

# Proteolysis, Histopathological Effects, and Immunohistopathological Localization of $\delta$ -Endotoxins of *Bacillus thuringiensis* subsp. *kurstaki* in the Midgut of Lepidopteran Olive Tree Pathogenic Insect *Prays oleae*

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## Abstract

Considering the fact that *Prays oleae* is one of the most pathogenic insects to the olive tree in the Mediterranean basin, particularly in Tunisia, the mode of action of Cry insecticidal toxins of *Bacillus thuringiensis* *kurstaki* in *Prays oleae* midgut was investigated. The proteolysis of *Bacillus thuringiensis*  $\delta$ -endotoxins in the midgut was a key step in determining their potency against *Prays oleae*. The latter's proteases activated the  $\delta$ -endotoxins early, yielding stable toxins. The in vitro and in vivo binding of these toxins to *Prays oleae* larvae midgut was studied immunohistochemically, evidencing a midgut columnar cell vacuolization, microvilli damage, and then a pass of epithelium cell content into the larvae midgut. Moreover, *Bacillus thuringiensis* toxins were shown to bind to the apical microvilli of the midgut epithelial cells. The in vitro study of the interaction of *Prays oleae* midgut proteins with biotinylated *Bacillus thuringiensis* toxins allowed the prediction of four suitable receptor proteins in *Prays oleae*.

**Index Entries:** *Prays oleae*; *Bacillus thuringiensis*; proteolysis; histopathological effect; binding; toxin localization.

## 1. Introduction

The olive moth, *Prays oleae*, is one of the most important insect pests to olives in the Mediterranean basin and from Mexico to southern America (1). This insect reduces the overall yields (10%) and quality of the fruit/oil. It could be controlled efficiently only by chemical pesticides, causing toxicity to consumers and other beneficial insects and increasing the costs of production. Thus, there was increasing demand for alternative methods of controlling this insect. Microbial bioinsecticides were shown to be an efficient tool for the control of plant pests. The Gram-positive, spore-forming bacterium *Bacillus thuringiensis* produces parasporal crystals composed of insecticidal crystal proteins (ICPs), named  $\delta$ -endotox-

ins. The *B. thuringiensis*  $\delta$ -endotoxins are the most valuable bioinsecticides used currently in commercial agriculture, forest management, and mosquito control (2). They exhibit a high specificity of insecticidal toxicity toward lepidopteran, coleopteran, and dipteran insect species (3). Among these  $\delta$ -endotoxins, cryA 1A are the commonly used ICPs because of their high activity and specificity to lepidopteran pests such as *P. oleae* and *Ephestia kuehniella* (4–6). During the insecticidal process of *B. thuringiensis*  $\delta$ -endotoxins, activation of protoxins by proteolytic cleavage and binding of the active toxin to specific insect receptors and its insertion into membrane play a key role in both toxicity and specificity (reviewed in ref. 2). So far, multiple toxin binding proteins have been reported for lepidoptera (6,7).

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Although the control of *P. oleae* by *B. thuringiensis* bioinsecticides was attractive, little is known about the mode of action of the correspondent Cry toxins in this insect gut or the selection of adequate bioinsecticides for efficient use and field application. In this study, we investigate the role of *B. thuringiensis* endotoxins proteolysis in activation, stability, and potency of these toxins toward *P. oleae* compared to other insects. Description of the interaction and organization of the epithelial cells in the larval midgut and the histopathological effects of exposure to Cry toxins would be a useful tool in this potency based on the *in vitro* and *in vivo* binding of these *B. thuringiensis* toxins to *P. oleae* larvae midgut. This report would contribute to the investigation of the mode of action of *B. thuringiensis* toxins in *P. oleae*.

## 2. Materials and Methods

### 2.1. Strain and Growth Conditions

*B. thuringiensis* subsp. *kurstaki* (serotype H3a, 3b, 3c) strain BNS3 was isolated from Tunisian soil and well characterized in our laboratory (8,9). This strain contains the genes *cryIAa*, *cryIAC*, *cry2Aa* and *cryIIa* encoding  $\delta$ -endotoxins. This strain is highly toxic to lepidopteran insect larvae, including *P. oleae* and *E. kuehniella* (4,10). T3 medium (11) was used for growth, sporulation and production of crystals into shaker flasks incubated in a rotary shaker set at 200 rpm during 64 h corresponding to the end of sporulation.

