Mosquito Resistance to Bacterial Larvicidal Toxins

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Abstract: Insecticide resistance to the microbial insecticides *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) represents a serious threat to their success. Available evidence indicates that the risk for resistance to *Bti* is low due to the makeup of its parasporal crystal, which contains Cyt1A, Cry4A, Cry4B, and Cry11A toxic proteins. Disrupting the toxin complex in *Bti* enables resistance to evolve, especially in the absence of the key factor, the cytolytic toxin, Cyt1A. Cross-resistance is widespread among mosquitocidal *Bacillus thuringiensis* Cry toxins and the mechanisms of Cry resistance in mosquitoes are not known. *Bacillus sphaericus* (*Bs*) is at higher risk for resistance due to its single-site action and field cases have been reported from a number of locations worldwide. Cross-resistance is reported among the various *Bs* isolates, although some isolates produce additional toxic proteins that can reduce cross-resistance and slow resistance evolution. Field and lab evolved resistant populations consistently show recessive and monofactorial inheritance of resistance. Resistant populations, however, have evolved a variety of molecular mechanisms causing that resistance. Traditional resistance management strategies with promise include rotations and mixtures of *Bti* and *Bs*, as well as untreated areas that provide natural refuges for susceptible alleles. Promising new strategies include genetic engineering to increase the toxin complexity targeted toward mosquito larvae, to enhance the host range of the mosquito control product, and to avoid the evolution of insecticide resistance. Regardless of the control strategy, a resistance-monitoring program alongside an integrative pest management approach is the best strategy to delay insecticide resistance.

Keywords: Bacillus thuringiensis israelensis, Bacillus sphaericus, resistance, inheritance, management.

INTRODUCTION

The important role played by mosquitoes in the spread of disease has long been recognized. Species of mosquitoes belonging to the genera *Anopheles, Aedes,* and *Culex* are intimately involved in the worldwide incidence of important human diseases such as malaria, dengue, filariasis, and multiple viral encephalitides. In addition, mosquitoes contribute a significant degree of discomfort and misery to humans and animals due to their blood-feeding habit. Early methods for controlling the spread of mosquito-borne diseases relied primarily upon synthetic chemical insecticides. These were widely deployed with excellent results, but their use ultimately caused adverse environmental effects and the evolution of insecticide resistance in many targeted mosquito species. The need to control mosquito populations continues, however the insecticides that can be safely used in such efforts are extremely limited. Because of this, interest has grown in the bacterial insecticides, primarily *Bacillus thuringiensis* subsp. *israelensis* de Barjac (*Bti*) and *Bacillus sphaericus* Neide (*Bs*), that produce crystalline proteins that are toxic toward mosquitoes and are safe for the environment.

Products based on *Bti* and *Bs* are developed for control of the immature stages of mosquitoes. Sensitive larvae ingest the crystalline inclusions, and the toxic proteins are released into the larval midgut following digestion and activation. The activated toxins bind to receptors on the larval midgut epithelium where they form pores, disrupting the osmotic balance of the cells, or in the case of *Bs*, are internalized [1-4]. Products based on *Bti* and *Bs* are marketed for mosquito larval control and are widely used in the USA and Europe. *Bs*, because of its better residual activity in polluted waters, has been broadly used against *Culex* species in the US, Central America, Brazil, India, Thailand and China. Unfortunately, the high cost of bacterial insecticides limits their more widespread adoption, particularly in the underdeveloped areas where the highest incidence of mosquito-borne disease occurs.

Although successful in controlling mosquito larvae and safe for the environment, bacterial insecticides will become ineffective if resistance evolves. Insecticide resistance is a consequence of strong directional selection pressure resulting from the repeated, intensive use of an insecticide. Rare, individual insects in a treated population may naturally possess genetic characteristics that reduce their sensitivity to an insecticide. Those individuals survive treatment in disproportionate numbers relative to their more sensitive counterparts and consequently their offspring are represented at higher frequencies in subsequent generations. Over time, with repeated treatments, the frequency of resistant individuals increases and the population may become difficult to control until the insecticide, and closely related insecticides, are useless for significantly reducing population numbers.

Historically, the patterns of insecticide use for controlling mosquitoes led to the evolution of insecticide resistance to DDT, BHC/cyclodienes, organophosphates, carbamates, and pyrethroids [5]. Not all species or even all populations of any species are resistant. But the pattern of adoption of new insecticides, followed by the eventual loss of use of that

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1875-4147/10

insecticide due to insecticide resistance, has occurred frequently enough and widely enough to serve as a grim reminder that insects, especially mosquitoes, have the genetic capacity to evolve and adapt to adverse conditions. As a result it is essential that new insecticides, such as the bacterial insecticides, be studied carefully and their field effects followed closely so that any undesirable consequences are detected or avoided. It is hoped that careful management and an understanding of the evolutionary patterns in mosquitoes in response to insecticide treatment will enable the safe and effective use of these materials.

In this article I will be discussing the current status of knowledge of resistance toward bacterial insecticides in mosquitoes. Field resistance and laboratory selection studies will be presented to illustrate the various factors that influence the evolution of resistance. The associated cross-resistance spectrums and their impact on future bacterial insecticides will be discussed. Mechanisms of resistance and fitness effects as they are currently understood are included. The final emphasis will be on a discussion of strategies that might be used to avoid or manage resistance.

Bacillus *thuringiensis* Subsp. *israelensis* de Barjac

Bti is a gram-positive, spore forming, aerobic bacterium that is found in a variety of habitats [6, 7]. During sporulation, *Bti* produces a spherical, parasporal inclusion that contains larvicidal proteins with activity against Nematoceran Dipterans; primarily mosquitoes and blackflies. Chironomid midges [8], fungus gnats [9], and crane flies [10] are also susceptible to a lesser degree.

The toxin composition of the parasporal body is unusual among the larger *Bt* family. Four large inclusions are observed in the parasporal body, composed of the proteins Cyt1A (27 kDa), Cry11A (72 kDa, formerly CryIVD), Cry4A (128 kDa, formerly CryIVA), and Cry4B (135 kDa, formerly CryIVB), contained in a lamellar envelope [11]. Two additional toxin genes, Cry10A and Cyt2A, are present on the plasmid of commercial strains of *Bti*, but no protein expression has been reported [12, 13]. Cry4A, Cry4B, and Cry11A share sequence homology with other Cry toxins in insecticidal strains of *Bt*, all of which are believed to have similar modes of action but can differ in their target specificity [14]. Cyt1A is unrelated in sequence to the Cry toxins and is cytolytic *in vivo* and *in vitro* [15].

Bti has been available as a commercial product for several decades and a variety of formulations, such as wettable powders, granules, flowable concentrate and briquettes, have been developed [7]. Although formulation advances have improved its activity, *Bti* has a relatively short life in heavily polluted waters, but it has a high level of safety for non-target organisms and humans [16].

Field Resistance to *Bti*

Natural variation in the susceptibility of populations and technical variation inherent in bioassay tests, need to be considered in the interpretation of bioassay data, since lethal concentration values (LC) can vary greatly between regions, laboratories and/or technicians and constrain data interpretation [17]. Thus, LC values that differ by 5-fold or less are not likely to reliably indicate resistance, and as a general guideline, differences of 10-fold or greater are necessary for proof of resistance (Georghiou personal comm.).

Natural variation in field susceptibility toward *Bti* was studied in populations of *C. pipiens* complex from California and the Mediterranean island of Cyprus. A sample of 31 populations from California showed a 3 – 4 fold range in LC values [18]. The 5 Cyprus populations showed greater variation, 10-fold, and higher mean LC values, than the California populations. Ten years later a subsequent study reported 3 – 4 fold range in LC values in *C. pipiens* populations from Cyprus [19]. These data suggest that LC values must exceed 5 – 10-fold before a population can be considered resistant to *Bti.*

Since 1982, *Bti* has been applied to more than 50,000 ha for the control of *Aedes vexans* field populations under the control of the Upper Rhine Valley mosquito control program [20]. After 10 years of treatments, the populations were sampled to determine any changes in susceptibility and no resistance was detected.

