Replication of *Bacillus thuringiensis* in larvae of the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae): Growth, sporulation and insecticidal activity of parasporal crystals

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Abstract

Spores and parasporal crystals of a *Bacillus thuringiensis* serovar *aizawai* strain were fed to fifth instar larvae of the Mediterranean flour moth, *Ephestia kuehniella*, under laboratory conditions. Viable numbers of *B. thuringiensis* cells in *E. kuehniella* larvae were estimated by dilution-plating on peptone-polymyxin agar medium, and combined spore and crystal insecticidal activity was bioassayed with larvae of *Bombyx mori*. *B. thuringiensis* rapidly grew and sporulated in the larval cadavers of *E. kuehniella* regardless of inoculation dose. Spores that were produced *in situ* in *E. kuehniella* cadavers persisted for at least 90 d. Insecticidal activity of the homogenates of *E. kuehniella* cadavers fluctuated along with the viable cell numbers and persisted for at least 90 d. To examine horizontal transmission of *B. thuringiensis* in *E. kuehniella* larval populations, *B. thuringiensis*-infected and healthy larvae were released into containers in various proportions, but no reliable transmission was detected under our experimental conditions.

Key words: *Bacillus thuringiensis*, *Ephestia kuehniella*, sporulation, parasporal crystal, horizontal transmission

INTRODUCTION

Bacillus thuringiensis, a facultative bacterial pathogen of insects in Lepidoptera, Diptera, Coleoptera, and other orders (Federici, 1994), has attracted much attention due to its use in pest control programs. Many attempts have been made to understand the mode of action and biochemical aspects of this bacterium. However, the ecology of *B. thuringiensis* has received relatively little attention.

Recent studies on the distribution of *B. thuringiensis* in the natural environment have shown that this bacterium could be isolated from insects (Dulmage, 1970; Burges, 1973; Vankova and Purrini, 1979; Chilcott and Wigley, 1993; Kaelin et al., 1994), sericultural farms (Aizawa et al., 1961; Ohba et al., 1979, 1981), soils (DeLucca et al., 1981; Ohba and Aizawa, 1986a, b; Martin and Travers, 1989; Chilcott and Wigley, 1993), stored products (DeLucca et al., 1982; Donovan et al., 1988;

Karamanlidou et al., 1991; Meadows et al., 1992; Kaelin et al., 1994), and the phylloplane of certain deciduous and coniferous leaves (Smith and Couche, 1991; Ohba, 1996). This suggests that *B. thuringiensis* is an indigenous organism in these environments.

Although simple nutrients serve for the growth and sporulation of *B. thuringiensis* in the laboratory (Lüthy et al., 1982), *B. thuringiensis* in the natural environment lives in biological interactions and physical extremes. Many authors have observed that spores of *B. thuringiensis* in soil failed to germinate and gradually decreased in number (Sekijima et al., 1977; Pruett et al., 1980; West et al., 1985; Petras and Casida, 1985; Akiba, 1986b). The reasons for this seem to involve the activities of soil-inhabiting microorganisms (Akiba et al., 1977; West et al., 1984, 1985; Yara et al., 1997), low nutrient availability (Saleh et al., 1970; West et al., 1985), low moisture content (West et al., 1985; Petras and Casida, 1985) and acidic

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conditions in natural soils (Saleh et al., 1970; Akiba et al., 1979). West et al. (1985) showed that *B. thuringiensis* would need a nutrient-rich environment to reproduce in nature. In view of this, susceptible host cadavers are more plausible candidates than soil to provide such an environment. A few observations on the replication of *B. thuringiensis* in host insects have been reported (Aizawa et al., 1962; Aizawa and Fujiyoshi, 1964; Utsumi, 1965; Prasertphon et al., 1973; Burges and Hurst, 1977; Aly, 1985; Akiba, 1986a), but there is little information about the formation of parasporal crystal toxin in natural settings.

Stored environments are a fruitful source/sink of *B. thuringiensis* (DeLucca et al., 1982; Donovan et al., 1988; Karamanlidou et al., 1991; Meadows et al., 1992; Kaelin et al., 1994), and there is a history of *B. thuringiensis* epizootics in populations of storage moths (Burges and Hurst, 1977; Krieg, 1987). Conversely, *B. thuringiensis* epizootics have rarely been observed in natural populations of insects under field conditions. Thus, without knowledge of ecological interactions between *B. thuringiensis* and its target organisms, it is difficult to conclude that *B. thuringiensis* has an ecological niche as a true insect pathogen in the environment.

