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Resistance to *Bacillus sphaericus* in *Culex pipiens* (Diptera: Culicidae): Interaction Between Recessive Mutants and Evolution in Southern France

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ABSTRACT In southern France, failure to control *Culex pipiens* L. with *Bacillus sphaericus* Neide toxin (*Bs*) was first detected in 1994, at the extreme east of the Languedoc-Roussillon coast. This failure was due to a single recessive mutant, *sp-1*^{R}. Two complementary strategies were used to test whether *sp-1*^{R} had invaded the *Bs*-controlled area by 1998. First, a strain (BP) was selected from resistant larvae sampled in the western part of the *Bs*-controlled area. In BP strain, resistance involved a single recessive gene, *sp-2*^{R}, distinct from *sp-1*^{R}, that conferred a similarly high resistance in the homozygous state (≈6,000-fold). Combining one copy of *sp-1*^{R} and one of *sp-2*^{R} conferred a > 100-fold resistance. Second, *Bs*-resistance was monitored among the offspring of field females crossed to *sp-1*^{RR} homozygous males. Females were sampled in 20 localities of southern France and three localities of the Llobregat delta (Barcelona, Spain) where *C. pipiens* control is also intensive. The 537 females in the study produced enough larvae to infer their genotype: 462 progenies were susceptible and the survival rate of 51 others was explained by the presence of *sp-1*^{R} and/or *sp-2*^{R}. The remaining 24 cases indicated that other factors could confer resistance when combined with *sp-1*^{R}. The current data showed that, even when recessive, resistant mutants can rapidly increase in frequency, providing some interactions that protect them from disappearance. We discuss the consequences of this finding on the current strategies aimed to avoid or delay resistance in the pests controlled with *B. sphaericus* or *B. thuringiensis* Berliner toxins.

KEY WORDS *Culex pipiens*, *Bacillus sphaericus*, *Bacillus thuringiensis*, resistance, gene identification, geographic distribution

PEST CONTROL USING highly specific toxins produced by *Bacillus thuringiensis* Berliner (*Bt*) or *B. sphaericus* Neide (*Bs*) is currently favored because of its supposed low environmental impact and the rarity of reported failures. The mode of actions of these toxins is still poorly understood except that they bind to specific receptors expressed in cell membranes of midgut epithelium (Zhu et al. 2000, Darboux et al. 2001). Similarly, the pattern of resistance evolution in natural populations remains unknown. Only two species, *Plutella xylostella* L. and *Culex pipiens* L., have developed resistance in the field and can provide some insights on possible characteristics of resistance to bacterial toxins as compared with conventional chemical insecticides. Resistance to *Bt* in *P. xylostella* and to *Bs* in *C. pipiens* share common characteristics. In the two species, most mutants are recessive and they can be of two types: mutants in which the binding between toxin and receptor is reduced, and mutants in which this step is not affected (Nielsen-Leroux et al. 1995, 1997; Ballester et al. 1999; Sayyed et al. 2000; Zhao et al. 2000). However, data on the local evolution of resistance is scarce due to the difficulty of identifying heterozygous recessive mutants in the absence of biochemical or molecular tests.

In southern France, an intensive mosquito control program has been conducted since 1969 along the Mediterranean coast of Languedoc-Roussillon province. During the nineties, *C. pipiens* control increasingly used Spherimos (*B. sphaericus* strain 2362), and the first failure occurred in 1994, in an underground-breeding site of Port-St-Louis (Fig. 1). A strain (SPHAE), derived from this population, developed a > 10,000 fold resistance to Spherimos after a few generations of selection (Nielsen-Leroux et al. 1997). SPHAE resistance was due to a single recessive gene (named *sp-1*^{R}), located on linkage group I at 22 recombination units from the sex factor. This resistance was not associated with any loss of binding affinity between the brush border membrane fractions and the *Bs* radiolabeled toxin. In 1996, the mosquito abatement organization (Entente Interdépartementale pour la Démoustication or EID) encountered difficulties in controlling *C. pipiens* with Spherimos in the town of Perpignan (≈200 km west of Port-St-Louis). Were the populations of Perpignan also resistant to *Bs* toxin and, if so, was this resistance due to the *sp-1*^{R} mutant isolated in 1994? What was the geographic

