Single cysteine substitution in *Bacillus thuringiensis* Cry7Ba1 improves the crystal solubility and produces toxicity to *Plutella xylostella* larvae

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**Summary**

Many *Bacillus thuringiensis* isolates have no demonstrated toxicity against insects. In this study, a novel holotype crystal protein gene cry7Ba1 was isolated from a ‘non-insecticidal’ *B. thuringiensis* strain YBT-978. The Cry7Ba1 protein showed high toxicity against *Plutella xylostella* larvae after the crystals were dissolved at pH 12.5, suggesting that the ‘non-insecticidal’ properties of this protein were due to insolubility in the normal insect midgut pH environment. After the C-terminal half of Cry7Ba1 was replaced by that of Cry1Ac or Cry1C proteins, the recombinant protein inclusions could be dissolved at pH 9.5, and exhibited high toxicity against *P. xylostella* larvae. This result proved the insolubility of Cry7Ba1 crystal was determined by the structure of its C-terminal half. Further, six mutations were constructed by substituting cysteine residues with serine. Solubility studies showed that the crystals from mutants C697S, C834S and C854S could be dissolved at lower pH (10.5, 9.5 and 11.5 respectively). Bioassays showed that crystals from mutant C834S were toxic to *P. xylostella* larvae. Our discoveries suggest that a single cysteine residue located in the C-terminal half of the protein determines the solubility and toxicity of some nontoxic crystal proteins. This study provides a strategy to isolate novel insecticidal crystal protein genes from ‘non-insecticidal’ *B. thuringiensis* strains.

**Introduction**

*Bacillus thuringiensis* is a rod-shaped, Gram-positive, spore-forming bacterium with characteristic parasporal crystals formed during the stationary phase of growth (Schnepf *et al*., 1998; Roh *et al*., 2007). These crystals comprise one or more insecticidal crystal proteins (ICPs or Crys) that are toxic to many insects, nematodes, mites, protozoa, etc. (Schnepf *et al*., 1998; van Frankenhuyzen, 2009). Given its strong and specific toxicity to a wide range of insects, *B. thuringiensis* has been developed as the leading biopesticide for use as an alternative or supplement to synthetic chemical pesticides (Rosas-Garcia, 2009). Additionally, cry genes may also be considered a key to generating transgenic crops with pest resistance (Kumar *et al*., 2008).

Due to the high insecticidal activity of *B. thuringiensis* and its ICPs, Bt-based insecticidal products have been widely studied and used in various fields. However, because of the extensive and continuous usage of Bt products, some insect populations have developed resistance to *B. thuringiensis* ICPs (Wright *et al*., 1997; Ferre and Van Rie, 2002; Akhurst *et al*., 2003; Tabashnik *et al*., 2009). Several strategies, such as refuges (Tabashnik, 2005), high dose or enhanced toxicity of Bt toxin (Pardo-Lopez *et al*., 2009), and plants with dissimilar *B. thuringiensis* toxins (Zhao *et al*., 2003), have been employed to overcome insect resistance. The discovery of novel *B. thuringiensis* strains and novel crystal proteins with high toxicity was considered to be one of the major approaches to control and overcome potential insect resistance problems (Gassmann *et al*., 2009). An increasing number of crystal protein genes have been identified (538 cry genes and 33 cyt genes, ‘Bacillus thuringiensis toxin nomenclature’ [http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/]) and classified according to the revised nomenclature system described by Crickmore and colleagues (1998). However, most of the reported cry genes were cloned from strains with high toxicity, specific activity or specific phenotypes (van Frankenhuyzen, 2009).

A large number of *B. thuringiensis* strains have been isolated and kept in laboratories or companies. However, a large portion of these isolates, especially most
B. thuringiensis strains recently reported as new serotypes, were demonstrated not to be toxic in any insects tested. These strains were considered as ‘non-insecticidal’ B. thuringiensis isolates (Du et al., 1994; Mizuki et al., 1999; Lee et al., 2000; Kitada et al., 2006). Du and colleagues (1994) showed that the crystal proteins of some B. thuringiensis strains were highly toxic in insects after solubilization and/or proteolytic activation, despite the same proteins earlier considered ‘non-insecticidal’ by using crystals during bioassays. Apart from insecticidal activity, other biological activities were also discovered from parasporal crystal proteins of ‘non-insecticidal’ B. thuringiensis strains. Several studies have reported the antibacterial action of the parasporal inclusion proteins from some ‘non-insecticidal’ strains (Yudina et al., 2003). Further, several ‘non-insecticidal’ toxin protein genes, such as cry31Aa1, cry33Aa1, cry41Aa1, cry45Aa1 and cry46Aa1, were found to be highly toxic to human cancer cells after the proteins have been dissolved and cleaved in vitro (Lee et al., 2001; Ohba et al., 2009). Additionally, crystal proteins with lectin activity with sheep erythrocytes have also been reported (Akao et al., 1999).

