bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 **Interactions between molecular chaperone P20 and Cyt2Ba7 toxin in *Bacillus***
2 ***thuringiensis***
3
4
5 Yongxia Shi[1,2], Mujin Tang[2], Yalin Liao[3], Wei Xu[3*]
6
7 [1]Health Quarantine Lab, Guangdong Inspection and Quarantine Technology Center,
8 Guangzhou, People's Republic of China 510700
9 [2]State Key Laboratory of Biocontrol, Sun Yat-sen University, Guangzhou, People's
10 Republic of China 510275
11 [3]School of Veterinary and Life Sciences, Murdoch University, Murdoch, West
12 Australia, Australia 6150
13
14 **Running title:** Molecular chaperone P20 and Cyt2Ba7 toxin in *Bacillus*
15 *thuringiensis*
16
17
18 *Corresponding author:
19 Dr. Wei Xu
20 School of Veterinary and Life Sciences, Murdoch University, Murdoch, WA 6150,
21 Australia
22 Tel: +61-0893606772
23 W.Xu@Murdoch.edu.au
24

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

25 **Keywords**: *Bacillus thuringiensis*, molecular chaperone, 20 kDa protein

26

27

28 **Abstract**

29 P20 or 20-kilodalton protein is a molecular chaperone protein in *Bacillus*
30 *thuringiensis* (*Bt*) which can increase yields and facilitates crystal formation of
31 various insecticidal crystal proteins (ICPs). In previous studies, a *B. thuringiensis*
32 insecticidal protein gene, *cyt*2Ba7, was cloned, expressed but its expression level is
33 very low in *B. thuringiensis*. In this study, various expression vectors were
34 constructed by incorporating *p*20 in forward or reverse direction in the upstream of
35 *cyt*2Ba7 and transformed into a *B. thuringiensis* acrystalliferous strain 4Q7. The
36 result showed that in the presence of P20, the expression of Cyt2Ba7 was
37 significantly increased. Especially when *p*20 gene was reversely inserted in the
38 upstream of *cyt*2Ba7 gene, the expression of Cyt2Ba7 was increased ~3.2 times
39 meanwhile more and bigger crystals were observed under electron microscopy. By
40 using purified Cyt2Ba7, P20 protein and P20-specific antiserum, immunoblotting
41 and ligand blot analysis demonstrated a strong binding affinity between P20 and
42 Cyt2Ba7. These results reveal that P20 can promote the crystal formation and
43 enhance the expression of Cyt2Ba7 as a molecular chaperone, which can be a
44 powerful tool to boost the ICPs production in *B. thuringiensis* and help develop more
45 effective insect control strategies.

46

47

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

48 **Introduction**

49 *Bacillus thuringiensis* (*Bt*) is a gram-positive soil bacterium which can produce
50 insecticidal crystal proteins (ICPs) that are composed of one or several toxic proteins
51 with insecticidal and cytolytic activities [Crickmore et al., 1998]. Cry and Cyt are
52 two major ICP groups but they do not share sequence homologies [Crickmore et al.,
53 1998; Palma et al., 2014; Rajamohan et al., 1998]. Cry proteins are not only toxic to
54 the larvae of lepidopteran, dipteran and coleopteran insects, but also to nematodes,
55 protozoan pathogens, animal-parasitic liver flukes and mites [Crickmore et al., 1998;
56 Zhang et al., 2016]. Cyt proteins are primarily lethal to dipteran insects and cytolytic
57 to a broad range of cells including erythrocytes [Crickmore et al., 1998; Thomas and
58 Ellar, 1983]. Its cytolytic properties are attributed to its affinity for unsaturated fatty
59 acids in cell membranes, in which it apparently aggregates, leading to the formation
60 of pores that cause cell lysis [Canton et al., 2014; Guerchicoff et al., 1997; Koni and
61 Ellar, 1993, 1994; Zhang et al., 2016]. Cyt proteins appear to have a different mode
62 of action and lack significant cross-resistance to Cry proteins, indicating that Cyt
63 proteins may play an even more important long-term role in managing resistance to
64 Cry proteins [Canton et al., 2014; Sayyed et al., 2001; Wirth et al., 2000; Wu and
65 Federici, 1993; Yu et al., 2002b].

