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Heterologous Expression of *Bacillus thuringiensis* Vegetative Insecticidal Protein-Encoding Gene *vip3LB* in *Photorhabdus temperata* Strain K122 and Oral Toxicity against the Lepidoptera *Ephestia kuehniella* and *Spodoptera littoralis*

Kaïs Jamoussi · Sameh Sellami · Lobna Abdelkefi-Mesrati ·
Alain Givaudan · Samir Jaoua

Abstract *Photorhabdus temperata* and *Bacillus thuringiensis* are entomopathogenic bacteria exhibiting toxicities against different insect larvae. Vegetative Insecticidal Protein Vip3LB is a *Bacillus thuringiensis* insecticidal protein secreted during the vegetative growth stage exhibiting lepidopteran specificity. In this study, we focused for the first time on the heterologous expression of *vip3LB* gene in *Photorhabdus temperata* strain K122. Firstly, Western blot analyses of whole cultures of recombinant *Photorhabdus temperata* showed that Vip3LB was produced and appeared lightly proteolysed. Cellular fractionation and proteinase K proteolysis showed that in vitro-cultured recombinant *Photorhabdus temperata* K122 accumulated Vip3LB in the cell and appeared not to secrete this protein. Oral toxicity of whole cultures of recombinant *Photorhabdus temperata* K122 strains was assayed on second-instar larvae of *Ephestia kuehniella*, a laboratory model insect, and the cutworm *Spodoptera littoralis,* one of the major pests of many important crop plants. Unlike the wild strain K122, which has no effect on the larval growth, the recombinant bacteria expressing *vip3LB* gene reduced or stopped the larval growth. These results demonstrate that the heterologous expression of *Bacillus thuringiensis* vegetative insecticidal protein-encoding gene *vip3LB* in *Photorhabdus temperata* could be considered as an excellent tool for improving *Photorhabdus* insecticidal activities.

Abbreviations
B.	*Bacillus*
P.	*Photorhabdus*
K122	*Photorhabdus temperata* strain K122
E.	*Ephestia*
S.	*Spodoptera*

Contribution Kaïs Jamoussi and Sameh Sellami contributed equally to this work and share first authorship.

K. Jamoussi · S. Sellami · L. Abdelkefi-Mesrati · S. Jaoua (✉)
Laboratory of Biopesticides, Centre of Biotechnology of Sfax,
P.O. Box: "1177", 3018 Sfax, Tunisia
e-mail: samir.jaoua@cbs.rnrt.tn

A. Givaudan
Laboratoire Ecologie Microbienne des Insectes et Interactions
Hôte-Pathogène UMR 1133 INRA-UMII, Université de
Montpellier 2, Place Eugène bataillon, 34095 Montpellier,
France

Introduction

Bacillus thuringiensis is an entomopathogenic bacterium widely used to control pest insects, from which most of the insecticidal toxins used in agriculture originate. For such purposes, this bacterium has been either employed as a direct agent or as a source of genes to generate commercial transgenic insect-resistant crops [1, 2]. *B. thuringiensis* produces parasporal crystalline δ-endotoxin inclusions, which are formed during sporulation [3, 4] and comprise Cry proteins which can be associated with Cyt proteins. So far, over 350 *B. thuringiensis* endotoxins have been identified [5–8]. These δ-endotoxins are the most valuable biopesticides used currently in different forms of commercially available biopesticides for agriculture and mosquito control [4, 9]. In addition to these δ-endotoxins, *B. thuringiensis* produces two common classes of secreted insecticidal proteins during its vegetative growth stage, namely,

vegetative insecticidal proteins (VIP) which include the binary toxins Vip1 and Vip2 with coleopteran specificity and Vip3 exhibiting lepidopteran specificity [10–12]. Vip3 toxins share no sequence similarity to Vip1 or Vip2. For example, the combination of Vip1 and Vip2 toxins expands their insecticidal properties especially against an agriculturally important pest, the Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) [10] and Vip3 against the European corn borer (*Ostrinia nubilalis*), two widespread corn pests, less affected by the δ-endotoxins [10]. Contrary to Cry toxin genes, only a limited number of *vip3* genes have been cloned and characterized previously [13, 14]. Vip3Aa1, the first-identified Vip3, is highly insecticidal to lepidopteran pests such as the fall armyworm *Spodoptera frugifera* and the cotton bollworm *Helicoverpa zea* [11], and many of these known Vip3 toxins have insecticidal activities similar to that of Vip3Aa1 [15–17]. Vip3A proteins must be activated by proteases prior to recognition at the surface of the midgut epithelium of specific 80 and 100 kDa membrane proteins different from those recognized by Cry toxins [12]. It was also observed that Vip3Aa1 kills insects by lysing insect midgut cells [18] via cell membrane pore formation [12]. The fact that Vip3 proteins act differently compared with Cry makes them good candidates for resistance-management strategies involving stacking or rotation of proteins with different insecticidal mechanisms.

