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Using the bio-insecticide *Bacillus thuringiensis israelensis* in mosquito control

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1. Introduction

Mosquito control is a major public health concern, as mosquitoes transmit many severe human diseases such as malaria, filariasis, dengue, yellow fever, West Nile virus and the chikungunya virus. These diseases represent a major health threat and economic burden in disease-endemic countries, and are currently in expansion due to increased worldwide exchanges, urbanization, and global warming. The only effective way of reducing the incidence of these diseases is to control the vector mosquitoes, mainly by application of insecticides to their breeding places. Since the 1950s, the massive use of chemical insecticides has led to undesired toxicity on non-target organisms and the selection of insecticide resistance mechanisms in mosquito populations (Hemingway & Ranson, 2000). A safe alternative to chemical insecticides is to spray toxins produced by the bacteria *Bacillus thuringiensis* subsp. *israelensis* (Bti) over mosquito breeding sites (Lacey, 2007). Bti represents today the best alternative to chemical insecticides in controlling mosquitoes. Bti toxins are safe for non-target species and human health, are believed to show low persistence in the environment, and so far no resistance was detected in mosquito populations. Bti is the only insecticide allowed against mosquito larvae in Europe. To insure a long-term efficiency of this bio-insecticide, it is however necessary to evaluate the risks associated to its intensive worldwide use. The two main risks are (1) the accumulation of spores and toxins in the environment, and possible proliferation of Bti a long time after spraying, which may have an impact on the whole ecosystem functioning, and (2) the evolution of resistance to Bti in mosquitoes, rendering the treatment inefficient. It is therefore necessary to develop monitoring tools to follow the fate of spores and toxins in the environment and the evolution of resistance in target mosquito populations. Here we review recent advances in our understanding of the mechanisms of Bti toxicity and of mosquito resistance. The chapter will be organized in three parts: the first part describes Bti structure and its fate in the environment, the second part describes the action of Bti toxins after ingestion by mosquito larvae and the diversity of mechanisms involved in mosquito resistance, and the third part is dedicated to the challenging objective of managing resistance in the field. We conclude in identifying issues that need further research.

2. Bti in the environment

2.1 What is Bti?

Bacillus thuringiensis subsp. *israelensis* (Bti), serotype H14, is a subspecies of the diversified *Bacillus thuringiensis* species, an entomopathogenic bacterium able to survive in the environment as a spore and producing insecticidal toxins within an inclusion body during the process of sporulation. Bti was first isolated from a water pond in the Negev desert

(Goldberg & Margalit, 1977) and was the very first strain described for having insecticidal activity outside Lepidoptera. Bti, like other *B. thuringiensis* subspecies, is a member of the *Bacillus cereus* complex. The characteristic of *B. thuringiensis* is the presence of an inclusion body or crystal (figure 1). The different subspecies are characterized by different flagellar H antigens (serotypes). However, the specificity to a given group of insects is a consequence of the particular set of proteins a strain is producing and there is thus no strict correlation between serotypes, the toxins they produce during sporulation as insecticidal crystal inclusions and the host range. Nevertheless, the specificity of the *B. thuringiensis* insecticidal proteins is central in the wide use of Bt as an alternative to chemical insecticides for the control of insect pests in forestry, agriculture, and public health. The serotype H14, Bti, produces 4 main toxins (Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa) specific to dipterans (mosquitoes, blackflies and chironomous midges). However, the 128-kb conjugative plasmid pBtoxis bearing the toxin genes carries three more genes for insecticidal proteins: Cry10Aa, Cyt2Ba and Cyt1Ca (Berry et al., 2002).

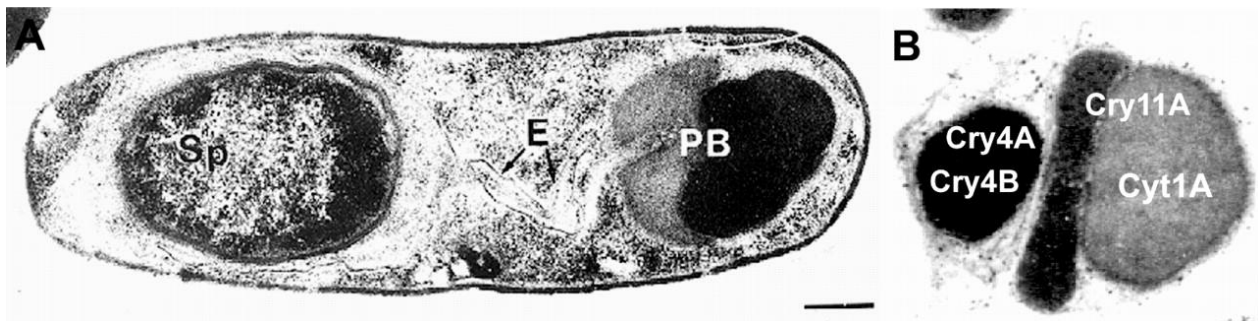


Figure 1. Ultrastructural section of a sporulated *Bacillus thuringiensis* subsp. *israelensis* cell (A) and of a purified inclusion body (B). Sp: spore; E: exosporium; PB: parasporal body. From Federici et al., 2003.

2.2 Structure and mode of action of Bti toxins.

Like other Bt toxins the mode of action of the Bti toxins is closely related to specific structure-function relationships. One particular feature of Bti is that its insecticidal activity relies on the combination of three distinct groups of toxins with respect to structure-function and thus specific mode of action, i.e. Cry4Aa+Cry4Ba, Cry11A and Cyt1Aa. This also shows at the level of the inclusion body which is a composite entity comprising three different crystal component, one for each the three groups mentioned above. Indeed, each group folds and accumulates separately into a specific sub-inclusion body of different shape assembled into a spherical parasporal body and held together by a lamellar envelope (Ibarra & Federici, 1986, Federici et al., 2003). The organization of the genes on pBtoxis reflects these differing structures with a separate monocistronic organization for each toxin gene. However, coevolution and selection for synergism can also be seen in this organization. Indeed, the Cyt1Aa1 is cytotoxic also to bacteria and to *B. thuringiensis* and must be properly folded into an inactivated intermediate state until activation by insect midgut proteases. This proper folding is mediated by a chaperone protein, P20, which is located in the Cry11Aa operon, along with the P19 protein. Interestingly, Cry11Aa does not require the P20 chaperone for folding whereas Cyt1Aa1 which requires it is located in a separate cistron in opposite orientation from the cry11Aa operon. This strongly suggests a coevolution of all these genes for synergism in

mosquitocidal activity which is also underlined by the divergence of the four major Bti toxins in toxicity and host range: Cry4Ba is active primarily against *Anopheles* and *Aedes*, and shows no toxicity to *Culex* species, in contrast to Cry4Aa toxin that is toxic to *Culex* larvae. Cry11 is the most toxic to *Aedes*, and Cyt1Aa shows low (*Aedes*, *Culex*) to non-toxicity at all (*Anopheles*). Cyt1Aa have a strong synergistic effect on the toxicity of Cry toxins in all mosquitoes

The 3-D structure of several Bti crystal proteins has been determined using x-ray chromatography. The crystal structure of Cry4Aa (Boonserm et al., 2006) and Cry4Ba (Boonserm et al., 2005) were analyzed with 2.8 and 1.75 Angström resolution, respectively. The overall structure of Cry4Aa and Cry4Ba resembles that of Cry1Aa (Grochulski et al., 1995) and Cry3Aa (Li et al., 1991; Galitsky et al., 1997) with a three-domain organization (figure 2). Domain I is composed of several α helices (the number varies between Cry4Aa and Cry4Ba) and is involved in the formation of a pore in the midgut epithelial cell membrane following insertion of the $\alpha 4$ and $\alpha 5$ transmembrane hairpin (Boonserm et al., 2005, 2006, Grochulski et al., 1995, Li et al., 1991; Galitsky et al., 1997). Domains II and III are composed of beta sheets and are involved in the specific recognition of the midgut receptors and in stability of the toxin (Boonserm et al., 2005, 2006, Grochulski et al., 1995, Li et al., 1991; Galitsky et al., 1997). Although this structure is very similar to that of Cry1 and Cry3 toxins, several differences are present between Cry4 and other toxins but also between Cry4Aa and Cry4Ab. A first set of differences appear with structural domain I.

