APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1995, p. 4343–4347 Vol. 61, No. 12
0099-2240/95/$04.00+0


# Isolation of Multiple Subspecies of *Bacillus thuringiensis* from a Population of the European Sunflower Moth, *Homoeosoma nebulella*

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Received 21 April 1995/Accepted 12 September 1995

**Five subspecies of *Bacillus thuringiensis* were isolated from dead and diseased larvae obtained from a laboratory colony of the European sunflower moth, *Homoeosoma nebulella*. The subspecies isolated were *B. thuringiensis* subspp. *thuringiensis* (H 1a), *kurstaki* (H 3a3b3c), *aizawai* (H 7), *morrisoni* (H 8a8b), and *thompsoni* (H 12). Most isolates produced typical bipyramidal crystals, but the *B. thuringiensis* subsp. *thuringiensis* isolate produced spherical crystals and the *B. thuringiensis* subsp. *thompsoni* isolate produced a pyramidal crystal. Analysis of the parasporal crystals by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the crystals from the *B. thuringiensis* subsp. *kurstaki* and *aizawai* isolates contained a protein of 138 kDa whereas those from *B. thuringiensis* subsp. *morrisoni* contained a protein of 145 kDa. The crystals from *B. thuringiensis* subsp. *thuringiensis* contained proteins of 125, 128, and 138 kDa, whereas those from *B. thuringiensis* subsp. *thompsoni* were the most unusual, containing proteins of 37 and 42 kDa. Bioassays of purified crystals conducted against second-instar larvae of *H. nebulella* showed that the isolates of *B. thuringiensis* subspp. *aizawai*, *kurstaki*, and *thuringiensis* were the most toxic, with 50% lethal concentrations ($LC_{50}$s) of 0.15, 0.17, and 0.26 μg/ml, respectively. The isolates of *B. thuringiensis* subspp. *morrisoni* and *thompsoni* had $LC_{50}$s of 2.62 and 37.5 μg/ml, respectively. These results show that a single insect species can simultaneously host and be affected by a variety of subspecies of *B. thuringiensis* producing different insecticidal proteins.**

The bacterium *Bacillus thuringiensis* is characterized by the production at sporulation of crystalline parasporal inclusions that contain insecticidal proteins. The spectrum of activity of these proteins includes insects in at least three different orders, Lepidoptera, Coleoptera, and Diptera, as well as nematodes (3, 15, 17, 27). Crystal proteins have been classified on the basis of their general insect specificity and amino acid sequences (18). CryI proteins are defined as those toxic to lepidopterous insects, with CryII proteins being toxic to both lepidopterous and dipterous insects. CryIII proteins are active against coleopterans, and CryIV proteins are toxic to dipterans in the suborder Nematocera. A newer type, CryV, is recognized on the basis of dual activity, with these proteins being active against both lepidopterous and coleopterous insects (27).

The life cycle of *B. thuringiensis* is divided into two major phases, vegetative growth and sporulation. During the first stage, growth is exponential when the bacteria are in a nutrient-rich environment. When nutrients become scarce, the bacteria sporulate, forming a spore that can remain viable in the environment for long periods until conditions favorable to vegetative growth return. The diversity of habitats from which subspecies of *B. thuringiensis* strains have been isolated indicates that the ecology of this bacterium is probably very complex. For example, *B. thuringiensis* has been isolated from habitats as different as soil (9, 22, 12), which is thought to be the preferred habitat, and grain mills (11), stored products (19), rearing facilities (13), the phylloplane (26), and diseased insects (15, 28). However, epizootics are very rare in nature (2, 6), adding to the complexity of the ecology of this bacterium.

Recent studies of the molecular biology of *B. thuringiensis* are beginning to provide insights into the possible origins of the diversity of subspecies and insecticidal proteins that characterize this bacterium. Insecticidal crystal protein genes have been shown to be borne on conjugative plasmids and arranged in clusters or in operons flanked by insertion sequences. This particular organization can facilitate recombination and exchange of plasmids, providing potentially important mechanisms for the generation of new specificities (2, 23). Efficient recombination can be achieved when a sufficient cell density is reached, which occurs when spores germinate in a suitable medium made by a dead insect larva (2). Meadow (23) suggested that *B. thuringiensis* would normally not germinate in the soil but would wait until favorable conditions for growth occur. Considering that insecticidal proteins are highly susceptible to UV light and crystals are inactivated within a few days under natural conditions, there is very little chance that a susceptible insect ingests enough active crystals, accompanied by spores, to cause its death. Nevertheless, the insecticidal activity of many proteins produced by *B. thuringiensis* indicates that there is a coevolution of the spores, crystal proteins, and insects.