### 2.2. Solubilization, Purification, and Labeling of $\delta$ -Endotoxins

The purification and solubilization of parasporal insecticidal crystals were carried out as reported by Lee et al. (12) with some modifications. After centrifugation of the spore crystal mixture, the pellet was washed three times with washing solution (1 M NaCl, 0.01% Tween 20) and six times with cold water (13). The pellet was then suspended in 50 mM Na<sub>2</sub>CO<sub>3</sub> (pH 9.6) and incubated during two hours at room temperature with constant agitation. Solubilized  $\delta$ -endotoxins, termed protoxins, were recovered in the supernatant after centrifugation for 10 min at 12,000g and their

concentration was determined using Bio-Rad protein assay. For binding assays,  $\delta$ -endotoxins obtained by solubilization of parasporal crystals, were activated by proteolysis and purified as described by Luo et al. (14) with some modifications. Activation was performed with 10  $\mu$ g/mL bovine pancreas trypsin (Amersham Pharmacia Biotech, France) at 37°C for 2 h. Activated toxins were loaded onto Mono Q XK 16/20 column (Amersham Pharmacia Biotech, France) and the fraction containing purified toxins was dialyzed against 0.1 M phosphate buffer (pH 6.8) and then loaded onto gel filtration column BioSilSec 250 (BIO-RAD, France) for further purification. Purified toxins were biotinylated using ECL<sup>TM</sup> protein biotinylation module according to manufacturer's protocol (Amersham Pharmacia Biotech, France).

### 2.3. Gut Extracts Preparation

Third instar larvae of *P. oleae* and *E. kuehniella*, kindly provided by the Institute of Olive Tree of Sfax (Tunisia), were chilled on ice during 30 min. The guts of *E. kuehniella* were excised and collected in MET buffer (Mannitol, 300 mM; EDTA, 5 mM; Tris, 20 mM; pH 7.2) whereas whole larvae of *P. oleae* (7–8 mm) were collected in the same buffer. They were then disrupted into a blender and centrifuged for 10 min at 13,000g. The supernatants were recovered and the protein contents were determined as described above.

### 2.4. Proteolysis Assay and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Solubilized protoxins were mixed with soluble proteins in the larvae extracts at a ratio of 50:1 (v/v) in a final volume of 50  $\mu$ L. The mixtures were incubated at room temperature with constant agitation for different incubation periods. Samples were separated by sodium dodecyl sulfate 10 % polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie blue dye.

### 2.5. Bioassays

Bioassays were carried out using third instar larvae of *P. oleae* under starvation for 20 h. Fifty microliters of *B. thuringiensis* crystal-spore sus-

pension containing 4 mg  $\delta$ -endotoxins were poured on the surface of one olive leaf. The latter were left in Petri dishes for 4 h then 10 larvae of *P. oleae* were introduced and incubated at room temperature in order to expose larvae to the diet containing the toxin. 10 mosquito larvae were fed with untreated olive leaves used as negative control in which immunohistochemical detection of BNS3 toxins is not expected to be positive.

## 2.6. Preparation and Sectioning of Insect Tissues

After exposure to the toxins of BNS3, *P. oleae* larvae were placed in 10% formol. Larvae were dehydrated in increasing ethanol concentrations, rinsed in 100% toluene, and embedded in paraffin wax. Five-micrometer sections were obtained and placed in carriers loaded with a mix of 1.5% egg albumin and 3% glycerol in distilled water. For histopathological localization of the effects of BNS3 toxins, the 5- $\mu$ m sections already de-paraffinated in 100% toluene were stained with hematoxylin-eosin (HE) as reported by Ruiz et al. (15).

