
Mode of Action of Bacillus thuringiensis δ-Endotoxin:
Relative Roles of Spores and Crystals in Toxicity
to Pieris, Lymantria and Ephestia Larvae

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The relative roles of spores and crystals of Bacillus thuringiensis in toxicity to Pieris rapae (fourth instars), Lymantria dispar (fourth and sixth instars) and Ephestia cautella (fourth instars) were studied using absolutely pure spores and crystals.

Spore-free pure crystals and its extract alone showed high toxicity even to Ephestia which has very low susceptibility to this bacterium. Although spores were far less toxic than crystals, the presence of spores enhanced the toxicity of crystals more than twofold. Mixtures of B. thuringiensis crystals and B. subtilis spores produced the same mortality as crystals alone, although B. subtilis spores as well as B. thuringiensis spores germinated and subsequently proliferated vigorously in the gut. Under the presence of antibiotics, no lethal effect of B. thuringiensis spores was observed. It is concluded that the presence of spores is not essential in the killing of any insects by δ-endotoxin. No systemic infection was found in diseased living larvae following any of our experimental treatments.

INTRODUCTION

We have extensively studied the mode of action of Bacillus thuringiensis δ-endotoxin on cultured cells in vitro (Nishiitsuji-Uwo et al., 1979, 1980) and silkworm in vivo (Nishiitsuji-Uwo and Endo, 1980; Endo and Nishiitsuji-Uwo, 1979). The bulk of lepidopterous insects are said to belong to Type II, which shows no general paralysis and little mortality within 48 hr after administration of B. thuringiensis. Although an absolute requirement for both spores and crystals to cause mortality has been maintained in so-called Type III insects, Ephestia kuehniella (Heimpel and Angus, 1959) and Galleria mellonella (Burges et al., 1976), crystals or their extract alone seemed to be toxic enough to kill larvae of other lepidopterous species.

Strictly speaking, however, conclusions on the roles of crystals and/or spores drawn from results by the preparations having contamination can not be judged absolutely doubtless. As far as we know, so far no one has succeeded to obtain pure crystals, which always inevitably contaminated with minor spores even if they are less than 0.01%. We prepared truly spore-free crystals as well as crystal-free spores. With these pure preparations we are now concerned with studies on insects classified to Type II, the common cabbageworm, Pieris rapae, the gypsy moth, Lymantria dispar (Heimpel and Angus, 1960) and the almond moth, Ephestia cautella (McGaughey,
This paper describes the relative roles of spores and crystals in the toxicity of *B. thuringiensis* to those insects.

**MATERIALS AND METHODS**

_Bacteria._ We used four strains of *B. thuringiensis* (*B.t*): two sporeless mutant strains, I-45 from *B.t* subsp. _aizawai_ and P-15 from subsp. _kurstaki* (Nishitsutsuji-Uwo et al., 1975), and their two original strains. Cells were grown in a medium containing 1% molasses, 0.5% casein, 1% polypeptone, 0.5% glucose and 0.3% NaCl at pH 7.0 for 4 days at 28°C. Harvests were washed with distilled water twice, then either lyophilized or subjected to further purification of crystals and spores. Both mutant strains are completely sporeless and has been used as a source of spore-free crystals. Method for purification of crystals and dissolution of δ-endotoxin were described elsewhere (Nishitsutsuji-Uwo et al., 1979). Plate counts showed absolutely no spore was viable in the crystal preparation.

The original strains were used as a source of spores, which were separated from crystals and cellular debris by the method of Goodman et al. (1967). Crystal-free spores were prepared by extracting the crystals contaminated in the spore fraction (0.1% contamination): the spore fraction was suspended in 20 mM carbonate-bicarbonate buffer (pH 9.6) containing partially purified digestive enzymes prepared from *Bombyx* gut juice (Nishitsutsuji-Uwo et al., 1979) at a concentration 1/50 of the spore weight and incubated at 35°C for 60 min with stirring. Under such conditions, crystals, if any, were easily dissolved out within 30 min. After centrifugation, spores were resuspended in the same reagent and extraction of the crystals was repeated three times. The resultant crystal-free spores were then washed four times with distilled water and lyophilized.

As a control, a strain PCI-219 of *B. subtilis* (*B.sub*) was cultured in a medium containing 0.1% glucose, 1% peptone, 0.3% NaCl, 0.5% beef extract and 1.5% agar at pH 7.2 for 10 days at 37°C. After washing with physiological saline solution three times, suspensions were treated with intermittent sterilization method at 65°C for 30 min three times. Spores were then washed with distilled water and lyophilized.