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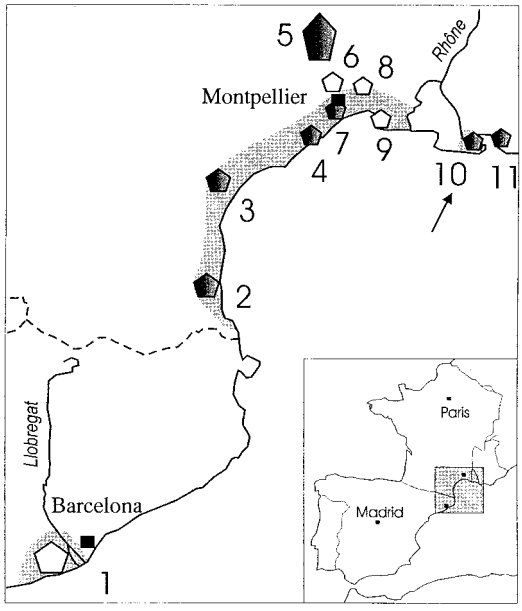

Fig. 1. Locations of *C. pipiens* field samples. General localization is given in inset, where squares figured the cities of Montpellier and Barcelona. Arrow designates the city of Port-St-Louis where larvae that gave the SPHAE strain were collected. Places where *C. pipiens* is controlled are indicated in gray. Pentagons, numbered as in Table 2, represent the areas where one or several samples were collected; they are white when all females were assigned as susceptible homozygous (see Tables 2 and 4). BP strain was isolated from a collection done in area 2.

distribution of *sp-1*^{R} in natural populations of southern France?

The current study was undertaken to investigate these questions in *C. pipiens* collections made during the 1997 summer. Two investigations were conducted: (1) the analysis of *Bs*-resistance characteristics of a strain derived from a Perpignan collection, and (2) a population survey of *sp-1*^{R} frequency in natural populations collected along the Mediterranean coast (Fig. 1). We showed that resistance to *Bs* toxin was present in many populations of southern France, and that *sp-1*^{R} was not the only factor involved. These results are discussed with regard of the sustainability of the pest control strategies using *Bs*-like toxins.

Materials and Methods

Mosquito Strains. Two laboratory strains were used: the susceptible reference strain S-LAB (Georghiou et al. 1966), and the *Bs*-resistant strain SPHAE (Nielsen-Leroux et al. 1997). To avoid sterility due to cytoplasmic incompatibilities (due to *Wolbachia pipientis* Hertig), we used endosymbiont-free males in crosses involving S-LAB and SPHAE strains (Guillemaud et al. 1997). A third strain, BP, was derived from a resistant sample collected after control failure in a suburb of Perpignan (Basse-Perpignan) in October 1997. This sample was maintained by exposing each generation larvae to 7 g/liter Spherimos.

Field Sampling. *C. pipiens* larvae or pupae were collected in 23 breeding sites between June and October 1997. For each sample, virgin females were released in a 30 by 30 by 30-cm cage in which SPHAE males (*sp-1*^{RR}) were regularly introduced until the end of the experiment. One week after the last female of a sample had emerged, chicken was offered every 2–3 d for blood feeding. Each blood-fed female was isolated in a 15-ml vial containing 1 ml of water, and covered with gauze on which was deposited a piece of cotton impregnated with honeyed water. Vials with egg-rafts were sorted out every day, and hatched larvae were transferred to 500-ml pans (one family per pan) and reared under normal laboratory conditions. For each sample, the number of females allowed to mate and the number of females that blood-fed and produced a fertile egg-raft were recorded.

Bioassays. Bioassays were done in plastic cups on batches of 20 third instars, and mortality was recorded after 48 h. The offspring of each field-collected female were exposed to 1 g/liter or 10 g/liter of Spherimos (Novo Nordisk, Bagsværd, Denmark), a flowable concentrate of spore and toxin mixture of *B. sphaericus* strain 2362, in a total volume of 100 ml. Two replicates per dose were done whenever possible, i.e., for the mothers that produced a minimum of 100 larvae (20 in control, and 2 × 20 exposed to each dose). Bioassays on SPHAE and S-LAB larvae were performed under the same conditions to assess *sp-1*^{RR} mortality and verify that no susceptible individuals could survive.

