Characterization of a New Highly Toxic Isolate of *Bacillus thuringiensis* from the Diapausing Larvae of Silkworm and Identification of cry1A 22 Gene

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**Abstract**

We have isolated 218 *Bacillus* isolates from the dissected guts of 100 diapausing larvae of the silkworm, *Bombyx mori*, collected from silkworm farmers in the Hangjiahu area of Zhejiang Province. Six isolates were identified as *Bacillus thuringiensis* strains. The strain named as W015-1 is highly toxic to the lepidopteran *Plutella xylostella*, and is deposited in the HITAR *Bacillus* Collections with Accession No. 20050509/W015. Strain W015-1 can synthesize bipyramidal crystals during sporulation as observed under light and scanning electron microscope. SDS-PAGE analysis showed that the dominant protein has a molecular mass of about 130 kD. The plasmid profile was revealed based on the technology of pulsed field gel electrophoresis (PFGE). W015-1 has a large plasmid profile very similar in size, number and banding pattern to the reference strain *Btk* HD73, and is completely different to *Btk* HD1 and *Bti* AND508. When we employed RFLP-PCR approach to identify the genotype of the strains, the results indicated that *Bt* strain W015-1 has a cry1A genotype with different enzyme cutting sites compared to the reference strain HD73. The full coding sequence of the crystal toxin was cloned (GenBank accession number EU282379) by combining the techniques of PCR-RFLP and inverted PCR and was designated as cry1Aa22 according to the nomenclature system proposed by Crickmore et al. Sequence analysis revealed that this gene contained an open reading frame of 3,534 nucleotides encoding a protein of 1,178 amino acid residues containing three typical toxin domains, and is highly homologous with the Cry1Ac family. There are three existing differences with the sequence of known cry 1Ac1, at 233 (T/R), 448 (M/I) and 1158 (K/E). We ligated the cry1Ac22 into *E. coli* expression vector pQE30 to construct pQE30-cry1Ac22 and then the recombinant plasmids were transformed into *E. coli* M15 to express an inclusion protein of about 133 kD. The inclusion of Cry1Ac22 can be hydrolysed to a trypsin-activated form with a molecular weight of about 80 kD. Larvicidal assays of the trypsin-activated form of Cry1Ac22 were carried out and was demonstrated high insecticidal activity against larvae of *Plutella xylostella* (LC50: 4.135×10⁸ cfu/mL; 95% FL: 3.368~5.122×10⁸ cfu/mL), which was much higher than that of model strain HD 73. W015-1 and the reference strains are dissimilar with differing in plasmid profiles, cry genotypes and crystal proteins. Thus, it is believed that *Br* W015-1 could be a potential biopesticide alternative to *Btk*.

**Keywords**

Silkworm; *Bombyx mori*; *B. thuringensis*; Parasporal crystal toxin; Cry genotype; cry1Ac22; Plasmid profile; Insecticidal activity

**Background**

With more than 160,000 species, Lepidoptera is recognized as the second most bio-diverse group of insects after Coleoptera, it includes many of the
most devastating pests of forests, crops, and stored products as well as some insects important to humans, such as the silkworm, *Bombyx mori*. The silkworm is a lepidopteran of economic value highly domesticated by man (Kristensen et al., 2007; Khajuria et al., 2009).

Sericulture has several thousand years of history in China. Asericulturist always faces the challenge of rearing silkworms free of bacterial infection which results in growth retardation and death. The infected larvae cause a severe symptom of diapause. The diapausing larvae of silkworm mostly are typically infected by the common soil bacterial called *Bacillus thuringiensis* (Ohba, 1996). The annual economic losses caused by *Bacillus thuringiensis* infection in the Hangjiahu regions of Zhejiang Province, known as the home of silk in China are incalculable.

