1 **Interactions between molecular chaperone P20 and Cyt2Ba7 toxin in *Bacillus***
2 ***thuringiensis***

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14 **Running title:** Molecular chaperone P20 and Cyt2Ba7 toxin in *Bacillus*
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Abstract

P20 or 20-kilodalton protein is a molecular chaperone protein in *Bacillus thuringiensis* (*Bt*) which can increase yields and facilitates crystal formation of various insecticidal crystal proteins (ICPs). In previous studies, a *B. thuringiensis* insecticidal protein gene, *cyt2Ba7*, was cloned, expressed but its expression level is very low in *B. thuringiensis*. In this study, various expression vectors were constructed by incorporating *p20* in forward or reverse direction in the upstream of *cyt2Ba7* and transformed into a *B. thuringiensis* acrySTALLIFEROUS strain 4Q7. The result showed that in the presence of P20, the expression of Cyt2Ba7 was significantly increased. Especially when *p20* gene was reversely inserted in the upstream of *cyt2Ba7* gene, the expression of Cyt2Ba7 was increased ~3.2 times meanwhile more and bigger crystals were observed under electron microscopy. By using purified Cyt2Ba7, P20 protein and P20-specific antiserum, immunoblotting and ligand blot analysis demonstrated a strong binding affinity between P20 and Cyt2Ba7. These results reveal that P20 can promote the crystal formation and enhance the expression of Cyt2Ba7 as a molecular chaperone, which can be a powerful tool to boost the ICPs production in *B. thuringiensis* and help develop more effective insect control strategies.

Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium which can produce insecticidal crystal proteins (ICPs) that are composed of one or several toxic proteins with insecticidal and cytolytic activities [Crickmore et al., 1998]. Cry and Cyt are two major ICP groups but they do not share sequence homologies [Crickmore et al., 1998; Palma et al., 2014; Rajamohan et al., 1998]. Cry proteins are not only toxic to the larvae of lepidopteran, dipteran and coleopteran insects, but also to nematodes, protozoan pathogens, animal-parasitic liver flukes and mites [Crickmore et al., 1998; Zhang et al., 2016]. Cyt proteins are primarily lethal to dipteran insects and cytolytic to a broad range of cells including erythrocytes [Crickmore et al., 1998; Thomas and Ellar, 1983]. Its cytolytic properties are attributed to its affinity for unsaturated fatty acids in cell membranes, in which it apparently aggregates, leading to the formation of pores that cause cell lysis [Canton et al., 2014; Guerchicoff et al., 1997; Koni and Ellar, 1993, 1994; Zhang et al., 2016]. Cyt proteins appear to have a different mode of action and lack significant cross-resistance to Cry proteins, indicating that Cyt proteins may play an even more important long-term role in managing resistance to Cry proteins [Canton et al., 2014; Sayyed et al., 2001; Wirth et al., 2000; Wu and Federici, 1993; Yu et al., 2002b].

A *cyt2* (*cyt2Ba7*) gene from a soil-isolated *B. thuringiensis* strain was cloned, expressed in *Escherichia coli* and purified to obtain the antiserum from rabbits [Yu et al., 2002b]. Purified Cyt2Ba7 also showed cytolytic activity to insect cells and mosquito larvicidal activity. However, the expression of Cyt2Ba7 in *B. thuringiensis*

is very low and can only be detected by specific antiserum [Yu et al., 2002b].

Improving the synthesis of ICP proteins in *B. thuringiensis* is critical since higher yields leads to higher insecticidal activity, which could be achieved by incorporating genetic elements to regulate protein synthesis at the transcriptional, translational and posttranslational levels [Adams et al., 1989; Diaz-Mendoza et al., 2012; Elleuch et al., 2016a; Elleuch et al., 2016b; Elleuch et al., 2015; Park et al., 2007; Wu and Federici, 1993].

