Inheritance and Mechanism of Resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from China and Brazil

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ABSTRACT Investigations on the inheritance and mechanism of resistance to *Bacillus sphaericus* Neide in *Culex quinquefasciatus* Say colonies, selected with strains C3-41 (RLCq1/C3-41) and 2362 (CqRL1/2362), were performed in China and Brazil, respectively. The progeny of reciprocal F1 crosses (susceptible female × resistant male and vice versa) from both resistant colonies responded alike in bioassays, indicating recessive inheritance. Data on larvae susceptibility from the backcross offspring between F1 and their respective susceptible and resistant parental colonies are consistent with a monofactorial and autosomal mode of inheritance. In vitro binding assays between ^{125}I binary (Bin2) toxin and the brush border membrane fractions (BBMF) from CqRL1/2362 and RLCq1/C3-41 larvae showed that resistance, in both colonies, is caused by a failure in the binding step of the *B. sphaericus* Bin2 toxin to its specific midgut receptor. The specific and saturable binding of Bin2 toxin to BBMF from F1 larvae (CqRL1/2362 X susceptible counterpart) confirms the recessive inheritance of the resistance gene. Further studies are needed to advance understanding of *B. sphaericus* resistance.

KEY WORDS *Bacillus sphaericus*, *Culex quinquefasciatus*, resistance, inheritance, receptor

Bacillus sphaericus NEIDE has been successfully used as a larvicide in mosquito control programs (Kumar et al. 1994, Regis et al. 1995, 2000, Skovmand and Bauduin 1997). *B. sphaericus* displays a specific toxicity toward some Culicine mosquitoes because of a crystal protein that is synthesized during bacterial sporulation (Davidson and Myers 1981, Yousten and Davidson 1982, de Barjac and Charles 1983). The crystal toxin or Bin toxin is composed of 51 - and 42-kDa polypeptides (BinB and BinA, respectively) that act in synergy (Broadwell et al. 1990, Davidson et al. 1990, Nicolas et al. 1993). *B. sphaericus* strains, such as 1593, 2362, 2297, C3-41, and IAB59, that display high toxicity contain Bin toxins, and only minor differences in their amino acid sequences have been reported (Berry et al. 1989, Humphreys and Berry 1998). Although they display different levels of toxicity against target mosquitoes, the strains 1593, 2362, and C3-41 contain an identical Bin2 toxin. The mode of action of Bin toxin on *Culex quinquefasciatus* Say larvae, the major target of *B. sphaericus*, has been partially elucidated. In vitro assays showed that the most important step is the binding of the Bin toxin to a single class of specific receptors present on larval midgut cells (Nielsen-LeRoux and Charles 1992, Silva-Filha et al. 1997). With regard to the mode of action on *Culex* larvae, the BinB component is responsible for the binding to the receptor, and the BinA component confers toxicity and might form pores in the epithelial cell membrane (Nicolas et al. 1993, Charles et al. 1997, Schwartz et al. 2001). The Bin toxin receptor in *C. pipiens* larvae midgut was recently identified and cloned as being a 60-kDa α-glucosidase (Silva-Filha et al. 1999, Darboux et al. 2001).

Despite the effective and specific mode of action of *B. sphaericus*, *C. quinquefasciatus* populations can develop resistance against this agent when subjected to strong selection pressure under laboratory or field conditions. Resistance was first observed under laboratory selection carried out in California when two colonies, selected independently, displayed resistance levels of 35- and >100,000-fold (Georghiou et al. 1992, Rodcharoen and Mulla 1994, Wirth et al. 2000). Field resistance was first reported in populations of *C. pipiens* complex in the South of France (Sinègre et al. 1994). Mosquito control programs in India (Rao et al. 1995), Brazil (Silva-Filha et al. 1995), and China (Yuan et al. 2000) demonstrated both low- and high-resistance levels to *B. sphaericus* among *Culex* populations intensively treated with larvicides.

Previous investigations on other highly resistant *Culex* colonies have demonstrated the existence of different mechanisms involved in the resistance to *B. sphaericus.* The high level of resistance (100,000-fold) in a laboratory-developed colony, GEO (Georghiou et al. 1992), was related to a failure in the binding of the *B. sphaericus* crystal toxin to its midgut

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receptor (Nielsen-LeRoux et al. 1995). This was also recently found for a *Culex* field colony (BP) from Southern France (Chevillon et al. 2001, Nielsen-LeRoux et al. 2002). The resistant colony SPHAE (>10,000-fold) from Southern France (Sinègre et al. 1994) and the highly resistant colony TUNIS, from Tunisia, exhibited resistance mechanisms that were not associated with changes in binding affinity between the toxin and the midgut receptors (Nielsen-LeRoux et al. 1997, 2002). These investigations demonstrated for each colony (GEO, SPHAE, BP, and TUNIS) that resistance to *B. sphaericus* was determined by a single major recessive gene. For each Mediterranean colony (SPHAE, BP, and TUNIS), one major recessive and sex-linked gene was implicated (Chevillon et al. 2001). It is likely that several genes, each encoding for different mechanisms of resistance, are present in natural *Culex* populations (Nielsen-LeRoux et al. 1997).

