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

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Abstract

Toxicity of the same lyophilized powder prepared identically of 16 combinations of four genes, *cry4Aa, cry11Aa, cyt1Aa* and *p20* from *Bacillus thuringiensis* subsp.*israelensis* (Bti) expressed in *Escherichia coli* were examined against three key mosquito vectors of diseases, namely *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 respectively listed mosquito species above: 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}), concluding that clone pVE4-ADRC is 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* as well in pVE4-DRC); the latter produced more *Cyt1Aa*, which is less toxic, at the expense of the more toxic *Cry11Aa*, thus quenching toxicity. On the other hand, the combination under pVRE4-DRC had an apparent correct stoichiometry to enhance toxicity. This observation implies that further toxicity fine-tuning could be reached by manipulating promoters to enhance toxicity in the recombinant systems.
In simulated semi-field experiments, the transgenic *Anabaena* PCC 7120 protected the toxins from premature degradation and better delivered the toxins to the larvae compared to commercial *Bti* preparation.

Key words: recombinant *Bti t*oxins, 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.

The most powerful environment 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 (-endotoxin) during sporulation. The crystal dissolves upon ingestion by mosquito larvae and its components are cleaved into the toxic polypeptides by specific proteases prevailing 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 within minutes or hours, depending on the concentration used.

Toxin stability and delivery are the 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, and to deliver the toxin to mosquito larvae in simulated semi-field experiments (Manasherob et al., 2003). This system must be field tested for sustainability in natural mosquito endemic habitats. It is anticipated that it will contribute to the reduction of mosquito populations and yield a long lasting solution to the prevalence of malaria in the tropics.

Other subsps. of *Bacillus thuringiensis* (Bt) (Bulla et al., 1980), all 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 prevailing in the basic larval mid-gut, 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 mosquito larvicidity of Bti resides in at least four major ICPs, of 134, 128, 72 and 27 kDa, encoded by *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, it 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) and 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 are necessary to avoid selection of resistance in the targets (Georghiou & Wirth 1997; Wirth et al., 1997; 2007). *Bti*'s limitations compromise its efficacy in natural environments (Ohana et al., 1987). Expressing its cry genes in other model systems may alleviate such limitations in effectively delivering the toxins 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 two 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, fear has been raised of persistence of *Bti* elements in the environment albeit without toxicity being able 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. Together with this is the need for development of 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), which also helps to deliver all its Cry proteins. The strategy of transferring *Bti*'s mosquito toxin genes for expression into alternative hosts that are eaten by mosquito larvae and multiply in their habitats is much safer and more economical than chemical insecticides.

Activity of the Insecticidal Crystal Proteins (ICPs) from Bti

The polypeptides and their genes

The larvicidal activity is included in several ICPs organized in a parasporal, proteinaceous crystalline body (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 a 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 ICPs toxicity is exhibited through the susceptible 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 susceptible insect midgut cells' 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 is much more toxic than each of the polypeptides alone. Various combinations within *Bti* are necessary to avoid selection of 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 these three proteins against three mosquito species display different synergy factors of between 2.5 - 15 (Crickmore et al., 1995; Poncet et al., 1995). The three Cry toxins are much more toxic than *Cyt1Aa* (Poncet et al., 1995; Delecluse et al., 1991), but the latter is the most synergistic to any of the other three and their combinations (Tabashnik, 1992; Canton et al., 2011). Cyt1Aa dose-response curve is 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* genes (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 studies have shown levels of resistance to specific combination of genes (Wirth

& Georghiou, 1997; Wirth et al., 1997; Wirth et al., 1998), information that is important in deciding desired larvicidal gene combinations for vector management. According to Wirth et al. (2001b), cross-resistance does not exist between cry19 of *B.t.* subsp. *jegathesan* and single or multiple *Bti* toxin genes. This is good news for sustaining the use of *Bti*-based biopesticides.

