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**Expression and genetic manipulation of *Bacillus thuringiensis* toxins for improved toxicity and development of a protocol for in vivo selection of toxin variants with improved activity**

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**SUBMITTED FOR THE AWARD OF DEGREE OF DOCTOR OF  
PHILOSOPHY**

**DEPARTMENT OF BIOCHEMISTRY  
SCHOOL OF LIFE SCIENCES  
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## **Declaration**

WORK NOT SUBMITTED ELSEWHERE FOR EXAMINATION

I hereby declare that this thesis has not been submitted in whole or in part to this or any other University for the award of a degree.

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Zenas Okon George

## Abstract

*Bacillus thuringiensis* (*Bt*) and its insecticidal toxins have been used in pest control for decades but there is a great concern about its future as a successful pest control agent due to the development of insect resistance and the narrow spectrum of activity of the toxins. To ensure the continuous relevance of *Bt* toxins in pest control, projects aimed at isolating novel *Bt* strains expressing toxins with improved activity are vigorously pursued and the genetic manipulation of existing toxins to improve their activity and overcome resistance is also undertaken. The aim of this project was to genetically manipulate the genes encoding Cry1Ah and Cry1Ie for improved activity aimed at countering resistance evolved by populations of *Plutella xylostella*. The project was also aimed at expressing *cry30Ea* and *cry40Da* genes cloned from highly mosquitocidal *Bt* strains S2160-1 and S2196 respectively and at developing a protocol for the in vivo selection of toxin variants with improved activity. Cry1Ah was successfully expressed in *E. coli* JM109 under the control of a *cry1Ac* promoter and ribosome binding site and in *Bt* IPS/78/11 under the control of the *cyt1Aa* promoter while Cry1Ie was also expressed in *E. coli* JM109. The expressed Cry1Ah and Cry1Ie toxins were found to be toxic to both susceptible (G88) and Cry1A resisitant (KARAK) populations of *Plutella xylostella* though there was significant cross resistance to Cry1Ah in KARAK. A genetically manipulated hybrid toxin CryAIA aimed at creating a novel toxin that captures the relatively broad spectrum of Cry1Ah but overcoming KARAK resistance was expressed but found to be non-toxic. Attempts to express *cry30Ea* and *cry40Da* were also not successful despite utilising different hosts and expression vector systems that have successfully been used in expressing other *cry* genes. Meanwhile, the strategy designed to enrich for more toxic *Bt* strains in vivo in from a mixed treatment in fact found that the non-toxic R128M strain dominated the toxic 431 strain.

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### **Dedication**

I dedicate this project to Almighty God who has been immeasurably merciful to me and the family He has given me.

## List of abbreviations

BBMV-----	Brush Border Membrane Vesicle
bp-----	base pairs
BSA-----	Bovine Serum Albumin
<i>Bt</i> -----	<i>Bacillus thuringiensis</i>
°C-----	Degrees Celsius
Cry-----	Crystal
Cyt-----	Cytolytic
kDa-----	kilo Dalton
DNA-----	Deoxyribonucleic Acid
DTT-----	Dithiothreitol
<i>E. coli</i> -----	<i>Escherichia coli</i>
g-----	gram
IPTG-----	Isopropyl -D-1-thiogalactopyranoside
L-----	Litre
LB-----	Luria Broth
LC <sub>50</sub> -----	Fifty percent Lethal Concentration
PAGE-----	Polyacrylamide Gel Electrophoresis
PCR-----	Polymerase Chain Reaction
SDS-----	Sodium Dodecyl sulphate
tRNA-----	transfer Ribonucleic Acid

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## **Chapter 1: Literature review, objectives and background to project**

### **1.1 Literature review**

#### **1.1.1 *Bacillus thuringiensis***

##### **1.1.1.1 Discovery and habitat**

*Bacillus thuringiensis* (*Bt*) was originally discovered in 1902 by a Japanese biologist Shigetane Ishiwatari who isolated it from diseased silkworm, *Bombyx mori*, but it was formally characterised in 1915 by Ernst Berliner of Germany who isolated it from diseased larva of *Ephestia kuhniella* (flour moth caterpillars) in Thuringia province and linked it to the cause of a disease called Schlaffsucht (Milner 1994). *Bacillus thuringiensis* is a Gram positive spore forming bacteria grouped into the *Bacillus cereus* group of *Bacilli* which produces proteinaceous insecticidal crystals during sporulation which is the distinctive feature between it and other members of the *Bacillus cereus* group (Read *et al.*, 2003, Rasko *et al.*, 2005).

The habitat of *Bacillus thuringiensis* is not distinct but ubiquitous as the bacteria has been isolated from soil (Hernández-Rodríguez and Ferré, 2009, Muleta *et al.*, 2009, Patel *et al.*, 2009, Saadaoui *et al.*, 2009, Vidal-Quist *et al.*, 2009, Baig *et al.*, 2010, Bozla an *et al.*, 2010, Gobatto *et al.*, 2010), faecal material of herbivorous animals (Maheswaran *et al.*, 2010), phylloplanes (Bizzarri and Bishop, 2008, Tilquin *et al.*, 2008, Noda *et al.*, 2009, Zhang *et al.*, 2009, Zhang *et al.*, 2010), food material (De Santis *et al.*, 2008, Ankolekar *et al.*, 2009), rhizosphere (Muleta *et al.*, 2009), dust (Hernández-Rodríguez and Ferré, 2009), insects (Gobatto *et al.*, 2010) and sea sediment (Baig and Mehnaz, 2010).

##### **1.1.1.2 Growth and response to biotic and abiotic factors**

*Bacillus thuringiensis* readily proliferates when environmental conditions like temperature and nutrients availability are favourable whilst the formation of spores have been shown to be triggered by internal and external factors including signals for nutrient starvation, cell density, and cell cycle progression (Hilbert and Piggot, 2004). The life cycle of *Bt* can be divided for convenience into phases and these are Phase I: vegetative growth; Phase II: transition to sporulation; Phase III: sporulation; and Phase IV: spore maturation and cell lysis (Hilbert and Piggot, 2004, Berbert-Molina *et al.*, 2008). Though strains of *Bacillus thuringiensis* have been isolated from numerous sources, its vegetative

proliferation in these samples is rather unclear as the most prevalent form isolated from most of these sources are dormant spores (Ankolekar *et al.*, 2009, Muleta *et al.*, 2009, Vidal-Quist *et al.*, 2009, Baig *et al.*, 2010, Baig and Mehnaz, 2010, Bozla an *et al.*, 2010, Gobatto *et al.*, 2010, Lietze *et al.*, 2010).

It has been shown that *Bt* is a pathogen in its own right and that its primary means of reproduction is in insect cadavers (Raymond *et al.*, 2010). Outside its primary means of reproduction (insect cadavers) *Bacillus thuringiensis* has been shown to be a poor competitor with other bacteria. Bizzarri and Bishop, (2008), demonstrated that *Bt* strains isolated in the vegetative form from the phylloplane of clover, 2810-S-4, and a laboratory strain, HD-1, were able to colonize clover to a density of about 1000 CFU/g of leaf when seeds were sown in sterile soil but only to a density of about 300 CFU/g of leaf in nonsterile soil showing about two-third reduction in population density. In another example, *Pseudomonas fluorescens* had the most significant antagonistic effect on the proliferation of *Bacillus thuringiensis* among seven other native soil bacterial isolates out of ten that showed various antagonistic effect on *Bt* (Rojas-Ruíz *et al.*, 2010). Jarosz, 1970 demonstrated that gut bacteria of *Galleria mellonella* larva, mainly dominated by *Streptococcus faecalis*, suppressed the proliferation of bacteria ingested with food (including *Bacillus thuringiensis*) through the production of bacteriocin. Takatsuka and Kunimi, 2000 showed that *Bacillus thuringiensis* was only able to proliferate in the gut of oriental tea tortrix, *Homona magnanima* Diakonoff when reared aseptically but fail to proliferate in normally reared larval cadavers. Raymond *et al.*, 2008b demonstrated that *Bacillus thuringiensis* showed an increased growth in the larva of *Plutella xylostella* when its groups member *Bacillus cereus* was present and further showed that the antibiotic producing *Bacillus cereus* strains suppressed the growth of other *Plutella xylostella* gut flora.

#### **1.1.1.3 Virulence and reproduction in insect host**

*Bacillus thuringiensis* has been tagged ‘an impotent pathogen’ because of its opportunistic pathogen behaviour under appropriate conditions based on the fact that *Bt* strains have been isolated from environments where their target insect are not normally associated with (Raymond *et al.*, 2010) and the supposed need for gut flora in *Bt* toxicity (Broderick *et al.*, 2009). Nevertheless,



*Bt* is well equipped as a pathogen that is capable to single handedly bypass the barriers posed by insects against bacterial infection (Ha *et al.*, 2005a, Ha *et al.*, 2005b, Vallet-Gely *et al.*, 2008). It has been shown that *Bt* produces virulence factors like phospholipases C (Palvannan and Boopathy, 2005, Martin *et al.*, 2010b), proteases (Hajaij-Ellouze *et al.*, 2006, Brar *et al.*, 2009, Infante *et al.*, 2010) and hemolysins (Gominet *et al.*, 2001, Nisnevitch *et al.*, 2010). The virulence factors listed above are controlled by the pleiotropic regulator PlcR and it has been demonstrated that cytotoxicity of *Bt* is PlcR dependent (Ramaraio and Lereclus, 2006). Deletion of the *plcR* gene has been shown to result in a drastic reduction in the virulence of *Bt* in orally infected insects (Salamitou *et al.*, 2000). The production of virulence factors by *Bt* is necessary but not enough for *Bt* to be called a pathogen (Fedhila *et al.*, 2003) but its production of proteins that have been proved beyond doubt to be independently insecticidal justifies it's name as an insect pathogen (Frankenhuyzen, 2009).

*Bacillus thuringiensis* exerts its toxicity through a multistep process that involves the solubilisation of *Bt* insecticidal crystal toxin in the insect midgut to release protoxins leading to the N-terminal and C-terminal cleavage of the protoxin by midgut proteases yielding an activated protease resistant core of about 60-70kDa (Bravo *et al.*, 2007). The activated toxin now bind to specific receptors which induces toxin insertion into the midgut epithelium columnar cells (Gomez *et al.*, 2007). Toxin insertion leads to the formation of lytic pores in microvilli of apical membranes (Bravo *et al.*, 2007). Subsequently, cells lyse and disruption of the midgut epithelium releases the cell content providing *Bacillus thuringiensis* spores a germinating medium leading to a severe septicaemia and insect death (de Maagd *et al.*, 2001, Bravo *et al.*, 2007).

### **1.1.2 *Bacillus thuringiensis* insecticidal toxins**

#### **1.1.2.1 Production, types and classification**

The life cycle of *Bt* has been shown to proceed through Phase I: vegetative growth; Phase II: transition to sporulation; Phase III: sporulation; and Phase IV: spore maturation and cell lysis (Hilbert and Piggot, 2004, Berbert-Molina *et al.*, 2008) and the production of insecticidal proteins deposited in crystals in the mother cell have been shown to mainly start from the onset of sporulation (Sedlak *et al.*, 2000, Xia *et al.*, 2005, Guidelli-Thuler *et al.*, 2009, Pérez-García

*et al.*, 2010). *cry*-genes have been shown to be transcribed from two overlapping promoters Btl and BtII by RNA polymerase that contain sporulation dependent sigma factors  $\sigma^E$  and  $\sigma^K$  (Sedlak *et al.*, 2000, Hilbert and Piggot, 2004) and a mutation in the consensus region of  $\sigma^E$  has been shown to inhibit transcription from Btl and BtII promoters (Sedlak *et al.*, 2000). It has also been shown that some *Bt* insecticidal proteins are produced and secreted into the culture medium during vegetative growth (Estruch *et al.*, 1996, Donovan *et al.*, 2001, Shi *et al.*, 2004, Bhalla *et al.*, 2005, Leuber *et al.*, 2006, Milne *et al.*, 2008, Singh *et al.*, 2010a, Abdelkefi-Mesrati *et al.*, 2011).

It has been shown that in some strains of *Bt*, the crystal proteins are localised on the surface of spores of mother cells (Wojciechowska *et al.*, 1999) while others are released from the mother cell spores after sporulation. Du and Nickerson, 1996 demonstrated that purified spores from a strain producing Cry1Ac toxin was able to cross-react with antibodies raised using a 65kDa protease activated core protein of Cry1Ac. Purified spores from crystal producing strains of *Bacillus thuringiensis* have been found to be lethal to insect pest though at a lower degree compared to the lethality of the crystal protein (Johnson and McGaughey, 1996, Tang *et al.*, 1996, Johnson *et al.*, 1998).

The insecticidal proteins in the crystalline bodies produced during sporulation have been shown to contain two types of insecticidal proteins namely Cry toxins and Cyt-toxins and there are between one to five Cry toxins produced and packaged into a single crystal or multiple crystals by a *Bt* strain (de Maagd *et al.*, 2001). The Cry toxins acquired the mnemonic Cry from the fact that they are found in the crystal while the Cyt-toxins acquired the mnemonic Cyt because of their in vitro cytolytic activity (Crickmore *et al.*, 1998).

Schnepf and Whiteley, 1981, confirmed that the insecticidal ability of *Bt* is as a result of the proteins that it produces by cloning and heterologously expressing the first toxin gene in *E. coli* which showed insecticidal activity to *Manduca sexta* just as the wild type *Bacillus thuringiensis* var. kurstali HD-1 that it was cloned from did. Since this discovery, a great number of other genes have been cloned and expressed and the process of *Bt* toxin gene discovery is still ongoing. In order to differentiate between one *Bt* insecticidal gene and the other, the discoverers of the genes gave them arbitrary names like 4.5, 5.3 and 6.6-kb-class genes (Kronstad and Whiteley, 1986), *bta* gene (Sanchis *et al.*, 1989), *cry* gene

(Donovan *et al.*, 1988) and Type A and Type B (Hofte *et al.*, 1988) among others. With a steady growth in the number of cloned and characterised novel insecticidal genes coming through, an attempt was made to organise the ever growing data. The first attempt to produce an organised systematic nomenclature of *Bt* insecticidal genes was dependent on the insecticidal activity of the protein they code for to assign a primary rank to the gene and with this system, genes that encode proteins toxic to lepidopteran insects were called *cryI* genes, while lepidopteran and dipteran protein genes were called *cryII* genes, *cryIII* genes were those ones that encoded proteins toxic to coleopterans and *cryIV* genes encoded proteins toxic to dipterans alone (Hofte and Whiteley, 1989). Though this system provided a framework for naming newly cloned novel toxins, it was short of a robust system of nomenclature that is able to accommodate new genes without ambiguity. The discovery of wild type gene like *cryIB* that code for toxins that are toxic to both lepidoptera and coleoptera (Bradley *et al.*, 1995) throw the system off balance as it did not have room to accommodate a toxin with such spectrum of activity. Also, toxins like CryIC that had toxicity to both dipterans and lepidopterans (Smith and Ellar, 1994) did not have a place in the (Hofte and Whiteley, 1989) system of nomenclature.

With the difficulty of accommodating newly discovered genes in the (Hofte and Whiteley, 1989) nomenclature system arising, there was a need to come up with a robust system of nomenclature and Crickmore *et al.*, 1998 came up with a system that is based on sequence similarity rather than function based. In the Crickmore *et al.* system, the mnemonic root was combined with a series of numerals and letters assigned in a hierarchical fashion to indicate degrees of phylogenetic divergence which was estimated by phylogenetic tree algorithms. The mnemonic Cyt was used for parasporal crystal proteins from *Bacillus thuringiensis* that exhibits hemolytic activity or any protein that has obvious sequence similarity to a known Cyt protein and mnemonic Cry was assigned to a parasporal crystal proteins from *Bacillus thuringiensis* that exhibits some experimentally verifiable toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein. With this system for naming Cry and Cyt proteins widely accepted, it has also been adopted for the naming of the vegetatively produced *Bacillus thuringiensis* toxins and this family of proteins has been given the mnemonic Vip. A website which hosts all the *cry*,

*cyt* and *vip* cloned genes has been established and is frequently updated as new genes are discovered.

#### 1.1.2.2 Structure of insecticidal toxins

The 3-D crystal structure of Cry-proteins including coleopteran specific Cry8Ea1 (Guo *et al.*, 2009), Cry3Aa (Li *et al.*, 1991) and Cry3Bb (Galitsky *et al.*, 2001), dipteran specific Cry4Aa (Boonserm *et al.*, 2006) and Cry4Ba (Boonserm *et al.*, 2005), lepidopteran specific Cry1Aa (Grochulski *et al.*, 1995), lepidopteran/dipteran specific Cry2Aa (Morse *et al.*, 2001) have been resolved through X-ray crystallographic methods of their activated forms. Also, the 3-D structure of Cyt-proteins have been resolved including activated Cyt2Ba (Cohen *et al.*, 2008) and unprocessed Cyt2Aa (Li *et al.*, 1996). Figure 1.1 is the crystal structure of Cry8Ea1 (Guo *et al.*, 2009) showing the three domain organisation typical of all resolved 3-D structures of Cry toxins while figure 1.2 is the 3-D structure of Cyt2Ba which shows overall similarity to 3-D structure of Cyt2Aa that had previously been resolved.

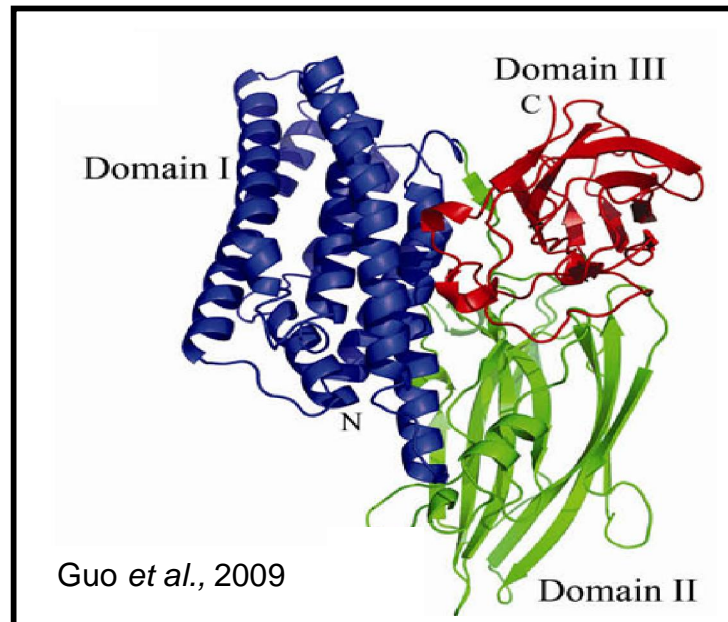
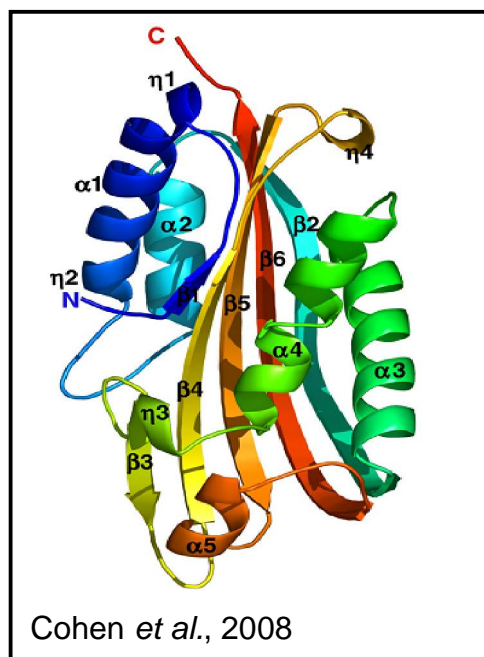


Figure 1.1: 3-D crystal structure of Cry8Ea determined at 2.20 Å (PDB ccode: 3EB7). The three domains of the protein are represented with different colours with domain I coloured blue, domain II coloured green while domain III is coloured red



**Figure 1.2:** Crystal structure of a monomer of Cyt2Ba determined at 1.80 Å (PDB code: 2RCI). The 'rainbow' colouring scheme is used in colouring the molecule starting with blue at the N-terminal and ending with red at the C-terminal

Though different Cry toxins have been shown to have specific targets in their insecticidal activity, the overall 3-D fold of many of them has been shown to be the same (see figure 1.1) , comprising of three domains (de Maagd *et al.*, 2003). Domain I has been shown to compose of seven  $\alpha$ -helices in which the central helix- 5 is hydrophobic and is encircled by six other amphipathic helices. The helical domain I has been shown to share structural similarities with other pore forming bacterial toxins like cytolyticin A (Mueller *et al.*, 2009), diphtheria toxin and colicin A (Parker and Pattus, 1993). Each of the outer helices of domain I is known to be amphipathic in nature and most of the helices are longer than 30 in length (Pigott and Ellar, 2007). Domain II is made up of three antiparallel  $\beta$ -sheets packed together to form a  $\beta$ -prism with pseudo three-fold symmetry (Li *et al.*, 1991). Two of the sheets are composed of four strands in a Greek key motif and are solvent exposed (Boonserm *et al.*, 2006). The third sheet packs against domain I and is arranged in a Greek-key-like motif with three strands and a short alpha-helix (Pigott and Ellar, 2007). The structure of domain II has been compared to those of other  $\beta$ -prism proteins with carbohydrate-binding

properties (de Maagd *et al.*, 2003), including vitelline (Shimizu *et al.*, 1994) and *Maclura pomifera* agglutinin (Lee *et al.*, 1998) and it reveals a great topological similarity. Domain III has been shown to contain two antiparallel  $\beta$ -sheets that adopt a  $\beta$ -sandwich fold with the jelly roll topology (Boonserm *et al.*, 2006). Both sheets are composed of five strands, with the outer sheet facing the solvent and the inner sheet packing against domain II. Two long loops extend from one end of the domain and interact with domain I (Grochulski *et al.*, 1995). Domain III shows less structural variability than domain II, and the main differences are found in the lengths, orientations, and sequences of the loops (Boonserm *et al.*, 2005). Domain III has been compared to other carbohydrate-binding protein domains and it shows great degree of similarity (de Maagd *et al.*, 2003) and similarity of domain III was also found with those of domain 4 of the pore-forming toxin aerolysin which is involved in maintenance and stability of the heptameric toxin complex (Lesieur *et al.*, 1999).

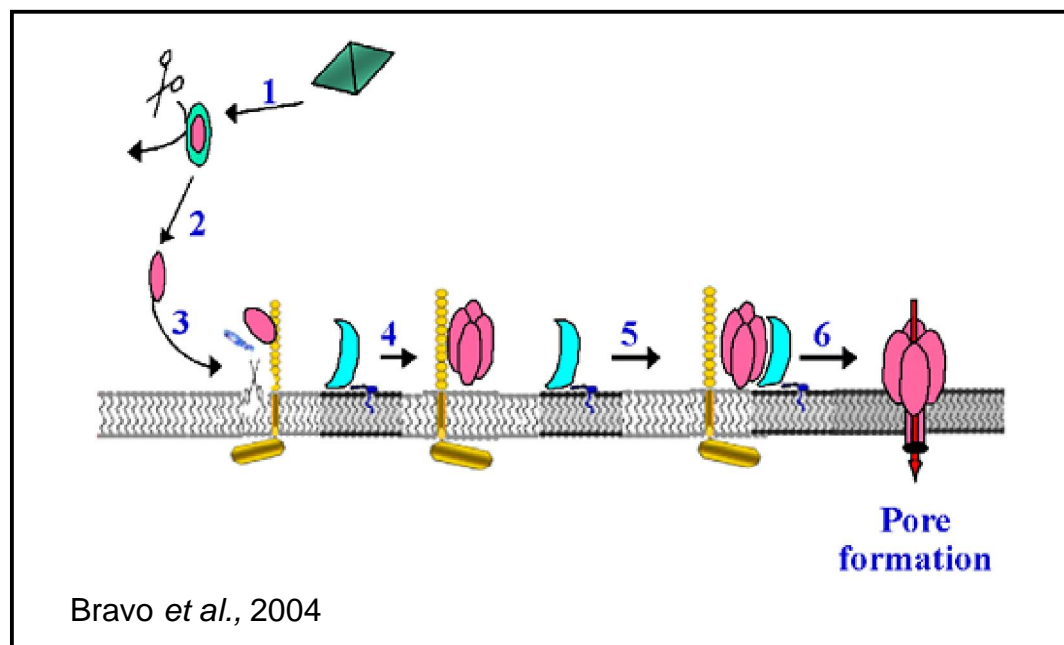
#### **1.1.2.3 Mechanisms of action of *Bacillus thuringiensis* toxins**

Proposed mechanisms of action of Cry and Cyt toxins from *Bt* include pore formation in which *Bt* toxins induce cell death by forming ionic pores following insertion into the membrane, causing osmotic lysis of midgut epithelial cells in their target insect (Knowles and Ellar, 1987, Haider and Ellar, 1989, Grochulski *et al.*, 1995, Schnepf *et al.*, 1998, Bravo *et al.*, 2004, Rausell *et al.*, 2004). Also, a relatively new mechanism of action of Cry toxins have been proposed which involves the activation of  $Mg^{2+}$ -dependent signal cascade pathway that is triggered by the interaction of the monomeric 3-domain Cry toxin with the primary receptor, the cadherin protein BT-R<sub>1</sub> (Zhang *et al.*, 2005, Zhang *et al.*, 2006, Soberón *et al.*, 2009). The triggering of the  $Mg^{2+}$ -dependent pathway has a knock-on effect and initiates a series of cytological events that include membrane blebbing, appearance of nuclear ghosts, and cell swelling followed by cell lysis (Zhang *et al.*, 2006). The  $Mg^{2+}$ -dependent signal cascade pathway activation by Cry toxins have been shown to be analogous to similar effect imposed by other pore forming toxins on their host cells when they are applied at subnanomolar concentration (Parker and Pattus, 1993, Nelson *et al.*, 1999, Menzies and Kourteva, 2000, Soberon *et al.*, 2009, Porta *et al.*, 2011).

Though the two mechanisms of action seem to differ, with series of downstream events following on from toxin binding to receptors on target cell membranes, they all agree to the fact that the toxins initially would have to be solubilised in an alkaline environment in vivo (Aronson *et al.*, 1991, de Maagd *et al.*, 2001, Soberon *et al.*, 2009) or in vitro (Lambert *et al.*, 1992, Bradley *et al.*, 1995, Zhang *et al.*, 2005, Zhang *et al.*, 2006) and activated by proteases before (Zhang *et al.*, 2005, Zhang *et al.*, 2006) or after binding (Gómez *et al.*, 2002, Bravo *et al.*, 2004, Jiménez-Juárez *et al.*, 2007, Soberon *et al.*, 2009) to receptors like cadherins. The midgut of lepidopteran and dipteran insects have been shown to be alkaline (Berebaum, 1980, Gringorten *et al.*, 1993) and it enhances the solubility of Cry toxins (Bravo *et al.*, 2004, Soberon *et al.*, 2009) and those of coleopteran are neutral or slightly acidic and in vitro solubilisation of Cry1Ba (Bradley *et al.*, 1995) and Cry7Aa (Lambert *et al.*, 1992) has been shown to enhance their activity to *Leptinotarsa decemlineata*.

With the pore forming model (Knowles and Ellar, 1987, Haider and Ellar, 1989, Grochulski *et al.*, 1995, Schnepf *et al.*, 1998, Bravo *et al.*, 2004, Rausell *et al.*, 2004), an ingested crystal toxin is solubilised in the alkaline environment of the insects midgut releasing protoxins which are initially processed by midgut proteases. The initial cleavage of the protoxin by the gut proteases results in the removal of the the C-terminal half and about 30 amino acid residues from the N-terminal thus releasing active toxin monomers which bind to receptors such as cadherin (Atsumi *et al.*, 2008, Bel *et al.*, 2009, Chen *et al.*, 2009, Fabrick *et al.*, 2009a, Muñoz-Garay *et al.*, 2009, Obata *et al.*, 2009, Pacheco *et al.*, 2009a, Arenas *et al.*, 2010) or proteins anchored to the membrane by GPI-anchored proteins such as aminopeptidase N (Arenas *et al.*, 2010). The initial binding of the activated toxins to receptors results in their conformational changes which facilitates a second cleavage that removes the N-terminal helix -1, by membrane-bound protease. The removal of helix -1 results in the formation of oligomers that are the membrane insertion competent (Bravo *et al.*, 2004). The binding of Cry toxins to the cadherin-like receptors have been shown to involve specific interactions of the variable loop regions in domain II and III with cadherin epitopes (Nair *et al.*, 2008, Chen *et al.*, 2009, Pacheco *et al.*, 2009a, Soberon *et al.*, 2009).

The oligomerised activated toxin that is bound to membrane receptors then insert the central hydrophobic helix -4 and 5 (Nair *et al.*, 2008) into the apical membrane of midgut cells causing osmotic shock, bursting of the midgut cells and finally ending in the insect death (Knowles and Ellar, 1987, Haider and Ellar, 1989, Grochulski *et al.*, 1995, Schnepf *et al.*, 1998, Bravo *et al.*, 2004, Rausell *et al.*, 2004). The pore formation model as proposed by Bravo *et al.*, 2004 for Cry1A toxins is presented in figure 1.3.



**Figure 1.3:** Model of the mode of action of Cry1A toxins

- 1: Crystal toxin solubilisation
- 2: Initial cleavage by gut proteases
- 3: Active toxin monomer binding to receptors and second cleavage by membrane bound proteases
- 4: Membrane insertional competent oligomer formation
- 5: Binding of oligomeric toxin to receptors
- 6: Lytic pore formation



Cyt-toxins have also been shown to effect killing of its insect targets through unspecific binding to midgut membrane lipids followed by membrane insertion which leads to pore formation and insect death (Li *et al.*, 1996, Cohen *et al.*, 2008, Zhao *et al.*, 2009, Rodriguez-Almazan *et al.*, 2011).

The activation of  $Mg^{2+}$ -dependent signal cascade pathway that is triggered by the interaction of the monomeric 3-domain Cry toxin with the primary receptor, the cadherin protein (Zhang *et al.*, 2005, Zhang *et al.*, 2006, Soberón *et al.*, 2009) has been shown to trigger a pathway involving stimulation of the stimulatory G protein  $\alpha$ -subunit and adenylyl cyclase (AC), increased cyclic adenosine monophosphate (cAMP) levels, and activation of protein kinase A (PKA). Activation of the AC/PKA signalling pathway initiates a series of cytological events that include membrane blebbing, appearance of nuclear ghosts, and cell swelling followed by cell lysis (Zhang *et al.*, 2005, Zhang *et al.*, 2006).

#### **1.1.2.4 Toxicity to insect pests and application as biological control agent**

With specific insecticidal effect of *Bt* toxins to insect pests in the orders Coleoptera (beetles and weevils) (López-Pazos *et al.*, 2010, Sharma *et al.*, 2010), Diptera (flies and mosquitoes) (Pérez *et al.*, 2007, Roh *et al.*, 2010), Hymenoptera (bees and wasps) (Garcia-Robles *et al.*, 2001, Sharma *et al.*, 2008), Lepidoptera (butterflies and moths) (Baig *et al.*, 2010, Darsi *et al.*, 2010) and non-insect species such as nematodes (Cappello *et al.*, 2006, Hu *et al.*, 2010), *Bt* toxins have taken centre stage as the major biological control agent because genetically modified crops expressing *Bt* toxins and insecticides formulated from *Bt* products are widely used and accepted. As published in the International Service for the Acquisition of Agri-biotech Applications (ISAAA), website (<http://www.isaaa.org/>), there is an increase to more than 1 billion hectares of accumulated GM crops between 1996 that they were commercialised to 2010 that the findings was published.

The increased popularity of biological control agents over synthetic chemicals because of the unselective lethal effect of chemical insecticidal agents (Moser and Obrycki, 2009, Kristoff *et al.*, 2010, Shah and Iqbal, 2010, Eriksson and Wikteliuss, 2011, Stevens *et al.*, 2011) and the development of resistance

(Ahmad *et al.*, 2008) has led to intense research in the isolation of new *Bt* strains, cloning of novel insecticidal protein genes and manipulation of existing ones to cope with emerging concerns on the future of *Bt* as biological control agent like the development of resistance (Pereira *et al.*, 2008, Gong *et al.*, 2010) or cross-resistance (Sayyed *et al.*, 2008, Xu *et al.*, 2010) and its narrow spectrum of activity (Kao *et al.*, 2003, Shu *et al.*, 2009).

Currently, about 600 insecticidal genes have been cloned from various *Bt* strains and deposited at the *Bt* toxin nomenclature website ([http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/)) and out of these, a greater number have been heterologously expressed and found to be either independently (Song *et al.*, 2003, Gonzalez-Cabrera *et al.*, 2006, Ibargutxi *et al.*, 2008, Xue *et al.*, 2008, Hu *et al.*, 2010) or in combination (Sharma *et al.*, 2010) toxic to insect species in one or more orders.

*Bacillus thuringiensis* and its insecticidal toxins have been used directly in the form of sprays (Ali *et al.*, 2010) or transgenic plants (Barton *et al.*, 1987, Vaeck *et al.*, 1987, Qaim and Zilberman, 2003, Walter *et al.*, 2010, Chen *et al.*, 2011) or indirectly (Hutchison *et al.*, 2010) as pesticidal agents to improve yields of agricultural crops.

Various assessments have been carried out to check for the safety of *Bt* toxins from sprays or transgenic plants to non-target species in the environment and it has been shown to be mostly environmentally friendly without known adverse effects (Kapur *et al.*, 2010, Walter *et al.*, 2010, Chen *et al.*, 2011, Randhawa *et al.*, 2011) though there is a laboratory observation that seem to implicate a commercial *Bt* strain *aizawai* in the reduction of reproduction in bumblebee (*Bombus terrestris*) workers when applied at a concentration of 0.1% through sugar water and pollen (Mommaerts *et al.*, 2010).

#### **1.1.2.5 Development of resistance and cross-resistance by insect pests to toxins**

The continuous relevance of *Bt* toxins in the control of insect and non-insect pests is threatened by the development of resistance by the pests in the field (Sayyed *et al.*, 2004) and laboratory reared populations (Pereira *et al.*, 2008, Fabrick *et al.*, 2009b). There have also been reports of insect populations resistant to a particular toxin showing resistance to other toxins that they have

not previously been exposed to a term known as 'cross-resistance' (Pereira *et al.*, 2008, Sayyed *et al.*, 2008, Gong *et al.*, 2010, Xu *et al.*, 2010).

The development of resistance to a toxins in the Cry1A group is of great economic importance because most commercialised *Bt* based insecticides contains *Bt* strains that naturally expressed Cry1A toxins or are engineered to do so (Ahmedani *et al.*, 2008).

There have been a number of proposed modes of resistance of insect pests to *Bt* toxins including reduction of binding of toxins to receptors in the midgut of insects, reduced solubilisation of protoxin, alteration of proteolytic processing of protoxins and toxin degradation and or precipitation by proteases (Bruce *et al.*, 2007). The understanding of the mechanism of action of *Bt* toxins (Knowles and Ellar, 1987, Haider and Ellar, 1989, Grochulski *et al.*, 1995, Schnepf *et al.*, 1998, Bravo *et al.*, 2004, Rausell *et al.*, 2004) have enhanced the experimental verification of some of the modes.

The most studied and experimentally verified mode of resistance is 'mode 1' which is characterized by recessive inheritance, resistance to and reduced binding by at least one Cry1A toxin, and negligible cross-resistance to Cry1C (Tabashnik *et al.*, 1998, Sayyed *et al.*, 2004, Heckel *et al.*, 2007). Cry1Ac and Cry1Ab toxins were shown to have a specific binding of 8 and 15% respectively to brush border membrane vesicles from a Cry1A toxin sensitive population of *Plutella xylostella* (LAB-UK) but these specific bindings were reduced to 0.6 and 1% respectively with brush border membrane vesicles prepared from a field population of *Plutella xylostella* (Karak) that has developed resistance to Cry1Ac toxin (Sayyed *et al.*, 2004). In another example of *Plutella xylostella* development of resistance to *Bt* toxins through reduced binding of toxins to receptors on its brush border membrane vesicles, Tabashnik *et al.*, 1994 demonstrated that a Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja resistant population of *Plutella xylostella* (NO-QA) showed a reduced binding to Cry1Ac. Kranthi, *et al.*, 2006 demonstrated that a Cry1Ac sensitive strain of cotton bollworm, *Helicoverpa armigera* SUS-G was able to inherit resistance from Cry1Ac resistant strains RES-Bt and RES-Ac. Gonzalez-Cabrera *et al.*, 2003 showed that brush border membrane vesicles from Cry1Ac resistant pink bollworm (*Pectinophora gossypiella*) did not bind to Cry1Ab which has been shown to have a common binding site as Cry1Ac. There have been other

reports implicating alteration in midgut proteins that bind to Cry toxins mutating and resulting in reduced binding (Jurat-Fuentes and Adang, 2006, Wang *et al.*, 2007, Fabrick *et al.*, 2009a).

Alteration of protease profile in the midgut of Cry1Ac resistant *Helicoverpa armigera* affected the proteolytic processing of Cry1Ac resulting in the production of 95 and 68kDa toxin as opposed to the active 65kDa toxin produced by midgut protease from susceptible population (Rajagopal *et al.*, 2009) is an evidence of linkage between improper processing of *Bt* toxin and development of resistance. Sayyed *et al.*, 2005 demonstrated that a field collected resistant population of *Plutella xylostella* (SERD4) which was subsequently selected in the laboratory using Cry1Ab and named Cry1Ab-SEL were more sensitive to trypsin-activated Cry1Ab compared to Cry1Ab protoxins. The brush border membrane vesicles from laboratory selected population of *Ostrinia nubilalis* resistant to Cry1F was found to be unaffected in its binding to the toxin and no differences in activity of luminal gut proteases or altered proteolytic processing of the toxin were observed in the resistant strain compared to the sensitive strain (Pereira *et al.*, 2010). With failure to implicate binding proteins or toxin processing proteins in the midgut of resistant strains of insects as the source of resistance, the full understanding of the mechanism of insect resistance to Cry toxins still have much to be discovered in years ahead.

### **1.1.3 Strategies for improving toxicity and overcoming insect pests resistance to *Bt* toxins**

#### **1.1.3.1 Use of synergistic proteins, other toxins and spores**

Apart from resistance by pests being a major threat to the future of *Bt* spores and its insecticidal toxins as biological control agent, the problem of efficacy and spectrum of activity (Regev *et al.*, 1996, de Maagd *et al.*, 2001) are other problems that are associated with *Bt* toxins. Only a small minority of a single toxin show activity that spans two to three insect orders like Cry1Ba which shows toxicity to Coleopterans, Dipterans and Lepidopterans (Zhong *et al.*, 2000). Most of the toxins cloned have narrow spectrum of activity (Kao *et al.*, 2003, Shu *et al.*, 2009) while some expressed toxins like Cyt1Aa show a weak toxicity to Dipteran like mosquito on their own but show synergistic activity when combined with other toxins like Cry4Ba and Cry11Aa (Pérez *et al.*, 2007).

To boost the efficacy of *Bt* insecticidal toxins and overcome resistance posed by insect pests, the use of other proteins like cadherin fragments have been shown to be a successful strategy (Chen *et al.*, 2007, Abdullah *et al.*, 2009, Pacheco *et al.*, 2009b, Park *et al.*, 2009a, Peng *et al.*, 2010). The use of a toxic compound, gossypol derived from cotton plant has also been used in combination with Cry1Ac to boost its efficacy against resistant population of *Helicoverpa zea* (Anilkumar *et al.*, 2009). Coexpression of chitinase a protein that is known to disrupt chitin which is a physical barrier in the peritrophic membrane in the midgut of insects have been shown to have an enhanced effect on the efficacy of Cry1Ac against *Helicoverpa armigera* (Ding *et al.*, 2008) and Cry1C against *Spodoptera littoralis* (Regev *et al.*, 1996). Also, the combination of Cry toxins have proven to be a very useful strategy employed in boosting efficacy and fighting resistance and this is called gene stacking or pyramiding (Jurat-Fuentes *et al.*, 2003, Kaur, 2006, Avisar *et al.*, 2009). The combination of Cry1Ac and Cry2Ab showed a synergistic effect to *Helicoverpa armigera* (Ibargutxi *et al.*, 2008).

It has also been shown that a mixture of crystal protein and spores from the same strain in a bioassay results in a synergistic insecticidal activity (Johnson and McGaughey, 1996, Tang *et al.*, 1996, Johnson *et al.*, 1998).

#### **1.1.3.2 Domain and loop swapping**

With insights gained into the structure of Cry toxins (Li *et al.*, 1991, Grochulski *et al.*, 1995, Galitsky *et al.*, 2001, Morse *et al.*, 2001, Boonserm *et al.*, 2005, Boonserm *et al.*, 2006) and their mechanism of action, (Zhang *et al.*, 2005, Zhang *et al.*, 2006, Bravo *et al.*, 2007, Gomez *et al.*, 2007, Pacheco *et al.*, 2009a) through molecular genetics studies of the interaction between the insect host and the toxins, specificity of toxins can be altered or broadened. Herrero *et al.*, 2004 demonstrated that replacing single residues in loops 2 and 3 of domain II with and residues 541–544 in domain III of Cry1Ca with alanine resulted in lower toxicity to *Spodoptera exigua* while their toxicity to *Manduca sexta* was not affected which underscores the detailed understanding of the importance of these residues to insect specificity providing a great tool for toxin manipulation. Swapping domain III of Cry1Ac, Cry1Ba and Cry1Ea which has low or no

toxicity to *Spodoptera exigua* with domain III of Cry1Ca have shown an improved toxicity to *Spodoptera exigua* (de Maagd *et al*, 2000).

Abdullah *et al*, 2003 remodelled domain II loops of Cry4Ba to resemble that of Cry4Aa and generated mutants that show improved toxicity to *Culex quinquefasciatus* and *Culex pipiens* up to >700-fold and >285-fold respectively. In a similar study, Cry19Aa, a mosquitocidal toxin with specificity toward *Anopheles stephensi* and *Culex pipiens* but with no measurable activity against *Aedes aegypti*, was made more than 42,000-fold more toxic to *Aedes aegypti* by engineering putative domain II loops 1 and 2 to resemble that of Cry4Ba (Abdullah and Dean, 2004). Also, Liu and Dean 2006 introduced mosquitocidal activity to Cry1Aa through rational design to the sequence of loops 1 and 2 based on a sequence alignment with Cry4Ba, a naturally occurring mosquitocidal toxin.

Replacement of domain III of Cry1Ba with that of Cry1Ac resulted in an improved activity to *Heliothis virescens* (Karlova *et al.*, 2005). Naimov *et al*, 2001 created a hybrid protein by replacing domain II of Cry1Ba with that of Cry1I which resulted in activity against Colorado Potato Beetle that is comparable to that of Cry3Aa.

#### **1.1.3.3 Site directed mutagenesis**

This is the use of cDNA technology to generate a point mutation at a predetermined position in a gene (Cammack *et al*, 2006) and DNA sequence of interest like promoters (Fleming *et al.*, 2010, Moustafa *et al.*, 2010). To effect an informed site directed mutagenesis, the sequence information and the structure of the protein targeted must be known at least to a reasonable extent and Cry toxins DNA and structural information is well studied and understood to a reasonable extent (de Maagd *et al.*, 2001, de Maagd *et al.*, 2003).

The understanding of the domain structure and function of Cry1Ac enhanced the use of site directed mutagenesis by Kim *et al*, 2008 to effect changes to domain I and II that resulted in mutants that showed improve activity to *Ostrinia furnacalis* and *Plutella xylostella*.

A triple mutant (N372A, A282G and L283S) in domain II loop of Cry1Ab resulted in a 36-fold increase in toxicity to *Lymantria dispar* and this correlated with an increased binding affinity of greater than 18-fold to brush border membrane

vesicles which also resulted in higher toxin concentration at the binding site (Rajamohan *et al.*, 1996).

#### **1.1.3.4 Directed evolution**

Natural evolutionary trend has been used in analysing the divergence and host specificity in Cry toxins (de Maagd *et al.*, 2001) and biotechnological technique like gene shuffling has been used in artificially directing the evolution of new genes with novel characteristics (Stemmer, 1994a, Stemmer, 1994b, Zhao and Arnold, 1997, Lassner and Bedbrook, 2001, Craveiro *et al.*, 2010). Craveiro *et al.*, 2010 used DNA shuffling technique to produce four gene variants from *cry11A12synth* and *cry11A12* genes that has toxicity against *Telchin licus licus* as opposed to non toxic product from the parent gene *cry11A12synth*. Shan *et al.*, 2011 used modern directed evolution techniques of error-prone PCR, staggered extension process (StEP) shuffling combined with Red/ET homologous recombination to investigate the insecticidal activity of Cry1Ac and isolated a toxin variant designated as T524N which has increased insecticidal activity against *Spodoptera exigua* larvae while its original insecticidal activity against *Helicoverpa armigera* larvae was still retained.

#### **1.1.3.5 Production of truncated toxins**

It is well understood that a Cry toxin mode of action involves the solubilisation of protoxins in the midgut of susceptible insects, activation of the solubilised protoxins by midgut proteases resulting in a truncated active toxin that binds to receptors being inserted into epithelial membrane creating lytic pores (Knowles and Ellar, 1987, Haider and Ellar, 1989, Grochulski *et al.*, 1995, Schnepf *et al.*, 1998, Bravo *et al.*, 2004, Rausell *et al.*, 2004) or activating Mg<sup>2+</sup>-dependent signal cascade pathway that is triggered by the interaction of the monomeric 3-domain Cry toxin with the primary receptor, the cadherin protein (Zhang *et al.*, 2005, Zhang *et al.*, 2006, Soberón *et al.*, 2009).

The understanding of the regions that are bound to receptors and how protoxins are processed to a functional form has led to creation of manipulated toxins that mimics the in vivo processing of toxins like truncated toxins that lack part of N-terminal sequence (Pardo-López *et al.*, 2009). Bravo *et al.*, (2004) demonstrated that the processing of a protoxin to an active toxin in the midgut

of susceptible insect involve the initial cleavage of the protoxin by proteases in the midgut followed by a second cleavage by membrane bound protease that removes the helix -1 while the toxin is bound to midgut receptors. Deletion in the amino-terminal region including helix -1 of Cry1A toxin resulted in Cry toxin that form oligomers in the absence of cadherin receptor that kill insects which has developed resistance to Cry1A toxins caused by mutations in the cadherin gene. The modified toxins were also effective against insects which had acquired reduced susceptibility to native *Bt* toxins due to diminished expression of cadherin protein by cadherin gene silencing with RNA interference (Soberón *et al.*, 2007). Morse *et al*, 2001 resolved the structure of Cry2Aa protoxin and observed that it has 49-amino acid residues preceding helix -1 at the N-terminal which is cleaved in vivo to generate an active toxin. Mandal, *et al*, 2007 demonstrated that in vitro truncation of Cry2Aa at the N-terminal of 42-amino acid residues resulted in an improved toxicity against *Spodoptera littoralis* and *Agrotis ipsilon*.

Apart from truncation of the N-terminal residues of domain I to generate toxins with improved activity, Wu *et al*, 2000 also created mutants (R345A, Y350, Y351) that involve the deletion and site specific mutation in loop I of Cry3A domain II which resulted in improved activity against *Tenebrio molitor*.

## **1.2 Objectives**

### **1.2.1 Overall objective**

The overall aim of this project is to clone and heterologously express and characterise wild type *cry1Ah*, *cry1le*, *cry30Ea* and *cry40Da* genes, genetically manipulate them to create variants with improved toxicity and to develop a protocol that can be used in selecting the toxin variants with improved toxicity.

### **1.2.2 Specific objectives**

#### **1.2.2.1 Design of expression systems for *cry1Ah*, *cry1le*, *cry30Ea* and *cry40Da* genes**

Under this objective, suitable expression systems would be designed for the expression of the genes in a range of hosts. pET vectors will be used as well as existing vectors used previously to express *Bt* toxins in *E. coli* and *Bt*. An existing vector like pGEM1AcP which has origin of replication in *E. coli* and a



native promoter of *cry1Ac* with ribosome binding site to its 3' end of the promoter would be manipulated for use in expressing the genes. Another existing vector pSV2, a *Bt* shuttle vector with origin of replication in *E. coli* and *Bt* would be manipulated to carry native promoter of *cry1Ac* gene and its ribosome binding site and used for expression of some of the genes in *Bt* host. Also pSVP27 a *Bt* shuttle vector with pSV2 backbone carrying the promoter of *cyt1A* would also be manipulated for the expression of some the genes in *Bt* host.

#### **1.2.2.2 Assay the heterologously expressed toxins for insecticidal activity**

This objective seeks to estimate the toxicity of the expressed proteins to ascertain if they have any improvement in toxicity compared to existing ones. Cry toxin sensitive and Cry1Ac-resistant populations of *Plutella xylostella* in which a range of toxins have been assayed with would be used in checking the toxicity of the expressed proteins. This would give insights into the cross-resistance effect observed in other Cry toxins.

#### **1.2.2.3 Genetical manipulation of the expressed toxins for desired activity**

Under this objective, the understanding of the genes *cry1Ah*, *cry1Ie*, *cry30Ea* and *cry40Da* would be exploited in manipulating them for improved toxicity and the overcoming of resistance and cross-resistance. To this end, techniques like domain swapping, truncation of the wild type gene, creation of fusion genes would be employed in creating new genes.

#### **1.2.2.4 Using toxin pairs to conduct bioassay to check for synergism**

In this objective, sublethal dose of toxin pairs Cry1Ie/Cry1Ah and Cry1Ie/Cry1Ac would be used in conducting bioassays against *Plutella xylostella* to check for synergistic activities between them. This method has been used in combating resistance developed by *Aedes aegypti* to Cry11Aa by combining Cry11Aa with Cyt1Aa (Pérez *et al.*, 2007) and *Culex quinquefasciatus* resistance to Cry4Ba by combining it with Cyt1Aa (Cantón *et al.*, 2011). Also, enhanced activity has been observed between Cry1Ab and Cry1Ac against *Chilo partellus* (Sharma *et*

*al.*, 2010), Cry1Ac and Cry2Ab against *Helicoverpa armigera* (Ibargutxi *et al.*, 2008).

#### **1.2.2.5 Develop an in vivo protocol for enriching for *Bt* strains expressing more active toxin**

This objective seeks to find out if in vivo passage of *Bt* strains generated from a mutant library through *Plutella xylostella* would enrich for mutant strains expressing more active toxins.

### **1.3 Background to project**

The background to this project is based on the abilities and disabilities of the insect pathogen, *Bacillus thuringiensis* (Raymond *et al.*, 2010). *Bacillus thuringiensis* produces proteinaceous insecticidal crystals during sporulation which is the distinctive feature between it and other members of the *Bacillus cereus* group (Rasko *et al.*, 2005). The proteinaceous crystal has been successfully linked to extrachromosomal DNA material (plasmid) by Ward and Ellar, 1983 who produced a plasmid cured strain that was acrySTALLIFEROUS and non-toxic to *Aedes aegypti* larvae. Schnepf and Whitely 1981, demonstrated that the source of toxicity of *Bacillus thuringiensis* var. kurstali HD-1 to *Manduca sexta* was a plasmid gene that coded for a protein that was found in the proteinaceous crystal. They cloned and heterologously express the gene from extracted plasmid DNA in *E. coli* and showed that the *E. coli* expressed protein was toxic to *Manduca sexta*.