Resistance of the BP strain were characterized with water suspensions of a lyophilized pellet of *B. sphaericus* strain 2362 provided by C. Nielsen-Leroux (Institut Pasteur, Paris, France). This preparation was expected to be more toxic than Spherimos (≈1,200 ITU/mg versus 120 ITU/mg for Spherimos, C. Nielsen-Leroux, personal communication). Bioassays were performed as described above, but in a total volume of 35 ml and with three to five replicates per dose. Mortality data were analyzed using the Probit software of Ratsira et al. (1993).

Assigning Genotype to Field Mothers. The problem was to explain the spherimos-mortality response observed in the progeny produced by a field-collected female crossed to a SPHAE male (*sp-1*^{RR}), that is to find the most likely genotype of this mother. The procedure followed was to screen the arrays of possible mother genotypes and, for each tested progeny, to reject the unlikely ones. Thus, a straightforward genotype assignation to the mother of a tested progeny required that all but one alternatives were rejected as a possible explanation for the observed mortality; when the progeny was tested at two doses (1 g/liter and 10 g/liter), results had to be consistent among doses.

In a first step, the analysis focused on the *sp-1* locus, restricting the possible mother genotypes to *sp-1*^{RR}, *sp-1*^{RS} and *sp-1*^{SS}. In absence of distorted segregation, each of these alternatives is characterized by the appropriate binomial distribution of *sp-1*^{RS} and *sp-1*^{RR}

genotypes in offspring, with the progenies of $sp\text{-}1^{SS}$, $sp\text{-}1^{RS}$ and $sp\text{-}1^{RR}$ mothers expected to contain 100, 50, or 0% of $sp\text{-}1^{RS}$ larvae, respectively. As $sp\text{-}1^{R}$ is totally recessive, the mortality response of $sp\text{-}1^{RS}$ or $sp\text{-}1^{RR}$ larvae are known: they are those observed for S-LAB or SPHAE strains, respectively. It is therefore possible to characterize each possible mother genotype by the Spherimos-mortality expected in its progeny, and hence to compute the probability that each of these characteristic expectations explains an observed progeny mortality. These probabilities were computed for each bioassay, and their distribution screened among all bioassays to detect the rejection cases ($P < 0.05$). For each bioassay, the 0.05 threshold was adjusted with the sequential Bonferroni method of Holm (1979) to take into account the high number of progenies tested at the same spherimos dose.

In a second step, the consideration of the distinct resistance genes of SPHAE and BP strains extended the array of possible mother genotypes to nine {*sp-1; sp-2*} combinations. There, the characterization of alternative mother genotypes by expectations on their progeny used the resistance data of S-LAB ($sp\text{-}1^{SS}sp\text{-}2^{SS}$), SPHAE ($sp\text{-}1^{RR}sp\text{-}2^{SS}$) and SPHAE x BP ($sp\text{-}1^{SS}$ $sp\text{-}2^{RR}$) larvae. Otherwise, the procedure followed was as described above.

Results

Characteristics of *Bs*-Resistance in BP Strain. The BP strain, selected from the resistant larvae collected in Perpignan, showed a ≈6,000-fold resistance to *Bs*-toxin. Under these bioassay conditions, BP and SPHAE larvae displayed the same resistance ratios relatively to S-LAB larvae (Table 1).

Inheritance of *Bs*-resistance in BP strain was examined by crossing BP females and S-LAB males. The dose-mortality response of F_1 offspring was similar to that of S-LAB.

Parallelism between mortality lines was not rejected ($\chi^2 = 12.01$, df = 7, $P = 0.10$), and offspring resistance ratio was ≈1 (Table 1). F_1 males were then backcrossed to BP females. The observed mortality curve of resulting offspring was similar to that expected if a single resistance gene was involved in BP strain ($P = 0.11$, Fig. 2A). Linkage of this gene with the sex factor was investigated via the change in sex ratio induced by a *Bs*-exposure killing susceptible larvae (68% mortality, on a total sample of 1,240 larvae). In *C. pipiens*, sex is coded by a locus with two alleles, *m* and *M*, such as *m*/*m* genotypes are females and *M*/*m* males (Gilchrist and Haldane 1947). F_1 males were thus *MS*/*mR* and BP females were *mR*/*mR*. Among the 403 survivors of the backcross, 185 were females (*mR*/*mR*, parental type) and 218 were males (*MR*/*mR*, recombinant type). Among adults of the control (not exposed to *Bs* toxin), females were less numerous than males (278 and 571, respectively), indicating a strong sex ratio distortion ($P < 0.001$). The sex ratio in favor of males was lower among survivors of *Bs*-exposure (1.18) than among control adults (2.05), the difference being significant ($P < 0.001$). Although the