_Insect._ We used three species, the common cabbageworm, *Pieris rapae*, the gipsy moth, *Lymantria dispar* and the almond moth, *Ephestia cautella*. All experiments were carried out at 25°C.

*Pieris rapae:* Pupae and adults were collected in the field and subjected to the long-day photoperiodic regime (16L:8D) at 25°C. Eggs were laid on the cabbage leaves and from the second instar onward larvae were reared on a semi-synthetic diet containing dried powders of cabbage leaves and chlorella (1:1) as major components (Sato, 1974).

Three to four milliliters of hot artificial diet were poured into a 200 ml icecream cup (transparent, plastic). After hardening, 0.3 ml of a toxic solution was applied on its surface removing the excess solution, if any, by filter paper and dried. Ten larvae of 1–2 days of the fourth instar were transferred into each cup, tissue paper was inserted under the lid and the cup was then held upside down. The tissue paper was changed every day and observation was continued for 7 days. Each duplex experiment was done with 6 serial dilutions. As toxic sources, autolyzed cultures of sporeless mutant I-45, P-15 and their original stocks were employed without any fur-
ther purification.

*Lymantria dispar*: In winter, egg-masses were collected in the field and stocked at 4°C in constant darkness. In spring eggs were subjected to a long-day photoperiodic regime (16L:8D) at 25°C. Larvae were reared with oak leaves (*Quercus* sp.).

For the feeding test, oak leaves soaked in a 0.25% Tween 80 solution containing either pure crystals or crystal-spore mixtures (1:1) were given to each of 13 larvae of the fourth instar with 5 serial dilutions. For the forced injection test, a sixth-instar larva was injected per os with 50 μl suspensions containing either pure crystals or crystal-spore mixtures. Four series of dilutions with each of 8 larvae were employed. Observation was continued for 5–6 days.

*Ephesia cautella*: Eggs were transferred into rice-bran autoclaved at 120°C for 20 min. For the experimental series with antibiotics, streptomycin and penicillin (500 μg each per gram of rice-bran) were added in the diet. The insects were reared at 25°C and the fourth instars were used in experiments.

Crystal extract, crystals and/or spores (5, 1, 0.1, 0.01 mg/g diet) were added to freshly prepared rice-bran with or without antibiotics (same doses used for cultivation) and mix thoroughly. One gram (or 0.5 g) of toxin-diet was put in a 5 ml Erlenmyer flask, into which 10–15 larvae were transferred. A glass-bead (15 mm diameter) was put on the top of each flask to prevent the animal’s escape. Observation was continued for 7 days. All experiments without antibiotics were repeated three times through three generations.

Method for electron microscopy of midgut epithelium was described elsewhere (Endo and Nishitstsui-Uwo, 1979).

**RESULTS**

1. *Pieris*. Whole cultures of sporeless mutant strains and their original strains of subsp. *aizawai* and *kurstaki* were tested against fourth instar larvae of *Pieris rapae* and third instar larvae of *Bombyx mori*. Results are shown in Table 1.

The *kurstaki*-subsp showed much stronger activity than the *aizawai*-subsp against

<table>
<thead>
<tr>
<th>Variety</th>
<th>Strain</th>
<th>LC50 (μg/g of diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-</td>
</tr>
<tr>
<td><em>Pieris</em> (4th instars)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aizawai</em></td>
<td>Sporeless</td>
<td>&gt;833</td>
</tr>
<tr>
<td></td>
<td>Original</td>
<td>&gt;833</td>
</tr>
<tr>
<td><em>kurstaki</em></td>
<td>Sporeless</td>
<td>&gt;833</td>
</tr>
<tr>
<td></td>
<td>Original</td>
<td>275</td>
</tr>
<tr>
<td><em>Bombyx</em> (3rd instars)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aizawai</em></td>
<td>Sporeless</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>Original</td>
<td>37.5</td>
</tr>
<tr>
<td><em>kurstaki</em></td>
<td>Sporeless</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Original</td>
<td>13.0</td>
</tr>
</tbody>
</table>

*a* LC50 (50% mortality by probit) does not express the ingested dose but the concentrations (μg) applied in one gram of diet,
the *Pieris*-larvae. Within 48 hr *Pieris* showed little mortality which is said to be characteristic to the Type II insects. Most of the larvae died in 3 to 5 days after the administration of the toxin. At 5-day point the sporeless mutant strains showed nearly the same activities as those of their original strains. Since we used whole culture, the number of crystals contained in the original strain was nearly half of the mutant strain when the doses were based on dried harvest weight. Based on crystal content, the LC$_{50}$ for 5 days of each original strain were increased nearly twofold from that of the mutant strain. These results indicate that the presence of spores resulted in a twofold increase in toxicity of crystals on *Pieris*-larvae.