Molecular chaperones are proteins that help in the folding/unfolding or the assembly/disassembly of other macromolecules to form new structures or complexes. However, they do not exist in the newly formed macromolecules and function [Hartl et al., 2011]. Therefore, molecular chaperones play a critical role in the conformational quality control of the proteome by interacting with, stabilizing and remodeling a wide range of polypeptides [Hartl et al., 2011]. A group of molecular chaperones including P20 [Wu and Federici, 1993], P19 [Manasherob et al., 2001], Orf1 and Orf2 [Tang et al., 2003], have been found and characterized in the synthesis of crystal proteins in *B. thuringiensis*, in which P20 is the mostly studied molecular chaperone in *B. thuringiensis*. It is an accessory protein in *B. thuringiensis* subsp. *Israelensis* (*Bti*), which was first described during a study of Cyt1A expression [Adams et al., 1989; Wu and Federici, 1993]. Investigations concentrated on the role of P20 in Cyt1A expression proved that it is necessary for Cyt1A crystallization and host cell viability [Adams et al., 1989; Wu and Federici, 1993]. Moreover, P20 also increased the production of other *B. thuringiensis* toxins [Elleuch et al., 2016a;

Elleuch et al., 2016b; Elleuch et al., 2015; Nisnevitch et al., 2006; Wang et al., 1997; Wu and Federici, 1995; Xu et al., 2001], which are poorly expressed without P20. An increase in their production in the presence of P20 prompted us to investigate if and how the 20-kDa chaperone enhances synthesis and crystallization of the Cyt2Ba7. In this study, *p20* and *cyt2Ba7* were incorporated in the same vectors and transformed to an acrystalliferous *B. thuringiensis* strain. Interestingly, Cyt2Ba7 production is increased and bigger crystals were formed in the presence of P20. Then we further applied binding assay study the protein-protein interactions between P20 and Cyt2Ba7.

Experimental Procedures

Bacterial strains, antiserum, media and plasmids

Bacterial strains and plasmids used in this study are all deposited in State Key Laboratory for Biocontrol (Sun Yat-sen University, China). *B. thuringiensis* subsp. strain 4Q7, a plasmid-free derivate of *B. thuringiensis* serotype israelensis (*Bti*), was obtained from *Bacillus* Genetics Stock Center, Columbus, Ohio. *E. coli* strain TG1 was grown in Luria-Bertani (LB) broth or agar plate at 37°C for plasmid propagation. Plasmids pHT3101, pMD18-T, pHT301 [Yu et al., 2002b] which contains the full-length *cyt2Ba7* gene with promoter, and pUP20 [Shi et al., 2006] which contains *cry1Ac* promoter and *p20* gene were used for plasmid construction. Bacteria were grown in LB medium at 37°C or G-tris medium at 30°C with ampicillin (100 µg/ml) or erythromycin (25 µg/ml). Cyt2Ba7-specific antiserum, purified P20 protein and P20-specific antiserum were prepared as described [Tang, 2003; Yu et al., 2002b]. All restriction enzymes and ligation enzymes were purchased from Roche (Germany).

Plasmids Construction

The full-length *cyt2Ba7* gene containing native promoter and ORF sequences were amplified from plasmid pHT301 [Yu et al., 2002b] by gene-specific primers; forward primer 5'-GTCGACGATAATGAGGTTATTTTGT-3' (*Sall*) and reverse primer 5'-GCAATGCATCTTACGATTTTATTGGAT-3' (*SphI*) as follows: 95 °C for 3 min; 35 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s; and final extension at

72 °C for 10 min. PCR products were ligated to vector pMD18-T and the ligation products were transformed into *E. coli* strain TG1 for DNA sequencing. The correct plasmid was digested by *Sall/SphI* and *cyt2Ba7* fragment was subsequently ligated into vector pUP20 [Shi et al., 2006] to construct plasmid pUP20CT (Fig. 1A). Then the fragment containing a *cry1Ac* promoter, a *p20* gene and a *cyt2Ba7* full length gene was digested by *BamHI/SphI* from pUP20CT and ligated to pHT3101 to obtain plasmid pHP20CT, which contains a *cry1Ac* promoter and a *p20* gene sequences upstream of full *cyt2Ba7* gene in the same transcription direction (Fig. 1A and B). Similarly, the *cry1Ac* promoter and *p20* gene were digested from the plasmid pUP20C by *KpnI/SphI* and then ligated into pHT301 [Yu et al., 2002b] to get plasmid pHP20CRT, which contains an upstream *cry1Ac* promoter and *p20* gene in a reverse transcription direction to *cyt2Ba7* gene (Fig. 1A and B). The plasmids pHT3101 (negative control), pHT301, pHP20CT and pHP20CRT were further electroporated into the acrySTALLIFEROUS strain 4Q7.

