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Effects of Protoplast Fusion on δ-endotoxin Production in *Bacillus thuringiensis* Spp. (H14)

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ABSTRACT

In this study, mutant forms of *Bacillus thuringiensis* spp. *israelensis* (H14) were produced. These mutants were identified when the cells were cultured on chloramphenicol plates and stained with crystal violet. Protoplasts of the mutants were isolated by enzymatic digestion (lysozyme) of the cell walls at the presence of an osmotic stabilizer. The protoplasts were induced to fuse to each other in the presence of PEG 6000. The frequency of regeneration and recombination was 80% and 2×10^{-4}, respectively. In order to survey the effect of protoplast fusion on production of toxin, anti-serum against pure toxin was raised in rabbit and was used in single radial immunodiffusion. The comparison of δ-endotoxin concentration between *B. thuringiensis* fusion and the wild type strains showed that *B. thuringiensis* fusion has 1.48 time more toxin than wild type. *Iran. Biomed. J. 6 (1): 25-29, 2002*

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INTRODUCTION

Bacillus thuringiensis spp. *israelensis* is a Gram-positive spore forming bacterium, that is well-known for its potent mosquitocidal crystal and is toxic to larvae of several dipteran insects [1-4]. During sporulation, strains of *B. thuringiensis* produce crystalline cytoplasmic protein inclusions that have been used for over 30 years as highly specific insecticides against certain species of *lepidoptera* and *diptera* [1-4]. *B. thuringiensis* contains several plasmids ranging in size from 5 to more than 300 kb. The chromosome or large plasmids are responsible for production of this toxin [5, 6]. However, the direct evidence showed that crystal protein genes are located on plasmids. Most δ-endotoxins are encoded by *Cry* genes that origin from gene cloning and hybridization (Southern blotting) [6]. Thus, there is a large family of related δ-endotoxins that are classified (19 classes) as *Cry* I to XVIIII, depending on their molecular relatedness and their activity against insects. More than 50 *Cry* genes related to over 20 different classes or subclasses have been identified [1, 5, 6]. *Cry* toxins structurally and functionally resemble to the colicin and diphtheria toxin thus they can be considered as members of Ion–channel protein family [4, 6].

Protoplast fusion is a versatile technique for inducing genetic recombination in a variety of prokaryotic and eukaryotic microorganisms, such as a*ctinomycetes* and *Bacillus* [7-9]. Protoplasts are prepared by treating mutant bacteria with a lytic enzyme such as lysozyme that removes the cell wall. As a result of this treatment, the cell content would be enclosed only by a cell membrane [10]. The protoplasts, a hypertonic medium, cause osmotic stability and survival. Then, in the presence of fusogenic agent such as polyethylene glycol (PEG), protoplasts are induced to fuse and form transient hybrids or diploids. During this hybrid state, the genomes may re-assort and genetic recombination can occur [10].

So far, an increasing number of recombinant strains have been formed [6]. In this study, the transfer of the genetic material has been achieved

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Abbreviations: Spp., Subspecies; HNB, Hypertonic Nutrient Broth; SMMAD, Sucrose Maleate Magnesium Albumin DNase; SMMD, Sucrose Maleate Magnesium DNase; RD, Regenerating Medium; NA, Nutrient Agar; NB, Nutrient Broth; BHIB, Brain Heart Infusion Broth; I.V., Intravenous; ELISA, Enzyme Linked Immunosorbent assay; SRID, Single Radial Immunodiffusion; HRP, Horse Radish Peroxidase; TMB, Tetra Methyl Benzidine.

by protoplast fusion and the effect of this technique has been assayed on the toxin production. Recombinant DNA technology offers promise to develop super strains of *B. thuringiensis* for more efficient production of crystals and spores [6].

MATERIALS AND METHODS

All chemicals were purchased from Sigma.

Organism. Two antibiotic resistant mutants of *B. thuringiensis* spp. *israelensis (*Catalogue no: T 14007 Pasteur B.t. 1884) were produced by UV radiation (245 LUX) and used for fusion experiments [11]. These mutants included chloramphenicol and crystal violet resistant strains. The cells were grown in hypertonic nutrient broth (HNB) at 37°C and harvested before stationary phase.

