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Nutrient conditions determine the localization of *Bacillus thuringiensis* Vip3Aa protein in the mother cell compartment

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Summary

Vip3Aa was first identified as a protein secreted during the vegetative growth phase of *Bacillus thuringiensis* (Bt) bacteria and which shows high insecticidal toxicity against lepidopteran insect pests (Estruch *et al*., 1996). Bt strains formulated as bio-insecticides only had low amounts of Vip3Aa secreted to the medium. Here, we report that Vip3Aa proteins produced by three different Bt strains, including an industrial strain, were indeed not secreted to the culture solution when grown in sporulation medium, but were retained in the mother cell compartment. In order to further investigate the Vip3Aa secretion and location, we grew the strains in rich medium. We found that in rich medium, a fraction of Vip3Aa was secreted, suggesting that Vip3Aa secretion is nutrient-dependent. Regardless of the growth conditions, we found that Vip3Aa retained in cell pellets exhibited high toxicity against *Spodoptera frugiperda* larvae. Hence, we speculate that the accumulation of Vip3Aa protein in the mother cell compartment under sporulation conditions could still be used as an efficient strategy for industrial production in commercial Bt strains.

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Introduction

Bacillus thuringiensis (Bt) bacteria grow exponentially and produce Vip proteins during the vegetative phase of growth under nutrient-rich conditions. In response to nutrient limitation, the bacteria enter a transition state and initiate sporulation to produce crystal proteins (Cry and Cyt) (Lereclus *et al*., 2000). The widespread agricultural use of these insecticidal proteins, as either Bt sprays or Bt crops, has led to the development of insect resistant to several Cry proteins, thereby posing a serious threat to the efficacy of this technology (Tabashnik and Carrière, 2017). However, thus far no resistance to Vip proteins has been reported (Tabashnik and Carrière, 2017; Tabashnik and Carrière, 2020). Although Cry and Vip toxins affect the same cells in the larval midgut tissue, these proteins have no structural homology and bind to different receptors in the larval midgut (Lee *et al*., 2003; Wang *et al*., 2018; Núñez-Ramírez *et al*., 2020), and no cross-resistance has been observed between these two proteins (Carrière *et al*., 2015; Chakroun *et al*., 2016a,b).

Vip3Aa insecticidal proteins show high toxicity to multiple lepidopteran insect pests (Chakrabarty *et al*., 2020; Shao *et al*., 2020). This protein is synthesized during the vegetative growth phase and is secreted into the medium (Estruch *et al*., 1996). However, the secretion of Vip3Aa is differentially regulated among Bt strains, and the duration of the presence of these proteins in the supernatant is variable (Cai *et al*., 2006; Argôlo-Filho and Loguercio, 2018). In addition, Vip3Aa is stabilized and protected by the P20 chaperone protein, allowing it to accumulate after exponential growth (Shi *et al*., 2006; Yu *et al*., 2011).

Industrial production of insecticidal proteins from Bt entails the production of large amounts of Bt biomass with high numbers of spores and entomopathogenic crystals composed of Cry proteins using low-cost medium and simple fermentation procedures (Jouzani *et al*., 2017; Rojas *et al*., 2018). However, the secretion of Vip3Aa into the medium results in low protein

concentrations, limiting its use as a formulated bioinsecticide (Hmani *et al*., 2018). In this study, we analysed the secretion of Vip3Aa protein in two native Bt strains, a control strain 4AP1 and an industrial strain KN11, as well as a biotechnological GFP-Vip3A transformed Bt HD73$^-$ strain. We found that in these strains, under sporulation conditions in which nutrients are limited, the Vip3Aa protein is not secreted but accumulates in the pellet containing the debris of the mother cell compartment. However, this protein was secreted into the medium when the cells were cultured in medium richer in nutrients. The GFP-Vip3A located in the pellet exhibited high toxicity against *S. frugiperda* larvae, suggesting that the Vip3Aa protein accumulated under sporulation conditions could be useful for efficient, cost-effective and easier industrial production of Vip3Aa containing strains.

Results

Vip3Aa protein is neither secreted into the medium nor accumulated in the crystal inclusions of Bt serovar fukuokaensis *4AP1*

We analysed the production of Vip3Aa in a standard strain, Bt serovar *fukuokaensis* 4AP1. This strain was cultured in sporulation medium (½ LB) to analyse secretion of Vip3Aa into the medium was analysed after incubation for various time periods, from 12 to 60 h. Even when the supernatant was concentrated 50-fold, no Vip3Aa protein was detected, as observed by Western blot analysis using anti-Vip3A antibody (upper panel of Fig. 1A). However, we found that this protein was present in the pellet that contained crystal inclusions and debris from the mother cells (lower panel of Fig. 1A). The crystal inclusions were purified and observed under a light microscope (Fig. 1B) and a scanning electron microscope (SEM) (Fig. 1C). The 4AP1 crystals appeared ellipsoidal (Fig. 1B and C). Western blot assays of this purified crystal sample showed that the Vip3Aa protein was not accumulated in the crystals (Fig. 1D). These crystals contained Cry9Aa protein, as observed in Western blot assays using an anti-Cry9A antibody (Fig. S1). Thus, these results strongly suggest that Vip3Aa is associated with the mother cell debris when bacteria were cultured in sporulation medium (½ LB).