These ‘non-insecticidal’ strains are ubiquitous in natural environments. However, whether these strains are really non-insecticidal or have certain insecticidal activity or even other activities is not clear. Previously, we isolated a B. thuringiensis strain YBT-978 from soil in China, which was identified as the standard H40 serotype strain of ssp. huazhongensis (Dai et al., 1996). This strain produces typical bipyramidal crystals of the 130 kDa major crystal protein, but the spore-crystal mixtures of this strain showed no toxicity to dipteran and lepidopteran insect larvae (including Bradysia odoriphaga, Plagiodes versicolora, Lasioderma serricorne, Tribolium castaneum, Helicoverpa armigera and Plutella xylostella). Thus, this strain was considered ‘non-insecticidal’ for more than 10 years. In the current study, we isolated a holotype cry gene cry7Ba1 from this ‘non-insecticidal’ strain. We found the crystal of Cry7Ba1 could not be dissolved at pH 9.5 but could be solubilized at pH 12.5, and the solubilized crystal protein exhibited high toxicity against P. xylostella larvae. We further proved that the insolubility of the Cry7Ba1 crystal was determined by the structure of its C-terminal half, and the key impact factor was several cysteine residues. Finally, we used C-terminal half replacement and single cysteine residue substitution to improve Cry7Ba1 solubility and produce its toxicity to P. xylostella larvae.

**Results**

**Dissolution of the crystals of strain YBT-978 needs a high pH, up to 12.5, and the dissolved crystal protein exhibit high toxicity to Plutella xylostella larvae**

To confirm the activity of strain YBT-978, bioassays were conducted against six kinds of insect larvae from lepidopteran and dipteran orders (P. xylostella, H. armigerar, B. odoriphaga, T. castaneum, P. versicolora and L. serricorne). The data showed that the spore-crystal mixture has no toxicity in any tested insect larvae.

The crystals of strain YBT-978 were difficult to dissolve at a normal pH (pH 9.5), at which most crystal can be dissolved, but dissolved well at a higher pH of 12.5 (Fig. 1A). Solubilized crystal proteins were used to test the insecticidal activity and proved to have high toxicity in the larvae of P. xylostella. The determined LC50 (50% lethal concentration) (95% confidence intervals) value was 4.3 (2.6–9.0) ng ml⁻¹. These results indicated that the crystal protein of YBT-978 is not a real ‘non-insecticidal’ crystal protein.
In order to analyse whether the crystal protein possesses any other biological activities, the antibacterial activity, human cancer cells activity and sheep erythrocytes activity were determined using solubilized and proteolytically active crystals. No significant difference was observed between treatment groups and control groups.

The 'non-insecticidal' crystals in strain YBT-978 are made up of Cry7Ba1 protein

To further analyse the 'non-insecticidal' crystal protein and its real activity, we cloned the putative gene. The crystals produced by strain YBT-978 had a typical bipyramidal shape as shown under microscopic observation (Fig. S1) and are comprised of a 130 kDa protein, as determined by SDS-PAGE (Fig. S1). The N-terminal amino acid sequence of this 130 kDa protein was determined to be MDIRNQNKYEVVYPA. Using an adaptor ligation PCR method, we obtained the encoding gene fragment of 500 bp. A bacterial artificial chromosome (BAC) library of *B. thuringiensis* strain YBT-978 was constructed. When the 500 bp fragment was used as a probe to screen the BAC library clones, one clone containing an approximately 60 kb fragment was obtained. After subcloning and DNA sequencing, a 3465 bp open reading frame (ORF) was identified that encoded a polypeptide of 1154 amino acid residues of predicted molecular mass of 130 456 Da (Fig. S2). The deduced amino acid sequence displayed similarity to Cry7A class of proteins with the highest respective identities of 57.9% and 57.1% to Cry7Ab1 and Cry7Aa1 respectively. The 130 kDa protein has a typical N-terminal half and C-terminal half and eight conserved blocks identified by CLUSTALW multiple sequence alignment, as described by Crickmore and colleagues (1998) (Fig. S3).

Cry7Ba1 crystals showed the same characteristics of dissolution and toxicity to that of strain YBT-978

The solubility experiment showed that the crystal protein of recombinant BMB0502 is also insoluble at normal pH environment (pH 9.5). However, similar to the crystal protein of strain YBT-978, the crystal of BMB0502 can be readily dissolved at higher pH (pH 12.5) (Fig. 2).

