Increased production of Coleoptera-specific delta-endotoxin by homologous expression of cry3Aa11 in Bacillus thuringiensis Mm2

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Abstract: The cry3Aa11 gene of Bacillus thuringiensis strain Mm2 (B. thuringiensis Mm2) was PCR-amplified and cloned into E. coli/B. thuringiensis shuttle vector pHT315. The recombinant plasmid carrying the cry3Aa11 gene was introduced into the parental B. thuringiensis Mm2. The toxin production capacities of parental and recombinant strains grown in DSM, containing either 50 mM or 200 mM inorganic phosphate (Pi), were compared by SDS-PAGE and western blot analyses. In comparison to the parental strain, recombinant B. thuringiensis Mm2 produced 4-fold more Cry3Aa11 toxin after 12 h of growth in the presence of 50 mM Pi. However, the 200 mM Pi concentration did not cause a drastic increase in toxin production by the recombinant strain. This study shows that higher toxin production could be obtained from the cultures during the early hours of growth by introducing the multicopy cry3Aa11 gene into the source organism.

Key words: Bacillus thuringiensis Mm2, Cry3Aa11 δ-endotoxin, homologous gene expression, cry3Aa11 gene

Introduction

Bacillus thuringiensis (B. thuringiensis) is a gram-positive, spore-forming bacterium with entomopathogenic properties. It produces insecticidal crystal proteins, delta-endotoxins, during the sporulation process, which are highly specific to their target insect larvae, completely biodegradable, and innocuous to humans, vertebrates, and plants. Therefore, B. thuringiensis is a viable alternative for the control of insect pests in agriculture and
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Important human disease vectors (1). Although a large number of B. thuringiensis strains have been isolated and many types of insecticidal crystal protein genes have been cloned, the search for novel B. thuringiensis strains is an active research area (2). So far, d-endotoxins from the members of B. thuringiensis comprise a group of 563 Cry and 34 Cyt proteins and are classified into Cry1 to Cry68 and Cyt1 to Cyt3, according to the degree of amino acid sequence homology (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

B. thuringiensis Mm2, isolated from the dead larvae of Melolontha melolontha (Coleoptera: Scarabaeidae), produces Cry3Aa11 delta-endotoxins, which are toxic against the larvae of certain Coleoptera (3). The cry3Aa gene encodes for a 73 kDa protein, which is proteolytically processed to a 65 kDa polypeptide during or after crystal formation; thus, the Cry3A protein can be visualized as 1 or 2 protein bands with SDS-PAGE (4). Unlike the other cry genes, the cry3A gene was found to be expressed during vegetative growth at a lesser extent than during the stationary phase (5-7). The activation of cry3A expression was independent of the key regulators or sigma factors involved in the initiation of sporulation. The promoter that directs the transcription of the anticoleopteran cry3A gene resembles σA vegetative stage promoters (8,9). The cry3Aa promoter is approximately a 635-bp DNA sequence, required for the full expression of the gene. It was shown that the cry3Aa promoter enhances the expression of other cry and vip genes and increases the activity of insecticidal strains (10-13).

The present study reports high amounts of toxin production at an earlier stage of incubation by multicopy cry3Aa11 introduced into B. thuringiensis Mm2 strains.

Materials and methods

Bacterial strain, plasmids, and media

The list of the strains and plasmids, and their sources, are given in Table 1. They were grown in Difco sporulation medium (DSM) (14).

Gene cloning and transformation

Amplification of cry3Aa11 from the genomic DNA of B. thuringiensis Mm2 was performed using forward (5'-gagctctgaaacgtagatgaacacctt-3') and reverse (5'-cggtaccttaattcactgga-3') primers. The primer design was made to include the 635-bp promoter region for further expression of the gene. The PCR product was then ligated to the pGEM-T vector (Promega). The recombinant molecules were amplified in E. coli DH5α and the insert was cloned to the SacI and KpnI restriction enzyme sites of pHT315 (Figure 1) (15). The recombinant pHT315 molecules isolated from E. coli DH5α were introduced into B. thuringiensis Mm2 via electroporation, as previously described (16). B. thuringiensis Mm2 cells were grown in 100 mL of brain heart infusion (BHI; Fluka) broth at 37 °C to an OD600 of 1.6. The cells were washed with 10 mL of cold dH2O and resuspended in 1 mL of cold sterile PEG 6000 (40% w/v) (Merck). The competent cells were stored at -80 °C. Added to 300 μL of competent B. thuringiensis Mm2 was 1 μg of pHT315AKC3, and the DNA-cell mixture was transferred to an ice-cold, 0.2-cm electroporation cuvette in a Bio-Rad MicroPulser™ electroporation apparatus. A pulse (2.5 kV for 2.5 ms) was applied only once. After electroporation, the cells were diluted 10 times with BHI broth and incubated at 37 °C on a rotary shaker at 100 rpm for 1 h. The cells were then plated on BHI agar containing erythromycin (25 μg/mL) and incubated at 30 °C for 16 h.