GFP-Vip3Aa fused protein was located in the cytoplasm and mother cell debris

To confirm the location of Vip3Aa protein during Bt growth under sporulation conditions, the *vip3Aa* gene was cloned from the 4AP1 strain and fused to green fluorescent protein (GFP). The constructed plasmid, p1Ac′*gfp-vip3A*, was transformed into the acrystalliferous Bt strain HD73 (HD73$^-$).

Analysis of fluorescence under a microscope showed that the GFP-Vip3Aa protein was located in the cytoplasm of the cells after 12 h of growth in ½ LB (Fig. 2A). After 24 h of growth, spore formation was evident, and some GFP-Vip3Aa was detected in the mother cell compartment (Fig. 2A). After 36 h of growth, the sporangium lysed, releasing spores (arrowhead in Fig. 2A); however, no fluorescent signal was detected in the spores. At 48 and 60 h, the GFP-Vip3Aa protein was retained in the mother cell debris (arrows in Fig. 2A). Western blot revealed that most GFP-Vip3As was present in the pellet throughout growth, except at 24 h (upper panel of Fig. 2B), whereas GFP-Vip3A proteins were only weakly detected in the supernatant of the culture medium at 12 h or 60 h (arrows in the lower panel of Fig. 2B). Two protein bands corresponding to the size of full-length GFP-Vip3Aa (~110 kDa) and cleaved Vip3Aa protein (~88 kDa) were detected in the supernatant with the anti-Vip3Aa antibody. These results confirmed that when Bt was cultured in ½ LB sporulation medium, the Vip3Aa protein was expressed in the cytoplasm during the vegetative growth phase and was associated with the mother cell compartment during sporulation.

Vip3Aa protein produced by the commercial Bt strain KN11 was associated with the mother cell compartment

The localization of Vip3Aa protein in the pellet containing mother cell compartment debris could have important implications for the use of Vip3Aa as a bioinsecticide. Therefore, we analysed the location of Vip3Aa under sporulation conditions in a Chinese commercial Bt strain, KN11, which contains the insecticidal genes *vip3Aa*, *cry1Ac*, *cry1Ia* and *cry2Ab* (Liu *et al*., 2019). This strain was cultured in ½ LB sporulation medium for 12–60 h. Light microscopic observation showed that after 12 h of growth, the KN11 strain was in the vegetative phase (Fig. 3A). After 24 h of growth, sporulation began, and spore formation was observed at one end of each cell. After 36 h of growth, we observed the release of spores (arrows in Fig. 3A). Released crystals were clearly observed after 48 h of growth (arrowheads in Fig. 3A). Western blot analysis using an anti-Vip3Aa antibody confirmed that the 88-kDa Vip3Aa protein was preferentially accumulated in the pellet samples, principally after 36 h of growth (Fig. 3B). Under these conditions, after 12 h of growth, only a weak signal of Vip3Aa was observed in the 50-fold-concentrated supernatant samples (Fig. 3B).

Secretion of Vip3A protein is regulated by metabolic growth conditions

Previously, it was shown that Vip3Aa protein was secreted into the medium when the cells were cultured

