High Resistance to *Bacillus sphaericus* Binary Toxin in *Culex pipiens* (Diptera: Culicidae): The Complex Situation of West Mediterranean Countries

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J. Med. Entomol. 39(5): 729–735 (2002)

ABSTRACT This study was aimed at clarifying the nature of the resistance to *Bacillus sphaericus* Neide (*Bs*) that *Culex pipiens* L. has developed in west Mediterranean countries, France, and Tunisia. Two recessive and sex-linked mutants, *sp-1*^{R} and *sp-2*^{R}, were previously detected in southern France. Here, the Tunisian resistance was also shown to involve a single recessive and sex-linked gene that was temporarily named *sp-T*^{R}. In addition, *sp-1*^{R}, *sp-2*^{R}, and *sp-T*^{R} were shown to separately confer a similar high resistance level (>5,000-fold) in the homozygous state. Knowing that *sp-1*^{R} resistance does not alter the binding of *Bs* binary toxin to its specific receptor, we investigated this character in *sp-2*^{RR} and *sp-T*^{RR} homozygotes. This was performed by in vitro experiments in which larval brush border membrane fractions (BBMF) were exposed to the ^{125}I-Bin2 toxin of *B. sphaericus* strain 1593. The toxin-receptor binding was found disrupted by *sp-2*^{R} but not by *sp-T*^{R}. Comparing the binding kinetics among nine *Culex pipiens* strains of diverse origins revealed that the *Bs* receptors of *sp-1*^{RR} and *sp-T*^{RR} homozygous larvae were displaying the highest affinity toward *Bs* binary toxins. These results are discussed with regard to alternative assumptions on the dynamics of high *Bs*-resistance and on the emerging possibilities to test them in a near future.

KEY WORDS *Culex pipiens*, *Bacillus sphaericus*, resistance, toxin-receptor binding, gene identification

SINCE THE LATE 1980s, a number of *Bacillus sphaericus* Neide (*Bs*) strains have been increasingly used worldwide to control *Culex pipiens* L. The success of this biopesticide is due to high specificity toward this mosquito and to a low risk of cross-resistance with the organophosphate insecticides previously used to control *C. pipiens* larvae (Nielsen-LeRoux et al. 1997, Wirth et al. 2000b). Several points are nevertheless raising concern on the long-term efficacy of *Bs*-control. First, spot field resistance cases were reported in India, Brazil, China, and France (Rao et al. 1995, Silva-Filha et al. 1995, Nielsen-LeRoux et al. 1997, Yuan et al. 1998, Yuan et al. 2000, Chevillon et al. 2001). Second, overseas dispersal of organophosphate resistance mutants is well documented in the *C. pipiens* species (last reviewed in Chevillon et al. 1999). Finally, *Bs*-resistance in Mediterranean France was recently shown to involve distinct and interacting factors (Chevillon et al. 2001). Two of them, *sp-1*^{R} and *sp-2*^{R}, separately confer a >5,000-fold resistance that is totally recessive, with the exception of a >100-fold resistance in *sp-1*^{RS} *sp-2*^{RS} double heterozygotes. In field populations, such an interaction will protect each recessive mutant from disappearing when both are rare, and hence will increase the probability that both establish themselves and spread. This prediction was supported by population data (Chevillon et al. 2001).

Further forecast on the evolution of *Bs*-resistance requires the identification of the genes and mechanisms that regulate this mosquito-bacterial interaction. *Bs*-toxicity relies on the production of a protein crystal formed by two major polypeptides, BinA and BinB, and is maximal when BinA and BinB are in equimolar ratios (Baumann et al. 1988, Porter et al. 1993, Priest et al. 1997). BinB initializes the binding of the binary toxin onto a specific receptor located in the membranes of mosquito midgut cells (Charles et al. 1997; Silva-Filha et al. 1997, 1999). BinA, either alone or with BinB, acts downstream to this binding event (Nicolas et al. 1993) in an unknown way that might involve the toxin internalization into midgut cells (Oei et al. 1992). One instance of high resistance level was due to a disruption of the toxin-receptor binding (Nielsen-LeRoux et al. 1995), and another low resistance case seemed achieved by a relatively low receptor concentration in midgut cells (Silva-Filha et al.

