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Toxicity of *Bacillus thuringiensis* Spores to the Tobacco Hornworm, *Manduca sexta*

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Toxicity of *Bacillus thuringiensis* spores to the tobacco hornworm, *Manduca sexta,* is described. The numbers of larvae killed were in relation to spore dry weight. At a surface application of 6.8 ng/cm^2, there was an 85% survival, but less than 50% survived at 68.2 ng/cm^2. Striking similarity of spores to parasporal crystals is revealed by slope of mortality curves, inhibition of stadial growth, and 50% lethal dose values based on protein content.

Bacillus thuringiensis is one species among the gram-positive spore-forming bacteria that forms a discrete intracellular glycoprotein crystal during sporulation (1, 3). The crystalline inclusion is toxic to lepidopteran larvae (4, 6, 12, 14) and is the basis for commercial formulation of *B. thuringiensis* as an insecticide. In addition, the spore exhibits insecticidal activity (13, 19, 20) and the spore coat protein appears to be biochemically homologous to the parasporal crystal (5, 7, 8, 11, 16-18). In our laboratory, we are interested in the insecticidal properties and biochemical mode of action of the parasporal crystal as well as the spore and also in the biological relationship between spore and crystal. Previously, we developed a highly sensitive, reproducible bioassay (14) that can be used to compare relative toxicities of crystals, spores, and other cellular components. Using this technique, we have determined the lethality of *B. thuringiensis* spores and report here the lethal doses and confidence limits of homogeneous spore preparation for the tobacco hornworm, *Manduca sexta.*

MATERIALS AND METHODS

Test organisms. *M. sexta* larvae were hatched from eggs received from J. P. Reinecke, Metabolism and Radiation Research Laboratory, U.S. Department of Agriculture, Fargo, N.D. Neonate larvae were used in all tests. The diet used was that of Yamamoto (21) as modified by Bell and Joachim (2). It contained Formalin and streptomycin to restrict the growth of microorganisms.

B. thuringiensis subsp. *kurstaki,* used in this study, was isolated from a commercial insecticidal formulation called Dipel (Abbott Laboratories, North Chicago, Ill.). Stock cultures of the organism were maintained on modified GYS (10) agar slants. Cells for experimental use were cultured in the same manner as described previously (14). Spores were separated from parasporal crystals and cellular debris by buoyant density centrifugation in Renografin gradients (15). Spores isolated in this manner were washed at least three times in distilled water and lyophilized to constant weight. Purity of the spore preparations was monitored at each step of the separation procedure by phase-contrast microscopy. The population of spores was 6.75 × 10^8 per mg (dry weight) and contained less than 0.01% of other cellular material. A portion of the spores was treated with 0.1% sodium azide (NaN_3) solution to prevent any cellular differentiation and then washed three times with distilled water. The NaN_3 treatment killed the spores and excluded the possibility of insect toxicity due to germination and subsequent proliferation of the *B. thuringiensis* cells. Mid-exponential vegetative cells, which are not insecticidal, were treated with NaN_3, washed with water, and used to determine any insect toxicity due to residual NaN_3. Both untreated and NaN_3-treated vegetative cells did not cause any adverse affects to the insect larvae.

Experimental treatments. Freshly prepared artificial diet was poured to a depth of 1.5 cm into an amber pill vial (4.3 by 7.8 cm, 30 dram [ca. 35.31 g]) with snap cap lid. Each lid has a small hole punched in the center to allow for air exchange. Dilutions of the spore preparation were made by homogenizing 400 μg of the purified spores in 5 ml of distilled water. Dilutions to give 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1 μg of spores per ml of solution were prepared, and 1 ml of each was applied uniformly to the diet surface (14.66 cm^2) and allowed to air dry. The applications resulted in surface treatments of 68.2, 54.6, 40.9, 27.2, 13.6, and 6.8 ng/cm^2. Each treatment was replicated in 10 separate containers.