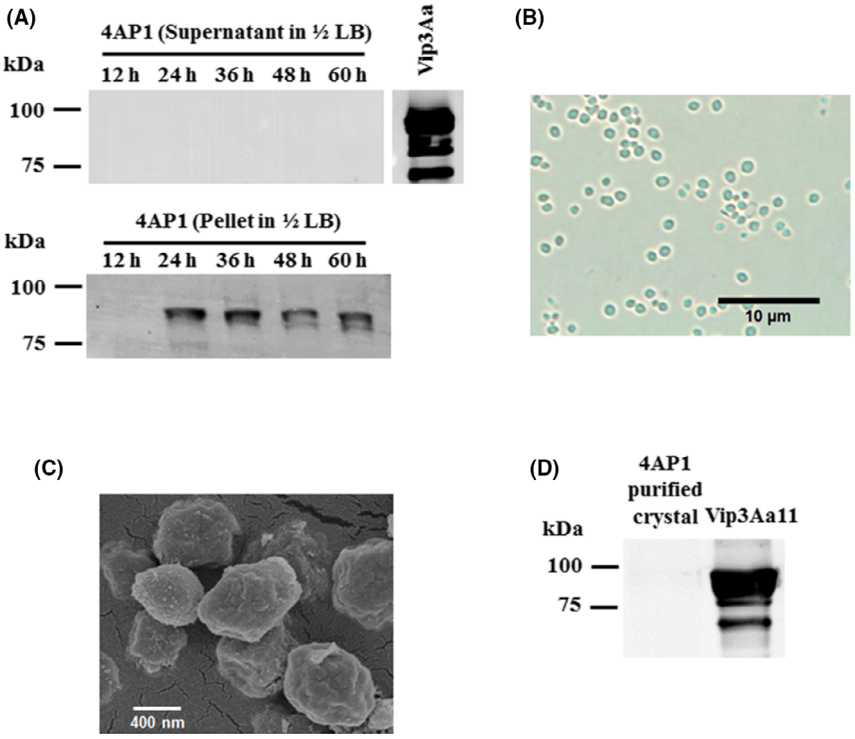

Fig. 1. Expression of Vip3Aa in Bt 4AP1 strain grown in sporulation ½ LB medium.A. Upper panel, Western blot analysis of 50-fold-concentrated supernatant of 4AP1 strain by using anti-Vip3A antibody. Lower panel, Western blotting analysis of pellet samples of 4AP1 strain by using the same antibody.B. Light microscopy of purified crystals of 4AP1 strain.C. Scan electronic microscopy of purified crystal of 4AP1 strain.D. Western blot analysis of purified crystals of 4AP1 strain detected with anti-Vip3A antibody. Vip3Aa11 protein was used as a positive control.

in a medium richer in nutrients, such as Terrific broth (Estruch *et al*., 1996; Chakroun *et al*., 2016a). To analyse the potential effect of growth conditions on the secretion of Vip3A, the KN11 strain was cultured in a medium rich in nutrients (complete LB). Light microscopic observation showed that the sporulation process was delayed by approximately 12 h since the vegetative growth phase was extended up to 36 h under these conditions, and released spores were observed after 48 h (Fig. 4A). Comparative analysis of Vip3Aa production in the pellet and 50 fold-concentrated supernatant by Western blotting showed that the pellet samples had lower Vip3Aa accumulation than the supernatant, particularly at 24–36 h of growth (Fig. 4B). These results indicated that bacterial growth in a medium richer in nutrients, such as complete LB medium, induces the secretion of Vip3Aa into the medium during the vegetative phase of growth.

To confirm that the secretion of Vip3Aa into the medium depends on the nutrient conditions, we analysed the secretion of GFP-Vip3Aa from the HD73$^-$ transformant strain when cultured in complete LB medium. The fluorescence microscopy images showed that the GFP-Vip3Aa protein was expressed in the cytoplasm of these cells at 12–60 h of growth and it was absent in the spores (Fig. 5A). Western blot analysis revealed that little Vip3Aa protein was associated with the pellet samples at 12 and 60 h of growth (arrows in the upper panel of Fig. 5B); however, it was clearly detected in the supernatant at 12–60 h (lower panel of Fig. 5B). We observed cleaved Vip3A bands (~68 kDa) with an anti-Vip3Aa antibody in the Western blot assays of the supernatant (arrows in the lower panel of Fig. 5B), suggesting that Vip3Aa is easily processed after secretion into the medium. The same degraded bands also observed when Vip3A proteins in KN11 were secreted into medium (Figs 3B and 4B). These analyses indicated that Vip3Aa is principally secreted into the medium when Bt bacteria are cultured in medium richer in nutrients, such as complete LB (Figs 4B and 5B), in contrast to ½ LB sporulation medium (Figs 1A, 2B and 3B).

Vip3Aa protein present in the pellet of the mother cell debris shows high insecticidal activity against Spodoptera frugiperda

The HD73$^-$ transformant strain expressing GFP-Vip3Aa protein was cultured in sporulation medium, and the

