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Insecticidal activity of microencapsulated Vip3Ag4 in *Bacillus megaterium*.

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Abstract: *Bacillus thuringiensis* (Bt) produces during its vegetative growth some insecticidal proteins that are secreted and diluted into the culture medium. These proteins were commonly known as vegetative insecticidal proteins (Vips) including binary Vpb/Vpa proteins (formerly known as Vip1/Vip2) with coleopteran activity, Vip (formerly Vip3) with activity against lepidopterans and Vpb4 proteins (formerly Vip4) also with coleopteran activity. The Vip proteins are highly toxic to different species of lepidopteran pests; however, their difficulty to be produced in a concentrated form has not allowed their development as formulated biopesticides, being relegated only to be produced in transgenic crops. In this work, we demonstrated that the gene encoding the Vip protein Vip3Ag4 could be successfully expressed in an asporogenic strain of *Bacillus megaterium* using (D)-xylose as a low-cost inductor. Under certain conditions (37 °C and induction with 0.5 % w/v xylose), active Vip3Ag4 protein is primarily produced in soluble form remaining encapsulated within the cell wall of *B. megaterium*. After treatment with lugol (1 % for 4 hours), induced cells were completely killed (fixed) but maintaining functional Vip3Ag4 protein, which produced above 95 % mortality against first instar larvae of *Chrysodeixis chalcites*, *Helicoverpa armigera*, *Spodoptera frugiperda*, *S. exigua*, *S. littoralis* and *Trichoplusia ni*. The fact that the recombinant Vip3Ag4 protein was successfully produced in a soluble and an active form in this bacterium (with a low-cost inductor), suggest that *B. megaterium* is one of the hosts of choice for the production of sprayable formulations in "killed-microbial pesticides", based on Vip proteins from Bt.

Keywords: *Bacillus thuringiensis*; Vip proteins; insecticidal proteins; *Bacillus megaterium*; biological control; insect pests

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1. Introduction

Bacillus thuringiensis (Bt) is a Gram-positive spore-forming bacterium that produces several insecticidal proteins of different types. The three-domain crystal (Cry) proteins, commonly known as δ-endotoxins, are not only the most abundant but also the best studied insecticidal proteins from this bacterium [1]. These proteins are synthesized during sporulation and aggregate to form parasporal crystals exhibiting pesticidal activity against species of different orders including Lepidoptera, Diptera, Coleoptera but also against some phytopathogenic nematodes [2]. Wild type strains of Bt serovar *israelensis* also produce other proteins commonly known as Cyt (cytolitic) proteins, which are syn-

thesized and crystallized together with Cry proteins exhibiting toxic activity against mosquitoes and black flies [3]. The mixed spores and parasporal crystals have been successfully used as the active material of a number of Bt-based formulates with insecticidal activity [1,4,5]. In addition, there are several *cry* genes that have been used, alone or in combination, for the construction of transgenic plants expressing Bt proteins with resistance against some insect pests, which are commonly known as Bt plants (e.g., Bt corn and Bt cotton) [6,7]. Other Bt proteins with insecticidal activity discovered later than Cry proteins, are produced during the vegetative growth phase and posteriorly secreted into the culture medium which are commonly known as vegetative insecticidal proteins [8]. This secretable proteins include binary Vpb1/Vpa2 (formerly Vip1/Vip2) proteins with toxic activity against coleopteran pests [9], the Vip proteins (formerly Vip3) with insecticidal activity against lepidopteran insects [8] and the last discovered, Vpb4 (formerly Vip4), with activity against *Diabrotica virgifera virgifera* (Coleoptera) [10]. Some of the genes encoding Vip3A proteins have been also used in the construction of transgenic plants. For example, cotton Cot102XCot67b co-expresses both Cry1Ab and Vip3Aa19 toxins to protect cotton from *Helicoverpa zea* and *Heliothis virescens* [11]. However, the Vip3 proteins have not been used as the active ingredient of insecticide formulations because they contain a signal peptide sequence driving their secretion into the culture medium [8]. This feature has prevented the production of these secretable insecticidal proteins in a concentrated form, necessary for example, for the production of a Vip-based formulation.