This paper presents partial results of epizootiology of *B. thuringiensis* in a Mediterranean flour moth population under simulated laboratory conditions. We investigated growth, sporulation and insecticidal activity of this bacterium in larvae of its susceptible host, *Ephestia kuehniella*. We also conducted research to determine the horizontal transmission ability of *B. thuringiensis* in *E. kuehniella* larval populations.

MATERIALS AND METHODS

Insect. *Ephestia kuehniella* used in this experiment originally were supplied as larvae by the Yokohama Plant Protection Station, Yokohama, Kanagawa, Japan, and were continuously reared in our laboratory. Approximately 200 eggs were put into a plastic container (15 cm in diameter, 9 cm in height) with 100 g wheat bran diet (wheat bran : glycerol : dried yeast = 10 : 2 : 1). For oviposition, newly emerged adults were

transferred into a cylindrical box (10 cm in diameter, 20 cm in height) with a Petri dish at the bottom as an oviposition substrate. Maintenance of the colony and experiments were conducted at 25°C, 68% RH, and a 16 : 8 (L : D)-h photoperiod.

Bacterium. *B. thuringiensis* serovar *aizawai* KH originally was isolated from larval cadavers of the Japanese silk moth, *Antheraea yamamai*, and continuously maintained in our laboratory. A mutant strain resistant to both two antibiotics, streptomycin and rifampicin, was obtained by the method of Yara et al. (1997). This strain was cultured in nutrient broth on a shaker at 30°C for 20 h. The culture was plated on nutrient agar and incubated at 30°C for 2 weeks. Cells on the plates were scratched and suspended into sterile distilled water (SDW), rinsed three times with SDW by centrifugation at $10,000 \times g$ for 20 min, and resuspended in SDW. The suspension was heated at 65°C for 15 min to kill any vegetative cells. The resulting suspension, designated as spore-crystal suspension, was stored at 4°C until subsequent use.

Mortality of *E. kuehniella* larvae. Fifth instar larvae (22-d old) were allowed to feed on droplets of spore-crystal suspension containing 5×10^8 colony forming units (CFU)/ml (high dose) or 5×10^7 CFU/ml (low dose). Forty-eight larvae which had ingested the spore-crystal suspension were individually transferred into a well of a 24-well tissue culture plate containing wheat bran. The larvae were reared at 25°C and a 16 : 8 (L : D)-h photoperiod and observed daily for mortality until pupation.

The number of spores ingested by larvae was determined with the drop plate method (Hoben and Somasogaran, 1982) using peptone-polymyxin agar medium (peptone 1%, NaCl 0.5%, polymyxin B 5 ppm, agar 1.5%, pH 8.5) containing 0.01% streptomycin and 0.005% rifampicin. Immediately after ingestion of droplet, ten larvae were placed individually in 1.5 ml conical microcentrifuge tubes containing 0.2 ml 0.05% Tween 80 solution. Each larva was homogenized with an electric hand homogenizer (Ikeda-Rika Co. Ltd., Tokyo) for 1 min, and then 0.8 ml 0.05% Tween 80 solution was added to each tube. Serial dilutions of the homogenate were made and spread onto peptone-poly-

myxin agar medium to estimate the number of spores in the samples.

Growth and sporulation of *B. thuringiensis* in *E. kuehniella* larvae. Fifth instar larvae (22-d old) were allowed to feed on droplets of spore-crystal suspension containing 5×10^8 colony forming units (CFU)/ml (high dose) or 5×10^7 CFU/ml (low dose). Larvae which had ingested the spore-crystal suspension were individually transferred into a well of a 24-well tissue culture plate containing wheat bran. The larvae were reared at 25°C and a 16 : 8 (L : D)-h photoperiod and observed daily for mortality until pupation. The dead larvae were individually transferred into 1.5 ml conical microcentrifuge tubes and incubated at 25°C and a 16 : 8 (L : D)-h photoperiod. In the high dose-treatment, on 0 and 1 d post ingestion (p.i.), living larvae were used for enumeration of *B. thuringiensis* cells in the larvae; on and after 2 d p.i., larvae that died between days 1 and 2 p.i. were used for this purpose. For the low dose treatment, living larvae were used only at ingestion, and larvae that died between days 2 and 3 p.i. were used for enumeration on and after 3 d p.i.