Expression of Cyt2Ba7

After inoculating 4Q7 transformed by pHT3101 (negative control), pHT301, pHP20CT and pHP20CRT in the G-Tris medium and cultured at 30°C, 1 ml medium was collected and centrifuged (15,000×g for 10 min) to harvest the cells at 12 h, 24 h, 36 h, 48 h, 60 h and 72 h after inoculation. The cell pellet was suspended in a 50 µl 1× Laemmli sample buffer containing sodium dodecyl sulfate (SDS) and boiled for 10 min, and then protein content was subjected to 12% SDS-PAGE for analysis [Shi

et al., 2006]. After separation, proteins were electroblotted onto the nitrocellulose membrane (Bio-Rad, Shanghai, China) by semi-dry blotting. Antiserum for Cyt2Ba7 was used in immunoblotting analysis [Yu et al., 2002b]. The expression levels of Cyt2Ba7 in 4Q7 (pHT301), 4Q7 (pHP20CT) and 4Q7 (pHP20CRT) were analyzed from 12 to 72 h to determine the highest expression time [Shi et al., 2006]. To compare the expression levels of Cyt2Ba7 in 4Q7 (pHT301), 4Q7 (pHP20CT) and 4Q7 (pHP20CRT), these three strains were grown in G-Tris medium at 30°C for 72 hours and harvested by centrifugation as above. The cell pellets were resuspended and calibrated to the same optical densities (OD₆₀₀) and 100 µl of each sample was incubated with 100 µl 2× Laemmli sample buffer and boiled. 50 µl of each sample was loaded to SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and photographed by using an Electrophoresis Documentation and Analysis 120 System (Eastman Kodak Co.). The protein bands were scanned and quantified using an ImageMaster TotalLab imaging system V3 (Amersham Pharmacia Biotech) [Shi et al., 2006]. At least three replicate were performed to statistically compare Cyt2Ba7 yields.

Transmission electron microscopy

B. thuringiensis and inclusion samples were purified and treated as previously described and then observed produced crystals under transmission electron microscopy JEM100cx-II (JEOL, Japan) [Nisnevitch et al., 2006; Park et al., 1998; Yu et al., 2002a].

Immunoblotting and ligand blot analysis

To study if there is a protein-protein interaction between P20 and Cyt2Ba7, fusion proteins Cyt2Ba7 and P20 were purified as described before [Tang, 2003; Yu et al., 2002b]. One microgram purified Cyt2Ba7 was analyzed by 12% SDS-PAGE gel (Fig. 4A) and then transferred electrophoretically to a nitrocellulose membrane. To visualize immunoreactive proteins, one membrane was allowed to react directly with P20-specific antiserum [1:1000] [Tang, 2003]. Another membrane with same preparation was allowed to react with 50 µg purified P20 first and then anti-P20 antiserum [1:1000]. After washing, both membranes were incubated with secondary antibody coupled to calf intestinal alkaline phosphatase (Ap) for developing.

Results and Discussion

Expression of Cyt2Ba7

The plasmid pHT301 possesses both native promoter and ORF sequences of *cyt2Ba7* (Fig. 1B). The plasmids pHP20CT and pHP20CRT both possess the native promoter of *cry1Ac*, *p20* gene, and full-length *cyt2Ba7* gene (with native promoter) but in different directions (Fig. 1B).

