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ORIGINAL PAPER

# Efficient screening and breeding of *Bacillus thuringiensis* subsp. *kurstaki* for high toxicity against *Spodoptera exigua* and *Heliothis armigera*

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**Abstract** *Spodoptera exigua* is one of the most renowned agricultural pest insects and relatively insensitive to *Bacillus thuringiensis* subsp. *kurstaki s*trains which are widely used commercial products to control lepidopterans such as *Heliothis armigera*. In the current study, we have developed a new and efficient approach to screen and breed a *B. thuringiensis* subsp. *kurstaki* strain exhibiting high toxicity against *S. exigua* while retaining its high toxicity against *H. armigera*. UV and diethyl sulfate methods were used for mutagenesis, followed by an agar plug plate diffusion assay for preliminary screening of Zwittermicin A over-producing mutants, from which we obtained a mutant strain, designated here as *B. thuringiensis* subsp. *kurstaki* D1-23, with high toxicity against *S. exigua*. The toxicity of D1-23 against *S. exigua* and *H. armigera* was improved by 115.4 and 25.9%, respectively, compared to its parental commercial strain BMB005.

**Keywords** *Bacillus thuringiensis* · *Spodoptera exigua* · Zwittermicin A · Mutagenesis · Screening

## Introduction

*Bacillus thuringiensis* (*B. thuringiensis*), a gram-positive bacterium, produces different types of delta-endotoxin during sporulation [1]. These delta-endotoxins are highly toxic to the larval forms of some harmful insects as well as harmless to most other organisms, including wildlife and beneficial insects [2].

*Spodoptera exigua* (Lepidoptera: Noctuidae), is a worldwide pest that attacks plant species, including important crops such as cotton, cabbage, maize, tomatoes, soybeans, and cauliflower [3]. *S. exigua* is relatively insensitive to *B. thuringiensis* subsp. *kurstaki* strains present in commercial *B. thuringiensis* products to kill lepidopterans [4], and has higher tolerance to *B. thuringiensis* compared to other lepidopteran species [5]. Although *B. thuringiensis* subsp. *aizawai* has high toxicity against *S. exigua*, it is less active against *Plutella xylostella* and *Heliothis armigera*. Therefore, *B. thuringiensis* strains have a limited market. Isolation of *B. thuringiensis* subsp. *kurstaki* strains with high toxicity against *S. exigua* will contribute to widen their insecticidal spectrum and provide a significant cost reduction for application of *B. thuringiensis* formulations.

Recent studies indicated that some strains of *B. thuringiensis* and *Bacillus cereus* produced Zwittermicin A in their culture supernatant [6, 7]. Zwittermicin A, a linear aminopolyol antibiotic, increases *B. thuringiensis* subsp. *kurstaki* activity at least 1.5-fold to optionally 1,000-fold, preferably from 100-fold to 400-fold [8]. For high production of Zwittermicin A, several methods for physical or chemical mutagenesis [8] and site-directed mutagenesis [9] were used to produce mutants of *B. cereus*. Agar plate diffusion bioassay was used to screen the mutants of *B. cereus* [8, 9], which was highly labor-intensive and less efficient. Up to now, there has been no report about improving the toxicity of *B. thuringiensis* by increasing the production of Zwittermicin A. In the present study, an efficient method was established for screening of high producing Zwittermicin A mutants of *B. thuringiensis* subsp. *kurstaki* which showed high toxicity against *S. exigua* and *H. armigera*.

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## Materials and methods

### Microorganisms

From our laboratory collection, 200 strains of *B. thuringiensis* subsp. *kurstaki* were used in the present study. *Erwinia herbicola* LS005 and *B. cereus* UW56 were kindly provided by Prof. Handelsman, from University of Wisconsin, USA.

### General inocula preparation method

Inocula were prepared as follows: one isolated colony was dispensed in 3 ml of Luria Broth (LB) medium [10] and incubated overnight at 30°C. Aliquots (0.2 ml) were used to inoculate 250-ml shake flasks containing 50 ml sterile LB medium. After 6 h of incubation at 30°C in a rotary shaker set at 200 rev $min^{-1}$, the culture broth was used to inoculate the studied media.

