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Vegetative insecticidal protein of *Bacillus thuringiensis* BLB459 and its efficiency against Lepidoptera

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ABSTRACT

Bacillus thuringiensis strain BLB459 supernatant showed a promising activity against Lepidopteran pests with extremely damages in the larvae midgut. Investigations of the genes that encode secreted toxin demonstrated that this strain harbored a *vip3*-type gene named *vip3*(459). Based on its original nucleotide and amino acid sequences, this gene was cloned into pET-14b vector and overexpressed in *Escherichia coli*. The expressed protein was purified and tested against different insects and interestingly the novel toxin demonstrated a remarkable activity against the stored products pest *Ephestia kuehniella* and the polyphagous insects *Spodoptera littoralis* and *Agrotis segetum*. As demonstrated, the acute activity of Vip3(459) protein against *A. segetum* can be due to its original amino acids sequence and the putative receptors of this toxin in the larvae midgut. These results demonstrated that this Vip3 toxin showed a wide spectrum of activity against Lepidoptera and support its use as a biological control agent.

1. Introduction

Bacillus thuringiensis is a Gram-positive bacterium which produces, during sporulation, crystalline inclusions containing one or more delta-endotoxins (Bravo et al., 2005). The latter are selectively toxic against a wide variety of insects (Bravo et al., 2007). These inclusions are solubilized in insect midgut, releasing proteins called δ-endotoxins that, upon proteolytic activation, exhibit a highly specific insecticidal activity (Höfte and Whiteley, 1989). In the past decades, many *B. thuringiensis* strains with different insects host spectra have been identified and their *cry* genes have been cloned in several microbes (Schnepf et al., 1998). Although industrial formulations of the Cry proteins have been used as biopesticides (Gelernter et al., 1990), most Cry proteins are not very effective in controlling some of agronomically important insects (Macintosh et al., 1990) such as the lepidopteran black cutworm (*Agrotis ipsilon*) attacking more than 50 crops including cereal grains (Rings, 1974). Extensive screening programs are being carried out by various groups to search for *B. thuringiensis* strains with new insecticidal spectra. These investigations have focused mainly on the identification of new insecticidal proteins that are expressed before and during sporulation. A second family of insecticidal proteins produced by *B. thuringiensis* during its vegetative growth phase (Vegetative insecticidal proteins: Vip) has been identified (Warren, 1997).

The Vip proteins have no sequence homology with the Cry toxins and have been classified into three groups according to their sequence homology: Vip1, Vip2 and Vip3. Vip1 and Vip2 proteins act as binary toxin and are toxic to Coleoptera (Shi et al., 2004) whereas Vip3 proteins are active against Lepidoptera. Genes coding for these type of proteins have been found to be very common among *B. thuringiensis* isolates (Bhalla et al., 2005; Abdelkefi-Mesrati et al., 2005; Beard et al., 2008; Hernández-Rodríguez et al., 2009; Yu et al., 2011).

The *vip3A* gene encodes an 88.5 kDa protein that is secreted into the supernatant fluid by *B. thuringiensis* cultures (Estruch et al., 1996). This protein possesses insecticidal activity against a wide spectrum of lepidopteran insects and displays acute bioactivity toward *Agrotis ipsilon*. Indeed, the activity of Vip3A protein against

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Agrotis ipsilon was 260 times higher than some of Cry1A proteins against this insect (Rings, 1974; Warren, 1997).

As the environment is diverse, hence the insecticidal proteins are also diverse showing differential insecticidal activities. Therefore, it is necessary to screen more *B. thuringiensis* isolates to clone and characterize *vip* genes and their variants. The screening of 200 *B. thuringiensis* collection permitted the selection of a promising strain named BLB459 having an exceptional *cry* genes content (Boukedi et al., 2016a). In the present study, and to identify the origin of the potentiality of the secreted proteins of BLB459 strain in controlling lepidopteran pests, we decided to investigate the *vip3* genes content and the effects of the corresponding protein on *E. kuehniella*, *S. littoralis* and *A. segetum* larvae.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. thuringiensis BLB459, known by its original *cry* genes content (Boukedi et al., 2016a), was used in the present study. For routine use in the laboratory, *B. thuringiensis* strains were grown in Luria-Bertani medium at 30 °C with shaking at 200 rpm.

To promote protein production during the vegetative stage, 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] (Donovan et al., 2001) at 30 °C then culture was centrifuged and the resulting supernatant was used for bioassays against Lepidopteran pests.

Acrystalliferous isolates (Cry-, Spo+) were obtained by plasmid curing from the *B. thuringiensis* BLB459, by cultivating the wild strain at 42 °C for five days.