Firstly we performed a time course study on the expression of Cyt2Ba7 in *B. thuringiensis* strain 4Q7 transformed with plasmids pHT301, pHP20CT and pHP20CRT (Fig. 2A, B and C) from 12 h to 72 h after inoculation. The immunoblot results showed that in all these three constructs, the expression of Cyt2Ba7 started from 24 h, reached the highest yield at 36 h and kept constant to 72 hours (Fig. 2). A weak band with molecular weight at around 58 kDa was observed in 4Q7 (pHP20CRT) but not in the other two constructs (Fig. 2C), which may be a dimer formation of Cyt2Ba7 [Cohen et al., 2008] and only be produced when the protein amount is high. There is no expression of Cyt2Ba7 in negative control, 4Q7 (pHT301) (Fig. 2). Then we compared the expression of Cyt2Ba7 in 4Q7 transformed with pHT301, pHP20CT and pHP20CRT at 72 h after inoculation. Using the integrated optical density (IOD) values, we observed that the expression level of Cyt2Ba7 in 4Q7 (pHP20CRT) was increased nearly 3.2 fold than that of 4Q7 (pHT301). Cyt2Ba7 in 4Q7 (pHP20CT) was increased nearly 0.7 fold than that of 4Q7 (pHT301) (Fig. 2B). This result suggests that when *p20* was inserted into the upstream of *cyt2Ba7* gene, no matter which direction, the expression of Cyt2Ba7 can

be significantly increased. It is very interesting that the expression of Cyt2Ba7 in 4Q7 (pHP20CRT) is much higher than that of 4Q7 (pHP20CT). The only difference between these two constructs is the direction of inserted *p20* gene and *cry1Ac* promoter. In pHP20CT, *p20* gene and *cry1Ac* promoter were inserted in a forward direction while in pHP20CRT, *p20* gene and *cry1Ac* promoter were inserted in a reverse direction (Fig. 1B). Why reversely inserted *cry1Ac* promoter and *p20* (pHP20CRT) increased the productivity of Cyt2Ba7 more significantly than forwardly inserted (pHP20CT) is unknown.

The crystals of Cyt2Ba7

Then we compared the 4Q7 transformed with pHT301, pHP20CT and pHP20CRT under electron microscopy. As anticipated, we did not see any crystals from negative control, 4Q7 (pHT3101) (Fig. 3A). In 4Q7 (pHT301), we observed very few and tiny crystals of Cyt2Ba7 (Fig. 3B). Similarly, a few small irregular crystals were also observed in 4Q7 (pHP20CT) (Fig. 3C). However, from 4Q7 (pHP20CRT), we observed more and bigger hexagonal-shaped crystals ranged from 0.6 μm to 1 μm (Fig. 3D, E and F). Similar results were reported on other ICPs before [Diaz-Mendoza et al., 2012; Nisnevitch et al., 2006; Park et al., 1998; Rang et al., 1996; Shao et al., 2001; Shao and Yu, 2004; Wang et al., 1997; Wu and Federici, 1993] and *Bacillus sphaericus* Bin toxin [Park et al., 2007] in *B. thuringiensis*. When *cry1Ac* promoter and *p20* were reversely inserted in the upstream of *cyt2Ba7*, the amount and size of Cyt2Ba7 crystals are both increased, suggesting the expression of P20

can help synthesize the crystals of Cyt2Ba7.

The interactions between Cyt2Ba7 and P20

The molecular mechanism how P20 helps increase the synthesis of Cyt2Ba7 and other ICPs is still unknown, which can be achieved at the transcriptional, translational and posttranslational levels [Adams et al., 1989; Diaz-Mendoza et al., 2012; Park et al., 2007; Wu and Federici, 1993]. Here we insert *p20* gene with two opposite directions into the upstream of *cyt2Ba7* gene and the results showed that the expression level of Cyt2Ba7 in reverse form (pHP20CRT) is much higher than that of forward form (pHP20CT). The crystals synthesized are also significantly increased in amount and size. Therefore we hypothesized that P20 plays a role at protein level. For example, in the protein folding to help Cyt2Ba7 form crystals and thus prevent proteolytic degradation. If this is the case, a protein-protein interaction between P20 and Cyt2Ba7 is required and essential. To investigate this hypothesis, we used previously purified P20 proteins from *E. coli* and prepared P20-specific antiserum [Tang, 2003]. Immunoblotting and ligand blot analysis results showed Cyt2Ba7 could not be detected by P20-specific antiserum directly (Fig. 4C). However, when P20 protein was added, Cyt2Ba7 can be detected by the P20-specific antiserum (Fig. 4B). These results suggest there is a protein-protein interaction between P20 and Cyt2Ba7.