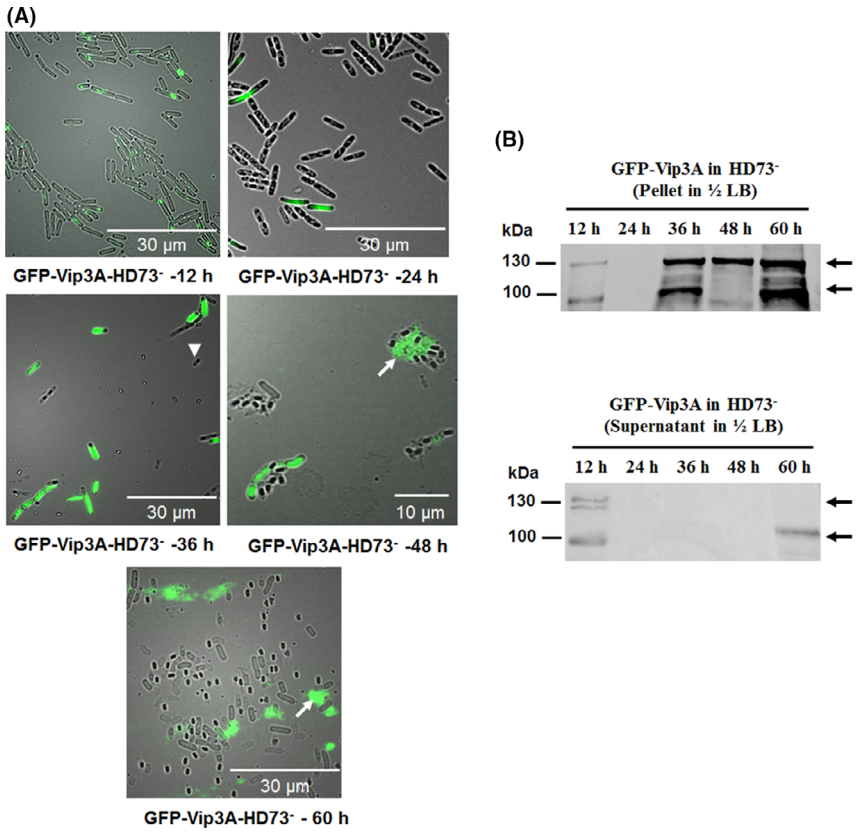

Fig. 2. Analysis of GFP-Vip3Aa fused protein expressed in HD73- transformant strain grown in ½ LB medium.A. Fluorescent microscopy of GFP-Vip3Aa fused protein expressed in transformed HD73- strain grown for different time periods. The arrows showed GFP-Vip3A proteins, and arrowhead showed the spore.B. Western blot analysis of protein in the pellet samples (upper panel) and in the concentrated supernatant samples (lower panel) detected with anti-Vip3A antibody. The arrows showed GFP-Vip3A and Vip3A cleaved proteins.

pellet containing the mother cell debris with the GFP-Vip3Aa protein, was suspended in 50 mM Na_2CO_3 buffer (pH 10). The concentration of Vip3Aa in this sample was 75 μg ml^{-1}. Toxicity assays against *S. frugiperda* neonates using GFP-Vip3Aa at two different concentrations (1 and 15 μg per gram of diet) showed mortality rates of 10.9% and 91.65%, respectively. The KN11 stran, used as a postive control, showed high insecticidal activity at both concentrations (75.8% and 96.2% mortality at 1 and 15 μg per gram of diet respectively). The negative control, GFP expressed in HD73$^-$ at the highest concentration (15 μg per gram of diet), showed only 3% mortality (Fig. 6).

Discussion

In this study, we showed that Vip3Aa protein produced by two different Bt strains, 4AP1 and KN11, was not secreted and remained attached to the mother cell debris when cultured under sporulation conditions, but was secreted when KN11 was cultured in a complete rich medium that delayed the sporulation process. We constructed a GFP-Vip3Aa fusion protein to analyse its localization. Although the *vip3Aa* gene in the GFP-Vip3Aa construct was expressed under the regulation of the *cry1Ac* promoter, this fusion protein showed a similar secretion pattern to the Vip3Aa protein of the two wild-type Bt strains analysed in this work. The expression of *vip3A* gene during both vegetative growth and sporulation phase corroborates the previous results (Perez-Garcia *et al*., 2010; Yang *et al*., 2012). These data suggest that the effect of nutrient conditions on Vip3A protein secretion is independent of gene expression controlled by the promoter.

Western blot revealed that the GFP-Vip3A was absent in the cultural pellet at 24 h when GFP-Vip3A transformant HD73$^-$ cultured in ½ LB, which could be attributed to the small amount of GFP-Vip3A that could not be recognized by the antibody, although it was observed under a fluorescent microscope. It is also noticed that the Vip3A protein was present in the supernatant after 12 h of growth, when the GFP-Vip3A containing HD73$^-$ and KN11 strains were cultured in ½ LB sporulation medium. This could be because the relatively high nutrient levels

