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[Communication]

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Expression of Mosquitocidal *Bacillus sphaericus* Binary Toxin and *B. thuringiensis cry11B* Genes in *B. thuringiensis* 4Q7

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Wild type *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* toxins have been used separately as active ingredients for bacterial insecticides to control mosquito larvae due to their comparable toxicity to chemical insecticides. Cry11B, recently cloned from *B. thuringiensis* subsp. *jegathesan*, shows higher toxicity against three major species of mosquito larvae than Cry11A, one of the major component of *B. thuringiensis* subsp. *israelensis* inclusion body. To determine whether the combination of *cry11B* and *B. sphaericus* binary toxins is as toxic as *B. thuringiensis* subsp. *israelensis* parental strain, *cry11B* and *B. sphaericus* binary toxin genes were co-expressed as an operon using *cyt1A* promoters/STAB-SD hybrid expression system in *B. thuringiensis* subsp. *israelensis* acrystalliferous strain 4Q7. However, unexpectedly, *B. sphaericus* binary toxins were barely produced, whereas relatively large amount of Cry11B was produced. When this strain was grown in four different media, NB+G and Peptonized Milk produced more toxin proteins and spores per unit of media than GYS and G-Tris. Toxicity of this strain against fourth instar *Culex quinquefasciatus* was ranged from of 8.3 to 45.7 ng/ml, with NB+G culture being the highest, and GYS culture was the lowest.

Key words : *Bacillus thuringiensis*, Bacillus sphaericus, Cry11B, Binary toxin, *Culex quiquefasciatus*, cyt1A promoter, STAB-SD

Introduction

Most biological control programs to suppress mosquito populations have relied on two entomopathogenic bacteria, *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus*. Both bacteria produce crystal proteins which are toxic to mosquito larvae during sporulation (Baumann *et al.*, 1991; Porter *et al.*, 1993; Charles *et al.*, 1996; Federici *et al.*, 2000). *B. thuringiensis* subsp. *israelensis* produces four different Cry proteins of Cry4A, Cry4B, Cry11A and Cyt1A, whereas several strains of *B. sphaericus* produce two proteins of 51.4- and 41.9-kDa, which are generally highly active against larvae of *Anopheles* and *Culex* species and are poorly or not toxic to larvae of *Aedes* species. Recently, a new strain of *B. thuringiensis* nearly as toxic as *B. thuringiensis* subsp. *israelensis* has been isolated and named as *B. thuringiensis* subsp. *jegathesan* (Seleena *et al.*, 1995; Kawalek *et al.*, 1995). One of the crystal proteins produced by this strain, Cry11B, was more active against three major mosquito species than Cry11A of *B. thuringiensis* subsp. *israelensis* (Délecluse *et al.*, 1995).

To enhance the activity of wild type strains of *B. thuringiensis* and *B. sphaericus*, basically two different methods have been used to combine the toxins of both organisms in the same host (Federici *et al.*, 2000). Most recombinants made to date have introduced Cry proteins of *B. thuringiensis* into *B. sphaericus*. For example, *cry4B* and *cry11A* genes of *B. thuringiensis* subsp. *israelensis* were transferred into *B. sphaericus* 2297 (Poncet *et al.*, 1994). More recently, *cry11A* gene of *B. thuringiensis* subsp. *israelensis* was integrated into *B. sphaericus* 2297 chromosome via homologous recombination (Poncet *et al.*, 1997), or both Cry11A and Cry11B toxins were produced in *B. sphaericus* 2297 (Servant *et al.*, 1999). However, none of these recombinants was better than parental strain of *B. thuringiensis* subsp. *israelensis* against *Aedes* species, and only a few were more toxic to *Culex* and *Anopheles* species than the parental *B. sphaericus* strains. The other method, producing *B. sphaericus* binary toxins in *B. thuringiensis* has been used less frequently. In the

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first attempt, recombinant strain where *B. sphaericus* binary toxin genes were under the control of their own promoter did not show a significant enhancement of toxicity (Bourgouin *et al.*, 1990). When *cytlA* promoters combined with STAB-SD mRNA stabilizing sequence (Park *et al.*, 1998; 1999; 2000; Park and Federici, 2000) were used to drive expression of *B. sphaericus* 2362 binary toxin, toxicity of *B. thuringiensis* subsp. *israelensis* producing *B. sphaericus* 2362 binary toxins was greatly improved (Bideshi *et al.*, unpublished data).

In this study, using *cytlA* promoters combined with STAB-SD mRNA stabilizing sequence, I constructed an artificial operon that contains both *B. sphaericus* 2362 binary toxin genes and *cry11B* of *B. thuringiensis*, each of which is highly toxic to mosquito larvae by itself, to decide whether this combination is enough to obtain as much toxicity as *B. thuringiensis* subsp. *israelensis* parental strain.