P20 has been reported to enhance the expression of many other ICP proteins from *B. thuringiensis*. For example, in the presence of P20, Cyt1Aa is highly expressed and

formed large ovoidal, lemon-shaped crystals [Wu and Federici, 1993]. P20 also help improve the yields of Cry1Ac [Shao et al., 2001], truncated Cry1C [Rang et al., 1996], Cry2A [Shao and Yu, 2004], Cry3A [Diaz-Mendoza et al., 2012], Cry4A [Wang et al., 1997], Cry4B [Elleuch et al., 2015] Cry11A [Park et al., 1998], Cyt2Ba [Cohen et al., 2008; Nisnevitch et al., 2006] and *B. sphaericus* Bin toxin [Park et al., 2007], which showed no or low homologies at amino acid sequences and very diverse structures. How P20 could increase the synthesis of these various proteins is still not clear. Interestingly, P20 could not promote the production of all ICP proteins. For example, P20 did not improve expression of Cry20Aa, a mosquitocidal protein [Lee and Gill, 1997]. In this study, we also found that there are certain bands on the top of Cyt2Ba7 sample (Fig. 4A), but they could not be detected by P20 and P20-specific antiserum (Fig. 4B), suggesting P20 has specificity and selectivity to bind the targeted proteins.

In summary, our study showed that P20 functions as a molecular chaperone to increase yields and facilitates crystal formation of Cyt2Ba7. Therefore it is promising for P20 to be used to increase the toxicity and mortality of *B. thuringiensis* strains to control insect pests. Many structures of ICPs including Cyt1Aa [Cohen et al., 2011], Cyt2Ba [Cohen et al., 2008] and Cry2Aa [Morse et al., 2001] have been reported. Therefore the structural functional and mutagenesis studies of P20 will assist us further explore the mechanism of P20. Better understanding P20 will pave the way to optimize its function and develop more

268 effective and efficient insect control strategies.

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References

- Adams LF, Visick JE, Whiteley HR: A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. J Bacteriol 1989;171:521-530.
- Canton PE, Lopez-Diaz JA, Gill SS, Bravo A, Soberon M: Membrane binding and oligomer membrane insertion are necessary but insufficient for *Bacillus thuringiensis* Cyt1Aa toxicity. Peptides 2014;53:286-291.
- Cohen S, Albeck S, Ben-Dov E, Cahan R, Firer M, Zaritsky A, Dym O: Cyt1Aa toxin: crystal structure reveals implications for its membrane-perforating function. J Mol Biol 2011;413:804-814.
- Cohen S, Dym O, Albeck S, Ben-Dov E, Cahan R, Firer M, Zaritsky A: High-resolution crystal structure of activated Cyt2Ba monomer from *Bacillus thuringiensis* subsp. *israelensis*. J Mol Biol 2008;380:820-827.
- Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH: Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiology and molecular biology reviews : Microbiol Mol Biol R 1998;62:807-813.
- Diaz-Mendoza M, Bideshi DK, Ortego F, Farinos GP, Federici BA: The 20-kDa chaperone-like protein of *Bacillus thuringiensis* ssp. *israelensis* enhances yield, crystal size and solubility of Cry3A. Lett Appl Microbiol 2012;54:88-95.
- Elleuch J, Jaoua S, Ginibre C, Chandre F, Tounsi S, Zghal RZ: Toxin stability improvement and toxicity increase against dipteran and lepidopteran larvae of *Bacillus thuringiensis* crystal protein Cry2Aa. Pest Manag Sci 2016a;72:2240-2246.
- Elleuch J, Jaoua S, Tounsi S, Zghal RZ: Cry1Ac toxicity enhancement towards lepidopteran pest *Ephestia kuehniella* through its protection against excessive proteolysis. Toxicon 2016b;120:42-48.
- Elleuch J, Zghal RZ, Ben Fguira I, Lacroix MN, Suissi J, Chandre F, Tounsi S, Jaoua S: Effects of the P20 protein from *Bacillus thuringiensis israelensis* on insecticidal crystal protein Cry4Ba. Int J Biol Macromol 2015;79:174-179.
- Guerchicoff A, Ugalde RA, Rubinstein CP: Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl Environ Microb 1997;63:2716-2721.
- Hartl FU, Bracher A, Hayer-Hartl M: Molecular chaperones in protein folding and proteostasis. Nature 2011;475:324-332.
- Koni PA, Ellar DJ: Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. J Mol Biol 1993;229:319-327.
- Koni PA, Ellar DJ: Biochemical characterization of *Bacillus thuringiensis* cytolytic delta-endotoxins. Microbiology 1994;140 (Pt 8):1869-1880.
- Lee HK, Gill SS: Molecular cloning and characterization of a novel mosquitocidal protein gene from *Bacillus thuringiensis* subsp. *fukuokaensis*. Appl Environ Microb 1997;63:4664-4670.
- Manasherob R, Zaritsky A, Ben-Dov E, Saxena D, Barak Z, Einav M: Effect of accessory proteins P19 and P20 on cytolytic activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* in *Escherichia coli*. Curr Microbiol 2001;43:355-364.
- Morse RJ, Yamamoto T, Stroud RM: Structure of Cry2Aa suggests an unexpected receptor binding