## 2.7. Immunohistochemical Localization of the BNS3 Toxins

Detection of BNS3 toxins was performed through modification of the technique developed by Ruiz et al. (15). The sections were de-paraffinated in 100% toluene and hydrated in decreasing concentrations of ethanol, washed in distilled water and stored in Tris-buffered saline TBS (66 mM NaCl, 1.6 mM KCl, 25 mM Tris base, pH 7.4). Antigen unmasking was carried out with 1 mg/mL trypsin in 0.2 N HCl for 5 min at room temperature. Trypsin was inactivated by adding 0.5 mM phenylmethylsulfonyl fluoride (PMSF) with incubation for 5 min and then the endogenous peroxidase activity was blocked by incubating the tissue section with successively 0.5%  $\text{H}_2\text{O}_2$  for 30 min and 6%  $\text{H}_2\text{O}_2$  for 15 min in methanol, at room temperature. The tissue samples were blocked with 3% bovine serum albumin (BSA) in TBS for 1 h at room temperature, rinsed with TBS, and incubated for 1 h at 37°C in humid chamber with 1:500 of the polyclonal antibody raised in rabbit against the toxins of BNS3 (16) diluted in TBS

with 1% BSA. The slides were rinsed three times with TBS, incubated for 40 min at 37°C in humid chamber with 1:200 of peroxidase-conjugated goat immunoglobulin (Ig)G (H+L) to rabbit IgG (Bio-Rad, France) and washed as above with TBS. Color development was achieved by incubation for 10 min in a freshly prepared substrate solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; DAKO, France) and 0.01% hydrogen peroxide in 10 mM Tris-HCl buffer, pH 7.6. Labeled slides were then rinsed with distilled water, dried and examined by light microscopy.

## 2.8. SDS-PAGE Ligand Blotting

*P. oleae* gut proteins prepared in MET buffer containing 0.1 mM PMSF were separated by electrophoresis in 7.5% polyacrylamide gel and blotted onto a nitrocellulose membrane by electrotransfer (Bio-Rad, France). Proteins were visualized by Ponceau S staining. The membranes were blocked with 5% milk for 1 h then reacted with biotinylated toxins (approx 1.5 nM) for 2 h at room temperature. After three 5-min washing cycles, membranes were incubated with streptavidin-peroxidase conjugate (1:1500 dilutions) supplied in ECL protein biotinylation module for 1 h, followed by washing as described above. Binding was visualized using luminol according to manufacturer's protocol (ECL; Amersham Pharmacia Biotech, France).

## 3. Results

### 3.1. Role of *P. oleae* Midgut Juice in BNS3 Protoxins Activation and Inactivation

Considering the fact that the midgut juice of the susceptible larvae includes all the necessary components for the *in vitro* activation of Cry protoxins (17), the analysis of the BNS3 Cry protoxins digestion by midgut juice of *P. oleae* at different incubation periods was realized. As shown in Fig. 1, after 15 min of incubation, an expected band of 62 kDa corresponding to the activated toxin was already evidenced. By increasing incubation time, this band became the major one and beyond 1 h, it remained stable for 13 h of incubation. The midgut juice of *E. kuehniella*, used

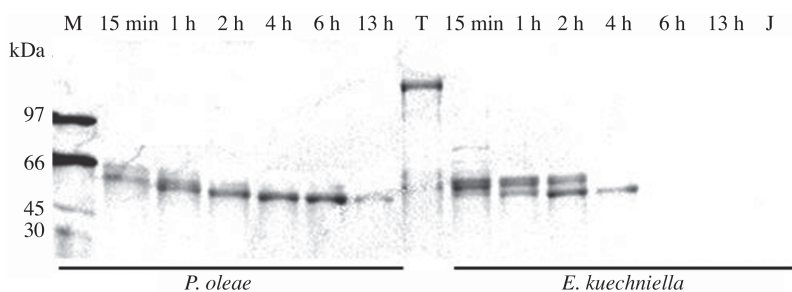


Fig. 1. Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis analysis of BNS3 protoxins digestion with *Prays oleae* and *Ephestia kuehniella* midgut juice proteases after different incubation periods. M, molecular mass markers (Pharmacia Biotech, France); T, BNS3 protoxin (130 kDa); J, larvae midgut juice incubated alone.

as control, needed longer incubation period for the complete activation of BNS3 protoxins and the activated toxins disappeared almost completely after 6 h incubation. Such results should give evidence on the best time efficiency of BNS3 toxins on *P. oleae* compared to *E. kuehniella*.

### 3.2. Histopathological Effects of *B. thuringiensis* Toxins in *P. oleae* Larvae

Figure 2A shows that the midgut wall of *P. oleae* is composed of an acellular peritrophic membrane that delimits the midgut lumen in ectoperitrophic and endoperitrophic space. It also consists of a pseudostratified columnar epithelial tissue with a brush border, a basement membrane, and muscular layers composed of packets of circular and longitudinal muscles (Fig. 2B). Mostly histopathological changes induced by BNS3 toxins included vacuolization of the cytoplasm, hypertrophy of the epithelial cells and their nucleus, brush border membrane impairment, vesicle formation in the apical region of cells toward the midgut lumen, and disintegration of the cells (Fig. 2C). The cells were disrupted at the apical region with lysis and leakage of cytoplasm material in the lumen (Fig. 2D).

