Curr Microbiol (2011) 63:289–294
DOI 10.1007/s00284-011-9976-3

Increase of the *Bacillus thuringiensis* Secreted Toxicity Against Lepidopteron Larvae by Homologous Expression of the *vip3LB* Gene During Sporulation Stage

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Received: 3 April 2011 / Accepted: 28 June 2011 / Published online: 10 July 2011

Abstract The Vegetative insecticidal Vip3A proteins display a wide range of insecticidal spectrum against several agricultural insect pests. The fact that the expression of *vip3* genes occurs only during the vegetative growth phase of *Bacillus thuringiensis* is a limiting factor in term of production level. Therefore, extending the synthesis of the Vip proteins to the sporulation phase is a good alternative to reach high levels of toxin synthesis. In this study, we have demonstrated that the maximal production of the secreted Vip3LB (also called Vip3Aa16) during the growth of the wild-type strain *B. thuringiensis* BUPM 95 is reached at the end of the vegetative growth phase, and that the protein remains relatively stable in the culture supernatant during the late sporulation stages. The *vip3LB* gene was cloned and expressed under the control of the sporulation dependant promoters BtI and BtII in *B. thuringiensis* BUPM 106 ($Vip3^-$) and BUPM 95 ($Vip3^+$) strains. The examination of the culture supernatants during the sporulation phase evidenced the synthesis of Vip3LB and its toxicity against the second-instars larvae of the Lepidopteron insect *Spodoptera littoralis* for the recombinant BUPM 106. Moreover, there was an increase of the Vip3LB synthesis level and an enhancement of the oral toxicity for the recombinant BUPM 95 resulting from the expression of the *vip3LB* gene during both the vegetative and sporulation phases and the relative stability of the Vip3LB protein.

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Introduction

Bacillus thuringiensis is a Gram-positive spore-forming bacterium that forms parasporal crystals in the cell during the stationary growth phase. These crystals contain one or more related insecticidal δ-endotoxins which have been used for many years as successful biological insecticides [18]. The expression of most of the *cry* genes is sporulation-dependent; *cry1Aa* is a typical example which is under the control of sporulation-linked promoters BtI and BtII [23]. Some of *B. thuringiensis* strains produce the Vegetative Insecticidal Protein called the Vip3, which is secreted into the culture supernatant during the vegetative growth [16]. The Vip3 protein is highly toxic to a range of lepidopteron insects, some of which are less sensitive to δ-endotoxins, such as *Agrotis ipsilon* [13] and *Spodoptera exigua* [14]. A study on the action mode of Vip3A has shown that the activated toxin can bind to Bruch Border Membrane Vesicles (BBMV) prepared from *Manduca sexta* [10]. Vip3A has been also shown to form pores in *M. sexta* midgut following activation by lepidopteron gut juice extracts to form a stable ion channels in the absence of any receptors in planar lipid bilayers. The analysis has demonstrated that the ion channels differed considerably in their principal conductance state and cation specificity from those formed by Cry1Ab [10]. These studies have thus indicated a very low cross resistance potential between Vip3A and currently deployed Cry toxins, supporting its use in effective resistance management strategies.

Due to the importance of the Vip3 proteins, many researches and several genetic manipulations have been carried out order to increase the effectiveness of the *vip3* genes of *B. thuringiensis* not only to improve the insecticidal activity but also to delay or prevent the development of resistance in target pest species. In this study, we develop strains that over-express *vip3LB* gene [1], also called *vip3Aa16* [7], in *B. thuringiensis*. Vip3LB synthesis was studied during the growth phase in the *B. thuringiensis* strain BUPM 95. On the other hand and in order to extend the synthesis of Vip3LB to the sporulation phase, the *vip3LB* gene was cloned and expressed under the control of the strong sporulation-dependant promoters BtI and BtII in *B. thuringiensis*. Oral toxicity tests were assayed against the second-instars larvae of *Spodoptera littoralis* to evaluate the insecticidal activities of the recombinant strains.