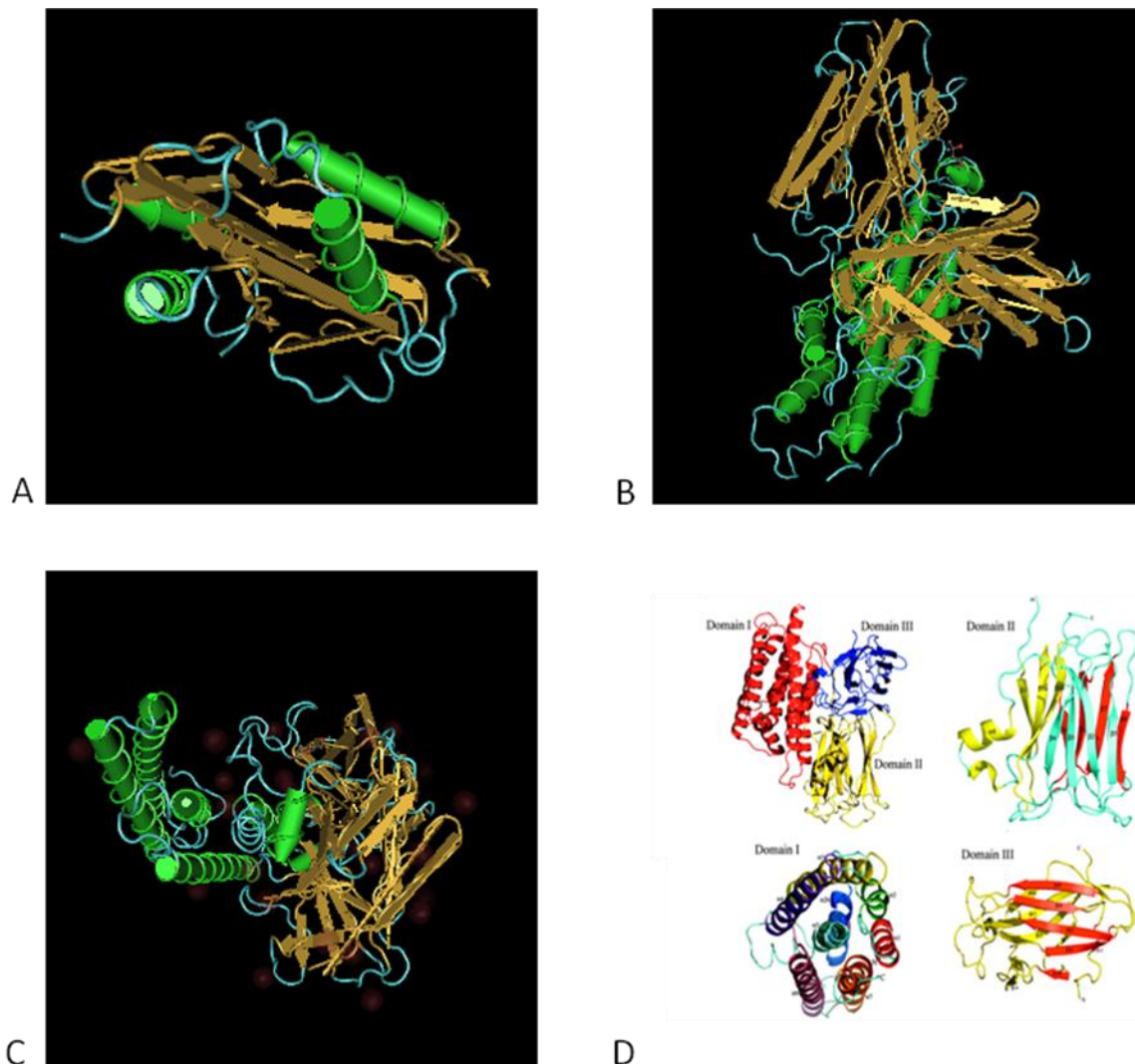


Figure 2. 3-dimensional structure of *Bacillus thuringiensis* subsp. *israelensis* toxins. A: Cyt2Ba (from Cohen et al 2008). B: Cry4Aa (from Boonserm et al., 2006), C: Cry4Ba (from Boonserm et al., 2005), D: Ribbon view of the complete Cry4Aa toxin and of the separate structural domains (from Boonserm et al., 2006)

Domain I of Cry4Aa comprises seven α -helices, like Cry1Aa or Cry3Aa (Grochulski et al., 1995, Li et al., 1991; Galitsky et al., 1997). However, the structure of the $\alpha 4$ - $\alpha 5$ loop is unique with the presence of a specific disulfide bond and a proline-rich motif rigidifying the hairpin and limiting the flexibility of the inter-helix loop (Boonserm et al., 2006). A consequence is that, unlike the very closely related Cry4Ba, Cry4Aa was unable to allow the release of calcein (Boonserm et al., 2006) which suggests a difference of channel behaviour, and thus of mode of action, between Cry4Aa and Cry4Ba. If Cry4Ba displays a $\alpha 4$ - $\alpha 5$ loop similar to that of Cry1 and Cry3, its domain I also exhibits a specific structure. Indeed, unlike the structurally related Cry toxins, and unlike Cry4Aa, domain I of Cry4Ba does not display seven α -helices but only five (Boonserm et al., 2005). Alpha helices 1 and 2 are absent, although the DNA sequence encoding this region is present in the toxin gene, which indicates that they are removed by proteolysis, probably in the process of crystallization. Nevertheless, Cry4Ba retains full mosquitocidal activity. A key difference is in structural domain II and more precisely in the loops connecting the β -sheets (Boonserm et al., 2005, 2006). Domain II is the most variable domain among all Cry toxins and these loops are involved in insect receptor specificity. Cry4Aa and Cry4Ba differ significantly in the size, especially loop2, and sequence of the domain II loops and exchanging loop 3 significantly increased toxicity of Cry4Ba to *Culex* while its activity against *Aedes* and *Anopheles* remained (Abdullah et al., 2003). This indicates that specificity for receptor binding in *Culex* is located mainly in loop 3 whereas specificity to *Aedes* and *Anopheles* is located in loops 1 and 3 (Abdullah et al., 2003). These data also further indicate that the closely related Cry4Aa and Cry4Ba toxins display different specificity and structures and that they act synergistically within the Bti inclusion body to increase the insecticidal host range. Cyt2Ba, is the other Bti toxin for which the 3-D structure was determined (Cohen et al., 2008). Unfortunately there is no 3-D structure available for Cyt1Aa, the major cytotoxin from Bti. Another 3-D structure of a Cyt2 toxin is available but not from Bti. It is the Cyt2Aa cytotoxin from *B. thuringiensis* subsp. *kyushuensis* (Li et al., 1996). Nevertheless, these structure analyses provide similar data and conclusion and one can assume that they might also apply to Cyt1Aa owing to the similarity of biochemical traits between Cyt1 and Cyt2 toxins. Unlike Cry4 toxins, the monomeric Cyt2 is organized as a β -sheet made of 6 antiparallel β -strands flanked on each side by two short α -helices (Li et al., 1996; Cohen et al., 2008). Still unlike Cry4 toxins, the transmembrane domain is made by the β -strands and not the α -helices which are involved in interprotein binding and oligomerisation (Du et al. 1999; Li et al., 1996; Gazit et al., 1997). These data on the structure of Cry and Cyt toxins from Bti clearly indicate major structural and functional differences and must be considered when addressing their respective mode of action and resistance to Bti toxins.

All Bti insecticidal proteins are produced as protoxins and all must be activated *in vivo* by insect midgut proteases prior insecticidal activity. Following this initial activation, the subsequent receptor binding step is still not fully resolved (figure 3).

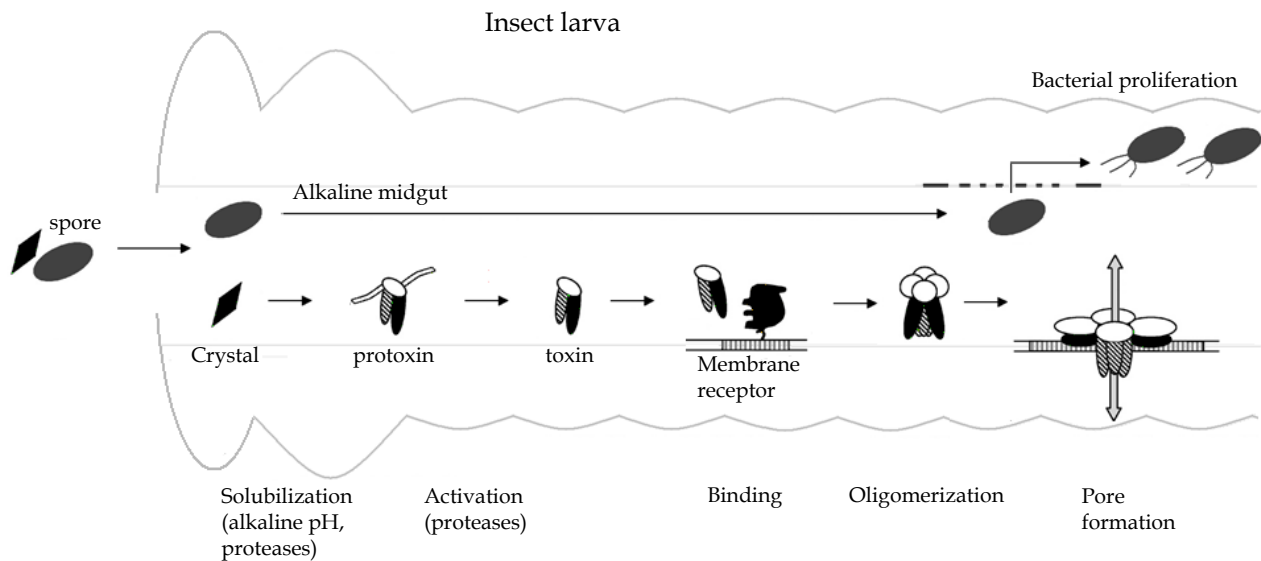


Figure 3: Model of the mode of action of Bti Cry toxin in insect midgut. The crystal is ingested by larva, protoxins are solubilized in the alkaline midgut and activated to toxins that bind to specific membrane receptors, oligomerize and form a pore allowing the bacteria to proliferate in the host larva. Another model was recently proposed that does not involve oligomerization and pore formation. After the death of the larva, bacteria are liberated in the environment and they sporulate still they encounter another host. The two main resistance mechanisms are modifications in the activity of midgut proteases and in the receptor sites. Adapted from Bravo et al., 2007.