The number of *B. thuringiensis* cells in the larvae at various times was determined with the drop plate method, as above. When the number of *B. thuringiensis* cells in the larvae was low, the pour and spread method was used with a 9 cm Petri dish. Five larvae were used at each sampling time. In order to estimate the number of spores, sample suspensions were heated at 65°C for 15 min and subjected to the drop plate method or the pour and spread method.

Insecticidal activity of parasporal crystals. Insecticidal activity of parasporal crystals was estimated by a bioassay using a hybrid race of the silkworm, *Bombyx mori* (Shunrei × Shogetsu). *B. mori* larvae were reared on Silkmate (Nihon Nosan-Kogyo Co. Ltd., Yokohama) at 25°C and a 16 : 8 (L : D)-h photoperiod. Groups of 30 newly molted second instar silkworms were transferred into 9-cm Petri dishes, each dish containing 10 artificial diet disks of Silkmate (8 mm in diameter, 1 mm in thickness). Twenty microliters of each concentration of the homogenate of *E. kuehniella* cadavers had been layered on the surface of the artificial diet disks. After 24 h, each larva was transferred

into a 30 ml cup with fresh diet and observed daily for mortality.

Transmission ability of *B. thuringiensis*. Fourth instar larvae (18-d old) were placed in plastic containers (95 mm in diameter, 55 mm in height) with wheat bran mixed with a spore-crystal suspension (1×10^9 CFU/1 g dried wheat bran). This dose was sufficient to cause 100% mortality of the larvae. After 24 h of feeding, each larva was rinsed with SDW. These larvae were designated as primary infected larvae and marked with a white paint marker. Primary infected larvae and healthy 4th instar larvae were released into plastic containers with 20 g wheat bran in various proportions and levels of crowding, as shown in Table 1. These containers were kept in an incubator at 25°C and a 16 : 8 (L : D)-h photoperiod. Two weeks after the start of adult emergence, dead larvae and pupae were recovered from the containers and checked for *B. thuringiensis* infection under phase-contrast microscopy. Infection was determined by the presence of spores and crystals in a smear of the recovered larva or pupa.

Statistical analysis. All data were analyzed by a one-way analysis of variance (ANOVA) and means were compared by Fisher's least significant difference (LSD). The frequency data were subjected to the arcsine square root-transformation before analysis.

RESULTS

Mortality of *E. kuehniella* larvae

By day 1 p.i., larvae exposed to the high dose of *B. thuringiensis* cells began to die, and 91% of the larvae were dead by day 5 p.i. (Fig. 1a). For larvae exposed to the low dose, mortality was observed at day 2 p.i., and 29% of the larvae were dead by day 5 p.i. (Fig. 1b).

Growth and sporulation of *B. thuringiensis* in *E. kuehniella* larvae

B. thuringiensis reproduced in the cadavers of *E. kuehniella* larvae regardless of dose (Fig. 2a, Fig. 3a). In the study of high-dose treatment, the mean density of total *B. thuringiensis* cells decreased approximately 1 log by day 1 p.i. Then cell density of *B. thuringiensis* began to increase rapidly to 3×10^5 CFU per larva by day 4 p.i., where it remained for 90 d. The average

Fig. 1. Cumulative mortality of *Ephestia kuehniella* larvae at various times post ingestion of spore-crystal suspension. Fifth instar larvae were allowed to feed on droplets of spore-crystal suspension containing 5×10^8 colony forming unit (CFU)/ml (a) or 5×10^7 CFU/ml (b).

spore density fluctuated along with total cell density, reaching 1×10^5 CFU per larva and maintaining this level for 90 d (Fig. 2a). When the low dose was applied, the mean density of total cells began to increase at day 3 p.i. to 2×10^5 CFU per larva by day 4 p.i., where it remained for 30 d. The average spore density fluctuated along with total cells density as in the previous case, reaching 1×10^5 CFU per larva and maintaining this level for 30 d (Fig. 3a).