### 3.3. Immunohistochemical Localization of *B. thuringiensis* Toxins

Binding of the BNS3 toxins to *P. oleae* larvae tissues was observed by a brownish staining at two locations, depending on exposure time of the larvae to the toxin (Fig. 3C,D). First, it was clear at the apical microvilli or through the epithelial

cells and then in the basal membrane. BNS3 toxins were also detected in the lumen and close to the peritrophic membrane just before binding to the midgut (Fig. 3B). Tissues not exposed to BNS3 toxins did not show the brownish coloration (Fig. 3A).

### 3.4. Functional Study of Activated *B. thuringiensis* Toxins on Larvae Extract of *P. oleae*

Protein blots of larvae extract of *P. oleae* were first stained with Ponceau S and then probed to biotinylated toxins of BNS3. As shown in Fig. 4, lane 3, proteins of 250 kDa and 75–70 kDa exhibited strong binding, whereas those of 105 kDa and 50 kDa bind moderately. When compared to the protein staining (lane 2), no proteins with MW higher than 75 kDa were detected. Based on these results, four proteins were presumed to be toxin receptors in *P. oleae* larvae.

## 4. Discussion

Elucidation of the toxicity events of *B. thuringiensis* toxins to the olive tree pathogen insect *P. oleae* was considered a key step in development of its bioinsecticides formulation. Indeed, in previous studies performed in our laboratory, *P. oleae* was shown as the most sensitive to *B. thuringiensis* BNS3  $\delta$ -endotoxins compared to other lepidopteran insects such as *E. kuehniella* (data not shown). Such higher susceptibility of *P. oleae* was hypothesized to be in part due to rapid activation of the BNS3 protoxins in *P. oleae* midgut larvae. In fact, previous reports suggested that  $\delta$ -endotoxin proteolysis is a major factor of toxic-