To further confirm the insecticidal activity of Cry7Ba1, bioassays were conducted using the same insect larvae used to test the toxicity of YBT-978 crystals. The data showed that the spore-crystal mixture of BMB0502 had no toxicity in any tested insect larvae, while the solution of Cry7Ba1 protein was highly toxic to *P. xylostella* larvae. The determined LC50 value of Cry7Ba1 protein was 3.9 (2.2–11.6) ng ml⁻¹, which was similar to that of the crystal protein dissolved from the crystals of strain YBT-978 [LC50 = 4.3 (2.6–9.0) ng ml⁻¹]. These results indicated that the Cry7Ba1 crystal protein is not a typical 'non-insecticidal' crystal protein, and if dissolved at high pH (12.5) can exhibit high toxicity in *P. xylostella* larvae in the soluble form.

The insolubility of Cry7Ba1 crystal is determined by the structure of its C-terminal half

Cry7Ba1 is a 130 kDa crystal protein and has typical an N-terminal half and a C-terminal half. It has been reported that the C-terminal half of the 130 kDa crystal proteins...
played an important role in crystal formation and stability (Schnepf et al., 1998). We therefore analysed the effect of Cry7Ba1 C-terminal half on its solubility. As shown in Fig. 3A and Fig. S4, the N- and C-terminal halves of Cry7Ba1 were substituted by the N- and C-terminal halves of Cry1C and Cry1Ac proteins respectively. SDS-PAGE analysis showed that all four recombinant proteins were expressed well in the acrystalliferous B. thuringiensis strain BMB171 (Fig. 3B). Microscopic observation showed that all recombinant proteins formed inclusion bodies. The solubility experiment showed that inclusions of the C-terminal chimerical recombinant proteins (with recombinant BMB0184 and BMB0185) could be dissolved in alkaline buffer (pH 9.5), similar to Cry1Ac and Cry1C crystals (Fig. 3D). However, when the N-terminal fragment of Cry7Ba1 was substituted with that of Cry1Ac and Cry1C (with recombinant BMB0183 and BMB0181), the inclusions were still insoluble in alkaline buffer (pH 9.5), but could be dissolved in a more alkaline buffer (pH 11.5 or 12.5) (Fig. 3C). These data suggested that the insolubility of Cry7Ba1 was determined by the structure of its C-terminal half.

To further determine the effects of C-terminal chimeras on the insecticidal activity of Cry7Ba1, the P. xylostella larva was selected as the target insect. Bioassay demonstrated that both C-terminal half chimerical recombinant proteins (with recombinant BMB0184 and BMB0185) showed high toxicity in P. xylostella larvae in both the crystal and soluble form (Table 1). In contrast, both of the N-terminal half chimerical recombinant proteins (with recombinant BMB0181 and BMB0183) only showed high toxicity in P. xylostella larvae in the soluble form and no toxicity in the crystal form (Table 1). These data indicated that C-terminal half substitution of Cry7Ba1 with the C-terminal half of Cry1Ac and Cry1C proteins produced its toxicity to P. xylostella larvae in the crystal form.

Specific Cysteine residues in the C-terminal half of Cry7Ba1 determine crystal solubility

It has been reported that Cysteine residues located in C-terminal half of crystal proteins is important for
maintaining crystal stability through disulfide bond formation (Schnepf et al., 1998). In the current study, we found seven Cys located in the C-terminal half of the Cry7Ba1 protein (Fig. 4A). To further analyse which part of the C-terminal half of Cry7Ba1 is the determining factor of stability, we replaced all Cys residues with serine residues to obtain six Cys mutations, with the exception of C840. SDS-PAGE (Fig. 4B) analysis and microscopic observation (Fig. 4C) showed that all six Cys substitution mutants produced 130 kDa proteins and also could form typical bipyramidal crystals in strain BMB171 similar to wild-type Cry7Ba1 proteins.

Protease digestion analysis was performed to determine regions of the Cry7Ba1 protein involved in toxicity. The crystal proteins of all mutants were digested to produce the normal 70 kDa toxic core when treated with

Table 1. Toxicity of Cry7Ba1 wild-type and recombinant proteins in P. xylostella larvae.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Products</th>
<th>Crystals</th>
<th>Solubility (pH)</th>
<th>Toxicity</th>
<th>Treatments</th>
<th>LC50 (95% CI) (ng ml⁻¹)</th>
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</thead>
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<tr>
<td>BMB0502</td>
<td>Cry7Ba1</td>
<td>Bip</td>
<td>12.5</td>
<td>Crystals</td>
<td>NT</td>
<td>3.90 (2.2–11.6)</td>
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<tr>
<td>BMB0181</td>
<td>NCRY7C–CCry7Ba</td>
<td>NIC</td>
<td>12.5</td>
<td>Crystals</td>
<td>NT</td>
<td>3.51 (1.08–8.07)</td>
</tr>
<tr>
<td>BMB0183</td>
<td>NCRY7A–CCry7Ba</td>
<td>NIC</td>
<td>12.5</td>
<td>Crystals</td>
<td>NT</td>
<td>3.66 (2.91–7.61)</td>
</tr>
<tr>
<td>BMB0184</td>
<td>NCRY7A–CCry7Ba</td>
<td>NIC</td>
<td>9.5</td>
<td>Crystals</td>
<td>NT</td>
<td>4.26 (3.85–10.20)</td>
</tr>
<tr>
<td>BMB0185</td>
<td>NCRY7A–CCry7Ba</td>
<td>NIC</td>
<td>9.5</td>
<td>Solution</td>
<td>4.32 (2.59–8.47)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Crystals</td>
<td>3.06 (1.58–9.35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Solution</td>
<td>4.39 (3.07–11.48)</td>
<td></td>
</tr>
</tbody>
</table>