The Cry proteins were shown to bind to midgut receptors but the nature of the receptors is not completely established. There is no report on the receptors recognized by Cry4Aa. However, with respect to Cry4Ba, cadherin AgCad1 of *Anopheles gambiae* (Hua et al., 2008; Park et al., 2009) and alpha-amylase from *Anopheles albimanus* (Fernandez-Luna et al., 2010) were shown to act as receptors. Furthermore, a series of other proteins, i.e. aminopeptidase, several alkaline phosphatase isoforms, flotillin, prohibitin, V-ATPase B subunit and actin were shown to bind Cry4Ba in *Aedes aegypti* (Bayyareddy et al., 2009). Data are also available on the membrane receptors of Cry11Aa. Cadherin (Chen et al., 2009a), aminopeptidase N (Chen et al., 2009b), alkaline phosphatase (Fernandez et al., 2009) and alpha-amylase (Fernandez-Luna et al., 2010) have all been described as Cry11Aa receptors in *Ae. aegypti*. Following binding, these proteins insert in the membrane to form a pore and more precisely an ionic channel triggering osmotic imbalance, cell death and ultimately insect death. However, important and not yet fully resolved steps are involved in this membrane insertion and permeation process. A first intermediate step seems to be oligomerization into a prepore structure with the probable involvement of membrane receptors. However, activated toxins are hydrosoluble intermediate forms which must undergo a conformational change to expose hydrophobic domains and insert stably into the membrane in order to form a transmembrane ionic channel. The two Cry4 toxins undergo this process in a way similar to that of the Cry1 or Cry3 proteins. The tight structure of the toxin is lost and the structural domain I moves freely from domains II and III allowing the bundle of α helices to reorganize at the contact of the lipid membrane. The highly hydrophobic helix $\alpha 5$ inserts into the membrane dragging along $\alpha 4$ which, with its free charged residues, will conduct ions through the membrane. This mode of action similar to that of Cry1 toxins requires more than one toxin to form a pore (figure 3). Atomic force microscopy analyses showed that four Cry4Ba toxins are required to form a pore (Puntheeranurak et al., 2005) exactly like Cry1Aa (Vie et al., 2001; Laflamme et al., 2008). Although Cry11Aa was shown to form pores in the membrane like Cry4 toxins, the exact mode of membrane insertion and permeation is still not fully described. Data available suggest that Cry11Aa could act in different ways. Proteolysis of the 72-kDa Cry11Aa was reported to result into

two inactive subunits of 32 kDa and 36 kDa. However, when these two subunits were mixed in equimolar amounts, full insecticidal activity was restored (Yamagiwa et al., 2002, 2004; Revina et al., 2004). This suggests that Cry11Aa could display at least under some conditions a binary-like action. However, there is no indication of whether this process is the normal mode of action of Cry11Aa, a particular mechanism or an intermediate step. Nevertheless, its mode of action must be rather complex owing to its interaction with Cyt1A. Cry11Aa was indeed reported to bind to Cyt1A which can facilitate pre-pore oligomerization (Perez et al., 2007) and act as an additional membrane-bond receptor, increasing thus the ability of Cry11Aa to insert in the membrane (Perez et al., 2005). Unlike the other Bti toxins, Cyt1A does not recognize a specific membrane receptor. It has the ability to insert by itself in the lipid bilayer (Butko et al., 1996; Gazit et al., 1997; Du et al., 1999) and form cationic channels pores through a process of detergent-like colloid-osmosis (Knowles et al., 1989, Manceva et al., 2005). Recently, domain homology with the *Erwinia* virulence factor (evf) was found which could explain this ability to integrate into lipid bilayers (Rigden, 2009). In addition to its own mosquitocidal and cytotoxic activity, Cyt1A was shown to act synergistically with the other Bti toxins (Wu & Chang, 1985; Ibarra and Federici, 1986; Federici et al., 2003; Crickmore et al., 1995; Perez et al., 2007, Soberon et al., 2010; Canton et al., 2011) but also with *Bacillus sphaericus* toxins (Wirth et al., 2000a). This synergistic effect is at the heart of the mode of action of Cyt1A and is explained by involvement of the N-terminal part of Cyt1Aa in protein-protein interaction with Cry toxins while the C-terminus of Cyt1A is involved in hydrophobic interaction and membrane insertion (Rodriguez-Almazan et al., 2011). Cyt1A binding to Bti Cry toxins provide a means for shunting the natural membrane receptors of these Cry proteins increasing the level of pore formation and membrane disruption, leading thus to synergism, but even more importantly leads immediately to the key aspect in insect control with Bt toxins: preventing and overcoming insect resistance.

2.3 Type and use of Bti formulations

The discovery of the pathogen activity of Bti against Dipteran vectors (mosquitoes and black flies) was rapidly followed by applications. From 1980s', thanks to combined efforts of World Health Organization (WHO), other institutions, and numerous research laboratories, several international programs were developed for the use of Bti and *Bacillus sphaericus* (Bs) (Lecadet, 1996). Primary powder formulation of Bti had virtually no residual effect against mosquito larvae beyond application, although the delta-endotoxin remained chemically stable in neutral and acid waters (Sinègre and al., 1980). Numerous trials and field observations show a quick decline in efficacy within few days in open conditions and a very low residual activity afterwards. This decline is mainly due to the quick sedimentation outside of the nutrition zone of the larvae, the inactivation by UV light of tryptophan residues essential for insecticidal activity (Pusztai et al., 1991; Padilla et al., 2006) or degradation in polluted or highly organic matter concentrated water. If several authors have reported recycling of Bti in larval cadavers in controlled or simulated conditions (Aly et al., 1985; Khawaled et al., 1990; Boisvert & Boisvert, 1999), evidence of such recycling under natural field conditions is scarce (but see Tilquin et al, 2008; de Melo-Santos et al, 2009). Numerous papers reviewed by Lacey (2007) have studied the biotic and abiotic factors influencing the larvicidal activity of Bti i.e. the specific susceptibility of the target species and their feeding strategies, the rate of ingestion, the density of larvae and their age, the dosage, temperature, solar radiation, depth of water, turbidity, tannin and organic content, vegetation coverage, etc. If the first Bti-based formulations were a technical powder more or less difficult to use due to the bad miscibility with water, various types of formulations were developed since 1981, adapted to the different mosquito species and habitats to be treated (Table 1).

Table 1: Formulations currently available on the European and USA market, their respective potency, doses of application, and main uses. The manufacturers recommend the higher dosage rates when late 3rd and early 4th instar larvae predominate, mosquito populations are high, water is heavily polluted, and/or algae are abundant.