Protoplast formation. The cells of two mutants were centrifuged at 3840 ×g at 18°C for 10 min at. The cell pellets were suspended in SMMAD buffer [0.5 M sucrose, 0.02 M maleate buffer pH 6.5, 20 mM $MgCl_2$, 1% BSA, DNase I 5 µg/ml] (pH 6.5) and their optical density was measured at 650 nm. Then, lysozyme was added at final concentration of 100µg/ml to SMMD buffer (same as SMMAD buffer without 1% BSA) and the cell suspension was incubated at 42°C for 30 min without shaking. Protoplast formation was confirmed by staining with methylene blue and then observed under light microscope [7, 12]. The protoplasts were centrifuged at 3840 ×g and then suspended in 1/5 to 1/10 volume of SMMAD buffer. The viability of protoplasts was determined by plating different dilution of protoplasts in regenerating medium (1.0 g NH_4NO_3, 3.5 g K_2HPO_4, 1.5 g KH_2PO_4, 2.0 % agar, 0.33 M sodium succinate, 5.0 g gelatin, 4.07 g $MgCl_2.6H_2O$, 5.0 g glucose (pH 7.3) [13]. Non-protoplasted viables were identified by plating different dilutions of the protoplasts on modified NA [14].

Protoplast fusion. Protoplast suspensions (0.1ml) were added to 0.9 ml of 40% PEG 6000 and shaked vigorously (30 s) and then left at room temperature for 2 min [5, 6, 8, 10].

Protoplast regeneration. The fusion mixture in SMMAD was diluted and plated on non-selective RD and allowed all protoplasts to regenerate. The plates were incubated at 37°C for 2 days. The colonies were replica plated by using sterile velvet and were incubated at 37°C for 1- 2 days [7].

Growth curve of organism. A volume of 3% of a pre-culture medium with optical density of 0.6 at 650 nm was added to BHIB and a sample was taken every 2 hours. Then, growth curve of the fusion and the wild strains of *B. thuringiensis* H14 was plotted.

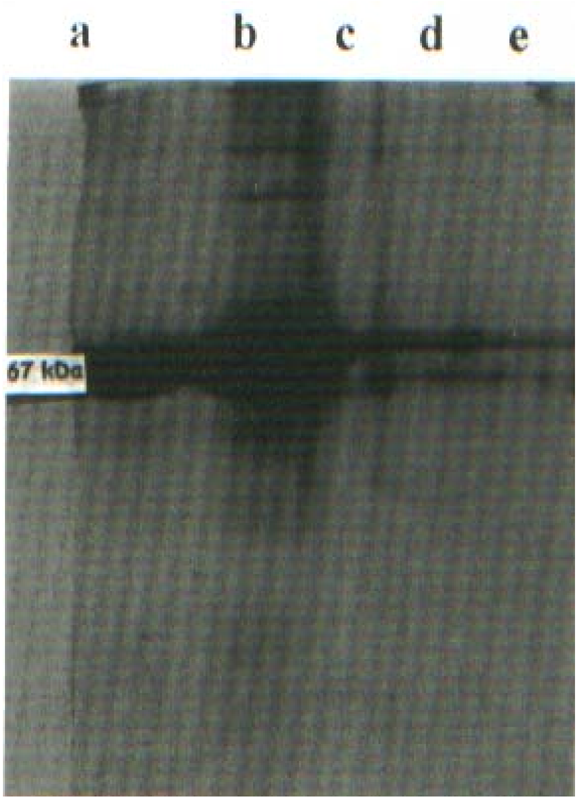

Fig. 1. SDS-PAGE of fractions from Sephadex G-100 gel filtration; electrophoresis in this condition revealed two proteins with 60-70 kDa molecular weight, (a) bovine serum albumin (67 kDa); (b) molecular weight markers; (c, d & e) purified δ-endotoxin (60-70 kDa)