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0022-2585/02/0729–0735$02.00/0

1995). In other cases, as for *sp-1*^{R} in Mediterranean France (Nielsen-LeRoux et al. 1997), resistance occurs despite normal toxin-receptor binding and its physiological basis remains unknown.

Here, the investigation of the complex basis of *Bs*-resistance was pursued by focusing on the French *sp-1*^{R} and *sp-2*^{R} resistance genes and on an uncharacterised resistance from Tunisia. This analysis was performed in three steps. First, inheritance of Tunisian resistance was characterized using, as complementary references, the sex-determining and *sp-1*^{R} loci, and lead us to temporarely name the recessive mutant *sp-T*^{R}. Second, the characteristics of kinetics of toxin-receptor binding interactions were analyzed in *sp-2*^{RR} and *sp-T*^{RR} homozygotes relative to susceptible reference homozygotes. Finally, these binding characteristics were compared with those previously obtained for various susceptible and resistant *C. pipiens* strains.

Materials and Methods

Mosquito Strains. The susceptible reference strain was S-LAB (Georghiou et al. 1966). Resistant reference strains were those originated from southern France, SPHAE and BP, that are homozygous for *sp-1*^{R} and *sp-2*^{R}, respectively (Nielsen-LeRoux et al. 1997, Chevillon et al. 2001). The TUNIS strain was derived from *Bs*-resistant larvae collected in 1997 in the basement of the Cité Olympique of Tunis by Dr. Gilbert Sinègre (Entente Interdépartementale pour la Démoustication, Montpellier, France). The initial sample displayed a 750-fold resistance (relatively to S-LAB), with a nonlinear dose-mortality response indicating heterogeneity. Present characterization of TUNIS strain was performed 18 generations later, with in-between generations having survived *B. sphaericus* treatments that gave 70–80% mortality.

Crosses and Bioassays. Inheritance of resistance in the TUNIS strain was studied relatively to S-LAB and SPHAE strains. As cytoplasmic incompatibilities occurred between these strains (due to *Wolbachia pipientis* Hertig), we used endosymbiont-free S-LAB and SPHAE males in crosses (Guillemaud et al. 1997). Bioassays used two preparations of *B. sphaericus* strain 2362: the liquid Spherimos formulation (initially Novo Nordisk, Denmark, now Valent BioScience, Walnut Creek, CA), and spore-crystal suspensions deriving from a lyophilized whole culture pellet prepared in Institut Pasteur (potency 120 ITU/mg). Bioassays were done in a total volume of 100 ml for Spherimos and of 35 ml for spore-crystal suspensions. For either product, batches of 20 third-instars were tested with ≥3 replicates per dose; mortality was recorded after 48 h, and mortality data were analyzed with PROBIT software (Raymond et al. 1993).

Preparation of Activated *B. sphaericus* Bin2 toxin. Crystal toxin was purified from the Cry minus strain 4Q2–81 of *B. thuringiensis* serovar *israelensis* transformed by plasmid pGSP10, this plasmid encoding the entire binary toxin of *B. sphaericus* strain 1593 (Bourgouin et al. 1990). *Bs*-strain 1593 produces exactly the same binary toxin as strain 2362 (referred as Bin2 toxin, Priest et al. 1997). Toxin was prepared as described in Silva-Filha et al. 1997. Crystals were obtained by ultra centrifugation of the spore-crystal culture on a sucrose gradient of the washed pellets. Crystals were alkali solubilized (50 mM NaOH), neutralized (HCl), dialyzed against phosphate buffer (20 mM, pH 8.5) and trypsin-activated (EC 3.4.21.4) (1% wt:wt, 52 IU, Serva, Heidelberg, Germany). Samples were concentrated/filtered with Ultrafree Biomax 30 (Millipore, Bedford, MA) to exclude the remaining trypsin. ^{125}Iodine labeling was performed according to Nielsen-LeRoux and Charles 1992, with the Chloramine-T method using Iodo-beads (Pierce, Rockford, IL, [http://www.piercenet.com]). Labeled and unlabeled toxins were centrifuged at 14,000 × *g* for 30 min, and were stored at 4°C in 20 mM phosphate buffer pH 7.4 with 0.02% NaN3 until required for binding experiments.

