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Evolution of resistance to the *Bacillus sphaericus* Bin toxin is phenotypically masked by combination with the mosquitocidal proteins of *Bacillus thuringiensis* subspecies *israelensis*

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Summary

Two insecticidal bacteria are used as larvicides to control larvae of nuisance and vector mosquitoes in many countries, *Bacillus thuringiensis* ssp. *israelensis* and *B. sphaericus*. Field studies show both are effective, but serious resistance, as high as 50 000-fold, has evolved where *B. sphaericus* is used against *Culex* mosquitoes. To improve efficacy and deal with even greater potential problems of resistance, we previously developed several recombinant larvicidal bacteria that combine the best mosquitocidal proteins of these bacteria. In the present study, we report laboratory selection studies using our best recombinant strain against larvae of *Culex quinquefasciatus*. This recombinant, Bti/BsBin, is a strain of *B. thuringiensis* ssp. *israelensis* engineered to produce a large amount of the *B. sphaericus* binary (Bin) toxin, which makes it more than 10-fold as mosquitocidal as the its parental strains. Here we show that larvae exposed to Bti/BsBin failed to develop significant resistance after 30 successive generations of heavy selection pressure. The highest level of resistance obtained at the LC_{95} level was 5.2-fold, but declined to less than two-fold at the 35th generation. Testing the selected populations against *B. sphaericus* alone showed resistance to Bin evolved, but was masked by combination with *B. thuringiensis* ssp. *israelensis*. These results suggest that recombinant bacterial strains have improved mosquito and vector management properties compared with the wild-type strains used in current commercial formulations, and should prove useful in controlling important human diseases such as malaria and filariasis on a long-term basis, even when used intensively under field conditions.

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Introduction

The use of commercial bacterial larvicides to control nuisance and vector mosquitoes has grown rapidly over the past two decades, and these are now used instead of synthetic chemical insecticides in many countries (Becker and Ludwig, 1993; Becker, 2000; Fillinger and Lindsay, 2006). Two bacteria are used as active ingredients in these larvicides, *Bacillus thuringiensis* ssp. *israelensis* (Bti) and *B. sphaericus* (Bs). Both have the advantage of being much more specific than chemical insecticides, having little effect on non-target organisms (Delécluse *et al.*, 2000). *Bacillus thuringiensis* ssp. *israelensis* typically kills only the larvae of mosquitoes, black flies, and to some extent, closely related nematoceran dipteran larvae such as those of chironomids (Glare and O'Callaghan, 2000). The target spectrum of Bs is more limited, restricted to mosquitoes, and even among these, it is ineffective against many. Most *Culex* species are highly sensitive to Bs, but within the genera *Aedes*, *Ochlerotatus* and *Anopheles*, some species are highly sensitive, whereas others show minimal sensitivity (Davidson *et al.*, 1984; Delécluse *et al.*, 2000).

Bacillus thuringiensis ssp. *israelensis* and Bs have advantages and disadvantages with respect to each other depending on the target mosquito species and habitat treated. For use against a broad range of species breeding in waters that are not highly polluted, formulations of Bti work best (Mulla *et al.*, 1982). *Bacillus thuringiensis* ssp. *israelensis*'s broad target spectrum is due to the combination of mosquitocidal proteins produced during sporulation, the major ones being Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa (Federici, 1999). The latter protein is particularly important because it synergizes the other three proteins, extends their target spectrum and delays the evolution of resistance to these (Ibarra and Federici, 1986; Wu *et al.*, 1994; Crickmore *et al.*, 1995; Georghiou and Wirth, 1997; Wirth *et al.*, 1997). On the other hand, Bs formulations are more effective than Bti in polluted waters,