Therefore, the aim of this work was to set up a microbial system suitable for the production of encapsulated and functional (insecticidal) Vip3 proteins using a safe bacterium that poses no risk to humans, terrestrial or aquatic wildlife if released into the environment. In the scientific literature there are several examples of systems using heterologous bacteria for production of different types of proteins including insecticidal Cry proteins from Bt. Probably the best known, is the use of non-pathogenic strains of the Gram-negative bacterium *Pseudomonas fluorescens*, for microencapsulating Bt crystal proteins [12], or even more, secretable Cry1I and Vip proteins [13]. This strategy has been initially used by the company Mycogen Corporation for two commercial products namely MVP™ (Mycogen Corporation) for control of Lepidoptera and M-TRAK™ to control beetles [14]. After induction of expression, *P. fluorescens* cells containing the Cry protein are killed by application of physical or chemical methods (e.g., chemical fixation with formaldehyde or lugol). This process significantly improves the effectiveness of the insecticidal protein by increasing their persistence in the environment and protecting them against the inactivation induced by the ultraviolet light from sun [15]. However, the natural presence of endotoxins (lipid A) in the membrane of the Gram-negative bacteria is in fact, a very inconvenient feature. Using a different approach, Lampel et al. (1994) introduced a *cry* gene in *Clavibacter xyli* var. *cynodontis*, an endophytic Gram-positive bacterium that naturally colonize the vascular system of various plants including maize. The *cry* gene introduced into this bacterium encodes the Cry1Ac protein, which is very toxic to *Ostrinia nubulalis* larvae [16]. Nonetheless, to our knowledge, there is not yet any Gram-positive bacterial system developed for the heterologous production and encapsulation of Vip3 proteins suitable for the production of biopesticide formulations.

In this work, we demonstrated that *B. megaterium* could be successfully used to produce and encapsulate Vip3 proteins. *B. megaterium* is an aerobic, spore-forming and non-pathogenic bacterium, is able to grow on a wide variety of substrates, it doesn't contain any endotoxin and is often found colonizing the leaves of cotton plants [17]. Moreover, *B. megaterium* has been described as a GRAS (Generally Recognized as Safe) organism by the US Food and Drug Administration [18]. Currently, there are available various expression vectors useful for transforming *B. megaterium* [19] that allow intracellular expression of proteins using (D)-xylose as a low-cost inductor of gene expression. In addition, the *B. megaterium* expression system has been also used to industrial production of a wide range of proteins with different purposes [20,21]. We used the shuttle vector pN-His-TEV1622

(Mobitec) for protein expression in *B. megaterium* and for the production of active micro-encapsulated Vip3 proteins, as a mean of obtaining an active concentrated ingredient for further development of novel Bt-based biopesticide formulations.

2. Materials and methods

2.1. Bacterial strains and growth conditions.

The Bt ZaB5.3 and *B. megaterium* WH320 (Mobitec) strains used in this work were cultured in Luria-Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl, pH 7.0) at 28 °C and 37 °C, respectively. *Escherichia coli* strain DH5α was used for routine DNA manipulations and also cultured in LB medium at 37 °C.

2.2. Amplification and cloning of vip3Ag4 gene.

Total DNA was obtained from the Bt strain ZaB5.3 as previously described by Ruiz de Escudero et al., (2006) [22]. The *vip3Ag4* gene (Acc. Num. HQ414237) [23] was obtained by polymerase chain reaction (PCR) using specific primers that hybridize and modify the sequence by site directed mutagenesis allowing the addition of restriction sites *Xma*I (5'-CCCGGGATGAACAAGAATAATACTAA-3') and *Kpn*I (5'-GGTACCTCAC-TTAATCGAAAAATTCC-3') immediately before and after the start (ATG) and stop (TGA) codons, respectively. Each PCR reaction was performed in a 25 µl mixture (final volume) using proof reading PrimeSTAR HS DNA polymerase (Takara) and 5 µl total DNA (100 ng) in an Eppendorf Mastercycler thermal cycler (Eppendorf) using the following cycling conditions: 4 min. initial denaturation at 94 °C, 35 amplification cycles (1 min. denaturation at 94 °C, 1 min. annealing at 50 °C and 2.5 min. extension at 72 °) with a final extension step at 72 °C for 10 min. The obtained PCR amplicon was electrophoresed in 1 % agarose gels, agarose purified using NucleoSpin Extract II kit (Macherey–Nagel), cloned in pGEM-T easy (Promega), sequenced and the construct designated as pGEM-Ag4. This modification facilitated the digestion with enzymes *Xma*I and *Kpn*I and the sub-cloning of modified *vip3Ag4* amplicon into pre-digested *E. coli*/*B. megaterium* shuttle expression vector pN-His-TEV1622 [21] obtaining the pN1622-Ag4 construct. In this construction, the *vip3Ag4* gene was introduced in frame and under the control of the xylose inducible promoter (P*xyl*A) and N-terminally fused to the poly-histidine tag encoded in the pN-His-TEV1622 vector. This construction and the empty vector (control without inserted gene) were transformed into protoplasts of *B. megaterium* WH320 following the manufacturer's instructions (Mobitec). The transformed cells of *B. megaterium* acquired tetracycline resistance and were selected in solid LB medium with tetracycline at a final concentration of 10 ug/ml (LB Tc10). A clone of the recombinant *B. megaterium* strain was selected and sequenced using sequencing forward (5′-TCCTTTGTTTATCCACCGAA-3′) and reverse (5′-TCGGGTTTCGCCACCTCTGA-3′) primers in order to verify the correct in frame N-terminal fusion to the polyhistidine-tag encoded in the pN-His-TEV1622 shuttle expression vector.