### Cultivation in shake flask level

The *B. thuringiensis* strains were grown in $H_8$ medium for delta-endotoxin production. $H_8$ medium contained (g $l^{-1}$ in distilled water) corn starch, 20.0; soybean flour, 25; peptone, 6; yeast powder, 3. The pH was adjusted to 8, and then 0.7 g of $MgSO_4 \cdot 7H_2O$ and 2 g of $CaCO_3$ were added before sterilization at 121°C for 20 min. Shake flask culture was carried out in 500-ml shake flask containing 20 ml $H_8$ medium, incubated at $30 \pm 0.5$°C on a rotary shaker at 200 rev $min^{-1}$, until 50% crystal was released.

### Cultivation in 3-L fermentor level

Fermentation was carried out using a 3-l fermentor (NBS, Edison, NJ, USA) with a working volume of 1.5 L. The temperature and agitation rate were maintained at $30 \pm 0.1$°C, 800 rev $min^{-1}$. Foaming was controlled by adding 1 ml of Antifoam A (Sigma) per liter of medium before sterilization. The dissolved oxygen was maintained over 20% saturation. Fermentation was stopped when 50% of the crystals were released.

### Investigating toxicity of *B. thuringiensis* subsp. *kurstaki* strains against *S. exigua*

Two hundred *B. thuringiensis* subsp. *kurstaki* strains were cultured in 500-ml shake flasks containing 20 ml $H_8$ medium. To estimate the strain's toxicity level, culture broths diluted 200-fold were bioassayed against neonate larvae of *S. exigua*. Agar plate diffusion bioassay detected Zwittermicin A relative concentration [11] and SDS-PAGE determined delta-endotoxin concentration as described by National Standards of P. R. China, GB/T 19567.3 [12].

### Treatment of vegetative cells with UV rays and diethyl sulfate

Survival curves were constructed for each mutagen using either the parental strain or the appropriate mutants. The mutagenesis by UV rays exposure according to the method of Ghribi et al. [13], or diethyl sulfate according to the method of Wang et al. [14], was followed by four to five generations in LB medium so that these mutants expressed their phenotypes. Survivors were plated on $H_8$ solid medium (H8 medium plus 2% agar) and scored after 24 h incubation at 30°C.

### Preliminary screening of Zwittermicin A over-producing mutants by agar plug plate diffusion method

The agar plug plate method for screening of mutants with improved capacity to produce Zwittermicin A is depicted in Fig. 1. The mutant colonies on the agar plug with larger diameter of inhibition zone than that of parental strain were selected and rescreened in 500-ml shake flasks.

### Rescreening of mutants with high toxicity against *S. exigua* in shake flask

The over-producing Zwittermicin A mutants from the preliminary screening were cultured in 500-ml shake flasks containing 20 ml $H_8$ medium. After the titers of delta-endotoxin and Zwittermicin A in the broths were determined, those mutants over-producing Zwittermicin A and high producing delta-endotoxin were finally bioassayed to validate their toxicity against *S. exigua*.

### Method for determination of delta-endotoxin

SDS-PAGE analysis of delta-endotoxin concentration in the broth was made as described by National Standards of P. R. China, GB/T 19567.3 [12]. Gels were photographed by the Gene-genius Gel imaging system and the amounts of delta-endotoxin were detected by the Gene Tools Analysis Software Version 3.02 (Synoptics).

### Method for determination of Zwittermicin A

*Bacillus cereus* UW56 was cultured in 0.5× trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) for 72 h at 28°C, culture supernatant was concentrated 10 times in a rotary evaporator at 45°C and then diluted into a series of concentrations with sterile 100 mM phosphate buffer (pH 7.0). Zwittermicin A relative concentration was tested by agar plate diffusion bioassay for inhibition of *E. herbicola* LS005 with 0.05× trypticase soy agar (TSA) (instead of the 0.001× TSA that was used by