Recently, the evolution of resistance was investigated in two field-collected *C. quinquefasciatus* colonies from China and Brazil, which under laboratory conditions, were subjected to strong selection pressure with *B. sphaericus* (Pei et al. 2002). After 13 and 46 generations were exposed to high concentrations of C3-41 (China) and 2362 (Brazil), these colonies developed >144,000- and 162,000-fold resistance to those strains, respectively (Pei et al. 2002). Both resistant colonies obtained after selection with *B. sphaericus* C3-41 and 2362 showed high levels of cross-resistance to *B. sphaericus* 2362 and C3-41, respectively, but displayed only a low cross-resistance to the strain IAB-59 (Pei et al. 2002). The main aim of the current study was to elucidate the mode of inheritance of resistance genes and the mechanism of resistance displayed by these colonies, highly resistant to strains C3-41 and 2362. Understanding these aspects is essential for improving resistance monitoring, detection, and management in vector control programs in regions such as China and Brazil that are particularly concerned with mosquito-borne diseases.

Materials and Methods

Mosquito Colonies. Four *C. quinquefasciatus* colonies were used in this work: (1) RLCq1/C3-41, a highly resistant (RR > 144,000) colony selected with *B. sphaericus* strain C3-41 and SLCq, its susceptible counterpart; (2) CqRL1/2362, a highly resistant (RR > 162,000) colony selected with *B. sphaericus* strain 2362 and CqSF, its susceptible counterpart. Each resistant colony and its susceptible counterpart were derived from the same colony established from a large number of field collected egg rafts as described in Pei et al. (2002). The resistant colonies, RLCq1/C3-41 and CqRL1/2362, were obtained after continuous laboratory selection pressure in China and Brazil, respectively (Pei et al. 2002).

Inheritance. All cross-experiments were undertaken between the resistant colonies and the respective susceptible counterparts from China (RLCq1/C3-41 and SLCq) and from Brazil (CqRL1/2362 and CqSF) independently. Resistant (R) and susceptible (S) pupae from each colony were kept separately until adult emergence. For reciprocal cross, 200 virgin individuals were used to form 50 pairs of S (female) × R (male) and 50 pairs of R (female) × S (male). The offspring of the former pairs (F1*a*) and that of the latter (F1*b*) were assayed separately for susceptibility to *B. sphaericus.* The back-crosses were undertaken between 50 virgin individuals ([female] or [male]) from F1 and 50 virgin individuals ([female] or [male]) from S and R parental colonies. Pairs composed by crosses of F1 × S parental colonies and F1 × R parental colonies were produced similarly, and the susceptibility of their offspring (BC) to *B. sphaericus* was analyzed separately. Surviving larvae from these bioassays were reared to adults to estimate the sex ratio and determine if gene inheritance was autosomal or sex-linked.

Bioassays. Progeny (F1 and BC) susceptibility to *B. sphaericus* was analyzed by bioassays performed with early fourth instars, according to standard method recommended by the WHO (1985). For all bioassays, larvae were exposed to serial dilutions of spore-crystal lyophilized powders of strains C3-41 or 2362 for 48 h. Plastic cups held 20 larvae in 100 ml of bacterial suspensions in water, and three replicates were performed for the six concentrations tested per bioassay. A control group tested with water only was run in each experiment, and the bioassay was repeated two or three times. Mortality data were analyzed using probit analysis as described by Finney (1971). Lines representing concentration–mortality responses of susceptible and resistant colonies, F1 and BC, were constructed using the LC_{50}'s and slopes estimated from probit analyses.

Binding Assays. In vitro assays between the radiolabeled binary (Bin2) toxin from *B. sphaericus* strains 1593 and brush border membranes fractions (BBMFs) of larvae midgut from *Culex* colonies were carried out. The BBMFs were obtained from fourth instars frozen and stored without buffer at −71°C. BBMF preparation was described in Silva-Filha et al. (1997) and is based on selective divalent cation precipitation and differential centrifugation in an ice-cold buffer (0.3 M Mannitol/5 mM EGTA/20 mM Tris-HCl, pH 7.4). The BBMF protein content was measured by the Bio-Rad protein assay (Hercules, CA), and the leucine aminopeptidase (LAP) and α-glucosidase activities, enzymatic markers of BBMFs, were measured as described in Silva-Filha et al. (1997, 1999). BBMFs from the following colonies were prepared: susceptible CqSL, resistant CqRL1/2362, F1 (CqSL × CqRL1/2362), susceptible SLCq, and resistant RLCq1/C3-41. BBMF were stored in aliquots at −71°C until required. The toxin Bin2 from the strain 1593 was obtained from crystals produced in a cry-minus strain (4Q2–81) of *B. thuringiensis* serovar. *israelensis*, transformed with the plasmid pGSP10 harboring genes encoding the Bin2 toxin (Bourgouin et al. 1990). Preparation of activated toxin and its radiolabeling with ^{125}I were previously described (Nielsen-LeRoux and Charles, 1992). Labeled and unlabeled toxins were stored at 4°C in