2.3. Amplification and cloning of vip3Ag4 gene.

B. megaterium strain transformed with the pN1622Ag4 construct was grown in 2×TY medium (37 °C and 200 RPM) with tetracycline at a final concentration of 10 ug/ml. After reaching an optical density of 0.3 at 600 nm, protein expression was induced by adding (D)-xylose (Merck) at a final concentration of 0.5 % (w/v). Induction of expression was continued overnight and from one liter of 2×TY medium, 500 ml were used for the extraction and purification of the recombinant protein and the remaining 500 ml for bioassays with the Vip3Ag4 protein encapsulated in the killed bacterium. Samples were centrifuged at 5000 ×*g* for 15 min at 4 °C and the obtained pellet weighed and resuspended with 3 mL sonication buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 3 mg/mL lysozyme, 25 U Benzonase (Novagen) and 100 µM phenylmethylsulfonyl fluoride (PMSF) per gram of pellet. Samples were further incubated at 37 °C for 60 min and sonicated on ice water with a

Branson analog sonifier 250 (Branson Ultrasonics Corporation) by applying two 1 min pulses with constant duty cycle at 60 W, separated by a 1 min cooling period. Insoluble material was pelleted by centrifugation at 12,000 ×*g* for 30 min at 4 °C and the soluble cellular fraction filtered through sterile 0.45 and 0.22 μm syringe filters. Then, protein purification was performed using Protino Ni-TED 2000 Packed Columns according to the manufacturer's instructions (Macherey–Nagel). Additionally, 500 ml of induced 2×TY supernatant were also passed through a fresh Protino Ni-TED 2000 Packed Columns (Macherey-Nagel) in an attempt to detect if the recombinant Vip3Ag4 protein was partially secreted to the culture medium. The resultant expressed (soluble) Vip3Ag4 protein was then analyzed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), stained with Coomassie brilliant blue R-250 (Sigma–Aldrich). The protein concentration was estimated by the Bradford method (Bradford, 1976) and after imaging (scanning) by SDS-PAGE gel densitometry using GelEval package (FrogDance software).

2.4. Cell fixation and counting.

The induced cells were harvested by centrifugation at 6000 RPM at 4 °C and fixed as previously described [24]. The cell pellet was incubated at room temperature in 1 % lugol for 4 h with gentle orbital shaking, washed three times with sterile distilled water and resuspended in 20 mL of sterile distilled water. Same 1 % lugol treatment was also conducted on control cell pellet containing the empty pN-His-TEV1622 shuttle expression vector. The estimation of the number of viable cells, in terms of colony-forming units per milliliter (CFU/ml) of culture medium, was performed immediately after induction step was completed by surface plating of serial dilutions in 9-cm Petri dishes containing LB Tc10 medium. Cell viability assay was also performed, by the streak plate technique, with a loopful of 1 % lugol treated and non-treated cells separately loaded onto plates containing LB Tc10 medium.