66 A *cyt*2 (*cyt*2Ba7) gene from a soil-isolated *B. thuringiensis* strain was cloned,
67 expressed in *Escherichia coli* and purified to obtain the antiserum from rabbits [Yu et
68 al., 2002b]. Purified Cyt2Ba7 also showed cytolytic activity to insect cells and
69 mosquito larvicidal activity. However, the expression of Cyt2Ba7 in *B. thuringiensis*

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

70 is very low and can only be detected by specific antiserum [Yu et al., 2002b].
71 Improving the synthesis of ICP proteins in *B. thuringiensis* is critical since higher
72 yields leads to higher insecticidal activity, which could be achieved by incorporating
73 genetic elements to regulate protein synthesis at the transcriptional, translational and
74 posttranslational levels [Adams et al., 1989; Diaz-Mendoza et al., 2012; Elleuch et al.,
75 2016a; Elleuch et al., 2016b; Elleuch et al., 2015; Park et al., 2007; Wu and Federici,
76 1993].
77 Molecular chaperones are proteins that help in the folding/unfolding or the
78 assembly/disassembly of other macromolecules to form new structures or complexes.
79 However, they do not exist in the newly formed macromolecules and function [Hartl
80 et al., 2011]. Therefore, molecular chaperones play a critical role in the
81 conformational quality control of the proteome by interacting with, stabilizing and
82 remodeling a wide range of polypeptides [Hartl et al., 2011]. A group of molecular
83 chaperones including P20 [Wu and Federici, 1993], P19 [Manasherob et al., 2001],
84 Orf1 and Orf2 [Tang et al., 2003], have been found and characterized in the synthesis
85 of crystal proteins in *B. thuringiensis*, in which P20 is the mostly studied molecular
86 chaperone in *B. thuringiensis*. It is an accessory protein in *B. thuringiensis* subsp.
87 *Israelensis* (*Bti*), which was first described during a study of Cyt1A expression
88 [Adams et al., 1989; Wu and Federici, 1993]. Investigations concentrated on the role
89 of P20 in Cyt1A expression proved that it is necessary for Cyt1A crystallization and
90 host cell viability [Adams et al., 1989; Wu and Federici, 1993]. Moreover, P20 also
91 increased the production of other *B. thuringiensis* toxins [Elleuch et al., 2016a;

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

92 Elleuch et al., 2016b; Elleuch et al., 2015; Nisnevitch et al., 2006; Wang et al., 1997;
93 Wu and Federici, 1995; Xu et al., 2001], which are poorly expressed without P20. An
94 increase in their production in the presence of P20 prompted us to investigate if and
95 how the 20-kDa chaperone enhances synthesis and crystallization of the Cyt2Ba7.
96 In this study, *p*20 and *cyt*2Ba7 were incorporated in the same vectors and
97 transformed to an acrystalliferous *B. thuringiensis* strain. Interestingly, Cyt2Ba7
98 production is increased and bigger crystals were formed in the presence of P20. Then
99 we further applied binding assay study the protein-protein interactions between P20
100 and Cyt2Ba7.
101

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

102 **Experimental Procedures**

103 *Bacterial strains, antiserum, media and plasmids*

104 Bacterial strains and plasmids used in this study are all deposited in State Key
105 Laboratory for Biocontrol (Sun Yat-sen University, China). *B. thurigiensis* subsp.
106 strain 4Q7, a plasmid-free derivate of *B. thuringiensis* serotype israelensis (*Bti*), was
107 obtained from *Bacillus* Genetics Stock Center, Colombus, Ohio. *E. coli* strain TG1
108 was grown in Luria-Bertani (LB) broth or agar plate at 37°C for plasmid propagation.
109 Plasmids pHT3101, pMD18-T, pHT301 [Yu et al., 2002b] which contains the full-
110 length *cyt*2Ba7 gene with promoter, and pUP20 [Shi et al., 2006] which contains
111 *cry*1Ac promoter and *p*20 gene were used for plasmid construction. Bacteria were
112 grown in LB medium at 37°C or G-tris medium at 30°C with ampicillin (100 µg/ml)
113 or erythromycin (25 µg/ml). Cyt2Ba7-specific antiserum, purified P20 protein and
114 P20-specific antiserum were prepared as described [Tang, 2003; Yu et al., 2002b].
115 All restriction enzymes and ligation enzymes were purchased from Roche
116 (Germany).

117

118 *Plasmids Construction*

119 The full-length *cyt*2Ba7 gene containing native promoter and ORF sequences were
120 amplified from plasmid pHT301 [Yu et al., 2002b] by gene-specific primers; forward
121 primer 5'-GTCGACGATAATGAGGTTATTTTGT-3' (*Sal*I) and reverse primer 5'-
122 GCATGCATCTTACGATTTTATTGGAT-3' (*Sph*I) as follows: 95 °C for 3 min; 35
123 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s; and final extension at