Preparation of Brush Border Membrane Fractions (BBMF). BBMF were prepared from whole larvae extracts, using a minimum of 3 g (wet weight) of fourth-instar larvae previously frozen in aliquots at −70°C (Silva-Filha et al. 1997). Enrichments in apical mid-gut membranes were surveyed by monitoring protein content and an apical membrane marker enzyme, leucine aminopeptidase (LAP). Protein content was determined by the Bio-Rad protein assay using bovine serum albumin (BSA) as standard. LAP activity (α-aminoacyl-peptide hydrolase, EC 3.4.11.1), was measured in 26 mM Tris-HCl, 260 mM NaCl, pH 7.8, using 2 mM l-leucine-4-p-nitroanilide (Sigma-Aldrich Chemie, Steinheim, Germany) as substrate.

Toxin–Receptor Binding Experiments. Binding experiments were run in duplicate in 1.5 ml poly-ethylene tubes, with 20 mM phosphate buffer pH 7.4 containing 0.15 M NaCl, 0.1% BSA and 0.02% NaN3 (PBS/BSA/Az). BBMF of different strains were always assessed side by side. At least two to three independent assays using duplicate samples were performed on different days with the same batch of toxin. Assays consisted of overnight incubations at room temperature (25°C) of 20 μg of BBMF into 100 μl of a solution of various concentrations of ^{125}I-labeled activated toxin. BBMF-bound toxin was separated from free toxin by centrifugation, and the radioactivity retained in pellets was measured with a liquid scintillation counter (Nielsen-LeRoux and Charles 1992). In saturation experiments (equilibrium binding assays), BBMF titration was performed with 2, 8, 24, 40, 50, or 100 nM of labeled activated toxin. Nonspecific binding was determined in parallel assays where titration by ^{125}I-Bin2 toxin was performed in the presence of an excess (1 μM) of unlabeled Bin2 toxin. Specific binding was calculated as the difference between total (without competitor) and nonspecific bindings. Binding data were analyzed using the Ligand program (Munson and Rodbard 1980) adapted for Apple Macintosh (Biosoft/Elsevier).

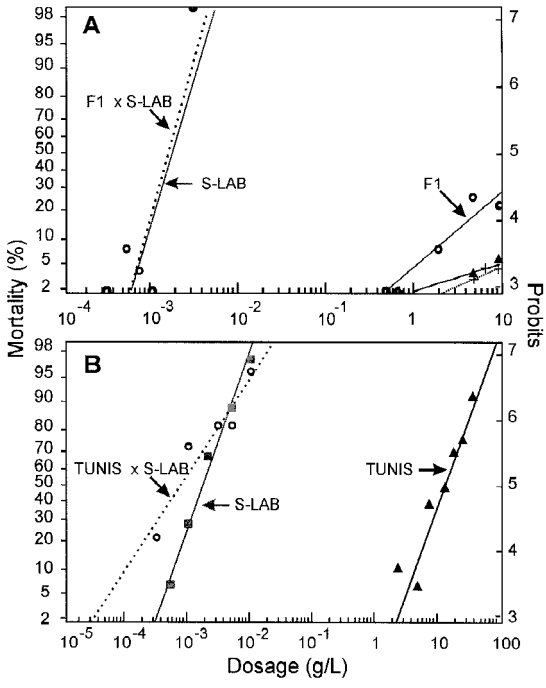

Fig. 1. Genetic characterization of TUNIS resistance (dose-mortality and Probit curves). (A) Spherimos doses in 100 ml characterized the mortality of S-LAB, TUNIS × SPHAE (F1), F1 × S-LAB, TUNIS (triangles) and SPHAE (crosses) larvae. (B) spore-crystal suspensions in 35 ml characterized the mortality of S-LAB, TUNIS and TUNIS × S-LAB larvae.

Results

Genetic Characterization of TUNIS Resistance. Using Spherimos, the LC50 computed for S-LAB larvae was 1.71 mg/liter (95% confidence interval [CI]: 1.35–2.19). By comparison, TUNIS and SPHAE, respectively, displayed 5 and 3% mortalities at the highest dose tested, i.e., 10 g/liter Spherimos (Fig. 1A). To determine how the TUNIS resistance was related to the *sp-1*^{R} mutant of SPHAE, we examined the dose-mortality response of the F1 larvae produced by crossing TUNIS females with SPHAE males. Linearity of this response was not rejected ($P = 0.28$), and F1-resistance was slightly lower than parental ones (Fig. 1A) with an estimate of ≈22,000-fold (CI: 12,600–40,400). Crossed to susceptible S-LAB males, F1 females were expected to produce resistant larvae in a proportion equal to the recombinant rate between these factors. The dose mortality response of the (F1 × S-LAB) offspring was similar to that of S-LAB (Fig. 1A), with a resistance ratio of 1 at the LC50 (LC50 = 0.90 mg/liter; CI: 0.30–2.73). The absence of survivors (i.e., recombinants) at doses ≥5 mg/liter (180 larvae tested) indicated that the resistance factors of TUNIS and SPHAE are either tightly linked or alleles of a same gene.