Since the discovery of the ability of *Bacillus thuringiensis* strains to selectively kill insect species in the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera and non-insect species such as nematodes it has been widely used as the major biological control agent and widely preferred to chemical insecticides.

The disability of some existing *Bacillus thuringiensis* toxins to overcome the resistance developed by some insect species in field (Sayyed *et al.*, 2004) and in laboratory reared populations (Pereira *et al.*, 2008, Fabrick *et al.*, 2009b) is eminent and increased research at the molecular level into the workings of the toxins has provided some insights into how some of the resistance traits could be managed (Soberón *et al.*, 2007, Bravo and Soberón, 2008, Pardo-López *et al.*, 2009).

The quest for new *Bacillus thuringiensis* strains with novel characteristics that are able to overcome resistance traits posed by insects in field and laboratory populations led to the isolation of a *Bt* strain *Bacillus thuringiensis* BT8 and cloning of the gene encoding *cry1Ah1* a 134kDa toxin from it by Xue, *et al*, 2008. The cloned *cry1Ah* was successfully expressed in an acrySTALLIFEROUS mutant of *Bacillus thuringiensis* HD73 and found to be highly toxic against Lepidopteran larvae of *Ostrinia furnicalis*, *Helicoverpa amigera*, *Chilo suppressalis* and *Plutella xylostella* with LC<sub>50</sub> values of 0.05, 1.48, 0.98µg/g and 1.52 µg/ml respectively. Unfortunately, a resistant strain of *Ostrinia furnacalis* (ACB-AbR) originally selected in the laboratory with Cry1Ab also developed resistance to other Cry1-toxins including Cry1Ah to which it had not previously been exposed. The cross-resistance level observed with Cry1Ah was 131-fold, Cry1Ac 36-fold and Cry1F 6-fold (Xu *et al*, 2010).

In another exploration, Cry1Ie which is a short form Cry toxin of approximately 81kDa was cloned from *Bacillus thuringiensis* isolate Btc007 and was heterologously expressed in *E. coli* BL21 (DE3) as a fusion protein using pET-21b vector. The *E. coli* expressed Cry1Ie was found to be toxic to *Ostrinia furnacalis*, with an LC<sub>50</sub> of 2.22µg/ml, *Plutella xylostella*, with an LC<sub>50</sub> of 0.20µg/ml and *Leguminivora glycinivorella*, with an LC<sub>50</sub> of 9.02µg/ml. It was found to be non-toxic to *Helicoverpa armigera* and *Spodoptera exigua* (Song *et al*, 2003).

Though Cry1Ie has a narrow spectrum of activity compared to Cry1Ah, it has an interesting aspect that could be exploited to create a new toxin with improved activity through molecular genetic techniques. It has been shown that a population of *Ostrinia furnacalis* (ACB-AbR) that has developed resistance to Cry1Ah as a result of its developing a resistance to Cry1Ab was still sensitive to Cry1Ie (Xu *et al*, 2010).

Cry1Ie belongs to the three domain Cry toxins but lacking C-terminal blocks 6-8 present in full length 130kDa cry-proteins like Cry1Ah. The C-terminal end of Cry toxins have been shown to be removed during proteolytic processing of inactive protoxins to an active form which initiates binding to brush border membrane of susceptible insect.

Still in the quest to isolate *Bt* strains with improved activity, two strains S2160-1 and S2196-1 were isolated from soil samples in Guangxi, China and found to

have activity to *Culex quinquefasciatus* and *Aedes Albopictus* comparable to that of *Bacillus thuringiensis* spp. *israelensis* which is a long standing mosquitocidal strain (Pérez *et al.*, 2007, Otieno-Ayayo *et al.*, 2008). The coding sequences of *cry30Ea* and *cry40Da* toxin genes were amplified using PCR from the isolated strains S2160-1 and S2196-1 respectively with deduced proteins of ~77.6kDa and ~73.5kDa respectively. However, SDS-PAGE analysis of the total protein from these strains suggested that these toxins were not expressed in the wild type strains (Zhang *et al.*, unpublished data). *cry30* genes have previously been cloned from mosquitocidal strains of *Bacillus thuringiensis* and heterologously expressed (Tan *et al.*, 2009, Zhu *et al.*, 2010). They have been found to form operons which splits into frames with the downstream frame having homology to C-terminal block 6-8 of full length *cry*-genes and the upstream frame having homology to block 1-5 of short *cry*-genes (Ito *et al.*, 2006). *cry40* gene has also been shown to form operon with *cry34* gene (Brown, 1993). It has been cloned from mosquitocidal strains *Bacillus thuringiensis* serovar *aizawai* (Ito *et al.*, unpublished data) obtained from the *Bacillus thuringiensis* nomenclature website ([http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/)) and *Bacillus thuringiensis* *thompsoni* (Brown, 1993).

## **Chapter 2: Materials and Methods**

### **2.1 Materials**

#### **2.1.1 Plasmids**

The plasmids used in this project were pSVP27 and pSV2 (Crickmore and Ellar, 1992) which are *Bt* shuttle vectors used in cloning genes in *E. coli* via their multiple cloning sites for expression in *Bt* as they have origin of replication in both *E. coli* and *Bt*. pSV2 has chloramphenicol resistance gene which is functional in *Bt* and ampicillin resistance gene which is functional in *E. coli*. pSVP27 in addition to the attributes of pSV2 also has *cyt1A* promoter which drives expression of genes inserted downstream of the promoter. Plasmid pGEM-T from Promega was also used in cloning of PCR products that are amplified using Taq DNA polymerase which adds adenine (A) to the 3' end of its PCR products. It has an origin of replication in *E. coli* and an ampicillin resistance gene. pGEM1AcP is another plasmid that was used in this project. It has in addition to the pGEM-T vector backbone, the promoter for *cry1Ac* including its ribosome binding site and coding sequence and was supplied by Neil Crickmore. Another plasmid that was used in this project that has the vector backbone of pGEM-T is p101 constructed by Vidisha Krishnan (unpublished data) by inserting the three gene *cry41Aa* operon and then deleting the first open reading frame. Also used was plasmid pET3a from Novagen which has origin of replication in *E. coli*, ampicillin resistance gene, T7 IPTG inducible promoter and multiple cloning sites.

#### **2.1.2 Bacterial strains**

The bacterial strains used for this project were *E. coli* JM109, *E. coli* BL21 DE3 supplied by Neil Crickmore and *E. coli* BL21 Rosetta from Promega which is chloramphenicol resistant due the pRARE plasmid that it harbours. pRARE plasmid is a plasmid that codes for tRNA genes that code for tRNAs that are rare in *E. coli* which enhances the heterologous expression of some non-*E. coli* genes in *E. coli* host. *E. coli* BL21 Rosetta was supplied by Jie Zhang of Institute of Plant Protection, Beijing, China.

Other bacterial strains used were acrySTALLiferous mutant of *Bacillus thuringiensis* sup. *israelensis* IPS 78/11, crystal producing wild type strain *Bacillus thuringiensis* HD73, acrySTALLiferous mutant of *Bacillus thuringiensis*

HD73<sup>-</sup>, 431 a genetically engineered *Bacillus thuringiensis* IPS 78/11 expressing wild type *cry1Ca* gene and R128M a genetically engineered *Bacillus thuringiensis* IPS 78/11 expressing mutated *cry1Ca* gene which were all supplied by Neil Crickmore.

### **2.1.3 Insect populations**

The insect populations used were of the species *Plutella xylostella* and were toxin sensitive population G88 which were maintained on either Chinese cabbage (*Brassica pekinensis*) or artificial diet and Cry1Ac-resistant population KARAK obtained from the University of Oxford, UK. KARAK was also maintained on Chinese cabbage (*Brassica pekinensis*)

### **2.1.4 Culture media and insect food**

The media used were 2x and 1x Luria broth and 1x Luria agar. Antibiotics concentrations were 50 or 100µg/ml for ampicillin, 5, 10, 17 or 20µg/ml for chloramphenicol and 50µg/ml for kanamycin.

The insect used in this project were fed with artificial diet or Chinese cabbage (*Brassica pekinensis*).

## **2.2 Methods**

### **2.2.1 Design of primers for DNA amplification**

PCR primers used in this project were designed using the programme PRIMER SELECT a part of DNASTAR software package. Some primers were designed to amplify exact sequence as found in the template while others were designed with changes when intending to introduce mutations. Primers were designed to have an optimum annealing temperature of between 50-60°C and this was done by altering the length of the primers.

### **2.2.2 PCR amplification of DNA**

Using appropriate primer pairs, Pfu Ultra<sup>TM</sup> Hotstart PCR master mix system or Taq PCR master mix or High Fidelity PCR master mix from Roche were used in the amplification of DNA fractions.

The reaction conditions for PCR amplification were set as follows:

Initial warming of lid 100°C for 4 minutes, initial denaturation 95°C for 2 minutes, annealing of primers 45°C or 50°C or 55°C or 60°C (depending on the primers' optimum annealing temperature) for 30 seconds, primer extension at 72°C for 1 minute per 1000bp of DNA to be amplified. The cycle from initial denaturation to primer extension was set to repeat for 29 cycles after which the sample was cooled to 4°C.

In table 2.1 below, a summary of the PCR reaction mix is shown.

Components	Volume
Distilled water	22.5µl
Forward primer	1µl of 100pmol/ µl
Reverse primer	1µl of 100pmol/ µl
DNA template	0.5µl
Pfu Ultra Hotstart 2x master mix or Taq PCR master mix or High Fidelity PCR master mix	25µl

Table 2.1: PCR master mix recipe

### 2.2.3 Purification of DNA from agarose gels

PCR reaction mixes were ran on 1% agarose gel and required bands excised and purified. The excised bands were placed in a clean microcentrifuge tube and purified according to the QIAquick Gel Extraction kit protocol. According to this protocol, three volume of buffer QG was added to one volume of excised gel in a microcentrifuge tube. The tubes were incubated for 10 minutes at 60°C with inversion every 3 minutes to enhance dissolution. After the incubation, one volume of isopropanol was added to one volume of gel and inverted to mix.

The mixes were transferred to MiniElute columns and centrifuged at 14,000xg for 1 minute. The flow through were discarded and 500µl of buffer QG were added to the columns and centrifuged at 14,000xg for 1 minute.

The flow through were discarded and 750µl of buffer PE were added to the columns and centrifuged for 1 minute at 14,000xg. The flow through were

discarded and the columns centrifuged again to remove any trace of buffer in the columns. The columns were then placed in clean 1.5ml centrifuge tubes and 10µl of buffer EB were added and allowed to stand for 1 minute after which they were centrifuged for 1 minute at 14,000xg to elute the DNA.

## **2.2.4 Transformation of bacterial strains with plasmids**

### **2.2.4.1 Transformation of *E. coli* strains**

*E. coli* JM109 were made competent for transformation by growing a loopful of its culture in 100ml of Luria broth for two hours at 37°C. The culture was centrifuged at 10k rpm in SLA-1500 rotor for 10 minutes to pellet cells. The pelleted cells were washed in 100ml of fridge-cold water and centrifuged at 10k rpm in SLA-1500 rotor for 10 minutes.

The cells were then suspended in 200µl of 4°C-distilled water. For *E. coli* JM109, 50µl of the cells were transferred into electroporation cuvette and 1µl of plasmid DNA was added, mixed and gently tapped to get the cells settled at the bottom of the cuvette. The cuvette was then placed in an electroporation machine and operated at the settings of 1.8 kV, 200 Ohms, 25µF for cells to take up the plasmid DNA. After electroporation, the cells were incubated in a shaker tank at 37°C in 1ml of Luria broth for 1 hour to resuscitate the cells. The 1ml culture was then centrifuged to get it concentrated by removing part of the supernatant and then plated onto Luria agar plate containing 100µg/ml of ampicillin.

For BL21 Rosetta, 1µl of plasmid DNA was added to 50µl of the ready-made competent cells and placed on ice for 45 minutes. The cells/plasmid mix was then placed in water bath at 42°C for 90 seconds and then placed on ice for 3 minutes. One millilitre of Luria broth was added to the cell/plasmid mix and incubated in water bath at 37°C for 1 hour. Five hundred microlitre of the transformation mix was spread plate on Luria agar plate containing 17µg/ml chloramphenicol and 50µg/ml of ampicillin.

### **2.2.4.2 Transformation of *Bt* strains**

*Bacillus thuringiensis* strains were made competent for transformation by growing a loopful of its culture in 100ml of Luria broth for four hours at 30°C. The culture was centrifuged at 10k rpm in SLA-1500 rotor for 10 minutes to get the cells pelleted. The pelleted cells were washed in 100ml of fridge-cold



electroporation buffer (272mM sucrose, 0.5mM MgCl<sub>2</sub>, 0.5mM K<sub>2</sub>HPO<sub>4</sub>, and 0.5mM KH<sub>2</sub>PO<sub>4</sub> pH7.2) (Peng *et al.*, 2009) and centrifuged at 10k rpm in SLA-1500 rotor for 10 minutes.

The cells were then suspended in 400µl of 4°C electroporation buffer. One hundred microlitre of the suspended cells were transferred into electroporation cuvette and 2µl of plasmid DNA was added, mixed and gently tapped to get the cells settled at the bottom of the cuvette. The cuvette was then placed in an electroporation machine and operated at the settings of 1.8 kV, 200 Ohms, 25µF for cells to take up the plasmid DNA. After electroporation, the cells were incubated in a shaker tank at 30°C in 1ml of Luria broth for 1 hour to resuscitate the cells. The 1ml culture was then centrifuged to get it concentrated by removing part of the supernatant and then plated onto Luria agar plate containing 5µg/ml of chloramphenicol.

#### **2.2.5 Rapid size screening of *E. coli* transformants**

Rapid size screening was conducted to quickly screen transformants for the presence of the plasmid.

To conduct rapid size screening, solution A as detailed below was pre-warmed to 37°C and 50µl pipetted into a 1.5ml Eppendorf tube. A colony from the transformants or a control colony harbouring the plasmid without insert was resuspended in the 50µl of solution A in 1.5ml Eppendorf tube and vortexed for 5-10 seconds. The tube was then incubated at 37°C for 5 minutes. After incubation, the tube was then placed on ice for at least 5 minutes and then centrifuged at 13,000xg for 5 minutes. After the centrifugation, 20µl of the supernatant was loaded on to 0.7% agarose gel.

From the gel, a colony with potential of having the insert will be higher up on gel than the control plasmid without insert and the colony with potential of harbouring the correct clone would be analysed further for confirmation.

#### solution A

Water	6.5ml
500mM EDTA	100µl
Sucrose	1g (this makes 30% concentration of sucrose)
10% SDS	250µl
0.4M NaOH	2.5ml
1M KCL	600µl
Bromophenol blue	to taste

### **2.2.6 Extraction of plasmid DNA from transformed bacterial strains**

#### **2.2.6.1 Extraction of plasmid from *E. coli* transformants**

Using the QIAprep spin miniprep kit protocol, 1.5ml of freshly grown *E. coli* culture was centrifuged at 14,000xg for 1 minute in a microcentrifuge tube and the supernatant discarded. The pelleted cells were resuspended in 250µl of buffer P1.

The P1 mix was vortexed and 250µl of buffer P2 was added and gently inverted for six times. Three hundred and fifty microlitre of buffer N3 was then added and inverted gently for six times. The mix was centrifuged for 10 minutes at 14,000xg and the supernatant transferred to QIAprep column and centrifuged for 1 minute. The column was washed with 500µl of buffer PB, centrifuged for 1 minute and the flow through discarded. The column was also washed by adding 750µl of buffer PE and centrifuged at 14,000xg for 1 minute and the flow through discarded. The column was centrifuged at 14,000xg for 1 minute to get rid of all remaining PE buffers. The column was then placed in a clean 1.5ml microcentrifuge tube and 50µl of buffer EB was added to the centre of the column, allowed to stand for 1 minute, and then centrifuged for 1 minute at 14,000xg to elute the plasmid DNA.

#### **2.2.6.2 Extraction of plasmid from *Bt* transformants**

Using the QIAprep spin miniprep kit protocol, 1.5ml of freshly grown transformed *Bt* culture was centrifuged at 14,000xg for 1 minute in a microcentrifuge tube and the supernatant discarded. The pelleted cells were resuspended in 250µl of buffer P1 containing 10µg/ml of lysozyme. The inclusion of lysozyme was to enhance the lysing of the thick cell wall of *Bt*.

The P1/lysozyme mix was vortexed and 250µl of buffer P2 was added and gently inverted for six times and the plasmid preparation continued as described in section 2.2.6.1.

### **2.2.7 Digestion of extracted plasmid DNA with restriction enzymes**

Plasmids extracted from transformants were checked by digesting 1µl of purified DNA with appropriate restriction enzyme in its buffer in a reaction mix in which distilled water was added to make a final volume of 10µl. The reaction mixes were then incubated at the restriction enzyme's manufacturer's recommended optimum temperature for 1 hour.

### **2.2.8 Expression and harvesting of protein and quantification of protein and spores**

#### **2.2.8.1 Expression and harvesting of protein from *E. coli* JM109**

The *E. coli* cells were grown in one litre of 2x Luria broth containing 100µg/ml of ampicillin and 17µg/ml of chloramphenicol for strain BL21 Rosetta and one litre of 2x Luria broth containing 100µg/ml of ampicillin for JM109.

The cultures were grown at 37°C for three days and the cells were observed under light microscope for presence of inclusion bodies. The cultures were centrifuged at 10k rpm in SLA-1500 rotor for 10 minutes and the supernatant discarded. The pelleted cells were suspended in 35ml of distilled water in a 50ml Falcon tube. The cells were sonicated for 5 minutes at full power pulsing at every 3 seconds. The sonicated cells were transferred into 50ml Oakridge tubes and centrifuged at 12k rpm in SS-34 rotor for 15 minutes and the supernatant discarded.

The pellets were resuspended in 35ml of distilled water and sonicated again at full power for 2 minutes pulsing at every 3 seconds. The sonication mixes were then centrifuged at 12k rpm in SS-34 rotor for 15 minutes and the supernatant discarded. The pellets containing the expressed proteins were resuspended in 5ml of distilled water.

Five microlitre of the suspended proteins were ran on 7.5% SDS-PAGE gel. The protein bands were visualised by treating the SDS-PAGE gel as follows: Fifty millilitre of solution containing 50% of ethanol, 10% of acetic acid and 40% of distilled water was added to the gel and heated in microwave for 30 seconds

and placed on a shaker for 5 minutes. The solution was drained out and 50ml of solution containing, 5% of ethanol, 7.5% of acetic acid, 87.5% of distilled water and 200µl of a solution of 0.25% coomassie blue in ethanol was added to the gel and heated for 30 seconds in microwave. The gel in this solution was placed on a shaker for about 1 hour for the protein bands to be visualised.

#### **2.2.8.2 Expression and harvesting of protein from *E. coli* BL21 (DE3)**

A loopful of *E. coli* BL21 (DE3) harbouring plasmid pET3a with the gene to be expressed were subcultured into 10ml of Luria broth containing 100 µg/ml of ampicillin and grown overnight. The 10ml overnight cultures were inoculated into 100ml of fresh Luria broth containing 100 µg/ml of ampicillin and grown to optical density of between 0.5 to 1.0. After the culture has attained the required optical density, it was then induced with IPTG to a final concentration of between 0.25-1.0nM and incubated at temperatures between 20°C to 37°C for 8 to 24 hours.

After growing the culture for the required length of time, it was centrifuged at 10k rpm in SLA-1500 rotor for 10 minutes and the supernatant discarded. The pelleted cells were suspended in 35ml of distilled water in a 50ml Falcon tube. The cells were sonicated for 5 minutes at full power pulsing at every 3 seconds. The sonicated cells were transferred into 50ml Oakridge tubes and centrifuged at 12k rpm in SS-34 rotor for 15 minutes and the supernatant discarded.

The pellets were resuspended in 35ml of distilled water and sonicated again at full power for 2 minutes pulsing at every 3 seconds. The sonication mixes were then centrifuged at 12k rpm in SS-34 rotor for 15 minutes and the supernatant discarded. The pellets containing the expressed proteins were resuspended in 5ml of distilled water and analysed on 7.5% SDS-PAGE gel as described in section 2.2.8.1.

#### **2.2.8.3 Expression and harvesting of protein from *Bt***

The *Bt* clones harbouring appropriate plasmid or a wild type *Bt* strain was grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates of 25ml Luria agar containing 5µg/ml of chloramphenicol were inoculated 1ml each with the 16 hour culture of the clones and grown at the temperature of between 20 to 30°C for 5 days.

After incubating the inoculated plates for 5 days, 2ml of sterile distilled water was added to the cultures on plates and carefully washed with a sterile spatula into SLA1500 rotor centrifuge tube and centrifuged at 10k rpm in SLA-1500 rotor for 10 minutes and the supernatant discarded. The pelleted cells were resuspended in 35ml of distilled water and sonicated for 5 minutes at full power pulsing at every 3 seconds. The sonicated cells were transferred into 50ml Oakridge tubes and centrifuged at 12k rpm in SS-34 rotor for 15 minutes and the supernatant discarded.

The pellets were resuspended in 35ml of distilled water and sonicated again at full power for 2 minutes pulsing at every 3 seconds. The sonication mixes were then centrifuged at 12k rpm in SS-34 rotor for 15 minutes and the supernatant discarded. The pellets containing the expressed proteins were resuspended in 5ml of distilled water and analysed on 7.5% SDS-PAGE gel as described in section 2.2.8.1.

#### **2.2.8.4 Estimation of protein concentration**

Concentration of harvested proteins were estimated by preparing three dilutions of the protein of unknown concentration and also three concentrations of BSA between 0.1 to 1.0mg/ml as standard.

The protein concentration of the unknown was estimated by band intensity comparison with the known BSA band intensity. To have a reliable estimation, the experiment was always repeated with more dilutions around the first estimated concentration.

For example, if 1:10 dilution of the unknown protein is observed to have the same band intensity as BSA 0.1mg/ml a further dilutions like 0.5:10, 1:10 and 1.5:10 of the unknown protein and 0.05, 0.1 and 0.15mg/ml of the BSA would be prepared and ran on SDS-PAGE gel again and a second comparison made.

#### **2.2.8.5 Quantification of spores**

To obtain spores, cultures from stock of *Bt* strains were grown on 5µg/ml chloramphenicol or 50µg/ml kanamycin nutrient agar plates for five days at 30°C, the growth was observed under the microscope to make sure they have sporulated. The sporulated cells were recovered in 20ml of distilled water and pasteurized at 70°C for 45 minutes in water bath to inactivate vegetative cells.

The spores were serially diluted and 100µl of the diluted concentration of  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  were separately plated in duplicate and incubated for 16 hours at 30°C for spores to germinate and form colonies. Dilutions with between 30 and 200 colonies per plates were counted and the number of spores/ml was estimated on the basis that a colony is formed from a single spore.

## **2.2.9 Characterisation of expressed proteins**

### **2.2.9.1 Alkaline solubility**

Forty microlitre of expressed proteins were centrifuged at 14,000xg for 2 minutes and supernatant discarded. The pellets were resuspended in 40µl of 50mM Na<sub>2</sub>CO<sub>3</sub> (pH 8.0, 8.5, 9.5, 10.5, 11.5 and 12) containing 10mMDTT. To check for the solubility the expressed proteins in Na<sub>2</sub>CO<sub>3</sub>, the mixtures were incubated at 37°C in water bath for 1 hour. After incubation, 10µl were pipetted from the total reaction mixtures and stored in an Eppendorf tube while the remaining reaction mixtures were then centrifuged and the supernatants pipetted into clean microcentrifuge tubes. Five microlitre of the supernatants and the total reaction mixtures were ran on SDS-PAGE gel to check for solubility.

### **2.2.9.2 Protease activation**

Forty microlitre of expressed proteins were centrifuged at 14,000xg for 2 minutes and supernatant discarded. The pellets were resuspended in 40µl of 1 or 2µg/ml of trypsin solution made in 50mM Na<sub>2</sub>CO<sub>3</sub> (pH 9.5 and 11.5). To allow for trypsin digestion, the mixtures were initially incubated at 37°C for 1 hour followed by another 1 hour after addition of 0.2µl of 1M DTT to the reaction mixtures. By so doing, the activity of trypsin was maintained as its activity would have been affected if DTT were to be added from the start of the experiment. Adding DTT after trypsin has completed its digestion was to make sure that the trypsinised protein is denatured for proper visualisation on SDS-PAGE gel because it has been shown that protease activated Cry toxins still have an intact 3D structure (Li *et al.*, 1991, Grochulski *et al.*, 1995, Galitsky *et al.*, 2001, Morse *et al.*, 2001, Boonserm *et al.*, 2005, Boonserm *et al.*, 2006, Guo *et al.*, 2009). After incubation, 10µl were pipetted from the total reaction mixtures and stored in an Eppendorf tube while the remaining reaction mixtures were

centrifuged and the supernatants pipetted into clean microcentrifuge tubes. Five microlitre of the total reaction mixtures and supernatants were ran on SDS-PAGE gel to check for solubility and activation.

#### **2.2.10 Preparation of insect diets**

Insect artificial diet preparation:

##### **Component A**

Wheat germ	32.0g
Casein	15.0g
Sucrose	13.0g
Yeast	6.4g
Salt Mixture W	4.3g
Cholesterol	0.4g
Methyl paraben	0.4g
Sorbic acid	0.7g

The above ingredients were measured and blended in 357ml of boiling distilled water containing 5.4g of agar. The blended mixture was then autoclaved.

##### **Component B**

Vitamin mixture Vanderzant	0.1g
Ascorbic acid	2.0g
Choline chloride	0.4g

The ingredients above making up component B were aseptically measured and dissolved in 50ml of sterile distilled water.

After autoclaving component A, it was allowed to cool to about 40°C after which component B was added and mixed by swirling. The diet was either prepared with rifampicin or without rifampicin depending on the strain of bacteria that would be assayed using the diet. Diet that contained rifampicin prepared by adding 1ml of 25mg/ml stock solution of rifampicin to a mixture of autoclaved component A and component B and mixed by swirling.

The diet was then dispensed 20ml each into 50ml plastic cups and covered with cling film and stored at 4°C and used within a month.

### **2.2.11 Maintenance of *Plutella xylostella* populations on artificial diets and cabbage (*Brassica pekinensis*) plants**

Eggs of *Plutella xylostella* were placed on either 6 week old Chinese cabbage leaves (*Brassica pekinensis*) in a glass cage or on 20ml of artificial diet in a 50ml cup covered with paper towel. The eggs on cabbage plant or diet were maintained at 28°C and 70% relative humidity with 16 hours light and 8 hours dark in an incubator where they hatch and grow from L1 to late L4 stage where they pupate.

Pupae were placed in a plastic cage that has an open top that is covered with cloth for aeration until adults emerge. The emerging adults were fed with 20% honey or 10% sucrose solution in water and they remain in the cage and mate and lay eggs on aluminium foil that is coated with cabbage juice.

### **2.2.12 Bioassay of expressed proteins (toxins) and/ or spores against *Plutella xylostella***

#### **2.2.12.1 Leaf dip assays**

The method of Iqbal *et al*, 1996 as modified by Sayyed *et al*, 2000 was used in which cabbage leaf discs (5cm diameter) were soaked in toxin dilution for 10 seconds, removed and held for 10 seconds and shaken to drain most the toxin solution. The toxin coated leaves were then air-dried for about 1 hour. The toxin solution were made in a solution of 50µl/L of triton-X100 which acts as surfactant enhancing the attachment of toxin to leave surfaces (Sayyed *et al*., 2000).

Ten third instar larvae of *Plutella xylostella* were exposed to two treated leaf discs in a 9cm Petri dish containing two 9cm No.1 Whatman filter paper soaked with 1ml of distilled water. The larvae were allowed to feed on the treated leaves for 3 days after which the number of dead larvae were counted. Fifty percent lethal concentration of the toxins were calculated using EPA PROBIT ANALYSIS PROGRAMME VERSION 1.5. This probit analysis software is used in calculating lethal concentration values and it requires a minimum of three doses of substance whose lethality is to be calculated.



#### **2.2.12.2 Diet dip assays**

The method of Iqbal *et al*, 1996 as modified by Sayyed *et al*, 2000 was used in which 20ml of diet that has been poured into a 50ml cup and allowed to set were aseptically divided into four and one quadrant soaked in toxin dilution for 10 seconds, removed and held for 10 seconds and shaken to drain most the toxin solution. The toxin coated diet quadrant were then air-dried for about 1 hour. The toxin solution were made in a solution of 50µl/L of triton-X100 which acts as surfactant enhancing the attachment of toxin to diet surfaces (Sayyed *et al*, 2000).

Ten third instar larvae of *Plutella xylostella* were exposed to 1 quadrant treated diet in a 20ml cup with perforated cap that allows for aeration of the cup while the larvae feed. The larvae were allowed to feed on the treated diet for 5 days after which the number of dead larvae were counted. Fifty percent lethal concentration of the toxins were calculated using probit software.

## Chapter 3: Expression, manipulation and characterization of Cry1Ie toxin

### 3.1 Introduction

Cry1Ie is a Cry toxin of approximately 81kDa cloned from *Bacillus thuringiensis* isolate Btc007. It has been heterologously expressed in *E. coli* BL21 (DE3) as a fusion protein using the pET-21b vector. The *E. coli* expressed Cry1Ie has been found to be toxic to *Ostrinia furnacalis*, with an LC<sub>50</sub> of 2.22µg/m, *Plutella xylostella*, with an LC<sub>50</sub> of 0.20µg/ml and *Leguminivora glycinivorella*, with an LC<sub>50</sub> of 9.02µg/ml. It has been found to be non-toxic to *Helicoverpa armigera* and *Spodoptera exigua* (Song *et al.*, 2003).

Though Cry1Ie has a narrow spectrum of activity compared to Cry1Ah (Xue, *et al.*, 2008), it has an interesting aspect that could be exploited to create a new toxin with improved activity through molecular genetic techniques. It has been shown that a population of *Ostrinia furnacalis* (ACB-AbR) that has developed cross resistance to Cry1Ah as a result of exposure to Cry1Ab was still sensitive to Cry1Ie (Xu *et al.*, 2010).

Cry1Ie belongs to the three domain Cry toxins but lacks C-terminal blocks 6-8 of full length 130kDa Cry proteins. With insights gained into the structure (Li *et al.*, 1991, Grochulski *et al.*, 1995, Galitsky *et al.*, 2001, Morse *et al.*, 2001, Boonserm *et al.*, 2005, Boonserm *et al.*, 2006) and mechanism of action (Bravo *et al.*, 2007, Gomez *et al.*, 2007) of Cry toxins through molecular genetics studies of the interaction between the insect host and the toxins, specificity of toxins can be altered or broadened. Liu and Dean, 2006 through genetic manipulation changed the Lepidopteran Cry1Aa to a Dipteran specific toxin. Swapping domain III of Cry1Ac, Cry1Ba and Cry1Ea which has low or no toxicity to *Spodoptera exigua* with domain III of Cry1Ca resulted in an improved toxicity to *Spodoptera exigua* (de Maagd *et al.*, 2000). Domain II of Cry1Ab has been shown to be involved in the insertion of the toxin into the brush border membrane vesicles of *Manduca sexta* and a mutation in the domain II residue F371 results in a mutant that lacks membrane insertional ability and thus toxicity (Nair *et al.*, 2008). Other modifications of Cry toxins like truncation of part of the N-terminal sequence (Franklin *et al.*, 2009, Muñoz-Garay *et al.*, 2009) have also been shown to be effective in creating new toxins with improved activity against certain insect population.

In this chapter, the expression of the 2160bp open reading frame of Cry1Ie in *E. coli*, its toxicity against susceptible and resistance populations of *Plutella xylostella* will be presented. The manipulation of Cry1Ie at the genetic level to produce a mutant toxin lacking its helix -1 and swapping of its domain II with that of Cry1Ah will also be covered. In addition, the synergistic studies conducted between Cry1Ie and Cry1Ah against susceptible and resistance populations of *Plutella xylostella* will be presented. Also presented here are the results from the expression, characterisation and toxicity testing of Cry1Ie mutants created through site directed mutagenesis.

## 3.2 Results

### 3.2.1 Construction of a plasmid for the expression of the *cry1Ie* gene

To construct a plasmid expressing Cry1Ie in *E. coli* as illustrated in figure 3.3, the DNA fragments needed are the coding sequence for Cry1Ie and a vector with all the expression vector elements like selective marker, *E. coli* origin of replication, ribosome binding site and a promoter that is able to drive the expression of Cry1Ie. Jie Zhang of Institute of Plant Protection, Beijing, China, supplied the coding sequence of Cry1Ie in a plasmid pETB-1IE shown in figure 3.2 while Neil Crickmore supplied the expression vector pGEM1AcP1Ac shown in figure 3.1. The vector pGEM1AcP1Ac is a vector designed to express Cry1Ac in *E. coli* hosts and it has the native promoter of *cry1Ac*, coding sequence of Cry1Ac and pGEM-T vector backbone.

Primer pair GEMF- 5' TCTCATGCAAACCTCAGGTTTAA 3'/GEMR- 5' AAGTTACCTCCATCTCTTTTATTTAAG 3' were designed using PRIMER SELECT programme software to amplify the required fragment of PGEM-T vector (Promega) backbone, native *cry1Ac* promoter and ribosome binding site hereafter designated as pGEM1AcP from pGEM1AcP1Ac. 5' phosphorylated primer pair IF-5' ATGAAACTAAAGAATCCAGATAAGC 3'/IR-5' CTACATGTTACGCTC- AATATGGA 3' were also designed to amplify the *cry1Ie* open reading frame from pETB-1IE.

Using Pfu Ultra Hotstart PCR Master Mix system according to the manufacturers amplification instructions, pGEM1AcP and Cry1Ie, were amplified and the PCR products were ran on 1% agarose gel and the required bands of 3566bp (pGEM1AcP) and 2160bp (Cry1Ie) were excised and purified

according to the procedure described in section 2.2.3 for the purification of DNA from agarose gels. Pfu Ultra Hotstart PCR Master Mix was PCR kit of choice because of Pfu polymerase's high fidelity property and the fact that it produces a blunt ended PCR product.

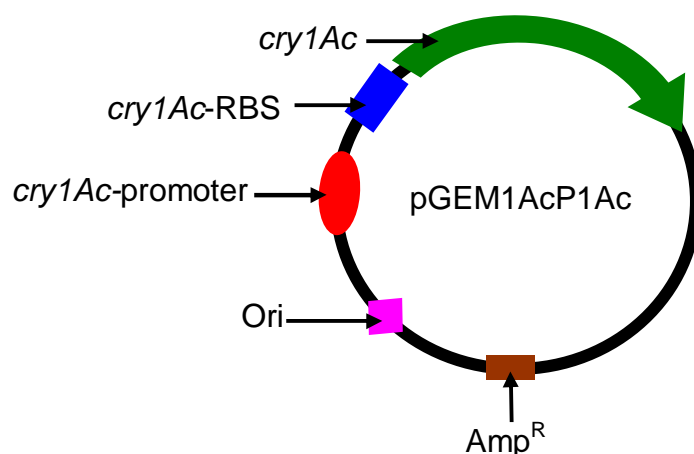


Figure 3.1: Schematic of pGEM1Ac plasmid showing *cry1Ac* coding sequence (green arrow), *cry1Ac*-ribosome binding site (blue block) and promoter (red oval), origin of replication in *E. coli* (pink block) and ampicillin resistance gene (brown block)

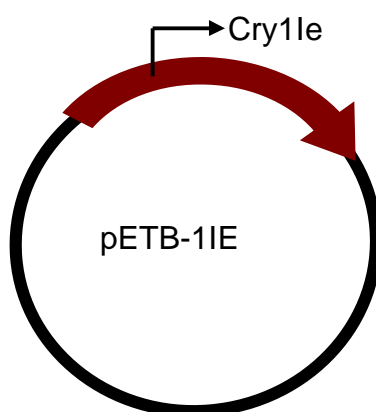


Figure 3.2: Schematic of plasmid pETB-1IE

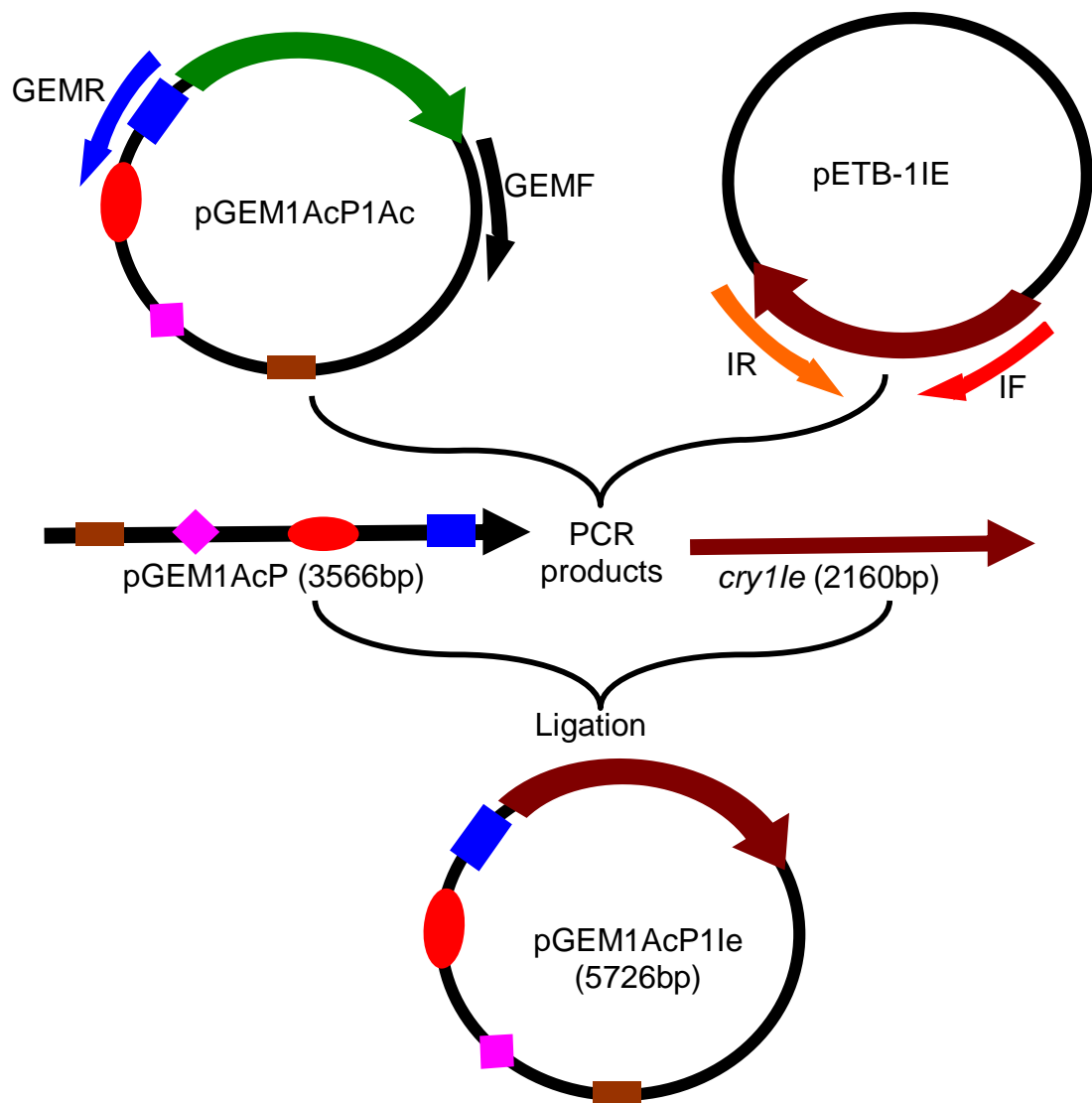


Figure 3.3: Schematic of the construction of the plasmid pGEM1AcP1Ie

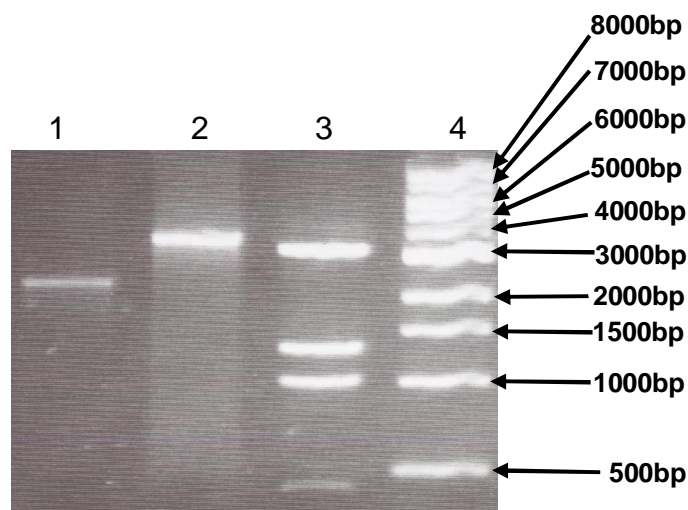
To construct the plasmid pGEM1AcP1le, the following volumes of reagent and DNA fragments were measured and mixed together as shown in table 3.1.

Components	Volumes
H <sub>2</sub> O	1.5µl
pGEM1AcP	1.0µl
Ligation buffer	5.0µl
Cry1le	2.0µl
T <sub>4</sub> Ligase	0.5µl

Table 3.1: Recipe of ligation mix for pGEM1AcP1le

The ligation mix was incubated at room temperature for 3 hours and then stored at 4°C overnight to enhance ligation.

*E. coli* JM109 was transformed with the ligation mix and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pGEM1AcP1le. Clones with the correct orientation of Cry1le were checked by digesting extracted plasmid with *Eco*RI and analysed on 1% agarose gel to check for required bands of 2997, 1266, 984 ,429 and 50 base pairs. Analysis of the *Eco*RI digest on agarose gel shown in figure 3.4 confirms that the plasmid pGEM1AcP1le was successfully constructed. Lane 3 of figure 3.4 has shown the presence of the expected bands of 2997, 1266, 984 ,429bp but the expected band of 50bp could not be detected on gel as it is too small.

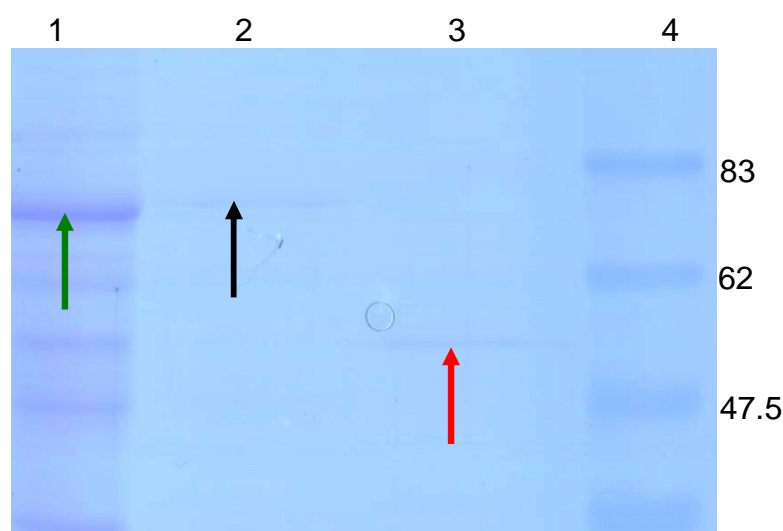


**Figure 3.4:** Gel picture showing the purified PCR fragments of *cry1le*, pGEM1AcP and digestion of pGEM1AcP1le extracted from *E. coli* JM109 with *EcoRI*. Lane 1: 2160bp *cry1le* coding sequence band, lane 2: 3565bp pGEM1AcP, lane 3: digested pGEM1AcP1le showing the expected bands of 2997, 1266, 984 and 429bp and lane 4: 1kb DNA marker

### 3.2.2 Expression and characterisation of Cry1le protein

The strain of *E. coli* JM109 harbouring the plasmid pGEM1AcP1le was grown in 2x Luria broth for 3 days at 37°C after which the total protein was harvested as described in section 2.2.8.1. The total protein was run on 7.5% SDS-PAGE gel and the protein band of ~81kDa was seen on gel.

The expressed proteins were tested for alkaline solubility and protease activation according to the method in section 2.2.9.1 and 2.2.9.2. The alkaline solubility test showed that expressed Cry1le is sparingly soluble in Na<sub>2</sub>CO<sub>3</sub> even at pH 11 while digestion with trypsin (a protease) gave a ~55kDa resistant core on SDS-PAGE gel. Figure 3.5 is a picture of SDS-PAGE gel showing expressed Cry1le band of 81kDa in lane 1, lane 2 shows Cry1le protein band from the Na<sub>2</sub>CO<sub>3</sub> solubility test at pH11 while lane 3 shows the presence of a resistant core from trypsin activation of Cry1le toxin.



**Figure 3.5:** Expressed Cry1Ie and its alkaline solubility and trypsin activation characterisation. Lane 1: Cry1Ie protein band of 81kDa (green arrow), lane 2: Na<sub>2</sub>CO<sub>3</sub> solubilised Cry1Ie band at pH11 (black arrow), lane 3: ~55kDa trypsin resistant core (red arrow) and lane 4: Protein marker

### 3.2.3 Toxicity testing of Cry1Ie against populations of *Plutella xylostella*

The potency of the expressed Cry1Ie against susceptible population of *Plutella xylostella* (G88) and Cry1Ac resistant population of *Plutella xylostella* (KARAK) was conducted using the leaf dip assay and diet dip assay as described in sections 2.2.12.1 and 2.2.12.2 and it was found to have an LC<sub>50</sub> of 0.319µg/ml against G88 with 95% confidence limit of 0.267-0.466µg/ml. It had an LC<sub>50</sub> of 4.176µg/ml against KARAK with 95% confidence limit of 3.299-4.477µg/ml.

### 3.2.4 Synergistic studies between Cry1Ie and Cry1Ac toxins

To check for synergism between Cry1Ie and Cry1Ac toxins, single concentration of the toxins were combined and used in assaying for the mortality of the exposed insect alongside assays that involved only the single concentration of individual toxin. Considering that the LC<sub>50</sub> of Cry1Ie and Cry1Ac against G88 population of *Plutella xylostella* was 0.319µg/ml and 0.037µg/ml respectively, a concentration of 0.15µg/ml for Cry1Ie and 0.03µg/ml



for Cry1Ac were used in combination and singly to conduct bioassays against G88. These concentrations were chosen such that they were below the  $LC_{50}$  of each of the toxins but also able to affect mortality when used individually.

Using the diet dip method described in 2.2.12.2, solutions containing a combination and individual concentration of the toxins were made and used in bioassay against G88.

On the fifth day, the number of death and live larvae were counted and recorded as shown in table 3.2.

Single or combination of Cry1Ie and Cry1Ac toxins ( $\mu\text{g/ml}$ )	Number of insect dead out of 20	Number of live insects out of 20	Total number of insects exposed
Cry1Ie ( $0.15\mu\text{g/ml}$ )	5 (25%)	15 (75%)	20
Cry1Ac ( $0.03\mu\text{g/ml}$ )	10 (50%)	10 (50%)	20
Cry1Ie ( $0.15\mu\text{g/ml}$ ) and Cry1Ac ( $0.03\mu\text{g/ml}$ )	9 (45%)	11 (55%)	20

Table 3.2: The number of dead and live larvae from the synergism studies between Cry1Ie and Cry1Ac toxins

The results presented in table 3.2 shows that there is no synergism between Cry1Ie and Cry1Ac because judging from the fact that Cry1Ie on its own kills 25% and Cry1Ac on the other hand kills 50% on its own, it would have been expected that synergism will result in killing of more than 75% (the sum of the percentage mortality from individual toxins). The 45% mortality observed with the mixture is lower than what would be expected even if the effect with mixture were to be additive which shows that there is no synergism.

### **3.2.5 Mutagenesis of Cry1Ie**

#### **3.2.5.1 Swapping domain II of Cry1Ie with domain II of Cry1Ah:**

##### **construction of pGEM1AcPIAI plasmid**

The observation that regions in domain II and III of Cry toxins are the determinants of the order of insects against which a toxin is active (de Maagd *et al*, 2000, Liu and Dean, 2006, Nair *et al*, 2008) was the basis for the swapping of domains between Cry1Ie and Cry1Ah. Also, the observation that a Cry1Ah resistant population of *Ostrinia furnacalis* (ACB-AbR) was still sensitive to Cry1Ie (Xu *et al.*, 2010) informed the domain swapping to produce a hybrid that targets a Cry1Ac and Cry1Ah resistant population of *Plutella xylostella* (KARAK) which belong to the same Lepidoptera order as *Ostrinia furnacalis*. The plasmid pGEM1AcPIAI was designed to express a hybrid Cry-protein carrying domain I of Cry1Ie, domain II of Cry1Ah and domain III of Cry1Ie hereafter known as CryIAI. The domain borders were mapped out by multiple sequence alignment of Cry1Ie protein sequence using ClustalW (Larkin *et al*, 2007) with Cry1-toxin sequences whose domain borders have already been determined viz: Cry1Ia and Cry1Ba (Naimov *et al*, 2001). To determine the borders between domains of Cry1Ie, its full amino acid sequence was aligned with full length of Cry1Ia and Cry1Ba amino acid sequences. Alanine-299 (green box of figure 3.6A) was chosen as the end of domain I for Cry1Ie because it aligns with Alanine-299 of Cry1Ia and Alanine-290 of Cry1Ba which Naimov *et al.*, (2001) has previously determined as the end of domain I. The same procedure was used in determining border between domain II and III.

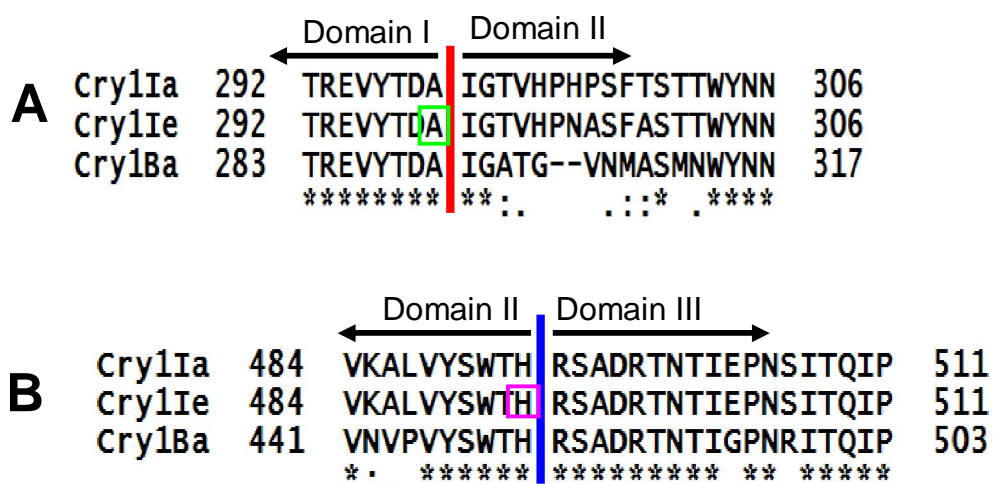


Figure 3.6: Multiple sequence alignment of segments of Cry1Ie, Cry1Ia, Cry1Ba, and Cry1Ea protein sequences using ClustalW software.