It should be noted that, despite being a large commercial market, *B. thuringiensis* has several disadvantages, limiting its use in biological control, such as its low persistence on the phylloplane, UV sensitivity of the toxins, restricted stability of Vip proteins during the sporulation growth stage, their dilutions in culture medium after being secreted and the high fermentation costs, as well as the fact that the prolonged application of *B. thuringiensis* formulations and the widespread planting of transgenic crops with *B. thuringiensis* endotoxins have raised concerns over the development of insect resistance [14, 19–25]. In order to overcome these problems, attempts have been made to stabilize or enlarge its performance essentially by enhancing crystal photoprotection [26] or cell stability by starch encapsulation [27]. On the other hand, recent strategies have exploited the heterologous expression of *B. thuringiensis* genes in novel hosts such as *Escherichia coli* [28–31], *Bacillus cereus* [32], *Gluconacetobacter diazotrophicus* [33], *Herbaspirillum seropedicae* [33, 34], *Methylobacillus xagellatum* [35] and *Bacillus subtilis* and *Bacillus licheniformis* [36], etc.

The newly described and promising entomopathogenic bacterium *Photorhabdus* is a genus of Gram-negative, and is member of the Enterobacteriaceae family. In addition to being pathogenic to a wide range of insects, *Photorhabdus* spp. are symbionts of Heterorhabditis entomopathogenic nematodes isolated from soil. The bacteria are carried in the gut of the nematodes during the symbiotic stage, and are released by the nematode into the insect blood system (hemocoel) during the pathogenic stage [37] where the bacteria replicate and release a range of toxins having both oral and injectable activities against insect hosts [38]. The prey (insect larvae) is probably killed by the combined action of the nematodes and the bacteria. Therefore, the "toxin complexes" Tc's (Tca, Tcb, Tcc and Tcd) and the "*Photorhabdus* insect-related" (Pir) toxins both have an oral activity against some insect species; however, other toxins such as the "Makes caterpillars floppy" toxins (Mcf1 and Mcf2) and the "*Photorhabdus* virulence cassettes (PVCs)" are only active by way of injection [39]. The exact biological role of the different *Photorhabdus* toxins in the infection process remains unclear.

vip3LB gene has been isolated from *Bacillus thuringiensis* strain BUPM95, encoding a Vip protein of 789 amino acids (88.5 kDa) [40, 41]. The aim of this study was to express *vip3LB* by *Photorhabdus temperata* K122, to determine the Vip3LB sub-cellular location, and then to test whether the recombinant *Photorhabdus temperata* K122 expressing both its endogenous toxins and Vip3LB protein shows oral toxicity against larvae of *Ephestia kuehniella* and *Spodoptera littoralis* species to ascertain their effectiveness in vivo. Possibilities of using *Photorhabdus* spp. in pest control will be discussed to formulate new biological control agents as alternatives to *Bacillus thuringiensis*.

Materials and Methods

Bacterial Strains and Plasmids

Photorhabdus temperata spp. *temperata* strain K122 is courtesy of Dr. D. Clarke [42]. It was grown in Luria-Bertani (LB) medium at 30°C. *Escherichia coli* strain Top10 (Invitrogen, USA) was used as the cloning host. It was grown in LB medium at 37°C. The ampicillin concentration used for bacterial selection was 60 μg/ml.

pBAD-*vip3LB* plasmid [40] is derived from pBAD-GFPuv vector (Clontech, USA) where the coding sequence of *B. thuringiensis* strain BUPM95 *vip3LB* gene [40] is under the control of the strong P_{BAD} promoter inducible with L-arabinose. The produced Vip3LB protein would be N-terminally supplemented with the (MARGSTSHMD) peptide as a result of cloning strategy. In order to obtain a control plasmid, pBAD plasmid was constructed by digesting pBAD-GFPuv vector with *Nhe*I and *Xba*I to delete *gfp* coding sequence and self-ligating the compatible cohesive ends generated by *Nhe*I and *Xba*I (data not shown).

