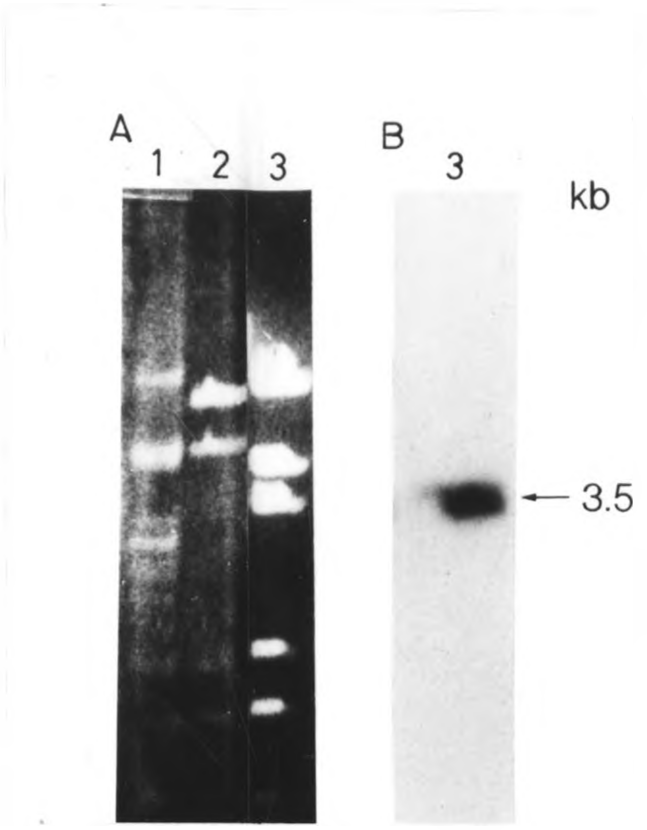


Fig. 3.5 **Localization of 3.5-kb fragment insert in plasmid pL12-7.4.**

Panel A: *B. thuringiensis L1-2* **plasmid DNA** digested with restriction endonuclease **1**, molecular size marker (Ms) are λ DNA digested with PstI **2**, pL12-7. 4, HindII; **3**, pL12-7. 4, HindII-PvuII

Panel B: Southern blot analysis of the gel blotted onto Hybond N and probed with Digoxigenin-labelled 1.9-kb PvuII pES1 fragment. **3.**

3.4 Subcloning of inserts into pBluescript SK

To sequence the *B. thuringiensis L1-2* gene, the insert was subcloned into **pBluescript SK** vector. The ***pBluescript SK*** vector represents the primary upgrades, and is offered with multiple cloning sites in both the forward (+) and reverse orientations. This allows easy cloning, and increase in expression applications. In addition ***pBluescript SK*** is provided in three reading frames to simplify subcloning with N-terminal tag. *A* series of derivatives were generated by deleting various segments of plasmid pL12-7.4 with various restriction endonuclease, fractionated by electrophoresis in 0.8 % (w/v) agarose gel in Tris-acetate buffer, and transferred to Hyond N (Amersham, UK) and probed Digoxigenin labelled 1.9 kb PvuII fragment from pES1. A restriction map was generated (**Fig 3.4**) based on which a number of subcloning and deletions were performed to determine the location of the cry gene. Complete PvuII-HindII endonuclease digestion of pL12-7.4 produced one internal insert fragment which when probed with 1.9-kb PvuII fragment from pES1, hybridized to a approximately 3.5-kb fragment (**Fig 3.5, lane 3**). It could be seen pES1 hybridized to plasmid DNA showing δ-endotoxin gene is localized on the 3.5-kb PvuII-HindII fragment. The 3.5-kb PvuII-HindII fragment was excised and subcloned into pBluescript SK^+ vector linearized with EcoRV in both orientations and transformed into *JM 105.* Both clones were sequenced and had the inserts in opposite orientations. pLPH6 was complemented and flipped to join pLPH7 at the HindII site (**Fig. 3.6**).

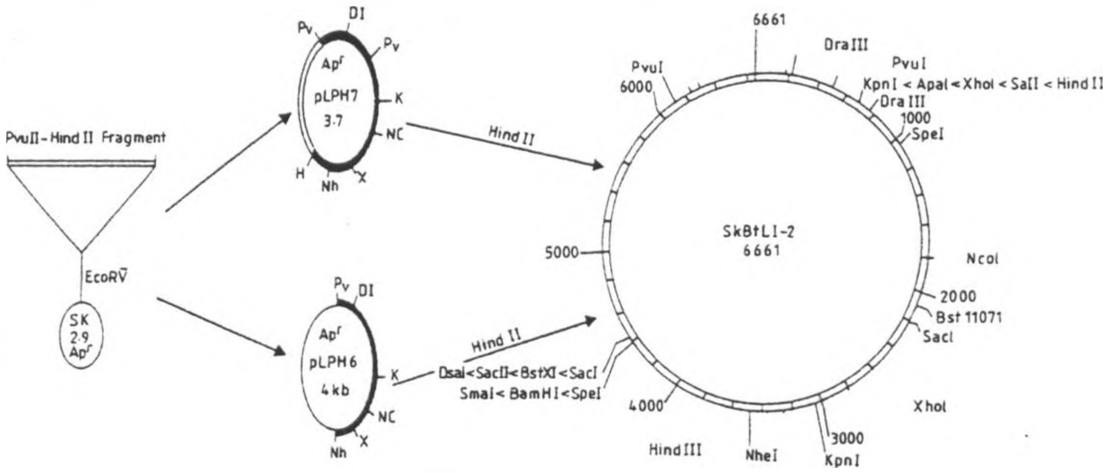


Fig. 3.6 Construction of pL12-7.4

Symbols ——, toxin gene; K=KpnI; A=ApaI; DI=DraIII; N=NdeI; Sp=SpeI; P=PvuII; NI=NcoI; S=SacI; X=XhoI; Nh=NheI; H=HindIII; Sa=SalI; H2=HincII; B=BamHI; Sm=SmaI; D=DsaI; SII=SacII; B2=BstXI; Pv=PvuI.

The insert DNA on both clones contained one NdeI, DraIII, SpeI, NcoI, BglI, SacI, AvaI, XhoI, KpnI BanI, PvuII, HindIII, SspI, NheI, HaeII two EcoRI, HaeIII, PstI and three AccI, XbaI, EcoRV (**Fig. 3.7**).

3.5 ExonucleaseIII/S1 nuclease shortening

Sets of overlapping deletion mutants were created for both pLPH6 and pLPH7 by unidirectional shortening using exonucleaseIII and S1 nuclease in a protocol adapted from Heinkoff (1984). Both pLPH6 and pLPH7 were digested with HindII to generate 5' overhang in the pBluescript SK$^+$ polylinker for exonucleaseIII digestion, and with the ApaI site in the pBluescript SK$^+$ multiple cloning region to create a 3' overhang for protection against exonucleaseIII digestion in two directions. After ligation, transformation and selection, three bacterial colonies were selected for each time interval. Plasmid DNA was isolated from each and digested with PvuII and XhoI, these endonuclease have restriction sites on the 5'- and 3'-termini of the polylinker-to ensure that the flanking sequencing primer binding sites had not been destroyed during the shortening reactions. The deletions of pLPH6 and pLPH7 derivatives were mapped by restriction analysis of subsequent *E. coli* transformants and suitable clones were prepared for double stranded sequencing. Sequencing templates were generated by subjecting CsCl-purified plasmid DNA to alkaline denaturation followed by ethanol precipitation. Sequencing was carried out using (α-^{35}S)dATP (Amersham, UK) and the Sequenase version 2.0 kit (US Biochemicals). The 3' end of clone pLPH7 insert was complemented and flipped to join clone pLPH6 at HindII site. The nucleotide sequence **(Fig. 3.8)** and complete restriction map of *B. thuringiensis L1-2* cry gene was generated **(Fig. 3.9)**.

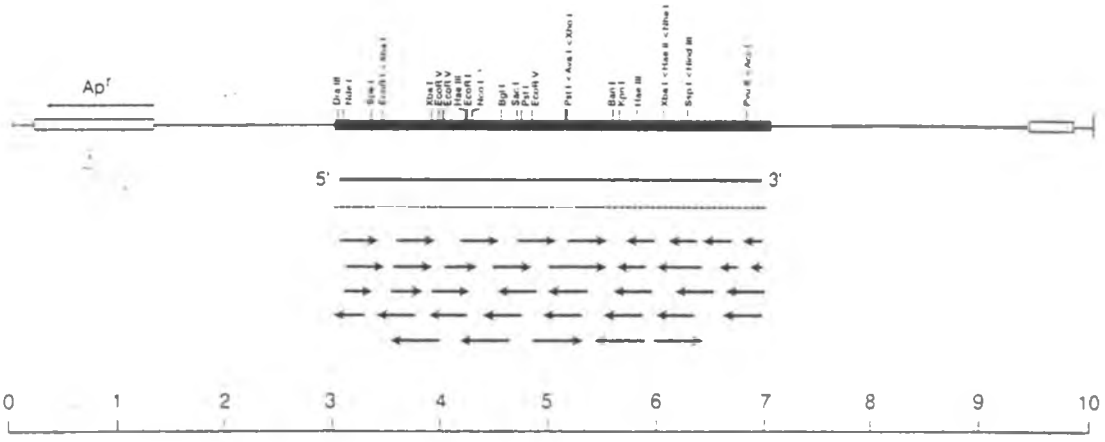


Fig. 3.7 Restriction endonuclease map and sequencing strategy of *B. thuringiensis L1-2*. Single lines and bold lines and open boxes represent vector DNA, *B. thuringiensis L1-2* insert DNA, and antibiotic resistance genes. The arrows beneath denote the extent and direction of sequence determination. The broken lines indicates the extent of nucleotide sequence shown in Fig. 3.8.

```
1    AACACCCTGGGTCAAAAATTGATATTTAGTAAAATTAGTTGCACTTTGTGCATTTTTTCATAAGATGAGTCATATGTTTTAAATTGTAGTAATGAAAAAC   100
101  AGTATTATATCATAATGAATTGGTATCTTAATAAAAGAGATGGAGGTAACTTATGGATAACAATCCGAACATCAATGAATGCATTCCTTATAATTGTTTA   200
                                                    M  D  N  N  P  N  I  N  E  C  I  P  Y  N  C  L
201  AGTAACCCTGAAGTAGAAGTATTAGGTGGAGAAAGAATAGAAACTGGTTACACCCCAATCGATATTTCCTTGTCGCTAACGCAATTTCTTTTGAGTGAAT   300
     S  N  P  E  V  E  V  L  G  G  E  R  I  E  T  G  Y  T  P  I  D  I  S  L  S  L  T  Q  F  L  L  S  E  F
301  TTGTTCCCGGTGCTGGATTTGTGTTAGGACTAGTTGATATAATATGGGGAATTTTTGGTCCCTCTCAATGGGACGCATTTCTTGTACAAATTGAACAGTT   400
       V  P  G  A  G  F  V  L  G  L  V  D  I  I  W  G  I  F  G  P  S  Q  W  D  A  F  L  V  Q  I  E  Q  L
401  AATTAACCAAAGAATAGAAGAATTCGCTAGGAACCAAGCCATTTCTAGATTAGAAGGACTAAGCAATCTTTATCAAATTTACGCAGAATCTTTTAGAGAG   500
       I  N  Q  R  I  E  E  F  A  R  N  Q  A  I  S  R  L  E  G  L  S  N  L  Y  Q  I  Y  A  E  S  F  R  E
501  TGGGAAGCAGATCCTACTAATCCAGCATTAAGAGAAGAGATGCGTATTCAATTCAATGACATGAACAGTGCCCTTACAACCGCTATTCCTCTTTTCGCAG   600
     W  E  A  D  P  T  N  P  A  L  R  E  E  M  R  I  Q  F  N  D  M  N  S  A  L  T  T  A  I  P  L  F  A  V
601  TTCAAAATTATCAAGTTCCTCTTTTATCAGTATATGTTCAAGCTGCAAATTTACATTTATCAGTTTTGAGAGATGTTTCAGTGTTTGGACAAAGGTGGGG   700
       Q  N  Y  Q  V  P  L  L  S  V  Y  V  Q  A  A  N  L  H  L  S  V  L  R  D  V  S  V  F  G  Q  R  W  G
701  ATTTGATGCCGCGACTATCAATAGTCGTTATAATGATTTAACTAGGCTTATTGGCAACTATACAGATTATGCTGTACGCTGGTACAATACGGGATTAGAA   800
       F  D  A  A  T  I  N  S  R  Y  N  D  L  T  R  L  I  G  N  Y  T  D  Y  A  V  R  W  Y  N  T  G  L  E
801  CGTGTATGGGGACCGGATTCTAGAGATTGGGTAAGGTATAATCAATTTAGAAGAGAATTAACACTAACTGTATTAGATATCGTTGCTCTGTTCCCGAATT   900
     R  V  W  G  P  D  S  R  D  W  V  R  Y  N  Q  F  R  R  E  L  T  L  T  V  L  D  I  V  A  L  F  P  N  Y
901  ATGATAGTAGAAGATATCCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAATTTTGATGGTAGTTTTCGAGGCTC  1000
       D  S  R  R  Y  P  I  R  T  V  S  Q  L  T  R  E  I  Y  T  N  P  V  L  E  N  F  D  G  S  F  R  G  S
1001 GGCTCAGGGCATAGAAAGAAGTATTAGGAGTCCACATTTGATGGATATACTTAACAGTATAACCATCTATACGGATGCTCATAGGGGTTATTATTATTGG  1100
       A  Q  G  I  E  R  S  I  R  S  P  H  L  M  D  I  L  N  S  I  T  I  Y  T  D  A  H  R  G  Y  Y  Y  W
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     S  G  H  Q  I  M  A  S  P  V  G  F  S  G  P  E  F  T  F  P  L  Y  G  T  M  G  N  A  A  P  Q  Q  R  I
1201 TTGTTGCTCAACTAGGTCAGGGCGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCTTTTAATATAGGGATAAATAATCAACAACTATCTGTTCT  1300
       V  A  Q  L  G  Q  G  V  Y  R  T  L  S  S  T  L  Y  R  R  P  F  N  I  G  I  N  N  Q  Q  L  S  V  L
```