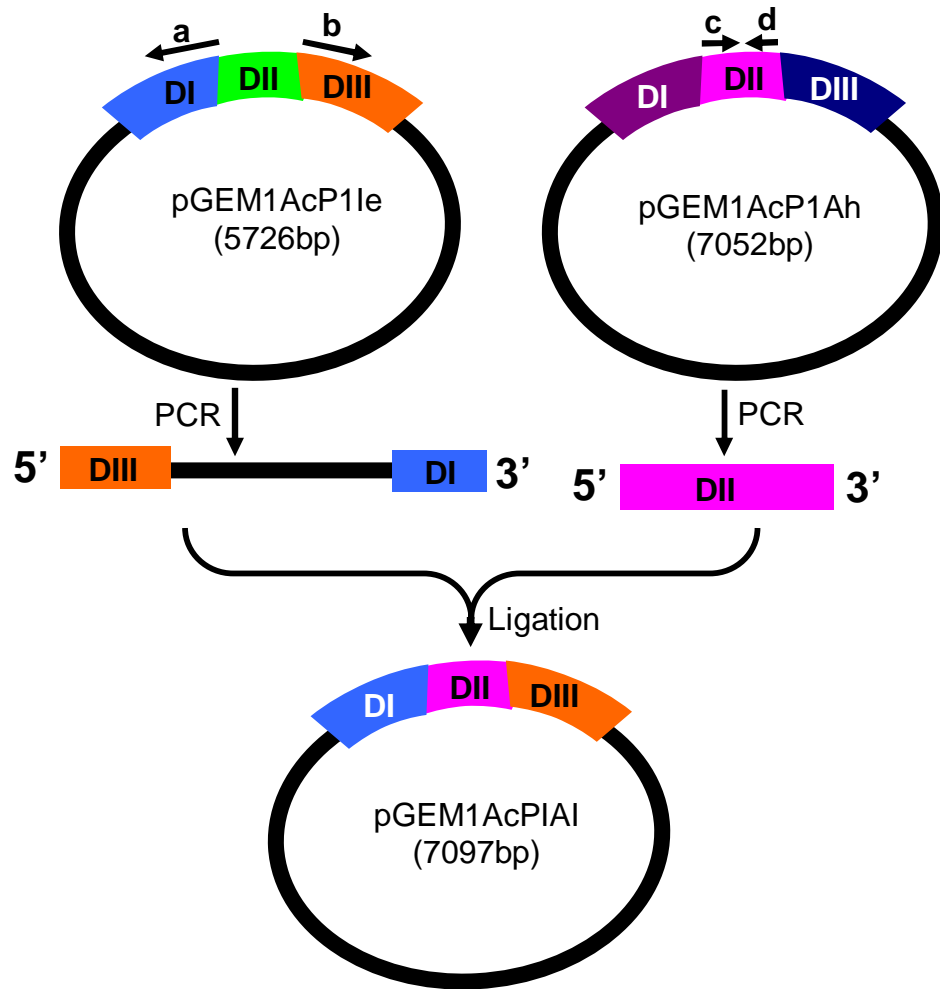
- A: The red line demarcates domain I and domain II and the green box is Alanine-299 which is the end of domain I for Cry1Ie.
- B: The blue line demarcates domain II and domain III and the pink box is Histidine-493 which is the end of domain II for Cry1Ie.

After mapping out the domain borders as shown in figure 3.6, primer pair 1AhDIIF 5' GTATTAGAAAATTTTGATGGTAGTTTT 3'/1AhDIIR 5' ATGTA-TCCAAGAGAACATAGGAGCT 3' were designed to amplify domain II of Cry1Ah from pGEM1AcP1Ah plasmid constructed in section 4.2.1. The primers were modified by addition of phosphate group to their 5' ends to allow ligation of their PCR product. Primer pair GEM1IeF 5' CGTAGTGCAGATCGTACAA 3' and GEM1IeR 5' TCGTCTGTATATACTTCTCTTGTAAG 3' were designed to amplify *cry1Ie* and its vector excluding its domain II as shown in figure 3.7. The primer pairs were used in conducting PCR and the amplified DNA sequences were gel purified according to the method described in section 2.2.3. The DNA fragments amplified using each primer pair were named as follows: 1AhDIIF/1AhDIIR product was named as 1AhDII (555bp) while that of GEM1IeF/GEM1IeR was named as GEM1Ie (5144bp). These PCR products were ligated to form the plasmid pGEM1AcPIAI.

The recipe in table 3.3 shows the ligation of the PCR fragments of 1AhDII, and GEM1Ie to form plasmid pGEM1AcPIAI.

Components	pGEM1AcPIAI
H <sub>2</sub> O	3µl
GEM1Ie	5µl
1AhDII	0.5µl
10x Ligase buffer	1µl
T <sub>4</sub> DNA ligase	0.5µl

Table 3.3: Recipe for the ligation of DNA fragments to form pGEM1AcPIAI expression vector

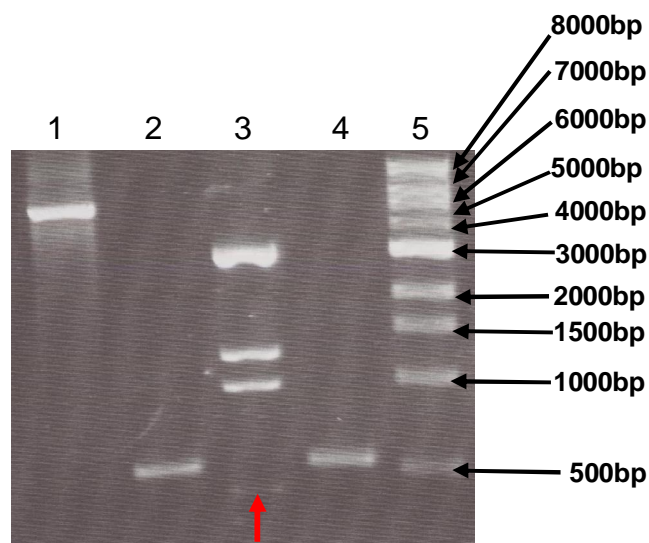


**Figure 3.7:** Schematic illustrating the construction of pGEM1AcPIAI plasmid from 1AhDII and GEM1Ie DNA fragments

The ligation mixes were incubated at room temperature for 3 hours and afterward incubated in the refrigerator at 4°C overnight. *E. coli* JM109 was transformed with the ligation mixes by electroporation and the transformants screened for colonies harbouring pGEM1AcPIAI plasmid. The presence of the 1AhDII in pGEM1AcPIAI was checked by conducting PCR using the primer pair 1AhDIIF/1AhDIIR with pGEM1AcPIAI extracted from transformants as the template. The correct orientation of 1AhDII in pGEM1AcPIAI was confirmed by digesting the extracted plasmid of pGEM1AcPIAI with *EcoRI* which gave the expected bands of 2997bp, 1248bp, 975bp and 429bp (pointed by red arrow) as shown in figure 3.8 except 50bp band which could not be detected as it is too small. When the plasmid pGEM1AcPIAI was successfully formed, *E. coli* BL21 Rosetta was also transformed with it for expression of CryIAI. The plan to

express CryIAI in *E. coli* BL21 Rosetta was informed by the fact that some *Bt* toxins had been found to express better in this host (Jie Zhang – personal communication).

Computational analysis of the hybrid *cryIAI* gene using EMBOSS Transeq tool an online programme at the EMBL-EBI website gave a putative protein with 756 amino acid residues. Compute pI/Mw tool (Bjellqvist *et al.*, 1993, Bjellqvist *et al.*, 1994) which is an online programme at ExPASy website used in computing the molecular weight of the deduced protein estimated the molecular weight of CryIAI to be approximately 80kDa.



**Figure 3.8:** Gel picture showing PCR products GEM1le, 1AhDII and confirmation of the presence of DNA fragment 1AhDII in pGEM1AcPIAI. Lane 1: 5144bp from GEM1leF/GEM1leR, Lane 2: 555bp from 1AhDIIF/1AhDIIR, Lane 3: Digestion of representative pGEM1AcPIAI with *EcoRI* showing band sizes of 2997bp, 1248bp, 975bp and 429bp (pointed by red arrow), Lane 4: PCR using primer pair 1AhDIIF/1AhDIIR with pGEMIAI as template and Lane 5: DNA marker

### **Expression of CryIAI protein from *E. coli* JM109 and *E. coli* BL21 Rosetta**

The strain of *E. coli* JM109 or *E. coli* BL21 Rosetta harbouring the plasmid pGEM1AcPIAI was grown in a 2x Luria Broth for 3 days at 37°C after which the total proteins were harvested as described in section 2.2.8.1.

The total proteins were ran on 7.5% SDS-PAGE gel but no protein band of ~80kDa was seen on gel (data not shown).

As the required band of 80kDa was not seen when the clones were grown at 37°C, they were then grown at other temperatures including 20°C, 25°C and 30°C. Total proteins were harvested from each set of culture and analysed on SDS-PAGE gel but again the expected bands were not seen.

### **Attempting expression of *cryIAI* in *Bt***

The plasmid pSV2 is a shuttle vector that has origins of replication for *Bt* and *E. coli*. It has multiple cloning sites, which includes a *Bam*HI site that was used for the cloning of the coding sequences of *cryIAI* gene including the native *cry1Ac*-promoter in its upstream region.

To construct plasmid pSV21AcPIAI, DNA fragments used were *Bam*HI linearized pSV2 and the *cry1Ac*-promoter/*cryIAI* region with *Bam*HI overhangs as shown in figure 3.9.

The sequence of native *cry1Ac*-promoter/*cryIAI* gene was obtained from the plasmid pGEM1AcPIAI previously constructed. Primer pair 1AcPF 5' gagctcggATcccaacaccctgg 3'/1AcPR 5' gatattGGaTcctgagtttgcattgag 3' were designed with base changes as shown with capital letters to introduce *Bam*HI sites at the 5' and 3' ends.

Using High Fidelity PCR Master system from Roche according to the manufacturers instructions, the required fragment was amplified from pGEM1AcPIAI plasmid and the PCR product was ran on 1% agarose gel and the required band of 2319bp was excised and purified as described in section 2.2.3. The PCR product was first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release 1AcPIAI with *Bam*HI overhangs. High Fidelity PCR Master system was used because of its high fidelity and the production of PCR products with 3' A-overhangs which aid cloning with the pGEM-T vector which has 3' T-overhangs.

Cloning the PCR products into PGEM-T was desirable because it allows for a proper digestion and release of the needed fragments.

pSV2 plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase. The phosphatase was included in the digestion mix to dephosphorylate the phosphate group at the 5' ends of the linearised pSV2 DNA fragment thus preventing it from self ligation. The linearised pSV2 was ran on 1% agarose gel and its band excised from the gel and purified according to the procedure described in section 2.2.3.

The gel purified DNA fragments of linearised pSV2 and 1AcPIAI were ligated according the recipe outlined in table 3.4.

Components	pSV21AcPIAI
H <sub>2</sub> O	5.0µl
pSV2	0.5µl
1AcPIAI	3.0µl
10x Ligase buffer	1.0µl
T <sub>4</sub> DNA ligase	0.5µl

Table 3.4: Recipe for ligation of DNA fragments to form plasmid pSV21AcPIAI

The ligation mix was incubated at room temperature for 3 hours and then incubated at 4°C overnight to enhance ligation.

*E. coli* JM109 was transformed with the ligation mix and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pSV21AcPIAI. Colonies whose plasmid size on agarose gel were higher than that of pSV2 which was used as a control, had the potential of having the insert 1AcPIAI. Plasmid DNA was extracted from clones with insert and the correct orientation of 1AcPIAI was confirmed by digesting extracted plasmid with *Nco*I (data not shown). AcrySTALLiferous strain of *Bt* IPS 78/11 was transformed with confirmed plasmid according to the method described in section 2.2.3.2. Successful transformation of IPS 78/11 was confirmed by extracting plasmids from IPS 78/11 clones, retransforming into *E.*



*coli* JM109 and then minipreping and digesting extracted plasmids alongside the original plasmid extracted from *E. coli* clones.

*E. coli* JM109 was transformed with the ligation mix and not directly into IPS 78/11 because the transformation efficiency of IPS 78/11 is very low and would be extremely difficult to obtain transformed clones with the ligation mix. *E. coli* JM109 on the other hand, is easily transformed with ligation mix.

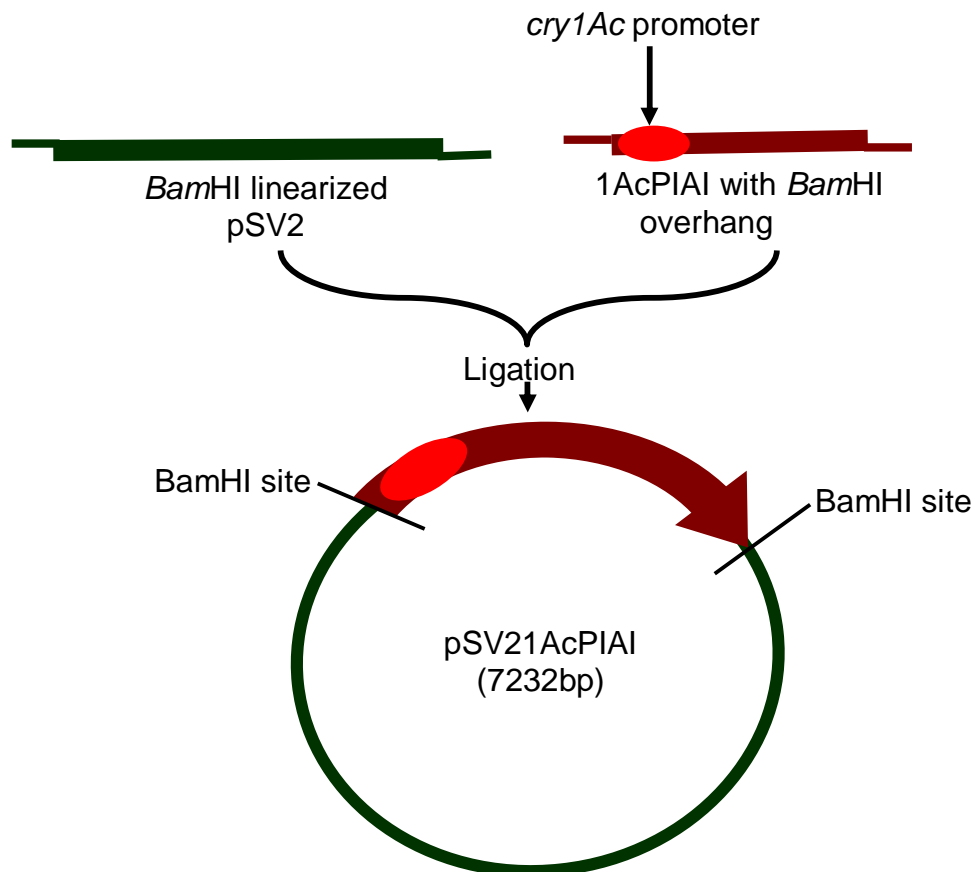


Figure 3.9: Schematic showing the construction of the plasmid pSV21AcPIAI

### **Expression of CryIAI protein from acrySTALLIFEROUS *Bt* IPS 78/11 under the control of native *cry1Ac*-promoter**

The clone of IPS 78/11 harbouring the plasmid pSV21AcPIAI was grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates of 25ml Luria agar with 5µg/ml chloramphenicol were inoculated 1ml each with the 16 hour culture of the clone and grown at 30°C for 5 days. Total protein were harvested as described in section 2.2.8.3 and ran on 7.5% SDS-PAGE gel but again the protein band of ~80kDa was not seen on gel (data not shown).

As the required band of ~80kDa was not seen when the clone was grown at 30°C, it was then grown at other temperatures including 20°C, 25°C and 37°C. Total protein was harvested from each set of culture and analysed on SDS-PAGE gel but the required band was still not seen.

### **Attempting expression of *cryIAI* gene in *Bt* utilising the *cyt1A*-promoter**

Plasmid pSVP27 is a vector for gene expression in *Bt* under the control of *cyt1A*-promoter constructed by fusing a sequence containing *cyt1A*-promoter to pSV2 and incorporating multiple cloning sites including *Bam*HI downstream of the promoter (Crickmore and Ellar, 1992).

To construct plasmids pSVP271AcRBSIAI aimed at expressing *cryIAI* gene under the control of native *cyt1A*-promoter in acrySTALLIFEROUS *Bt*, DNA fragments used were *Bam*HI linearized pSVP27 and sequence containing *cry1Ac*-ribosome binding site/*cryIAI* gene with *Bam*HI overhangs as shown in figure 3.10.

Sequence of *cry1Ac*-Ribosome binding site/*cryIAI* gene was obtained from the plasmid pGEM1AcPIAI previously constructed through PCR. Primer pairs RIAIF 5' aatgGatCCgtatcttaataaaagagatgg/RIAIR 5' gatattGGaTcctgagttgcatgag 3' were designed with base changes as shown with capital letters to introduce *Bam*HI sites at 5' and 3' ends using PRIMER SELECT programme.

Using High Fidelity PCR Master system from Roche according to the manufacturers amplification instructions, needed fragment was amplified from pGEM1AcPIAI plasmid and the PCR product was ran on 1% agarose gel and required band of 2194bp was excised and purified according to the PCR products purification procedure described in section 2.2.3. The PCR product was first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was

transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release 1AcRBSIAI fragment with *Bam*HI overhangs.

pSVP27 plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase. The linearised pSVP27 was ran on 1% agarose gel and its band excised and purified according to the procedure described in section 2.2.3.

The gel purified DNA fragments of linearised pSVP27 and 1AcRBSIAI were ligated according the recipe outlined in table 3.5.

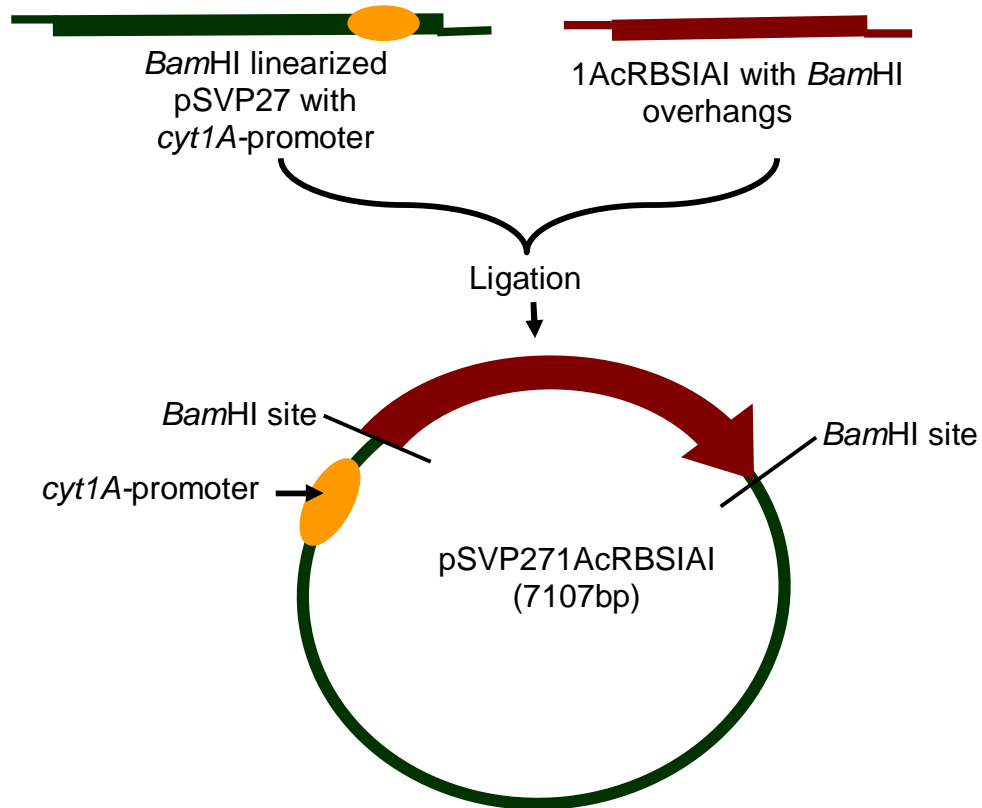
Components	pSVP271AcRBSIAI
H <sub>2</sub> O	5.0µl
pSVP27	0.5µl
1AcRBSIAI	3.0µl
10x Ligase buffer	1.0µl
T <sub>4</sub> DNA ligase	0.5µl

Table 3.5: Recipe for ligation of DNA fragments to form plasmid pSVP271AcRBSIAI

The ligation mixes were incubated at room temperature for 3 hours and then incubated at 4°C overnight.

*E. coli* JM109 was transformed with the ligation mix and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pSVP271AcRBSIAI. Colonies whose plasmid size on agarose gel were higher than that of pSVP27 which was used as control, had the potential of having the insert 1AcRBSIAI. Plasmid DNA was extracted from clones with insert and the correct orientation of 1AcRBSIAI was confirmed by digesting extracted plasmid from pSVP271AcRBSIAI with *Nco*I and *Bam*HI (data not shown). The plasmid was successfully formed and acrySTALLIFEROUS strain of *Bt* IPS 78/11 was transformed with the confirmed plasmid. Successful transformation of IPS 78/11 was confirmed by extracting plasmids from IPS

78/11 clones, retransform *E. coli* JM109 and then miniprep and digest extracted plasmids alongside the original plasmid.



**Figure 3.10:** Schematic showing the construction of the plasmid pSVP271AcRBSIAI

### **Expression of CryIAI protein in acrySTALLIFEROUS *Bt* IPS 78/11 under the control of *cyt1A* promoter**

The clone of IPS 78/11 harbouring the plasmid pSVP271AcRBSIAI was grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates of 25ml Luria agar with 5µg/ml of chloramphenicol were inoculated 1ml each with the 16 hour culture of the clone and grown at 30°C for 5 days. Total protein was harvested as described in section 2.2.8.3 and ran on 7.5% SDS-PAGE gel no protein band of 80kDa was seen on gel (data not shown).

As the required band of 80kDa was not seen when the clone was grown at 30°C, it was then grown at other temperatures including 20°C, 25°C and 37°C. Total protein was harvested from each set of culture and analysed on SDS-PAGE gel but the required band was not still seen.

### 3.2.5.2 Truncation of N-terminal $\alpha$ -helix

To construct the plasmid pGEM1AcP1le which is a plasmid designed to express truncated sequence of *cry1le* gene, primer pair 1le F 5' GTTCCTTTTGCTGGACAAATAGCTAGCCTC 3'/1le R 5' CATAAGTTACCTCCATCTCTTTTATTAAG 3' was designed such that Cry1le protein sequence will be deleted from amino acid position 2(Lysine) to amino acid position 75(Glycine) inclusive. This deletion targeted the possible protease recognition site of glycine outside the helix  $\alpha$ -1 region of Cry1le domain I which when recognised, the amino acid to its carboxyl end is cleaved (Lightwood *et al.*, 2000, Walters *et al.*, 2008). The pore forming model of Cry toxin mode of action has demonstrated that following the initial cleavage of protoxins by gut proteases that removes the C-terminal half and about 30 amino acid residues from the N-terminal, the processed toxins then bind to receptors resulting in their conformational changes which facilitates a second cleavage by membrane bound protease that removes the N-terminal helix  $\alpha$ -1 (Bravo *et al.*, 2004). The processed toxins lacking helix  $\alpha$ -1 and the C-terminal half have been shown to form oligomers that are capable of inserting into insect midgut epithelial membrane in the absence of cadherin (Gomez *et al.*, 2002, Soberon *et al.*, 2007). The need to create a modified Cry1le with deleted helix  $\alpha$ -1 was to target a Cry1Ac/Cry1Ah resistant population of *Plutella xylostella* (KARAK) because it has been shown that a modified Cry1Ab that lacks helix  $\alpha$ -1 was active against a resistant population of *Pectinophora gossypiella* whose resistance has been linked to cadherin gene deletions (Muñoz-Garay *et al.*, 2009). Also, Sayyed *et al.*, 2005 demonstrated that a field collected resistant population of *Plutella xylostella* (SERD4) which was subsequently selected in the laboratory using Cry1Ab and named Cry1Ab-SEL were more sensitive to trypsin-activated Cry1Ab compared to Cry1Ab protoxins. The amino acid position 75(Glycine) was selected for inclusion in the deletion because multiple sequence alignment of Cry1le amino acid sequence with other Cry1-toxin amino acid sequences

with known helix -1 position (Grochulski *et al*, 1995) showed that Glycine-75 a protease recognition site is just outside helix -1 as shown in figure 3.11.

Using Pfu Ultra Hotstart PCR Master Mix system according to the manufacturers instructions, primer pair 1le F/1le R, was used in conducting PCR using pGEM1AcP1le as template as illustrated in figure 3.12. The PCR mix was ran on 1% agarose and a product with band size of 5501bp was excised and purified according to the PCR products purification procedure described in section 2.2.3. Three microlitre of the purified PCR product was self ligated in the presence of 4.5µl of water, 2µl of 10x ligation buffer and 0.5µl of T4 DNA ligase. The ligation mix was incubated at room temperature for 3 hours and then at 4°C overnight. *E. coli* JM109 was transformed with the ligation mix and the successful formation of plasmid pGEM1AcP1le was confirmed by digestion of the plasmid extracted from the transformants with *EcoRI* restriction enzyme as shown in figure 3.13.

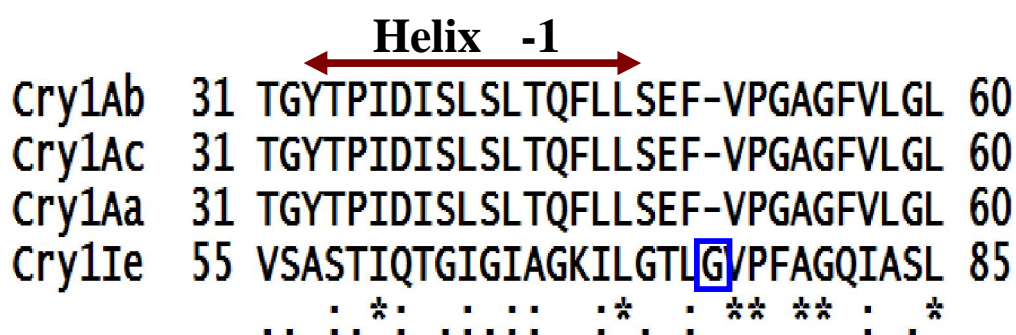


Figure 3.11: Multiple sequence alignment of Cry1Ab, Cry1Ac, Cry1Aa and Cry1Ie protein sequences for determination of protease recognition sites after helix -1 segments of their domain I. The brown double-headed arrow indicates the helix -1 segment, the 'G' in the blue box is Glycine-75 of Cry1Ie

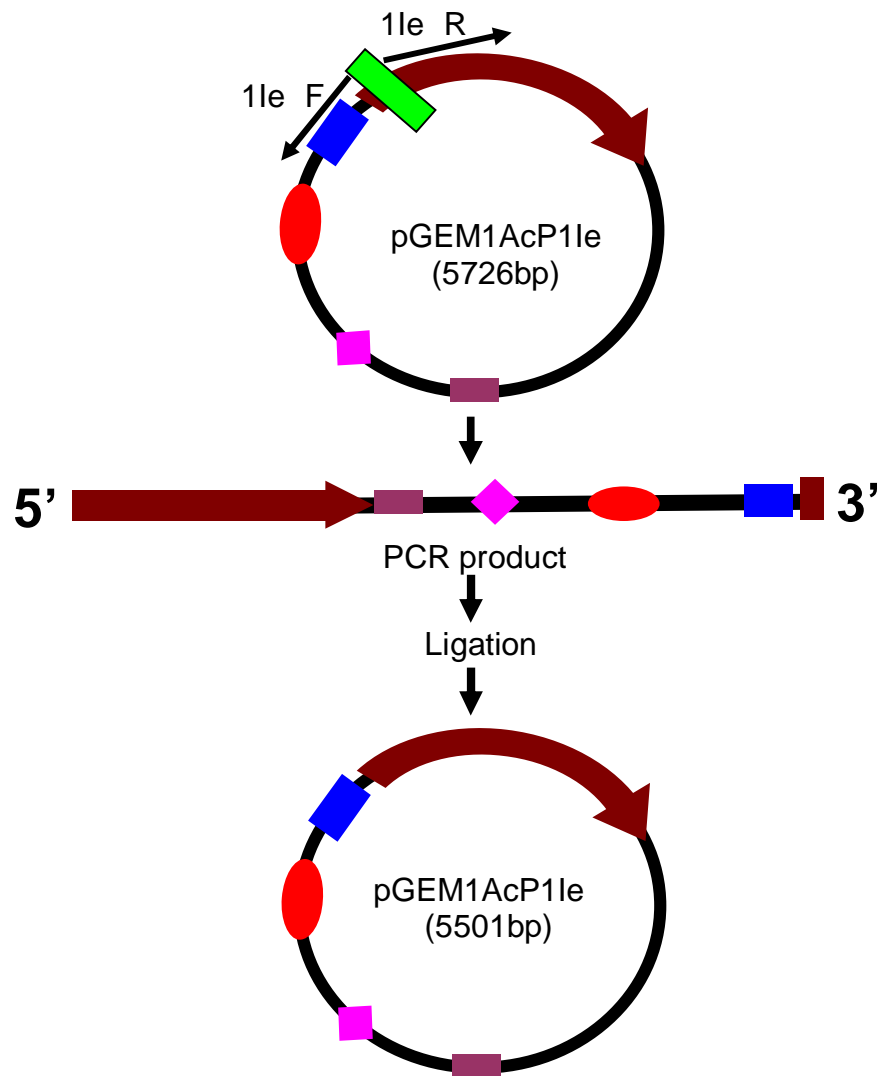
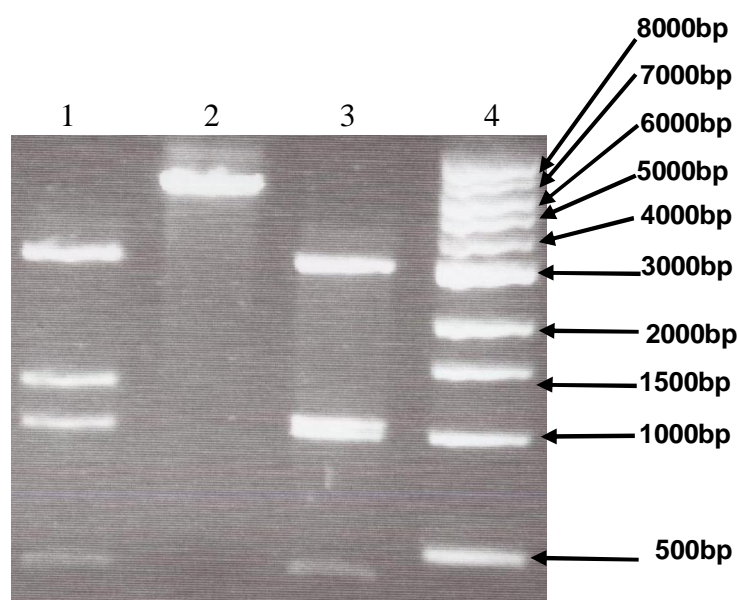


Figure 3.12: Schematic for the construction of the plasmid pGEM1AcP1le . The PCR primers were designed to amplify the gene deleting the green block

- Ampicillin resistance gene
- ◆ Origin of replication in *E. coli*
- *cry1Ac* promoter
- *cry1Ac* ribosome binding site



**Figure 3.13:** Gel picture of PCR product ligated to form the plasmid pGEM1AcP1le and confirmation of its formation by digesting a representative plasmid with *EcoRI* restriction enzyme. Lane 1: digestion of pGEM1AcP1le plasmid with *EcoRI* as control showing band sizes of 2997bp, 1266bp, 984bp and 429bp, Lane 2: PCR product (5501bp) of pGEM1AcP1le, Lane 3: digestion of representative pGEM1AcP1le plasmid with *EcoRI* showing band sizes of 2997bp, 1044bp, 984bp and 429bp and Lane 5: DNA marker

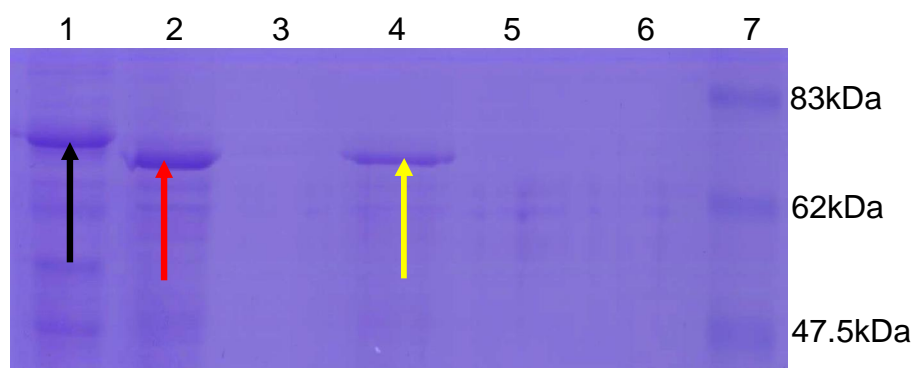
### **Expression and characterisation of Cry1le**

The strain of *E. coli* JM109 harbouring the plasmid pGEM1AcP1le was grown in a 2x Luria Broth for 3 days at 37°C after which the total protein was harvested as described in section 2.2.8.1. The total protein was run on 7.5% SDS-PAGE gel and a protein band of ~73kDa (the predicted size of Cry1le) was seen on gel as shown in figure 3.14 lane 2.

The expressed protein was tested for alkaline solubility and protease activation according to the method in section 2.2.9.1 and 2.2.9.2. The alkaline solubility test showed that expressed Cry1le was not soluble in 50mM Na<sub>2</sub>CO<sub>3</sub> at pH11



as shown in lane 3 of figure 3.14 while digestion with trypsin (a protease) did not give a resistant core of ~50-60kDa on SDS-PAGE gel but showed that the protein was completely degraded as shown in lane 5 of figure 3.14.



**Figure 3.14:** Expressed Cry1Ie and its solubility and trypsin characterization. Lane 1: 81kDa wild type Cry1Ie (pointed by black arrow), Lane 2: ~73kDa Cry1Ie (pointed by red arrow), Lane 3: Supernatant from alkaline solubility test of Cry1Ie at pH11, Lane 4: Total sample from alkaline solubility test of Cry1Ie at pH11, Lane 5: Supernatant from trypsin activation test of Cry1Ie, Lane 6: Total sample from trypsin activation test of Cry1Ie and lane 7: Protein molecular weight marker

### **Toxicity testing of expressed Cry1Ie**

The toxicity of Cry1Ie was tested against G88 and KARAK populations of *Plutella xylostella* according to the method described in sections 2.2.13.1 but there was no mortality to either the toxin sensitive G88 or the Cry1Ac resistant KARAK at concentrations as high as 100µg/ml.

### 3.2.5.3 Site directed mutagenesis

Based on preliminary results obtained by Liu *et al*, 2010 (unpublished data), it was observed that two Cry1Ie mutants viz: Y442H and A522V had the potential of improved toxicity towards *Plutella xylostella* compared to the wild type Cry1Ie. We decided to confirm this finding using our own Cry1Ie expression system.

To create the mutant Y442H, mutagenic PCR primers Y4F 5' TATTATCTA-GGG**CAT**GCTGGAGTTG 3'/Y4R 5' AAAATTATCAGATGCAATCGGAAG 3' were designed using PRIMER SELECT programme such that Tyrosine-442 codon TAT of the wild type *cry1Ie* is replaced with CAT which codes for Histidine by changing the first 'T' of the codon to a 'C' as shown with the red letters in the forward primer Y4F. A522V mutant was created by designing a mutagenic primer A5F 5' TGTCTTCAGGT**GTC**GCTGTAGTG 3'/A5R 5' GATTGAACGCTTTTACTAATGGTA 3' that changed the first 'C' of GCC Alanine-522 codon to 'T' creating GTC which codes for Valine as shown with the red lettering in the forward primer A5F.

Using Pfu Ultra Hotstart PCR Master Mix system according to the manufacturer's instructions, primer pair Y4F/Y4R or A5F/A5R, was used in conducting PCR using pGEM1AcP1Ie as template and the PCR mix was ran on 1% agarose. The PCR product with band size of 5726bp was excised and purified according to the PCR products purification procedure described in section 2.2.3. Six microlitre of each of the purified PCR products were self ligated in the presence of 1.5µl of water, 2µl of 10x ligation buffer and 0.5µl of T4 DNA ligase. The ligation mixes were incubated at room temperature for 3 hours and then at 4°C overnight. *E. coli* JM109 were transformed with the ligation mixes and the successful formation of plasmid pGEM1AcPY442H or pGEM1AcPA522V was confirmed by digestion of the plasmid extracted from the transformants with *Hae*III restriction enzyme with pGEM1AcP1Ie plasmid as control. Successful creation of the mutants was confirmed by sequencing of the mutant plasmids (data not shown).

Creation of the double mutant Y442H+A522V was done by using the primers for A522V (A5F/A5R) to conduct PCR with Y442H mutant plasmid as template and its successful formation of the plasmid pGEM1AcPY442H+A522V was also by digestion of plasmid extracted as described for Y442H and A522V and sequencing.

### Bioinformatic analysis of wild type Cry1Ie and its Y442H, A522V and Y442H+A522V mutants

Since the 3D structure of Cry1Ie has not been resolved at the moment, its amino acid sequence was submitted online to the SWISS-MODEL Workspace (Arnold *et al.*, 2006; Schwede *et al.*, 2003; Guex and Peitsch, 1997) a web based method of modelling 3D structures of proteins using templates with resolved structures. Results from SWISS-MODEL showed that Cry1Ie has a 46.31% strict identity to the template 3eb7B whose structure has been determined at 2.30 Å and deposited in the SWISS-MODEL library. The predicted 3D structure produced by the SWISS-MODEL programme was that of a typical three domain Cry-protein as shown in figure 3.15.

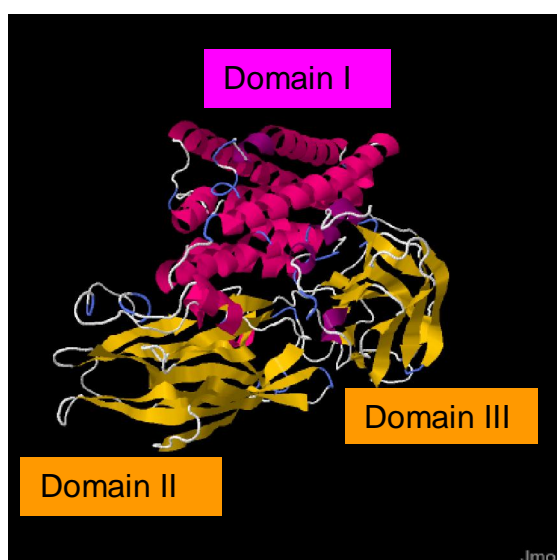


Figure 3.15: SWISS-MODEL predicted 3D structure of Cry1Ie. Domain I is coloured red while domains II and III are coloured gold

The replacement of Tyrosine with Histidine in mutant Y442H is a highly conservative replacement with both having aromatic side chains that are reactive while the replacement of Alanine with Valine in mutant A522V is semi conservative replacement as they both have hydrocarbon side chains but the

difference is that the side chain of Alanine is smaller than that of Valine (Berg *et al*, 2006).

The secondary structure prediction by SWISS-MODEL software did not assign structures to the points where the Y442H and A522V mutations were made but predicted loop structures for regions around them as shown appendix 2. Predictions by PSIPRED for wild type Cry1Ie and 3eb7B (Cry8Ea) showed that the Y442H and A522V mutations are within loop regions as shown in figure 3.16 and 3.17 respectively. The location of the Tyrosine-442 and Alanine-522 in the 3D structure showed that they are part of the exposed loop regions as shown in figures 3.18 and 3.19 and exposed loops have been shown in other Cry toxins to be involved in binding to midgut receptors. Therefore, it is possible that this replacement will affect toxicity.

To find out the percentage similarity between the wild type Cry1Ie and 3eb7B, pairwise sequence alignment was conducted using BLASTP 2.2.24+ programme and the result (appendix 1) showed that they share 48% strict identical amino acids, 65% replacement that are conservative and 3% gaps. The percentage of shared identical amino acid between Cry1Ie and 3eb7B (Cry8Ea) obtained with BLASTP 2.2.24+ programme is 48% while that of SWISS-MODEL programme is 46.31% which gives a difference of 1.69%. This difference is as a result of the length of amino acids used by the two programmes in their predictions. While BLASTP 2.2.24+ programme use the sequences from amino acid position 1 for both Cry1Ie and Cry8Ea, SWISS-MODEL programme start at amino acid position 56 for Cry1Ie and 64 for 3eb7B. The alignment between Cry1Ie and 3eb7B has shown an overall even distribution of conserved regions between them though there is not much conservation at the loop regions where the mutations Y442H and A522V are made. This is not surprising because loop regions varies among Cry toxin which give rise to different host specificities observed for different Cry toxins.







Figure 3.18: 3D structure of Cry1Ie showing the position of Tyrosine-442 (black arrow). Domain I is coloured red while domain II and III are gold coloured



Figure 3.19: 3D structure of Cry1Ie showing the position of Alanine-522 (red arrow). Domain I is purple coloured while domain II and III are coloured green

### Expression and characterisation of Y442H, A522V and Y442H+A522V mutant proteins

The strains of *E. coli* JM109 harbouring the plasmid pGEM1AcPY442H or pGEM1AcPA522V or pGEM1AcPY442H+A522V was grown in a 2x Luria broth for 3 days at 37°C after which the total protein were harvested as described in section 2.2.8.1. The total proteins were ran on 7.5% SDS-PAGE gel and the protein band of ~81kDa was seen on gel for all the clones as shown in figure 3.20.

The expressed proteins were tested for alkaline solubility and protease activation according to the method in section 2.2.9.1 and 2.2.9.2. The alkaline solubility test showed that expressed Y442H, A522V and Y442H+A522V were sparingly soluble in 50mM Na<sub>2</sub>CO<sub>3</sub> pH11 while digestion with trypsin (a protease) gave a ~55kDa resistant core on SDS-PAGE gel as shown in figure 3.21.

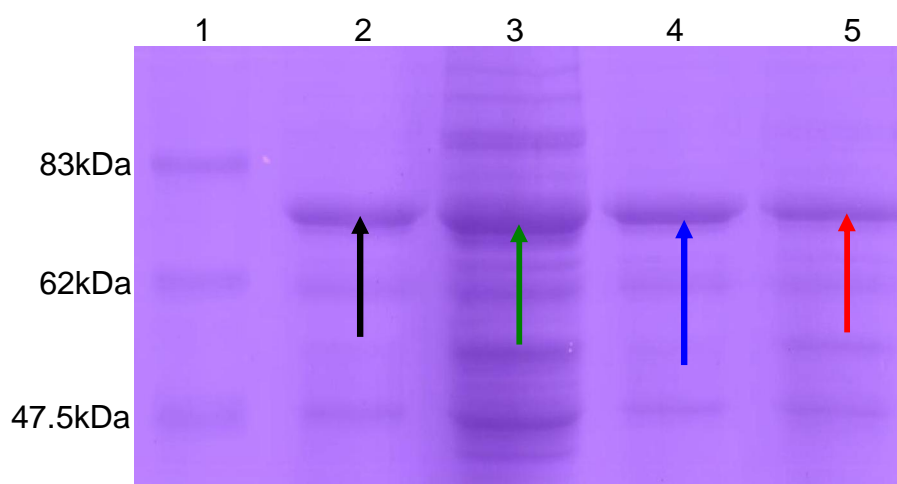
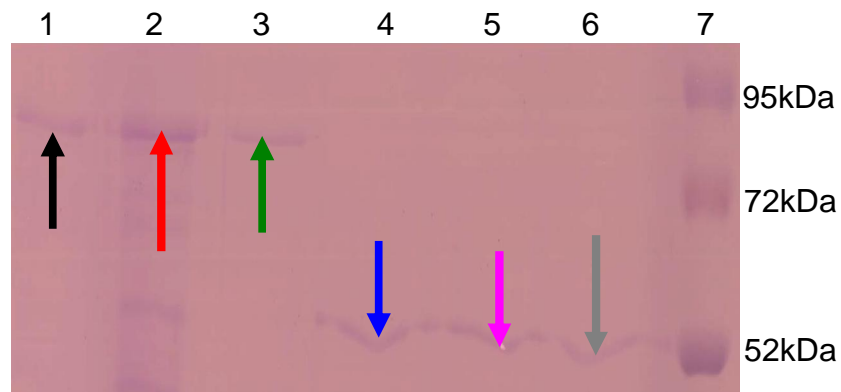


Figure 3.20: Gel picture of expressed Y442H, A522V and Y442H+A522V. Lane 1: Protein molecular weight marker, Lane 2: 81kDa wild type Cry1Ie (pointed by black arrow), Lane 3: 81kDa Y442H mutant (pointed by green arrow), Lane 4: 81kDa A522V mutant (pointed by blue arrow) and Lane 5: 81kDa Y442H+A522V (pointed by black arrow)





**Figure 3.21:** Gel picture of alkaline solubility and protease digestion characterization of expressed Y442H, A522V and Y442H+A522V Lane 1: Supernatant from alkaline solubility test of Y442H at pH11 showing presence of 81kDa band (pointed by black arrow), Lane 2: Supernatant from alkaline solubility test of A522V showing presence of 81kDa band (pointed by red arrow), Lane 3: Supernatant from alkaline solubility test of Y442H+A522V showing presence of 81kDa band (pointed by green arrow), Lane 4: Supernatant from trypsin activation test of Y442H showing presence of ~55kDa band (pointed by blue arrow), Lane 5: Supernatant from trypsin activation test of A522V showing presence of ~55kDa band (pointed by pink arrow), Lane 6: Supernatant from trypsin activation test of Y442H+A522V showing presence of ~55kDa band (pointed by gray arrow), Lane 7: Protein molecular weight marker

**Toxicity testing of Cry1Ie, Y442H, A522V and Y442H+A522V against sensitive population of *Plutella xylostella* (G88).**

The toxicity of the expressed mutants Y442H, A522V and Y442H+A522V were tested against the susceptible population of *Plutella xylostella* (G88) using the method described in section 2.2.13.2 and the results are as shown in table 3.6. To determine the lethal concentrations of the toxins that are able to cause 50% mortality, three different concentrations for each toxin were used in duplicate to conduct the assay and the assays were repeated for at least four times until reproducible results were obtained. For Cry1Ie, the three concentrations used were 0.15, 0.25 and 0.35µg/ml while 0.18, 0.6 and 1.8µg/ml were for Y442H mutants. The concentrations used for A522V were 0.1, 0.8 and 1.8µg/ml and that of the double mutant Y442H+A522V were 0.1, 0.8 and 1.8µg/ml.

Toxins	LC <sub>50</sub> (µg/ml)	95% Confidence limits
Cry1Ie	0.319	0.267-0.466
Y442H	0.420	0.295-0.582
A522V	0.386	Not determined
Y442H+A522V	0.401	0.281-0.551

Table 3.6: Toxicity testing of Cry1Ie, Y442H, A522V and Y442H+A522V against G88

### **3.3 Discussion**

The expression of the open reading frame of wild type *cry1Ie* under the control of the native *cry1Ac*-promoter was successful as the protein band of approximately 80kDa was seen on SDS-PAGE gel. The characterization of the expressed protein also showed that it folded properly as it was found to be soluble, though sparingly, in alkaline solution of Na<sub>2</sub>CO<sub>3</sub> at pH11. Its activation by trypsin resulted in approximately 55kDa band on SDS-PAGE gel and its toxicity to *Plutella xylostella* gave an LC<sub>50</sub> of 0.319 µg/ml (95% confident limit

0.267-0.466) which is comparable to the LC<sub>50</sub> of 0.20µg/ml of the fusion form expressed by Song *et al*, 2003.

The strategy used in producing the truncated Cry1Ie known as Cry1Ie<sub>tr</sub> was found to be successful at the expression stage as the expected protein band of approximately 73kDa was seen on SDS-PAGE gel. The characterisation of the expressed truncated Cry1Ie protein revealed that the protein probably did not fold properly as it failed to demonstrate the typical characteristics of Cry proteins. It was found that it was not soluble in alkaline solution of Na<sub>2</sub>CO<sub>3</sub> and the protease resistant core of 50-60kDa band was not seen on SDS-PAGE gel when supernatant from its protease activation experiment was analysed. The SDS-PAGE analysis of the total reaction mix from the protease activation experiment shows that the protein was completely digested to fragments that could not be detected on a gel thus failing to produce the resistant core of approximately 50-60kDa. Though the full length Cry1Ie protein was sparingly soluble in alkaline solution of Na<sub>2</sub>CO<sub>3</sub> it gave the trypsin resistant core of approximately 55kDa and was toxic to *Plutella xylostella*. Cry1Ie<sub>tr</sub> on the other hand could not be solubilised or activated and it was not toxic to *Plutella xylostella*. Susceptible insect midgut proteases Cry toxin activation has been shown to be an important step that prepares a protoxin for activity against susceptible insects. Yamaguchi *et al.*, (2010) showed that Cry8Da toxin was only able to bind to brush border membrane vesicles of Japanese beetles (*Popillia japonica* Newman) when activated using midgut juice extracted from the beetles. In a Cry1Ac resistant population of *Helicoverpa armigera*, it was observed that there was a down regulation of HaSP2 (*Helicoverpa armigera* serine protease 2) and that the protease resistant core of 95 and 68kDa was observed when the protease extracted from this resistant population was used in activating Cry1Ac (Rajagopal *et al*, 2009) which underscores the importance of proper protease activation to toxin toxicity. In the case of Cry1Ie<sub>tr</sub>, its impotency to susceptible and Cry1Ac resistant population of *Plutella xylostella* is not a case of the insects midgut protease regulation but more likely as a result of its improper folding. Adamo *et al*, 2000 demonstrated that the deletion of the first six N-terminal segment of h4 plasma membrane Ca<sup>2+</sup> pumps did not affect its activity but the mutants in which 15-75 amino acid residues were deleted from the N-terminal reduced its activity to undetectable levels. Also

Chow *et al*, 2003 showed that the secondary and tertiary structure of apomyoglobin (apoMb) is dependent on its length and that at short length, non-native  $\beta$ -sheet conformation and self-associated amyloid-like species were generated while as the length increases,  $\alpha$ -helix structures gradually takes over. With the length of a protein directly affecting its folding and properties, it is reasonable to conclude that the deletion of Cry1Ie from amino acid position 2-75 has adversely affected its folding and structure which results in the generation of a non-functional protein.

The genetically manipulated CryIAI which is a hybrid protein made by swapping of domain II of Cry1Ie with that of Cry1Ah did not result in expression of the protein that it codes for. Though all the upstream elements like the *cry1Ac* promoter, *cyt1A*-promoter, ribosome binding sites that have been successfully used in expressing the parent genes of *cry1Ie* and *cry1Ah* were supplied, it still did not express. *cyt1A*-promoter has been used in the expression of Cry2A (Crickmore and Ellar, 1992) while *cry1Ac*-promoter has been used in expression of Cry11A with *Bacillus brevis* as host (Roh *et al*, 2010). The use of different hosts and varying of culturing conditions like temperature did not rescue the expression of *cryIAI* and this could be as result of the fact that the hybrid gene created cannot express a functional protein. Reports have also shown that a nonsense and missense mutation in *Chlamydia trachomatis* serovar L2 and D strains results in the formation of pseudogenes (Giles *et al*, 2009). The non-expression of the *cryIAI* hybrid can also be that the protein it is coding for is toxic to the cell even at level that could not be detected on SDS-PAGE though this has not been confirmed. Kemble *et al*, 2006 demonstrated that Protein tyrosine kinase Src are toxic to bacteria cells and creates a non-toxic mutant that could be expressed in bacterial host.