We report here the isolation of five different subspecies of *B. thuringiensis* producing a diversity of insecticidal proteins from a single population of the European sunflower moth, *Homoeosoma nebulella*. The simultaneous presence of these five subspecies with different characteristics shows that diversity can exist within single insect species in narrow and well-defined habitats.

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TABLE 1. Properties of five different subspecies of *B. thuringiensis* isolated from *H. nebulella*

| Subspecies | Serotype | Strain | Crystal shape | Mass (kDa) of parasporal protein(s) | $LC_{50}$ (μg/ml) |
|---|---|---|---|---|---|
| *thuringiensis* | H 1a | HnD | Spherical | 125, 128, 138 | 0.26 ± 0.12 |
| *kurstaki* | H 3a3b3c | HnF | Bipyramidal | 138 | 0.17 ± 0.05 |
| *aizawai* | H 7 | HnA, HnB | Bipyramidal | 138 | 0.15 ± 0.05 |
| *morrisoni* | H 8a8b | HnE | Bipyramidal | 145 | 2.62 ± 0.13 |
| *thompsoni* | H 12 | HnC | Pyramidal | 37, 42 | 37.5 ± 0.05 |

## MATERIALS AND METHODS

**Rearing of *H. nebulella*.** Larvae of *H. nebulella* were reared in containers (8 cm high and 10 cm in diameter), 15 to 20 larvae per container, and fed a semidefined diet (21) supplemented with sunflower oil (wheat germ, 37.5 g/liter; yeast extract, 37.5 g/liter; maize flour, 142.5 g/liter; ascorbic acid, 12.5 g/liter; sorbic acid, 1.5 g/liter; agar-agar, 16.25 g/liter; sunflower oil, 0.5 ml/liter). After preparation, the containers were held in growth chambers at 25°C with a relative humidity of 65% and a photoperiod of 15 h (3,000 lux). Larvae were provided with corrugated cardboard, in which they pupated after about 16 days of growth on the diet. After pupation, the pupae were collected and placed in adult emergence containers (25 cm high, 20 cm long, and 20 cm wide), which were held in the same growth chambers as the larvae. After mating, eggs were laid on velvet strips and then collected and placed in small plastic containers (5 cm high and 8 cm in diameter) until they hatched.

**Isolation of subspecies and serotypes of *B. thuringiensis* from *H. nebulella*.** Dead larvae collected from different containers were pooled and triturated in sterile distilled water and held at 80°C for 5 min to kill vegetative cells and nonsporeforming bacteria. From these pools of larvae, numerous colonies of *B. thuringiensis* were isolated on HCT medium [tryptone, 5 g/liter; casein hydrolysate, 2 g/liter; $K_2HPO_4$, 12.5 mM; $MgSO_4$, 12.5 mM; $MnSO_4$, 0.05 mM; $ZnSO_4$, 1.2 mM; $Fe_2(SO_4)_3$, 1.2 mM; $H_2SO_4$, 0.5%; $CaCl_2$, 25 mM] complemented with 0.3% glucose. The colonies were divided into six different morphological groups based on colony morphology and crystal shape as observed by light microscopy.

**Serotyping.** Colonies of crystalliferous bacteria were sent to the Laboratory of Entomopathogenic Bacteria, Pasteur Institute, Paris, France, for serotyping with antiflagellar antibodies raised against flagellar (H) antigens as previously described (10).

**Culturing of isolates.** An overnight preculture was grown at 28°C with vigorous shaking (250 rpm) in 2 ml of HCT medium complemented with 0.3% glucose. This culture was then added to 100 ml of the same medium in 500-ml flasks and grown under the same conditions as the preculture until sporulation and lysis were complete. Five flask cultures were prepared for each isolate. The cultures were checked for the presence of parasporal crystals by phase-contrast microscopy.