The spore-forming bacterium *Bacillus thuringiensis* synthesizes parasporal crystal toxin during sporulation. *Bt* toxin usually works in the insect midgut, where *Bt* protoxins are activated by gut protease to produce activated toxins, which bind to specific receptors to confer toxicity. Large numbers of studies show that the reactions between *Bt* toxins and insect gut are determined by many gene products expressed in the insect gut. These include many proteins and enzymes involved in *Bt* protoxin activities, toxins bound to receptors and toxin degradation products. These results imply that there are interacting systems for *Bt* toxin functions existing in the insect midgut. Changes in these systems might cause particular *Bt* toxin specificity and efficacy, and could affect *Bt* toxic lethal action to a variety of insects (Crickmore et al., 1998; Knowles, 1994).

Silkworm was the first lepidopteran to have its complete genome sequenced and has become the model insect species for Lepidoptera research (Mita et al., 2004). Mining highly toxic *Bt* strains from the midgut of diseased silkworm larvae and identifying the *Bt* toxin functional gene has significant importance for studying insect resistance to *Bt* toxins.

In this study, we collected 100 diapausing silkworm larvae from different farms in the Hangjiahu region in Zhejiang Province. Sodium acetate & temperature separation was used to isolate *Bacillus* isolates from the midgut tissue and slime of collected samples. The *Bacillus thuringiensis* strains were further characterized by using staining, crystal shape observation, SDS-PAGE, plasmid profile and bioassay to make clear the genotype and *Bt* toxin genes.

1 Results

1.1 Isolation of *Bacillus* strains and *Bacillus thuringiensis* isolates

We dissected 100 diapausing silkworm larvae collected from Hangjiahu of Zhejiang Province and used midgut tissue and slime to isolate the *Bacillus* strains using sodium acetate & temperature separation. From 218 bacillus strains were harvested, six isolates were further identified to be *Bacillus thuringiensis* based on parasporal crystal formation observed under the oil lens optical microscope and scanning electron microscope. Larvicidal assays were carried out using crude proteins and the results indicated that *Bt* strains W015–1 was highly toxic to the lepidopteran *Plutella xylostella* of lepidopteran insects (data not shown). It was deposited in the HITAR *Bacillus* Collections with Accession No. 20050509W015.

1.2 Parasporal inclusion morphology of W015–1 isolate

Strain W015–1 was grown in BP solid medium at 30°C for 3 days until the parasporal crystal were observed through oil lens light microscopy, and then the crystals were examined by scanning electron microscopy (SEM). It was clear that the parasporal crystals from *Bt* W015–1 were typically bipyramidal in shape (Figure 1). SDS-PAGE analysis indicated that the intact parasporal crystal of W015–1 has a dominant polypeptide of about 130 kD after grown for 20 hours during the sporulation stage (Figure 2). The crystal proteins of W015–1 were processed into about 80 kD fragments with 1 µmol/L trypsin treatment (Figure not shown).

1.3 Plasmid profiles of W015–1

*Bacillus thuringiensis* commonly harbors a varied number of large plasmids with different molecular mass. Most of the *cry* genes are located on these
Figure 1 Scanning electron micrograph of the spores and crystal proteins from *Bt* W015-1 (6.6 mm×15 k)
Note: A, B, C are spore, paraspore and bacterial cell, respectively

Figure 2 SDS-PAGE profiles of parasporal inclusion proteins of *Bt* W015-1
Note: M: Protein molecular weight marker; Lane 1-9: *Bt* W015 parasporal inclusion protein grown after 4 h, 16 h, 18 h, 20 h, 22 h, 26 h, 30 h, 34 h and 48 h

large plasmids (Carlson et al., 1996). Therefore, plasmid size and number are usually considered as a tools to identify the strain characteristics (Procar et al., 1999; Vilas-Boas et al., 2004). In this study, the Pulsed-Field Gel Electrophoresis (PFGE) was employed to compare plasmid profiles of *Bt* W015-1 and the reference strains *Bti* AND508, HD1 and HD73. The plasmid profiles of *Bt* W015-1, in size and number are the same as HD71, but significantly different from *Bti* AND508 and HD1 (Figure 3).

