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# Biological characteristics of *Bacillus thuringiensis* strain Bt11 and identification of its *cry*-type genes

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**Abstract** A novel strain of *Bacillus thuringiensis* Bt11, isolated from soil samples in China, was classified and characterized in terms of its crystal proteins, *cry* genes content. The Bt11 strain showed high toxicity against *Spodoptera exigua* and *Helicoverpa armigera* neonates. Bt11 strain shares morphological and biochemical characteristics with the previously described *Bacillus thuringiensis* subsp. *kurstaki*. SDS-polyacrylamide gel electrophoresis revealed that crystals were composed of several polypeptides ranging from 20 to 130 kDa, of which the 35, 80, and 130 kDa proteins were the major components. PCR-RFLP with total DNA from strain Bt11 and specific primers for *cry1*, *cry2*, *cry3*, *cry4/10*, *cry7*, *cry8*, *cry9*, and *cry11* genes revealed that *cry1Aa*, *cry1Ab*, *cry11a*, and *cry9Ea* genes were present.

**Keywords** *Bacillus thuringiensis*, insecticidal activity, biochemical reaction, PCR-RFLP

## 1 Introduction

*Bacillus thuringiensis* (Bt) is a gram-positive spore-forming bacterium with entomopathogenic properties. Bt can produce proteinaceous parasporal inclusion bodies during the sporulation phase (Gill et al., 1992). The crystal proteins of Bt have been found to have a toxic activity toward a target organism. Bt is of special economic importance because its products play a growing role in the biological control of agricultural and forestry pests and

insects that are vectors of human and animal diseases. This entomopathogenic property has led to a great interest in finding new Bt isolates, and consequently, growth within-species variation has been found. So far, more than 40000 Bt isolates have been isolated (Zhang, 2001), and 82 different serovars have been reported (Lecadet et al., 1999).

Although serotyping remains as a valuable classification of Bt strains, some other methods have been developed to analyze the protein composition of the crystals by polyacrylamide gel electrophoresis as well as the quick and easy characterization of toxin genes by PCR. These methods allow the analysis of a large number of samples, but the bioassay remains as an essential tool to determine the insecticidal activity or a wider spectrum of activity.

The purpose of this study was to characterize a new Bt strain and identify its *cry* genotype, including crystal protein composition, *cry* gene content, and the evaluation of their biological activity against a number of insects.

## 2 Methods

### 2.1 Bt strains and insects

Bt11 strain was stored in our laboratory, and HD-73 standard strain used in this study was obtained from the Chinese Academy of Agricultural Plant Protection Institute. Larvae of *Spodoptera exigua* and *Helicoverpa armigera* were obtained from laboratory colonies and reared on the artificial diet.

### 2.2 Bioassays of insecticidal activity

A modified diet overlay assay method (Zhao et al., 2003; Guo, 2005) was employed to test the susceptibility of neonates to Bt11. In each cup, 0.1 mL Bt11 solution was applied and distributed over the diet surface. Cups were allowed to air dry for 1.5–2 h before infesting it with the neonates of *Helicoverpa armigera* and *Spodoptera exigua*.

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Ten neonates were transferred into each cup. The cups were covered with lids and held at  $(27\pm 1)^{\circ}\text{C}$ ,  $(50\pm 2)\%$  RH, and a photoperiod of 16:8 (L:D) h for 4 d to determine the mortality or growth inhibition. The POLO program (LeOra Software, 1997) was used for probit analysis of concentration-mortality response data (Russell et al., 1977). Mortality was adjusted using Abbott's formula (Abbott, 1925) for each probit analysis.

### 2.3 Morphology observation and physiological and biochemical analyses of Bt11

Bt cultures were grown on a 1/2-LB medium (tryptone 0.5%, yeast extract 0.25%, sodium chloride 0.5%, pH 7.0) at  $30^{\circ}\text{C}$  for 38 h with agitation until 70%–90% crystal off, with the number of crystal unit volume counted. Observation was made on the morphology of Bt11 strain staining reaction. The method was used to analyze the physiological and biochemical properties, following Dai and Wang (1997) and Yu (1990).

