Mosquitocidal Bacterial Toxins: Diversity, Mode of Action and Resistance Phenomena

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Bacteria active against dipteran larvae (mosquitoes and black flies) include a wide variety of Bacillus thuringiensis *and* B. sphaericus *strains, as well as isolates of* Brevibacillus laterosporus *and* Clostridium bifermentans. *All display different spectra and levels of activity correlated with the nature of the toxins, mainly produced during the sporulation process. This paper describes the structure and mode of action of the main mosquitocidal toxins, in relationship with their potential use in mosquito and/or black fly larvae control. Investigations with laboratory and field colonies of mosquitoes that have become highly resistant to the* B. sphaericus *Bin toxin have shown that several mechanisms of resistance are involved, some affecting the toxin/receptor binding step, others unknown.*

Key words: *Bacillus thuringiensis - Bacillus sphaericus - Brevibacillus laterosporus - Clostridium bifermentans* - toxin binding - receptor - resistance

The control of mosquitoes and black flies is a part of the prevention of important vector born diseases like malaria, yellow fever, dengue, and filariasis, including onchocerciasis. The intensive use of chemical insecticides led to the development of resistant insect populations, resulting in a reduced control and often to a negative impact on various non-target organisms and on the environment in general. Therefore, alternative control measures including microbial control have been developed. Particularly, naturally occurring bacteria are successfully used against the larval stages of mosquitoes and black flies.

DIVERSITY OF MOSQUITOCIDAL BACTERIAL TOXINS

Entomopathogenic bacteria, namely *Bacillus thuringiensis* (*Bt*), have been known from the early 1900's but the control of dipteran species has been envisaged only since the discovery of *B. thuringiensis* serovar *israelensis* (*Bti*) in 1977 (Goldberg & Margalit 1977) and a highly toxic strain of *B. sphaericus* (*Bsp*) strain 1593 (Singer 1974).

Since that time, several screening programmes, the goal of which was to isolate a number of new mosquitocidal strains, have been developed. These resulted in the identification of a wide variety of Gram-positive bacteria, including both *Bt* and *Bsp* isolates and other bacteria, for which no insecticidal activity had been reported previously: *Brevibacillus laterosporus* and *Clostridium bifermentans*.

All strains isolated have been characterized for their level of mosquitocidal activity and specificity. For most of these bacteria, the factors responsible for the insecticidal activity have then been identified and characterized, and main toxicity is due to the presence of protein inclusion bodies produced during sporulation of the bacteria. These toxic factors are named Cry and Cyt toxins for *Bt* and Bin toxins for *Bsp*.

Bacillus thuringiensis strains

The diversity of toxin produced is the reason for the high specificity of these bacteria. However, *Bti* covers the largest activity spectra covering larvae from many Culicidae (mosquito) genera: *Culex*, *Aedes*, and *Anopheles*, with a higher activity on the two former. *Bti* crystals are also toxic against Simuliidae (Undeen & Nagel 1978). In contrast, they have no effect on vertebrates and non-target invertebrates (Miura et al. 1980). However, upon solubilisation, the crystals display non-specific cytolytic and haemolytic activities (Thomas & Ellar 1983).

Bti inclusions are composed of four major proteins with molecular weight of 135, 125, 68, and 28kDa. Since the discovery of *Bti*, several other *Bt* strains displaying mosquitocidal activity have been isolated. These strains, differing from *Bti* by either their serotype, mosquitocidal activity or polypeptide composition, can be classed into three groups. Class 1 includes strains with larvicidal and haemolytic activities as well as crystal polypeptides similar to those of *Bti*. Class 2 contains few

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strains which are nearly as toxic as *Bti* but produce different polypeptides, *B. thuringiensis* serovar *jegathesan* (*Btjeg*) (Delécluse et al. 1995) and *B. thuringiensis* serovar *medellin* (*Btmed*) (Orduz et al. 1998). Class 3 includes a number of strains, which synthesise polypeptides different from those found in *Bti*, but are only weakly active.

The *Btjeg* inclusions are composed of six major proteins with molecular weight of 80, 74-70, 65, 37 26 and 16kDa (Ragni et al. 1996). Genes encoding the 80, 74-70, 65, and 26kDa polypeptides have been cloned. The 80kDa protein is 58% identical to the *Bti* Cry11Aa, and has therefore been designated Cry11Ba. In comparison with other proteins contained in the crystals, this is the one which have, from far, the highest mosquitocidal activity. The 26kDa protein is 66% identical to Cyt2Ba from *Bti* and has been therefore named Cyt2Bb (Cheong & Gill 1997).