1.4 Identification of cry-type toxin genes
The polymerase chain reaction-fragment length polymorphism (PCR-RFLP) was used to identify the cry genotype of W015-1 (All tested universal primers not listed in this paper). The primer pairs of K5un2/K3un2 and K5un3/K3un3 produced PCR fragments in size of 1.6 kb and 1.4 kb, respectively (Figure 4A). Both PCR amplicons were digested with *Pst* I and *Xba*I, *Pst* I and *EcoR* I, respectively (Figure 4B). The 1.6 kb fragments digested by *Pst* I and *Xba*I are 820 bp, 550 bp and 320 bp in size which are obviously larger than those of expecting sizes (801 bp, 518 bp and 322 bp). Similarly, The 1.4 kb fragments digested by *Pst* I and *EcoR* I are 800 bp, 470 bp and 280 bp in size which are a little larger than those of expecting sizes (726 bp, 434 bp, 244 bp and 59 bp), it is clear that 59 bp digested fragment...
was lacking in the strain *Bt* w015–1. Although the PCR amplicon of W015–1 is the same size as that of HD73, the RFLP patterns of the strains are different in size and number. The results indicated that *Bt* W015–1 has a different cry genotype than that of standard strain HD73. Purified PCR amplicons were ligated into pMD18–T vector to construct the recombinant plasmid pMDK2 and pMDK3 for sequencing (Beijing Genome Institute, Beijing, China). The sequence analysis revealed that deduced amino acid residues of the amplicons generated by the above primer was highly similar to *cry1Ac1*, which implied that the W015–1 strain contains a *cry1Ac* type gene.

### 1.5 Cloning of *cry1Ac* gene

In order to obtain the full length sequence of Cry1Ac from W015–1, inverted PCR was used to amplify the sequences through primer pair cry15/cry13 designed by primer software based on the sequences of plasmids pMDK2 and pMDK3.

The restriction endonucleases *Nde* I, *Sal* I, *Bgl* II and *Bam* H I were used to completely digest the plasmids of W015–1, and then inactivated the restriction enzymes were inactivated in a 65°C water bath for 15 min, followed by the addition of T4 DNA ligase to randomly connect the digested fragments. The inverse PCR produced the 1.6 kb fragment (Figure 5) based on the *Nde* I digestion fragment as template. This product was ligated into the vector pMD18–T to construct the recombinant plasmid pMDIS for sequencing. The sequences of recombinant pMDK2, pMD1K3 and pMDIS were assembled into a spliced DNA sequence of 3,772 bp, which contained a 3,534 bp open reading frame (ORF). The primer pairs of E1A5/E1A3 were designed based on the assembled sequence to amplify the full length sequence of the ORF of about 3.5 kb (Figure 6). This further confirmed that the *cry1Ac* gene existed in the strain W015–1.

The sequence of this gene deposited in the GenBank with accession number EU282379 was designated as *cry1Ac22* based on the nomenclature system proposed by Crickmore et al (1998). The coding sequence of *cry1Ac22* with 3,534 bp in length encodes a putatively weak acidic polypeptide of 1,178 amino acid residues with estimated molecular weight of 133 kD and iso-electric point of 5.04, which includes 30.90% hydrophilic amino acids, 32.64% hydrophobic amino acids, 13.83% acidic amino acids and 11.43% basic amino acids. The deduced amino acid sequence of *cry1Ac22* has 99% similarity to *cry1Ac1*, whereas three sites of 233 (T/R), 448 (M/I) and 1158 (K/E) are differences existing between *cry1Ac22* and *cry1Ac1*. Multiple sequence alignment and conserved domain of *cry1Ac22* were generated by using clustalW and *proDom* programs (http://www.ebi.ac.uk/Tools/clustal w2/index.html, http://prodrom.prabi.fr/prodom/ current/html/form.php). The results indicated that the *cry1Ac22* toxic protein has three protein domains and five conserved blocks that are typical features of cry 1A proteins (Figure 7). Three-dimensional structure prediction revealed that Cry1Ac22, whose
three-dimensional structure was identified by X-Ray analysis, is highly similar to cry1Aa and cry3Aa (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1).

