THE PATHOGENICITY OF STRAINS OF *BACILLUS THURINGIENSIS* TO LARVAE OF *AEDES* AND TO *CULEX* MOSQUITOES[1]

I. M. HALL AND K. Y. ARAKAWA

Department of Entomology, University of California, Riverside 92502

and

H. T. DULMAGE AND J. A. CORREA

U.S. Department of Agriculture, ARS, SR, Subtropical Texas Area, Cotton Insects Unit, Brownsville TX 78520.

ABSTRACT. Formulations prepared from 127 strains of *Bacillus thuringiensis*, including representatives from 16 variety/serotype groupings plus 2 unidentified strains, all of which were grown on a standardized medium, were assayed against larvae of *Aedes aegypti*, *Ae. triseriatus*, *Culex tarsalis*, *Cx. pipiens* and *Cx. quinquefasciatus*. Preparations from most strains were no more than moderately pathogenic to a host at high concentration levels (100 μg/ml). However, formulations from 26 strains showed marked effect against the most susceptible hosts, *Ae. triseriatus*, *Cx. tarsalis* and *Ae. aegypti*, with many found to have LC-50's well below 1 μg/ml. The most pathogenic formulations, labeled HD-169/R-567B and HD-96/R-574D, had LC-50's of 0.04 and 0.06 μg/ml, respectively, against *Ae. triseriatus*. *Cx. pipiens* and *Cx. quinquefasciatus* were much less susceptible, with only 23 formulations able to cause 50% mortality at concentrations between 4–10 μg/ml.

INTRODUCTION. Although increasingly toxic formulations of *Bacillus thuringiensis* Berliner have been developed into marketable microbial insecticides for use against susceptible agricultural insects during the past 2 decades, there has been no concerted effort to develop formulations of a similar nature for use against mosquitoes. This has been due in part to the apparent long-held belief that few bacteria are important pathogens of mosquitoes (Chapman et al. 1972). Furthermore, it has been a natural response to published reports that certain laboratory and/or commercially produced strains of *B. thuringiensis* isolated from other hosts were either not pathogenic to mosquito larvae against which they were tested (Metalnikov and Chorine 1929; Kellen and Lewallen 1960), or were only moderately effective at high dosage levels (Dunn, P. H. 1953, M.S. Thesis, Ohio State University, Columbus); Liles and Dunn 1959; Shaikh and Morrison 1966). Lack of interest in seriously exploiting the varied *B. thuringiensis* materials for mosquito control has continued despite the report of promising results with strains in laboratory and field tests against *Aedes*, *Culex* and *Anopheles* (Lavrentyev et al. 1965), and the discovery of the bicrystalliferous bacillus isolate BA-068 [now known to be a strain of *B. thuringiensis* var. *thuringiensis*—Serotype 1] from *Cx. tarsalis* and the report that it is highly effective against species of *Aedes* (Reeves 1970; Reeves and Garcia 1971a,b). More recently, Singer (1975) reported that several strains of *B. thuringiensis* showing activity against species of *Culex* were less effective than a strain of the non-crystalliferous *B. sphaericus* that he had isolated from mosquitoes. With his contention that the latter species offers far greater promise as a mosquito control agent, the potential of *B. thuringiensis* for use against mosquitoes based on present published information has remained very much in doubt.

During 1972, in the the course of bioassaying different entomogenous microorganisms against larvae of several species of mosquitoes, we tested 2 commercial prep-

[1] This paper reflects the results of research only. Mention of a pesticide or proprietory product in this paper does not constitute a recommendation for use by the U.S.D.A., nor does it imply registration under F.I.F.R.A. as amended.

arations of *B. thuringiensis* [IMC 10001.3 and DIPEL® WP] to determine if they possessed greater pathogenicity than the older less toxic materials screened by Kellen and Lewallen (1960) and Shaikh and Morrison (1966). The IMC 10001.3 material, which was never identified to strain, proved to be at least moderately effective against larvae of several mosquitoes, causing mortalities of 59–94% at a concentration of 10 μg/ml. Even better results were obtained with DIPEL®, formulated from the HD–1 strain (*B. thuringiensis* var. *kurstaki*, Serotype 3a,b). These results suggested that even more effective agents lethal to mosquitoes might exist among the many untried strains of *B. thuringiensis*. This paper is a report on the testing of 127 strains of the bacillus under uniform assay procedures against larvae of 5 species of *Aedes* and *Culex* during 1974–75.

MATERIALS AND METHODS. The mosquitoes used in the assays were *Ae. aegypti*, *Ae. triseriatus*, *Cx. tarsalis*, *Cx. quinquefasciatus* and *Cx. pipiens*. The first 4 species were from stocks reared for several years at Riverside, and the *Cx. pipiens* colony was started from field-collected insects from southern California. The colonies were maintained using standard mosquito rearing techniques in a temperature and humidity controlled rearing room to assure maximum vigor of the progency selected for testing.