Insecticidal activity

The insecticidal activity in the homogenate of larval cadavers of *E. kuehniella* fluctuated along with viable cell number (Fig. 2b, Fig. 3b). In the high-dose treatment, insecticidal activity began to increase at day 3 p.i., reached the maximum (60% of the test insects died) at day 4 p.i., and maintained this level for 90 d (Fig. 2b). When the low dose was applied, insecticidal activity was detected at day 3 p.i., increased rapidly to the maximum (60% of the test insects died) at day 5 p.i., and maintained this level for 30 d (Fig. 3b).

Fig. 2. Change in the number of total cells (●) and spores (○) of *Bacillus thuringiensis* (a) and increase in its insecticidal activity in *Ephestia kuehniella* larvae (b) at various times post ingestion (p.i.) of spore-crystal suspension. Fifth instar larvae were allowed to feed on droplets of spore-crystal suspension containing 5×10^8 colony forming unit (CFU)/ml. On 0 and 1 d p.i., living larvae were used, and on and after 2 d p.i., larvae that died between days 1 and 2 p.i. were used. The vertical lines at each point indicate the standard error.

Transmission ability of *B. thuringiensis*

The rates of adult emergence of *E. kuehniella* in each experimental treatment ranged from 50% to 78%, and were not significantly different among treatments containing only healthy larvae and those containing both primary infected and healthy larvae ($p > 0.05$), regardless of the initial crowding of larvae at release (Table 1). Although a small fraction of test larvae (0.9%) was infected with *B. thuringiensis* when 16 primary infected larvae and 144 healthy larvae were released into a container, no reliable transmission was observed in the range of crowding used in this experiment (Table 1).

DISCUSSION

One of important features of pathogens is the production of infective propagules in their hosts. Previous studies on the replication of *B.*

Fig. 3. Change in the number of total cells (●) and spores (○) of *Bacillus thuringiensis* (a) and increase in its insecticidal activity in *Ephestia kuehniella* larvae (b) at various times post ingestion (p.i.) of spore-crystal suspension. Fifth instar larvae were allowed to feed on droplets of spore-crystal suspension containing 5×10^7 colony forming unit (CFU)/ml. Living larvae were used immediately p.i., and on and after 3 d p.i., larvae that died between days 2 and 3 p.i. were used. The vertical lines at each point indicate the standard error.

thuringiensis in susceptible hosts have reported only whether susceptible hosts provided *B. thuringiensis* with a suitable environment for growth and/or sporulation (Aizawa et al., 1962; Utsumi, 1965; Prasertphon et al., 1973; Katagiri and Shimazu, 1974; Burges and Hurst, 1977; Aly, 1985; Akiba, 1986a). We showed, for the first time, that *B. thuringiensis* can produce infective propagules in the larval cadavers of one of its hosts, *E. kuehniella*, regardless of the dose applied. We also found that these infective propagules persisted for at least 90 d in the cadavers. In conjunction with long persistence of spores and insecticidal activity of *B. thuringiensis* in stored products (Kinsinger and McGaughey, 1976), *B. thuringiensis* has an advantage as a true insect pathogen in stored environments because long-lived-infective stages or reservoirs are an important aspect of host-pathogen interrelationships (Anderson and May, 1981; Tanada and Fuxa, 1987; Hochberg, 1989).

Previous works showed that there were sev-

eral cases in which *B. thuringiensis* could neither grow nor sporulate in insects (Aizawa et al., 1962; Prasertphon et al., 1973; Akiba, 1986a). Relatively little is known about factors affecting growth and sporulation of *B. thuringiensis* in insects. However, Akiba (1986a) observed that *B. thuringiensis* multiplied and sporulated in the larval cadavers of both the silkworm and the fall webworm in spring when the density of intestinal bacteria was low, whereas its multiplication was suppressed in autumn when the density of intestinal bacteria was high. This suggests that multiplication of *B. thuringiensis* in susceptible host insects is affected by the activities of intestinal bacteria. In addition, we observed that *B. thuringiensis* grew well in *Homona magnanima* (Oriental tea tortrix) larvae which had been reared aseptically, but could not grow in the larvae reared unseptically (Takatsuka and Kunimi, unpublished). This observation supports Akiba's findings. In view of this, the reason for our results of rapid growth and sporulation of *B. thuringiensis* in the cadavers of *E. kuehniella* larvae may be due to low density of intestinal bacteria in the larvae used in our experiment. Generally, insects reared under laboratory conditions are thought to have a simple microflora and low density of intestinal bacteria (Brooks, 1963). Stored environments should resemble laboratory conditions in which the environment is controlled with relatively constant temperature and humidity. Therefore, susceptible insects that inhabit stored environments might generally provide *B. thuringiensis* with environments for its reproduction. It is necessary to investigate replication of *B. thuringiensis* in insects inhabiting stored environments.

