Different Toxicity of the Novel *Bacillus thuringiensis* (Bacillales: Bacillaceae) Strain LLP29 Against *Aedes albopictus* and *Culex quinquefasciatus* (Diptera: Culicidae)

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ABSTRACT *Bacillus thuringiensis* (Bt) (Berliner) strain LLP29 produces a crystal protein Cyt1Aa6 toxic to mosquito vectors of human diseases. However, the susceptibility of *Culex quinquefasciatus* (Say) in the current study was 8.25 times higher than that of *Aedes albopictus* (Skuse) with this single protein Cyt1Aa6 purified from LLP29. To understand the mechanism of the novel mosquitocidal protein, the binding characteristic of brush border membrane vesicles from the two tested mosquitoes was investigated. Enzyme-linked immunosorbent assay showed that Cyt1Aa6 bound to the two mosquitoes' brush border membrane vesicles. However, the titer of *Ae. albopictus* was a little higher than that of *Cx. quinquefasciatus*, with 3.21 and 2.91, respectively. Ligand Western blot analysis showed Cyt1Aa6 toxin specifically bound to the same three proteins (i.e., 68, 54, and 26 kDa) in the two mosquitoes, but one another protein, approximately to 37 kDa, could just be detected in *Cx. quinquefasciatus*. However, little difference was found in the test of immunohistochemistry. Cyt1Aa6 was detected in the midguts of both mosquitoes with histopathological changes. It would of great importance to the knowledge of the novel toxin against to *Cx. quinquefasciatus* and *Ae. albopictus*.

KEY WORDS *Bacillus thuringiensis*, receptor binding, ELISA, immunohistochemistry, *Aedes albopictus*

Mosquitoes are the most important group of arthropods of medical and veterinary importance. It is the bloodsucking habit that accounts for their importance as disease vectors. Among the disease pathogens transmitted by mosquitoes are those causing the diseases malaria, yellow fever, dengue, and so on. Therefore, mosquito management is an essential component for the control of mosquito-borne diseases.

Bacillus thuringiensis (Bt) (Berliner) have been the subject of intensive studies because of their insecticidal activity. Of them, *B. thuringiensis* subsp. *israelensis* (Bti) harboring Cry4, Cry10, Cry11, Cyt1, and Cyt2 toxins were toxic to *Aedes*, *Culex*, *Anopheles*, *Mansonia*, and *Simulium* larvae (Poncet et al. 1997, Fernandez–Luna et al. 2010), which is believed that Cry toxin-induced membrane pore formation is responsible for the toxicity. The molecular mechanism of pore formation involves recognition and subsequent binding of the toxins to membrane receptors (Likitvivatanavong et al. 2011). Besides cadherin, APN, ALP proteins, and α-amylase have been identified as important receptor in mosquitoes (Fernandez–Luna et al. 2010, Likitvivatanavong et al. 2011). In any case, specific receptors are necessary for Cry toxin action. However, receptor expression levels have also been shown to correlate with Cry toxin activity (Likitvivatanavong et al. 2011).

LLP29 was a novel and useful mosquitocidal *B. thuringiensis* strain isolated from phylloplanes of *Magnolia denudata* from Wuyi Mountain, Fujian Province of China (Zhang et al. 2010, 2011). In addition, *cyt1Aa6* was a novel gene toxic to mosquito larvae (Zhang et al. 2009). Cyt1Aa6 purified from LLP29 was toxic to *Aedes albopictus* and *Culex quinquefasciatus*, two ubiquitous mosquitoes in southern China, which have been implicated as the major vector of dengue fever and Bancroftian filariasis, respectively. However, its mosquitocidal activity against *Cx. quinquefasciatus* was much higher than that against *Ae. albopictus*, confirming the previous report that *Cx. quinquefasciatus* was more susceptible to *B. thuringiensis* than *Ae. albopictus* (Zhang et al. 2007, 2011). To further understand the difference in activity level between the two mosquitoes, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry and ligand blot assays of Cyt1Aa6 toward the two tested mosquitoes were in-

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vestigated in this current study. It not only would be of great value in understanding the action mechanism of this new toxin, but also might lead to the development of new insecticides with high mosquitocidal activity.

Materials and Methods

Insects and Antibody. The larvae of *Ae. albopictus* and *Cx. quinquefasciatus* used in this study were kindly provided from the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences. The mosquitoes were reared in our lab in an environment-controlled room at 28°C and 85% relative humidity (RH) with a photoperiod of 14:10 (L:D) h (Zhang et al. 2012). The Cyt1Aa6 antibody of the female New Zealand White rabbit was developed, purified and stored in our lab (Zhang et al. 2012).