Effect of protoplast fusion on toxin production. A semi quantitative assay of toxin production in two strains was determined by pre-culturing in BHIB at 37°C for 24 hours. When the OD_{650} reached 0.6, the cells were harvested. Then 3–10% inoculum was added to fresh BHIB (200 ml) and incubated for 5 days at 37°C. When cell growth was completed and the autolysis was accomplished, the cells were centrifuged at 11000 ×g, at 4°C for 10 min and the total mass was measured. Cell debris and spores were removed by CCl_4 centrifugation and the total protein concentration was estimated using Lowry's method [15]. The toxins (wild and fusion) were purified by Sephadex G-100 gel filtration (50 mM Tris buffer, pH 8 [16, 17]. The purity of toxins was assayed by SDS-PAGE electrophoresis (Fig. 1). Pure toxins were injected (i.v.) into rabbits and the antisera were titrated with sandwich ELISA [18]. The wells of microtiter plate (Denmark Maxisorb) were coated with 100 μl of the purified endotoxin (8 μg/ml). In this assay, anti-rabbit HRP and TMB were used as 2nd Ab conjugated and substrate, respectively. Finally, the OD of the product was determined at 450 nm with ELISA reader. In this technique, antisera against δ-endotoxin were added to the agarose gel, then mutant and wild type proteins were diluted and reposed in the wells of SRID gel and incubated for 24 hours.

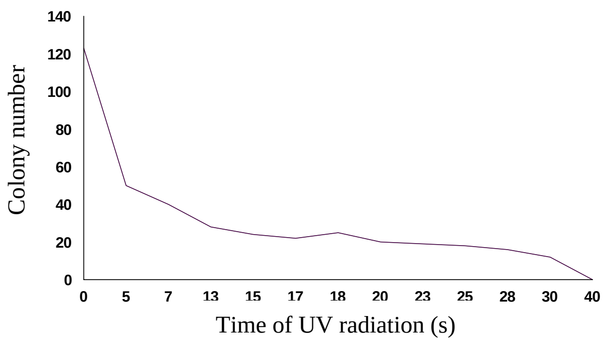

Fig. 2. Survival curve of *B. thuringiensis* (H14) versus UV. After 5-7 s UV radiation, 50% of bacteria survived; however after 40 s of UV radiation all bacteria were died.

RESULTS AND DISCUSSION

The survival of *B. thuringiensis* H14 treated with UV is shown in Figure 2. When radiation time was reached to 44 seconds, no growth was observed. The growth curve of *B. thuringiensis* H14 in BHIB is shown in Figure 3. The results show that the growth of both fusion and wild strains were identical. Two antibiotic resistant mutants of *B. thuringiensis* H14 were obtained by UV radiation. One mutant was resistant to 0.0001% (w/v) chloramphenicol and the other one to 0.0003% (w/v) crystal violet. The frequency of the regeneration and the recombination were shown to be 80% and 2×10^{-4}, respectively. Bacteria subcultured in a medium containing enough glycine (1.5%) are more sensitive to lysozyme. This effect is due to the replacement of D-alanine residues with glycine in peptidoglycan that interferes with cross-linking [13].

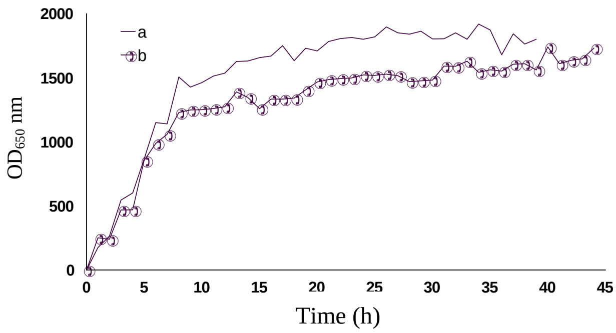

Fig. 3. Growth curve of *B. thuringiensis* (H14). (a) wild type; (b) fusion type.

