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Minireview

Variations in the mosquito larvicidal activities of toxins from *Bacillus thuringiensis* ssp. *israelensis*

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Summary

Comparing activities of purified toxins from *Bacillus thuringiensis* ssp. *israelensis* against larvae of seven mosquito species (vectors of tropical diseases) that belong to three genera, gleaned from the literature, disclosed highly significant variations in the levels of LC_{50} as well as in the hierarchy of susceptibilities. Similar toxicity comparisons were performed between nine transgenic Gram-negative species, four of which are cyanobacterial, expressing various combinations of *cry* genes, *cyt1Aa* and *p20*, against larvae of four mosquito species as potential agents for biological control. Reasons for inconsistencies are listed and discussed. Standard conditions for toxin isolation and presentation to larvae are sought. A set of lyophilized powders prepared identically from six *Escherichia coli* clones expressing combinations of four genes displayed toxicities against larvae of three mosquito species, with levels that differed between them but with identical hierarchy.

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Introduction

Mosquitoes cause enormous public health menace by transmitting various tropical diseases and by being a nuisance (Service, 2004). Many species of the genera *Anopheles*, *Aedes* and *Culex* are vectors of, e.g. malaria (WHO, 2006), yellow fever, dengue fever, haemorrhagic fever and lymphatic filariasis (Gyapong and Twum-Danso, 2006). Chemical insecticides used in vector control programmes harm the environment (Tabashnik, 1994) with adverse impacts on man and nature. To deal with these problems and limitations, alternative technologies such as biological control are called for. The Gram-positive spore-forming bacterium *Bacillus thuringiensis* ssp. *israelensis* is the most powerful and environment-friendly component in malaria integrated vector management (Fillinger *et al*., 2003). This bacterium forms a parasporal crystalline toxic body (the δ-endotoxin) that is widely used as a commercial bio-pesticide against larvae of noxious arthropod species of the suborder *Nematocera*, including mosquitoes, black flies and chironomid midges (Margalith and Ben-Dov, 2000).

The larvicidity of *B. thuringiensis* ssp. *israelensis* resides in at least four major crystal pro-toxic proteins, of 134, 128, 72 and 27 kDa, encoded by *cry4Aa*, *cry4Ba*, *cry11Aa* and *cyt1Aa* respectively, and all mapped on the 128 kb plasmid known as pBtoxis (Ben-Dov *et al*., 1999; Berry *et al*., 2002). The level of toxicity depends on the capacity of the target species to activate the pro-toxin by breaking it down into the active toxic components using specific proteases prevailing in the basic larval mid-gut. Subsequent steps involve toxin binding to receptors (Feldmann *et al*., 1995; Yamagiwa *et al*., 2001; Boonserm *et al*., 2005; 2006; Fernandez *et al*., 2005; 2006; Chayaratanasin *et al*., 2007), insertion into the membrane and aggregation, leading to the formation of gated, cation-selective channels (Knowles *et al*., 1989; Aronson and Shai, 2001). Lethality is due to destruction of the *trans*-membrane potential, with subsequent osmotic lysis of cells lining the mid-gut (Knowles and Ellar, 1987).

Despite the low toxicity of Cyt1Aa against exposed larvae, it is highly synergistic with the Cry toxins and their combinations *in vitro* (Wu *et al*., 1994; Crickmore *et al*., 1995; Wirth *et al*., 1997; 2007; Pérez *et al*., 2005) due to different modes of action (Butko, 2003). The latter are structurally unrelated to Cyt1Aa, but resemble in their membrane-perforating ability (Margalith and Ben-Dov, 2000). The Cry toxins bind to membrane proteins whereas Cyt1Aa binds to the unsaturated phospholipids (Butko, 2003). Cry11Aa, for example, binds to a 148 kDa protein in *Anopheles stephensi* (Feldmann *et al*., 1995) and to a 65 kDa alkaline phosphatase in cells of the *Aedes aegypti* digestive system (Fernandez *et al*., 2006). Various combinations of Cry toxins with Cyt1Aa alleviate selection of resistance in the targets (Georghiou and Wirth, 1997; Wirth *et al*., 1997; 2007).