318 epitope. Structure 2001;9:409-417.

319 Nisnevitch M, Cohen S, Ben-Dov E, Zaritsky A, Sofer Y, Cahan R: Cyt2Ba of *Bacillus thuringiensis*
320 *israelensis*: activation by putative endogenous protease. Biochem Bioph Res Co
321 2006;344:99-105.

322 Palma L, Munoz D, Berry C, Murillo J, Caballero P: *Bacillus thuringiensis* toxins: an overview of
323 their biocidal activity. Toxins 2014;6:3296-3325.

324 Park HW, Bideshi DK, Federici BA: The 20-kDa protein of *Bacillus thuringiensis* subsp. *israelensis*
325 enhances *Bacillus sphaericus* 2362 bin toxin synthesis. Curr Microbiol 2007;55:119-124.

326 Park HW, Ge B, Bauer LS, Federici BA: Optimization of Cry3A yields in *Bacillus thuringiensis* by
327 use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence.
328 Appl Environ Microb 1998;64:3932-3938.

329 Rajamohan F, Lee MK, Dean DH: *Bacillus thuringiensis* insecticidal proteins: molecular mode of
330 action. Prog Nucleic Acid Re 1998;60:1-27.

331 Rang C, Bes M, Lullien-Pellerin V, Wu D, Federici BA, Frutos R: Influence of the 20-kDa protein
332 from *Bacillus thuringiensis* ssp. *israelensis* on the rate of production of truncated Cry1C
333 proteins. FEMS Microbiol Lett 1996;141:261-264.

334 Sayyed AH, Crickmore N, Wright DJ: Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* is toxic
335 to the diamondback moth, *Plutella xylostella*, and synergizes the activity of Cry1Ac towards
336 a resistant strain. Appl Environ Microb 2001;67:5859-5861.

337 Shao Z, Liu Z, Yu Z: Effects of the 20-kilodalton helper protein on Cry1Ac production and spore
338 formation in *Bacillus thuringiensis*. Appl Environ Microb 2001;67:5362-5369.

339 Shao Z, Yu Z: Enhanced expression of insecticidal crystal proteins in wild *Bacillus thuringiensis*
340 strains by a heterogeneous protein P20. Curr Microbiol 2004;48:321-326.

341 Shi YX, Yuan MJ, Chen JW, Sun F, Pang Y: [Effects of helper protein P20 from *Bacillus thuringiensis*
342 on Vip3A expression]. Acta microbiologica Sinica 2006;46:85-89.

343 Tang MJ: Cloning , Expression and its Functional Study of Accessary Protein Genes from *Bacillus*
344 *thuringiensis*; in: Molecular Biology and Biochemistry. Sun Yat-sen University, 2003, vol
345 PhD.

346 Tang MJ, Zeng S, Chen J, Shi Y, Xu W, Yuan M, Pang Y: Effects of Helper Proteins Encoded by p19
347 and orf1-orf2 genes on cyt1Aa protein expression in acrySTALLIFEROUS strain of *Bacillus*
348 *thuringiensis*. Entomologia sinica 2003;10:9.

349 Thomas WE, Ellar DJ: *Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: effects on insect
350 and mammalian cells in vitro and in vivo. J Cell Sci 1983;60:181-197.

351 Wang X, Vennison SJ, Huirong L, Ben-Dov E, Zaritsky A, Boussiba S: Mosquito larvicidal activity of
352 transgenic *Anabaena* strain PCC 7120 expressing combinations of genes from *Bacillus*
353 *thuringiensis* subsp. *israelensis*. Appl Environ Microb 1997;63:4971-4974.

