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(RESEARCH ARTICLE)

Promising active bioinsecticides produced by *Bacillus thuringiensis* strain BLB427

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Abstract

Bacillus thuringiensis is gaining worldwide importance in the control of pests in agriculture and public health. Characterization of new isolated strains with original, higher and broader spectrum of activity is an ever developing field. Parasporal inclusions from BLB427, a new *B. thuringiensis* strain isolated from Tunisian soil, contained 2 major Cry proteins having molecular weights of about 130-135 kDa and 65-70 kDa corresponding to *cry1*-type and *cry2*-type genes, respectively. These toxins exhibited high toxicities to *Ephestia kuehniella* and *Spodoptera littoralis* larvae, with LC_{50} values of about 81.91 µg/cm² (+/- 15.30) and 79.70 µg/cm² (+/- 36.73), respectively. BLB427 supernatant showed a promising activity against Lepidopteran pests due to the presence of *vip3*-type gene in this strain. The high toxicity of BLB427 supernatant compared to that of BUPM95, used as control, was due in part to the high copy number of *vip3* gene of BLB427 compared to that of *vip3Aa16* of BUPM95, as demonstrated by Real-time PCR. This gene was cloned, sequenced and the comparison with the most known *vip3* genes in databases, demonstrated that *vip3*(427) gene and the corresponding protein showed differences that can influence the efficiency of the Vip3(427) toxin against Lepidoptera. After overexpression of Vip3(427) protein in *Esherichia coli* and its purification using His-Trap column, the toxin showed a promising toxicity against the lepidopteran pest *S. littoralis* with extremely damages in the larvae midgut traduced by the vacuolization of the apical cells, the damage of microvilli and the disruption of epithelial cells. The results described in the present study proved that *B. thuringiensis* BLB427 strain could be of a great interest for lepidopteran biocontrol by using its δ-endotoxins and/or Vip3 toxins in bioinsecticides formulations.

Keywords: *Bacillus thuringiensis*; biocontrol; Delta-endotoxins; Vip3; *Ephestia kuehniella*; *Spodoptera littoralis*.

1. Introduction

Biomolecules derived from *Bacillus thuringiensis* are gaining worldwide importance as environmentally desirable alternatives to synthetic chemicals for pests control in public health and agriculture [1]. This Gram-positif soil bacterium is known by its ability to produce crystalline inclusions during sporulation. These parasporal inclusions are solubilized in the midgut of the target larvae, releasing proteins called δ-endotoxins (or Cry) that, upon proteolytic activation, exhibit a highly specific insecticidal activity [2-4].

A second family of insecticidal proteins produced by this bacterium during its vegetative stage of growth has been identified [5]. The Vegetative insecticidal proteins (Vip) have been classified into three groups according to their sequence homology: Vip1 and Vip2 proteins that act as binary toxin and are toxic to Coleoptera [6] and Vip3 proteins known by their activity against Lepidoptera [7].

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Due to their high efficiency in the biological control of pests, the repetitive use of some Cry toxins contributed to the resistance emergence to these Cry proteins in some insect populations. As example, we cited the resistance of *Heliothis virescens* to Cry1Ac and Cry2Aa toxins, and the resistance of the diamondback moth *Plutella xylostella* to Cry1Ac [8, 9].

The use of Vip3 toxins in combination with Cry proteins can be classified as a good strategy to ovoid these problems of resistance emergence. In fact, one of the interesting features of the Vip3 protein is that it shares no sequence homology with the δ-endotoxins [7]. Moreover, in ligand blotting experiments with brush border membrane vesicles from the tobacco hornworm, *Manduca sexta*, activated Cry1Ab and Vip3A bound to different molecules receptors which demonstrated that resistant pests to Cry toxins can be killed by the second type of bioinsecticide (Vip3).

Moreover, isolation of new *B. thuringiensis* strains and cloning of novel insecticidal protein genes (*cry* and *vip*) are imperative for increasing the diversity of toxins and overcoming potential problems associated with resistance.

