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RESPONSE OF THREE MOSQUITO SPECIES TO RECOMBINANT BACTERIAL TOXINS FROM *Bacillus thuringiensis* SUBSP. *israelensis* EXPRESSED IN TWO MODEL SYSTEMS

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Abstract

Toxicity of lyophilized powders prepared prepared 16 combinations of four genes, *cry4Aa, cry11Aa, cyt1Aa* and *p20* from *Bacillus thuringiensis* subsp.*israelensis* (Bti) expressed in *Escherichia coli* were examined against three mosquito vectors of diseases, *Culex quinquefasciatus* Say, *Anopheles arabiensis* Patton, and *Aedes aegypti* Linnaeus, followed by simulated studies using transgenic *Anabaena* PCC7120 expressing the most toxic combination of genes. The following clones were the most toxic to the three mosquito species: pVE4-ADRC expressing all four genes (LC_{50s} of 0.59, 3.2, and 0.68 μg ml^{-1}); pVE4-ARC expressing *cry4Aa, cyt1Aa, p20* (LC_{50s} of 0.93, 6.2 and 0.87 μg ml^{-1}), and pVE4-AD expressing *cry4Aa* and *cry11Aa* (LC_{50s} of 1.51, 7.5 and 1.3 μg ml^{-1}). The clone pVE4-ADRC was undoubtedly the most effective. The role of appropriate promoter(s) in enhancing toxicity was demonstrated by comparing expression of the same gene combination under a strong *E. coli* promoter (PA1) either singly, in pVRE4-DRC or two (the second preceding *cyt1Aa* in pVE4-DRC). The latter produced more *Cyt1Aa*, which is less toxic, at the expense of the more toxic *Cry11Aa*, thus reducing toxicity. On the other hand, the combination of toxins under pVRE4-DRC control had an enhanced toxicity. This observation implies that further toxicity fine-tuning could be reached by manipulating promoters to enhance toxicity in recombinant systems. In simulated semi-field experiments, transgenic *Anabaena* PCC 7120 protected the toxins from premature degradation and better delivered the toxins to the larvae compared to commercial Bti preparations.

Key words: recombinant Bti toxins, A. *aegypti*, *An. arabiensis, C. quinquefasciatus*, western blot, LC_{50}

Introduction and Literature Review

With the ever-emerging elusive mosquitoes resistant to chemical pesticides (Chandre et al., 1999a; Martinez-Torres et al., 1998) and drug-resistant *Plasmodium* spp. (Björkman & Bhattarai, 2005; Lopes et al., 2002), coupled with emergence of arboviral infections (Gould & Higgs, 2009) and the growing fear of the inter-relation in transmission of various vector-borne diseases, increased efforts in emerging alternative technologies for integration in malaria control are desired.

A powerful environmentally friendly component of this approach is *Bacillus thuringiensis* subsp. *israelensis* (Bti) de Barjac, discovered by Goldberg and Margalit (1977). This soil inhabiting gram-positive bacterium produces a parasporal, proteinaceous crystal (δ-endotoxin) during sporulation. The crystal dissolves upon ingestion by mosquito larvae and its components are cleaved into toxic polypeptides by specific proteases present in the basic larval mid-gut. The active toxins then bind to receptors in the gut epithelium of susceptible species and cause paralysis and death, depending on the concentration used.

Toxin stability and delivery are limiting factors in Bti's efficiency to control mosquitoes in field conditions. It has been demonstrated that the cyanobacterium (blue-green algae) *Anabaena* PCC 7120 expressing the toxin genes is a novel way to simultaneously protect the insecticidal crystal proteins (ICPs) from sunlight inactivation (Manasherob et al., 2002) and from sinking into bottom sediments. *Anabaena* has been used to deliver the toxins to mosquito larvae in simulated semi-field experiments (Manasherob et al., 2003). This system must be field tested for effectivenes in natural mosquito habitats. It is anticipated that this toxin expression and delivery system will contribute to the reduction of mosquito populations and yield a long lasting solution to the prevalence of malaria in the tropics.

Other subspecies of *Bacillus thuringiensis* (Bt) (Bulla et al., 1980), have been characterized by the formation of a parasporal crystal protein (the δ-endotoxin) during sporulation (de Barjac & Sutherland, 1990). Susceptibility is based on the capacity of the target species to dissolve the crystal by specific proteases in the basic larval mid-gut of many species, thus exposing the toxins, which bind to specific receptors on the gut membrane (Yamagiwa et al., 2001; Boonserm et al., 2005, 2006; Fernandez et al., 2006; Chayaratanasin et al., 2007). Lethality is believed to be due to destruction of the trans-membrane potential, with the subsequent osmotic lysis of cells lining the midgut (Knowles & Ellar, 1987).

The toxicity of Bti resides in at least four major ICPs, of 134, 128, 72 and 27 kDa, encoded by the genes *cry4Aa, cry4Ba, cry11Aa* and *cyt1Aa,* respectively, all mapped on the 128 kb plasmid known as pBtoxis (Ben-Dov et al., 1999; Berry et al., 2002). These ICPs differ in toxicity levels and specificity against different species of mosquitoes (Margalith & Ben-Dov, 2000). Despite the low toxicity of Cyt1Aa against exposed larvae, this protein is highly synergistic with the Cry toxins and their combinations *in vitro* (Crickmore et al., 1995; Wirth et al., 1997; Wirth et al., 2007) due to different modes of action (Butko, 2003). Cyt1 Aa has been found to restore toxicity of *B. sphaericus* against *Culex quinquefasciatus* (Wirth et al., 2000). Cyt1Aa synergizes the Cry toxins when expressed simultaneously in transgenic *E. coli* and *Anabaena* PCC 7120 (Khasdan et al., 2001 and 2003, respectively), and with the heterologous mosquito larvicidal binary toxin of *B. sphaericus* (Wirth et al., 2000a; 2000b; 2005). Various combinations of Bti Cry toxins with Cyt1Aa may be necessary to avoid selection for resistance in the target mosquitoes (Georghiou & Wirth 1997; Wirth et al., 1997; 2007). Bti's limitations can compromise its efficacy in natural environments (Ohana et al., 1987). Expressing cry genes in other model systems may alleviate such limitations by effectively delivering the toxins in optimal combinations to the targets (Margalith & Ben-Dov, 2000).