**Purification of parasporal crystals.** Parasporal crystals and spores were harvested by centrifugation at 4,000 × *g* at 4°C for 15 min. The pellet was resuspended in 250 ml of 1 M NaCl and vigorously shaken until formation of a spore-rich foam, which was then removed with a spatula. The suspension was centrifuged again as above. The pellet was resuspended in 10 ml of sterile distilled water containing 1 mM phenylmethylsulfonyl fluoride and stored on ice prior to sonication at 100 W for 30 s to disrupt aggregates of crystals. This suspension was then layered on top of a discontinous 30 to 70% Renografin gradient. The gradient was centrifuged at 16,000 × *g* for 90 min at 4°C. The band containing purified crystals was collected, diluted fivefold in distilled water, and centrifuged at 20,000 × *g* for 30 min at 4°C. This step was repeated three times. The last pellet was resuspended in 1 ml of sterile distilled water containing 1 mM phenylmethylsulfonyl fluoride. The purity of the sample was checked by light microscopy, and the protein concentration was determined by the bicinchoninic acid method (Pierce MicroBCA protein kit). Light microscopy showed the presence of more than 20 crystals for one spore. Samples were partitioned into aliquots and stored at −20°C.

**Electron microscopy.** Transmission electron microscopy was carried out on purified crystals. The crystals were embedded in 4% agar, fixed in 5% glutaraldehyde in 0.2 M cacodylate buffer (2 h), and then postfixed in 1% $OsO_4$ in the same buffer. Specimens were dehydrated through an ethanol-propylene oxide series and embedded in Epon 812. Ultrathin sections were cut on Ultratom IV ultramicrotome, stained with uranyl acetate and lead citrate, and examined and photographed with a JEOL 200 electron microscope. Scanning electron microscopy was performed on purified crystals placed on coverslips. The crystals were dehydrated, coated with gold, and then examined with a Zeiss DSM 950 scanning electron microscope.

**Crystal protein composition.** The major proteins in the crystals were characterized with respect to size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified crystals were lysed in electrophoresis sample buffer (0.06 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue), incubated for 5 min in boiling water, and separated in 10% polyacrylamide gels at 60 V overnight (20). Gels were stained with 0.1% Coomassie blue R-250 in 7% (vol/vol) acetic acid containing 50% methanol. Molecular weight markers Low and High (Bethesda Research Laboratories) were used as standards.

**Determination of toxicity.** Toxicity was determined through bioassays against second-instar larvae of *H. nebulella*. Serial dilutions of purified parasporal inclusions (six dilutions of 1/10 for each crystal preparation) were evenly applied to the surface of artificial diet identical to that used for the rearing, at a rate of 20 μl/176 $mm^2$. This corresponds to the surface of the compartment containing one larva. Mortality was determined at 7 days posttreatment. Controls were treated in similar manner except that no purified paraporal inclusions were applied to the diet. For each concentration, 25 to 30 larvae were tested per replicate and three replicates were conducted for each isolate. The 50% lethal concentrations ($LC_{50}$s) were determined by probit analysis after correction for control mortality by the method of Abbott (1).

## RESULTS

**Isolation of different serotypes of *B. thuringiensis* from dead larvae of *H. nebulella*.** The subspecies of *B. thuringiensis* reported in this study were isolated after an outbreak of disease in a laboratory colony of *H. nebulella* reared for six to seven generations with no significant mortality. Over the course of a week, 60 to 80% of the population, several hundred larvae, died. In the course of examining several larvae for the cause of death, it was noticed that most contained bacterial spores and crystals. Subsequently, 10 larvae were collected, and from these larvae, numerous colonies of *B. thuringiensis* were isolated on HCT medium. The number of colonies was too large for them to be examined individually, and so they were separated into six different morphological groups based on colony morphology and crystal shape as observed by light microscopy. Then a representative isolate of each group was sent to Institut Pasteur for serotyping. Of these six isolates, two were of *B. thuringiensis* subsp. *aizawai* (H 7) and the remaining four consisted of one isolate each of *B. thuringiensis* subspp. *thuringiensis* (H 1a), *kurstaki* (H 3a3b3c), *morrisoni* (H 8a8b), and *thompsoni* (H 12). For clarity, a strain name was given to each isolate representative of a group. This strain names are presented in Table 1 along with the major properties of the isolates.

