Bacillus thuringiensis novel toxin Epp is toxic to mosquitoes and prodenia litura larvae

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Abstract

As a pathogenic bacterium, *Bacillus thuringiensis* (*Bt*) has become an alternative to chemical insecticides in commercial agricultural to control forestry pests and mosquitoes. To prevent pest resistance, many novel *Bt* strains have been isolated. Strain S3580-1 (WGS: VHPX0000000) used in this research and originally isolated from Hainan Qixianling National Forest Park (China) showed significant toxicity to *Culex pipiens pallens*. Here, using whole genome sequencing, assembly, and bioinformatics analysis, the predicted S3580-1CG_5163 (GenBank Accession No. MK124137) gene-encoded protein was found to share low homology with known toxins designated by the *Bt* toxin nomenclature system. It was considered to be an ETX/MTX2-type toxin and was designated Epp. Bioinformatics analysis showed that the predicted S3580-1CG_5163 gene-encoded protein Epp shared low identity with other known toxic protein sequences containing Cry-ETX/MTX conserved domains at the amino acid level, but significant similarity at the structural level. In addition, bioassays showed that Epp was toxic against *Spodoptera litura* (LC₅₀ 296.133 µg/mL; 95% FL 200.555–471.318 µg/mL) and *Cx. pipiens pallens* (LC₅₀ 322.193 µg/mL; 95% FL 238.217–477.243 µg/mL). On pathological observation, the peritrophic membrane of *Cx. pipiens pallens* larvae was degraded causing the midgut structure to become incomplete, resulting in larval death. Further bioassays are required to fully elucidate the insecticidal spectrum of the ETX/MTX2-type toxin Epp, and thereby provide future research directions.

Keywords *Bacillus thuringiensis* · S3580-1 · ETX/MTX · Epp · Prodenia litura (*Spodoptera litura*) · *Cx. pipiens pallens*

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Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium that produces insecticidal crystal proteins (ICPs) that are toxic to some invertebrate populations during sporulation. As a pathogenic bacterium, *Bt* has become a substitute for chemical pesticides in commercial agricultural to control forestry pests and mosquitoes [10, 11]. However, under high continuous selective pressure, many pests have developed resistance to *Bt* products [15]. Given the successful use of conventional pesticides and concerns regarding resistance, the simplest but most effective strategy might be to screen more alternative strains to prolong the activity of effective pesticides. It has been shown that different ICPs have different toxic effects on mosquito larvae [14].

ICP family members containing the crystal (Cry)-*Clostridium* epsilon toxin ETX/*Bacillus* mosquitocidal toxin MTX2 conserved domain (Cry-ETX/MTX) exhibit different toxicity to different insect species. For example, Cry15, Cry23, Cry33, and Cry38 toxins were toxic to Coleoptera larvae; Cry51 toxins (Cry-ETX/MTX-type) were toxic to both Coleoptera and

Hemiptera larvae [5]; Cry45 (Parasporin-4) and Cry64 (Parasporin-5) have larvicidal activities against human cancer cells; Cry60 toxins (Cry-ETX/MTX) were active against Diptera larvae [9, 13]; Cry75Aa1 homologous proteins containing the Mtx2 domain were active against Lepidoptera and Coleoptera [3]; while Cry64Ba/Cry64Ca (both harboring ETX/MTX2) showed toxicity against two important hemipteran rice pests, *Laodelphax striatellus* and *Sogatella furcifera* [6].

Hainan Institute of Tropical Agricultural Resources (HITAR) isolated the novel toxin found in this study from *Bt* S3580-1 (CGMCC No.13278), which was isolated from Qixianling National Forest Park, Hainan, China, and the toxin showed apparent toxicity to *Culex pipiens pallens*. Using whole-genome sequencing, sequence assembly, and bioinformatics analysis, we found that the predicted toxin gene had low homology with the reported *Bt* toxins, which suggested that it might be a novel toxic protein. Elucidation of the biological function of this novel mosquitocidal toxin would further enrich the target insect profiles of Cry-ETX/MTX protein family members and would provide new resources for mosquito control and Lepidoptera insect prevention.