A study from New York documented the susceptibility of *Culex pipiens* to various insecticides, including *Bti* [21]. That study reported 2 populations with reduced susceptibility toward *Bti* relative to a susceptible *C. quinquefasciatus* reference colony. The population from Syracuse showed a resistance ratio of 34-fold toward *Bti*. The second population from Albany was more sensitive than the Syracuse population, with resistance ratios of 5.7 and 14 at the LC_{50} and LC_{95}, respectively. Records indicated that *Bti* had been used for 4 years in the county at the time these collections were made, although no records of treatment history for those specific sites were available [21]. The significance of these data is somewhat limited due to the lack of information on the level of selection that may have been exerted on the populations, and the absence of data for additional field populations, especially any populations with higher susceptibility. Although no field control failure has been reported in relation to these populations, it should be noted that the levels of resistance were high enough to indicate field resistance.

Laboratory Selection for Resistance to *Bti*

Several attempts to study the evolution of resistance to *Bti* in laboratory populations of mosquitoes have been undertaken (Table **1**). The earliest report involved the selection of 4 populations of *C. quinquefasciatus* from interior (collections 1 and 2) and coastal areas (collections 3 and 4) of southern California (Table **1**) [22]. Collections 1, 3, and 4 were selected at the LC_{90} for 60, 12, and 11 generations, respectively. Collection 2 was selected more gradually, starting with a lower lethal concentration and gradually increasing the selection pressure for 36 generations. Regardless of the origin of the collection or the level of selection pressure, only modest levels of resistance were attained. Collection 1 reached the highest level of resistance of 16.5-fold at the LC_{95} in generation 46, after which resistance declined, staying around 12-fold. Resistance was unstable in the absence of selection pressure, declining by 50% in 3 generations. Collections 2, 3, and 4 attained 4.4, 4.1, and 5.9-fold resistance at the LC_{95}.

C. pipiens from Egypt were selected for 20 generations at the LC_{90} and a maximum of 2.8-fold resistance was observed

Table 1. Laboratory Selection Studies of the Evolution of Resistance to *Bacillus thuringiensis* subsp. *israelensis*

	Colony	Location	Selection Material	Number of Generations	Maximum Level of Resistance	Reference
Culex quinquefasciatus						
	1	CA	**Bti**	60	16.5	[22]
	2	CA	Bti	36	4.4	[22]
	3	CA	Bti	12	4.1	[22]
	4	CA	Bti	11	5.9	[22]
	India	India	Bti	20	2-3	[24]
Culex pipiens	Egypt	Egypt	Bti	20	2.8	[23]
Aedes aegypti	USA	Georgia	Bti	15	1.1	[25]
	Sri Lanka	Sri Lanka	Bti	15	1.1	[25]
	Brazil	Brazil	Bti	15	2	[25]
	Recombinant *Bacillus thuringiensis* bacterial toxins					
Culex quinquefasciatus	Cry11A	CA, USA	Cry11Aa	28	>900	[26]
	CqAB	CA, USA	Cry4Aa, Cry4Ba	25	>120	[26]
	Cq4ABD	CA, USA	Cry4Aa, Cry4Ba, Cry11Aa	28	91	[26]
	Cq4ABDCytA	CA, USA	**Bti**	28	3.2	[26]

at the LC_{50} [23]. *C. quinquefasciatus* from India showed very similar resistance levels after 20 generations of laboratory selection, 2-3 fold resistance [24].

Three colonies of *Aedes aegypti*, consisting of a laboratory strain from Georgia (USA) and collections from Sri Lanka and Brazil, were selected with *Bti* for 15 generations at the LC_{50} [25]. The resistance ratio for the laboratory colony, and the colony from Sri Lanka, reached 1.1-fold and were not significant. A slight, but statistically significant shift of 2-fold was reported for the Brazil collection.

One hypothesis that developed from the early studies with *Bti* was that resistance evolved more slowly and to lower levels because *Bti* produces a complex parasporal crystal with at least 3 diverse Cry toxins and a cytolytic toxin. These diverse toxins might act at different receptors, making the evolution of resistance to all 4 materials very difficult. The availability of recombinant *Bt* strains expressing the different toxin genes found in *Bti* enabled selection of parallel lines exposed to one (Cry11A), two (Cry4 and Cry4B), three (Cry4A, Cry4B and Cry11A), or all 4 native *Bti* toxins (Cry4A, Cry4B, Cry11A, and Cyt1A) [26]. After 28 generations of selection, resistance evolved in the populations exposed to 1, 2, or 3 Cry toxins, but little or no resistance evolved to wild-type *Bti* [26]. Resistance levels were inversely related to the number of toxins used in selection (Fig. **1**). The highest resistance observed was in the single toxin selected line, colony Cq11A (formerly CryIVD), exposed to Cry11A. Resistance was >900-fold at the LC_{95}. Resistance levels reached >120-fold in Cq4AB, selected with Cry4A and Cry4B, whereas resistance was 91-fold in the colony Cq4AB11A, exposed to Cry4A, Cry4B and Cry11A. The line selected with wild-type *Bti*, colony Cq4AB11ACytA exposed to Cry4A, Cry4B, Cry11A, and Cyt1A, showed 3.2-fold resistance. These data suggested that increasing the number of Cry toxins delayed the evolution of resistance somewhat, but the presence of the Cyt1A toxin, combined with the 3 Cry toxins was essential to delay the evolution of resistance.

Cross-Resistance

The small number of *Bti* resistant colonies has limited the information on cross-resistance among *Bt* mosquitocidal strains and their component Cry toxins to a handful of studies and a small number of colonies. The Cry11A resistant colony above was examined for cross-resistance to a number of wild-type *Bt* strains that each produce a mixture of both Cry and Cyt toxins (Table **2**). Cross-resistance was detected toward *B. t. jegathesan* (6.7-fold), *B. t. kyushuensis* (8.2-fold), and *B. t. fukuokaensis* (8.1-fold). [27].

The cross-resistance patterns to different Cry toxins and combinations of Cry-toxins, including *Bti*, were reported for the 4 Bti selected lines (Table **2**) [28]. The Cq11A line showed cross-resistance at the LC_{95} to Cry4A and Cry4B (41.6-fold), to Cry4A, Cry4B and Cry11A (13.5-fold), but no resistance to *Bti* (1.1-fold). The Cq4AB line showed cross-resistance to Cry11A (350-fold), to Cry4A and Cry4B and Cry11A (16.2-fold), and a low but significant resistance

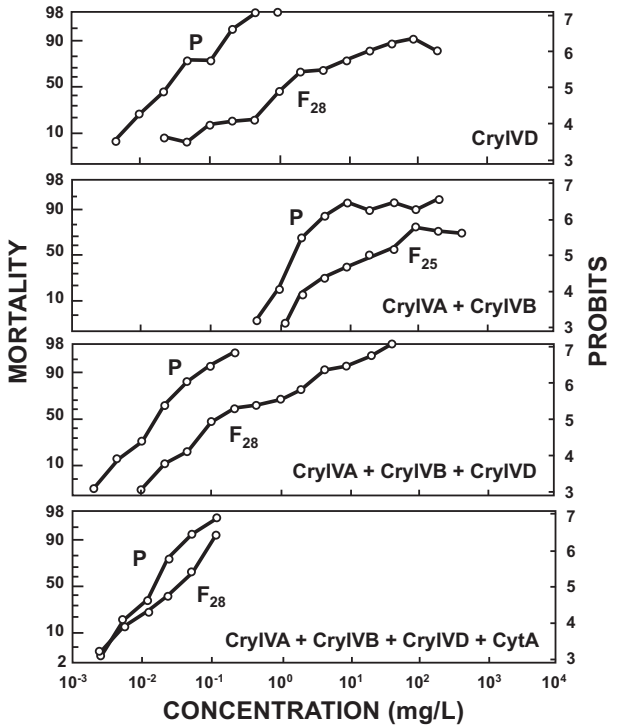

Fig. (1). Dose-mortality regression lines obtained in concurrent tests on the parental CqSynP line and the selected lines (generations F25 or F28) of *C. quinquefasciatus* tested with the respective selecting toxin or toxin combinations of *Bti.* Georghiou GP, Wirth MC. Appl Environ Microbiol 1997; 63: 1095-1101. Reproduced with permission from American Society of Microbiology.