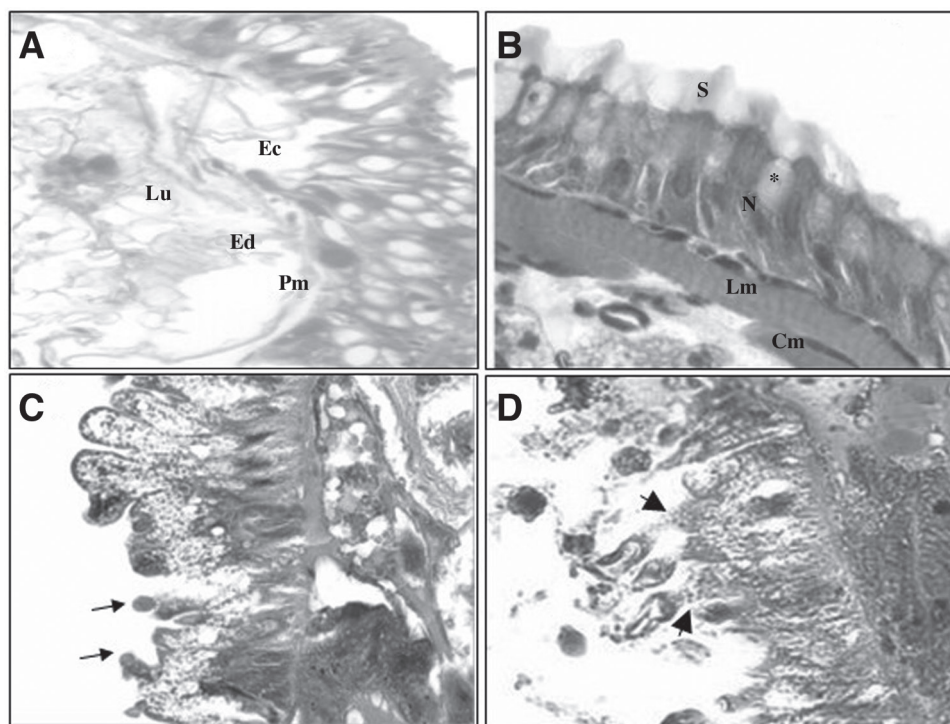


Fig. 2. General aspects of the midgut of *Prays oleae* (A,B) and histopathological effects of BNS3 toxins on it (C,D). C: Vesicle formation in the apical region of cells. In D, arrows indicate lysis of columnar cells. Lu, lumen; Pm, peritrophic membrane; Ec, ectoperitrophic spaces; and Ed, endoperitrophic spaces; S, striated border; \*, goblet-shaped cavity; N, nucleus; Cm, circular muscles; Lm, longitudinal muscles. B and C, Magnification  $\times 100$ ; A and D, Magnification  $\times 40$ .

ity (17). This evidence of the early activation of the *B. thuringiensis* strain BNS3 Cry protoxins by proteolysis in *P. oleae* midgut starting from 15 min is given. Moreover, higher recalcitrance of activated toxins to further proteolysis in *P. oleae* midgut is added as a major factor in *P. oleae* sensitivity. Intramolecular processing of Cry protoxins was previously described, and results reported by Miranda et al. (18) supported the idea that proteolytic processing of Cry toxins is not only involved in their activation, but also could be implicated in insect resistance. It is therefore clear that prediction of toxicity of *B. thuringiensis* toxins to different insects could be achieved by assessment of the efficiency—based on rapidity and stability—of their toxin activation in the midgut of the different insects (17).

On the other hand, the level of morphological lesions caused by these toxins on the intestinal

epithelium could explain the susceptibility of *P. oleae* to *B. thuringiensis* toxins. Different histological alterations on midgut epithelium of *P. oleae* were observed. Presence of secretory vesicles and cytoplasmic extrusions, which are normally seen in the cell apex of midgut larvae, were previously described in *Anticarsia gemmatalis* (19), *Hyalophora cecropia* (20), *E. kuehniella* (21), and *Apis mellifera* (22). These authors suggested that the cytoplasmic loss phenomenon is probably correlated with cell degeneration resulting from epithelial renewal, whereas others (23,24) considered that these features are related to high secretory enzymatic activity. However, in the case of *P. oleae*, this phenomenon was especially observed after exposure of the larvae to the BNS3 toxins, confirming the involvement of these toxins in cell degeneration and alteration of permeability of the membranes, which conducts to larvae death. The

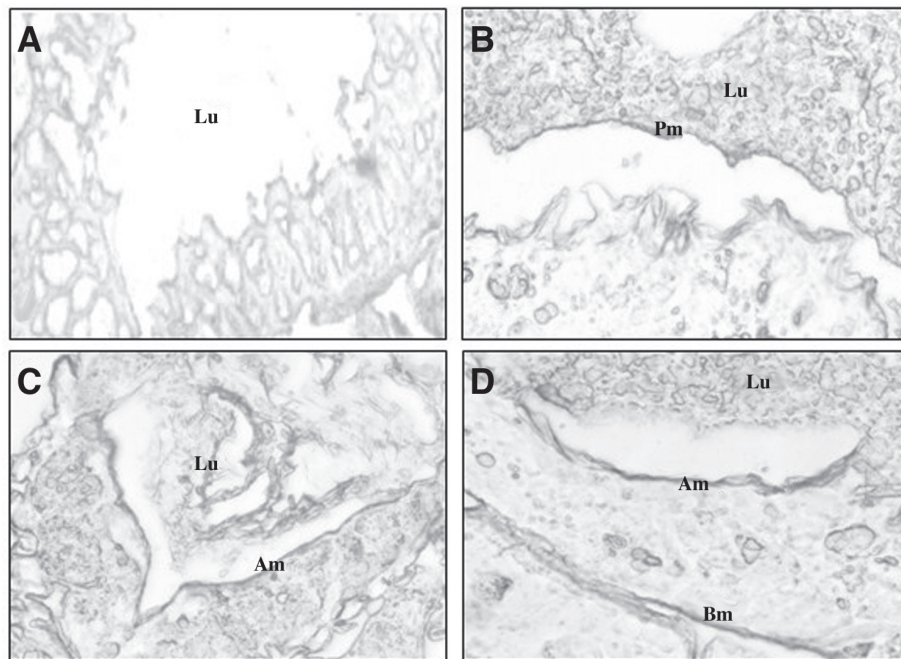


Fig. 3. Immunohistochemical localization of BNS3 toxins in the midgut of *Prays oleae*. (A) Section through the midgut epithelium of control larvae not exposed to BNS3 toxins. (B) Toxin localized in the lumen and close to peritrophic membrane. Lu, lumen; Pm, peritrophic membrane. (C) Midgut with clear signal on the apical microvilli and through epithelial cells. (D) Localization of the toxin on both apical (Am) and basal (Bm) membranes of the epithelial cells. Magnification  $\times 100$ .