Materials and methods

Strain, plasmid, and culture medium

Bacillus thuringiensis S3580-1 was isolated from Qixianling National Forest Park of Hainan Province in 2007 and was maintained by Hainan Institute of Tropical Agricultural Resources (HITAR). *Escherichia coli* strain DH5 α and expression vector plasmid pET30a were used for cloning and expression of the *Epp* gene, respectively, and were provided by the Laboratory of Molecular Virology and Mycology, College of Life Science and Technology, Guangxi University.

E. coli BL21 (DE3) cells (Trans, Beijing, China) were used for toxic gene expression. *E. coli* and *Bt* strains were both cultured in Luria–Bertani medium at 37 °C and 28 °C, respectively. Kanamycin (50 mg/L) was added to the culture medium of *E. coli* BL21 (DE3) carrying pET30a strain.

Morphological observation of *Bt* S3580-1 crystal protein under a scanning electron microscope

Bt S3580-1 cells cultured at 28 °C with shaking for 3 days were collected by centrifugation at 15294g for 10 min at 4 °C; then, the cells were washed three times with precooled 1 M NaCl and resuspended in precooled ultrapure water. The mixture of spores and crystals was then placed on a clean coverslip and dried under a vacuum at 4 °C. The sample was fixed with 1% osmium tetroxide (OsO₄) and placed on a metal sample stage, which was metallized to 20–30 nm thickness in a vacuum coating gold-plating instrument. Finally, the

sample was observed and photographed using S-3400N equipment at a working voltage of 20–30.0 kV and an appropriate working distance.

Construction of the gene expression vector

The genomic DNA of *Bt* strain S3580-1 was extracted with the E.Z.N.A.® Bacterial DNA Kit (Omega Biotek Inc., Guangzhou, China) according to the manufacturer's instructions. Based on the genomic DNA, specific primers containing restriction enzyme sites were used for PCR amplification of the *epp* gene (forward and reverse primers were 3580-chr-5163F (*Bam*HI): GCGGGATCCatgaaaagtcttgaatcccaag; 3580-chr-5163R (*Sal*I): CGCGTCGACTTAAGATGTGCTTACCGACGTGG, respectively). The PCR products of the *epp* gene were purified by a Universal DNA Purification Kit (Catalog No. DP214, TIANGEN Biotech (Beijing) Co., Ltd., China), digested with restriction enzymes *Bam*HI and *Sal*I (FastDigest, Thermo Scientific) at 37 °C, then ligated to vector pET30a digested with the same enzymes. The final construct was transformed into DH5 α competent cells.

The positive clone was sequenced after identification by PCR and double enzyme digestion, and the sequence was analyzed using the by Vector NTI Advance 11.0 software. The identified positive recombinant plasmid was transformed into competent cells of the protein expression host BL21(DE3), and PCR was used to confirm successful construction of the expression strain.

Online analysis of the ETX/MTX2-type toxin Epp

The Epp protein was analyzed and predictions were made using various analytical software and tools. The protein molecular weight and isoelectric point of the gene-encoded amino acid sequence were analyzed through online software (<http://isoelectric.ovh.org/>), gene secondary structure was predicted by SOPMA software, the tertiary conserved domain was constructed by SWISS-MODEL, and the Epp functional domain was analyzed with online software PFAM. Multiple sequence alignment of Epp proteins and other Cry-ETX/MTX-type *Bt* toxic proteins was carried out using BioEdit, and the phylogenetic tree was constructed using MEGA 4.0 software.

Expression of the ETX/MTX2-type toxin Epp

To optimize induction of the expression conditions, the expression strain pET30a-epp was induced with IPTG (50 mg/mL, Sigma, USA) at a final concentration of 0.2 mM. The expression strain without IPTG and the expression vector without target genes, treated with IPTG, were included as negative and positive controls, respectively. The expressed proteins were detected by SDS-PAGE.

MALDI-TOF mass spectrometry identification of the ETX/MTX2-type toxin Epp

The target protein band detected by SDS-PAGE, as described above, was identified by MALDI-TOF MS (AB 4800 MALDI-TOF-TOF Plus, USA) following gel band treatment, as previously described by Shevchenko and colleagues [12]. The mass spectrum (MS) and tandem mass spectrometry MS/MS information for the protein were collected in the reflection mode, and the ion fragment fingerprint spectrum of each parent ion was obtained by collision-induced dissociation (CID). Finally, the MS and MS/MS data were comprehensively analyzed by the Mascot search algorithm based on the known protein database as the research library for mass spectrometry data.

