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RESEARCH ARTICLE

Identification of a novel *enhancin-like* gene from *Bacillus thuringiensis*

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Abstract **An *enhancin-like* gene was cloned from *Bacillus thuringiensis* (*Bt*) strain GS8 isolated from soil samples in china. The sequence analysis revealed that an open reading frame (ORF) of 2202 nucleotides encoding a protein containing 733 amino acids with a molecular mass of 84 kDa. The enhancin-like protein showed 100% identity to Bel protein (FJ644935) and 23%–41% identity to viral enhancin proteins; in the 252 to 261 amino-acid sequence of enhancin-like protein, a conserved metal binding motif (HEIAH) similar to that in the reported bacterial enhancin-like proteins was found (HEXXH in viral enhancin protein), which indicated that the enhancin-like protein belongs to metalloprotease. The purified enhancin-like protein was fed together with Cry9Ea to *Spodopera exigua* and *Trichoplusia ni* larvae, but no significant increase in toxicity was observed.**

Keywords *Bacillus thuringiensis*, *enhancin-like* gene, Cry9Ea protein, synergism

Introduction

Being a ubiquitous gram-positive spore-forming soil bacterium, *Bacillus thuringiensis* (*Bt*) may produce a parasporal crystal during the stationary phase of its growth cycle (Gill et al., 1992). These crystals encoded by *cry* gene, also called *δ*-endotoxins, are the main insecticidal activity substances of *B. thuringiensis* and are highly specific to their target insects. *Bt* toxin is safe to humans, vertebrates, and plants (Schnepf et al., 1998). *Bt* has been extensively used as bioinsecticide to control crop pests in commercial agriculture and forest management and is also a key source of genes for transgenic expression to provide pest resistance in plants (Höfte et al., 1989). With further research on *Bt*, more insecticidal activity substances have been found, such as vegetative insecticidal proteins (VIPs), enhancing factor ZWA, viral enhancin proteins, etc.(Yin et al., 2007). As a synergistic factor, the viral enhancin protein was first described from the granuloviruses (GVs) of the armyworm, *Pseudaletia unipuncta* (PsunGV) (Tanada et al., 1980). Since then, synergistic activity has been associated with several GVs, and enhancin genes have been identified in several other GVs and Nucleopolyhedrovirus (NPVs) (Roelvink et al., 1995; Hayakawa et al., 1999; Li et al., 2003). Enhancin has a conserved metal binding motif, and the action mode of viral enhancin protein has been widely accepted, which has been identified as a metalloprotease (Bischoff and Slavicek, 1997). Generally, enhancin can disrupt the protective peritrophic matrix (PM), allowing virion access to the underlying epithelial cells of the insect gut (Lepore et al., 1996). The PM has a lattice structure formed by chitin and insect intestinal mucin (IIM), and viral enhancin protein targets the IIM for degradation and then facilitate GV and NPV infection and decrease larval survival time, acting as a synergic factor (Wang and Granados, 1997). Viral enhancin was also found to increase *Bt* larvicidal activities toward insect larvae (Xu et al., 2003).

Interestingly, *Bacillus cereus* group genomes were found to have a *Bacillus enhancin-like* (*bel*) gene, encoding a peptide with 20%–30% identity to viral enhancins (Galloway et al., 2005; Fang et al., 2009). Amino acid sequence alignment

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revealed that homology between bacterial and viral enhancins was high in the N-terminal region of the protein, and the HEIAH metal binding motif was conserved (HEXXH in viral enhancin protein) (Hajaij-Ellouze et al., 2006). Previous studies showed that Bel protein can enhance the toxicity of Cry1Ac protein to *Helicoverpa armigera* larvae by degrading the IIM, which is similar to the findings for viral enhancin.

In the present study, to understand the biochemical activity of *Bt* enhancin, a novel *enhancin-like* gene from *Bt* was cloned and expressed in *E. coli*, the enhancin-like protein was purified, and an engineering *Bt* strain harboring the *enhancin-like* gene was constructed. *In vivo* observations were done to show the function of the enhancin-like protein as a synergist of Cry9Ea toxicity against *Spodopera exigua* and *Trichoplusia ni* larvae.

Materials and methods

Materials

Strains, plasmids, and media

The strains and plasmids used in this study are listed in Table 1. *E. coli* strains and *Bt* strains were maintained on LB media (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1% agar) at 37°C and 30°C, respectively.