In this study, the optimum amount of glycine for culture medium was estimated to be 1.5%. The physiological status of the organisms at the time of protoplast formation (Fig. 4) is a major factor in determining the protoplast yield [7, 10, 13]. The cells were harvested 45 hours after incubation. The temperature during bacterial growth and lysozyme treatment can affect the regeneration frequency and the increase of recombination. The bacteria incubated at 37°C and then treated with lysozyme in 42°C. The presence of 1% BSA in SMMD buffer increased the yield of regeneration from 10 to several hundred folds. The presence of 0.5% gelatin in the regeneration medium increased the regeneration. A requirement for Ca^{2+} is also well established. The optimal concentration of Ca^{2+} in the medium was 0.01M [7, 13]. In addition, the pH of buffers was controlled and maintained at 6.5 because the pH is an important factor during preparation of protoplast and fusion. Regeneration on RD medium may stabilize the formation of the diploids and to increase the frequency of subsequent genetic recombination [7, 13, 19]. The total protein concentration in fusion strain, in different times of experiments, was higher than wild strain. The existence of δ-endotoxin in total proteins was confirmed with specific anti-sera against the toxin by SRID method. In this technique, anti-sera against δ-endotoxin were added to agarose gel. The specificity and titer (1:8000) of antisera were determined by ELISA. The average total protein of wild type was 1.583 mg/ml and the fusion type was 2.346 mg/ml. This comparison showed that the capability of protein production in fusion type is higher than the wild type (the ratio was 1.48). In order to investigate the relationship between the increase of total protein and the production of toxin in the fusion strain, SRID method was used. The solubilized mutant and the wild type proteins were diluted and reposed in the wells of SRID gel after 24 hours of incubation. The ring diameter of protein for fusion type (1/4 dilution) was equal to the ring diameter of the protein for the wild strain (0.8 mm of 1/1 dilution). This results demonstrated that the increase of protein production was partly due to the increase of the toxin production. The above results demonstrate the capacity of the protein production including endotoxin. Because of the advantages of this toxin in biological field and the importance of this product as an insecticide large scale production of this toxin would be very valuable. Our technique identified a strain of *B. thuringiensis* that can produce δ-endotoxin in large scale for industrial use.

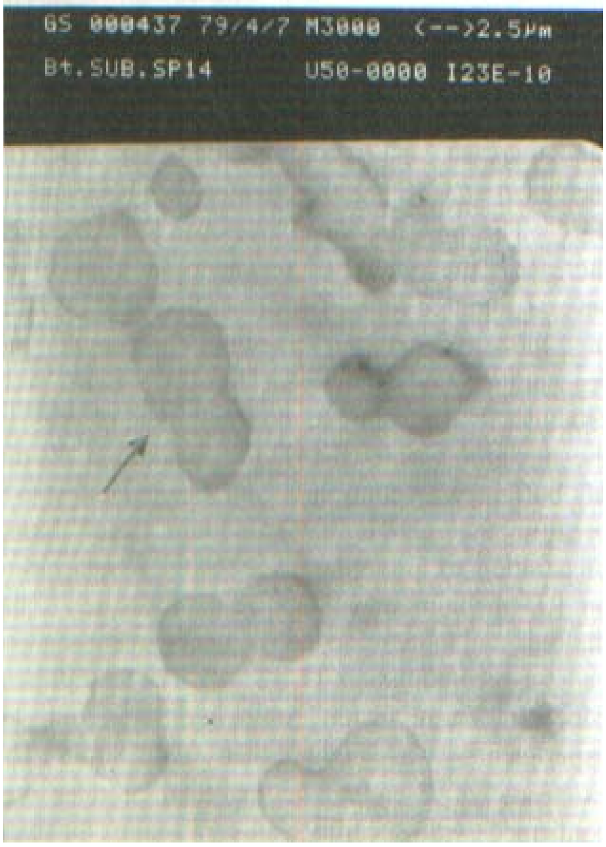

Fig. 4. Electron microphotograph showing of protoplast fusion between two mutant strains of *B. thuringiensis* (H14) (one resistant to crystal violet and the other resistant to chloramphenicol).

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