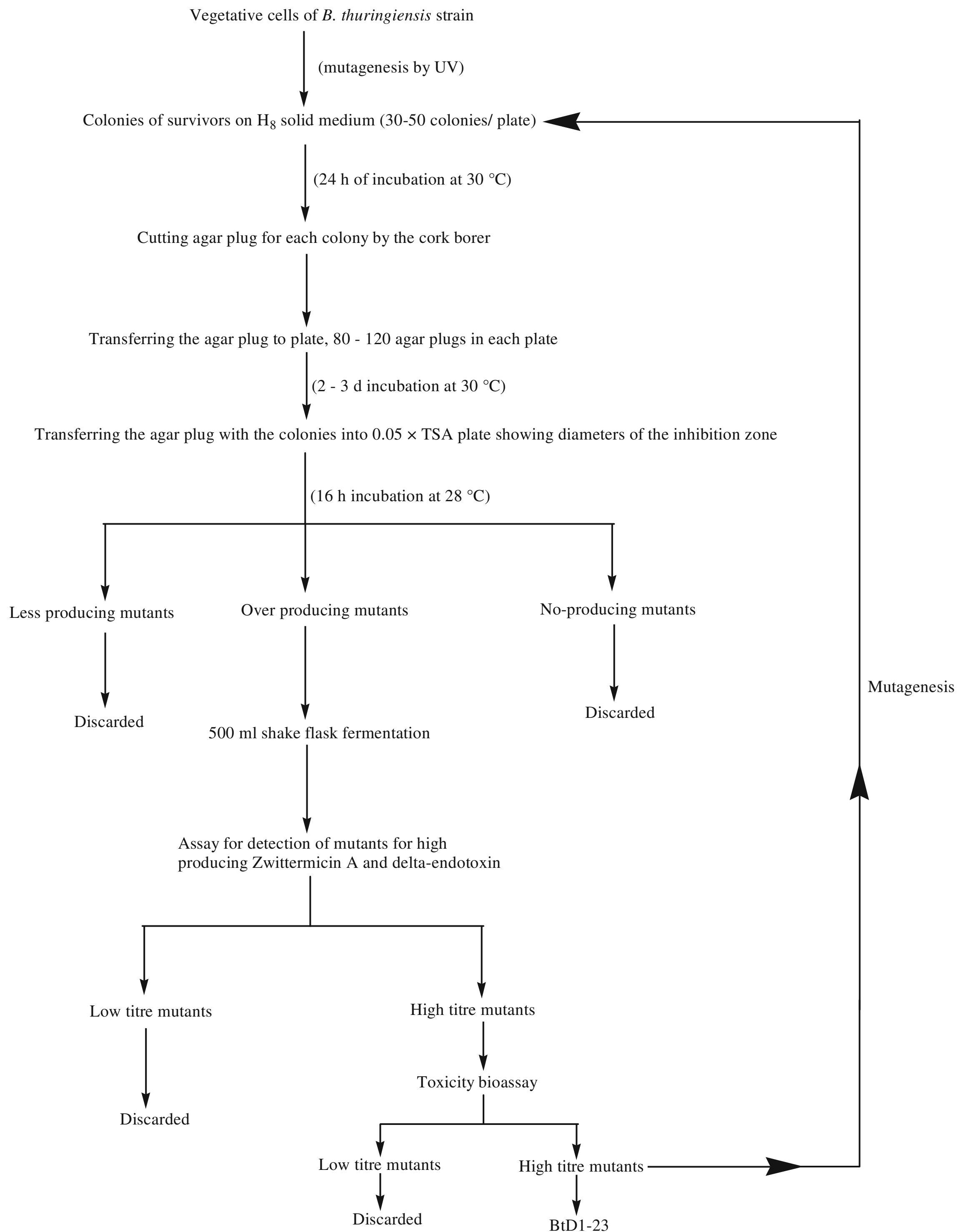


**Fig. 1** Efficient procedure for screening of *Bacillus thuringiensis* mutants over-producing Zwittermicin A

Stabb et al. [11], and plates were incubated for 24 h at 28°C prior to measuring the diameters of the inhibition zones. Zwittermicin A concentration of *B. cereus* UW56 culture supernatant was set as 10 units $ml^{-1}$, the mathematical relationship between the logarithm of Zwittermicin A concentration (logM) and the square of diameter of inhibition zone ($D$) was set up according to our trial and followed a linear equation: $D^2 = 3.64 \ \log M - 2.26$ (1.0 cm = $D$ = 2.6 cm), $R^2 = 0.9956$. The Zwittermicin A relative concentration of *B. thuringiensis* broth was determined according to the above equation with three replications.

Toxicity bioassay

To estimate the toxicity level, bioassays were conducted against neonate larvae of *S. exigua* and *H. armigera* as described by National Standards of P. R. China, GB/T 19567.3 [12]. For each dose, 120 neonate larvae of *S. exigua* and 120 neonate larvae of *H. armigera* were tested. All tests were performed with spore–parasporal inclusion suspensions with three replications. Statistical analysis of data was summarized by probit analysis (SAS Institute, SAS V8.1).