Table 1. Toxicity of *B. sphaericus* strains C3-41 or 2362 against *C. quinquefasclatus* 4th instar larvae (L4) from resistant (R) and susceptible (S) colonies and its respective offspring (F1)

Colony (China)	Colonies tested against C3-41			Colony (Brazil)	Colonies tested against 2362		
	No. L4	LC_{50} (CI)[a]	RR[b]		No. L4	LC_{50} (CI)[a]	RR[b]
RLCql/C3-41	1,260	>840	>144,000	CqRL/2362	1,440	>4,320	>162,000
SLCq	960	0.006 (0.005–0.008)	1.0	CqSL	1,440	0.027 (0.016–0.039)	1.0
F1*a*[c]	1,260	0.008 (0.006–0.009)	1.3	F1*a*[c]	1,440	0.462 (0.244–0.715)	17.1
F1*b*[c]	1,260	0.010 (0.008–0.013)	1.7	F1*b*[c]	1,440	0.401 (0.145–0.678)	14.8

[a] LC_{50} is the concentration that kills 50% of larvae after 48 h of exposure.
[b] Resistance level is the ratio of the LC_{50} of the colony to that of the susceptible colony.
[c] F1*a* is the offspring from (R) female X (S) male and F1*b* from (S) female X (R) male.

20 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl and 0.02% sodium azide (PBS/Az). Saturation assays were performed in duplicate, at room temperature, in a total volume of 100 μl of PBS/Az (pH 7.5) buffer containing 0.1% bovine serum albumin (PBS/Az/BSA). Six increasing concentrations of ^{125}I-toxin (2–150 nM) were incubated with 20 μg of BBMFs. Nonspecific binding was determined in parallel by incubating another set of samples in the presence of an excess (1 μM) of the respective unlabeled toxin. Incubation was conducted overnight at room temperature, and the BBMF-bound toxins were separated from free toxins by centrifugation. Counting was performed in a liquid scintillation counter with scintillation cocktail, and data were analyzed using the Ligand program (Munson and Rodbard 1980).

Results

Inheritance of Resistance. The susceptibility of the heterozygous offspring (F1) resulting from crosses between the resistant colony RLCq1/C3-41 and its susceptible counterpart SLCq was similar to that of susceptible parental colony SLCq (Table 1). The concentration–mortality regression line of F1 was similar to that of the SLCq (Fig. 1A). The F1 from the cross between the resistant CqRL1/2362 colony and the

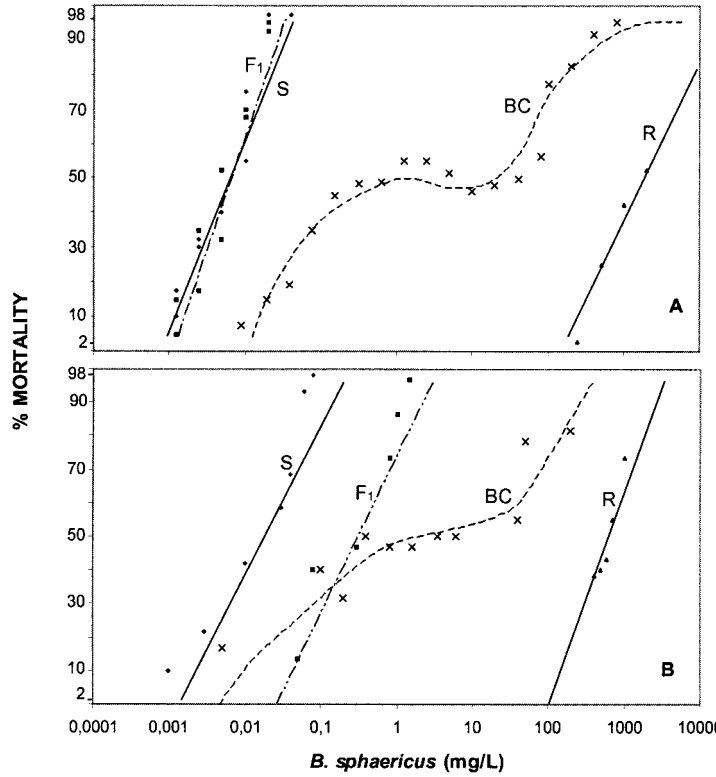

Fig. 1. Dose-mortality lines of *B. sphaericus* strains C3-41 (A) and 2362 (B) against fourth-instar *C. quinquefasciatus* larvae from susceptible (S) colonies, resistant (R) colonies, their offspring (F1), and the offspring from the back-cross F1 × parental resistant colony (BC).