Linkage of TUNIS resistance with the sex factor (*Mm* in males and *mm* in females, (Gilchrist and Haldane 1947) was tested using the spore-crystal suspension of strain 2362 (Institut Pasteur). With this product, LC50 of TUNIS larvae was 15.0 g/liter (CI: 13.3–16.6) and was slightly higher than those of BP and SPHAE larvae (Table 1). In these bioassay conditions, LC50 estimates were much lower for either (TUNIS × S-LAB) offspring or S-LAB larvae with respective values of 0.79 mg/liter (CI: 0.38–1.27) and 1.73 mg/liter (CI: 1.51–2.00). Moreover, the similarity of S-LAB and (TUNIS × S-LAB) mortality responses in Fig. 1B confirmed that TUNIS resistance was recessive. The larvae produced by backcrossing (TUNIS × S-LAB) males with TUNIS females were exposed 48 h to 0.1 g/liter of spore-crystal suspensions, i.e., a dose expected to induce 50% mortality if TUNIS resistance was mono-factorial. This selection killed 49% of 226 exposed larvae, and significantly biased the sex-ratio of resulting adults ($P < 0.001$): 10 out of the 119 survivors were males compared with 66 out of 138 nonexposed mosquitoes in control. Overall, these results showed that TUNIS resistance involved one major recessive gene, located at 8.4 (CI: 4.1–14.8) recombination units from the sex factor and temporally named *sp-T*^{R}.

Table 1. Pair-wise comparisons of resistance levels among three West-Mediterranean *Culex pipiens* strains, using spore-crystal suspensions of *Bs*-strain 2362 in a total volume of 35 ml

	BP[a]	SPHAE[a]	TUNIS
BP	—	1.07 (0.76–1.52)[b]	1.44 (1.19–1.75)
SPHAE	1.07 (0.76–1.52)[c]	—	1.34 (1.09–1.66)
TUNIS	1.74 (1.09–2.79)	2.19 (1.33–3.61)	—

[a] BP and SPHAE data from Chevillon et al. 2001.
[b] Above diagonal, resistance ratios (95% confidence interval) were computed at LC_{50}; the value expected for identity is 1.
[c] Below diagonal, computations of resistance ratio were performed at LC_{95}; the value expected for identity is 1.

Toxin-Receptor Binding in TUNIS and BP Compared with Susceptible S-LAB Strain. For the three mosquito strains S-LAB, TUNIS and BP, the enrichment in apical midgut membranes was about three- to four-fold from the first to last step of BBMF preparation according to LAP (leucine amino-peptidase) activity and protein content (details not shown). At the final step, the LAP specific activities were 0.069, 0.065, and 0.050 U/min/mg BBMF proteins in S-LAB, TUNIS, and BP preparations, respectively.

In vitro direct binding experiments gave similar results for S-LAB and TUNIS strains (Fig. 2). In either case, the curve of specific binding displayed a plateau indicating that available receptors were saturated by 40–100 nM of Bin2 toxin. Nonspecific binding was linear, increased proportionally to the concentration in labeled toxin, and reached a maximum of 35% of total binding at the highest concentration used (100 nM ^{125}I-Bin2). In addition, the linearity of the Scatchard plots (see inserts in Fig. 2) indicated that the labeled toxin bound to a single class of specific sites in either S-LAB or TUNIS strains. Among three independent experiments, the average dissociation constant of the toxin-BBMF complex was estimated as $K_d = 6.3 \pm 0.2$ nM in S-LAB and as 3.7 ± 1.1 nM in