Type of Formulation	Product Name	Potency (UTI/mg)	Registered dose/surface unit	Main uses
Bti alone				
Technical powder (TP)	VectoBac® technical Powder Aquabac® primary powder	6000 7000		Manufacturing use product intended for formulation into end-use products
Suspension concentrate (SC)	VectoBac® 12AS AquaBac® XT Teknar®HD-P	1200 1200 1200	0.25-1-2 pts/acre 0.25-1-2 pts/acre 0.25-1-2 pts/acre	All mosquito breeding sites by ground or by air application: irrigation ditches, roadside ditches, flood water, standing ponds, woodland pools, snow melt pools, pastures, catch basins, storm water retention areas, tidal water, salt marshes and rice fields. Standing water containing mosquito larvae, in fields growing crops (alfalfa, almonds, asparagus, corn, cotton, dates, grapes, peaches and walnuts) Polluted water (such as sewage lagoons, animal waste lagoons).
Water dispersible granule (WG)	VectoBac® WDG Aquabac® DF 3000	3000 3000	1.75-7-14 oz/acre 1.75-7-14 oz/acre	Liquid formulations are also recommended for control of black fly in streams and nuisance flies (<i>Psychoda</i> spp., <i>Chironomus</i> spp.) in sewage treatment facilities utilizing trickling filter systems.
Granule (GR)	VectoBac® G Aquabac® 200G Aquabac® 400G Mosquito Bits®	200 200 400 200	2.5-10-20 lb/acre 2.5-10-20 lb/acre 1.5-5-8 lb/acre 0.5 lb/2178 ft²	
Tablet (TB)	VectoBac® DT	3400	1 tab./50 l	Artificial containers (terracotta, concrete, iron, plastic), flower pots, catch basins, and a variety of small breeding sites.
Briquette (BR)	Mosquito Dunk® Summit Bti Briquets™	7000 7000	1 br/25-100 ft². 1 br/25-100 ft².	Outdoor applications near the household where water collects and remains for periods of time, , ditches, tree holes, roof gutters for collecting rainwater, flower pots, animal watering troughs, etc. or indoor use such as elevator shafts, basements that flood, sump pumps and any drainage areas within buildings
Bti + <i>Bacillus sphaericus</i>				
Granule (GR)	VectoMax® CG VectoMax® G VectoMax® WSP	nc & 50 nc & 50 nc & 50	5-20 lbs/acre (0.5-2 lbs/1000 ft² in used tires) 1 pouch/50 ft²	Sewage effluent, sewage lagoons, oxidation ponds, septic ditches, animal waste lagoons, impounded wastewater associated with fruit and vegetable processing, drainage ditches, roadside ditches, retention, detention, and seepage ponds, tidal water, salt marshes, mangroves, estuaries, natural and man made aquatic sites , waste tires stockpiled in dumps, landfills, recycling plants, and other similar sites. Briquets are recommended in quite small man-made and natural water holding receptacles.
Briquette (BR)	FourStar™ Briquets 45 FourStar™ Briquets 90 FourStar™ Briquets 180	70 & 60 70 & 60 70 & 60	1 br/≤100 ft² 1 br/≤100 ft² 1 br/≤100 ft²	

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The different formulations are aimed to favour a better contact with larvae by taking into account their bioecology and the specificity of their habitats. The type of formulation influences highly the efficacy and the persistence of Bti depending on the toxin content, how effectively the material reaches the target, and settling rate, storage conditions, means of application and frequency of treatments, and production factors, especially the medium in which the bacterium is grown. When Bti is applied as a liquid (after mixing a suspension concentrate or a water dispersible granule in water) or a ready-for-use granule formulations directly in open field, its biological efficacy declines generally quickly after 24 to 48 hours. Such formulations are used where short action is required, by instance for the control of univoltine species like *Ochlerotatus caspius* and *Oc. detritus* in Mediterranean or Atlantic temporary flooded saltmarshes or *Oc. rusticus*, *Aedes vexans*, or *Oc. sticticus* in fresh water breeding sites as in Rhone-alpine or upper Rhine valleys. According of the size of the breeding site, the products are applied by ground using hand, or shoulder-carried manually-operated compression sprayers or knapsack sprayer with gooseneck lance, portable or vehicle-mounted power-operated mistblowers. Airborne applications (helicopters, fixed-wing aircrafts) may be justifiable in emergency situations but also in routine mosquito control where large or inaccessible areas must be treated quickly (World Health Organisation, 1996), by instance in many US counties, along the French Western Mediterranean coast and in Thessalonica plain, in the upper Rhine valley, the Po and Elbra deltas, etc.). On the contrary, ready-for-use tablets, pellets, briquets or specific slow release granules are designed to persist for several weeks. They are above all used in urban or periurban areas for the control of plurivoltine species like *Culex* or *Aedes* spp. (*Stegomyia*) in small natural or anthropic peridomestic breeding sites like containers, flower pots, catch basins, etc. Bti-based products are now used all over de world. They represent now the main larvicides used in natural areas in Europe. Urban and periurban mosquito vector control strategies under subtropical and tropical conditions depend of the target species and are based on the use of a panel of insecticides for controlling adults and/or larvae. Where they are recommended, larvicides used are generally long lasting chemicals like organophosphates whereas the use of bioinsecticides and insect growth regulators is still limited (WHO, 2009). However, Bti is used in alternance where resistance to chemical insecticides appears, or to replace them when they are prohibited and/or removed from the market as in the French overseas territories. The long-lasting or slow release Bti-based products are now able to compete with them. Different extemporaneous formulations were also tested and used. Before Bti granule formulation was available on the market, some mosquito control operators like EID Rhône-Alpes (France) applied by helicopter sand granule mixed beforehand with Bti-based wetttable powder and vegetal oil. Such method was used to improve the penetration of the product into the canopy for the control of wooded breeding sites. The frozen granule formulation "IcyPearls" produced by Becker (2003) and used against *Ae. vexans* larvae in the upper Rhine River Valley of Germany has certain advantages over Bti sand granules. The ice pellets melt on the water surface where the microbial toxins are slowly released. Applied by helicopter, the ice formulation results in increased swath widths and the cost of application is consequently reduced.

2.4 Effect of environmental variables on Bti persistence and proliferation

Numerous studies have assessed the persistence of Bti toxicity after treatment. Several environmental factors such as solar radiation, temperature, type of substrate, presence of vegetation, salinity, pollution, water height, were shown to influence the persistence of Bti toxicity in the environment (reviewed in Lacey, 2007). Depending on all these factors, Bti

toxicity was shown to decrease with highly variable patterns in the field, from a few days to several weeks. In contrast, very few studies looked at the fate of Bti spores in the environment after spraying (Hajaij et al., 2005; Tilquin et al., 2008), and no studies exist so far on the fate of Bti toxins in the environment. Reduced toxicity over time does not mean that Bti spores and toxins are quickly eliminated in the environment: they may accumulate, or even proliferate in soil, or in decaying vegetation at the bottom of mosquito breeding sites (Tilquin et al., 2008; de Melo-Santos, 2009). If many studies have investigated the fate of Cry toxins produced by genetically modified plants (GMPs) and released into agricultural soils (e.g., Clark et al., 2005), such studies are missing for Bti toxins. It is however important to be able to follow the fate of each toxin in the environment after spraying, because the acute toxicity of Bti is due to the synergistic action of toxins. If some toxins are more rapidly eliminated than others in the environment, mosquito populations might be in contact long after spraying with only one or two of the most persistent toxins, thereby favouring the evolution of resistant mechanisms to these toxins, a first step toward the evolution of resistance to Bti. There is therefore an urgent need for developing an easy-to-use immunological test for detecting each of the main Bti toxins in environmental samples. Indeed, if commercial ELISA kits are available for detecting each of the Bt toxins introduced in GMPs, such immunological tests are not available so far for Bti toxins. Until now, few studies have evaluated the persistence of Bti spores in the environment following spraying (Hajaij et al., 2005). In this study, to follow the fate of Bti spores after spraying, environmental samples were collected several times after treatment, plated on a nutrient-rich medium and Bt-like colonies (i.e., sporulate cells containing crystal inclusions visualized using a phase-contrast microscope) were counted. Besides being a fastidious method, it does not allow discriminating between Bti and other Bt strains potentially naturally present in the environment. Recently, a method involving whole DNA extraction from environmental samples and amplification of the Cry4 (A and B) genes present only in Bti by real-time quantitative PCR (RT-qPCR) was proposed for an accurate and precise quantification of the Bti present in treated sites (Guidi et al., 2010); this method does not require bacterial cultivation and allows the quantitative detection of Bti spores directly from environmental samples. This molecular tool allows monitoring the fate of Bti spores and possible recycling in the environment after a treatment.

2.5 Ecological risk

2.5.1 Effect of Bti on non-target species

Bti products present low risk for the human health through direct or indirect exposure. Laboratory studies have demonstrated that Bt and Bt products are non infectious and are toxic to mammals only at a dose higher or equal to 10^8 colony forming unit per mouse (Siegel, 2001). The pH and presence of receptors in the midgut determine the specificity of the larvicide action of Bti. Since the discovery of its insecticidal potential in 1976, the innocuity of Bti for micro- and macro-invertebrates, fishes, batracians, and other vertebrates sharing the same habitats as mosquito larvae is well established at dosage rates used at the operational scale (Boisvert & Lacoursière, 2004; Lacey & Merritt, 2004). All the studies carried out in laboratory and in field conditions show that Bti has a main target effect on the Diptera Nematocera i.e. Culicidae, Simuliidae, and Chironomidae (Ali, 1981, Garcia et al., 1981, Merritt et al., 1989, Boisvert & Boisvert, 2000, He & Ong, 2000). Amongst the 77 scientific papers reviewed by Boisvert and Lacoursière (2004), negative effects (mortality, population reduction) were observed on 15% of the 616 identified taxa of non target aquatic organisms after Bti treatment against mosquitoes or black flies. Amongst these 98 taxa, 62% were exposed to concentrations of Bti 5 to 1000 fold the recommended dosage rate and 45% were chironomids. The other impacted taxa belong to Diptera, Trichoptera, Plecoptera, Ephemeroptera, Lepidoptera, and Hemiptera as well as some worms, Crustacea, Gasteropoda, Fish or Algae, all after exposure to extreme overdosage.