Preparation of Brush Border Membrane Vesicles of *Ae. albopictus* and *Cx. quinquefasciatus*. Brush border membrane vesicles (BBMV) from dissected midguts of fourth-instar *Ae. albopictus* and *Cx. quinquefasciatus* larvae were prepared and quantified as reported (Bradfords 1976, Wolfersberger et al. 1987).

Enzyme-Linked Immunosorbent Assay. As an independent test of peptide specificity, ELISA was performed by comparing nonspecific binding with bovine serum albumin (BSA). The enzymatic reaction was stopped with 6 M HCI and the absorbance read at 490 nm with an Amersham Pharmafa LKB Ultraspec II (Perez et al. 2005, Zhang et al. 2012).

Ligand Blot Assays. To determine whether there would be different in special binding sites, 70 mg of Cyt1Aa6 protein from *Ae. albopictus* and *Cx. quinquefasciatus* larvae were run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membrane. Furthermore, Ligand Western blot assays were carried out (Mendoza et al. 2002, Zhang et al. 2012).

Immunohistochemistry Analysis. To further understand the difference between the two mosquitoes, immunohistochemistry of Cyt1Aa6 toward the two tested mosquitoes were investigated as described (Zhang et al. 2012). Thirty fourth-instar tested larvae were removed and fixed in the same fixative overnight at 4°C, dehydrated, and routinely embedded in paraffin with 16 h of processing. Sections were cut with 6-μm in thickness. For detecting receptors in situ, sections were deparaffinized and pretreated by hydrolytic autoclaving in 10 mM citrate buffer (pH 6.0) for 30 min at 97°C to retrieve antigens. Thereafter, sections were incubated with Cyt1Aa6 protein for 2 h at 37°C and preblocked with serum-free blocking solution (1% BSA) for 30 min before primary antibody incubation. Rabbit-anti-Cyt1Aa6 (1:200) was used as the primary antibody for 2 h at room temperature, followed by a secondary goat-anti-rabbit-AP-Red (ZSGB-BIO) antibody for 1 h at 37°C. Phosphate-buffered saline or normal rabbit serum was used instead of primary antibody as a negative control.

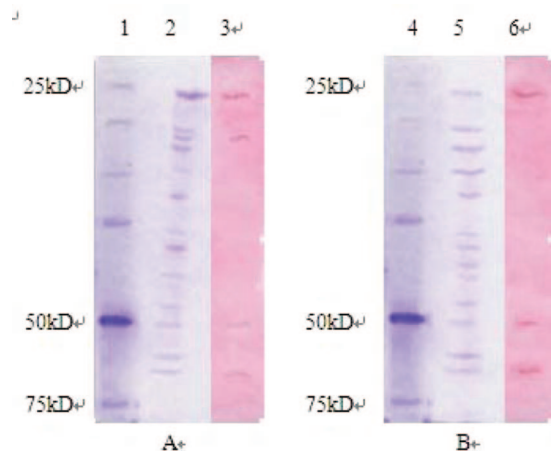

Fig. 1. Ligand Blot Assays. BBMV proteins of *Cx. quinquefasciatus* (lane 2 in A) and *Ae. Albopictus* (lane 5 in B) were solubilized in SDS loading buffer, separated by SDS-PAGE, and blotted to nitrocellulose membrane, respectively (lane 3 in A and lane 6 in B). Protein blots were probed with HRP conjugated Cyt1Aa6 toxin (lane 3, 6) and detected with DAB. Positions of molecular size markers are indicated on the left (lane 1). (Online figure in color.)

Results

Enzyme-Linked Immunosorbent Assay. ELISA was performed by comparing nonspecific binding with BSA as an independent test of peptide specificity. To investigate the difference of Cyt1Aa6 in direct binding, ELISA was also performed by comparison of specific binding with *Ae. albopictus* and *Cx. quinquefasciatus.* Binding of receptor proteins to wells coated with purified Cyt1Aa6 was detected by anti-Cyt1Aa6-habit conjugated with horseradish peroxidase (HRP). Cyt1Aa6 bound to BBMV of *Ae. albopictus* and *Cx. quinquefasciatus* coated wells, which confirmed that Cyt1Aa6 specifically associated with the two tested mosquitoes. However, the titer for *Ae. albopictus* (with the outer diameter [OD] value of 3.21) was a little higher than that for *Cx. quinquefasciatus* (with the OD value of 2.91). *Cx. quinquefasciatus* had a lower specificity for BBMV in vitro, which finding differed from the result of the bioassay.