Inclusions of *Btmed* are composed of four different and major proteins of 94, 70-68, 30 and 28kDa (Restrepo et al. 1997). The 94kDa protein, named Cry11Bb (as it is 83% identical to Cry11Ba) is the most toxic component, whatever the mosquito species is, with a level of activity comparable to that of Cry11Ba (Orduz et al. 1998). No mosquitocidal activity has been found yet for the two other proteins (Cry29A and Cry30A, respectively), but it cannot be excluded that they interact with other *Btmed* crystal components to enhance their activity (A Delécluse, pers. commun.).

Bacillus sphaericus strains

The binary toxin (Bin) is the most important of the *Bsp* toxins owing to its predominant role in determining the overall toxicity of strains. All high-toxicity strains produce binary toxin as a part of their mosquitocidal arsenal. This crystal toxin is composed of two separate proteins BinA (42kDa) and BinB (51kDa). Genes encoding binary toxin of highly toxic strains are grouped in four types. Several studies have shown that, when produced in *Escherichia coli*, both BinA and BinB are required for toxicity to mosquito larvae and that an equimolar ratio of both proteins yields the greatest toxicity (Broadwell et al. 1990). It is interesting to note however, that the 42kDa (BinA) produced alone in recombinant *B. thuringiensis* is toxic to mosquitoes (Nicolas et al. 1993). During the vegetative stage other mosquitoacidal toxins are produced, called Mtx1 (100kDa; Tanabalu et al. 1991), Mtx2 and Mtx3 (Liu et al. 1996), the activity of which are lower than the crystal proteins.

Brevibacillus laterosporus

B. laterosporus, formerly *Bacillus laterosporus*, is a spore-forming bacterium characterized by its ability to produce canoe-shaped lamellar bodies, adjacent to the spore (Montaldi & Roth 1990). Recently, two strains – 16-92 (isolate 921) and LAT006 (isolate 615) have been identified – that are highly toxic for mosquitoes (Orlova et al. 1998). These two strains are more toxic for the mosquitoes in the species *Ae. aegypti* and *A. stephensi*, and less active against *C. pipiens*. Both produce crystalline inclusions of various shapes and sizes (Smirnova et al. 1996) responsible for the toxicity. Purified *B. laterosporus* 615 crystals are as toxic as those from *Bti*, to *Ae. aegypti* and *A. stephensi*. The natures of the crystal components responsible for this activity have not yet been elucidated.

Clostridium bifermentans

Screening programs, aimed to identify new strains of insecticidal bacteria, led to the description, in 1990, of the first anaerobic isolate, CH18, having a high mosquitocidal activity. This strain was isolated from a mangrove swamp soil from Malaysia and identified as *C. bifermentans* serovar *malaysia* (*Cbm*; de Barjac et al. 1990). More recently, a new mosquitocidal *C. bifermentans* strain was reported, *C. bifermentans* serovar. *paraiba* (*Cbp*), isolated from a secondary forest floor in 1997 (Seleena et al. 1997). The insecticidal spectrum of both *C. bifermentans* strains is similar and their toxicity comparable to *Bti* strains, although the toxic factor(s) are not the same as in *Bti*; for these reasons they resemble class-2 *B. thuringiensis* strains. Both strains are characterized by a high toxicity to *Anopheles* larvae followed, in order of increasing larval susceptibility, by *Aedes* and *Culex*. *Simulium* sp. are also susceptible to *Cbm* whole cultures, but in a much less extent than the other Culicidae species. *Clostridium* is a very diverse group of bacteria that includes some human pathogenic species. For this reason, special considerations were undertaken to determine the safety of the *Cbm* strain as a potential bioinsecticide. All tests conducted with non-target invertebrates and vertebrates confirmed the innocuity of this isolate (Thiéry et al. 1992).

Little is known concerning the insecticidal components of either *C. bifermentans* strain and, at present, only the *Cbm* strain has been extensively studied, with some controversial results. First, and in contrast to *Bt*, *B. sphaericus* or *B. laterosporus* strains, the *Cbm* isolate does not produce parasporal bodies or any other morphological phenotype that could be associated with toxicity. Amorphous structures are observed close to the spores and it was suggested that they could be responsible for toxicity (de Barjac et al. 1990). Toxicity is greatly reduced or inactivated by physical or chemical treatments. In addition, treatment of crude sporulated cell extracts with proteinases or with antibodies raised against a crude extract of *Cbm*

cells leads to a total inactivation of the mosquitocidal activity (Nicolas et al. 1990), indicating a proteinaceous origin of the toxic factor(s). In this way, and by comparison of the crude extracts of one non-mosquitocidal *C. bifermentans* strain and the *Cbm* strain, four major proteins were identified. Those putative toxins include a doublet of 66-68kDa and two other small proteins of 18 and 16kDa (Nicolas et al. 1993). The genes encoding those four proteins have already been cloned and sequenced (Barloy et al. 1996). PCR and western blotting analysis, showed that those four genes are also present and functional in the *Cbp* strain but have not yet been cloned. The doublet of 66-68kDa belonged to the Cry family, but cloned proteins expressed in *Bt* were not toxic for mosquito larvae.

