ACTIVITY ON THE BETA-EXOTOXIN OF *BACILLUS THURINGIENSIS* VAR. *THURINGIENSIS* IN THE *ESCHERICHIA COLI* DNA REPAIR ASSAY FOR BACTERIAL GENOTOXICITY[1,2]

George E. Cantwell,[3] Robert J. Argauer,[4] and William W. Cantelo[3]
Agriculture Research Service, USDA
Beltsville, MD 20705
(Accepted for publication Jan. 9, 1985)

ABSTRACT

The beta-exotoxin of an experimental preparation of *Bacillus thuringiensis* var. *thuringiensis* was bioassayed against neonate Colorado potato beetle larvae and its lethal concentrations determined. The amount of active ingredient in the preparation was determined by liquid chromatography. The *Escherichia coli* DNA Repair Assay was used to determine the DNA damaging potential of the beta-exotoxin, which was found to be negative in this system.

Key Words: *Bacillus thuringiensis,* var. *thuringiensis,* beta-exotoxins, *E. coli* DNA assay, genotoxicity, Colorado potato beetle.

J. Entomol. Sci. 20(1) 104-108 (January 1985)

INTRODUCTION

Cells exposed to agents that modify their DNA tend to protect themselves by excising the altered DNA portion and then resynthesizing the correct sequence. DNA polymerase has been implicated in this repair process (D'Alisa et al. 1971). In 1971 Slater et al. described a simple procedure for evaluating the carcinogenic or mutagenic potential of chemicals in a bacterial culture based on the greater sensitivity of a mutant *Escherichia coli* strain deficient in DNA polymerase. In this procedure the effects of the test chemical on the mutant strain deficient in DNA polymerase (pol A−), as compared with the parent strain (pol A+), is expressed as inhibition of growth.

In our test, this system, the *E. coli* DNA Repair Assay was used to determine the DNA damaging potential of the beta-exotoxin from *Bacillus thuringiensis* var. *thuringiensis (B.t.t.).* This exotoxin, which was described as a nucleotide-like heat stable molecule by Farkas et al. (1969), shows great promise for controlling insects in the order Coleoptera. However, this material has not yet been approved for use in the United States by the EPA, and the question of its safety has not been fully answered.

MATERIALS AND METHODS

The beta-exotoxin used in this test was produced by a commercial fermentation process[5] utilizing *Bacillus thuringiensis* var. *thuringiensis (B.t.t.).* The resulting

[1] Mention of a commercial or proprietary product in this paper does not constitute an endorsement of the product by the U. S. Department of Agriculture.

[2] The study director for the DNA repair assay was S. Louie of the testing facility Intox Laboratories, Redfield, AR 72132.

[3] Vegetable Lab., Horticulture Science Inst.

[4] Analytical Chemistry Lab., Agric. Environ. Quality Inst.

[5] *B.t.* material furnished by Biochem Products, Montchanin, DE 19710 originating from Roger Bellon Labs. as strain #19.

experimental product was centrifuged and its cell-free supernatant filtered through a 0.45μ filter and autoclaved at 15 psi for 20 minutes. This material was then bioassayed for activity aginst 1st-instar Colorado potato beetle larvae *Leptinotarsa decemlineata* (Say) (CPB). The amount of *B.t.t.* exotoxin in an aliquot of the supernatant was quantitated by liquid chromatography. An aliquot was also used in the *E. coli* DNA Repair Assay.

Bioassay: The supernatant, after being filtered and autoclaved, was sprayed with an atomizer on tomato foliage at 10-fold serial water dilutions of 10^{-2} to 10^{-5}. After drying, each leaf was placed in a petri dish along with ten 1st-instar CPB larvae. Controls were water sprayed. Each test was replicated six times. Mortality was recorded at four days and the data were subjected to a probit analysis to determine the regression of insect mortality on the concentration of the exotoxin.

Liquid chromatography: A DuPont Model 848 liquid chromatograph with a 254 nm absorbance detector was equipped with a Rheodyne Model 70-10 six-port sample injection valve filled with a 50 microliter sample loop. The column was 4.4 mm $\times$ 15 cm stainless steel filled with C-18 five micron hypersil (Shandon). The mobile phase used for the chemical quantitation was 0.1% trifluoroacetic acid in 0.05M KH_2PO_4. Gelman Sciences 0.45 micron Acro LC 13 disposable filters were used to filter solutions prior to chromatography. The amount of thuringiensin in the experimental formulation obtained from Solvay and Cie was verified by comparing peak heights to a secondary standard of 55% AI obtained from Abbott Laboratories. We subsequently obtained a primary analytical grade thuringiensin standard from Abbott and confirmed the actual concentration of thuringiensin in the secondary standard at 55% AI and that the toxic primary peak in the secondary standard corresponded to that peak attributed to thuringiensin in the primary standard.

Chromatographic fractions of the eluate were collected at retention times, before, under, and after the major peak which included the unresolved satellite peak. These fractions were bioassayed against neonate CPB larvae for biological activity.