Fig. 3.8 Nucleotide sequence of *B. thuringiensis L1-2* structural gene and flanking regions

```
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1401  AATAACAACGTGCCACCTAGGCAAGGATTTAGTCATCGATTAAGCCATGTTTCAATGTTTCGTTCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAG  1500
        N  N  N  V  P  P  R  Q  G  F  S  H  R  L  S  H  V  S  M  F  R  S  G  F  S  N  S  S  V  S  I  I  R  A
1501  CTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTT  1600
        P  M  F  S  W  I  H  R  S  A  E  F  N  N  I  I  A  S  D  S  I  T  Q  I  P  A  V  K  G  N  F  L  F
1601  TAATGGTTCTGTAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATAACATTCAGAATAGAGGGTATATTGAAGTT  1700
        N  G  S  V  I  S  G  P  G  F  T  G  G  D  L  V  R  L  N  S  S  G  N  N  I  Q  N  R  G  Y  I  E  V
1701  CCAATTCACTTCCCATCGACATCTACCAGATATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAACGTTAATTGGGGTAATTCATCCA  1800
        P  I  H  F  P  S  T  S  T  R  Y  R  V  R  V  R  Y  A  S  V  T  P  I  H  L  N  V  N  W  G  N  S  S  I
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        F  S  N  T  V  P  A  T  A  T  S  L  D  N  L  Q  S  S  D  F  G  Y  F  E  S  A  N  A  F  T  S  S  L
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        N  L  V  T  Y  L  S  D  E  F  C  L  D  E  K  R  E  L  S  E  K  V  K  H  A  K  R  L  S  D  E  R  N
2201  TTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCGGAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTT  2300
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```
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2801  GGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATAAAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCT  2900
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3001  TGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAGAAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTGA  3100
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3101  TTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAAACAACCAACGTTCGGTCCTTGTTGTTCCGGAATGGGAAGCA  3200
        F  N  N  G  L  S  C  W  N  V  K  G  H  V  D  V  E  E  Q  N  N  Q  R  S  V  L  V  V  P  E  W  E  A
3201  GAAGTGTCACAAGAAGTTCGTGTCTGTCCGGGTCGTGGCTATATCCTTCGTGTCACAGCGTACAAGGAGGGATATGGAGAAGGTTGCGTAACCATTCATG  3300
        E  V  S  Q  E  V  R  V  C  P  G  R  G  Y  I  L  R  V  T  A  Y  K  E  G  Y  G  E  G  C  V  T  I  H  E
3301  AGATCGAGAACAATACAGACGAACTGAAGTTTAGCAACTGCGTAGAAGAGGAAATCTATCCAAATAACACGGTAACGTGTAATGATTATACTGTAAATCA  3400
        I  E  N  N  T  D  E  L  K  F  S  N  C  V  E  E  E  I  Y  P  N  N  T  V  T  C  N  D  Y  T  V  N  Q
3401  AGAAGAATACGGAGGTGCGTACACTTCTCGTAATCGAGGATATAaCGAAGCTCCTTCCGTACCAGCTGATTATGCGTCAGTCTATGAAGAAAAATCGTAT  3500
        E  E  Y  G  G  A  Y  T  S  R  N  R  G  Y  N  E  A  P  S  V  P  A  D  Y  A  S  V  Y  E  E  K  S  Y
3501  ACAGATGGACGAAGAGAGAATCCTTGTGAATTTAACAGAGGGTATAGGGATTACACGCCACTACCAGTTGGTTATGTGACAAAAGAATTAGAATACTTCC  3600
        T  D  G  R  R  E  N  P  C  E  F  N  R  G  Y  R  D  Y  T  P  L  P  V  G  Y  V  T  K  E  L  E  Y  F  P
3601  CAGAAACCGATAAGGTATGGATTGAGATTGGAGAAACGGAAGGAACATTTATCGTGGACAGCGTGGAATTACTCCTTATGGAGGAATAGTCTC  3693
        E  T  D  K  V  W  I  E  I  G  E  T  E  G  T  F  I  V  D  S  V  E  L  L  L  M  E  E  *
```

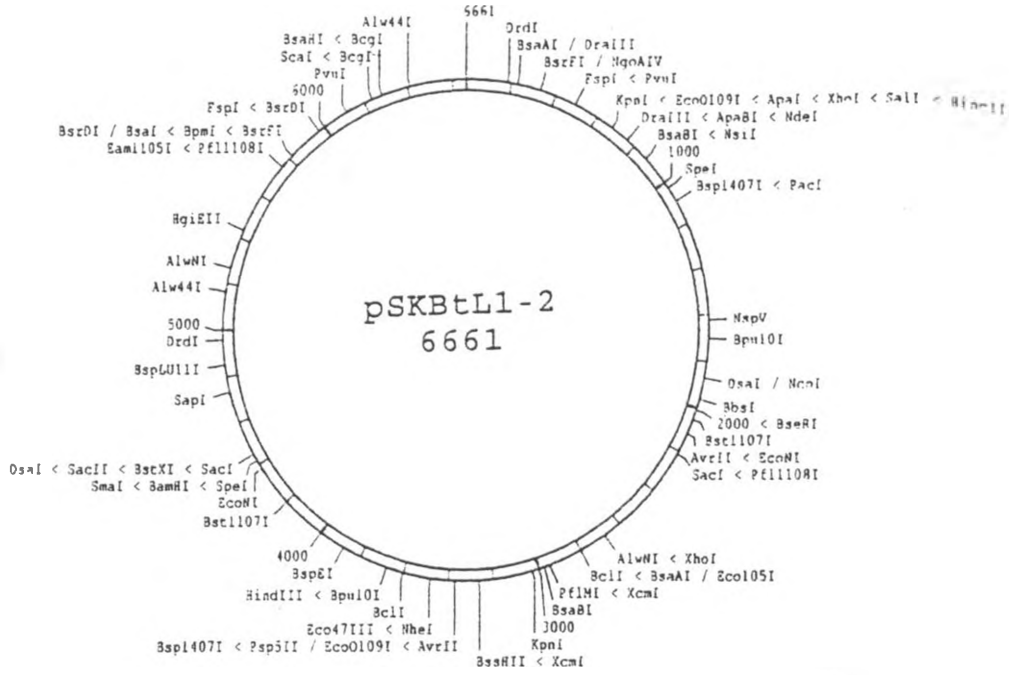


Fig. 3.9 Complete Restriction endonuclease map and restriction endonuclease sites of *B. thuringiensis L1-2* in pBluescriptSK$^+$ vector

3.6 *Nucleotide sequencing*

The nucleotide sequence of the pL12-7.4 and the deduced amino acid sequence are shown **(Fig. 3.8)**. A 3534 bp large open reading frame (ORF) starting at ATG (position 152) and terminating at TAG (position 3686) was found which was capable of coding 1178 amino acid residues with a calculated molecular mass of M_r ~ 130,000 -to 140,000 **(Fig. 3.8)** which corresponded with the estimated size of the insecticidal protein of *B. thuringiensis L1-2* as observed on SDS-polyacrylamide gel **(Fig. 3.12)**.

3.7 *Sequence comparison of B. thuringiensis L1-2 with other B. thuringiensis genes*

The deduced amino acid sequence of *B. thuringiensis L1-2* was compared with sequence in the GenEMBL data base using TFASTA and BESTFIT programmes from Genetic Computer Group sequence analysis package (Devereux *et al.*, 1984). Homology was found between *B. thuringiensis Ghad* (Herrera *et al.*, 1994), *B. thuringiensis HD-73 kurstaki* (Adang et al., 1985); *B. thuringiensis HD-588 kenyae* (Von Tersch et al., 1991); *B. thuringiensis Ps81gg kurstaki and Ps85a1 kurstaki* (J. S Feitelson unpublished data), *B. thuringiensis L1-2* was found to be closest to crylA(c). The alignment of the deduced amino acid sequence with the others of crylA(c) revealed that DNA differences occur at 444 *(Ghad and HD-73 kurstaki)*, at 1089, 1092 and 1098 (*HD-588 kenyae and Ps81gg kurstaki)* and at 1323/4/5 (*HD-588 kenyae and Ps81gg kurstaki)*, therefore it was found to be identical to *B. thuringiensis Ps85a1 kurstaki*. Amino acids differences were found to occur at 148 (*B. thuringiensis Ghad and HD-73 kurstaki)* and 206 (*B. thuringiensis HD-588 kenyae).*

Comparisons of the deduced nucleotide sequences of the cry IA(c) genes from *B. thuringiensis var kurstaki HD73, B. thuringiensis L1-2, B. thuringiensis HD 588 kenyae, B. thuringiensis Ps81gg* kurstaki although there are amino acid sequence differences at position 366 and 442, *B. thuringiensis Ps85a1 kurstaki, B. thuringiensis Ghad* **(Table 3)**. These results further confirmed that *B. thuringiensis L1-2* was

identical to *B. thuringiensis Ps81gg kurstaki* and *Ps85a1 kurstaki*. However, the ORF revealed that the sequence of cloned *B. thuringiensis L1-2* gene was found to be similar to that of the classic *B. thuringiensis HD-73 kurstaki* cryIA(c) gene but having one amino acid difference at position 148 and five additional DNA differences which did not result in amino acid changes **(Fig. 3.10)**.

	1				50
B. t. L1-2	MDNNPNINEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF
Ps85a1-Kur	MDNNPNINEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF
Ghad	MDNNPNINEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF
HD73-Kur	MDNNPNINEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF
HD588-Ken	MDNNPNINEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF
Ps81gg-Kur	MDNNPNINEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF

	51				100
B. t. L1-2	VPGAGFVLGL	VDIIWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAISRL
Ps85a1-Kur	VPGAGFVLGL	VDIIWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAISRL
Ghad	VPGAGFVLGL	VDIIWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAISRL
HD73-Kur	VPGAGFVLGL	VDIIWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAISRL
HD588-Ken	VPGAGFVLGL	VDIIWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAISRL
Ps81gg-Kur	VPGAGFVLGL	VDIIWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAISRL

	101				150
B. t. L1-2	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLLAV
Ps85a1-Kur	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLLAV
Ghad	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLFAV
HD73-Kur	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLFAV
HD588-Ken	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLLAV
Ps81gg-Kur	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLLAV

Fig. 3.10 **Amino acid sequence alignment of *B. thuringiensis* genes** from *B. thuringiensis var kurstaki HD-73, B. thuringiensis HD 588 kenyae, B. thuringiensis Ps81gg kurstaki, B. thuringiensis Ps85a1 kurstaki, B. thuringiensis Ghad* and *B. thuringiensis L1-2*. The amino acids are identified by the single letter code and the underlined letters represent amino acid differences.