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

124 72 °C for 10 min. PCR products were ligated to vector pMD18-T and the ligation
125 products were transformed into *E. coli* strain TG1 for DNA sequencing. The correct
126 plasmid was digested by *Sal*I/*Sph*I and *cyt*2Ba7 fragment was subsequently ligated
127 into vector pUP20 [Shi et al., 2006] to construct plasmid pUP20CT (Fig. 1A). Then
128 the fragment containing a *cry*1Ac promoter, a *p*20 gene and a *cyt*2Ba7 full length
129 gene was digested by *Bam*HI/*Sph*I from pUP20CT and ligated to pHT3101 to obtain
130 plasmid pHP20CT, which contains a *cry*1Ac promoter and a *p*20 gene sequences
131 upstream of full *cyt*2Ba7 gene in the same transcription direction (Fig. 1A and B).
132 Similarly, the *cry*1Ac promoter and *p*20 gene were digested from the plasmid
133 pUP20C by *Kpn*I/*Sph*I and then ligated into pHT301 [Yu et al., 2002b] to get
134 plasmid pHP20CRT, which contains an upstream *cry*1Ac promoter and *p*20 gene in a
135 reverse transcription direction to *cyt*2Ba7 gene (Fig. 1A and B). The plasmids
136 pHT3101 (negative control), pHT301, pHP20CT and pHP20CRT were further
137 electroporated into the acrystalliferous strain 4Q7.
138
139 *Expression of Cyt2Ba7*
140 After inoculating 4Q7 transformed by pHT3101 (negative control), pHT301,
141 pHP20CT and pHP20CRT in the G-Tris medium and cultured at 30°C, 1 ml medium
142 was collected and centrifuged (15,000×g for 10 min) to harvest the cells at 12 h, 24 h,
143 36 h, 48 h, 60 h and 72 h after inoculation. The cell pellet was suspended in a 50 μl
144 1× Laemmli sample buffer containing sodium dodelyl sulfate (SDS) and boiled for
145 10 min, and then protein content was subjected to 12% SDS-PAGE for analysis [Shi

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

146 et al., 2006]. After separation, proteins were electroblotted onto the nitrocellulose
147 membrane (Bio-Rad, Shanghai, China) by semi-dry blotting. Antiserum for Cyt2Ba7
148 was used in immuoblotting analysis [Yu et al., 2002b]. The expression levels of
149 Cyt2Ba7 in 4Q7 (pHT301), 4Q7 (pHP20CT) and 4Q7 (pHP20CRT) were analyzed
150 from 12 to 72 h to determine the highest expression time [Shi et al., 2006].
151 To compare the expression levels of Cyt2Ba7 in 4Q7 (pHT301), 4Q7 (pHP20CT)
152 and 4Q7 (pHP20CRT), these three strains were grown in G-Tris medium at 30°C for
153 72 hours and harvested by centrifugation as above. The cell pellets were resuspended
154 and calibrated to the same optical densities (OD600) and 100 μl of each sample was
155 incubated with 100 μl 2× Laemmli sample buffer and boiled. 50 μl of each sample
156 was loaded to SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and
157 photographed by using an Electrophoresis Documentation and Analysis 120 System
158 (Eastman Kodak Co.). The protein bands were scanned and quantified using an
159 ImageMaster TotalLab imagining system V3 (Amersham Pharmacia Biotech) [Shi et
160 al., 2006]. At least three replicate were performed to statistically compare Cyt2Ba7
161 yields.
162
163 *Transmission electron microscopy*
164 *B. thuringiensis* and inclusion samples were purified and treated as previously
165 described and then observed produced crystals under transmission electron
166 microscopy JEM100cx-II (JEOL, Japan) [Nisnevitch et al., 2006; Park et al., 1998;
167 Yu et al., 2002a].

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

168 *Immunoblotting and ligand blot analysis*

169 To study if there is a protein-protein interaction between P20 and Cyt2Ba7, fusion
170 proteins Cyt2Ba7 and P20 were purified as described before [Tang, 2003; Yu et al.,
171 2002b]. One microgram purified Cyt2Ba7 was analyzed by 12% SDS-PAGE gel
172 (Fig. 4A) and then transferred electrophoretically to a nitrocellulose membrane. To
173 visualize immunoreactive proteins, one membrane was allowed to react directly with
174 P20-specific antiserum [1:1000] [Tang, 2003]. Another membrane with same
175 preparation was allowed to react with 50 μg purified P20 first and then anti-P20
176 antiserum [1:1000]. After washing, both membranes were incubated with secondary
177 antibody coupled to calf intestinal alkaline phosphatase (Ap) for developing.

178

179

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

180 **Results and Discussion**

181 *Expression of Cyt2Ba7*

182 The plasmid pHT301 possesses both native promoter and ORF sequences of *cyt*2Ba7
183 (Fig. 1B). The plasmids pHP20CT and pHP20CRT both possess the native promoter
184 of *cry*1Ac, *p*20 gene, and full-length *cyt*2Ba7 gene (with native promoter) but in
185 different directions (Fig. 1B).