Table 1. Mortality curves observed with lyophilized pellet suspension in a total volume of 35 ml

Strain or cross	Slope ± SE	Linearity[a]	LC_{50}[b] (CI)	LC_{95}[b] (CI)	RR_{50}[c] (CI)	RR_{95}[d] (CI)
S-LAB	2.63 ± 0.22	NS	1.74 (1.52–2.02)	7.34 (5.63–10.5)	—	—
BP	2.96 ± 0.42	***	10,379 (8,787–12,260)	37,393 (25,279–55,961)	5,958 (4,825–7,356)	5,095 (3,137–8,274)
SPHAE	3.85 ± 0.53	***	11,124 (9,654–12,827)	29,767 (21,609–41,335)	6,386 (5,074–8,037)	4,056 (2,424–6,786)
BP × S-LAB[e]	2.10 ± 0.72	***	3.02 (1.58–5.78)	18.40 (4.24–0.836)	1.73 (1.05–2.87)	2.51 (0.80–7.82)
C = BP × SPHAE	0.92 ± 0.10	***	227.2 (141.2–364.8)	13,902 (5,697–34,905)	130 (107–158)	1,894 (1,282–2,798)
C × S-LAB	1.55 ± 0.21	*	3.73 (2.69–5.18)	43.1 (22.25–86.89)	2.14 (1.72–2.67)	5.88 (3.72–9.30)

[a] Chi-square test for linearity of mortality curves: NS stands for nonsignificant, * for $P < 0.05$ and *** for $P \leq 0.0005$.
[b] In mg/liter.
[c] Resistance ratio relatively to S-LAB at LC_{50}.
[d] Resistance ratio relatively to S-LAB at LC_{95}.
[e] In crosses, female parents are indicated first.

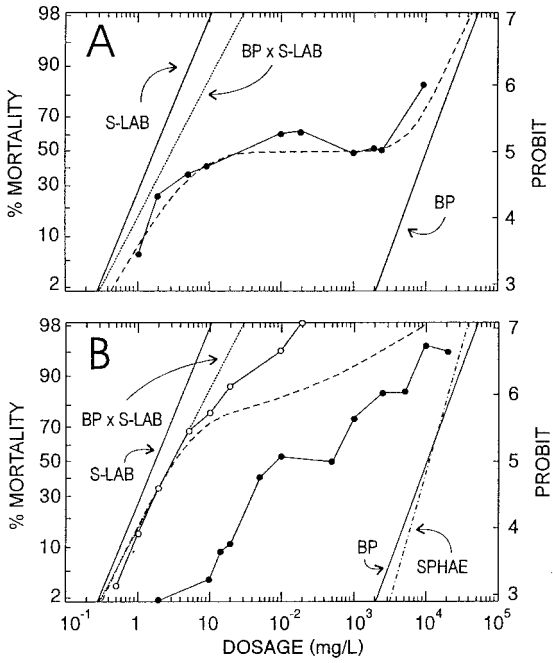

Fig. 2. *Bs*-resistance in BP strain: inheritance (A) and interaction with the *sp-1*^{R} allele of SPHAE (B). Mortality lines of S-LAB, BP, SPHAE, and (BP x SLAB) are given for reference. (A) Linked black dots represent the mortality observed for the (BP x S-LAB) x BP backcross and the dashed line the expectation assuming that a single resistance gene is involved. (B) Black and white dots represents the mortality observed for (BP x SPHAE) and (BP x SPHAE) x S-LAB crosses, respectively. The dashed line represents the (BP x SPHAE) x S-LAB expected mortality assuming that BP and SPHAE resistance involved two independent genes.

strong distortion in sex ratio rendered the interpretation difficult, the decrease in male frequency among survivors indicated a linkage in BP strain between the sex factor and *Bs*-resistance gene.