	151				200
B. t. L1-2	QNYQVPLLSV	YVQAANLHLS	VLRDVSVFGQ	RWGFDAATIN	SRYNDLTRLI
Ps85a1-Kur	QNYQVPLLSV	YVQAANLHLS	VLRDVSVFGQ	RWGFDAATIN	SRYNDLTRLI
Ghad	QNYQVPLLSV	YVQAANLHLS	VLRDVSVFGQ	RWGFDAATIN	SRYNDLTRLI
HD73-Kur	QNYQVPLLSV	YVQAANLHLS	VLRDVSVFGQ	RWGFDAATIN	SRYNDLTRLI
HD588-Ken	QNYQVPLLSV	YVQAANLHLS	VLRDVSVFGQ	RWGFDAATIN	SRYNDLTRLI
Ps81gg-Kur	QNYQVPLLSV	YVQAANLHLS	VLRDVSVFGQ	RWGFDAATIN	SRYNDLTRLI
	201				250
B. t. L1-2	GNYTDYAVRW	YNTGLERVWG	PDSRDWVRYN	QFRRELTLTV	LDIVALFPNY
Ps85a1-Kur	GNYTDYAVRW	YNTGLERVWG	PDSRDWVRYN	QFRRELTLTV	LDIVALFPNY
Ghad	GNYTDYAVRW	YNTGLERVWG	PDSRDWVRYN	QFRRELTLTV	LDIVALFPNY
HD73-Kur	GNYTDYAVRW	YNTGLERVWG	PDSRDWVRYN	QFRRELTLTV	LDIVALFPNY
HD588-Ken	GNYTDHAVRW	YNTGLERVWG	PDSRDWVRYN	QFRRELTLTV	LDIVALFPNY
Ps81gg-Kur	GNYTDYAVRW	YNTGLERVWG	PDSRDWVRYN	QFRRELTLTV	LDIVALFPNY
	251				300
B. t. L1-2	DSRRYPIRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIERS	IRSPHLMDIL
Ps85a1-Kur	DSRRYPIRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIERS	IRSPHLMDIL
Ghad	DSRRYPIRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIERS	IRSPHLMDIL
HD73-Kur	DSRRYPIRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIERS	IRSPHLMDIL
HD588-Ken	DSRRYPIRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIERS	IRSPHLMDIL
Ps81gg-Kur	DSRRYPIRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIERS	IRSPHLMDIL

	301				350
B. t. L1-2	NSITIYTDAH	RGYYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQQRI
Ps85a1-Kur	NSITIYTDAH	RGYYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQQRI
Ghad	NSITIYTDAH	RGYYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQQRI
HD73-Kur	NSITIYTDAH	RGYYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQQRI
HD588-Ken	NSITIYTDAH	RGYYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQQRI
Ps81gg-Kur	NSITIYTDAH	RGYYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQQRI
	351				400
B. t. L1-2	VAQLGQGVYR	TLSSTLYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
Ps85a1-Kur	VAQLGQGVYR	TLSSTLYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
Ghad	VAQLGQGVYR	TLSSTLYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
HD73-Kur	VAQLGQGVYR	TLSSTLYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
HD588-Ken	VAQLGQGVYR	TLSSTFYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
Ps81gg-Kur	VAQLGQGVYR	TLSSTFYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
	401				450
B. t. L1-2	YRKSGTVDSL	DEIPPQNNNV	PPRQGFSHRL	SHVSMFRSGF	SNSSVSIIRA
Ps85a1-Kur	YRKSGTVDSL	DEIPPQNNNV	PPRQGFSHRL	SHVSMFRSGF	SNSSVSIIRA
Ghad	YRKSGTVDSL	DEIPPQNNNV	PPRQGFSHRL	SHVSMFRSGF	SNSSVSIIRA
HD73-Kur	YRKSGTVDSL	DEIPPQNNNV	PPRQGFSHRL	SHVSMFRSGF	SNSSVSIIRA
HD588-Ken	YRKSGTVDSL	DEIPPQNNNV	PPRQGFSHRL	SHVSMFRSG.	SSSSVSIIRA
Ps81gg-Kur	YRKSGTVDSL	DEIPPQNNNV	PPRQGFSHRL	SHVSMFRSG.	SSSSVSIIRA

	451				500
B. t. L1-2	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFNGSVISG	PGFTGGDLVR
Ps85a1-Kur	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFNGSVISG	PGFTGGDLVR
Ghad	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFNGSVISG	PGFTGGDLVR
HD73-Kur	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFNGSVISG	PGFTGGDLVR
HD588-Ken	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFNGSVISG	PGFTGGDLVR
Ps81gg-Kur	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFNGSVISG	PGFTGGDLVR
	501				550
B. t. L1-2	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
Ps85a1-Kur	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
Ghad	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
HD73-Kur	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
HD588-Ken	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
Ps81gg-Kur	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
	551				600
B. t. L1-2	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII
Ps85a1-Kur	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII
Ghad	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII
HD73-Kur	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII
HD588-Ken	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII
Ps81gg-Kur	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII

	601				650
B. t. L1-2	DRFEFIPVTA	TLEAEYNLER	AQKAVNALFT	STNQLGLKTN	VTDYHIDQVS
Ps85a1-Kur	DRFEFIPVTA	TLEAEYNLER	AQKAVNALFT	STNQLGLKTN	VTDYHIDQVS
Ghad	DRFEFIPVTA	TLEAEYNLER	AQKAVNALFT	STNQLGLKTN	VTDYHIDQVS
HD73-Kur	DRFEFIPVTA	TLEAEYNLER	AQKAVNALFT	STNQLGLKTN	VTDYHIDQVS
HD588-Ken	DRFEFIPVTA	TLEAEYNLER	AQKAVNALFT	STNQLGLKTN	VTDYHIDQVS
Ps81gg-Kur	DRFEFIPVTA	TLEAEYNLER	AQKAVNALFT	STNQLGLKTN	VTDYHIDQVS
	651				700
B. t. L1-2	NLVTYLSDEF	CLDEKRELSE	KVKHAKRLSD	ERNLLQDSNF	KDINRQPERG
Ps85a1-Kur	NLVTYLSDEF	CLDEKRELSE	KVKHAKRLSD	ERNLLQDSNF	KDINRQPERG
Ghad	NLVTYLSDEF	CLDEKRELSE	KVKHAKRLSD	ERNLLQDSNF	KDINRQPERG
HD73-Kur	NLVTYLSDEF	CLDEKRELSE	KVKHAKRLSD	ERNLLQDSNF	KDINRQPERG
HD588-Ken	NLVTCLSDEF	CLDEKRELSE	KVKHAKRLSD	ERNLLQDSNF	KDINRQPERG
Ps81gg-Kur	NLVTYLSDEF	CLDEKRELSE	KVKHAKRLSD	ERNLLQDSNF	KDINRQPERG
	701				750
B. t. L1-2	WGGSTGITIQ	GGDDVFKENY	VTLSGTFDEC	YPTYLYQKID	ESKLKAFTRY
Ps85a1-Kur	WGGSTGITIQ	GGDDVFKENY	VTLSGTFDEC	YPTYLYQKID	ESKLKAFTRY
Ghad	WGGSTGITIQ	GGDDVFKENY	VTLSGTFDEC	YPTYLYQKID	ESKLKAFTRY
HD73-Kur	WGGSTGITIQ	GGDDVFKENY	VTLSGTFDEC	YPTYLYQKID	ESKLKAFTRY
HD588-Ken	WGGSTGITIQ	GGDDVFKENY	VTLSGTFDEC	YPTYLYQKID	ESKLKAFTRY
Ps81gg-Kur	WGGSTGITIQ	GGDDVFKENY	VTLSGTFDEC	YPTYLYQKID	ESKLKAFTRY

	751				800
B. t. LI-2	QLRGYIEDSQ	DLEIYLIRYN	AKHETVNVPG	TGSLWPLSAQ	SPIGKCGEPN
Ps85a1-Kur	QLRGYIEDSQ	DLEIYLIRYN	AKHETVNVPG	TGSLWPLSAQ	SPIGKCGEPN
Ghad	QLRGYIEDSQ	DLEIYLIRYN	AKHETVNVPG	TGSLWPLSAQ	SPIGKCGEPN
HD73-Kur	QLRGYIEDSQ	DLEIYLIRYN	AKHETVNVPG	TGSLWPLSAQ	SPIGKCGEPN
HD588-Ken	QLRGYIEDSQ	DLEIYLIRYN	AKHETVNVPG	TGSLWPLSAQ	SPIGKCGEPN
Ps81gg-Kur	QLRGYIEDSQ	DLEIYLIRYN	AKHETVNVPG	TGSLWPLSAQ	SPIGKCGEPN
	801				850
B. t. LI-2	RCAPHLEWNP	DLDCSCRDGE	KCAHHSHHFS	LDIDVGCTDL	NEDLGVWVIF
Ps85a1-Kur	RCAPHLEWNP	DLDCSCRDGE	KCAHHSHHFS	LDIDVGCTDL	NEDLGVWVIF
Ghad	RCAPHLEWNP	DLDCSCRDGE	KCAHHSHHFS	LDIDVGCTDL	NEDLGVWVIF
HD73-Kur	RCAPHLEWNP	DLDCSCRDGE	KCAHHSHHFS	LDIDVGCTLL	NEDLGVWVIF
HD588-Ken	RFAPHLEWNP	DLDCSCRDGE	KCAHHSHHFS	LDIDVGCTDL	NEDLGVWVIF
Ps81gg-Kur	RCAPHLEWNP	DLDCSCRDGE	KCAHHSHHFS	LDIDVGCTDL	NEDLGVWVIF
	851				900
B. t. LI-2	KIKTQDGHAR	LGNLEFLEEK	PLVGEALARV	KRAEKKWRDK	REKLEWETNI
Ps85a1-Kur	KIKTQDGHAR	LGNLEFLEEK	PLVGEALARV	KRAEKKWRDK	REKLEWETNI
Ghad	KIKTQDGHAR	LGNLEFLEEK	PLVGEALARV	KRAEKKWRDK	REKLEWETNI
HD73-Kur	KIKTQDGHAR	LGNLEFLEEK	PLVGEALARV	KRAEKKWRDK	REKLEWETNI
HD588-Ken	KIKTQDGHAR	LGNLEFLEEK	PLVGEALARV	KRAEKKWRDK	REKLEWETNI
Ps81gg-Kur	KIKTQDGHAR	LGNLEFLEEK	PLVGEALARV	KRAEKKWRDK	REKLEWETNI