Susceptibility of mosquito larvae to purified B. thuringiensis *ssp.* israelensis *toxins*

Seven species of mosquitoes that belong to three genera are most commonly used in bioassays to estimate activities of the four crystal proteins and synergies among them (Margalith and Ben-Dov, 2000). Table 1 summarizes all LC_{50} values of purified toxins and their combinations against at least one species of each of the three genera, gleaned from the literature reported to date.

The most conspicuous observations are the huge variation of the LC_{50} values and apparent inconsistencies between toxicities of different larvicidal Cry and Cyt1Aa toxins against various mosquito species obtained in different assays. It can thus be concluded that the materials used in the bioassays dramatically affect the outcome. The toxins are usually purified from different bacterial species (original, recombinant, Gram-positive or -negative) grown under varying culture conditions (medium, time, temperature, aeration) and presented to larvae of varying instars (1st–4th) under varied densities (0.17–10 larvae ml^{-1}) and during various periods (12–48 h). For example (Table 1), toxicity of Cry11Aa against larvae of the same three species was an order of magnitude lower (LC_{50} between 8.6 and 135 ng ml^{-1}) in the hands of one group (Revina *et al*., 2004) than that (121.5–372.4 ng ml^{-1}) obtained by another team (Delécluse *et al*., 1995). Strikingly, the respective numbers for *Culex pipiens* alone were 8.6 and 372.4! Similar discrepancies are also true for Cry4Ba, Cyt1Aa and combination of Cry4Aa + Cry4Ba.

In an attempt to further clarify these differences, the data summarized in Table 1 was normalized (Table 2) so that LC_{50} in bioassays against *Ae. aegypti* (to which data are complete) were set in each case to a value of 1.00. Despite some clarifications, many inconsistencies persist. For example, relative toxicities of purified Cry4Aa and Cry4Ba to larvae of four *Anopheles* species vary up to 1900- and 15-fold respectively, whereas against two *Culex* species LC_{50} fluctuates up to about 12- and 50-fold respectively (Table 2). Relative LC_{50} values of purified Cry11Aa against *An. stephensi* vary up to 7.9-fold, whereas against *C. pipiens* they vary up to about 3.8-fold only.

Table 1. Summary of LC_{50} values (ng ml^{-1}) of toxins from *B. thuringiensis* ssp. *israelensis* against larvae of seven mosquito species (three genera).

	Culex		*Anopheles*					
Cry/Cyt combination	*quinque fasciatus*	*pipiens*	*stephensi*	*dirus*	*gambiae*	*quadrim aculatus*	*Aedes aegypti*	Reference[a]
Cry4Aa		251	1296				563	Poncet *et al*. (1995)
	345			159			2390	Boonserm *et al*. (2006)
		400	7400				1600	Delecluse *et al*. (1993)
	980				1170		1360	Angsuthanasombat *et al*. (1992)
	980	400				> 80 000	600	Abdullah *et al*. (2003)
Cry11Aa		268	455				287	Poncet *et al*. (1995)
		8.6	135				10.8	Revina *et al*. (2004)
		372.4	326				121.5	Delecluse *et al*. (1995)
Cyt1Aa		1200	6300				880	Thiery *et al*. (1997)
	400	600	2700				1000	Juárez-Pérez *et al*. (2002)
Cry4Aa + Cry11Aa		110	165				108	Poncet *et al*. (1995)
Cry4Ba		> 50 000	17				145	Poncet *et al*. (1995)
		> 50 000	550				300	Delecluse *et al*. (1993)
	> 80 000	> 20 000				25	61	Abdullah *et al*. 2003
	24 500				790		940	Angsuthanasombat *et al*. (1992)
Cry4Aa + Cry4Ba		36	16				52	Poncet *et al*. (1995)
		63	300				82.6	Delecluse *et al*. (1993)
	180				380		280	Angsuthanasombat *et al*. (1992)
Cry4Ba + Cry11Aa		12 495	196				207	Poncet *et al*. (1995)
Cry4Aa + Cry4Ba + Cry11Aa		84	114				100	Poncet *et al*. (1995)

a. All toxins were obtained by purification (described in each reference). Recorded are only preparations bioassayed against at least one species of each of the three genera.