In the present study, we characterized a new isolated *B. thuringiensis* strain, named BLB427 [10], having a promising future as a biological control agent. In fact, Cry and Vip3 proteins of BLB427 demonstrated an interesting activity against different lepidopteran pests such as *Ephestia kuehniella* and *Spodoptera littoralis*.

2. Material and methods

2.1. Bacterial strains and growth conditions

New *B. thuringiensis* isolate BLB427 was obtained from Tunisian bioinsecticide-free soil sample [10]. *B. thuringiensis* BUPM95 [11] and *B. thuringiensis* subsp. *kurstaki* HD1 [12] were used as reference strains. For laboratory routine use, *B. thuringiensis* strains were grown in Luria-Bertani medium (LB) [13] at optimum conditions (30°C, 200 rpm). T3 medium [14] was used to detect the parasporal crystals formed during *B. thuringiensis* growth.

During the vegetative stage of *B. thuringiensis* growth and in order to promote protein production, cells were grown in PY Broth [1.2% (w/v) peptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 54 mM K_2HPO_4 and 16 mM KH_2PO_4] [15] at optimum temperature then the culture was centrifuged and the supernatant was used for bioassays against Lepidopteran pests.

2.2. *B. thuringiensis* crystal solubilization and protein content

Total spores and crystals from *B. thuringiensis* strains were treated as reported by Saadaoui et al. [16]. The solubilized proteins were then analyzed by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (10% SDS-PAGE) and visualized by Coomassie blue staining [17].

2.3. Protein quantification

The concentrations of *B. thuringiensis* solubilized crystal proteins and purified Vip3 toxins were measured with the Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

2.4. Bioassays against Lepidopteran larvae

A free ingestion technique was used to evaluate the toxicity to *E. kuehniella*, *S. littoralis* and *A. segetum* larvae of *B. thuringiensis* crystal proteins, supernatant extracts and purified Vip3(427) protein as described by Abdelkefi-Mesrati et al. [18, 19] ; Ben Hamadou-Charfi et al. [20] and Boukedi et al. [10, 21]. All the experiments were done in triplicate in the presence of a negative control set maintained in the same conditions of temperature (23 °C), relative humidity (65%) and photoperiod (18 h light and 6 h dark). Larval mortality was scored after 5 days and fifty percent lethal concentrations (LC_{50}) were calculated by probit analysis using programs written in the R. language [22].

2.5. Plasmid DNA isolation and PCR amplification

B. thuringiensis DNA was extracted using the alkali lysis method including a lysozyme treatment step as described by Sambrook et al. [13].

For the detection of *vip3*-like genes, PCR reactions were done in a thermocycler ''Gene Amp PCR System 2700'' (Applied Biosystems) using *B. thuringiensis* DNA as template and (V1, V2) as primers (Table 1).

Using *B. thuringiensis* DNA, iQ SYBER Green Supermix (BIORAD) and primers V13 and PS21 (Table 1), Real-time quantitative PCR reactions were accomplished to amplify *vip3*-type genes. The detection of the amplified products was performed with C1000 thermal cycler (Bio-Rad) during 45 cycles [23].

Table 1 Sequences of primers used in PCR.

Primer	Sequence (5' ➔ 3')	Reference
VipM1 (f)	AAGATGCA*TATGAACAAGAATAATA	[24]
VipM2 (b)	GATG*GAT CCCGATCTTACTTAATAG	[24]
V1 (f)	ATGAACAAGAATAATACTA	[11]
V13 (f)	CAAGCCGCAAATCTTGTGGA	[21]
V2 (b)	TCTATTTGCAGACTTAGCGC	[11]
V3 (b)	TTACTTAATAGAGACATCGT	[11]
PS21 (b)	ATGGCTTGTTTCGCTACATC	[21]

(f) forward primers; (b) backward primers.