E. coli. DNA Repair Assay: Escherichia coli K-12 strains W3110, DNA repair-competent and P3478 DNA polymerase-deficient were used in this test. Solutions of the *B.t.t.* supernatant were prepared in sterile deionized water at doses of 0.1, 0.5, 1.0, 5.0, and 10.0 mg/10 μl. (v/v).

Bacterial growth media were Vogel-Bonner-Glucose medium with and without agar and a top agar medium consisting of 0.6 g agar, 0.6 g NaCl and 100.0 ml H_2O. Each bacterial strain was exposed to the *B.t.t.* exotoxin both with and without the addition of mammalian liver microsomal enzyme preparation. This increases the usefulness of the assay system (by allowing metabolic activation to occur on the test plate) because some carcinogens or mutagens require metabolic activation which is beyond the enzymatic capability of the bacterium.

Negative controls consisted of sterile deionized water tested against both strains with and without mammalian activation; positive controls consisted of methyl methanesulfonate without activation and 2-acetylaminofluorene in dimethyl sulfoxide (DMSO) with metabolic activation.

The procedure for disc assay in this test was similar to that described by Longnecker et al. (1974) in which DMSO was used as the solvent.

RESULTS AND DISCUSSION

Biological Activity: In order to confirm the presence of the beta-exotoxin and its degree of biological activity in the supernatant, bioassays were conducted by feeding the supernatant to neonate CPB. Mortality was recorded at 4 d. A probit analysis of these data is given in Table 1. Feeding *ad libitum* on a dilution of the supernatant of ca. 2 ppt resulted in 95% kill of 1st-instar larvae. Chromatography indicated that the supernatant contained 0.5 g/liter active ingredient. The actual concentration of active ingredient necessary to achieve 95% kill was approximately 1 ppm.

Table 1. Dose rates (ppt) and 95% fiducial limits of concentrations of beta-exotoxin supernatant of *Bacillus thuringiensis* var. *thuringiensis* on neonate Colorado potato beetles at 4 d.

		95% fiducial limits	
	Dose/ppt	Lower	Upper
LC10	0.086	0.045	0.131
LC25	0.168	0.106	0.233
LC50	0.353	0.259	0.460
LC75	0.741	0.569	1.011
LC95	2.155	1.491	3.720

Bioassay of chromatographic fractions: CPB first instar larvae fed very little on tomato leaves sprayed with the mobile phase used in the chemical quantitation. This material was phytogoxic to the plant. Normal feeding occurred when 0.01M KH_2PO_4 was substituted as the mobile phase for bioassay of the chromatographic fractions. The retention time for the peak attributed to beta-exotoxin however did change from 7 minutes in Figure 1 to 2.5 minutes. A 7 μg AI injection of an Abbott standard formulation was chromatographed using 0.01 M KH_2PO_4 as the mobile phase. Fractions of the eluate which were collected at retention times before, under, and after the major peak and which included the unresolved satellite peak showed biological activity in that fraction containing the peak attributed to beta-exotoxin when bioassayed.

E. coli: DNA Repair Assay: The diameters in mm of zones of inhibition were zero for both Pol A+ with and without activation and Pol A− with and without activation at each of the test dose levels. Hence, direct activity in preferentially inhibiting the growth of the DNA polymerase mutant strain was not found with the beta-exotoxin of *B.t.t.* without metabolic activation or when metabolic activation was added to the assay procedure.

Preferential inhibition of the mutant was demonstrated with methyl methanesulfonate, the direct acting positive control, and 2-acetylaminofluorene, the activated positive control. Statistical analyses of the data (Wilcoxon 1960) from the positive control groups indicated that a significant difference ($P = 0.020$) existed between the zones of inhibition measured for the two strains with metabolic activation. A significant difference ($P = 0.028$) also was recorded for MMS without metabolic activation (Table 2).