Biological activity assay of the ETX/MTX2-type toxin Epp crude protein against *Spodoptera litura* and *Cx. pipiens pallens* larvae

To extract protein, 300 mL of pET30a-epp-expressing cells induced by IPTG (50 mg/mL, Sigma, USA) at 28 °C for 16 h was centrifuged 3213g for 25 min at 4 °C. The supernatant was discarded and the precipitation was resuspended in 15 mL of ddH₂O containing 1% protease inhibitor PMSF. The cells were precooled on ice for 10 min, and then proteins were extracted using Ultrasonic equipment (Sonics&Materials, Inc. Newtown, CT, USA).

The proteins were treated with trypsin (1:300, from Porcine pancreas) at 37 °C for 30 min, then diluted across five concentration gradients prior to the bioassay. The susceptible larvae of *S. litura* used for the bioassay were artificially fed on cassava leaves grown in a greenhouse in the laboratory. The cassava leaves were dipped into the trypsin-treated protein samples of each concentration for 30 s, then allowed to dry at room temperature and subsequently placed adaxially on moist tissue paper in glass petri dishes. Ten 2nd instar larvae were placed in each dish, and lids were placed on the dishes. Leaves dipped in ddH₂O were included as a negative control.

Mosquito larvicidal assays against 3rd instar larvae of *Cx. pipiens pallens* were performed as described previously [16]. All of the tested mosquito larvae were provided by the insectarium in the Institute of Life Science, Jiyang College of Zhejiang A&F University. The serial-treated protein samples were transfer to plastic cups with supplemented ddH₂O, and 15 mosquito larvae were placed in each container. Double-distilled H₂O and mosquitocidal strain *Bt* S2160-1 from our laboratory [16] were included as negative and positive controls, respectively. They were maintained at 28 °C and 70% relative humidity with a 12 h light/dark cycle. Mortality was recorded after incubation for 72 h, and the 50% lethal concentration (LC₅₀) and 95% confidence interval were determined by Probit analysis using IBM Statistics SPSS 19.0 software.

Multiple sequences alignment of ETX/MTX2 conserved domains

According to the previous references and the known toxins containing ETX/MTX2 conserved domain in the *Bt* toxin nomenclature system, the corresponding protein sequences were acquired from NCBI database (<https://www.ncbi.nlm.nih.gov/>). Subsequently, each acquired toxin sequence was analyzed in SMART (<http://smart.embl-heidelberg.de/>), respectively, so as the ETX/MTX2 conserved domain position of each toxin was confirmed. Finally, multiple sequences alignment of these ETX/MTX2 conserved domain sequences were carried out based on Vector NTI Advance 11.0 software.

Construction of phylogenetic tree of ETX/MTX2-type toxins

Firstly, the file of above ETX/MTX2-type toxins protein sequences was transferred into FASTA formation file used BioEdit software. Then opened file with MEGA 7 software, clicked Align and chose Edit/Build Alignment to create a new alignment, selected OK to build a protein sequence alignment and Save As. Subsequently, opened the alignment file, back to the Home page, clicked Phylogeny to choose Construct/Test Neighbor-Joining Tree, setting the tree parameters with resetting the No. of Bootstrap Replications as 1000, other parameters were keep default. Click Compute to complete the construction. Finally, view the result with Bootstrap consensus tree and save the file as filename.mts, which can be shown in MEGA 7.

Observations of the pathological midgut tissue of mosquitoes

The dead mosquito larvae from the bioassay were immediately fixed in 10% neutral formalin solution for more than 24 h. Since the mosquito larvae are small, the entire larva was placed directly into the dehydration box, which was later placed in a hanging basket in a dehydrator (Leica ASP300S), followed by gradient alcohol dehydration and then waxing. Subsequently, the wax-impregnated mosquito larvae were embedded using an embedding machine (Leica EG1150C), labeled, and then cooled on a cold table at −20 °C. After the wax had solidified, the wax block was taken out from the embedding frame and trimmed, then immediately sliced on a paraffin slicer (Leica RM2245) to a thickness of ~4 to 10 µm. The slices were dewaxed, washed with distilled water, then placed in hematoxylin dyeing solution for 1~2 min, followed by washing with tap water. The slices were differentiated by treatment with hydrochloric acid for 30 s and then rinsed with tap water. The slices were then stained for 5 min in Yihong dyeing solution and dehydrated; then, transparency was ensured by sequential treatment with a gradient