where many different important *Culex* species breed. Bs also has longer residual activity than Bti formulations in these habitats (Davidson *et al*., 1984; Nicholas *et al*., 1987; Kramer, 1990; Charles *et al*., 1996). The principal protein responsible for Bs activity is the binary toxin, commonly referred to as Bin, which like those of Bti is produced during sporulation (Davidson *et al*., 1990). Bin is a very potent mosquitocidal protein consisting of two separate proteins that work together, BinA and BinB, which are, respectively, the toxic and binding moieties (Charles *et al*., 1996). Sensitivity to Bs is primarily dependent upon the presence of α-glucosidase, the 'receptor' or docking protein for BinB, on the midgut microvillar brush border membrane of sensitive species (Darboux *et al*., 2001). Unlike Bti's parasporal body, which assembles outside the exosporium membrane and dissociates from the spore upon cell lysis, Bin forms on the internal surface of this structure and remains associated with the spore after lysis (Charles *et al*., 1996), which may account for its longer residual activity. Unfortunately, because Bin is in essence a single toxin, resistance to it can evolve quickly. In fact, where Bs has been used intensively for control of *Culex* species in China and Thailand, very high levels of mosquito resistance, as high as 50 000-fold, have evolved within a few years (Yuan *et al*., 2000; Mulla *et al*., 2003).

Although bacterial larvicides have the advantage of being much better than chemical insecticides for the environment, they remain expensive, and thus are not used widely in most developing countries. The rapid evolution and potential spread of resistance to Bs also presents problems for widespread and intensive use. In previous studies, we demonstrated that Bti's Cyt1Aa can delay the evolution of resistance to Bs Bin (Wirth *et al*., 2005). Thus, to reduce the cost of bacterial insecticides and combine their most potent mosquitocidal properties into individual strains, we used genetic engineering techniques to produce a recombinant Bti strain that produces a large amount of Bs Bin. Aside from being at least 10-fold more toxic than wild-type Bti or Bs, as this strain contains Cyt1Aa, it has the potential to avoid the development of resistance to the Bin protein (Park *et al*., 2005). The purpose of the present study was to test the hypothesis that combining Bs Bin with Bti proteins in a recombinant Bti strain could delay the evolution of resistance. Here we show that even under very heavy selection pressure, resistance is delayed very significantly, suggesting that such recombinant bacterial strain should have a long life under operational field conditions aimed at controlling nuisance and vector mosquitoes.

Results

During this study, we induced the evolution of resistance by selecting the Bti/BsSel (sensitive) strain of *Culex quinquefasciatus* with increasing concentrations of the Bti/BsBin recombinant bacterial strain. After 35 generations, as described below, no significant resistance was apparent to this recombinant. To determine whether Cyt1Aa was masking any resistance, at the end of the 25th generation, we tested the selected Bti/BsSel mosquito strain for resistance to the individual mosquitocidal strains producing individual Bti or Bs proteins in the recombinant, namely, the strains Cry4Aa + Cry4Ba, Cry11Aa, all three Bti Cry proteins, Cyt1Aa, or the technical powder of Bs 2362.

Over the course of this study, resistance ratios reached a maximum of 5.2-fold at the LC_{95} by generation 21 in the Bti/BsSel colony, the colony selected with the Bti/BsBin recombinant, but declined to < 2-fold by generation 35 (Fig. 1; Table S1). Statistically significant changes in lethal concentration values, based on non-overlapping fiducial limits, were detected in generations 12, 19, 21 and 25.