2.6. Cloning and sequencing of *vip3*-type gene

The open reading frame (ORF) of the *vip3*-type gene was PCR amplified, using total DNA isolated from *B. thuringiensis* strain BLB427 as template, primers V1 and V3 (Table 1) and DNA polymerase (Amersham). The obtained fragment, having a molecular weight rounding 2.37 kb, was purified from the agarose gel and the *vip3* ORF was cloned in pGEM-Teasy vector (Promega) generating a recombinant plasmid pGEM*vip3*(427). As reported by Sambrook et al. [13], *E. coli* cells (Top10) transformation was performed then selection of recombinant cells was performed on plates containing LB medium supplemented with ampicillin (100 mg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (40 mg/ml) and Isopropyl β-D-thiogalactoside (80 mg/ml).

The *vip3*(427) gene sequencing was carried out using a recombinant plasmid, the taq DyeDeoxy Terminator Cycle Sequencing kit and a 3700 ABI Prism DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequence was subjected to a blast nucleotide homology search against the nucleotide database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Blast.cgi).

2.7. Over-expression of *vip3*(427) in *Escherichia coli* and protein purification

The *vip3*(427) gene (GenBank Accession No. MG659728) overexpression was achieved by cloning its ORF in the pET-14b vector (Novagen). As described by Abdelkefi-Mesrati et al. [24] and using VipM1 and VipM2 primers (Table 1), restriction enzyme sites *Nde*I and *BamH*I were created by PCR upstream the initiation codon (ATG) and downstream the stop codon of the gene, respectively. After being cloned into pGEM-Teasy vector (Promega), the open reading frame of the gene (2.37 kb) was recovered by digesting it with the restriction enzymes *Nde*I and *BamH*I. Then, the gene was cloned in frame in its 5' end with the His-tag sequence of the *E. coli* expression vector pET-14b (Novagen) and the recombinant plasmid, named pET-*vip3*(427), was transformed into *E. coli* BL21(DE3). As described by Abdelkefi-Mesrati et al. [24], recombinant cells were grown in Luria-Bertani medium supplemented with ampicillin (100 mg/ml) and induced using IPTG.

The sonication of the recombinant cells was done in buffer [PBS 1X (pH 7.5); 4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride] [24] and after centrifugation, the supernatant was loaded onto His-Trap column (Amersham) preequilibrated with a binding buffer (PBS 1X, imidazole 40 mM). Bound proteins were then eluted using elution buffers containing increasing concentrations of imidazole in PBS 1X as described by Abdelkefi-Mesrati et al. [24].

2.8. Preparation and sectioning of insect tissues

After exposure to the *B. thuringiensis* Vip3(427) toxins, lepidopteran larvae were placed in 10% formaldehyde [19]. Larvae were then dehydrated in increasing ethanol concentrations, rinsed in toluene (100%) and embedded in paraffin wax. Sections (5 μm) were obtained and placed in carriers loaded with a mix of 1.5% egg albumin and 3% glycerol in distilled water. After being de-paraffinated in 100% toluene, sections were stained with hematoxylin eosin for histopathological localization of the effects of *B. thuringiensis* Vip3 toxins on midgut larvae.

3. Results and discussion

3.1. BLB427 crystal protein content

BLB427 is one of the promising *B. thuringiensis* strains isolated by Boukedi et al. [10]. This strain was characterized by a special plasmid profile compared to the collection of about 200 *B. thuringiensis* isolates [10]. To compare the compositions of parasporal proteins extracted from *B. thuringiensis* BLB427, BUPM95 and HD1, SDS-PAGE analysis was employed. Parasporal inclusions from BLB427 contained 2 major Cry proteins having molecular weights of about 130-135 kDa and 65-70 kDa corresponding to *cry1*-type and *cry2*-type genes, respectively (Fig. 1). This profile was similar to that detected for BUPM95 and HD1 used as reference strains (Fig. 1) and give us an idea about the *cry*-type genes that BLB427 may contain and the possible efficiency of this strain to control lepidopteran pests.