Materials and Methods

Construction of an artificial operon

The construction of an artificial operon was based on 9.8-kb pPFT11Bs (Park *et al.*, unpublished data) in which *cytlA* promoters plus STAB-SD sequence and *cry11B* open reading frame were inserted into pHT3101 (Lereclus *et al.*, 1989). The pPFT11Bs was digested with *Xba*I, filled-in with Klenow fragment, and treated with Calf Intestinal Alkaline Phosphatase (New England Biolabs, MA). *B. sphaericus* 2362 toxin genes were obtained by PCR using a 43-base oligomer 5-AACTGCAGCTTGT-CAACATGTGAAGATTAAAGGTAACTTT CAG-3, as the forward primer, and a 27-base oligomer 5-CACTATAA TCCAAAAGT ACTTAAATTG-3, as the reverse primer. Plasmid containing *B. sphaericus* 2362 binary toxin genes, pHBS (Bideshi *et al.*, unpublished data), was used as a template. PCR was performed with the Vent® DNA Polymerase (New England Biolabs, MA) for 30 cycles as follows: 94°C for 1 min, 55°C for 1min, and 72°C for 3 min. The 3.2-kb PCR product was then purified and digested with *Hpa*I to remove *B. sphaericus* toxin promoter region. Final product of 2.8-kb fragment, which contains whole binary toxin open reading frame and transcription termination sequence of *B. sphaericus* 2362, was inserted into pPFT11Bs *Xba*I/Klenow fragment site using T4 DNA ligase (Fig. 1). The recombinant plasmid, pBS-11BL, was confirmed by restriction endonuclease analysis.

Electroporation of 4Q7

An overnight culture of *B. thuringiensis* subsp. *israelensis* acrystalliferous strain 4Q7 was incubated on ice for 10 min and harvested at 1,000 × g for 10 min. Pellets were washed twice with ice-cold sterilized double distilled water and resuspended in 40% PEG 6000 (w/v) at a density 100 times that of the original culture. Then 300 μl of this cell suspension was mixed with 5 μg of pBS-11BL DNA and held on ice for 10 min. Electroporation was performed with a 0.2 cm cuvette in BTX electroporation system set at 480 Ω and 2.3 kV. After the pulse, the culture was added to 3 ml of BHI and incubated with shaking for 1 hr at 37°C. Transformed cells were plated on BHI supplemented with 25 μg of erythromycin per ml for growth.

Production of Cry11B and *B. sphaericus* binary toxins

To select an optimal growth medium, toxin yields for 4Q7/pBS-11BL strain were compared by using the following four liquid media: glucose-yeast extract-salts (GYS) [0.1% glucose, 0.2% yeast extract, 0.05% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.002% $MgSO_4$, 0.005% $MnSO_4$, 0.008 % $CaCl_2$], G-Tris [0.08% $CaCl_2$, 0.0025% $FeSO_4$, 0.005% $CuSO_4$, 0.005% $ZnSO_4$, 0.05% $MnSO_4$, 0.2% $MgSO_4$, 2% $(NH_4)_2SO_4$, 2% glucose, 0.5% 1M Tris (pH 7.5), 0.15% yeast extract, 0.5% K_2HPO_4], Peptonized Milk (1% peptonized milk, 1% dextrose, 0.2% yeast extract, 1.216 mM $MgSO_4$, 0.072 mM $FeSO_4$, 0.139 mM $ZnSO_4$, 0.118 mM $MnSO_4$) and Nutrient Broth plus Glucose (NB+G) [0.8% nutrient broth, 0.15% yeast extract, 0.005% $MnCl_2$, 50 mM $NaHPO_4$, 1.22 mM $MgSO_4$, 0.68 mM $CaCl_2$, 0.5% glucose]. Two control strain 4Q7/pPFT11Bs and 4Q7/pPHSP-1 (Park *et al.*, unpublished data; Bideshi *et al.*, unpublished data), which produce, respectively, Cry11B and *B. sphaericus* binary toxins using *cytlA* promoters/STAB-SD expression system, were grown only in GYS. After 5 days of culture at 30°C, 1 ml of each culture was collected and pelleted at 15,000 × g for 5 min. The pellets were resuspended in 60 μl of 5 × Laemmli protein sample buffer and boiled for 5 min. 10 μl of each sample was analyzed using 10% sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE).