	901				950
B. t. L1-2	VYKEAKESVD	ALFVNSQYDQ	LQADTNIAMI	HAADKRVHSI	REAYLPELSV
Ps85a1-Kur	VYKEAKESVD	ALFVNSQYDQ	LQADTNIAMI	HAADKRVHSI	REAYLPELSV
Ghad	VYKEAKESVD	ALFVNSQYDQ	LQADTNIAMI	HAADKRVHSI	REAYLPELSV
HD73-Kur	VYKEAKESVD	ALFVNSQYDQ	LQADTNIAMI	HAADKRVHSI	REAYLPELSV
HD588-Ken	VYKEAKESVD	ALFVNSQYDQ	LQADTNIAMI	HAADKRVHSI	REAYLPELSV
Ps81gg-Kur	VYKEAKESVD	ALFVNSQYDQ	LQADTNIAMI	HAADKRVHSI	REAYLPELSV
	951				1000
B. t. L1-2	IPGVNAAIFE	ELEGRIFTAF	SLYDARNVIK	NGDFNNGLSC	WNVKGHVDVE
Ps85a1-Kur	IPGVNAAIFE	ELEGRIFTAF	SLYDARNVIK	NGDFNNGLSC	WNVKGHVDVE
Ghad	IPGVNAAIFE	ELEGRIFTAF	SLYDARNVIK	NGDFNNGLSC	WNVKGHVDVE
HD73-Kur	IPGVNAAIFE	ELEGRIFTAF	SLYDARNVIK	NGDFNNGLSC	WNVKGHVDVE
HD588-Ken	IPGVNAAIFE	ELEGRIFTAF	SLYDARNVIK	NGDFNNGLSC	WNVKGHVDVE
Ps81gg-Kur	IPGVNAAIFE	ELEGRIFTAF	SLYDARNVIK	NGDFNNGLSC	WNVKGHVDVE
	1001				1050
B. t. L1-2	EQNNQRSVLV	VPEWEAEVSQ	EVRVCPGRGY	ILRVTAYKEG	YGEGCVTIHE
Ps85a1-Kur	EQNNQRSVLV	VPEWEAEVSQ	EVRVCPGRGY	ILRVTAYKEG	YGEGCVTIHE
Ghad	EQNNQRSVLV	VPEWEAEVSQ	EVRVCPGRGY	ILRVTAYKEG	YGEGCVTIHE
HD73-Kur	EQNNQRSVLV	VPEWEAEVSQ	EVRVCPGRGY	ILRVTAYKEG	YGEGCVTIHE
HD588-Ken	EQNNQRSVLV	VPEWEAEVSQ	EVRVCPGRGY	ILRVTAYKEG	YGEGCVTIHE
Ps81gg-Kur	EQNNQRSVLV	VPEWEAEVSQ	EVRVCPGRGY	ILRVTAYKEG	YGEGCVTIHE

	1051				1100
B. t. L1-2	IENNTDELKF	SNCVEEEIYP	NNTVTCNDYT	VNQEEYGGAY	TSRNRGYNEA
Ps85a1-Kur	IENNTDELKF	SNCVEEEIYP	NNTVTCNDYT	VNQEEYGGAY	TSRNRGYNEA
Ghad	IENNTDELKF	SNCVEEEIYP	NNTVTCNDYT	VNQEEYGGAY	TSRNRGYNEA
HD73-Kur	IENNTDELKF	SNCVEEEIYP	NNTVTCNDYT	VNQEEYGGAY	TSRNRGYNEA
HD588-Ken	IENNTDELKF	SNCVEEEIYP	NNTVTCNDYT	VNQEEYGGAY	TSRNRGYNEA
Ps81gg-Kur	IENNTDELKF	SNCVEEEIYP	NNTVTCNDYT	VNQEEYGGAY	TSRNRGYNEA
	1101				1150
B. t. L1-2	PSVPADYASV	YEEKSYTDGR	RENPCEFNRG	YRDYTPLPVG	YVTKELEYFP
Ps85a1-Kur	PSVPADYASV	YEEKSYTDGR	RENPCEFNRG	YRDYTPLPVG	YVTKELEYFP
Ghad	PSVPADYASV	YEEKSYTDGR	RENPCEFNRG	YRDYTPLPVG	YVTKELEYFP
HD73-Kur	PSVPADYASV	YEEKSYTDGR	RENPCEFNRG	YRDYTPLPVG	YVTKELEYFP
HD588-Ken	PSVPADYASV	YEEKSYTDGR	RENPCEFNRG	YRDYTPLPVG	YVTKELEYFP
Ps81gg-Kur	PSVPADYASV	YEEKSYTDGR	RENPCEFNRG	YRDYTPLPVG	YVTKELEYFP
	1151				1200
B. t. L1-2	ETDKVWIEIG	ETEGTFIVDS	VELLLMEE*S	HAN.....	.....
Ps85a1-Kur	ETDKVWIEIG	ETEGTFIVDS	VELLLMEE*.	.....	.....
Ghad	ETDKVWIEIG	ETEGTFIVDS	VELLLMEE*S	HANSGLNIVF	KSIVQEQHYK
HD73-Kur	ETDKVWIEIG	ETEGTFIVDS	VELLLMEE*S	HANSGLNIVF	KSIVQEQHYK
HD588-Ken	ETDKVWIEIG	ETEGTFIVDS	VELLLMEE*S	HANSGLNIVF	KSIVQEQHYK
Ps81gg-Kur	ETDKVWIEIG	ETEGTFIVDS	VELLLMEE*.	.....	.....

Table 3. Comparisons of the nucleotide sequences of the cryIa(c) genes from *B. thunngiensis var kurstaki HD-73.. B. thunngiensis L1-2.. B thunngiensis HD 588 kenyae , B thunngiensis Ps81gg kurstaki., B thunngiensis Ps85a1 kurstaki., B thunngiensis Ghad* Underlined letters represent nucleotide differences

Nucleotide	HD588 *ken*	Ps81gg	*B. t L1-2*	Ps85a1	HD73	Ghad
444	G	G	G	G	T	T
978	C	C	C	C	A	C
981	T	T	T	T	G	T
1002	G	G	G	G	T	G
1020	C	C	C	C	T	C
1089	C	C	G	G	G	G
1092	T	T	C	C	C	C
1098	T	T	A	A	A	A
1323	-	-	T	T	T	T
1324	-	-	A	A	A	A
1325	-	-	A	A	A	A

3.8 Bioassays of the B. thuringiensis L1-2 crylA(c) δ-endotoxin.

3.8.1 Glossina

Insecticidal activities of recombinant inclusion body preparations using *E. coli LK111 (pL12-7.4)* were carried out on adult female *G. m. morsitans*. Effective dose estimates were obtained by Probit analysis of the percent mortality observed after 54 h in the recombinant *B. thuringiensis L1-2*. The endotoxin caused mortalities in *G. m. morsitans* (LC_{50} = 425.5 μg/ml **(Table 4)**. Bioassay using wild type *B. thuringiensis L1-2* protoxin inclusion body preparation against adult *G. m. morsitans* were determined in diet incorporation assays and the effective dose estimates were obtained after 24 h. Different concentrations (0, 50, and 75 μg/ml) of the protoxin were tested for each protein suspension. The protoxins caused mortalities in *G. m. morsitans* LC_{50} = 74 μg/ml) **(Table 5)**. The flies showed mortality 20 h after initial exposure to the toxin. No mortality was scored on the control even after 54 h exposure.

Table 4: Toxicity of Recombinant *Bacillus thuringiensis L1-2* crystals on Tsetse.

Crystal Protein (μg/ml)	% Flies surviving after T					
	0	20	40	44	48	54
0	100	100	100	100	100	100
50	100	100	69	44	33.5	0
75	100	86	31	10	0	0

T = Time in h

Table 5: Toxicity of wild type *Bacillus thuringiensis L1-2* crystals on Tsetse.

Crystal Protein (μg/ml)	% Flies surviving after T								
	0	3	6	9	12	15	18	21	24
25	100	100	85	50	20	10	0	0	0
50	100	100	85	50	30	0	0	0	0
100	100	100	75	50	0	0	0	0	0

T = Time in h

3.9 Analysis of the recombinant B. thuringiensis L1-2 protein expressed in E. coli

Recombinant pL12-7.4 was used in all analyses of *B. thuringiensis L1-2* protein and for the preparation of protein extracts for insect bioassay. Toxicity of purified *B. thuringiensis L1-2* crystals and *E. coli* inclusions (pL12-7.4) showed that these extracts had contained biological activities similar to crystal protein of the purified wild type *B. thuringiensis L1-2*. Analysis of crude protein extracts from *E. coli* LK111 (pL12-7.4) showed that the insert of plasmid-encoded pL12-7.4 directed the synthesis of a protein having an apparent mobility of approximately M_r ~130,000 to- 140,000 (**Fig. 3.11**). In addition, the protein was detected by immunoblot analysis using antiserum raised against *B. thuringiensis L1-2* crystal protein. Recombinant *B. thuringiensis L1-2* showed cross-reactivity with antibodies raised against wild type *B. thuringiensis L1-2* **(Fig. 3.12).** This result indicated that the M_r ~ 130,000 - 140,000 was immunologically similar to the protoxin molecules obtained by alkali solubilization of *B. thuringiensis L1-2* parasporal crystals. From the bioassay results presented in (**Tables 4 and 5**), it is shown that the wild type *B. thuringiensis L1-2* is more active compared to the recombinant *B. thuringiensis L1-2,* this could be explained by the implication of the spore in the intoxication of susceptible insects. Hempel and Angus (1959), in their studies classified susceptible Lepidotera into three broad types according to thier response to *B. thuringiensis* intoxication. Types I and II insects require only the delta endotoxin crystals and differ from each other by the gut pH changes that occur and the length of incubation period necessary to affect mortilty. Type I insects die 1-7 hours. TypeII insects die from starvation in 2-4 days, however, septicemia from opportunist bacteria in gut can significantly shorten the the incubation period. Type III insects require 2-to 4-days incubation period (Dubois and Dean, 1995). Dean and Adang (1992) had relagated the influence of the spore in the intoxication process. None the less interest in using spore-toxin combinations to maximize the lethality of *B. thuringiensis* preparation has continued (Dubois and Dean, 1995). Wolfsberger, 1990 in thier studies have also found that with the availability of pure CryIA (a) and CryIA(c) insecticidal crystal proteins that differ in insecticidal activity and specificity towards gypsy moth, their studies have now allowed

the determination whether insecticidal crystal proteins -bacteria combinations are specific for *B. thuringiensis* spores or whether other bacteria could also act synergistically with the insecticidal crystal proteins and significantly increase lethality compared with intoxication with crystal proteins alone. For many years *B. thuringiensis* spores were assumed to play a specific role in the mechanism of intoxication in susceptible insects (Li *et al*., 1987).

These previous studies have corrobrated with the findings in these studies. The bioassay results have confirmed that the wild type *B. thuringiensis L1-2* which has crystal insecticidal proteins- spores combination has higher lethality against these insects than *recombinant B. thuringiensis L1-2*. These differences in lethality could reflect the presence of spores in the wild type *B. thuringiensis L1-2* which might have contributed to the high toxicity. There could be some synergism between the insecticidal proteins and the spores which could have enhanced the lethality of wild type *B. thuringiensis L1-2*. Analysis of protein extracts derived from recombinant *B. thuringiensis L1-2* **(Fig 3.11 and 3.12)** show the M_r ~ 130,000 sub unit, yet the wild type *B. thuringiensis L1-2* show the M_r 130,000 as well as M_r ~ 45,000 subunit. This is because *B. thuringiensis* crystal protein is composed of several subunits which may be encoded by different genes but the recombinant *B. thuringiensis L1-2* shows only the M_r ~ 130,000 because a purified CrylA(a) M_r ~ 130,000 was used to screen the *B. thuringiensis L1-2* plasmid libraray and only homologous size protein would be seen.

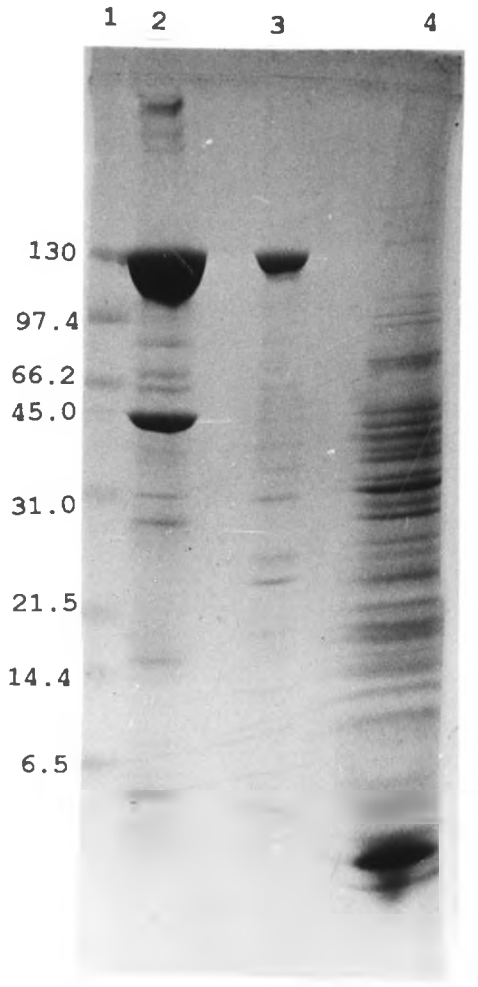


Fig. 3.11 SDS-polyacryamide gel electrophoresis (SDS-PAGE) of protein extracts from *B. thuringiensis L1-2 wild type* and recombinant strains.