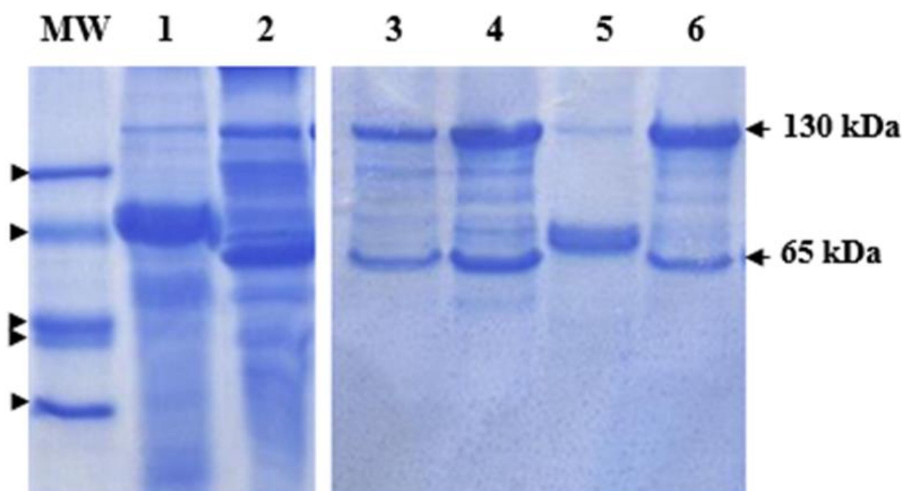

Figure 1 Analysis of *B. thuringiensis* parasporal inclusions content.

Analysis of *B. thuringiensis* proteins found in the crystal by SDS-PAGE. Lanes: MW: Molecular weight marker (97; 66; 45; 30; 20,1 kDa); 1, Crystal proteins of BLB459; 2, Crystal proteins of HD1; 3, Crystal proteins of BLB384; 4, Crystal proteins of BLB427; 5, Crystal proteins of BLB459; 6, Crystal proteins of BUPM95.

3.2. Insecticidal activity of BLB427 delta-endotoxins against Lepidoptera

Bioassays against lepidopteran pests were conducted using parasporal inclusions of BLB427 and HD1 as reference and the insecticidal activity was assessed using LC_{50} values [22]. Table 2 revealed that parasporal proteins of BLB427 exhibited high toxicities to *E. kuehniella* and *S. littoralis* larvae, with LC_{50} values of about 81.91 µg/cm^2 (+/- 15.30) and 79.70 µg/cm^2 (+/- 36.73), respectively. According to the results showed in Table 2, we presumed that Cry toxins of BLB427 were slightly more active against the cited Lepidoptera than those of the reference strain, HD1. This result supports the use of this strain in the biological control of Lepidoptera.

Table 2 *Bacillus thuringiensis* parasporal proteins efficiency against Lepidopteran larvae

	Parasporal proteins of *B. thuringiensis* strains	
	BLB427	**HD1**
LC_{50} against *E. kuehniella* (µg/cm^2 of semolina)	81.91 (+/- 15.30)	71.08 (+/- 11.44)
LC_{50} against *S. littoralis* (µg/cm^2 of artificial diet)	79.70 (+/- 36.73)	112.80 (+/- 12.26)

3.3. Insecticidal activity of BLB427 supernatant

To know if BLB427 strain produces soluble insecticidal molecules, the insecticidal activity of its supernatant against lepidopteran pests was evaluated. The Duncan grouping test performed after ANOVA analysis revealed that mortality caused by BLB427 supernatant was statistically higher than that of the reference strain *B. thuringiensis* BUPM95 as demonstrated in Figure 2. Moreover, the both supernatant strains are significantly more toxic against *S. littoralis* and *E. kuehniella* than *A. segetum*, according to the Duncan grouping test (Table 3). Some *B. thuringiensis* strains are able to secrete vegetative insecticidal proteins known by their acute activity against lepidopteran pests [7, 21]. For that, we suggested to investigate the presence of genes encoding such proteins in *B. thuringiensis* strain BLB427.