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (a) and scanning electron micrograph (SEM) (b) analyses of spores and crystal mixtures from *Bacillus thuringiensis* (Bt) strain S3580-1

of alcohol concentrations and a transparent agent. After drying, the slices were subjected to neutral gum sealing. Finally, the slices were observed by microscopy (Leica DM3000), and images were collected using the image analysis system (Leica DMC4500).

Results and analysis

Toxic protein prediction analysis of mosquitocidal strain *Bt* S3580-1

Bt S3580-1 strain (CGMCC No.13278) was isolated from the soil of Qixianling National Forest Park in Hainan Province of China. The crude spore/crystal protein (Fig. 1a) of this strain had high mosquitocidal activity against *Cx. pipiens pallens* larvae. Scanning electron microscopy revealed that *Bt* S3580-1 strain produced numerous bipyrimal crystal proteins (Fig. 1b). To identify the virulence factors of *Bt* strain S3580-1, genomic DNA was isolated and sequenced using high-throughput sequencing technology. After splicing and assembly of the original raw sequence data, a total of

6,128,480 nucleotide base pairs with a GC content of 34.91%, and 6181 protein-coding sequences, were predicted.

The BLAST alignment local database was constructed using amino acid sequences of the Bt toxic protein collected from public data resources. Through BLASTP and BLASTN alignment programs, the predicted toxic genes were screened, with an e-value < 0.0001 set as the significance level threshold. Then, the predicted toxic protein genes were aligned again using BLASTP on the NCBI and PFAM databases, and SWISS-MODEL tertiary domain analysis was also performed. By combining these data and analyzing the typical toxic protein characteristics, five candidate toxic protein genes were finally predicted in mosquitocidal strain *Bt* S3580-1 (Table 1).

The S3580-1CG_5163 (GenBank Accession No. MK124137) sequence encoding the candidate toxic protein had the highest identity (~81%) with the hypothetical protein annotated “Clostridium epsilon toxin ETX/*Bacillus* mosquitocidal toxin MTX2,” and had the highest homology (~31.4%) with the known toxin protein Cry75Aa2 (ASY04852.1). Subsequently, its amino acid sequence was submitted to the *Bacillus thuringiensis* toxin nomenclature committee system. It was considered to be an ETX/MTX2-type toxin, and was designated Epp.

Table 1 Physicochemical properties of the toxic proteins identified in *Bacillus thuringiensis* (Bt) strain S3580-1

No.	Predicted gene	Amino acids	Molecular weight	Theoretical pI
1	S3580-1CG_2118	738	81,546.8	5.83
2	S3580-1CG_5163	356	39,677.0	4.69
3	S3580-1p1G_126	599	67,629.9	7.66
4	S3580-1p1G_404	432	48,627.6	4.35
5	S3580-1p2G_89	373	42,386.0	5.67

Construction of the expression vector for ETX/MTX2-type toxin Epp

Using the genomic DNA of *Bt* strain S3580-1 as the template, the full-length S3580-1CG_5163 (*epp*) gene (1071 bp) was amplified using specific primers (Fig. 2a). After purification and digestion, the *epp* gene was ligated to pET30a and then transformed into DH5 α competent cells. The recombinant plasmid pET30a-*epp* was extracted and identified by restriction enzyme digestion with *Bam*HI and *Sal*II (Fig. 2b).

The sequencing of the positive clone showed that the full-length *epp* gene was 1071 bp, which was consistent with the predicted gene sequence obtained by genomic sequencing, and encoded 357 amino acids with a deduced molecular mass of 39.7 kDa, which agreed with the protein bands observed in the spore/crystal mixture (Fig. 1a), demonstrating that this cloning gene was correct. To express Epp protein in vivo, the recombinant plasmid pET30a-*epp* was transferred into the competent cells of the expression host BL21 (DE3), and the positive clone pET30a-*epp* was identified based on PCR validation (Fig. 2c).