Advantages and limitations of Bti in biocontrol of mosquitoes

Biological control agents active against mosquito larvae include several species of fish, nematodes, fungi, protozoa, viruses and bacteria (Scholte et al., 2004; Floore, 2006; Lacey, 2007; Futami et al., 2011). Bti was the first subspecies of *Bt* found to be toxic to dipteran larvae, and is much more effective against many species of mosquito and black fly larvae than any previously known bio-control agent. It is highly specific and hence safe to the environment (Murthy, 1997). No resistance has been detected to-date toward *Bti* in field populations of mosquitoes, despite over three decades of extensive field usage (Margalith & Ben-Dov, 2000). It has therefore been integrated into many vector control

programs worldwide.

Application of Bti for mosquito control is limited by short residual activity of current preparations under field conditions. The major reasons are: (a) sinking of the protoxin to the bottom of the water body (Manasherob et al., 2003); (b) adsorption onto silt particles and organic matter (Ohana et al., 1987); (c) consumption by non-target/ non-susceptible aquatic organisms; (d) inactivation by sunlight (Hoti & Balaraman, 1993; Liu et al., 1993). Furthermore, the persistence of Bti elements in the environment may have the potential to induce resistance in mosquitoes (Tiquin et al., 2008; Paris et al., 2011). Efforts are being made to improve effectiveness of Bti by prolonging its activity, as well as by targeting delivery of the active ingredient in the larval feeding zone. These improvements are being facilitated by development of new formulations utilizing conventional and advanced tools in molecular biology and genetic engineering. It is also necessary to develope culture conditions for Bti that maintain the integrity of the protoxin when used in the conventional way for mosquito control (Otieno-Ayayo et al., 1993). The strategy of transferring Bti's toxin genes for expression into alternative hosts that are eaten by mosquito larvae is much safer and more economical than chemical insecticides. These alternative hosts may also multiply in their habitats to prolong the effectiveness of the Bti application.

Activity of the Insecticidal Crystal Proteins (ICPs) from Bti

The polypeptides and their genes

The larvicidal activity is produced by several ICPs organized in a parasporal, proteinaceous (δ-endotoxin) synthesized during sporulation (de Barjac & Sutherland, 1990). The specific mosquitocidal properties are attributed to complex, synergistic interactions between three proteins (Poncet et al., 1995), Cry4Aa (125 kDa), Cry4Ba (130kDa) and Cry11Aa (68-72 kDa) (Donovan et al., 1988; Höfte & Whitley, 1989), and the non-specific Cyt1Aa, which is hemolytic and cytotoxic (Höfte & Whiteley, 1989; Tabashnik, 1992; Crickmore et al., 1995; Poncet et al., 1995). At least one accessory protein, P20, involved in δ-endotoxin production (Visick & Whiteley, 1991; Xu et al., 2001; Shao & Yu, 2004), seems to stabilize both Cyt1Aa and Cry11Aa in recombinant *E. coli* and Bt by a post-transcriptional mechanism, probably protection from proteolysis by interaction with Cyt1Aa while the latter is synthesized (Visick & Whiteley, 1991; Wu & Federici, 1995). In addition, it stimulates production of Cry4Aa in recombinant *E. coli* (Yoshisue et al., 1992). All genes involved with δ-endotoxin production are located on the 128 kb (pBtoxis) plasmid (Ben-Dov et al., 1999; Berry et al., 2002), have been cloned and expressed, their sequences deciphered and toxicities examined (Margalith & Ben-Dov, 2000).

Mode of action of toxins

The toxicity of the ICPs has been demonstrated through applications to the larval mid-gut (Gill et al., 1992; Knowles & Dow, 1993). A two-step model was proposed for the action of Bt processed toxins (Knowles & Ellar, 1987): binding to midgut cell receptors (Van Rie et al., 1990; Yamagiwa et al., 2001; Feldmann et al., 1995) and pore formation disrupting membrane permeability. Consequently, an uncontrolled efflux of ions, which disturbs the osmotic equilibrium, leading to colloid osmotic cytolysis (Knowles & Ellar, 1987).

Synergistic interactions of toxic polypeptides

The ICPs differ qualitatively and quantitatively in their toxicity levels and against different species of mosquitoes (Poncet et al., 1995). The crystal complex is much more toxic than each of the polypeptides alone. Various combinations of toxins are necessary to avoid selection for resistance in the targets (e.g., Wirth et al., 1997), and expression of such in other model systems is necessary to alleviate limitations in delivering the toxins to the targets. Synergism among Bti proteins has been demonstrated and widely studied (Wu and Chang, 1985; Poncet et al., 1994). All combinations of the three Cry proteins against three mosquito species had different synergy factors of between 2.5 - 15 (Crickmore et al., 1995; Poncet et al., 1995). The three Cry toxins were much more toxic than *Cyt1Aa* (Poncet et al., 1995; Delecluse et al., 1991), but Cyt1 Aa was the most synergistic to any of the three Cry proteins and their combinations (Tabashnik, 1992; Canton et al., 2011). The Cyt1Aa concentration-response curve was different from those of the Cry toxins, indicating different mechanisms of action (Crickmore et al., 1995). Synergism has also been demonstrated between Bti's *cyt1Ab* and *B. sphaericus* toxins (Wirth *et al*., 2001a). This suggests that the host range of a selected model system can be expanded by the interaction of gene combinations

across species.