Protein Analysis

Protein extracts were suspended in Laemmli sample buffer and boiled for 5 min. They were analysed by SDS-PAGE on a 9% polyacrylamide separating gel as described by Laemmli [43] and stained using Coomassie blue or transferred to nitrocellulose membranes 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). Purified 6His-Vip3LB and rabbit polyclonal antibody against 6His-Vip3LB were prepared in our laboratory (data not shown).

P. temperata K122 Transformation by Electroporation

Transformation of the recipient strain *P. temperata* K122 was carried out as described by Tounsi et al. [44]. *P. temperata* K122 was grown in LB medium with shaking at 30°C overnight. The LB medium of 100 ml was inoculated with 1 ml of overnight culture and incubated with shaking to an $OD_{600\ nm}$ of 0.2. The bacterial culture was kept on ice for 90 min and centrifuged, and the cell pellet was washed three times with a sterile buffer (4 mM Hepes pH 7; 10% glycerol); the bacteria were finally suspended in approximately 150 μl. The appropriate volume preparation was added to 0.5 μg plasmid DNA and incubated on ice for 5 min. Electroporation was performed with an ice-cold 0.2-cm electroporation cuvette (Bio-Rad) in a Bio-Rad Gene Pulser apparatus set at 100 Ω, and 2.1 kV, with the pulser controller at 25 μF. The culture was added to 1 ml LB medium and incubated at 30°C for 3 h. Transformed cells were selected on LB plate containing 60 μg/ml ampicillin after incubation for 48 h at 30°C.

Proteolysis by Proteinase K

Whole cultures of *P. temperata* K122 were subjected to proteolysis by proteinase K to examine the extracellular Vip3LB protein. Stationary phase cultures of the recombinant strains of *P. temperata* K122 were diluted in a 50-ml fresh LB medium and were incubated at 30°C for 3 h to an $OD_{600\ nm}$ of 0.5. L-arabinose was added to the cultures to obtain a final concentration of 0.1% and incubation was prolonged for 4 h at 30°C to a final $OD_{600\ nm}$ of 2.5. one millilitre aliquots was transferred to 1.5-ml tubes, and proteinase K was added to each tube to the final concentrations of 2, 6, 10, 20 and 30 μg/ml; the tubes were incubated at 20°C for 15 min. In order to stop the reactions, PMSF (4 mM final concentration) and 0.5 ml of Laemmli sample buffer concentrated three times were added, and the tubes were heated at 100°C for 5 min. Samples were analysed by SDS-PAGE on a 9% polyacrylamide separating gel and Coomassie blue staining.

Cellular Fractionation

Recombinant *P. temperata* K122 strains, exponentially growing ($OD_{600\ nm}$ of 0.5), were induced with 0.1% L-arabinose for approximately 4 h to reach an $OD_{600\ nm}$ of 2.5. Whole culture extracts were obtained by adding Laemmli sample buffer concentrated four times to a fraction of the total culture. Each culture was centrifuged at $14,000 \times g$ for 20 min, and the supernatant was saved as an extracellular fraction and was both precipitated with 10% trichloroacetic acid (TCA) and suspended in 1:100 volume of Laemmli sample buffer; the cell pellet was suspended in 1:5 volume of Laemmli sample buffer.

For disruption by sonication, bacterial pellets were suspended in 5 ml of 20 mM phosphate buffer pH 7.5, and the mixture was sonicated three times for 30 s each time to disrupt cells. The homogenate was centrifuged at $14,000 \times g$ for 20 min at 4°C. The pellet contained the bacterial insoluble fraction and the supernatant contained the soluble one.

Bioassays

A free ingestion technique was used to assess the oral toxicity of *P. temperata* K122 (pBAD) and *P. temperata* K122 (pBAD-*vip3LB*) to *Ephestia kuehniella* (Lepidoptera: Pyralidae) and *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae. The expression of recombinant bacteria was induced by adding 0.1% L-arabinose when *P. temperata* K122 cultures grown in LB medium without antibiotic and at 30°C reached $OD_{600\ nm}$ of 0.5. Cultures were achieved 4 h post-induction with an $OD_{600\ nm}$ of 2.5–3. For *S. littoralis* assays, the diet is a derivative of the previously described artificial semi-solid diet [45] consisting of mixture of wheat germ, beer yeast, maize semolina, ascorbic acid, nipagine, agar and water. Samples of whole cultures of the recombinant strains, as well as LB medium as a control, were incorporated into 1.5 g of artificial diet cubes with a surface area of about 1 cm^2 previously placed into a plate (12 × 12 cm).Treated larvae food blocks were allowed to dry for 15 min, and then a set of 10 s-instar larvae of *S. littoralis* was placed on each food block. The plates were kept in the insect culture room under controlled conditions of temperature 23°C, relative humidity of 65% and a photoperiod of 18 h light and 6 h dark. The experiment was replicated three times.