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, 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 are genetically manipulatable (Shestakov & Khyen, 1970; Wolk et al., 1984). In as much as some cyanobacteria may produce toxic blooms, strain PCC 7120 of Anabaena species, which is the delivery system exploited in this study, is non-toxic (Rouhiainen et al., 1995).

Further suitability of *Anabaena* PCC 7120 is due to its 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 at once.

b) The high copy number of the *Escherichia coli-Anabaena* shuttle plasmid constructed raises the number of gene copies per cell, tandem promoters—the transcription rate, and appropriate Ribosome Binding Site (RBS)—translation efficiency in the transgenic organism.

c) Codon usage of Anabaena sp. resembles that of the four *Bti* cry genes.

Materials and Methods

Media used in this study

All media used in this study were autoclaved 20 min at 121^{o}C, 1.5 Atm, and filter sterilized antibiotics added after cooling to approx. 40^{o}C prior to use, when required.

LB Medium

This complex, undefined medium used for *Bti* and *E. coli* consists of 1% Bacto Tryptone, 0.5% Yeast Extract, 1% NaCl, and solidified with 1.5% bacteriological agar when required.

BG-11 Medium

This defined medium for *Anabaena* PCC 7120 consists 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 $ZnSO_4$. The liquid BG-11 medium was solidified with 1.5% bacteriological agar when required.

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 is 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 period, bacteria were inoculated onto fresh agar plates, incubated at 37^{o}C overnight and stored at 4^{o}C. The bacteria were sub-cultured into fresh plates every two months. For extended storage, *E. coli* cultured to mid-log phase were stored in well-mixed aliquots containing 20% glycerol (200 µl into 400 µl freshly concentrated culture) at -86^{o}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 as for expression of larvicidal activity was harvested by centrifugation, washed and overnight freeze dried in a LABCONCO 2.5 Plus FreeZone freeze-drier LABCONCO Corporation, Kansas City, Missouri), operating at up to 0.009 Torr, up to -85°C (at the lower level).

Storage of competent cells

Competent cells were stored in 0.1 M $CaCl_2$ buffer at 4°C for less than a month (preferably used after overnight storage or at least within a week).

Bacterial growth conditions

For E. coli plasmid isolation

For plasmid DNA isolation and purification, *E. coli* was grown in 5 ml medium containing appropriate antibiotics) in test tubes incubated at 37°C with shaking at 200 rpm in a shaker incubator. Bacteria were harvested after overnight culture and used for plasmid isolation.

For competent E. coli cells preparation

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

For expression of Bti toxin genes in transgenic E. coli

Media for the recombinant *E. coli* strains were supplemented with 100 g ml^{-1} Ampicillin (Amp), 10 µg ml-1 Tetracyclin (Tet), 10 µg ml-1 Chloramphenicol (Cm) (when required). A colony was inoculated into test tube containing 5 ml LB and incubated overnight in a shaker (250 rpm) at 37°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 each. Induction with IPTG to a final concentration of 0.1 mM was done at the exponential stage. The biomass was 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.

For Anabaena PCC 7120 and its 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, used for the studies was cultured as described by Khasdan et al., 2003 and Manasherob et al., 2003. For bioassays, 2.5 L glass columns were used (30oC) under continuous illumination (85 mol photon m-2 sec-1) and furnished with air containing 1.5% CO2.

Microbial growth measurements

Bacteria

Growth of *E. coli* and *Bti* was measured either 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, or by using a Klett-Summerson colorimeter to determine Klett Units.

Cyanobacteria

Cyanobacterial growth was determined by estimating chlorophyll-a (Mackinney, 1941). Blank reading was set with distilled water. To 200 µl sample was added 800 µl methanol and mixed by hand before incubation at 70°C for 2 min 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, harvested by spinning at 3500 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 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 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 incubated for 1 hr at 37°C. 100 µl of the cells was 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 'vortexed'.