Table 2. Relative LC_{50} values of purified toxins against larvae of six mosquito species (two genera).

	Culex		*Anopheles*			
Cry/Cyt combination	*quinquefasciatus*	*pipiens*	*stephensi*	*dirus*	*gambiae*	*quadrimaculatus*
Cry4Aa		0.45	2.30			
	0.14			0.07		
		0.25	4.63			
	0.72				0.86	
	1.63	0.67				> 133
Cry11Aa		0.93	1.59			
		0.80	12.50			
		3.07	2.98			
Cyt1Aa		1.36	7.16			
	0.40	0.60	2.70			
Cry4Aa + Cry11Aa		1.02	1.53			
Cry4Ba		> 344.83	0.12			
		> 166.67	1.83			
	> 1311.48	> 327.87				0.41
	26.06				0.84	
Cry4Aa + Cry4Ba		0.69	0.31			
		0.76	3.63			
	0.64				1.36	
Cry4Ba + Cry11Aa		60.36	0.95			
Cry4Aa + Cry4Ba + Cry11Aa		0.84	1.14			

LC_{50} values from Table 1 were divided each by the one obtained by the same team with larvae of *Ae. aegypti*.

For the sake of completeness, Table 3 summarizes data from five additional publications, all with purified toxins (except one, with spore-crystal powder). They evaluate toxicities against *A. aegypti* only, but of varying larval instars, between 1st and 4th, thus explaining the wide range of LC_{50} values obtained. When purified Cry11Aa produced in recombinant *B. thuringiensis* ssp. *kurstaki* was used against 4th instar larvae of *Culex quinquefasciatus* however, similar values were indeed obtained in two different studies by the same team and some synergy observed with Cyt1Aa (Chang *et al*., 1992; 1993). Toxicity of Cry11A produced in recombinant *B. thuringiensis* ssp. *israelensis* was several-fold higher against 4th instar larvae of *C. pipiens* (Yamagiwa *et al*., 2004).

Toxicity of recombinant Gram-negative bacteria expressing cry *and* cyt

Many more Gram-negative species were engineered to express several *cry* and *cyt* genes in attempts to overcome limitations (such as low efficacy and short residual activity) encountered in biological control of mosquitoes (Margalith and Ben-Dov, 2000). Table 4 sums up results of 14 toxicity studies with 9 such species, 4 of which are cyanobacteria, against larvae of 4 species in all 4 instar stages, fed in variable manners.

Transgenic *Agmenellum quadruplicatum* PR-6 and *Synechocystis* PCC 6803 expressing *cry4Ba* under P_{cpcB} and P_{psbA} promoters respectively, displayed very low

Table 3. Larvicidities of purified toxin combinations from recombinant *B. thuringiensis* against *A. aegypti* (complementary to Table 1).