Table 2. Cross-Resistance Spectra Toward Wild-Type and Recombinant Microbial Toxins in Mosquitoes Resistant to *Bacillus thuringiensis* Subsp. *israelensis*

Colony	Selection Material	Cross-resistance spectra toward *B. thuringiensis strains* (resistance ratio at LC_{50} or LC_{95}).					
			israelensis	***jegathesan***	***kyushuensis***	***fukuokuensis***	Reference
Cq11A	Cry11Aa		3.0/1.1/5.7	6.7/2.8	8.2	8.1	[27, 28]
Cq4AB	Cry4Aa+ Cry4Ba		3.2	2.3			[27, 28]
Cq4AB11A	Cry4Aa + Cry4Ba + Cry11Aa		1.2	3.5			[27, 28]
Cq4AB11ACytA	Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa		3.2	5.1			[27, 28]
Cross-resistance to recombinant bacterial toxins. (resistance ratio at LC_{50} or LC_{95}).							
		Cry11Aa	**Cry4Aa + Cry4Ba**	**Cry4Aa +Cry4Ba + Cry11Aa**	**Cry11B**	**Cry19A**	
Cq11A	Cry11Aa	> 913	41.6	13.5	6.8/53.1	2.4	[27, 28, 30, 31]
Cq4AB	Cry4Aa+ Cry4Ba	>350	11.0	16.2	80.7	2.6	
Cq4AB11A	Cry4Aa + Cry4Ba + Cry11Aa	185	12.9	91.3	347	3.7	
Cq4AB11ACytA	Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa	30.1	10.2	8.1	11.8	1.0	

to *Bti* (3.2-fold). The Cq4AB11A line showed high resistance to Cry11A (185-fold), and to Cry4A and Cry4B (12.9-fold) but no significant resistance to *Bti* (1.2-fold). The Cq4AB11ACytA line, selected with *Bti*, showed resistance to Cry11A (30.1-fold), Cry4A and Cry4B (10.2-fold), and Cry4A, Cry4B and Cry11A (8.1-fold). Resistance to *Bti* was 3.2-fold. When recombinant *Escherichia coli* expressing the insecticidal toxins and genes from *Bti* were assayed against these resistant lines, similar patterns of resistance were reported, although the levels of resistance differed [29].

Cross-resistance toward *B. t. jegathesan* and 2 recombinant strains expressing 2 of its Cry toxins was examined in the 4 Bti selected lines. Cross-resistance to *B. t. jegathesan* was low in all lines. Resistance ratios at the LC_{95} were 6.7/2.8, 2.3, 3.5, and 5.1 for Cq11A, Cq4AB, Cq4AB11A, and Cq4AB11ACytA, respectively [27; 30]. Cross-resistance toward Cry11B was 6.8/53.1, 80.7, 347, and 11.8 for the same respective lines. Interestingly, no cross-resistance was detected toward Cry19A from *B. t. jegathesan* when the resistant lines were assayed with that material. Resistance ratios were 2.4, 2.6, 3.7, and 1.0 [31].

Based on the results of these diverse studies, *Bti* in its native form shows a unique capacity to avoid the evolution of resistance in mosquito populations compared to most conventional insecticides. This conclusion applies primarily to *C. quinquefasciatus*, which has been the most intensively studied mosquito species. Highly limited data on *Aedes aegypti* and a single study done was conducted using *C. pipiens* suggest that the same may be true for other mosquito species. The studies using recombinant *Bti* strains provide the strongest evidence that it is the complex mixture of toxins and their specific interactions that reduce the risk for resistance, most importantly the presence of Cyt1A [26, 32]. The strong cross-resistance observed among most mosquitocidal Cry toxins, and the presence of high levels of Cry toxin resistance in the *Bti*-selected line, Cq4A11ACytA, indicate that Cry resistance does evolve in mosquitoes selected with *Bti,* but that resistance is suppressed to some degree by the interactions between the Cry toxins, and is strongly suppressed by the presence of Cyt1A. The direct impact of Cyt1A was demonstrated when *C. quinquefasciatus* were selected with Cry11A, Cyt1A or a mixture of Cry11A and Cyt1A (Fig. **2**) [33]. High levels of resistance evolved in the 2 lines selected with a single toxin, but only 8.1-fold resistance was observed with the mixture.

Given the number of different mosquito populations and species that may be targeted with *Bti*, it cannot be concluded that resistance will not occur, only that the relative risk is low. The 2 New York field populations that demonstrated resistance should remind us that the evolution of resistance is an ongoing risk for any treated mosquito population [21].

Bacillus sphaericus Neide

Bs is a gram-positive, spore–forming, aerobic bacterium that is found in a variety of soil and aquatic habitats [7]. Some isolates of *Bs* produce a highly insecticidal crystal that contains proteins that are toxic to mosquito larvae, primarily *Culex* and *Anopheles* species, and which are poorly active toward most *Aedes* species [4]. Among the various *Bs* isolates that have been examined, three show high activity against mosquito larvae and have been used for mosquito control products: 1593, 2362, and C3-41. Other *Bs* strains may lack the crystal toxins but contain other toxins that are of interest and may have a role in resistance [34, 35]. Most commercial formulations of *Bs* are based on isolate 2362 [7], however isolates 1593 and C3-41 have been used for wide scale field control in India and China [36, 37]. For more details on the various isolates see the review by Charles *et al*. [4].

The primary agent responsible for *Bs* toxicity is the binary toxin known as the Bin toxin. Bin contains 2 components, the toxin domain known as BinA (42 kDa) and the binding domain known as BinB (51 kDa), equal amounts of which are necessary for maximal activity [2, 38, 39]. The Bin toxin receptor in *Culex pipiens* complex is a 60-kDa α-glucosidase that is bound to the epithelial cell membrane by a glycosylphosphatidylinositol anchor [40, 41]. Because Bin targets a single receptor in the mosquito midgut, it is essentially a single-site acting insecticide, which predisposes the evolution of insecticide resistance relative to multi-site