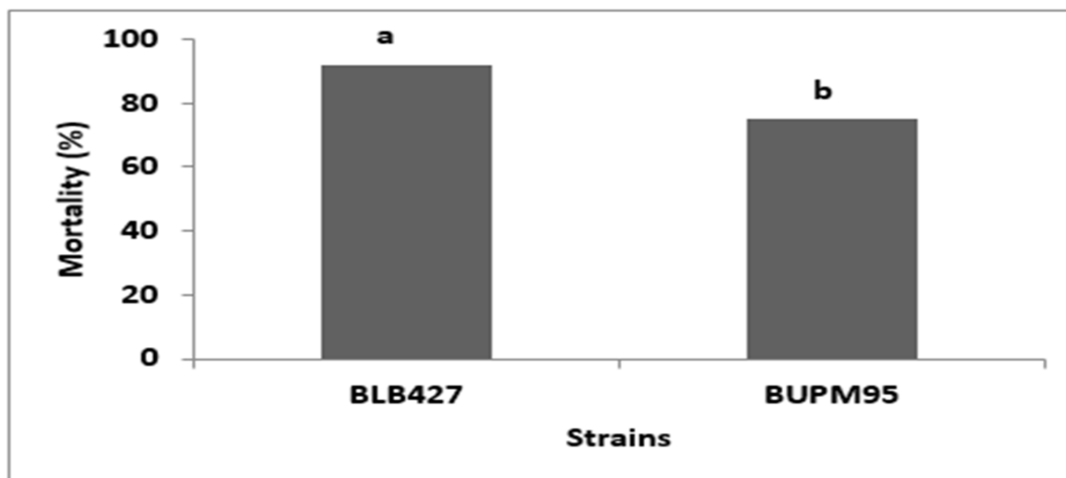

Figure 2 Means of *B. thuringiensis* supernatants efficiency against Lepidoptera**.**

* Means with the same letter are not significantly different, at p = 0.05 upon Duncan multiple range test.

3.4. Evidence of *vip3* gene presence in BLB427 strain (PCR and quantitative PCR)

Using (V1, V2) primers (Table 1) and Polymerase Chain Reaction, we investigated the presence of *vip3*-type genes in the new isolated *B. thuringiensis* BLB459. And as suspected, BLB459 DNA amplification showed a fragment of about 0.4 kb corresponding to a *vip3*-type gene and demonstrated that this strain is able to produce Vip3 toxins in contrast to the negative control which did not lead to any amplification product (Data not shown). To compare the *vip3*-type genes copy numbers between *B. thuringiensis* BLB427 and the reference strain BUPM95 [11], we decided to amplify identical quantities of DNA extracted from each strain by Real-time quantitative PCR. As shown in figure 3, fluorescence emerged early in the case of BLB427 DNA with a threshold cycle (Ct) of about 23 (Fig. 3A) while the Ct detected for BUPM95 DNA was around 29 (Fig. 3B). The detected differences between the mean Ct values of the samples tested demonstrated that BLB427 strain contains a higher copy-number of *vip3* gene than BUPM95 and such result can explain the differences in the efficiency of the supernatants of these two strains against Lepidoptera (Table 3).

Table 3 Insecticidal activities of *B. thuringiensis* supernatants.

Larvae	**Strains**	
	BLB427	**BUPM95**
E. kuehniella	93.10 ± 3.42% a*	75.30 ± 1.044% a
S. littoralis	95.60 ± 0.79% a	77.40 ± 1.70% a
A. segetum	87.50 ± 1.37% b	72.50 ± 3.57% b

R2 = 0.964; F = 38.49; Coeff. Var. = 2.77; (Pr>F) < 0.001

* Means in the same column not followed by the same letters are significantly different at p = 0.05 upon Duncan multiple range test.

