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CROSS-RESISTANCE TO *BACILLUS SPHAERICUS* STRAINS IN *CULEX QUINQUEFASCIATUS*

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ABSTRACT. Laboratory and field-collected strains of *Culex quinquefasciatus* that were selected with *Bacillus sphaericus* strain 2362 in the laboratory for about 100 generations and attained 37- and 31-fold resistance, respectively, did not show marked cross-resistance to *Bacillus thuringiensis* spp. *israelensis*; only 2–3-fold tolerance was noted. However, they showed significant levels of cross-resistance to strains 1593 (6–22-fold) and 2297 (4–12-fold) of *B. sphaericus*. Possible mechanisms and management implications are discussed.

INTRODUCTION

It is known that arthropods showing resistance to a given pesticide may also manifest resistance to related compounds to which they have not been exposed. Cross-resistance in insects, defined as protection from more than one insecticide through the action of a single mechanism (Georghiou 1965, Scott 1990), is usually shown to insecticides with similar mode(s) of action.

The endospore-forming bacterium *Bacillus sphaericus* Neide is known to be a heterogeneous group of subspecies or strains. On the basis of DNA–DNA hybridization (Krych et al. 1980) and auxanogram studies (de Barjac et al. 1980), this species consists of a number of strains pathogenic and nonpathogenic to mosquito larvae. This microbial pest control agent (MPCA) has already been used in operational mosquito control programs in some countries. Strains 1593, 2297, and 2362 have been extensively studied and the latter strain was granted registration for mosquito control in the United States by the Environmental Protection Agency in 1991. The toxins produced by strains 2362 and 1593 are essentially the same structurally, whereas the restriction pattern of the toxins of strain 2297 is different from the toxins of the other 2 strains (Baumann et al. 1987, Arapinis et al. 1988). The 51 and 42 kDa protein toxins act as a binary toxin, with both being required for expression of toxicity in mosquito larvae (Broadwell et al. 1990). The mode of action and pathogenicity of these strains have been studied at the gross and ultrastructural scale. It is evident that the toxic strains of *B. sphaericus* generally express the same pathologic effects and exhibit similar modes of action (Broadwell and Baumann 1987; Davidson 1988; 1989; Nielsen-LeRoux and Charles 1992). The intoxication process involves: 1) ingestion of the crystal/spore/cell complex; 2) dissolution in the midgut by alkaline pH; 3) activation of the protoxins (51 and 42 kDa) into the toxic form (43 and 39 kDa) by gut proteases; 4) binding of the binary toxin to the specific receptors on the brush border membrane of epithelial cells in the gastric ceca and posterior midgut regions; 5) toxin internalization and exertion of toxicity by an unknown mechanism; and 6) cell lysis (Baumann et al. 1991, Porter et al. 1993). The pathologic effects then involve basal separation and sloughing of midgut epithelial cells, swelling of midgut, paralysis, and death within 4–48 h depending on doses ingested (Davidson and Yousten 1990).

Bacillus thuringiensis ssp. *israelensis* de Barjac (*B.t.i.*), another endospore-forming bacterium used in mosquito control, produces different types of toxin proteins. During sporulation, this bacterium produces 3 major types of parasporal inclusions that contain crystals composed of at least 5 polypeptides of approximately 134, 128, 58, 70, and 27 kDa (Whiteley and Schnepf 1986, Federici et al. 1990) that are toxic to a broader spectrum of dipteran hosts than *B. sphaericus*, (i.e., toxic to mosquitoes, black flies, and some other nematocerans). Toxins of *B.t.i.* are also gut poisons affecting midgut epithelium as the primary target site and induce a similar initial pathologic syndrome as *B. sphaericus* toxin. The intoxication process of *B.t.i.* (Porter et al. 1993) includes essentially the same steps as those of *B. sphaericus* strains and the treated larvae die within 4–24 h depending on doses ingested.

Due to practical considerations in control programs and the management of resistance in mosquitoes, it is of interest to find out whether the resistance developed to certain strain(s) of a microbial agent would confer cross-resistance to the other strains of the same species or different species of toxin-producing organisms. The present study was carried out to investigate the potential development of cross-resistance conferred by resistance to *B. sphaericus* strain 2362 to other strains of *B. sphaericus* and to *B.t.i.*

MATERIALS AND METHODS

Strains of the southern house mosquito, *Culex quinquefasciatus* Say, resistant and susceptible

Table 1. Susceptibility to *Bacillus thuringiensis* ssp. *israelensis* and strains of *B. sphaericus* in *Culex quinquefasciatus* selected with *B. sphaericus* 2362 for about 100 generations.