Materials and Methods

Bacterial Strains and Plasmids

Escherichia coli strain Top10 (Invitrogen, USA) was used as the cloning host. It was grown at 37°C in Luria–Bertani (LB) medium [17] supplemented with ampicillin (60 μg/ml). *B. thuringiensis* strains: BUPM 95 (harbours the wild *vip3LB* gene) [1] and BUPM 106 (without a *vip3* gene, unpublished data) were isolated from soil samples collected from Tunisia. LB medium was used for the growth of *B. thuringiensis* strains at 30°C.

Construction of pHT-*spo-vip3LB* Plasmid

The *vip3LB* gene was amplified by PCR using as template the BUPM 95 DNA and the primers PS12 (5′-CTAGTATC GATTAGCTGAAAAGGAAGGTCGACATGAACAAGA ATAATACTAAATT-3′) and PS14 (5′-ATACTGAATTC CCCGGGAACTTAAGATCTCTTAATAGAGACATCGT AAAAATG-3′). The forward primer brings the *Cla*I recognition site, the *vip3LB* Shine-Dalgarno sequence and *Sal*I site in the 5′ region. The reverse primer was designed from the 3′ region of the gene and contained *Bgl*II site, the stop codon, *Xma*I and *Eco*RI sites. The endogenous *vip3LB* stop codon was omitted. In addition, we dispose of a plasmid called pHT-*spo-cry1Ia* deriving from the pHT*Blue* plasmid [22] where the ORF of the *cry1Ia* gene was placed downstream the transcription regulatory region containing the promoters BtI–BtII (GenBank: U87793) and upstream the terminator region of *cry1Ia* (GenBank: AJ315121). *cry1Ia* coding sequence (ORF) was then replaced by that of the *vip3LB* gene after a double digestion with *Cla*I and *Eco*RI. The resulting plasmid, called pHT-*spo-vip3LB*, was transferred to *E. coli* cells Top10. The pHT*Blue* plasmid is a derivative of the much known pHT3101 plasmid [11] where the multiple cloning sites of the latter was substituted by that of the pBluescript II KS + plasmid. We have transferred either pHT*Blue* or pHT-*spo-vip3LB* plasmid to either the *B. thuringiensis* wild-type strains BUPM 106 ($Vip3^-$) or BUPM 95 ($Vip3^+$) by electroporation as described by Schurter et al. [19] with minor modifications.

Trichloroacetic Acid (TCA) Precipitation Assay and Protein Analysis

A 1:10 supernatant volume of 100% TCA was added to each culture supernatant sample, obtained after the centrifugation of the collected aliquots during the growth time of the *B. thuringiensis* strains and incubated for 30 min on ice. Subsequent to centrifugation at 7000 rpm for 20 min at 4°C, the supernatant was carefully removed, and a 1:40 (eventually 1:50 and 1:60) supernatant volume of Laemmli sample buffer (1×) was added to the protein pellet. The pH was adjusted during protein solubilisation by adding small NaOH 5 M volumes. Protein extracts were then boiled for 5 min, analysed by SDS-PAGE on a 9% polyacrylamide separating gel as described by Laemmli [9], transferred to nitrocellulose membrane and probed with rabbit polyclonal antibody raised against Vip3LB. Horseradish peroxidase-labelled goat anti-rabbit was used as secondary antibody to visualize Vip3LB by enhanced chemiluminescence using ECL^+ kit (Amersham Biosciences, France).