	LC_{50} (ng ml^{-1}) against *A. aegypti* larvae				
Cry/Cyt Combination	Crickmore *et al*. (1995) (3rd instar)	Pérez *et al*. (2005) (4th instar)	Wu *et al*. (1994) (1st instar)	Park *et al*. (1999) (1st instar)	Beltrão and Silva-Filha (2007) (4th instar)[b]
Cry4Aa	1125				13 010
Cry4Ba	467				120
Cry11Aa	224	236	85	73	1350
Cyt1Aa	1209	1245	60		
Cry11Aa + Cyt1Aa[a]	118 (1:1)	12.3 (1:0.2)	14.8 (1:1)		
Cry4Aa + Cyt1Aa[a]	75 (1:1)				
Cry4Ba + Cyt1Aa[a]	62 (1:1)				
Cry4Ba + Cry11Aa[a]	173 (1:1)				
Cry4Aa + Cry4Ba + Cry11Aa[a]	125 (1:1:1)				
Cry4Aa + Cry4Ba + Cyt1Aa[a]	77 (1:1:1)				

a. Toxins were mixed in the proportions (w/w) indicated in parentheses.
b. Powders of recombinant *B. thuringiensis* ssp. *israelensis* containing spore/crystal mixtures.

Table 4. Comparative larvicidities of recombinant Gram-negative bacteria (excluding *E. coli*), as potential agents for controlling mosquito larvae, expressing toxin genes from *B. thuringiensis* ssp. *israelensis*.

Cyanobacterial strain	Toxin gene(s)	Mode of feeding or (single dose)	Larval species instar	% mortality in 24 h or (LC_{50}; cells ml^{-1})	% mortality in 48 h	References
Agmenellum quadruplicatum PR-6	*cry4Ba*	Sonicated cells (10 mg protein ml^{-1})	*Ae. aegypti* 2nd	ND	45	Angsuthanasombat and Panyim (1989)
A. quadruplicatum PR-6	*cry11Aa*	2×10^7 cells ml^{-1} in 12 h intervals	*C. pipiens* 1st	0	0	Murphy and Stevens (1992)
A. quadruplicatum PR-6	*cry11Aa*	2×10^7 cells ml^{-1} in 12 h intervals	*Ae. aegypti* 1st	45	75	Stevens *et al.* (1994)
Anabaena PCC 7120	*cry4Aa* + *cry11Aa* + *p20*	(Single dose)	Ae. aegypti 3rd	(3.3×10^5)		Wu *et al.* (1997)
Anabaena PCC 7120	*cry4Aa* + *cry11Aa* + *cyt1Aa* + *p20*	(Single dose)	*Ae. aegypti* 4th	(3.5×10^4)		Khasdan *et al.* (2003)
Ancylobacter aquaticus	*Cry4Ba*	(Single dose)	*Ae. aegypti* 2nd	(4.1×10^7)		Yap *et al.* (1994b)
Asticcacaulis excentricus	*cry11Aa* + *p20*	(Single dose)	*Ae. aegypti* 1st	(6.83×10^5)		Armengol *et al.* (2005)
Caulobacter crescentus	*cry4Ba*	(3.2×10^8)	*Ae. aegypti* 2nd	16	32.5	Thanabalu *et al.* (1992)
C. crescentus	*cry4Ba*	(Single dose)	*Ae. aegypti* 2nd	(2.2×10^6)		Yap *et al.* (1994a)
Enterobacter amnigenus	*cry4Ba*	(1×10^8)	*Ae. aegypti* 2nd	ND	63–100	Tanapongpipat *et al.* (2003)
E. amnigenus	*cry4Ba*	(Single dose)	*Ae. aegypti* 2nd	(7×10^5)		Khampang *et al.* (1999)
		(Single dose)	*An. dirus* 2nd	(8.7×10^5)		
P. putida	*cry11Aa* + *p20*	(Single dose)	*Ae. aegypti* 3rd	(3.9×10^4)		Xu *et al.* 2001
Synechocystis PCC 6803	*cry4Ba* or *cyt1Aa*	Soluble proteins (1 mg ml^{-1})	*Ae. aegypti* 3rd	ND	30	Chungjatupornchai (1990)
Synechococcus PCC 7942	*cry4Ba*	7.5×10^7 cells ml^{-1}, daily	*C. restuans* 1st	0	20–90	Soltes-Rak *et al.* (1993)