186 Firstly we performed a time course study on the expression of Cyt2Ba7 in *B.*
187 *thruingiensis* strain 4Q7 transoformed with plasmids pHT301, pHP20CT and
188 pHP20CRT (Fig. 2A, B and C) from 12 h to 72 h after inoculation. The immunoblot
189 results showed that in all these three constructs, the expression of Cyt2Ba7 started
190 from 24 h, reached the highest yield at 36 h and kept constant to 72 hours (Fig. 2). A
191 weak band with molecular weight at around 58 kDa was observed in 4Q7
192 (pHP20CRT) but not in the other two constructs (Fig. 2C), which may be a dimer
193 formation of Cyt2Ba7 [Cohen et al., 2008] and only be produced when the protein
194 amount is high. There is no expression of Cyt2Ba7 in negative control, 4Q7
195 (pHT3101) (Fig. 2). Then we compared the expression of Cyt2Ba7 in 4Q7
196 transformed with pHT301, pHP20CT and pHP20CRT at 72 h after inoculation.
197 Using the integrated optical density (IOD) values, we observed that the expression
198 level of Cyt2Ba7 in 4Q7 (pHP20CRT) was increased nearly 3.2 fold than that of 4Q7
199 (pHT301). Cyt2Ba7 in 4Q7 (pHP20CT) was increased nearly 0.7 fold than that of
200 4Q7 (pHT301) (Fig. 2B). This result suggests that when *p*20 was inserted into the
201 upstream of *cyt*2Ba7 gene, no matter which direction, the expression of Cyt2Ba7 can

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

202 be significantly increased. It is very interesting that the expression of Cyt2Ba7 in
203 4Q7 (pHP20CRT) is much higher than that of 4Q7 (pHP20CT). The only difference
204 between these two constructs is the direction of inserted *p*20 gene and *cry*1Ac
205 promoter. In pHP20CT, *p*20 gene and *cry*1Ac promoter were inserted in a forward
206 direction while in pHP20CRT, *p*20 gene and *cry*1Ac promoter were inserted in a
207 reverse direction (Fig. 1B). Why reversely inserted *cry*1Ac promoter and *p*20
208 (pHP20CRT) increased the productivity of Cyt2Ba7 more significantly than
209 forwardly inserted (pHP20CT) is unknown.
210

211 *The crystals of Cyt2Ba7*

212 Then we compared the 4Q7 transformed with pHT301, pHP20CT and pHP20CRT
213 under electron microscopy. As anticipated, we did not see any crystals from negative
214 control, 4Q7 (pHT3101) (Fig. 3A). In 4Q7 (pHT301), we observed very few and
215 tiny crystals of Cyt2Ba7 (Fig. 3B). Similarly, a few small irregular crystals were also
216 observed in 4Q7 (pHP20CT) (Fig. 3C). However, from 4Q7 (pHP20CRT), we
217 observed more and bigger hexagonal-shaped crystals ranged from 0.6 μm to 1μm
218 (Fig. 3D, E and F). Similar results were reported on other ICPs before [Diaz-
219 Mendoza et al., 2012; Nisnevitch et al., 2006; Park et al., 1998; Rang et al., 1996;
220 Shao et al., 2001; Shao and Yu, 2004; Wang et al., 1997; Wu and Federici, 1993] and
221 *Bacillus sphaericus* Bin toxin [Park et al., 2007] in *B. thuringiensis*. When *cry*1Ac
222 promoter and *p*20 were reversely inserted in the upstream of *cyt*2Ba7, the amount
223 and size of Cyt2Ba7 crystals are both increased, suggesting the expression of P20

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

224 can help synthesize the crystals of Cyt2Ba7.

225

226 *The interactions between Cyt2Ba7 and P20*

227 The molecular mechanism how P20 helps increase the synthesis of Cyt2Ba7 and
228 other ICPs is still unknown, which can be achieved at the transcriptional,
229 translational and posttranslational levels [Adams et al., 1989; Diaz-Mendoza et al.,
230 2012; Park et al., 2007; Wu and Federici, 1993]. Here we insert *p*20 gene with two
231 opposite directions into the upstream of *cyt*2Ba7 gene and the results showed that the
232 expression level of Cyt2Ba7 in reverse form (pHP20CRT) is much higher than that
233 of forward form (pHP20CT). The crystals synthesized are also significantly
234 increased in amount and size. Therefore we hypothesized that P20 plays a role at
235 protein level. For example, in the protein folding to help Cyt2Ba7 form crystals and
236 thus prevent proteolytic degradation. If this is the case, a protein-protein interaction
237 between P20 and Cyt2Ba7 is required and essential. To investigate this hypothesis,
238 we used previously purified P20 proteins from *E. coli* and prepared P20-specific
239 antiserum [Tang, 2003]. Immunoblotting and ligand blot analysis results showed
240 Cyt2Ba7 could not be detected by P20-specific antiserum directly (Fig. 4C).
241 However, when P20 protein was added, Cyt2Ba7 can be detected by the P20-specific
242 antiserum (Fig. 4B). These results suggest there is a protein-protein interaction
243 between P20 and Cyt2Ba7.