Bioassays

The toxicity assays against *S. littoralis* were carried out by integrating 150 μl of the 48 h culture supernatants of either the recombinant strains BUPM 106 (pHT*Blue*), BUPM 106 (pHT-*spo-vip3LB*), BUPM 95 (pHT*Blue*) and BUPM 95 (pHT-*spo-vip3LB*) into 1.5 g of artificial semi-solid diet (a mixture of wheat germ, beer yeast, maize semolina, ascorbic acid, nipagine, agar and water) cubes with a surface area of about 1 cm^2 and placed in a Petri dish [15]. Ten second-instars larvae were added to each dish, the plates were kept in the insect culture room under controlled conditions of temperature 25°C, relative humidity of 65% and a photoperiod of 16 h light and 8 h dark. The experiment was replicated three times. The LB medium and 150 μl of culture supernatants of the recombinants BUPM 106 (pHT*Blue*) and BUPM 95 (pHT*Blue*) were used as controls. For these tests, the larval weights were recorded periodically. Statistical analyses were carried out using Excel software to calculate the mean values of three experiments and their standard deviations.

Results

Vip3LB Synthesis in the Wild-Type Strain *B. thuringiensis* BUPM 95

In order to determine the time course of Vip3LB protein synthesis during the growth of the wild strain BUPM 95, the supernatants were collected throughout the vegetative and sporulation phases, then the proteins were precipitated with the TCA, suspended in a Laemmli sample buffer to be concentrated 40 fold and analysed by Western blot. Vip3LB was detected starting from the beginning of the growth and its amount increased during the logarithmic phase, reaching its maximal level at the end of this phase and then remained relatively stable during the late stages of the sporulation phase (until 53 h) despite the presence of proteases during sporulation [5] (Fig. 1).

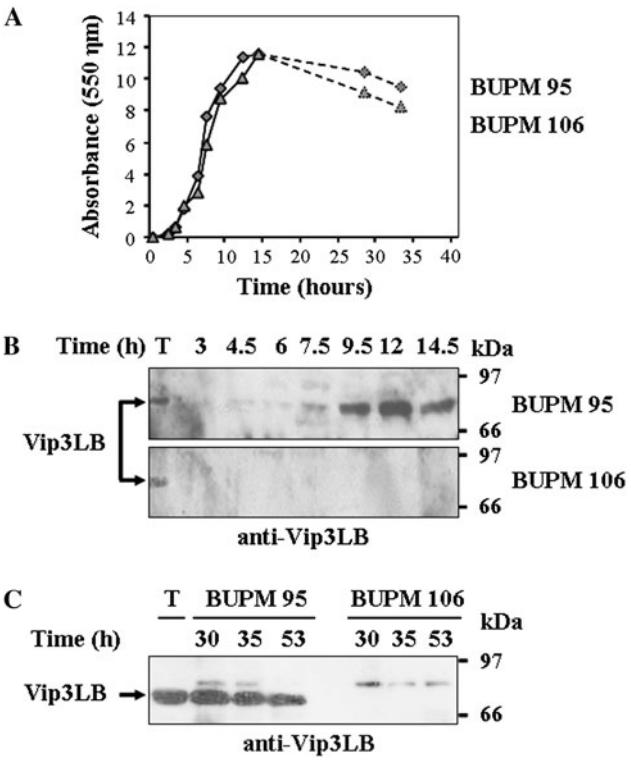

Fig. 1 Synthesis of the Vip3LB (88.5 kDa) during the growth of the wild-type strain BUPM 95. Time is indicated as hours after culture start. *T*, purified 6His tagged Vip3LB used as control. **a** Growth curves of bacteria cultured in liquid LB medium in a rotary shaker; *filled diamond* BUPM 95 and *filled triangle* BUPM 106. *Dashed lines* are presented essentially to indicate the start time of the stationary phases. Western blot analysis with anti-Vip3LB antibody for the presence of Vip3LB: **b** in the 40 fold concentrated culture supernatant proteins during exponentially growth phase and **c** in the 60 fold concentrated culture supernatant proteins during stationary growth phase