susceptible colony CqSF was found to be 15- to 17-fold less susceptible than its susceptible parental (Table 1). However, its regression line was clearly close to that of the susceptible parental colony and very far from that of the resistant CqRL1/2362 colony (Fig. 1B). Data indicated that *B. sphaericus* resistance, in this case, is incompletely recessive. The reciprocal F1s had very similar LC_{50}'s: ≈0.01 mg/l for F1*a* and F1*b* from the cross RFCq2/C3-41 and ≈0.4 mg/l for F1*a* and F1*b* from the cross CqRL1/2362 (Table 1). There was no consistent difference in mortality between sexes in either F1; therefore, the F1 data were pooled for estimating concentration–mortality regression lines. Bioassay results of the offspring (BC) showed that high doses of *B. sphaericus* kill no >50% of larvae from back-cross F1 × parental resistant, while lower doses achieved close to 100% mortality of offspring from back-cross F1 × parental susceptible, and ≈75% of the cross F1 × F1 progeny (Fig. 1). Data are consistent with a monofactorial mode of inheritance. Concentration–mortality lines for the back-cross (F1 × parental resistant) offspring showed a plateau at 50–55% mortality for RLCq1/C3-41 and at 45–50% mortality for the CqRL1/2362 strain (Fig. 1), confirming that the resistance to *B. sphaericus* in both colonies is caused by a single major recessive gene. The sex ratio found among the survivors from the *B. sphaericus* exposures showed a similar number of females and males in all cases, indicating that inheritance of resistance in the China and Brazil colonies is autosomal and not sex linked.

Binding Assays. Saturation assays showed that the Bin2 ^{125}I-toxin binds specifically and with high affinity to the BBMFs from CqSL and SLCq, the susceptible colonies of Brazil and China, respectively. Binding of the toxin increased according to the concentrations employed, attaining a saturation plateau in the range of 100–150 nM of labeled toxin (Fig. 2A). Data on the Scatchard plot indicated that the ^{125}I -toxin binds to a single class of receptors present in the BBMF of CqSL (Fig. 2A). The dissociation constant (K_d) of the complex formed by the ^{125}I -toxin and the receptors was 9.8 ± 3.2 nM, and the B_{max}, estimating the concentration of receptors available, was 6.3 ± 1.2 pmol/mg of BBMFs. Saturation assays employing BBMFs from the resistant colony CqRL1/2362 showed a very weak specific binding of the ^{125}I-toxin to the midgut membranes. The specific binding was at the same level as the nonspecific binding recorded (Fig. 2B). The data obtained do not fit to a Ligand-receptor model, and the K_d and B_{max} could not be calculated for those assays. Specific binding was observed between the ^{125}I-toxin and BBMFs from F1. Data on the Scatchard plot indicated that toxin also binds to a single receptor class (Fig. 3). The saturation plateau was achieved in the range of 50–100 nM of ^{125}I -toxin employed (Fig. 3). The K_d of the complex formed by the ^{125}I -toxin and the receptors was 7.9 ± 2.1 nM, and the B_{max} was 3.8 ± 1.6 pmol/mg of BBMFs. Saturation assays between the 1593 ^{125}I-labeled Bin2 toxin and the BBMFs from the resistant RLCq1/C3-41 colony from China showed (as