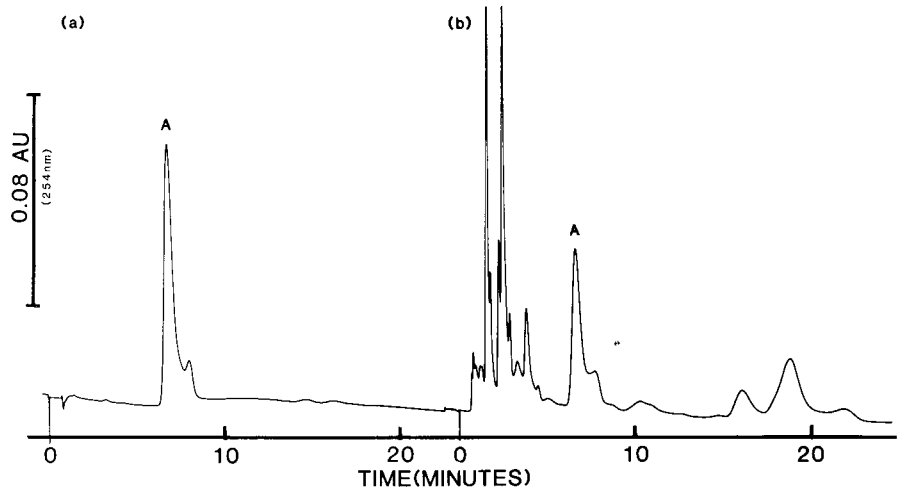

Fig. 1. Chromatograms of beta-exotoxin (thuringiensin) – A
(a) ABG-6146, 55% AI, Abbott Laboratories, North Chicago, Illinois. Amount injected: 7 μg AI (not used in the mutagenicity or bioassay tests).
(b) Experimental formulation from Solvay and Cie S.A., Biochem Products, Brussels, Belgium. Amount injected: 50 μl of a 1:5 dilution.
Mobile phase: 0.1% trifluoroacetic acid 0.05 M KH_2PO_4 at 2 ml/minute column: 4.4 mm × 15 cm 5 μ Shandon C-18.

Table 2. Direct acting and S-9 activated positive controls in *E. coli* DNA repair assay. Diameter in mm of zones of inhibition averaged from four plates.

Strain	Bact. only	DMSO* 10 μl	MMS* 10 μl	Bact. only	DMSO 50 μl	MMS 10 μl
W3110 Pol A+	0	0	41†	0	0	0 a‡
P3478 Pol A−	0	0	59 b†	0	0	7 b‡

DMSD = dimethyl sulfoxide, MMS = methyl methanesulfonate.
† Significantly different (P = 0.028) from the other 2 treatments in the same row.
‡ Significantly different (P = 0.020) from the other 2 treatments in the same row.

Our results indicated that the beta-exotoxin was not toxic to either the parent or mutant strains of *E. coli* under the conditions used in this test. However, in this assay system, definitive conclusions can be drawn only when the growth of the pol A− strain is actually inhibited by the chemical. Negative results may be due to inactivity of the chemical or inability of the agent to penetrate the bacterial cell wall.

Additional *in vitro* studies of the beta-exotoxins from vars. *morrisoni* and *thuringiensis* conducted by Cantwell et al (1982, 1983) utilizing the *Salmonella/ Microsomal* Assay resulted in similar findings of non-mutagenicity, thereby adding evidence to that of this test. *In vivo* or long-term animal studies remain to be done.

One must also be aware of the possibility that the heat-stable exotoxin as produced by *B.t.t.* strain 19 and used in our test may be dissimilar from those produced by other strains. Dulmage (1981) has presented evidence to indicate that, based upon insecticidal activities, there may be three groups or categories of "B-exotoxins" produced by the many strains of *B.t.* Dulmage further states that workers in the International Cooperative Program on the spectrum of activity of *Bacillus thuringiensis* found that some of the B-exotoxin like compounds appear to differ among themselves, and from thuringiensin, and that "Apparently, there was more than one kind of B-exotoxin."

LITERATURE CITED

Cantwell, G. E., E. Dougherty, and W. W. Cantelo. 1983. Activity of the B-exotoxin of *Bacillus thuringiensis* var. *thuringiensis* against the Colorado potato beetle (Coleoptera: Chrysomelidae) and bacterial mutagenic response as determined by the Ames test. Environ. Entomol. 12: 1424-7.

Cantwell, G. E., R. E. Gingrich, and S. E. Kunz. 1982. Activity of a "thermostable exotoxin" of *Bacillus thuringiensis* subsp. *morrisoni* in the *Salmonella/*microsomal assay for bacterial mutagenicity. J. Invertebr. Pathol. 40: 350-8.

D'Alisa, R. M., G. A. Curphey III, H. S. Carr, and H. S. Rosenkranz.. 1971. "Reversion" of DNA polymerase-deficient *Escherichia coli.* Mol. Gen. Genet. 110: 23-6.

Dulmage, H. T., and Cooperators. 1981. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control. *In* "Microbial Control of Pests and Plant Diseases, 1970 - 1980" (H. D. Burgess, ed.). pp. 193-222. Academic Press, London.

Farkas, J., K. Sebesta, K. Horska, Z. Samek, J. Dolejs, and F. Storm. 1969. The structure of exotoxin of *Bacillus thuringiensis* var. *gelechiae.* Collect. Czech. Chem. Commun. 34: 1118-20.

Longnecker, D. S., T. J. Curphey, S. T. James, D. S. Daniel, and N. J. Jacobs. 1974. Trial of a bacterial screening system for rapid detection of mutagens and carcinogens. Cancer Res. 34: 1658-63.

Slater, E. E., M. D. Anderson, and H. S. Rosenkranz. 1971. Rapid detection of mutagens and carcinogens. Cancer. Res. 31: 970-3.

Wilcoxon, F. as cited in R. G. Steel, and J. H. Torrie. 1960. Procedures of Statistics. McGraw-Hill Book Co., Inc. New York, NY. p. 405.