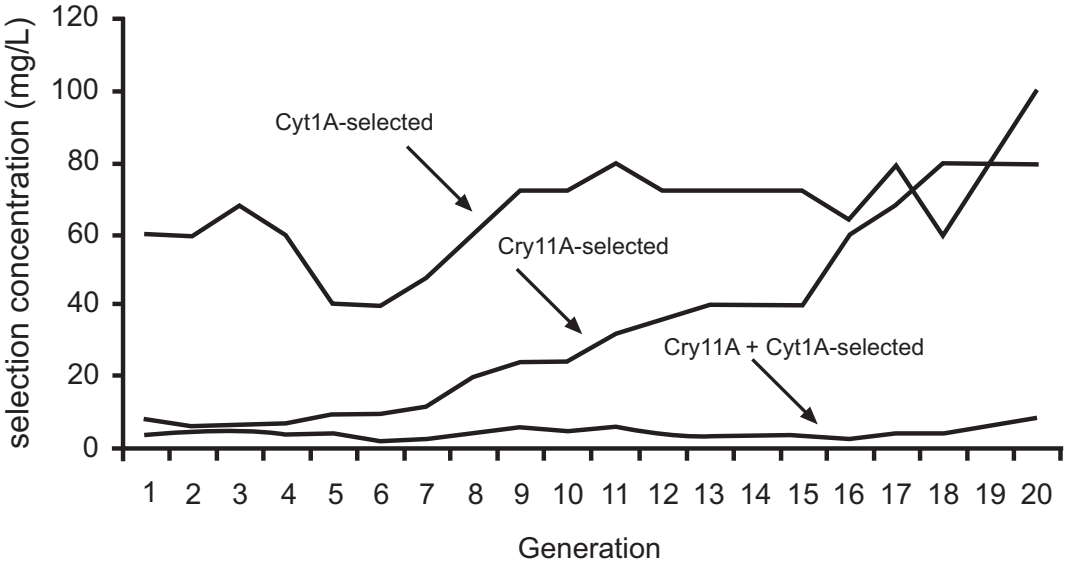

Fig. (2). Increases in selection concentration of *B. thuringiensis* strains producing Cyt1A, Cry11A, or a combination of these two toxins required to achieve resistance in three corresponding lines of *C. quinquefasciatus.* Mortality in the line selected for resistance to Cry11A averaged 70% for generations 1 to 10 and then declined in subsequent generations to approximately 50%, regardless of the increase in strain concentration. Mortality in the lines selected with Cyt1A or the Cyt1A plus Cry11A combination remained high and, thus, the concentration of toxin was essentially constant from one generation to the next. Wirth MC, Park H-W, Walton WEW and Federici BA. Appl Environ Microbiol. 2005; 71: 185-189. Reproduced with permission from American Society of Microbiology.

Table 3. Reports of Field-Evolved Resistance to Different Isolates of *Bacillus sphaericus* in *Culex pipiens* Comples

Bs isolate	Location	Length of Treatment	Level of Resistance	Reference
2362	France	na	RR_{50} = 70	[43]
1593	India	2 years, 35 cycles	RR_{50} = 146	[36]
Spherix™ (B101)	India	1 year	Site1 RR_{50} = 1.8 Site 2 RR_{50} = 7.0 Site 3 RR_{50} = 8.3 Site 4 RR_{50} = 2.4	[44]
2362	Brazil	2 years	RR_{50} = 9.7	[45]
C3-41	China	8 years	RR_{50} = 22,672	[37]
2362	France	> 2 years	RR_{50} = 5958	[46]
2362	Tunisia	na	$RR_{(?)}$ = 750	[47]
2362	Thailand	19 treatments	Control failure	[49]
2362	Thailand	5 treatments	RR_{50} = 125,000	[49]

acting insecticides [42].

Field Resistance to *B. sphaericus*

A total of 9 reports of field-evolved resistance to *Bs* in populations of *Culex pipiens* complex are documented in the literature, although some reports document several different sites. Some of these field resistant strains were brought into the lab and underwent further laboratory selection pressure with *Bs*; the results of which will be discussed separately. The earliest report of field-evolved resistance to *Bs* 2362 came from a field population of *C. pipiens* from southern France with a history of treatment and a reported resistance ratio at the LC_{50} (RR_{50}) of 70 [43] (Table **3**). This was followed by additional reports of high resistance in India, (146-fold) toward isolate 1593 in field populations of C. *quinquefasciatus* [36] and another report of variable levels of resistance in 4 sites toward the commercial preparation, Spherix™, *Bs* isolate B-101, serotype H5a 5b (1.8 – 8.3-fold) [44]. Low-level field resistance (9.7 fold) was reported in Brazil toward isolate 2362 after 2 years of treatment [45]. Following 8 years of treatment with *Bs* isolate C3-41; resistance levels of 22,672-fold were detected in *C. quinquefasciatus* from China [37]. An additional case of resistance after 2 years of treatment was reported from France. Resistance levels were measured at 5958-fold [46]. Resistance levels of 750-fold were reported in a Tunisian population but little information on treatment history was provided [47]. Rapid field control failure after 5 and 19 cycles of treatments with *Bs* isolate 2362, and resistance levels of 125,00-fold, were reported in *C. quinquefasciatus* from 2 locations in Nonthaburi, Thailand [48, 49].

The number of cases of *Bs* field resistance from different continents provides abundant evidence that *C. pipiens* complex populations controlled with *Bs* have a high risk for the evolution of insecticide resistance.

Laboratory Selection for Resistance to *B. sphaericus*

Most reports on laboratory selections involved field collected *C. pipiens* complex, with or without a history of exposure to *Bs* products. The first report detailed the selection of 2 colonies for 80 generations with *Bs* 2362, one colony from laboratory culture for 25 years prior to selection (LAB) and the other a recent field collection (JRMM) (Table **4**) [50]. LAB first revealed resistance in generation 20 with a RR_{50} of 8.1. Resistance eventually reached 37-fold by generation 80. JRMM evolved a low level of resistance (RR_{50} = 4.4) in generation 5 and resistance subsequently fluctuated between 27.4 – 30-fold. Resistance reached 27.4-fold in generation 80. A field collection (ADAK) showing low levels of resistance was selected for 6 generations with Spherix™ and achieved 52,00-fold resistance [44]. A field-resistant collection (KOCHI) with an initial resistance level of 146-fold, was selected using *Bs* 1593 for 18 generations [36]. Final resistance levels were 6,223 and 31,325 at the LC_{50} and the LC_{95}, respectively. High levels of resistance rapidly evolved to *Bs* 2362 in a field collection from Fresno, CA (BSR) [51]. Resistance was evident by generation 5 and 7000-fold resistance was observed by generation 12 (Fig. **3**). Laboratory selections were undertaken with the SPHAE colony [52]. Field evolved resistance was 70-fold but laboratory selection subsequently increased it to 23,000-fold. That same report documented further laboratory selection of the KOCHI strain from India using 1593 [52]. Resistance increased from 146-fold in the field to 31,325 after 7 generations of laboratory selection. A second field resistant collection (BP) from southern France, whose initial resistance levels were not reported, was selected with *Bs* 2362 to a RR_{50} of 5,958 [46]. An uncharacterized field resistant collection from Tunisia (TUNIS) was selected with *Bs* 2362 for 18 generations and attained > 5,000-fold resistance [47]. Parallel laboratory selection studies were undertaken using 3 different isolates, C3-41, *Bs* 2362, and IAB-59 against field-collected, low-level resistant larvae and field-collected, susceptible larvae [34]. The low-level resistant larvae were divided into 2 colonies and selected with C3-41 (RLCq1/C3-41) and IAB59 (RLCq2/IAB59), whereas the field-collected, susceptible larvae were selected with 2362 (CqRL1/2362) and IAB-59 (CqRL2 /IAB59).