2.2. Protein quantification

The concentrations of soluble proteins found in *B. thuringiensis* supernatants and purified Vip3(459) toxin were measured with the Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

2.3. Bioassays

A free ingestion technique was used to assess the toxicity to *E. kuehniella*, *S. littoralis* and *A. segetum* larvae of *B. thuringiensis* supernatant extracts and purified Vip3(459) protein as described by Abdelkefi-Mesrati et al. (2011a,b) and Boukedi et al. (2015). The experiments were replicated three times with the presence of a negative control set maintained in the same conditions of temperature 23 °C, relative humidity of 65% and a photoperiod of 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 (Venables and Smith, 2004).

2.4. Preparation and sectioning of insect tissues

After exposure to the *B. thuringiensis* supernatant extract, *S. littoralis* larvae were placed in formol solution (10%) then tissues were dehydrated using increasing ethanol concentrations and rinsed in 100% toluene solution. After paraffin wax embedding, five-micrometer sections were prepared and placed on slides coated with a mix of 1.5% egg albumin and 3% glycerol in distilled water. Then, tissues were de-paraffinated using 100% toluene solution and slides were stained with hematoxylineosin as described by Ruiz et al. (2004).

2.5. DNA extraction and PCR amplification

DNA was extracted from *B. thuringiensis* strains using the alkali lysis method including a lysozyme treatment step as described by Sambrook et al. (1989). Based on the published sequences of *vip3*-type genes, different oligonucleotides were designed (Table 1). In the PCR reaction, V1 and V2 primers (Table 1) were used for the detection of *vip3*-like genes using DNA extracted from *B. thuringiensis* strains as template and a "Gene Amp PCR System 2700" (Applied Biosystems) (Jaoua et al., 1996).

Using *B. thuringiensis* DNA, iQ SYBER Green Supermix (BIORAD) and (V13/PS21) primers (Table 1), Real-time quantitative PCR reactions were accomplished to amplify *vip3*-type genes. The amplification and detection of PCR products were performed with C1000 thermal cycler (Bio-Rad) during 45 cycles (Wielinga et al., 2011).

2.6. Cloning and sequencing of vip3-type gene

The putative *vip3*-type gene of BLB459 was PCR amplified, using total DNA isolated from *B. thuringiensis* strain as template, primers V1 and V3 (Table 1) and DNA polymerase (Amersham). The amplified fragment, with a molecular weight of about 2.37 kb, was purified from the agarose gel. Then, the *vip3* open reading frame (ORF) was cloned in pGEM-Teasy vector (Promega) generating a recombinant plasmid pGEM*vip3*(459). *E. coli* cells (Top10) transformation was performed as reported by Sambrook et al., (1989), then selection of *E. coli* transformants was performed on LB medium plates containing ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (40 μg/ml) and Isopropyl β-D-thio-galactoside (80 μg/ml).

The *vip3*(459) gene sequencing was carried out using the recombinant plasmid, the taq DyeDeoxy Terminator Cycle Sequencing kit and a 3700 ABI Prism DNA sequencer (Applied Biosystems, Foster City, CA) according to instructions of the manufacturer. Then, the obtained 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(459) in Escherichia coli and protein purification

Overexpression of *vip3*(459) (GenBank Accession No. JN990981) was achieved by cloning the ORF in the pET-14b vector (Novagen). Restriction enzyme sites *Nde*I and *BamH*I were created by PCR, respectively, upstream the initiation codon (ATG) and downstream the stop codon of the *vip3* gene using primers VipM1 and VipM2 as described by Abdelkefi-Mesrati et al. (2009). After being cloned into pGEM-Teasy vector (Promega), the 2.37 kb fragment (ORF) was recovered by digesting it with *Nde*I and *BamH*I restriction enzymes then cloned in frame in its 5' end with the His-tag sequence of the *E. coli* expression vector pET-14b (Novagen). The recombinant plasmid, named pET-*vip3*(459), was transformed into *E. coli* BL21(DE3) then recombinant cells were grown Luria-Bertani medium supplemented with 100 μg/ml ampicillin and induced using IPTG, as described by Abdelkefi-Mesrati et al. (2009).

The cell pellet was resuspended in sonication buffer [PBS 19 (pH 7.5); 4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride] then sonicated (Abdelkefi-Mesrati et al., 2009) and centrifuged. To purify the Vip3(459) toxin fused with six histidine, the supernatant was loaded onto His-Trap column (Amersham) preequilibrated with a binding buffer (PBS 1X, imidazole 40 mM) then bound proteins were eluted using elution buffers containing increasing concentrations of imidazole in PBS 1X (Abdelkefi-Mesrati et al., 2009).