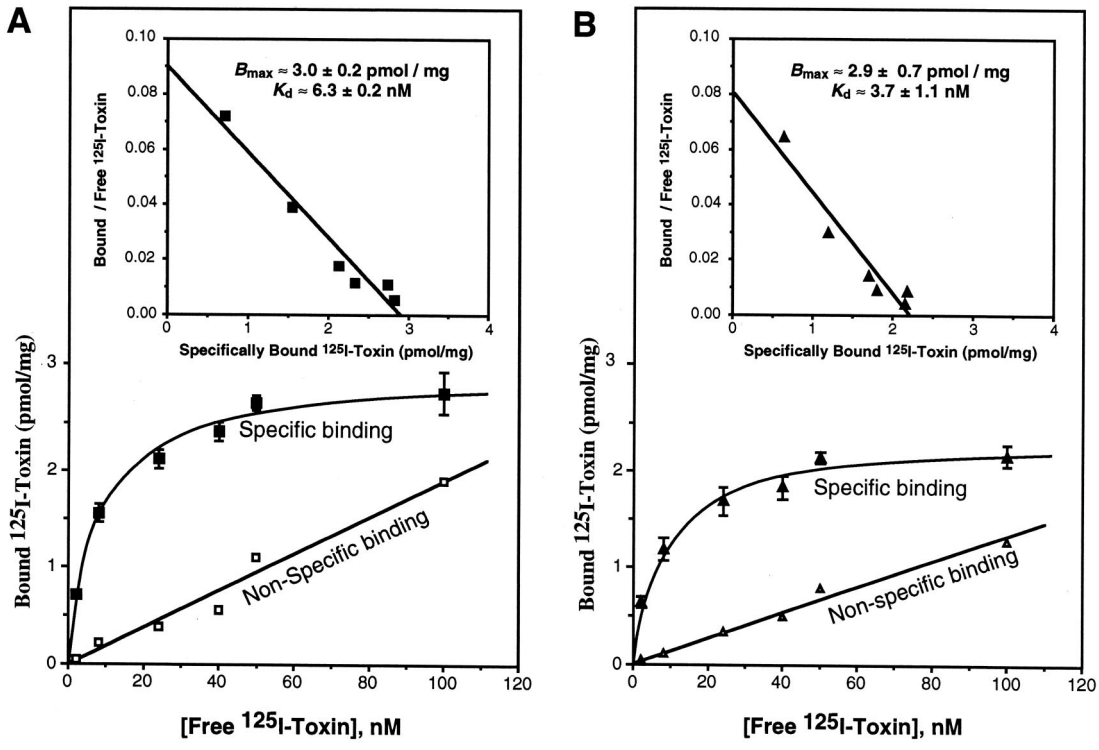

Fig. 2. Direct binding experiments of ^{125}I-labeled *B. sphaericus* Bin2 toxin to *C. pipiens* larval BBMFs from susceptible S-LAB (A) and resistant TUNIS (B) strains. Each point is the mean of duplicate samples ± SEM. Nonspecific binding (open squares and triangles) was determined in parallel assays with an excess (1 μM) of unlabelled toxin. Specific binding (filled squares and triangles) is the difference between total binding (data not shown) and nonspecific binding. Inserts (top) represent the same specific binding in Scatchard coordinates.

TUNIS. Meanwhile, the average maximum binding capacity of BBMF was estimated as *B*max = 3.0 ± 0.2 pmol/mg in S-LAB and as 2.9 ± 0.7 pmol/mg in TUNIS.

Results were clearly different for the BP strain. In that case, no saturation could ever be established, and the nonspecific binding exceeded the specific one whatever the concentration in binary toxin. For 50 nM of ^{125}I-Bin2 toxin, specific binding in BP was only ≈6% of those observed in TUNIS and S-LAB strains (data not shown).

Discussion

High *Bs*-resistance (i.e., > 5,000 fold) has been detected six times in the *C. pipiens* complex. No genetic information is available for Indian and Chinese resistance cases (Rao et al. 1995, Yuan et al. 2000), whereas a single factor was shown to be involved in the Californian colony (Wirth et al. 2000b) as well as in each of the three west Mediterranean colonies (Nielsen-LeRoux et al. 1997, Chevillon et al. 2001; current study). West Mediterranean mutants *sp-1*^{R}, *sp-2*^{R}, and *sp-T*^{R} are all sex-linked and separately confer the same totally recessive *Bs*-resistance (Table 1; Fig. 1; Chevillon et al. 2001). In this study, binding experiments clearly discriminated the *sp-2*^{R} mutant of BP strain from the other two. In BP, as in the Californian GEO strain, the physiological explanation of resistance is likely to be the absence of a functional receptor to the *Bs* toxin. As a single class of functional receptors was identified (Table 2), one can expect that it is also a mutated *sp-2* allele that confers the high resistance level observed in GEO strain. Moreover, these data provide a candidate identity for *sp-2:* the gene encoding the α-glycosidase specific receptor recently identified (Silva-Filha et al. 1999) and sequenced (Darboux et al. 2001) from the IP susceptible strain. Comparison in receptor sequences will allow testing of this hypothesis as well as to verify whether *sp-2*^{R} has effectively invaded west Mediterranean France as suspected from bioassay surveys (Chevillon et al. 2001).