Synergistic studies between Cry1Ie and Cry1Ac showed that there is no synergism between them. With individual  $LC_{50}$  of 0.319 and 0.037 for Cry1Ie and Cry1Ac respectively, and the use of their combination at individual doses that will result in lethality below  $LC_{50s}$ , a synergistic effect could not be observed. Synergism has been variously reported between Cry toxins including Cry1Ab and Cry1Ac (Sharma *et al*, 2010), Cry1Ac and Cry2Ab (Ibargutxi *et al.*, 2008) and Cry1Ac and Cry2Aa (Yunus *et al.*, 2011). It has also been reported between Cry toxins and Cyt toxins including Cry4Ba and Cyt1Aa (Canton *et al*,

2011) and Cry10Aa and Cyt1Aa (Hernandez-Soto *et al*, 2009). Ibargutxi *et al*, 2008, suggested that the synergism observed between Cry1Ac and Cry2Ab was as a result of special interaction between them while Canton *et al*, 2011 showed that synergism between Cry11Aa and Cyt1Aa results from specific interaction between them and created a mutant Cyt1Aa that results in reduced synergism. The absence of synergism between Cry1Ie and Cry1Ac might be that these specific interactions is also absent.

The sequence information obtained from the sequencing of Cry1Ie mutants Y442H, A522V and Y442H+A522V showed that they were all successfully engineered at the DNA level and SDS-PAGE of total proteins from each of the mutants showed that the proteins were all expressed. Alkaline solubility and protease activation characterisation of the expressed mutants showed that they still posses characteristics typical of Cry toxins which means that they were properly expressed and folded. The toxicity testing of the mutants did not show any significant difference in toxicity improvement compared to the wild type Cry1Ie. Point mutations have been shown to enhance the toxicity of Cry toxins like Cry1Aa (Lebel *et al*, 2009), Cry3A (Wu *et al*, 2000) and these has been effected by studying the gene carefully and determining where to effect the mutation but the Cry1Ie mutants created here was as a result of random mutation that was effected through an error prone PCR (Liu *et al*, 2010 – unpublished data). Bioinformatics analysis of wild type Cry1Ie and the mutants Y442H, A522V and Y442H+A522V showed that the mutated points fall on regions that have been shown to be involved in toxin binding to brush border membrane vesicles of a susceptible host (Fernandez *et al*, 2005, Gomez *et al*, 2006, Atsumi *et al*, 2008, Obata *et al*, 2009) Though the mutations Y442H, A522V and Y442H+A522V did not affect the stability of their expressed proteins, it did not however result in any substantial increase in toxicity. The inability of the mutants to show remarkable increase in toxicity might be due to the fact that the replacements were very conserved for Y442H mutant and moderately conserved for A522V thus making no difference (Berg *et al*, 2006). On the other hand, it might be that the mutated regions are not involved in Cry1Ie binding to receptors in the gut of *Plutella xylostella*.

The results from this chapter show that *cry1Ac*-promoter is capable of driving the expression of a functional Cry1Ie toxin and its mutants except CryIAI which

might be as result of the fact that the manipulated toxin could not fold correctly. The truncation of Cry1Ie from amino acid position 2 to 75 did not produce a functional protein as bioassay against sensitive population of *Plutella xylostella* showed no sign of toxicity at concentration as high as 100µg/ml. The non toxicity of Cry1Ie is likely to be as a result of misfolding as characterisation of the expressed protein fall short of those of typical Cry toxin. Moreover, the mutants Y442H, A522V and Y442H+A522V though properly expressed were not of any significant improvement in toxicity to *Plutella xylostella* compared to the wild type Cry1Ie as the amino acid substitutions were conservative. The inconsistency between the toxicity of Y442H and A522V observed here compared to the preliminary finding of Liu *et al* could be as a result of our using a different population of *Plutella xylostella* for the toxicity assays or it could be that their preliminary results were not valid.

## Chapter 4: Expression, manipulation and characterization of Cry1Ah toxin

### 4.1 Introduction

Cry1Ah1 is a 134KDa protein cloned from *Bacillus thuringiensis* BT8 by Xue, *et al.*, 2008. It was successfully expressed in an acrySTALLIFEROUS mutant of *Bacillus thuringiensis* HD73 and found to be highly toxic against Lepidopteran larvae of *Ostrinia furnicalis*, *Helicoverpa amigera*, *Chilo suppressalis* and *Plutella xylostella* with LC<sub>50</sub> values of 0.05, 1.48, 0.98 and 1.52 µg/ml respectively.

A resistant strain of *Ostrinia furnacalis* (ACB-AbR) originally selected in the laboratory with Cry1Ab also developed resistance to other Cry1-toxins including Cry1Ah which it had not been previously exposed but was not resistance to Cry1Ie. The cross-resistance level observed with Cry1Ah was 131-fold, Cry1Ac 36-fold and Cry1F 6-fold (Xu *et al.*, 2010).

The development of cross-resistance among these toxins was not surprising because pair wise sequence alignment between Cry1Ab1 and Cry1Ac1, Cry1Ab1 and Cry1Ah1, and Cry1Ab1 and Cry1Fa1 using ClustalW programme (Larkin *et al.*, 2007) revealed a high level of identity of 87%, 82% and 71% respectively. Moreover, the 3-D crystal structures of representative Cry toxins (Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Ba and Cry8Ea1) display a high level of similarity with a three-domain organization.

With the successful mapping of epitopes in domain II and III involved in Cry1A binding to midgut receptors of susceptible insects (Liang and Dean, 1994, Gomez *et al.*, 2006), and also with the identification of midgut molecules that interact with these toxins (Abdullah *et al.*, 2006), exchange of toxin domains and segment have been used in creating novel toxins with improved properties (Nakamura, 1990, de Maagd *et al.*, 1996, Naimov, *et al.*, 2001).

Synergistic effects resulting from the combination of *Bacillus thuringiensis* spores and Cry1A toxins (Johnson and McGuaghey, 1996), different crystal toxins including Cry1A toxins (Sharma *et al.*, 2010), crystal and cytolytic toxins (Hernandez-Soto *et al.*, 2009), crystal proteins and plant substances (Anilkumar *et al.*, 2009), crystal toxins and cadherin receptor fragments from insects (Chen *et al.*, 2007, Park *et al.*, 2009) have been shown and these have been exploited in boosting the toxicity of Cry proteins and overcoming insect resistance.

Other modifications of Cry toxins like truncation of part of the N-terminal (Franklin *et al.*, 2009, Muñoz-Garay *et al.*, 2009), creation of hybrid toxins (Xia *et*

*al*, 2009) and site directed mutagenesis (Wu and Aronson, 1992, Rajamohan *et al*, 1996, Wu *et al*, 2000, Padilla *et al*, 2006, Wang *et al*, 2008) have proven to be effective in boosting the toxicity of Cry toxins including Cry1A toxins and help in fighting resistance.

In this chapter, the expression of Cry1Ah toxin in *E. coli* and IPS 78/11 (an acrySTALLIFEROUS *Bacillus thuringiensis*) hosts, its toxicity against susceptible and resistance populations of *Plutella xylostella* will be presented. In addition, the manipulation of Cry1Ah at the genetic level to produce a mutant toxin lacking helix -1 and swapping of its domains I or II with that of Cry1Ie will also be covered. Moreover, the synergistic studies conducted between Cry1Ah and Cry1Ie against *Plutella xylostella* will be presented.

## **4.2 Results**

### **4.2.1 Construction of plasmid for expression of Cry1Ah in *E. coli***

To construct the plasmid expressing Cry1Ah in *E. coli*, the DNA fragments needed are the coding sequence for *cry1Ah* and a vector with all the expression vector elements like selection marker, *E. coli* origin of replication, ribosome binding site and a promoter that is able to drive the expression of Cry1Ah. Jie Zhang of Institute of Plant Protection, Beijing, China, supplied the coding sequence of *cry1Ah* in a plasmid pSXY422-1Ah as shown in figure 4.1 while Neil Crickmore supplied the expression vector pGEM1AcP1Ac. The vector pGEM1AcP1Ac is a vector designed to express Cry1Ac in *E. coli* hosts and it has the native promoter of *cry1Ac* and coding sequence of *cry1Ac* with pGEM-T vector backbone.

Primer pairs GEMF- 5' TCTCATGCAAACCTCAGGTTTAA 3'/GEMR- 5' AAGTTACCTCCATCTCTTTTATTTAAG 3' were designed using PRIMER SELECT programme to amplify the required fragment of PGEM-T vector (Promega) backbone, native *cry1Ac*-promoter and ribosome binding site hereafter designated as pGEM1AcP from pGEM1AcP1Ac. Primer pairs AF- 5' ATGGAGATAGTGAATAATCAGAATC 3' and AR- 5' CTATTCCTCCATAAGGA-GTAATTC 3' were also designed to amplify *cry1Ah* open reading frame from pSXY422-1Ah as shown in figure 4.2.

Using Pfu Ultra Hotstart PCR Master Mix system according to the manufacturers amplification instructions, pGEM1AcP and *cry1Ah*, were



amplified and PCR products were ran on 1% agarose gel and required bands of 3566bp (pGEM1AcP) and 3486bp (*cry1Ah*) were excised and purified according to the procedure described in section 2.2.3 for the purification of DNA from agarose gel. Pfu Ultra Hotstart PCR Master Mix was PCR kit of choice because of Pfu polymerase high fidelity property and ability to proof read and produce a blunt ended PCR product.

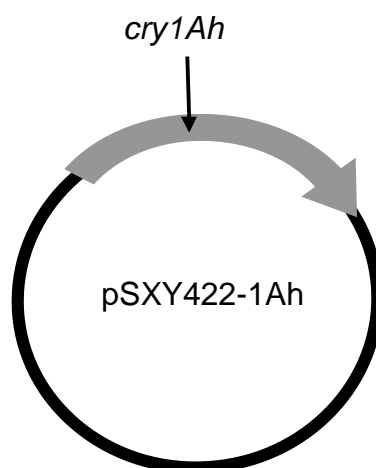
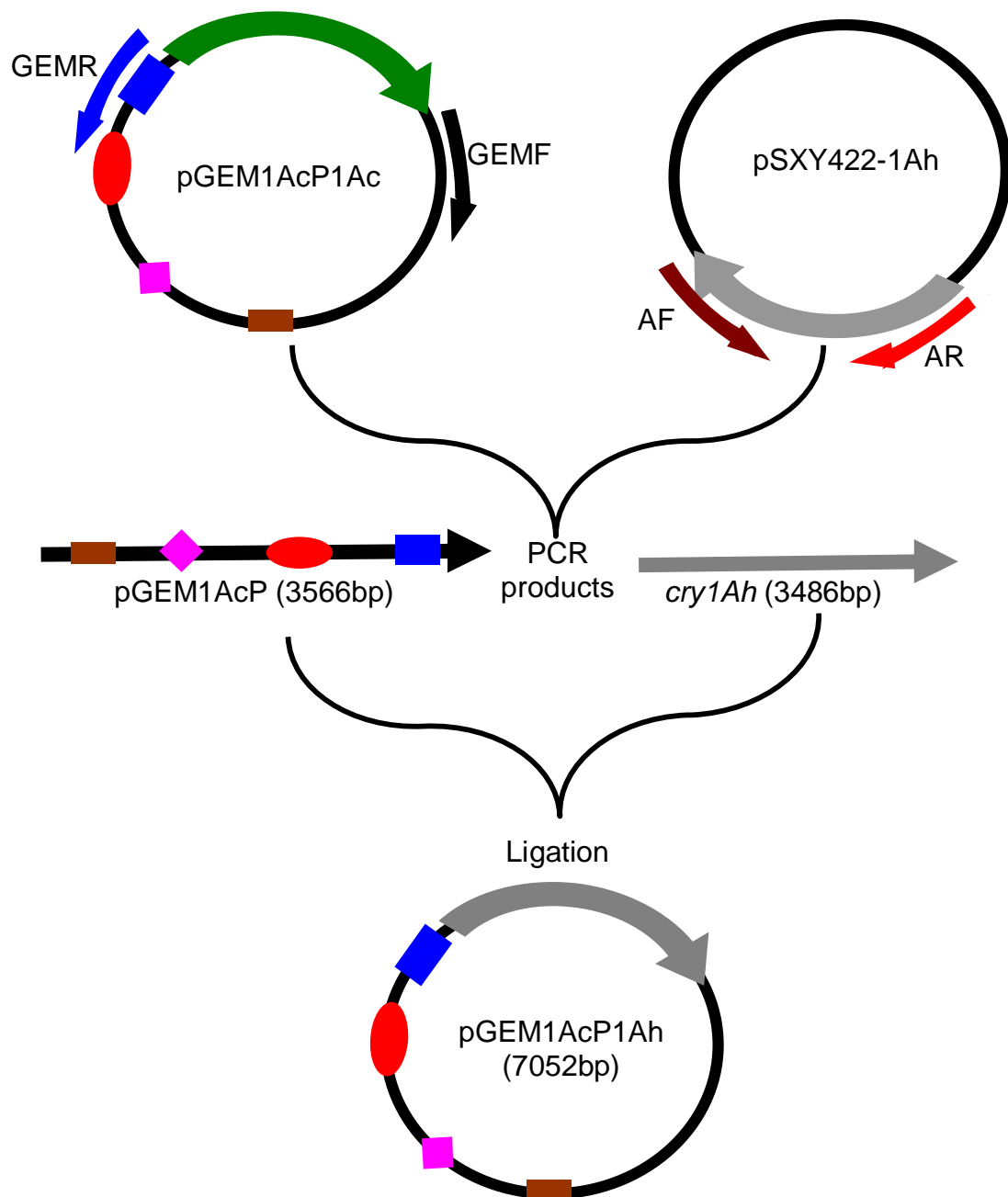


Figure 4.1: Schematic representation of the plasmid pSXY422-1Ah

## Construction of pGEM1AcP1Ah



**Figure 4.2:** Schematic for construction of plasmid pGEM1AcP1Ah

- *cry1Ac* ribosome binding site
- *cry1Ac* promoter
- ◆ Origin of replication in *E. coli*
- Ampicillin resistance gene

To construct the plasmid pGEM1AcP1Ah, the following volumes of reagent and DNA fragments as shown in table 4.1 were measured and mixed together.

Components	Volumes
H <sub>2</sub> O	-
pGEM1AcP	1µl
Ligation buffer	5µl
<i>cry1Ah</i>	4µl
T <sub>4</sub> Ligase	1µl

**Table 4.1:** Recipe of ligation mix for pGEM1AcP1Ah

The ligation mix was incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 was transformed with the ligation mix and many transformants were screened to check for colonies harbouring pGEM1AcP1Ah. The correct orientation of *cry1Ah* was checked by digesting extracted plasmid with *EcoRI* and running on 1% agarose gel to check for required bands of 2997bp, 2841bp, 1164bp and 50bp which was successful as all the expected bands were seen on gel as shown in figure 4.4 except the 50bp which could not be detected as it is too small.

#### **4.2.2 Construction of plasmid for expression of Cry1Ah in *Bt***

##### **Construction of pSVP271AcRBS1Ah**

Plasmid pSVP27 is a vector for gene expression in *Bt* under the control of *cyt1A*-promoter constructed by fusing a sequence containing *cyt1A*-promoter to pSV2 and incorporating multiple cloning sites including *Bam*HI downstream of the promoter (Crickmore and Ellar, 1992).

To construct plasmids pSVP271AcRBS1Ah, aimed at expressing *cry1Ah* gene under the control of native *cyt1A*-promoter in acrySTALLIFEROUS *Bt*, DNA fragments used were *Bam*HI linearized pSVP27 and sequence containing *cry1Ac*-Ribosome binding site/*cry1Ah* gene with *Bam*HI overhangs.

Sequence of *cry1Ac*-Ribosome binding site/*cry1Ah* gene was obtained from the plasmid pGEM1AcP1Ah previously constructed. Primer pairs RAhF 5' aatgGatCCgtatcttaataaaagagatgg/RAhR 5' gatattGGaTcctgagtttgcagag 3' were designed with base changes as shown with capital letters to introduce *Bam*HI sites at 5' and 3' ends using PRIMER SELECT programme.

Using High Fidelity PCR Master system from Roche according to the manufacturers amplification instructions, needed fragment was amplified from pGEM1AcP1Ah plasmid and the PCR product was run on 1% agarose gel and required band of 3553bp was excised and purified according to the PCR products purification procedure described in section 2.2.3. The PCR product was first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release 1AcRBS1Ah fragment with *Bam*HI overhangs.

pSVP27 plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase. The phosphatase was included in the digestion mix to dephosphorylate the phosphate group at the 5' ends of the linearised pSVP27 DNA fragment thus preventing it from re-ligation. The linearised pSVP27 (5588bp) was run on 1% agarose gel and its band excised and purified.

The gel purified DNA fragments of linearised pSVP27 and 1AcRBS1Ah were ligated according the recipe outlined in table 4.2 and the schematic for the construction of the plasmid pSVP271AcRBS1Ah is as shown in figure 4.3.

Components	pSVP271AcRBS1Ah
H <sub>2</sub> O	4.0µl
pSVP27	0.5µl
1AcRBS1Ah	4.0µl
10x Ligase buffer	1.0µl
T <sub>4</sub> DNA ligase	0.5µl

Table 4.2: Recipe for ligation of DNA fragments to form plasmid pSVP271AcRBS1Ah

The ligation mix was incubated at room temperature for 3 hours and then stored at 4°C overnight to enhance ligation.

*E. coli* JM109 was transformed with the ligation mix and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pSVP271AcRBS1Ah. Colonies whose plasmid size on agarose gel were higher than that of pSVP27a which was used as control, had the potential of having the insert 1AcRBS1Ah. Plasmid DNA were extracted from clones with insert and the correct orientation of 1AcRBS1Ah was confirmed by digesting extracted plasmid with *EcoRI* and checking for required bands of 7412bp and 1697bp as shown in figure 4.4. AcrySTALLiferous strain of *Bt* IPS 78/11 was transformed with the confirmed plasmid according to the method in section 2.2.3.2. Successful transformation of IPS 78/11 was confirmed by extracting plasmids from IPS 78/11 clones, retransforming *E. coli* JM109 with the extracted plasmid and then minipreped and digest extracted plasmids from transformed *E. coli* JM109 alongside the original plasmid.

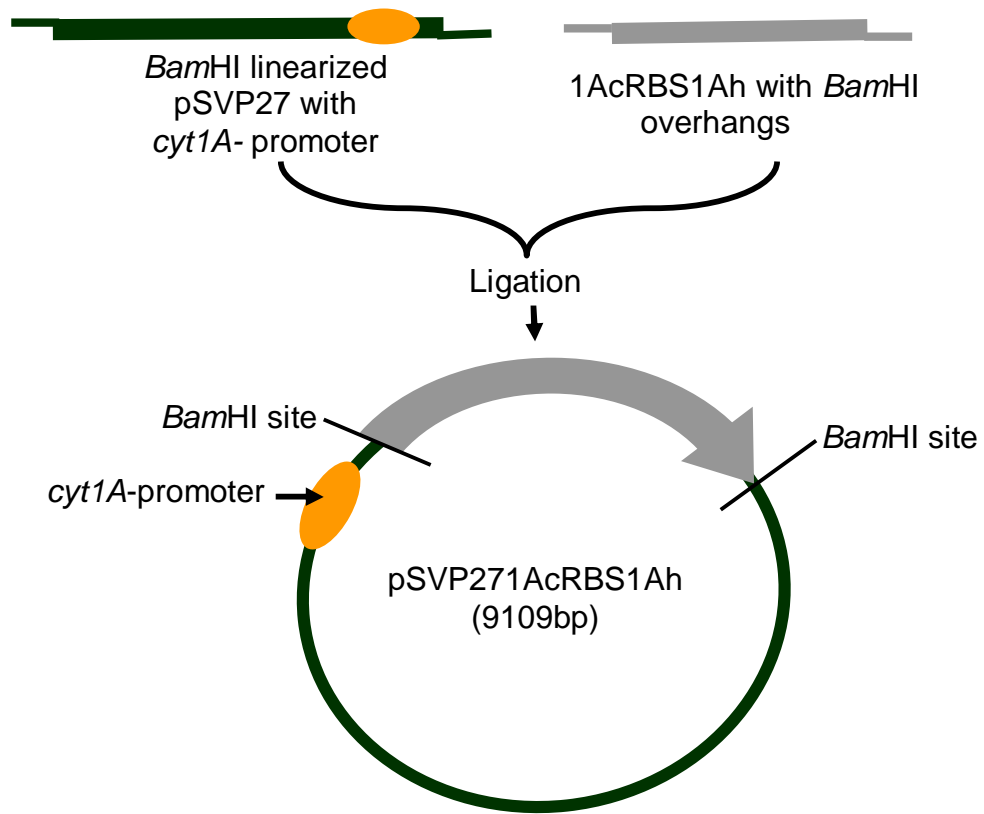
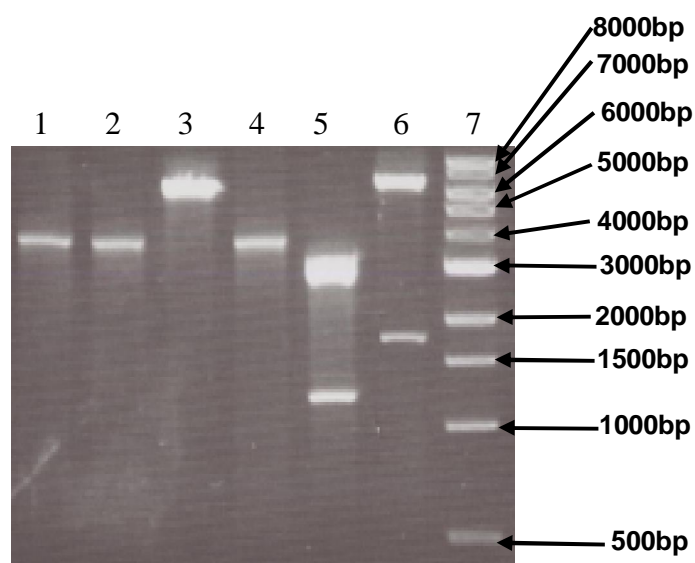


Figure 4.3: Schematic showing the construction of the plasmid pSVP271AcRBS1Ah



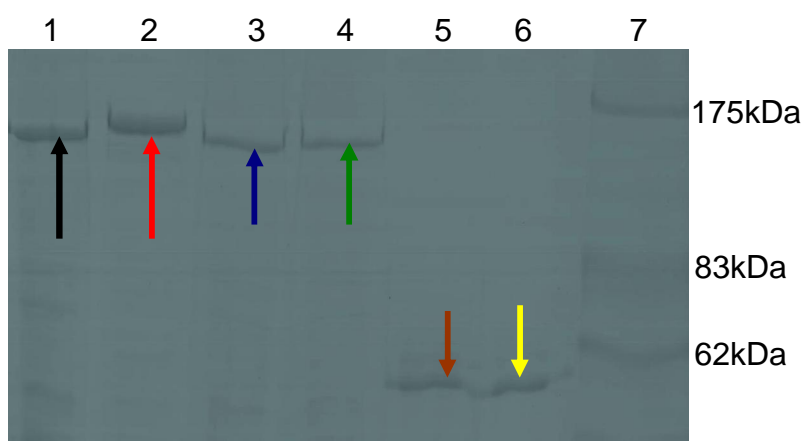
**Figure 4.4:** Gel picture showing the purified PCR fragments of *cry1Ah*, pGEM1AcP, purified *Bam*HI linearized pSVP27, purified 1Ac-Ribosome binding site/*cry1Ah* with *Bam*HI overhangs, *Eco*RI digested pGEM1AcP1Ah and pSVP271AcRBS1Ah. Lane 1: 3486bp *cry1Ah* band, Lane 2: 3566bp pGEM1AcP band, Lane 3: 5588bp *Bam*HI linearized pSVP27 band, Lane 4: 3521bp 1AcRBS1Ah with *Bam*HI overhang band, Lane 5: *Eco*RI digestion of pGEM1AcP1Ah showing 2997/2841bp and 1164bp bands, Lane 6: *Eco*RI digestion of pSVP271AcRBS1Ah showing 7412bp and 1697bp bands and Lane 7: DNA marker

#### 4.2.3 Expression and characterisation of Cry1Ah protein

The strains of *E. coli* JM109 harbouring the plasmid pGEM1AcP1Ah were grown in a 2x Luria Broth for 3 days at 37°C after which the total protein were harvested as described in section 2.2.8.1. The total protein was ran on 7.5% SDS-PAGE gel and the protein band of ~134kDa was seen on gel as shown in figure 4.5. Also, the clone of IPS 78/11 harbouring the plasmid pSVP271AcRBS1Ah was grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates of 25ml Luria agar with 5µg/ml concentration of chloramphenicol

were inoculated 1ml each with the 16 hour culture of the clone and grown at 30°C for 5 days. Total protein was harvested as described in section 2.2.8.3 and ran on 7.5% SDS-PAGE gel and the protein band of ~134kDa was seen on gel as shown in figure 4.5.

The expressed proteins were tested for alkaline solubility and protease activation according to the method in sections 2.2.9.1 and 2.2.9.2. The alkaline solubility test showed that both the *E. coli* JM109 and *Bt* IPS 78/11 expressed Cry1Ah were soluble in 50mM Na<sub>2</sub>CO<sub>3</sub> pH11 while digestion with trypsin (a protease) gave a ~60kDa resistant core on SDS-PAGE gel as shown in figure 4.5.



**Figure 4.5:** Gel picture of Cry1Ah expressed in *E. coli* and *Bt* and their pH11 alkaline solubility and trypsin activation. Lane 1: *E. coli* JM109 expressed Cry1Ah (black arrow), Lane 2: IPS 78/11 expressed Cry1Ah (red arrow), Lane 3: Na<sub>2</sub>CO<sub>3</sub> solubilised *E. coli* expressed Cry1Ah (blue arrow), Lane 4: Na<sub>2</sub>CO<sub>3</sub> solubilised IPS 78/11 expressed Cry1Ah (green arrow), Lane 5: Trypsin activated core from *E. coli* expressed Cry1Ah (brown arrow), Lane 6: Trypsin activated core from IPS 78/11 expressed Cry1Ah (yellow arrow) and Lane 7: Protein molecular weight marker



#### **4.2.4 Toxicity testing of Cry1Ah against populations of *Plutella xylostella***

The potency of the *E. coli* JM109 and *Bt* IPS 78/11 expressed Cry1Ah against artificial diet reared susceptible population of *Plutella xylostella* (G88) and cabbage reared Cry1Ac resistant population of *Plutella xylostella* (KARAK) was conducted using the diet dip assay and leaf dip assay as described in sections 2.2.13.2 and 2.2.13.1. Using the leaf dip method, three doses 49, 51 and 53µg/ml of the *E. coli* JM109 expressed Cry1Ah were used in bioassay against resistant *Plutella xylostella* population and the assays were repeated at least four times until a reproducible result was obtained. The doses of the *E. coli* JM109 expressed Cry1Ah used in bioassay with the sensitive G88 population were 1.0, 1.5 and 2.5µg/ml while the doses of the *Bt* IPS 78/11 expressed Cry1Ah used in bioassay against G88 populations were 1.0, 1.5 and 2.5µg/ml. From the assays, it was found that the *E. coli* JM109 expressed and the *Bt* IPS 78/11 expressed proteins had an LC<sub>50</sub> of 1.417µg/ml (95% confidence limit 0.867-1.917µg/ml) and 1.502µg/ml against G88 respectively. The *E. coli* JM109 expressed Cry1Ah had an LC<sub>50</sub> of 50.620µg/ml against KARAK with 95% confidence limits of 48.932-51.760µg/ml.

#### **4.2.5 Synergistic studies between Cry1Ah and Cry1Ie toxins**

To check for synergism between Cry1Ah and Cry1Ie toxins, a single concentration of each toxin was combined and used in assaying for the mortality of the exposed insect alongside assays that involve only the single concentration of an individual toxin. Considering that the LC<sub>50</sub> of Cry1Ah and Cry1Ie against G88 population of *Plutella xylostella* was 1.417µg/ml and 0.319µg/ml respectively, a concentration of 0.4µg/ml for Cry1Ah and 0.15µg/ml for Cry1Ie was used in combination and singly to conduct bioassay against G88. These concentrations were chosen such that they were below the LC<sub>50</sub> of each of the toxins but also able to affect mortality when used individually.

Using the diet dip assay method described in 2.2.12.2, solutions containing a combination and individual concentrations of the toxins were made and used in bioassay against G88.

On the fifth day, the number of death and live larvae were counted and recorded as shown in table 4.3

Single or combination of Cry1Ah and Cry1Ie toxins	Number of insect dead out of 20	Number of live insects out of 20	Total number of insects exposed
Cry1Ah (0.4µg/ml)	10 (50%)	10 (50%)	20
Cry1Ie (0.15µg/ml)	5 (25%)	15 (75%)	20
Cry1Ah (0.4µg/ml) and Cry1Ie (0.15µg/ml)	13 (65%)	7 (35)	20

**Table 4.3:** The number of dead and live larvae from the synergistic studies between Cry1Ah and Cry1Ie toxins

The results presented in table 4.3 shows that there is no synergism between Cry1Ie and Cry1Ac because judging from the fact that Cry1Ah on its own kills 50% and Cry1Ie on the other hand kills 25% on its own, it would have been expected that synergism will result in killing of more than 75% (the sum of the percentage mortality from individual toxins). The 65% mortality observed with the mixture is lower than what would be expected even if the effect with mixture were to be additive which shows that there is no synergism.

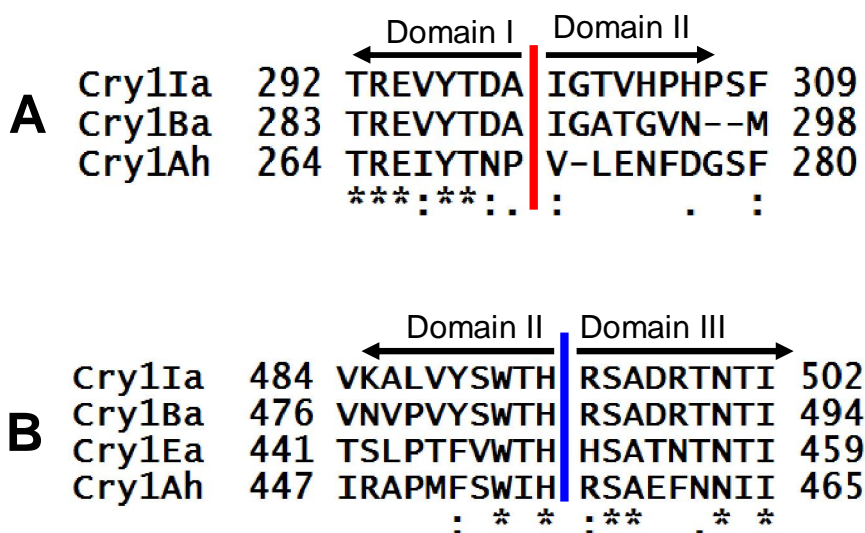
#### **4.2.6 Manipulation of Cry1Ah for improved activity**

##### **4.2.6.1 Swapping domain II of Cry1Ah with domain II of Cry1Ie and domain I of Cry1Ah with domain I of Cry1Ie**

##### **Construction of pGEM1AcPAIA and pGEM1AcPIAA plasmids**

The research findings that regions in domain II and III of Cry toxins are the determinants of the species of insects a toxin is toxic against (Nair *et al*, 2008 and Liu and Dean, 2006) and the level of toxicity (de Maagd *et al*, 2000) was the basis for the swapping of domains between Cry1Ah and Cry1Ie. Moreover, it has been reported by Xu *et al.*, 2010 that there was no cross resistance to Cry1Ie by a Cry1Ah resistant population of *Ostrinia furnacalis* (ACB-AbR) and furthermore Cry1Ah has been shown to have a broader spectrum of activity compared to Cry1Ie (Song *et al.*, 2003, Xue *et al.*, 2008). Considering the broad spectrum activity of Cry1Ah and the sensitivity of ACB-AbR to Cry1Ie, the

domain swapping was aimed at producing a hybrid toxin that is able to overcome the resistance posed by a Cry1Ah resistant population of *Plutella xylostella* (KARAK) while capturing the broad spectrum activity of wild type Cry1Ah. The plasmid pGEM1AcPAIA was designed to express a hybrid Cry-protein carrying domain I of Cry1Ah, domain II of Cry1Ie and domain III of Cry1Ah hereafter known as CryAIA while the plasmid pGEM1AcPIAA was designed to express a hybrid Cry-protein carrying domain I of Cry1Ie and domain II and III of Cry1Ah. The domain borders were mapped out by multiple sequence alignment of Cry1Ah protein sequence using ClustalW programme (Larkin *et al*, 2007) with Cry1-toxin sequences viz: Cry1Ia, Cry1Ba and Cry1Ea (Naimov *et al*, 2001) in which their domain borders have already been determined as shown in figure 4.6.



**Figure 4.6:** Multiple sequence alignment of segments of Cry1Ah, Cry1Ia, Cry1Ba, and Cry1Ea protein sequences using ClustalW software.

A: The red line demarcates between domain I and domain II.

B: The blue line demarcates between domain II and domain III.

After mapping out the domain borders, primer pair 1leDIIF 5' ATTGGGACA-GTACATCCAAATG 3'/1leDIIR 5' ATGTGTCCAAGAATATACCAATGC 3' were designed to amplify domain II of *cry1le*. The primers were modified by addition of phosphate group to their 5' ends to enhance the ligation of their PCR product. Primer pair GEM1AhF 5' CGTAGTGCTGAATTTAATAATATAATT 3'/GEM1AhR 5' TGGGTTTGTATAAATTTCTCTTG 3' were designed to amplify *cry1Ah* and its vector excluding its domain II. Primer pair GEMF- 5' TCTCATGCAAACCTCAGGTTTAA 3'/GEM1leR 5' TGCGTCTGTATATACTTC-TCTTGTAAG 3' were designed to amplify the plasmid pGEM1AcP1le excluding domain II and III of Cry1le. Also primer pair 1AhDIIF 5' GTATTAGAAAATTT-TGATGGTAGTTTT 3'/AR- 5' CTATTCCTCCATAAGGAGTAATTC 3' were designed to amplify domain II and III of Cry1Ah. The primer pairs were used in conducting PCR and the amplified DNA sequences were gel purified from gel according to the method described in section 2.2.3. The DNA fragments amplified using each primer pair were named as shown in table 4.4.

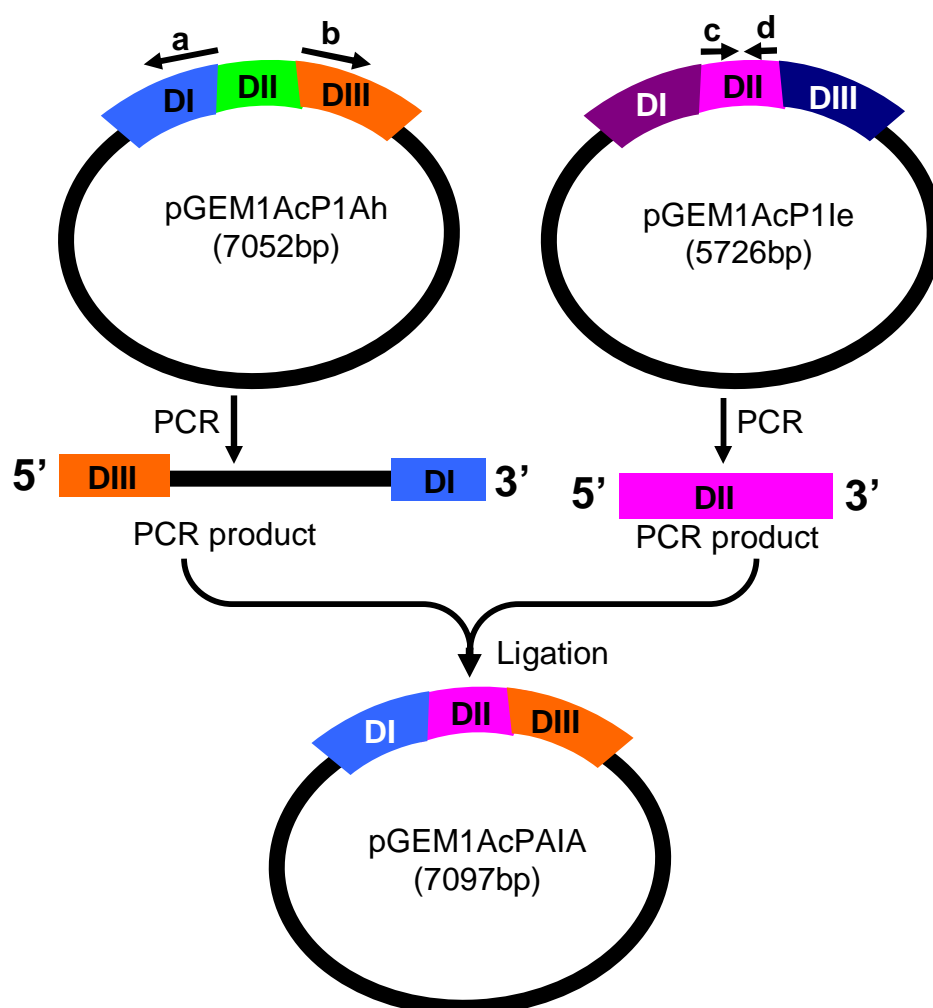
Primer pair	Template	PCR product name	PCR product size (bp)
1leDIIF/1leDIIR	pGEM1AcP1le	1leDII	582bp
GEM1AhF/ GEM1AhR	pGEM1AcP1Ah	GEM1Ah	6497bp
GEMF/ GEM1leR	pGEM1AcP1le	GEM1leDI	5144bp
1AhDIIF/ AR	pGEM1AcP1Ah	1AhDII_III	2673bp

Table 4.4: Tabular presentation of primer pair and their PCR product name and size including the template they are amplified from

The recipe in table 4.5 shows the ligation of the PCR fragments of leDII/GEM1Ah and GEM1leDI/1AhDII\_III to form plasmids pGEM1AcPAIA and pGEM1AcPIAA respectively and figure 4.7 is a schematic for the construction of these plasmids.

Components	pGEM1AcPAIA	pGEM1AcPIAA
H <sub>2</sub> O	3.0µl	3.5µl
GEM1leDI	-	4.0µl
1leDII	0.5µl	-
GEM1Ah	5.0µl	-
1AhDII_III	-	1.0µl
10x Ligase buffer	1.0µl	1.0µl
T <sub>4</sub> DNA ligase	0.5µl	0.5µl

Table 4.5: Recipe for the ligation of DNA fragments to form pGEM1AcPAIA and pGEM1AcPIAA expression vectors

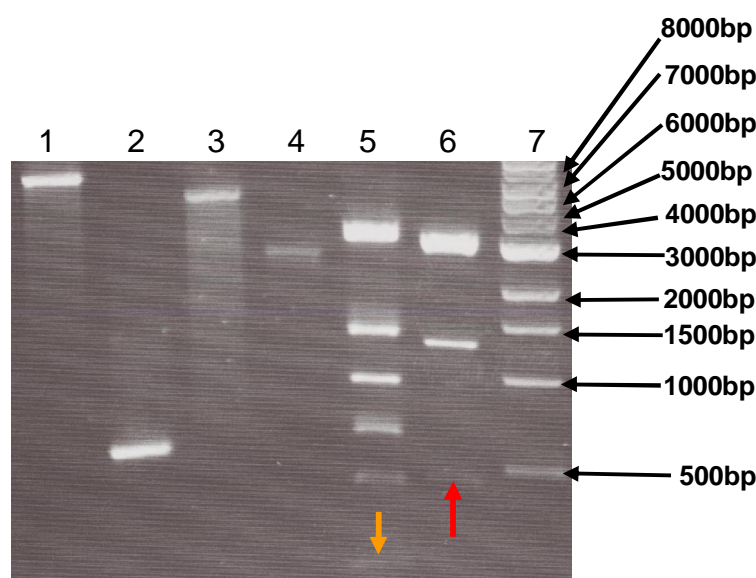


**Figure 4.7:** Schematic illustrating the construction of pGEM1AcPAIA plasmid from 1leDII and GEM1Ahe DNA fragments. The construction of pGEM1AcPIAA is the same except that different domains are swapped. a= GEM1AhR; b= GEM1AhF; c= 1leDIIIF; d= 1leDIIIR; DI= domain I; DII= domain II; DIII= domain III

The ligation mixes were incubated at room temperature for 3 hours and afterward incubated in the refrigerator at 4°C overnight. *E. coli* JM109 were transformed with the ligation mixes by electroporation and the transformants screened for colonies harbouring pGEM1AcPAIA or pGEM1AcPIAA plasmid. The presence of the 1leDII in pGEM1AcPAIA was confirmed by conducting PCR

using the primer pair AF/1leDIIR with pGEM1AcPAIA extracted from transformants as the template while the correct orientation of 1AhDII\_III in pGEM1AcPIAA was confirmed by conducting PCR using IF/1AhDIIR primer pair with extracted pGEM1AcPIAA as template. A further confirmation of construction of pGEM1AcPAIA was by digesting extracted plasmid with *Afl*III while plasmid pGEM1AcPIAA was further confirmed by digesting plasmid extracted from its transformant with *Eco*RI. The expected bands from pGEM1AcPAIA and pGEM1AcPIAA restriction digestion were observed on agarose gel as shown in figure 4.8 except a 50bp band from pGEM1AcPIAA digestion which could not be detected because of its small size.

Computational analysis of the hybrid *cryA*/IA and *cryI*/AA genes using EMBOSS Transeq tool an online programme at the EMBL-EBI website gave a putative protein with 1170 and 1189 amino acid residues respectively. Compute pI/Mw tool (Hughes *et al*, 1993, Bjellqvist *et al*, 1994, Gasteiger *et al*, 2005) which is an online programme at ExPASy website used in computing the molecular weight of the deduced proteins gave their molecular weights to be approximately 132kDa and 134kDa respectively.



**Figure 4.8:** Gel picture showing PCR products GEM1Ah, 1leDII, GEM1leDI, 1AhDII\_III, digestion of pGEM1AcPAIA and pGEM1AcPIAA plasmids to confirm the presence of DNA fragment 1leDII in pGEM1AcPAIA and 1AhDII\_III in pGEM1AcPIAA. Lane 1: 6497bp gel purified GEM1Ah band, Lane 2: 582bp 1leDII fragment, Lane 3: 5144bp GEM1leDI band, Lane 4: 2673bp 1AhDII\_III band, Lane 5: *AfIII* digestion of pGEM1AcPAIA showing 3225bp, 1336bp, 912bp, 614bp, 418/403bp and 171bp (orange arrow), Lane 6: *EcoRI* digestion of pGEM1AcPIAA showing 30093/2997bp, 1248bp and 429bp (red arrow) and Lane 7: DNA marker

### **Expression of CryAIA or CryIAA proteins in *E. coli* JM109**

The clones of *E. coli* JM109 harbouring the plasmid pGEM1AcPAIA or pGEM1AcPIAA were grown in a 2x Luria broth for 3 days at 37°C after which the total proteins were harvested as described in section 2.2.8.1.

The total proteins were run on 7.5% SDS-PAGE gel but the protein band of ~132kDa for CryAIA or ~134kDa for CryIAA was not seen on the gel. When the strains were grown at other temperatures including 25°C and 30°C, a protein band of ~132kDa was seen for strain harbouring pGEM1AcPAIA but the required band of ~134kDa was not seen from strain harbouring pGEM1AcPIAA.



The expressed CryAIA protein was tested for pH11 alkaline solubility and protease activation according to the method in section 2.2.9.1 and 2.2.9.2. The alkaline solubility test showed that expressed CryAIA was not soluble in 50mM Na<sub>2</sub>CO<sub>3</sub> pH 9-11 as the protein band could not be seen on gel when the supernatant was ran. Digestion of CryAIA with trypsin (a protease) did not give a resistant core of ~60kDa on SDS-PAGE gel but showed that the protein was degraded by trypsin as shown in figure 4.9.

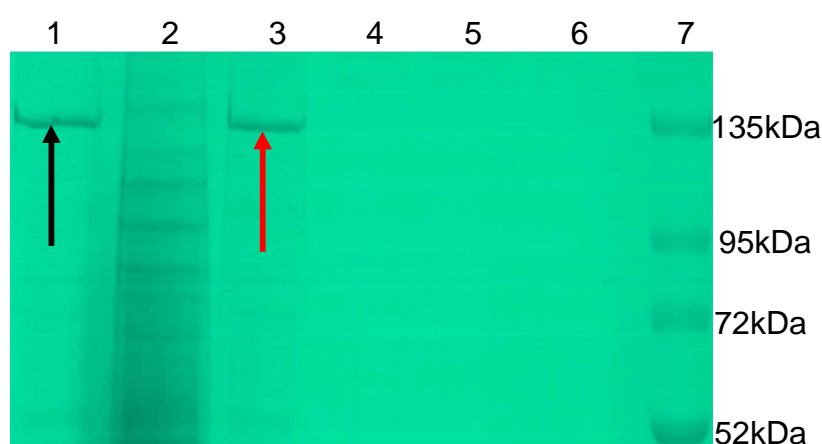


Figure 4.9: SDS-PAGE gel picture of expressed CryAIA, total protein from *E. coli* JM109 strain harbouring plasmid pGEM1AcPIAA, alkaline solubility test and trypsin digestion of CryAIA. Lane 1: Expressed CryAIA protein band (black arrow), Lane 2: Total protein from *E. coli* strain harbouring plasmid pGEM1AcPIAA Lane 3: Total sample from alkaline solubility test of CryAIA at pH11 showing protein band of 132kDa, Lane 4: Supernatant from alkaline solubility of CryAIA at pH11, Lane 5: Supernatant from trypsin activation test of CryAIA, Lane 6: Total sample from trypsin activation test of CryAIA and lane 7: Protein molecular weight marker

### **Toxicity testing of expressed CryAIA**

The toxicity of CryAIA was tested against G88 and KARAK populations of *Plutella xylostella* according to the method described in sections 2.2.13.1 but there was no mortality to either the toxin sensitive G88 or the Cry1Ac resistant KARAK at concentrations as high as 100µg/ml.

### **Attempting expression of CryIAA in *E. coli* BL21 Rosetta**

As the required bands of 134kDa was not seen when the clone of *E. coli* JM109 harbouring the plasmid pGEM1AcPIAA, *E. coli* BL21 Rosetta was then transformed with the plasmid for expression of CryIAA. The plan to express CryIAA in *E. coli* BL21 Rosetta was informed by the fact that some *Bt* toxins had been found to express better in this host (Jie Zhang – personal communication). The strain of *E. coli* BL21 Rosetta harbouring the plasmid pGEM1AcPIAA was grown in a 2x Luria Broth for 3 days at 37°C after which the total protein were harvested as described in section 2.2.8.1.

The total protein was ran on 7.5% SDS-PAGE gel and the protein band of ~134kDa for CryIAA was not seen on gel. As the required band of ~134kDa was not seen when the clone was grown at 37°C, it was then grown at other temperatures including 20°C, 25°C and 30°C. Total protein was harvested from each set of culture and analysed on SDS-PAGE gel but again the required band was not seen (data not shown).

### **Attempting expression of *cryIAA* gene utilising native *cry1Ac*-promoter with *Bt* shuttle vector backbone pSV2 and IPS 78/11 acrySTALLIFEROUS *Bt* as host**

To construct plasmid pSV21AcPIAA aimed at expressing *cryIAA* gene under the control of native *cry1Ac*-promoter in acrySTALLIFEROUS *Bt*, DNA fragments used were *Bam*HI linearized pSV2 and sequence of native *cry1Ac*-promoter/*cryIAA* gene with *Bam*HI overhangs.

Sequence of native *cry1Ac*-promoter/*cryIAA* gene was obtained from the plasmid pGEM1AcPIAA previously constructed. Primer pairs 1AcPF 5' gagctcggATcccaacaccctgg 3'/1AcPR 5' gatattGGaTcctgagttgcatgag 3' were designed with base changes as shown with capital letters to introduce *Bam*HI sites at 5' and 3' ends using PRIMER SELECT programme.

Using High Fidelity PCR Master system from Roche according to the manufacturers amplification instructions, needed fragment was amplified from pGEM1AcPIAA plasmid and the PCR product was ran on 1% agarose gel and required band of 3763bp was excised and purified according to the PCR products purification procedure described in section 2.2.3. The PCR product was first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release 1AcPIAA with *Bam*HI overhangs. High Fidelity PCR Master system was used because of its high fidelity and the production of PCR products with 3' A-overhangs which aid cloning with the pGEM-T vector which has 3' T-overhangs. Cloning the PCR products into PGEM-T was desirable because it allows for a proper digestion and release of the needed fragments.

pSV2 plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase. The phosphatase was included in the digestion mix to dephosphorylate the phosphate group at the 5' ends of the linearised pSV2 DNA fragment thus preventing it from self ligation. The linearised pSV2 was ran on 1% agarose gel and its band excised and purified according to the procedure described in section 2.2.3.

The gel purified DNA fragments of linearised pSV2 and 1AcPIAA were ligated according the recipe outlined in table 4.6 while figure 4.10 is a schematic showing the construction of the plasmid pSV21AcPIAA.

Components	pSV21AcPIAA
H <sub>2</sub> O	5.0µl
pSV2	0.5µl
1AcPIAA	3.0µl
10x Ligase buffer	1.0µl
T <sub>4</sub> DNA ligase	0.5µl

Table 4.6: Recipe for ligation of DNA fragments to form plasmid pSV21AcPIAA

The ligation mix was incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 was transformed with the ligation mix and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pSV21AcPIAA. Colonies whose plasmid size on agarose gel was higher than that of pSV2 which was used as control, had the potential of having the insert 1AcPIAA. Plasmid DNA were extracted from clones with insert and the correct orientation of 1AcPIAA was confirmed by digesting extracted plasmid with *Nco*I and ran on 1% agarose gel to check for required bands (data not shown). AcrySTALLIFEROUS strain of *Bt* IPS 78/11 was transformed with confirmed plasmid according to the method in section 2.2.3.2. Successful transformation of IPS 78/11 was confirmed by extracting plasmids from IPS 78/11 clones, retransform *E. coli* JM109 and then miniprep and digest extracted *E. coli* JM109 plasmids alongside the original plasmid.