MODE OF ACTION OF MOSQUITOCIDAL BACTERIAL TOXINS

Among all these various toxins, very little is known about the mode of action. For *Bti*, the only thing which has been demonstrated is the binding of the toxins on the midgut brush border membrane, after ingestion and protoxin activation within the mosquito larval midgut. Charles and de Barjac (1981, 1983) showed that the histopathological changes on the gut epithelia of *Ae. aegypti* induced by crystal protein of *Bti* were similar to those found in Lepidoptera. This was an indication that the mode of action of the δ-endotoxins could be uniform irrespective the *Bt* subspecies and the order of the target insect. Nevertheless, even if some receptors have been identified in caterpillars for lepidopteran-active Cry toxins, nothing has been found for mosquito-active Cry or Cyt toxins.

Despite *Bti* having been the first mosquitocidal organism studied, some steps of the mode of action of *Bsp* Bin toxin has been investigated with a better success. Evidence that a specific receptor was involved in binding of the toxin to midgut apical cell membranes was provided by Nielsen-LeRoux and Charles (1992), in experiments with radiolabelled Bin toxin and isolated brush border membrane fractions (BBMF) of *C. pipiens*. Similar experiments with *Ae. aegypti*, a naturally resistant species, gave no specific binding, whereas a specific receptor was also shown to be present in *A. gambiae* and *A. stephensi* species (Silva-Filha et al. 1997). *In vitro* binding assays seem to confirm that binding affinity is correlated with the *in vivo* susceptibility of larvae: the affinity of binding to BBMF is higher for *C. pipiens* larvae than for *A. gambiae*, and the affinity of BBMF binding is higher for *A. gambiae* than for *A. stephensi* larvae (Silva-Filha et al. 1997). In *C. pipiens* larvae, it was shown that BinB was mainly responsible for the binding to the receptor, while BinA had a very low affinity for the receptor (Charles et al. 1997). Recently, the receptor was identified as a 60kDa protein attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Silva-Filha et al. 1999). Microsequencing indicated that this molecule had a strong homology with insect maltases, and enzymatic activity suggested that it could be a α-glucosidase (Silva-Filha et al. 1999). After cloning and sequencing, this was recently confirmed (I Darboux, C Nielsen-Le Roux, J-F Charles, D Pauron, in preparation).

After binding on the receptor, cytopathological events such as appearance of large vacuoles (and/or cytolysosomes) in *C. pipiens* midgut cells and mitochondria swelling, may suggest that the toxin might exert its effects at the cell membrane itself. The electrophysiological effects of the native Bin toxin and its individual components, BinA and BinB, have been investigated in cultured *C. pipiens* cells using the patch clamp technique (Cokmus et al. 1997). The authors reported a reduction in whole-cell membrane resistance, suggesting that the toxin create pores or channels in the cell membrane. *In vitro* assays using electrophysiological measurements in planar lipid bilayers and permeabilisation measurements in liposomes have confirmed that the Bin toxin forms channel in artificial phospholipid membranes, mainly due to the BinA component, although BinB may also, to a lesser extent, cause channel formation (J-L Schwartz, J-F Charles, C Berry, G Menestrina, in preparation).

RESISTANCE PHENOMENA

It was thought that development of insect resistance against *Bsp* and *Bti* would not appear, due to possible multi-site interactions between the pathogens and their targets. Indeed, no records of field resistance have been found to *Bti*, because of the presence of the four different toxins with putative different modes of action. For *Bsp*, the Bin toxin has to be considered as an one site-acting molecule, because of the single receptor interaction with BinB component (at least in *C. pipiens*). For *Bsp* cases of resistance have been recorded during the last four years, in Brazil (10 fold-resistance; Silva-Filha et al. 1995), in India (150 fold; Rao et al. 1995) and in France on *C. pipiens* (10,000 fold; Sinègre et al. 1994). More recently, two other reports from China (25,000 fold) and Tunisia (ca. 2,000 fold; Z Yuan, G Sinègre, pers. commun.), confirmed that resistance to *Bsp* may develop in the field when this bacteria is used intensively. Before records of field resistance to *Bsp*, active laboratory selections for resistance had been done in two different laboratories in California (>100,000 fold, Georghiou et al. 1992; about 37 fold, Rodcharoen & Mulla 1994).

In order to understand, and eventually prevent resistance to *Bsp*, studies on mode of action and the related mechanisms of resistance have been done on some of the populations.