Strain[1]	Slope ± SEM	LC_{50} (95% CI)[2]	LC_{90}[2]	χ^2 (df)	RR (at LC_{50})[3]	RR (at LC_{90})[3]
B. sphaericus 2362						
L-S	3.0 ± 0.2	0.008 (0.008–0.009)	0.022	1.6 (3)	—	—
L-SEL	5.6 ± 0.3	0.304 (0.271–0.332)	0.513	10.3 (4)	37.0 (33.4–40.9)	23.6 (20.3–27.3)
F-S	2.5 ± 0.3	0.014 (0.004–0.025)	0.048	8.7 (2)	—	—
F-SEL	6.4 ± 0.5	0.444 (0.424–0.466)	0.707	0.6 (3)	31.1 (25.3–38.3)	14.9 (11.6–19.2)
B. thuringiensis ssp. *israelensis*						
L-S	3.5 ± 0.2	0.107 (0.100–0.114)	0.245	2.5 (3)	—	—
L-SEL	4.1 ± 0.2	0.246 (0.204–0.287)	0.501	16.9 (4)	2.3 (2.1–2.5)	2.0 (1.8–2.4)
F-S	2.1 ± 0.2	0.076 (0.048–0.105)	0.314	7.2 (4)	—	—
F-SEL	3.2 ± 0.2	0.203 (0.126–0.313)	0.508	21.5 (3)	2.6 (2.1–3.3)	1.6 (1.3–2.1)
B. sphaericus 1593						
L-S	5.9 ± 0.5	0.007 (0.006–0.009)	0.012	6.6 (2)	—	—
L-SEL	5.7 ± 0.3	0.159 (0.143–0.175)	0.267	5.8 (4)	21.6 (19.5–23.9)	22.0 (19.2–25.3)
F-S	3.6 ± 0.2	0.016 (0.012–0.020)	0.036	12.6 (4)	—	—
F-SEL	3.1 ± 0.2	0.097 (0.081–0.115)	0.248	9.3 (5)	6.1 (5.4–6.9)	6.8 (5.6–8.2)
B. sphaericus 2297						
L-S	0.9 ± 0.1	0.009 (0.007–0.011)	0.244	1.6 (3)	—	—
L-SEL	3.1 ± 0.2	0.109 (0.068–0.283)	0.287	66.6 (3)	11.7 (8.7–15.9)	1.2 (0.7–1.9)
F-S	3.3 ± 0.2	0.011 (0.008–0.015)	0.027	18.4 (4)	—	—
F-SEL	2.9 ± 0.3	0.045 (0.018–0.061)	0.123	29.1 (5)	4.0 (3.4–4.7)	4.5 (3.6–5.5)

[1] Strains of *B. sphaericus*, L-S = susceptible laboratory, L-SEL = selected laboratory, F-S = susceptible field-collected, F-SEL = selected field-collected.
[2] 48 h, mg/liter, n = 900–1,575.
[3] 95% CI (confidence interval) in parentheses.

to *B. sphaericus*, used in this study were obtained from the previous selection studies by Rodcharoen and Mulla (1994): the selected laboratory strain (L-SEL), the susceptible nonselected laboratory strain (L-S), the selected field-collected strain (F-SEL), and the susceptible nonselected field-collected strain (F-S). The L-SEL and F-SEL strains were selected with a powder preparation of *B. sphaericus* strain 2362 (ABG-6232, Abbott Laboratories, North Chicago, IL) and had developed a maximum and stable level of resistance of 37- and 31-fold in F_{80} and F_{60}, respectively, to *B. sphaericus* strain 2362 (Rodcharoen and Mulla 1994) (Table 1). The L-S and F-S strains, which were cultured without exposure to *B. sphaericus*, did not show any marked change in their susceptibility to this agent. The L-S and F-S strains were used in bioassays for comparisons of susceptibility of the resistant strains to each microbial agent. Cross-resistance experiments were conducted during generations 95–109 of the L-SEL, 95-112 of the L-S, 90–104 of the F-SEL, and 101–114 of the F-S. During these tests, the 2 selected strains were subjected to selection pressure (at the LC_{80} level) with *B. sphaericus* strain 2362 to maintain the resistance level. The 2 selected strains showed stable levels of resistance (37- and 31-fold) to *B. sphaericus* strain 2362 throughout the cross-resistance tests. It was assumed that the resistance in both selected strains had reached a plateau of maximum level.

The *B. sphaericus* preparations used for determination of cross-resistance were standard (lyophilized) preparations of *B. sphaericus* strains 1593 (RB 80) and 2297 (SPH 84) (provided by Institut Pasteur, Paris, France), and an aqueous suspension of *B.t.i.* (Vectobac 12 AS, Abbott Laboratories). Due to the waxy and flaky nature of the standard preparations of strains 1593 and 2297, their stock suspensions in deionized water were sonicated in order to assure good suspensions. Stock suspensions of Vectobac 12 AS in deionized water were mixed by vigorous shaking because this formulation suspends readily in water on shaking. Bioassays were conducted according to the procedures of Rodcharoen and Mulla (1994). Each material was tested against the resistant strains concomitantly with their susceptible counterparts, with a 48-h exposure period for *B. sphaericus* strains and 24 h for *B.t.i.* At least 3 tests were done on different cohorts for each mosquito strain–microbial agent pair. Probit regressions were esti-

mated by using the POLO-PC computer program (Russell et al. 1977, LeOra Software 1987). Resistance ratios (RR) at LC_{50} and LC_{90} along with their 95% CI for each resistant strain to each material were calculated by comparing the strain's LC values with those of the unselected counterpart, using the method described by Robertson and Preisler (1992).