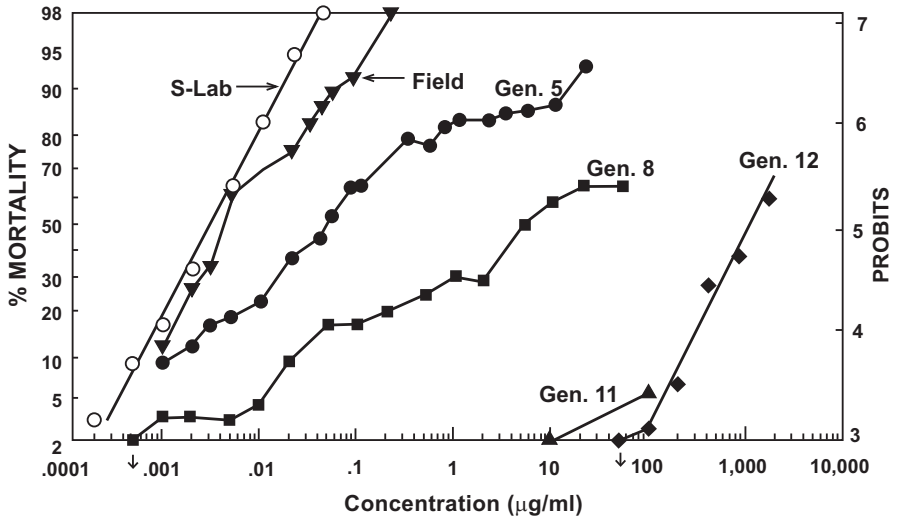

Fig. (3). Dose-response regression lines of the field-collected strain of *C. quinquefasciatus* and generations 5, 8, 11, and 12 of the *C. quinquefasciatus* strain Bs-R toward *B. sphaericus* 2362. S-Lab is the susceptible reference colony. Wirth MC, Georghiou GP, Malik JI, and Abro GH. J Med Entomol 2000; 37: 534-540.

After 13 and 18 generations of selection, the RLCq1/C3-41 showed >144,00-fold resistance to C3-41 and RLCq2/IAB59 showed 46.3-fold resistance to IAB-59 [34]. CqRL1/2362 evolved >162,00-fold resistance to *Bs* 2362 and CqRL2/IAB59 evolved 5.7-fold resistance to IAB-59. The CqRL2/IAB59 colony was eventually selected to very high levels of resistance (RR_{50} = 40,000) [35].

There is a single report of laboratory selection of *Anopheles stephensi* Liston using Spherix™. After 4 cycles of selection the colony, Sarojini Nagar, attained more than 18,000-fold resistance at the LC_{50} [53].

Laboratory selection studies provided a warning of the risk of resistance associated with *Bs*, and the first laboratory reports appeared just as the first case of field resistance was detected. The populations developed by laboratory selections have been invaluable in documenting the levels of resistance that mosquitoes can reach under intensive selection pressure and they also provided researchers with relatively homogenous populations that could be investigated for their cross-resistance spectra, their patterns of inheritance, and ultimately used to identify the receptor for *Bs* as well as the molecular basis of *Bs* resistance.

Inheritance of Resistance to *B. sphaericus*

Information on the inheritance of resistance has been reported for 9 selected colonies, including ADAK, BSR, SPHAE, BP, Tunisia, CqRL1/2362, RLCq2/IAB59, RLCq1/C3-41, and CqRL2/IAB59 (Table **4**). All colonies showed recessive inheritance of *Bs*-resistance in F1 hybrid offspring resulting from crosses between a resistant and a susceptible parent (Fig. **4**) [35, 44, 46, 47, 51, 54, 55]. Resistance was a monofactorial character in all colonies except ADAK, whose bioassay data for the backcross generation was not consistent with a monofactorial pattern of inheritance [44]. Three colonies reported that *Bs*-resistance was a sex-linked trait; SPHAE, BP, and Tunisia [46, 47, 54]. The remaining colonies showed no linkage between the sex factor and the *Bs*-resistance trait [35, 44, 51, 55].

Inheritance of *Bs* resistance in *An. stephensi* was found to be recessive, monofactorial and showed no sex linkage [56].

Cross-Resistance to Various *Bs* Isolates

Although a relatively small number of *Bs* isolates are used in commercial larvicides, a much larger number of isolates have been identified and tested. Some of these isolates, in addition to the more commonly used isolates 2362, 1593, and C3-41, have been assayed to determine the cross-resistance spectra of selected colonies. This information is useful for identifying isolates that could potentially be used against resistant populations and reveals common patterns of resistance and susceptibility among the different resistant populations.

In general, mosquito larvae that were selected with *Bs* showed high cross-resistance to isolates 1593, 2297, 2362, IAB-881, IAB-872 and C3-41 [51, 52, 57-59] (Table **4**). Low to moderate cross-resistance was reported to isolates IAB-59, 47-6B, and C3-41 [34, 52]. It should be noted that the *Bs* resistant colonies with low to moderate resistance to their selection isolate, LAB and JRMM showed low to moderate cross-resistance to other isolates, as would be expected for more weakly resistant insects [50, 52, 57]. However in the high resistance insects, very high levels of cross-resistance were observed, as stated above, with some interesting exceptions. For example, cross-resistance to isolate 2297 varied among the high resistance mosquito colonies. KOCHI was reported to have only moderate cross-resistance to 2297 (18.2-fold) [58], whereas BSR [51], RLCq1/C3-41, RLCq2/IAB59 [34] had very high cross-resistance (1,000 – 48,000-fold). SPHAE revealed low cross-resistance to IAB-59 (2.7-fold), while KOCHI [52], BSR [51], RLCq1/C3-41, and CqRL1/2362 [34, 59] reported moderate levels of cross-resistance (14.7 – 26-fold). LPG-1 and 47-6B were tested on 2 resistant lines, RLCq1/C3-41 and RLCq2/IAB59, and no cross-resistance was observed [59].

Interestingly, mosquitoes selected for resistance to IAB-59 evolved low to moderate (5.7 – 46.3) levels of resistance -

Table 4. Laboratory Selections, Inheritance, and Cross-Resistance Toward Various Isolates of *Bacillus sphaericus* in Mosquitoes

Colony (Location)	Bs Isolate	Generations Selected	Resistance Levels	Inheritance	Cross-Resistance Levels (RR)	References
***Culex pipiens* complex**						
LAB (CA, USA)	Bs 2362	F80	RR_{50} = 37		1593 (21.6) 2297 (11.7) Bti - none	[50]
JRMM (CA, USA)	Bs 2362	F80	RR_{50} = 27.4		1593 (6.8) 2297 (4.5) Bti – none IAB-59 (8.2) IAB-881 (5.1) IAB-872 (10.6)	[57, 52]
ADAK (India)	Bs B-101	F6	RR_{50} = 52,000	Recessive Not monofactorial Not sex-linked	Bti - none	[44]
Kochi (India)	Bs 1593	F7	RR_{50} = 6,223 RR_{50} = 31,325		2362 (4,125) 2297 (18.2) Bti – none IAB-881 (>270) IAB-872 (>409) IAB-59 (14.7)	[36, 52, 58]
BSR (CA, USA)	BS 2362	F12	RR_{50} = 7,000	Recessive Single Locus Not sex linked	1593 (> 1,000) 2297 (>1,000) IAB-59 (16.0) 2173 (=ISPC5) (none) Bti – none IAB-881 (>35) IAB-872 (>173)	[51, 52]
SPHAE (France)	Bs 2362	F8	RR_{50} = 23,000	Recessive Single locus Sex-linked	IAB-881 (2285) IAB-872 (3478) IAB-59 (2.7)	[52, 54]
BP (France)	Bs 2362	N. A.	RR_{50} = 5,958	Recessive Single locus Sex linked		[46]
Tunis (Tunisia)	Bs 2362	F18	RR_{50} = 5,000	Recessive Sex linked		[52]
RLCq/C3-41 (China)	C3-41	F13	RR_{50} = >144,000		2362 (108,000) IAB-59 (23.7) 2297 (48,000) 1593 (85,700) 47-6B (1.9) Bti - none	[34, 59]

Table 4. Contd….