The oral toxicity assays against *E. kuehniella* were carried out using different doses of recombinant *P. temperata* K122 cells. The latter were mixed with 1 g of wheat

semolina placed in a Petri dish, dried at room temperature and then ground. Ten second-instar larvae were added to each dish and were incubated at 25°C. The experiment was replicated four times. For these tests, the larval weights were recorded periodically by using a suitable microbalance. Statistical analyses were carried out using Excel software to calculate the mean values of three or four experiments and their standard deviations.

Results

Expression of Vip3LB by *P. temperata* K122 and Examination of its Sub-cellular Location

In order to examine the production of Vip3LB by *P. temperata* K122 and its cellular location, plasmids pBAD and pBAD-*vip3LB* were introduced into *P. temperata* K122 by electroporation. Recombinant bacteria were cultured in LB medium with shaking at 30°C, and *vip3LB* expression was induced by 0.1% L-arabinose. SDS-PAGE and Western blot analysis of the whole cultures showed that Vip3LB, N-terminally supplemented with the (MARGSTSHMD) peptide, was produced and that its amount was similar to the highly synthesized bacterial proteins. Furthermore, Vip3LB appeared slightly proteolysed (Fig. 1a).

In order to localize Vip3LB protein, total proteins of exponentially growing *P. temperata* K122, whether expressing or not Vip3LB, were fractionated into cell-associated and extracellular proteins. Western blot analysis using antibodies directed against Vip3LB and comparison of signal intensities showed that approximately all the produced Vip3LB was present in the cell-associated fraction, and a very low amount was found in the extracellular fraction and cell washing fraction (Fig. 1b). We next investigated whether Vip3LB was secreted into the culture medium of *Photorhabdus*, but it was not soluble nor it was adsorbed to the outer membrane. Various proteinase K concentrations were added to samples of whole cultures of the exponentially growing cells of *P. temperata* K122, whether expressing or not Vip3LB. The separation of proteins by SDS-PAGE and the comparison of Vip3LB amounts stained with Coomassie blue showed no significant reduction after proteinase K treatment (Fig. 1c). In order to test the solubility of Vip3LB expressed in *P. temperata* K122, the culture pellets of the recombinant strains harbouring pBAD or pBAD-*vip3LB* were disrupted by sonication, then the cleared extracts were centrifuged at $14,000 \times g$ for 20 min and pellets (insoluble fractions) and supernatants (soluble fractions) were analysed by SDS-PAGE and Coomassie blue staining. Vip3LB protein was found to be soluble inside bacteria (Fig. 1d).
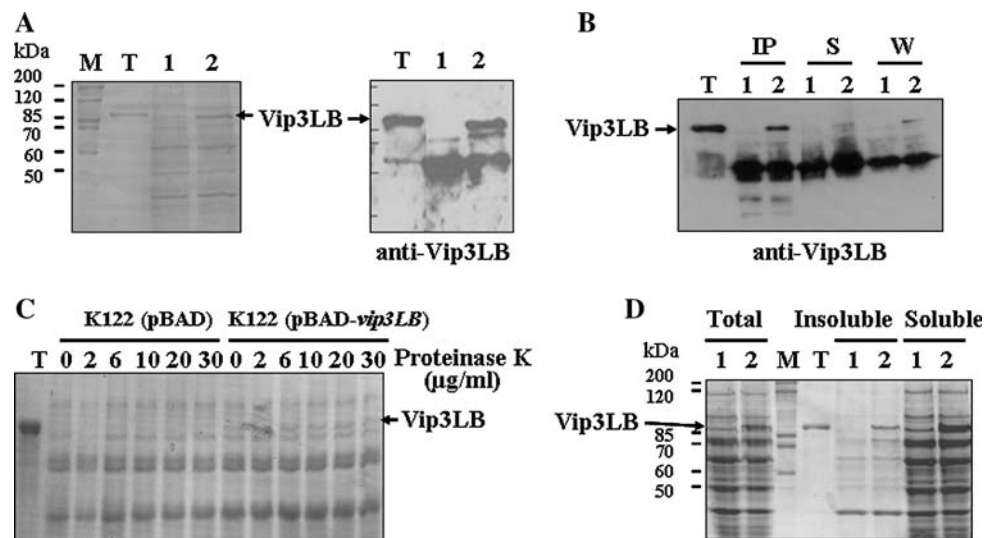

