HOST RANGE AND SELECTED FACTORS INFLUENCING THE MOSQUITO LARVICIDAL ACTIVITY OF THE PG-14 ISOLATE OF *BACILLUS THURINGIENSIS* VAR. *MORRISONI*

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ABSTRACT. Laboratory bioassay of the PG-14 isolate of *Bacillus thuringiensis* var. *morrisoni* (serotype 8a:8b) against early fourth instar larvae of 8 species of mosquitoes revealed a range of susceptibilities similar to the susceptibilities of these species to *Bacillus thuringiensis* var. *israelensis* (serotype 14). The most susceptible species were: *Culex quinquefasciatus, Cx. salinarius, Anopheles albimanus* and *Aedes aegypti*. The least susceptible species tested was *An. quadrimaculatus*. Separate bioassays of PG-14 against the four instars of *Ae. aegypti* demonstrated a strong negative correlation ($R = -0.97$) between larval age and susceptibility. Temperature significantly affected the stability of larvicidal toxin in aqueous suspensions of PG-14. Larvicidal activity of a bacterial suspension was nearly completely eliminated after 132 days of storage at 31°C, but was essentially unchanged for those suspensions stored at 4°C.

INTRODUCTION

Just a little more than 10 years ago the pathogens and parasites of mosquitoes thought to have the greatest biological control potential were certain fungi and mermithid nematodes (Chapman 1974). Only low to moderate mosquito larvicidal activity was observed in most of the *Bacillus thuringiensis* strains (Reeves and Garcia 1970, Hall et al. 1977). Since the isolation of the highly larvicidal *Bacillus thuringiensis* var. *israelensis* (serotype 14) by Goldberg and Margalit (1977), several additional isolates of this serotype and others have been discovered that are also larvicidal for mosquitoes. The PG-14 isolate of *B. thuringiensis* var. *morrisoni* (serotype 8a:8b), found by Padua et al. (1984), displays larvicidal activity similar to that of *B. thuringiensis* (H-14) toward the mosquito species tested thus far (Padua et al. 1984, Ibarra and Federici 1986). In addition to mosquito larvicidal activity, similarities in parasporal inclusion morphology and protein composition, between *B. thuringiensis* (H-14) and the PG-14 isolate of *B. thuringiensis* (H-8a8b) have also been reported (Ibarra and Federici 1986). Curiously, other isolates of *B. thuringiensis* (8a:8b) that have demonstrated larvicidal activity for certain Lepidoptera have exhibited little or no larvicidal activity toward mosquitoes (Hall et al. 1977, Larget and de Barjac 1981). The objectives of our study were to elucidate further the host range and other factors that may influence the activity of the PG-14 isolate.

MATERIALS AND METHODS

The inoculum used in this study was freshly prepared each week from an aqueous suspension of a stock culture of *B. thuringiensis* (PG-14) in the following manner: 2 drops of the stock culture (1.19×10^8 spores/ml) of *B. thuringiensis* (PG-14) were spread onto Tryptose Blood Agar Base (TBAB) in each of 10 Petri plates. The plates were then incubated for 5 days at 32°C after which time bacterial growth was scraped from the plates with a small rubber spatula into a flask containing 100 ml of sterile distilled water. Care was taken not to transfer agar with the bacteria. After thoroughly suspending the bacteria in the distilled water, a small amount of the suspension (5 ml) was pasteurized (80°C, 12 min), serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and plated on TBAB using standard procedures in order to determine viable spore count. The plates were incubated at 32°C for 24 hr after which time the colonies were counted. The bacterial suspensions were stored for 1 to 5 days at 4°C until they were used for testing.

The bioassay procedure that was utilized for determining comparative larvicidal activity of *B. thuringiensis* (PG-14) against 8 species of mosquitoes was identical to that used by Lacey and Oldacre (1983). Early fourth instars of *Anopheles quadrimaculatus* Say, *Anopheles albimanus* Weidemann, *Aedes aegypti* (Linn.), *Ae. taeniorhynchus* (Weidemann), *Culex quinquefasciatus* Say, *Cx. salinarius* Coquillett, and *Cx. tarsalis* Coquillett were obtained from colonies maintained at the USDA Insects Affecting Man and Animals Research Laboratory