a. LC50, 50% lethal concentration; 95% CI, 95% confidence intervals ranges. The LC50 unit is the amount of proteins per millilitre of contaminated diet.
N, N-terminal half of Crystal proteins; C, C-terminal half of Crystal proteins; NT, No toxicity. Bip, bipyramidal crystals in shape. NIC, No integrity crystals observed.

Fig. 4. The expression and crystal formation of cysteine mutants in BMB171.
A. The schematic and position of cysteine residues locate in the C-terminal half of Cry7Ba1.
B. SDS-PAGE detected the expression of Cry7Ba1 cysteine mutants in BMB171. 1, C697S; 2, C834S; 3, C854S; 4, C1059S; 5, C1101S; 6, C1154S; 7, 8, Cry7Ba1. M, the standard proteins molecular marker. Molecular mass marker positions (in kDa) are indicated to the right.
C. The crystal morphology of Cry7Ba1 cysteine mutants.

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trypsin similar to the wild-type Cry7Ba1 (Fig. S5A). CD spectra were also obtained to investigate whether the mutations introduced significant structural changes. The CD spectra of the wild-type Cry7Ba1 and six Cys mutants indicated that there was no global or significant variation in the secondary and tertiary structure due to these mutations (Fig. S5B).

We further determined the solubility of these six mutants at different pH. Three mutants (C697S, C834S and C854S) could be dissolved in low alkaline buffer (pH 10.5, 9.5 and 11.5 respectively) (Fig. 5); however, other mutants were still insoluble in alkaline buffer at pH 9.5, and were only dissolved in higher alkaline buffer (pH 12.5) (Fig. 5). These data suggest that specific Cys residues located in the C-terminal half of Cry7Ba1 are involved in crystal solubility.

### Cysteine substitution in Cry7Ba1 recovers its toxicity in *P. xylostella* larvae in crystal form

To determine the effects of single Cys substitution on the insecticidal activity of Cry7Ba1, bioassays were performed, and demonstrated that all single Cys substitution mutants showed high toxicity in *P. xylostella* larvae in the soluble form (Table 2). Further, toxicity was similar to that of wild-type Cry7Ba1. These data indicated that the single Cys substitution did not affect the insecticidal activity of Cry7Ba1. When using crystal and spore mixture for bioassays, five mutants showed no toxicity in *P. xylostella* larvae (Table 2). However, the mutant C834S showed high toxicity in *P. xylostella* larvae in both crystal and soluble form with an LC$_{50}$ value of 4.39 (2.74–10.16) ng ml$^{-1}$ and 3.07 (2.20–4.98) respectively (Table 2). This result indicated that a single Cys substitution in the C-terminal half of Cry7Ba1 could produce its toxicity to *P. xylostella* larvae in the crystal form.

### Discussion

A large number of *B. thuringiensis* strains have been isolated, however, many of these isolates were considered ‘non-insecticidal’ strains (Du et al., 1994; Mizuki et al., 1999; Lee et al., 2000; Kitada et al., 2006). It is known that crystals are typically dissolved in the alkaline midgut of insects and are then able to exert their toxicity in target insects (Soberon et al., 2009). Several studies have also shown that the crystal proteins of some *B. thuringiensis* strains were highly toxic to tested insects after solubiliza-
tion, although these were formerly considered to be ‘non-insecticidal’ when using crystals during bioassays (Du et al., 1994; Bradley et al., 1995). Here, we found crystals of YBT-978 and Cry7Ba1 were insoluble at pH 9.5, but could be dissolved at a higher pH of 12.5 (Figs 1 and 2), and the solubilized crystal exhibited high toxicity in *P. xylostella* larvae (Table 1). These results indicated that Cry7Ba1 was not a real non-insecticidal protein, and its ‘non-insecticidal’ designation was due to the insolubility of the protein at alkaline pH.