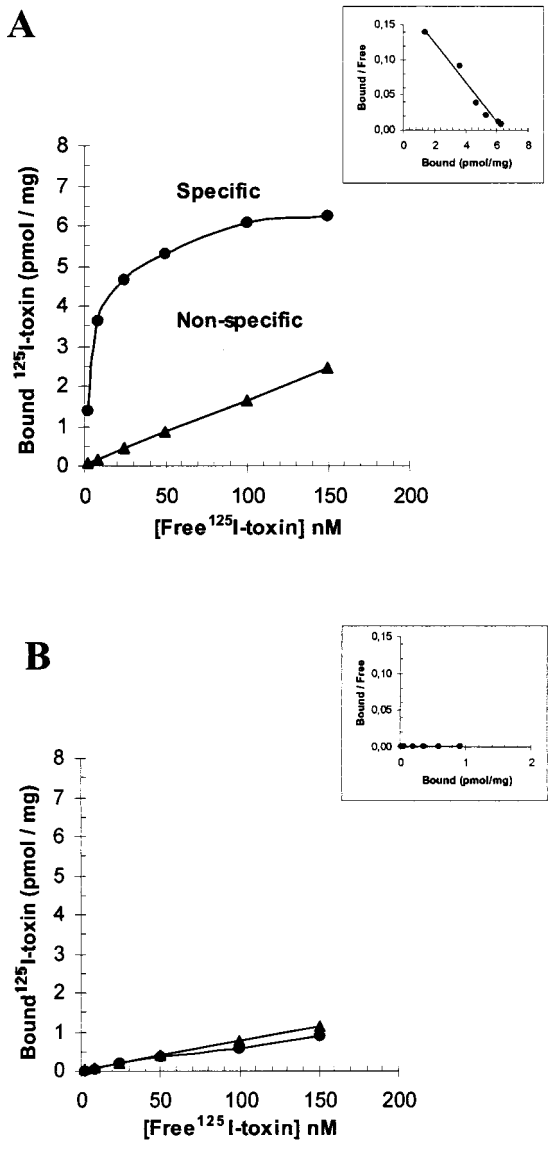

Fig. 2. In vitro saturation binding assays between *B. sphaericus* 1593 Bin2 toxin and BBMFs from fourth-instar *C. quinquefasciatus* larvae. Twenty micrograms of BBMFs was incubated for 16 h at room temperature in PBS/Az/BSA buffer, with increasing concentrations of ^{125}I-labeled toxin. Nonspecific binding was obtained from a similar set of incubations, carried out in the presence of 1 μM of free toxin. Each value is the mean of duplicate samples and data on Scatchard plot is shown in the upper side of each graphic. Assays were conducted using BBMFs from a (A) susceptible colony (CqSL) and (B) a 2362 *B. sphaericus*-resistant colony (CqRL1/2362).

was the case with the CqRL1/2362 colony) that toxin binding was lost throughout the selection and that no specific binding was found as early as in generation F8, displaying a 80,000-fold resistance (data not shown).

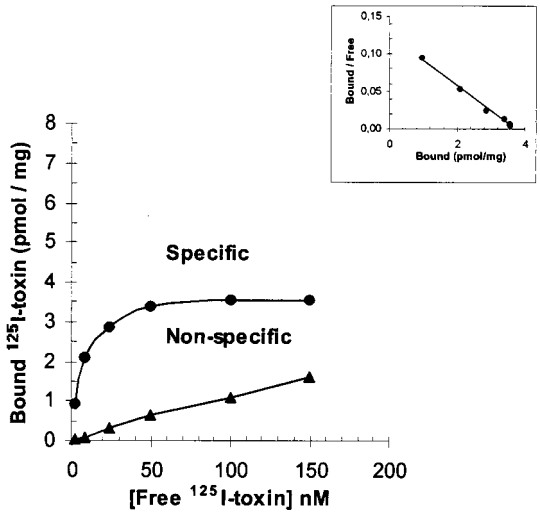

Fig. 3. In vitro saturation binding assays between *B. sphaericus* 1593 Bin2 toxin and BBMFs from fourth-instar *C. quinquefasciatus* larvae, offspring (F1) from the cross between a susceptible colony (CqSL), and a *B. sphaericus*-resistant colony (CqRL1/2362). Twenty micrograms of BBMFs was incubated for 16 h at room temperature in PBS/Az/BSA buffer, with increasing concentrations of ^{125}I-labeled toxin. Nonspecific binding was obtained from a similar set of incubations, carried out in the presence of 1 μM of free toxin. Each value is the mean of duplicate samples, and data on Scatchard plot are shown in the upper side of each graph.

Discussion

Understanding the genetic basis of resistance is fundamental for developing strategies to manage insect resistance to *B. sphaericus*. In this investigation, reciprocal crosses between susceptible and *B. sphaericus*–resistant *C. quinquefasciatus* colonies, established in independent laboratory selection procedures in China and Brazil, showed that *B. sphaericus* resistance is inherited as a recessive and autosomal trait. In both resistant colonies, an absence of sex linkage was observed such that either the female or male parent was capable of transmitting the resistance character to hybrids in the F1. For the colony RLCq1/C3-41, resistance is completely recessive, whereas for the CqRL1/2362, it is incompletely recessive, and it is possible that minor modifier gene(s) might also be involved. The single back-cross method was used to determine whether *B. sphaericus* resistance among those colonies was controlled by one locus (monogenic) or many (polygenic), as described by Georghiou (1969). The dose–response lines for the back-crosses showed clear plateaus at the 50% mortality level, indicating that in both mosquito colonies, *B. sphaericus* resistance is inherited in a simple Mendelian manner, as a single major gene. The observed dose–response lines for both back-crosses differed from those observed for polygenic inheritance, which tend to have slopes approximately parallel to those of the back-cross parents (e.g., Liu et al. 1981, Halliday and Georghiou 1985). In these resistant colonies, RLCq1/C3-41 and CqRL1/2362, as well as for colonies SPHAE and GEO that were previously studied, the *B. sphaericus* resistance was always inherited as a recessive trait under the control of a single locus (Nielsen-LeRoux et al. 1997, Wirth et al. 2000). This trait has an important role in the management of resistance because resistance development can be significantly delayed by the introduction of susceptible mosquitoes from untreated refuges within the treated area or from surrounding untreated areas. In this model, the interruption of *B. sphaericus* selection pressure, associated or not with the introduction of another control agent, can restore the population susceptibility, as previously observed in *B. sphaericus* control programs (Silva-Filha et al. 1995, Yuan et al. 2000). However, a recent study on the resistant *C. pipiens* populations intensively treated with *B. sphaericus* in the South of France provided information on the evolution of this phenomenon under field conditions (Chevillon et al. 2001). This work showed that resistance detected in two zones of the *Bs*-treated area are caused by two recessive mutant genes, and both alleles (sp-1^R and sp-2^R), although recessive, can be widespread and reach significant frequencies in some of the treated localities. The interaction between the resistant mutants remains unclear because heterozygous individuals possessing one copy of each mutant displayed a 100-fold resistance, less than the 6,000-fold level of resistance displayed when only one of those genes is present in the homozygous state (Chevillon et al. 2001). The use of the entomopathogen *B. thuringiensis* serovar. *israelensis*, for short periods in rotation with *B. sphaericus*, may be an alternative to eliminate resistant genotypes and to dilute the frequency of the resistance gene. *B. sphaericus*–resistant *Culex* colonies do not display cross-resistance to that agent because of its distinct mode of action on mosquito larvae (Nielsen-LeRoux and Charles 1992, Silva-Filha et al. 1995, Rao et al. 1995, Rodcharoen and Mulla 1996, Wirth et al. 2000, Pei et al. 2002).