**Morphology and ultrastructure of parasporal crystals.** The paraporal crystals produced by the different isolates of *B. thuringiensis* obtained from *H. nebulella* were of three types: bipyramidal, pyramidal, and spherical. Crystals produced by HnA, HnB, HnD, and HnE were bipyramidal (Table 1; Fig. 1). The parasporal inclusion produced by HnE appeared to be slightly different from the others in that it was surrounded by a thick envelope (Fig. 1A). The crystals produced by HnC were triangular in cross section when observed by transmission electron microscopy (Fig. 1B) and pyramidal as observed by scanning electron microscopy (Fig. 1C) as already reported (8). HnD produced parasporal inclusions with no definite shape as seen by transmission electron microscopy (Fig. 1D). However, scanning electron microscopy revealed these inclusions to be essentially spherical (Fig. 1E).

**Parasporal crystal protein composition.** SDS-PAGE analysis of the protein content of the parasporal crystals showed that crystal proteins were distributed in five different classes with

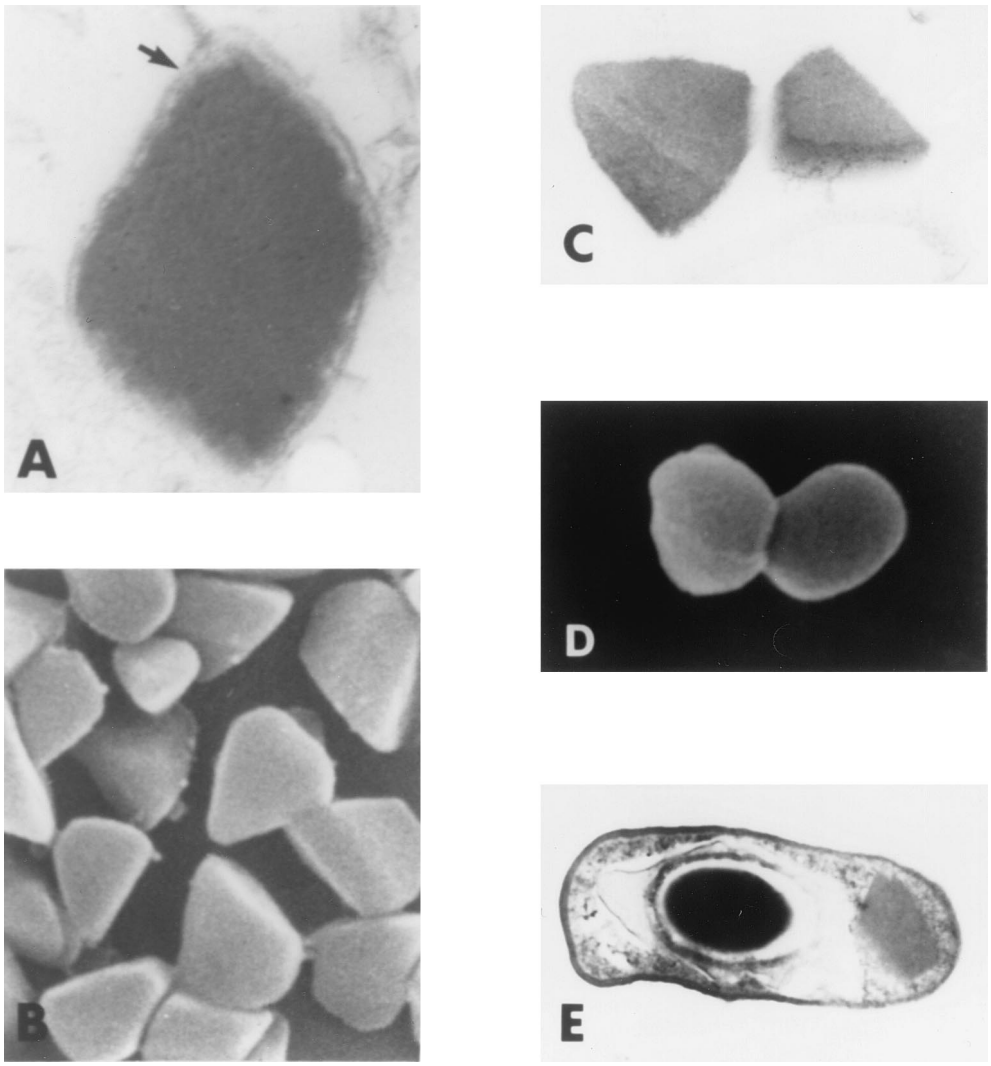


FIG. 1. Ultrastructure and morphology of purified parasporal inclusions. (A) Ultrastructure of the purified parasporal inclusion from HnE observed by transmission electron microscopy. The arrow indicates the thick envelope surrounding the parasporal inclusion. (B) Ultrastructure of the purified parasporal inclusion from HnC observed by transmission electron microscopy. (C) Morphology of the purified parasporal inclusions from HnC observed by scanning electron microscopy. (D) Ultrastructure of the purified parasporal inclusions from HnD observed by scanning electron microscopy. (E) Morphology of the purified parasporal inclusions from HnD observed by transmission electron microscopy.

respect to their size (Table 1; Fig. 2). HnA, HnB, and HnF exhibited a single major protein of 138 kDa (Fig. 2, lanes A, B, and F), a size characteristic of CryI proteins. The crystal from HnC contained two crystal proteins with apparent masses of 37 and 42 kDa (lane C). The crystal from HnD also showed a somewhat atypical profile, with a major protein of 138 kDa and two additional proteins with masses of 125 and 128 kDa (lane D). The crystal produced by HnE migrated at a single position corresponding to a size of 145 kDa (lane E).