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

Culture and sonication of cells

E. coli strains were cultured and induced as described earlier in this study, 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.

Sources of insects for bioassays

*Source and rearing of Ae. aegypti Linnaeus 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 3rd instar stage of development.

Source and rearing of *An. arabiensis* Patton larvae

An. arabiensis eggs were obtained from a laboratory colony, courtesy of 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, made in Germany and supplied by Martons, P.O. Box 12711 Jacobs, 4026, Kwa Zulu Natal, South Africa, till the larvae were in their third instar stage.

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 the tested species (*Ae. aegypti* and *An. arabiensis*) were introduced into each cup, with each test replicated at least thrice, in different days. 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 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 response and the main results are reported in this study.

Results

All 15 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; and see section 3.3 below). 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.

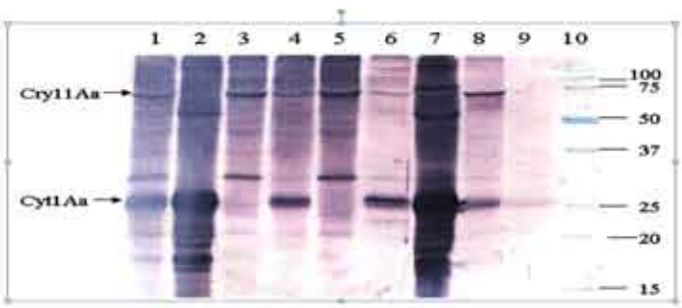
Expression of *Bti* toxin genes in *E. coli*

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 different promoter systems yielded different stoichiometries (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).



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.

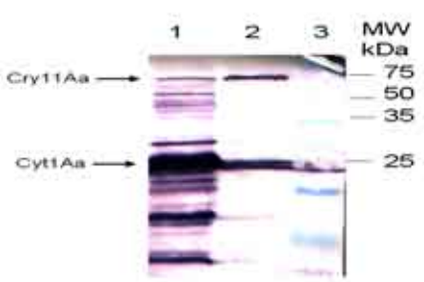
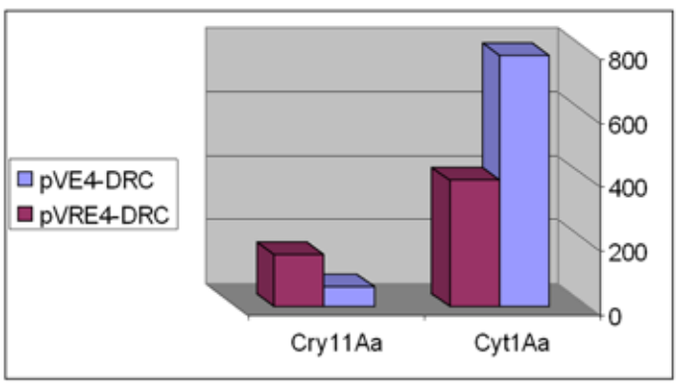


Figure 1

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.



(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 different stoichiometry establishes that more expression of *Cry11Aa* and less of *Cyt1Aa* cause higher toxicity than vice versa.

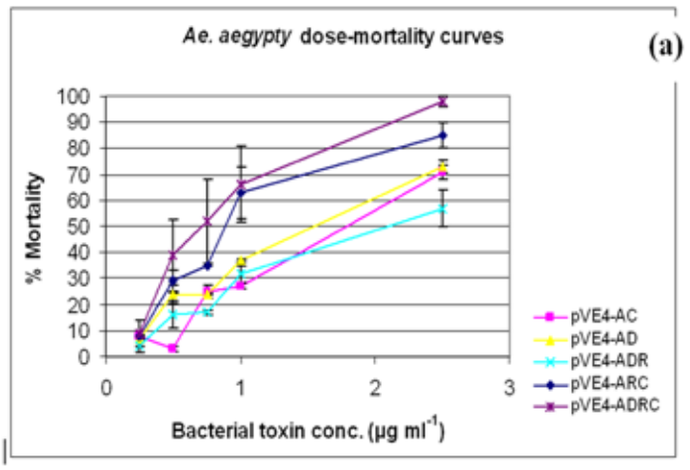
Toxicity against all three susceptible mosquito larvae of recombinant clones pHE4-A (and pHE4-AR) expressing *cry4Aa* (and *p20*) were low; it rose significantly upon addition of cyt1Aa (in pVE4-AC)