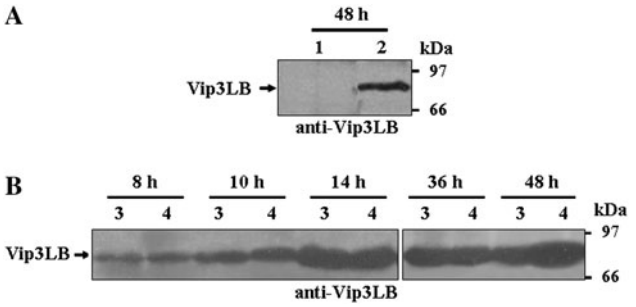

Fig. 2 Synthesis of the Vip3LB protein in the wild-type and the recombinant strains of *B. thuringiensis*. Western blot analysis of the Vip3LB (88.5 kDa) in the 50 fold concentrated supernatant proteins using anti-Vip3LB antibody. *M* molecular weight markers (HMW-SDS, Amersham). **a** Sporulation growth phase (48 h) of BUPM 106 (pHT*Blue*) (*1*) and BUPM 106 (pHT-*spo-vip3LB*) (*2*). **b** Vegetative (8, 10 and 14 h) and sporulation (36 and 48 h) growth phases of BUPM 95 (pHT*Blue*) (*3*) and BUPM 95 (pHT-*spo-vip3LB*) (*4*)

Homologous Expression of *vip3LB* Gene Under the Control of the BtI and BtII Promoters

The relative stability of Vip3LB, synthesized during the logarithmic phase, during the late stages of the sporulation phase encouraged us to extend its synthesis to the sporulation phase. Thus, the *vip3LB* gene was cloned and expressed under the control of the two strong promoters BtI and BtII [20] into the shuttle vector pHT*Blue* [22]. In order to verify the expression of *vip3LB* gene via BtI and BtII, each one of the plasmids pHT*Blue* and pHT-*spo-vip3LB* was individually transferred to both the wild-type strains BUPM 106 ($Vip3^{-}$) or BUPM 95 ($Vip3^{+}$). The latter carries the wild *vip3LB* gene and its own promoter working during the logarithmic phase. The four recombinant strains were grown in liquid LB medium, aliquots were collected during the exponentially and stationary growth phases and the proteins were precipitated with TCA to be concentrated 50 fold and then analysed by Western blot using rabbit antibodies directed against Vip3 protein (Fig. 2). Compared with the control strain BUPM 106 (pHT*Blue*), the Western blot did not show any band corresponding to the 88.5 kDa Vip3LB protein at 14 and 23 h of time culture (data not shown), but showed the expected one at 48 h, demonstrating the presence of the Vip3LB protein and the expression of the corresponding gene by BtI and BtII (Fig. 2a). On the other hand, the amounts of the Vip3LB protein produced by BUPM 95 (pHT*Blue*) and BUPM 95 (pHT-*spo-vip3LB*) increased during the culture course between 8 and 14 h but they are alike at each culture time and remain clearly preserved after 36 h (Fig. 2b). However, the Vip3LB protein yield improved in the recombinant strain BUPM 95 (pHT-*spo-vip3LB*) at 48 h, compared with that of BUPM 95 (pHT*Blue*) which remains approximately constant (Fig. 2b). We have also constructed the

plasmid pHT-*Prv-vip3LB* where the *vip3LB* coding sequence and its upstream native promoter, as described by Mesrati et al. [2], were cloned in the plasmid pHT-*spo-cry1Ia* after the deletion from the latter of the *spo* promoter BtI–BtII and the *cry1Ia* gene. The Western blot analysis of BUPM 106 (pHT-*Prv-vip3LB*) supernatant using antibodies anti-Vip3LB showed signals corresponding to Vip3LB protein during the exponential growth phase which were less important than those of the wild-type BUPM 95. These signals decreased weakly at 24, 48 and 72 h, demonstrating that the absence of the BtI–BtII promoters was followed by the absence of the expression during the sporulation stage. Moreover, the pHT*Blue* plasmid and its derivatives have a low copy number, about four per equivalent chromosome [3]; this is a serious limitation for the homologous expression during the exponentially growing phase by the native promoter (data not shown). During the sporulation phase, the improvement of the Vip3LB amount clearly results from the BtI and BtII controlled expression of the *vip3LB* carried by pHT-*spo-vip3LB*. Thus, the accumulation of Vip3LB during both the vegetative and sporulation phases is due to the expression of *vip3LB* under the control of the native promoter and the BtI–BtII promoters, respectively.