One neonate larva of *M. sexta* was placed on the treated surface in each container. After introduction of the larvae to the treated diet surface, they were held at a constant temperature (27°C) and humidity (60%) until the observation period ended. A mortality count was made after 7 days of exposure to the treated surface. All larvae surviving on day 10 were pooled according to treatment and weighed, and the average weight per larva was recorded. Larvae were character-

ized according to instar, if dead, or by measuring the size of the head capsule and converting to instar or stage of growth, if alive.

RESULTS AND DISCUSSION

The effect of a homogeneous preparation of *B. thuringiensis* spores is given in Table 1. The numbers of larvae killed were in relation to spore dry weight. NaN_3-treated spores produced similar results. At a surface application of 6.8 ng/cm^2, there was an 85% survival, but less than 50% survived at 68.2 ng/cm^2. Mean lethal end points and the 95% confidence limits are given in Table 2. The mean 50% lethal dose value for spores against neonate *M. sexta* larvae is 7.4×10^{-8} g/cm^2. Interestingly, the slope of the mortality curve for larvae fed whole spores (curve not shown) is not significantly different from the 2.51 slope of the mortality curve reported earlier for parasporal crystals (14).

An extreme reduction in weight of surviving larvae on treated surfaces at day 10 is also shown in Table 1. At the lowest dose tested (6.8 ng/cm^2), the surviving larvae were only 41% as heavy as the control insects for the same period. The highest dosage (68.2 ng/cm^2) resulted in a 97% weight reduction; i.e., the survivors weighed only 3% as much as the untreated insects. Significantly, larvae that were fed spores exhibited the same degree of restricted growth through the developmental stages (2nd instar) as larvae treated with parasporal crystals (14). Control larvae molted to 4th and 5th instar by day 10 (Table 1). Physical evidence showing only a small amount of feeding activity in tests with both the spores and crystals indicates an inhibition of feeding. Certainly, the pathological effects were not the result of proliferating vegetative cells that outgrew from germinated spores, because NaN_3-treated spores caused the same limited growth and ultimate death.

TABLE 1. *Mortality of M. sexta after treatment with B. thuringiensis spores*[a]

Dose			Mortality after 7 days		Surviving larvae at 10 days		
Suspension (μg/ml)	Diet surface (ng/cm^2)	Protein (ng/ml)	Percent	Instar	Avg wt per larva (mg)	Wt reduction (%)	Instar
Control	0	0	0		302		4, 5
0.10	6.8	12.8	0		124	59	3
0.20	13.6	25.5	15	2	50	83	2
0.40	27.2	51.0	20	2	34	87	2
0.60	40.9	76.5	25	2	20	93	2
0.80	54.6	102.0	35	2	12	96	2
1.00	68.2	127.5	55	2	10	97	2

[a] Mortality after 7 days on a diet surface treated with *B. thuringiensis* spores and average weight per larva of survivors at 10 days. Each treatment was replicated 10 times and was initiated with neonate larvae.

TABLE 2. *Lethal doses and confidence limits of B. thuringiensis spores for M. sexta*

Lethal doses[a]			95% confidence limits (μg/cm^2)	
End point	μg/ml per 14.66 cm^2	ng/cm^2	Upper	Lower
50	1.08	73.66	2.53	.75
95	7.99	545.42	137.36	3.10
99	18.29	1,247.61	739.42	5.41

[a] Slope, 1.89; intercept, 4.93.

If spore dry weight is converted to extractable protein content based on the Lowry procedure (9), 138 ng of spore protein is required to achieve a mean lethal dose of 50%. This figure compares well with the 50% lethal dose value of 112 ng for parasporal crystal protein. We have already shown that the spore coat contains the same glycoprotein subunit as the parasporal crystalline protoxin (L. A. Bulla, Jr., K. J. Kramer, and L. I. Davidson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I 62, p. 165), and Delafield et al. (5) reported that spore protein and parasporal crystal have similar immunological properties. Together with these findings and with the demonstration that membrane fractions of outer spore layers are toxic to *Pieris brassicae* larvae (13), the similar 50% lethal dose values based on protein content that we calculate for spores and parasporal crystals provide strong evidence that both crystals and spores contain a similar, if not the same, insecticidal polypeptide.

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