Colony (Location)	Bs Isolate	Generations Selected	Resistance Levels	Inheritance	Cross-Resistance Levels (RR)	References
CqRL1/2362 (Brazil)	Bs 2362	F46	RR_{50} > 162,000	Recessive Monofactorial Not sex linked	C3-41 (32,000) IAB-59 (26) Bti – none	[34, 55]
RLCq2/IAB59 (China)	IAB-59	F18	RR_{50} = 46.3	Recessive Monofactorial Not sex linked	2362 (132,000) C3-41 (>144,000) 2297 (45,000) 1593 (116,000) 47-6B (1.2) LPG-1 (2.8) Bti - none	[34, 55, 59]
CqRL2/IAB59 (Brazil)	IAB-59	F12	RR_{50} = 5.7		2362 (5.5) C3-41 (57.1) Bti – none	[34]
CqRL2/IAB59 (Brazil)	IAB-59	F72	RR_{50} = 40,000	Recessive	2362 (69,000) Bti – none	[35]
Anopheles stephensi						
Sarojini Nagar (India)		F4	RR_{50} > 18,000	Recessive Monofactorial Not sex linked	Bti – none	[24, 53]

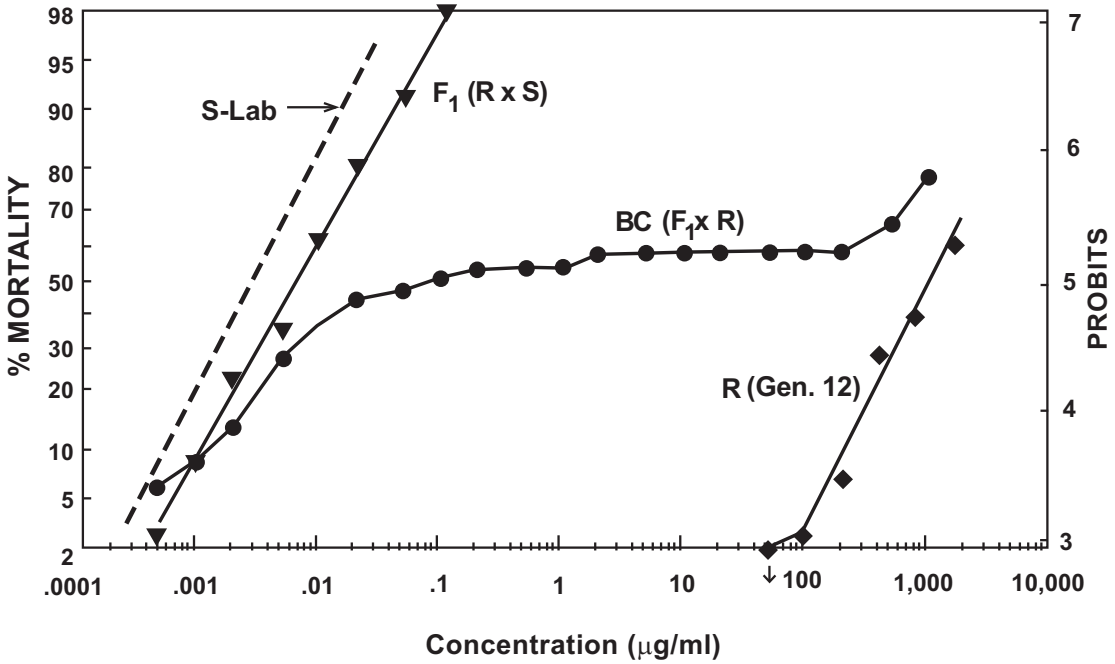

Fig. (4). Dose-response regression lines for the *C. quinquefasciatus* colonies used in the genetic crosses. S-Lab is the susceptible reference line. R is the *B. sphaericus* resistant colony (gen. 12) F1 (R X S) are the offspring of the cross BSR ♀ X Slab ♂, and BC (F1 X R) are the offspring of the backcross (BSR♀ X Slab ♂) F1♀ X BSR ♂. Wirth MC, Georghiou GP, Malik JI, and Abro GH. J Med Entomol 2000; 37: 534-540.

but were highly cross-resistant to 2362, C3-41, 1593, and C3-41 (57.1 – 144,000-fold) [34, 59]. CqRL2/IAB59 initially evolved low resistance, 5.7-fold after 12 generations of selection and showed much higher cross-resistance to C3-41 (57.1-fold). That colony eventually evolved very high levels of resistance after 72 generations of selection, reaching 40,000-fold and showed even higher cross-resistance to isolate 2362 (69,000) [33].

Because the activity of all highly active *Bs* isolates depends primarily on the Bin toxin acting at the α-glucosidase receptor, it is not surprising to find high levels of cross-resistance among various *Bs* isolates in highly *Bs* resistant mosquitoes. Recently, two new toxins, Cry48A and Cry49A, were identified in IAB-59 [60]. Cry48A and Cry49A act as a binary toxin; they exhibit high activity to both susceptible and Bin-resistant *C. quinquefasciatus* when presented in a 1:1 molar ratio. Their presence may explain the high activity of IAB-59 toward Bin-resistant *Culex* [60] and the slower evolution of resistance associated with that isolate [34].

Cross-Resistance to *Bacillus thuringiensis* Subsp. *israelensis* (Bti)

No cross-resistance to the bacterial strain *Bti* has been reported in either *C. pipiens* complex or *An. stephensi* (Table **4**).

Mechanism of Bs-Resistance in Mosquito Larvae

The receptor for Bin toxin in wild-type *C. pipiens* was identified as a α-glucosidase that is anchored to the epithelial membrane by a glycosylphosphatidylinositol (GPI); an allele known as *cpm1* [40, 41]. The first mechanism of *Bs* resistance was identified as the failure of Bin toxin to bind to the brush-border membrane of midgut epithelial cells [61] in the BSR colony (Table **5**). Subsequently, loss of binding was identified in 3 other colonies, BP from France [54], CqRL1/2362 from Brazil [55], and RLCq1/C3-41 from China [55]. However 3 colonies that were tested for binding changes showed no loss of binding. The SPHAE colony from France showed no loss of binding and no further information on mechanism is available [34]. The low-level field resistant colony from Brazil (FIELD) showed a small decrease in receptor concentration but no change in binding affinity [45]. The Tunisia colony, TUNIS, showed no loss of binding and the underlying mechanism of *Bs* resistance is unknown [48].

Among the 4 colonies whose mechanisms of resistance involved failure to bind Bin, 4 different molecular mechanisms have been identified. A mutation that causes the premature termination of translation and leads to loss of the glycosylphosphatidylinositol membrane anchor that holds the α-glucosidase Bin receptor to the cell membrane, was reported in colony BSR ($cpm1_{GEO}$) [62]. A 19-nucleotide deletion in the amino acid sequence of the α-glucosidase gene originates a premature stop codon and prevents the synthesis of a full-length receptor in colony CqRL1/2362 ($cqm1_{REC}$) [63]. In the case of BP, two mechanisms of Bin resistance evolved. $cpm1_{BP}$ shows 5 amino acid substitutions and a nonsense mutation. $cpm1_{bp}$-del has a 198 base pair internal deletion [64]. Whereas $cpm1_{BP}$ was secreted into the extracellular space because of lack of the GPI anchor, $cpm1_{BP}$-del does not interact with the Bin toxin [64]. Although loss of binding was identified in the RLCq1/C3-41 colony, no molecular basis for the failure to bind has been identified [55].

Fitness Costs Associated with *Bs*-Resistance

The proportion of genes that an individual leaves in the population gene pool determines its reproductive success or fitness [65]. The reproductive success of a *Bs*-resistant individual therefore has significant influence on the evolution of resistance in mosquito population. In the presence of the insecticide, resistant individuals have a distinct advantage over susceptible individuals due to the genes that enable them to survive exposure to the insecticide. However in the absence of insecticide exposure, the resistance genes are not likely to confer a particular advantage, and may confer a distinct disadvantage, such that the resistant individual has lower fitness than a susceptible individual. The reduction is fitness may accelerate the decline in frequency of resistance alleles in the absence of insecticide. Fitness costs were found to be associated with conventional insecticide resistance in mosquitoes [66-69].