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(IAMARL), and third instars of *Psorophora columbiae* (Dyar and Knab) were collected from recently flooded ponds. Due to the wide spectrum of susceptibilities of the mosquito species tested, a broad range of bacterial concentrations were required for the bioassays. These were produced by serially diluting the stock suspension. Dilution ratios from 0.2 to 0.8 were used and final dilutions of the stock ranged from 10^{-2} to 10^{-6}. With the exception of *Ae. taeniorhynchus,* the larvae were exposed to 5–8 concentrations of a bacterial suspension of *B. thuringiensis* (PG-14) (mean spore count of undiluted stocks, $2.47 \pm 0.58 \times 10^8$ spores/ml) in 100 ml well water for 24 hr (27°C) without the addition of food. The conditions under which *Ae. taeniorhynchus* larvae were tested were identical to those for the other species except that 0.5% salt water (the normal rearing medium) was utilized in place of well water. The range of dilutions was determined for each species during preliminary exposures. Three cups, each containing 20 larvae, were used for each dilution and control for each replicate test. At least 3 replicate tests for each species were run on separate dates.

The effect of larval age on susceptibility was assessed with bioassays of *B. thuringiensis* (PG-14) against the four larval instars of *Ae. aegypti* in a manner similar to that for the species susceptibility tests. Larvae that were harvested from laboratory colonies (27°C) 24, 48, 72 and 96 hr posthatch were late first, second, third and fourth instars, respectively. Due to low survivorship of early instars when preliminary tests were run without food, all of the larvae were provided with 0.5 ml of a 1% suspension of finely ground hog chow at the beginning of each test.

The effect of continuous exposure to an approximate LC_{95} (under standard bioassay conditions) of PG-14 on mortality progression was determined by treating early fourth instars of *Ae. aegypti* with 1.7×10^4 spores/ml of *B. thuringiensis* (PG-14) and observing the progression of resultant mortality over a 48 hr period. Ten cups of 20 larvae were checked every 2 hours for the first 12 hr after treatment and thereafter every 4 hr until the test was terminated after 48 hr. All other treatment conditions were as in the species susceptibility tests. Three such tests were replicated during a 2 wk period.

Suspensions of PG-14 in distilled water were stored at 4 and 31°C and bioassayed against early fourth instars of *Ae. aegypti* after 2, 8, 15, 29, 104, and 132 days of storage. On each test date, 8 ml of a 10^{-5} dilution (calculated LC_{99} based on original toxicity) of each suspension was bioassayed against the larvae in 5 cups containing 20 larvae/cup in the same manner as the host range and larval age tests. Five additional cups containing 20 larvae each were left untreated as controls.

The mortality data obtained in the host range and larval age tests were subjected to probit analysis after correcting for control mortality with Abbott's formula. Linear regression analysis was also performed on the larval age bioassay data and toxin stability test data. The data from individual test dates from the toxin stability tests were analyzed using the T-test.

RESULTS

The results of the laboratory bioassays of *B. thuringiensis* (PG-14) against early fourth instar larvae of anopheline and culicine larvae are presented in Table 1. The most susceptible species were *Cx. quinquefasciatus, Cx. salinarius, An. albimanus* and *Ae. aegypti.* The least susceptible species tested were *Ps. columbiae* and *An. quadrimaculatus.* Of particular interest was the rather elevated susceptibility of *An. albimanus;* it was over 14 times more susceptible to the PG-14 toxin than was *An. quadrimaculatus.*

The bioassays of *B. thuringiensis* (PG-14) against the four instars of *Ae. aegypti* demonstrated a strong negative correlation between larval age and susceptibility ($R = -0.97$; $Y = -2.11 + 2.26\ X$; $P \leq 0.05$). First instar larvae were over 25 times more susceptible than were fourth instars. The respective LC_{50} values in spores/ml $\times 10^3$ for first, second, third, and fourth instars were: 0.24, 1.75, 5.69 and 6.45. The disparity among the LC_{50} for fourth instars in Table 1 and that obtained in the larval age susceptibility tests may be due to the addition of food in the larval age tests.

The effect of continuous exposure to an LC_{95} of *B. thuringiensis* (PG-14) on the progression of mortality of *Ae. aegypti* is presented in Fig. 1. Over 75% of the larvae died within the first

Table 1. Comparative larvicidal activity of *Bacillus thuringiensis* (PG-14) against early fourth instars of 8 species of mosquitoes (27°C, 24 hr exposure).

Test species	LC_{50}[1]	LC_{95}
Anopheles quadrimaculatus	46.42 f	188.76 d
Anopheles albimanus	3.16 c	10.24 b
Aedes aegypti	4.51 d	16.60 bc
Aedes taeniorhynchus	5.53 d	22.56 c
Culex quinquefasciatus	0.85 a	3.26 a
Culex salinarius	1.96 b	4.06 a
Culex tarsalis	7.99 e	22.61 c
Psorophora columbiae	11.28 e	130.38 d

[1] Spores (10^3)/ml. LC values in the same column followed by the same letter have 95% fiducial limits that overlap.