A number of laboratory studies have shown levels of resistance to specific combinations of genes (Wirth & Georghiou, 1997; Wirth et al., 1997; Wirth et al., 1998). Information of this type is important in determining optical larvicidal gene combinations for vector management. According to Wirth et al. (2001b), cross-resistance does not exist between cry19 of *B.t.* subspecies *jegathesan* and single or multiple *Bti* toxin genes.

Selecting alternative model systems

***Escherichia coli*, a molecular biology tool for recombinant protein production**

The Gram negative bacterium, *E. coli*, is one of the most widely used hosts for the production of heterologous proteins (Baneyx, 1999) because its genetics, biochemistry, and metabolic pathways are far better understood than those of any other microorganism (Terpe, 2006). *E. coli* is thus widely used for recombinant protein production for industrial and research applications.

Anabaena PCC 7120

The organisms considered for toxin delivery should multiply in mosquito-breeding habitats, produce the toxic proteins efficiently, protect them from degradation, and efficiently deliver them to the larvae. Photosynthetic cyanobacteria are attractive candidates for this purpose (Boussiba & Zaritsky, 1992; Boussiba et al., 2000). They are ubiquitous, float in the upper water layer and resist adverse conditions (Porter et al., 1993). They are used as natural food sources for mosquito larvae (Merritt et al., 1992; Avissar et al., 1994) and can be genetically manipulated (Shestakov & Khyen, 1970; Wolk et al., 1984). Strain PCC 7120 of *Anabaena* species, the delivery system used in this study, is non-toxic and does not produce toxic blooms (Rouhiainen et al., 1995).

The suitability of *Anabaena* PCC 7120 is due to the following advantages (from Margalith and Ben-Dov, 2000):

a) Multicellular organisms such as *Anabaena* more efficiently deliver toxicity than unicellular because larger amounts of toxin are carried in a single aggregate.
b) The high copy number of the *Escherichia coli-Anabaena* shuttle plasmid raises the number of gene copies per cell. Tandem promoters—the transcription rate, and appropriate Ribosome Binding Site (RBS)—improve translation efficiency in the transgenic organism.
c) Codon usage of *Anabaena* sp. resembles that of Bti for the four cry genes.

Materials and Methods

Media

All media used in this study were autoclaved 20 min at 121°C, 1.5 Atm, and filter sterilized. Antibiotics were added after the media cooled to approx. 40°C when required. All liquid media were solidified with 1.5% bacteriological agar when required.

LB Medium

This complex, undefined medium was used for Bti and *E. coli* and consisted of 1% Bacto Tryptone, 0.5% Yeast Extract, 1% NaCl.

BG-11 Medium

This defined medium for *Anabaena* PCC 7120 consisted of the following (in mM): 17.65 $NaNO_3$, 0.18 K_2HPO_4, 0.3 $MgSO_4$, 0.25 $CaCl_2$, 0.19 Na_2CO_3, 0.003 Na_2Mg EDTA, 0.029 citric acid, and 0.03 ferric ammonium citrate, pH8, and the following trace minerals (in µM): 46 H_3BO_3, 0.17 $Co(NO_3)2$, 0.32 CSO4, 9.2 $MnCl_2$, 1.6 Na_2MoO_4, and 0.77 $ZnSO_4$.

Pharma medium

This is a cottonseed-derived protein nutrient from Southern Cotton Oil Company (POB 80367, Memphis, TN 38108, USA) used for rearing *Ae. aegypti*. Powder was suspended in distilled water at a concentration of 1.5g L^{-1} and autoclaved.

Microorganisms

Strains of (cyano)bacteria

Plasmids and the cloned Bti genes are listed in Table 1.

Bacterial storage conditions

For routine use over short periods, bacteria were inoculated onto fresh LB agar plates, incubated at 37°C overnight and stored at 4°C. The bacteria were sub-cultured into fresh plates every two months. For extended storage, *E. coli* were cultured to mid-log phase in liquid LB medium and then stored in well-mixed aliquots containing 20% glycerol (200 µl into 400 µl freshly concentrated culture) at -86°C.

Table 1
Plasmids used in this study: List of transgenic E. coli clones used in this study. All the clones were transformed to XL-Blue MRF', a commercial clone from clone library.

Plasmid	***Bti* gene(s) cloned**	**Source/Reference**
pHE4-A	*cry4Aa*	Ben-Dov et al., 1995
pVE4-AC	*cry4Aa, cyt1Aa*	Khasdan et al., 2001
pVE4-AD	*cry4Aa, cry11Aa*	Ben-Dov et al., 1995
pVE4-ADC	*cry4Aa, cry11Aa, cyt1Aa*	Khasdan et al., 2001
pVE4-ADR	c*ry4Aa, cry11Aa, p20*	Ben-Dov et al., 1995
pVE4-ADRC	*cry4Aa, cry11Aa, p20, cyt1Aa*	Khasdan et al., 2001
pHE4-AR	*cry4Aa, p20*	Ben-Dov et al., 1995
pVE4-ARC	*cry4Aa, p20, cyt1Aa*	Khasdan et al., 2001
pRM4-C	*cyt1Aa*	Manasherob et al., 2001
pHE4-D	*cry11Aa*	Ben-Dov et al., 1995
pVE4-DC	*cry11Aa, cyt1Aa*	Khasdan et al., 2001
pHE4-DR	*cry11Aa, p20*	Ben-Dov et al., 1995
pVE4-DRC	*cry11Aa, p20, cyt1Aa*	Khasdan et al., 2001
pVRE4-DRC	*cry11Aa, p20, cyt1Aa*	Khasdan et al., 2001
pHE4-R	*p20*	Ben-Dov et al., 1995
pRM4-RC	*p20, cyt1Aa*	Manasherob et al., 2001
pUHE24	-	Deuschle et al., 1986 (via S.Leu)

Storage as lyophilized powder
Bacteria grown for expression of larvicidal protein toxins were harvested by centrifugation, washed and overnight freeze dried in a LABCONCO 2.5 Plus FreeZone freeze-drier (LABCONCO Corporation, Kansas City, Missouri), operating at maxima of 0.009 Torr, and -85^{o}C (at the lower level).