## Results

Screening of *B. thuringiensis* subsp. *kurstaki* strain with high toxicity against *S. exigua*

The investigation of toxicity of 200 *B. thuringiensis* subsp. *kurstaki* strains against neonate *S. exigua* larvae is shown in Table 1. Results in Table 1 clearly showed that the culture broth toxicity against *S. exigua* was closely related to the production of delta-endotoxin and Zwittermicin A. High toxicity was observed because of the synergetic effect of compounds Zwittermicin A and delta-endotoxin. All the 20 strains with high toxicity against *S. exigua* (mortality $\geq$80%) produced over 10 units $ml^{-1}$ Zwittermicin A and 2.0 mg $ml^{-1}$ delta-endotoxins (Table 1). Those strains with high toxicity against *S. exigua* were bioassayed against neonate larvae of *S. exigua*. At an infected diet concentration of 2.5 μl culture broth per ml, five strains (BMB005, BMB101, BMB159, BMB163 and BMB190) had 100% mortality, which was higher than the toxicity of other strains (data not shown). The bioassay results showed that BMB005 had the highest toxicity, and its 50% lethal concentration ($LC_{50}$) to *S. exigua* was 0.623 μl $ml^{-1}$. Although the Zwittermicin A relative concentration of BMB159 and BMB190 was as high as 30 units $ml^{-1}$, they were not as toxic as BMB005. This is likely to their lower (~30%) delta-endotoxin concentration than that of BMB005. Therefore, BMB005 was chosen as the parental strain for mutagenesis.

Selection of higher activity strains from *B. thuringiensis* subsp. *kurstaki* BMB005

Synergetic effect of compounds Zwittermicin A and delta-endotoxin showed high toxicity against *S. exigua*. On the basis of this principle, we presented a novel model for selecting strains having high toxicity against *S. exigua*. According to our model, three rounds of mutagenesis by UV rays exposure were applied on the parental strain BMB005. Over 3,300 survivors were screened on 0.05× TSA plates for Zwittermicin A over-producing mutants, and 95 mutants obtained in this way were rescreened in shake flasks (Table 2). By this selection, a mutant, BtU3-17, with the highest toxicity against *S. exigua,* was obtained. Its $LC_{50}$ to *S. exigua* was 0.455 μl $ml^{-1}$ which decreased by 36.9%, its Zwittermicin A relative concentration was 40.89 units $ml^{-1}$ which increased by twofold, and its delta-endotoxin concentration was 4.37 mg $ml^{-1}$ without a significant decrease of 0.06%, all as compared to that of BMB005 at the shake flask level (Table 3).

To obtain mutants with even higher toxicity against *S. exigua*, two rounds of mutagenesis by diethyl sulfate were performed on BtU3-17. Over 2,500 survivors were screened on 0.05× TSA plates for Zwittermicin A over-producing mutants, and 121 mutants obtained in this way were rescreened at the shake flask level (Table 2). The mutant BtD1-23 with the highest toxicity against *S. exigua* was obtained. Its $LC_{50}$ to *S. exigua* was 0.305 μl $ml^{-1}$ which decreased by 104.3%, its Zwittermicin A relative concentration was 78.25 units $ml^{-1}$ which increased by 2.72-fold, and its delta-endotoxin concentration was 4.18 mg $ml^{-1}$ which decreased by 10.1%, all as compared to that of BMB005 at the shake flask level (Table 3).

**Table 1** Investigation of toxicity of 200 *Bacillus thuringiensis* subsp. *kurstaki* strains against *Spodoptera exigua*

| No. of strains | Zwittermicin A relative concentration[a] | Delta-endotoxin concentration[b] | Toxicity against *S. exigua*[c] |
|---|---|---|---|
| 2 | ++ | ++ | ++ |
| 10 | + | ++ | ++ |
| 8 | ++ | + | ++ |
| 9 | − | ++ | + |
| 7 | + | + | + |
| 35 | − | + | − |
| 33 | ++ | − | +/− |
| 72 | + | − | +/− |
| 24 | − | − | − |

[a] ++ above 25 units $ml^{-1}$, + 10–25 units $ml^{-1}$, − below 10 units $ml^{-1}$

[b] ++ above 3.0 mg $ml^{-1}$, + 2.0–3.0 mg $ml^{-1}$, − below 2.0 mg $ml^{-1}$

[c] ++ above 80% mortality, + 20–80% mortality, − below 20% mortality, infected diet concentration was 5 μl culture broth per ml