1, Protein size markers; **2**, M_r ~130,000 crystal protein (wild type)

3, protein extracts from recombinant *B. thuringiensis L1-2* ; **4**, *E. coli* LK111 protein

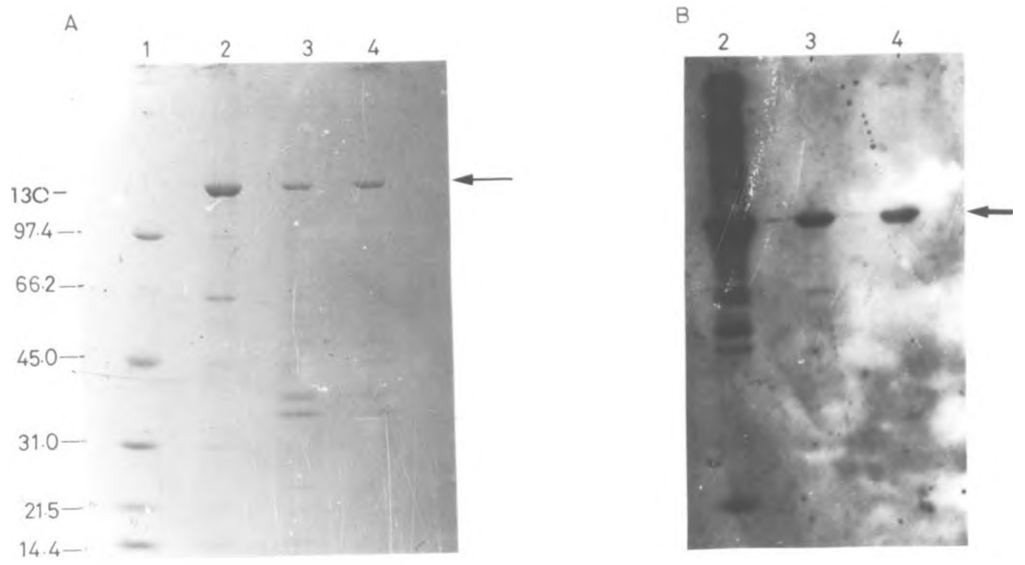


Fig. 3.12 **Immunological analysis of recombinant *B. thuringiensis L1-2* proteins**

Panel A:

1. Protein size marker; **2.** Wild type *B. thuringiensis Tikki*

3. Recombinant B. thuringiensis *Tikki*; **4.** recombinant *B. thuringiensis L1-2.*

Panel B: Immunoblot analysis of recombinant *B. thuringiensis L1-2* and *Tikki*

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

A strain of *E. coli* pL12-7.4 has been isolated which carry recombinant plasmids and produce protein antigens that react with antibodies specific for the crystal protein of *B. thuringiensis*. The recombinant plasmids isolated from pL12-7.4 transformed culture of *E. coli* strain *LK111* which is ampicillin resistance. When pL12-7.4 was identified with anti-crystal antibodies, the intact polypeptide fragments had electrophoretic mobilities similar to those of the crystal protein fragment of *B. thuringiensis*. Protein extracts of pL12-7.4 were found to be toxic to adult *G. m. morsitans* and larvae of *C. partellus*. These results suggests that the DNA insert of recombinant pL12-7.4 encodes polypeptides possessing properties similar to those of the crystal protein of *B. thuringiensis*. It also encodes sufficient information to produce dipteran and lepidopteran active toxins. The toxic proteolytic fragment of the crystal protein gene has been confirmed in the M_r ~ 130,000 to-140,000 range. The major difference between *B. thuringiensis L1-2* and other *B. thuringiensis* studied so far is that this isolate has shown dual toxicity to adult dipteran and lepidopteran insects, whereas the M_r ~ 130,000 to- 140,000 has always been shown to be toxic to lepidopteran larvae. The studies found *B. thuringiensis L1-2* sequence were found to be almost identical to the cry1A(c) gene found in *B. thuringiensis* subsp. *kurstaki HD-73* (Adang *et al.*, 1985; Höfte and Whiteley, 1989), however a lysine replaced phenylalanine at position 148, while the DNA changes at positions 978 (A to C), 981 (G to T), 1002 (T to G) and 1020 (T to C) did not result in any amino acid changes. The sequence located upstream from **ATG** start codon showed strong homology to the cryIA(c) upstream region, including the transcription start sites in *B. thuringiensis* and *E. coli* (Wong *et al.*, 1983).

Comparison of the nucleotide sequence of the *B. thuringiensis* cryIA protein Open reading frame (ORF) revealed only four additional DNA differences none of which resulted in any amino acid change indicating that the protein encoded by the gene of *B. thuringiensis L1-2* is identical to that of *B. thuringiensis HD-73* cryIA(c) gene. This apparently minor change in DNA difference may have resulted in this interesting and

potentially important difference between this M_r ~130,000 to- 140,000 with other lepidopteran insects, but the most surprising finding is the high homology found not only among the coding sequences of this gene, but also in its flanking regions as indicated by a comparison of the restriction endonuclease map of *B. thuringiensis L1-2* gene. The high homology thus indicates that the δ-endotoxin gene of *B. thuringiensis L1-2* is almost identical to that of *B. thuringiensis* subsp. *kurstaki HD-73* and that this gene is widely spread and this gene is expressed in biologically active form in *E. coli.* By comparing the hybridization of gene-specific probe to digests of plasmid DNA and chromosomal DNA, at all level of hybridization stringency, there is evidence indicating that the δ-endotoxin gene from *B. thuringiensis L1-2* is located on the chromosome as well as on the plasmid, but with higher copy number on the plasmid than chromosomal gene but since most *B. thuringiensis* cry genes are localized on large plasmids and are often flanked by inverted repeat, transposon-like structures, it is therefore conceivable that recombination events between the *B. thuringiensis HD-73*, *B. thuringiensis Kenyae HD 558-2* may account for the apparent homology with the *B. thuringiensis L1-2* cryIA(c) gene.

It is generally known that the sporulating soil bacterium *B. thuringiensis* produces proteinaceous crystalline inclusions which are often toxic to lepidopteran, dipteran, and coleopteran insect larvae. However, the crystal production in *B. thuringiensis L1-2* is quite unusual in that it produces a proteinaceous crystalline inclusion which is toxic to lepidopteran larva and to adult dipteran. To analyze this unique behavior of *B. thuringiensis L1-2*, the following approaches were undertaken to study what could be behind this uniqueness. The first approach was to answer the question that the phenomenon observed could be caused by an altered phenotype of the bacterium due to a possible mutation in the chromosome and not by the crystal protein gene.

The nucleotide sequence have revealed that *B. thuringiensis L1-2* is made of M_w ~130,000 protein, suggesting that there is no change in the phenotype. Although, various cry proteins are known to maintain some homology, five blocks of amino acid sharing a high degree of homology are found to be located within the active toxin segments of most cry classes and it has been observed that the fifth conserved block demarks the C-terminus of the active toxins in the M_w 130,000 cry proteins. The nucleotide sequence of

this bacterium, *B. thuringiensis L1-2,* has also confirmed that it contain five conserved amino acid blocks.

More than 150 *B. thuringiensis* have been isolated in Kenyan soils by ICIPE scientists. *B. thuringiensis M44-2, an* ICIPE isolate has been characterized and is being used to control Lepidopteran stemborers *(Chilo partellus)* in agricultural fields in Nyanza and coastal provinces of Kenya. Lepidoptera stemborers are among the most damaging pests of cereal crops in the tropics. *Chilo partellus, Busseola fusca* and *Eldana saccharina* are the major pests of maize, sorghum, millets and other cereals grown in Africa. These stemborers are susceptible to various insecticidal crystal proteins (δ-endotoxins) produced by a number of *B. thuringiensis* strains.

B. thuringiensis is an entomopathogenic micro-organism of outstanding scientific interest, having a ubiquitous distribution and ability to produce insecticidal parasporal inclusion, which exhibits specific toxicity towards certain susceptible insect larvae. This uniqueness has spurred investigations into its use as a natural biological control in agriculture, forestry and human health for the elimination of disease vectors making it the leading organism used in industry as microbial pesticide. The use of *B. thuringiensis* appears likely to increase dramatically in the future as farmers move towards more environmentally acceptable agricultural practices but there are commercialization limitations for example the rapid inactivation in the field, development of resistance, and its high specific host spectrum need to be addressed.

There is substantial justification for promoting environmentally safe methods for integrated pest management programs. *B. thuringiensis* is now the most widely used biologically produced pest agent. It represent about 2% of the global insecticide market. While the use of biological pesticides in agriculture remains significantly behind that of synthetic chemical pesticides, several environmental and safety considerations favour the future, development of *B thuringiensis*

B. thuringiensis cry proteins that have been studied thus far are not pathogenic to mammals, birds, amphibians, or reptiles, but are very specific to the groups of insects and pests against which they have activity. These cry-based pesticides generally have low costs for development and registration than for novel chemical pesticides. The mode of

action of action for the cry proteins differs completely from the modes of action of known chemical pesticides, making *B. thuringiensis* cry proteins key components of integrated pest management strategies aimed at preserving natural enemies of pests and managing insect resistance.

Traditional animal and crop protection programs have relied mainly on the spraying of a number of highly effective organophosphates and synthetic insecticides. These insecticides are available in a variety of formulations, but their efficacy against these insect pests are restricted by the feeding behavior of the insects and potential insect resistance to these chemicals. Over 500 species of insects have become resistant to one or multiple synthetic chemical insecticides. Furthermore, the prolonged use of chemical pesticides are not ecologically sound, due to their effects on a wide range of non-target organisms resulting in unbalanced insect communities, and the disruption of resident natural enemies which allows the development of secondary pests many of which were present in insignificant numbers before the use of broad-spectrum pesticides.

There are other ostentatious issue that need to be addressed, for example, how to find alternative delivery systems for these cry proteins. *B. thuringiensis* crystal genes have been introduced into *E. coli, B. subtilis, B. megaterium* and *Pseudomonas fluorescens*. Fermentations of recombinant pseudomonads are now being used to produce concentrated aqueous biopesticide formulations consisting of cry inclusion encapsulated in dead cells. These encapsulated forms of the cry proteins have been reported to show improved persistence in the environment. Live endophytic or epiphytic bacteria have been used as hosts of *B. thuringiensis* crystal genes in the field. The rationale for using these bacteria as hosts has been because these bacteria can propagate themselves at the site of feeding and continue to produce crystal proteins thus prolonging the persistence of cry proteins in the field. Similarly, cry genes have been transferred into other plant colonizers including *Azospirillum* spp., *Rhizobium leguminosarum, Pseudomonas cepacia,* and *Pseudomonas flourescens.*

*D*elivery system will have to be sought for the dipteran-active toxins of *B. thuringiensis L1-2* because this cry toxins has been found to be active against adult dipteran (tsetse) in the laboratory- selection experiments. Tsetse are important pests of

livestock and man, however, tsetse have adrenotrophic viviparity method of reproduction and feed only on bloodmeal, unlike the other dipteran (mosquitoes and black fly), which are larvicidal and have aquatic feeding zones. Attempts have been made to *B. thuringiensis* for tsetse control but delivery system has been the major set back, Other studies have found *B. thuringiensis* toxins to play vital role in the delay of resistance to synthetic pyrethroids which are now under investigation for control of livestock pests. Since laboratory selection experiments have found *B. thuringiensis L1-2* to be toxic to tsetse, a combination of pyrethroids and *B. thuringiensis L1-2* could be introduced as pesticide -impregnated ear tags and this could offer possible delivery system for *B. thuringiensis* toxins for tsetse fly control. *B. thuringiensis* used in combination with chemicals such as endosulfan have been used as part of pyrethroid resistance management strategy. In theory, those insects with the potential to survive the chemical insecticides are likely to encounter a lethal dose of *B. thuringiensis*. Consequently, fewer individuals are likely to pass on their genetic advantage to the next generation. This effect can also be compounded if the chemical is used at doses low enough for the natural predators and parasites to continue to function. If successful, such combinations would extend the possible use time into stage two of the livestock animals. Also the use of *B. thuringiensis* would avoid early-season selection for resistance to chemicals in tsetse fly, as well as conserving any natural control, allowing resistance levels to drop permitting continued use of these valuable pyrethroid products.

