MIXTURES AS A TOOL FOR THE MANAGEMENT OF INSECTICIDE RESISTANCE TO THE BACTERIAL INSECTICIDE *BACILLUS THURINGIENSIS* ISRAELENSIS.

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Abstract—Mixtures of insecticides have been proposed as one tool for the management of insecticide resistance. The concept of mixtures relies on the hypothesis that organisms may be able to overcome a single toxicant but are unlikely to be able to overcome several independently acting toxicants. Mixtures have been successfully used to manage antibiotic resistance in medicine, and have been proposed to manage insecticide resistance in arthropods. Insecticide mixtures have been tested in theoretical models as well as in laboratory selection experiments, and the results are promising.

The bacterial toxins of *Bacillus thuringiensis israelensis* (Bti) are toxic to mosquitoes and blackflies. Bti expresses four different toxin proteins: 1) CryIVA (125 kDa), 2) CryIVB (135 kDa), 3) CryIVD (65 kDa) and 4) CytA (28 kDa). Because resistance has been slow to develop to Bti, both in the field and in laboratory selection experiments, it has been hypothesized that the four toxins may act as a natural mixture, delaying the development of resistance. We undertook a series of experiments, designed to test whether resistance would develop more easily in response to selection pressure with a single Bti toxin, compared to selection with mixtures of two, three and four toxins. These experiments demonstrated that resistance developed more rapidly, and to a higher level, when mosquitoes were selected with a single Bti toxin. Further, we observed that no resistance developed in response to selection with four toxins, whereas resistance developed in lines selected with one, two and three toxins. The loss of a single toxin, the CytA toxin, from the original four toxins in Bti, resulted in significant levels of resistance in mosquitoes under selection pressure. The role of the CytA toxin was further investigated through bioassay tests, singly, and in combination with the other CryIV toxins. The results indicate that the CytA toxin, which differs significantly from the CryIV toxins in its gene sequence and mode of action, may play an important role in delaying resistance to Bti.

INTRODUCTION

Insecticide mixtures have been discussed as one possible strategy for the management of insecticide resistance (Curtis, 1985; Georghiou, 1983, 1994; Tabashnik, 1989; McGaughey and Whalon, 1992). In order for this approach to be effective the mechanisms of resistance to each component in the mixture must be different. The mixture must be used when the frequency of each resistance mechanism is sufficiently low that both mechanisms do not occur in the same individual. Under these conditions, an insect which survives one insecticide will be killed by the other insecticide.

Computer simulations have been used to evaluate some of the factors influencing the success of insecticide mixtures (Curtis *et al.*, 1978; Taylor and Georghiou, 1979; Curtis, 1985; Mani, 1985). These models demonstrated that if resistance is a fully recessive trait, mixtures can delay resistance considerably. Only the rare, double homozygotes can survive. But any deviation from recessive inheritance would adversely affect a mixture strategy. For example, when inheritance is intermediate in dominance, resistance develops rapidly under selection with a mixture due to the development of linkage disequilibrium between the resistance mechanisms. But intermediate expression of dominance can be rendered functionally recessive by the choice of an appropriate dose of insecticide which kills heterozygotes as well as susceptibles (Curtis *et al.*, 1978; Curtis, 1981; Taylor and Georghiou, 1979).

Additional factors are critical to the success of insecticide mixtures. The rate of decay of each of the chemicals in a mixture must be equal in order to avoid exposure to one chemical in the absence of the other (Curtis, 1985). The mixture strategy also depends on moderate numbers of insects escaping exposure (Curtis, 1985). Curtis *et al.* (1993) have shown that insecticide mixtures have a decided advantage in the case of malaria vectors which are exposed to impregnated bednets. Under these conditions, treatment affects only half of the population, the host seeking females. Synergistic interactions between the components of a mixture would increase the toxicity of each component and enhance the utility of a mixture (Georghiou, 1983), but such fortuitous interactions between chemicals are rare.

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Unfortunately, practical and economic considerations limit the situations in which a mixture strategy may be appropriate. Many populations of pest insects already possess genes for resistance to a variety of insecticides (Georghiou and Lagunes-Tejeda, 1991), reducing the available options for mixtures. The cost-effectiveness of mixtures is a further difficulty since a mixture can require twice the amount of insecticide per treatment compared to conventional treatments. However nature may have fortuitously provided us with a natural mixture in the insecticidal crystal proteins produced during sporulation by the bacterium *Bacillus thuringiensis (Bt)*. Different strains of *Bt* have been shown to be insecticidal to different insect groups. For example, *Bt kurstaki, Bt aizawai,* and *Bt entomocidus* are insecticidal to Lepidoptera, *Bt tenebrionis* is insecticidal to Coleoptera, and *Bt israelensis* is insecticidal to Diptera (Höfte and Whitely, 1989). Each isolate produces a variety of different insecticidal proteins (Höfte and Whitely, 1989).