Further investigation of *Bs*-resistance in BP strain was done by analyzing the dose-mortality response of the offspring produced by crossing BP females and SPHAE males. Clearly, offspring were much less resistant than parents and more than S-LAB (Table 1; Fig. 2B). This result indicates that the *Bs*-resistance factors present in BP and SPHAE strains are different, and that they interact to confer resistance. Two hypotheses can be envisaged to explain this interaction. Resistance in BP strain could be due to a mutation at a second gene (*sp-2*), distinct from *sp-1*, so that *sp-1*^{RS}*sp-2*^{RS} are resistant. Alternatively, resistance in BP strain could be due to a second resistance allele at the *sp-1* gene (*sp-1*$^{R'}$), so that *sp-1*$^{RR'}$ heterozygotes are less resistant than either homozygote. The dose-mortality response of the offspring of (BP x SPHAE) females crossed with S-LAB males was thus investigated, as these offspring were expected to be partially resistant under the two-gene hypothesis, and completely susceptible under the one-gene hypothesis. This offspring was resistant as compared with S-LAB (Table 1). Mortality was higher at the three highest doses than expected if *sp-1* and *sp-2* genes segregated independently (Fig. 2B), with these differences being significant ($P < 0.05$). This is in agreement with the finding that both genes were sex-linked. In conclusion, SPHAE and BP strains contained different resistance mutants (*sp-1*^{R} and *sp-2*^{R}, respectively) which are probably coded by two linked genes. The larvae carrying one *sp-1*^{R} and one *sp-2*^{R} copies are less resistant than *sp-1*^{RR} and *sp-2*^{RR} homozygotes.

Monitoring *sp-1*^{R} Frequency in Natural Populations. This study was done at a time when data on the resistance of BP strain were not available, and the experiment was set up for allowing the identification of the three known genotypes at the *sp-1* locus (*sp-1*^{SS}, *sp-1*^{RS}, or *sp-1*^{RR}). Thus, females that derived from field-collected larvae were crossed with SPHAE males (*sp-1*^{RR} homozygotes), and their offspring was exposed to Spherimos doses that killed all susceptible (*sp-1*^{SS} and *sp-1*^{RS}) but none or few resistant (*sp-1*^{RR}) phenotypes. Because the concentration of *Bs* toxin in different stocks of Spherimos may vary, the mortality induced by the chosen doses was tested on S-LAB and SPHAE larvae: it was 100% at both doses for S-LAB, and 0 and 3% for SPHAE at one and 10 g/liter, respectively.

Although our original objective was to genotype 50 females of each field sample, this was not possible because many females did not take a blood-meal, did not lay eggs after blood feeding, or laid egg-rafts that did not hatch (Table 2). All blood-fed females that did not produce larvae had empty spermathecae, indicating that mating had not occurred. Out of the 3,394 females released in cages, 665 (20%) produced larvae. However, 128 families were discarded from analyses because bioassays were carried out on <20 larvae, or because mortality was >5% in control. Thus, only 537 families could be considered. Among them, 462 displayed a 100% mortality at both Spherimos doses, indicating that mothers were susceptible homozygotes. For the remaining 75 families, a first assessment of mother genotypes was done in considering that *sp-2*^{R} did not exist, and we then examined how the presence of *sp-2*^{R} could modify our conclusions. In both cases, the spherimos independent mortality of the 75 partly resistant progenies was considered equal to the average mortality observed over all bioassay controls (e.g., 4%, 496 dead out of 12,291 larvae).

Inferences Considering Only sp-1^R. Distorted segregation in *sp-1*^{RS} mother was considered unlikely, and the spherimos mortality responses of *sp-1*^{RS} and *sp-1*^{RR} offspring were considered equal to those observed in the same conditions for SLAB and SPHAE larvae, respectively. Thus, the expected mortality in the offspring of *sp-1*^{RR} mothers was four and 7%, and that of *sp-1*^{RS} mothers was 52 and 54% at one and 10 g/liter, respectively. 16 progenies led to a straightforward assignation of maternal genotype with 10 field-collected mother inferred as *sp-1*^{RS} and six as *sp-1*^{RR} (Table 2). Inference on mother genotype was not possible for 59 partly resistant progenies, either because of inconsistent outputs among doses, or because