Another possibility is to use sex pheromones as delivery vehicle for *B. thuringiensis* toxins into tsetse fly. This approach has been successful for the control for the oriental fruit moth, *Grapholita molesta* in stone fruits. Others studies have attempted to incorporate fungus into tsetse traps and this approach to control of tsetse has proved successful. A combination of fungus and *B. thuringiensis* toxin could be used for more effective control of tsetse and resistance management strategy.

B. thuringiensis has evolved to produce large quantities of crystal proteins making it a logical host for developing new improved cry biopesticide. *B. thuringiensis* toxin genes are currently being transferred to crop plant genomes to overcome field degradation problems of conventional *B. thuringiensis* applications and to improve the efficiency of

these toxins. Several cry genes have been introduced into plants, starting with tobacco and now many including many major crop species like potato, cotton and maize. However, potential for insect resistance to *B. thuringiensis* toxin. In the past it was hoped that insects would not develop resistance to *B. thuringiensis* toxins, since *B. thuringiensis* and insects co-evolved. Starting in the mid 1980's, however, a number of insect populations of several different species with different levels of resistance to *B. thuringiensis* crystal proteins have been obtained by laboratory-selection experiments, using either laboratory-adapted insects, or insects collected from the wild populations. Example of insects resistant to individual cry protein toxins include Indian meal moth (*Plodia Interpunctella),* almond moth (*Cadera cautella*), Colorado Potato beetle (*Leptinotarsa decemlineata),* cottonwood leaf beetle (*Chrysomela scripta*), cabbage looper *(Trichoplusia ni),* cotton leafworm (*Spodoptera littoralis),* beet armyworm (*Spodoptera exigua*), tobacco budworm (*Heliothis virescens*), European corn borer (*Ostrinia nubilalis).* Understanding the mechanism the of resistance to *B. thuringiensis* toxin need to be studied in great depth as this could be helpful for the management of rapid onset of insect resistance.

Given the severe impact of mosquitoes-and black fly-borne human diseases, there is considerable interest in identifying additional dipteran-active toxins that could be used to control these insect pests. Since its discovery, *B. thuringiensis var israelensis* has proved to be one of the most effective and potent biological pesticide for the control of mosquitoes and black flies. Its discovery came at an auspicious moment because of the mounting resistance of mosquitoes and black flies to chemical pesticides

The expansion in the insecticidal activity spectrum of *B. thuringiensis* has been achieved by the availability of techniques for cloning, manipulations and transfer of the toxin gene, allowing for genetic manipulation of *B. thuringiensis,* leading to creation of combinations of genes more useful for a given purpose than those known to occur in natural isolates. A conjugation-like system has been used to transfer cry-encoding plasmids from one strain to another, but most cry genes are not readily transmissible by this process. One successful approach for constructing strains with broader or improved insecticidal activities has been the application of recombinant DNA technology to plant

biology and crop production.

The technical advances in plant biotechnology has translated into commercial products that provide significant advantages and benefits to farmers, processors and consumers. The inevitable growth of world population and demand for food and clear sensitivity expressed by consumers for environmentally sustainable agricultural production methods are key factors that underline the important role these new products will have in assuring a safe and affordable food supply. Plant biotechnology will provide the agricultural community with unique, effective and environmentally friendly manner to control insect pests which cause great economic losses to farmers.

The expression of *B. thuringiensis* of cry genes in *planta* could offer several benefits. several cry genes have been introduced into plants, starting with tobacco now many including major crop species. There are many problems encountered with the expression of these toxin genes in planta and they problems need to be addressed, for example, when unmodified crystal protein genes are fused with expression signals used in the plant nucleus, protein production is quite poor when compared to similar transcription units containing typical plant marker genes. The relatively A+T rich *Bacillus* DNA contain a number of sequences that could be deleterious to gene expression in plants, such as splice sites, poly-A addition sites, mRNA degradation signals and transcription termination sites, as well as codon usage biased away from that used in plants, but when the *Bacillus* sequences are extensively modified using synonymous codons bias more like that of a plant, expression has been found to improve dramatically.

In transgenic plants, selection pressure could be restricting the expression of the crystal protein genes to certain tissues of the crop, those tissues most susceptible to pest damage. Selection pressure will allow certain parts of the plant to be fully protected providing a form of spatial refugia.

Because the toxins are produced continuously and apparently persist for some time in plant tissue, fewer applications of other insecticide are needed, reducing field management costs. Like *B. thuringiensis* based biopesticides, such "enhanced seed systems" are less harmful to the environment than chemical pesticides and typically do not affect beneficial predatory and parasitic insects. The plant delivery system also

expands the range of pests targeted for control with cry proteins, including the sucking and boring insects, root-dwelling insects and nematodes.

Genetic manipulation of *B. thuringiensis* to create combinations of genes more useful for a given purpose than those known to occur in natural isolated may be desirable as certain combinations of *B. thuringiensis* cry proteins have been shown to exhibit synergistic effects.

A variety of shuttle vectors, some employing *B. thuringiensis* plasmid replicons have been used to introduce cloned cry genes into *B. thuringiensis* plasmid vector system employing *B. thuringiensis* site-specific recombination systems have been developed to construct recombinant *B. thuringiensis* strains for new Biopesticide products.

A concern that has been associated with increased use of *B. thuringiensis* sprays and the production of *B. thuringiensis* toxin-expressing transgenic is the increased risk of resistance *to B. thuringiensis* toxins. In response to this concern research on the judicious use of *B. thuringiensis* product are being promoted which include strategies which will try to prevent or diminish the selection of the rare individuals carrying resistance genes and hence will keep the frequency of resistance genes below levels resulting in inefficient insect control.

Proposed strategies include the use of multiple toxins referred to as stacking pyramiding, crop rotation, high or ultra-high dosages, and spatial or temporal refugia, site- or temporal-specific gene expression, inducible gene expression, crop mosaics, multilines and mixtures of resistant and susceptible genotypes. Crystal proteins gene expression could also be triggered by the feeding of the insect itself by using wound-inducible promoters driving cry gene expression. If plants would only express *B. thuringiensis* toxin in response to specific damage thresholds, this may provide a mechanism to diminish toxin exposure to insects. Alternatively, toxin expression could be induced by the application of a chemical. In this way, the growers (farmers) would have the option to have cry toxins present in the crops only when insect densities exceed an economic threshold.

Alternative management option is the rotation of plants (crops) or sprays of a particular *B. thuringiensis* with those having another toxin type that binds to a different

receptor. This strategy has potential value when fitness costs is associated with resistance. Fitness costs have been reported in resistance *Plutonella xylostella* larvae. Insects resistance to one cry toxin type would be at disadvantage during the next growth season when a different toxin type is used, resulting in decrease of the frequency of the corresponding resistance gene.

Another attractive resistance management tactic is the high-dose strategy combined with refugia or areas free of toxin-expressing plants. The principle is to express cry toxins at such a dose that nearly all heterozygotes, which are the most common carriers of resistance, will be killed, consequently, the frequency of resistance gene will be kept low. On transgenic plants, the most sensitive stage, the first instar larvae, is exposed to toxin, unless older larvae could move from toxin-free to toxin-expressing plants. It has been reported that inclusion of refuge plants in cages with transgenic plants resulted in slower evolution of resistance in *Plutella xylostella*. Depending on the crop, refugia may naturally present or may need to be created by the planting of non-transgenic plants. Refugia should be uncontaminated, and there should be random mating between resistant and susceptible insects.

A specific planting strategy that has been recommended to reduce selection is to use seed mixture of toxin-expressing and toxin-free plants to provide "pre-packaged" refugia. Another valuable option for resistance management, in combination with the use of refugia, is the expression of multiple proteins in crops, or incorporation of multiple proteins in *B. thuringiensis* sprays, provided these toxins have different modes of action with respect to the insect's mechanism of resistance. Cry toxins that recognize different receptors in the same target species could be deployed in this strategy, since they are less prone to cross-resistance. Such an effect has been observed with *B. thuringiensis var israelensis*, strain that naturally combines toxins with different modes of action. Additionally, *B. thuringiensis* cry toxins could be combined with other insecticidal proteins. This "multiple attack" strategy exploits the fact that within a population, if insects homozygous for one resistance gene are rare, then insect homozygous for multiple genes are very rare. A critical condition for the success of this strategy is that each of the insecticides on its own should have should have high mortality for susceptible

homozygotes. An example includes European corn bearer, *Ostrinia nubilalis* where cryIA(b) and cryIB(a), both highly active, bind to different receptors.

Although the adaptation of such strategies will undoubtedly prolong the durability of pest resistance within a particular transgenic crop, these approaches do not address the critical issue of how to limit the consequences of *B. thuringiensis* resistance when it does evolve, particularly the evolution of resistance in polyphagous pests. These polyphagous pests are capable of attacking a wide range of plants. Highly mobile polyphagous pests such as *heliothis* or *Spodoptera*, which might develop resistance on one crop species and then disperse to infest other crops, could rapidly nullify the effectiveness of pest resistance in a wide range of transgenic crops, particularly if they express the same insecticidal protein. Under current conditions, pests which develop resistance on transgenic plants are also likely significantly cross-resistance to *B. thuringiensis* bioinsecticides because the large majority of transgenic plants incorporate cryIA proteins.

Rarely can *B. thuringiensis* be used alone throughout the season for many reasons: high insect pressures which cannot be controlled sufficiently with *B. thuringiensis*, multiple pest species that are not controlled by *B. thuringiensis*, or the desire to rotate pesticide chemistry to minimize selection pressure for resistance. These problems lead to the use of *B. thuringiensis* in a program for total pest control. The program approach is used in situations where early season pest pressure is predominately from lepidoterous pests offer an opportunity to exploit the selective advantage of *B thuringiensis*. By choosing to use *B. thuringiensis* early in the season, the natural enemy fauna is allowed to build and maintain a pressure against other pests, such as aphids, thus suppressing the need to intervene with conventional chemistry. This tactic also prevents the build up of resistance to conventional chemicals, such as the pyrethroids, which are best held back until the later part of the growing season when the risk of damage to the harvestable product demands high control levels and broad spectrum activity.

This study was aimed at obtaining an understanding of *B. thuringiensis* genes, their major characteristics, and ability to produce insecticidal crystalline proteins (ICPs) during sporulation. A library constructed of plasmid DNA fragments from *B. thuringiensis L1-2* in pEcoR251 vector was screened for genes expressing insecticidal activities. Two

insecticidal crystal protein genes from these isolates, *B. thuringiensis L1-2* and *Tikki* have been sequenced in ICIPE. The data obtained from these sequences have been useful in the classification and characterization of these genes.

We propose to continue with this project by carrying out similar work using a truncated version of the cryIA(c) from *B. thuringiensis L1-2*. The crosses of transgenic inbred maize would offer protection to maize and other cereal plants against tropical pests, *Busseola fusca*, *Sesamia calamistis* and *Eldana saccharina*.

Future work will emphasize on the development and deployment of transgenic organisms expressing *B. thuringiensis* δ-endotoxin genes and results obtained from these work will augment the use of naturally occurring microbial insecticides in integrated pest management programs. Plans are also underway for future investigations of the dual toxicity role played by this cryIA(c) gene from *B. thuringiensis L1-2*.

APPENDIX A

CHEMICALS, BUFFERS, STOCK SOLUTIONS AND MEDIA REQUIREMENTS

All media, buffers and stock solutions were sterilized by autoclaving at 120° C for 20 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22 μm membrane filters (Millipore).

All solutions (Molar or %) are made volumetrically.

A.1 Buffers and stock solutions

A.1.1 Ampicillin (100 mg/ml)

Dissolve 2 g Ampicillin in 20 ml distilled water. Filter sterilize and store aliquot at -20° C. Dilute 1/1000 into media (1 μl/ml) for final concentration of 100 μg/ml.