2.5.2 Effect of Bti on ecosystems

The activity persistence of Bti and the induced side-effects on non target organisms depend on the type and the characteristics of the formulation, the frequency of application as well as the environmental factors such as the temperature, the water depth or the vegetation. A 5-years study including three years of intensive Bti treatments (six applications on three months between 1991 and 1993) showed a reduction of the taxonomic richness and the total number of invertebrates (Hershey et al., 1998; Niemi et al., 1999). These changes should have interfered with the trophic network involving the invertebrates but did not affect the populations of zooplankton and nesting birds. In a 4-years study conducted between 1998 and 2002 by Lagadic et al. (2002) in Morbihan, France, the health, the number, and the abundance of non target aquatic invertebrates present in mosquito breeding sites treated annually with Bti (VectoBac®12AS) were monitored. Bti was sprayed by ground using knapsack sprayers. No significant effect was observed on both sentinel species *Nereis diversicolor* and *Chironomus salinarius*. Even analysis at the community level showed that the environmental fluctuations had a more important impact on the community structure and that no Bti effect was detectable. A comparative study with VectoBac® 12AS and VectoBac®WG carried out in the same habitats between 2006 and 2007 did not demonstrate any effect on aquatic invertebrates namely on groups representing a trophic interest for the birds. These results allow considering that Bti treatments at the recommended registered dose did not present unacceptable risks for non target aquatic invertebrates and the trophic chains. In the frame of a *in situ* study (European project n°LIFE99 ENV/F/000489) carried out from 2000 till 2003 in Grande Camargue, southern France, on the impact of five treatment campaigns with VectoBac®12AS, comparatively with temephos, an organophosphate larvicide, on temporary flooded saltmarshes favourable to *Ochlerotatus caspius* and *Oc. detritus*, no significant effect of Bti on Chironomidae and other invertebrate taxa were observed during the three successive stocking stages characterising these habitats: a first colonization stage by *Ochlerotatus* spp., then Baetidae and Chironomidae is followed by a maturity stage with a dominance of Chironomidae and Baetidae and a higher taxonomic richness, and finally by a senescence stage where sedentary (Crustacean), allochthonous, and predatory taxa are predominant. In another study carried out also in Camargue, Franquet and Fayolle (2002) did not highlight effect of Bti on flora and non target fauna in similar temporary flooded breeding sites of *Oc. caspius*. No proliferation of phytoplanktonic algae was observed after the disappearance of the mosquito larvae and some other taxa playing the role of filter-feeders. In cases of overdosage at 8 L/ha VectoBac®12AS (1200 UTI/mg), adverse effects on population dynamic of chironomids were observed, immediately after the treatment, or 2 and 8 days after, although the persistence of Bti is low. The dose of 3 L/ha seems to be the maximum acceptable dosage for the fauna in such temporary breeding sites.

A recent study focused on the development and use of methods for assessing the environmental risk of Bti and another bio-insecticide, spinosad, in the context of mosquito control in coastal wetlands of two French ecoregions: Morbihan (south Brittany) and Grande Camargue (southern France) (Duchet et al., 2008, 2010). *Daphnia pulex* and *Daphnia magna* were used as model species in laboratory bioassays and as sentinel species in field studies performed in *in situ* microcosms (enclosures). The scientific approach aimed at determining whether the toxic effects (lethal and non lethal) that affect individuals can be detected at the population level. Modelling with RAMAS GIS was used to simulate effects of the 2 larvicides on population dynamics, and extinction risk of the exposed populations was estimated. Bti showed no significant impact on both *D. pulex* and *D. magna* population dynamics, a result confirmed by experiments conducted in microcosms that showed no impact of Bti on *D. pulex* population even when exposed to the highest concentration of 0.50 $\mu\text{L.L}^{-1}$ (Duchet et al., 2008). Combination of laboratory bioassays and field microcosm studies provides a sound and

reproducible methodological framework that could be used to define a strategy for the risk assessment of bio-larvicides used for mosquito control in coastal wetlands.

Ecological and structural impact of insecticides on the trophic networks and aquatic and terrestrial communities could result from the massive reduction of one or more organism groups. Some studies have been published on these effects associated to the temporary disappearance of mosquito larvae after Bti treatments. Mosquito larvae are only a part of the diet of aquatic and terrestrial predators. Mammals (e.g. mole, shrew), birds (ducks, seagulls, starling, minah bird, nightjars, swallows), batrachians (frogs, salamanders), fishes (salmons, trouts, stickle back, miller's stumb, perches, brill, bass, zebrafish, chub), spiders, insects (odonates, plecoptera, megalopetra, trichoptera, coleoptera, hymenoptera, diptera) and microinvertebrates (platyhelminthes, leech, crustaceans) can eat larvae and adults of mosquitoes and black flies (Peckarsky, 1984; Crosskey, 1990; Davies, 1991). But even in the case of high abundances, mosquito represents less than 5% of the diet of birds (Bourassa, 2000) probably because their respective activity periods do not overlap. However, using the common house martin *Delichon urbica* species as a model, Poulain et al. (2010) assessed the effect of Bti spraying on foraging rates and chick diet prior to and during three years of Bti spraying in the Camargue, France. Intake of Nematocera (including midges and mosquitoes) and their predators (spiders and dragonflies) decreased significantly at treated sites concurrently with increase of flying ant intake. Small preys were significantly more taken at treated relative to control sites where the foraging rates were also higher. As a result, clutch size and fledgling survival were significantly lower at treated sites relative to control. Bats feed at sunset during the activity period of mosquitoes. However, the bolus of *Myotis daubentoni* contains only 0 to 8.25% Culicidae and the bolus of *Pipistrellus nathusii* 4.86-9.91% Culicidae and Chaoboridae (Arnold et al., 2002). Several studies have shown that invertebrate predators and detritivores eat mosquito larvae killed by Bti without negative impact on their growth and emergence (Aly & Mulla, 1987; Wipfli & Merritt, 1994a, 1994b)

3. Evolution of Bti resistance in mosquitoes

Resistance to Bti toxins is nothing else than the interruption at one place or at several places of the cascade of events known as "mode of action". This interruption of the mode of action, i.e. mechanisms of resistance, can occur at different levels including toxin lack of activation, toxin proteolysis, precipitation of the toxin, modification of the receptor but also through a lack of pore formation after specific binding (Frutos et al., 1999). However, the most frequently encountered mechanisms of resistance were receptor mutation and altered binding on one hand and lack of proteolytic toxin activation on the other hand. It is also important to consider that almost all these accounts are laboratory works under forced artificial conditions where resistance can be easily selected. With respect to Bti, there is, despite a significant amount of research over the last twenty years, no resistance to the whole Bti crystal in laboratory or in the field. The reason for that is a direct consequence of the mode of action of Cyt1Aa and its interaction with the other Bti toxins. Resistance to individual Bti toxins was easily developed under laboratory conditions. High levels of resistance were established for Cry11Aa, Cry4Aa+Cry4Ba and Cry4Aa+Cry4Ba+Cry11Aa and in all cases the addition of Cyt1Aa overcame the resistance established (Georghiou & Wirth, 1997; Wirth et al., 1997, 2005a, 2010; Khasdan et al., 2001; Federici et al., 2003). Conversely, evolution of resistance could not be obtained when Cyt1Aa was present. This positive impact of Cyt1Aa on the prevention of resistance or overcoming of established resistance was also observed with *B. sphaericus* toxins (Wirth et al., 2000a, 2004, 2010). Furthermore, addition of Cyt1Aa to *B. sphaericus* resulted in a 3600-fold increase of toxicity to *Ae. aegypti* (Wirth et al., 2000b). This increase of toxicity of *B. sphaericus* toxins was linked to the Cyt-mediated insertion of *B. sphaericus* Bin toxins in the membrane (Federici et al., 2003). It is this same cooperative effect

of Cyt-mediated membrane insertion that prevents resistance to Bti toxins by shunting mutating receptors or making the common resistance mechanism of receptor mutation inefficient. Synergism, combinational mode of action and counter-resistance effect are unique evolutionary traits of Bti which makes it the ideal biocontrol agent and explain why it is still the most efficient tool for mosquito control. A major point to address today is thus how to further improve the product and even more importantly how to make it sustainable.

In natural mosquito populations, no consistent resistance has been detected even after long periods of repeated treatment with Bti toxins, but recent studies suggested that moderate Bti resistance may occur locally (Paul et al., 2005; Boyer et al., 2007; Paris et al., 2010). Furthermore, we obtained in only 22 generations of selection using environmental Bti (i.e., field collected leaf litters containing Bti toxins), a strain of *Ae. aegypti* resistant only moderately to commercial Bti (2-fold), but 4-fold to environmental Bti and up to 30-fold to individual Cry toxins (Paris et al., 2011a). Furthermore, in this leaf litter collected several months after Bti spraying, no Cyt1Aa could be detected using specific antibodies, although high levels of Cry toxins were detected, suggesting that Cyt1Aa toxins might be more prone to rapid degradation than Cry toxins in the environment. This raises concerns about a possible accumulation of Cry toxins in the environment, and the evolution of resistance to Cry toxins in mosquito populations, which is a first step towards the evolution of resistance to the full Bti mixture.