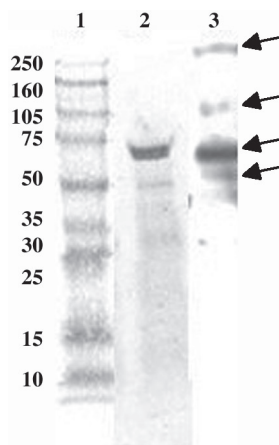


Fig. 4. Functional study of activated BNS3 toxins on larvae extract of *Prays oleae*. Lane 1, mass molecular weights (Paharmacia Biotech, France). Lane 2, Red ponceau stained blot of larvae extract after protein transfer. Lane 3, BNS3 toxins binding to larvae extract of *P. oleae*. Arrows show the different bands corresponding to the presumed receptors of BNS3 toxins in *P. oleae*.

immunohistochemical study performed in order to investigate the *in situ* binding of activated BNS3 toxins to epithelium cells gave evidence of binding on the lumen, through the peritrophic membrane, at the apical microvilli, and in the basal membrane. Such results are similar to those reported to *Aedes aegypti* and *Culex quinquefasciatus* larvae (15), and suggest that the midgut is the first site of the action of BNS3 toxins in *P. oleae*. The immunolocalization of the toxin in the basal membrane could be due to its leakage from the midgut lumen to the basal area after disruption of the midgut tissue. The authors (15) showed that in addition to the midgut, toxin binding was also observed in the posterior midgut and in the malpighian tubules. Further investigations of the binding of BNS3 toxins through the same sections in *P. oleae* would be performed, because the report suggested that different larvae showed the same sites of binding, which indicated

a possible relationship with the mode of action of the toxin.

In vitro binding study allowed the identification of several proteins with different molecular sizes that bind to BNS3 toxins. These presumed receptors seem to have different affinities to these toxins, because a strong signal was observed in the case of 250-kDa and 105-kDa proteins. It was previously suggested by Segura (25) that Cry1 toxins could have more than one receptor in the midgut apical microvilli of *Ae. aegypti* and *Cx. quinquefasciatus*, and so *P. oleae* might also share more than one receptor to the BNS3 toxins.

Finally, this study showed that toxin activation as well as interaction with brush border membranes of the midgut affected both potency and specificity of *B. thuringiensis* BNS3 toxins against *P. oleae*. Further morphophysiological studies of the cell types found in this insect would be very useful in increasing the effectiveness of techniques used in its control. On the other hand, advanced investigation of the interaction between *B. thuringiensis* toxin and the receptors of these larvae could contribute to the design of more toxic biological control agents.