Expression and identification of the ETX/MTX2-type toxin Epp

After optimization of the expression conditions, ETX/MTX2-type toxin Epp was induced at 28 °C with 0.2 mM IPTG and shaking at 200 r/min for 15 h (Fig. 3). The target band was then excised from the gel following SDS-PAGE and subjected to MALDI-TOF mass spectrometry (AB 4800 MALDI-TOF-TOF Plus, USA). The data obtained by mass spectrometry were as follows: the molecular weight of the protein, 39.652 kDa; protein isoelectric point (pI), 4.69; peptide sequence MS1: FYQVDIR, MS2: GTYANYNPVQTNLFGR; protein score (confidence interval %), 100%. These data were consistent with those expected for the target protein, Epp.

Fig. 3 SDS-PAGE analysis of Epp expression. M: PageRuler Prestained Protein Ladder (Thermo Scientific); pET: pET30a vector induced with IPTG; -: Epp protein without IPTG induction; +: Epp protein with IPTG induction; arrow: target protein

Structure and homologous phylogenetic tree analysis of ETX/MTX2-type toxin Epp

Both the secondary and tertiary structures of Epp were analyzed online. The secondary structure of Epp included an alpha helix (Hh) (50 (14.04%)), extended strand (Ee) (105 (29.49%)), beta-turn (Tt) (24 (6.74%)), and a random coil (Cc) (177 (49.72%)), respectively (Fig. 4a). The random coil structure constituted most of the structure, followed by the extended strand structure. The online homology building model of Epp using SWISS-MODEL also showed the corresponding tertiary structure (Fig. 4b). In addition, PFAM analysis revealed that Epp contained a *Clostridium* epsilon toxin ETX/*Bacillus* mosquitocidal toxin MTX2 conserved domain, from position 67 to 336 (Fig. 4c). As reported, Mtx2 was a mosquitocidal toxin produced during the vegetative stage.

In the *Bt* delta-endotoxin nomenclature committee system, there are several known Cry proteins that contain ETX/MTX

Fig. 2 Construction of the pET30a-*epp* expression vector. **a** Amplification of the S3580-1CG_5163 gene with specific primers. **b** Identification of recombinant plasmid pET30a-5163 by *Bam*HI and *Sal*II restriction analysis. **c** PCR validation of expression vector pET30a-*epp*

Fig. 4 Sequence analyses of Epp. **a** The secondary structure of Epp. **b** The simulated spatial 3D structure of Epp. Cry51Aa1 (PDB code 4PKM) was selected as a model for homology modeling in SWISS-MODEL (global model quality estimation [GMQE] 0.35). **c** Conserved domain of Epp

conserved domains. Multiple sequence alignments with different *Bt* Cry-ETX/MTX-type toxins showed that although these proteins were all ETX/MTX-type toxic proteins, the level of consensus between their sequences was low (Fig. 5a). In addition, phylogenetic analysis of these known Epp proteins revealed that Epp resided in the same branch as Cry64Ca1, indicating that the evolutionary relationship between Epp and Cry64Ca1 was close, while other ETX/MTX-type toxic proteins resided in different branches, illustrating that their evolutionary relationships were distant (Fig. 5b).

Preliminary determination of the biological activity of Epp protein

Preliminary determination of the biological activity of EPP protein against mosquitoes *Cx. pipiens pallens* and *S. litura* larvae was performed. Total protein from the positive control strain *Bt* S2160-1 had no insecticidal activity against *S. litura*, but high mosquitocidal activity against *Cx. pipiens pallens*, with all of the larvae dying within 12 h. By contrast, in the negative control (ddH₂O), only one larva died and the rest appeared healthy. The expressed target protein Epp showed specific insecticidal activity against *S. litura* and *Cx. pipiens*

pallens, among which the corrected mortality rate against *S. litura* larvae was 44% after incubation for 48 h (LC₅₀ 296.133 µg/mL; 95% FL 200.555–471.318 µg/mL), and the adjusted mortality rate of *Cx. pipiens pallens* larvae was 40% after exposure for 48 h (LC₅₀ 322.193 µg/mL; 95% FL 238.217–477.243 µg/mL).