The relationships between the other two west Mediterranean mutants, *sp-1*^{R} and *sp-T*^{R}, were likely to provide further insights on two interconnected issues, namely the complexity of *Bs-C. pipiens* interactions and resistance dynamics. Three a priori alternatives existed. *sp-T*^{R} could have resulted from *sp-1*^{R} migration across Mediterranean Sea between 1994 (when detected in southern France) and 1997 (when detected in northern Tunisia). Alternatively, *sp-1*^{R} and *sp-T*^{R} could have been independently created by distinct mutation events affecting either the same gene or

Table 2. Binding characteristics of *Bs*-binary toxin (Bin2 from Strain 1593) with the brush border membrane fractions (BBMF) prepared from diverse susceptible and resistant *Culex pipiens* strains

Mosquito strains	Resistance status[a]	Binding characteristics[b]	
		K_d (nM)	B_{max} (pmol / mg)
IP	Susceptible	15 ± 2^{c}	5 ± 1^{c}
S-LAB	Susceptible	6 ± 0^{d}	3 ± 0^{d}
Madurai	Susceptible	17 ± 2^{e}	2 ± 1^{e}
JRMM-S	Susceptible	$7 \pm 1^{e,f}$	$13 \pm 1^{e,f}$
S-SYN	Susceptible	$11 \pm 1^{f,g}$	$7 \pm 2^{f,g}$
Brazil-S	Susceptible	$12 \pm 2^{f,h}$	$9 \pm 1^{f,h}$
Brazil-R	Low-level resistant	$11 \pm 1^{f,h}$	$7 \pm 1^{f,h}$
GEO	High-level resistant	No specific binding[c]	
SPHAE	High-level resistant	$3 \pm 1^{f,i}$	$16 \pm 1^{f,i}$
TUNIS	High-level resistant	4 ± 1^{d}	3 ± 1^{d}
BP	High-level resistant	No specific binding[d]	

[a] Qualitative comparison in Bs-resistance status where low-level resistant and high-level resistant design 10- to 100-fold resistant and >5,000-fold resistant strains, respectively.

[b] Values from binding assays performed using BBMFs prepared from whole larvae and overnight incubation with toxins (as described in *Materials and Methods*).

[c] Yuan et al. 2001.

[d] Present study.

[e] Nielsen-LeRoux et al., unpublished data.

[f] Assays performed using BBMFs prepared from dissected midguts and incubated with toxins for 90 min.

[g] Nielsen-LeRoux et al. 1995.

[h] Silva-Filha et al. 1995.

[i] Nielsen-LeRoux et al. 1997

two linked genes; with this last possibility requiring a more complex basis of *Bs-C. pipiens* interactions. Unfortunately, binding experiments failed to provide more categorical clues than genetics regarding *sp-1*^{R}–*sp-T*^{R} relationships. On the one hand, the marginal difference in sex-resistance linkage between SPHAE and TUNIS strains ($P = 0.02$) is not enough to confidently rule out the hypothesis that *sp-1*^{R} and *sp-T*^{R} are identical. However, neither *sp-1*^{R} nor *sp-T*^{R} prevented the activated toxin from binding on its receptor and they apparently did not recombine, but this is not enough to confidently rule out the hypothesis of tightly linked genes. Indeed, clarifying whether or not *sp-1*^{R} and *sp-T*^{R} are alleles of a single gene will require identifying and comparing their molecular or physiological bases. This task looks difficult as realistic assumptions on resistance physiology are still lacking when *Bs*-toxin receptors are functional. To date, a single alternative is retained because of its report in Lepidoteran resistances to *B. thuringiensis* Berliner (Ferré et al. 1995, Oppert et al. 1997): the improper digestion (i.e., activation) of the protoxin into BinA and BinB active components (Nicolas et al. 1990) could interrupt the chain of events rendering it toxic before the binding step. Although not investigated for *sp-T*^{R}, this possibility was rejected for *sp-1*^{R} (Nielsen-LeRoux et al. 1997).