1.6 Expression of cry1Ac22 gene and bioassay analysis

The cry1Ac22 was ligated into pQE30 to construct recombinant plasmid pQE30-cry1Ac22. The expression of cry1Ac22 was induced by IPTG in the strain E. coli M15. SDS-PAGE analysis indicated that the band of 133 kD inclusion protein was present in the whole cell lysate with IPTG induction and no band was present without IPTG induction (Figure 8). The inclusion of cry1Ac22 can be hydrolysed to a trypsin-activated form with a molecular weight of about 80 kD with the 1 µmol/L trypsin treatment (Figure 8). Bioassay of a trypsin-activated form of cry1Ac22 was carried out and the results showed that expressed cry1Ac22 protein exhibited high toxicity to second instar larvae of Plutella xylostella (LC50: 4.135×10^8 cfu/mL; 95% FS: 3.368~5.122×10^8 cfu/mL).

2 Methods and Materials

2.1 Collection and anatomy of Silkworm

One hundred diseased and dead silkworm larvae with brown or black diapause symptoms were collected from farms in the Hangjiahu region of Zhejiang Province and placed in individual 15 mL tubes. The larvae were dissected to obtain the midgut tissue and slime for isolating bacteria.

2.2 Isolation of Bacillus and Bacillus thuringiensis

Bacillus isolates were obtained with the use of high temperature sodium acetate according to Xie et al. (2009) and Hossain et al. (1997). The midgut tissue and slime were completely dissolved in 20 mL BPA medium incubated for 4–5 hours at 30°C with shaking at 220 r/min and then moved to a water bath at 75°C for 15 min, 1 mL of dissolved solution and was proportionally diluted in 9 mL sterilized water to a final dilution ratio of 10^2 and 10^3 for plating. The isolates were grown in NB solid medium plate for three days (beef extract 5 g, peptone 10 g, NaCl 34 g, distilled water 1,000 mL, pH value 7.0~7.4, 1.5% (w/v) agar, at 121°C for 20 min autoclave sterilization). Single colonies were re-plated and then used to observe the morphology and parasporal crystal through oil lens optical microscopy and scanning electronic microscope. The isolates were stored in 50% glycerin solution at low temperature refrigeration.

2.3 Strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 2. B. thuringiensis W015–1 was isolated from the diapausing silkworm larvae. B. thuringiensis subsp. kurstaki and B. thuringiensis subsp. israelensis were used as reference strains. pQE30 and pMD 18T were the vectors for cloning and expression of cry gene. Bt strains were grown in BP medium (Lecadet et al., 1980) and G-Tris medium (Aronson and Thompson, 1971) at 30°C. All E. coli strains were grown at 37°C in Luria-Bertani (LB) medium and Terrific Broth (TB) medium (12 g Bacto-tryptone, 24 g yeast extract, 4 mL glycerol ddH₂O to 900 mL). All culture medium was autoclaved at 121°C for 20 min before using. For solid media 1% agar was added. Ampicillin (100 µg/mL) or Kanamycin (12.5 µg/mL) were added to culture media as required.
Table 1: Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Characteristics</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. thuringiensis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bt</em> W015−1</td>
<td>Wild strain of <em>B. thuringiensis</em></td>
<td>This lab</td>
</tr>
<tr>
<td><em>Bt</em> subsp. <em>kurstaki</em> HD73</td>
<td>Model strain of <em>B. thuringiensis</em> harboring <em>cry1Ac1</em> gene</td>
<td>This lab</td>
</tr>
<tr>
<td><em>Bt</em> subsp. <em>kurstaki</em> HD1</td>
<td>Model strain of <em>B. thuringiensis</em></td>
<td>This lab</td>
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<tr>
<td><em>Bt</em> subsp. <em>israelensis</em> AND508</td>
<td>Mutant is a derivative of AND406 cured of pTX14−2 and pTX14−3</td>
<td>This lab</td>
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<td><strong>Escherichia coli</strong></td>
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<tr>
<td><em>E. coli</em> JM110</td>
<td>Dam, dcm, supE44, hsdR17, thi, leu, rpsL1, lacY galK, galT, arat0nA thr, tsx, D (lac-proAB) (F0, traD36, proAB, lacI q ZDM15)</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMD18−T</td>
<td>AmpR, AT-easy clone vector</td>
<td>Takara</td>
</tr>
<tr>
<td>pMDK2</td>
<td>AmpR pMD18−T vector harboring PCR fragment by primer pairs of K5un2/K3un2</td>
<td>This study</td>
</tr>
<tr>
<td>pMDK3</td>
<td>AmpR pMD18−T vector harboring PCR fragment by primer pairs of K5un3/K3un3</td>
<td>This study</td>
</tr>
<tr>
<td>pMDIS</td>
<td>AmpR pMD18−T vector harboring PCR fragment by primer pairs of cry1I5/cry1I3</td>
<td>This study</td>
</tr>
<tr>
<td>pQE−30</td>
<td>AmpR, T5 promoter, expression vector</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>pQE1Ac22</td>
<td>AmpR, pQE−30 vector carrying <em>cry1Ac22</em> gene</td>
<td>This study</td>
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</table>