Although resistance mechanisms to Bti toxins have not been well characterized yet in dipteran insects, resistance to other Cry toxins have been intensively studied in lepidopteran insects resistant to transgenic crops producing *Bacillus thuringiensis* Cry toxins (Ferre & Van Rie, 2002; Bravo et al. 2007). To date, changes in the activity of the midgut proteases involved in toxin activation, and modifications in specific membrane receptors are the two main mechanisms described for resistance to Cry toxins (Bravo et al., 2007; Oppert et al., 1997), but recent studies suggest that genes involved in insect immunity and in membrane cell regeneration might also be involved in resistance to Bti (Paris et al., in prep).

3.1 The role of proteases in resistance

The mode of action of Bti toxins in the gut of susceptible insects is complex, involving many steps in the conversion of protoxins to toxins. In the crystal, protoxins interact through hydrogen bonding, disulfide linkages, and hydrophobic interactions. In the alkaline midgut of insect larvae, the protoxins are hydrolyzed to toxins by proteases, mostly serine-proteases. Both the bacterium and the insect produce proteases able to solubilize and activate Bti protoxins, and in the lab, mammalian trypsin and chymotrypsin can also activate the protoxins (Oppert, 1999). However, insect proteases appear to be key in determining toxin specificity; for example, Bt *aizawai* protoxins are toxic to both lepidopteran and dipteran insects, but their incubation with lepidopteran proteases yields a 55kDa protein toxic only to lepidopterans, while their incubation with dipteran proteases results in a 52kDa protein toxic only to dipterans (Haider et al., 1986). The role of proteases in resistance to Bt toxin Cry1Ca1 was recently demonstrated in *Spodoptera frugiperda*: a serine-protease gene found to be down regulated in the larval midgut of intoxicated larvae was further shown by RNAi-mediated knockdown to be involved both in reduced protoxin activation in the midgut and reduced susceptibility of insects to toxins in bioassays (Rodriguez-Cabrera et al., 2010). In contrast with their role in toxin activation, proteases were shown in several studies to be involved in Bt toxin degradation and/or sequestration in resistant insects, but this mechanism has not been reported so far in mosquitoes. Altogether, the role of proteases in conferring resistance to Bti toxins is ambiguous as both the type and activity level (decreased or increased activity) of gut proteases might be involved in resistance. A comparison of the genes differently expressed in Bti resistant and susceptible mosquitoes was performed using high-throughput sequencing technology (Digital Gene Expression Tag Profiling). This method is based on

sequencing of 20 pb fragments in the 5'-terminal region of mRNA anchored on specific restriction sites, long enough to identify the genes and quantify expression levels, but not long enough to investigate alternative splicing events and sequence variations. Out of a total of 138 genes differently expressed, nearly a quarter of those with known function were proteases, and among them, about half were under-expressed in resistant insects and half were over-expressed (Paris et al, in prep). Further understanding of the role of proteases in resistance will involve investigating the precise role of a set of candidate proteases, either by using specific inhibitors, or gene silencing approach (siRNA specific), or heterologous expression in *E. coli*, and then test the activity of the target protease on Bti protoxins (activation/degradation). The ultimate validation would be testing toxicity on mosquito larvae after protoxin processing by the candidate protease (bioassays).

3.2 The role of membrane receptors in resistance

Cry toxins bind to the brush border membrane cells in the midgut on specific membrane receptors, leading to pore formation, ionic imbalance, cell lysis, bacterial proliferation in host tissues, and septicemia (figure 3). An alternative mode of action for Cry toxins was recently proposed that involves a cellular signaling pathway following toxin binding rather than pore formation, and cytotoxicity can eventually be due to the combined effects of osmotic lysis and cell signaling, but initial toxin binding to specific membrane receptors remains a key process (Zhang et al 2006; Pigott & Ellar, 2007; Soberon et al., 2009, 2010). Genetic resistance to Cry toxins involve change in the sequence of the specific receptor gene so that binding is no more effective (Tabashnik et al., 1997), and/or down regulation of the receptor gene so that binding is considerably reduced or suppressed. Alternative splicing of the receptor gene due to transposable elements was shown to confer resistance to *Bacillus sphaericus* binary toxins in *Culex pipiens* populations (Darboux et al., 2007) but such mechanism has not been demonstrated so far for Bti toxins. Cross-resistance mechanisms between different Cry toxins have been reported, presumably involving shared membrane receptors (Siqueira et al., 2004; Zhao et al., 2001; Xu et al., 2010; Likitvivanavong et al., 2011). In lepidopterans, four types of receptors for Cry1A have been identified: cadherin, glycosylphosphatidylinositol (GPI)-anchored APN (aminopeptidase N), GPI-anchored ALP (alkaline phosphatase) and glycolipids (reviewed in Pigott & Ellar, 2007). Cry1Ab toxin binds to the abundant but low affinity GPI-anchored proteins ALP or APN and concentrates in the microvilli membrane where it then binds to cadherin receptor with high affinity. This binding mechanism was named 'ping-pong' because it involves going from GPI-anchored proteins to cadherin and back to GPI-anchored proteins before membrane insertion (Gomez et al., 2010). The specific receptors of Bti toxins in mosquito midguts have been much less studied. To date, one cadherin was identified as Cry4B receptor and one APN as Cry11B receptor in *Anopheles* (Hua et al., 2008; Abdullah et al., 2006; Zhang et al., 2008), and one cadherin as Cry11B receptor, one APN as Cry11A receptor and one other ALP as Cry4B receptor in *Aedes* (Chen et al., 2009a, Fernandez-Luna et al., 2006, Bayyareddy et al., 2009). Another cadherin was found to be down-regulated and to exhibit genomic signature of selection in a Bti resistant *Aedes aegypti* strain, suggesting its implication in resistance, but there is not so far validation of its role as a receptor for Cry toxin (Bonin et al., 2009).

3.3 Other mechanisms involved in mosquito resistance

Zhang et al. (2005) suggested that cell death following Cry toxins binding to membrane receptors is a more complex cellular response than the simple osmotic lysis previously assumed. They propose a pathway involving G protein, adenylyl cyclase and adenosine monophosphate, and resulting in the activation of protein kinases A that initiate membrane blebbing, cell swelling and cell lysis (Zhang et al., 2006). The crucial role of protein kinases A in this cell death pathway was demonstrated by using specific inhibitors (Zhang et al., 2006). Using two-dimensional gel electrophoresis

and mass spectrometry method, actin was identified as a binding protein for the Cry1Ac toxin in the lepidopteran species *M. sexta* (McNall & Adang, 2003), *Heliothis virescens* (Krishnamoorthy et al., 2007) and *Helicoverpa armigera* (Chen et al., 2010), and for the Cry4B toxin in *Ae. aegypti* (Bayyareddy et al., 2009). Because actin is located within the cell, it is unlikely to be a membrane receptor for Cry toxin, and binding presumably occurs after the penetration of the toxin into the epithelial barrier of the midgut (McNall & Adang, 2003). Toxin binding to actin could lead to disruption of its normal function in maintenance of the cytoskeleton architecture, causing loss of cell shape and integrity (Krishnamoorthy et al., 2007). Changes in the expression of genes involved in membrane cell remodelling and epithelium repair, and genes involved in immunity, were observed in Bti resistant mosquitoes (Paris et al., in prep). A more efficient repair or replacement of damaged midgut cells could be involved in resistance. Similarly, a more efficient immune response to bacterial invasion may be selected for in insects fed with Bti, but this mechanism alone is unlikely to confer resistance, because Bti toxins alone are able to kill susceptible mosquito larvae, although radiated spores are 20-30% less efficient than unirradiated spores (Becker, 2002).

3.4 The genetic basis of resistance

Because Bti contains many toxins with different modes of action that act in synergy to confer acute toxicity, various mechanisms are likely to be simultaneously involved in resistance: behavioural avoidance of toxins, physiological changes in the larval midgut (pH, protease activity), and genetic changes in specific receptors. Mosquito larvae are particle feeders and Bti tend to sink more or less rapidly in mosquito breeding sites where it is sprayed. In the field, Bti toxicity is lost within a week against *Anopheles* which feeds in surface (Kroeger et al., 1995), whereas *Aedes* species are still killed up to 4 weeks after treatment (Marcombe et al., 2010), suggesting that differences in feeding behaviour might explain this difference in susceptibility (Lacey, 2007). Changes in the type and/or level of expression of the midgut proteases can also be involved, as well as modifications in the expression and/or sequence of the specific membrane receptors. All these mechanisms are likely to act together in resistant insects. For example, a resistant strain of *Ae. aegypti* exhibited slower larval development as compared to the susceptible strain (Paris et al., 2011b). This difference in larval development time might be attributed to differences in feeding behaviour, and/or to changes in proteases modifying metabolic efficiency, and/or to pleiotropic effects of modified receptors. So far the study of resistance to Bt toxins in insects was based on the study of a few candidate proteins, either toxin receptors or proteases, using proteomic approaches (2D-DIGE, ligand binding...). Resistance to Bti can involve both mutations (in specific proteases, specific membrane receptors), transcriptional regulation (up or down regulation of proteases, of membrane receptors and other co-factors involved in toxin binding, of immune genes...) and post-transcriptional changes (alternative splicing of mRNA...). Given the variety of mechanisms involved in resistance to the multiple toxin contained in Bti, a global screening of genomic /transcriptomic/ proteomic changes in resistant insects should be favoured to a candidate gene approach, in order to tackle all the genes simultaneously involved and their interactions (Bonin et al., 2008, 2009, Paris et al., 2010). Bti containing a mixture of toxins with various modes of action, the resistance is likely to involve many loci with various levels of dominance and various degrees of epistatic interactions. The selection of fully resistant genotypes is likely to take longer than for monolocus resistance, because recombination disrupts advantageous resistant allele combinations at each generation. However, the multigenic basis of Bti resistance is not a guarantee that resistance will not evolve in natural populations. Indeed, high resistance levels evolved in field *Culex pipiens* populations treated with *Bacillus sphaericus*, despite the fact that resistance involves at least two recessive loci. Complex interactions between these two loci appear to protect each recessive mutant from disappearing when both are rare, leading to a rapid increase in