Growth and toxicity of 4Q7/pBS-11BL in four different media

Growth of the recombinant bacteria was measured by determination of the number of spores produced per ml of media, whereas toxicity was determined by median lethal concentrations (LC_{50}) against fourth instar larvae of *Culex quinquefasciatus*. One milliliter of culture broth was placed in a 1.5 ml tube, heated at 60°C for 20 min (Adams *et al.*, 1994), diluted, and plated on nutrient agar. Colonies were counted after 20 hr of growth at 30°C, and data were analyzed with the Super ANOVA program (Abacus Concepts, CA). Bioassay was performed using 50 fourth instar

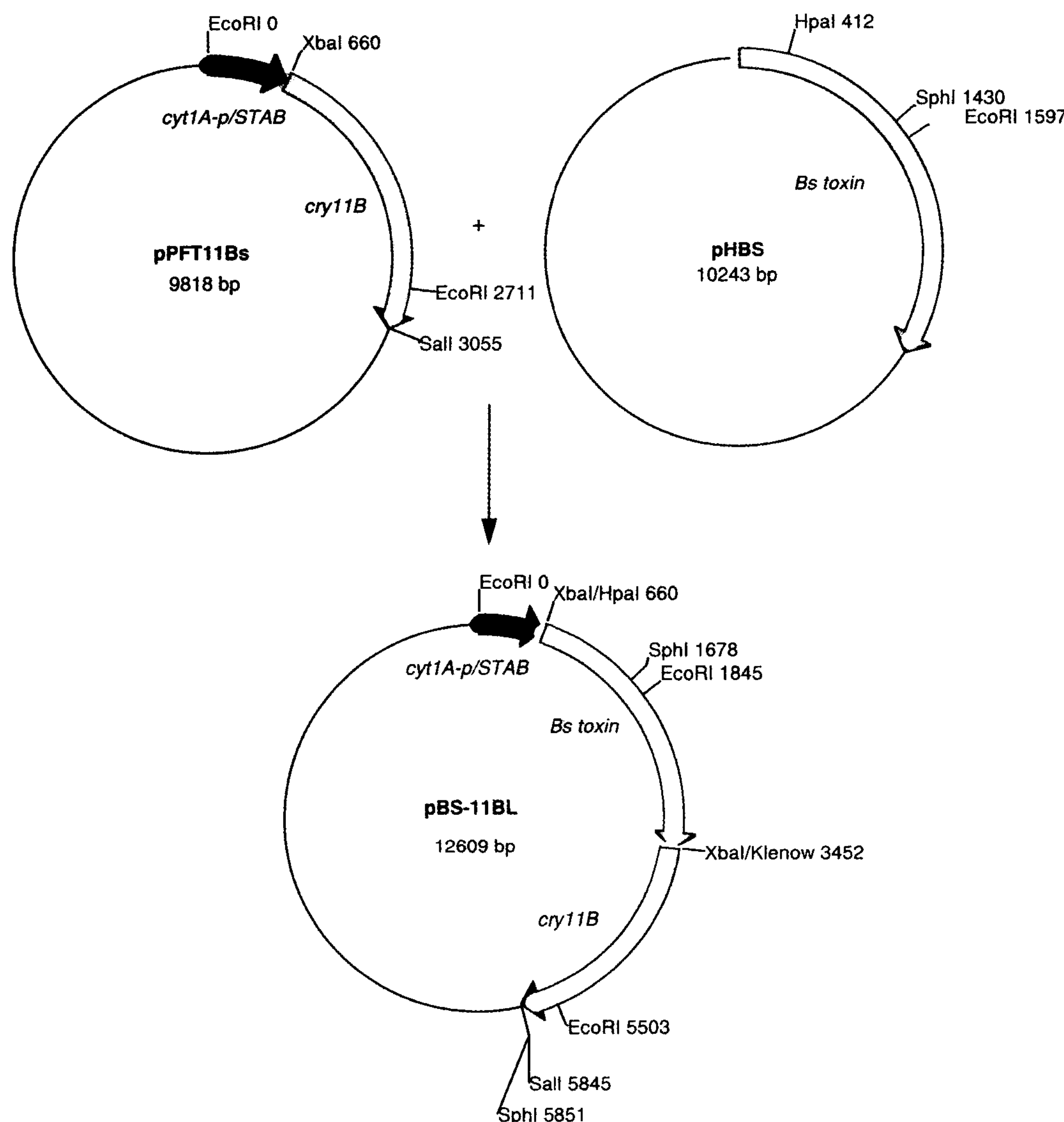

Fig. 1. Construction map of pBS-11BL for expressing *B. thuringiensis* subsp. *jegathesan cry11B* and *B. sphaericus* 2362 binary toxin genes. The vector, pPFT11Bs, containing *cyt1A* promoters/STAB-SD sequence and *cry11B* gene was digested with *Xba*I, and filled-in with Klenow fragment. The 3.2-kb PCR fragment containing *B. sphaericus* binary toxin gene, amplified using pHBS as a template, was digested with *Hpa*I. Final 2.8-kb *B. sphaericus* binary toxin gene without its own promoter was ligated with pPFT11Bs cut with *Xba*I to generate recombinant plasmid, pBS-11BL.

larvae of *C. quinquefasciatus* per concentration per medium. Six to seven different concentrations per medium were used to calculate the LC_{50}. Mortality was recorded after 24 hr of incubation at 28°C, and data was analyzed using Probit analysis (POLO-PC; CA).