Improvement of the Oral Toxicities of the *B. thuringiensis* Recombinant Strains

The toxicity of the BUPM 106 (pHT*Blue*), BUPM 106 (pHT-*spo-vip3LB*), BUPM 95 (pHT*Blue*) and BUPM 95 (pHT-*spo-vip3LB*) culture supernatants were evaluated against second-instars larvae of *S. littoralis* (Fig. 3). The growth curves of *S. littoralis* larvae revealed that the supernatant of BUPM 106 (pHT*Blue*) strain, lacking *vip3LB* and used as negative control, was non-toxic towards *S. littoralis* since the growth of the larvae was the same in the presence or the absence of this strain BUPM 106. Furthermore, it was demonstrated that the supernatant of the strain BUPM 106 (pHT-*spo-vip3LB*) expressing the Vip3LB protein causes a remarkable inhibition of the growth of *S. littoralis*. Thus, it was concluded that *B. thuringiensis* Vip3LB protein affects clearly the growth of *S. littoralis* second-instars larvae (Fig. 3a). On the other hand, and compared to the control curve (without bacteria), BUPM 95 (pHT*Blue*) harbouring the wild *vip3LB* gene expressed during the vegetative growth caused an inhibition of the growth of the larvae, proving the activity of Vip3LB against *S. littoralis*. Moreover, an important improvement of the oral toxicity was observed in the presence of the BUPM 95 (pHT-*spo-vip3LB*) culture supernatant which accumulates the Vip3LB synthesis during both the vegetative and sporulation phases due to the vegetative and the BtI–BtII promoters, respectively.

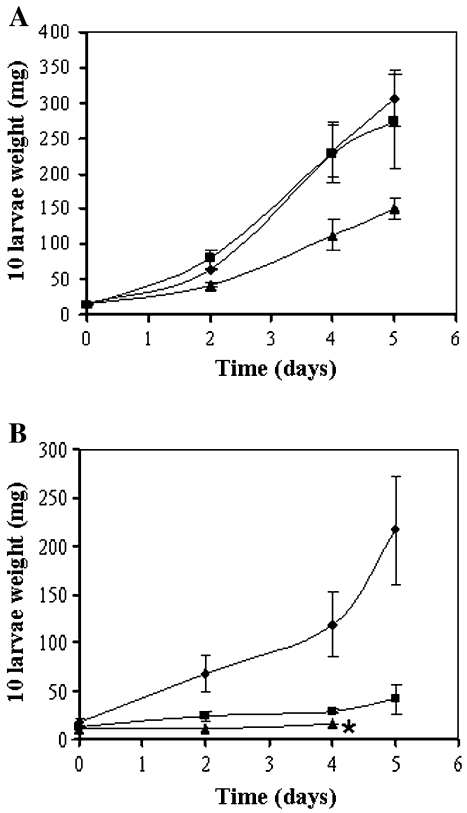

Fig. 3 Effects of the BUPM 106 (pHT-*spo-vip3LB*) and BUPM 95 (pHT-*spo-vip3LB*) *B. thuringiensis* recombinant strains on the growth (larval body weight) of *S. littoralis* larvae. Each measurement consisted in weighing randomly chosen 10 second-instars larvae and was replicated three times. Each data point is a mean of three experiments; *error bars* depict a standard deviation of the mean values. **a** *Filled diamond* without bacteria, *filled square* BUPM 106 (pHT*Blue*) and *filled triangle* BUPM 106 (pHT-*spo-vip3LB*). **b** *Filled diamond* without bacteria, *filled square* BUPM 95 (pHT*Blue*) and *filled triangle* BUPM 95 (pHT-*spo-vip3LB*). Death of the larvae: *asterisk*

These larvae were died after 4 days compared with the control larvae fed with the supernatant of BUPM 95 (pHT*Blue*) where no mortality was detected (Fig. 3b). These results prove the importance of the Vip3LB synthesis during the sporulation phase for the insecticidal activity.