Ligand Blot Assays. In vitro binding experiments were carried out to elucidate the difference of the receptor components of the two tested mosquitoes in the binding. SDS-PAGE analysis of BBMV purified from *Ae. albopictus* and *Cx. quinquefasciatus* demonstrated that the protein composition was not significantly different (Fig. 1). Most of the protein composition was detected in the two mosquitoes' BBMV. To determine whether there would be differences in special binding sites between the two tested mosquitoes, ligand blotting experiment was performed. However, it was different in the binding character. Ligand Western blot analysis of SDS-PAGE size-separated proteins showed Cyt1Aa6 toxin specifically bound to the same three proteins (i.e., 68, 52, and 26 kDa) in the two mosquitoes, but another protein, approximately to 37

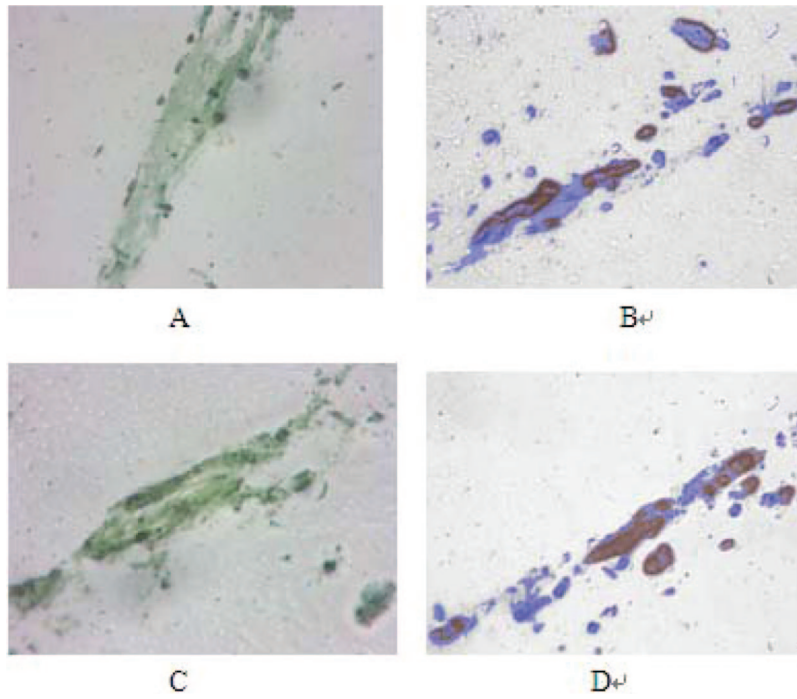

Fig. 2. Cyt1Aa6 is examined by immunohistochemistry staining analysis. Immunohistochemistry was performed on paraffin sections of normal *Cx. quinquefasciatus* and *Ae. albopictus* with the antigen retrieval method in the presence or absence of rabbit anti-Cyt1Aa6 primary antibody. (A) *Cx. quinquefasciatus* in the absence of rabbit anti-Cyt1Aa6 primary antibody as a negative control. (B) *Cx. quinquefasciatus* in the presence of rabbit anti-Cyt1Aa6 primary antibody. (C) *Ae. albopictus* in the absence rabbit anti-Cyt1Aa6 primary antibody as a negative control in *Ae. albopictus*. (D) *Ae. albopictus* in the presence rabbit anti-Cyt1Aa6 primary antibody. (Online figure in color.)

kDa, could be identified to be the Cyt1Aa6 receptor on the midgets of *Cx. quinquefasciatus*, but no receptor could be identified on *Ae. albopictus* (Fig. 1).

Immunohistochemistry Localization of the Cyt1Aa6 Toxin on the Midgut Ephitelial Cells. Immunohistochemistry was performed on paraffin sections of normal *Cx. quinquefasciatus* and *Ae. albopictus* with the antigen retrieval method in the presence or absence of rabbit anti-Cyt1Aa6 primary antibody. As shown in Fig. 2, Cyt1Aa6 was detected in the midguts of both mosquitoes with histopathological changes. The general histopathological changes of midguts induced by the Cyt1Aa6 toxin in the mosquito larvae gut epithelia included brush border membrane impairment and disintegration of midgut. However, little difference was found, and this may not be the reason why Cyt1Aa6 was more toxic against *Cx. quinquefasciatus*.