Transmission is a key factor that determines whether a disease becomes epizootic (Anderson and May, 1981; Andreadis, 1987). Our results indicate that *B. thuringiensis* has little, if any, transmission ability in our system. This may be partially due to a relatively small yield of *B. thuringiensis* propagules in the *E. kuehniella* larva, because the number of pathogen units produced by an infected larva is one of important parameters that determines the dynamics of a disease (Anderson and May, 1981).

Table 1. The percentage of *Ephestia kuehniella* emerging as adults and mortality of test larvae caused by *Bacillus thuringiensis* in each treatment

Percentage of primary infected larvae	No. of primary infected larvae released	No. of healthy larvae (test larvae) released	Percentage of test insects emerging as adults ^a	Percent mortality of test larvae caused by <i>B. thuringiensis</i> ^a
0	—	36	78.3±8.6 b	0
10	4	36	76.7±11.7 b	0
0	—	72	73.6±5.1 b	0
10	8	72	72.8±5.5 b	0
0	—	108	72.6±3.7 b	0
10	12	108	71.7±2.6 b	0
0	—	144	50.6±3.2 a	0
10	16	144	50.0±2.7 a	0.9±0.6
0	—	32	77.5±9.0 c	0
20	8	32	76.9±7.9 bc	0
0	—	64	71.9±5.0 abc	0
20	16	64	70.6±3.4 ab	0
0	—	96	72.9±2.9 abc	0
20	24	96	72.5±2.4 abc	0
0	—	128	68.0±3.0 a	0
20	32	128	67.5±2.8 a	0
0	—	28	77.5±9.0 b	0
30	12	28	76.9±7.9 ab	0
0	—	56	74.3±3.3 ab	0
30	24	56	74.3±2.1 ab	0
0	—	84	72.8±2.7 ab	0
30	36	84	71.4±2.7 ab	0
0	—	112	70.5±2.6 a	0
30	48	112	70.9±2.2 ab	0
0	—	24	76.4±7.4 b	0
40	16	24	74.3±8.1 b	0
0	—	48	71.7±3.8 a	0
40	32	48	70.8±4.7 a	0
0	—	72	70.6±4.2 a	0
40	48	72	70.8±4.7 a	0
0	—	96	71.7±2.5 a	0
40	64	96	71.9±3.0 a	0

^aMean±SE.Means followed by the same letters within the entire column are not significantly different by Fisher's LSD, $p>0.05$.

Another possible reason could be that propagules of *B. thuringiensis* produced in the larvae were infective but not transmissible under our experimental conditions. *E. kuehniella* larvae killed by *B. thuringiensis* were desiccated and had a hard integument, suggesting that *B. thuringiensis* propagules in the cadavers may not be released into host feeding habitats where transmission could occur. Hochberg (1989) divided a pathogen population into two distinct

subpopulations, transmissible stages which actually infect hosts in a habitat, and protected stages which are non-transmissible but long-lived. He theoretically showed that translocation of protected stages to the host habitat had significant effects on the dynamics of host-pathogen interactions. In our system, some process such as cannibalism in a dense population would be required for transition from protected stages to transmissible stages. It is neces-

sary to discover or induce such a process and to quantify its effect on transmission dynamics. In contrast with our results, Knell et al. (1996) demonstrated that the transmission rate, in the sense of Anderson and May (1981), of *B. thuringiensis* was very high in a *Plodia interpunctella* larval population. The discrepancy between our and their results might be due to the differences in insects and crowding in each experiment. Crowding was much higher in their experiment than in our experiment. Given the host threshold density for pathogen transmission (Anderson and May, 1981), a scenario to explain the discrepancy could be envisaged that the density of larval populations in our experiment was below the transmission threshold. More work, particularly, to determine transmission dynamics over a wide range of host population densities, is required for the explanation of observed sporadic diseases caused by *B. thuringiensis* in stored environments (Burges and Hurst, 1977; Krieg, 1987).

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