Insect rearing and virus production

Larvae of *S. exigua* and *T. ni* were obtained from laboratory colonies and reared on the artificial diet. *Trichoplusia ni granulovirus* (*TnGV*) was amplified in *T. ni* larvae, and occlusion bodies were purified using SDS incubation and repeated centrifugation. *TnGV* enhancin was purified by gel filtration chromatography with a sephacryl S-300 column (Peng et al., 1999).

Methods

Cloning of the enhancin-like gene from Bt strain

The DNA fragment containing the *enhancin-like* gene (2.2 kb) was obtained from *Bt* strain GS8 via PCR amplification with the Primer 1: GCCGGATCCATGTATACAATGTTTTTCCTC and Primer 2: GGCGAATTCTTATTCATTATATAAGCTATC, which were designed based on the *enhancin-like* gene sequence of GenBank. The reaction mixture was cycled 33 times at 94°C for 45 s; 55°C for 45 s; 72°C for 2 min. The PCR product was purified by electrophoresis, digested with *Bam*HI&*Eco*RI, and ligated with the similarly digested pET-30a vector to yield the recombinant vector, pET30a-*enhancin-like*, which has a His-tag at the C terminus of the protein to facilitate protein purification. DNA sequencing was finished by Sangon (Shanghai, China).

Sequence analysis

Annotation, comparison, and alignment of sequences were performed using the National Center for Biotechnology Information BLAST search services and Clustal Alignment Program.

Expression of the enhancin-like gene in E . coli

The recombinant expression vector, pET30a-*enhancin-like*, was transformed to the *E. coli* strain BL21 (DE3), resulting in BL21 (pET*enhancin-like*). When OD_{600} reached 0.6, the BL21 (pET*enhancin-like*) cultures were induced by 1 mmol/L Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were further cultivated at 37°C and then harvested by centrifugation at 4°C at 10000 r/min. The cell pellets were resuspended in a buffer (20 mmol/L Tris-HCl, 0.5 mol/L NaCl, pH 7.5), and the pellets were collected. Finally, the expressed products were separated and identified with routine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al., 1992).

Purification of soluble enhancin-like protein and antibody production

All purification procedures were performed at 4°C. The cells were resuspended in the buffer (20 mmol/L Tris-HCl, 0.5 mol/L NaCl, pH 7.5) and disrupted by sonication on ice; the cell debris was removed by centrifugation. Urea solution was added, and the precipitate was removed by centrifugation. The entire sample was loaded onto a Ni-nitrilotriacetic

Table 1 Strains and plasmids used in this study

Strains and plasmids	Characteristics	Origin
Strains		
E. coli TG1	*supE hsd*Δ5 *thi* Δ*(lAc-proAB)* F' [*traD*36 *proAB*$^+$ *lAcI*^{q}*lAcZ*Δ*M*15]	stored in this laboratory
SCS110	*Rpsl thr leu endA dcm supE44 proAB*	by Dr. Zhang Jie
E. coli BL21	F- *omp*T *hsd*SB(rB- mB-) gal *dcm* λ(DE3)	stored in this laboratory
HD73$^-$	*Bt* acrystalliferous mutant strain	by Dr. Zhang Jie
GS8	Wild strain	stored in this laboratory
HD9EA	*Bt* strain, HD73$^-$with pSXY-9EA	stored in this laboratory
Plasmids		
pET30a	Kna^R *E. coli* expression vector, 5.4 kb	stored in this laboratory
pSXY422b	Amp^R Er^R *Bt-E. coli shuttle* expression vector	by Dr. Zhang Jie

acid (Ni-NTA) agarose column that had been equilibrated in sonication buffer. The flow rate was maintained at 0.5 mL/min. Enhancin-like protein was eluted using 15 mmol/L, 60 mmol/L, or 500 mmol/L imidazole, respectively. Approximately, 10 mL enhancin-like protein was purified, fractions were analyzed by SDS-PAGE, and the protein concentration was determined by the Bradford assay (Sangon of Shanghai, China).

Antiserum was collected after the boost injections. Preimmune serum collected from the same rabbit prior to immunization was used as a control. Preparation of antiserum was done by Biologic Academy of Sciences of Hebei.

Construction of an engineering strain HD73⁻(pSXY422b-enhancin-like)

Plasmid pET30a-*enhancin-like* was digested with *Bam*HI& *Eco*RI, and the resultant 2.2 kb fragment containing the *enhancin-like* gene was cloned into the same sites in the *Bt-E. coli* shuttle vector pSXY422b (Song, 2001) to generate recombinant plasmid pSXY422b-*enhancin-like*. Next, plasmid pSXY422b-*enhancin-like* was transformed into *E. coli* SCS110 to demethylated. Finally, pSXY422b-*enhancin-like* purified from *E. coli* SCS110 was transformed to *Bt* acrystalliferous mutant HD73⁻ by electroporation (Macaluso and Mettus, 1991).