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

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

246 formed large ovoidal, lemon-shaped crystals [Wu and Federici, 1993]. P20 also help
247 improve the yields of Cry1Ac [Shao et al., 2001], truncated Cry1C [Rang et al.,
248 1996], Cry2A [Shao and Yu, 2004], Cry3A [Diaz-Mendoza et al., 2012], Cry4A
249 [Wang et al., 1997], Cry4B [Elleuch et al., 2015] Cry11A [Park et al., 1998], Cyt2Ba
250 [Cohen et al., 2008; Nisnevitch et al., 2006] and *B. sphaericus* Bin toxin [Park et al.,
251 2007], which showed no or low homologies at amino acid sequences and very
252 diverse structures. How P20 could increase the synthesis of these various proteins is
253 still not clear. Interestingly, P20 could not promote the production of all ICP proteins.
254 For example, P20 did not improve expression of Cry20Aa, a mosquitocidal protein
255 [Lee and Gill, 1997]. In this study, we also found that there are certain bands on the
256 top of Cyt2Ba7 sample (Fig. 4A), but they could not be detected by P20 and P20-
257 specific antiserum (Fig. 4B), suggesting P20 has specificity and selectivity to bind
258 the targeted proteins.
259
260 In summary, our study showed that P20 functions as a molecular chaperone to
261 increase yields and facilitates crystal formation of Cyt2Ba7. Therefore it is
262 promising for P20 to be used to increase the toxicity and mortality of *B.*
263 *thuringiensis* strains to control insect pests. Many structures of ICPs including
264 Cyt1Aa [Cohen et al., 2011], Cyt2Ba [Cohen et al., 2008] and Cry2Aa [Morse et al.,
265 2001] have been reported. Therefore the structural functional and mutagenesis
266 studies of P20 will assist us further explore the mechanism of P20. Better
267 understanding P20 will pave the way to optimize its function and develop more

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

268 effective and efficient insect control strategies.

269

270 **Acknowledgements**

271 This project was supported by National Natural Sciences Foundation of China
272 (No.39900092). Dr Wei Xu is the recipient of an Australian Research Council
273 Discovery Early Career Researcher Award (DECRA) (DE160100382).