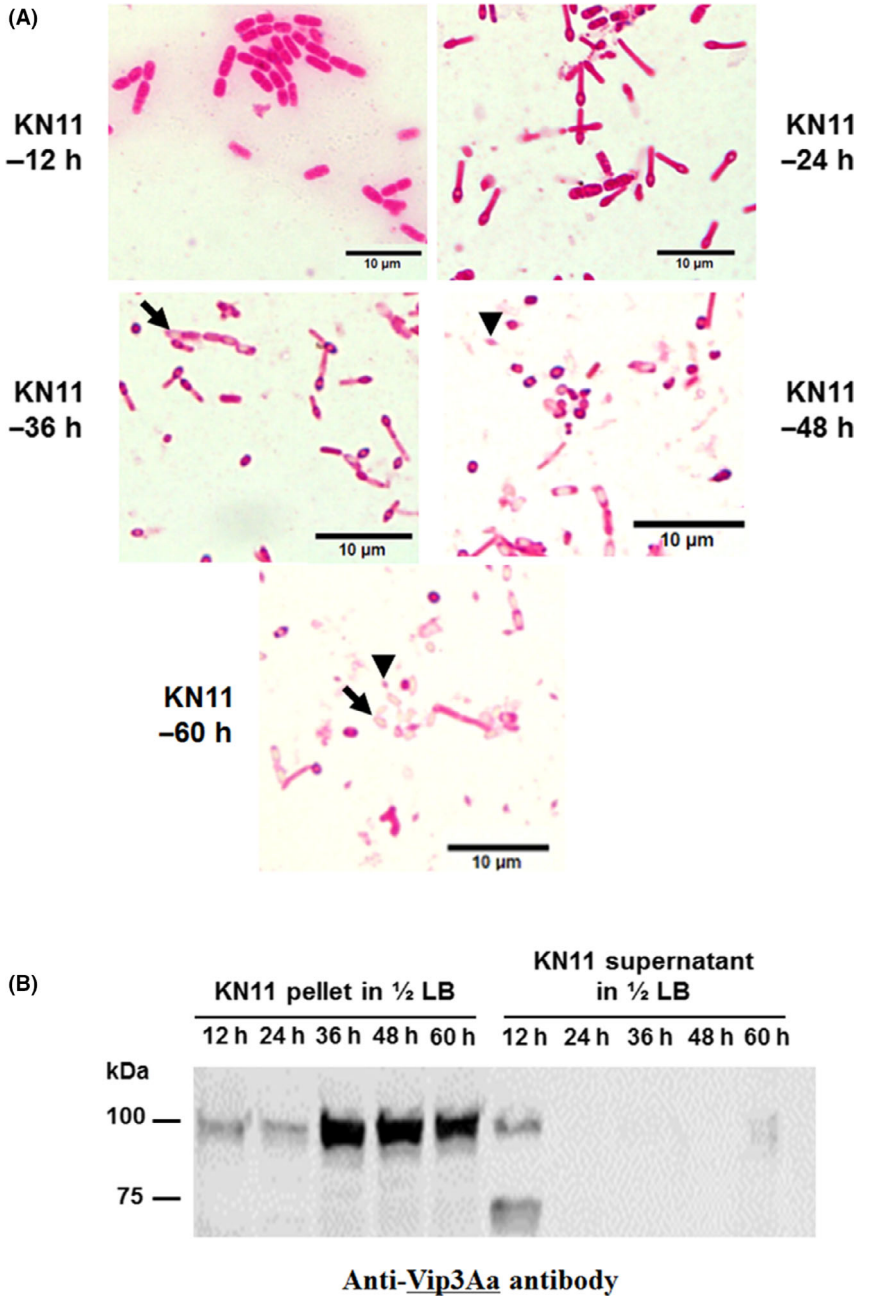

Fig. 3. Analysis of Vip3Aa protein expressed in the industrial Bt KN11 strain grown in sporulation ½ LB medium.A. Light microscopy of KN11 strain stained with carbolfuchsin (crystals are pointed with arrowhead and spores with arrows).B. Western blot analysis of protein expressed in the pellet and the concentrated supernatant samples by using anti-Vip3A antibody.

present during 12 h after transfer of Bt strains to ½ LB sporulation medium. Otherwise, the detection of Vip3A in supernatant at 60 h could be owing to most mother cells of Vip3A containing HD73^{-} were lysed and some of the Vip3A proteins might be released and dissolved into the medium. The handicap principle applied to biofilms in *Bacillus subtilis* could give another explanation for differential expression of Vip3A proteins. The secretion of signals within the microbial communities is condition-dependent (Harris and Kolodkin-Gal, 2019). The expression of Vip3A protein might be regulated by some secreted factors responding to extreme environmental conditions such as nutrient limitation.

A number of proteases are secreted into the culture medium by *Bacillus* strains during growth (Harwood and Cranenburgh, 2008). Vip3Aa protein is sensitive to

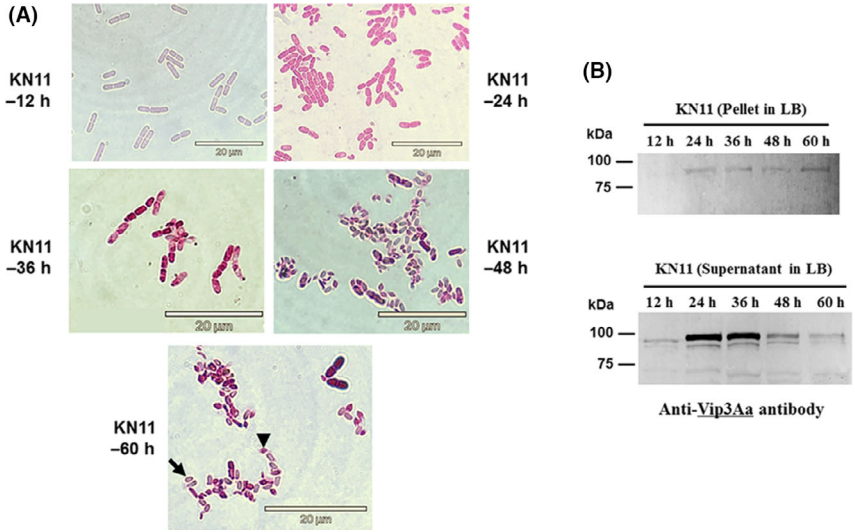

Fig. 4. Analysis of Vip3Aa protein expressed in the industrial Bt KN11 strain grown in LB medium.A. Light microscopy of KN11 strain stained with carbolfuchsin (The spore and crystal were pointed with an arrow and arrowhead respectively).B. Western blot analysis of proteins expressed in the pellet (upper panel) and in the supernatant (lower panel), and samples were detected by using anti-Vip3A antibody.