2.5. Preliminary insect bioassays using microencapsulated Vip3Ag4 protein.

B. megaterium strains harboring pN1622Ag4 and empty pN-His-TEV1622 vector as control were growth over night reaching stationary phase. Bioassays were conducted by adding 100 μl of 1 % lugol fixed bacterial suspensions on the surface of solidified artificial diet for lepidopterans [25]. The suspensions were immediately distributed into the surface of 9-cm diameter petri dishes with a sterile Drigalsky spatula and allowed to completely dry for 30 minutes in the laminar flow hood. Then, 25 neonate larvae of the following lepidopteran pest species, namely *Chrysodeixis chalcites*, *Helicoverpa armigera*, *Spodoptera frugiperda*, *S. exigua*, *S. littoralis* and *Trichoplusia ni*, were added to each plate and incubated at 25 °C, 60 ± 5 % RH, and a 16:8 (light/dark [h]) photoperiod. Mortality was registered at 3 and 5 days after treatment.

3. Results

3.1. Vip3Ag4 insecticidal gene cloning in B. megaterium

The analysis of the pN1622Ag4 construct was performed by restriction endonuclease digestion with XmaI and KpnI enzymes, which produced two DNA fragments of the expected size and corresponding to the pN-His-TEV1622 (6589 bp) and the vip3Ag4 insecticidal gene insert (2364 bp). Furthermore, the analysis performed by PCR showed the amplification of the vip3Ag4 gene amplicon of approximately 2.4 kb (Figure 1). Full-length sequencing analysis of the insert demonstrated that no mismatches were present into the vip3Ag4 coding sequence of the pN1622Ag4 construct.

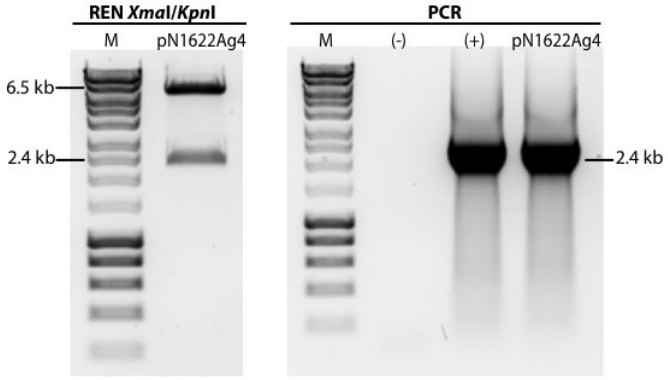

Figure 1. a) Analysis REN pN1622Ag4 construction, b) PCR analysis of the construction pN1622Ag4, M) Molecular weight marker Hyperladder I (Labolan) , (-) Negative control and positive (+) control.

3.2. Gene expression in B. megaterium.

SDS–PAGE analysis showed that the Vip3Ag4 protein was successfully expressed in B. megaterium and produced a major protein of the expected size (approx. 90 kDa) that was found in both the soluble and insoluble fractions (pellet), indicating that recombinant B. megaterium produced both insoluble and soluble fractions (Figure 2). The protein yield estimated reached 3,1 mg per 5 grams of cell pellet (1042,3 µg/mL of elution buffer) from 500 ml of 2×TY medium. In addition, attempts to purify the recombinant protein from the culture medium failed showing no detectable amounts of recombinant Vip3Ag4 protein by SDS-page gel electrophoresis analysis (data not shown).

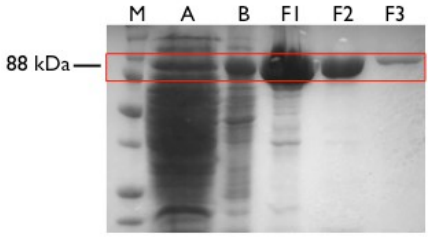

Figure 2. Polyacrylamide gel electrophoresis analysis the recombinant Vip3Ag4 protein expressed in B. megaterium. M) Molecular weight marker (BioRad), A) Cellular pellet, B) Cellular supernatant with soluble Vip protein (after sonication). F1, F2 and F3 purified soluble fractions of 6× His-tagged Vip3Ag4 protein (Protino Ni-TED 1000).

3.4. Fixation, counting and viability analysis

The amount of viable B. megaterium cells after induction was $1x10^8$ CFU/ml. Approximately 5g (wet weight) of pellet per 500 ml of culture medium were obtained. Once the cells were fixed with 1 % lugol, they were unable to grow on LB Tc10 medium.