A.1.2 Antibody-AP conjugate solution for colorimetric detection NBT/X-phosphate

Dilute anti-digoxigenin-AP conjugate 1:5000 in Buffer 2

A.1.3 Antibody-AP conjugate solution for chemiluminescence detection:

Dilute Anti-digoxigenin-AP conjugate 1:10,0000 in Buffer 2

A.1.4 ATP (0.1 M)

Dissolve 60 mg Adenosine triphosphate in 4.0 ml sterile distilled water. Adjust pH to 7.0 with 0.1 M NaOH (spot a few μl on pH paper to check pH) make up to 5 ml. Freeze 0.1 ml aliquot at -70° C. Discard remainder once defrosted.

A.1.5 Bovine Serum Albumin (1 %)

Dissolve 100 mg BSA (Fraction V) in 10 ml of sterile distilled water. Filter sterilize, aliquot into sterile microfuge tubes and store at -20° C.

A.1.6 $CaCl_2$ (1 M)

Dissolve 14.7 g $CaCl_2.2H_2O$ in 100 ml distilled water. Autoclave

A.1.7 Colour substrate solution:

Colour substrate solution is freshly prepared, by taking 45 µl NBT solution and 35 µl X-Phosphate solution and adding to 10 ml buffer 3.

A.1.8 Deoxyribonucleoside triphosphates (dNTPs) cocktail

Containing: 0.125 mM of each dNTP (Boehringer Mannheim)

A.1.9 Digoxigenin labelling and detection system for Nucleic Acid analysis.

A.1.9.1 Buffer 1:

150 mM NaCl

100 mM Maleic acid, pH 7.5

pH adjusted with solid or concentrated NaOH, autoclaved.

A.1.9.2 Blocking reagent stock solution:

Blocking reagent (Boeringer Mannheim)	10 g
Buffer 1	100 ml

Blocking reagent is dissolved in Buffer 1 to a final concentration of 10 % (w/v) with shaking and heating either on a heating block or microwave oven. The blocking reagent must be heated while it dissolves in the Maleic acid buffer. Boiling causes the reagent to coagulate. Autoclave and store at 27° C, 4° C or -20° C.

A.1.9.3 Buffer 2

Blocking Reagent stock solution diluted 1:10 in Buffer 1

(final concentration = 1 % Blocking reagent)

A.1.9.4 Buffer 3

100 mM Tris-HCl pH 9.5

100 mM NaCl

50 mM $MgCl_2$

Buffer 3 is prepared from corresponding stock solutions to avoid $MgCl_2$ precipitation. If pH 9.5 is adjusted $Mg(OH)_2$ will precipitate otherwise. For preparation dilute a 1 M Tris-HCl pH 9.5 and add NaCl and $MgCl_2$.

A.1.10 Dithiothreitol (DTT) (1 M)

Dissolve 3.09 g in 20 ml of 10 mM sodium-acetate (pH 5.2). Filter sterilize and freeze aliquot at -20° C

A.1.11 EDTA (0.5 M, pH 8.0) (Sambrook et al., 1989)

EDTA $2H_20$	168.1 g
Distilled water	1000 ml

EDTA will only dissolve when pH has been adjusted to 8.0 by NaOH

A.1.12 Ethidium bromide solution

(2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide)

A solution of 10 mg/ml was made in distilled water and stored in a dark bottle. Dissolve 0.1 g of ethidium bromide in 10 ml of distilled water. Shake well to dissolve.

A.1.13 Exonuclease III Shortening solutions (Henikoff, 1984)

A.1.13.1 S_1 (10X) nuclease buffer.

NaOAc (3.0 M) pH 4.6	1.1 ml
NaCl (5.0M)	5.0 ml
$ZnSO_4$	30.0 mg
Glycerol	5.0 ml

A.1.13.2 Exonuclease III buffer

Tris-Cl (1 M, pH 8.0)	660 µl
$MgCl_2$ (0.1 M)	66.4 µl
Distilled water	9.27 ml

A.1.14 Gel loading buffer (6X)

Bromophenol blue (0.25 %)	0.25 g
Xylene cyanol FF (0.25 %)	0.25 g
Ficoll (Type 400; Pharmacia) in water	15.0 g
Distilled water	100 ml

The solution was stored at 27° C

A.1.15 Glucose (20 % (w/v)

Dissolve 20 g of Glucose in 100 ml distilled water. Autoclave.

A.1.16 Glycerol (100 %)

Sterilize by autoclaving

A.1.17 Isopropanol (salt saturated)

Isopropanol was saturated with aqueous 5 M NaCl, 10 Mm Tris-HCl and 1 mM EDTA (pH 8.0).

A.1.18.1 Ligation buffer (10X)

The buffer was made according to the following table and stored at -70° C

Stock solution	final conc	conc/1 ml
Tris-HCl (1 M, pH 7.6)	500 mM	500 µl
$MgCl_2$ (1 M)	50 mM	50 µl
ATP (0.1 M)	10 mM	100 µl
DTT (0.5 M)	50 mM	100 µl
Distilled water		250 µl

A.1.18.2 Ligase dilution buffer

The buffer was made according to the following table and stored at - 20°C

Stock solution	final conc	conc. /10 ml
Tris-HCl (1 M, pH 7.6)	20 mM	0.2 ml
EDTA (0.5M, pH 8.0)	1.0 mM	2.0 µl
DTT (0.5M)	5.0 mM	10 µl
KCl (1 M)	60 mM	0.6 ml
Glycerol 44 % (v/v)		4.4 ml
Distilled water		4.788 ml

A.1.18.3 Ligase reaction mix

10X ligation buffer	240 µl	
T_4 DNA ligase,(Boehringer Mannheim); 1 U/µl	32 µl	
Distilled water		1568 µl

A.1.19 $MgCl_2$ (1 M)

Dissolve 20. 3 g $MgCl_2.6H_2O$ in 100 ml distilled water. Autoclave.

A.1.20 $MgSO_4$ (1 M)

Dissolve 24.7 g $MgSO_4$ $7H_2O$ in 100 ml distilled water. Autoclave.

A.1.21 $NaCl_2$ (5 M).

Dissolve 292.2 g of $NaCl_2$ in 800 ml of distilled water. Adjust the volume to 1000 ml. Autoclave.

A.1.22 NaOH (10 N)

Dissolve 40 g NaOH in 100 ml distilled water. Autoclave.

A.1.23 N-lauroylsarcosine:

10 % (w/v) filtered through a 0.2-0.45 μm membrane

A.1.24 Phenol (TE saturated)

Phenol (500 g, Merck) was melted at 65° C and 0.6 g of 8-hydroxyquinoline, 7.5 ml NaOH (2 M), 130 ml water, and 6 ml Tris-HCl (1 M, pH 7.6) was added. The phenol was left overnight to liquefy (or 4° C until the solution is clear) phase was approximately pH 7.6. The phenol was stored under TE (1X) at -20° C.

A.1.25 Phenol:Chloroform

Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris.Cl (pH 7.6). Store in dark bottle at 4° C

A.1.26 Potassium acetate (5 M) (pH 7.5).

Dissolve 491 g of Potassium acetate in 900 ml in distilled water. Adjust the pH with glacial acetic acid). Add distilled water to 1000 ml.

A.1.27 Probe stripping solution:

0.2 N NaOH

0.1 % SDS.

A.1.28 Sodium acetate (3 M, pH 5.2)

Sodium acetate $3H_2O$	204.05 g
Distilled water	400 ml

Adjust pH to 5.2 with glacial acetic acid, and then make up to 500 ml.

A.1.29 SDS 10 % (laurel sulphate)

10 g of SDS in 100 ml of distilled water. Heat to about 80° C to dissolve. Add few drops of HCl to pH = 7.2.

A.1.30 SSC (20X)

NaCl (3 M)	175.3 g
Sodium citrate (0.3 M)	88.2 g
Distilled water	1000 ml

Adjust pH to 7.0 with NaOH (10N). Autoclave.

A.1.31 S_1 nuclease reaction mix (enough for 16 tubes)

S1 (10X) nuclease buffer	53 µl
S1 nuclease (60 U)	1.5 µl
Distilled water	347 µl

A.1.31.1 S_1 nuclease stop buffer

Tris base (no HCl)	300 mM
EDTA (pH 8.0)	50 mM

A.1.32 Southern transfer solution

A.1.32.1 Denaturation solution

0.4 M NaOH	16 g
1 M NaCl	58 44 g
Distilled water	1000 ml

A.1.32.2 Denhardt's solution (10X) (Sambrook et al., 1989)

Ficoll	1 g
Polyvinylpyrrolidone	1 g
BSA (Fraction V)	1 g
Distilled water	100 ml

The solution was stored in aliquot (10 ml) at - 20° C

A.1.32.3 Depurination solution

0.25 M HCl	21.35 ml
Distilled water	1000 ml

A.1.32.4 Neutralization solution

0.5 M Tris-HCl pH 7.5

3 M NaCl

*A.1.32.5 Standard hybridization buffer (*5X SSC)

0.1 % Sodium-lauroylsarcosine

0.02 % SDS

1 % Blocking Reagent

*A.1.32.6 Prehybridization solution (*SSC 6X)

SDS	0.5 g
Denhardt's (50X)	10 ml
EDTA (0.5M, pH 8.0)	2 ml

A.1.32.7 Washing solution (SSC 2X)

0.1 % SDS

A.1.32.8 Washing solution (SSC 0.1X)

0.1 % SDS

A.1.33 Transformation and storage buffer (TSB)

LB medium, containing:

10 % (w/v) PEG M_r 4,000

5 % (v/v) DMSO

10 mM $MgCl_2$

10 mM $MgSO_4$

A.1.34 Tris-acetate buffer (50X TAE)

Tris	242 g
Acetic acid	57.1 ml
EDTA (0.5 M, pH 8 0)	100 ml (or 37.2 g EDTA)
Distilled water	1000 ml

A.1.35 Tris-base (1 M)

Dissolve 121.1 g Tris in 1000 ml distilled water.

A.1.36 Tris-borate buffer (10X TBE)

Tris (99 mM)	108.9 g
Boric acid (99 mM)	55.65 g
EDTA (2.5 mM)	20 ml or 9.3 g
Distilled water	1000 ml

A.1.37 Tris-HCl (1 M).

Dissolve 121.1 g in 800 ml distilled water. Adjust pH to required level with concentrated HCl. Three solutions are generally required.

pH 7.4 - add about 70 ml HCl

pH 7.6 - add about 60 ml HCl ¦ check pH

pH 8.0 - add about 42 ml HCl ¦

A.1.38 TE (Tris-EDTA) buffer (10X)

Use Tris and EDTA stock solutions to make 10 mM Tris, 1 mM EDTA, pH 7.6.

Tris	121 g
EDTA (0.5 M, pH 8.0)	200 ml
HCl	60 ml
Distilled water	1000 ml

A.1.39 Water

Water Glass double distilled water was treated overnight with 0.1 % diethyl pyrocarbonate (DEPC) at 37° C, and then autoclaved. All glassware, tips and micro centrifuge tubes were similarly treated.