274

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

275 **References**

276 Adams LF, Visick JE, Whiteley HR: A 20-kilodalton protein is required for efficient production of the
277 *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. J
278 Bacteriol 1989;171:521-530.
279 Canton PE, Lopez-Diaz JA, Gill SS, Bravo A, Soberon M: Membrane binding and oligomer
280 membrane insertion are necessary but insufficient for *Bacillus thuringiensis* Cyt1Aa toxicity.
281 Peptides 2014;53:286-291.
282 Cohen S, Albeck S, Ben-Dov E, Cahan R, Firer M, Zaritsky A, Dym O: Cyt1Aa toxin: crystal
283 structure reveals implications for its membrane-perforating function. J Mol Biol
284 2011;413:804-814.
285 Cohen S, Dym O, Albeck S, Ben-Dov E, Cahan R, Firer M, Zaritsky A: High-resolution crystal
286 structure of activated Cyt2Ba monomer from *Bacillus thuringiensis* subsp. israelensis. J Mol
287 Biol 2008;380:820-827.
288 Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH: Revision
289 of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiology
290 and molecular biology reviews : Microbiol Mol Biol R 1998;62:807-813.
291 Diaz-Mendoza M, Bideshi DK, Ortego F, Farinos GP, Federici BA: The 20-kDa chaperone-like
292 protein of *Bacillus thuringiensis* ssp. *israelensis* enhances yield, crystal size and solubility of
293 Cry3A. Lett Appl Microbiol 2012;54:88-95.
294 Elleuch J, Jaoua S, Ginibre C, Chandre F, Tounsi S, Zghal RZ: Toxin stability improvement and
295 toxicity increase against dipteran and lepidopteran larvae of *Bacillus thuringiensis* crystal
296 protein Cry2Aa. Pest Manag Sci 2016a;72:2240-2246.
297 Elleuch J, Jaoua S, Tounsi S, Zghal RZ: Cry1Ac toxicity enhancement towards lepidopteran pest
298 *Ephestia kuehniella* through its protection against excessive proteolysis. Toxicon
299 2016b;120:42-48.
300 Elleuch J, Zghal RZ, Ben Fguira I, Lacroix MN, Suissi J, Chandre F, Tounsi S, Jaoua S: Effects of the
301 P20 protein from *Bacillus thuringiensis israelensis* on insecticidal crystal protein Cry4Ba.
302 Int J Biol Macromol 2015;79:174-179.
303 Guerchicoff A, Ugalde RA, Rubinstein CP: Identification and characterization of a previously
304 undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl Environ Microb
305 1997;63:2716-2721.
306 Hartl FU, Bracher A, Hayer-Hartl M: Molecular chaperones in protein folding and proteostasis.
307 Nature 2011;475:324-332.
308 Koni PA, Ellar DJ: Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-
309 endotoxin. J Mol Biol 1993;229:319-327.
310 Koni PA, Ellar DJ: Biochemical characterization of *Bacillus thuringiensis* cytolytic delta-endotoxins.
311 Microbiology 1994;140 (Pt 8):1869-1880.
312 Lee HK, Gill SS: Molecular cloning and characterization of a novel mosquitocidal protein gene from
313 *Bacillus thuringiensis* subsp. *fukuokaensis*. Appl Environ Microb 1997;63:4664-4670.
314 Manasherob R, Zaritsky A, Ben-Dov E, Saxena D, Barak Z, Einav M: Effect of accessory proteins
315 P19 and P20 on cytolytic activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* in
316 *Escherichia coli*. Curr Microbiol 2001;43:355-364.
317 Morse RJ, Yamamoto T, Stroud RM: Structure of Cry2Aa suggests an unexpected receptor binding

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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.

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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.

372

373

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

374 **Figure Legends**

375 **Fig. 1.** Plasmids constructing procedures (A) and maps (B) of the recombinant
376 plasmids pHT301, pHP20CT and pHP20CRT. The plasmid pHT301 possesses
377 *cyt*2Ba7 gene (with native promoter and ORF sequence). The plasmids pHP20CT
378 and pHP20CRT both possess the promoter of *cry*1Ac, *p*20 gene, and *cyt*2Ba7 gene
379 (with its native promoter) but in different directions. Arrows represent transcription
380 direction of different genes.

381

382 **Fig. 2.** Expression time courses study of Cyt2Ba7 protein in 4Q7 transformed with
383 plasmids pHT301(A), pHP20CT (B), pHP20CRT (C) and the expression comparison
384 (D) of Cyt2Ba7 protein in these three strains at 72 h by using Cyt2Ba7 specific
385 antiserum. M, protein marker; 1, 1 ml cell pellets of 72 h of 4Q7 (pHT3101); 2-7 are
386 1 ml cell pellet collected from 12 h, 24 h; 36 h, 48 h; 60 h and 72 h respectively. In
387 (B) and (C), lane 8 is cell pellets of 72 h of 4Q7 (pHT301).

388

389 **Fig. 3.** Transmission electron microscopies of crystals of Cyt2Ba7. Arrow indicates
390 the crystals formed by Cyt2Ba7. (A) 4Q7 (pHT3101); (B) 4Q7 (pHT301); (C) 4Q7
391 (pHP20CT); (D) 4Q7 (pHP20CRT); (E) and (F), crystals of Cyt2Ba7 in 4Q7
392 (pHP20CRT). "C" stands for crystals of Cyt2Ba7 while "S" stands for spores of *B.*
393 *thuringiensis*. The bar stands for 1 μm.