Bt israelensis (Bti) expresses a mixture of four major proteins in a parasporal body during sporulation. These proteins have approximate molecular weights of 125, 135, 72 and 27 kDa and have been classified as CryIVA, CryIVB, CryIVD and CytA (Höfte and Whitely, 1989). Three of the proteins are classified as Cry-type proteins on the basis of their nucleotide sequence and their similarity to other Cry-type proteins produced by *Bt* isolates. CryIVA and CryIVB share considerable sequence homology, but CryIVD has only limited sequence homology with these two proteins (Höfte and Whitely, 1989). The Cry proteins are believed to bind to specific receptors on the brushborder membrane of epithelial cells in a sensitive insect's midgut. After binding, the toxins insert into the cell membrane, disrupting osmotic balance (Knowles and Ellar, 1988). The CytA protein has no sequence homology with the CryIV proteins, as well as a more general mode of action. The CytA protein is more broadly cytolytic to a variety of invertebrate and vertebrate cells (Höfte and Whitely, 1989). Thus it appears that *Bti* is composed of a heterogeneous mixture of insecticidal proteins.

A project was undertaken to determine whether the development of resistance to *Bti* in the mosquito *Culex quinquefasciatus* was affected by the number of *Bti* toxins present in the selection mixture. The possible role of the CytA protein in suppressing resistance was also investigated. Based on this information, rational resistance management strategies for *Bti*-based insecticides may be developed.

MATERIALS AND METHODS

Bti toxins used

Toxin powders from batch cultures were used for both bioassay and selection. Each powder consisted of a sporulated, lyophilized powder. CytA (Wu *et al.*, 1994) and CryIVD (Chang *et al.*, 1992) were provided by B. A. Federici and S. S. Gill, respectively, University of California, Riverside.

The following three toxin combinations were provided by A. Delécluse from strains maintained at the Pasteur Institute, Paris: CryIVA+CryIVB (Delécluse *et al.*, 1993), CryIVA+CryIVB+CryIVD (Delécluse *et al.*, 1991) and CryIVA+CryIVB+CryIVD+CytA, known as the IPS80 formulation.

Strains used

A "synthetic" population (BT-SYN) of *C. quinquefasciatus* was produced by combining larvae from 19 different collections of *Culex pipiens quinquefasciatus* using approximately 500 first instar larvae from each collection. A population of several thousand adults was established from these larvae and maintained at this level for seven months before the selection experiments were initiated. The following selection series were conducted:

Selection series	Selecting toxins	Generations selected
I	CryIVD	28
II	CryIVA+CryIVB	24
III	CryIVA+CryIVB+CryIVD	28
IV	CryIVA+CryIVB+CryIVD+CytA	28
V	Unselected Control (BT-SYN	0

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Stock suspensions of the toxins were prepared with distilled water in 125 ml flasks in which 25 glass beads were placed to facilitate homogenization. Suspensions containing both CytA and one or more CryIV toxin were prepared by combining powders in a 1:3 ratio of CytA to CryIV on the basis of weight. Suspensions were agitated for 5 min. by shaking and vortexing. Ten fold dilutions were made from stock suspensions and these, as well as the remainder of the stocks, were kept frozen at −20°C when not in use.

Bioassay Method

Bioassays were conducted in 8 oz. plastic cups in 100 ml of distilled water using 20 early-fourth instar larvae per cup. A minimum of 5 concentrations giving mortality within the range of 2% and 98% were used. Tests were replicated on 5 different days. Adjustments to control and test cups were made with distilled water. Mortality was determined after 24 hours. Data were subjected to probit analysis (Finney, 1971) using the program of Raymond (1985).

Bioassays on each selected line were performed on every third generation concurrently with bioassays on the unselected (BT-SYN) colony, using the same stock solutions in order to provide more accurate comparisons of changes in susceptibility (Generations 1 - 28). Tests with the CytA protein were conducted at a later stage. Therefore the relative resistance ratios reported for some of the selected lines differ from those reported for generation 28.

Selection Method

Selections were done in enameled pans with 1000 early-fourth instar larvae in 1000 ml distilled water. A minimum of 5000 larvae were selected for each strain in each generation. The desired concentration of *Bti* toxin was added and the surviving larvae were recovered after 24 hours. Generations were maintained separately.