Storage of competent cells
Competent cells were stored in 0.1 M $CaCl_2$ buffer at 4^{o}C for less than a month. Usually these cells were used after overnight storage or at least within a week.

Bacterial growth conditions
E. coli plasmid isolation
For plasmid DNA isolation and purification, *E. coli* cells were grown in 5 ml LB medium containing appropriate antibiotics) in test tubes incubated at 37^{o}C with shaking at 200 rpm in a shaker incubator. Bacteria were harvested after overnight culture and used for plasmid isolation.

Competent E. coli cells preparation
Presumed host bacteria were cultured on LB agar (without antibiotics) overnight and a single colony was transferred to a starter tube containing 5 ml LB medium. These tubes were cultured at 37^{o}C, 200 rpm to mid log phase and diluted 1:150 into a flask culture maintained in same conditions up to OD_{600} of 0.2-0.5 nm before harvesting and processing for competence.

Expression of Bti toxin genes in transgenic E. coli
Media for recombinant *E. coli* strains were supplemented with 100 μg ml^{-1} Ampicillin (Amp), 10 μg ml^{-1} Tetracyclin (Tet), 10 μg ml^{-1} Chloramphenicol (Cm) (when required). A colony was inoculated into a test tube containing 5 ml LB and incubated overnight in a shaker (250 rpm) at 37^{o}C. The cultures were diluted (1:150) into 250 ml flasks containing 30 ml medium, brought to exponential growth phase after about 2h (determined by spectro-photometry), and diluted (1:150) into 2 liter flasks containing 600 ml medium. Induction with IsoPropyl-β-ThioGalactoside (IPTG) to a final concentration of 0.1 mM was done at the exponential stage. The bacteria were harvested by centrifugation after 12 hr and washed twice with double distilled water. The resultant paste was freeze-dried overnight and ground into fine powder for bioassay.

Anabaena PCC 7120 and the recombinant strain
Anabaena PCC 7120 and its recombinant

clone (pSBJ2 # 11; containing toxicity of 6 AA ITU mg^{-1}) expressing *cry4Aa, cry11Aa* and *p20* from *Bti*, were cultured as described by Khasdan et al., (2003) and Manasherob et al., (2003). For bioassays, 2.5 L glass columns were used (30°C) under continuous illumination (85 µmol photon m^{-2} sec^{-1}) and furnished with air containing 1.5% CO_2.

Microbial growth measurements

Bacteria

Growth of *E. coli* and *Bti* was measured by introducing 1 ml of culture into a disposable micro-cuvette and measuring optical density using UVIKON 860, Software Version 8611 (from Kontron Instruments, supplied by Lumitron Electronic Instruments Ltd, Israel) at OD_{600} nm. Alternatively, a Klett-Summerson colorimeter was used to determine cell density in Klett Units.

Cyanobacteria

Cyanobacterial growth was determined by estimating chlorophyll-a (Mackinney, 1941). A blank reading was set with distilled water. 200 µl of sample was added to 800 µl methanol and mixed by hand before incubation at 70°C for 2 min. The mixture was then centrifuged at 13,000 rpm for 1 min. The optical density of the supernatant was determined at 665 nm for chlorophyll-a. The chlorophyll-a content was calculated using the equation: C (µg ml^{-1}) = OD_{665}* Dilution factor * 13.9.

Recombinant DNA methods

Standard molecular techniques (Sambrook et al., 1989) were applied in most procedures.

Preparation of competent cells

Cells harvested from above mentioned culture conditions were cooled in ice for 10 minutes. They were then harvested by centrifugation at 350 rpm for 5 minutes at 4°C, and washed twice in ice-cold 0.1 M $CaCl_2$ before concentrating 50-100 times in the same solution. For increased competence, the cells were stored overnight at 4°C before use.

***E. coli* plasmid DNA isolation and purification**

E. coli plasmid DNA was isolated and purified from 5 ml of culture using QIAprep®Spin Mini prep kit (from QIAGEN, supplied by Westburg (Israel) Ltd) following the instructions from the supplier. Plasmid DNA concentration and purity was determined by capillary method using GeneQuant spectrophotometer from Amersham Biosciences. Procedures were according to the user manual.

Transformation of *E. coli* cells by heat-shock method

In a sterile Eppendorf tube, 1 µl of plasmid vector DNA was mixed with 50-100 µl of overnight competent cells and incubated on ice for 2 min. The cells were then heat shocked by transferring the tube to a 42°C heat system for 2 min followed by incubation on ice for 2 min. To the heat-shocked cells was added 1 ml of LB medium without antibiotic and the tube incubated for 1 hr at 37°C. 100 µl of the cells were plated on LB agar plates containing appropriate antibiotics and incubated at 37°C overnight.

Protein and biochemical methods

Purification of Bti **Crystals**

Bti crystals were separated from spores and purified by the method of Otieno-Ayayo et al., (1993), modified from Pendleton and Morrison (1966). When large quantities were purified, the spore/crystal complex was processed in a blender rather than by vortexing.

Processing of recombinant *E. coli* strains for protein analysis

Culture and sonication of cells

E. coli strains were cultured and induced as described earlier, harvested and washed. The biomass was concentrated 25-fold and disrupted by sonication (SONICS vibra cellTM from SONICS and Materials Inc.) at 100% energy pulsing the cells for 2 min 30 sec (with regimes of 5 pulses of 30 seconds each, alternating with 30 sec breaks between pulses).