Analysis of expression of enhancin-like gene in the engineering HD strain

The strain HD73⁻(pSXY422b-*enhancin-like*) was cultured in 1/2 LB medium at 30°C with agitation (Liu et al., 2009) for 18 h, and the cells were collected every two hours. Cell extracts were prepared by resuspending cell pellets in 50 μL of sterilized water and 50 μL of 2 × SDS sample buffer. Samples were loaded on a denaturing gradient acrylamide gel, and the proteins were separated by SDS-PAGE. For Western blotting analysis, proteins were transferred from the acrylamide gel to a Polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 3% BSA in 1 × TBS buffer for 1 h at room temperature and then incubated with the enhancin-like protein antibodies (1∶1000) at room temperature for 1 h. Goat antirabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody at a dilution of 1∶2000. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for color development.

Cry9Ea crystal protein preparation

The Cry9Ea crystal protein was purified from recombinant strain HD9EA (a HD73⁻ derivative containing the full *cry9Ea* gene in the vector pSXY422b). Recombinant *Bt* strains were grown in 1/2 LB medium supplemented with Erythromycin and Ampicillin for 30 h at 30°C. Spores and crystals were separated by centrifugation at 8000 r/min for 10 min and then washed four times with 1 mol/L NaCl. Crystal solubilization was carried out in carbonate buffer (50 mmol/L Na_2CO_3, 50 mmol/L EDTA, β- mercaptoethanol, pH 10.0) for 6 h with constant shaking on the ice. After centrifugation to eliminate insoluble material, 3 mol/L NaAc-HAc (pH 4.6) was added into the supernatant to precipitate the crystal protein. After 4 h, crystals were separated by centrifugation at 10000 r/min for 10 min and then washed twice with deionized water. Finally, the crystal protein was dissolved in 50 mmol/L Na_2CO_3. The protein concentration was determined by the Bradford assay.

Effects of enhancin-like with Cry9Ea toxin on S. exigua and T. ni

Bioassays were carried out by the diet incorporation method, as described by Gunning et al. (2005). Enhancin-like protein was mixed with the Cry9Ea toxin at different ratios (one group received enhancin-like:Cry9ea at the following ratios: 0∶10, 2∶10, 4∶10, 6∶10, 8∶10, 10∶10; the other group use enhancin of TnGV as control.) in phosphate-buffered saline buffer. The number of neonate larvae of per dilution used was 10, and four replicates were conducted with each dilution. After 3 days of incubation at 26.5°C, mortalities for each treatment were recorded. All the bioassays were conducted three times for each treatment.

Results

Cloning of *enhancin-like* gene from *Bt* strain GS8

Using the genome DNA of *Bt* strain GS8 as template, the expected 2200-bp PCR product of *enhancin-like* was obtained (Fig. 1). The PCR product purified and ligated with pET-30a vector and recombinant plasmid pET30a-*enhancin-like* was transformed into *E. coli* resulting BL21 (pET30a-*enhancin-like*).

Sequence analysis of *enhancin-like* gene

DNA sequence showed that the *enhancin-like* gene has an open reading frame of 2202 nucleotides that encodes a protein containing 733 amino acids with a molecular mass of 84 kDa. Sequence comparison showed the enhancin-like protein 22%–25% identity to viral enhancin proteins, 30% identity with the enhancin-like protein from *Yersinia pestis*, and 95%–96% identity to the *B. cereus* group enhancin-like proteins. The enhancin-like protein was analyzed using the NCBI Conserved Domain Database. Amino acid sequence alignment revealed that the homology among bacterial enhancin-like proteins, and viral enhancin proteins was high in the N-terminal regions of the proteins. In the 252 to 261 amino-acid sequence of enhancin-like protein, a conserved metal binding motif (HEIAH) similar to that in the reported bacterial enhancin-like proteins was found (HEXXH in viral enhancin protein) (Fig. 2). There was also a conserved glutamic acid in

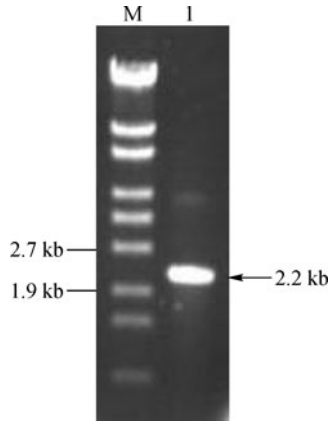

Figure 1 PCR amplification of *enhancin-like* gene of *Bt* strain GS8. M: marker; 1: PCR product of *enhancin-like* gene.

the enhancin-like protein. The presence of these features indicated that the enhancin-like protein has the structural requirements defining a metalloprotease, which is similar to the viral enhancin proteins.