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### References

1. Arambourg, Y. (1986) *Traité d'entomologie oléicole*. Conseil oléicole international, Madrid.
2. Schnepf, E., Crickmore, N., Van Rie, J., et al. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**, 775–806.
3. Höfte, H. and Whiteley, H. R. (1989) Insecticidal crystal protein of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**, 242–255.
4. Tounsi, S., Dammak, M., Rebai, A., and Jaoua, S. (2005) Response of larval *Ephestia kuehniella* (Lepidoptera/Pylalidae) to individual *Bacillus thuringiensis kurstaki* toxins and toxin mixtures. *Biol. Control* **35**, 27–31.
5. De Maagd, R. A., Bravo, A., and Crickmore, N. (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world? *Trends Genet.* **17**, 193–199.
6. Knight, P. J. K., Crickmore, N., and Ellar, D. J. (1994) The receptor for *Bacillus thuringiensis* Cry 1A(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Mol. Microbiol.* **11**, 429–436.
7. Gill, S. S., Cowles, E. A., and Francis, V. (1995) Identification, isolation and cloning of a *Bacillus thuringiensis* Cry 1Ac toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem.* **270**, 27,277–27,282.
8. Jaoua, S., Zouari, N., Tounsi, S., and Ellouz, R. (1996) Study of  $\delta$ -endotoxins produced by three recently isolated strains of *Bacillus thuringiensis*. *FEMS Microbiol. Lett.* **145**, 349–354.
9. Tounsi, S., J'Mal, A., Zouari, N., and Jaoua, S. (1999) Cloning and nucleotide sequence of a novel cry 1Aa-type gene from *Bacillus thuringiensis* subsp. *kurstaki*. *Biotechnol. Lett.* **21**, 771–775.
10. Tounsi, S., Dammak, M., Zouari, N., Rebai, A., and Jaoua, S. (2006) Evidence of the effect of  $\delta$ -endotoxin ration in *Bacillus thuringiensis* crystals on the toxicity against *Ephestia kuehniella*. *Biol. Control* **37**, 243–246.
11. Travers, R. S., Martin, P. A. W., and Reichelderfer, C. F. (1987) Selective process for efficient isolation of soil *Bacillus* species. *App. Environ. Microbiol.* **53**, 1263–1266.
12. Lee, I. H., Je, Y. H., Chang, J. H., et al. (2001) Isolation and characterization of a *Bacillus thuringiensis* ssp. *kurstaki* strain toxic to *Spodoptera exigua* and *Culex pipiens*. *Current Microbiol.* **43**, 284–287.
13. Ibarra, J. E. and Federici, B. A. (1986) Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* **165**, 527–533.
14. Luo, K., Banks, D., and Adang, M. J. (1999) Toxicity, binding, and permeability analyses of four *Bacillus thuringiensis* Cry 1  $\delta$ -endotoxins using brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. *Appl. Environ. Microbiol.* **65**, 457–464.
15. Ruiz, L. M., Segura, C., Trujillo, J., and Orduz, S. (2004) In vivo binding of the Cry11Bb toxin of *Bacillus thuringiensis* subsp. medellin to the midgut of mosquito larvae (Diptera: Culicidae). *Mem. Inst. Oswaldo Cruz.* **99**, 73–79.
16. Zouari, N. and Jaoua, S. (1997) Purification and immunological characterization of particular delta-endotoxins from strains of *Bacillus thuringiensis*. *Biotechnol. Lett.* **19**, 825–829.
17. Lightwood, D. J., Ellar, D. J., and Jarrett, P. (2000) Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac delta-endotoxin. *Appl. Environ. Microbiol.* **66**, 5174–5181.

18. Miranda R., Fernando Z. Z., and Bravo A. (2001) Processing of Cry1Ab d-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *spodoptera frugiperda* midgut proteases: role in protoxin activation. *Insect Biochem. Mol. Biol.* **31**, 115–1163.
19. Levy, S. M., Falleiros, A. M. F., Moscardi, F., Gregorio, E. A., and Toledo, L. A. (2004) Morphological study of the hindgut in larvae of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae). *Neotropical Entomol.* **33**, 427–431.
20. Anderson, E. and Harvey, W. R. (1966) Active transport by the *Cecropia* midgut: II. Fine structure of the midgut epithelium. *J. Cell. Biol.* **31**, 107–134.
21. Smith, D. S., Compher, K., Janners, M., Lipton, C., and Wittle, L. W. (1969) Cellular organization and ferritin uptake in the mid-gut epithelium of a moth *Ephestia kuehniella*. *J. Morphol.* **127**, 41–72.
22. Jimenez, D. R. and Gilliam, M. (1990) Ultrastructure of the ventriculus of the honey bee, *Apis mellifera* (L.): cytochemical localization of acid phosphatase, alkaline phosphatase, and non-specific esterase. *Cell Tissue res.* **261**, 431–443.
23. Terra, W. R. and Ferreira, C. (1994) Insect digestive enzymes: properties, compartmentalization and function. *Comp. Biochem. Physiol.* **109B**, 1–62.
24. Cristofolletti, P. T., Ribero, A. F. and Terra, W. R. (2001) Apocrine secretion of amylase and exocytosis of trypsin along the midgut of *Tenebrio molitor* larvae. *J. Insect Physiol.* **47**, 143–155.
25. Segura, C. (2001) Study on the mode of action of *Bacillus thuringiensis subsp. medellin* toxins. PhD Thesis, University of Antioquia, Medellin Colombia.