The LAB and JRMM selected colonies that showed 37 and 31-fold resistance to *Bs*, respectively, were examined for effects on fitness [70]. Both *Bs*-resistant colonies showed significantly lower fecundity and fertility. However the resistant colonies showed significantly higher survival rates and a greater percentage of emergence than the control colonies, possibly due to lower larval densities. The overall

Table 5. Mechanisms of Resistance to *Bacillus Sphaericus* Reported in *Culex Pipiens* Complex

Colony	Location	Resistance Level	Resistance Allele	Mechanism of Resistance	Reference
BSR	California, USA	> 10,000	$cpm1_{GEO}$	Mutation causing loss of the GPI membrane anchor	[61, 62]
SPHAE	France	> 10,000	unknown	No loss of binding, mechanism is unknown	[54]
BP	France	5958	$cpm1_{BP}$ $cpm1_{BP}$.del	Nonsense mutation, loss of GPI anchor Transposon-mediated insertion, loss of membrane anchor	[47, 64]
Field	Brazil	9.7	unknown	Decrease in concentration of receptor	[45]
TUNIS	Tunisia	> 5,000	Unknown	No loss of binding, mechanism is unknown	[47]
CqRL1/2392	Brazil	> 162,000	$cqm1_{REC}$	19 nucleotide deletion that causes premature stop codon	[55, 63]
RLCq1/C3-41	China	> 144,000	unknown	Loss of binding	[55]

yield of adults and the yield of females per raft were lower because of the reductions in fecundity and fertility. *Bs*-resistant males were reported to develop more slowly than their control counterparts, whereas resistant females developed more quickly [70].

A second study of fitness in highly *Bs*-resistant mosquitoes reported slightly different effects [71]. The CqRL1/2362 resistant mosquitoes demonstrated delayed oviposition, showed longer embryonic development time, laid significantly fewer eggs, and showed lower hatching rates than the parental colony. The latter 2 effects resulted in a lower yield of adults.

Both studies concluded that the resistant mosquitoes were at a disadvantage in the absence of the insecticide, which combined with immigration of susceptible individuals from other areas in the field, might result in the decline in the frequency of resistance alleles in the absence of insecticide exposure. Some evidence for this is seen in 2 reports, both of which observed the rapid reversal of resistance to *Bs* in the field when treatment stopped. The Recife, Brazil population became susceptible within 16 months of treatment cessation [72], whereas the field population in China, resistance declined from 22,676 to 5.78-fold within 6 months of stopping treatment [37].

MANAGEMENT OF RESISTANCE TO BACTERIAL INSECTICIDES IN MOSQUITO POPULATIONS

The principles of insecticide resistance management have been well studied experimentally and theoretically. The goal of such strategies is to delay, ideally for an indefinite time, the evolution of resistance. Georghiou [73] classified resistance management strategies into 3 broad categories; management by moderation, management by saturation, and management by multiple attacks. Management by moderation recognizes the value of susceptible genes in insect populations and seeks to preserve that susceptibility by limiting the selection pressure that is applied. For example, susceptible alleles can be preserved by maintaining areas of refuge, in which no treatment occurs and from which insects can provide a source of susceptible alleles to dilute the frequency of resistance alleles in nearby treated areas. This strategy was employed to manage Cry toxin resistance in genetically modified cotton, [74, 75] but since refuge size requirements can be large and setting aside untreated mosquito breeding sites is counter to the need for high levels of mosquito control to disrupt disease transmission, large untreated areas are not well suited for management of vector borne diseases. However many breeding sites in a treated area are unreachable or not identified, thus some areas escape treatment and serve as *de facto* refuges. Management by saturation indicates saturation of insect defenses by highly targeted insecticides that can kill heterozygous resistant insects while they are relatively rare in populations. One example of saturation was the use of synergism to suppress detoxication enzymes. This has been used effectively in both agricultural and household pest control and may be applicable in concept to vector control. Management by multiple attacks involves control through the use of several independently acting materials that can be incorporated into rotations or mixture strategies. This strategy also has application to vector control.

Bti presents a low risk for resistance because it produces a diverse mixture of insecticidal toxins that interact with one another synergistically to increase toxicity toward *Ae. aegypti, C. pipiens,* and *An. stephensi* [76, 77]. In addition to increasing activity against mosquitoes, these synergies were shown to slow the rate of evolution of resistance. Cyt1A plays the most significant role, since it was demonstrated that resistance evolves to Cry toxins in CytA's absence [26] and that resistance is suppressed if Cyt1A is combined with the Cry toxins [32]. Furthermore, when Cyt1A is combined with a single Cry toxin, the mixture can significantly delay resistance and reduce the level of resistance compared to the resistance that evolves toward either individual toxin [33]. An additional benefit is the short residual that *Bti* has after application that significantly limits the selection pressure exerted on populations. Inevitably some breeding sites escape treatment so that some proportion of the populations escapes exposure. These natural characteristics place *Bti* within 2 categories of resistance management, moderation due to its short residual and the likely presence of refuges due to incomplete coverage, and saturation of the mosquito's defenses by the synergy of its components. These traits are strongly advantageous to avoiding resistance, although there is no guarantee that resistance will never evolve due to the great variability in field conditions and insect populations.

Bs lacks this inherent capacity to delay resistance. It targets a single receptor in the mosquito larval midgut, and this single-site action places *Bs* at high risk for selecting resistance. However the cases of *Bs* field resistance have revealed variable rates and levels of resistance evolution resulting from the different treatment practices and environmental conditions unique for each location. Although many of the cases of field-resistance show the rapid evolution of *Bs* resistance [36, 37, 43, 48, 49], treatment with *Bs* does not inevitably lead to resistance. This was observed in a program in Recife, Brazil where 2 sites were followed over a 3-year period during which bi-monthly *Bs* treatments occurred [78]. The sites included urban breeding sites such as septic tanks and areas along the banks of the Pinheiros River. Along the river, 25 treatments with *Bs* were followed by 5 cycles of treatment with *Bti*. These sites were monitored because of the risk for resistance but no significant changes in susceptibility were detected from either location. The authors concluded that 2 factors were important: the urban developmental sites that escaped treatment with *Bs* due to logistical difficulties and the *Bti* treatment cycles along the river that followed *Bs* treatments. They also concluded that monitoring was essential to ensuring the effectiveness of *Bs*.

Bti/Bs rotation and mixture strategies were tested in the laboratory to determine whether they were effective at reversing pre-existing *Bs* resistance [79]. A *Bs* resistant colony was selected with *Bti* for 10 generations and resistance declined more than 50%. However when the *Bs* selections were resumed, *Bs* resistance increased although resistance remained lower than before the *Bti* selections. When the *Bs* resistant colony was selected with *Bti* and *Bs* in rotation, alternating every generation for 30 generations, *Bs* resistance declined about 80%. When the *Bs* resistant colony was selected with a mixture of *Bs* and *Bti*, at a 2:1 ratio for the first 5 generations and a 1:1 ratio thereafter, substantial declines in *Bs* resistance were observed. The authors

concluded that both rotation and mixture strategies were equally effective tactics for reversing *Bs* resistance.