RESULTS AND DISCUSSION

The L-SEL and F-SEL, which possessed 37- and 31-fold resistance to *B. sphaericus* strain 2362, respectively, did not show any marked resistance to *B.t.i.* compared with their susceptible counterparts (L-S and F-S). Only about 2-fold tolerance, both at the LC_{50} and LC_{90} levels, was detected in the 2 strains (Table 1). This low level of tolerance, although statistically significant (95% CI of RRs did not include the integer 1 [Robertson and Preisler 1992]), is usually considered to be due to biological variations or experimental errors and not a real development of resistance. It is known that both *B.t.i.* and *B. sphaericus* toxins bind to specific receptors on the surface of midgut brush border membranes (BBM) after they are released from their envelope and activated (Hofmann et al. 1988, Nielsen-LeRoux and Charles 1992). Toxins of the 2 bacteria, however, were found to bind to different classes of specific receptors on BBM, as binding assays showed that *B. sphaericus* toxin failed to bind to midgut BBM of *Aedes aegypti* (Linn.), which is refractory to *B. sphaericus* but highly susceptible to *B.t.i.* (Davidson 1989, Nielsen-LeRoux and Charles 1992). Moreover, *Cx. quinquefasciatus* larvae, which were highly resistant (>100,000-fold, Georghiou et al. 1992) and no longer had functional receptors for *B. sphaericus,* were still susceptible to *B.t.i.* (Nielsen-LeRoux et al. 1994).

Therefore, in addition to the differences in structure and biochemistry between *B. sphaericus* and *B.t.i.* toxins, the differences in specific receptors for these toxins could explain the absence of cross-resistance between the 2 microbial agents in *Cx. quinquefasciatus.* In terms of resistance management, *B.t.i.* would be a good larvicide of choice, in addition to larvicidal oils and insect growth regulators, to use in rotation with *B. sphaericus* in mosquito control programs, because it would help prevent or delay development of resistance in mosquitoes to either microbial agent. All evidence to date shows that there is little or no development of resistance to *B.t.i.* in mosquitoes even though *B.t.i.* has been used in operational programs for about 15 years.

The L-SEL strain (37-fold resistance to *B. sphaericus* 2362) showed a significant level of 21.6-fold (at LC_{50}) resistance to *B. sphaericus* strain 1593, compared with its susceptible counterpart (L-S). The F-SEL (31-fold resistance to *B. sphaericus* 2362) showed only 6.1-fold (at LC_{50}) resistance to strain 1593, compared with the F-S (Table 1). To *B. sphaericus* strain 2297, a lower level of resistance (at LC_{50}) of 11.7-fold was detected in L-SEL, whereas a 4-fold resistance was found in F-SEL (Table 1). The RRs at the LC_{90} level are in general about the same as those at LC_{50}.

In vitro binding assays conducted by Nielsen-LeRoux et al. (1994) showed that the binary toxin of *B. sphaericus* (strain 1593) failed to bind to larval midgut brush border membrane fractions (BBMFs) of a *Cx. quinquefasciatus* strain >100,000-fold resistant to strain 2362 (Georghiou et al. 1992). These authors suggested that the larvae highly resistant to strain 2362 had lost the functional receptors for *B. sphaericus* strain 1593 toxin. In a 10-fold resistant (to strain 2362) field-strain of *Cx. quinquefasciatus* (Silva-Filha et al. 1995), however, there was no change in binding affinity (Nielsen-LeRoux et al. 1994). The level of resistance developed in L-SEL and F-SEL in the present study is considered to be moderate and it is possible that these resistant strains may have partial alteration or reduction in receptor sites and binding affinities. Studies are underway to determine the specific receptors and binding affinities in these strains. It is speculated that any changes in receptor sites or binding affinities to one of the *B. sphaericus* strains such as 1593 or 2362 will invest some degree of cross-resistance to the other strain because these strains produce similar binary toxins (Baumann et al. 1988) which bind to a single class of specific receptors (Nielsen-LeRoux and Charles 1992). The degree of cross-resistance to strain 2297, which produces a different type of toxins (Baumann et. al. 1988), may be less pronounced, as found in our studies. The higher levels of cross-resistance to strain 1593 than to strain 2297 in both L-SEL and F-SEL strains is therefore due to close affinity between strains 1593 and 2362, as they are in the same H-serotype and phage-type (H5a,5b, phage-group 3), whereas strain 2297 is in a different group (H25, phage-group 4). Strains 1593 and 2362 were also found to be more effective than strain 2297.

The possible cross-resistance among strains of *B. sphaericus* give us a warning sign that this agent should be used properly to prevent or delay development of resistance in mosquitoes, as resistance to one strain will also render other strains less effective in mosquito control programs.

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