2. *Lymantria*. Fourth-instar larvae were fed with graded dosages of preparations containing either spore-free pure crystals or mg/mg mixtures of pure crystals and spores of *B.t* subsp. *kurstaki*. In the feeding test (µg/ml of toxin-solution applied on diet) the LC$_{50}$ for pure crystals was 22 µg/ml at 3-day, 8.6 µg/ml at 4-day and 3.6 µg/ml at 5-day, for the crystal-spore mixture (based on crystal weight) was 15, 2.6 and 0.6 µg/ml respectively (Table 2). With the lapse of time, enhancement of the toxicity

### Table 2. Effect of Pure Crystals and Crystal-Spore Mixtures of *B. thuringiensis subsp. kurstaki on Larvae of *Lymantria dispar*

<table>
<thead>
<tr>
<th>Feeding test (LC$_{50}$ µg/ml)$^a$</th>
<th>1-</th>
<th>2-</th>
<th>3-</th>
<th>4-</th>
<th>5-</th>
<th>6-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystals</td>
<td>&gt;1000</td>
<td>-</td>
<td>22</td>
<td>8.6</td>
<td>3.6</td>
<td>-</td>
</tr>
<tr>
<td>Crystal-spore mixtures (based on crystal weight) (&gt;500)</td>
<td>&gt;1000</td>
<td>-</td>
<td>30</td>
<td>(2.6)</td>
<td>(0.6)</td>
<td>-</td>
</tr>
<tr>
<td>Forced injection (LD$_{50}$ µg/larva)$^b$</td>
<td>Crystals</td>
<td>&gt;25.0</td>
<td>5.0</td>
<td>1.3</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Crystal-spore mixtures (based on crystal weight) (8.0)</td>
<td>16.0</td>
<td>2.6</td>
<td>2.6</td>
<td>1.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

$^a$ Fourth-instar larvae were fed oak leaves soaked in toxic solution. LC$_{50}$ expresses the lethal concentration (µg/ml) applied on leaves (50% mortality).

$^b$ Sixth-instar larvae were injected per os with 50 µl of toxic suspensions. LD$_{50}$ expresses the lethal dose (µg) per larva (50% mortality).

### Table 3. Effect of Pure Crystals, Crystal-Extract and Spores of *B. t* and *B. sub* on Larvae of *Euphestia cautella*

| Preparation           | *Euphestia* | *Bombbyx*$^e$ |  |  |
|-----------------------|-------------|---------------|  |  |
|                       | LC$_{50}$a | LC$_{50}$b    |  |  |
| 7 days (µg/g)         | 7 days      | 5 days        |  |
| + antibiotics         | + antibiotics |             |  |
| Crystal               | 175±5       | 96            | 0.39±0.06 |
| Crystal-extract       | 74±8        | 29            | 35.0±2.7  |
| Spore (*B. t*)        | 267±115     | 5000          | 35.1±2.7  |
| Crystal+Spore (*B. t*)| 74±30       | 160           | 0.64±0.06 |
| Spore (*B. sub*)      | (mortality 0) | (mortality 0) |  |
| Crystal+Spore (*B. sub)| 380±40     | 176           |  |

$^a$ LC$_{50}$ (50% mortality by probit) was not expressed as the ingested dose but as the concentration (µg) applied on one gram of diet.

$^b$ Streptomycin and penicillin were added into diets (500 µg each per g) from egg stage to the end of experiments.

$^e$ Third-instar larvae one day after moultling.
of crystals by the presence of the spores became obvious. The same was true in the
forced injection test (sixth instars).

3. *Ephesia*. Pure preparations of crystal, crystal extract, spore of *B.t* or *B.sub*
and crystal-spore mixtures (subsp. *aizawai*) were tested against fourth-instar larvae of
*Ephesia cautella*. Results are shown in Table 3. In general, the susceptibility of
*Ephesia*-larvae to killing by *B.t* is extremely low—it is about several hundred times
less than those of *Bombyx*, *Pieris* and *Lymantria* larvae.