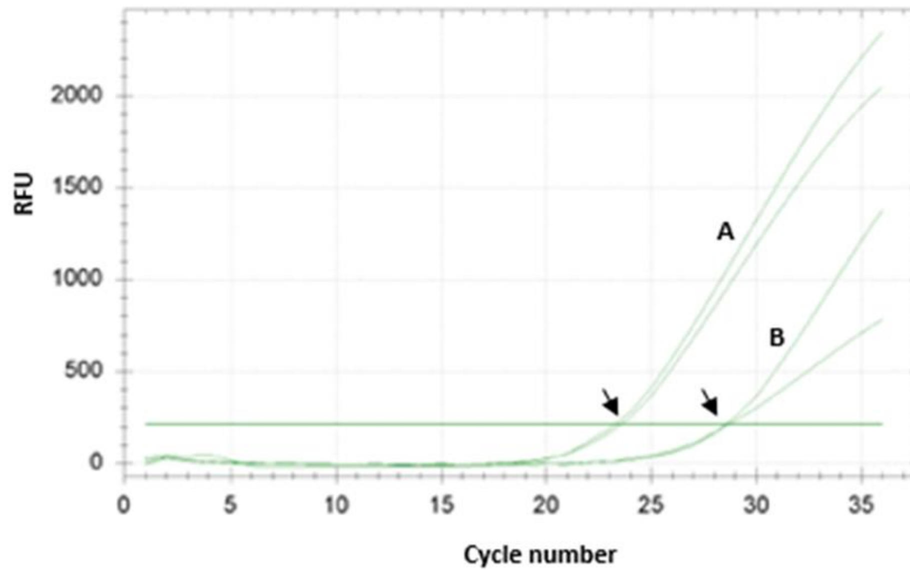

Figure 3 The amplification curves of *vip3*-type gene as recorded in Real-time quantitative PCR reactions. Reactions operated using DNA extracted from *B. thuringiensis* strains BLB427 (A) and the reference strain BUPM95 (B).

RFU: relative fluorescence units.

3.5. Cloning and sequence analysis of the BLB427 *vip3* gene

To investigate the main reasons of the higher toxicity of BLB427 supernatant against Lepidoptera compared to that of the reference strain BUPM95, we decided to clone and sequence the *vip3* gene of this isolate and compare the corresponding protein with Vip3Aa16 of *B. thuringiensis* BUPM95 [11]. The amplified PCR fragment corresponding to the ORF (open reading frame) of *vip3*(427) was cloned in the pGEMTeasy vector and the resultant recombinant plasmid was used for the *vip3* gene sequencing. The obtained sequence corresponds to an ORF of 2370 pb that encodes a protein of about 789 AA residues with a predicted molecular mass rounding the 88.5 kDa. The search for sequence similarity, using Blast, demonstrated that *vip3*(427) harbored some differences compared to the other reported *vip3* genes. This result was confirmed with multiple alignments using the program CLUSTALW. When compared with the most known *vip3*-type genes, there was substitutions at different positions resulting in the modification of the corresponding amino acid (Figure 4). These modifications can have positive or negative effects on the toxicity of the Vip3(427) protein against Lepidoptera.