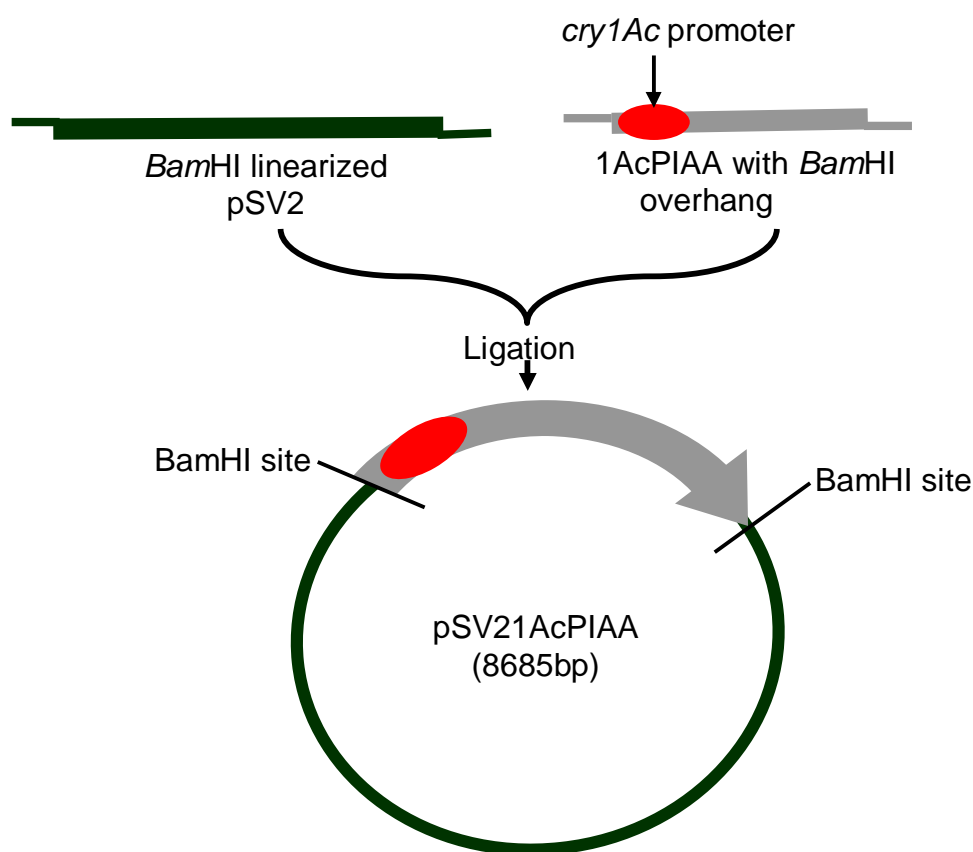


Figure 4.10: Schematic showing the construction of the plasmid pSV2APIAA

### **Expression of CryIAA protein in acrySTALLIFEROUS *Bt* IPS 78/11 under the control of native *cry1Ac*-promoter**

The clone of IPS 78/11 harbouring the plasmid pSV21AcPIAA was grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates with 25ml Luria agar containing 5µg/ml concentration of chloramphenicol were inoculated 1ml each with the 16 hour culture of the clone and grown at 30°C for 5 days. Total protein was harvested as described in section 2.2.8.3 and ran on 7.5% SDS-PAGE gel but no protein band of ~134kDa was seen on gel (data not shown).

As the required bands of ~134kDa was not seen when the clones were grown at 30°C, they were then grown at other temperatures including 20°C, 25°C and 37°C. Total proteins were harvested from each set of culture and analysed on SDS-PAGE gel but the required band was still not seen.

### **Attempting expression of *cryIAA* gene utilising *cyt1A*-promoter of plasmid pSVP27 with IPS 78/11 acrySTALLIFEROUS *Bt* as host**

To construct plasmid pSVP271AcRBSIAA aimed at expressing *cryIAA* gene under the control of native *cyt1A* promoter in acrySTALLIFEROUS *Bt*, DNA fragments used were *Bam*HI linearized pSVP27a and sequence containing 1Ac-Ribosome binding site/*cryIAA* gene with *Bam*HI overhangs.

Sequence of 1Ac-Ribosome binding site/*cryIAA* gene was obtained from the plasmid pGEM1AcPIAA previously constructed through PCR. Primer pair RIAAF 5' aatgGatCCgtatcttaataaaagagatgg 3'/RIAAR 5' gatattGGaTcctgagtttgcag 3' were designed with base changes as shown with capital letters to introduce *Bam*HI sites at 5' and 3' ends using PRIMER SELECT programme.

Using High Fidelity PCR Master system from Roche according to the manufacturers amplification instructions, needed fragment was amplified from pGEM1AcPIAA plasmid and the PCR products were ran on 1% agarose gel and required band of 3638bp was excised and purified according to the PCR products purification procedure described in section 2.2.3. The PCR product was first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release RIAA fragment with *Bam*HI overhangs.

High Fidelity PCR Master system was used because of its high fidelity and the production of PCR products with 3' A-overhangs which aid cloning with the pGEM-T vector which has 3' T-overhangs. Cloning the PCR products into PGEM-T was desirable because it allows for a proper digestion and release of the needed fragments.

pSVP27 plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase. The phosphatase was included in the digestion mix to dephosphorylate the phosphate group at the 5' ends of the linearised pSVP27 DNA fragment thus preventing it from re-ligation. The linearised pSVP27 was run on 1% agarose gel and its band excised and purified according to the procedure described in section 2.2.3.

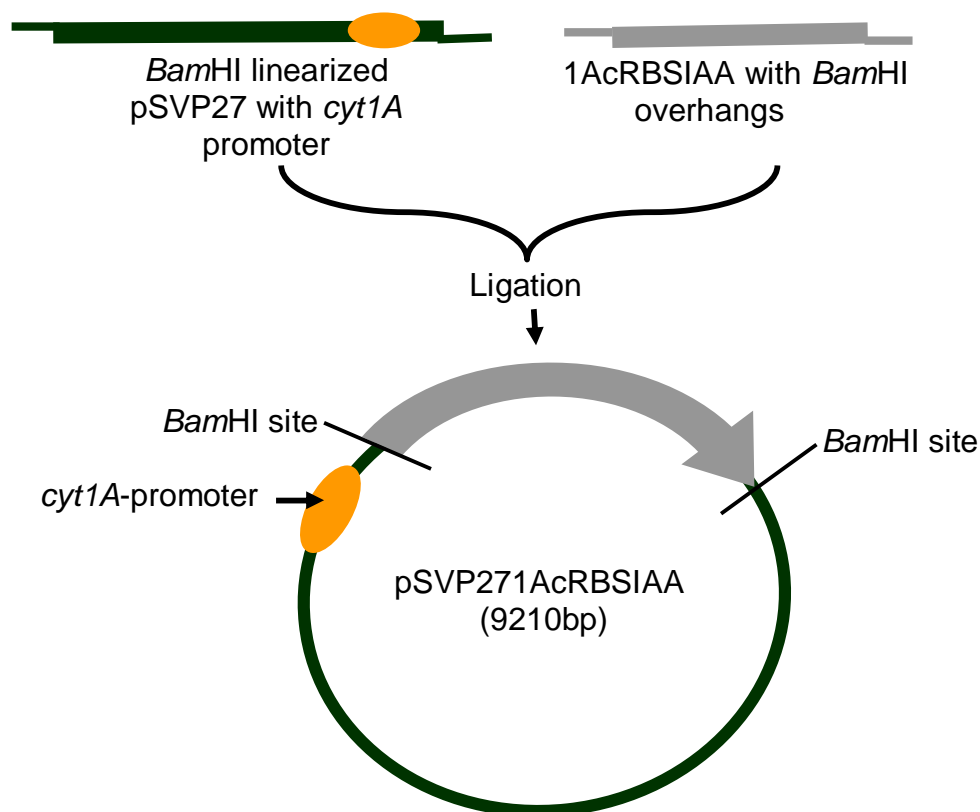
The gel purified DNA fragments of linearised pSVP27 and 1AcRBSIAA were ligated according the recipe outlined in table 4.7 while figure 4.11 is a diagrammatical illustration of pSVP271AcRBSIAA plasmid construction.

Components	pSVP271AcRBSIAA
H <sub>2</sub> O	5.0µl
pSVP27	0.5µl
1AcRBSIAA	3.0µl
10x Ligase buffer	1.0µl
T <sub>4</sub> DNA ligase	0.5µl

Table 4.7: Recipe for ligation of DNA fragments to form plasmid pSVP271AcRBSIAA

The ligation mix was incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 was transformed with the ligation mix and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pSVP271AcRBSIAA. Colonies whose plasmid size on agarose gel were higher than that of pSVP27 which was used as control, had the potential of having the insert 1AcRBSIAA. Plasmid DNA were extracted from clones with insert and the correct orientation of 1AcRBSIAA was confirmed by digesting extracted plasmid from pSVP271AcRBSIAA with *Nco*I and *Bam*HI and run on 1% agarose gel to check for required bands (data not shown). AcrySTALLiferous strain of *Bt* IPS 78/11 was transformed with confirmed plasmid according to the method in section 2.2.3.2. Successful transformation of IPS 78/11 was confirmed by extracting plasmids from IPS 78/11 clones, retransform *E. coli* JM109 and then minipreped and digest *E. coli* JM109 extracted plasmids alongside the original plasmid.



**Figure 4.11:** Schematic showing the construction of the plasmid pSVP271AcRBSIAA

#### **Expression of CryIAA protein in acrySTALLIFEROUS *Bt* IPS 78/11 under the control of native *cyt1A*-promoter**

The clone of IPS 78/11 harbouring the plasmid pSVP271AcRBSIAA was grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates of 25ml Luria agar containing 5µg/ml concentration of chloramphenicol were inoculated 1ml each with the 16 hour culture of the clone and grown at 30°C for 5 days. Total protein was harvested as described in section 2.2.8.3 and run on 7.5% SDS-PAGE gel but the protein band of ~134kDa was not seen on gel (data not shown).

As the required band of ~134kDa was not seen when the clone was grown at 30°C, it was then grown at other temperatures including 20°C, 25°C and 37°C. Total proteins were harvested from each set of culture and analysed on SDS-PAGE gel but the required bands were still not seen.

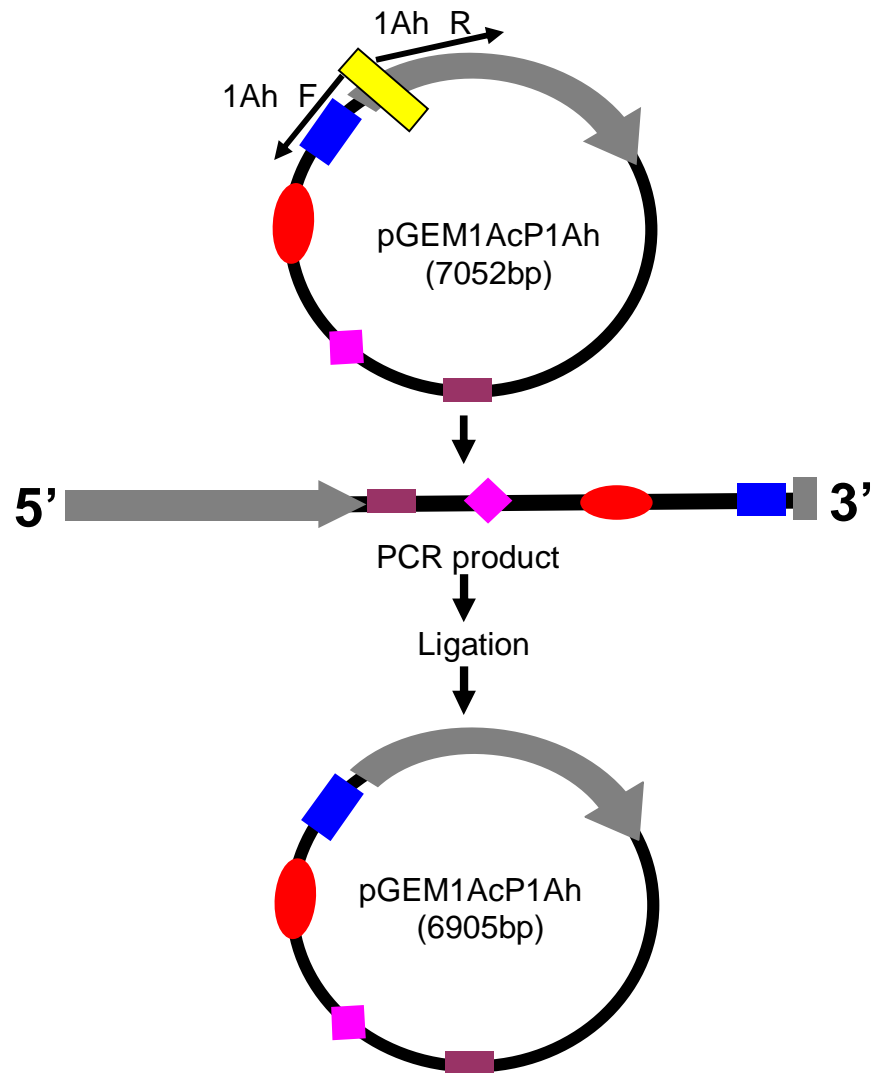


#### 4.2.6.2 Truncation of N-terminal $\alpha$ -helix

To construct the plasmid pGEM1AcP1Ah which is a plasmid designed to express truncated sequence of *cry1Ah* gene, primer pair 1Ah F 5' GTCCC-AGGTGCGGGGTTT 3' and 1Ah R 5' CATAAGTTACCTCCATCTCTTTT-ATTAAG 3' were designed such that Cry1Ah protein sequence will be deleted from amino acid position 2(Glutamic acid) to amino acid position 50(Phenylalanine) inclusive. This design took into consideration the amino acid phenylalanine-50, which is a protease recognition site. Its recognition by protease results in cleavage of the neighbouring amino acid to its carboxyl side (Walters *et al*, 2008; Lightwood *et al*, 2000). According to the pore forming model of Cry toxin mode of action, the initial cleavage of protoxins by gut proteases that removes the C-terminal half and about 30 amino acid residues from the N-terminal is followed by the processed toxins binding to receptors resulting in their conformational changes which facilitates a second cleavage by membrane bound protease that removes the N-terminal helix  $\alpha$ -1 (Bravo *et al.*, 2004). Truncated toxins lacking helix  $\alpha$ -1 have been shown to form oligomers that are membrane insertion competent even in the absence of cadherin (Gomez *et al*, 2002, Soberon *et al*, 2007). The need to create a modified Cry1Ah with deleted helix  $\alpha$ -1 was to target our Cry1Ac/Cry1Ah resistant population of *Plutella xylostella* (KARAK) because Sayyed *et al.*, 2005 demonstrated that a field collected resistant population of *Plutella xylostella* (SERD4) which was subsequently selected in the laboratory using Cry1Ab and named Cry1Ab-SEL were more sensitive to trypsin-activated Cry1Ab compared to Cry1Ab protoxins. Moreover, it has been shown that a modified Cry1Ab that lacks helix  $\alpha$ -1 was active against a resistant population of *Pectinophora gossypiella* whose resistance has been linked to cadherin gene deletions (Muñoz-Garay *et al.*, 2009). The amino acid position 50(Phenylalanine) was selected for inclusion in the deletion because multiple sequence alignment of Cry1Ah amino acid sequence with other Cry1-toxin sequences with known helix  $\alpha$ -1 position showed that phenylalanine-50 a protease recognition site is just outside helix  $\alpha$ -1 (Grochulski *et al*, 1995) as shown in figure 4.12.

Computational analysis of the truncated gene *cry1Ah* using EMBOSS Transeq tool an online programme at the EMBL-EBI website gave a putative protein with





**Figure 4.13:** Schematic for the construction of the plasmid pGEM1Ah . The PCR primers were designed to amplify the gene deleting the yellow block

- Ampicillin resistance gene
- Origin of replication in *E. coli*
- cry1Ac* promoter
- cry1Ac* ribosome binding site

### Expression and characterisation of Cry1Ah

The strain of *E. coli* JM109 harbouring the plasmid pGEM1AcP1Ah was grown in a 2x Luria Broth for 3 days at 37°C after which the total protein was harvested as described in section 2.2.8.1. The total protein was ran on 7.5% SDS-PAGE gel but the protein band of ~126kDa was not seen on gel.

As the required band of ~126kDa was not seen when the clone was grown at 37°C, it was then grown at other temperatures including 20°C, 25°C and 30°C. Total proteins were harvested from each set of culture and analysed on SDS-PAGE gel but the required band was not still seen (data not shown).

### 4.3 Discussion

The expression of the open reading frame of wild type Cry1Ah under the control of the native *cry1Ac* and *cyt1A*-promoter was successful as the protein band of approximately 134kDa was seen on SDS-PAGE gel. The characterization of the expressed proteins also showed that it folded properly as it was found to be soluble in alkaline solution of Na<sub>2</sub>CO<sub>3</sub> even at pH8.5. Its activation by trypsin resulted in the observation of an approximately 60kDa band on SDS-PAGE gel. The toxicity to *Plutella xylostella* with an LC<sub>50</sub> of 1.417 µg/ml (95% confident limit 0.867-1.917µg/ml) for *E. coli* JM109 expressed and 1.502µg/ml for the IPS 78/11 expressed which is comparable to the LC<sub>50</sub> of 1.52µg/ml of its expression in acrySTALLIFEROUS mutant of *Bacillus thuringiensis* HD73 by Xue *et al*, 2008. This further confirms that the protein was expressed in a stable and active form in both *E. coli* JM109 and IPS 78/11.

The strategy used in producing the hybrid toxin CryAIA was found to be successful at the expression stage as the expected protein band of approximately 132kDa was seen on SDS-PAGE gel. The characterisation of the expressed CryAIA protein revealed that the protein did not fold properly as it failed to demonstrate typical characteristics of Cry-proteins. It was found that it was not soluble in alkaline solution of 50mM Na<sub>2</sub>CO<sub>3</sub> and the protease resistant core of 50-60kDa band was not seen on SDS-PAGE gel when supernatant from its protease activation experiment was analysed. The SDS-PAGE analysis of the total reaction mix from the protease activation experiment shows that the protein was completely digested to fragments that could not be detected on gel

thus failing to produce the resistant core of approximately 50-60kDa. The toxicity testing of CryAIA showed that it was not toxic to susceptible and Cry1Ac resistant population of *Plutella xylostella*. Susceptible insect midgut protease activation of Cry toxin have been shown to be an important step that prepares a protoxin for activity against susceptible insects. Yamaguchi *et al*, 2010 showed that Cry8Da toxin was only able to bind to brush border membrane vesicles of Japanese beetles (*Popillia japonica* Newman) when activated using midgut juice extracted from the beetles. In a Cry1Ac resistant population of *Helicoverpa armigera*, it was observed that there was a down regulation of HaSP2 (*Helicoverpa armigera* serine protease 2) and that the protease resistant core of 95 and 68kDa was observed when the protease extracted from this resistant population was used in activating Cry1Ac (Rajagopal *et al*, 2009). In the case of CryAIA, its impotency to susceptible and Cry1Ac resistant population of *Plutella xylostella* is not a case of the insects midgut protease regulation but as a result of its improper folding. Singh *et al.*, 2010 and Henderson *et al.*, 2010 demonstrated that a missense mutation of dystrophin protein results in its instability and conformational changes and the fact that CryAIA has been expressed but failed the characterisation test shows that its conformation and stability has been affected by the domain swap.

The genetically manipulated *cryIAA* which is a toxin gene made by swapping of domain I of *cry1Ah* with that of *cry1Ie* and *cry1Ah* which is *cry1Ah* mutant designed to to express truncated Cry1Ah did not result in expression of the proteins they code for. Though all the upstream elements like the *cry1Ac*-promoter, *cyt1A*-promoter and ribosome binding site that have been successfully used in expressing the parent genes of *cry1Ah* and *cry1Ie* were supplied, they were not still expressed. *cyt1A*-promoter has been used in the expression of Cry2A (Crickmore and Ellar, 1992) while *cry1Ac*-promoter has been used in expression of Cry11A with *Bacillus brevis* as host (Roh *et al*, 2010). The use of different hosts and varying of culturing conditions like temperature did not rescue the expression of *cryIAA* or *cry1Ah* and this could be as result of the fact that the hybrid and the truncated genes created have resulted in proteins that have not folded properly.

Synergistic studies between Cry1Ah and Cry1Ie showed that there is no synergism between them. With individual LC<sub>50</sub> of 1.417 and 0.319µg/ml for

Cry1Ah and Cry1Ie respectively, and the use of their combination at individual doses that will result in lethality below LC<sub>50</sub>, a synergistic effect could not be observed. Synergism has been reported between Cry1Ac and Cry2Aa (Yunus *et al.*, 2011), Cry1Ac and Cry2Ab (Ibargutxi *et al.*, 2008) and Cry1Ab and Cry1Ac (Sharma *et al.*, 2010). Ibargutxi *et al.*, 2008, suggested that the synergism observed between Cry1Ac and Cry2Ab was as a result of special interaction between them. Carmona *et al.*, 2011 made a mutant Cry1Ab-D136N/T143D that has a dominant negative phenotype inhibiting the toxicity of wild type Cry1Ab and other Cry1 toxins including Cry1Aa, Cry1Ac and Cry1Fa against *Manduca sexta*. Since Cry1Ab-D136N/T143D was able to inhibit activity of other Cry1 toxins, they concluded that the dominant negative phenotype was as a result of hetero-oligomers formed between Cry1Ab-D136N/T143D and the other Cry1 toxins. Synergism has also been reported between Cry toxins and Cyt toxins including Cry4Ba and Cyt1Aa (Canton *et al.*, 2011) and Cry10Aa and Cyt1Aa (Hernandez-Soto *et al.*, 2009). Canton *et al.*, 2011 showed that synergism between Cry11Aa and Cyt1Aa results from specific interaction between them and created a mutant Cyt1Aa that resulted in reduced synergism. The absence of synergism between Cry1Ah and Cry1Ie might be that these specific interactions are also absent.

The results from this chapter show that *cry1Ac* and *cyt1A*-promoters are capable of driving the expression of a functional Cry1Ah toxin and also its non-functional mutant CryAIA. It has also been shown that the strategy leading to the construction of the *cry1AA* and *cry1Ah* mutants did not result in the formation of functional genes as their putative proteins were not seen on gel despite different methods employed to enhance their expression. The hybrid protein CryAIA engineered by swapping domain II of Cry1Ah with that of Cry1Ie did not produce a functional protein as bioassay against sensitive population of *Plutella xylostella* showed no sign of toxicity at concentration as high as 100µg/ml. The non toxicity of CryAIA is likely to be as a result of misfolding as characterisation of the expressed protein fall short of those of typical Cry toxin.

## **Chapter 5: Attempting expression of coding sequences for *cry30Ea* and *cry40Da* toxin genes**

### **5.1 Introduction**

The coding sequences of *cry30Ea* and *cry40Da* toxin genes were amplified using PCR from *Bacillus thuringiensis* strains S2160-1 and S2196-1 respectively isolated from soil samples in Guangxi, China. The SDS-PAGE analysis of the total protein from these strains did not show the ~77.6kDa and ~73.5kDa bands of their respective deduced proteins. Total proteins prepared from these strains were found to be toxic to mosquito strains *Culex quinquefasciatus* and *Aedes Albopictus* (Zhang *et al*, unpublished data).

Other *cry30* genes like *cry30Aa1* (Juárez-Pérez *et al*, 2003), *cry30Ba1* (Ito *et al*, 2006), *cry30Fa1* (Tan *et al*, 2009) and *cry30Ga1* (Zhu *et al*, 2010) have been cloned from mosquitocidal strains of *Bacillus thuringiensis* and heterologously expressed in acrySTALLIFEROUS strains of *Bacillus thuringiensis* and *E. coli* BL21. A *cry40* gene cloned from a *Bacillus thuringiensis* strain has also been expressed in *Bacillus subtilis* under its native promoter (Brown, 1993) and *E. coli* under IPTG inducible *tac* promoter (Brown and Whiteley, 1992). Brown, 1993 also showed that the DNA sequence upstream of *cry40* gene start site had consensus with those of other *cry*-genes which are sporulation sigma factor dependent. Ito *et al*, 2006 have shown that Cry30Ba has the five conserved blocks found in a typical Cry toxin. It has also been shown that Cry30Fa1 has the conserved five blocks typical of Cry toxins but it lacks the C-terminal half found in 130kDa Cry toxins (Tan *et al*, 2009).

To study the molecular characteristics and toxicity of the proteins possibly encoded by *cry30Ea* and *cry40Da* genes, heterologous expression was attempted and this chapter is focusing on the various methodologies utilised in attempting to express and characterise their proteins.

### **5.2 Results**

#### **5.2.1 Bioinformatics analysis of *cry30Ea* and *cry40Da* DNA and deduced amino acid sequences**

To apply molecular genetics techniques in manipulating the *cry30Ea* and *cry40Da* sequences for heterologous expression, bioinformatics analysis like pair wise and multiple sequence alignments were carried out. Tables 5.1 and

5.2 below summarise the similarities and differences between their deduced protein sequences with known expressed proteins of their class. The deduced amino acid sequence from *cry30Ea* and *cry40Da* DNA sequences were 688 and 658 amino acids respectively. Compute pI/Mw tool (Hughes *et al*, 1993; Bjellqvist *et al*, 1994, Gasteiger *et al*, 2005) which is an online programme at ExPASy website used in computing the molecular weight of the deduced proteins of Cry30Ea and Cry40Da estimate their molecular weight to be approximately 77.6kDa and 73.5kDa respectively. BLASTP 2.2.24+ programme (Altschul *et al*, 1997, Altschul *et al*, 2005) was used in conducting pair wise and multiple alignment of the deduced amino acid sequence.

Proteins	Similarities with Cry30Ea	Positives with Cry30Ea	Gaps
Cry30Aa1	76% (519/689)	84% (573/689)	0% (2/689)
Cry30Ba1	56% (388/694)	70% (481/694)	5% (37/694)
Cry30Ca1	67% (461/695)	77% (535/695)	2% (14/695)
Cry30Fa1	70%(479/690)	79%(539/690)	0% (5/690)
Cry30Ga1	59% (406/690)	72% (492/690)	4% (28/690)

Table 5.1: BLASTP 2.2.24+ programme pair wise alignment results of Cry30Ea1 deduced protein sequence with sequences of five members of Cry30 Cry toxins that has been heterologously expressed



Proteins	Similarities with Cry40Da	Positives with Cry40Da	Gaps
Cry40Aa1	60% (399/672)	72% (479/672)	3% (23/672)
Cry40Ba1	53% (375/677)	66% (443/677)	4% (30/677)

Table 5.2: BLASTP 2.2.24+ programme pair wise alignment results of Cry40Da1 deduced protein sequence with sequences of two members of Cry40-toxins that have been heterologously expressed

## 5.2.2 Exploring different expression vector systems and hosts for the expression of *cry30Ea* and *cry40Da* genes

### 5.2.2.1 Utilizing native *cry1Ac*-promoter with pGEM vector backbone and *E. coli* JM109 as host

The native promoter of *cry1Ac* in pGEM-T vector backbone as described in chapter 3 and 4 was used in attempting expression of *cry30Ea* and *cry40Da* DNA sequences with *E. coli* JM109 as host. The needed DNA fragments were pGEM1AcP which consist of pGEM-T vector backbone and *cry1Ac*-promoter from pGEM1AcP1Ac plasmid and the coding sequence of *cry30Ea* and *cry40Da* genes obtained from *Bacillus thuringiensis* strains S2160-1 and S2196-1 respectively.

From the sequence information obtained from Wenfei Zhang who supplied *Bacillus thuringiensis* strains S2160-1 and S2096-1, primer pairs F30 5' ATGAATTCTTATCAAAATACAAATG 3'/R30 5' TTAGTTCACTGTACAAG-CTACTAC 3' and F40 5' ATGAATTCATATCAAAATACAAATG 3'/R40 5' TTAATTGATAAATAAATCGTTCACC 3' for amplification of the coding sequences of *cry30Ea* and *cry40Da* genes respectively were designed using PRIMER SELECT programme. The primers for amplification of pGEM1AcP fragment from pGEM1AcP1Ac plasmid have been described in chapter 3. For simplicity the PCR product sequences for *cry30Ea* and *cry40Da* would be referred to as 30Ea and 40Da respectively.

Using Pfu Ultra Hotstart PCR Master Mix system according to the manufacturers instructions pGEM1AcP, *cry30Ea* and *cry40Da* sequences were amplified and PCR products were ran on 1% agarose gel and required bands of 3566bp (pGEM1AcP), 2067bp (30Ea) and 1977bp (40Da) were excised and purified according to the PCR products purification procedure described in section 2.2.3. The purified bands of pGEM1AcP, 30Ea and 40Da were used in the construction of the plasmids pGEM1AcP30Ea and pGEM1AcP40Da as shown in figure 5.1.

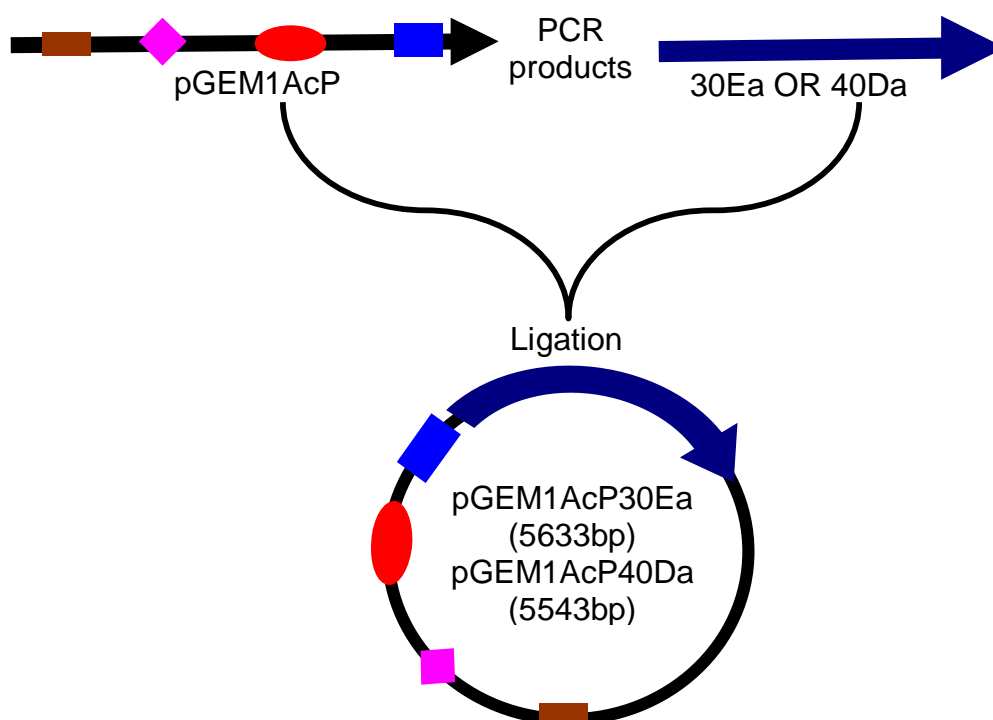


Figure 5.1: Schematic illustrating the construction of pGEM1AcP30Ea or pGEM1AcP40Da plasmids from the ligation of the PCR products of pGEM1AcP, and 30Ea or 40Da

- *cry1Ac* ribosome binding site
- *cry1Ac* promoter
- ◆ Origin of replication in *E. coli*
- Ampicillin resistance gene

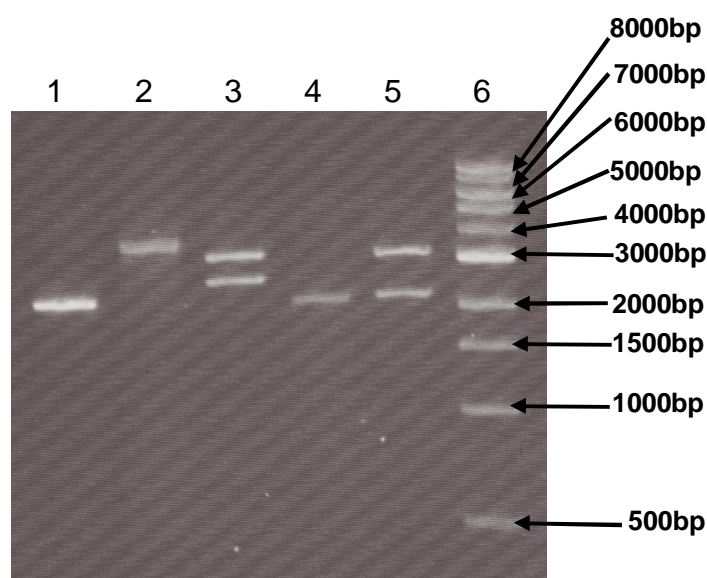
To construct the plasmids pGEM1AcP30Ea or 40Da, the following volumes of reagent and DNA fragments as shown in table 5.3 were measured and mixed together.

Components	Volumes
H <sub>2</sub> O	1µl
pGEM1AcP	1µl
Ligation buffer	5µl
30Ea or 40Da	3µl
T <sub>4</sub> Ligase	1µl

Table 5.3: Recipe of ligation mixes for pGEM1AcP30Ea or 40Da

The ligation mixes were incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 were transformed with the ligation mixes and many transformants were screened to check for colonies harbouring the plasmid pGEM1AcP30Ea or pGEM1AcP40Da. Clones with the correct orientation of 30Ea or 40Da was checked by digesting extracted plasmid with *Eco*RI. As shown in lane 3 of figure 5.2, the construction of pGEM1AcP30Ea was confirmed when digested plasmid ran on gel gave expected bands of 2997 and 2413bp though expected bands of 173 and 50bp were not seen as they are too small to be detected. Also lane 5 of figure 5.2 confirms the construction of pGEM1AcP40Da as expected bands of 2997 and 2099bp were seen on gel though expected bands of 224 and 50bp were not seen as they are too small to be detected.



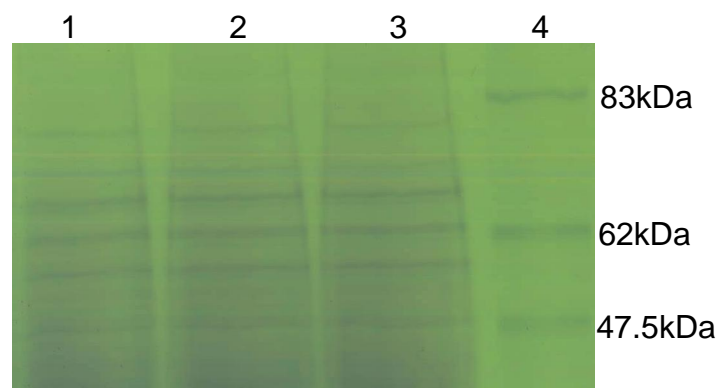
**Figure 5.2:** Picture of gel showing 30Ea, pGEM1AcP, *Eco*RI digestion of pGEM1AcP30Ea, 40Da, *Eco*RI digestion of pGEM1AcP40 plasmid. Lane 1: 2067bp fragment of 30Ea, Lane 2: 3566bp band of pGEM1AcP, Lane 3: *Eco*RI digested pGEM1AcP30Ea showing band sizes of 2997bp and 2413bp, Lane 4: 1997bp fragment of 40Da, Lane 5: *Eco*RI digested pGEM1AcP40Da showing band sizes of 2997bp and 2099bp and Lane 6: DNA marker

### **Expression of Cry30Ea or Cry40Da proteins in *E. coli* JM109**

The strains of *E. coli* JM109 harbouring the plasmid pGEM1AcP30Ea or pGEM1AcP40Da was grown in a 2x Luria Broth for 3 days at 37°C after which the total protein were harvested as described in section 2.2.8.1.

The total proteins were ran on 7.5% SDS-PAGE gel but the protein bands of 77.6kDa and 73.5kDa from *cry30Ea* and *cry40Da* genes were not seen on gel as shown in figure 5.3.

As the required bands of 77.6kDa and 73.5kDa were not seen when the clones were grown at 37°C, they were then grown at other temperatures including 20°C, 25°C and 30°C. Total proteins were harvested from each set of culture and analysed on SDS-PAGE gel and the required bands were not still seen (data not shown).



**Figure 5.3:** Pictures of gel showing total proteins from *E. coli* JM109 harbouring plasmids pGEM1AcP30Ea or pGEM1AcP40Da and total protein from *E. coli* JM109 without the plasmids. Lane 1: Total protein for *E. coli* JM109 harbouring pGEM1AcP30Ea, Lane 2: Total protein for *E. coli* JM109 harbouring pGEM1AcP40Da, Lane 3: Total protein for *E. coli* JM109 without the plasmids and Lane 4: Protein marker

#### 5.2.2.2 Utilising pET3a vector and *E. coli* BL21 (DE3) as host

pET3a is a member of pET series of vectors from Novagen which expresses proteins under a T7 promoter. It has ribosome binding site, *NdeI* cloning site, T7 gene for 10 leader peptide, *Bam*HI cloning site, T7 terminator, ampicillin resistance (*bla*) ORF, and pBR322 origin of replication. The *Bam*HI cloning site was used in the cloning of *cry30Ea* and *cry40Da* genes and this requires the introduction of *Bam*HI sites at their 5' and 3' ends of their sequences.

Primer pairs 30BamF 5' gagatggGgAtCCcttatgaattc 3'/30BamR 5' gatattGGaTcctgagtttgcag 3' and 40BamF 5' gagatggGgAtCCcttatgaattc 3'/40BamR 5' gatattGGaTcctgagtttgcag 3' were designed using PRIMER SELECT programme with changes to introduce the *Bam*HI sites shown in uppercase letters within the primer sequences.

Cloning the orfs via the *Bam*HI site means that Cry30Ea and Cry40Da proteins from the formed plasmids would have a higher molecular weight than 77.6kDa and 73.5kDa respectively because *Bam*HI sites is preceded by the 10 amino

acid T7 gene leader peptide and the *Bam*HI fragments of *cry30Ea* and *cry40Da* inserts form a continuous frame with the leader peptide as shown in figure 5.4.

**A**

ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGC GgA tCC ctt atg aat.....  
M A S M T G G Q Q M G R G S V M N

**B**

ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGC GgA tCC ctt atg aat.....  
M A S M T G G Q Q M G R G S V M N

**Figure 5.4:** Fused DNA sequence of the T7 leader peptide and *Bam*HI cloned *Cry30Ea* (A) and *Cry40Da* (B) sequences and their corresponding amino acid sequences

With the fusion of the leader peptide, the molecular weight of the deduced protein from the *cry30Ea* and *cry40Da* genes were calculated using the Compute pI/Mw tool (Hughes *et al*, 1993, Bjellqvist *et al*, 1994 and Gasteiger *et al*, 2005) and they were estimated to be approximately 79kDa and 75kDa respectively.

Using High Fidelity PCR Master system from Roche according to the manufacturers amplification conditions, *cry30Ea* and *cry40Da* sequences were amplified using pGEM1AcP30Ea and pGEM1AcP40Da plasmids constructed in section 5.2.2.1 as template and the PCR products were ran on 1% agarose gel and required bands of 2011bp (*cry30Ea*) and 2021bp (*cry40Da*) were excised and purified according to the PCR products purification procedure described in section 2.2.3. The PCR products were first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release *cry30Ea* and *cry40Da* fragments with *Bam*HI overhangs. The fragments of *cry30Ea* and *cry40Da* sequences released from pGEM-TBam30Ea and pGEM-TBam40Da with *Bam*HI overhangs will be known as Bam30Ea and Bam40Da for clarity.

pET3a plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase. The phosphatase was included in the digestion mix to dephosphorylate the phosphate group at the 5' ends of the linearised pET3a DNA fragment thus preventing it from re-ligation. The linearised pET3a was ran on 1% agarose gel and purified from its band excised from the gel according to the procedure outlined in section 2.2.3.

The gel purified DNA fragments of linearised pET3a, Bam30Ea and Bam40Da were ligated according the recipe outlined in table 5.4 while figure 5.5 shows the schematic illustrating the construction of the plasmids pET3aBam30Ea and pET3aBam40Da.

Components	pET3aBam30Ea	pET3aBam40Da
H <sub>2</sub> O	3.5µl	3.5µl
pET3a	1µl	1µl
Bam30Ea	4µl	-
Bam40Da	-	4µl
10x Ligase buffer	1µl	1µl
T <sub>4</sub> DNA ligase	0.5µl	0.5µl

Table 5.4: Recipe for ligation of DNA fragments to form plasmids pET3aBam30Ea and pET3aBam40Da

The ligation mixes were incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 were transformed with the ligation mixes and many transformants were screened to check for colonies harbouring the plasmid pET3aBam30Ea and pET3aBam40Da. Clones with the correct orientation of Bam30Ea or Bam40Da were confirmed by digesting extracted plasmid with *Eco*RI and *Bam*HI. *E. coli* BL21(DE3) were transformed with confirmed plasmids and

confirmed by extracting and digesting plasmids from transformants. The plasmids pET3aBam30Ea and pET3aBam40Da were successfully constructed as the expected bands were seen on gel as shown in figure 5.6.

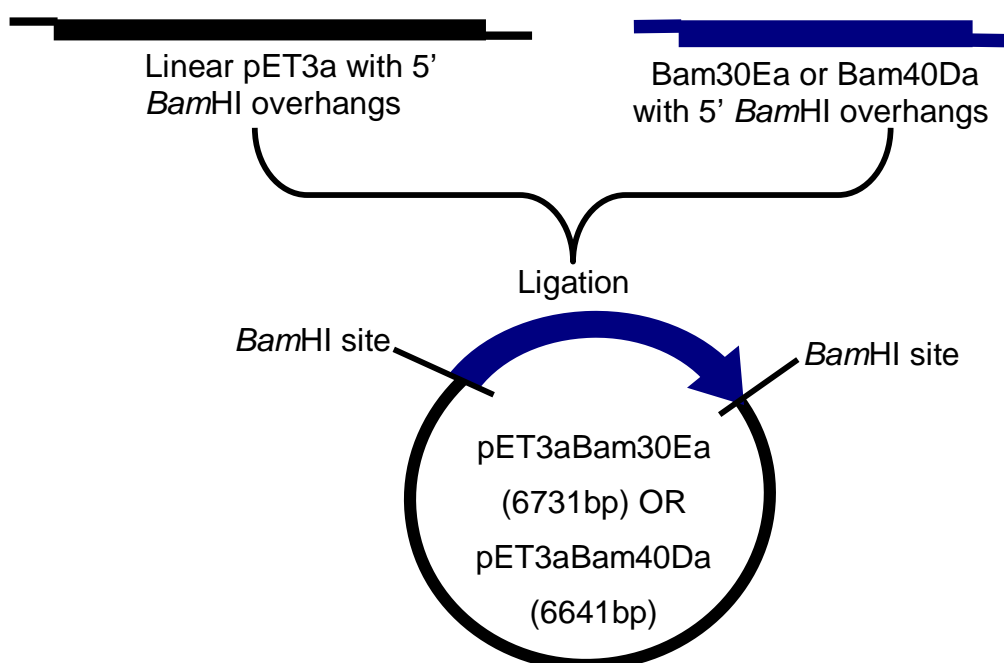
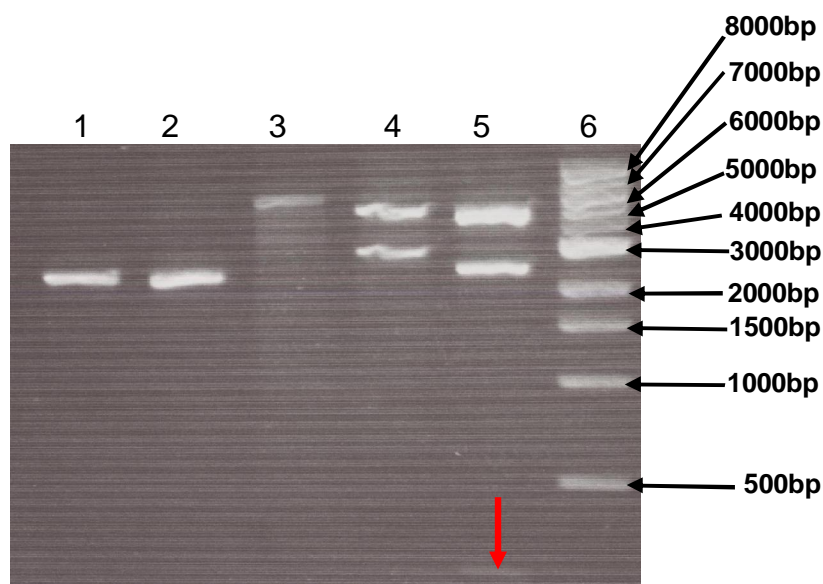


Figure 5.5: Schematic illustrating the construction of plasmids pET3aBam30Ea or pET3aBam40Da from ligation of *Bam*HI linearised pET3a and Bam30Ea or Bam40Da DNA fragments





**Figure 5.6:** Gel picture showing Bam30, Bam40, pET3a, *EcoRI* digestion of pET3aBam30Ea and pET3aBam40Da. Lane 1: 2090bp band of Bam30Ea, Lane 2: 2000bp band of Bam40Da, Lane 3: *Bam*HI linearized pET3a, Lane 4: *EcoRI* digested pET3aBam30Ea showing band sizes of 4139bp and 2592bp, Lane 5: *EcoRI* digestion of pETBam40Da showing band sizes of 4139bp, 2278bp and 224bp pointed by red arrow and Lane 6: 1Kb DNA marker

### **Expressing Cry30Ea and Cry40Da under the control of T7 promoter through IPTG induction**

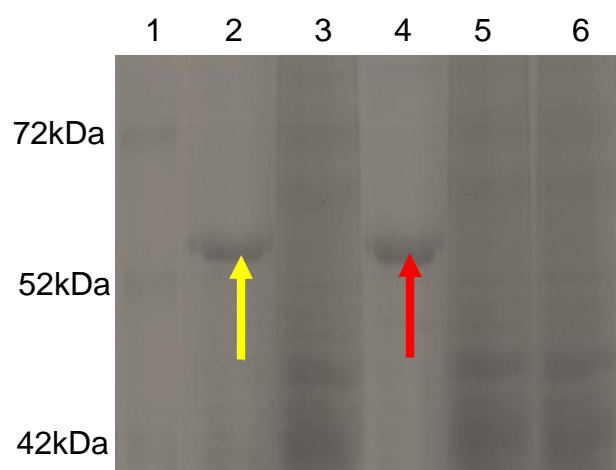
*E. coli* BL21 (DE3) harbouring pET3aBam30Ea or pET3aBam40Da plasmid were subcultured into 10ml of Luria broth containing 100 µg/ml of ampicillin and grown overnight. The 10ml overnight cultures were inoculated into a fresh Luria broth containing 100 µg/ml of ampicillin and grown to optical density of 1.066 and 0.682 for pET3aBam30Ea and pET3aBam40Da respectively. At the mentioned optical densities, 50µl of 1M IPTG was added to the cultures and grown at 37°C for 16 hours. Total proteins were harvested according to the method outlined in section 2.2.8.2.

The total proteins were ran on 7.5% SDS-PAGE gel and the protein bands of 77.6kDa and 73.5kDa from *cry30Ea* and *cry40Da* genes were not seen on gel

but a band of ~62kDa was observed for both pET3aBam30Ea and pET3aBam40Da clones.

To confirm that the observed band of ~62kDa were from the genes *cry30Ea* and *cry40Da*, the BL21 (DE3) clones with the pET3aBam30Ea or pET3aBam40Da plasmids and without the plasmid were grown as previously described but in 200ml Luria broth and when they attained optical density of 0.8, the cultures were divided into two equal portions and one portion was induced with IPTG while the other half was not induced.

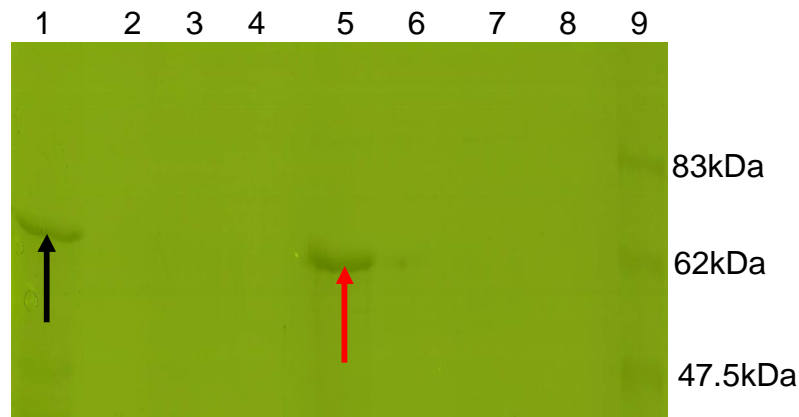
Total proteins were harvested and ran on 7.5% SDS-PAGE gel and the ~62kDa bands were still observed for the IPTG induced strains having the pET3aBam30Ea or pET3aBam40Da plasmids but not with the uninduced strains and the BL21 (DE3) without the plasmids as shown in figure 5.7.



**Figure 5.7:** SDS-PAGE gel picture of the induced and non-induced strains of *E. coli* BL21 (DE3) harbouring plasmids pET3aBam30Ea and pET3aBam40Da. Lane 1: Protein molecular weight marker, Lane 2: ~62kDa (pointed by yellow arrow) protein band from strain harbouring pET3aBam30Ea induced with IPTG, Lane 3: Total protein from strain harbouring pET3aBam30Ea without IPTG induction, Lane 4: ~62kDa (pointed by red arrow) protein band from strain harbouring pET3aBam40Da induced with IPTG, Lane 5: Total protein from strain harbouring pET3aBam40Da without IPTG induction, Lane 6: Total protein from IPTG induced *E. coli* BL21 (DE3) without the plasmids

### **Characterisation of the expressed proteins**

The expressed proteins were tested for alkaline solubility at pH11 and protease activation according to the method in section 2.2.9. The results show that the ~62kDa protein was not seen when the supernatant from the alkaline solubility test was ran on SDS-PAGE gel but the ~62kDa band was still present in the pellet meaning that the proteins are not soluble in alkali. Results from SDS-PAGE gel analysis of the total reaction mixes and supernatants from the protease activation experiments show that the protease resistant core of about 50-60kDa typical of Cry-proteins was not seen meaning that the proteins were destroyed by protease as shown in figure 5.8.



**Figure 5.8:** SDS-PAGE Gel picture showing alkaline solubility and protease activation characterisation of the ~62kDa protein band. Lane 1: Total reaction sample from 50mM  $\text{Na}_2\text{CO}_3$  pH11 solubility test of ~62kDa band from pET3aBam30Ea strain showing the presence of ~62kDa band (pointed by black arrow), Lane 2: Supernatant from 50mM  $\text{Na}_2\text{CO}_3$  pH11 solubility test of ~62kDa band from pET3aBam30Ea strain indicating absence of the ~62kDa band, Lane 3: Total reaction sample from trypsin activation test of ~62kDa band from pET3aBam30Ea strain indicating absence of resistant core, Lane 4: Supernatant from trypsin activation test of ~62kDa band from pET3aBam30Ea strain indicating absence of resistant core, Lane 5: Total reaction sample from 50mM  $\text{Na}_2\text{CO}_3$  pH11 solubility test of ~62kDa band from pET3aBam40Da strain showing the presence of ~62kDa band (pointed by red arrow), Lane 6: Supernatant from 50mM  $\text{Na}_2\text{CO}_3$  pH11 solubility test of ~62kDa band from pET3aBam40Da strain indicating absence of the ~62kDa band, Lane 7: Total reaction sample from trypsin activation test of ~62kDa band from pET3aBam40Da strain indicating absence of resistant core, Lane 8: Supernatant from trypsin activation test of ~62kDa band from pET3aBam40Da strain indicating absence of resistant core and Lane 9: Protein molecular weight marker

From the characterisation results of the expressed ~62kDa proteins, it was thought that changing the culturing conditions like concentration of IPTG, culture temperature after inductions and time of growth after inductions, a functional protein could be obtained. With this assumption considered, the clones were cultured at 37°C, induced with IPTG to concentration of 0.25mM, 0.5mM and 1mM, grown after induction at temperatures of 20°C, 25°C and 30°C and grown for 8 hours, 12 hours and 24 hours after induction.

The proteins from these cultures were harvested and they all show the ~62kDa protein on SDS-PAGE gel but were neither soluble in alkaline solution nor produce a resistance core from protease activation experiments as summarised in table 5.5.