In a separate study, the effect of mixtures and rotations on the evolution of resistance in susceptible mosquito populations was tested. *C. quinquefasciatus* larvae were selected with *Bs* alone, *Bs* alternating with *Bti* every other generation, or *Bs* and *Bti* in a mixture [80]. The colony selected with *Bs* alone showed resistance levels of 11.1 fold at the LC_{95} after 33 generations of pressure. When *Bs* and *Bti* were mixed, in a 2:1 ratio for the first 5 generations and subsequently at a 1:1 ratio, no significant resistance evolved to *Bs*, *Bti* or to the mixture of *Bs* and *Bti*. However, the colony selected with the rotation series of *Bs* and *Bti* rapidly evolved resistance to *Bs*, but not to *Bti*. *Bs* resistance was first detected in generation 10, with 3.5 fold at the LC_{95}, and reached 43.8-fold by generation 25. This level of resistance exceeded that observed in the colony selected with *Bs* alone. The data suggest that the mixture strategy is preferable to rotating *Bs* and *Bti* to avoid resistance in susceptible populations.

In Thailand, field populations of *C. quinquefasciatus* were successfully treated in the field with a mixture of *Bs* and *Bti*, and no control problems were observed after 9 treatments in 9 months, whereas control problems appeared after 9 treatments with *Bs* alone and control failure occurred after 17 treatments [49].

Different isolates of *Bs* may vary in their capacity to select for resistance. Selection studies in the laboratory using *Bs* isolate IAB59 revealed slower rates of resistance evolution and lower levels of resistance than observed in parallel selection lines with isolates 2362 and C3-41 [34]. Resistance to IAB59 was 5.7 and 46-fold after 18 and 12 generations of selection, whereas resistance levels in the same number of generations was > 100,000-fold for C3-41 and 2362. Although a later report showed that high levels of resistance could eventually evolve to IAB59 (40,000-fold), the process was slower than for the other isolates. IAB59 is known to produce Mtx toxins in addition to the Bin toxins [4], but as these toxins are primarily present only during vegetative phase, their contribution to strain activity in their native form is limited. As mentioned previously, Cry48A and Cry49A, were identified in IAB-59 and show activity to susceptible and Bin-resistant larvae [60]. Their presence may explain the high activity of IAB-59 toward Bin-resistant *Culex* [60]. Laboratory studies have shown that the activity of *Bs* can be enhanced by artificially combining it with naturally co-occurring toxins such as the Mtx toxins or with toxins from other bacteria with mosquitocidal activity such as *Bti*. For example, recombinant bacteria expressing Mtx toxins from *Bs* during sporulation synergized *Bs* and Cry toxins against susceptible and resistant mosquitoes [81]. Cyt1A from *Bti* was also shown to suppress high levels of *Bs* resistance [82] and *Bti* Cry toxins can interact synergistically with *Bs* and reduce resistance [83]. For example, a combination of *Bs* and *Bti* (5:1) was sufficient to completely suppress high levels of *Bs* resistance. In addition, Cyt1A or *Bti* combined with *Bs*, extended activity to *Aedes aegypti*, an important vector species that is not naturally susceptible to *Bs* [83, 84]. These data and others suggest that engineering *Bs* or *Bti* to express additional mosquitocidal toxins may prove an effective strategy for managing insecticide resistance and extending the host range of *Bs*.

Engineering *Bs* or *Bti* to express additional toxins has been investigated and some of the recombinants showed enhanced activity [for a review see 85]. However problems with stability have limited the utility of *Bs* recombinants, while *Bti* has proved more amenable to engineering. Although some early recombinants showed no particular improvement in activity relative to the wild-type strains, recent molecular genetic techniques enabled engineering for the expression of high levels of Bin toxins and *Bti*'s own toxin complement in a recombinant *Bti* strain. That strain showed increased activity against both susceptible and resistant mosquitoes [86]. Laboratory selection studies have validated the design of this recombinant as effective for avoiding the evolution of resistance [87].

Other researchers have successfully transferred the toxin-coding plasmid from *Bti* into *Bs*, and showed that the recombinant strain was able to overcome *Bs* resistance in mosquitoes [88]. Stability of the recombinant was not sustainable. Cry4A from *Bti* was cloned and expressed in *Bs* and the recombinant suppressed *Bs* resistance in larvae [89]. An acrystalliferous strain of *Bti* was engineered to express Bin, Cry11B and Cyt1A, thus incorporating toxins from *Bs*, *Bti*, and *B.t. jegathesan* [90]. That recombinant had greater activity than either *Bs* or *Bti*. *B. t. morrisoni* PG-14, which naturally produces the 4 major endotoxins found in *Bti* plus a 144 kDa Cry protein with Lepidopteran activity, was engineered to express Bin [91]. That recombinant showed improved activity against *C. quinquefasciatus* but not against *Ae. aegypti. B. t. jegathesan* was also engineered to express Bin and Cyt1A, in addition to its normal complement of endotoxins, and a 17-fold improvement in activity against *C. quinquefasciatus* was observed [91]. These examples illustrate the potential of this strategy to produce recombinant bacterial strains with novel combinations of endotoxins that have higher activity and possess resistance management properties.

Regardless of the approach taken to controlling mosquito populations and managing resistance, an essential and sometimes overlooked aspect is the need to monitor population susceptibility. There are very few reports of long-term monitoring of treated populations with the exception of *Ae. vexans* in the Rhone Valley treated with *Bti* [20]. Resistance monitoring is particularly critical for *Bs*-treated populations because of the known risk for resistance evolution in treated populations. Since *Bs* resistance is reported as a recessive trait in all populations investigated to-date, it is undetectable in standard bioassay tests if the frequency of the resistance allele is at low to moderate frequencies. This is due to the high frequency of the resistance allele in the heterozygous state whose phenotype is indistinguishable from that of susceptible insects. However, identification of the alleles for Bin resistance enables the use of diagnostic PCR assays to detect the resistance allele at low frequency. This approach was successfully demonstrated on *C. quinquefasciatus* field populations from treated and untreated areas in Recife, Brazil [92]. This test successfully detected the $cpm1_{REC}$ allele in the three areas that were sampled, two of which were nontreated and one from a *Bs*-treated area. As might be

expected, frequencies of the $cpm1_{REC}$ allele were low, 0.003 - 0.006, in the untreated populations, but were significantly higher in the treated population (0.053 – 0.055) [92]. The sensitivity of this approach combined with the identification of *Bs*-resistance alleles from various parts of the world; make diagnostic PCR a valuable tool for resistance management.

Considerable progress has been made in our understanding of the evolution of insecticide resistance to bacterial insecticides in mosquito populations. This knowledge has enabled the development of various hypothetical strategies for avoiding or managing resistance, although few controlled field studies of resistance management strategies for mosquitocidal bacteria exist. This area requires additional research before practical management programs can be widely implemented, but such data are critical to the viability of current and future insecticides. Any management plan should include monitoring the treated population's susceptibility over time, combined with a strategy to disrupt the continuous selection pressure from an individual insecticide by using one or more of the strategies discussed above (refuges, using insecticides with different modes of action, recombinant bacterial insecticides etc.). For example, an integrated approach might include insecticide treatments with 2 or more unrelated insecticides (bacterial insecticides, adulticides, insect growth regulators) with treatments targeted at different life stages (larvicides versus adulticides), combined with physical methods (abatement of seepages, cleaning weed infested irrigation channels, periodic drying of breeding sites) and medical treatments for the at-risk community. An integrated approach, designed specifically for the local conditions and limitations, will preserve the life-expectancy of insecticides in the field, delay of the evolution of insecticide resistance, and provide the efficacy and safety needed to protect the environment while controlling vector populations of mosquitoes.

Acknowledgements. Jeffrey Johnson is gratefully acknowledged for his help with figures. The US National Institute of Heath (AI45817), the U.S. Department of Agriculture (S-300), the Agricultural Experiment Station at the University of California, Riverside, and the United States- Israel Binational Science Foundation (2001-042) supported this work.

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Received: 00 00, 2010 Revised: 00 00, 2010 Accepted: 00 00, 2010