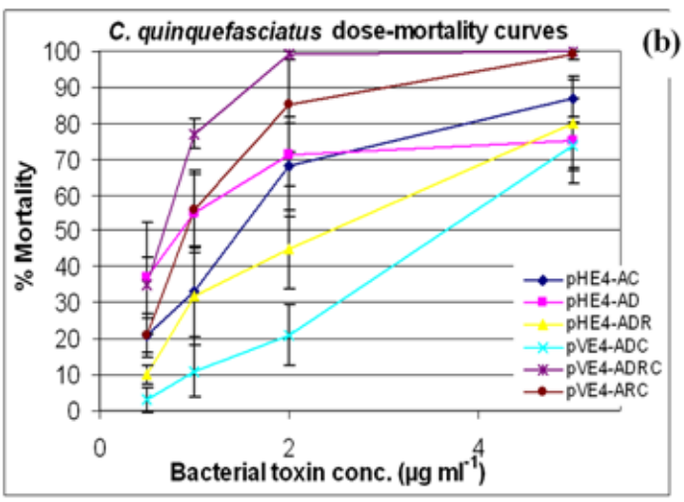
and even more so when *p20* was included as well (in pVE4-ARC).

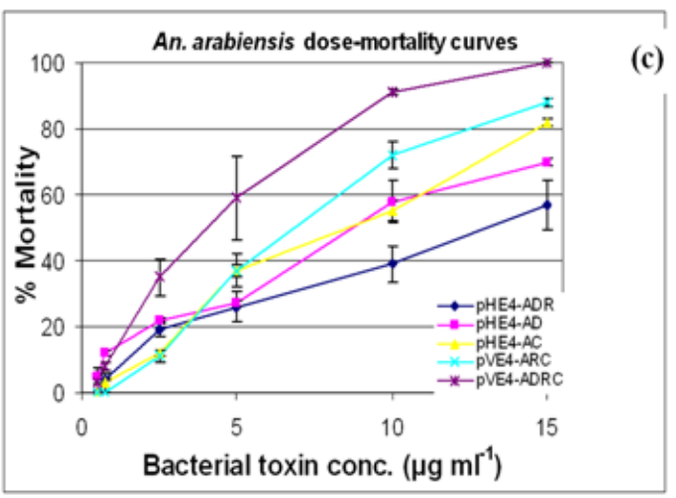
Recombinant clones, pHE4-ADR and pHE4-AD expressing *cry4Aa* and *cry11Aa* with and without p20 respectively were moderately toxic against all three susceptible mosquito species; they displayed comparable toxicities against *Ae. aegypti*, but the latter clone was 1.5 and 2.1 times more toxic against *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. Values of LC_{50} 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 Cyt1Aa contribution to toxicity of Cry4Aa and Cry11Aa against all three mosquito species is demonstrated by this series of bioassays: pVE4-ADRC was 1.9 - 2.5 more toxic than pVE4-AD and 2.7 - 5.3 more 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 against the target mosquito larvae (Figure 3). An ANOVA analysis of variance to test the model of comparison of the contributions of combinations, concentrations and the interactions between them were highly, highly significant at $\alpha = 0.05$ (Prob < 0.0001).







Hierarchy of toxicities against several mosquito species

In general higher and comparable toxicities of the six most toxic clones were achieved against *Ae. aegypti* and *C. quinquefasciatus* and 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), with Ae. aegypti and *C. quinquefasciatus* responding at par.