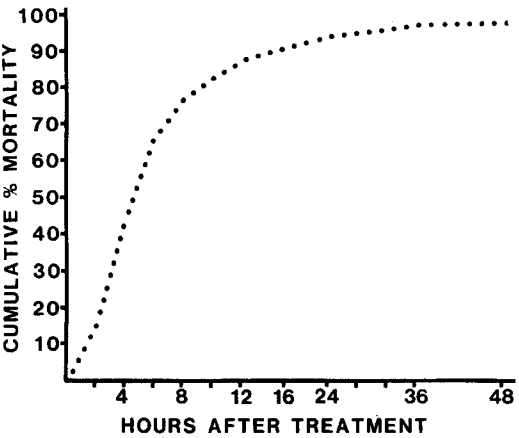

Fig. 1. Effect of continuous exposure to the PG-14 isolate of *Bacillus thuringiensis* on the progression of mortality of fourth instar *Aedes aegypti* under laboratory conditions (27°C; no food; mean spore count: 1.44×10^4 spores/ml).

8 hr of exposure. At the 24 hr point (the standardized exposure period for our bioassays) an average of 93.7 ± 0.73% (s.e.) mortality was observed.

The comparative efficacy of *B. thuringiensis* (PG-14) after storage in distilled water at 4 and 31°C is presented in Fig. 2. A small but significant loss of larvicidal activity in the suspension stored at 31°C was observed after only 15 days of storage. A steady decline in activity was observed for the 31°C suspension over the 132 days of storage ($R = -0.99$; $Y = 99.96 - 0.65X$; $P \leq 0.001$). Larvicidal activity of the suspension that was stored at 4°C remained unchanged. Control mortality ranged from 0 to 2%.

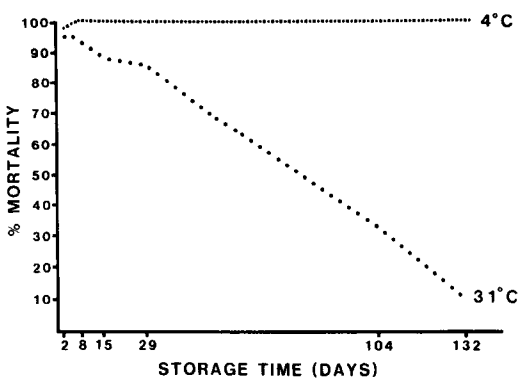

Fig. 2. Effect of storage of aqueous suspensions of the PG-14 isolate of *Bacillus thuringiensis* at 4 and 31°C on larvicidal activity when bioassayed against fourth instar *Aedes aegypti* (27°C; no food; 1.6×10^4 viable spores/ml).

DISCUSSION

A number of isolates of *Bacillus thuringiensis* display variable larvicidal activity toward mosquitoes. Even strains that are highly toxic to Lepidoptera contain variable amounts of mosquitocidal toxin (Hall et al. 1977, Ignoffo et al. 1981, Larget and de Barjac 1981). In some strains exhibiting elevated mosquito larvicidal activity, host specificity also appears to be variable; for example, the 73-E-10-16 isolate of *Bacillus thuringiensis* var. *darmstadiensis* (serotype 10), demonstrates pronounced larvicidal activity against *Cx. quinquefasciatus*, but is considerably less active toward other species (Padua et al. 1980, Lacey and Oldacre 1983).

The results of bioassays of the PG-14 isolate of *B. thuringiensis* (H-8a8b) and of *B. thuringiensis* (H-14) against *Ae. aegypti* and *Culex molestus* Forskal that were reported by Padua et al. (1984) indicated comparable larvicidal activity for both varieties. Studies conducted by Lacey and Singer (1982) with the IPS-78 preparation of *B. thuringiensis* (H-14) against early fourth instars of *Ae. aegypti* revealed an LC_{50} of 0.203 mg/liter. The corresponding spore count was 8.6×10^3 spores/ml of bioassay water. This compares with an LC_{50} of 4.5×10^3 spores/ml reported here for the PG-14 isolate against the same species (Table 1).