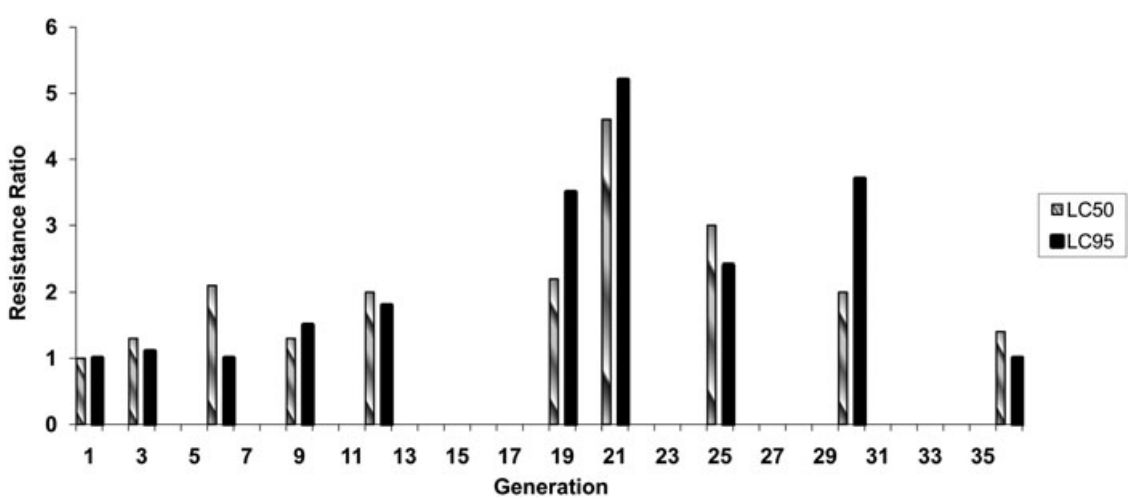

Fig. 1. Resistance ratios at the LC_{95} level for fourth-instar *C. quinquefasciatus* treated with the recombinant bacterial strain Bti/BsBin.

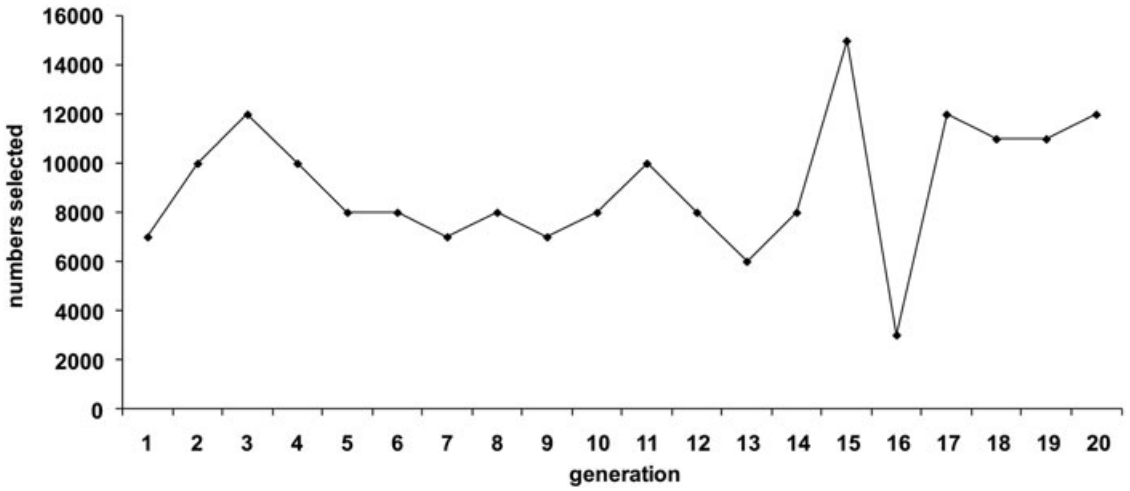

Fig. 2. Total number of fourth-instar *C. quinquefasciatus* treated in each generation of selection pressure with the recombinant bacterial strain Bti/BsBin.

With the exception of generation 21, those changes represented shifts in susceptibility of 3.5-fold or less. The selection concentration gradually increased from 40 ng ml^{-1} to 800 ng ml^{-1} at generation 20. However, the lyophilized crystal spore recombinant powder showed loss of activity over the 36 months of this study and the lethal concentration values were higher towards both the susceptible and selected colonies. For example, the LC_{50} to RecSyn was 7.35 ng ml^{-1} in generation 3, but in generation 35, the LC_{50} reached 54.4 ng ml^{-1}. Some fluctuations were likely due to periodic fluctuations in larval sensitivity but the roughly sevenfold decline in activity probably resulted from long-term storage.

On average, eight generations were selected per year with a mean of 9050 larvae treated per generation (Fig. 2). The only exception was generation 16, in which selection was suspended. Mortality levels fluctuated between 40% and 90%, but were generally maintained above 60% (Fig. 3). These mortality fluctuations reflect declines in susceptibility of the targeted population and subsequent increases in the selection concentration. For example, low average mortality in generation 13 led to relatively large increases in selection concentration in generations 14 and 15 until a satisfactory level of selection pressure, averaging 80%, was achieved (Fig. 3).