RESULTS

After 28 generations of selection pressure, high levels of resistance were observed in the one toxin (CryIVD), two toxin (CryIVA+CryIVB) and three toxin (CryIVA+CryIVB+CryIVD) selected lines, but not in the four toxin (CryIVA+CryIVB+CryIVD+CytA) selected line (Figure 1). The resistance ratio was highest in the CryIVD-selected line, greater than 913-fold at the LC95. Lower resistance was detected in the two-toxin and three-toxin selected lines, 122-fold and 91-fold at the LC95, respectively. Resistance was only 3-fold in the four-toxin selected line.

When each of the selected lines was bioassayed with a 1:3 ratio of CytA and the CryIV toxin(s) used for selection, resistance was suppressed to less than 5-fold in all cases (Figure 2).

DISCUSSION

The level of resistance achieved in the different selected lines was inversely related to the number of toxins in the selection mixture. Resistance was highest in the single-toxin selected line and progressively lower as additional toxins were present in the selection mixture. These results suggest that a combination of differing Bti toxins is preferable to selection with a single toxin. However, because significant levels of resistance were observed in the one, two and three toxin-selected lines, these mixtures of CryIV toxins were ineffective in preventing resistance. In contrast, no significant resistance was observed in the four-toxin selected line. Because the three and four-toxin selected lines differed in the presence of the CytA protein, this observation further suggests that CytA may have played a role in the lack of resistance in this line. However it is not possible to know from these data whether the absence of any one of the other four toxins would have produced a similar result.

When the CytA protein was artificially recombined with the CryIV proteins, resistance to CryIV toxins was suppressed.These results provide direct evidence that the CytA protein has a significant

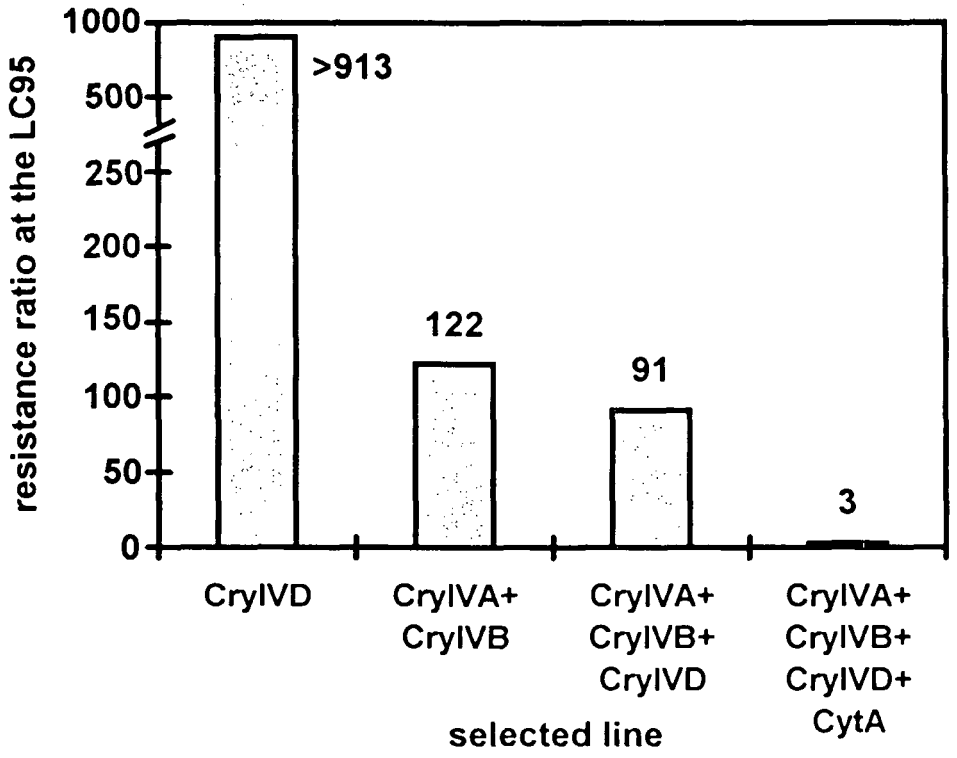

Figure 1. Relative resistance ratios (at the LC 95) of the one, two, three and four *Bti* toxin selected lines of *Culex quinquefasciatus*, after 28 generations of selection pressure.