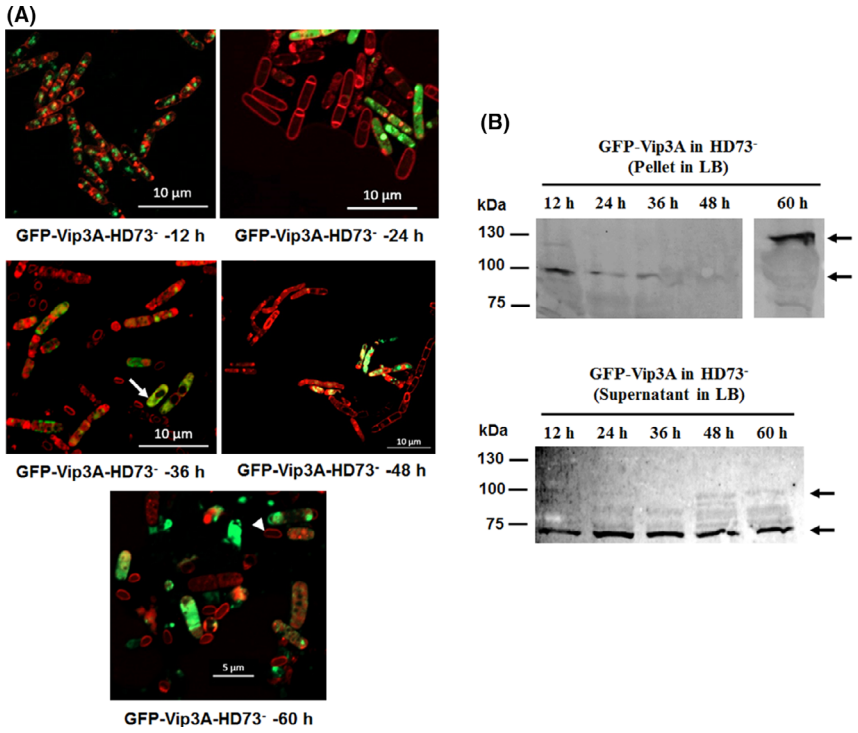

Fig. 5. Analysis of GFP-Vip3Aa fused protein expressed in HD73⁻ transformant strain grown in LB medium.A. Confocal laser scanning microscopy of GFP-Vip3Aa fused protein expressed in HD73⁻ transformant strain grown for different time periods. Cells were stained with lipophilic probe FM4-64 staining dye that selectively stain cytomembranes with red fluorescence. The arrows showed GFP-Vip3A protein, and arrowhead showed spore.B. Western blot analysis of protein expressed in the pellet samples (upper panel) and in the concentrated supernatant samples (lower panel) detected with anti-Vip3A antibody. The arrows showed GFP-Vip3A and Vip3A cleaved proteins.

proteases and was found to be cleaved by SDS-PAGE (Caccia *et al*., 2014; Bel *et al*., 2017). Thus, it is possible that GFP-Vip3Aa is processed after secretion into the medium or is sensitive to the proteases secreted from Bt when separated by SDS-PAGE, leading to degraded bands were detected in Western blot. However, the

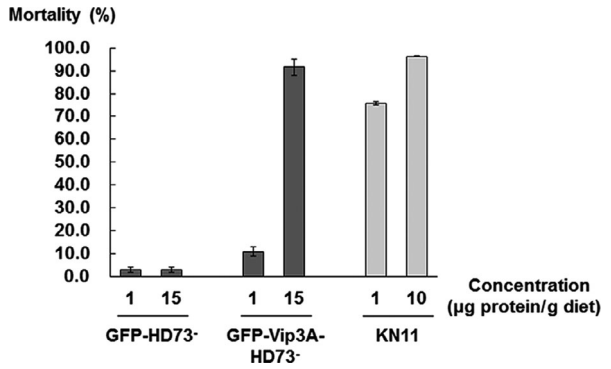

Fig. 6. Insecticidal activity of Bt strain expressing GFP-Vip3Aa protein and KN11 strain in ½ LB sporulation medium against *S. frugiperda* neonates. GFP expressed in HD73- transformant strain was used as negative control obtained with same method as the GFP-Vip3Aa containing strain.

GFP-Vip3A or Vip3A protoxin associated in the cultural pellet can be detected no matter in HD73- or in KN11 cultured in sporulation conditions, indicating that the Vip3A proteins were relatively stable in pellet. This might be resulted from the strain pellet protect the Vip3A protein from proteases in cultural medium. More importantly, the association of Vip3A in the pellet of cultural products gives possibility to achieve it together with Cry proteins in Bt strains (Fig. S1) during commercial production.