After 25 generations of selection pressure, the selected colony, Bti/BsSel, was evaluated for resistance to the various wild-type strains and component toxins that were present in the recombinant strain. Bti/BsSel demonstrated no significant resistance to Bti technical powder IPS 80

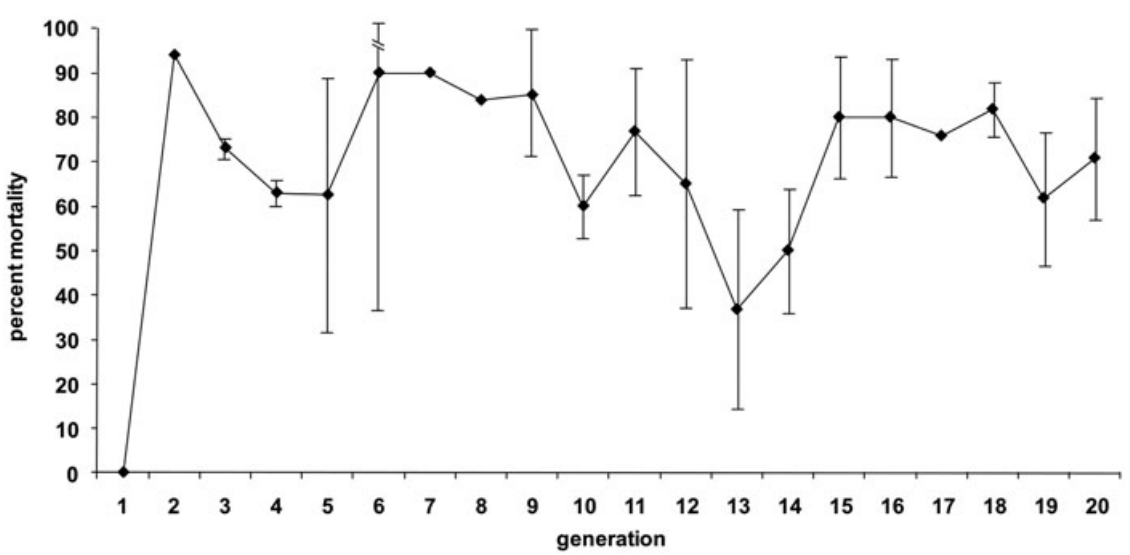

Fig. 3. Per cent mortality in each generation of fourth-instar *C. quinquefasciatus* treated with the recombinant bacterial strain Bti/BsBin.

(Table S2). In contrast, higher levels of resistance were obtained in Bti/BsSel when larvae of this strain were assayed using the technical powder of Bs 2362, the Bs strain used in commercial preparations. Resistance ratios were 17.4 and > 9000 at the LC_{50} and LC_{95} respectively. Significant resistance was also detected in the Bti/BsSel colony to the Bti strains that synthesized one or more of its Cry proteins. Resistance ratios of 19.1 were measured at the LC_{50} for Cry11Aa, 24.5 at the LC_{50} for Cry4Aa + Cry4Ba, and 4.0 at the LC_{50} for Cry4Aa + Cry4Ba + Cry11Aa (Table S2). However, no significant resistance was detected in Bti/BsSel to the Bt strain producing only Cyt1Aa.

Discussion

In previous studies, we have shown that Cyt1Aa can overcome resistance in *C. quinquefasciatus* that evolved in response to selection with recombinant strains of Bti that produced its Cry proteins alone or in combination in the absence of Cyt1Aa (Wirth *et al.*, 1997). Combining Cyt1Aa with these mutated Bti strains overcame most of this resistance. We obtained a similar result by combining Cyt1Aa with Bs 2362 against *C. quinquefasciatus* larvae resistant to the strain (Wirth *et al.*, 2005). In the latter study, we showed that after 20 generations of selection with Bs alone, the resistance level exceeded 1000-fold. These studies, especially in light of the significant levels of resistance that have evolved during operational mosquito control programmes in China and Thailand (Yuan *et al.*, 2000; Mulla *et al.*, 2003), provided the impetus to construct recombinant Bti strains with improved efficacy obtained through the addition of Bs Bin, and which had the possibility of delaying resistance to the latter due to the presence of Cyt1Aa in these strains. Here we tested the most potent of these strains, Bti/BsBin (Park *et al.*, 2005) for its capacity to delay resistance, and have shown that under heavy selection pressure in the laboratory the evolution of resistance was not only delayed, but that which did evolve was not statistically significant. Under our selection regime, there was no possibility, as there would be under field conditions, for wild-type *C. quinquefasciatus* to mate with the colony selected with Bti/BsBin. The implication of these results, because of the likelihood that populations of treated and non-treated mosquitoes will mate, is that resistance will not evolve to such a recombinant bacterium for many years in operational control programmes, be they for nuisance or vector mosquito populations.