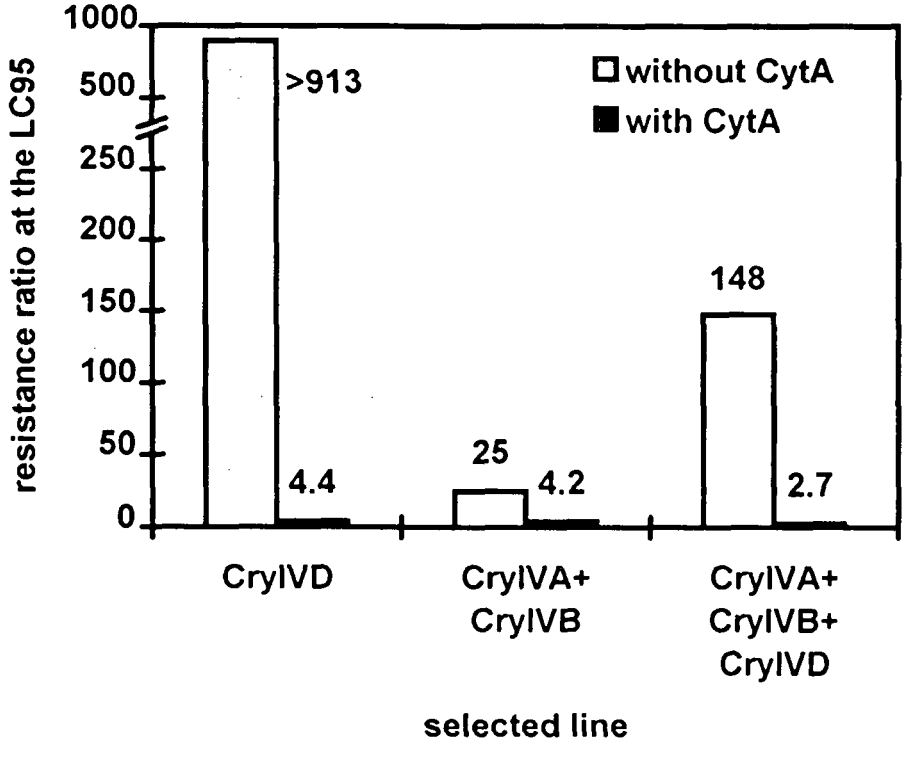

Figure 2. Relative resistance ratios (at the LC 95) in the one, two and three *Bti* toxin selected lines of *Culex quinquefasciatus* in the presence or absence of the CytA toxin.

effect on resistance to CryIV toxins and further indirect evidence that it may be responsible for the lack of resistance in the four toxin selected line.

This research did not directly addressed the question of whether the combination of CytA with one or more CryIV toxins would be effective in either preventing or delaying resistance. Additional experimentation is underway to test this hypothesis. But the results from other attempts to select for resistance with *Bti* are consistent with our observation that resistance is slow to develop to the CryIV + CytA toxin mixture that naturally occurs in *Bti*. For example, laboratory selection with *Bti* resulted in only low levels of resistance in *Culex quinquefasciatus* (Georghiou, 1983), and no resistance in *Aedes aegypti* (Goldman *et al.*, 1986). Field control of *Aedes vexans* with *Bti* for more than 10 years (Becker and Ludwig, 1993) has failed to change the population's sensitivity to *Bti*.

The results of selection with *Bti* are in contrast to those with other isolates of *Bt*. Field resistance to *Bt kurstaki* has been reported in *Plutella xylostella* from the Philippines, Hawaii, Japan, Florida and Malaysia (Tabashnik *et al.*, 1991; Hama *et al.* 1992; Shelton *et al.*, 1993). Laboratory selection of *Plodia interpunctella* collected from the field, indicates that this species can also develop resistance to *Btk* (Kinsinger and McGaughey, 1979). Although *Bt kurstaki* contains a mixture of several different CryI and CryII toxins (Tabashnik, 1994), the detection of field and laboratory resistance demonstrates that this mixture of Cry toxins was not effective in avoiding resistance. In contrast, mixtures of unrelated *Bt* toxins, that differ in nucleotide sequence and mode of action, such as the CryIV and CytA proteins in *Bti*, may provide a means of managing resistance to this important insecticide.

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REFERENCES

Becker, N., and Ludwig, M. (1993). Investigations on possible resistance in *Aedes vexans* after a 10-year application of *Bacillus thuringiensis israelensis*. *J. Amer. Mosq. Control Assoc.* 9(2):221–224.

Chang, C., Dai, S.-M., Frutos, R., Federici, B. A., and Gill, S. S. (1992). Properties of a 72-kilodalton mosquitocidal protein from *Bacillus thuringiensis* subsp. *morrisoni* PG-14 expressed in *Bacillus thuringiensis* subsp. *kurstaki* by using the shuttle vector pHT 3101. *Appl. and Environ. Microbiol.* 58:507–512.