Expression and purification of *enhancin-like* gene in *E. coli* and protein purification

Expression of the *enhancin-like* gene was tested in BL21 (pET30a-*enhancin-like*). SDS-PAGE analysis showed that the molecular weight of expressed enhancin-like protein was about 84 kDa showed in arrow, which was in agreement with the result expected (Fig. 3). For the ultrasonic broken cells, the expression product was detected in pellet. To gain further insight into the biologic function of *enhancin-like* gene, purified fusion protein was obtained by affinity chromatography.

Construction of an engineering strain HD73-(pSXY422b-*enhancin-like*)

The recombinant plasmid pSXY422b-*enhancin-like* was successfully transformed to HD73$^-$, resulting in an engineering strain HD73$^-$(pSXY422b-*enhancin-like*). Antibody against the purified recombinant protein was generated and showed specificity to the engineering strain, suggesting that *enhancin-like* gene could be expressed 84 kDa protein as the predicted size in HD73$^-$(pSXY422b-*enhancin-like*) (Fig. 4).

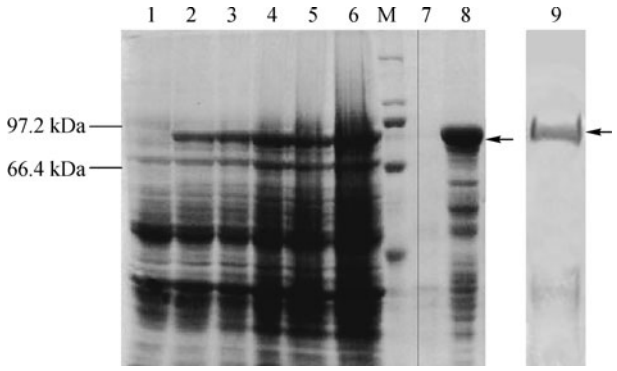

Figure 3 SDS-PAGE showing the induced expression of protein in *E. coli*. M is protein marker; 1–6 are the soluble fraction of *E. coli*; 7 and 8 are the supernatant and pellet the ultrasonic broken cells, respectively, and 9 is the purification of the *enhancin-like* protein.

Pathogenicity in insects

To gain further insight into the biologic function of the *enhancin-like* gene, enhancin-like protein was used to test its enhancement to Cry9Ea toxicity toward to *S. exigua* and *T. ni* larvae. During insect bioassays, the enhancing effect depended on crystal protein and the ratio of the Cry protein to the enhancin protein. Cry9Ea protein showed highly virulence to *T. ni* but low toxicity to *S. exigua*. With the Cry9Ea (0.1 μg/mL) protein plus the enhancin, the mortality caused by the Cry9Ea protein was increased significantly, which was significantly different from that with the Cry9Ea treatment alone at the same Cry9Ea dosage. On the other hand, when the enhancin-like protein was mixed with the Cry9Ea protein at different ratios, no significant increase in the larval mortality was observed (Fig. 5). In controls, insecticidal activity was not observed when insects fed on the *enhancin-like* protein alone. This indicated that the enhancin-like protein had the same structural requirements defining a metalloprotease, which is similar to the viral enhancin proteins; however, it failed to enhance the activity of cry9Ea protein to insects.

Discussion

More recently, a large number of enhancing factors were

B. thuringiensis	ISGAGGAYYGTNWTANSTDST.KMWLDKLS..WGTLHEIAHGYQAGFDNQGIFTG.EVSN
B. anthracis	ISGAGGAYYGANWTANSTDST.KMWLDKLS..WGTLHEIAHGYQAGFDNQGIFTG.EVSN
B. cereus	ISGAGGAYYGTNWTANSTDST.KMWLDKLS..WGTLHEIAHGYQAGFDNQGIFTG.EVSN
Y. pestis	KGGGGAAYYSNNWIASSSGSINTFWLSPNATNWGCLHEIAHGYQGGFIDDKYFSTREVWN
TnGV	AGGPGGAYYGPFWTAPASSNLGDYLRISPTN.WMVIHELGHAYDFVFTVNTILIE.IWNN
HaGV	AGGPAEHYYTDAYIANSADTLGPFLLSTITN.WPALHQIGHGYDLNFTNHTVLIE.VWSS
PuGV	AGGPGDHYYSDSYIANSANTLGPFLLSTITN.WPALHQIGHGYDHHFTNNTSLKE.VWSS

Figure 2 Amino acid sequence alignment of enhancing. The highly conserved metalloprotease zinc binding domain (HEXXH) in the region of positions 252 to 261 is shown in the box.