An interesting aspect of our results is that when the Cry4Aa, Cry11Aa or Bs Bin strains were tested against the selected Bti/BsSel mosquito strain in the absence of Cyt1Aa at the end of the 3 years of selection, high levels of resistance to all of these proteins was apparent. Thus, resistance did evolve during the selection series, but its phenotypic expression was masked in the presence of Cyt1Aa. This pattern of resistance evolution to the components of selection using a mixture of mosquitocidal toxins has been previously observed in mosquitoes selected with wild-type Bti (Wirth and Georghiou, 1997), wild-type *B. thuringiensis* ssp. *jegathesan* (Wirth *et al.*, 2004a), and mosquitoes selected with a 3:1 mixture of Cry11Aa and Cyt1Aa (Wirth *et al.*, 2004b). Despite the evolution of resistance to underlying components, all the mixtures, natural or recombinant, remained highly active and selected mosquitoes remained susceptible to the mixtures. Interestingly, the Bti/BsBin-selected population failed to uniformly evolve resistance to Bs. Thus, the wide range in lethal concentration values indicates a highly heterogenous population consisting of both susceptible and resistant individuals, a pattern not observed in strains selected solely with Bs. From a practical standpoint, this still means that resistance should not be a problem, provided that Cyt1Aa is present. The specific mechanism by which Cyt1Aa masks the resistance is not fully understood. But it is known that Cyt1Aa forms lesions in the lipid portion of the microvillar membrane, which enables Bs Bin to cross this membrane without binding to it in the absence of the α-glucosidase receptor (Federici *et al.*, 2003). In the case of Cry11Aa, Cyt1Aa apparently acts as a binding domain for this protein (Perez *et al.* 2005). Cyt1Aa is not known to bind directly to Cry4Aa or Cry4Ba, so if it is enabling these to be toxic as resistance to them evolves, it likely overcomes resistance through its lipophilic properties, enabling these proteins to enter the microvillar membrane and form pores.

In other recent studies, to avoid the use of recombinant DNA technology for marketing reasons, in other words, a possible lack of acceptance by the public of recombinant organisms for mosquito control, it has been suggested that resistance-delaying products could be formulated by simply combining wild-type strains of Bti and Bs (Zahiri and Mulla, 2003; Zahiri *et al.*, 2003). There are two problems with such a strategy: (i) the products would not be as potent as the Bti/Bs recombinant tested here (Park *et al.*, 2005), especially as there would be two spores per unit of combined product, and (ii) the differential settling properties of the two different wild-type strains would still lead to selection with Bs. The latter could perhaps be overcome through devising formulations that maintained the products of the two bacterial species together, but this would not improve the efficacy of such formulations compared with recombinant strains that have been developed (Park *et al.*, 2003; 2005).

Over the past few years, several other Cry proteins have been shown to be mosquitocidal, such as Cry11Ba, isolated from *B. thuringiensis* ssp. *jegathesan* (Delécluse *et al.*, 1995; Seleena *et al.*, 1995; Charles *et al.*, 1996)

and the Mtx toxins of Bs, which are synthesized during vegetative growth (Thanabalu *et al*., 1991; Delécluse *et al*., 2000). These represent what is possibly a very rich source of mosquitocidal proteins that can be genetically manipulated and recombined to provide new types of recombinant bacteria for mosquito control. *Bacillus sphaericus* Bin and Cry proteins, as far as is known, have very different modes of action, although resistance to both is delayed by Cyt1Aa. Thus, including Cyt1Aa in recombinant bacteria with other types of mosquitocidal proteins that bind to the microvillar membrane may well provide a range of new larvicidal products that are potent, delay resistance, and are much more compatible with the environment than synthetic chemical insecticides.