Both the genetic traits and mechanism of resistance play an important role in a population response to selection pressure. In this work, we also compared the ability of the 1593 Bin toxin (Bin2) from *B. sphaericus* to bind the BBMFs from *C. quinquefasciatus* larvae to elucidate the mechanism of resistance observed for the colony CqRL1/2362 and RLCq1/C3-41. Saturation assays showed that the 1593 Bin toxin specifically binds to the BBMFs from the parental colonies (CqSL and SLCq), and the levels of binding (K_d and B_{max}) detected for these colonies were similar to other *Culex* populations previously investigated (Nielsen-LeRoux and Charles 1992, Nielsen-LeRoux et al. 1995, Silva-Filha et al. 1997). However, the 1593 Bin toxin did not show a saturable and specific binding to the BBMFs from either the resistant colony, CqRL1/2362, or RLCq1/C3-41. These assays showed that there was no clear toxin–receptor interaction, and as a consequence, the toxic action cannot be accomplished, because functional binding sites are not available in the midgut apical membranes of those larvae. The resis-

tance mechanism observed in this work for both 2362- and C3-41-resistant colonies was previously described for GEO, another laboratory-selected colony highly resistant to *B. sphaericus* (Nielsen-LeRoux et al. 1995), and recently for BP, selected under field conditions (Nielsen-LeRoux et al. 2002). Data indicate that the failure of the toxin to bind to the receptor might be the most common resistance mechanism to Bin toxin found among *Culex* populations exposed to this agent. Until now, only two reports of highly resistant *C. pipiens* colonies, SPHAE, field-selected in the Southern France (Nielsen-LeRoux et al. 1997), and TUNIS, field selected in Tunisia, show functional receptors for the Bin toxin in the midgut epithelium (Nielsen-LeRoux et al. 2002). A third resistant field-selected colony from Brazil also showed saturable toxin binding; however, because the resistance level displayed by this colony was very low (10-fold), resistance would not be expected to be strictly associated with the loss of functional receptors (Silva-Filha et al. 1995). In this work, investigation of F1 larvae (offspring from CqRL1/2362 X CqSL) showed that Bin2 toxin binds specifically to the BBMFs, which also confirms that resistance is recessive. In addition, the binding parameters indicate that individuals from F1 display different levels of susceptibility to *B. sphaericus.* This variation is reflected by a lower concentration of receptors (B_{max}), while the affinity (K_d) is similar to that of the susceptible colony. Data suggest that the decrease in the binding in those cases is not caused by a mutation in the binding site itself but more likely to a reduction of available receptor molecules (α-glucosidases). This is in agreement with previous binding studies with the GEO colony (Nielsen-LeRoux et al. 1995) and with recent findings on the molecular basis of *B. sphaericus* resistance. These experiments demonstrated that a loss of membrane anchoring of the receptor is responsible for the absence of toxin binding to the epithelial cells from larvae midgut (Darboux et al. 2002). Nevertheless those α-glucosidases are present in the larval midgut as soluble form and it is likely to play its physiological role. A previous study showed that *B. sphaericus* resistance in CqRL1/2362 colony was not associated with an important decrease in biological fitness (Oliveira et al. 2003), and further studies are necessary to elucidate the molecular basis of the mechanism of resistance in those colonies as well as to design tools for the detection of resistance genes among natural vector populations that are potential targets of *B. sphaericus* treatments.

Acknowledgments

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References Cited

Berry, C., J. Jackson-Yap, C. Oei, and J. Hindley. 1989. Nucleotide sequence of 2 toxin genes from *Bacillus sphaericus* IAB-59- Sequence comparisons between 5 highly toxinogenic strains. Nucleic Acids Res. 17: 7516.

Bourgouin, C., A. Delécluse, F. de la Torre, and J. Szulmajster. 1990. Transfer of the toxin protein genes of *Bacillus sphaericus* into *Bacillus thuringiensis subsp. israelensis* and their expression. Appl. Environ. Microbiol. 56: 340–344.