A.1.40 Lumigen™ PPD solution:

Stock solution of Lumigen™ PPD (sold premixed at 10 mg/ml) diluted 1:100 in buffer 3

A.1.41 Lumi-Phos™ 530

Solution is sold ready to use

A.1.42 Restriction endonuclease dilution buffer

A.1.42.1 Boehringer Mannheim restriction endonuclease incubation buffer set

Buffers (Buffer components)

Final concentration	A	B	L	M	H
Tris-acetate	33	-	-	-	-
Tris-Cl	-	10	10	10	50
Mg-acetate	10	-	-	-	-
$MgCl_2$	-	5	10	10	10
K-acetate	66	-	-	-	-
NaCl	-	100	-	50	100
DTE	-	-	1.0	1.0	1.0
DTT	0.5	-	-	-	-
pH at 37° C	**7.9**	**8.0**	**7.5**	**7.5**	**7.5**

A.1.42.2 Universal restriction endonuclease buffer (10X)

The buffer is made according to the following table and stored at 20° C

Stock solution	Salt concentration			
	0	50	100	150
Tris-HCl	1.0 ml	1.0 ml	1.0 ml	1.0 ml
$MgCl_2$	1.0 ml	1.0 ml	1.0 ml	1.0 ml
DTT (0.5 M)	0.2 ml	0.2 ml	0.2 ml	0.2 ml
BSA (10 mg/ml)	1.0 ml	1.0 ml	1.0 ml	-
Glycerol	4.4 ml	4.4 ml	4.4 ml	3.8 ml
NaCl (5 M)	-	1.0 ml	2.0 ml	3.0 ml
Distilled H_2O	2.4 ml	1.4 ml	0.4 ml	-

A.1.43 SDS-Polyacrylamide gel electrophoresis (Laemmli, 1970)

A.1.43.1 Monomer (Acrylamide-bis-acryamide) stock solution

Acrylamide solution: 40 % stock solution:

0.8 % (30 % acrylamide:0.8 % bis = 39:1) (BDH Electran)

The solution was filtered through Whatman's paper (No 1) and stored in dark at 4° C.

A.1.43.2 Resolving gel buffer:

1.5 M Tris-Cl pH 8.8	18.17 g
Distilled H_2O	100 ml

A.1.43.3 Stacking gel buffer:

0.5 M Tris-HCl pH 6.8	6.06 g
Distilled H_2O	100 ml

A.1.43.4 Electrophoresis buffer (10X)

250 mM Tris base	15.15 g
192 mM Glycine	72.05 g
10 % SDS pH 8.0	350 µl
Distilled H_2O	5000 µl

The pH should be approximately 8.3

A.1.43.5 Sample treatment buffer:

125 mM Tris-Cl pH 6.8;

10 % (w/v) SDS;

1 % (v/v), ß-mercaptoethanol;

20 % (v/v) Glycerol;

0.002 % (w/v), Bromophenol blue.

A.1.43.6 Coomassie Brilliant Blue staining solution

Coomassie Blue R250 (0.25 %, w/v)	2.5 g
Destaining solution	1000 ml

The solution was stirred vigorously to dissolve the dye and then filtered through Whatman's paper (No 1).

A.1.43.7 Destaining solution

Glacial Acetic acid	250 ml
Methanol	750 ml
Distilled water	1500 ml

A.1.43.8 SDS-PAGE separating gel preparation

Stock	15 %	4 %
Monomer	9.0 ml	2.4 ml
Separating gel Buffer	4.6 ml	4.6 ml
Dist. H_2O	4.2 ml	10.8 ml
10 % (w/v) SDS	180 µl	180 µl
10 % (w/v) APS	90 µl	130 µl
TEMED	10 µl	10 µl

A.1.43.9 Stacking Gel (3.13 %)

Stock	
Monomer	1.02 ml
Stacking gel Buffer	2.5 ml
Distilled water	6.1 ml
10 % SDS	100 µl
10 % APS	50 µl
TEMED	10 µl

A.1.44 Plasmid preparation solutions (Maxi- and mini- Plasmid)

Three solutions are used in this method and are made up as follows:

A.1.44.1 Solution I

Stock solution /1000 ml	final conc.	
Tris-HCl pH 8 (1 M)	0.25 M	250 ml
Glucose (20 %, w/v)	0.50 M	455 ml
EDTA (0.5 M, pH 8 0)	0.10 M	200 ml
Water		95 ml

A.1.44.2 Solution II

Stock solution /1000 ml	**final conc.**	
NaOH (10 N)	0.2 N	20 ml
SDS (10 %, w/v)	1 %	10 ml
Distilled Water		970 ml

A.1.44.3 Solution III

Stock solution /500 ml	**final conc.**
Potassium acetate	3 M 300 ml of 5M K-acetate or 147.0 g
Acetic acid	57.5 ml

A.2 Media

A.2.1 Luria - Bertani medium (LB)

Bacto tryptone	16 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000 ml

For agar, add 15 g/l. Autoclave as usual.

A.2.2 Modified Nutrient Broth (MNB)

Tryptose	5 g
Glucose	5 g
Ammonium sulphate	2 g
Calcium chloride	0.01 g
Manganese sulphate	0.001 g
Magnesium sulphate	0.001 g
Iron sulphate	0.001 g
Zinc sulphate	0.001 g
Distilled water	1000 ml

Adjust pH to pH 7.0 using H_2SO_4 then Autoclave

A.2.3 SPY MEDIUM

Make SPY buffer first

$(NH_4)_2SO_4$	3 g
K_2HPO_4	14 g
KH_2PO_4	6 g
NaCitrate-H_2O	1 g
Yeast Extract	1 g
Distilled water	1000 ml

Autoclave as usual and before using add the following

$MgSO_4$-$7H_2O$ (MW = 246.48)	0.5 g
50 % Glucose stock solution	10 ml

A.2.4 2 X Yeast-tryptone broth/agar (2X YT).

Bacto tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	1000 ml

A.3 Media additives

Media were cooled to 50° C before addition of antibiotics, XGal and IPTG. Plates containing these additives were stored for no longer than one week at 4° C.

A.3.1 LB medium plates containing X-gal

40 µl X-gal (20 mg/ml in Dimethylformamide stored at -70° C) and 7 µl IPTG (23.8 mg/ml in H_2O) was spread onto the surface of LB + 100 mg/ml ampicillin (LB + 100 mg/ml ampicillin) plates, at least 30 min before use.

A.3.2 X-Gal (5-bromo-chloro-3-indolyl-B-galactoside)

XGal	0.2 g
DMSO	10 ml

The solution was stored at -70° C

A.3.3 IPTG (Isopropyl-ß-D-thio-galactopyranoside)

IPTG (100 mM)	23.4 mg
Distilled water	1.0 ml

The solution was stored in aliquot at -70° C.

APPENDIX B

B.1 BACTERIAL/PLASMID STRAINS, GENOTYPES, AND REFERENCES

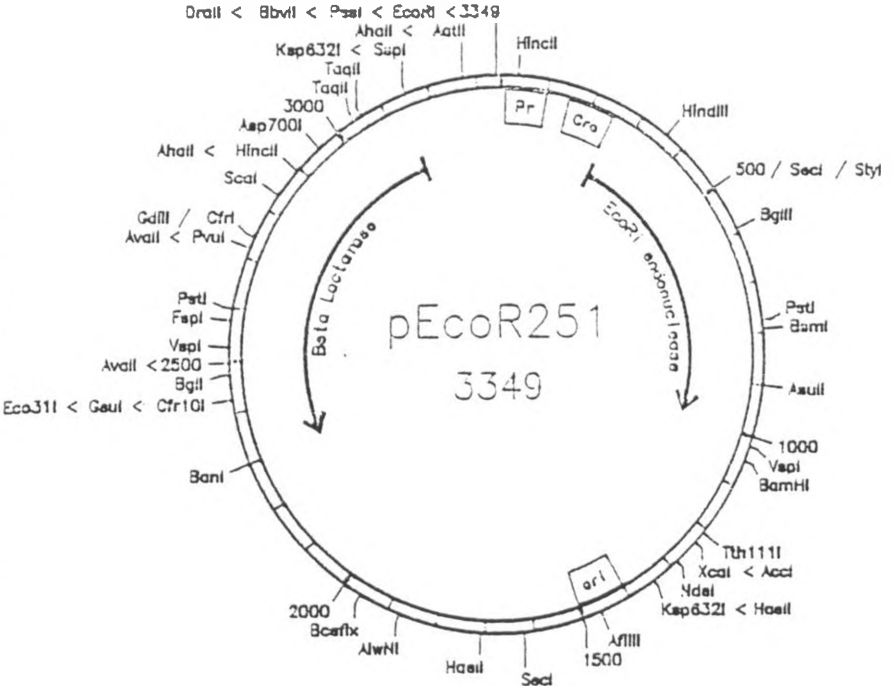
Strains	Relevant characteristics (genotypes)	References
E. coli LK111	lacI^q lacZ m15 lacY$^+$ F- thi1 thr$^-$1 leuB6 lacY1 tonA21 supE44 lambda- r_k^- m_k^+ lacI^q lacZ M15 recA$^+$ (K514 derivative)	Zabeau and Stanley (1982)
E coli K514	lacY$^+$ suppE44 r_k^-, m_k^+ tonA21 thr$^-$1 leuB6	
E. coli JM103	(lac pro) thi strA supE hsdR$^-$ F'tra36, endA sbcB proAB lacI	Messing *et al.*, 1981
E. coli JM105	(lac pro) thi strA supE	Yanisch-Perron *et al.*, 1985
JM109	endA sbcB hsdR F'tra36 proAB lacI	

E. coli LK111, JM105 and JM109 thus have ß-galactosidase activity only when transformed with a plasmid carrying the lac Z' gene, which is able to complement the enzymatically inactive lacZDM15 gene product. Such plasmids (pBluescriptSK$^+$) are usually constructed so that cloning allows the the inactivation of the lacZ' gene, resulting in blue/white selection of colonies without/with inserts on plates containing X-gal. This strain was maintained on LB plates/stabs at 4° C, and grown preparatively at 37° C in LB medium.

APPENDIX C

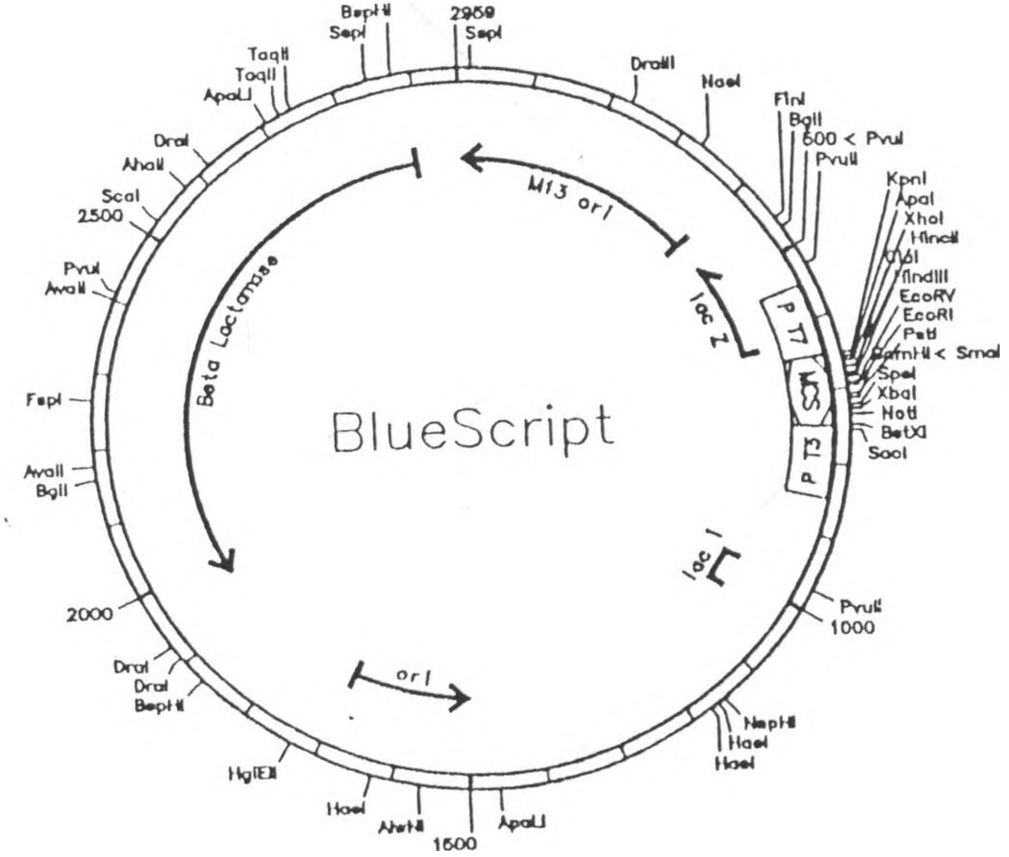
C.1 ONE- AND THREE-LETTER CODES USED FOR AMINO ACIDS

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



PLASMIDMAP of: pEcoR251.Seq check: 6248 from: 1 to: 3349

Zappe February 23, 1990 15:30



Reference: Stratagene, San Diego, California

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