Induction of Antibacterial Activity against *Bacillus thuringiensis* in the Common Cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae)

Shyuichiro INAGAKI, Minoru MIYASONO, Makiko YAMAMOTO,
Katsuaki OHBA, Takeo ISHIGURO, Reiji TAKEDA and Yoshiyuki HAYASHI

*Aburahi Laboratories, Shionogi & Co., Ltd., Gotanda 1405,
Koka-cho, Shiga 520-34, Japan*

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The larvae of *Spodoptera litura* were 2,500-fold more resistant to infection with the vegetative cells of *Bacillus thuringiensis* var. *kurstaki* HD-1 than *Bombyx mori* larvae. The injected bacteria were eliminated or maintained at low levels in the hemolymph of *S. litura*, while rapid growth was observed in the hemolymph of *B. mori*. Antibacterial activity against the vegetative cells of *B. thuringiensis* was induced in the hemolymph of *S. litura* upon bacterial inoculation, but it was not detected in the hemolymph of *B. mori*.

Key words: *Spodoptera litura*, *Bombyx mori*, *Bacillus thuringiensis*, antibacterial activity, infection

INTRODUCTION

In microbial control of insect pests, a gram-positive bacterium *Bacillus thuringiensis* is most widely used. The main insecticidal principle of the bacterium resides in the parasporal crystal proteins, δ -endotoxins that are produced during the spore formation (HÖFTE and WHITELEY, 1989). However, it is believed that the spore germination contributes to the pathogenicity leading to larval death (HEIMPEL and ANGUS, 1959; ELLAR, 1990). In this context it is noteworthy that *B. thuringiensis* must contend with the immune system of insects (EDLUND et al., 1976). *B. thuringiensis* is resistant to antibacterial proteins like cecropins produced by insects, but other spore formers such as *B. megaterium* are not (HULTMARK et al., 1982).

The common cutworm *Spodoptera litura*, a major pest to a variety of crops, is known not to be susceptible to many varieties of *B. thuringiensis*. This insect has been shown to be resistant to δ -endotoxins of *B. thuringiensis* var. *kurstaki* HD-1 (INAGAKI et al., 1992).

In this study, we report that *S. litura* is much more resistant to infection with this bacterium than the silkworm *Bombyx mori* that is highly susceptible to var. *kurstaki* HD-1. In addition, we show that antibacterial activity against *B. thuringiensis* is induced in the hemolymph of *S. litura* after injection of the bacterial cells.

MATERIALS AND METHODS

Insects. The larvae of *S. litura*, which had been collected in the field and maintained in the laboratory, were reared at 25°C on an artificial diet described by OYAMA and KAMANO (1976). The larvae of *B. mori* (Shunrei × Shogetsu) were reared at 25°C on an artificial diet (ISHIGURO and MIYASONO, 1979). The sixth instar larvae of *S. litura* and the fourth instar larvae of *B. mori* were used for experiments.

Bacteria. *B. thuringiensis* var. *kurstaki* HD-1, kept in the bacterial collection of this laboratory, was used. A streptomycin-resistant mutant was isolated from a wild-type strain. For the preparation of the vegetative cells, cultures were grown in a GYS medium with shaking at 30°C, and were harvested at an optical density at 600 nm of 0.5–0.7. The cells were collected by centrifugation, washed with saline (130 mM NaCl, 50 mM KCl, 1 mM CaCl₂) three times, and resuspended in saline.

Injection of bacteria and collection of hemolymph. The larva was chilled on ice for 5–10 min and injected into the hemocoel with 5 µl suspension of the bacteria using a glass capillary attached to a microsyringe. One to three days later, hemolymph was collected in an ice-cooled tube containing a few crystals of phenylthiourea and used for assays of viable cell number and antibacterial activity. For the experiment on *in vivo* trace of viable cell number, the streptomycin-resistant mutant was injected. In the insect mortality and *in vitro* experiments, the wild type strain was injected.

Assay of antibacterial activity. Antibacterial activity against *B. thuringiensis* vegetative cells was determined by the method of NATORI (1977) with slight modifications. The bacteria (*str*⁻: 5×10^2 cells) were injected and the hemolymph was collected at the time indicated. The hemolymph was centrifuged at 6,000 rpm for 10 min. The supernatant was used immediately or stored at -20°C until used. The hemolymph (10 µl), which was diluted with saline if needed, was added to 10 µl of the bacterial suspension (*str*⁺: 3×10^2) and then incubated at 30°C for 60 min, followed by cooling in an ice bath to stop the reaction. The mixture (10 µl) was spread on Mueller-Hinton agar (Eiken Chemical Co., Tokyo) plate containing streptomycin (100 µg/ml). The plates were incubated for 18–24 h at 37°C and then colonies were counted and the number of colonies on test and control plates were compared.

RESULTS

In preliminary experiments, the injection of the vegetative cells (10^2 cells/larva) of *B. thuringiensis* var. *kurstaki* HD-1 into larval hemocoel caused a lethal effect on *B. mori* larvae, but it did not kill *S. litura* larvae. Thus, we examined quantitatively the effect of the injected bacteria on the mortality of the two kinds of insects (Table 1). In *S. litura* larvae, the lethal dose causing 50% mortality (LD₅₀) was 51,600 cells/g of body weight; in *B. mori*, 20 viable cells were sufficient to cause 50% mortality. The variation in sensitivity represented a 2,500-fold difference in LD₅₀ values between *S. litura* and *B. mori*.