Results and Discussion

4Q7/pBS-11BL produced comparable amount of Cry11B to 4Q7/pPFT11Bs, while, unexpectedly, very little amount of *B. sphaericus* toxins compared to the control strain 4Q7/pPHSP-1 (Fig. 2, lane 2) was observed in all four media. Among four different media, Peptonized Milk and NB+G yielded more toxin proteins than the other two media, GYS and G-Tris (Fig. 2, lanes 3 - 6). Yield from G-Tris was the lowest (Fig. 2, lane 5). It is not clear at this time why this recombinant strain produces very little amount of *B. sphaericus* toxin. However, the experiments to elucidate this mystery are currently in progress by construction of a series of *B. sphaericus* plus *cry11B* operon.

The spore count data were generally consistent with SDS-PAGE analysis. Peptonized Milk and NB+G produced more spores than GYS and G-Tris, and no signif-

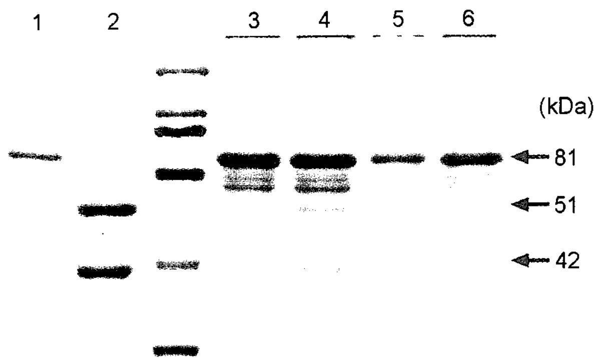

Fig. 2. SDS-PAGE analysis of *B. thuringiensis* subsp. *israelensis* acrystalliferous strain 4Q7 producing Cry11B and *B. sphaericus* binary toxins. Lane M, molecular mass standard; lane 1, 4Q7/pPFT11Bs grown in GYS; lane 2, 4Q7/pPHSP-1 grown in GYS; lane 3, 4Q7/pBS-11BL grown in Peptonized Milk; lane 4, 4Q7/pBS-11BL grown in NB+G; lane 5, 4Q7/pBS-11BL grown in G-Tris; lane 6, 4Q7/pBS-11BL grown in GYS.

Table 1. Toxicity versus number of spores produced per unit media for *B. thuringiensis* 4Q7 strain producing *B. thuringiensis* Cry11B and *B. sphaericus* binary toxin using pBS-11BL

Media	Number of spores/ml [a]	LC_{50}^{b}
GYS	1.0×10^6 (2.3×10^5) a	45.7 (39.5 - 56.7)
G-Tris	7.0×10^5 (1.0×10^5) a	25.1 (20.6- 31.4)
Peptonized Milk	2.0×10^6 (5.8×10^4) b	37.9 (32.1- 46.7)
NB+G	1.6×10^6 (3.8×10^5) b	8.3 (6.7- 1.0)

[a] Standard deviations are shown in parenthesis and the same letters followed by the value are not significantly different at P = 0.05.
[b] Bioassays were performed using 4th instar larvae of *Culex quinquefasciatus*. Mortality was recorded after 24 hr of incubation. Confidential limits are shown in parenthesis.

icant difference in number of spores was observed between two highest yielding media and between two lowest yielding media (Table 1).

Although there was no significant difference in toxin yield and growth between Peptonized Milk and NB+G, toxicity was much higher in NB+G (8.3 ng/ml) than Peptonized Milk (37.9 ng/ml) (Fig. 2; Table 1). Moreover, the poorest yielding media G-Tris showed higher toxicity than Peptonized Milk. This may be due to the higher toxin yield per unit medium in G-Tris, and therefore, optimal medium regards growth and toxicity for 4Q7/pBS-11BL strain was NB+G.

The artificial operon used in this study to express *cry11B* and *B. sphaericus* binary toxin genes did not synthesize both toxin proteins effectively. However, Cry11B and *B. sphaericus* binary toxin combination is promising because LC_{50} of 4Q7/pBS-11BL against *C. quinquefasciatus* obtained using NB+G medium was similar with that of *B. thuringiensis* subsp. *israelensis* IPS-82 parental strain (Park *et al.*, unpublished data).

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