The laboratory bioassays presented in our study demonstrate a range of susceptibilities similar to, but not identical to the susceptibilities of these species to the toxin of *B. thuringiensis* (H-14). Larval feeding strategy and toxin settling rate are believed to be the primary reasons for observed differences in susceptibility to *B. thuringiensis* (H-14) between anopheline and culicine larvae under laboratory and field conditions (reviewed in Lacey 1985). The disparity between LC_{50} values of PG-14 for *An. quadrimaculatus* and *An. albimanus* is greater than that reported in other studies conducted at the IAMARL with *B. thuringiensis* (H-14) and *B. thuringiensis* (H-10) (Lacey and Singer 1982, Lacey and Oldacre 1983). The activity of PG-14 toward *An. albimanus* is comparable to its activity against the culicines that were tested; a phenomenon that is not normally observed with *B. thuringiensis* (H-14). Considering that this species is the principal vector of malaria in Mexico and Central America, further investigation of PG-14 against *An. albimanus* is warranted, especially under field conditions. The susceptibility of *Ae. aegypti* to the toxin of *B. thuringiensis* (H-14) is reported as being similar to or greater than that of *Cx. quinquefasciatus* (Tyrell et al. 1979, Ignoffo et al. 1981, Lacey and Singer 1982). However, we observed over a 5-fold greater susceptibility of *Cx. quin-*

quefasciatus to the PG-14 isolate. These data suggest slightly different modes of action for the toxins of *B. thuringiensis* (H-14) and PG-14.

The correlation between larval age and dosage has been reported by a number of authors for *Bacillus* pathogens of mosquitoes (reviewed in Lacey 1985). In addition to their probably greater physiological sensitivity to bacterial toxins, there is a larger ratio of toxin to biomass with younger larvae compared to that of the same number of older instars exposed to the same amount of toxin. Prolonged abatement of larval populations might, therefore, be possible using sustained-release formulations of PG-14 or other efficacious bacteria that provide rapid release of inoculum for control of older larvae, followed by a steady release of considerably less toxin for the continued control of subsequent generations.

The stability of the larvicidal toxin when stored in aqueous suspension at 4°C is similar to that reported for suspensions of *B. thuringiensis* (H-14) by Ignoffo et al. (1983). The decline in larvicidal activity of the suspension that was stored at 31°C was probably due to microbial degradation of the toxin. Growth of other bacteria in the suspension may have been promoted by the warm temperature and inclusion of a small amount of medium during the scraping of the plates.

Our study on the effect of continuous exposure to an LC_{95} of PG-14 on the pattern of mortality progression of *Ae. aegypti* indicates that the larvicidal moiety of this isolate is rapidly acting. The data obtained in these tests are similar to those reported by a number of researchers for *B. thuringiensis* (H-14) (Goldberg and Margalit 1977, Ignoffo et al. 1981, Lacey and Lacey 1981, Lacey and Singer 1982). The average percent mortality observed after 24 hr (94%) corroborates the calculated LC_{95} (24 hr bioassay) which was based on serial dilution of fresh stock to obtain the approximate required spore count/ml.

Spore count alone is not totally reliable as a measure of dosage or toxicity. The media employed and fermentation conditions can result in increased toxin production without necessarily resulting in a concomitant rise in spore count. Weight of primary powders or lyophilized bacterial preparations offers a convenient and simplified means of measuring relative larvicidal activity of different preparations. This is especially useful in determining relative efficacies of commercial products and for estimating relative cost for mosquito abatement when two or more products are considered. However, when primary powders from separate sources are compared, a variety of determinants (media, fermentation conditions, isolate of bacterium utilized, powder preparation procedures, etc.) are collectively compared. In order to determine quantitatively the effect of strain or isolate of bacterium on innate larvicidal activity all other conditions must be equal or nearly equal. Comparison of data generated in this study on PG-14 with that reported for the 73-E-10-16 isolate of *B. thuringiensis* (H-10) by Lacey and Oldacre (1983) is facilitated by the virtually identical conditions under which inoculum was prepared and the tests were conducted. Comparison of PG-14 with *B. thuringiensis* (H-14), however, should be repeated against a spectrum of pest and vector mosquitoes using primary powders or whole cultures produced under identical conditions for a more accurate assessment of their similarities and differences.

The benefits of continued search for additional strains of *B. thuringiensis* and other bacteria with larvicidal activity for mosquitoes are apparent. In addition to providing efficacious microbial control agents, new sources of genetic material will also be available. By incorporating the genes for toxins from the various isolates such as PG-14, 73-E-10-16 and *B. thuringiensis* (H-14), with demonstrated efficacy against one or more target mosquitoes, an engineered bacterium could be produced with even greater activity than that observed with *B. thuringiensis* (H-14).

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Ms. Pamela Kaylor, Mr. Scott Burgard and Mr. Tok Fukuda with bioassays and to Ms. Susan W. Avery for help with statistical analyses. We thank Dr. Dan Molloy, New York State Science Service and Dr. Jeffrey Lord, IAMARL, for their review of the manuscript and for providing useful comments and suggestions.

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