The bioassay results showed Cry7Ba1 is highly toxic to *P. xylostella*. This was much different from previously reported members of Cry7A, which were found to be toxic only to Coleoptera. Cry7Ba1 has typical N-terminal and C-terminal halves. The C-terminal half of Cry7Ba1 show much higher homology (87.9%) with Cry7Ab. However, the N-terminal half of Cry7Ba1 showed quite lower similarity (37.0%) to that of Cry7A (Fig. S5). It’s known that the N-terminal half of Cry toxins is the core toxic fragment and determined the specificity (Schnepf et al., 1998). The low similarity between the N-terminal half of Cry7Ba1 and other members of Cry7A may explain their distinct insecticidal spectrum.

Some studies have reported that the C-terminal half of the crystal proteins play an important role in crystal formation and stability (Schnepf et al., 1998). In this study, we found the C-terminal half substitution of Cry7Ba1 with that of Cry1Ac and Cry1C improved the crystal solubility and produced its toxicity to *P. xylostella* larvae (Table 1). This information provides a novel understanding of the biology function of the C-terminal half of the crystal proteins from *B. thuringiensis*.

The Cys residues located in the C-terminal half of 130 kDa class Cry proteins are important to maintain crystal stability via disulfide bond formation (Agaisse and Lereclus, 1995; Kadokura et al., 2003). At the beginning, we want to replace all the 7 Cys residues with serine residues in the C-terminal half of Cry7Ba1. However, we cannot obtain the mutant C840S with several attempts to try. The solubilization test showed crystals from mutants, namely C697S, C834S and C854S, could be dissolved in lower alkaline buffers (pH 10.5, 9.5 and 11.5 respectively) (Fig. 5). These results indicated that some Cys residues located in the C-terminal half of Cry7Ba1 are responsible for Cry7Ba1 insolubility. The crystals from mutant C834S produced toxicity to *P. xylostella* larvae with a similar level to that of wild-type Cry7Ba1 protein. However, crystals from mutants C697S and C854S still showed no toxicity (Table 2). It is clear that C834S is solubilized at pH 9.5 in contrast to C697S and C854S (Fig. 5), which may explain the lack of toxicity of these two proteins. Our findings revealed a novel function of Cys residues, suggest that a single Cys substitution in the C-terminal half is significant to improve the solubility and produce toxicity of some ‘non-insecticidal’ crystals. This phenotype determined by Cys may also exist in the C-terminus of other 130 kDa class Cry proteins (e.g. Cry1, Cry4, Cry8, Cry9, etc.).

Furthermore, the Cry7Ba1 were insoluble at pH 9.5, which was much different from previously reported members of Cry7Aa (soluble at pH 7.5). We analysed the C-terminal of Cry7Aa in comparison to Cry7Ba1. Except the C1059, all the other 6 Cys are conserved in both toxins (Fig. S6). We also predicted the disulfide bonds formation in the C-terminal of both toxins by using unified software for Cysteine State and Disulfide Bond Partner Prediction (http://clavius.bc.edu/clotelab/DiANNA/). The highest scores of disulfide bond formation in C-terminal of Cry7Aa are in C1086-C1138 (0.98671) (Table S1). While, the highest scores of disulfide bond formation in

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**Table 2.** Toxicity of Cry7Ba1 Cysteine mutants in *P. xylostella* larvae.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Nucleotide switch</th>
<th>Crystals</th>
<th>Solubility (pH)</th>
<th>Treatments</th>
<th>LC50 (95% CI) (ng ml⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C697S</td>
<td>TGT→AGT</td>
<td>Bip</td>
<td>10.5</td>
<td>Crystals</td>
<td>NT</td>
</tr>
<tr>
<td>C834S</td>
<td>TGC→AGC</td>
<td>Bip</td>
<td>9.5</td>
<td>Solution</td>
<td>3.77 (2.62–7.98)</td>
</tr>
<tr>
<td>C854S</td>
<td>TGT→AGT</td>
<td>Bip</td>
<td>11.5</td>
<td>Crystals</td>
<td>NT</td>
</tr>
<tr>
<td>C1059S</td>
<td>TGT→AGT</td>
<td>Bip</td>
<td>12.5</td>
<td>Solution</td>
<td>3.21 (1.89–8.99)</td>
</tr>
<tr>
<td>C1101S</td>
<td>TGT→AGT</td>
<td>Bip</td>
<td>12.5</td>
<td>Crystals</td>
<td>NT</td>
</tr>
<tr>
<td>C1154S</td>
<td>TGT→AGT</td>
<td>Bip</td>
<td>12.5</td>
<td>Solution</td>
<td>3.15 (1.98–5.07)</td>
</tr>
</tbody>
</table>

a. LC₅₀, 50% lethal concentration; 95% CI, 95% confidence intervals ranges. The LC₅₀ unit is the amount of proteins per millilitre of contaminated diet.

C, Cysteine residue; S, Serine residue. NT, No toxicity. The underlined Nucleotide means the position which has been switched in the mutations. Bip, bipyramidal crystals in shape.
C-terminal of Cry7Ba is in C834-C1101 (0.72567) (Table S2). This molecular feature might be responsible for the significant differences in solubility between these closely related Cry proteins. It also corroborated the C834 in the C-terminal of Cry7Ba is responsible for Cry7Ba1 insolubility in low pH.