Broadwell, A. H., L. Baumann, and P. Baumann. 1990. Larvicidal properties of the 42 and 51 kilodalton *Bacillus sphaericus* proteins expressed in different bacterial hosts: evidence for a binary toxin. Curr. Microbiol. 21: 361–366.

Charles, J.-F., M. H. Silva-Filha, C. Nielsen-LeRoux, M. Humphreys, and C. Berry. 1997. Binding of the 51- and 42-kDa individual components from the *Bacillus sphaericus* crystal toxin on mosquito larval midgut membranes from *Culex* and *Anopheles* sp. (Diptera: Culicidae). FEMS Microbiol. Lett. 156: 153–159.

Chevillon, C., C. Bernard, M. Marquine, and N. Pasteur. 2001. Resistance to *Bacillus sphaericus* in *Culex pipiens* (Diptera: Culicidae): interaction between recessive mutants and evolution in the Southern France. J. Med. Entomol. 38: 657–664.

Darboux, I., C. Nielsen-LeRoux, J.-F. Charles, and D. Pauron. 2001. The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae) midgut: molecular cloning and expression. Insect Biochem. Mol. Biol. 31: 981–990.

Darboux, I., Y. Pauchet, C. Castella, M. H. Silva-Filha, C. Nielsen-Leroux, J.-F. Charles, and D. Pauron. 2002. Loss of the membrane anchor of the target receptor as a mechanism of bioinsecticide resistance. Proc. Nat. Acad. Sci. USA. 99: 5830–5835.

Davidson, E. W., and P. Myers. 1981. Parasporal inclusions in *Bacillus sphaericus.* FEMS Microbiol. Lett. 10: 261–265.

Davidson, E. W., C. Oei, M. Meyer, A. L. Bieber, J. Hindley, and C. Berry. 1990. Interaction of the *Bacillus sphaericus* mosquito larvicidal proteins. Can. J. Microbiol. 36: 870–878.

de Barjac, H., and J.-F. Charles. 1983. Une nouvelle toxine active sur les moustiques, présente dans des inclusions cristallines produites par *Bacillus sphaericus.* C. R. Acad. Sc. Paris (série III) 296: 905–910.

Finney, D. J. 1971. Probit analysis—a statistical treatment of the sigmoid response curve. Cambridge University Press, Cambridge, United Kingdom.

Georghiou, G. P. 1969. Genetics of resistance to insecticides in houseflies and mosquitoes. Exp. Parasitol. 26: 224–255.

Georghiou, G. P., J. I. Malik, M. Wirth, and K. Sainato. 1992. Characterization of resistance of *Culex quinquefasciatus* to the insecticidal toxins of *Bacillus sphaericus* (strain 2362). University of California, Mosquito Control Research, Annual Report 1992. University of California, Riverside, CA.

Halliday, W. R., and G. P. Georghiou. 1985. Inheritance of resistance to permethrin and DDT in the Southern house mosquito (Diptera: Culicidae). J. Econ. Entomol. 78: 762–767.

Humphreys, M. J., and C. Berry. 1998. Variants of *Bacillus sphaericus* binary toxin: implications of differential toxicity strains. J. Invertebr. Pathol. 71: 184–185.

Kumar, A., V. P. Sharma, P. K. Sumodan, D. Thavaseluam, and R. H. Kamat. 1994. Malaria control utilising *Bacillus sphaericus* against *Anopheles stephensi* in Panaji, Goa. J. Am. Mosq. Control Assoc. 10: 534–539.

Liu, M.-Y., Y.-J. Tzeng, and C.-N. Sun. 1981. Diamondback moth resistance to several synthetic pyrethroids. J. Econ. Entomol. 74: 393–396.

Munson, P. J., and D. Rodbard. 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107: 220–239.

Nielsen-LeRoux, C., and J.-F. Charles. 1992. Binding of *Bacillus sphaericus* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. Eur. J. Biochem. 210: 585–590.

Nicolas, L., C. Nielsen-LeRoux, J.-F. Charles, and A. Delécluse. 1993. Respective role of the 42- and 51-kDa component of the *Bacillus sphaericus* toxin overexpressed in *Bacillus thuringiensis.* FEMS Microbiol. Lett. 106: 275–280.

Nielsen-LeRoux, C., J.-F. Charles, I. Thiery, and G. P. Georghiou. 1995. Resistance in the laboratory population of *Culex quinquefasciatus* to *Bacillus sphaericus* binary toxin is due to a change in the receptor on midgut brush-border membranes. Eur. J. Biochem. 228: 206–210.

Nielsen-LeRoux, C., F. Pasquier, J.-F. Charles, G. Sinègre, B. Gaven, and N. Pasteur. 1997. Resistance to *Bacillus sphaericus* involves different mechanisms in *Culex pipiens* mosquito larvae (Diptera: Culicidae). J. Med. Entomol. 34: 321–327.