Fig. 1 Heterologous expression of *vip3LB* by *P. temperata* K122 and the subcellular location of the corresponding protein. Exponentially growing cultures of recombinant strains were analysed. Lanes: M, molecular weight markers (HMW-SDS, Amersham); T, purified 6His tagged Vip3LB used as control; 1, *P. temperata* K122 (pBAD); 2, *P. temperata* K122 (pBAD-*vip3LB*). **a** SDS-PAGE and immunoblotting of whole cultures, using rabbit polyclonal antibody raised against Vip3LB, of *P. temperata* K122 (pBAD) and *P. temperata* K122 (pBAD-*vip3LB*) used for the toxicity assays. **b** Subcellular location of Vip3LB. Proteins of total cultures of recombinant strains were fractionated into intracellular proteins (IP) concentrated 5 times, extracellular proteins (or culture supernatant) (S) and bacteria washing fraction (W) each concentrated 100 times; equal volumes (20 μl) of each bacterial fraction were analysed by SDS-PAGE and immunoblotting using polyclonal antibody raised against Vip3LB. **c** Proteinase K susceptibility of Vip3LB in total cultures of the recombinant *P. temperata* K122 strains. The reactions were stopped by adding PMSF (4 mM) and Laemmli sample buffer and by heating the samples at 100°C for 5 min. **d** Total, insoluble and soluble protein expression were monitored by SDS-PAGE

These results demonstrate that in vitro-cultured *Photorhabdus temperata* K122 (pBAD-*vip3LB*) appears not to release Vip3LB into the culture medium and accumulates this protein inside the bacteria in a soluble state.

Oral Toxicity of Recombinant *P. temperata* K122 Expressing VIP3LB Against *Ephestia kuehniella* and *Spodoptera littoralis*

The potential insecticidal effects of *Photorhabdus temperata* K122, whether expressing or not the *B. thuringiensis*, vegetative insecticidal protein Vip3LB, towards growth rate expressed as weight evolution of *E. kuehniella* and *S. littoralis* were assessed by incorporating them in the larvae diets (Fig. 2). Both *E. kuehniella* and *S. littoralis* larvae, fed on diet without or with *Photorhabdus temperata* K122 not expressing *B. thuringiensis* Vip3LB, showed good and comparable growths (Fig. 2), indicating a limited toxicity of *P. temperata* K122 against these insects. On the other hand, *E. kuehniella* larvae fed on diet supplemented with cultures of *P. temperata* K122 (pBAD-*vip3LB*) expressing Vip3LB, showed that even with 5.7×10^7 CFU per gram of diet, there was a marked *E. kuehniella* larvae growth inhibition, but with 2.2×10^8 CFU, growth was stopped during the first 7 days. The toxicity was more importantly pronounced with 4×10^8 CFU (Fig. 2a). On the other hand, the growth of *S. littoralis* larvae, fed on base diet containing 1.7×10^7 CFU of *P. temperata* K122 (pBAD-*vip3LB*) expressing Vip3LB, was stopped completely (Fig. 2b).

We could thus conclude that *P. temperata* K122 expressing *B. thuringiensis* vegetative insecticidal protein Vip3LB affects clearly the growth of both *E. kuehniella* and *S. littoralis* second-instars larvae. Such growth inhibition appears to be proportional to CFU number of the recombinant *P. temperata*. It is noteworthy that *P. temperata* K122 showed very limited consequences on the development of both insect larvae.