Estimation of total protein and sample preparation

Total protein was estimated from the lysates at OD_{595} using Bradford's method (Bradford, 1976). Calculated quantities of protein from the lysates were denatured in sample buffer (40% glycerol, 4% mercaptoethanol, 0.1% bromophenol blue and 8% SDS) by boiling for 5 minutes prior to loading onto the gel for electrophoresis using Laemmli's method (Laemmli, 1970).

Sources of insects for bioassays

*Source and rearing of Ae. aegypti larva*e

Dry strips of paper bearing eggs of *Ae. aegypti*, received from the Centre for Biological Control of BGU (courtesy of the late Prof. Yoel Margalith), were submerged in 1 l sterile tap water supplemented with 1.5 g of Pharmamedia and incubated at 28 ±0.5°C for hatching and larval growth (Khawaled et al., 1988) until the 3rd instar stage.

Source and rearing of *An. arabiensis* **and** *C. quinquefasciatus* **larvae**

Anopheles arabiensis and *Culex quinquefasciatus* eggs were obtained from a laboratory colonies, courtesy of the National Institute of Health Sciences, Harare-Zimbabwe and University of Nairobi in Nairobi-Kenya. The eggs were hatched at 28°C and maintained on Tetramin Baby Fish Food, Tetra GmbH D-49304 Melle, Germany and supplied by Martons, P.O. Box 12711 Jacobs, 4026, Kwa Zulu Natal, South Africa, until the larvae were in the third instar.

Laboratory bioassays

Bio-assays were performed in 125 ml disposable cups, each containing 100 ml of sterile distilled water. The test organism were suspended in sterile distilled water and serially diluted in the disposable cups. Twenty early third instar larvae of each mosquito species (*Ae. aegypti* and *An. arabiensis*) were introduced into each cup. Each test replicated at least thrice, on different days. Bioassays with *C. quinquefasciatus* were performed with the powders only. The test mosquitoes were deprived of any other food except *Anabaena* PCC 7120, either the transgenic (for test) or wild type (for control) and the respective *Bti* formulations as a known active control. The cups were incubated at 28°C for 24h and survival/mortality recorded before killing in hot water and discarding using environmentally sanitary procedures. Probit analysis was used to determine the concentration response.

Results

All 16 possible combinations with *cry4Aa, cry11Aa, cyt1Aa* and *p20* have been prepared in *E. coli* (Ben-Dov et al., 1995; Manasherob et al., 2003; Khasdan et al., 2001), and the most toxic clones were transformed into *Anabaena* PCC 7120 (Wu et al., 1997; Khasdan et al., 2003). A battery of chaperonins accompanied production of the recombinant toxins by *E. coli*, albeit to a lesser extent than in protease deficient *E. coli* strains.

Expression of Bti **toxin genes in** *E. coli*

Production of the polypeptides varied from one combination to another (Khasdan et al., 2001) and depended on the promoter(s) used. For instance, great variation (and different patterns of cross-reacting polypeptides) in the turnover of Cry11Aa was observed in clones with different gene combinations (Figure 1). The same gene combinations but with different promoter systems yielded different proportions of the Cry11Aa and Cyt1Aa proteins (Figure 2). It was evident that the integrity of the proteins produced by the recombinant *E. coli*, especially of Cry11Aa, i.e. in pVRE4-DRC and pVE4-DRC, varied greatly depending on whether the genes were regulated by a single strong *E. coli* promoter in the former or when cyt1Aa had in addition a second promoter in the latter.

Mosquito larvicidity of clones expressing all combinations of four genes

Larvae of three different mosquito species, namely *C. quinquefasciatus*, *Ae. aegypti* and *An. arabiensis*, exhibited varying levels of susceptibility to *E. coli* expressing genes and combination of genes from Bti. The responses of *C. quinquefasciatus* and *Ae. aegypti* were however very close, and more pronounced than of *An. arabiensis* (Figure 3).

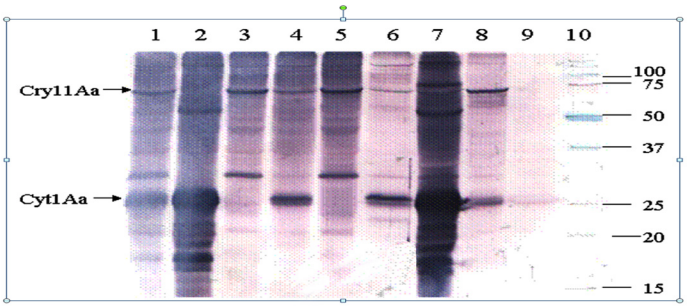
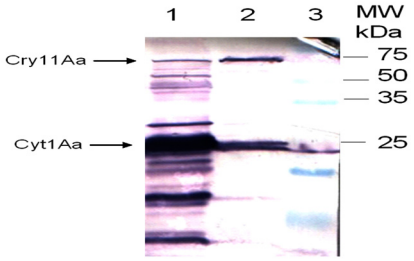


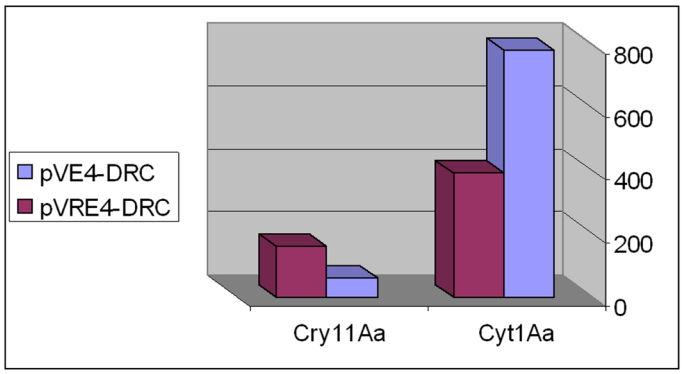
Figure 1
Western blot analysis for the expression of Bti toxic proteins genes by E. coli. The protein bands reacted to antibodies developed against whole crystal, with primary detection using anti-rabbit anti-IGg: Lane 1, pVE4-ADRC; L2, pVE4-ARC; L3, pVE4-AD; L4, pVE4-AC; L5, pUHE4-ADR; L6, pVE4-ADC; L7,pVE4-DRC; L8, pVRE4-DRC; L9, pUHE24; L10, MW markers.