**Parasporal crystal toxicity.** The toxicities of purified parasporal crystals from the different isolates of *B. thuringiensis* to *H. nebulella* are presented in Table 1. All the strains produced crystal proteins toxic to *H. nebulella*; however, this toxicity varied greatly depending on the isolate. Second-instar larvae of *H. nebulella* were highly susceptible to crystal proteins produced by HnA and HnB, HnF, and HnD, with $LC_{50}$s of 0.15, 0.17, and 0.26 μg/ml, respectively, at the cutoff point of 7 days. The two isolates of *B. thuringiensis* subsp. *aizawai*, HnA and HnB, were similar in their toxicity to *H. nebulella*. Parasporal crystals from HnE and HnC were less toxic, with $LC_{50}$s of 2.62 and 37.5 μg/ml, respectively.

## DISCUSSION

In the present report, we have demonstrated the isolation of five subspecies of *B. thuringiensis* from a single laboratory colony of the sunflower moth *H. nebulella*. Although we cannot be certain of the source of these isolates, it is probable that they were either contaminants of the insects used to establish the colony, contaminants of the diet, or a combination of these two possibilities. It is well known that spores of *B. thuringiensis* can be isolated from insects and grain dust and can remain viable for long periods in indoor areas such as rearing facilities (7, 11). Since five subspecies were isolated more or less simultaneously, it seems possible that these originated from different sources, although we do not know which sources. While it might be suggested that the commercial formulations served as a source for the isolates, this is very unlikely, since three of the

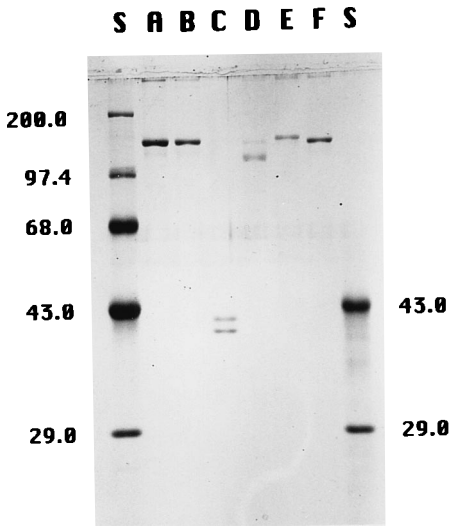


FIG. 2. Protein composition of purified parasporal inclusions determined by SDS-PAGE. Lanes: S, molecular mass standards; A and B, purified parasporal inclusions from HnA and HnB; C, purified parasporal inclusions from HnC; D, purified parasporal inclusions from HnD; E, purified parasporal inclusions from HnE; F, purified parasporal inclusions from HnF.

five subspecies described in this work, *B. thuringiensis* subspp. *morrisoni* (HnE), *thuringiensis* (HnD), and *thompsoni* (HnC), are not used in commercial products. Thus, we suggest that the five subspecies we isolated originated from either the insects, grain dust, or both and built up gradually in our colony and rearing facility over time, the high density of the larval population favoring their development to a point at which they caused a noticeable outbreak.