354 Wirth MC, Federici BA, Walton WE: Cyt1A from *Bacillus thuringiensis* synergizes activity of
355 *Bacillus sphaericus* against *Aedes aegypti* (Diptera: Culicidae). Appl Environ Microb
356 2000;66:1093-1097.

357 Wu D, Federici BA: A 20-kilodalton protein preserves cell viability and promotes CytA crystal
358 formation during sporulation in *Bacillus thuringiensis*. J Bacteriol 1993;175:5276-5280.

359 Wu D, Federici BA: Improved production of the insecticidal CryIVD protein in *Bacillus thuringiensis*
360 using cryIA(c) promoters to express the gene for an associated 20-kDa protein. Appl Environ
361 Microb 1995;42:697-702.

362 Xu Y, Nagai M, Bagdasarian M, Smith TW, Walker ED: Expression of the p20 gene from *Bacillus*
363 *thuringiensis* H-14 increases Cry11A toxin production and enhances mosquito-larvicidal
364 activity in recombinant gram-negative bacteria. Appl Environ Microb 2001;67:3010-3015.
365 Yu J, Xie R, Tan L, Xu W, Zeng S, Chen J, Tang M, Pang Y: Expression of the full-length and 3'-
366 spliced cry1Ab gene in the 135-kDa crystal protein minus derivative of *Bacillus*
367 *thuringiensis* subsp. *kyushuensis*. Curr Microbiol 2002a;45:133-138.
368 Yu J, Xu W, Zeng S, Zhang X, Liu J, Xie R, Pang Y: Cloning and expression of cyt2Ba7 gene from a
369 soil-isolated *Bacillus thuringiensis*. Curr Microbiol 2002b;45:309-314.
370 Zhang Q, Hua G, Adang MJ: Effects and mechanisms of *Bacillus thuringiensis* crystal toxins for
371 mosquito larvae. Insect Sci 2016; 00: 1-16.

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Figure Legends

Fig. 1. Plasmids constructing procedures (A) and maps (B) of the recombinant plasmids pHT301, pHP20CT and pHP20CRT. The plasmid pHT301 possesses *cyt2Ba7* gene (with native promoter and ORF sequence). The plasmids pHP20CT and pHP20CRT both possess the promoter of *cry1Ac*, *p20* gene, and *cyt2Ba7* gene (with its native promoter) but in different directions. Arrows represent transcription direction of different genes.

Fig. 2. Expression time courses study of Cyt2Ba7 protein in 4Q7 transformed with plasmids pHT301(A), pHP20CT (B), pHP20CRT (C) and the expression comparison (D) of Cyt2Ba7 protein in these three strains at 72 h by using Cyt2Ba7 specific antiserum. M, protein marker; 1, 1 ml cell pellets of 72 h of 4Q7 (pHT3101); 2-7 are 1 ml cell pellet collected from 12 h, 24 h; 36 h, 48 h; 60 h and 72 h respectively. In (B) and (C), lane 8 is cell pellets of 72 h of 4Q7 (pHT301).

Fig. 3. Transmission electron microscopies of crystals of Cyt2Ba7. Arrow indicates the crystals formed by Cyt2Ba7. (A) 4Q7 (pHT3101); (B) 4Q7 (pHT301); (C) 4Q7 (pHP20CT); (D) 4Q7 (pHP20CRT); (E) and (F), crystals of Cyt2Ba7 in 4Q7 (pHP20CRT). “C” stands for crystals of Cyt2Ba7 while “S” stands for spores of *B. thuringiensis*. The bar stands for 1 μ m.

Fig. 4. Immunoblot and ligand blot analysis of Cyt2Ba7. (A) SDS-PAGE analysis of

396 1 µg purified Cyt2Ba7. (B) Immunoblot analysis of Cyt2Ba7 after incubated with 50
 397 µg purified fusion P20 protein and then P20-specific antiserum. (C)
 398 Cyt2Ba7incubated with P20-specific antiserum but without purified P20 protein. M:
 399 Standard protein marker.
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Figure 1

Figure 2

Figure 3

Figure 4