Toxicities of this series of 16 clones were reported previously, in wet form against *Ae. aegypti* (Khasdan et al., 2001) and as dry powder against

C. quinquefasciatus (Wirth et al., 2007). The same set of powders was used here to evaluate relative toxicities of the whole series against *Ae. aegypti* and *An. arabiensis* as well. Recombinants pHE4-R, pRM4-C and pRM4-RC expressing *p20, cyt1Aa* and both, respectively, consistently failed to display any toxicity (even at 200 µg dry weight ml-1) towards susceptible larvae of all three mosquito species.



(a)



(b)

Figure 2

(a) *Immunoblot analysis of E. coli clones pVE4-DRC and pVRE4-DRC expressing cry11Aa, cyt1Aa and p20 from Bti. Lane 1, pVE4-DRC; L2: pVRE-DRC, L3: MW markers; (b) Histogram of EZQuant-gel quantification of Cry11Aa and Cyt1Aa of blot (a).*

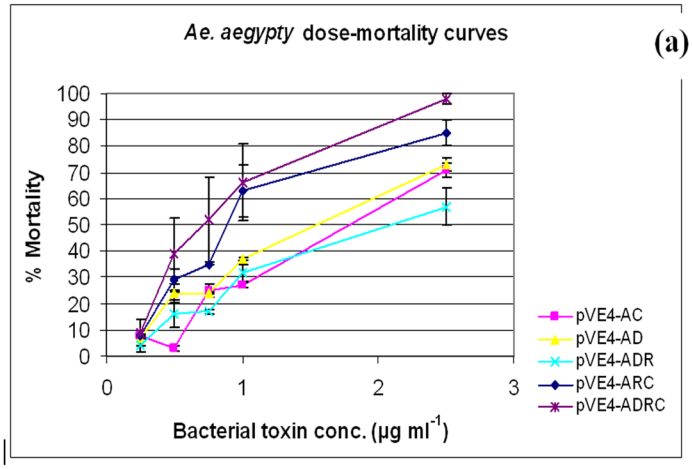
The clones expressing *cry11Aa* alone or in combination with *p20* or with *cyt1Aa* (pHE4-D, pHE4-DR and pVE4-DC, respectively) displayed similar, moderate toxicities (LC_{50} of about 6.5 µg ml^{-1}) against *C. quinquefasciatus* but not against *An. arabiensis* and *Ae. aegypti*. Disparity in toxicities was observed in two clones in which all three genes were combined, depending on the way of their construction: when cyt1Aa was added to pHE4-DR as the third gene without an additional promoter to form a single operon, the resultant clone pVRE4-DRC displayed similar toxicity against *C. quinquefasciatus* but much higher against *Ae. aegypti*. On the other hand, when *p20* and *cyt1Aa* were added with a second, identical promoter PA1 to clone pHE4-D, the resultant pVE4-DRC was less toxic to both and not at all to *An. arabiensis,* as was pVRE4-DRC. Consistently, clone pVRE4-DRC produces lower levels of Cyt1Aa and higher levels of Cry11Aa than pVE4-DRC (compare lanes 2 and 1 respectively, in Figure 2). This differential expression of these proteins demonstrated that higher expression of *Cry11Aa* and less of *Cyt1Aa* produced higher toxicity than vice versa.

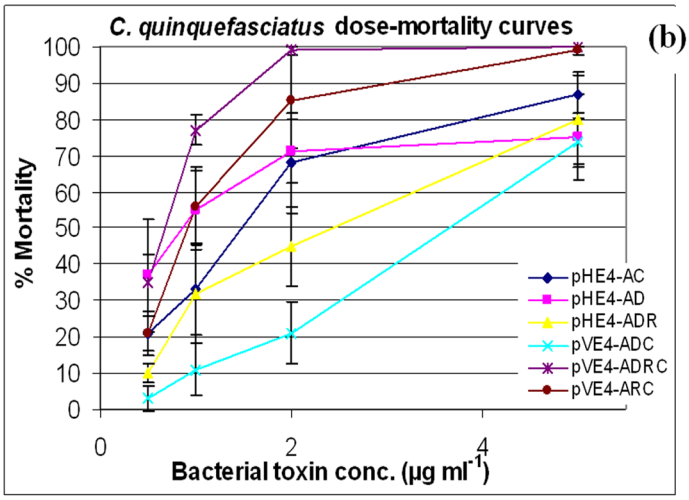
Toxicity of recombinant clones pHE4-A (and pHE4-AR) expressing cry4Aa (and p20) against all three susceptible mosquito larvae was low. Toxicity rose significantly upon addition of cyt1Aa (in pVE4-AC) and even more so when p20 was included in pVE4-ARC. .

Recombinant clones, pHE4-ADR and pHE4-AD expressing *cry4Aa* and *cry11Aa* with and without p20 respectively were moderately toxic against all three mosquito species. They had comparable toxicities against *Ae. aegypti*, but pHE4-AD was 1.5 and 2.1 times more toxic against *An. arabiensis* and *C. quinquefasciatus*, respectively.

Highest toxicity levels were achieved in pVE4-ADRC and pVE4-ARC producing Cry4Aa, Cyt1Aa and P20, with and without Cry11Aa, respectively. LC_{50} values of both clones against *C. quinquefasciatus* and *Ae. aegypti* were between 0.6 – 0.9 µg ml^{-1}, and between 3.2 – 6.2 µg ml^{-1} against *An. arabiensis*. The same constructs lacking p20 (pVE4-ADC and pVE4-AC) were less toxic, most likely because cells expressing *cyt1Aa* lose viability (Douek et al., 1992) unless co-expressed with *p20* (Manasherob et al., 2001). The presence of the regulatory protein P20 is also important in increasing the production of Cry4Aa (Yoshisue et al., 1992) and of Cyt1Aa (Manasherob et al., 2001; Wu & Federici, 1993). The significance of the Cyt1Aa contribution to toxicity of Cry4Aa and Cry11Aa against all three mosquito species was demonstrated by this series of bioassays: pVE4-ADRC was 1.9 - 2.5 more toxic than pVE4-AD and 2.7 - 5.3 more toxic than pVE4-ADR.