The formulations assayed were prepared from the collection of *B. thuringiensis* strains maintained at Brownsville. They were produced by submerged fermentation in medium B–4, a Proflo®-based substrate, in shake flasks according to the procedures described by Dulmage (1971). Dry formulations were derived from the beers by the acetone-coprecipitation procedure of Dulmage et al. (1970). This method eliminated most, if not all, of any β-exotoxin produced by a strain. Formulations were designated by HD-No. (strain number in Dulmage's culture collection) followed by an R-No. designating the fermentation run in which the material was produced. No diluent was added to the final preparation, which, thus, consisted of the spore-δ-endotoxin complex, lactose and miscellaneous insoluble fermentation residues, primarily unused cottonseed flour.

The 127 cultures tested included strains from 16 varieties of *B. thuringiensis*, plus 2 of unknown variety and listed as "autoagglutinate—cannot be serotyped" by DeBarjac (personal communication) (Table 1).

The assays were conducted in waxed-surface paper food containers, each holding 100 ml of nonchlorinated industrial-grade tap water. For much of the study, tests were initiated against 5 groups of 20 second-instar (or early 3rd-instar, on occasion) larvae per container for a total of 100 larvae per treatment. This was deemed necessary to compensate for variable nonassay mortality in the *Cx. tarsalis* population. However, in later tests, increased host vigor greatly reduced this problem, permitting reduction to 10 larvae per container or 50 larvae per treatment.

Following setup with water and larvae, each unit of a 5-replicate group was treated with an assay suspension which, upon further dispersion in the container

Table 1. Number of strains of *Bacillus thuringiensis* assayed per variety and serotype.

B. thuringiensis variety	Serotype (De Barjac)	No. of strains tested
thuringiensis	1	27
finitimus	2	2
alesti	3a	10
kurstaki	3a, b	15
sotto	4a, b	1
dendrolimus	4a, b	9
kenyae	4a, c	3
galleriae	5a, b	35
canadensis	5a, c	1
entomocidus	6	3
subtoxicus	6	1
aizawai	7	9
morrisoni	8	2
tolworthi	9	3
darmstadiensis	10	3
toumanoffi	11	1
Autoagglutinate—cannot be serotyped		2
		127

water, would give a specific bacillus formulation concentration to which the test larvae would be exposed. Similar, but untreated, 5-replicate groups were set up as controls. All groups were kept together on trays in the laboratory at 25° ± 3°C. With the assay containers uncovered, natural aeration proved to be satisfactory. However, resultant evaporation required replenishment of water to the 100 ml level at least once during a test run. The larvae were fed a small quantity of triturated tropical fish food applied by a small shaker over the surface of the water in each container at 24–48 hr intervals.

The starting and maximum pathogen concentration used in the study was 100 μg/ml. Counts of living larvae were made at 24 or 48 hr intervals, depending upon host response, and the cumulative mortality in each unit of a group was recorded. The mortality data for each assay grouping were summed and corrected by Abbott's formula when a test was terminated at the end of 7 days or the onset of pupation among check larvae and test survivors. Preparations causing 80%+ mortality were retested at lower dosages until LC–50(±2%) levels were determined. In a few instances when assays were unable to give results within this range, LC–50's were estimated with the use of probability-log graphs.

RESULTS AND DISCUSSION. The results revealed that the mosquito species used in the assays fell into 2 groupings relative to their susceptibilities to the more pathogenic *B. thuringiensis* strains. The 3 most susceptible mosquitoes were the 2 *Aedes* and *Cx. tarsalis*. Strains of *B. thuringiensis* showing LC–50's at 2 μg/ml and lower against these species are listed by variety and serotype within specific dosage levels in Table 2.

The most susceptible mosquito was *Ae. triseriatus*, against which 21 strains of the bacillus in 6 varieties or serotypes produced LC–50's of 2 μg/ml and below. The most pathogenic formulations were HD–169/R–567B [var. *kurstaki*, Serotype 3a,b] with an LC–50 of 0.04 μg/ml and HD–96/R–574D [var. *thuringiensis*, Serotype 1] with an LC–50 of 0.06 μg/ml. These were followed by HD–14/R–358A [var. *thuringiensis*, Serotype 1] and HD–125/R–542A [var. *tolworthi*, Serotype 9] with LC–50's of 0.1 μg/ml, and HD–13/R–547C [var. *tolworthi*, Serotype 9] and HD–1/R–102 [var. *kurstaki*, Serotype 3a,b], derived from the HD–1 strain used as the base ingredient of the current commercial *B. thuringiensis* microbial insecticides, at 0.2 μg/ml. Among 4 formulations with LC–50's of 0.3 μg/ml was HD–225/R–531C [var. *thuringiensis*, Serotype 1], which is the same as the isolate BA–068 reported by Reeves (1970) and Reeves and Garcia (1971a,b) to be highly pathogenic to species of *Aedes*.