Discussion

The examination of the excretion time course of the vegetative insecticidal protein Vip3LB during the growth of the wild-type strain BUPM 95 has shown that the maximal Vip3LB amount outside bacteria was reached at the end of the vegetative phase. Interestingly, the Vip3LB protein remains present in the culture supernatant during the late stages of sporulation phase (until 53 h), although the expression of the *vip3* gene starts at the logarithmic phase

as it corroborates with the results provided by Estruch et al. [8]. Therefore, the expression of the *vip3LB* gene and/or the potential stability of the corresponding protein to the proteases during the sporulation phase [12] can explain the persistence of the Vip3LB during this phase. In fact, Vip3LB protein can be produced during the stationary phase since the *vip3LB* transcripts were detected at the middle of the vegetative growth phase as well as the middle of the sporulation phase [2], although it's known to be a vegetatively expressed gene. In addition, a nucleotide sequence partially homologous to the consensus sequence for the σ^{35} holoenzyme of *B. thuringiensis* was found upstream the *vip3LB* transcription starting point and then suggested that the *vip3LB* gene could be transcribed by sigma factor σ^{35} [2]. We highlight that BtI is active between t2 and t6 of sporulation depending on the alternative transcriptional factor σ^{35}, and BtII is active from about t5 onwards depending on another alternative sigma factor σ^{28} [6] (tn is n hours after the end of the exponential phase).

Based on these arguments, we have cloned and expressed the *vip3LB* coding sequence downstream the BtI and BtII promoters in BUPM 106 which lacks the *vip3LB* gene and BUPM 95 harbouring the endogenous *vip3LB* gene controlled by its own vegetative promoter. Thus, comparing with the control recombinant strains, the assessment of the Vip3LB in the concentrated culture supernatants by Western blot analysis revealed the presence of the 88.5 kDa Vip3LB protein after 48 h of the BUPM 106 (pHT-*spo-vip3LB*) culture. This result has demonstrated the effectiveness of the BtI and BtII expression system, as it was also described by Arora et al. [4] and Song et al. [21] who expressed a signal peptide truncated Vip3-encoding genes under the control of the same promoters. Furthermore, Arora et al. [4] produced the corresponding protein inside *B. thuringiensis* in the form of inclusion bodies and the bioassays showed that the protein causes the mortality of the lepidopteron larvae *Chilo partellus* after a 48 h. Moreover, Song et al. [21] found that the synthesised protein does not form inclusion bodies and has found a lower toxicity towards *Helicoverpa armigera*, *S. exigua* and *Plutella xylostella* than that of the wild-type Vip3Aa7.

Interestingly, this expression system showed a significant improvement of the Vip3LB amount in the recombinant strain BUPM 95, compared with the wild-type strain BUPM 95, as a result of the accumulation of the protein during both the vegetative and the sporulation phases due to the joint functioning of the vegetative and the BtI–BtII promoters, respectively. Moreover, we noticed an improvement of the toxicity in the supernatant of the recombinant strain BUPM 95 where the second-instars larvae died after 4 days compared with the control larvae fed with the supernatant of BUPM 95 where we have detected a weight loss but not mortality.

In this study, the Vip3LB protein was over-produced, in the constructed recombinant *B. thuringiensis* strains, during the sporulation phase in parallel with the synthesis of the crystalline inclusions or δ-endotoxins. A clear enhancement of the insecticidal activity was evidenced due to the extended expression of the *vip3LB* gene during the sporulation phase and also the stability of the Vip3LB protein. These findings demonstrate the possible simultaneous production of both the Vip3LB and the δ-endotoxins which can be applied as an emulsion or a wettable powder for biocontrol.

Acknowledgment We are grateful to Dr. L. Abdelkefi-Mesrati for providing the polyclonal antibody against Vip3LB. This study was supported by grants from the Tunisian « Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (MESRS) » .

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