toxicity (Angsuthanasombat and Panyim, 1989; Chungjatupornchai, 1990). Using tandem promoters (its own and P_{lac}) in *Synechococcus* PCC 7942 slightly improved the toxicity against 1st instar larvae of *Culex restuans* (Soltes-Rak *et al.*, 1993). Tandem strong promoters (P_{psbA} and P_{A1}) and favourable codon usage in the filamentous cyanobacterium *Anabaena* PCC 7120 raised efficiency of expression of *cry4Aa*, *cry11Aa* and *cyt1Aa* (Wu *et al.*, 1997; Khasdan *et al.*, 2003). The higher level of Cyt1Aa in the clone expressing *p20* as well than without (regulated by the same tandem promoters) indicate that P20 protects *Anabaena* from the deleterious action of Cyt1Aa, as it does in *Escherichia coli* (Douek *et al.*, 1992; Khasdan *et al.*, 2001; Manasherob *et al.*, 2001). Recombinant *Pseudomonas putida* expressing *cry11Aa* was not toxic to larvae unless coexpressed with *p20*, in which case toxicity resembled that of *E. coli* (Xu *et al.*, 2001).

The product of *p20* stabilizes both Cyt1Aa or Cry11A in recombinant *E. coli* (as well as in *B. thuringiensis*; Wu and Federici, 1993; 1995) by a post-transcriptional mechanism; substantially more Cyt1Aa/Cry11Aa was produced in recombinant *E. coli* carrying *p20* than in those without it (McLean and Whiteley, 1987; Adams *et al.*, 1989; Visick and Whiteley, 1991). Some larvicidity was obtained when *cry11Aa* was expressed together with *p20* but not alone (Ben-Dov *et al.*, 1995). Cry11Aa is thus apparently degraded and partially stabilized by P20. Similarly, Cry11Aa alone formed parasporal inclusions in an acrystalliferous recombinant *B. thuringiensis* species, and higher levels were observed in the presence of P20 (Chang *et al.*, 1992; 1993; Wu and Federici, 1995). Increased production of the fused Cry4Aa-ΔLacZ in *E. coli* when expressed with *p20 in trans* was also observed (Yoshisue *et al.*, 1992).

Toxicity of *cry4Ba* expressed in the ubiquitous Gram-negative *Caulobacter crescentus* varied 100-fold according to the regulatory region constructed to drive its expression (Thanabalu *et al.*, 1992; Yap *et al.*, 1994a). The same gene was also efficiently expressed in *E. coli* and formed phase-bright insoluble inclusions, which were highly toxic to *Ae. aegypti* larvae (Angsuthanasombat *et al.*, 1987; Chungiatupornchai *et al.*, 1988; Delécluse *et al.*, 1988; Ward and Ellar, 1988). Toxicities of Cry4Ba against *Ae. aegypti* and *C. quinquefasciatus* larvae were synergized by Cyt2Aa when their encoding genes were coexpressed in *E. coli* (Promdonkoy *et al.*, 2005).

To test whether bioassays with recombinant strains under identical conditions will reduce variability, the same batch of standard freeze-dried powders that had been assayed with *C. quinquefasciatus* (Wirth *et al.*, 2007) was exploited here to evaluate relative toxicities against *Ae. aegypti* and *Anopheles arabiensis* as well, and the bioassays were conducted with larvae of the same instar raised

Table 5. Toxicities of six transgenic *E. coli* strains carrying combinations of four genes from *B. thuringiensis* ssp. *israelensis* against three mosquito species.