### 2.4 Growth characteristics

The wild-type strain Bt11 and the standard strain HD-73 were grown on a 1/2-LB medium at  $30^{\circ}\text{C}$  with agitation, and samples were collected every two hours, respectively. To examine the growth phase, the optical density at 600 nm ( $\text{OD}_{600}$ ) of appropriately diluted culture was measured using a spectrophotometer.

### 2.5 SDS-PAGE analysis

Cell extracts were prepared by resuspending cell pellets in  $40\ \mu\text{L}$  sterilized water and  $40\ \mu\text{L}$   $2\times$  SDS sample buffer. Samples were loaded on a denaturing gradient (10% to 5%) arylamide gel, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al., 1989).

### 2.6 Identification of *cry*-type genes by PCR-RFLP

The PCR primers in this work were designed following Kuo and Chak (1996) and Song et al. (1998). PCR was performed by predenaturing at  $94^{\circ}\text{C}$  for 4 min, followed by

30 cycles at  $94^{\circ}\text{C}$  for 1 min,  $52^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min, with the final extension at  $72^{\circ}\text{C}$  for 10 min. The genotype analysis was carried out by the PCR-RFLP following Kuo and Chak (1996) and Song et al. (1998).

## 3 Results

### 3.1 Insecticidal activity of Bt11 strain

The bioassay results indicated that the Bt11 strain was highly toxic against the neonates of *Helicoverpa armigera* and *Spodoptera exigua* (Table 1). The mortality of *Helicoverpa armigera* and *Spodoptera exigua* were observed as 100% at the concentration of  $5.9\times 10^7$  spores  $\cdot\text{mL}^{-1}$ .

### 3.2 Morphological observation

Under the light microscopy ( $1000\times$ ), the parasporal inclusions isolated from Bt11 had a diamondoid, spherical, and squarish morphology, and the spores appeared as oval (Fig. 1).

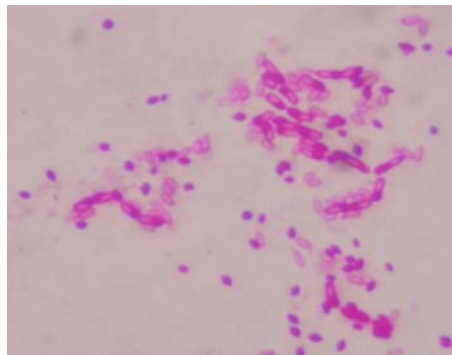


Fig. 1 The spore and crystal of Bt11 strain

### 3.3 Physiological and biochemical characteristics

The physiological and biochemical characteristics of the Bt11 strain showed the similarity to those of *Bacillus thuringiensis* subsp. *kurstaki* (Table 2).

Table 1 Bioassay results of Bt11 strain

colony	number of larvae tested	treatment concentration/spores $\cdot\text{mL}^{-1}$	corrected mortality/%
<i>Helicoverpa armigera</i>	50	$5.9\times 10^7$	100
<i>Spodoptera exigua</i>	50	$5.9\times 10^7$	100

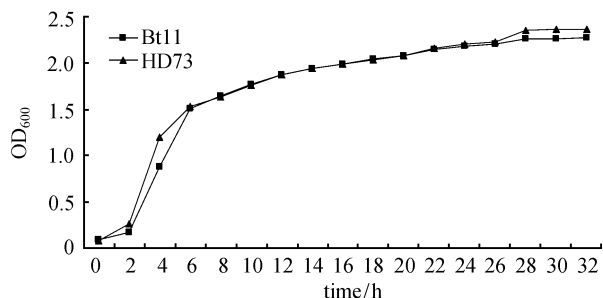
Table 2 Physiological and biochemical characteristics of Bt11 strain

strain	myco-dema	V . P reaction	lecithinase	salicin	surcrose	cellobiose	mannose	starch hydrolysis	protein hydrolysis	urease
Bt11	-	+	+	+	-	+	-	+	+	+

Note: "+" represents positive; "-" represents negative.

### 3.4 Growth characteristics of Bt11 strain

Compared with the growth curve of Bt11 strain and HD-73 strain (Fig. 2), the growth characteristics of the wild-type strain Bt11 showed no significant difference with those of the standard strain HD-73. Both of them could be observed in three distinct phases: lag phase after 0–2 h, logarithmic phase after 2–16 h, and stationary phase after 16 h.