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

The results thus show that the dose-mortality curve patterns in all the three species is similar (Figure 3, a-c) irrespective of the individual LC_{50} values. In general, *An. arabiensis* was the least responsive, while in general there were no differences in response of *Aedes* and *Culex*. Comparing the first four in order of hierarchy and response by different mosquito species under concentrations of toxic bacteria ranging between 0.5 and 15µg ml^{-1}, depending on the level of toxicity (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

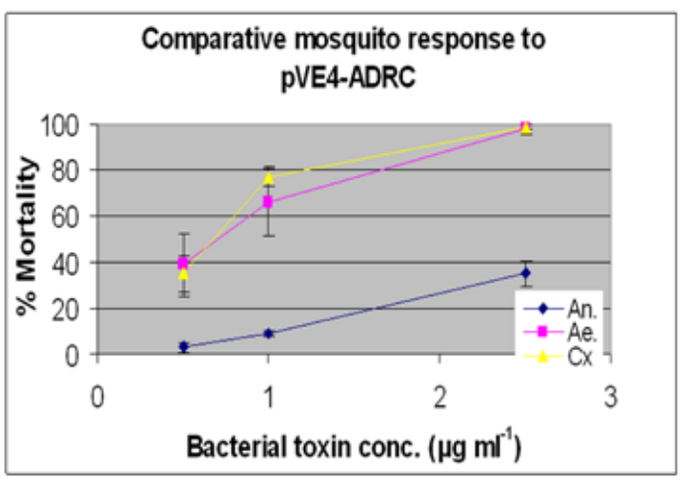


Figure 4.
Comparative mosquito response to (pVE4-ADRC) the most toxic combination of toxins.

both cases followed by pHE4-ADR, while pHE4-ADR was fourth in *An. Arabiensis*.

In comparing all concentration levels, it was evident that important as cry4Aa may be, it did not show 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) were not significant in all the three species (and are 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 led to having 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).

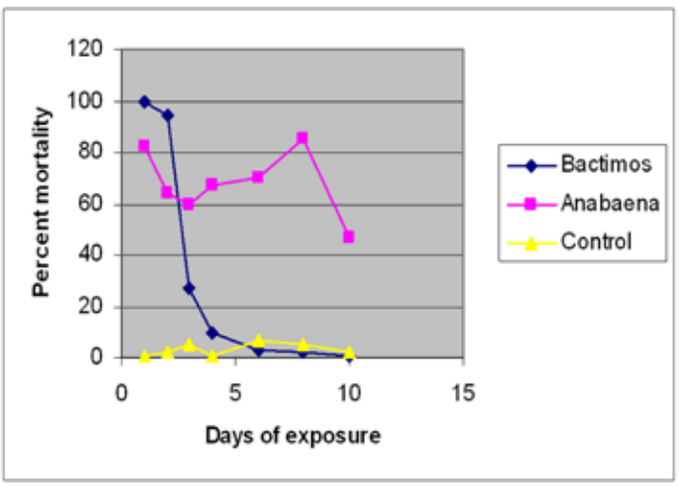


Figure 5
Relationships between cholophyll and protein concentrations with toxicity of Anabaena PCC 7120 at different (column) culture stages.