Discovery and application of novel crystal protein genes was considered to be one of the major approaches to control and overcome potential resistance of certain pests (Kumar et al., 2008; Gassmann et al., 2009). A growing number of cry and cyt genes have been identified using different strategies (Beron et al., 2005; Guo et al., 2008; Noguera and Ibarra, 2010). The discovery of cry7Ba1 gene provides a framework for isolating novel and highly toxic crystal protein genes from ‘non-insecticidal’ B. thuringiensis strains.

Experimental procedures

Bacterial strains, plasmids and media

The strains and plasmids used in this study are listed in Table 3. Bacillus thuringiensis strain YBT-978 is the standard H40 serotype strain and was named as subspecies huazhongiensis after our university (Dai et al., 1996). An acrystalliferous B. thuringiensis strain BMB171 was used for heterologous protein expression (He et al., 2010). For regular culture, all Escherichia coli strains and B. thuringiensis strains were maintained on Luria–Bertani (LB) medium and supplemented with appropriate antibiotics at 37°C and 28°C respectively. For crystal protein preparation, B. thuringiensis strains were grown in ICPM liquid medium (0.6% Tryptone, 0.5% Glucose, 0.1% CaCO₃, 0.1% MgSO₄·7H₂O and 0.05% KH₂PO₄, pH 7.0) at 28°C until cell lysis. Ampicillin (100 μg ml⁻¹) and erythromycin (25 μg ml⁻¹) were added when required.

Crystal protein preparation, quantification, solubilization and activation

Bacillus thuringiensis strains were grown in ICPM medium until cell lysis. Spores and crystals were harvested and washed twice in 1 M NaCl and twice in cold sterilized water. These washed mixtures were used to analyse the crystal protein profiles by SDS-PAGE. Protein concentration was determined by the method of Lowry and colleagues (1951). Crystals were solubilized by incubation in 1 M Na₂CO₃, 0.1 M NaCl, 2 mM EDTA and 10 mM DTT, pH 9.5–12.5 at 37°C for

<table>
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<th>Strains or plasmids</th>
<th>Characteristics</th>
<th>Origin or references</th>
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<td>Escherichia coli strains</td>
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<td>DH5x</td>
<td>supE44 lacU169 (ø80 lacZ M15) Hsd R17 recA1 end A1 gyr A relA1</td>
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<td>EMB0491</td>
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<td>Bacillus thuringiensis strains</td>
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<td>BMB171</td>
<td>Acrystalliferous mutant, ssp. He et al. (2010)</td>
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<td>YBT-978</td>
<td>ss. huazhongensis, H₄₀ (Dai et al., 1996)</td>
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<td>BMBlc</td>
<td>BMB171 derivative, harboured cry1C gene</td>
<td>Stored in this lab</td>
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<td>BMB0632</td>
<td>BMB171 derivative, harboured cry1Ac gene</td>
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<td>BMB0502</td>
<td>BMB171 derivative with plasmid pBMB0495</td>
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<td>BMB0503</td>
<td>BMB171 derivative with plasmid pH304</td>
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<td>BMB171 derivative with plasmid pBMB0181</td>
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<tr>
<td>pUC18</td>
<td>A clone vector, oriE. coli, Amp', 2.7 kb</td>
<td>Stored in this lab</td>
</tr>
<tr>
<td>pH304</td>
<td>A E. coli to Bt shuttle vector, Amp', Erm'; 6.6 kb</td>
<td>Arantes and Lereclus (1991)</td>
</tr>
<tr>
<td>pBeloBAC11</td>
<td>A BAC vector, oriE. coli, Cmr'</td>
<td>Luo and Wing (2003)</td>
</tr>
<tr>
<td>pBMB0491</td>
<td>Derivative of pBeloBAC11, containing a 60 kb genome fragment of YBT-978, which harboured cry7Ba1 gene, Amp', Cmr'</td>
<td>This study</td>
</tr>
<tr>
<td>pBMB0495</td>
<td>Derivative of pH304, containing a 5.2 kb genome fragment of YBT-978, which harboured cry7Ba1 gene, Amp', Erm', 11.8 kb</td>
<td>This study</td>
</tr>
<tr>
<td>pBMB0496</td>
<td>Derivative of pUC18, containing a 5.2 kb genome fragment of YBT-978, which harboured the full cry7Ba1 gene, Amp', 8.1 kb</td>
<td>This study</td>
</tr>
<tr>
<td>pBMB0181</td>
<td>Recombinant gene (cry1C-N-terminal + cry7Bal-C-terminal) cloned into pH304 at the site of EcoRI and BamHI</td>
<td>This work</td>
</tr>
<tr>
<td>pBMB0183</td>
<td>Recombinant gene (cry1Ac-N-terminal + cry7Bal-C-terminal) cloned into pH304 at the site of Sphl and BamHI</td>
<td>This work</td>
</tr>
<tr>
<td>pBMB0184</td>
<td>Recombinant gene (cry7Bal-N-terminal + cry1Ac-C-terminal) cloned into pH304 at the site of Sall and EcoRI</td>
<td>This work</td>
</tr>
<tr>
<td>pBMB0185</td>
<td>Recombinant gene (cry7Bal-N-terminal + cry1C-C-terminal) cloned into pH304 at the site of Sall and EcoRI</td>
<td>This work</td>
</tr>
</tbody>
</table>
2 h. Protoxins were digested with trypsin at a protease: pro-toxin ratio of 1:20 (w/w) for 2 h at 37°C, and analysed by 10% SDS-PAGE.