*Without antibiotics* (three repeats, Table 3, left column)

Pure crystals were found to be quite toxic to *E. cautella* and dissolved crystal toxin
(based on the protein weight) showed 2–3 times stronger activity than the crystal itself.
Since the protein weight of the dissolved toxin was about one third of the crystal
weight, the former activity was estimated the same as the latter's.

Spore alone also killed *E. cautella* although their activity was a half of the crystal’s.
A mixture of crystal-spore (mg/ml) showed two (based on mixture weight) or four
(based on crystal weight) times higher effect than that of the crystals alone. Since
the pH of the midgut juice of *E. cautella* is nearly neutral (pH 7.3), spores were able
to germinate easily and proliferated vigorously inside the peritrophic membrane.
Spores germinated even in the rice-bran within 24 hr after the preparation. According
to Sutter and Raun (1967), in the European-corn-borer, *Ostrinia nubilalis* after the
ingestion of crystal-spore mixture, the midgut epithelial cells slough off into the lumen
and thus exposed areas of the basement membrane to attack by vegetative rods. If
the bacterial proliferation itself contribute to *Ephesia*-lethality, (including other Type
II insects), *B. sub* spores—harmless to lepidopterous larvae—can be substituted for
*B.t* spores in our bioassay.

Mixtures of *B.t* crystal and *B.sub* spores produced the same mortality as crystal
alone, although *B.sub* spores also germinated and bacterial proliferation was vigorous
inside the peritrophic membrane. We are thus led to conclude that the effect of *B.t*
spores enhancing the lethality of crystals cannot be explained by a general bacterial
proliferation.

*With antibiotics* (Table 3, middle column)

When a minimum inhibitory concentration test (MIC) was made, streptomycin
killed *B.t* subsp. *kurstaki* in 15.6 μg/ml and *aizawai* in 31.3–15.6 μg/ml. Penicillin
has no sterilizing activity on this bacterium. By rearing the larvae on a diet contain-
ing both antibiotics we not only inhibited the germination of *B.t* spores (by strepto-
mycin) but killed most of the bacteria that would otherwise have been present in
the diet or the gut. For instance, *B.sub* PCI-219 was totally killed by penicillin (0.003
μg/ml-MIC) as well as streptomycin (0.39 μg/ml-MIC).

When larvae were reared and bioassayed with the diet containing antibiotics,
they were apparently not affected by the spore alone and nearly zero mortality was
observed. When *B.t* crystal-*B.t* spore mixture were supplied, the mortality was sim-
ilar to those obtained with *B.t* crystal-*B.sub* spore mixture or with crystal alone. Thus,
in the presence of antibiotics spores have no lethal effect at all. According to
Somerville and Pockett (1975) spores of *B. thuringiensis* contain a toxin active against
lepidopterous larvae. This toxin can be solubilized by extraction with reagents which
dissolve the protein crystal and it is inactivated by crystal-specific antiserum. In
the present experiment, since toxicity of crystal did not decrease under the presence of
antibiotics, if the spore contains the same toxic component(s) present in the crystal
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as Somerville and Pockett say, that must be present in spores in very small amount. Clearly the spore substance(s) play a minor role, if any, in the insecticidal effect on Ephesia. The crystals dosed or produced by germination and subsequent multiplication of bacteria seem to be the primary cause of Ephesia-mortality.

In the presence of antibiotics the toxic effects of crystals or crystal extract were more than twice as high as those in the absence of antibiotics in our experiments. On the other hand, Somerville et al. (1970) reported that crystal solutions had a surprisingly small lethal effect on larvae of Trichoplusia ni and Pseudaletia unipuncta raised on a diet containing aureomycin. The reason why is at present unclear.

Germination of spores

Spores, irrespective of species (B.t or B.sub) often germinated within 24 hr even in the diet, unless antibiotics were present. Inside the peritrophic membrane many bacterial rods were observed (Figs. 1 and 2) accompanying the cell division. Bacterial rods sometimes invaded the intracellular spaces (Fig. 1 arrow) of midgut epithelial cells 24 hr after the administration of 2 mg crystals +2 mg spores or 10 mg spores per g rice-bran. A few rods already contained a crystal with lattice fringes (Fig. 3). A mass of crystals was very often observed inside the peritrophic membrane (Fig. 2).

The neutrality (pH 7.3) of Ephesia's midgut juice, unlike those of Bombyx (pH 10–11), Lymantria (8.9) and Pieris (8.3) is presumably the reason why these crystals remained indissolved. And that, in turn, is why Ephesia's susceptibility to B.t induced death is so low.