Comparative toxicities of BtD1-23 and BMB005 in 3-L fermentor level

BMB005 and BtD1-23 were processed in three separate batches. The delta-endotoxin and Zwittermicin A production of BMB005 were 5.14 mg $ml^{-1}$ and 22.05 units $ml^{-1}$, respectively. On the other side, the delta-endotoxin production of BtD1-23 decreased by 7.6% whereas Zwittermicin A production of BtD1-23 increased by 2.73-fold, as

Table 2 Breeding of *Bacillus thuringiensis* subsp. *kurstaki* BMB005

| Step | Mutagen | Parental strain | No. of experiments | No. of survivors | No. of mutants screened on 0.05× TSA plate[a] | No. of mutants screened in shake flasks | Isolated |
|---|---|---|---|---|---|---|---|
| 1 | UV | BMB005 | 3 | 15,450 | 3,345 | 95 | BtU3-17 |
| 2 | DES | BtU3-17 | 2 | 1,340 | 2,541 | 121 | BtD1-23 |

*DES* Diethyl sulfate

[a] Picked out the colonies in the plates where the lethal frequency of mutagenesis was above 80%

Table 3 Comparative toxicities of the parental strain BMB005 and its mutants BtU3-17and BtD1-23 at the shake flask level

| Strains | Delta-endotoxin concentration (mg $ml^{-1}$) | Zwittermicin A relative concentration (units $ml^{-1}$) | $LC_{50}$ to *S. exigua*[a] (μl $ml^{-1}$) |
|---|---|---|---|
| BMB005 | 4.65 | 21.06 | 0.623 (0.460–0.880) |
| BtU3-17 | 4.37 | 40.89 | 0.455 (0.285–0.565) |
| BtD1-23 | 4.18 | 78.25 | 0.305 (0.234–0.362) |

The $LC_{50}$ unit was the amount of culture broth per ml of infected diet

[a] The 95% confidence intervals ranges are shown in parentheses

Table 4 Comparative toxicities of BMB005 and BtD1-23 in 3-l fermentor level

| Strains | Period of fermentation (h) | Broth titer | | | |
|---|---|---|---|---|---|
| | | $LC_{50}$ to *H. armigera*[a] (μl $ml^{-1}$) | $LC_{50}$ to *S. exigua*[a] (μl $ml^{-1}$) | Delta-endotoxin concentration (mg $ml^{-1}$) | Zwittermicin A relative concentration (units $ml^{-1}$) |
| BMB005 | 29 | 0.107 (0.066–0.149) | 0.603 (0.442–0.862) | 5.14 | 22.05 |
| BtD1-23 | 31 | 0.085 (0.044–0.126) | 0.280 (0.207–0.305) | 4.75 | 82.15 |

The $LC_{50}$ unit was the amount of culture broth per ml of infected diet. The data were the mean values of three batch experiments

[a] The 95% confidence intervals ranges are shown in parentheses

compared to BMB005. The BtD1-23 showed higher toxicity against *S. exigua* and *H. armigera* as compared to BMB005. BtD1-23 showed $LC_{50}$ to *S. exigua* and *H. armigera* were 0.280 and 0.085 μl $ml^{-1}$, which decreased by 115.4 and 25.9%, respectively. And its period of fermentation was prolonged by 2–3 h (Table 4).

## Discussion

In the present study, we screened *B. thuringiensis* subsp. *kurstaki* strains exhibiting high toxicity against *S. exigua* by selecting Zwittermicin A over-producing strains. Our results proved that the toxicity of *B. thuringiensis* subsp. *kurstaki* against *S. exigua* and *H. armigera* could be enhanced by a high amount of Zwittermicin A accumulated in culture broth. The above viewpoint was also supported by the Outtrup et al. [8] in which Zwittermicin A significantly enhanced the toxicity of *B. thuringiensis* subsp. *kurstaki* against *S. exigua*, but only slightly increased the toxicity against *H. armigera*. We were able to screen up to 1,000 mutants within a week, as compared to less than 100 strains by the conventional in vivo bioassay [15] and the in vitro midgut epithelial tissue assay [16] previously used to screen and breed *B. thuringiensis* with high toxicity against *S. exigua*. So, our novel approach is not only effective but also less labor-intensive for efficient selection of *B. thuringiensis* subsp. *kurstaki* strains with high toxicity against *S. exigua*.

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