Discussion

Control of insect pests of public health importance through the application of chemical pesticides is one of the measures to control transmission of vector borne diseases. However, development of resistance and environmental concerns limit their use. Biological control through the use of parasitoids and pathogens is one of the alternatives to the use of chemical pesticides for control of insects of public health importance.

Until now, *B. thuringiensis* has been developed as alternative tools for the control of vector mosquitoes, and their efficacy has been successfully demonstrated. LLP29 is a novel Bti strain, harboring *cry4Aa4*, *cry10Aa4*, *cry11Aa4*, *cyt1Aa6*, and *cyt2Ba15* genes, which could have potential as a biocontrol agent in controlling mosquitoes and preventing mosquito-borne diseases (Zhang et al. 2009, 2010, 2011). However, its mosquitocidal activity against *Cx. quinquefasciatus* was 8.25 times higher than that against *Ae. albopictus*, confirming the previous report that *Cx. quinquefasciatus* was more susceptible to *B. thuringiensis* than *Ae. albopictus* (Mulla 1990; Zhang et al. 2007, 2011). The reasons for the difference in activity level between the two mosquitoes were further studied here.

Ligand Western blot analysis showed Cyt1Aa6 toxin specifically bound to the same three proteins (i.e., 68, 54, and 26 kDa) in the two mosquitoes, except for one another protein, approximately to 37 kDa. Meanwhile, little difference was found in the test of immunohistochemistry. Cyt1Aa6 was detected in the midguts of both mosquitoes with histopathological changes. Present in vivo experiments showed that Cyt1Aa6 had sensitivity and specificity bound to the BBMV of *Cx. quinquefasciatus* and *Ae. albopictus* (Zhang et al. 2012),

and the epithelial cells of the midgut could be considered as the main target of this toxin (Ruiz et al. 2004), which was the same as previous reports that there was a good correlation between a socalled binding constant measured as described above with BBMV and toxicity (Aronson and Shai 2001).

However, there was also some difference detected. As just listed, there was one another protein, approximately to 37 kDa that could just be detected in *Cx. quinquefasciatus.* It may be one of the important reasons why Cyt1Aa6 was more toxic against *Cx. quinquefasciatus* than *Ae. albopictus.* ELISA showed that Cyt1Aa6 bound to the two mosquitoes' BBMV, but the titer of *Ae. albopictus* was a little higher than that of *Cx. quinquefasciatus* (3.21 and 2.91), respectively, which was different with the result of the bioassay. This indicated a higher specificity for BBMV of *Ae. albopictus* in vitro than that of *Cx. quinquefasciatus,* comparing to the specificity observed in vivo. It was the same as the result of Aminopeptidase N (APN) that the presence of APN activity was not directly correlated with toxin binding and no clear relationship could be found between APN activity and the toxicity of Cry proteins (Lorence et al. 1997, Jenkins et al. 1999, Gilliland et al. 2002, Likitvivatanavong et al. 2011). Study on the infection difference between them showed that such difference might be because of infection barrier; furthermore, the receptor on the midgut might be presumed as the primary factors responding to infection. It may also be because of either differential activation by midgut proteases or differential stability with the Cyt1Aa family (Zhang et al. 2009, 2010). Research of the receptor on the mosquito's midgut may help us understanding the mechanisms on the different toxicity of the novel *B. thuringiensis* isolate LLP29 against *Ae. albopictus* and *Cx. quinquefasciatus.*

The binding to the receptor determined the specificity and efficiency of the toxic effect. Many toxin receptors had been recently studied in some insects, such as APN, cadherin-like proteins, and so on (Buzdin et al. 2002, Pigott and Ellar 2007). *Ae. aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from Bti (Chen et al. 2009).

Proteins with molecular weights of 65 and 62 kDa were reported capable of binding with the mosquitocidal toxins Cry4B and Cry11A, which had been isolated earlier from BBM of *Ae. aegypti.* The protein with the molecular weight of 65 kDa, which might be of an unknown class of endotoxin receptors, was the only component of *Ae. aegypti* larvae BBM capable to specifically bind mosquitocidal toxins Cry4B and Cry11A of *B. thuringiensis* (Buzdin et al. 2002). However, it was also detected binding with Cyt1Aa6. It might was suggested that during evolution mosquitocidal toxins should adapt for the binding to membrane proteins of another class more suitable for providing the effective interaction of the toxins with BBM of Diptera (Buzdin et al. 2002). Except for the one of 65 kDa, the binding proteins in present data presented would be further studied, which might be great value in understanding the mechanism of this toxin and could provide a potential molecular genetic strategy for use of the novel toxin and the new *B. thuringiensis* strain.

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