Effect on the midgut structure of *Cx. pipiens pallens* larvae of Epp protein

The effects of Epp on the midgut structure of the 3rd instar *Cx. pipiens pallens* larvae were analyzed using Epp protein-treated and untreated mosquito larvae. The *Cx. pipiens pallens* larvae that died within 36 h and the negative control were fixed immediately and paraffin-embedded so that the morphology could be analyzed under a microscopy. The results showed that the midgut structure of the negative control was

Fig. 5 Multiple sequence alignment and the corresponding phylogenetic tree of Cry-ETX/MTX-type toxins. **a** Multiple sequence alignments were based on the ETX/MTX conserved domain sequences of Cry proteins. **b** Phylogenetic tree of Cry-ETX/MTX-type toxins was constructed by Neighbor-Joining Tree of MEGA 7 (Bootstrap Replications is 1000)

Fig. 6 Effect of Epp protein on the midgut of *Culex pipiens pallens* larvae. **a** The midgut transection of untreated *Culex pipiens pallens* larvae. **b** The midgut transection of Epp-treated *Culex pipiens pallens* larvae

intact, while the peritrophic membrane of *Cx. pipiens pallens* larvae that had died following Epp protein treatment was degraded. It is proposed that the midgut structure became incomplete (Fig. 6), causing an imbalance in cell osmotic pressure, resulting in larval death.

Discussion

Cry protein is a vital virulence factor in *Bt* strains and exhibits specific insecticidal activity against Lepidoptera, Diptera, and Coleoptera pests. Cry proteins can be divided into several types based on their characteristics, such as the 3D conserved domain, ETX/MTX-like, and Bin-like [1]. Among the known *Bt* toxic proteins, there are more than 10 Cry-ETX/MTX-type, which exhibit different insecticidal effects against Coleoptera, Lepidoptera, Diptera, Hemiptera, and even human cancer cells [3, 9, 13]. Interestingly, researchers found that some Cry-ETX/MTX toxins were accompanied by other proteins. For example, the Cry15Aa toxic protein was associated with a 40 kDa protein, but the individual Cry15Aa protein also exhibited hemolytic and insecticidal activity against *Manduca sexta*, indicating that the 40 kDa protein is not necessary for its toxicity [4, 8].

At the amino acid sequence level, the Cry-ETX/MTX toxins share lower identity, but at the structural level, almost all Cry-ETX/MTX toxins show high similarity to the tail region of aerolysin-like pore-forming toxin (PFT) proteins, which are responsible for oligomerization and pore formation [2]. Whereas, Cry-ETX/MTX toxins share lower structural similarities to the head region of aerolysin-like PFT proteins, which are responsible for specific binding to receptors [7]. Although the structure of Cry-ETX/MTX toxin is significantly different from the three domain toxins, these toxins are still thought to act via insertion into the membranes of target cells to form pores [2]. In this research, the *epp* gene was cloned and bioinformatics analysis was performed on its encoded protein Epp, along with assays of its biological function. Multiple sequence alignments of different *Bt* Cry-ETX/MTX-type toxins showed that Epp protein indeed shared low amino acid level identity with other known Cry-ETX/

MTX-type toxic proteins. However, evolutionary analysis revealed that Epp protein resided in the same branch as Cry64Ca1, illustrating a close evolutionary relationship, while all other ETX/MTX-type toxic proteins resided in different branches, indicating that their evolutionary relationships were distant.

Here, the biological activities of Epp protein against mosquitoes *Cx. pipiens pallens* and *S. litura* larvae were determined based on the analysis of crude protein. Although Epp protein exhibited specific insecticidal activity against *Cx. pipiens pallens* and *S. litura*, this may not be completely representative of its insecticidal activity. Therefore, in future experiments, we need to further purify the Epp protein prior to biological activity determination to confirm its LC₅₀ and 95% confidence intervals for the target insects.

Furthermore, the insecticidal profile of requires further research against other Lepidopteran, Dipteran, Coleopteran larvae, and cancer cells. It was worth noting that other toxic proteins, including a mosquitocidal toxin and a 43.8 kDa ICP, have also been predicted in *Bt* strain S3580-1. In subsequent experiments, we need to perform bioinformatic analysis of these predicted toxins and determine their biological functions. It will also be interesting to analyze the possible synergy between these toxic proteins that contributes to the various insecticidal activities. This study highlights some of the key characteristics of *Bt* strain S3580-1 and provides an experimental basis for further investigations.