DISCUSSION

There are many literatures on the lethality to Lepidoptera of the spores and δ-endotoxin crystals of B. thuringiensis. Conclusions about the relative importance of spores and crystals vary depending on the insect species used for assay. Nor are they always the same for a given species, presumably reflecting differences in the purity and activity of crystals. Whereas crystal-free spores are easy to prepare, it is likely that we are the first workers to use the sporeless mutant of B.t to get truly spore-free crystals. Variations in the mildness of extraction procedures probably cause comparable variation in the activity of crystal extract (Nishitsuji-Uwo et al., 1977). In the present study, the sporeless mutant assured absolute purity (spore-free) of the crystals and the crude extract of δ-endotoxin which we used retained activity as great as that of intact crystals.

In our hands the relative role of crystals and spores in the mortality of the so-called Type II insects is as follows: crystal-spore mixture (1:1) > crystal extract ≥ crystal>spore. Similar results were obtained by Fast (1977) in the spruce budworm, Choristoneura fumiferana. According to him, since the regression coefficients were indistinguishable between purified crystals, purified spores or mg/mg mixtures of the two, indicating a common or similar mode of action, spores play little or no role in mortality of the spruce budworm induced by B.t insecticides. The increase in toxicity of crystals by the presence of spores has been reported in many insects such as, Ephesia cautella and Ploidia interpunctella (McGaughey, 1978), Trichoplusia ni, Colias eurytheme and Pseudaletia unipuncta (Somerville et al., 1970) and Galleria mellonella (Burgess et al., 1976). From our data on pH of midgut juice and bioassay of six
Fig. 1. Proliferation of bacteria in the midgut *Ephesia* larva (Administration of crystals and *B. t* spores). Many bacterial rods (B) are seen accompanying the cell division. Sometimes they invaded to the intracellular cytoplasm of columnar cell or to the intercellular spaces (arrow). C; columnar cell. MV; microvilli. ×6,600.

Fig. 2. Proliferation of bacteria (B) and crystal mass in the lumen of midgut of *Ephesia* larva (Administration of crystals and *B. sub* spores). A mass of crystals (Cr) was often observed inside the peritrophic membrane. They were supposedly indissolved crystals fed with diet, under the neutral gut juice (pH 7.3). ×8,300.

Fig. 3. Crystal inclusion of bacterial rod in the lumen of mid gut of *Ephesia* larva (Administration of *B.t* spores). A few bacteria already contained crystal inclusion (Cr) with lattice fringes. ×40,000.

lepidopterous species (unpublished data), the lower the pH of midgut juice, the lower the susceptibility to crystals and the higher enhancement of toxicity of crystals by the addition of spores were generally observed.

Schesser et al. (1977) and Schesser and Bulla (1978) reported on the toxicity of spores of *B.t* to the tabacco hornworm, *Manduca sexta*. They observed that the spores and crystals were nearly equally toxic and that larvae were killed even by the
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NaNO₃-treated dead spores. They concluded that the pathological effects were not the result of proliferating cells that grew from germinated spores and they agreed with the postulation of Somerville and Pocquet (1975) that the spore toxin is chemically very similar to the crystal toxin. In our experiments on E. cautella, when no antibiotics were employed, both spores of B.t and B.sub germinated in the diet as well as in the gut and proliferated vigorously. Proliferation of B. sub did not kill the insect and there was no increase of mortality by adding these spores to the crystals. Therefore we do not deny the possibility that a large amount of spore toxin or some toxic substance(s) produced by proliferation of B.t might be concerned in the ultimate death of lepidopterous larvae. However, the primary cause in killing any insects seems to derive from the crystals dosed or produced subsequently in the multiplying bacteria. We never observed any systemic infection in the diseased living larvae.

So far, two species of Lepidoptera, Ephesia kuehniella (Heimpel and Angus, 1959) and Galleria mellonella (Burges et al., 1976) have been described as Type III insects, said to die by the ingestion of crystals in the presence of spores. The spruce budworm was also once thought to be a Type III insect (Smirnoff, 1963, 1974). However, it has become clear that crystal or its extract alone is enough to kill this insect (YamvriAs and Angus, 1970; Fast, 1977). Ephesia cautella is closely related to Ephesia kuehniella—same genus—yet, the former belongs to Type II (McGaughey, 1977) and the latter to Type III. We surmise that the principal mode of δ-endotoxin action does not differ from species to species and there is only sequential differences from species to species on general pathology in terms of larval behavior, hemolymph chemistry and associated histopathology in the midgut epithelium as those we found in Type I and II insects. We will discuss these subjects in separate reports.

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