Table 2: Primers for gene identification, cloning and expression

<table>
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<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Origin and reference</th>
</tr>
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<tbody>
<tr>
<td>K5un2</td>
<td>AGGACCAGGATTACAGGAGG</td>
<td>Kuo and Chak (1996)</td>
</tr>
<tr>
<td>K3un2</td>
<td>GCTGTCACGCAGGATTATAGCCAC</td>
<td>Kuo and Chak (1996)</td>
</tr>
<tr>
<td>K5un3</td>
<td>CAATGCGTACCTTACATTGTTTAAGTAT</td>
<td>This study</td>
</tr>
<tr>
<td>K3un3</td>
<td>CCTCCGTGAAATTCCCTGTCCT</td>
<td>This study</td>
</tr>
<tr>
<td>cry1I5</td>
<td>TTGCCTTAGCGACAAGGAAAT</td>
<td>This study</td>
</tr>
<tr>
<td>cry1I3</td>
<td>AATGTGCCAGTACCGGGGGTTC</td>
<td>This study</td>
</tr>
<tr>
<td>E1A5</td>
<td>CAGGGATCC AGAGATGGAAGGTAACTTATG</td>
<td>This study</td>
</tr>
<tr>
<td>E1A3</td>
<td>ACGCGTCGAC TGAGACTATTCCTCCATAAG</td>
<td>This study</td>
</tr>
</tbody>
</table>

2.4 Observation of parasporal crystal by Scanning Electron Microscope (SEM)

*Bt* strains were grown in BP medium at 30°C for 3 days until sporulation was complete as examined by light microscope with an oil-immersion lens. The spores and crystals were collected by centrifugation at 4°C at 12,000 g for 10 min, and the precipitate was washed three times with ice-cold sterilized double-distilled water. The spore-crystal suspensions were placed on aluminum mount and fixed in 1% OsO4 after the samples were air-dried overnight. The samples were then coated with gold in an IB−5 ion coater (HITACHI, Japan). The SEM observation was conducted on a HITACHI S−3400N (HITACHI Japan) at a voltage 15 kV following the machine instructions for the devise (Zhang et al., 2009).

2.5 Plasmids profiles of *Bt* strains

*Bt* W015−1 was grown to the final OD600 value of 2.0 at 30°C in LB medium with shaking at 220 r/min. The strain cells were pelleted by centrifugation at 10, 000 g for 5 min and re-suspended in GTE buffer [2.5 mmol/L Glucose, 25 mmol/L Tris-Cl, 10 mmol/L...
EDTA (pH 8). Lysozyme was added to the suspension at a final concentration of 10 mg/mL and incubated 1 hour at 37°C for enzymatic lysis of cell walls. Lysozyme (1.0% SDS; 0.8 mol/L NaOH) was added at 2 × volume and mixed gently by inverting the suspension 6 to 8 times. A half volume of 3 mol/L sodium acetate was added and incubated on ice bath for more than 4 h. After centrifugation at 4°C at 12,000 g for 15 min, the supernatant was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then the plasmid DNA was precipitated from the aqueous phase with 2 volumes of cold 96% ethanol.