Unlike most secreted proteins, Vip3Aa is not N-terminally processed during secretion into the medium (Estruch *et al*., 1996; Chakroun *et al*., 2016a). However, a previous study indicated that Vip3V (Vip3Aa) protein cloned from a Bt subsp. *kurstaki* strain contained a putative signal peptide, and a cleavage site was predicted between amino acids 10 and 11 (Doss *et al*., 2002). We examined an amino acid sequence alignment of Vip3Aa sequences from different Bt strains (Estruch *et al*., 1996; Doss *et al*., 2002); however, we could not detect signal peptides in these sequences using the SignalP 5.0 server. Some studies have shown that proteins lacking any known signal peptides or secretion motifs are secreted via several non-classical pathways (Zhao *et al*., 2017; Su *et al*., 2020). For instance, an autolytic enzyme lacking signal peptides can be transported to the cell wall of *B. subtilis* (Potvin *et al*., 1988; Kuroda and Sekiguchi, 1990). However, the mechanism of Vip3Aa secretion remains unknown. Our work suggests a potential novel secretion system since Vip3Aa does not contain a canonical signal peptide and its secretion depends on bacterial growth conditions.

Overall, our results indicate that the localization of Vip3Aa in the mother cell pellet compartment could be exploited for the production of commercial Bt strains in relatively low-cost media, providing an efficient strategy to recover high concentrations of this insecticidal protein. Our results also suggest that Vip3Aa could be formulated with Cry proteins that accumulate under sporulation conditions and could be exploited as an efficient alternative for designing novel formulations for effective pest control.

Experimental procedures

Strains and culture conditions

Bt strains serovar *fukuokaensis* BGSC 4AP1 was obtained from the Bacillus Genetic Stock Center (BGSC) and acrystalliferous HD73^{-} was conserved in our laboratory. A commercial wettable powder of Bt strain KN11 (32 000 IU mg^{-1}) was obtained from Wuhan Kernel Biotech.

The 4AP1 and HD73^{-} strains transformed with p1Ac′*gfp-vip3A* were cultured in 5 ml of LB (1% tryptone, 1% NaCl and 0.5% yeast extract) for 8 h at 30°C. Then, 1 ml of this culture was transferred to 100 ml of ½ LB sporulation medium (0.5% tryptone, 0.5% NaCl and 0.25% yeast extract) or complete LB medium and cultured for varying time periods (up to 60 h) at 30°C with agitation at 220 rpm. For the KN11 strain, 100 mg of KN11 powder was suspended in 1 ml of double distilled sterile H_2O (ddH_2O), and this sample was then used as the inoculum to culture the KN11 strain in ½ LB sporulation medium or complete LB medium as described above. At the selected time points, a 100 ml sample of culture was centrifuged at 12 000 × *g* for 10 min at 4°C, and the culture supernatant was separated from the pellet. The supernatant was treated with a protease inhibitor (1 mM phenylmethylsulfonyl fluoride; PMSF of final concentration) and concentrated 50-fold (to 2 ml) by dialysis against PEG8000 for further analysis. The pellet was suspended in 10 ml of 50 mM Na_2CO_3–$NaHCO_3$ (pH 10) for Western blot analysis. As described in the 'Western blotting' section, similar volumes of pellet or 50-fold concentrated supernatant samples from each time point were used for comparative analysis of Vip3Aa production.

For 4AP1 crystal purification, the 4AP1 strain was cultured for 48 h in sporulation medium, centrifuged at 6000 × *g* at 4°C for 10 min and purified as previously reported (Mounsef *et al*., 2014). Briefly, the obtained pellet was washed twice with 1 M NaCl containing 0.01% Triton X-100 and suspended in 30 ml saline solution (0.7% NaCl) supplemented with 10% hexane (final concentration) in a 50 ml centrifuge tube. The sample was sonicated for 10 min at 40% power in 3 s pulses at 5 s intervals to dispel clumps and then centrifuged at 6000 × *g* for 10 min. The final pellet was washed once with cold distilled water and used for SDS-PAGE, light microscopy and scanning electron microscopy.

Microscopy analysis

Crystals formed by 4AP1 were observed using an Echo Revolve Hybrid Microscope (Beijing Cycloud Biotechnology). These crystals were fixed with 2.5% glutaraldehyde for 48 h and then sent to the Laboratory of Electron Microscopy at the Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences for sample preparation for observation using a scanning electron microscope (HITACH, Japan).

KN11 cells were cultured in different medium for different time periods. For analysis, 100 ml samples were collected at each time point, pelleted by centrifugation at 12 000 × *g* and washed once with double distilled water (ddH_2O). This pellet sample was suspended in ddH_2O and mixed with carbolfuchsin staining buffer for light microscopy observation using an Echo Revolve Hybrid Microscope.