IPTG conc. (mM)	Temperature (°C)	Time of incubation after IPTG induction	Protein size on SDS-PAGE (~kDa)	Alkaline solubility	Protease activation
0.25	20	24 hours	62	Not Soluble	No protease resistant core
0.5	20	8 hours	62	Not Soluble	No protease resistant core
1.0	20	16 hours	62	Not Soluble	No protease resistant core
0.5	25	24 hours	62	Not Soluble	No protease resistant core
0.5	30	8 hours	62	Not Soluble	No protease resistant core

Table 5.5: Summary of the changes in IPTG concentration, temperature of growth after IPTG induction and time of growth after IPTG induction and characteristics of expressed proteins

### 5.2.2.3 Utilising native *cry1Ac*-promoter with *Bt* shuttle vector backbone pSV2 and IPS 78/11 acrySTALLIFEROUS *Bt* as host

The plasmid pSV2 is a shuttle vector that has origin of replication in *Bt* and *E. coli*. It has multiple cloning sites, which includes a *Bam*HI site that was used for the cloning of the coding sequences of *cry30Ea* or *cry40Da* genes carrying native *cry1Ac*-promoter in their upstream regions.

To construct plasmids pSV21AcP30Ea and pSV21AcP40Da aimed at expressing *cry30Ea* or *cry40Da* genes under the control of native *cry1Ac*-promoter in acrySTALLIFEROUS *Bt*, DNA fragments used were *Bam*HI linearized pSV2 and sequence of native *cry1Ac*-promoter/*cry30Ea* or *cry40Da* genes with *Bam*HI overhangs.

Sequence of native *cry1Ac*-promoter/*cry30Ea* or *cry40Da* genes were obtained from the plasmids pGEM1AcP30Ea and pGEM1AcP40Da previously constructed in section 5.2.2.1. Primer pair 1AcPF 5' gagctcggATcccaacaccctgg 3'/1AcPR 5' gatattGGaTcctgagtttgcattgag 3' were designed with base changes as shown with capital letters to introduce *Bam*HI sites at 5' and 3' ends using PRIMER SELECT programme. This primer pair was the same for both *cry30Ea* and *cry40Da* genes as the regions they bind to in the template plasmids pGEM1AcP30Ea and pGEM1AcP40Da are the same because they share the same vector backbone pGEM1AcP.

Using High Fidelity PCR Master system from Roche according to the manufacturers amplification instructions, needed fragments were amplified from pGEM1AcP30Ea and pGEM1AcP40Da plasmids and the PCR products were ran on 1% agarose gel and required bands of 2259bp (*cry30Ea*) and 2169bp (*cry40Da*) were excised and purified according to the PCR products purification procedure described in section 2.2.3. The PCR products were first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release 1AcP30Ea and 1AcP40Da fragments with *Bam*HI overhangs. The fragments of 1AcP30Ea and 1AcP40Da sequences released from pGEM-T1AcP30Ea and pGEM-T1AcP40Da with *Bam*HI overhangs would now be known as 1AcP30Ea and 1AcP40Da for clarity.

pSV2 plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase.

The linearised pSV2 was ran on 1% agarose gel and purified from its band excised from the gel according to the procedure outlined in section 2.2.3.

The gel purified DNA fragments of linearised pSV2, 1AcP30Ea and 1AcP40Da were ligated according the recipe outlined in table 5.6 while Figure 5.9 shows a diagrammatic illustration of how the plasmids pSV21AcP30Ea and pSV21AcP40Da were made.

Components	pSV21AcP30Ea	pSV21AcP40Da
H <sub>2</sub> O	5.0µl	5.5µl
pSV2	0.5µl	0.5µl
1AcP30Ea	3.0µl	-
1AcP40Da	-	2.5µl
10x Ligase buffer	1.0µl	1.0µl
T <sub>4</sub> DNA ligase	0.5µl	0.5µl

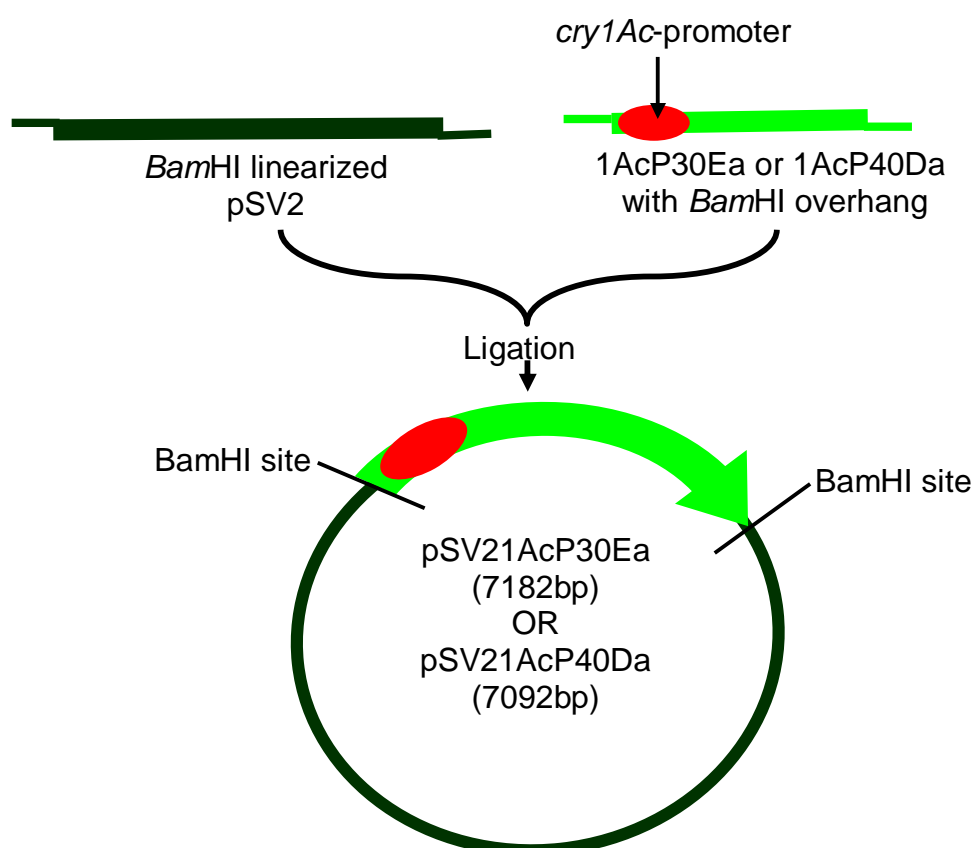
Table 5.6: Recipe for ligation of DNA fragments to form plasmids pSV21AcP30Ea and pSV2AP40Da

The ligation mixes were incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 were then transformed with the ligation mixes and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pSV21AcP30Ea and pSV21AcP40Da. Colonies whose plasmid size on agarose gel were higher than that of pSV2 which was used as control, had the potential of having the insert 1AcP30Ea and 1AcP40Da. Plasmid DNA were extracted from clones with insert and the correct orientation of 1AcP30Ea or 1AcP40Da were confirmed by digesting extracted plasmid from pSV21AcP30Ea with *Hae*III and *Bam*HI and plasmid from pSV21AcP40Da with *Eco*RI and *Bam*HI (data not shown). The plasmids pSV21AcP30Ea and pSV21AcP40Da were successfully and acrySTALLIFEROUS strain of *Bt* IPS 78/11 were transformed with the confirmed plasmids according to the method in section 2.2.3.2. Successful transformation of IPS 78/11 was confirmed by extracting plasmids from IPS 78/11 clones, retransform *E. coli*



JM109 and then miniprep and digest extracted plasmids alongside the original plasmid.



**Figure 5.9:** Schematic showing the construction of the plasmids pSV2AP30Ea and pSV2AP40Da

### **Expression of Cry30Ea or Cry40Da proteins in acrySTALLIFEROUS *Bt* IPS 78/11 under the control of native *cry1Ac*-promoter**

The clones of IPS 78/11 harbouring the plasmid pSV21AcP30Ea or pSV21AcP40Da were grown in 5ml of Luria broth for 16 hours 30°C. Five Petri plates of 25ml Luria agar with 5µg/ml concentration of chloramphenicol were inoculated 1ml each with the 16 hour culture of the clones and grown at 30°C

for 5 days. Total protein were harvested as described in section 2.2.8.3 and ran on 7.5% SDS-PAGE gel but the protein bands of 77.6kDa and 73.5kDa from *cry30Ea* and *cry40Da* genes were not seen on gel as shown in figure 5.10.

As the required bands of 77.6kDa and 73.5kDa were not seen when the clones were grown at 30°C, they were then grown at other temperatures including 20°C, 25°C and 37°C. Total proteins were harvested from each set of culture and analysed on SDS-PAGE gel and the required bands were not still seen (data not shown).

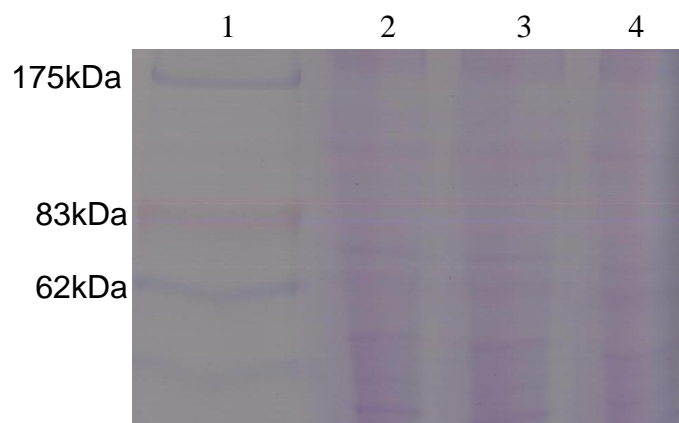


Figure 5.10: Picture of SDS-PAGE gel showing total proteins from *Bt* IPS 78/11 strains harbouring pSV21AcP30 or pSV21AcP40Da plasmids. Lane 1: Protein molecular weight marker, Lane 2: Total protein from *Bt* strain IPS 78/11 harbouring pSV21AcP30Ea, Lane 3: Total protein from *Bt* strain harbouring pSV21AcP40Da and Lane 4: Total protein from *Bt* strain IPS 78/11 without the plasmid

#### 5.2.2.4 Utilising *cyt1A*-promoter of plasmid pSVP27 and IPS 78/11 acrystalliferous *Bt* as host

Plasmid pSVP27 is a vector for gene expression in *Bt* under the control of *cyt1A* promoter constructed by fusing a sequence with *cyt1A* promoter to pSV2 and incorporating multiple cloning sites including *Bam*HI downstream of the promoter (Crickmore and Ellar, 1992).

To construct plasmids pSVP271AcRBS30Ea and pSV271AcRBS40Da aimed at expressing *cry30Ea* or *cry40Da* genes under the control of native *cyt1A*-promoter in acrystalliferous *Bt*, DNA fragments used were *Bam*HI linearized pSVP27 and sequence containing 1Ac-Ribosome binding site/*cry30Ea* or *cry40Da* genes with *Bam*HI overhangs.

Sequence of 1Ac-Ribosome binding site/*cry30Ea* or *cry40Da* genes were obtained from the plasmids pGEM1AcP30Ea and pGEM1AcP40Da previously constructed in section 5.2.2.1. Primer pairs R30\_40F 5' aatgGatCCgtatctt-aataaaagagatgg 3'/R30\_40R 5' gatattGGA~~Tcct~~gagtttgcagtag 3' were designed with base changes as shown with capital letters to introduce *Bam*HI sites at 5' and 3' ends using PRIMER SELECT programme. This primer pair was the same for both *cry30Ea* and *cry40Da* genes as the regions they bind to in the template plasmids pGEM1AcP30Ea and pGEM1AcP40Da are the same because they share the same vector backbone pGEM1AcP.

Using High Fidelity PCR Master system from Roche according to the manufacturers amplification instructions, needed fragments were amplified from pGEM1AcP30Ea and pGEM1AcP40Da plasmids and the PCR products were ran on 1% agarose gel and required bands of 2134bp (*cry30Ea*) and 2044bp (*cry40Da*) were excised and purified according to the PCR products purification procedure described in section 2.2.3. The PCR products were first cloned into a pGEM-T vector from Promega and *E. coli* JM109 was transformed with the ligation mix. Plasmids extracted from transformants harbouring positive clones were digested with *Bam*HI to release 1AcRBS30Ea and 1AcRBS40Da fragments with *Bam*HI overhangs.

pSVP27 plasmid was extracted from the clone of *E. coli* JM109 harbouring it and the extracted plasmid was digested with *Bam*HI in the presence of phosphatase. The linearised pSVP27 was ran on 1% agarose gel and purified

from its band excised from the gel according to the procedure outlined in section 2.2.3.

The gel purified DNA fragments of linearised pSVP27, 1AcRBS30Ea and 1AcRBS40Da were ligated according the recipe outlined in table 5.7 while figure 5.11 illustrates how the plasmids pSVP271AcRBS30Ea and pSVP271AcRBS40Da were constructed.

Components	pSVP271AcRBS30Ea	pSVP271AcRBS40Da
H <sub>2</sub> O	5.0µl	5.5µl
pSVP27	0.5µl	0.5µl
1AcRBS30Ea	3.0µl	-
1AcRBS40Da	-	2.5µl
10x Ligase buffer	1.0µl	1.0µl
T <sub>4</sub> DNA ligase	0.5µl	0.5µl

Table 5.7: Recipe for ligation of DNA fragments to form plasmids pSVP271AcRBS30Ea and pSVP271AcRBS40Da

The ligation mixes were incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 were then transformed with the ligation mixes and many transformants were rapid size screened as described in section 2.2.5 to check for colonies harbouring the plasmid pSVP271AcRBS30Ea and pSVP271AcRBS40Da. Colonies whose plasmid size on agarose gel were higher than that of pSVP27 which was used as control, had the potential of having the insert 1AcRBS30Ea and 1AcRBS40Da. Plasmid DNA were extracted from clones with insert and the correct orientation of 1AcRBS30Ea and 1AcRBS40Da were confirmed by digesting extracted plasmid from pSVP271AcRBS30Ea with *Hae*III and *Bam*HI and plasmid from pSVP271AcRBS40Da with *Eco*RI and *Bam*HI (data not shown). The plasmids pSVP271AcRBS30Ea and pSVP271AcRBS40Da were successfully formed and acrySTALLIFEROUS *Bt* IPS 78/11 were transformed with confirmed plasmids

according to the method in section 2.2.3.2. Successful transformation of IPS 78/11 was confirmed by extracting plasmids from IPS 78/11 clones, retransform *E. coli* JM109 and then minipreped and digest extracted plasmids alongside the original plasmid.

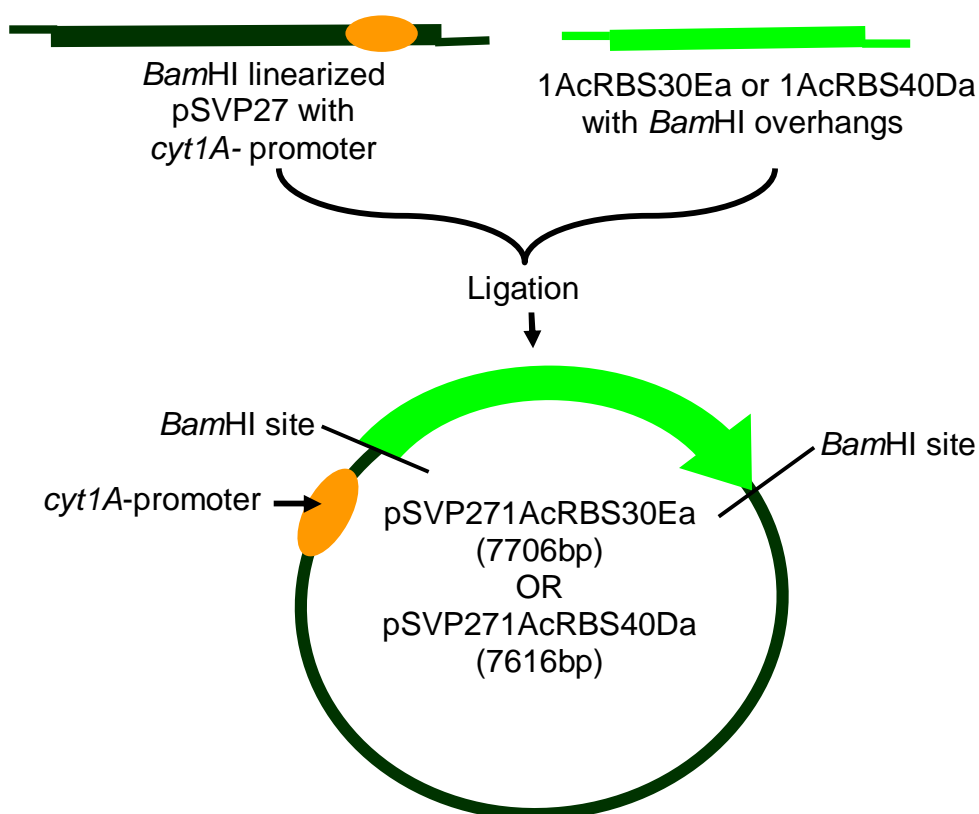


Figure 5.11: Schematic showing the construction of the plasmids pSVP271AcRBS30Ea and pSVP271AcRBS40Da

### **Expression of Cry30Ea or Cry40Da proteins in acrystalliferous *Bt* IPS 78/11 under the control of native *cyt1A*-promoter**

The clones of IPS 78/11 harbouring the plasmid pSVP271AcRBS30Ea or pSVP271AcRBS40Da were grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates of 25ml Luria agar with 5µg/ml concentration of chloramphenicol were inoculated 1ml each with the 16 hour culture of the clones and grown at 30°C for 5 days. Total protein were harvested as described in section 2.2.8.3 and ran on 7.5% SDS-PAGE gel but the protein bands of 77.6kDa and 73.5kDa from *cry30Ea* and *cry40Da* genes were not seen on gel (data not shown).

As the required bands of 77.6kDa and 73.5kDa were not seen when the clones were grown at 30°C, they were then grown at other temperatures including 20°C, 25°C and 37°C. Total proteins were harvested from each set of culture and analysed on SDS-PAGE gel and the required bands were not still seen (data not shown).

#### **5.2.2.5 Replacing ORF-2 of *cry41A* operon with the coding sequence of *cry30Ea* or *cry40Da* with *E. coli* JM109 as host**

To construct the expression vectors p101BamBam30Ea and p101BamBam40Da for the expression of *cry30Ea* and *cry40Da* genes, the plasmid p101 which is a plasmid constructed by inserting the operon of *cry41Aa1* gene lacking ORF-1 into pGEM-T vector (Vidisha Krishnan-unpublished data) was used. p101 plasmid carries a putative promoter and ribosome binding site, orf-2 (*Cry*-gene block 1-5) and orf-3 (*Cry*-gene block 6-8) of *cry41Aa1* and orf-2 is known to be a short *cry*-gene while orf-3 is the carboxyl end of a 130kDa Cry toxin (Yamashita *et al*, 2005). Since the C-terminal (block 6-8) segment of *cry*-genes have been shown to enhance crystal formation and stability of Cry proteins (Naimov, *et al*, 2006, Song *et al*, 2008), the replacement of orf-2 (block 1-5) of p101 with the short *cry*-genes (*cry30Ea* and *cry40Da*) may hypothetically enhance their expression and crystal formation since the plasmid will provide them with their lacking orf-3 (block 6-8). *cry30Ea* and *cry40Da* are usually split-genes meaning that their open reading frames normally form operons comprising of two or more reading frames (Juarez-Perez *et al.*, 2003;

Ito *et al*, 2006). One of the open reading frames contains the conserved sequence of most *cry*-genes designated as blocks 1-5 while the frame following this is another conserved sequence of *cry*-genes designated as block 6-8 (Juarez-Perez *et al*, 2003, Yamashita, *et al*, 2005, Ito *et al*, 2006). To facilitate the cloning of *cry30Ea* or *cry40Da* genes into the orf-2 position of p101 which will supply them with their lacking blocks 6-8, PCR primer pair p101BamF 5' CCGTGTGCAACTATCCCTGAC 3'/ p101BamR 5' ATCCTCCATTCCATTTCTATCC 3', which amplify p101, excluding the orf-2 of *cry41Aa1* were designed. The primers were designed such that the PCR product when ligated will have a *Bam*HI site through which *cry30Ea* or *cry40Da* genes could be inserted.

*Bam*HI sites were also created at the 5' and 3' ends of *cry30Ea* and *cry40Da* genes through their PCR amplification with primers designed to introduce the *Bam*HI sites. The primer pair 30\_40F 5' GAGATGGG**GGATCC**CTTATGA ATTC 3' and 30\_40R 5' GATATT**GGATCC**TGAGTTTGCATGAG 3' were designed with introduction of *Bam*HI sites as shown by the blue fonts. The *Bam*HI sites present at both 5' and 3' ends were introduced in such a way that when *cry30Ea* or *cry40Da* with *Bam*HI overhangs is ligated to *Bam*HI digested p101Bam, their start codon will be seven base pairs away from the ribosome-binding site. The primer pair 30\_40F/30\_40R was suitable for amplifying *cry30Ea* and *cry40Da* because they were in the same vector and share similar bases at their 5' ends.

Using the primer pair p101BamR/p101BamF and p101 as template, Pfu Ultra Master mix was used in the amplification of a segment of p101 designated as p101Bam (6086bp) while the primer pair 30\_40F/30\_40R was used in the amplification of *cry30Ea* and *cry40Da* from pGEM1AcP30Ea and pGEM1AcP40Da respectively using Taq polymerase master mix. The purified PCR products of p101Bam, *cry30Ea* and *cry40Da* are shown on figure 5.13. The use of Pfu Ultra Master mix for p101Bam amplification was to capitalize on the 3' 5' proofreading ability of Pfu thus producing a blunt ended PCR product while Taq polymerase master mix was used for amplification of *cry30Ea* and *cry40Da* because the 3' addition of adenosine (A) by Taq polymerase was to be used in direct cloning of the PCR product into pGEM-T vector for ease of digestion with *Bam*HI and final cloning into p101Bam.

The PCR product of 6086bp from the primer pair p101BamF/p101BamR was gel purified according to the method described in section 2.2.3 and 3µl of it was ligated in the presence of 5.5µl of water, 1µl of 10x ligase buffer and 0.5µl of ligase.

*E. coli* JM109 were transformed with the ligation mixes according to the method outlined in section 2.2.4.1. The colony harbouring the formed plasmid of p101Bam was confirmed by digesting extracted plasmid with *Bam*HI giving a single band of 6086bp. The PCR products from the primer pair 30\_40F/30\_40R of 2111bp and 2020bp with pGEM1AcP30Ea and pGEM1AcP40Da respectively as templates were first cloned into pGEM-T vector, which enhanced their digestion with *Bam*HI. The digestion fragments of 2096bp for *cry30Ea* and 2005bp for *cry40Da* from pGEM-TBam30Ea and pGEM-TBam40Da respectively were gel purified and ligated to *Bam*HI digested p101Bam plasmid as shown in table 5.8. Figure 5.12 illustrates the construction of the plasmids p101BamBam30Ea or p101BamBam40Da from *Bam*HI linearised p101Bam and *cry30Ea* or *cry40Da* with *Bam*HI overhangs.



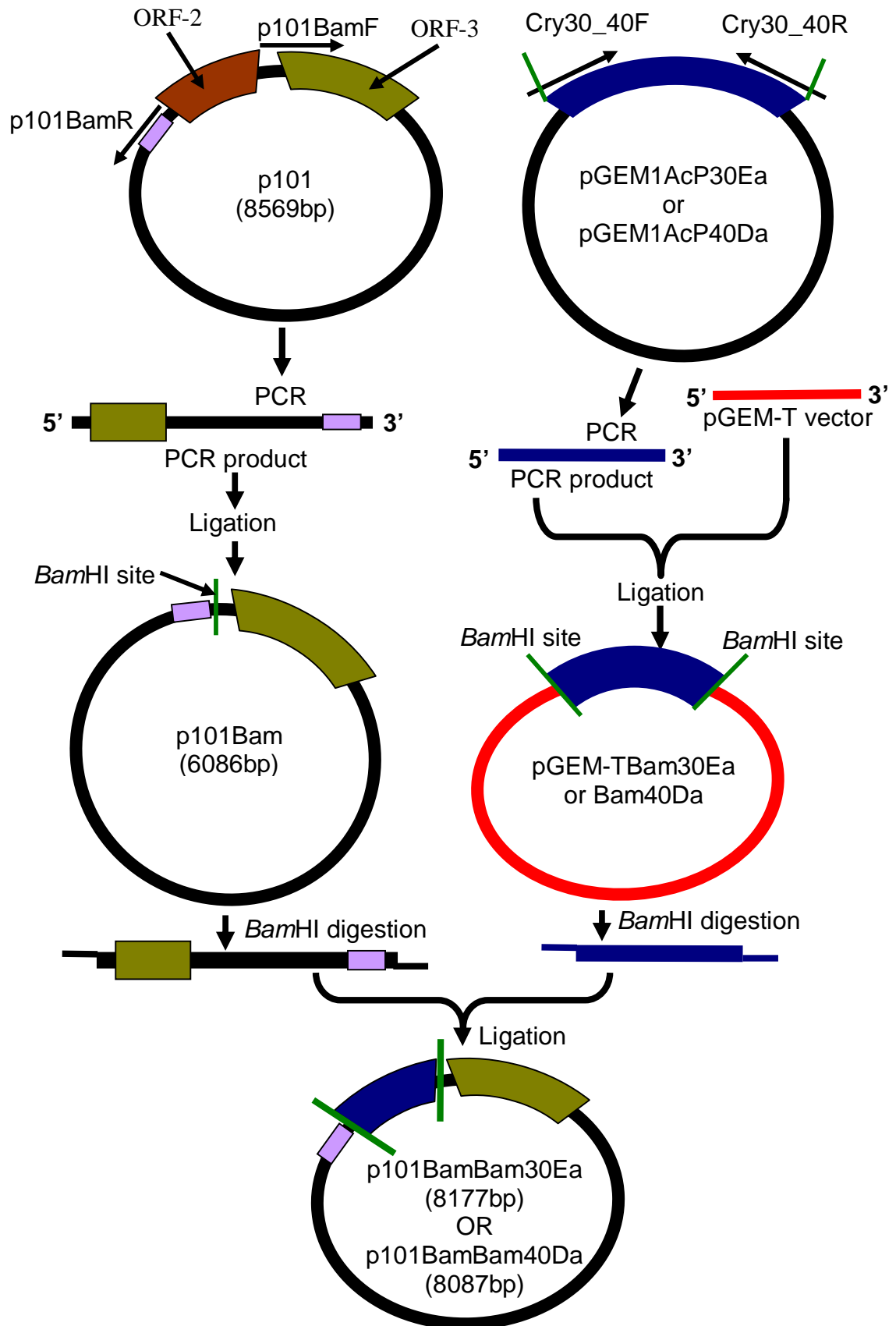


Figure 5.12: Schematic for the construction of p101BamBam30Ea and p101BamBam40Da

Components	Plasmids	
	P101BamBam30Ea	P101BamBam40Da
H <sub>2</sub> O	4.5µl	4.5µl
Bam30Ea	3µl	-
Bam40Da	-	3µl
p101Bam	1µl	1µl
10x ligase buffer	1µl	1µl
Ligase	0.5µl	0.5µl

Table 5.8: Recipe for the ligation of Bam30Ea and Bam40Da respectively to *Bam*HI linearized p101Bam for the formation of p101BamBam30Ea and p101BamBam40Da plasmids

*E. coli* JM109 was transformed with the ligation mixes according to the protocol in section 2.2.4.1. The colonies harbouring p101BamBam30Ea and p101BamBam40Da were confirmed by digestion of plasmids extracted from transformants with *Bam*HI. The correct orientation of *cry30Ea* in p101BamBam30Ea was confirmed by digestion with *Nde*I while that of *cry40Da* in p101BamBam40Da was confirmed by digestion with *Nco*I. The plasmids p101BamBam30Ea and p101BamBam40Da were successfully formed as the expected bands from their restriction digestion gave the expected bands as shown in figure 5.14 though the expected band of 54bp from p101BamBam30Ea digestion with *Nde*I could not be seen on gel as it is too small to be detected.

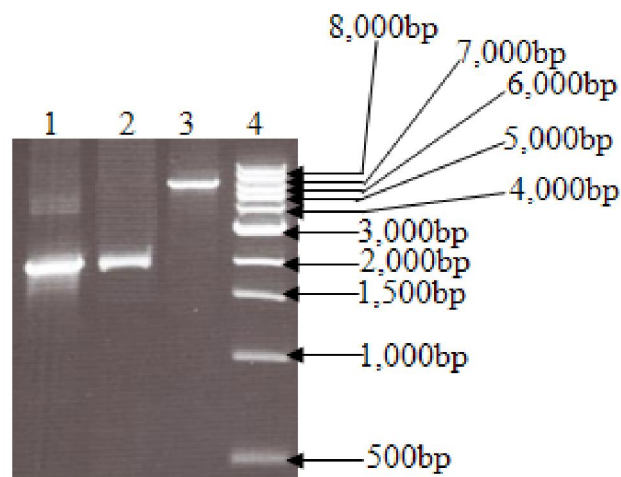
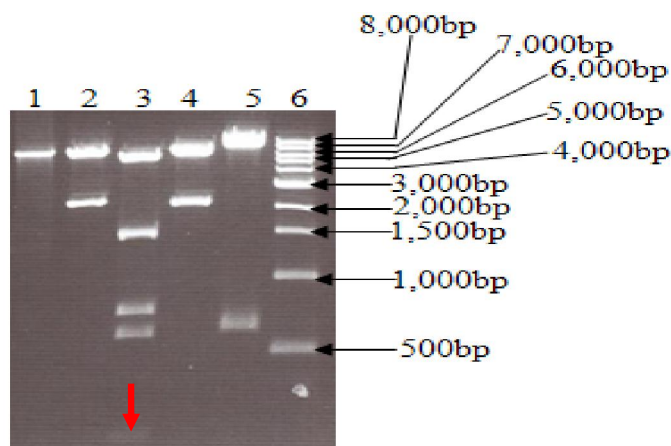


Figure 5.13: Gel picture showing the PCR products of *cry30Ea* (2111bp), *cry40Da* (2020bp) and p101Bam (6086bp) from primer pairs Cry30\_40F/Cry30\_40R and p101BamF/p101BamR. Lane 1: PCR product of 2111bp using primer pairs 30\_40F/30\_40R with pGEM1AcP30Ea as template, Lane 2: PCR product of 2020bp using primer pairs Cry30\_40F/Cry30\_40R with and pGEM1AcP40Da as template, Lane 3: PCR product of 6086bp using primer pair p101BamF/p101BamR with p101 as template, Lane 4: DNA marker

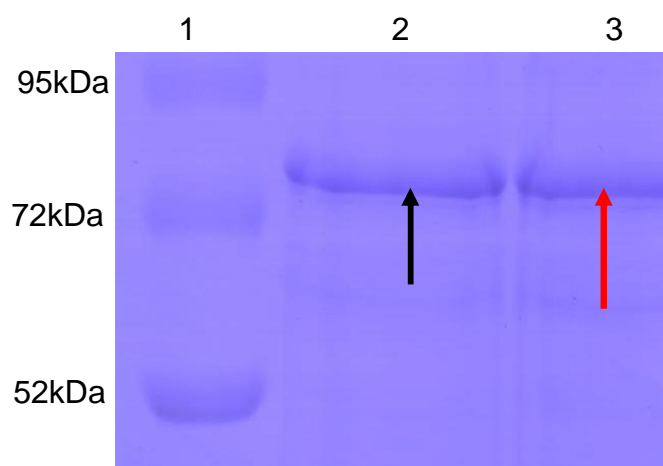


**Figure 5.14:** Gel picture confirming the successful construction of the plasmids p101Bam, p101BamBam30Ea and p101BamBam40Da. Lane 1: p101Bam digestion with *Bam*HI producing a single band of 6086bp, Lane 2: digestion of p101BamBam30Ea with *Bam*HI producing Bam30Ea fragment (2096bp) and linear p101Bam (6086bp), Lane 3: Digestion of p101BamBam30Ea with *Nde*I producing band sizes of 5265bp, 1396bp, 714bp, 577bp and 171bp (pointed by red arrow), Lane 4: Digestion of p101BamBam40Da with *Bam*HI producing Bam40Da fragment (2005bp) and linear p101Bam (6086bp), Lane 5: Digestion of p101BamBam40Da with *Nco*I producing band sizes of 7441bp and 646bp and Lane 6: DNA marker.

### **Expression of Cry30Ea or Cry40Da proteins in *E. coli* JM109**

The strains of *E. coli* JM109 harbouring the plasmid p101BamBam30Ea and p101BamBam40Da were grown in a 2x Luria broth for 3 days at 37°C after which the total protein were harvested as described in section 2.2.8.1. The total proteins were ran on 7.5% SDS-PAGE gel but the protein bands of 77.6kDa and 73.5kDa from *cry30Ea* and *cry40Da* genes were not seen on gel but a band which was about 84kDa was seen for both clones as shown in figure 5.15.

The ~84kDa bands were excised and sent for sequencing and the sequencing results showed that they were product of ORF3.



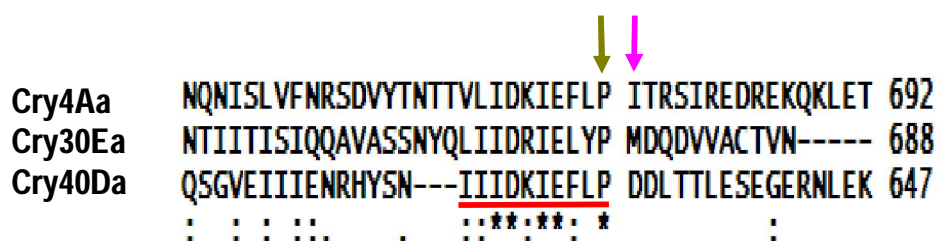
**Figure 5.15:** Picture of SDS-PAGE gel showing total proteins from *E. coli* JM109 strains harbouring plasmids p101BamBam30Ea or p101BamBam40Da. Lane 1: Protein molecular weight marker, Lane 2: Total protein from *E. coli* JM109 strain harbouring plasmid p101BamBam30Ea showing ~84kDa band (pointed by black arrow) and Lane 3: Total protein from *E. coli* JM109 strain harbouring plasmid p101BamBam40Da showing ~84kDa band (pointed by red arrow)

#### **5.2.2.6 Coupling the coding sequence of *cry30Ea* and *cry40Da* at the C-terminal end to the C-terminal of Cry4Aa to make a hybrid gene using the pET3aBam30Ea or pET3aBam40Da plasmids and *E. coli* BL21 (DE3) as host**

Considering the fact that Cry30Ea and Cry40Da are short Cry-proteins lacking the C-terminal half (blocks 6-8) of a full length 130kDa Cry-proteins and always form an operon with an ORF having homology to C-terminal of full length Cry-protein, we decided to fuse their proteins with the C-terminal half of Cry4Aa, which is a full length Cry toxin, to form a hybrid protein. The C-terminal (blocks 6-8) of Cry proteins have been shown to facilitate crystallisation and its linkage to Cry30Ea and Cry40Da may enhance their expression and folding.

To determine where about in the C-terminal of *cry30Ea* and *cry40Da* to start the fusion of C-terminal of *cry4Aa*, multiple sequence alignment using ClustalW

programme (Larkin *et al*, 2007) was conducted using the deduced protein sequences for Cry30Ea and Cry40Da and the protein sequence of Cry4Aa. From the multiple sequence analysis, a conserved region close to the C-terminal end of the Cry30Ea and Cry40Da deduced protein sequences was observed as seen in figure 5.16 underlined with red line. A proline which is the the last amino acid in the observed conserved block pointed by the dark yellow arrow in figure 5.16 was the last amino acid for Cry30Ea and Cry40Da that will be used in the fusion while the beginning of the C-terminal of Cry4Aa used for the fusion was the isoleucine pointed by the pink arrow in figure 5.16 which follows the end of the conserved block.



**Figure 5.16:** Multiple sequence alignment indicating the ends of Cry30Ea and Cry40Da and beginning of Cry4Aa C-terminal used in the fusion protein construction. The dark yellow arrow points to the end of C-terminal conserved blocks for Cry30Ea and Cry40Da, the pink arrow points to the beginning of Cry4A C-terminal used in the fusion while the red line shows the C-terminal conserved region from the three protein sequences

With the ends of Cry30Ea and Cry40Da sequences determined through the multiple sequence alignment, PCR primers 304AF 5' GGATCCGGC-TGCTAACAAAG 3'/304AR 5' TGGATATAATTCGATTCTGTCAATAATT 3' for Cry30Ea and 404AF 5' GGATCCGGCTGCTAACAAAG 3'/404AR 5' CGGTAAAAATTCAATTTTGTCAATG 3' for Cry40Da were designed to amplify the truncated *cry30Ea* and *cry40Da* including the pET3a vector in pET3aBam30Ea and pET3aBam40Da plasmids respectively excluding the sequence starting from methionine (Cry30Ea) and aspartic acid (Cry40Da). Primers pair 4ACF 5' ATTACTCGTTCTATAAGAGAGGATAGAGAG 3'/4ACR 5'

TCACTCGTTCATGCAAATTAATTCAATG were also designed to amplify Cry4Aa C-terminal starting from isoleucine down to its stop codon. For simplicity, the PCR product using 304AF/304AR primers with pET3aBam30Ea as template will be designated as pET3a30Ea, while the PCR product using 404AF/404AR with pET3aBam40Da as template will be designated as pET3a40Da. Also the C-terminal fragment amplified from Cry4Aa gene using primer pair 4ACF/4ACR will be designated as 4Aa.

Using Pfu Ultra Hotstart PCR Master Mix system according to the manufacturers amplification instructions, pET3a30Ea, pET3a40Da and 4Aa sequences were amplified and PCR products were ran on 1% agarose gel and required bands of 6680bp (pET3a30Ea), 6542bp (pET3a40Da) and 1515bp (4Aa) were excised and purified according to the PCR products purification procedure described in section 2.2.3.

The gel purified PCR products of 6680bp (pET3a30Ea), 6542bp (pET3a40Da) and 1515bp (4c) were ligated according the recipe outlined in table 5.9 while figure 5.17 gives a diagrammatic presentation of how the pasmids pET3a30Ea-4Aa and pET3a40Da-4Aa were constructed.

Components	pET3a30Ea-4Aa	pET3a40Da-4Aa
H <sub>2</sub> O	1.5µl	1.5µl
4Aa	5µl	5µl
pET3a30Ea	2µl	-
pET3a40Da	-	2µl
10x Ligase buffer	1µl	1µl
T <sub>4</sub> DNA ligase	0.5µl	0.5µl

**Table 5.9:** Recipe for ligation of DNA fragments to form plasmids pET3a30Ea-4Aa and pET3a40Da-4Aa

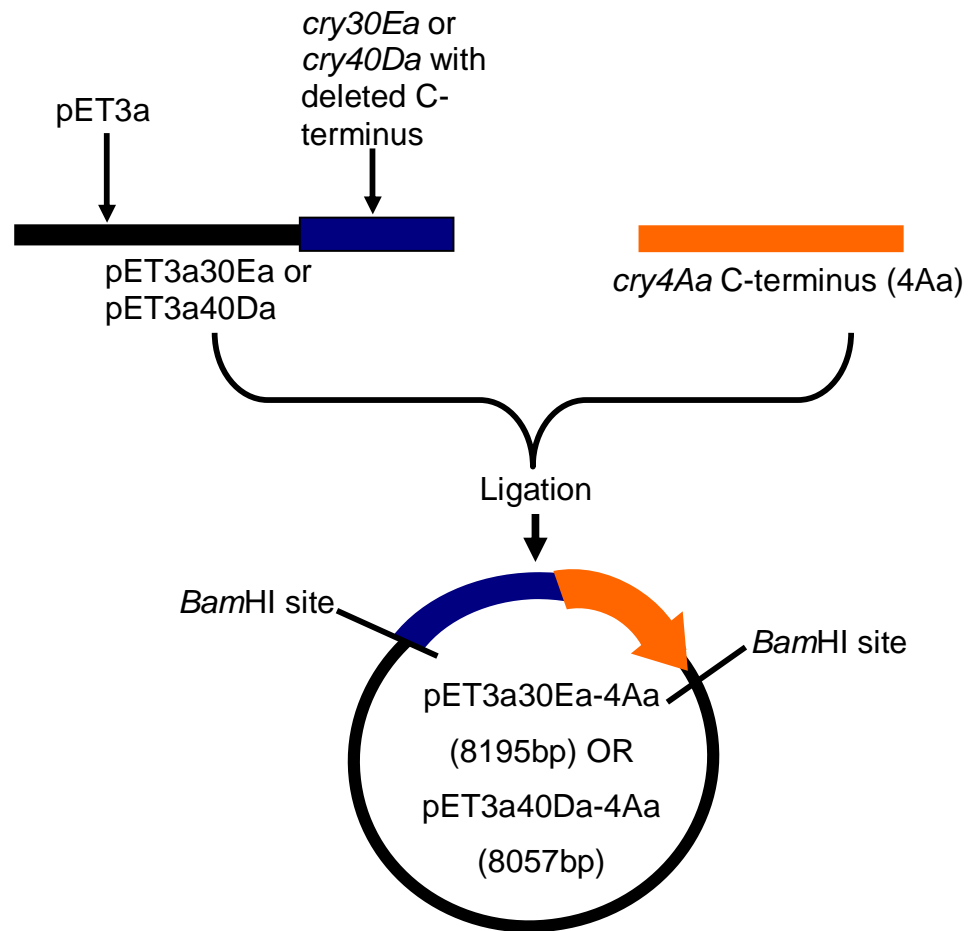
The ligation mixes were incubated at room temperature for 3 hours and then stored at 4°C overnight.

*E. coli* JM109 were transformed with the ligation mixes and many transformants were screened to check for colonies harbouring the plasmid pET3a30Ea-4Aa

and pET3a40Da-4Aa. Clones harbouring plasmid pET3a30Ea-4Aa with the correct orientation of *cry4Aa* C-terminal fragment (4Aa) were confirmed by digesting extracted plasmid with *EcoRI* and *BamHI* (data not shown). The construction of the plasmid pET3a40Da-4Aa was not successful with the first ligation. To enhance the construction of the plasmid pET3a40Da-4Aa, a new ligation mix of 20µl volume was made and incubated at 15°C for 4 hours and then at 4°C for 16 hours. The ligation mix was then extracted in 1:1 mixture of ligation mix to phenol-chloroform solution. This was to help detach any ligase molecules that could be bound to the ligating DNA fragments thus reducing the molecular weight of the ligation product and easing its transportation through the *E. coli* cell wall. The ligation mix/phenol-chloroform mixture was centrifuged at 14,000xg for 10 minutes and the supernatant was carefully extracted and used in transformation of *E. coli* JM109. The construction of the plasmid pET3a40Da-4Aa was not still successful.

*E. coli* BL21(DE3) were transformed with confirmed pET3a30Ea-4Aa plasmid and transformed *E. coli* BL21(DE3) was confirmed by extracting and digesting plasmids from transformants.





**Figure 5.17:** Schematic illustrating the strategy used in the construction of plasmids pET3a30Ea-4Aa or pET3a40Da-4Aa from ligation of pET3a30Ea or pET3a40Da to cry4Aa C-terminus (4Aa)

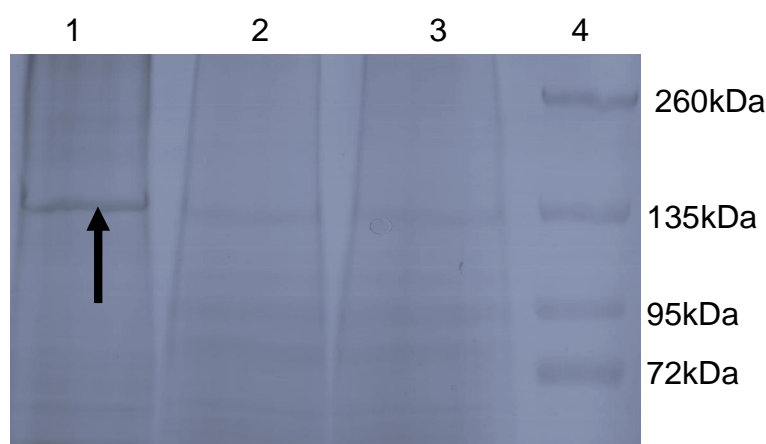
### **Expressing *cry30Ea-4Aa* gene from plasmid pET3a30Ea-4Aa under the control of T7 promoter through IPTG induction**

*E. coli* BL21 (DE3) harbouring pET3a30Ea-4Aa plasmid was subcultured into 10ml of Luria broth containing 100µg/ml of ampicillin and grown overnight. The 10mls overnight culture was inoculated into a fresh 100ml Luria broth containing 100µg/ml of ampicillin and grown to optical density of 0.802. At this optical density, 50µl of 1M IPTG was added to the culture and grown at 37°C for 16 hours. Total protein was harvested according to the method described in

section 2.2.8.2. The total protein was run on 7.5% SDS-PAGE gel and the expected protein band of about 133.5kDa was seen on gel, though very faint.

To confirm that the observed band of ~134kDa was from the gene *cry30Ea-4Aa*, the BL21 (DE3) clone with the pET3a30Ea-4Aa plasmid and BL21 (DE3) without the plasmid were grown as previously described but in 200mls Luria broth and when they attained optical density of 0.7, the cultures were divided into two equal portions and one portion was induced with IPTG while the other half was not induced.

Total proteins were harvested and ran on 7.5% SDS-PAGE gel and the ~134kDa band was still observed with the IPTG induced clone having the pET3a30Ea-4Aa plasmid but not with the uninduced strain and the BL21 (DE3) without the plasmids as shown in figure 5.18.



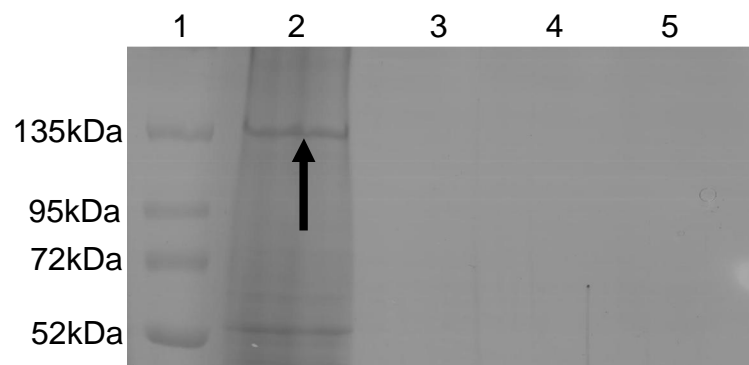
**Figure 5.18:** SDS-PAGE picture of the induced and non-induced strain of *E. coli* BL21 (DE3) harbouring the plasmid pET3a30Ea-4Aa. Lane 1: Total protein from *E. coli* BL21 (DE3) harbouring plasmid pET3a30Ea-4Aa induced with IPTG showing presence of ~134kDa band (pointed by black arrow), Lane 2: Total protein from *E. coli* BL21 (DE3) harbouring plasmid pET3a30Ea-4Aa but not induced with IPTG, Lane 3: Total protein from *E. coli* BL21 (DE3) without the plasmid pET30Ea-4Aa and Lane 4: Protein molecular weight marker

### **Characterisation of the expressed fusion protein**

The expressed protein was tested for alkaline solubility and protease activation according to the method in section 2.2.9. The results in figure 5.19 shows that the ~134kDa protein was not seen when the supernatant from the pH11 alkaline solubility test was ran on SDS-PAGE gel but the ~134kDa band was still present in the pellet meaning that the protein is not soluble in alkaline solution. Results from SDS-PAGE gel analysis of the total reaction mixes and supernatants from the protease activation experiments show that the protease resistant core of about 50-60kDa typical of Cry-proteins was not seen. This means that the protein was degraded by protease.

From the characterisation results of the expressed ~134kDa protein, it was hoped that by changing the culturing conditions like concentration of IPTG, culturing temperature after inductions and time of growth after inductions, a functional protein could be obtained. With this assumption considered, the clone was cultured at 37°C, induced with IPTG to concentration of 0.25mM, 0.5mM and 1mM, grown after induction at temperatures of 20°C, 25°C and 30°C and grown for 8 hours, 12 hours and 24 hours after induction.

The proteins from these cultures were harvested and they all show the ~134kDa protein on SDS-PAGE gel but were neither soluble in alkaline solution nor produce a resistance core from protease activation experiments as shown in table 5.10.



**Figure 5.19:** SDS-PAGE Gel picture showing the ~134kDa, alkaline solubility and protease activation test. Lane 1: Protein molecular weight marker, Lane 2: Total reaction sample from 50mM Na<sub>2</sub>CO<sub>3</sub> pH11 solubility test of ~134kDa band from pET3a30Ea-4Aa strain showing the presence of ~134kDa band (pointed by black arrow), Lane 3: Supernatant from 50mM Na<sub>2</sub>CO<sub>3</sub> pH11 solubility test of ~134kDa band from pET3a30Ea-4Aa strain indicating absence of the ~134kDa band, Lane 4: Total reaction sample from trypsin activation test of ~134kDa band from pET3a30Ea-4Aa strain indicating absence of resistant core, Lane 5: Supernatant from trypsin activation test of ~134kDa band from pET3a30Ea-4Aa strain indicating absence of resistant core.

IPTG conc. (mM)	Temperature (°C)	Time of incubation after IPTG induction	Protein size on SDS-PAGE (~kDa)	Alkaline solubility	Protease activation
0.25	20	24 hours	134	Not Soluble	No protease resistant core
0.5	20	8 hours	134	Not Soluble	No protease resistant core
1.0	20	16 hours	134	Not Soluble	No protease resistant core
0.5	25	24 hours	134	Not Soluble	No protease resistant core
0.5	30	8 hours	134	Not Soluble	No protease resistant core

Table 5.10: Summary of the changes in IPTG concentration, temperature of growth after IPTG induction and time of growth after IPTG induction and characteristics of expressed proteins

#### **5.2.2.7 Attempting co-expression of *cry30Ea* and *cry40Da* genes in crystal toxin producing strain of *Bt***

It was reported by Slim *et al*, 2010 (Contributed paper, SIP Meeting, Turkey-2010) that a Cry protein that is not usually expressed could be expressed if a *Bt* strain that is already capable of expressing and forming a crystalline inclusion body of Cry toxins is transformed with a plasmid carrying the non-expressing toxin gene.

Wild type *Bt* strain Cry<sup>+</sup>HD73 which expresses Cry1Ac were transformed with plasmids pSV21AcP30Ea, pSV21AcP40Da, pSVP271AcRBS30Ea and pSVP271AcRBS40Da constructed in sections 5.2.2.3 and 5.2.2.4 for expression of *cry30Ea* and *cry40Da*.

Successful transformation of Cry<sup>+</sup>HD73 was confirmed by extracting plasmids from transformants that were chloramphenicol resistance, retransforming *E. coli* JM109 with extracted plasmids and then minipreping transformed *E. coli* JM109 and digesting extracted plasmids alongside the original plasmids.