In our study, we did not determine how or even if the spores remained in association with larvae. If this happens, they may remain on the cuticle or be passed through the gut, eventually being transmitted to the adults and eggs. It can be envisioned that spores from several subspecies could accumulate in a colony through such mechanisms, killing only a few insects now and then. However, the death of a larva caused by one subspecies would provide a source of nutrients for all spores, and thus all subspecies that occupied this ecological niche would benefit. A similar feature was recently reported for the navel orangeworm, *Amyelois transitella*, in which *B. thuringiensis* subsp. *aizawai* was shown to multiply at a high rate, totally invading the dead insect body (14). These observations demonstrate that a dead insect represents a suitable growth medium for *B. thuringiensis* and strongly suggest that the role of the inclusion body is to kill the insect, providing *B. thuringiensis* with an excellent medium for growth (14).

Aside from these observations, our results could have practical applications. For example, the isolates of *B. thuringiensis* subspp. *aizawai* (HnA and HnB), *kurstaki* (HnF), and *thuringiensis* (HnD) were toxic to *H. nebulella* and thus represent potential microbial control agents for use against this insect. The tests conducted under laboratory conditions with purified crystals may not be representative of the actual toxicity of a strain under natural conditions when spores are present. Further bioassays with spore-crystal mixtures should therefore be conducted both under laboratory and field conditions in comparison with the activity of commercialized strains such as HD-1 to determine the exact potential of the strains described in this report to control *H. nebulella*. Although *H. nebulella* cannot yet be considered a major pest in France, it causes significant losses to sunflowers in Eastern Europe and Asia. Additionally, the closely related species *H. electella*, the American sunflower moth, is a major pest of commercial sunflowers in North America, and products based on *B. thuringiensis* are already used for its control (4). The increase in acreage devoted to sunflowers in France may result in a greater threat from *H. nebulella*, making the availability of several active *B. thuringiensis* strains an important issue.

The other *B. thuringiensis* strains isolated from *H. nebulella*, although less toxic to this insect, might represent a source of new types of crystal protein genes. HnC apparently produces insecticidal crystal proteins that are similar to those described by Calabrese et al. (8) and Brown and Whiteley (5). In addition, all strains correspond to the same serotype, *B. thuringiensis* subsp. *thompsoni*. Although the protein sizes were the same and a partial sequencing of the corresponding genes yielded a nucleotide sequence similar to those published by Brown and Whiteley (data not shown), the crystal was pyramidal as described in the original report by Calabrese et al. (8) whereas the crystal shape reported by Brown and Whiteley (5) was cuboidal. The reason for this difference is unknown, but these proteins nevertheless represent a new family of endotoxins. As such, it would be interesting to further investigate their characteristics and their distribution among subspecies of *B. thuringiensis.*

HnE is also of interest, because of the 145-kDa protein produced by this isolate. This protein is larger than most other CryI proteins but similar in size to the largest protein that occurs in the PG-14 mosquitocidal isolate of this subspecies (24, 25). PG-14 is known mostly for its mosquitocidal activity, which is due to a complex of CytA and CryIV proteins very similar to that of *B. thuringiensis* subsp. *israelensis* (16, 17). However, in addition to the CryIV and CytA mosquitocidal proteins, the 145-kDa protein produced by PG-14 is toxic to lepidopterous insects (24). Unlike the mosquitocidal protein genes, which are located on the same plasmid, the 145-kDa protein gene is borne by a different plasmid (24). The occurrence of this atypical CryI protein in two different isolates of the same serotype indicates that the proteins may be similar or the same and could represent a Cry protein with novel lepidopteran specificity or toxicity.

## ACKNOWLEDGMENTS

This work was supported by grants (ATP 21/90 and ATP 31/90) from CIRAD.

We are very grateful to Lerry Lacey and especially to Brian A. Federici for critical review of the manuscript. We are indebted to Yves Pivot for his help in rearing *H. nebulella* and conducting bioassays and to P. Louisor (CIRAD-CA) for her help in isolating the strains from *H. nebulella*. We thank M. Dollet (CIRAD-LPRC) for giving us access to the transmission electron microscope.

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