	Mosquito larvicidal activity, LC_{50} (μg ml^{-1})[b] [relative values][c]		
Strains[a] (genes)	*C. quinquefasciatus*[d]	*Ae. aegypti*[e]	*An. arabiensis*[f]
pVE4-ADRC (*cry4Aa*, *cry11Aa*, *p20*, *cyt1Aa*)	0.6 (0.4–0.8) [0.9]	0.7 (0.6–0.7) [1.0]	3.2 (2.4–4.4) [4.7]
pVE4-ARC (*cry4Aa*, *p20*, *cyt1Aa*)	0.9 (0.7–1.3) [1.1]	0.9 (0.8–0.9) [1.0]	6.2 (5.7–6.8) [7.1]
pHE4-ADR (*cry4Aa*, *cry11Aa*, *p20*)	3.1 (1.2–8.3) [1.7]	1.8 (1.6–2.1) [1.0]	11.2 (8.5–16.0) [6.1]
pVE4-ADC (*cry4Aa*, *cry11Aa*, *cyt1Aa*)	4.2 (2.0–9.1) [0.7]	6.4 (5.8–7.1) [1.0]	20.4 (17.5–25.0) [3.2]
pVE4-AC (*cry4Aa*, *cyt1Aa*)	1.5 (1.2–1.7) [1.2]	1.3 (0.8–1.8) [1.0]	7.0 (4.9–9.4) [5.6]
pHE4-AD (*cry4Aa*, *cry11Aa*)	1.5 (0.6–4.0) [1.2]	1.3 (1.1–1.6) [1.0]	7.5 (5.2–11.3) [5.8]

a. Clones designated pHE4- stem from Ben-Dov and colleagues (1995) and those designated pVE4- stem from Khasdan and colleagues (2001).
b. LC_{50} values represent the results of at least three bioassays performed with the same powder preparation (Wirth *et al.*, 2007). Numbers in parentheses are 95% confidence limits, as determined by probit analysis.
c. Values normalized to LC_{50} against larvae of *Ae. aegypti*.
d. Values taken from Wirth and colleagues (2007).
e. Performed as described before (e.g. Khasdan *et al.*, 2001) but with the same powders as used in Wirth and colleagues (2007).
f. Larvae of *An. arabiensis* were obtained from the National Institutes of Health Sciences, Harare, Zimbabwe. The eggs were hatched and larvae reared to the 3rd instar stage at 28 ± 0.5°C and relative humidity of 70%, on ground dog biscuit and Tetramin Baby Fish Food (Tetra GmbH D-49304 Melle, Germany).

similarly. The powders were prepared identically from each of 15 clones expressing all combinations of four genes (*cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20*) from the same promoter in the same *E. coli* strain (Khasdan *et al.*, 2001). All four recombinants with each of the four genes alone, four out of six with two genes each and one of the four with three gene combinations displayed very low toxicities (if at all) against at least one of these mosquito species. Thus, the more toxin genes are coexpressed in *E. coli*, the higher is the likelihood that it would be effective.

Toxicities of the six clones that displayed measurable LC_{50} values against all three species are recorded in Table 5. Mosquito larvicidities of these clones against *Ae. aegypti* were comparable to those against *C. quinquefasciatus* (1.3-fold more toxic on the average) but significantly higher (5.4-fold on the average) than against *An. arabiensis*. Moreover, the toxicity hierarchal order was identical, i.e. pVE4-ADRC > pVE4-ARC > pVE4-AC = pHE4-AD > pHE4-ADR > pVE4-ADC. The results are also consistent with the notion that Cyt1Aa is synergistic to the Cry toxins (Wu *et al.*, 1994; Crickmore *et al.*, 1995; Wirth *et al.*, 1997; Pérez *et al.*, 2005), provided it is co-produced with the helper P20 (Wu and Federici, 1993; Manasherob *et al.*, 2001).