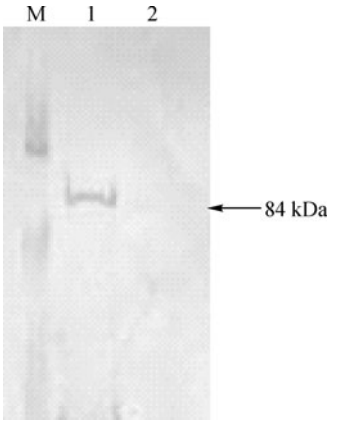

Figure 4 Analysis of enhancin-like protein from HD73$^-$ (pSXY422b-*enhancin-like*) by western blotting. M is protein marker; 1 is HD73$^-$(pSXY422b-*enhancin-like*), and 2 is HD73$^-$.

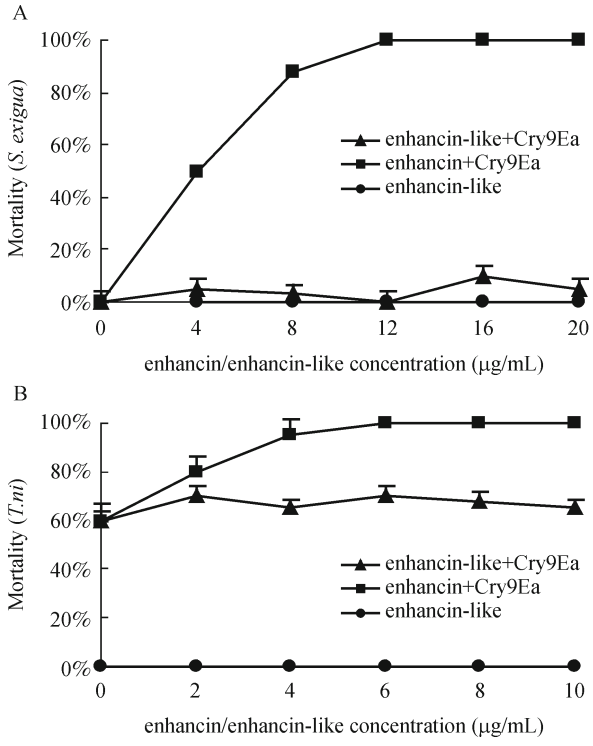

Figure 5 A and B are the synergistic effects between the enhancin-like protein and Cry9Ea toward to *S. exigua* and *T. ni*.

reported in the GVs and NPVs (Yin et al., 2007). Viral enhancin strongly enhanced larval baculovirus infection, acting as a synergic factor. It was also found that the enhancin increases *Bt* larvicidal activities toward lepidopteran larvae (Granados et al., 2001). Enhancin is not essential for growth of virus in cell culture or infected animals but has been shown to decrease larval survival time and improve the larvicidal activities. The previous work on the degradation of PM proteins by a baculovirus enhancin demonstrated IIM, the major PM protein in *T. ni* larvae, is the target substrate for the enhancin (Wang et al., 1994). The degradation of mucin led to the disruption of this intestinal barrier and supported the proposed mode of action for enhancins (Wang and Granados, 1997). Enhancin has a great potential for use in a cost-effective baculovirus pesticide. Until recently, it has been found that bacterial *enhancin* genes encode a peptide with 20%–30% identity to viral enhancin protein. Sequencing analysis revealed glutamic acid was rich in bacterial enhancin, a conserved metal binding motif (HEIAH) similar to that in the reported viral enhancin proteins (HEXXH in viral enhancin protein). Phylogenetic analysis exhibited amino acid identities with the enhancins from baculovirus and bacterial. The grouping of NPV and bacterial enhancins suggested a common or related origin of their *enhancin* genes (Slavicek and Popham, 2005). *Bel* gene was found to have an activity similar to that of the viral *enhancin* gene. A *bel* knockout mutant was constructed by using a plasmid-free *B. thuringiensis* derivative, BMB171. The 50% lethal concentration of this mutant plus the *cry1Ac* insecticidal protein gene was about 5.8-fold higher than that of the BMB171 strain. When purified Bel was mixed with the Cry1Ac protein and fed to *Helicoverpa armigera* larvae, the mortality rate rose significantly. Microscopic observation showed a significant disruption detected on the midgut PM of *H. armigera* larvae after they were fed with Bel. In vitro degradation assays showed that Bel digested the IIM of *T. ni* and *H. armigera* larvae to various degrading products. These results implied Bel toxicity enhancement depends on the destruction of midgut PM and IIM, which is similar to the case with viral enhancin. This discovery showed that Bel has the potential to enhance insecticidal activity of *B. thuringiensis*-based biopesticides and transgenic crops (Fang et al., 2009).