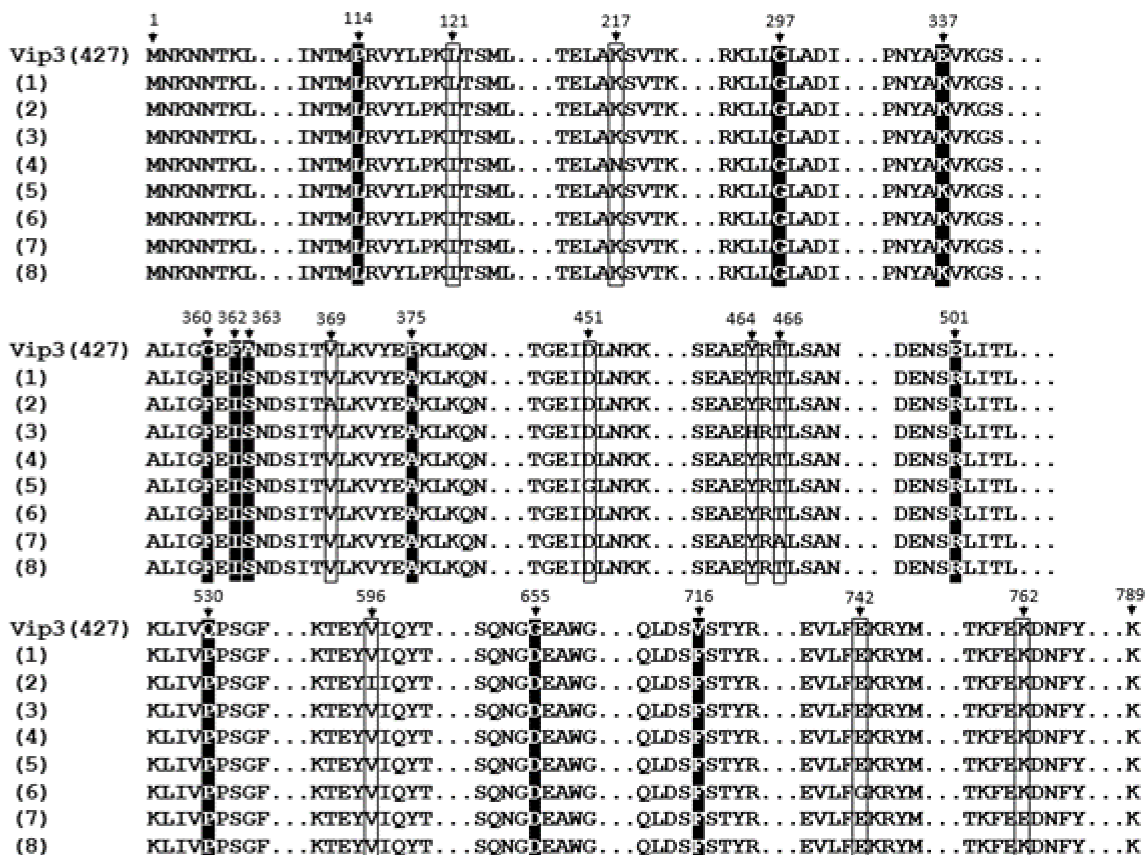

Figure 4 Comparison of the Vip3(427) amino acid sequence with those encoded by the other *vip3*-type genes. GenBank Accession Numbers of the *vip3* genes and their corresponding proteins: 1, Vip3Aa16 (AAW65132); 2, Vip3(459) (JN990981), 3, Vip184 (AAO32350.1); 4, Vip3BR (AAW62286.1); 5, Vip3 (CAI96522.1); 6, Vip3 (CAA76665.1); 7, Vip3 (AAU89707.1); 8, Vip3V (AAN60738.1).

Vertical downward arrows indicated amino acids positions, the black boxes represented the residues showing variations and only regions containing differences are presented.

3.6. Over-expression of *vip3*(427) in *Escherichia coli*, protein purification and study of the toxin efficiency on Lepidoptera eradication

The *vip3* gene of *B. thuringiensis* strain BLB427 was cloned in the pET-14b vector as described in Material and Methods section. The recombinant strain *E. coli* (pET-*vip3*(427)) was induced by IPTG than cells were sonicated. Supernatant proteins were analyzed by SDS-PAGE and showed the presence of a protein with high amount and having a molecular weight of about 90-kDa which corresponds to the expressed Vip3 protein. Based on the six-histidine tail fused at the N-terminal end of Vip3(427) protein, we purified the toxin using the His-trap column and an increasing gradient of imidazole.

When tested against first instar larvae of *S. littoralis*, purified Vip3(427) protein showed an interesting toxicity with an LC_{50} of about 239.52 (+/-41.52) ng/cm^2. Negative control set was done by the exposure of larvae to the buffer solution and the treatment did not affect larval growth increase, weight gain, or morphology. When compared to the purified Vip3Aa16 of BUPM95 having an LC_{50} against *S. littoralis* of about 305 (+/-95) ng/cm^2 [19], the purified Vip3(427) was slightly more active against this lepidopteran pest. This difference can be due to the variations detected on the amino acid sequences described above.