The next most susceptible mosquito was *Cx. tarsalis*, against which 19 bacillus strains in 7 varieties or serotypes produced formulations that had LC–50's at or below 2 μg/ml. Most effective were HD–158/R–563C [var. *galleriae*, Serotype 5a,b] and HD–13/R–547C with LC–50's of 0.2 μg/ml, and HD–96/R–547D with an LC–50 of 0.3 μg/ml. Among formulations causing 50% mortality at 0.4 μg/ml was HD–1/R–102, and in the group that followed was HD–225/R–531C with an LC–50 of 0.8 μg/ml.

Only 7 strains of *B. thuringiensis* in 2 varieties or serotypes were found to produce formulations highly pathogenic to *Ae. aegypti*. The most effective was HD-125/R-542A with an LC-50 of 0.3 μg/ml, followed by HD-13/R-547C at 0.4 μg/ml, and HD-15/R-358B, HD-22/R-361C and HD-39/R-559A [all strains of var. *thuringiensis*, Serotype 1] at 0.5 μg/ml. Against this host, HD-225/R-531C caused 50% mortality at 2 μg/ml, and HD-1/R-102 had an LC-50 of 4 μg/ml.

Far less susceptible to the *B. thuringiensis* strains were *Cx. pipiens* and *Cx. quinquefasciatus*. Products from strains that caused 50% mortality at 4-10 μg/ml are presented in Table 3. With a few exceptions, they differed from those found to be effective against the much more susceptible mosquitoes (Table 2). Moreover, the comparative HD-1/R-102 and HD-225/R-531C were relatively ineffective against these hosts, causing 4% and 39% mortal-

ity, respectively, to *Cx. pipiens* and 33% and 9% mortality, respectively, to *Cx. quinquefasciatus* at the maximum concentration of 100 μg/ml.

Of the 16 strains from 7 varieties or serotypes producing materials found to have some pathogenicity against *Cx. pipiens*, the most effective formulations were HD-112/R-525C and HD-127/R-531B [both var. *aizawai*, Serotype 7] with LC-

Table 2. LC-50's of formulations of highly pathogenic strains of *Bacillus thuringiensis* varieties against larvae of the most susceptible test mosquitoes, *Aedes aegypti*, *Ae. triseriatus* and *Culex tarsalis*.

LC-50 (ug/ml)	Variety	Serotype	*Ae. aegypti* HD-#/R-#	*Ae. triseriatus* HD-#/R-#	*Cx. tarsalis* HD-#/R-#
0.04	*kurstaki*	3a,b		169/567B	
0.06	*thuringiensis*	1		96/574D	
0.1	*thuringiensis*	1		14/358A	
	tolworthi	9		125/542A	
0.2	*kurstaki*	3a,b		1/102	
	galleriae	5a,b			158/563C
	tolworthi	9		13/547C	13/547C
0.3	*thuringiensis*	1		39/559A	96/574D
				225/531C	
	kurstaki	3a,b		164/565C	
	tolworthi	9	125/542A	124/541B	
0.4	*thuringiensis*	1			15/358B
					22/361C
	kurstaki	3a,b			1/102
					169/567B
	kenyae	4a,c		136/580A	
	entomocidus	6			198/542C
	tolworthi	9	13/547C		125/542A
0.5	*thuringiensis*	1	15/358B	15/358B	14/358A
			22/361C	22/361C	
			39/559A		
	kurstaki	3a,b		89/573A	164/565C
	tolworthi	9			124/541B
0.6	*thuringiensis*	1			39/559A
0.7	*thuringiensis*	1		103/524C	
0.8	*thuringiensis*	1		17/360A	225/531C
	kenyae	4a,c		5/545A	
1.0	*thuringiensis*	1		18/360B	
	aizawai	7		128/539B	
	tolworthi	9	124/541B		
2.0	*thuringiensis*	1	225/531C		
	kurstaki	3a,b			89/573A
	kenyae	4a,c			136/580A
	galleriae	5a,b			161/564C
	entomocidus	6		9/546B	
				198/542C	
	aizawai	7			111/525B
					127/531B

Table 3. LC-50's of formulations of the most pathogenic strains of *Bacillus thuringiensis* varieties against larvae of the least susceptible test mosquitoes, *Culex pipiens* and *Cx. quinquefasciatus*.