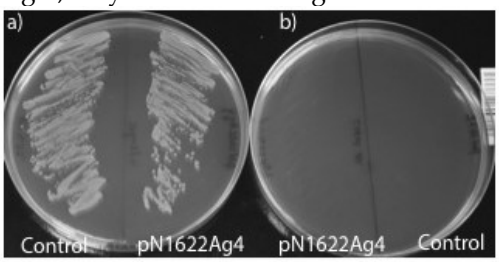

Figure 3. Viability analysis in Petri plates with Lb Tc10 cells of B. megaterium pN1622Ag4 control. a) Untreated cells and b) Fixed (killed) cells with Lugol 1 %.

3.5. Preliminary bioassays

Plate (preliminary) bioassays showed that induced cells (lugol fixed) maintained functional Vip3Ag4 protein, which produced above 95 % mortality against first instar larvae of Chrysodeixis chalcites, Helicoverpa armigera, Spodoptera frugiperda, S. exigua, S. littoralis and Trichoplusia ni (Figure 4).

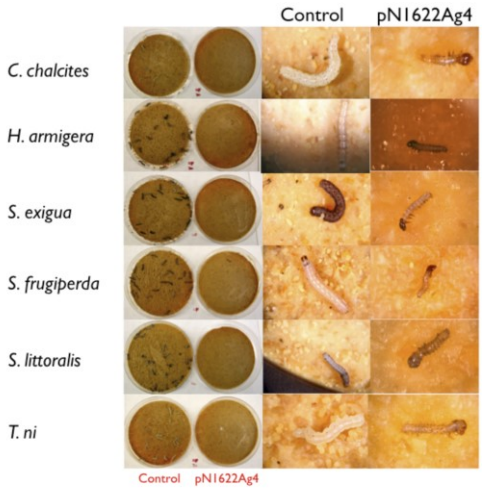

Figure 4. Plate assay performed with *B. megaterium* control (empty plasmid) and cell-encapsulated Vip3Ag4 protein after 5 days.

4. Discussion

Bacillus thuringiensis synthesizes an extraordinary diversity of insecticidal proteins and has demonstrated its potential and safety as a biocontrol agent for more than four decades [2,26]. These insecticidal proteins include both crystal and secretable proteins highly toxic against a wide range of invertebrate species [1,27-29]. Several natural *B. thuringiensis* strains have been incorporated successfully in the production of sprayable biopesticides wherein the active ingredient is a mixture of spores and crystal proteins. Genes encoding insecticidal proteins have also been used in novel insecticidal formulations and in the construction of transgenic crops [30]. However, vegetative or secreted insecticidal proteins (Vpb1/Vpa2, Vip3 and Vpb4) are synthesized during the vegetative growth phase and subsequently secreted and diluted into the culture medium [8,9,31]. This feature has prevented the production of Bt-based biopesticide sprays where the active ingredient is constituted by a secretable insecticidal protein, limiting their production exclusively to transgenic crops, and suggesting that novel methods must be used in order to develop Vip3-based formulations suitable for the biocontrol of insect pests [32]. One option, successfully assessed in this work, was the expression and encapsulation of active vegetative insecticidal protein Vip3Ag4 in the asporogenic mutant strain WH320 of *B. megaterium* (Mobitec). This bacterium is able to produce high levels of intracellular recombinant proteins, it doesn't produce significant amounts of alkaline proteases [19], is suitable for the production of proteins from small to industrial scale [21] and is non-pathogenic [33]. Moreover, this expression system has a wide range of plasmids suitable for the production of not only intracellular but also extracellular recombinant proteins that could be induced with low-price substrates as xylose. In this work, we demonstrated for the first time that the gene encoding a Vip protein could be successfully expressed in this asporogenic strain of *B. megaterium* using xylose as a low-cost inductor of protein expression and retaining toxic activity against susceptible lepidopteran larvae after fixing the cells with lugol. Additional studies need to be performed in order to describe the protection potential of this method over abiotic factors including UV light, storage conditions (e.g., time of storage, optimum temperatures, salinity, and pH). However, the fact that the recombinant

Vip3Ag4 protein was successfully produced in a soluble and an active form in this bacterium, with a low-cost inductor, suggest that *B. megaterium* is one of the heterologous hosts of choice for the commercial production of Vip proteins from *B. thuringiensis* in "killed microbial pesticides".

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