The 130 kDa protein of YBT-978 was separated and purified by SDS-PAGE, and transferred to a PVDF membrane (Milli-pore). The 130 kDa band was deduced according to a Prestained Protein Molecular Weight Marker (Fermentas) and excised and submitted to Midwest Analytical Incorporated (St. Louis, MO, USA) for N-terminal amino acid sequencing.

Construction of YBT-978 genomic DNA library

The BAC library of B. thuringiensis strain YBT-978 was constructed by using a BAC vector pBeloBAC11 as the method described by Luo and Wing (2003). To estimate the insert sizes of BAC clones, they were cleaved with NotI and then separated by PFGE with a Bio-Rad CHEF III instrument. Finally, 1038 clones were randomly selected and constituted the YBT-978 BAC library, which harboured 30–50 kb genomic fragments achieved by partial digestion with HindIII.

Cloning the cry7Ba1 gene from strain YBT-978

A special PCR method based on adaptor ligation was used to clone the target cry gene from strain YBT-978, as previously described (Siebert et al., 1995). The primers used in this study are listed in Table 4. According to the detected N-terminal acid sequence, primers 130P1 and 130P2 were designed corresponding to N-terminal amino acid sequence from position 1 (M) to 9 (Y) and 7 (N) to 15 (A) respectively. Primers AD-L and AD-S were synthesized to form the adaptor, and AP1 and AP2 were used as adaptor primers. Primers AD-L and AD-S were mixed at 70°C for 10 min and then cooled slowly to form adaptors that were subsequently ligated to the ends of the YBT-978 genomic DNA partially digested by Scal. These ligation products were used as template for nested PCR. Primers AP1 and 130P1 were used for the first round amplification, while AP2 and 130P2 were used for the second round. A 500 bp PCR product was obtained from this amplification that was then cloned and sequenced. Two primers 130S1 and 130A1 were designed based on the 500 bp PCR products to screen the BAC library. One positive clone harbouring a 60 kb YBT-978 genome DNA was isolated, and this fragment was then subcloned, sequenced, and a 5.2 kb fragment containing the full length of crystal protein gene cry7Ba1 was obtained. Finally, this 5.2 kb fragment was inserted into the SalI site of the cloning vector pUC18 and the shuttle vector pHT304 to generate plasmids pBMB0496 and pBMB0495 for sequencing and heterologous expression respectively.

Sequence analysis

DNA sequences were determined and primers were synthesized by Beijing AuGCT Biotechnology. The sequences obtained were analysed using the Vector NTI Suite of programs from InforMax (Bethesda, MD, USA) and the protein sequences were compared with other crystal proteins using BLAST and CLUSTALW.

Transformation of E. coli and B. thuringiensis

Transformation of E. coli was carried out using CaCl2-treated competent cells. The transformation of B. thuringiensis was...
performed by electroporation with the Bio-Rad gene pulser set, as previously described (Peng et al., 2009).

Construction of the chimeras proteins

The C-terminal region of Cry7Ba1 contains 537 aa (from 618 to 1154), the C-terminal region of Cry1Ac contains 567 aa (from 612 to 1178), and the C-terminal region of Cry1C contains 571 aa (from 618 to 1189). The promoters of Cry7Ba, Cry1Ac and Cry1C combined with their N-terminal half coding region were amplified by PCR. Also, the C-terminal half coding regions combined with their terminators of Cry7Ba, Cry1Ac and Cry1C were amplified by PCR. The primers used here are listed in Table 4. Then, four chimeras genes were constructed by Ligation. Figure S4 showed an example of recombinant plasmid pBMB0183 Construction. The sequence of chimaera genes was finally confirmed by automated DNA sequencing.

Construction of Cysteine substitution mutants

The seven cysteine codons of C697, C834, C840, C854, C1059, C1101 and C1154 located in the C-terminal half of Cry7Ba1 were mutated to serine residues. The plasmid pBMB0495 was used as the template DNA. Codon mutation was performed by site-directed mutagenesis according to the QuikChange manufacturer’s manual (Stratagene). Mutations were confirmed by automated DNA sequencing.