Nielsen-LeRoux, C., N. Pasteur, J. Pretre, J.-F. Charles, H. B. Sheikh, C. Chevillon. 2002. High resistace to *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae): the complex situation of west-mediterranean countries. J. Med. Entomol. 39: 729–735.

Oliveira C.M.F., M. H. Silva-Filha, J. Beltrán, C. Nielsen-Leroux, G. Pei, Z. Yuan and L. Regis. 2003. Biological fitness of *Culex quinquefasciatus* (Diptera: Culicidae) larvae resistant to *Bacillus sphaericus.* J. Am. Mosq. Control Assoc. 19: 125–129.

Pei, G., C.M.F. Oliveira, Z. Yuan, C. Nielsen-Leroux, M. H. Silva-Filha, J. Yan, and L. Regis. 2002. A strain of *Bacillus sphaericus* causes a slower development of resistance in *Culex quinquefasciatus.* Appl. Environ. Microbiol. 68: 3003–3009.

Rao, D. R., T. R. Mani, R. Rajendran, A.S.J. Joseph, and A. Gajanana. 1995. Development of a high level of resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. J. Am. Mosq. Control Assoc. 11: 1–5.

Regis, L., M. H. Silva-Filha, C.M.F. Oliveira, E. M. Rios, S. B. Silva, and A. F. Furtado. 1995. Integrated control measures against *Culex quinquefasciatus,* the vector of filariasis in Recife. Mem. Inst. Oswaldo Cruz. 90: 115–119.

Regis, L., C.M.F. Oliveira, M. H. Silva-Filha, S. B. Silva, A. Maciel, and A. F. Furtado. 2000. Efficacy of *Bacillus sphaericus* in control the filariasis vector *Culex quinquefasciatus* in an urban area of Olinda, Brazil. Trans. Royal Soc. Trop. Med. Hygiene. 94: 488–492.

Rodcharoen, J., and M. S. Mulla. 1994. Resistance development in *Culex quinquefasciatus* to the microbial agent *Bacillus sphaericus.* J. Econ. Entomol. 87: 1130–1140.

Rodcharoen, J., and M. S. Mulla. 1996. Cross-resistance to *Bacillus sphaericus* strains in *Culex quinquefasciatus.* J. Am. Mosq. Control Assoc. 12: 247–250.

Schwartz J.-L., L. Potvin, F. Coux, J.-F. Charles, C. Berry, M. J. Humphreys, A. F. Jones, I. Bernhart, M. Dalla Serra, and G. Menestrina. 2001. Permeabilization of model lipid membranes by *Bacillus sphaericus* mosquitocidal binary toxin and its individual components. J. Membrane Biol. 184: 171–183.

Silva-Filha, M. H., L. Regis, C. Nielsen-LeRoux, and J. F. Charles. 1995. Low-level resistance to *Bacillus sphaericus* in a field treated population of *Culex quinquefasciatus.* J. Entomol. 88: 525–530.

Silva-Filha, M. H., C. Nielsen-LeRoux, and J.-F. Charles. 1997. Binding kinetics of *Bacillus sphaericus* binary toxin on midgut brush border membranes of *Anopheles* and *Culex* sp. larvae (Diptera: Culicidae). Eur. J. Biochem. 90: 299–303.

Silva-Filha, M. H., C. Nielsen-LeRoux, and J.-F. Charles. 1999. Identification of the receptor of *Bacillus sphaericus* crystal toxin in the brush border membrane of the mosquito *Culex pipiens* (Diptera: Culicidae). Insect Biochem. Molec. Biol. 29: 711–721.

Sinègre, G., M. Babinot, J. M. Quermei, and B. Gaven. 1994. First occurrence of *Culex pipiens* resistance to *Bacillus sphaericus* in Southern France. Proceedings, 8th European Meeting of Society for Vector Ecology, 5–8 September 1994, Barcelona, Spain. Society for Vector Control, Santa Ana, CA.

Skovmand, O., and S. Bauduin. 1997. Efficacy of a granular formulation of *Bacillus sphaericus* against *Culex quinquefasciatus* and *Anopheles gambiae* in West Africa Countries. J. Vector Ecol. 22: 43–51.

[WHO] World Health Organization. 1985. Informal consultation on the development of *Bacillus sphaericus* as microbial larvicide. TDR/BCV/SPHAERICUS 85.3, 1–24. WHO, Geneva, Switzerland.

Wirth, M. C., G. P. Georghiou, J. I. Malik, and G. H. Abro. 2000. Laboratory selection for resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from California, USA. J. Med. Entomol. 37: 534–540.

Yousten, A. A., and E. W. Davidson. 1982. Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. Appl. Environ. Microbiol. 44: 1449–1455.

Yuan, Z. M., Y. M. Zhang, and E. Y. Liu. 2000. High-level field resistance to *Bacillus sphaericus* C3-41 in *Culex quinquefasciatus* from Southern China. Biocontrol Sci. Technol 10: 43–51.

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