Histological observations of Vip3(427) effects on *S. littoralis* were studied on first instar larvae which had been fed a diet containing the purified protein. We detected extensive damages in the midgut of larvae treated with the Vip3(427) toxin (Fig. 5B) in contrast to the negative control corresponding to the midgut of untreated larvae that showed uniform morphology with well-defined epithelial cells and unaffected apical microvilli membrane (Fig. 5A). We noticed that main histopathological modifications included brush border membrane destruction, intensive vacuolization of the cytoplasm, and vesicle secretion in the apical region of cells toward the midgut lumen (Fig. 5B). This kind of mode of action is

similar to that described for other *B. thurigiensis* Vip3 toxins active against Lepidoptera such as Vip3Aa16 *of B. thuringiensis* BUPM95 that caused serious damages in the midgut larvae of the lepidopteran pest *S. littoralis* [19].

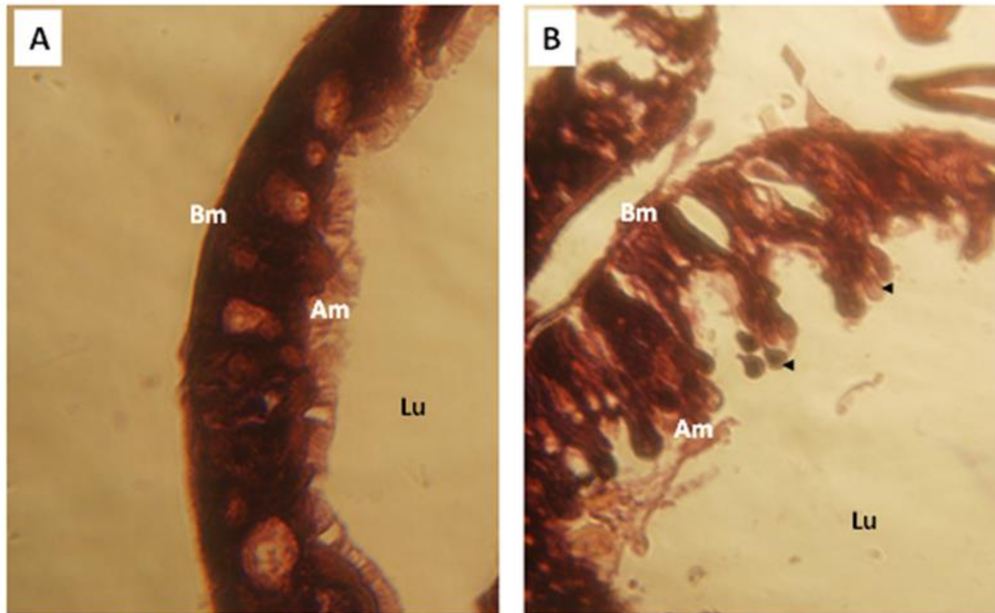

Figure 5 Histopathological effects of *B. thuringiensis* Vip3(427) toxin on Lepidopteran midgut larvae. A: General aspects of the midgut larvae of *S. littoralis*; B: Histopathological effects of purified Vip3(427) toxin on the midgut larvae of the lepidopteran pest *S. littoralis*. Am, Apical membrane; Bm, Basement membrane; Lu, Lumen. Magnification 40X. Arrowhead indicates vesicle formation.

4. Conclusion

The present study was undertaken to investigate the insecticidal potency of *B. thuringiensis* strain BLB427. Results showed that this strain is a promising bioinsecticide. In fact, BLB427 produces 2 types of insecticidal proteins, Cry and Vip3 toxins having an interesting activity against lepidopteran pests such as *S. littoralis* which supports their use as a biological control agent against a large spectrum of Lepidoptera especially in case of resistance emergence in the target larvae species.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors; Dr. Boukedi H., Dr. Hmani M., Dr. Ben Khedher S., Pr. Tounsi S. and Pr. Abdelkefi-Mesrati L. have declared that there is no conflict of interest with the publication of this manuscript, institution as well as product that is mentioned in the manuscript.

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