The HD73$^-$ strain transformed with GFP-Vip3Aa was cultured in different medium for different time periods, and samples (100 ml) were collected by centrifugation at 12 000 × *g* and washed once with ddH_2O. For analysis, 2 μl sample cultured in ½ LB were suspended in ddH_2O for observation under Echo Revolve Hybrid Microscope, as well as 2 μl of sample cultured in LB was mixed with 1 μl of FM4-64 (100 μg ml^{-1}, Thermo Fisher Scientific) to stain the cell wall, and the samples were analysed under a confocal laser scanning microscope 980 (Zeiss, Germany).

*Construction of p1Ac′*gfp-vip3A

Plasmid p1Ac′*gfp* was constructed in our laboratory. This construct contains the *cry1Ac* promoter driving a *gfp* gene in the pHT315 vector with kanamycin resistance (Yang *et al*., 2012; Deng *et al*., 2015). The *vip3Aa51* gene was amplified using genomic DNA of the 4AP1 strain as a template in a PCR using PrimeSTAR DNA Polymerase (TAKARA Biomedical Technology [Beijing]) with primers gfp-Vip3A-F and gfp-Vip3A-R (Table S1). The linear plasmid p1Ac′*gfp* was amplified with pHT1618K-F and pHT1618K-R primers (Table S1) using Phusion Master Mix (Thermo Fisher Scientific). The *vip3A* gene was inserted into the p*1Ac′gfp* plasmid downstream of the *gfp* gene by homologous recombination using Master Assembly Mix (Clone Smarter Technologies, Beijing, China), according to the manufacturer's instructions. The final recombinant plasmid was transformed into *E. coli* DH10b competent cells; p1Ac′*gfp-vip3A* was purified and was confirmed by DNA sequencing. The plasmid was transformed into *E. coli* ET-competent cells for demethylation and then transformed into the Bt HD73$^-$ strain via electroporation (Yang *et al*., 2012).

Western blotting

An aliquot (40 μl) of the supernatant or pellet obtained from the Bt 4AP1, GFP-Vip3A containing HD73$^-$, or KN11 strain cultured for different time periods or purified crystal samples from the 4AP1 strain was mixed with 10 μl of 5× loading buffer (with β-mercaptoethanol) (Beijing Solarbio Science & Technology) and boiled for 15 min. Then, 10 μl (for 4AP1 and KN11) or 20 μl (for HD73$^-$) of the pellet was loaded on and separated by SDS-PAGE, and either stained with Coomassie brilliant blue or subjected to Western blot assays. For the assays, the separated proteins were electro-transferred to PVDF membranes. After blocking with 5% skim milk in phosphate-buffered saline (PBS) for 1 h, the membranes were probed with an anti-Vip3Aa11 polyclonal antibody (Wang *et al*., 2018) at a 1: 5000 dilution in PBS (containing 0.1% Tween-20) for 1 h at room temperature followed by incubation for 1 h with the FITC-labelled goat anti-rabbit secondary antibody (Beijing Solarbio Science & Technology) at 1:2000 dilution in PBST. Membranes were washed twice with PBST and PBS (10 min per wash) and analysed using a Typhoon 9410 Laser Scanning Imaging System.

Insecticidal bioassay

The insecticidal activity of GFP-Vip3Aa protein expressed in HD73$^-$ transformant strain and KN11 strain collected after 36-h growth were tested against *S. frugiperda* neonates. GFP protein expressed in HD73$^-$ strain was used as negative control. *S. frugiperda* eggs were obtained from the Institute of Plant Protection, Cangzhou Academy of Agricultural and Forestry Sciences, Hebei Province. The diet was obtained from Cotton Insect Research group of Institute of Plant Protection, Chinese Agricultural Academy of Sciences. The concentration of GFP-Vip3Aa, Vip3Aa (from KN11) and GFP proteins used in these bioassays was determined after SDS-PAGE analysis by using BSA as standard and quantification of the densitometry of the bands by ImageJ software. Bioassays were performed with different concentrations of Vip3Aa protein in 3 mL Na_2CO_3 buffer applied to 15 g diet in 24-well plates. Units are μg of GFP-Vip3A or GFP protein per g of diet. One larvae was placed into each well. The percentage of mortality was recorded after 7 days at 27±1°C with a 16:8 h light: dark cycle and 65±5% relative humidity. Three repetitions were performed from these bioassays.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. primers used for construction p1Ac'*gfp-vip3A* plasmid.

Fig. S1. Western blot analysis of purified crystals of Bt strain 4AP1 with anti-Cry9Aa antibody.

Fig. S2. (A) Alignment of Vip3A proteins from four *B. thuringiensis* strains. (B) Signal peptides of Vip3Aa protein from 4AP1 strain predicted by using SignalP server 5.0. (C) Signal peptides of Vip3Aa protein from KN11, AB88 or Bt subsp *kurstaki* strain (Vip3V protein) predicted by using SignalP server 5.0 (http://www.cbs.dtu.dk/services/SignalP/).