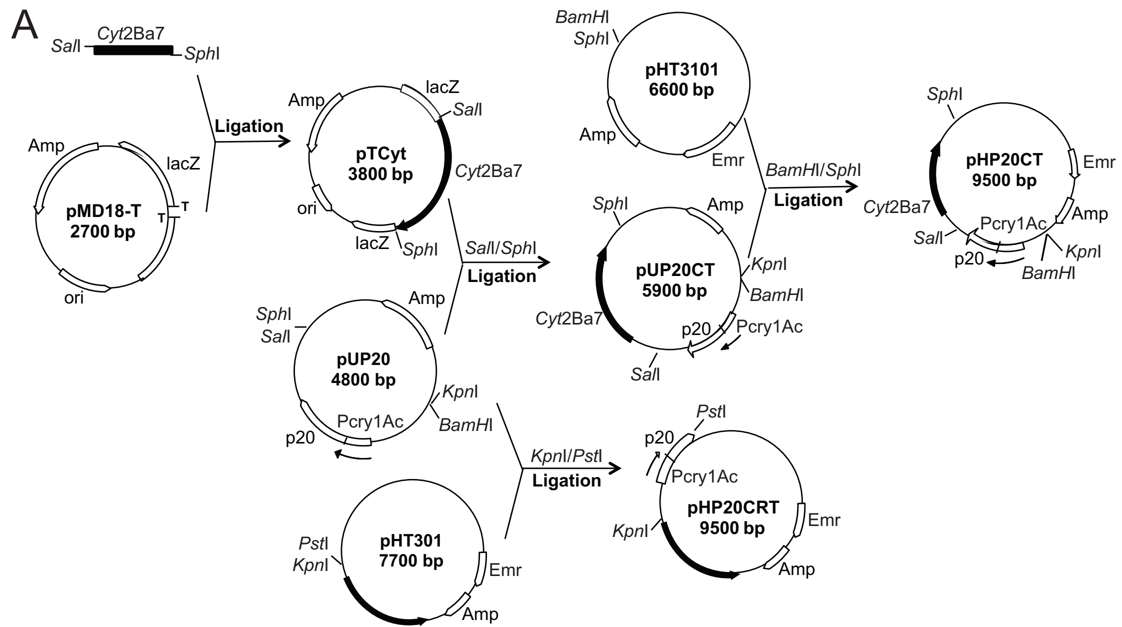
394

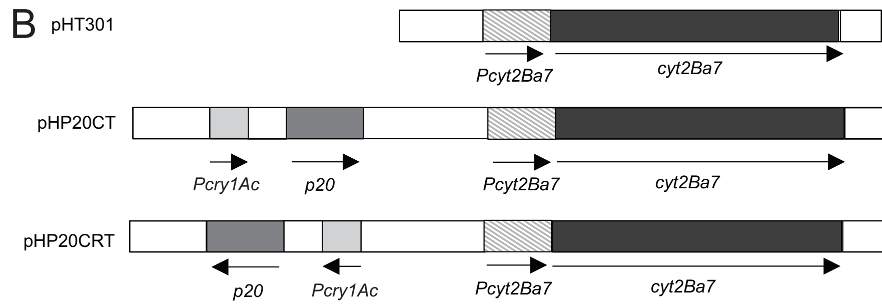
395 **Fig. 4.** Immunoblot and ligand blot analysis of Cyt2Ba7. (A) SDS-PAGE analysis of

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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.
400

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.