In this study, the *enhancin-like* gene was analyzed by PCR from 163 *Bt* strains. The results indicated that *enhancin-like* gene existed commonly in *Bt* (data not shown). A novel *enhancin-like* gene was cloned from *Bt* strain GS8. The *enhancin-like* protein had high conservation in the N-terminal regions of the proteins and a conserved zinc binding motif (HEIAH); this zinc binding signature site also included a conserved aspartic acid residue two E and D downstream of the HEXXH sequence. The structural analysis indicated that the enhancin-like belonged to a metalloprotease, which is similar to other baculovirus enhancins. This result suggested that the bacterial enhancins may have evolved to an activity similar to viral enhancins consistent with the homology. Phylogenetic analysis showed that bacterial enhancins have a related origin of NPV *enhancin* genes (Ivanova et al., 2003). Our finding of the *enhancin-like* gene in *B. thuringiensis* provides further support for this hypothesis. If the *B. cereus* ancestor resided in the guts of insects and was this insect virus's hosts, there may have been an opportunity for an exchange of genetic material between these bacteria enhancins and NPVs.

In general, proteins expressed in *E. coli* had a lower insecticidal activity, which may be caused by formation of inclusion. In recent years, many *cry* genes are used broadly for the construction of engineering strains and insecticidal transgenic plants, so as to increase the insecticidal activity or delay the insect resistance development. Here, we constructed a *Bt* engineering strain that can express the soluble target protein. Although the discovery of new *cry* genes encoding toxins with new insecticidal specificities or that bind to different receptors in the insect gut would be of great value, it may not be the most appropriate answer for future resistance management strategies because broad-spectrum resistance to *Bt* toxin has already been observed in some cases. Therefore, many research projects and screening programs are currently underway to identify new insecticidal proteins with different mode of action. Bacterial enhancins have developed different infection strategies to overcome some host-specific barrier, such as a robust host peritrophic matrix structure, and it has profound potential effects on the construction of engineering strains and insecticidal transgenic plants, so as to increase insecticidal activity or delay insect resistance development (Huang and Lin, 2001). The construction of *Bt* engineering strain will provide a basis for the future activity determination of *enhancin-like*.

We expected that this *enhancin-like* protein would have the function as a synergist of Cry9Ea toxicity against *S. exigua* and *T. ni* larval according to the reports of Fang, etc. (Fang et al., 2009). However, larval bioassays indicated that this *enhancin-like* protein did not enhance infection of *Bt*. Neither *enhancin-like* alone nor their combination with Cry9Ea protein will not induce the mortality, compared with the enhancin, which is in accordance with the previous studies. Enhancins from *Yersinia* and *Bacillus* sp. were recloned into a baculovirus vector to create occluded recombinant virus expressing enhancin, and they failed to enhance the natural AcMNPV infection under conditions where the delivery of VEF enabled enhancement (Galloway et al., 2005); a large number of *B. thuringiensis* and *B. cereus* genes encoding potential extracellular virulence factors were positively regulated by the pleiotropic transcriptional activator PlcR (Hajaij-Ellouze et al., 2006); and no significant reduction in mortality was found with the mutant strain, indicating that *B. thuringiensis enhancin* is not essential for virulence toward *G. mellonella* larvae. These results imply that the enhancin-like protein obtained from Bt may not increase Cry proteins' infection universally, and the synergism of the *Bt* enhancin-like is specific to the toxicity of Cry proteins. Although no significant reduction in virulence was observed about the enhancin-like protein, this may not exclude *Bacillus enhancin-like* playing a role in pathogenesis, and the mode action of this protein still needs further researches.

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