Conclusion

Here, we found a wild strain S3580-1 with the insecticidal activity against the larvae of mosquitoes and prodenia litura. The whole genome sequencing was conducted to detect the genes that are responsible for the toxicity. The novel toxin gene of Epp (GenBank Accession No. MK124137) was identified through bioinformatics analysis and successfully expressed in *E. coli* cells for function verification. It was considered to be an ETX/MTX2 type toxin in *Bacillus thuringiensis* toxin nomenclature committee. We confirmed

that the candidate toxin of Epp showed high toxic activity against *Culex pipiens pallens* and *Spodoptera litura* larvae.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Adang MJ, Crickmore N, Jurat-Fuentes JL (2014) Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. In: Dhadialla TS, Gill SS (eds) Insect midgut and insecticidal proteins, Advances in insect physiology, vol 47. Elsevier, Philadelphia, pp 39–88
- Bokori-Brown M, Savva CG, Fernandes da Costa SP, Naylor CE, Basak AK, Titball RW (2011) Molecular basis of toxicity of *Clostridium perfringens* epsilon toxin. FEBS J 278:4589–4601
- Bowen DJ, Chay CA, Flasinski S, Yin Y (2017) Novel insect inhibitory proteins. Monsanto Technology LLC, US Patent Application. 2017; No.A120170044568
- Brown KL, Whiteley HR (1992) Molecular characterization of two novel crystal protein genes from *Bacillus thuringiensis* subsp. *thompsoni*. J Bacteriol 174:549–557
- Baum JA, Sukuru UR, Penn SR, Meyer SE, Subbarao S, Shi X, Flasinski S, Heck GR, Brown RS, Clark TL (2012) Cotton plants expressing a Hemiptera-active *Bacillus thuringiensis* crystal protein impact the development and survival of *Lygus hesperus* (Hemiptera: Miridae) nymphs. J Econ Entomol 105:616–624
- Liu Y, Wang Y, Shu C, Lin K, Song F, Bravo A, Soberón M, Zhang J (2018) Cry64Ba and Cry64Ca, two ETX/MTX2-type *Bacillus thuringiensis* insecticidal proteins active against Hemipteran pests. Appl Environ Microbiol 84:e01996–e01917
- Moar W, Evans A, Kessenich CR, Baum JA, Bowen DJ, Edrington TC, Haas JA, Kouadio JK, Roberts JK, Silvanovich A (2017) The sequence, structural, and functional diversity within a protein family and implications for specificity and safety: the case for ETX_MTX2 insecticidal proteins. J Invertebr Pathol 142:50–59
- Naimov S, Boncheva R, Karlova R, Dukiandjiev S, Minkov I, de Maagd RA (2008) Solubilization, activation, and insecticidal activity of *Bacillus thuringiensis* serovar *thompsoni* HD542 crystal proteins. Appl Environ Microbiol 74:7145–7151
- Palma L, Muñoz D, Berry C, Murillo J, Caballero P (2014) *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins 6:3296–3325
- Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol J 9(3):283–300
- Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesti-cidal crystal proteins. Microbiol Mol Biol Rev 62(3):775–806
- Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1(6):2856–2860
- Sun YJ, Zhao Q, Xia LQ, Ding XZ, Hu QF, Federici BA, Park HW (2013) Identification and characterization of three previously undescribed crystal proteins from *Bacillus thuringiensis* subsp. *Jegathesan*. Appl Environ Microbiol 79(11):3364–3370
- Wirth MC (2010) Mosquito resistance to bacterial larvicidal toxins. Open Toxicol J 3:126–140
- Yuan Z, Zhang Y, Cai Q, Liu EY (2000) High-level field resistance to *Bacillus sphaericus* C3-41 in *Culex quinquefasciatus* from south-ern China. Biocontrol Sci Tech 10:41–49
- Zhang W, Crickmore N, George Z, Xie L, He YQ, Li Y, Tang JL, Tian L, Wang X, Fang X (2012) Characterization of a new highly mosquitocidal isolate of *Bacillus thuringiensis*—an alternative to *Bti*? J Invertebr Pathol 109:217–222

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