Table 2. Contingencies of the females involved in population survey

Origin		Treatments steps				Genotype inferences[a]			
Area[b]	Locality	Mating[c]	Larvae[d]	Test[e]	2dose[f]	*sp-1*^{SS}	*sp-1*^{RR}	*sp-1*^{RS}	x[g]
1	Llobregat-1	90	11	7	7	7	—	—	—
1	Llobregat-2	180	12	7	7	7	—	—	—
1	Llobregat-3	77	18	14	14	14	—	—	—
2	Perpignan-1	70	68	60	59	56	—	1	3
2	Perpignan-2	118	15	12	12	10	—	—	2
2	Perpignan-3	190	48	35	35	34	—	—	1
2	Perpignan-4	259	62	52	52	41	—	—	11
3	Narbonne-1	190	32	21	12	20	—	—	1
3	Narbonne-2	135	14	10	3	9	—	—	1
4	Sète	153	63	62	62	41	—	8	13
5	Sumène	136	23	18	3	18	—	—	—
5	St Bauzille de P.	120	5	4	0	4	—	—	—
5	Notre Dame	263	33	28	4	27	—	—	1
6	Les Matelles	131	50	40	5	40	—	—	—
6	Prades-le-Lez	159	15	13	2	13	—	—	—
7	Près d'Arène	114	18	14	1	14	—	—	—
7	Lattes	100	29	21	21	20	—	—	1
7	Maurin	183	26	24	2	24	—	—	—
8	St Brès	295	31	29	29	28	—	—	1
9	Le Grau du Roi	51	15	13	13	13	—	—	—
10	Port-St-Louis	100	31	17	17	1	5	—	11
11	Martigues-1	151	24	21	21	9	1	1	10
11	Martigues-2	129	22	15	15	12	—	—	3
	Total	3,394	665	537	396	462	6	10	59

[a] Inferences assuming *sp-1*^{R} as the only resistance factor present.
[b] Numbered as in Fig. 1 and Table 4.
[c] Virgin females allowed to mate with SPHAE males.
[d] Females that produced fertile egg rafts.
[e] Bioassays performed on progeny large enough to infer maternal genotype.
[f] Bioassays performed on large progenies and that include two spherimos® doses.
[g] Offspring mortality-response incompatible with all three possible mother genotypes at *sp-1* locus.

the observed mortality significantly rejected ($P < 0.05$) both *sp-1*^{RS} and *sp-1*^{RR} assumptions.

Inferences Considering Both sp-1^{R} *and sp-2*^{R} *Resistance Genes.* This enlarged array of possible mother genotypes introduced two new resistant genotypes in possible progenies (Table 3). The dose mortality response of *sp-1*^{RS}*sp-2*^{RS} offspring should be equivalent to that of (BP x SPHAE) ones while that of *sp-1*^{RR}*sp-2*^{RS} larvae is more likely to resemble that of SPHAE. Although (BP x SPHAE) offspring were not tested with Spherimos, their mortality at one and 10 g/liter Spherimos may be roughly estimated from the mortality curves of Fig. 2B, considering the conditions killing 3% of SPHAE as equivalent: i.e., ≈3 g/liter of *Bs* lyophilized suspension in 35 ml and 10 g/liter Spherimos in 100 ml. With these extrapolations, the Sphe-

Table 3. Mother genotypes and corresponding expectations in progenies

Mother genotype[a]	Progeny composition[b]				Expected mortality, %	
					1 g/liter	10 g/liter
H_1: *sp-1* only	*sp-1*^{RS}		*sp-1*^{RR}			
sp-1^{SS}	1		0		100 (100)[c]	100 (100)
sp-1^{RS}	0.5		0.5		50 (52)	52 (54)
sp-1^{RR}	0		1		0 (4)	3 (7)
H_2: *sp-1* & *sp-2*	*sp-1*^{RS} *sp-2*^{SS}	*sp-1*^{RS} *sp-2*^{RS}	*sp-1*^{RR} *sp-2*^{SS}	*sp-1*^{RR} *sp-2*^{RS}		
sp-1^{SS}*sp-2*^{SS}	1	0	0	0	100	100
sp-1^{SS}*sp-2*^{RS}	0.5	0.5	0	0	77 (79)	93 (95)
sp-1^{SS}*sp-2*^{RR}	0	1	0	0	54 (58)	85 (89)
sp-1^{RS}*sp-2*^{SS}	0.5	0	0.5	0	50 (52)	52 (54)
sp-1^{RS}*sp-2*^{RS} [d]	0.25	0.25	0.25	0.25	39 (42)	48 (51)
sp-1^{RS}*sp-2*^{RR}	0	0.5	0	0.5	27 (31)	44 (48)
sp-1^{RR}*sp-2*^{SS}	0	0	1	0	0 (4)	3 (7)
sp-1^{RR}*sp-2*^{RS}	0	0	0.5	0.5	0 (4)	3 (7)
sp-1^{RR}*sp-2*^{RS}	0	0	0	1	0 (4)	3 (7)

[a] At one (H1) or two loci (H2). SPHAE paternal genotypes are *sp1*^{RR} under H1, and *sp1*^{RR} *sp2*^{SS} under H2.
[b] Assuming no segregation bias in heterozygous females.
[c] The analysis used the values in parentheses that take into account the mortality observed in controls.
[d] Assuming a segregation independence between *sp-1* and *sp-2* genes.