Curtis, C. F. (1981). Possible methods of inhibiting or reversing the evolution of insecticidal resistance in mosquitoes. *Pestic. Sci.* 12:557–564.

Curtis, C. F. (1985). Theoretical models of the use of insecticide mixtures for the management of resistance. *Bull. Entomol. Res.* 75:259–265.

Curtis, C. F., Cook, L. M., and Wood, R. J. (1978). Selection for and against insecticide resistance and possible methods of inhibiting the evolution of resistance in mosquitoes. *Ecol. Entomol.* 3:273–287.

Curtis, C. F., Hill, N., and Kasim, S. H. (1993). Are there effective resistance management strategies for vectors of human diseases. *Biol. J. of Linn. Soc.* 48:3–18.

Delécluse, A., Charles, J.-F., Klier, A., and Rapaport, G. (1991). Deletion by *in-vitro* recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. *J. Invert. Pathol.* 173:3374–3381.

Delécluse, A., Poncet, S., Klier, A., and Rapoport, G. (1993). Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal negative strain of *Bacillus thuringiensis* subsp. *israelensis*. *Appl. and Environ. Microbiol.* 59:3922–3927.

Finney, D. (1971). *Probit Analysis*. Cambridge University Press. Cambridge, England. 333 p.

Georghiou, G. P. (1983). Management of resistance in arthropods. G. P. Georghiou and T. Saito. eds. *Pest Resistance to Pesticides*. Plenum. Publishing. Corp. New York. 769–792.

Georghiou, G. P. (1994). Principles of insecticide resistance management. *Phytoprotection* (suppl.) 75:51–59.

Georghiou, G. P. and Lagunes-Tejeda, A. (1991). *The Occurrence of Resistance to Pesticides in Arthropod.* FAO. Rome. 318 p.

Goldman, I. R., Arnold, J., and Carlton, B. C. (1986). Selection for resistance to *Bacillus thuringiensis* subsp. *israelensis* in field and laboratory populations of the mosquito *Aedes aegypti*. *J. Invert. Pathol.* 47:317–324.

Hama, H., Suzuki, K., and Tanaka, H. (1992). Inheritance and stability of resistance to *Bacillus thuringiensis* formulations in the Diamondback moth *Plutella xylostella* (Linnaeus) (Lepidoptera:Yponomeutidae). *Appl. Entomol. Zool.* 27(3):355–362.

Höfte, H. and Whitely, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53 (2):242–255.

Kinsinger, R. A. and McGaughey, W. H. (1979). Susceptibility of populations of the Indianmeal moth and the Almond moth to *Bacillus thuringiensis*. *J. Econ. Entomol.* 72:346–349.

Knowles, B. H., and Ellar, D. J. (1988). Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* (-endotoxin with different insect specificities. *Biochim. Biophys. Acta.* 924:509–518.

Mani, G. S. (1985). Evolution of resistance in the presence of two insecticides. *Genetics.* 109:761–783.

McGaughey, W. H. and Whalon, M. E. (1992). Managing insect resistance to *Bacillus thuringiensis* toxins. *Science.* 258:1451–1455.

Raymond, M. (1985). Presentation d'un programme "Basic" d'analyse log-probit pour micro-ordinateur. Cah. O.R.S.T.R.O.M. Ser. Med. & Parasitol. 23:117–121.

Shelton, A. M., Robertson, J. L., Tang, J. D., Perez, C., Eigenbrode, S. D., Preisler, H. K., Wilsey, W. T., and Cooley, R. J. (1993). Resistance of Diamondback moth (Lepidoptera:Plutellidae) to *Bacillus thuringiensis* subspecies in the field. *J. Econ. Entomol.* 86:697–705.

Tabashnik, B. E. (1994). Evolution of resistance to *Bacillus thuringiensis*. *Ann. Rev. Entomol.* 39:47–79.

Tabashnik, B. E. (1989). Managing resistance with multiple pesticide tactics: theory, evidence and recommendations. *J. Econ. Entomol.* 82(5):1263–1269.

Tabashnik, B. E., Cushing, N. L., Hinson, N., and Johnson, M. W. (1991). Field development of insect resistance to *Bacillus thuringiensis* in the Diamondback moth (Lepidoptera:Plutellidae). *J. Econ. Entomol.* 83:1671–1676.

Taylor, C. E. and Georghiou, G. P. (1979). Genetic and biological influences in the evolution of insecticide resistance. *J. Econ. Entomol.* 70(3):319–323.

Wu, D., Johnson, J. J., and Federici, B. A. (1994). Synergism of mosquitocidal toxicity between CytA and CryIVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. *Mol. Microbiol.* 13:965–972.