**Fig. 2** The growth curve of Bt11 strain and standard strain HD-73

### 3.5 SDS-PAGE analysis

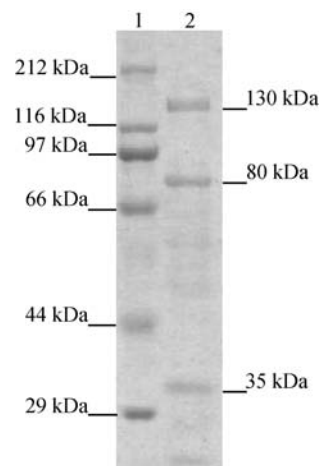
SDS-PAGE analysis of the protein composition demonstrates that Bt11 strain contained several proteins ranging from 20 to 130 kDa (Fig. 3). Proteins with molecular masses of 35, 80, and 130 kDa were observed.

### 3.6 Identification of *cry*-type genes

DNA templates were used for PCR amplification with different universal oligonucleotide primers. The results of RFLP pattern analysis after different digestion of the PCR products are shown in Table 3 and Fig. 4. The sizes of the restriction fragments, the RFLP patterns of the PCR products, and the predicted *cry*-type genes are listed in Table 3. As a result, we detected *cry1*, *cry1I*, *cry2*, and *cry9* genes in Bt11 strain.

## 4 Discussion

Insect pests cause a considerable crop loss in agricultural production. According to the FAO statistics, the economic



**Fig. 3** SDS-PAGE analysis of isolate Bt11

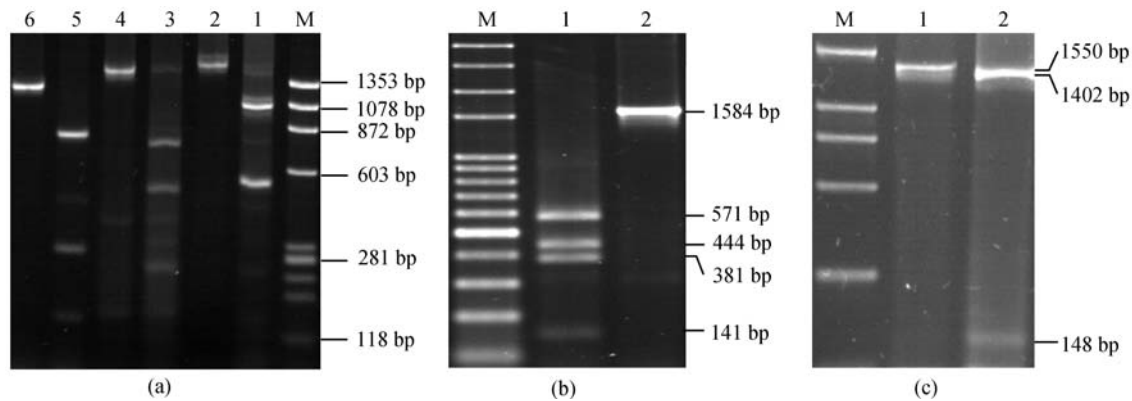
Note: 1 represents protein molecular weight marker (High) (212, 116, 97, 66, 44 kDa); 2 represents Bt11 strain.

loss that insect pests create every year reaches as high as 14% of the total amount of world agricultural production. Therefore, biological agricultural chemicals that are highly effective, safe, low in poison, low in residue, and low cost are to be developed. *Bacillus thuringiensis* has a broad application prospect in the biological control of insect pests. Therefore, seeking for highly effective strains has a vital significance to the production of biological pesticides. The *Bacillus thuringiensis* crystal has various shapes including diamondoid, long diamondoid, spherical, circle or ellipse, mosaic shape, amorphous (Li et al., 1983; Li et al., 1989), and triangle. In our research, we did not find the triangle parasporal crystal, but other parasporal crystals shapes could be seen obviously. It has been reported that the shapes of parasporal crystals of Bt were responsible for their insecticidal activity (Li et al., 1983). Therefore, it is significantly vital that we study the formation, shape, and structure of the parasporal crystal of Bt and make further research on the relationship between toxicity and mechanism of Bt.