frequency of the resistant alleles in natural populations (Chevillon et al., 2001). In the case of resistance to Bti in mosquitoes, the genes involved and the dominance level of the resistant alleles are not yet known. Further characterization of mechanisms underlying resistance to Bti is essential in order to develop an effective resistance management of field populations.

3.5 Consequence of resistance on insect fitness: the cost of resistance

When affecting genes with an important function, resistance alleles may have pleiotropic effects and reduce the fitness of resistant individuals in the absence of insecticide. The spread and the evolution of resistance in populations depend not only on the selective advantages linked to level of resistance, but also the negative fitness costs associated with the resistance. Resistance costs can be estimated in two ways. The first one consists of directly comparing the life-history traits associated with fitness, such as survival, reproduction or behavior, of susceptible and resistant individuals (Gassmann et al., 2009). The second way consists of monitoring the changes in resistance allele frequencies in space (through transects between resistant and susceptible populations) or time (over several generations without insecticide). The main advantage of this method is that it takes into account the fitness costs expressed at all the life-stages of the resistant phenotype. The cost of resistance to Cry toxins has been intensively studied in lepidopteran or coleopteran pests resistant to transgenic Bt crops expressing Cry toxin genes (reviewed in Gassmann et al., 2009). About 70% of studies detected costs associated with Cry toxin resistance, affecting various phenotypic and life history traits such as development time, mass, survival or fecundity. In contrast, only two studies of the fitness cost of resistance to Bti toxins in mosquitoes have been undertaken so far, on *Culex pipiens* (Saleh et al., 2003) and *Aedes aegypti* (Paris et al., 2011b) laboratory strains. Larval selection with Bti in *C. pipiens* over 20 generations caused a 44.8% reduction in female fecundity, but no significant reduction in fertility (egg hatchability) or in adult longevity was found; resistance quickly decreased by 58% after 3 generations without selection (Saleh et al., 2003). In *Ae. aegypti* selected for 22 generations using leaf litters containing Bti, 40% reduction in female fecundity, 68% decrease in egg survival after 4 months desiccation, and 17% increase in larval development time were observed; resistance was totally lost after only 5 generations without selection (Paris et al., 2011b). The magnitude of variation in fitness traits measured in the laboratory may be more or less transposed to natural mosquito populations, depending on the ecological conditions experienced in the field. For example, in temperate regions, eggs will usually persist for a long time in the environment (usually during winter), and the fitness cost in terms of egg survival to long-term desiccation will be expressed. In contrast, it is presumably of less importance in tropical regions where the successive mosquito generations usually do not require egg diapause. However, in tropical regions with many successive generations, the costs on larval development time and female fecundity will be expressed. Finally, for a similar period of time (e. g., 4 months), the resistance level is reduced to the level of the susceptible strain in both environments, even though different fitness costs are expressed and involved in the counter-selection of resistant individuals. Therefore, the rapid decline in resistance observed in laboratory conditions is promising as it opens up perspectives for effective management strategies. However, compensatory mutations decreasing fitness costs are likely to be selected for, especially in natural populations with a large standing variation. The selection of allelic combinations conferring both resistance and reduced fitness costs from the genetic diversity already present within the population is the first mechanism to act in recently treated areas. Then, new mutations which decrease fitness costs may appear and accumulate. Finally, the replacement of the costly resistant allele by a less costly resistant allele may occur at the same or at another locus (Roush & McKenzie, 1987; Labbe et al., 2009).

4. Future directions and challenges: managing Bti resistance in the field

4.1 Monitoring resistance in natural populations

4.1.1 Toxicological tests (bio-assays)

So far, no consistent resistance to Bti has been reported in natural populations, even in regions heavily treated since decades (Becker & Ludwig, 1993). The lack of evidence for Bti resistance in treated mosquito populations does not mean that resistance to individual toxins is not arising in the field (Paris et al., 2010). Indeed, resistance to individual toxins might evolve in field populations if toxins have different persistence in the environment. If some toxins are more persistent than others, the selective pressure at play in natural populations may be quite different from the full toxins mixture tested in the laboratory. An *Ae. aegypti* strain selected in the laboratory with leaf litter collected in the field long after Bti treatment (Tilquin et al., 2008) showed high levels of resistance to individual Cry toxins (up to 30-fold resistance) but only limited resistance to Bti mixture (2-fold resistance, Paris et al., 2011a). This resistance to particular Bti toxins might represent a 'first step' toward Bti resistance in regions treated exclusively with this bio-insecticide. In light of these results, we propose to monitor resistance in field populations using separate toxins rather than the full mixture, in order to detect resistance at the earliest step. A preliminary survey of 16 treated populations belonging to 5 mosquito species (*Ae. rusticus*, *Ae. vexans*, *Ae. sticticus*, *Ae. cinereus* and *Culex pipiens*) have shown that each mosquito species presents only limited difference in its level of tolerance to individual Bti toxins (i. e. less than 2-fold difference in LC_{50}). We found evidence for increased resistance to Cry4A and Cry11 in one population of *Aedes sticticus* out of three (more than 10-fold difference in LC_{50}), although no such evidence was found with Bti. It is therefore important that mosquito control agencies periodically check the level of resistance in treated populations to individual toxins, because it is the first step to resistance to the full Bti mixture. Undertaking bioassays on field populations is not an easy task, because it requires testing many doses with many replicates in order to determine the LC_{50} . We therefore propose to develop a simple toxicological test involving only two diagnostic doses per toxin and per mosquito species. Only when a field collected population of larvae will present more than 10-fold resistance to an individual toxin as compared to populations of the same species, a more rigorous F2 screen test involving to create many F2 progeny from isofemale lines, and test for mortality on F2 larvae using the highest diagnostic dose, will be performed to further evaluate the frequency of resistant alleles in that particular population.

4.1.2 Monitoring change in resistance allele frequencies

Identifying genes involved in resistance to Bti is a challenging ongoing research area, and so far, no diagnostic mutation is known for Bti resistance. When available, such molecular diagnostic tool will allow the mosquito control agencies to efficiently monitor the frequency of resistant alleles in treated populations, and to adapt their treatment strategy in order to delay the evolution of resistance in the field.

4.2 Strategies for resistance management

4.2.1 Taking advantage of the cost of resistance

Although the commercial Bti mix is a combination of several toxins, the evolution of resistant alleles in treated populations will probably occur sooner or later, if no management strategy is in place to slow down their frequency. The high cost of resistance to Bti expressed at various stages of the mosquito's life opens up perspectives for managing Bti