Toxicity of heterologous proteins in recombinant species is usually poorer due to weak expression of the δ-endotoxin genes by the original promoters, low stability and proteolytic cleavage of polypeptides and non-formation or malformation of crystals. The amount of active heterologous proteins expressed depends however, on various additional factors, including regulation of replication (i.e. plasmid copy number), transcription (promoter structure and σ factors), translation (efficiency of ribosomal binding site, U-rich sequence and codon usage) and mRNA stability (stem-loop structure at the 3′ end, and 5′ mRNA stabilizer) (Chandler and Pritchard, 1975; Ikemura, 1981; Studier and Moffatt, 1986; Vellanoweth and Rabinowitz, 1992; Soltes-Rak *et al.*, 1993; 1995; Yap *et al.*, 1994a; Agaisse and Lereclus, 1995; Baum and Malvar, 1995; Dong *et al.*, 1995; Guerchicoff *et al.*, 1996; Liu *et al.*, 1996; Park *et al.*, 1999; 2003).

Additional sources of toxicity variations

In addition to the conditions listed above that can be modified at will and hence reduce bioassay fluctuations, at least two natural features are likely to raise variability of the results, feeding behaviour of the larvae and the structure and density of their gut toxin receptors (not only between different genera but also between certain species of the same genus). For example, surface feeding (at the air–water interface) by *Anopheles* larvae limits their ingestion of the toxin (whether pure or in bacteria), which quickly settles at the bottom of the water body, whereas bottom feeders, such as *Aedes* and *Culex*, filter-feed at all levels of the water column (Merrit *et al.*, 1992; Service, 2004), enhancing the interaction with the introduced toxin. This may partially explain the higher susceptibility and lower variability in LC_{50} values of *Aedes* and *Culex* to the larvicidal proteins than of *Anopheles* (Tables 1, 2, 4 and 5).

Toxicity comparisons with several mosquito species indicate existence of different receptors or of their concentrations on the midgut epithelium. For example (Tables 1 and 2), Cry4Aa displayed high toxicity against *C. pipiens*, less against *Ae. aegypti*, and low against *An. stephensi* (Poncet *et al.*, 1995), but high toxicity against *Anopheles dirus*, less against *C. quinquefasciatus*, and lowest against *Ae. aegypti* (Boonserm *et al.*, 2006). The range of target mosquitoes affected by Cry4Ba differs

from that of Cry4Aa, possibly by using distinct binding sites for interaction with the host receptors (Boonserm *et al*., 2006), but the specific receptors for Cry4Aa and Cry4Ba still remain to be determined. Involvement of their domains II and III in toxin-receptor recognition/binding has been shown recently (Yamagiwa *et al*., 2001; Boonserm *et al*., 2005; 2006; Beltrão and Silva-Filha, 2007; Chayaratanasin *et al*., 2007).

Concluding remarks

The huge variation of LC_{50} values obtained in bioassays of mosquito larvicidal Cry and Cyt1Aa proteins against various mosquito species seems to result from vast differences in the procedures involved. These can be divided into several categories, strain and growth of the producing bacteria, isolation of the toxins and bioassays conditions, as follows.

The toxin-producing bacteria. These can be either the original *B. thuringiensis* subspecies or recombinant strains. The transgenic species can either be Gram-negative or -positive, the latter from either the same or different genus. Protein folding and solubility are affected by the producer species, which sometimes dramatically modify the final toxicity attributes. Different culture conditions, such as medium composition and pH, growth temperature, mode of aeration and period of cultivation till harvest, all affect the amounts of toxins produced, although not their specific activities.

Toxin purification and processing. The mode of purification depends on the consistency at which the toxin is synthesized *in vivo*. The toxins, produced as crystals in the original species, end up in recombinant strains as inclusion bodies, crystals or soluble proteins. Solubilization methods vary immensely, sometimes require pH shifts, and toxicity level depends on the mode of pro-toxin activation (protease species and incubation conditions).

Bioassays: larvae and conditions. Susceptibility of larvae is age-dependent: 4th instars require an order of magnitude higher concentration to be affected than 1st instars, for example, due to changes in their size and protease composition/contents. Literature-recorded bioassays vary dramatically in the densities of larvae (0.17–10 ml^{-1}) and periods of exposure to the toxin (12–48 h). Food availability, water quality and temperature are factors to be considered.

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