To examine whether this difference of insect responses to the inoculated bacteria is due to the difference in the bacterial growth in the two insects, we traced the number of the viable bacteria in their hemolymph (Table 2). When 6×10^2 or 6×10^3 cells were injected into *S. litura* larvae, the viable cells in the hemolymph were eliminated or maintained at low levels after the inoculation. Significant bacterial growth was

Table 1. Lethal effect of vegetative cells of *Bacillus thuringiensis* var. *kurstaki* HD-1 injected into hemocoel of *Spodoptera litura* and *Bombyx mori*

Insect	LD ₅₀ ^a (95% FL)
<i>S. litura</i>	51,600 (42,300–82,700)
<i>B. mori</i>	20.7 (15.0–48.0)

Mortality was recorded 3 d after injection.

^a LD₅₀ was calculated by log-probit analysis and expressed as the number of bacteria injected per g of body weight.

Table 2. Growth of *B. thuringiensis* in the hemolymph of *S. litura* and *B. mori*

Insect	Dose injected (cells/larva)	Viable cell number/larva		
		Days after injection		
		1	2	3
<i>S. litura</i>	6×10^2	ND ^a	ND	ND
	6×10^3	2×10^3	1×10^2	2×10^2
	6×10^4	4×10^4	1×10^7	1×10^9
<i>B. mori</i>	4×10	1×10^6	5×10^8	1×10^9
	4×10^2	8×10^6	4×10^9	2×10^{11}

^a Not detected.

Fig. 1. Antibacterial activity against vegetative cells of *B. thuringiensis* in the hemolymph of *S. litura* (A) and *B. mori* (B). Hemolymph was collected 48 h after the bacterial inoculation and used for assays. ○: normal larvae; ●: injected larvae.

observed when more cells (6×10^4) were injected. In contrast to *S. litura*, rapid growth of the bacteria was observed in the hemolymph of *B. mori* even when much smaller numbers of the bacteria (4×10 cells) were injected.

Then, we examined antibacterial activity against *B. thuringiensis* in the hemolymph of the two kinds of insects using *in vitro* assay systems (Fig. 1). Hemolymph was collected 48 h after the inoculation and used for assays. The bacterial cells were not grown in the hemolymph from normal larvae of *S. litura* in 60 min, but they were not killed.

Fig. 2. Changes in antibacterial activity against *B. thuringiensis* in the hemolymph of *S. litura*. ○ : normal larvae; ● : injected larvae.

Fig. 3. Effect of the dilution of immune hemolymph from *S. litura* on the antibacterial activity against *B. thuringiensis*.

The hemolymph from *S. litura* injected with the bacteria killed 75% of the bacterial cells in 10 min and over 90% in 60 min. In contrast to *S. litura*, the hemolymph from *B. mori* larvae did not kill the bacterial cells. Significant growth of the cells was observed during the incubation with the hemolymph from both normal and injected larvae of *B. mori*.

Some characters of antibacterial activity of *S. litura* hemolymph were determined. Induction kinetics showed that weak activity appeared 1 d after the inoculation and that the maximum level was attained in 2 d later (Fig. 2). As shown in Fig. 3, significant antibacterial activity was detectable even in 16-fold dilution of the immune hemolymph.

DISCUSSION

In this study, the pathogenicity of *B. thuringiensis* for *S. litura* and *B. mori* larvae was compared by injecting vegetative cells of the bacteria into their hemocoel. We found that *S. litura* was 2,500-fold more resistant to the infection than *B. mori*. The injected bacteria grew rapidly in the hemolymph of *B. mori*, while they did not grow or

were eliminated in the hemolymph of *S. litura*. These results indicate that *S. litura* larvae have defense mechanisms against *B. thuringiensis*.

Many investigators have shown humoral defense reactions against bacteria in many insects (BOMAN and HULTMARK, 1987). Thus, we examined antibacterial activity against *B. thuringiensis* in the hemolymph of *S. litura*, and compared it with that of *B. mori*. The hemolymph from normal *S. litura* larvae showed bacteriostatic activity against *B. thuringiensis* vegetative cells, and bactericidal activity was induced after the bacterial inoculation. Both activities were not detected in any hemolymph of *B. mori* larvae. These results suggest that bacteriostatic and inducible bactericidal activities are the factors involved in the resistance of *S. litura* to the infection with the bacteria. Although the chemical nature and the mode of action of the bactericidal factor against *B. thuringiensis* induced in *S. litura* are not yet characterized, the factor will provide a new insight into the interaction between *B. thuringiensis* and the defense mechanisms of insects, because this bacterium is shown to be highly resistant to cecropins and attacins, which are two families of antibacterial proteins that are induced in a variety of lepidopteran insects including *B. mori* (HULTMARK et al., 1982, 1983; MORISHIMA et al., 1990). The bactericidal activity, however, began to appear in *S. litura* 1 d after the inoculation when the bacteria grew to high titers in the hemolymph of *B. mori*. It is probable that early elimination of the bacteria in *S. litura* is due to cellular mechanisms such as phagocytosis (CHEUNG et al., 1978; KURIHARA et al., 1992). Thus, we cannot elucidate the significance of the bactericidal activity in the resistance of *S. litura* to the bacteria. Further studies are therefore needed to clarify this problem.

S. litura larvae were known to have low susceptibility to the δ -endotoxins produced by *B. thuringiensis* var. *kurstaki* HD-1 used in this work (INAGAKI et al., 1992). Therefore, it is interesting that this insect develops resistance to both δ -endotoxins and infection with the bacteria, while *B. mori* is sensitive to both.

The study on the defense mechanisms against the infection with *B. thuringiensis* will contribute to the use of the bacterium as an agent for biological control of insect pests. Research is now in progress on the isolation and characterization of the antibacterial factor in the hemolymph of *S. litura*.

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