resistance before the resistant alleles spread into populations. The cornerstone of resistance management is the 'high dose-refuge' (HDR) strategy in which some sites are heavily treated while adjacent sites remain untreated, allowing the persistence of a population of susceptible insects. The principle of the HDR strategy is to conserve non treated refuges in proximity of treated areas to promote survival and dispersion of susceptible insects (spatial refuges). Its success depends principally on the counter-selection of resistance alleles in refuges due to fitness cost. Alternatively, the treatment can be interrupted during a period of time, allowing the competition between susceptible and resistant phenotypes to slow down the evolution of the resistant alleles (temporal refuges). This strategy is particularly effective when resistance is at least partly recessive, and relies on the competitive superiority of susceptible individuals in untreated sites (due to the cost of resistance) that will invade the adjacent treated areas and mate with the few surviving resistant individuals. The success of this strategy therefore depends on the cost of resistance, the level of dominance of the resistant alleles, and the migration rate of insects between untreated and treated sites. The latter determines the proportion of refuges required, and their spatial arrangement. The size and disposition of these refuges have been widely modeled but only in situations involving a single, bi-allelic resistance locus (e.g., Tabashnik & Croft, 1982; Caprio, 2001). In the case of resistance to Bti, a mixture of toxins with various modes of action, the resistance is likely to involve many unlinked loci with various levels of dominance (Bonin et al., 2009; Paris et al., 2010). Further characterization of mechanisms underlying resistance to Bti is essential in order to develop an effective resistance management of field populations. Paris et al. (2011b) have shown that the diapause of mosquito eggs for four months is long enough to counter-select resistance alleles suggesting that Bti resistance can be slowed down in temperate climates, at least for mosquito species overwintering as eggs. This could partly explain the lack of resistance detection in European countries that have been using Bti for decades. The fitness costs observed in the laboratory when the selective pressure was relaxed appear to be sufficient to counter-select resistant individuals over just five generations (Paris et al., 2011b). This suggests that in tropical regions with a rapid turnover of generations, just a few generations with no treatment would limit resistance evolution. The ability to easily counter-select resistant individuals by exploiting the resistance costs expressed in tropical or in temperate environments opens up interesting perspectives for ensuring the long-term effectiveness of this environmentally safe bio-insecticide against mosquitoes. However, it would not be possible to stop anti-mosquito treatment altogether for several consecutive generations in tropical regions where mosquitoes represent a major threat to human health. An alternative to HDR strategy is the regular release of susceptible adult males bred in the lab in treated populations. Indeed, mosquito males are not disease vectors and they do not bite mammals, so that their release will not generate any nuisance. Furthermore, Bti targets larvae so that these released susceptible adult males are not affected by treatment, and efficiently compete with local males to mate with females, thereby diluting resistant alleles in the next generation. Finally, combining chemical insecticides against adults together with Bti against the larvae every few generations could contribute to effective Bti resistance management in tropical regions.

4.2.2 Combining the use of Bti with other bio-insecticides

Following the discovery of Bti, other *B. thuringiensis* strains and toxins active against mosquitoes have been searched for intensively. There are today at least 19 type toxins with mosquitocidal activity in addition to the Cry4, Cry11A and Cyt toxins, i.e. Cry16Aa, Cry17Aa, Cry19Aa, Cry19Ba, Cry20Aa, Cry20Ba, Cry24Aa, Cry24Ba, Cry24Ca, Cry25Aa, Cry27Aa, Cry29Aa, Cry30Aa, Cry30Ba, Cry30Ca, Cry30Da, Cry30Fa, Cry30Ga, Cry39Aa, Cry40Aa, Cry40Ba, Cry44Aa, Cry48Aa, Cry49Aa, Cry52Ba, Cry54Aa, Cry56Aa, Cry60Aa, Cry60Ba, which belong to at least 10 identified *B. thuringiensis* serotypes, i.e. *morrisoni*, *fukuokaensis*, *jegathesan*, *kyushuensis*, *higo*, *medellin*, *entomocidus*, *sotto*, *aizawai* and *malayensis*, but

also from *Clostridium bifermentans* subsp. *malaysia* and from *Bacillus sphaericus* (Lee & Gill, 1997; Juarez-Perez, et al., 2003; Ito et al., 2006; Beron & Salerno, 2007; Barloy et al., 1996; Hwang et al., 1998; Ohgushi et al., 2005; Tan et al., 2009; Jones et al., 2007; Zhu et al. 2010; Padua & Federici, 1990; Lacey et al., 1988; Lacey, 2007; Federici et al., 2003, Poopathi & Abidha, 2010). However, despite a high number of toxins and strains identified the efficiency of Bti for controlling mosquitoes was not reached. The only strain which could truly compete with Bti was *B. thuringiensis* subsp. *morrisoni* strain PG-14 (Padua & Federici, 1990; Lacey et al., 1988; Lacey, 2007). PG-14 produces the same toxins as in Bti in addition to a 144-kDa non-mosquitocidal toxin. However the 144-kDa toxin gene is borne on a different plasmid than those coding for the Bti toxins. PG-14 is therefore an initially non-mosquitocidal strain which acquired mosquitocidal activity by transfer of the conjugative pBtoxis plasmid from Bti. The efficiency of Bti is closely linked to its specific combination of toxins displaying synergisms for both toxicity (acute effect) and sustainability (resistance avoidance). As Bti, all these Bt subspecies produce a cocktail of Cry and Cyt toxins, and the mode of action is likely to involve the same mechanisms if not the same binding receptors: cross-resistance might evolve rapidly if these insecticides are used in combination with Bti. Prospects for improvement might therefore be on complementary products with a mode of action radically different from Bt toxins. Many other bio-insecticides have proven to be efficient against mosquitoes, including the binary toxins produced by *Bacillus sphaericus* (Bs). Although Bs toxins also bind to larval midgut receptors, there is no cross resistance to Bti within Bs resistant *Culex* populations, and there is even evidence for an increased sensitivity to Bti (Rao et al., 1995). Valent BioSciences Corporation (ill., USA) recently developed a new granule product, under the commercial name VectoMax® G (table 1), combining Bti and Bs in a specific toxin ratio into every micro particle. This mixture exhibits extended residual control and good efficacy in polluted water contrary to Bti. The neuro-toxic spinosad produced by the actinomycete *Saccharopolyspora spinosa* also exhibits efficient mosquitocidal activity (Bond et al., 2004). Plant compounds are a main source of natural insecticides (Després et al., 2007) and their efficiency has been evaluated against mosquitoes. Saponins and essential oils with larvicidal, repellent, or oviposition deterrent effects on mosquitoes have been described (Kiran et al., 2007, Senthilkumar et al., 2009). Seed extract from *Moringa oleifera* was shown to efficiently kill mosquito larvae without toxicity to non-target organisms (Ferreira et al., 2009), as well as sodium anacardate extracted from cashew nut shells (Farias et al., 2009). These bio-insecticides present the advantage of showing different specificity towards the various mosquito species co-existing in treated areas, and of action modes and target sites very different from those of Bti. The combined or sequential use of these bio-insecticides with Bti can allow efficient mosquito control together with limited selective pressure for resistance evolution in treated populations.

4.2.3 What future for mosquitocidal Bt products?

An important element to consider when addressing the potential future of mosquitocidal products is that only two biopesticides are currently registered for mosquito control: Bti and *B. sphaericus*. The immediate consequence is that, considering the time and cost of toxicology analyses and registration, there might be at best little incentive to develop novel products when Bti is a perfect biocontrol agent. Ways for improving Bti are not on the toxins per se for two main reasons. First, there is little room, if any, to improve the activity of a set of toxins capable to synergize and to delay resistance. Secondly, any modification of the toxins would result into a new product to register following a long process of deregulation and delay, eliminating thus the benefits of having a product already registered and relying on 30 years of toxicological and environmental analyses. Ways of improvement are therefore in the mode of delivery and in the increase of the life time of the sprayable product. These aspects have been investigated soon after the development of Bti as a successful biocontrol agent. Expression of Bti toxins in different species of cyanobacteria and fresh-water bacteria

such as *Caulobacter*, *Asticcacaulis* or *Ancylobacter* (Angsuthanasombat & Panyim, 1989; Chungjatupornchai, 1990; Liu et al., 1996; Murphy and Stevens, 1992; Sangthongpitag et al., 1996; Soltes-Raket et al., 1995; Thanabalu et al., 1992; Xiaoqiang et al., 1997; Xudong et al., 1993; Yap et al., 1994). The objective in this case was to integrate the Bti toxins in a permanent way in the chain food of mosquito larvae without resorting to sprays. Another opportunity offered by genetic engineering is the expression along with the Cry and Cyt toxins of chitinases or chitinase-like genes. These proteins also often referred to as enhancing factors or enhancin, were shown to effectively increase the toxicity of Bt toxins (Thamthiankul et al., 2004; Liu et al., 2002; Fang et al., 2009). However, this approach, although technically efficient is from a regulatory standpoint moving away from a biopesticide approach to a transgenic approach and is thus liable to the GMO regulation. Besides the delivery mode, the other way of engineering explored in the past few years was to improve the level of production and to stack Bti and *B. sphaericus* toxins or extended synergism (Park et al., 2003, 2005; Wu & Federici, 1993, 1995; Federici et al., 2003, 2007). Nevertheless, these approaches also generate recombinant strains which must be considered under the time consuming and expensive GMO regulation. Besides, GMO is still the subject of public debates which may further delay the use of genetically improved products. The solution in the near future might therefore be on the improved management, monitoring and formulation of the existing Bti product which is after all the best mosquito control product currently available.

5. Conclusion

Bti appears to be a safe and efficient bio-insecticide against mosquitoes. Although Bti still comes at a higher cost than chemical insecticides, presumably due to its so far limited use, there is no technical reason for this high cost. The successful use of food processing organic industrial wastes such as chicken feathers as a nutrient medium to grow Bti (Poopathi & Abidha, 2007) opens avenues to a future low-cost production of this bio-insecticide, with the additional benefit of effective recycling of bio-organic wastes from the environment. The most important threat to the long-term use of this efficient mosquito-control tool is the evolution of resistance in treated populations. There is an urgent need to develop efficient, easy-to-use and low-cost diagnostic tools to evaluate the fate of Bti in the environment, and to monitor Bti resistance evolution in mosquito populations.

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