#### **Expression of Cry30Ea or Cry40Da proteins in Cry<sup>+</sup>HD73 clones harbouring the plasmids pSV21AcP30Ea, pSV21AcP40Da, pSVP271AcRBS30Ea or pSVP271AcRBS40Da**

The clones of Cry<sup>+</sup>HD73 harbouring the plasmid pSV21AcP30Ea, pSV21AcP40Da, pSVP271AcRBS30Ea or pSVP271AcRBS40Da were grown in 5ml of Luria broth for 16 hours at 30°C. Five Petri plates of 25ml Luria agar with 5µg/ml concentration of chloramphenicol were inoculated 1ml each with the 16 hour culture of the clones and grown at 30°C for 5 days. Observation of the growth on plates under the light microscope shows that the cells have sporulated and formed bipyramidal crystals. Total protein were harvested as described in section 2.2.8.3 and ran on 7.5% SDS-PAGE gel but the protein bands of 77.6kDa and 73.5kDa from *cry30Ea* and *cry40Da* genes were not seen but the 130kDa band of Cry<sup>+</sup>HD73 native Cry1Ac protein was seen on gel (data not shown).

As the required bands of 77.6kDa and 73.5kDa were not seen when the clones were grown at 30°C, they were then grown at other temperatures including 20°C, 25°C and 37°C. Total proteins were harvested from each set of culture and

analysed on SDS-PAGE gel but the required bands were not still seen (data not shown).

### 5.3 Discussion

Despite the various strategies utilised to enhance the expression of *cry30Ea* and *cry40Da* genes that were amplified from mosquitocidal strains of *Bacillus thuringiensis* S2160-1 and S2196-1 respectively, a functional protein could not be expressed.

The utilisation of native *cry1Ac*-promoter in pGEM-T vector backbone with *E. coli* JM109 as host that could not express *cry30Ea* and *cry40Da* genes has been found to be very effective in expressing other *cry*-genes including Cry1Ac (unpublished data), Cry1Ie, Cryle, Cry1IeA522V, Cry1IeY442H, Cry1Ah and CryAIA as described in chapter 3 and 4 of this thesis. Among these *cry1Ac*-promoter controlled expressed proteins are short wild type Cry-protein Cry1Ie; full-length wild type Cry-protein Cry1Ac and Cry1Ah; genetically manipulated short Cry-protein Cryle, Cry1IeA522V and Cry1IeY442H; and full length genetically manipulated Cry-protein CryAIA.

Expression of *cry30Ea* and *cry40Da* genes utilising *cry1Ac* or *cyt1A*-promoters in acrySTALLIFEROUS *Bt* was not successful despite the changes in culturing conditions. *cyt1A*-promoter has been used in the expression of Cry1Ah as described in chapter 3 and has been used in the expression of Cry2A (Crickmore and Ellar, 1992). *cry1Ac*-promoter has been used in expression of Cry11A with *Bacillus brevis* as host (Roh *et al*, 2010).

Though the expression of *cry30Ea* and *cry40Da* genes under the control of T7 promoter gave a protein band that was associated with the clones harbouring the plasmids pET3aCry30Ea or pET3aCry40Da, the observed protein bands of ~62kDa fall short of the expected band of 77.8kDa and 73.5kDa respectively for Cry30Ea and Cry40Da thus casting doubts on the source of the ~62kDa bands. Normally there should have been a difference in band size between the protein expressed from *cry30Ea* and *cry40Da* genes but this was not the case as ~62kDa observed was the same for both clones. Moreover, alkaline solubility characterisation and protease activation failed to prove that the observed ~62kDa bands were Cry toxins. The observed results cast doubts on the functional integrity of the *cry30Ea* and *cry40Da* genes because T7 promoter has

been used in expressing functional Cry toxins like Cry1Ie, a short Cry-protein (Song *et al*, 2003), Cry2A and its mutant and a full length Cry-protein (Mandal *et al*, 2007). Vip3Ba1 a member of a class of insecticidal protein expressed during vegetative growth of some strains of *Bacillus thuringiensis* has been cloned from a strain of *Bacillus thuringiensis* and expressed in *E. coli* under the control of T7 promoter (Rang *et al*, 2005). The use of *E. coli* BL21 Rosetta which has been shown to express other Cry toxins did not rescue the expression of *cry30Ea* or *cry40Da* genes in a complete and functional form.

The function of each of the domains in a Cry protein has been extensively studied and the C-terminal (blocks 6-8) has been found to be cysteine rich and function in the stability and enhancement of crystal formations through disulphide bonds (Wabiko and Yasuda, 1995, Naimov *et al*, 2006, Song *et al*, 2008). The stability of Cry30Ea could not be achieved despite the construction of hybrid protein that was made up of *cry30Ea* gene and a C-terminal of *cry4Aa* gene. Despite the strategy designed to make sure that the transition from Cry30Ea into Cry4Aa C-terminal in the hybrid protein was as close to the continuation of Cry4Aa as possible, the hybrid protein could only produce a required band of ~134kDa protein that turn out to be non-functional.

Though *cry30Ea* and *cry40Da* genes have a high level of similarity to members of their groups in the *Bacillus thuringiensis* toxin nomenclature as reviewed by Crickmore *et al*, 1998 and hosted at the web link ([http://www.lifesci.-sussex.ac.uk/Home/Neil\\_Crickmore/Bt/](http://www.lifesci.-sussex.ac.uk/Home/Neil_Crickmore/Bt/)), it might be that they are pseudogenes. Pseudogenes are basically open reading frames that are homologous to functional genes but cannot be expressed due to mutation that preclude their expression or lack of some flanking control region in the promoter (Cammack *et al*, 2006). Martin *et al*, 2010 computational analysis of the genome of *Bacillus anthracis* reveals pseudogenes with promoters that maintain transcriptional activity. It has been reported that a frame shift in the coding region of a novel tomato class I basic chitinase gene results in the formation of a pseudogene (Baykal *et al*, 2006). Reports have also shown that a nonsense and missense mutation in *Chlamydia trachomatis* serovar L2 and D strains results in the formation of pseudogenes (Giles *et al*, 2009). In the case of *cry30Ea* and *cry40Da*, it does not seem to be the lack of some flanking control region in the promoter that is rendering them non functional because upstream elements like



*cry1Ac*, *cyt1A*, T7 promoters and ribosome binding site have been supplied to them and they cannot still be expressed whereas these upstream elements have been used in the expression of other genes. It is likely that their pseudogene status is as result of mutation within the open reading frame which could be nonsense, missense, insertion or deletion as pairwise sequence alignment with members of their groups as shown in appendices 3 and 4 indicates differences like insertions and deletions.

With the numerous methods that have been used to express other genes failing to expression *cry30Ea* and *cry40Da* genes, the functional integrity of these open reading frames is therefore questionable and they can rather be seen as pseudogenes that have been accumulated in the genome of the strains S2160-1 and S2196-1 respectively during evolutionary process.

## Chapter 6: Development of a protocol for in vivo selection of toxin variants with improved activity

### 6.1 Introduction

The creation of Cry toxin mutant libraries has been achieved through directed evolution techniques like DNA shuffling (Craveiro *et al.*, 2010), error prone PCR (Shu *et al.*, 2007) and a combination of error-prone PCR, staggered extension process (StEP) shuffling and Red/ET homologous recombination (Shan *et al.*, 2011). Though these methods might create vast number of mutants, selecting for a variant with improved toxicity is always laborious, time consuming and expensive if all the variants created are to be individually tested for activity. With the perceived cost involved in screening every mutant created through directed evolution techniques, screening of such libraries is often through a random selection of a few which has a possibility of leaving out mutants with improved toxicity that was sort for in the first place. To exemplify the amount of time and resources that could be wasted in assaying every individual in a mutant library, out of 35 clones that were selected and assayed by Shu *et al.*, 2007, 21 clones showed reduced toxicity, 6 clones showed similar toxicity as the wild type while 8 clones showed increased toxicity meaning that about 77% of time and resources were wasted.

Considering the high cost associated with screening every strain in a mutant library, we were interested in investigating the possibility of whether a strain expressing a more active toxin could be enriched for in a mixed pool in vivo which could be utilised in screening mutant libraries for strains with improved activity. This interest was based on the possibility of a strain expressing a more active toxin germinating and replicating faster than a strain expressing a less active toxin in vivo. As demonstrated by Du and Nickerson, 1996 the presence of BBMV's from *Manduca sexta* increases the rate and completeness of Cry<sup>+</sup> HD73 spore germination but not Cry<sup>-</sup> HD73 spores. They also showed that toxin receptors of 115 to 120kDa could be purified from Cry<sup>+</sup> spores/solubilised BBMV's complex which shows that there is specific interaction between the Cry<sup>+</sup> spores and the insect gut receptors. Moreover, they demonstrated that spores from a crystal producing strain of *Bt* were activated for germination at alkaline pH 10.3 while non-crystal producing spores could not be activated under the same condition. Raymond *et al.*, 2008a also demonstrated that four

strains of *Bacillus thuringiensis* var. *kurstaki* and one of *Bacillus thuringiensis* var. *tenebrionis* were capable of growing in alkaline pH. Meanwhile, studies have shown that the pH of midgut of insect orders like Lepidoptera and Diptera range from 8.0 to 12.4 (Berenbaum, 1980, Gringorten *et al*, 1993) which would be a suitable in vivo environment to test for spore germination. From these findings, Cry<sup>+</sup> strains are better adapted to germination and reproducing in the midgut of lepidopteran than Cry<sup>-</sup> strains. The mode of action of three-domain Cry toxin has been proposed to involve a multi-step process of toxin crystal dissolution in the alkaline environment of the insect midgut to release protoxins. The released protoxins have been shown to be first processed by midgut proteases which removes the C-terminal half and approximately 30 amino acid residues from the N-terminal revealing the active toxins which then bind to receptors on epithelial cells of susceptible insect gut. This binding culminates into a second cleavage by membrane bound proteases which removes the N-terminal helix -1. The toxins which at this stage are bound to specific receptors, form oligomers which insert into the epithelial cell membrane (Bravo *et al.*, 2004, Bravo *et al.*, 2007, Gomez *et al.*, 2007). Toxin insertion leads to the formation of lytic pores in microvilli of apical membranes (Bravo *et al.*, 2007). Subsequently, cells lyse and disruption of the midgut epithelium releases the cell contents providing *Bacillus thuringiensis* spores a germinating medium leading to a severe septicaemia and insect death (de Maagd *et al.*, 2001, Bravo *et al.*, 2007). From these findings, it was thought that since Cry<sup>+</sup> spore germination rate was enhanced by BBMV binding and *Bt* strains are capable of growing in alkaline environment as found in some insect guts, a strain with more active toxin would bind, germinate, replicate and dominate its less toxic counterpart in an in vivo pool.

Studies have shown that crystals formed by *Bacillus thuringiensis* ssp. *finitimus* during sporulation are associated with the spores of the mother cells (Wojciechowska *et al*, 1999) while in other strains of *Bt* the crystals are dissociated from the mother cell spores after sporulation. Though the spores are separated from the crystals in many species, in the situation of an insect gut, it has been suggested that there is high possibility of having spores and their crystals still lying close together (Raymond *et al.*, 2010). Moreover, traces of toxins could still be detected from samples of purified spores (Johnson and

McGaughey, 1996, Tang *et al*, 1996, Johnson *et al*, 1998) which shows how difficult separating spores from their crystal toxins could be and hence how likely it is to find them together. Du and Nickerson, 1996 also showed that purified spores from a *Bt* strain producing Cry1Ac toxin were able to cross-react with antibodies raised using a 65kDa protease activated core portion of Cry1Ac which means that toxins could be detected on spore surfaces. Crystal toxins have also been purified from extracted spore coat proteins (Aronson *et al.*, 1982, Johnson *et al.*, 1998). Purified spores from crystal producing strains of *Bacillus thuringiensis* have been found to be lethal to insect pests albeit at a much lower degree compared to the lethality of the crystal protein though the purified spores were still found to have some traces of toxins which could support their toxicity (Johnson and McGaughey, 1996, Tang *et al*, 1996, Johnson *et al*, 1998). It has also been shown that a mixture of crystal protein and spores from the same strain in a bioassay can result in a synergistic insecticidal activity (Johnson and McGaughey, 1996, Tang *et al*, 1996, Johnson *et al*, 1998) which could be as a result of familial interaction between the spores and the crystals. With the idea of close proximity between spores and its crystals, it was thought that spores from a strain whose toxin binds to insect gut and creates a lesion would be highly likely to be the one to get into the haemocoel and cause septicaemia hence the strain that will dominate a mixed infection. Moreover, since it is a toxin with higher activity that will bind better and better effect the lesion, it was thought that this could also be a means of enriching for a strain with more active toxin in vivo.

The possibility of enriching for a strain of *Bt* with more active toxin in an in vivo mixed pool was also based on the findings that there exists competition and reproduction between pathogenic and non-pathogenic strains of *Bacillus* species in an in vivo mixed infection. Raymond *et al.*, 2007 reported that *Bacillus cereus*, which is a non-insect-pathogenic *Bacillus*, dominated its toxin producing, Lepidopteran toxic counterpart *Bacillus thuringiensis kurstaki* in an in vivo mixed infection with *Plutella xylostella* as host. The dominance of *Bacillus cereus* agreed with the prediction that selection for virulence in a group infection involving related individuals will depend on group's cooperation that will result in low energy cost (Brown *et al.*, 2002). Also, as reported by West *et al.*, (2007), individuals in a group can benefit from public goods that are produced by

related members of the group who are themselves negatively affected because they expend more energy than the benefiting members of the group thus having a reduced fitness. It was therefore expected that *Bacillus cereus* will dominate the crystal producing *Bacillus thuringiensis kurstaki* as the energy cost of *Bacillus cereus* reproduction is expected to be lower than that of the crystal producing *Bacillus thuringiensis kurstaki*. With competition observed between related species, it was thought that two crystal producing strains will have the same energy cost and that dominance will therefore be controlled by ability to bind to gut receptors and activation for germination that has been reported to favour toxic Cry<sup>+</sup> strains.

From the above, a hypothesis was proposed that *Bt* strain expressing a toxin with high toxicity would be preferentially activated for germination in the presence of its non or less functional Cry<sup>+</sup> counterpart in an in vivo mixed infection, and that a cadaver from this mixed treatment will be dominated by *Bt* strain with higher toxicity. In this chapter, the results from in vivo experiments conducted to test germination and replication ability of Cry<sup>+</sup> and Cry<sup>-</sup> spores of *Bt* in single and mixed infection, germination and replication ability of two Cry<sup>+</sup> strains of *Bt* with one expressing an active toxin and the other expressing a non-toxic variant in single and mixed infections will be investigated.

## **6.2 Single and mixed treatment of *Plutella xylostella* with crystal and non-crystal producing *Bt* strains and recovery of spores from dead and living larvae**

This set of experiment was conducted to see whether a toxic crystal producing strain of *Bt* would out compete an acrySTALLiferous non-toxic strain in vivo. To enhance the recovery of *Bt* spores from insect cadavers, wild type *Bt* strain Cry<sup>+</sup>HD73 (crystalliferous) and its plasmid cured strain Cry<sup>-</sup>HD73 (acrySTALLiferous) were transformed with plasmids pSV2 and pUBpUC which confer chloramphenicol and kanamycin resistance respectively. The transformed strains hereafter named as Cry<sup>+</sup>HD73/SV2, Cry<sup>-</sup>HD73/SV2, Cry<sup>+</sup>HD73/pUBpUC and Cry<sup>-</sup>HD73/pUBpUC were subcultured on 5µg/ml chloramphenicol or 50µg/ml kanamycin agar plates and single colonies from each strain were picked, cultured and stored. These stocks were used throughout these experiments.

To obtain spores from Cry<sup>+</sup>HD73/SV2, Cry<sup>-</sup>HD73/SV2, Cry<sup>+</sup>HD73/pUBpUC, Cry<sup>-</sup>HD73/pUBpUC, stock cultures were grown on 5µg/ml chloramphenicol or 50µg/ml kanamycin agar plates for five days at 30°C, growth was observed under the microscope to make sure they had sporulated. The sporulated cells were recovered in 20ml of distilled water and pasteurized at 70°C for 45 minutes in water bath to inactivate vegetative cells.

The spores were serially diluted and 100µl of the 10<sup>-6</sup> dilution was plated in duplicate for each strain for spore count estimation as described in section 2.2.8.5. The inoculated plates were incubated overnight at 30°C and colonies counted as shown in table 6.1.

Strain	Colonies		Average colonies	Number of spores/ml of sample
	Plate 1	Plate 2		
Cry <sup>+</sup> HD73/SV2	46	42	44	4.4 X 10 <sup>8</sup>
Cry <sup>-</sup> HD73/SV2	112	106	109	1.1 X 10 <sup>9</sup>
Cry <sup>+</sup> HD73/pUBpUC	110	90	100	1.0 X 10 <sup>9</sup>
Cry <sup>-</sup> HD73/pUBpUC	532	488	500	5.0 X 10 <sup>9</sup>

Table 6.1: Quantification of spores/crystals in Cry<sup>+</sup>HD73/SV2, Cry<sup>-</sup>HD73/SV2, Cry<sup>+</sup>HD73/pUBpUC, Cry<sup>-</sup>HD73/pUBpUC

The number of spores/ml for each strain was calculated using the formula

$$\frac{\text{Average number of colonies}}{\text{Plated dilution} \times \text{amount plated}}$$

The spore concentrations were prepared in 30ml of water containing 50µl/L triton using either a single strain or a combination of two strains as shown in table 6.2. The spore dilutions were exposed to 3<sup>rd</sup> instar larvae of *Plutella xylostella* as described in material and methods section 2.2.13.1.

Treatment type	Strains of <i>Bacillus thuringiensis</i> and spore concentration
Control	No Cry <sup>+</sup> HD73/pUBpUC or Cry <sup>-</sup> HD73/pUBpUC
single	Cry <sup>+</sup> HD73/SV2(1000/ml)
single	Cry <sup>-</sup> HD73/SV2(1000/ml)
single	Cry <sup>+</sup> HD73/pUBpUC(1000/ml)
single	Cry <sup>-</sup> HD73/pUBpUC(1000/ml)
mixed	Cry <sup>+</sup> HD73/SV2(1000/ml)+ Cry <sup>-</sup> HD73/SV2(1000/ml)
mixed	Cry <sup>+</sup> HD73/pUBpUC(1000/ml) + Cry <sup>-</sup> HD73/pUBpUC(1000/ml)
mixed	Cry <sup>-</sup> HD73/pUBpUC(1000/ml) + Cry <sup>+</sup> HD73/SV2(1000/ml)
mixed	Cry <sup>+</sup> HD73/pUBpUC(1000/ml) + Cry <sup>-</sup> HD73/pUBpUC(2000/ml)
mixed	Cry <sup>+</sup> HD73/pUBpUC(2000/ml) + Cry <sup>-</sup> HD73/pUBpUC(2000/ml)

**Table 6.2:** Types of *Plutella xylostella* treatment including strains and quantity of spores/ml in each sample

After exposing five 3<sup>rd</sup> instar larvae of *Plutella xylostella* to the treated cabbage leaf discs for seven days, the dead and live larvae were recovered and larvae from each disc were homogenised in 0.85%(w/v) NaCl solution. The homogenates were pasteurized at 70°C for 45 minutes to kill vegetative cells. The spore suspensions from strains with pSV2 plasmid were plated out on chloramphenicol plates for single colonies while those from stains with pUBpUC plasmid were plated out on kanamycin plates for single colonies. The plates

were incubated at 30°C overnight. Single *Bacillus thuringiensis* colonies from the chloramphenicol plate could not be recovered as contaminants possibly from the insect's gut or cabbage leaf discs outgrow and covered the plate but single colonies of *Bacillus thuringiensis* from the kanamycin plates were subcultured and incubated at 30°C for five days. On the fifth day, the growths were observed under the light microscope for spores and crystals and the results was as shown in table 6.3.

Treatment	Total number of kanamycin resistance colonies observed	Number of Cry <sup>+</sup> strains	Number of Cry <sup>-</sup> strains
Control	0	0	0
Cry <sup>+</sup> HD73/pUBpUC(1000/ml)	46	46	0
Cry <sup>-</sup> HD73/pUBpUC(1000/ml)	50	0	50
Cry <sup>+</sup> HD73/pUBpUC(1000/ml) and Cry <sup>-</sup> HD73/pUBpUC(1000/ml)	112	74	38
Cry <sup>-</sup> HD73/pUBpUC(1000/ml) and Cry <sup>+</sup> HD73/SV2(1000/ml)	62	0	62
Cry <sup>+</sup> HD73/pUBpUC(1000/ml) and Cry <sup>-</sup> HD73/pUBpUC(2000/ml)	82	42	40
Cry <sup>+</sup> HD73/pUBpUC(2000/ml) and Cry <sup>-</sup> HD73/pUBpUC(1000/ml)	96	11	85

Table 6.3: Strains and number of cells recovered from exposed larvae of *Plutella xylostella*



From the results presented in table 6.3, the control experiment in which the insects were not exposed to any of the *Bt* strains did not produce any kanamycin resistant *Bt* which was in line with what was expected. The reliability of the results was also bolstered by the fact that the strain of *Bt* recovered from insects exposed to a particular strain of *Bt* in the single treatment was the same as the strain they were exposed to. The recovery of only Cry<sup>-</sup>/kanamycin resistance strain from a mixed treatment that involved Cry<sup>+</sup>/chloramphenicol resistance strain and Cry<sup>-</sup>/kanamycin resistance strain, showed that there was no in vivo plasmid transfer between the two strains of *Bt* used in the mixed treatment. Dominance of the Cry<sup>-</sup> strain was not observed when concentration of spores for both Cry<sup>-</sup> and Cry<sup>+</sup> strains were at 1000/ml but surprisingly its dominance was observed when the Cry<sup>-</sup> spore concentration was at 1000/ml with the Cry<sup>+</sup> at 2000/ml but not when Cry<sup>-</sup> concentration was at 2000/ml and the Cry<sup>+</sup> at 1000/ml. The dominance of the Cry<sup>-</sup> strain could be as a result of the fact that the fitness of the Cry<sup>+</sup> which produces toxin that kills the insect is reduced while the Cry<sup>-</sup> is only on the receiving side and therefore gained fitness (West *et al.*, 2007). With no clear dominance between the Cry<sup>-</sup> and Cry<sup>+</sup> strains of *Bt* used in the above experiment, a competition between toxic and non-toxic crystal producing *Bt* strains was investigated to find out if toxicity could enrich for *Bt* strains recovered from in vivo experiments involving two crystal producing *Bt* strains which are expected to have same metabolic load.

### **6.3 Single and mixed treatment of *Plutella xylostella* with toxic and non-toxic crystal producing *Bt* strains and selection of spores from dead and living larvae**

Spores of *Bt* used in this experiments were from the strains 431 and R128M, while spore free toxin was from *E. coli* JM109 strain engineered to express Cry1Ca toxin. 431 is an engineered strain made by transforming the acrySTALLIFEROUS *Bt* strain IPS 78/11 with a *Bt* shuttle vector carrying the wild type *cry1Ca* gene. The vector confers chloramphenicol and kanamycin resistance to transformed clones. It expresses the wild type *cry1Ca* gene and forms bipyrAMIDAL crystalline inclusion bodies on sporulation and its spore/crystal mix is lethal to susceptible *Plutella xylostella*. R128M on the other hand is an engineered strain made by transforming the acrySTALLIFEROUS *Bt* strain IPS 78/11

with a *Bt* shuttle vector carrying a mutated *cry1Ca* gene in which arginine at position 128 has been mutated through base change to methionine which renders the toxin inactive. The vector confers chloramphenicol resistance to transformed clones. It expresses the mutated *cry1Ca* gene and forms bypyramidal crystalline inclusion bodies on sporulation but its spore/crystal mix is not lethal to *Plutella xylostella*. Wild type Cry1Ca expressed in *E. coli* JM109 is toxic to *Plutella xylostella*.

The *Bt* strains, 431 and R128M were subcultured to single colonies and a colony picked for each strain and stored in the freezer as stock. Inoculum was taken from the pure stock culture and plated out on 50µg/ml kanamycin agar plate (431) or 5µg/ml chloramphenicol agar plate (R128M) and incubated at 30°C for 5 days to allow for sporulation and crystal formation. On the fifth day, observation of the cultures under the light microscope confirmed that they had sporulated and formed crystals. Crystal proteins and spores were harvested and quantified according to the method described in sections 2.2.8.4 and 2.2.8.5. Based on the spore count and crystal protein concentration estimates, 1 millilitre of both 431 and R128M spore/crystal mixes were estimated to contain approximately  $5.0 \times 10^9$  spores and 667µg of crystal protein.

*E. coli* JM109 harbouring the plasmid for the expression of the wild type Cry1Ca was grown in Luria broth containing 100µg/ml of ampicillin for 3 days and the expressed Cry1Ca was harvested as described in section 2.2.8.1. Its concentration was estimated according to the method described in section 2.2.8.4 to be 1667µg/ml.

Sublethal doses of spore/crystal mix of 431 were made using sterile distilled water as shown in table 6.4. The sublethal doses of 431 were made based on a toxicity assay that was conducted using its spore/crystal mix. R128M spore/crystal mix was also diluted to similar spore concentrations as 431 as shown in table 6.4. A bioassay as described in section 2.2.13.2 was conducted to estimate the  $LC_{50}$  for *E. coli* expressed Cry1Ca and it was found to be approximately 2µg/ml.

Strain	Spore count/ml	Corresponding toxin concentration (µg/ml)
431 or R128M	1,000	0.00013
431 or R128M	5,000	0.00067
431 or R128M	10,000	0.00133
431 or R128M	20,000	0.00267
431 or R128M	50,000	0.00667
431 or R128M	100,000	0.01334

**Table 6.4:** Dilutions of spore/crystal mixes of 431 and R128M

To test the in vivo activation/germination and replication of the spores of 431 and R128M, the spore/crystal mix dilutions of table 6.4 were made in 30ml of triton water and used in *Plutella xylostella* assays according to the method described in section 2.2.13.2. The assay was left for 5 days after which each larva whether dead or alive was crushed in 50µl of sterile distilled water in 1.5ml Eppendorf tubes and left at room temperature for 5 days to enhance sporulation of *Bt* cells in the cadaver. On the fifth day, the larvae were homogenised in 500µl of sterile distilled water. The homogenates were pasteurised at 62°C for 30 minutes to get rid of vegetative cells and possible gut flora. One hundred microlitre of the homogenates from each larva was plated onto 50µg/ml kanamycin agar plate (431) or 10µg/ml chloramphenicol agar plate (R128M) and incubated overnight at 30°C for single colony counts. The number of spores recovered for 431 and R128M were approximately the same and is represented in table 6.5. As control, insects were also reared with the same diet that was used in assaying 431 and R128M and the cadavers were also harvested as described for 431 and R128M but no spores were recovered when control samples were plated on chloramphenicol plates. The absence of spores from

the control samples confirmed that the spores recovered from 431 and R128M treated cadavers were from 431 and R128M.

Spore count/ml (431 or R128M) Used in assay	Spores recovered from plates
1,000	-
5,000	+
10,000	++
20,000	+++++
50,000	+++++++
100,000	+++++++++

Table 6.5: Tabular representation of the spores recovered from larvae of *Plutella xylostella* exposed to varying concentrations of 431 or R128M. (- means no spores recovered while + represents the relative number of spores recovered)

As shown in table 6.5, no spores were recovered for both 431 and R128M at spore concentration of 1,000/ml possibly because the concentration was too low which reduces the probability of insect ingestion or to initiate an infection. It was also observed that the number of spores recovered from cadavers was directly proportional to the spore concentration used in the assay.

To test the in vivo competition ability between the spores of 431 and R128M, spore dilutions of  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $5 \times 10^4$  and  $10^5$  were made in 30ml of triton water containing  $2 \mu\text{g/ml}$  of *E. coli* expressed Cry1Ca and used in *Plutella xylostella* assay according to the method described in section 2.2.13.2. The *E. coli* expressed Cry1Ca was added to effect the killing of about 50% of the larvae as the spore concentrations for 431 used in these experiments were sublethal. The

killing of about 50% of the assayed population was necessary because *Bt* spores were only recovered in good number from dead larvae even at spore concentrations of  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $5 \times 10^4$ . The assay was left for 5 days after which each larva was crushed in 50 $\mu$ l of sterile distilled water in 1.5ml Eppendorf tubes and left at room temperature for 5 days to enhance sporulation of *Bt* cells in the cadaver. On the fifth day, the larvae were homogenised in 500 $\mu$ l distilled water. The homogenates were pasteurised at 62°C for 30 minutes to get rid of vegetative cells and possible gut flora. One hundred microlitre of the homogenates from each larva was first plated onto 10 $\mu$ g/ml chloramphenicol agar plate because both 431 and R128M are chloramphenicol resistant and incubated overnight at 30°C for single colonies. Single colonies picked from chloramphenicol plates were each streaked on a 50 $\mu$ g/ml kanamycin agar plate and then a 10 $\mu$ g/ml chloramphenicol agar plate and grown overnight at 30°C to differentiate between 431 (which are kanamycin resistant) from R128M (which are kanamycin sensitive). As control, insects were also reared on the same batch of diet that was used in with 431 and R128M but the diet was treated with triton water containing 2 $\mu$ g/ml of the spore free *E. coli* expressed Cry1Ca. Samples from the the control experiment were plated on chloramphenicol plates but no colonies were recovered which bolstered the fact that the spores recovered from 431/R128M treated cadavers were actually from 431 and R128M. The different strains recovered from the cadavers treated with 431 and R128M in the presence of 2 $\mu$ g/ml of *E. coli* expressed Cry1Ca were as shown in tables 6.6 to 6.12.

Spore ratio (431:R128M)	Larval status	431	R128M	Number of colonies observed from each larva
10,000:50,000 (1:5)	Dead	0	10	10
	Dead	0	10	10
	Dead	2	8	10
Total		2	28	30
Percentage		6.7%	93.3%	
50,000:10,000 (5:1)	Dead	10	0	10
	Dead	0	50	50
	Dead	44	0	44
	Dead	50	0	50
Total		104	50	154
Percentage		67.5%	32.5%	

Table 6.6: Tabular presentation of strains of *Bt* spores recovered from dead larvae of *Plutella xylostella* infected with 431 and R128M at the ratios of 10,000:50,000 (1:5) and 50,000:10,000 (5:1)

Spore ratio (431:R128M)	Larval status	431	R128M	Number of colonies observed from each larva
20,000:100,000 (1:5)	Dead	1	9	10
	Dead	0	10	10
	Dead	0	10	10
Total		1	29	30
Percentage		3.3%	96.7%	
100,000:20,000 (5:1)	Dead	0	10	10
	Dead	8	2	10
	Dead	0	10	10
	Dead	37	5	42
	Dead	50	0	50
Total		95	27	122
Percentage		77.9%	22.1%	

Table 6.7: Tabular presentation of strains of *Bt* spores recovered from dead larvae of *Plutella xylostella* infected with 431 and R128M at the ratios of 20,000:100,000 (1:5) and 100,000:20,000 (5:1)

Spore ratio (431:R128M)	Larval status	431	R128M	Number of colonies observed from each larva
5,000:5,000 (1:1)	Dead	10	2	10
	Dead	0	8	8
	Dead	0	9	9
	Dead	0	9	9
	Dead	0	20	20
Total		10	48	58
Percentage		17.2%	82.8%	

Table 6.8: Tabular presentation of strains of *Bt* spores recovered from dead larvae of *Plutella xylostella* infected with 431 and R128M at the ratio of 5,000:5,000 (1:1)



Spore ratio (431:R128M)	Larval status	431	R128M	Number of colonies observed from each larva
10,000:10,000 (1:1)	Dead	0	20	20
	Dead	0	20	20
	Dead	0	20	20
	Dead	0	7	7
	Dead	11	9	20
	Dead	20	0	20
	Dead	15	5	20
	Dead	0	11	11
	Dead	0	12	12
	Dead	12	8	20
Total		58	112	170
Percentage		34.1%	65.9%	

Table 6.9: Tabular presentation of strains of *Bt* spores recovered from dead larvae of *Plutella xylostella* infected with 431 and R128M at the ratio of 10,000:10,000 (1:1)

Spore ratio (431:R128M)	Larval status	431	R128M	Number of colonies observed from each larva
100,000:100,000 (1:1)	Dead	17	3	20
	Dead	0	20	20
	Dead	8	12	20
	Dead	11	9	20
	Dead	4	16	20
	Dead	2	18	20
	Dead	0	3	3
	Dead	0	5	5
	Dead	4	1	5
	Dead	2	8	10
Total		48	95	143
Percentage		33.6%	66.4%	

Table 6.10: Tabular presentation of strains of *Bt* spores recovered from dead larvae of *Plutella xylostella* infected with 431 and R128M at the ratio of 100,000:100,000 (1:1)

Spore ratio (431:R128M)	Larval status	431	R128M	Number of colonies observed from each larva
200,000:200,000 (1:1)	Dead	1	19	20
	Dead	7	13	20
	Dead	8	12	20
	Dead	5	15	20
	Dead	0	20	20
	Dead	1	19	20
	Dead	2	18	20
	Dead	2	18	20
	Dead	0	20	20
	Dead	0	20	20
Total		26	174	200
Percentage		13%	87%	

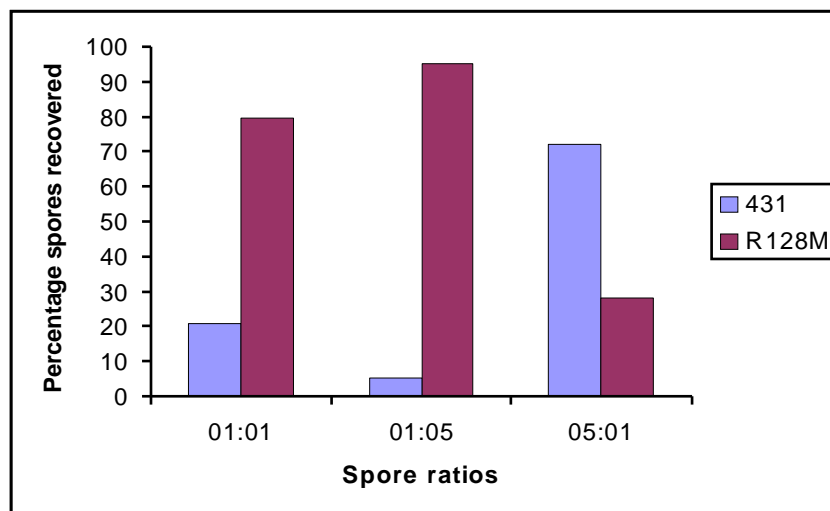
Table 6.11: Tabular presentation of strains of *Bt* spores recovered from dead larvae of *Plutella xylostella* infected with 431 and R128M at the ratio of 200,000:200,000 (1:1)

Spore ratio (431:R128M)	Larval status	431	R128M	Number of colonies observed from each larva
1,000,000:1,000,000 (1:1)	Dead	1	19	20
	Dead	0	20	20
	Dead	1	19	20
	Dead	1	19	20
	Dead	3	17	20
	Dead	0	20	20
	Dead	6	14	20
	Dead	0	20	20
	Dead	4	16	20
	Dead	1	19	20
Total		17	183	200
Percentage		8.5%	91.5%	

Table 6.12: Tabular presentation of strains of *Bt* spores recovered from dead larvae of *Plutella xylostella* infected with 431 and R128M at the ratio of 1,000,000:1,000,000 (1:1)

Spore ratio (431:R128M)	431	R128M	Total
1:1	159 (20.6%)	612 (79.4%)	771
1:5	3 (5%)	57 (95%)	60
5:1	199 (72.1%)	77 (27.9%)	276

**Table 6.13:** Sum of all the spores of 431 and R128M recovered from the different ratios of treatment



**Figure 6.1:** A bar graph for percentage of 431 and R128M spores recovered from cadavers of insects exposed to spore ratios of 1:1, 1:5 and 5:1

The results shown in tables 6.6 to 6.12 showed that larval death was low at lower spore concentrations while much death was recorded as the spore concentration increased. It was also observed that fewer spores (less than 100) were recovered from cadavers exposed to lower spore concentrations. When fewer spores (less than 100) were recovered from a larva, all the spores were analysed but when many colonies were recovered from a larva, only 20 colonies picked from different areas of the plate were further analysed. From tables 6.6 and 6.7 in which 431 and R128M were used at the ratio of 1:5 and 5:1, it was observed that in most cadavers the strain with a larger proportion

was the one that was recovered in greater numbers though there were few cases where the lesser strain was recovered in greater numbers. The observed trend of dominance of strain with larger proportion is also shown in figure 6.1 where a bar chart of the percentage of strains recovered from each ratio is presented. A general trend was that R128M seems to dominate more than 431. From tables 6.8 to 6.12 where 1:1 ratio of 431/R128M was used at spore concentrations of 5,000 (table 6.8), 10,000 (table 6.9), 100,000 (table 6.10), 200,000 (table 6.11) and 1000,000 (table 6.12), it was observed that in most of the cases, the strains recovered was dominated by R128M and this dominance increase as the spore concentration increases. To test the significance of these results a G test of independence was conducted using spores recovered from cadavers that were exposed to different spore ratios (table 6.13) to test the null hypothesis that there is no difference between the fitness of 431 and R128M. From the test a positive G-value of 13.19 with a *P*-value of 0.001367 was obtained which shows that the null hypothesis is not true and was rejected meaning that there is a difference in fitness between 431 and R128M. The null hypothesis could have been accepted if a negative G-value was obtained. The dominance of R128M could be as result of synergistic interaction between its spores and the Cry1Ca toxin that was used or as a result of the fact that 431 has a different plasmid which might have had a negative impact on its fitness. These results do not support the hypothesis that a *Bt* strain expressing a toxin with high toxicity will be preferentially enriched in vivo as R128M that was recovered in higher number in most cases is non-toxic.

Since 431 and R128M were made by transforming an acrySTALLIFEROUS *Bacillus thuringiensis* spp. *israelensis* (*Bti*) with a plasmid carrying wild type *cry1Ca* (431) and mutated *cry1Ca* (R128M) genes and judging from the fact that the wild type *cry1Ca* gene was originally cloned from *Bacillus thuringiensis* spp. *aizawai* (*Bta*), synergism between *E. coli* expressed Cry1Ca and spores of 431, R128M and other *Bt* spores was investigated. This investigation was based on an aspect of the hypothesis which relies on a familial interaction between spore and expressed toxin which may only take place between related spores and crystals. As such it would be expected that spores from a *Bti* strain expressing a *Bta* toxin may not have spore-associated toxin, germinate better or synergise compared with a *Bta* strain (its normal host) expressing Cry1Ca.

#### **6.4 Synergism between spores from 431 and *E. coli* expressed Cry1Ca; spores from R128M and *E. coli* expressed Cry1Ca; spores from Cry<sup>-</sup>IPS 78/11 and *E. coli* expressed; Cry<sup>-</sup>HD73 and *E. coli* expressed Cry1Ca.**

Synergism studies were conducted between *E. coli* expressed Cry1Ca and spores from the non-toxic *Bt* strains R128M, IPS 78/11 and Cry<sup>-</sup>HD73 and spores from the toxic strain 431. Late third instar larvae of Cry toxin sensitive population of *Plutella xylostella* were exposed to 2µg/ml of the *E. coli* expressed Cry1Ca (about LC<sub>20</sub> dose) in combination with 100,000 spores/ml of the various *Bt* strains. The 100,000 spores/ml of 431 was a sublethal dose to *Plutella xylostella*. The larvae were also exposed to a solution containing only 2µg/ml of the *E. coli* expressed Cry1Ca as well as solutions containing only 100,000 spores/ml of the *Bt* strains. The assay method used in testing the synergism was as described in section 2.2.13.2. From previous experiments the 2µg/ml of Cry1Ca toxin added was expected to effect a 20% mortality while the 100,000 spores/ml solutions of R128M, IPS 78/11 and 431 are not expected to effect mortality but the Cry<sup>-</sup>HD73 spores at 100,000 spores/ml are expected to effect about 1% mortality. On the fifth day, the number of dead and live larvae were counted and recorded as shown in table 6.14.

Toxin and/or spore/crystal mix	Number of dead insects out of 20	Number of live insects out of 20
Cry1Ca (2µg/ml)	4	16
431 (100,000 spores/ml) + Cry1Ca (2µg/ml)	11	9
431 (100,000 spores/ml)	0	20
R128M (100,000 spores/ml) + Cry1Ca (2µg/ml)	15	5
R128M (100,000 spores/ml)	0	20
IPS 78/11 (100,000 spores/ml) + Cry1Ca (2µg/ml)	12	8
IPS 78/11 (100,000 spores/ml)	0	20
CryHD73 (100,000 spores/ml) + Cry1Ca (2µg/ml)	14	6
CryHD73 (100,000 spores/ml)	2	18

Table 6.14: Display of the number of dead and live larvae from the synergism studies between spores of 431, R128M, CryIPS 78/11, CryHD73 and *E. coli* JM109 expressed Cry1Ca



Toxin and or spore/crystal mix	Expected mortality	Observed mortality	P-value	Statistical significance of results
431 (100,000 spores/ml) + Cry1Ca (2µg/ml)	20%	55%	0.0242	Significant
R128M (100,000 spores/ml) + Cry1Ca (2µg/ml)	20%	75%	0.0006	Extremely significant
IPS 78/11 (100,000 spores/ml + Cry1Ca (2µg/ml)	20%	60%	0.0112	Significant
Cry <sup>-</sup> HD73 (100,000 spores/ml) + Cry1Ca (2µg/ml)	21%	70%	0.0001	Extremely significant

Table 6.15: Tabular presentation of the expected and observed mortality from the spore/crystal synergistic experiments and the calculated *P*-values with their statistical significance

From the Fisher's exact test of the expected and observed mortality resulting from the synergism studies shown in table 6.15, all the spores from the different *Bt* strains synergised *E. coli* expressed Cry1Ca but the synergism observed with R128M and Cry<sup>-</sup>HD73 spores were judged to be extremely significant as their *P*-values were 0.0006 and 0.0001 respectively.

## 6.5 Discussion

The exposure of *Plutella xylostella* larvae to toxic crystalliferous and non-toxic acrySTALLIFEROUS *Bacillus thuringiensis* in a mixed treatment was to find out if the toxin producing strain will always reproduce and out compete the acrySTALLIFEROUS non-toxic strain in the insect or vice versa. Results from the treatments in which the insects were exposed to a mixture of 2000 spores/ml of Cry<sup>+</sup> and 1000 spores/ml of Cry<sup>-</sup> showed that the Cry<sup>-</sup> dominates the *Bt*

population pulled from the cadavers making up 88.5% of the *Bt* isolated compared to Cry<sup>+</sup> 11.5% which was in line with previous experimental results by Raymond *et al*, 2007 which showed that *Btk* a toxin producing strain was dominated by its non-crystal producing counterpart *Bc* in an in vivo experiments. The dominance of the Cry<sup>-</sup> could also be explained in the light of the theory of cooperation that exist among related individuals in a group (West *et al.*, 2007). With the cooperation theory, the Cry<sup>-</sup> could be seen as being passive in producing substances that kill the insect but only benefit by feeding on the cadaver while the Cry<sup>+</sup> strain expends its energy to kill at a cost to its fitness. In a set of mixed treatment in which both Cry<sup>+</sup> and Cry<sup>-</sup> were at 1000 spores/ml, out of 112 colonies recovered from treated larvae and observed, 74 were Cry<sup>+</sup> while 38 were Cry<sup>-</sup>. Results from another mixed treatment in which Cry<sup>+</sup> was at 1000 spores/ml and Cry<sup>-</sup> at 2000 spores/ml showed that out 82 colonies recovered, 42 were Cry<sup>+</sup> while 40 were Cry<sup>-</sup>. It would have been expected that since Cry<sup>-</sup> was able to dominate the Cry<sup>+</sup> even when it was at half the Cry<sup>+</sup> concentration, that it would even show more degree of dominance at ratios 1:1 and 1:2 but this was not the case as the Cry<sup>+</sup> dominates the *Bt* population pulled from the cadavers from 1:1 treatment while Cry<sup>+</sup> and Cry<sup>-</sup> were at equal proportion when cadavers from 1:2 treatment were analysed. Possible reason for the dominance of the Cry<sup>+</sup> strain could be that the insect fed on the Cry<sup>+</sup> strain and later stopped eating as the sequence of events leading to the killing of insect larvae by *Bt* toxin has been shown to proceed from toxin ingestion which culminates into loss of appetite (Ibagutxi *et al*, 2006). Therefore, if after ingesting the Cry<sup>+</sup> strain, the larvae lost appetite then this can give the Cry<sup>+</sup> the edge of germination and replication over the Cry<sup>-</sup>. A possibility that the insect could feed more on a single strain from a leaf disc treated with a solution of mixed strains is because feeding on any of the strain deposited on a leaf disc is a random event and may not be random at all times as live insect keep moving around. The inconsistency in the number of a specific strain recovered from the larvae treated with the toxic crystalliferous and non-toxic acrySTALLIFEROUS strains of *Bacillus thuringiensis* could also be that the competition that was observed between *Btk* and *Bc* (Raymond *et al.*, 2007) is not existing between the strains used in these experiments.

Using the strains 431 and R128M which are both crystal producing strains, the results obtained from their single strain treatment of *Plutella xylostella* showed that at low concentration of 1,000 spores/ml there was no larval death and no *Bt* cells could be recovered from the insect cadavers but when the spore concentration was increased to 5000 spores/ml, there was recovery of spores from the cadavers though no death was registered. The observed increase in number of colonies recovered from cadavers of insects that were alive at the point of harvest which were treated with high concentration of spores could be that the bacteria were able to regulate their virulent factors to their advantage in what is termed 'Quorum sensing' (de Kievit and Iglewski, 2000, Williams *et al*, 2000, Schauder and Bassler, 2001) or may simply be that the insect immune system could not cope with these high numbers.

The in vivo mixed treatment results showed that when 431 and R128M were combined at the ratio of 1:5 or 5:1, the dominating strain recovered from the insect cadaver was the one with the higher proportion in the mixture which is not in line with the hypothesis that 431 by virtue of it producing a more toxic toxin will, germinate and reproduce more than the non-toxic R128M. In treatments where there was a 1:1 ratio of the toxins, and at concentration ranging from 5,000-100,000 spores/ml, R128M was showing some degree of dominance but with concentrations of 200,000 and 1,000,000 spores/ml still at 1:1 ratio, R128M was clearly the dominating strain.

Synergism of Cry toxin by spores of crystal producing *Bt* strains have been reported (Miyasono *et al*, 1994, Johnson *et al*, 1996, Tang *et al*, 1996, Liu *et al*, 1998). Synergism was conducted to check if the spores from different strains of *Bt* can synergise the activity of *E. coli* JM109 expressed Cry1Ca. Results showed that all the spores from the different strains used synergise the activity of Cry1Ca irrespective of their strain background. The one tail *P*-value statistical analysis of the expected and observed number of live and dead larvae showed that the synergism between *E. coli* expressed Cry1Ca and the spores from the non-toxic mutant R128M was extremely significant while synergism between *E. coli* expressed Cry1Ca and spores from 431, Cry<sup>-</sup>HD73 and Cry<sup>-</sup>IPS 78/11 were just considered to be significant (table 6.14). The higher synergism observed with spores of R128M compared to that of 431 was quite interesting because the in vivo competition between them at 1:1 ratio in the presence of the *E. coli*

expressed Cry1Ca showed that R128M dominates 431 in most of the cases which points to the existence of special interaction between R128M and the *E. coli* expressed Cry1Ca. The acrySTALLiferous mutant IPS 78/11 was obtained from *Bacillus thuringiensis* var. *israelensis* IPS 78/11 by curing the toxin encoding plasmid (Ward and Ellar, 1983). It lacks the ability to form crystals during sporulation and thus lost toxicity to mosquito larvae unlike the wild type. The lack of crystal production and non-toxicity did not incapacitate its ability to synergise the activity of Cry1Ca which means that synergism is not only as result of interaction with the toxin on spore surfaces (Johnson *et al.*, 1998). The mechanism of synergism of Cry toxin by spores of acrySTALLiferous mutants could be explained in the light of the fact that the Cry toxin at a low dose though not lethal enough to effect an independent mortality is able to cause the suppression of the immune barrier creating a suitable environment for the germination of the acrySTALLiferous mutant spores which results in septicaemia and subsequent death of the insect (Johnson *et al.*, 1998, Gatehouse, 2008, Raymond *et al.*, 2010) or it could be that the toxins simply give the spores access to the haemolymph by breaking down the gut cell wall. On the other hand, there might be other proteins or substances on the surface of spores that enhance the activity of Cry toxins. Another acrySTALLiferous mutant Cry<sup>HD73</sup> produced by growing the Cry1Ac expressing *Bacillus thuringiensis* HD73 at 42°C which lost its ability to form crystals during sporulation and also its toxicity was also able to synergise Cry1Ca. SDS-PAGE analysis of the acrySTALLiferous mutants confirmed that they do not express Cry proteins and therefore their synergising Cry1Ca is not due to Cry toxin from spore surfaces but other mechanism.

These results suggest that exposing *Plutella xylostella* to *Bt* strains expressing toxin variants will not enrich for *Bt* strains with improved toxicity as 431 which was independently toxic to *Plutella xylostella* population used in these experiments could not dominate R128M which showed no independent toxicity.

## Chapter 7: General discussion and Further work

### 7.1 General discussion

The expression of the open reading frames of wild type *cry1Ie*, *cry1Ah* under the control of the native *cry1Ac*-promoter and *cry1Ah* under the control of *cyt1A*-promoter were successful as the protein band of approximately 80kDa for Cry1Ie and 134kDa for Cry1Ah were seen on SDS-PAGE gels. Also, the manipulation of Cry1Ie to produce a truncated mutant and Cry1Ah for the replacement of its domain II with that of Cry1Ie were also successful. The characterization of the expressed wild type Cry1Ie and Cry1Ah protein also showed that they folded properly as they were found to be soluble in an alkaline solution of Na<sub>2</sub>CO<sub>3</sub>. Their activation by trypsin resulted in the observation of a protease resistant band on SDS-PAGE which also confirmed that they were properly expressed and folded. Toxicity to *Plutella xylostella* with an LC<sub>50</sub> of 0.319 µg/ml (95% confident limit 0.267-0.466) for Cry1Ie and 1.417µg/ml with 95% confidence limit of 0.867-1.917µg/ml for Cry1Ah are comparable to the LC<sub>50</sub> of 0.20µg/ml of the fusion form of Cry1Ie expressed under the control of T7 promoter by Song *et al*, 2003 and the LC<sub>50</sub> of 1.52µg/ml of Cry1Ah expressed in acrySTALLIFEROUS mutant of *Bacillus thuringiensis* HD73 by Xue *et al*, 2008.

For the mutants Cry1Ie and CryAIA the strategy used in producing them were found to be successful at the expression stage as the expected protein band of approximately 73kDa for Cry1Ie and 134kDa for CryAIA were seen using SDS-PAGE. The characterisation of these manipulated toxins reveal that they did not fold properly as they failed to demonstrate typical characteristics of Cry-proteins. Their non-toxicity to *Plutella xylostella* was further proof that the manipulation has resulted in their lost of properties of Cry toxins demonstrated by their parent toxins. Adamo *et al*, 2000 demonstrated that the deletion of the first six N-terminal segments of h4 plasma membrane Ca<sup>2+</sup> pumps did not affect its activity but the mutants in which 15-75 amino acid residues were deleted from the N-terminal reduced its activity to undetectable levels. Also Chow *et al*, 2003 showed that the secondary and tertiary structure of apomyoglobin (apoMb) is dependent on its length and that at short length, non-native  $\beta$ -sheet conformation and self-associated amyloid-like species were generated while as the length increases,  $\alpha$ -helix structures gradually takes over. With the length of a protein directly affecting its folding and properties, it is reasonable to conclude

that the deletion of Cry1Ie from amino acid position 2-75 had adversely affected its folding and structure which results in the generation of a non-functional protein. The swapping of domain II of Cry1Ah with that of Cry1Ie in the construction of CryAIA, though resulting in the production of a protein band that looked normal on an SDS-PAGE gel turned out to be non functional and this can be seen as a large scale missense mutation. Mashimo *et al*, 2010 demonstrated that a missense mutation in Voltage-Dependent Sodium Channel (Na<sub>v</sub>1.1) which still appeared to be normal conferred susceptibility to Febrile Seizures in rats. The genetically manipulated *cryIAI cryIAA* and *cry1Ah* , did not result in expression of the proteins they code for. Though all the upstream elements like the *cry1Ac*-promoter, *cyt1A*-promoter, ribosome binding sites that have been successfully used in expressing the parent genes of *cry1Ie* and *cry1Ah* were supplied, they still did not express.