The results demonstrate that mosquito larvicidal activity of the δ-endotoxin gene products of *Bti* in transgenic *E. coli* depends not only on the number of genes but also on the expression patterns and varies with target mosquito species larvae (Figure 3). An analysis of variance to test the comparison of the contributions of combinations, concentrations and the interactions between them was highly, highly significant at $\alpha = 0.05$





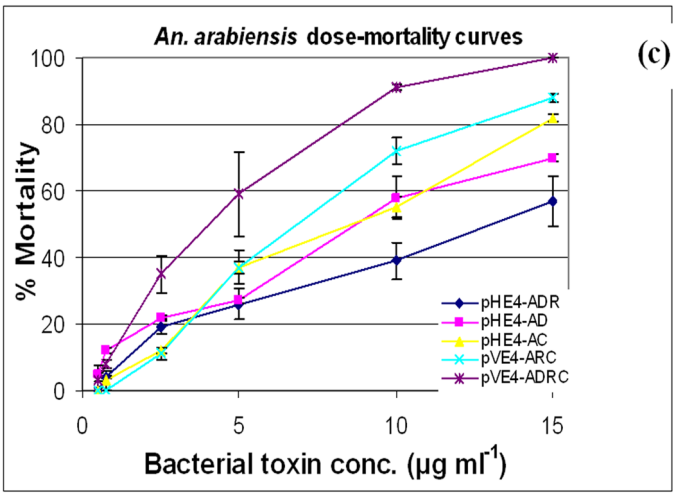


Figure 3
Concentration-mortality toxicity curves against larvae of (a) Ae. aegypti; (b) C. quinquefasciatus; (c) An. arabiensis.

Hierarchy of toxicities against several mosquito species

In general, higher toxicities of the six most toxic clones were achieved against *Ae. aegypti* and *C. quinquefasciatus*. These clones were less toxic but in the same hierarchy against *An. arabiensis*. A comparison of the responses of the three mosquito species showed a lower performance against *A. arabiensis* (Figure 3).

The results thus show that the concentration-mortality curve patterns in all the three species were similar (Figure 3, a-c) irrespective of the individual LC_{50} values. In general, *An. arabiensis* was the least susceptible, while in general there were no differences in response of *Aedes* and *Culex*. Comparing the first four clones in order of hierarchy and response by different mosquito species under concentrations of toxic proteins ranging between 0.5 and 15µg ml^{-1}, (Figure 3), pVE4-ADRC was the most toxic in all the three species, followed by pVE4-ARC and pHE4-AD in descending order. Clone pHE4-AC was third in *An. arabiensis* and in *C. quinquefasciatus*, in both cases followed by pHE4-ADR, while pHE4-ADR was fourth in *An. arabiensis*.

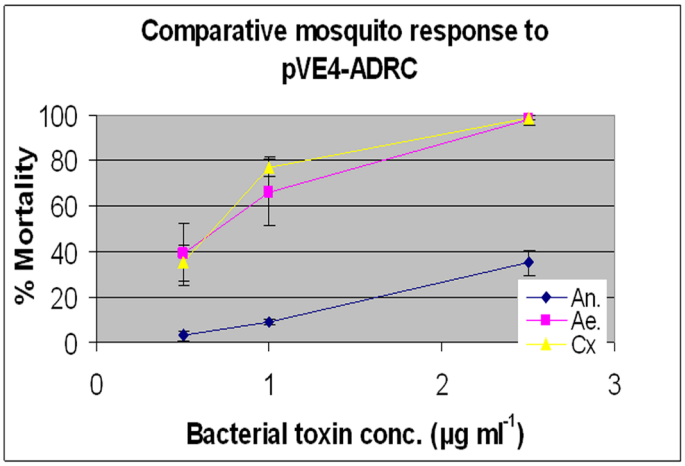


Figure 4.
Comparative mosquito response to (pVE4-ADRC) the most toxic combination of toxins.

It was evident that as important as cry4Aa may be, it did not show significant toxicity to *An. arabiensis* on its own (LC_{50} 23 µg ml^{-1} on average). It was even less toxic to *Ae. aegypti* and *C. quinquefasciatus*. The toxicity of Cyt1Aa with and without p20 (pRM4-RC and pRM4C, respectively) was not significant in all the three species (data not presented here)

Toxicity of transgenic *Anabaena* PCC7120 to *Ae. aegypti*

Initial studies were done using various batches of *Anabaena*, harvested at varying growth stages. This produced test materials with considerably varying toxicity. There was a sharp drop of toxicity at the end of the lag phase, after which toxicity (inverse of LC_{50}) rose with culture age, together with protein and chlorophyll synthesis (Figure 5). There was significant increase in toxicity from the stationary phase to the harvest time (at ca. 13 days).

Comparative bioassays in controlled (outdoor) light conditions

The recombinant *Anabaena* PCC 7120 expressing *cry4Aa, cry11Aa* and *p20* has shown high potency in the laboratory and relatively higher persistence in semi-field trials when compared to commercial Bti. The toxicity of the transgenic *Anabaena*'s persisted for 8 days, but considering the lower initial concentration of chlorophyll, it lasted for at least 12 days (Fig. 5).