Experimental procedures

Bacterial strains

Two wild-type bacteria, Bti (IPS-80, Institut Pasteur, Paris, France) and Bs 2362 (Valent Biosciences, Libertyville, IL), and five recombinant strains that produce one or more toxin(s) from Bti and/or Bs were used for these tests. The recombinant strains were named after the toxin or toxins that each produced, including Cry11Aa that was produced in an acrystalliferous strain of *B. thuringiensis* ssp. *kurstaki* (Chang *et al*., 1992), Cry4Aa + Cry4Ba (Delécluse *et al*., 1993), Cry4Aa + Cry4Ba + Cry11Aa (Delécluse *et al*., 1991), Cyt1Aa (Wu and Federici, 1993) and Bti/BsBin (Park *et al*., 2005) that were produced in Bti. Initially, Bti/BsBin was grown on nutrient agar plates, but this was changed to liquid media due to the need for larger amounts of material for the selections and bioassays. Bti/BsBin was 10-fold more active when grown on agar plates than when grown in the 4 l fermentor, probably because of a loss in efficiency in the scale-up. However, since a large quantity of powder was required for these studies, we decided to proceed with the lower activity powder.

Bacterial strains were grown on solid or liquid media as described previously (Delécluse *et al*., 1991; Chang *et al*., 1992; Wu and Federici, 1993; Park *et al*., 2005). Sporulated cells were washed in 1 M NaCl and/or distilled water, sedimented, and the pellets were lyophilized. Bioassays and selections used suspensions of lyophilized crystal/spore powders that were prepared by weight in distilled water and homogenized by shaking with approximately 25 glass beads. Stock suspensions were prepared monthly and 10-fold serial dilutions were prepared weekly as needed. All suspensions were stored at –20°C when not in use.

Mosquito colonies

Two colonies of *C. quinquefasciatus* were involved in this study. A large synthetic population of *C. quinquefasciatus*, named RecSyn, was established in the laboratory using larvae from a large field collection obtained in 2003, and supplemented with larvae from three other synthetic populations previously established in the laboratory, including CqSyn (Georghiou and Wirth, 1997), SynP (Wirth *et al*., 2004b) and SynC (Wirth *et al*., 2005). The RecSyn colony was allowed to interbreed for 3 months before testing and selection commenced. The second colony, Bti/BsSel (Sel for selected), was derived from RecSyn by selection using the recombinant bacterial strain Bti/BsBin.

Bioassay and selection procedures

Bioassay procedures involved feeding groups of 20 early fourth instars with different concentrations of suspended crystal/spore mixtures in 250 ml plastic cups containing 100 ml of deionized water. Six or more concentrations that produced mortality between 0 and 100% plus an untreated control were used for each dose–response test and replicated a minimum of five times on five different days. Control mortality was very rare, never exceeding 1%, and was corrected for using Abbott's formula. Mortality was determined at 24 or 48 h, depending on the material tested. Tests that were held for 48 h received a small amount of food after 24 h. Data were analysed using Probit Analysis (Finney, 1971). Resistance ratios (RR) were calculated by dividing the lethal concentration value at the 50% or 95% level for the selected line, by the concurrently measured lethal concentration values predicted for RecSyn. The selected and unselected lines were bioassayed every third generation. However, testing of generation 15 was skipped due to a shortage of personnel.

Selection procedures were similar to bioassay procedures except groups of 1000 early fourth instars were fed a suspension of lyophilized spore/crystal powder in 1 l of deionized water for 48 h. Food was added to the selection container after 24 h. Survivors were subsequently transferred to clean water, fed, and used to continue the colonies. Generations were maintained separately through generation 20, after which generations overlapped. Selection pressure was suspended for generation 16 but resumed for generation 17.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Change in susceptibility for *Culex quinquefasciatus* selected and assayed with the recombinant bacterial strain Bti/Bs.

Table S2. Cross-resistance spectrum in generation 25 of *Culex quinquefasciatus* selected with Bti/Bs and assayed against different wild-type strains of *Bacillus thuringiensis*, *B. sphaericus* and mutant strains of *B. thuringiensis*.

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