Table 4. Characteristics of partly resistant progenies

Maternal origin		Distribution		Resistance factors detected		
Area	Locality	Sample size	Frequency, %	*sp-1*^{R}	*sp-2*^{R}	Others[a]
1	Llobregat-1	7	0			
1	Llobregat-2	7	0			
1	Llobregat-3	14	0			
2	Perpignan-1	60	7	+	+	
2	Perpignan-2	12	17		+	+
2	Perpignan-3	35	3		+	
2	Perpignan-4	52	21	+?[b]	+	+
3	Narbonne-1	21	5		+	
3	Narbonne-2	10	10		+	
4	Sète	62	44	+?	+	+
5	Sumène	18	0			
5	St Bauzille de P.	4	0			
5	Notre Dame	28	4		+	
6	Les Matelles	40	0			
6	Prades-le-Lez	13	0			
7	Près d'Arène	14	0			
7	Lattes	21	5			+
7	Maurin	24	0			
8	St Brès	29	3		+	
9	Le Grau du Roi	13	0			
10	Port-St-Louis	17	94	+		+
11	Martigues-1	21	57	+	+	+
11	Martigues-2	15	20		+	+

[a] Offspring mortality incompatible with any expectation of Table 3.
[b] Offspring mortality was also compatible with assumptions excluding *sp-1*^{R}.

rimos mortality of *sp-1*^{RS}*sp-2*^{RS} larvae was estimated as 54% at 1 g/liter, and 85% at 10 g/liter. Accordingly, the expected mortalities characterizing the alternative assumptions on mother genotypes would be as in Table 3. This permitted to attribute a genotype to 51 of the 75 mothers of a partly resistant progeny (details not shown). Mortality data observed in the remaining 24 resistant families differed from expectations at either one or 10 g/liter, or at both doses. They interestingly defined two subgroups, as the 11 cases recorded for mothers from Port-St-Louis (area 10 on Fig. 1) differed in mean mortality (5 ± 6% at 1 g/liter and 56 ± 33% at 10 g/liter) from the 13 cases observed for mothers collected elsewhere (71 ± 26% at 1 g/liter and 80 ± 23% at 10 g/liter). Although it remains possible that these 24 cases rely on the approximate evaluation of expected mortalities, their differentiation in subgroups would rather indicate the presence of additional factors conferring *Bs*-resistance when associated with *sp-1*^{R}.

In conclusion, mortality observed in the offspring of field females crossed with SPHAE males indicated that *sp-1*^{R} was not the only factor of resistance to *Bs* present in southern France, as expected from the results obtained on the BP strain. The analysis considering that both *sp-1*^{R} and *sp-2*^{R} mutants were involved improved the efficacy of mother genotype inferences from 21 to 68%, indicating the probable presence of *sp-2*^{R}. Table 4 summarized the overall results. It showed that *Bs*-resistance was apparently absent in Spain and in only seven of the 20 French samples, with *sp-2*^{R} apparently present over the whole *Bs*-controlled area, and *sp-1*^{R} in several females collected at the eastern border (Port-St-Louis and Martigues) and in one from the western border (Perpignan). In addition, possible carriers of factors, distinct from *sp-1*^{R} and *sp-2*^{R}, that confer *Bs*-resistance when associated with *sp-1*^{R} were particularly abundant in Port-St-Louis (65%) and in Sète (10%).

Discussion

We designed crosses between field-collected females and SPHAE males to monitor the *sp-1*^{R} geographical distribution 3 yr after its first detection in Port-St-Louis (area 10, Fig. 1). During these 3 yr, occasional operational problems were reported in areas 10 and 2 (C. Lagneau, EID, personal communication), at the eastern and western borders of the *Bs*-controlled area. Our survey detected several factors that conferred resistance when combined with *sp-1*^{R}, and showed that they were widespread and had already reached high frequencies in some localities (Table 4). The contrast between the rare control failures encountered and the extensive distribution of resistance factors raised several remarks regarding the sustainability of *C. pipiens* control by *B. sphaericus* (*Bs*), and, by extension, that of similar systems using *Bt* toxins.