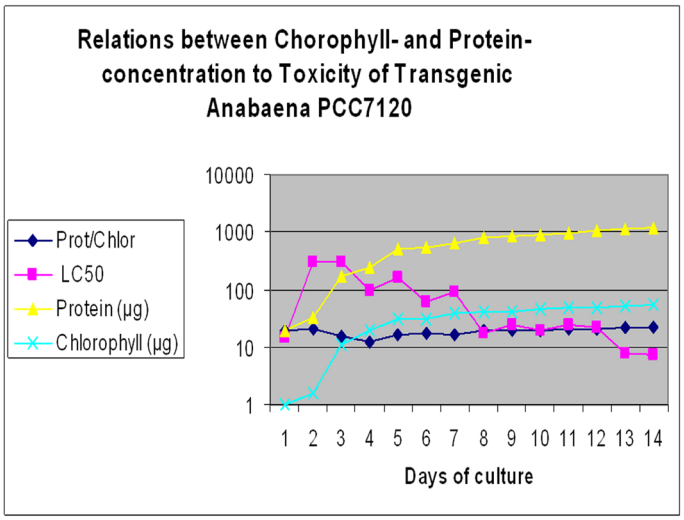


Figure 5
Relationships between cholophyll and protein concentrations with toxicity of Anabaena PCC 7120 at different (column) culture stages.

Discussion

Lyophilized powders of recombinant *E. coli* strains expressing *cry4Aa*, *cry11Aa, cyt1Aa* and *p20* individually showed varying toxicity levels (moderate, low or none) against larvae of *C. quinquefasciatus, Ae. aegypti* and *An. arabiensis* (Figures 3 a-c). Cyt1Aa was the least toxic of the four ICPs of Bti, but was the most active synergist in combination with any of the other three and with their combinations (not p20) (Tabashnik, 1992; Wu et al., 1994; Crickmore et al., 1995; Wirth et al., 1997, 2007; Otieno-Ayayo et al., 2008), most likely due to different mechanisms of action (Butko, 2003). Furthermore, Cyt1Aa binds differently when used alone as in combination with the Cry's: it is dispersed when applied separately, whereas together, it preferentially associates with the other toxins, which might explain the synergy between them. Binding of Cry11Aa to the membrane of midgut epithelial cells is enhanced by membrane-embedded Cyt1Aa, just as it interacts with its natural receptor (Pérez et al., 2005). Cyt1Aa thus enhances Cry11Aa toxicity and suppresses resistance of the target organisms with mutations in the Cry11Aa receptor (Pérez et al., 2005). Enhancement of Cry11Aa toxicity by Cyt1Aa, e.g., moderate synergy against *Ae. aegypti* (Crickmore et al., 1995), was also found in this study. This nsynergism was demonstrated with pVRE4-DRC against *C. quinquefasciatus* and *Ae. aegypti* but not against *An. arabiensis* (Figures 3 a-c). Consistently, toxicity of pure Cry11Aa has been similar against *Ae. aegypti* and *C. pipiens* and lower against *An. stephensi (Poncet et al*., 1995). Toxicity of Cry11Aa-free inclusions was similar to that of the wild-type crystals against *An. stephensi* but half as high as against *C. pipiens* and *Ae. aegypti* (Poncet et al., 1993). On the other hand, our clone pVE4-ARC expressing *Cry4Aa, Cyt1Aa* and *P20* had higher and similar toxicities against *C. quinquefasciatus* and *Ae. aegypti* but only about half the toxicity against *An. arabiensis* than pVE4-ADRC expressing Cry11Aa in addition to the other three proteins.

The next challenge will be to design a releasable transgenic organism, which will satisfy the minimum toxicity demands in addition to demonstrating a mechanism for minimising the possibilities of resistance and horizontal gene transfer.

Role of promoter system on gene expression and subsequent toxicity

It was interesting to note that two clones with the same gene combination had very different levels of toxicity. These clones, pVRE4-DRC and pVE4-DRC expressed the same three genes (cry11Aa, p20, and cyt1Aa) but were derived differently. In pVRE4-DRC, cyt1Aa was added as the third gene without an additional promoter to form a single operon, whereas in pVE4-DRC, p20 and cyt1Aa were added with the promoter PA1 to the original clone pHE4-D (Ben-Dov et al., 1995) expressing *Cry11Aa* from another PA1 promoter (Khasdan et al., 2001). The clone

pVRE4-DRC produces a lower level of Cyt1Aa but a higher level of Cry11Aa in comparison to pVE4-DRC (compare lanes 2 and 1 in Figure 2). In western blot patterns quantified by *EZQuant-Gel 2.11* (Figure 2 b), the density ratio of Cry11Aa in pVE4-DRC compared to pVRE4-DRC was 1:2 (with total value of 1:3). The Cyt1Aa ration of pVE4-DRC to pVRE4-DRC was 1:3 (with total value of 1:4). This analysis only provided qualitative comparisons and may not be considered valid quantification of absolute densities because of exposure saturation in several lanes. *Clone* pVRE4-DRC was much more toxic with an LC_{50} of about 4.7 μg ml^{-1} and 4.2 μg ml^{-1}, whereas pVE4-DRC had LC_{50s} of > 200 μg ml^{-1} and 15.5 μg ml^{-1} against *C. quinquefasciatus* and *Ae. aegypti* respectively. Both clones were not toxic against *An. arabiensis*. The different expression pattern had implications on toxicity, in that enhanced production of Cry11Aa and less Cyt1Aa resulted in higher toxicity than *vice versa*.

This study concludes that: (a) The type, strength and tandem number of promoter used for gene expression was important for polypeptide pattern, intensity and subsequent toxicity of the desired products; (b) Of the 16 possible combinations, the 6 most toxic clones displayed a consistent hierarchy in all the three mosquito spp. tested; (c) The combination clone with all four genes was consistently the most toxic; (d) *Anabaena* PCC 7120 is quite promising as a candidate for field release; and (e) Recombinant *Anabaena* performed better in simulated semi-field conditions than *Bti*.

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