Mechanisms of resistance to *Bsp*

In vitro binding investigations between the toxin and midgut BBMF (brush-border membrane fractions) from three resistant *Culex* populations gave some knowledge about the mechanisms of resistance. For the high-level resistant lab-selected colony, no binding was found, meaning that the receptor was not functional (Nielsen-LeRoux et al. 1995). For the high-level resistant population from France and the low-level resistant population from Brazil (both field-selected), no changes were found in binding kinetics (Silva-Filha et al. 1997). Furthermore, the gut juice proteases from this colony were able to proteolyse the protoxins to the activated forms. Then, if the *Bsp* crystal toxin has selected highly resistant individuals possessing a mutation influencing the initial toxin-binding in one case, in the other case the same toxin selected highly resistant individuals expressing their resistance at another level of the intoxication process. However, it is not excluded that the receptor molecule could also be involved in the resistance from France, but at another site than the binding site. This indicates that different genes can be involved in the resistance to *Bs*, depending on various factors like the origin of *Culex* populations, the frequency of the resistance genes and the conditions of selection.

Inheritance of resistance to *Bsp*

The genetically basis of *Bsp* resistance have been investigated on the two high-level resistant populations, from France and from California, by crossing homozygous resistant colonies with susceptible homozygous and backcross experiments between F_1 and the resistant colonies. This indicated that resistance was due to one major gene, sex linked for the colony from France but autosomal for the colony from California (Nielsen-LeRoux et al. 1995, 1997, Wirth et al. 2000). In other populations such as the low-level Brazilian one, resistance is also supposed to be recessive, because of the fast decline in resistance when *Bsp* treatments were interrupted (Z Yuan, pers. commun.).

Although resistance is recessive in all studied cases, high-level resistance may constitute a major threat to the future use of *Bsp* toxins for mosquito control. However, it seems that in some areas, even with intensively field applications (e.g. in Cameroon, Tanzania, Brazil and India), decrease in susceptibility has not occurred. In southern France, *Bsp* had been used for eight years from March to October with 1-2 treatments per month. Resistance occurred faster in closed breeding sites (interior underground cellar). This was also the case in Tunis, meaning that in such breeding sites only low migration of susceptible *Culex* individuals from non-treated areas could occur. In Recife (Brazil), the 10-fold resistant population was found in open drains and covered cesspitts in a small area where all breeding sites were treated during a two year period with a total of 37 treatments. In Cochin (India) resistance occurred in different kinds of open breeding sites after about two years (35 treatments) and in Doungguan (China) after eight years with about 36 treatments per year. Then, it seems that key elements for appearance of resistance are the selection pressure in time and in dose, and the genetic background of the populations.

Cross-resistance to *Bsp*

In the above mentioned treated areas only three different *Bsp* strains were used, 2362, 1593 and C3-41, all belonging to serotype H5a5b, which express the same crystal toxin (identical amino acid compositions; Berry et al. 1989, Z Yuan, pers. commun.). These strains are used in most commercial *Bsp* formulations.

Investigations on the level of cross-resistance among natural *Bsp* strains have been done by testing the toxicity of several highly active *Bsp* strains on some of the above mentioned *Bs*-resistant *Culex* colonies. For the laboratory-selected low-level resistant colony from California, cross-resistance was found to strain 2297 (Rodcharoen & Mulla 1996). This was also the case for the field-selected population from India (Poncet et al. 1997). However, among five other *Bsp* strains isolated from Ghana and Singapore, we have found at least two strains, which seem to confer only a very low level of cross-resistance. These strains are presently under investigation (Nielsen-LeRoux et al. unpublished data). There is no cross-resistance to *Bti* within the populations resistant to *Bs*, and there is even evidence for an increased susceptibility to *Bti* (Rao et al. 1995, Silva-Filha et al. 1995, Z Yuan, pers. commun.). This is in agreement with the finding that the crystal toxin from *Bsp* and the crystal toxins from *Bti* do not compete for the same binding sites.

CONCLUSIONS AND PERSPECTIVES

There is evidence for development of resistance to any bacterial toxin, as soon as its mode of action implies only one toxin, or toxins with identical mode of action (binding on the same receptor). *Bsp* belongs to this category. This microbial insecticide has therefore to be used in a reasonable way in integrated control program. Monitoring of the susceptibility of the treated mosquito populations before and during treatments is necessary. Other

measures to be taken are to multiply the control methods and/or the insecticides. For example, *Bti* could be an alternative in certain conditions and formulations. In addition, other *Bsp* strains or recombinant *Bsp* expressing additional toxins from other mosquitocidal bacteria have to be considered. Nevertheless, there is a risk in introducing the *Bsp* crystal toxin genes alone into natural mosquito larval food (e.g. Cyanobacteria), because this would expose the larvae to a continuous selection pressure. Besides to this, further understanding on the mode of action, on the receptor identification for other mosquito species and on the putative intracellular activity of the *Bsp* crystal toxin, may give good tools to identify other mechanisms of resistance, in order to predict and reduce resistance.

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