401

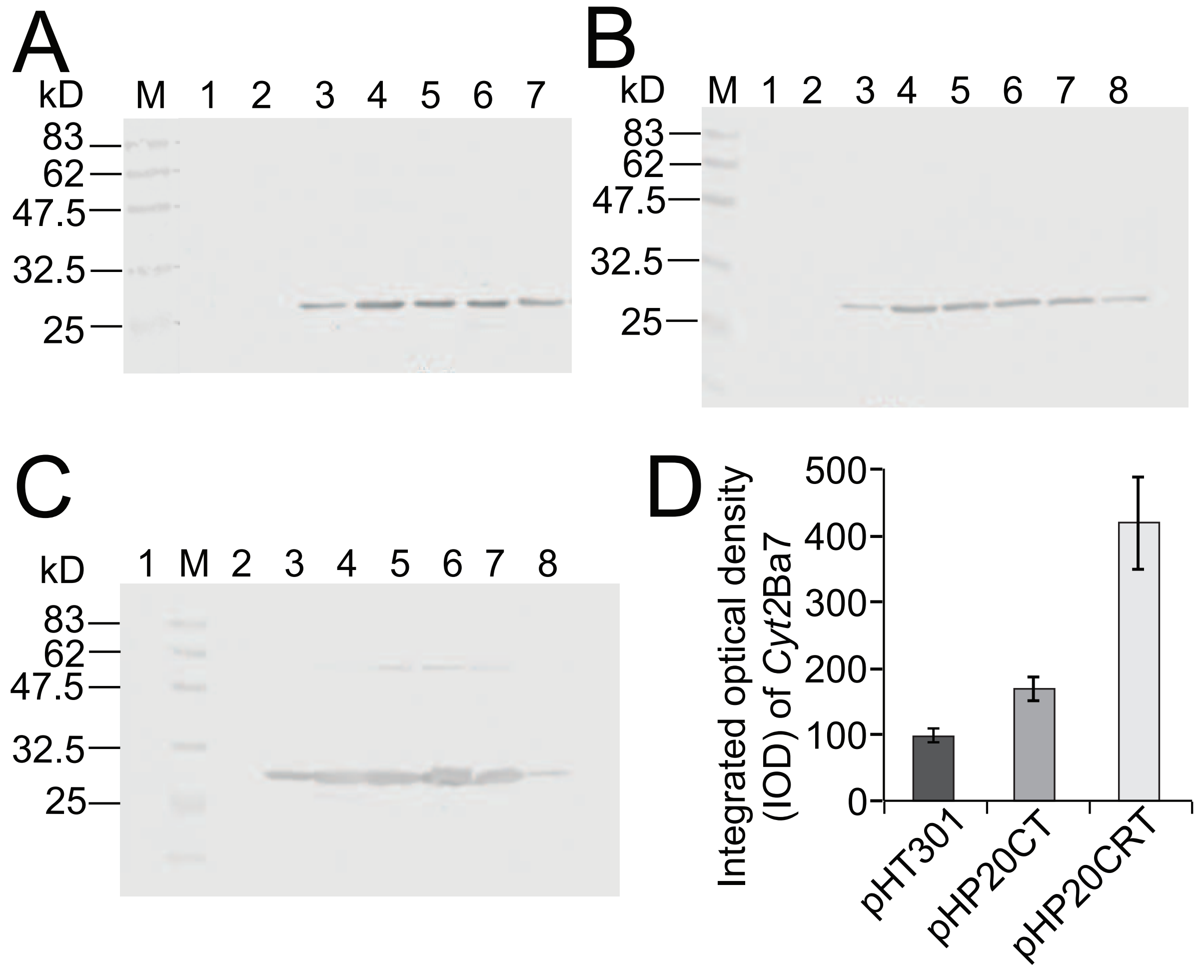
402

403

404 Figure 1

405

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



406

407

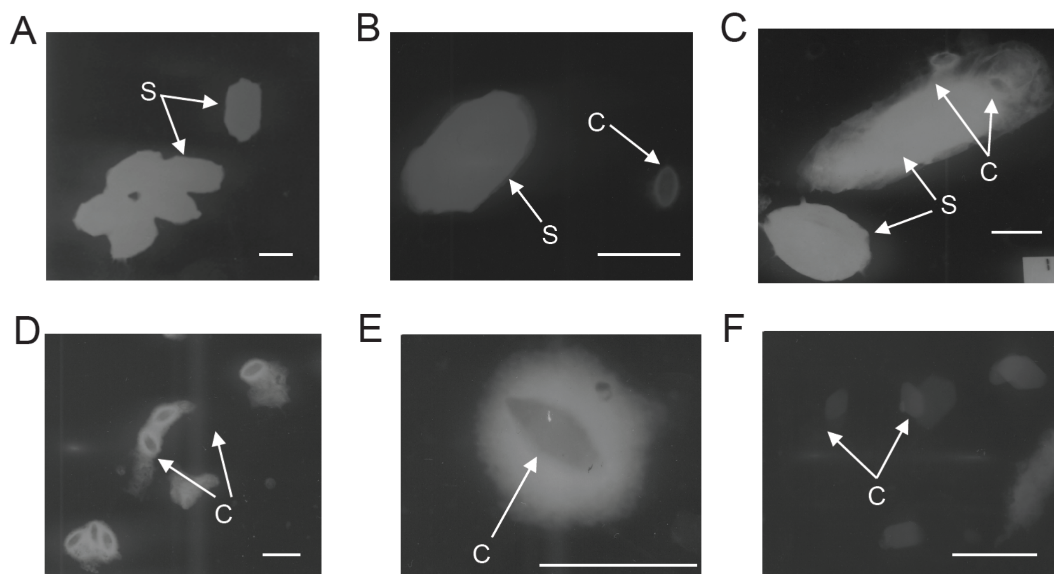
408

409 Figure 2

410

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

411



412

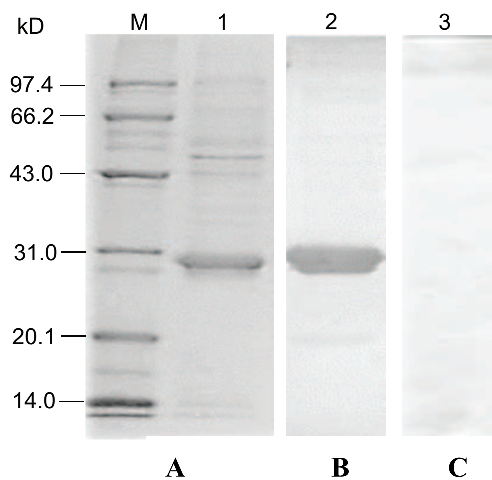
413

414

415 Figure 3

416

bioRxiv preprint doi: https://doi.org/10.1101/129833; this version posted April 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



417
418
419
420
421
422 Figure 4
423
424