Discussion

In a previous study, a novel *vip3LB* gene was identified in the BUPM95 strain of *B. thuringiensis* [40]. In this article, the heterologous expression of *vip3LB* in *Photorhabdus temperata* K122 was described. In this study, using polyclonal antibodies against *B. thuringiensis* Vip3LB, we evidenced that the Vip3LB protein, expressed in *P. temperata* strain K122, was found in the bacterial pellet and was not released outside the cell. Since secretion sequences are located at the N-termini of proteins, it is possible that the failure of *Photorhabdus temperata* K122 to secrete Vip3LB is due to blocking of the normal Vip3 secretion signal because an additional peptide sequence was added
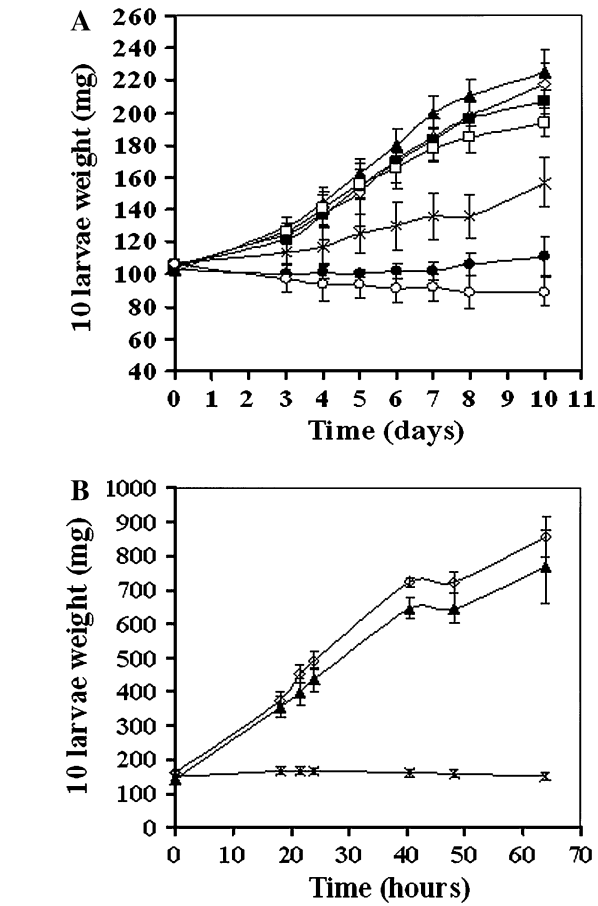

Fig. 2 The effect of *Photorhabdus temperata* K122 expressing Vip3LB and added to artificial diet or wheat semolina on the growth (larval body weight) of *Ephestia kuehniella* (**a**) or *Spodoptera littoralis* (**b**) second-instar larvae, respectively. For time course experiment, insect larvae were incubated with whole culture of *P. temperata* K122 harbouring either pBAD as a control or pBAD-*vip3LB* expressing Vip3LB for different time periods. Each measurement consisted in weighing randomly chosen 10 s-instar larvae and was replicated three and four times for *S. littoralis* and *E. kuehniella*, respectively. Each data point is a mean of three or four experiments; error bars depict a standard deviation of the mean values. **a** (▲) Without bacteria; K122 (pBAD): (◇) 4×10^7, (■) 1.6×10^8 and (□) 2.8×10^8 (CFU/g); K122 (pBAD-*vip3LB*): (x) 5.7×10^7, (●) 2.2×10^8 and (○) 4×10^8 (CFU/g). **b** (▲) Without bacteria; (◇) K122 (pBAD) 1.7×10^7 (CFU/g); (x) K122 (pBAD-*vip3LB*) 1.7×10^7 (CFU/g)

during cloning to the N-terminus of the Vip3 protein. The protein size corresponded to the excepted one, demonstrating that Vip3LB was stably maintained inside the bacterial cell without degradation nor secretion. Vip3LB was mainly soluble. These findings demonstrate that the recombinant *P. temperata* synthesizes and accumulates Vip3LB inside its cell under a soluble state and does not release it into the culture medium. Moreover, the insecticidal activity study of the recombinant *Photorhabdus temperata* K122 expressing *B. thuringiensis* vegetative insecticidal protein Vip3LB towards *E. kuehniella* and *S. littoralis* showed a clear larval growth inhibition. The

fact that the intracellular Vip3LB is active against larvae would mean that the bacteria are disrupted probably inside larval gut releasing Vip3LB insecticidal protein.

According to their published data, Tounsi et al. have evidenced the oral toxicity of *P. temperata* strain K122 against the *Prays oleae* larvae and have demonstrated, for the first and unique time, its improvement by heterologous expression of *B. thuringiensis cry1Aa* and *cry1Ia* genes [44]. All these factors contribute to increasing efforts to find alternative and novel approaches for pest insect control. Thus, the heterologous expression of *B. thuringiensis* Vip toxins in *Photorhabdus* could be considered as an excellent tool not only for improving *Photorhabdus* insecticidal activities, but also as a suitable *B. thuringiensis* Vip delivery system or a biological formulation by encapsulation of this soluble insecticidal protein. This experiment could indicate that other biological encapsulation bacteria could be selected for future formulations.

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