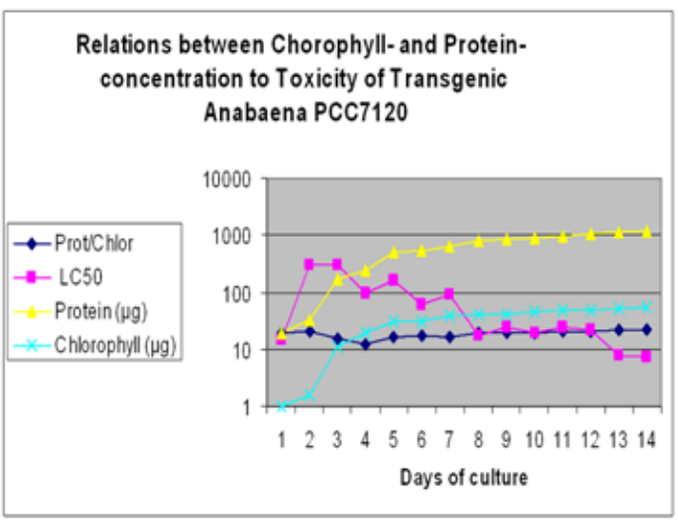


Figure 6
Persistence of toxicity against Ae. aegypti larvae in tap water at 40% shade in summer. Range of light intensity outdoors was 1900-2100 mmol photon m-2 s-1; cholorophyll range 1.0 to < 0.1 µg per ml

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* . Endurance of the transgenic *Anabaena*'s toxicity remained for 8 days, but considering the lower initial concentration of chlorophyll, it lasted for at least 12 days.

Discussion

Lyophilized powders of recombinant *E. coli* strains expressing, individually, *cry4Aa*, *cry11Aa, cyt1Aa* and *p20* showed varying toxicity levels (moderate, low or none) against larvae of susceptible *C. quinquefasciatus, Ae. aegypti* and *An. arabiensis* (Figures 3 a-c). Cyt1Aa is the least toxic of the four ICPs of Bti, but is the most active synergist with any of the other three and with their combinations (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). Such enhancement of Cry11Aa toxicity by Cyt1Aa, e.g., moderate synergy against Ae. aegypti (Crickmore et al., 1995), was also found here (with pVRE4-DRC) against *C. quinquefasciatus* and *Ae. aegypti* but not against An. arabiensis (Fiures 3 a-c). Consistently, toxicity of pure Cry11Aa is similar against *Ae. aegypti* and *C. pipiens* and lower against *An. stephensi (Poncet et al*., 1995). Toxicity of Cry11Aa-free inclusions is 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* displayed higher and similar toxicities against *C. quinquefasciatus* and *Ae. aegypti* but only about half toxicity against *An. arabiensis* than pVE4-ADRC expressing in addition *Cry11Aa*.

The main challenge of the near future is to come up with a releasable transgenic organism, which will satisfy the minimum toxicity demands in addition to demonstrating a water-tight mechanism for dealing with possibilities of resistance and horizontal gene transfer.

Role of promoter system on stoichiometry and subsequent toxicity

It was interesting to note that two clones with the same gene combination showed very different levels of toxicity. These clones, pVRE4-DRC and pVE4-DRC expressing the same three genes (cry11Aa, p20, and cyt1Aa) 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 (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 respectively in Figure 2). On analysis of western blot patterns by *EZQuant-Gel 2.11* (Figure 2 b), the density ratio of Cry11Aa in pVE4-DRC: pVRE4-DRC was found to be 1:2 (with total value of 1:3), while Cyt1Aa ration of pVE4-DRC: pVRE4-DRC was 1:3 (with total value of 1:4). This analysis was however used only to give qualitative comparisons and not 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 displayed toxicity of > 200 μg ml^{-1} and 15.5 μg ml^{-1} against *C. quinquefasciatus* and *Ae. aegypti* respectively, but both clones were not toxic against *An. arabiensis* (data not presented here). This different stoichiometry had implications on toxicity, in that more 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 is important for polypeptide pattern, intensity and subsequent toxicity of the desired products; (b) Of the 16 possible combinations, the 6 most toxic ones displayed a consistent hierarchy in all the three mosquito spp. tested; (c) The combination with all four genes was consistently the most toxic; (d) *Anabaena* PCC 7120 is quite promising as a candidate

for field release; (e) Recombinant *Anabaena* performs better in simulated semi-field conditions than *Bti* , and (f) Limited toxin degradation (preliminary results not presented here) does not affect toxicity.

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