CHEF Mapper® XA Pulsed Field Electrophoresis System (Bio-Rad, USA) was employed to detect the plasmid profiles of Bt W015-1 in accordance with the manufacturer’s instructions. Parameters were set for automatic separation of a plasmid DNA with size range of 15 kb to 500 kb (voltage: 6 V/cm, running time: 20 hours, initial switch interval time: 1.19 s, final switch interval time: 44.69 s). The plasmid DNA was subjected to electrophoresis in 1% low-melting-point agarose (Amresco, USA) at 14°C with 0.5×TBE buffer (45 mmol/L Tris•Cl, 1 mmol/L EDTA). The plasmids DNA was then stained in ethidium bromide (5 µg/mL) after electrophoresis for 30 min, followed by destaining in double-distilled water (at 4°C) for 2 hours. Water was changed three times in the course of decoloring and plasmids were then observed under ultraviolet light.

2.6 SDS-PAGE analysis of Bt strains
Bt W015-1 was inoculated in 5 mL of liquid LB medium and grown overnight at 30°C with shaking at 200 r/min, at 12 hours post inoculation, the mixture was transferred into Eppendorf tubes with 200 mL BP medium by 1% volume ratio to continue culturing at 30°C while shaking at 200 r/min. A 1 mL spore-crystal mixture was harvested every two hours SDS-PAGE analysis after centrifugating at 4°C at 12,000 g for 5 min and re-suspended the pellet was in 100 µL sterilized water after removing the supernatant. After 25 µL NaOH (0.5 mol/L) was added, samples were placed at room temperature for 10 min and 63 µL of a 3×loading sample buffer (3.63 g Tris, 0.3 g bromophenol blue, 6 g SDS, 30 mL glycerol, 15 mL b-mercaptoethanol were dissolved in 100 mL double-distilled water and pH 6.8) was added to the suspension. The suspension was boiled at 100°C for 5 min and then centrifugated at 12,000 g for 5 min. The supernatant was loaded onto 7.5% gel immediately for SDS-PAGE analysis. Laemmli’s electrophoresis procedures were followed in this study (1970).

2.7 Identification of cry genotype by PCR-RFLP
DNA templates for PCR were prepared following Yu’s (2006) method. Thirty four primer pairs were used to identify the cry gene (Xie et al., 2009), including the primer pairs of K5un2/K3un2 and K5un3/K3un3 for screening the Bt strain cry-type genes (Table 2) (Kuo and Chak, 1996). 50 µL of PCR reaction volume sample contains 5 µL 10× PCR buffer (promega, USA), 0.5 µL (4 U/µL) Taq polymerase (promega, USA), 1 µL dNTP mixture (to 250 µmmol/L final concentration), 1 µL each primer, 1 µL (50~100 ng) template DNA, after adding 40.5 µL ddH2O. The PCR was performed in a PTC-200 Thermo Cycler (MJ Research, USA) with the procedures as follows: pre-denaturation at 94°C for 5 min, followed by 30 cycles (94°C 1 min, 53°C 1min and 72°C 3 min), and finished at 72°C for 10 min. PCR products were examined by 1% agarose gel electrophoresis and purified using the TIANgen Midi Purification Kit (Tiangen, Beijing, China). PCR-RFLP analysis followed the procedures of Kuo and Chak (1996a). Table 3 shows the PCR-RFLP banding patterns of cry1Ac1revealed by using 2% agarose gel electrophoresis (Kuo and Chak, 1996).