Synergistic studies between Cry1Ie and Cry1Ac or Cry1Ah showed that there is no synergism between Cry1Ie and either of Cry1Ac or Cry1Ah. With individual LC<sub>50</sub> of 0.319, 0.037 and 1.417µg/ml for Cry1Ie, Cry1Ac and Cry1Ah respectively, and the use of their combination at individual doses that will result in lethality below LC<sub>50</sub>, a synergistic effect could not be observed. Synergism has been variously reported between and among many Cry toxins including Cry4Ba and Cyt1Aa (Canton *et al*, 2011), Cry10Aa and Cyt1Aa (Hernandez-Soto *et al*, 2009), Cry1Ab and Cry1Ac (Sharma *et al*, 2010). Canton *et al*, 2011 showed that synergism between Cry11Aa and Cyt1Aa results from specific interaction between them and created a mutant Cyt1Aa that results in reduced synergism. The absence of synergism between Cry1Ie and Cry1Ac or Cry1Ah might be that these specific interactions are also absent.

Bioinformatic analysis of wild type Cry1Ie and the increased toxicity mutants Y442H, A522V and Y442H+A522V showed that the mutated points fall on regions that have been shown to be involved in toxin binding to brush border membrane vesicles of susceptible host (Fernandez *et al*, 2005, Gomez *et al*, 2006, Atsumi *et al*, 2008, Obata *et al*, 2009) Though the mutations Y442H, A522V and Y442H+A522V did not affect the stability of their expressed proteins, it did not however result in any substantial increase in toxicity. Though the initial screen by Liu *et al.*, 2010 (unpublished data) showed increase in activity for the mutants, it might be that the initial screen data is not reliable. The inability of the

mutants to show remarkable increase in toxicity might be due to the fact that the replacements were very conserved for Y442H mutant and moderately conserved for A522V thus making no difference (Berg *et al*, 2006).

Despite the various strategies utilised to enhance the expression of *cry30Ea* and *cry40Da* genes that were amplified from mosquitocidal strains of *Bacillus thuringiensis* S2160-1 and S2196-1 respectively, a functional protein could not be expressed. Though *cry30Ea* and *cry40Da* genes have a high level of similarity to members of their groups in the *Bacillus thuringiensis* toxin nomenclature as reviewed by Crickmore *et al*, 1998 and hosted at the web link ([http://www.lifesci.sussex.ac.uk/Home/Neil\\_Crickmore/Bt/](http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/)), it might be that they are pseudogenes. Pseudogenes are basically open reading frames that are homologous to functional genes but cannot be expressed due to mutation that preclude their expression (Cammack *et al*, 2006). Martin *et al*, 2010's computational analysis of the genome of *Bacillus anthracis* revealed pseudogenes with promoters that maintain transcriptional activity. It has been reported that a frame shift in the coding region of a novel tomato class I basic chitinase gene results in the formation of a pseudogene (Baykal *et al*, 2006). Reports have also shown that nonsense and missense mutations in *Chlamydia trachomatis* serovar L2 and D strains resulted in the formation of pseudogenes (Giles *et al*, 2009). In the case of *cry30Ea* and *cry40Da*, it does not seem to be the lack of some flanking control region in the promoter that is rendering them non functional because upstream elements like *cry1Ac*, *cyt1A*, T7 promoters and ribosome binding site have supplied to them and they cannot still be expressed whereas these upstream elements have been used in the expression of other genes. It is likely that their pseudogene status is as result of mutation within the open reading frame which could be missense, insertion or deletion as pairwise sequence alignment with members of their groups indicates differences like insertions and deletions (appendices 3 and 4).

Synergism between spores from *Bt* strains 431, R128M, Cry<sup>+</sup>IPS 78/11 and Cry<sup>-</sup>HD73 and *E. coli* expressed Cry1Ca showed that the spores from the different *Bt* strains all synergise the activity of *E. coli* expressed Cry1Ca. Surprisingly the highest synergistic effect with an extremely significant *P*-value of 0.0006 was observed with the spores of R128M which is a non-toxic mutant of Cry1Ca expressed in acrySTALLIFEROUS *Bt* IPS 78/11. In vivo competition between toxic

crystal producing strain 431 and non-toxic crystal producing strain R128M showed that neither of the strain dominated a population of *Bt* pooled from *Plutella xylostella* that were exposed to a mixture of the strains. Also, there was no dominance observed between Cry<sup>-</sup>HD73 and Cry<sup>+</sup>HD73 when analysis of the cardavars of *Plutella xylostella* in which a mixture of the strains were exposed to was carried out.

## **7.2 Further work**

With the sequence alignment strategy employed in creating the mutant Cry1Ie resulting in the production of a non-functional protein, a different strategy can be employed in carrying out a further work on the truncation experiment. This can be the use of BBMV associated protease from midgut of *Plutella xylostella* to digest the parent Cry1Ie and then sequencing the N-terminal of the resistant core which would help in determining where to truncate the protein thus mimicking in real terms the activated toxin. This can also be applied in the truncation of Cry1Ah.

As revealed by multiple sequence alignment between Cry30Ea and Cry30 toxins that there exist mutations like insertion and deletions, Cry30Ea can be manipulated by ether deleting or inserting portions that are either absent or present in those ones that have been successfully expressed in functional forms.



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## Appendices

### Appendix 1: BLASTP 2.2.24+ pairwise alignment between Cry8Ea and Cry1Ie

>lcl|25025 Cry1Ie  
Length=719

Score = 625 bits (1613), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 334/700 (48%), Positives = 457/700 (65%), Gaps = 24/700 (3%)

Cry8Ea	37	LQNMNYKEYLRMSEGYDSEYSGSPEVLISERDAVKTAISLVGTILGKLGVPPLVGPIVSLY	96
		L+N+N++++LRMSE S P V S ++T I + G ILG LGVP G I SLY	
Cry1Ie	34	LKNINHEDFLRMSE-----HESIDPFVSAS---TIQTGIGIAGKILGTLGVFPFAGQIASLY	86
Cry8Ea	97	STLIDVLWPGGKSQWEIFMEQVEALINQKIAEYARAKALAELEGLGNNYQLYLTALEEWQ	156
		S ++ LWP GKSQWEIFME VE LI+QKI+ YAR ALA+L+GLG+ +Y +LE W	
Cry1Ie	87	SFILGELWPKGKSQWEIFMEHVEELIDQKISTYARNIALADLKGLGDALAVYHESLESWI	146
Cry8Ea	157	ENPSSTRVLRDVRNRFEILDSLFTQYMPSFRVTGYEVPLLSVYAQAANLHLLLLLKDASIF	216
		+N ++ R V++++ L+ LF Q +PSF V+G EVPLL +YAQAANLHLLLL+DAS+F	
Cry1Ie	147	KNRNNARATSVVKSQYIALELLFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLLRDASVF	206
Cry8Ea	217	GEEWGFSTTAINNYNRQMSLIAQYSDHCVQWYRTGLDRLKGSNAKQWVEYNRFRREMTL	276
		G+EWG S + I+ +YNRQ+ + YSDHCV+WY TGL+ L+G+NA+ WV YN+FR++MTL	
Cry1Ie	207	GKEWGLSNSQISTFYNRQVERTSDYSDHCVKWYSTGLNNLRGTNAESWVRYNQFRKDMTL	266
Cry8Ea	277	SVLDIMTLFPMYDMRTYPMETKAQLTREVTYTDPIGAIG-----AQGSWY-DSAPSFNTLE	330
		VLD++ LFP YD YP++T +QLTREVTYTD IG + A +WY ++APSF+ +E	
Cry1Ie	267	MVLDLIALFPSYDTLVYPIKTTSQLTREVTYDAIGTVHPNASFASTTWYNNNAPSFSIAIE	326
Cry8Ea	331	STFIRGKHLFDFITRLSIYTGRSSFSASNYLKKWIGHQISSQPIGGSIQTYGTTSYGSS	390
		S +R HL DF+ +++IY+ S +S + Y+ W GH++ + IGG + T T G+T+ S	
Cry1Ie	327	SAVVRNPHLLDFLEQVTIYSLLSRWSNTQYMNMWGGHRLEFRTIGGVLNTSTQGSTNTS-	385

Cry8Ea	391	VIATQQIGFTGFDVYKTLSTAGVLFAYTSKYYGVSKVVFDAIYPDNKYKTTFTYNPGSEG	450
		I + FT DVY+T S AG+ T GV +V F + + Y G G	
Cry1Ie	386	-INPVTLPFTSRDVYRTESLAGLNLFLTQPVNGVPRVDFHWKFATLPIASDNFYLYGAG	444
Cry8Ea	451	IGAQEKDSEVELPPETLDQPNYEAYSHRLNYVTFIRNPDVP--VFSWTHRSADRTNTVYS	508
		+G Q +DSE ELPPET QPNYE+YSHRL+++ I V V+SWTHRSADRTNT+	
Cry1Ie	445	VGTQLQDSENELPPETTGQPNYESYSHRLSHIGLISASHVKALVYSWTHRSADRTNTIEP	504
Cry8Ea	509	DKITQIPVVKASDGPKPSANEVG-HYLGGDPISFNSSGSTGVIRLNINSPLSQYRVRIR	567
		+ ITQIP+VKA + +A G + GGD + ++G+ G IR+NIN P +Q+YRVRIR	
Cry1Ie	505	NSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRVNINPPFAQRYRVRIR	564
Cry8Ea	568	YCSSVDFDLDVVRGGTTVNNGRFNKSAPNVGWQSLKYENFKFASFSTPFTFNQAQDTLKI	627
		Y S+ D G +N G F+ + N G + L Y+ F+ F+TPF+F+ Q T I	
Cry1Ie	565	YASTTDLQFHTSINGKAINQGNFSATM-NRG-EDLDYKTFRTVGF'TTPFSFSDVQSTFTI	622
Cry8Ea	628	SVRNFSSIVGGSVVYIDRIELIPVNATYAEQDLDSAKKAVNTLFTNTK-DGLRPGVTDY	686
		NFSS G+ VYIDRIE +PV TYEAE D + A++ V LFT+T GL+ V DY	
Cry1Ie	623	GAWNFSS---GNEVYIDRIEFVPVEVTYEAEYDFEKAQEKVTALFTSTNPRGLKTDVKDY	679
Cry8Ea	687	EVNQAANLVECLSDDLYPNEKRLLFDAVKEAKRLSEARNL 726	
		++Q +NLVE LSD+ Y +EKR LF+ VK AK++ RN+	
Cry1Ie	680	HIDQVSNLVESLSDEFYLDKRELFEIVKYAKQIHIERNM 719	

## Appendix 2: SWISS-MODEL secondary structure predictions for Cry1Ie and 3eb7B (Cry8Ea)

Cry1Ie	56	SASTIQ	TGIGIAGKIL	GTLGVPFAGQ	IASLYSFILG	ELWPKGKSQW
3eb7B	64	is--erdavk	taislvgtil	gklgvplvgp	ivslystlid	vlwpggksqw
Cry1Ie		hhhhh	hhhhhhhhhh	hh	hhhhhhhhhh	hh h
3eb7B		h hhhhhh	hhhhhhhhhh	hh	hhhhhhhhhh	hh h
Cry1Ie	102	EIFMEHVEEL	IDQKISTYAR	NIALADLKGL	GDALAVYHES	LESWIKNRNN
3eb7B	112	eifmeqveal	inqkiaeyar	akalaelegl	gnnyqllyta	leewqenpss
Cry1Ie		hhhhhhhhhh	hhhh	hhhhhhhhhh	hhhhhhhhhh	hhhhhh
3eb7B		hhhhhhhhhh h	hhhh	hhhhhhhhhh	hhhhhhhhhh	hhhhhh
Cry1Ie	152	ARATSVVKSQ	YIALELLFVQ	KLPSFAVSGE	EVPLLPIYAQ	AANLHLLLLR
3eb7B	162	trvlrdvrnr	feildslftq	ympsfrvtgy	evpllsvyaq	aanlhllllk
Cry1Ie		hhhhhhhhhh	hhhhhhhhhh	hhh	hh	hhhhh hhhhhhhhhhh
3eb7B		hhhhhhhhhh	hhhhhhhhhh	hhh	hh	hhhhh hhhhhhhhhhh
Cry1Ie	202	DASVFGKEWG	LSNSQISTFY	NRQVERTSDY	SDHCVKWYST	GLNNLRGTNA
3eb7B	212	dasifgeewg	fsttainny	nrqmsliagy	sdhcvqwyr	gldrlkgsna
Cry1Ie		hhhhhhhhhh	hhhhhhhh	hhhhhhhhhh	hhhhhhhhhh	hhhh h
3eb7B		hhhhhhhhhh	hhhhhhhh	hhhhhhhhhh	hhhhhhhhhh	hhhh h
Cry1Ie	252	ESWVRYNQFR	KDMTLMVLDL	IALFPSYDTL	VYPIKTTSQL	TREVYTD AIG
3eb7B	262	kqwveyrnfr	remtlsvldi	mtlfpmydmr	typmetkaql	trevytdpig
Cry1Ie		hhhhhhhhhh	hhhhhhhhhh	hhhhhh	sss	sss
3eb7B		hhhhhhhhhh	hhhhhhhhhh	hhhhhh	sss	sss

Cry1Ie	302	TVHPNASFAS	TTWYNNNAPS	FSAIESAVVR	NPHLLDFLEQ	VTIYSLLSRW
3eb7B	312	aigagq-----	-sw-ydsaps	fntlestfir	gkhlfdfitr	lsiytgrssf
Cry1Ie				hhhhhh	ssss	ssssssssss
3eb7B		sss s ss	hhhhhh	ssss	ssssssssss	
Cry1Ie	352	SNTQYMMMWG	GHRLEFRTIG	GVLNTSTQGS	TNT--SINPV	TLPFTSRDVY
3eb7B	356	sasnylkkwi	ghqissqpig	gsiqqtqtygt	tsgssviatq	qigftgfdvy
Cry1Ie		ssssssss	ssssssss	ssssssss	sss	ssss sss
3eb7B		ssssssss	ssssssss	ssssssss	sss	ssss sss
Cry1Ie	400	RTESLAGLNL	FLTQPVNGVP	RVDFHWKFAT	LPIASDNFY	LYAGVGTQL
3eb7B	406	ktlstagvlf	aytskyyygvs	kvvfdaiypd	nkykttftyn	pgsegigaqe
Cry1Ie		ssssssssss	s	ssssssss	ssssssss	ssss
3eb7B		ssssssssss	s	ssssssss	ssssssss	ssss
Cry1Ie	450	QDSENELPPE	TTGQPNYESY	SHRLSHIGLI	SASHVKALVY	SWTHRSADRT
3eb7B	456	kdsevelppe	tldqpnyey	shrlnyvtfi	rn--pdvpvf	swthrsadrt
Cry1Ie		ssshhh		ssssssssss	ssss	ssss
3eb7B		ssshhh		ssssssssss	ssss	ssss
Cry1Ie	500	NTIEPNSITQ	IPLVKAFNLS	SGAAVVRGPG	FTGGDILRRT	NTGTFGDIRV
3eb7B	504	ntvysdkitq	ipvvkasdgp	kp-sanevgh	ylggdpisfn	ssgstgvirl
Cry1Ie		sss	sss sss	hh	sss	sss sss
3eb7B		sss	sss sss	hh	sss	sss sss

Cry1Ie	550	NINPPFAQRY	RVRIRYASTT	DLQFHTSING	KAINQGNFSA	TM--NRGEDL
3eb7B	553	ninsplsqky	rvriryCSSV	dfldlvvrgg	ttvnngfrnk	sapnvgwqsl

Cry1Ie		sss	ss	ssssssssss	ssssssss	ssssssss
3eb7B		sss	ss	ssssssssss	ssssssss	ssssssss

Cry1Ie	598	DYKTFRTVGF	TTPFSFSDVQ	STFTIGAWNF	SSG---NEVY	IDRIEFVPVE
3eb7B	603	kyenfkkasf	stpftfnqag	dtlkisvrnf	ssivggsvvy	idrielipvn

Cry1Ie		sssss	ss	ssssssss	sss	ssssssssss
3eb7B		sssss	ss	ssssssss	sss	ssssssssss

Cry1Ie		
3eb7B		-

Cry1Ie
3eb7B

### Appendix 3: Pairwise sequence alignments between Cry30Ea and other Cry30 toxins

>lcl|19517 Cry30Aa1  
Length=688

Score = 1053 bits (2724), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 519/689 (76%), Positives = 573/689 (84%), Gaps = 2/689 (0%)

Query	1	MNSYQNTNEYEILDASQKNSTMSNRYPRCPLANNPQVPLQNTSYKDWLNMCTITPLCTP	60
		MNSY+N NEYEILD SQKNS MSNRY + PL NNP+VPLQNT+YKDWLNMCTITPLCTP	
Sbjct	1	MNSYENKNEYEILDTSQKNSNMSNRYSKYPLTNNPKVPLQNTNYKDWLNMCTITPLCTP	60
Query	61	VETVSDYVAAFVPGSIFGAMPGPAAVGLFLSSFSTIIPILWPNDTTPIWKEFTKQGL	120
		++T S VA I V G+IF +MPGPAAVGL L SFSTIIPILWPND TPIWKEFTKQGL	
Sbjct	61	IDTDSKLVATAIKVIGAIFKSMPGPAAVGLVLKSFSTIIPILWPNDKTPIWKEFTKQGL	120
Query	121	QLFRPELGRDAIEIIGNDVQAEYNALKTMMQDFETKFATWDLNRTRANAIAATTEFN SVK	180
		QLFRPELGRDAIEIIGNDVQAEYN+L+ MM+DFE KFA W+ NRTRANAIA TT F++V	
Sbjct	121	QLFRPELGRDAIEIIGNDVQAEYNSLEIMMRDFENKFANWESNRTRANAIAVTTAFSTVN	180
Query	181	NQIIRLQERFLIAAENRPAFLNLYAQTANIDLILYQRGAANGDKWLEDINNRSISPFSSK	240
		QIIRL+ERFLIA ENRPAFLNLYAQTANIDLILYQRG+ GDKW+ DINNRS SPFSSK	
Sbjct	181	TQIIRLKERFLIAPENRPAFLNLYAQTANIDLILYQRGSVYGDKWVADINNRSSTPFSSK	240
Query	241	DYYQDLKLKIKNYTNYCAETYRNSLNILKNKSDIQWSIYNGYRRVATLGALDLVALFPNY	300
		DYYQ LK KIK+YTNYCAETYRNSL ILKNK IQW IYN YRR A LGALDLVALFPNY	
Sbjct	241	DYYQSLKGKIKDYTNYCAETYRNSLTILKNKPHIQWDIYNRYRREAILGALDLVALFPNY	300
Query	301	DICIYPIQTQTELTRKVMPFSFYSERLPKGNIE TWENSLTHPPSLFTWLKKLDPYTKSER	360
		DICIYP QT+TELTRKVMPFSFY + L + +IET EN LTHPPSLFTWL +L+ YT ER	
Sbjct	301	DICIYPTQTRTELTRKVMPFSFY LQALQQRDIETVENQLTHPPSLFTWLNELNLYTIRER	360



Query	361	FNPALVASLCGLHAALSYTPQNGPEFAGPFQGI LGTKTTPLISFDNQFVYELFLTQYRH	420
		FNP L+VASL GL A YT QN + P QG + T IS N ++Y+LF++QYRH	
Sbjct	361	FNPVLQVASLSGLQATSRYT-QNTTTISNPVQGPVREGTPTKISLANYYIYKLFMSQYRH	419
Query	421	PNDCYISGKPKITFYISDYYGNSRPNK-EYSSNIQLSSVITSYMNGPQNASTSNNISIK	479
		PNDC ISG +++FY SDYYG P YS+ ++VI +YMNGPQNA SN+ISI	
Sbjct	420	PNDCLPISGINEMSFYRSYDYGAGGPAPVHYSAGESPTNVIKTYMNGPQNALISNDISIN	479
Query	480	QTKHILSDIKMIYTQIGGIYPSHDFGYSAFWTHTSVDPDNLIVPNRITQIPAVKAYSLTS	539
		+T HILSDIKM Y++ GG+YP +DFGYSAFWTHTSVDPDNLIVPNRITQIPAVKAYSLTS	
Sbjct	480	ETSHILSDIKMNYSRITGGVYPLYDFGYSAFWTHTSVDPDNLIVPNRITQIPAVKAYSLTS	539
Query	540	PARVIAGPGHTGGDLVALLNNNLEAGRMQIQCKTGSFTGASRRYGLRMRYAANSQFTVNL	599
		PARVI GPGHTGGDLVALLN+ ++G MQIQCKTGSFTG SR+YGLRMRYAANS FTV+L	
Sbjct	540	PARVIVGPGHTGGDLVALLNSGTQSGTMQIQCKTGSFTGSPSRQYGLRMRYAANSFATVSL	599
Query	600	SYVLSGTTYGTSTFITESTFSRLNNIIPDLKYEEFKYKEYSQIITMTLPANTIITISIQQ	659
		SY L G T GTSF+TE+TFSR NNIIPDLKYEEFKYK+Y QIITMTLPANTIITIS+QQ	
Sbjct	600	SYTLLGQTRGTSFVTENTFSRSNNIIPDLKYEEFKYKDYLQIITMTLPANTIITISMQQ	659
Query	660	AVASSNYQLIIDRIELYPMDQDVVACTVN	688
		A N QLIIDRIE YPMDQ VVACTVN	
Sbjct	660	ATGLLNNQLIIDRIEFYPMDQGVVACTVN	688

>lcl|19518 Cry30Ba1  
Length=683

Score = 722 bits (1863), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 388/694 (56%), Positives = 481/694 (70%), Gaps = 37/694 (5%)

Query	1	MNSYQNTNEYEILDASQKNSTMSNRYPRCPLANNPQVPLQNTSYKDWLNMCTITPLCTP	60
		MN YQ EYEILDA S M N Y R PLANNPQVPLQNTSYKDWLNMCTI PLCTP	
Sbjct	1	MNPYQKKTEYEILDALPNYSNMVNAYSRYPLANNPQVPLQNTSYKDWLNMCTINPLCTP	60
Query	61	VETVSDYVAAFVPGSIFGAMPGPAAVGLFLSSFSTIIPILWPNDTTPIWKEFTKQGL	120
		+ D +AA I V GSI +PGPG A+G L +FSTI+PILWPN T IW +F ++GL	
Sbjct	61	INIDIDSLAASIADVGSILALIPGPGEAIGFVLGTFSTILPILWPNGETKIWTDFAEERGL	120
Query	121	QLFRPELGDAIEIIIGNDVQAEYNALKTMMQDFETKFATWDLNRTRANAIAATTEFNNSVK	180
		QLFRPELG+DAIEI+ V++ YNALK M++FE F W NRTR+NA +F+SV+	
Sbjct	121	QLFRPELGQDAIEILVTGVKSGYNALKKNRMENFEQAFTKWKGNRTRSNAEQVIRDFDVSVR	180
Query	181	NQIIRLQERFLIAAENRPAFLNLYAQTANIDLILYQRGAANGDKWLEDINNRSISPF-SS	239
		+++I L+ ++I EN+PAF+NLYAQTANIDLILYQRGA GD W +DIN SISPF S	
Sbjct	181	DKVIDLKN DYMINPENKPAFINLYAQTANIDLILYQRGAVYGGDWEKDING-SISPFWGS	239
Query	240	KDYYQDLKLKIKNYTNYCAETYRNSLNILKNKSDIQWSIYNGYRRVATLGALDLVALFPN	299
		KDYY+ LK KI+ YTNYCAETYRNSLNILKNK +I W YN YRR ATLGALDLVALFPN	
Sbjct	240	KDYYESLKTKEEYTNCAETYRNSLNILKNKPNISWDTYNKYRREATLGALDLVALFPN	299
Query	300	YDICIYPIQTQTELTRKVMPSPFYSERLPKGN----IETWENSLTHPPSLFTWLKKLDPY	355
		YD+ +YP T+TELTRK+YMPSF L + N +E EN+LTHPPSLFTWL +L+ Y	
Sbjct	300	YDMHLYPAATKTELTRKIYMPSPF---GLQQSNYFQSLEGLENALTHPPSLFTWLNELNLY	356
Query	356	TKSERFNPALVASLCGLHAALSYTPQNGPEFAGPFQGILGKTTPPLISFDNQFVYELFL	415
		T E FNPAL+V+SL GL A YT QN P QG+ +T I +N FVY+L +	
Sbjct	357	TVRENFNPALQVSSLSGLQARSRYT-QNPTILDNPAQGVNNGTSTQ-IGLNNLFVYKLSM	414
Query	416	TQYRHPNDCYSISGKPKITFYISDYYGNSRPNKEYSSNIQLSSVITSYMNGPQNASTSNN	475
		+QY HPNDC SI+G +TFY SDY GN+ + Y + ++VI ++MNGPQ AS+SNN	
Sbjct	415	SQYHHPNDCSSIAGISDMTFYKSDYNGNASATQTYQAGRNTNNVINTFMNGPQKASSSNN	474

Query	476	ISIKQTKHILSDIKMIYTQIGGIYPSHDFGYSAFWHTSVDPDNLIVPNRITQIPAVKAY	535
		ISIKQTKHILSDIKMIY + GG+Y +DFGYSAFWHTSVDPDNLIVPNRITQIPAVKA	
Sbjct	475	ISIKQTKHILSDIKMIYFRITGGMYQVYDFGYSAFWHTSVDPDNLIVPNRITQIPAVKAL	534
Query	536	SLTSPARVIAGPGHTGGDLVALLNNNLEAGRMQIQCKTGSFTGASRRYGLRMRYAANSQF	595
		+++ ++V+ GP GGD+ L L+A I+ KT A+ RY +R+RYA+N+	
Sbjct	535	GISTDSKVVGKPVFIGGDLALL---KLQA---TIRIKTDH---ANTRYKIRVRYASNANT	585
Query	596	TVNLSYVLSGTTYGTSTF---ITESTFSRLNNIIPDLDKYEEFKYKEYSQIITMTLPANTI	652
		+ LS + + T +F IT ST S +L+Y++F+Y + M P+	
Sbjct	586	PIVLSSLQN--TLTVTFPQTITHTSTIS-----ELQYKDFQYVTFPGEFIMDKPS---	632
Query	653	ITISIQQAVASSNYQLIIDRIELYPMDQDVVACT	686
		I ++I + V + + IDRIE P+ Q V+ T	
Sbjct	633	IDVAI-RGVQNDNRNDIWDRIEFLPITQSVLDYT	665

>lcl|19519 Cry30Ca1  
Length=688

Score = 891 bits (2303), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 461/695 (67%), Positives = 535/695 (77%), Gaps = 14/695 (2%)

Query	1	MNSYQNTNEYEILDASQKNSTMSNRYPRCPLANNPQVPLQNTSYKDWLNMCTITPLCTP	60
		MN Y N N+ EIL+AS NS MS YP+ PLA++ Q ++N +YK+W+N C+TI CTP	
Sbjct	1	MNLYGNKNDSEILNASSNNSNMSTTYPKYPLAHSRQDSMENMNYKEWINQCETINTFCTP	60
Query	61	VETVSDYVAFIGVPGSIFGAMPGPGAAGVGLFLSSFSTIIPILWPNDTTPIWKEFTKQGL	120
		++T + VAA IG G+I +PGPG A+G LS+F+++IP LWP+DT IW +FTKQGL	
Sbjct	61	IDTDINSVAATIGAVGAILALIPGPGEAIGFVLSTFTSLIPYLWPSDTKKIWGDFTKQGL	120
Query	121	QLFRPELGRDAIEIIGNDVQAEYNALKTMMQDFETKFATWDLNRTRANAIAATTEFNQSVK	180
		QLFRPELG DAIEIIGNDVQ+EYN+LKT MQ+FE F W R RA A+A T +F+SV+	
Sbjct	121	QLFRPELGNDIEIIGNDVQSEYNLKTFTMQNFEDSFDTWKKYRNRATAVAVTNDFSSVR	180

Query	181	NQIIRLQERFLIAAENRPAFLNLYAQ TANIDLILYQRGAANGDKWLEDINNRSISPF-SS	239
Sbjct	181	+QIIRL++RFLI EN+PAFL LYAQTAN DLILYQRGA D+W DIN RSISP S	239
Query	240	KDYYQDLKLIKINYTNYCAETYRNSLNILKNKSDIQWSIYNGYRRVATLGALDLVALFPN	299
Sbjct	240	KDYY L KIK YTNYCAETYRNSLNILKNK++I W YN YRR TLGALDL ALFPN	299
Query	300	YDICIYPIQTQTELTRKVMPSPFYSERLPKGN----IETWENSLTHPPSLFTWLKKLDPY	355
Sbjct	300	YDICIYPIQT+TELTRKVMPSF L + N +E EN+LTHPPSLFTWL +L+ Y	356
Query	356	TKSERFNPALVSLGLHAALSYTPQNGPEFAGPFQGIL-GTKTTPPLISFDNQFVYELF	414
Sbjct	357	TIRENFNPALLVSSLSGLQAISRYT-QNPNRISNPAQGVNRGTPTQ--IGLNNLFVYKLS	413
Query	415	LTQYRHPNDCYSISGKPKITFYISDYGNRPNKEYSSNIQLSSVITSYMNGPQNASTSN	474
Sbjct	414	L+QY HPN+CYSI+G +TFY SDY GN+ + Y + ++ I ++MNGPQ AS+SN	473
Query	475	NISIKQTKHILSDIKMIYTQIGGIYP SHDFGYSAFWHTSVDPDNLIVPNRITQIPAVKA	534
Sbjct	474	NISIKQTNHILSDIKMIYSRTGGTYP SYDFGYSAFWHTSVNPDNLIVPNRITQIPAVKA	533
Query	535	YSLTSPARVIAGPGHTGGDLVALLNNNLEAGRMQIQCKTGSFTGASRRYGRLMRYAANSQ	594
Sbjct	534	LTSPA+VIAGPGHTGGDLVALLN +AGRMQIQCKTGSFTGASRRYG+R+RYAAN+	593
Query	595	FTVNLSY-VLSGTTYGT SFITESTFSRLNNIPTDLKYEEFKYKEYSQIITMTLPANTII	653
Sbjct	594	TV+LSY V G T T+FITE TF R NN IPTDLKYEEFKYKEY+QIITMT P NTI+	653
Query	654	TISIQQAVASSNYQLIIDRIELYPMDQDVVACTVN 688	
Sbjct	654	TI+IQQ A N QLIIDRIE YPMDQ VV CTVN 688	

>lcl|15419 Cry30Fa1  
Length=687

Score = 951 bits (2458), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 479/690 (70%), Positives = 539/690 (79%), Gaps = 5/690 (0%)

Query	1	MNSYQNTNEYEILDASQKNSTMSNRYPRCPLANNPQVPLQNTSYKDWLNMCTITPLCTP	60
		M YQ+ NEYEILD K S + N Y R PLANNPQVPLQNTSYKDWLNMCTITPLCTP	
Sbjct	1	MKPYQSENEYEILDTLPKYSNIVNVYSRYPLANNPQVPLQNTSYKDWLNMCTITPLCTP	60
Query	61	VETVSDYVAAFVPGSIFGAMPGPAAVGLFLSSFSTIIPILWPNDTTPIWKEFTKQGL	120
		++ S VA IG+ G+IF AMPGPG+AVGLFL +FSTIIPILWPND TPIWKEFTKQGL	
Sbjct	61	IDIDSKLVATAIGILGAIFKAMPGPASAVGLFLKTFSTIIPILWPNDNTPIWKEFTKQGL	120
Query	121	QLFRPELGRDAIEIIGNDVQAEYNALKTMMQDFETKFATWDLNRTRANAIAATTEFN	180
		SVK QLFRPELGRDAIEIIGNDVQ+ +NALK M DFETKF WD +RT+ NA T F V	
Sbjct	121	QLFRPELGRDAIEIIGNDVQSGFNALKDHMNDFETKFEIWDKDRTQTNATYLITAF	180
		GVVN	
Query	181	NQIIRLQERFLIAAENRPAFLNLQAQTANIDLILYQGAANGDKWLEDINNRSISPF-SS	239
		+II L+ +FLI N+PAFLNLQAQTANIDLILYQGA GD W + IN+ SISPF SS	
Sbjct	181	GKIIDLKNQFLINPANQPAFLNLQAQTANIDLILYQGA VYGDWAKAINDSSISPFNSS	240
Query	240	KDYYQDLKLKIKNYTNYCAETYNLSNLILKNKSDIQWSIYNGYRRVATLGALDLVALFPN	299
		+ +Y LK KIK YTNCAETYNLS ILKN+ +IQW IYN YRR ATLGALDLVALFPN	
Sbjct	241	QIFYDSLKAKIKEYTNYCAETYNLSLTILKNQPNIQWDIYNRYRREATLGALDLVALFPN	300
Query	300	YDICIYPIQTQTELTRKVMPSPFYSERLPKGNITWENSLTHPPSLFTWLKKLDPYTKSE	359
		YDIC YPI T+TELTRKVMPSPFY + L NIET EN LTHPPSLFTWL +L+ YT E	
Sbjct	301	YDICKYPISTKTELTRKVMPSPFYQLALQHSNIETLENQLTHPPSLFTWLNELNLYTIRE	360
Query	360	RFPNPALEVASLCGLHAALSYTPQNGPEFAGPFQGI LGTKTTPLISFDNQFVYELFLTQYR	419
		FNPAL+V+SL GL A YT + P QGI P+I +N F+Y+L ++QYR	
Sbjct	361	NFPNPAQVSSLSGLQAKYRYTQNSTILPNPPAQGITNGTPIPIIGLNNLFYKLSMSQYR	420

Query	420	HPNDCYSISGKPKITFYISDYYGNSRPNKEYSSNIQLSSVITSYMNGPQNASTSNNISIK	479
		HPNDC I+G +TFY SDY GN+ + Y + ++VI ++MNGPQNAS+SNNISI	
Sbjct	421	HPNDCVPIAGISDMTFYKSDYNGNASATQTYQAGRNSNNVIDTFMNGPQNASSSNNISIN	480
Query	480	QTKHILSDIKMIYTQIGGIYP SHDFGY SFAWTHTSVDPDNLIVPNRITQIPAVKAYSLTS	539
		QT HILSDIKM Y + GG+Y DFGYSFAWTHTSVD DNLIVPNRITQIPAVKA L+S	
Sbjct	481	QTNHILSDIKMNYARSGGVY---DFGY SFAWTHTSVDLDNLIVPNRITQIPAVKANFLSS	537
Query	540	PARVIAGPGHTGGDLVALLNNNLEAGRMQIQCKTGSFTGASRRYGLRMRYAANSQFTVNL	599
		PARVIAGPGHTGGDLVALLN +AG+MQIQCKTGS TGASRRYG+RMRYAAN+ FTV+L	
Sbjct	538	PARVIAGPGHTGGDLVALLNGGTQAGKMQIQCKTGSSTGASRRYGIRMRYAANNAFTVSL	597
Query	600	SYVL-SGTTYGTSFITESTFSRLNNIIPDLDKYEEFKYKEYSQIITMTLPANTIITISIQ	658
		SY L G T GT+FITE TFSR NNIIPDLDKYEEFKYKEY+QIIT+T P NTI+TI+I+	
Sbjct	598	SYTLQGGNTIGTTFITERTFSRPNNIIPDLDKYEEFKYKEYNQIITVTSPQNTIVTIAIR	657
Query	659	QAVASSNYQLIIDRIELYPMDQDVVACTVN	688
		Q N QLIIDRIE YP+DQD AC VN	
Sbjct	658	QLNPIPNDQLIIDRIEFYPVDQDAFACPVN	687

>lcl|20005 Cry30Ga1  
Length=664

Score = 787 bits (2032), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 406/690 (59%), Positives = 492/690 (72%), Gaps = 28/690 (4%)

Query	1	MNSYQNTNEYEILDASQKNSTMSNRYPRCPLANNPQVPLQNTSYKDWLNMCTITPLCTP	60
		MN YQN NEY+ILD S M N Y PLANNPQVPLQNTSYKDWLNMCTITPLCT	
Sbjct	1	MNLYQNENEYKILDVLPNYSNMVNAYSSYPLANNPQVPLQNTSYKDWLNMCTITPLCTT	60
Query	61	VETVSDYVAAFVPGSIFGAMPGPAAVGLFLSSFSTIIPILWPNDTTPIWKEFTKQGL	120
		+++ + VAA IGV SI G + GPG A+GL L +FS+IIP LWP + T IW+EFT +GL	
Sbjct	61	IDSDINSVAAAIGVIAIIIGLRGPGEAIGLILGTFSSIIPFLWPENKTIIWEEFTHRGL	120
Query	121	QLFRPELGRDAIEIIIGNDVQAEYNALKTMMQDFETKFATWDLNRTRANAIAATTEFN	180
		L RPEL IEII N ++ YNAL+ + +FE +FA W + +A +++++	
Sbjct	121	NLIRPELTPAEIEIILNPLKGSYNALREQLVNFEREFAIWAGAKNQATTGDLRRISAIE	180
Query	181	NQIIRLQERFLIAAENRPAFLNLYAQNTANIDLILYQGAANGDKWLEDINNRSISPFSSK	240
		II+L+ + ++ N+PA L+LYAQNTANIDLIL+QGA GD+W + N+ I +S+	
Sbjct	181	GAIQLKNQLTVSEANKPALLSLYAQNTANIDLILFQRGAKYGDEWAKYARNQPIPFKTSR	240
Query	241	DYYQDLKLIKKNYTNCAETYRNSLNILKNKSDIQWSIYNGYRRVATLGALDLVALFPNY	300
		+YY L KIK YTN A TYRN LN +KN +I W +N YRR TL ALDLVALFPNY	
Sbjct	241	EYYASLIEKIKTYTNDIAGTYRNLNLIKNIQNISWDTFNEYRRGMTLSALDLVALFPNY	300
Query	301	DICIYPIQTQTELTRKVYMPFSFYSERLPK-GNIETWENSLTHPPSLFTWLKKLDPYTKSE	359
		DICIYPIQT+TELTRK+YMPSFY + L + GN+E+ EN LTHPPSLFTWL +L+ YT SE	
Sbjct	301	DICIYPIQTKTELTRKIYMPFSFYLQALQQSGNLESLENQLTHPPSLFTWLNELNLYTISE	360
Query	360	RFPNPALEVASLCGLHAALSYTPQNGPEFAGPFQGI LTKTTPLISFDNQFVYELFLTQYR	419
		FNPA+ P QGI G P I +N F+Y+L ++QY	
Sbjct	361	NFNPAI-----LPNPAQGITGGTPIP-IGLNNLFYIKLSMSQYH	398
Query	420	HPNDCYSISGKPKITFYISDYGNRPNKEYSSNIQLSSVITSYMNGPQNASTSNNISIK	479
		PN CY I+G +TFY SDY GN+ + Y + ++VI ++MNGPQNASTSNNISIK	
Sbjct	399	DPNGCYPIAGISDMTFYKSDYNGNASTTQPYHAGRNSNNVIDTFMNGPQNASTSNNISIK	458

Query	480	QTKHILSDIKMIYQTQIGGIYPSHDFGYSTFAWHTSVDPDNLIVPNRITQIPAVKAYSLTS	539
		+TKHILSDIKM+Y++ G+Y GYSFAWT TSV+PDNLIVPNRITQIPAVKA L S	
Sbjct	459	ETKHILSDIKMVYSR-SGVY---SLGYSTFAWTCTSVNPDNLIVPNRITQIPAVKANLLNS	514
Query	540	PARVIAGPGHTGGDLVALLNNNLEAGRMQIQCKTGSFTGASRRYGLRMRYAANSQFTVNL	599
		PARVIAGPGHTGGDLVALLN+ ++GRM+I+CKTGSFT SRRYG+RMRYAAN+ FTV+L	
Sbjct	515	PARVIAGPGHTGGDLVALLNSGTQSGRMEIKCKTGSFTETTSRRYGIRMRYAANNAFTVSL	574
Query	600	SYVL-SGTTYGTSTFITESTFSRLNNIIPDLDKYEEFKYKEYSQIITMTLPANTIITISIQ	658
		SY L G G +F TE TF R NNIIPDLDKYEEFKYKEY+QIITMT P NTI+TI++	
Sbjct	575	SYTLQGGNPIGITFGTERTFLRTNNIIPDLDKYEEFKYKEYNQIITMTAPQNTIVTIAVY	634
Query	659	QAVASSNYQLIIDRIELYPMDQDVVACTVN	688
		Q+ S N QLIIDRIE YPMDQ V AC +N	
Sbjct	635	QSTPSLNNQLIIDRIEFYPMDQGVEACKMN	664



## Appendix 4: Pairwise sequence alignments between Cry40Da and other Cry40 toxins

>lcl|Cry40Aa1 Cry40Aa1  
Length=666

Score = 743 bits (1919), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 399/672 (60%), Positives = 479/672 (72%), Gaps = 23/672 (3%)

Query	2	NSYQNTNGYEILESSSNNTNTPNRYPFANDPNIFPIILNDCPGKPWQDTWKSISNFI SVM	61
		NSY+N N YEILESSSNNTN PNRYPFAND ++ + NDC G W + W+S S+	
Sbjct	1	NSYENKNEYEILESSSNNTNMPNRYPFANDRDMSTMSFNDCQGISWDEIWESAETITSIG	60
Query	62	VSMAGFISSPGVLIGIPALLGIVNLLIPSSGSPVAALSICDLLSIIRKEVDESVLNDAVA	121
		+ + F+ P L GI L I+ LIP++ SV+ALSICDLLSIIRKEV +SVL+DA+	
Sbjct	61	IDLIEFLMEPS-LGGINTLFSIIGKLIPTNHQSVSALSICDLLSIIRKEVADSVLSDAIC	119
Query	122	DF-NGKLTNYKEYYLSSLQEWLSAGKP----NDSRLSNVVEYFKKSEEGFNEILAGSLSR	176
		F +GKL NY+EYYL L+ WL GKP N+S + +V+YF+ SE FNEIL GSL+R	
Sbjct	120	RFLDGKLNKREYYLPYLEAWLKDGLKPLQKTNNSDIGQLVKYFELSERDFNEILGGSLAR	179
Query	177	QNAQIILLPTFAQAANVQLLLLRDAVQYKKEWGALLSAEKVGSELISP-----TIDYGQ	230
		NAQIILLP F + QLLLRDAVQY+++W LSAE V SELISP T DY +	
Sbjct	180	NNAQIILLPYFCASCKQLLLLRDAVQYEEQWFPFLSAENVRSELISPNSGCDFTGDYYE	239
Query	231	RLKDKIAQYAKYCVFWYQEGLNQIKEEGAGTTTWLKFNFRRREMTLAVLDIIALFPIYDF	290
		RLK KIA+Y YC +WYQ GLNQIK+ G G TW KFNKFRREMTL VLDIIA+F YDF	
Sbjct	240	RLKCKIAEYTDYCEYWYQAGLNQIKQAGTGADTWAKFNKFRREMTLTVLDIIAIFQTYDF	299
Query	291	EKYPLGTNVELTREIYTDVPVGYSRGNRWEGLF--FNSLEANGTRGPGLVTWLQAIDIY	348
		+KYPL T+VELTREIYTDVPVGY G Y W ++ FN+LEANGTRGPGLVTWL++I IY	
Sbjct	300	KKYPLPTHVELTREIYTDVPVGYSSGTYSWLKYWTGAFNTLEANGTRGPGLVTWLRSIGIY	359

Query	349	SHPVFTQPGYLIGWGGTRHYEDYTKGNGAFQRMSTTSNDPHSISFGNTDIFKISSLARV	408
		+ V Y GW GTRHYEDYT GNG FQRMSTTSND ISF N+DIFKI S A +	
Sbjct	360	NEYV---SRYFSGWVGTRHYEDYTTGNGNFQRMSTTSNDLRDISFPNSDIFKIESKAIM	416
Query	409	ELQPFVGYSHIPRYRTSRAEFFPTTLNLTLLYERNSSGY-SQTIESVLPIDKDLPPSARNY	467
		L + + P YR SRAEF +T LY+ +SG S TI S LPGI K+ PS R+Y	
Sbjct	417	NLVGEIN-ARPEYRVSRAEFSESTAFIYLYDAGNSGLSSMTITSKLPGI-KNPEPSYRDY	474
Query	468	SHRLSNAACVQYETSVVNVFGWTHTSMTNRNPIYDPKITQIPAVKAFALENG--AYVSAG	525
		SHRLSNAACV S +NV+GWTHTSMT++ N IYDPKITQIPAVKAF + + V AG	
Sbjct	475	SHRLSNAACVGAGNSRINVYGWTHTSMSKYNLIYDPKITQIPAVKAFDISDTGPGQVIAG	534
Query	526	PGDTGGDVVTLPYLGRLLKIRLTPAPTNNKNYRVIRYATSYGASLMVQRWSPSGSESDFYFG	585
		PG TGG+VV+LPY RLKIRL PA TNKNY VR+RY ++ L+V+RWSPS + YF	
Sbjct	535	PGHTGGNVVSLPYYSRLKIRLIPASTNKNYLVRVRYTSTSNRLLVERWSPSSIINSYFF	594
Query	586	SSPTGPYPPTFGYMNLTVTTFNQSGVEIIENRHYSNIIIDKIEFLPDDLTTLESEGERNL	645
		TGP +FGY++TLVTTFNQ GVEIII+N + I +DK+EF+P + T LE EG+++L	
Sbjct	595	LPSTGPGDSFGYVDTLVTTFNQPGVEIIIQNLD-TPINVDKVEFIPVNSTALEYEGKQSL	653
Query	646	EKTKKAVNDLFI 657	
		EK + VNDLF+	
Sbjct	654	EKAQDVVNDLFI 665	

>lcl|Cry40Ba1 Cry40Ba1  
Length=666

Score = 648 bits (1671), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 357/677 (53%), Positives = 443/677 (66%), Gaps = 30/677 (4%)

Query	1	MNSYQNTNGYEILESSSNNTNTPNRYPFANDPNIFPIILNDCPGKPWQDTWKSISNFISV	60
		MNSYQN N YEIL+SS NNTN PNRYPFAND ++ P+ NDC G PW D W+S ++F +	
Sbjct	1	MNSYQNKNEYEILKSSPNNTNIPNRYPFANDRDMSPMSWNDQCQSPWNDVWESTASFTGI	60
Query	61	MVSMAGFISSPGVLIGIPALLGIVNLLIPSSGPSVAALSICDLLSIIRKEVDESVLNDV	120
		+ + F+ P + GI L ++ L+PS G +VA+LSICDLLSIIRKEVDESVL+DA	
Sbjct	61	GIDLITFLGEPsi-TGINLLFSVIGKLLPS-GQNVASLSICDLLSIIRKEVDESVLSDAY	118
Query	121	ADFNGLTNYKEYYLSSLQEWLSAGKPNDSRL-SNVVEYFKKSEEGFNEILAGSLSRQNA	179
		DFNG + NY+ YYL+SL++WL AGKP +L ++V ++F+ SE FN +L GSLSR	
Sbjct	119	GDFNGVVNNYQTYYLTSKKKWLDAKPTTGQLLTDVTKHFEFSEREFNALLKGSLSRPKG	178
Query	180	QILLLPTFAQAANVQLLLLRDAVQYKKEWGALLSAEKVGSELISPTIDYGQRLKDKIAQY	239
		+ILLLPT+ Q AN+ LLLLRD VQYK W L E V SELISP+ DY K+++A++	
Sbjct	179	EILLLPTYTQGANLHLLLRDFVQYKAVWEKELRTENVESELISPSFDYEGHFKEQLAEH	238
Query	240	AKYCVFWYQEGLNQIKEEGAGTTTWLKFNFRRREMTLAVLDIIALFPIYDFEKYPLGTNV	299
		+C+ WYQ GLNQIKE G T WLKFNFRRREMTL+VLDIIA+FP YDFE Y T++	
Sbjct	239	INHCITWYQAGLNQIKESGTSTENWLKFNFRRREMTLSVLDIIAIFPTYDFENYKSETHI	298
Query	300	ELTREIYTDVPVGYSRGNRYWEGLFS--FNSLEANGTRGPGLVTWLQAIDYSHPVFTQPG	357
		EL+RE+YTDVPVGY+ WE + FN+LEANGTRGPGLVTWL+ IDI++ V G	
Sbjct	299	ELSREYVYTDVPVGYN----GWEQNLTNNGFNTLEANGTRGPGLVTWLKKIDIFTDEVTEYSG	354
Query	358	Y-----LIGWGGTRHYEDYTKGNQAFQRMSTTSNDPHSISFGNTDIFKISSLARVELQP	412
		+ L GW GTRHYE YT + QR+SGTTSND +I F N+ IF I+SLAR L	
Sbjct	355	WSPVAILRGWAGTRHYEITYTGSSNTLQRISGTTSDNDVSNIDFINSRIFIITSLARYALAG	414
Query	413	FVGYS--IPRYRTSRAEFFPTTLNLTLLYERNSSGY-SQTIESVLPIDKDLPPSARNYSH	469
		+ PRYR SR EF T T LYE NS G S TIES LPG+ +Y +	
Sbjct	415	AAAGNPGSPRYRVSRVEFRSTGRYTFLYEVNSPGISSMTIESKLPGVKN--ATGFTDYFN	472

Query	470	RLSNAACVQYETSVVNVFGWTH TSMTRNNPIYPDKITQIPAVKAFALENGAYVSAGPGDT	529
		RLSNAACVQ+ TS VNV+GWTH SM N +YP+KITQIPAVKA+ + + V AGPG T	
Sbjct	473	RLSNAACVQFGTSRVNVYGWTHISMGE GNYVYPNKITQIPAVKAW EIRGTSSVVAGPGHT	532
Query	530	GGDVVTLPYLGRLKIRLTPAPT NKNYRVRIRYATSYGASLMVQRW-----SPSGSES	581
		GG++V + Y I+ T K YRVIRYA+ L ++RW +	
Sbjct	533	GGNLVKMSYHSVWSIKFT CQQL-KRYRVRIRYASDGNCQLAMRRWRGGPGYVQEARHTVQ	591
Query	582	DYFGSSPTGPYP TFGYMN TLVTTFNQSGVEII IENRHYSNIIIDKIEFLPDDLTTLESEG	641
		F S T Y +F Y++ ++ I+ + IDKIEF+PDDLTTLE E	
Sbjct	592	RTFSGSMT--YDSFKYLDIFTMPAEDYTFDLTIDLES GGALYIDKIEFIPDDLTTLEYEE	649
Query	642	ERNLEKTKKAVNDLFIN	658
		ERNLEKTK AVNDLF N	
Sbjct	650	ERNLEKTKNAVNDLFTN	666