This contrast first strengthens how limited a forecast on resistance is without a precise characterization of the genetic diversity involved. Here, the presence of other factors than those isolated in SPHAE (*sp-1*^{R}) and BP (*sp-2*^{R}) strains was suspected but no mean allowed to identify them. Moreover, the low fecundity success, that decreased our sampling performance by almost 80%, confirmed that the "F2-screen" approach, designed to characterize the genetic diversity involved in *Bt*-resistance of agricultural pests (Andow and Alstad 1998), is not appropriate for *C. pipiens* from

southern France where eurygamy (e.g., inability to mate in laboratory conditions) is widespread (Pasteur et al. 1977). Clearly, identifying the genetic diversity involved in *Bs*-resistance requires diagnostic tools, such as molecular or biochemical markers of resistance, that will allow to by-pass crosses.

In addition, a curious interaction was found between two co-occurring resistance genes: *sp-1*^{R}, detected in Port-St-Louis in 1994 (SPHAE strain), and *sp-2*^{R}, isolated in Perpignan in 1997 (BP strain). Present data indicated that *sp-1*^{R} and *sp-2*^{R} are likely to be encoded by distinct and physically linked genes. Both were showed to confer a similarly high resistance (≈6,000-fold) in the homozygote state and to be totally recessive, except that the heterozygotes carrying one copy of each were in fact resistant (130-fold at LC_{50} and 1,800-fold at LC_{95}). The mechanism underlying the *sp-1*^{R} - *sp-2*^{R} interaction remains to be elucidated, a difficult task due to the poor understanding of the mode of action of bacterial toxins. Presently, it is known that, after solubilization and activation, the toxins produced by either *B. sphaericus* or *B. thuringiensis* bind to specific insect receptors located in the midgut brush border cells, with this binding step being invoked as the major cause of their specificity in species toxicity (Hoffmann et al. 1988, Charles et al. 1996), and known to lead to death via osmotic cell lysis in the case of *Bt* toxins (Knowles 1994). Resistance may be due to any mutation interrupting an event along this chain, or else to increased detoxification. In *C. pipiens*, alteration in the toxin-receptor binding was reported as a *Bs*-resistance cause (Nielsen-Leroux et al. 1995), and cloning and heterologous expression recently identified this receptor as an α-glucosidase (Darboux et al. 2001). In addition, the toxin-receptor binding was found unmodified by *sp-1*^{R} (Nielsen-Leroux et al. 1997), and the *sp-1*^{R} - *sp-2*^{R} interaction indicates that they probably affect subsequent steps along the process of *Bs*-toxicity. Further investigation of this interaction, associating analyses of resistance genetics, binding studies and receptor sequencing, may give a better insight on the mode of action of *Bs* binary toxin.

Most importantly, the association of this particular *sp-1*^{R} –*sp-2*^{R} interaction and the unsuspected wide distributions of resistance factors is unlikely to be incidental, as the resistant *sp-1*^{RS} *sp-2*^{RS} combination counts among the most likely genotypes in populations where both are rare. The discovery of a combination of distinct recessive factors conferring resistance raises thus new questions on the sustainable strategies of *Bs*- and *Bt*-resistance management. A present model to delay operational problems regarding insect pests that feed on *Bt*-transgenic crops is the high-dose-refuge strategy (Gould 1998). The "high-dose" underlying argument is that, resistance to bacterial toxins being usually recessive, resistant mutants will mostly be found in heterozygotes killed by high doses. However, coexistence of different resistance mutants within populations was reported for the *Bt*-resistance developed by *P. xylostella* (Tabashnik et al. 1997) as for the *Bs*-resistance developed by *C. pipiens* (current study). If interactions between recessive mutants, as that described here for *sp-1*^{R} and *sp-2*^{R}, is a common mechanism, these coexisting mutants will "protect" and help each other to establish themselves. This may restrict the present enthusiasm for high-dose strategies, as long as the possibility of multiple interacting mutations remains unexplored in theoretical frameworks modeling the evolution of resistance to bacterial toxins.

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