Table 3 Typical banding pattern of cry1Ac1 by PCR-RFLP analysis

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Sizes of PCR productions (bp)</th>
<th>The size of restricted fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>K5un2/K3un2</td>
<td>1,600</td>
<td>322,801,518 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>K5un3/K3un3</td>
<td>1,450</td>
<td>726,434,244,59 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: PCR amplicon was digested with restricted enzymes; a: PstI/HpaI; b: PstI/EcoRI

2.8 Cloning of cry type gene
The PCR products amplified by primer pairs K5un2/K3un2 and K5un3/K3un3 were ligated into the Easy Vector pMD18T to construct recombinant
2.9 Expression of cry1Ac22 and bioassay
The primer pair E1A5/E1A3, was designed based on the full sequence of cry1Ac22, in which the BamHI and SalI restrictive endonuclease sites were introduced into the 5′ end of the forward and reverse primers, respectively. The sequence confirmed gene was ligated into the prokaryotic expressing vector pEQ30 to make the recombinant vector pEQ30–cry1Ac22. The recombinant plasmid was further transformed into Escherichia coli M15 and then incubated in TB medium adding 12.5 µg/mL Kanamycin at 37°C for about 1.5 h until the value of OD600 reached about 0.5 IPTG was added to the mixture at the final concentration of 1 µmol/L for inducing Cry1A22 for 4–10 hours at 30°C. The expression cells were collected by centrifugation at 12,000 g at 4°C, for 10 min and completely suspended in an equal volume of TE. Lysozyme was then added to digest at the final concentration of 20 mg/mL and incubated at 37°C while shaking at 250 r/min for 30 min. The cells and proteins were re-collected by centrifugation, the pellet was washed with an equal volume of 1 mol/L NaCl three times and then broken up by ultrasonic treatment (Model VC–130, Sonics and Materials Inc, USA) for 20 min. The concentration of expressed Cry1Ac22 protein was measured by the Lowry assay with a standard marker protein of bovine serum albumin (BSA) (Lowry et al., 1951). The expressed proteins were dissolved in 50 mmol/L Na2CO3 (pH 10.0) with 1 mol/L trypsin added to digest during incubation at 37°C for 1 hour. SDS-PAGE procedures were the same as that mentioned above.

To bioassay, 8 cm diameter disks of Chinese cabbage leaves were immersed in different concentrations of trypsin treated proteins, containing 0.02% Triton X–100, for 10 sec and air dried naturally at room temperature for 2 hours. Each leaf disks was placed in an individual Petri dish (10 cm diameter) lined with moistened filter paper and 10 second-instar larvae of Plutella xylostella were introduced into each Petri dish. The experiment was replicated six times and sterilized water were used for reference and blank. Larvae assays were performed at 26°C and 65% relative humidity for 96 hrs, with a photoperiod of 14 hrs light/10 hrs dark. The LC50 with the 95% confidence intervals were estimated by SPSS software for windows (SPSS Inc., Chicago, USA) (Sayyed et al., 2001).

3 Conclusions
In this paper, we isolated and characterized Bacillus thuringiensis W015–1, from the diapausing silkworm larvae. It showed high insecticidal activity against the lepidopteran, P. xylostella. Bt W015–1 synthesizes a bipyramidal crystal with a molecular weight of 130 kD during sporulation. The plasmid profiles of Bt W015–1 are similarity to the reference HD73 whereas there were dissimilarities in size and number to HD1 and Bti. The cry genotype of W015–1 has obvious differences in the restricted enzyme sites with that of model strain HD73.

We cloned the cry1Ac22 gene that has a length of 3,537 bps encoding 1,178 amino acid residues. Cry1Ac22 has high amino acid sequence identity to Cry1Ac1 with the differences existing in the sites of 233 (T/R), 448 (M/I) and 1158 (K/E). The Cry1Ac22 inclusion protein, with a molecular weight of 133 kD, was expressed in E. coli induced by IPTG. The trypsin-activated form of the recombinant protein was found to have high insecticidal activity against larvae of Plutella xylostella compared to that of model strain HD73.

With respect to the reference strains W015–1 has
different plasmid profiles, cry genotypes and crystal proteins. Thus, it is believed that Bt W015–1 could be used as a potential biopesticide alternative to Btk.

Authors’ contributions

LX, WFZ and ZML conducted all the research for this paper. YGC was involved in collecting the diseased silkworm larvae. LX and WFZ jointly completed the data analysis and manuscript preparation. J.X. participated in experimental management and reviewed the manuscript. XF coordinated the project and was fully involved in the experimental design, data analysis and manuscript preparation. All authors read and approved the final manuscript.

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