Circular dichroism (CD) spectroscopy

CD spectra of wild-type and mutant crystal proteins were collected with an Aviv Circular Dichroism spectrophotometer-model 62A DS (Lakewood, NJ, USA) in a 21-Q-1 quartz cuvette at 25°C using STAR STATIONARY 3.0 software. The purified crystal proteins were solubilized at 2 mM in carbonate buffer (30 mM Na2CO3, 20 mM NaHCO3; pH 10). Data shown were based on scanning from 450–180 nm at 1.0 nm steps and averaged from five scans.

Microscopic observation

An optical microscope was used to observe crystal formation. Bacillus thuringiensis strains were cultivated on ICPM agar plates for more than 32 h at 28°C and cells were then stained with basic fuchsin by a simple staining procedure and observed with an oil immersion lens. For transmission electron microscopic observation, samples were treated by following the methods described by Shao and colleagues (2001) and examined using a Hitachi 7000 FA electron microscope.

Bioassays

Larvae of P. xylostella, B. odoriphaga, P. versicolora and L. serricorne were fed with leaves immersed in the crystal protein solution for 30 min and dried at room temperature. Larvae of H. armigera and T. castaneum were fed a diet treated with crystal protein solution by covering the surface the leaves. Single-dose assays were performed by incubating larvae for 3, 5 and 7 days with a high concentration (100 ng ml⁻¹) of crystal protein mixture or solutions were tested to estimate toxicity initially. Five appropriate concentrations were used to calculate the LC50 for 3 days.

Acknowledgements

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References


**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** The crystal protein profile (A) and crystals morphology (B) of YBT-978. Additional Supporting Information may be found in the online version of this article.

**Fig. S2.** Nucleotide sequence of 5235 bp DNA fragment inserted into recombinant pBMB0496 and deduced amino acid sequence of Cry7Ba1 protein. 15 amino acid sequences in shadow are the same as the N-terminal amino acid sequence of Cry7Ba1 protein. 15 amino acid sequences in shadow are the same as the N-terminal amino acid sequence determined by micro sequencing. The putative ribosome-binding site (RBS, boxed), BTI (continuously underlined) and BTII promoters (discontinuously underlined) are indicated. The arrows present the inverted sequence (IR) that is considered to be the terminor of cry7Ba1.

**Fig. S3.** Alignment of amino acid sequences in eight conserved blocks of Cry7Ba1, Cry1Aa1, Cry4Aa1, Cry7Aa1 and Cry1Aa2. The consensus of eight conserved blocks as reported by Schnepf and colleagues (1998). Conserved amino acids are those that fall underlined) and BTII promoters (discontinuously underlined) are indicated. The arrows present the inverted sequence (IR) that is considered to be the terminor of cry7Ba1.

**Fig. S4.** The schematic diagram of the Recombinant plasmid pBMB0183 Construction. The primers used here are listed in Table 4. The promoters of cry1Ac gene combined with its N-terminal half coding region were amplified by PCR using primers p2-F and p2-R. The amplified product was...
then digested and inserted in SphI and Sall sites of pHT304 to generate plasmid pBMB0182. The C-terminal half coding regions combined with the terminators of cry7Ba gene were also amplified by PCR using primers p3-F and p3-R. The amplified product were then digested and inserted in Sall and BamHI sites of pBMB0182 to generate plasmid pBMB0183. The sequence of chimera genes were finally confirmed by automated DNA sequencing.

**Fig. S5.** There are no significant difference among the Cysteine substitution in the C-terminal half of and Cry7Ba1 in the activation pattern and secondary or tertiary structure.

A. SDS-PAGE detected the Cry7Ba1 and its Cysteine muteins after digestion with trypsin. Lane 1, Cry7Ba1; lane 2, C697S; lane 3, C834S; lane 4, C854S; lane 5, C1059S; lane 6, C1101S; lane 7, C1154S; lane M, protein standards. Molecular mass marker positions (in kDa) are indicated to the left.

B. The CD spectra of Cry7Ba1 and its muteins. The spectra of the purified toxins (2 mM) within the range from 180 to 450 nm were measured at 25°C.

**Fig. S6.** The alignment between the C-terminal half of Cry7Aa and Cry7Ba. The black arrow indicted the position of Cys residues, which are conserved in both Cry7Aa and Cry7Ba. The red arrow indicted the position of Cys residues which are specific in Cry7Ba.

**Table S1.** Disulfide Bonds Prediction in the C-terminal half of Cry7Aa by using a trained Neural Network.

**Table S2.** Disulfide Bonds Prediction in the C-terminal half of Cry7Ba by using a trained Neural Network.

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