LC-50 (ug/ml)	Variety	Serotype	*Cx. pipiens* HD-#/R-#	*Cx. quinquefasciatus* HD-#/R-1#
4.0	*aizawai*	7	112/525C	
5.0	*aizawai*	7	127/531B	
	morrisoni	8		12/547B
	tolworthi	9		124/541B
	Autoagglutinate	not serotyped		43/543A
6.0	*aizawai*	7	111/525B	
			128/539B	
7.0	*galleriae*	5a,b	193/572A	
			195/572C	
	darmstadiensis	10	146/543C	
8.0	*entomocidus*	6	198/542C	
	aizawai	7	143/538B	
9.0	*morrisoni*	8	12/547B	
10.0	*alesti*	3a	79/106	
	galleriae	5a,b	129/539C	
			190/571B	
			192/571D	
			197/572D	
	entomocidus	6		110/525A
				198/542C
	aizawai	7		143/538B
	tolworthi	9	124/541B	
	darmstadiensis	10		146/543C

50's of 4 μg/ml and 5 μg/ml, respectively. Formulations of only 7 strains from 5 varieties or serotypes, or other grouping (non-serotyped autoagglutinate), were found to have some effect against *Cx. quinquefasciatus,* the least susceptible of the 5 test mosquitoes. The most pathogenic ones were HD-12/R-547B [var. *morrisoni,* Serotype 8], HD-124/R-541B [var. *tolworthi,* Serotype 9] and HD-43/R-543A [non-serotyped autoagglutinate] with LC-50's of 5 μg/ml.

The data indicate that formulations of 5 bacillus strains were highly pathogenic to *Ae. aegypti, Ae. triseriatus* and *Cx. tarsalis,* with LC-50's between 0.1 and 0.6 μg/ml (Table 2). They are HD-15/R-358B, HD-22/R-361C and HD-39/R-559A [in var. *thuringiensis,* Serotype 1], and HD-125/R-542A and HD-13/R-547C [in var. *tolworthi,* Serotype 9]. HD-124/R-541B [also in var. *tolworthi,* Serotype 9] was almost as pathogenic to the 3 mosquitoes, being only slightly less so against *Ae. aegypti* with an LC-50 of 1 μg/ml, but it also was the only formulation of the group showing effect against *Cx. pipiens* (LC-50 = 10 μg/ml) and *Cx. quinquefasciatus* (LC-50 = 5 μg/ml).

Because of the large number of larvae involved in the assays, microscopic examination of each cadaver was not undertaken. However, random checking revealed that septicemia was commonplace, indicating that actual invasion of the host body did take place at some stage in the infection process, with subsequent multiplication of bacterial cells within the tissues and hemolymph.

Although the method of harvesting in the production of the HD strains was in-

tended to remove β-exotoxin produced by any of the strains, analysis of samples from other production runs of most of the strains that were tested by C. Beegle (personal communication) indicated that 11 formulations might possess this unwanted toxin. Most of these strains, HD-2, HD-26, HD-27, HD-28, HD-41 and HD-138 [in var. *thuringiensis*, Serotype 1], HD-116 [in var. *morrisoni* Serotype 8] and HD-199 [in var. *darmstadiensis*, Serotype 10], showed very little effect against the mosquitoes. The only strains listed in Tables 2 and 3 suspected of possessing β-exotoxin were HD-12, HD-125 and HD-146. The most pathogenic strain, HD-125, was retested to compare the effects of autoclaved (spores and δ-endotoxin destroyed) and unautoclaved suspensions, and it was determined that the high mortality causing factors were removed by the heat treatment, thus negating any possible effect on the mosquitoes by any β-exotoxin that might have been present.

The data from our study confirm the variability of activity of strains of *B. thuringiensis* against *Aedes* and *Culex* larvae suggested by Reeves (1970). Of the 127 samples assayed, most were unable to meet our minimum requirement of causing 80%+ mortality at a dosage concentration of 100 μg/ml. However, 26 strains from several varieties or serotypes, including HD-1 and HD-225 (=BA-068), showed marked effect against larvae of *Aedes aegypti, A. triseriatus* and *Cx. tarsalis*, mostly at concentrations below 1 μg/ml. A few of these (5), plus an additional 18 strains were found to have LC-50's in the 4-10 μg/ml range against the much more resistant larvae of *Cx. pipiens* and *Cx. quinquefasciatus*.

Since the HD cultures were grown in a medium intended to create stable activity levels against lepidopterous larvae rather than mosquitoes, the determination that some of the strains possess the capability of causing high mortality to *Cx. tarsalis, Ae. triseriatus* and *Ae. aegypti*, all of which are major mosquito pests, is considered to be of significance. These strains, plus those that performed best, albeit relatively poorly, against the more resistant *Cx. pipiens* and *Cx. quinquefasciatus*, will be regrown in media designed to increase greatly levels of toxicity. They then will be reassayed to determine any increases in activity against the hosts used in this study, as well as other species. From this effort, it is hoped that several highly active strains of *B. thuringiensis* can be selected for large scale production for testing in the field against susceptible mosquito hosts.

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