Wild *Bacillus thuringiensis* strain Bt11 was preliminary classified as a subspecies of *Bacillus thuringiensis* isolated from the soil samples in China, which further enriched the resources of Chinese Bt, simultaneously providing new resources of Bt strains for the development of new highly

**Table 3** *cry*-type gene contents of the isolate Bt11

gene	primer	product size/bp	restriction enzyme	RFLP results/bp
<i>cry1Aa</i>	K5un2 /K3un2	1635	<i>Pst</i> I & <i>Xba</i> I	1117, 518
	K5un3 /K3un3	1463	<i>Eco</i> R I & <i>Pst</i> I	726, 493, 244
<i>cry1Ab</i>	K5un2 /K3un2	1557	<i>Pst</i> I & <i>Xba</i> I	1039, 518
	K5un3 /K3un3	1463	<i>Eco</i> R I & <i>Pst</i> I	726, 493, 244
<i>cry1Ia</i>	S5uni /S3uni	1584	<i>Bsp</i> 119 I & <i>Ban</i> I	141, 444, 571, 47, 381
<i>cry2Ab</i>	S5un2 /S3un2	1231	<i>Hinc</i> II & <i>Msp</i> I	791, 297, 143
<i>cry9Ea</i>	S5un9 /S3un9	1550	<i>Msp</i> I & <i>Cla</i> I	1402, 148



**Fig. 4** Garose gel electrophoresis of PCR-RFLP

Note: (a) represents the result of Bt11 *cry1* and *cry2* gene PCR-RFLP, with M represents  $\Phi$ X174-*Hae* III digest DNA marker; 1, 2, 3, 4, 5 and 6 representative of Bt11-K2PCR/*Pst* I & *Xba* I, Bt11-K2PCR, Bt11-K3PCR/*Eco*R I & *Pst* I, Bt11-K3PCR, Bt11-S2PCR/*Hinc* II & *Msp* I and Bt11-S2PCR, respectively. (b) represents the result of Bt11 *cry1* gene PCR-RFLP with M, 1 and 2 representing 100 bp DNA marker, Bt11-S1iPCR/*Bsp*119 I & *Ban* I and Bt11-S1iPCR, respectively. (c) represents the result of Bt11 *cry9* gene PCR-RFLP with M, 1 and 2 representing DNA marker DL2000, Bt11-S9PCR and Bt11-S9PCR/*Msp* I & *Cla* I, respectively.

effective bioinsecticides. PCR is the most effective and rapid method used to identify the aimed DNA sequence (Saiki et al., 1988), which has already been used to identify the genes coded for parasporal crystal proteins and forecast their insecticidal activity (Ceron et al., 1994). In our research, the PCR method was applied to identify *cry*-type genes in Bt11 strain. The results indicated that the Bt11 strain simultaneously included *cry1Aa*, *cry1Ab*, *cry1Ia*, *cry2Ab*, and the *cry9Ea* genes. Whether it includes other kinds of *cry* genes besides the identified genes needs further researches.

It is reported that Cry1Aa has certain toxicity to the larvae of *Trichoplusia ni*, and Cry1Ab has higher toxicity to both *Helicoverpa armigera* and *Trichoplusia ni* larvae. However, both Cry1Aa and Cry1Ab are not so poisonous to *Spodoptera exigua* larvae (Dankocsik et al., 1990; De Maagd et al., 1996; Iracheta et al., 2000; Liao et al., 2002). Cry2Ab has higher toxicity to *Helicoverpa armigera* and *Trichoplusia ni*. Cry1Ia has higher toxicity to *Bombyx mori* and *Plutella Xylostella*, but low toxicity to *Spodoptera litura*. The biological activity of Cry9Ea has not been reported. The results of our research indicated that the Bt11 strain has high insecticidal toxicity to *Spodoptera exigua*, *Helicoverpa armigera*, and *Trichoplusia ni* larvae, which indicates that the Bt11 strain can harbor many kinds of *cry* genes. In the future, we will clone all the *cry* genes identified in Bt11 strain to provide beneficial gene resources for transgenic plants as well as the construction of genetically engineered bacteria.

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