A complementary interest of this study was to challenge our vision on how variation in binding data among functional receptors may relate with the output of *Bs-C. pipiens* interactions. Table two recapitulated the estimates of the B_{max} and K_d parameters among six susceptible, one slightly resistant (≈10-fold) and four highly (>5,000-fold) resistant strains. In nine of the 11 strains listed, binding data characterized a single class of specific receptor; the exceptions concerning the highly resistant strains from California (GEO) and France (BP) for which binding was almost absent. As two different protocols were used to compute these estimates (see footnote *f* to Table 2), there was a possibility that this protocol difference affected more the distribution in binding estimates than strain differences. This possibility was partially rejected as the estimates driven for the susceptible IP strain were congruent among protocols ($Kd = 20 \pm 5$ or 15 ± 2 nM and $Bmax = 7 \pm 1$ or 5 ± 1 pmol/mg, respectively, for BBMFs from dissected midguts or from whole midguts). Among strain variation brought two remarks. First, a decrease in receptor concentration had seemed a valid explanation for a low resistance level (Silva-Filha et al. 1995), but the increased number of characterized strains now questions this mechanism and the possibility to detect it by a *B*max decrease (Table 2). Second, the receptors associated with *sp-1*^{R} and *sp-T*^{R} emerged as those with the highest affinities for *Bs*-toxins out of nine examples ($Kd \approx 3$–4 nM for the SPHAE and TUNIS strains, and ≥7 nM for other susceptible or slightly resistant, Table 2). Although caution is required by the low *K*d variation and the still restricted dataset, we can wonder whether this common property does not signal either a common resistance physiology, selecting for a receptor with enhanced affinity, or a common mutational origin, as their physical linkage increases the likelihood that a particular receptor hitch-hiked with a recent *sp-1*^{R} allele. Further investigation of binding kinetics and receptor sequence diversities would provide indirect tests of such hypotheses. Nevertheless, it is worth noting that the possibility of a tight physiological interaction between *sp-1* and *sp-2* resistances received other indirect supports. For instance, *sp-1*^{RS} *sp-2*^{RS} double heterozygotes showed a >100-fold resistance although each resistance was totally recessive otherwise (Chevillon et al. 2001). Moreover, resistance to *Bs*-toxins of GEO and SPHAE larvae is overcome in the presence of *B. thuringiensis* Berliner pore-forming Cyt toxins (serovars *medelin* or *israelensis* in [Thiéry et al. 1998, Wirth et al. 2000a], respectively), which suggests that the absence of pore-formation was a common limiting factor of *Bs*-intoxication in both *sp-2* and *sp-1* resistance cases. The latest results in *Bs*-toxicity are likely to help test these assumed interactions of *sp-1*^{R} physiology with the binding event and/or the cell entrance of *Bs*-toxin component(s). Up to recently, BinA and BinB were known to act in synergy with a pivotal role of BinB in initializing the toxin-receptor binding and a suspected pivotal role of BinA in subsequent step(s). Then, (Yuan et al. 2001) identified one BinA amino acid residue whose nature determines the stability and the fatal evolution of receptor-toxin complexes, and so, with a very low dependence on BinB composition. Investigating whether these results (driven with IP susceptible strain) rely on the endocytosis of BinA, BinB, and/or

the receptor, and whether they persist with *sp-1*^{RR} larvae is likely to improve our understanding of how *sp-1*^{R} interferes with the intoxication process. Altogether, comparative studies on *Bs*-toxin composition and on highly resistant *C. pipiens*, start to efficiently complement each other to greater understanding on the physiological bases of this bacteria-mosquito interaction, and hence on key features of *Bs*-resistance dynamics.

Acknowledgments

We thank G. Sinègre for providing the TUNIS collection; C. Magnus and C. Bernard for technical help; and C. Berticat, I. Darboux, D. Pauron, M. Raymond, and J. Scott for helpful comments on earlier drafts. This is contribution 2002-002 of Institut des Sciences de l'Évolution de Montpellier (ISEM).

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Received for publication 5 September 2001; accepted 18 January 2002.