Determination of cultural growth

Cultural growth was monitored by absorbances at 600 nm and dry cell weight (DCW) determination. For this, 5 mL of culture was passed through a cellulose nitrate membrane filter (0.2 μm pore size, Whatman) under vacuum. Filters were dried at 80 °C for 24 h and weighed. DCW values tabulated herein represent the mean of 3 independent measurements.

SDS-PAGE

A slightly modified version of the procedure of Armelle (17) was used for protein extraction. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was performed and protein concentration was measured by the method of Bradford (18), with bovine serum albumin (BSA) as the standard. Into each well, 5 μg of total protein was loaded. Proteins were stained by Coomassie Brilliant Blue. PageRuler™ Unstained Protein Ladder (Fermentas) was used as the size marker.
Western blot analysis

Cry3Aa11 from *B. thuringiensis* Mm2 was partially purified using the procedure of Kaelin et al. (19). Western blot analysis was then carried out as described by Okay et al. (20). Mixed with aluminum hydroxy gel (VBR Co., Ankara) was 0.5 mL of Cry3Aa11 protein. The pH of the mixture was adjusted to 6.5 and it was incubated at 4 °C for 1 h by shaking once every 10 min. At 2-week intervals, 3 BALB/c mice received 2 subcutaneous injections of 0.5 mL per animal. Their sera were collected and pooled 14 days after the second injection and stored at –20 °C. Protein mixtures of parental and recombinant *B. thuringiensis* Mm2 were extracted from their cultures, harvested at different incubation times, and run on SDS-polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane by the modified method of Towbin et al. (21) using a semidry blotting system (Cleaver Scientific Ltd, UK). Western blot analysis was carried out using the primary antibodies raised against Cry3Aa11 and rabbit antimouse IgG conjugated to alkaline phosphatase (Sigma) as the secondary antibody. An AP Conjugate Substrate Kit (Bio-Rad, USA) was used for color detection. PageRuler™ Prestained Protein Ladder Plus (Fermentas) was used as the size marker.

Protein quantification

Gels were photographed using the Vilber Lourmat Gel imaging system. DECODON Delta 2D
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Version 3.3 (Germany) software was used for gel-based relative quantitation of protein bands. In this method, a bovine serum albumin (BSA; 66.2 kDa) band from the protein size marker was chosen as the standard. As the concentration of BSA (200 μg/mL) in the protein ladder was known, the amount of Cry3Aa11 protein in the SDS-PAGE gels was calculated accordingly, by comparing the density and size of the bands with that of 66.2-kDa BSA protein.

Results and discussion

To date, different cry genes encoding insecticidal crystal proteins have been cloned and expressed in various hosts such as E. coli, B. subtilis, B. megaterium, Pseudomonas cerea, P. cepacia, P. fluorescens, Cyanobacteria, and Rhizobium (22,23). Theoduloz et al. (24) cloned and expressed the cry1Aa gene in B. subtilis and B. licheniformis and obtained an expression potency comparable to the source organism. An increase in the gene copy number makes it possible to increase Cry toxin yield (25). B. thuringiensis Mm2, the source organism of the cry3Aa11 gene, was selected as the ultimate host for the introduction and multicopy expression of the cry3Aa11 gene.

Amplified via PCR from the genomic DNA of B. thuringiensis Mm2 was 2.5 kb of the cry3Aa11 gene with its own promoter. After cloning into pGEM-T, the cry3Aa11 gene was next cloned into the shuttle vector pHT315. The resulting recombinant plasmid, pHTAKC3, was first introduced to E. coli DH5α. pHTAKC3 molecules isolated from recombinant E. coli DH5α cells were introduced into B. thuringiensis Mm2 via electroporation. Recombinants were selected on BHI agar plates containing erythromycin. Plasmids were isolated from putative recombinants and verification of recombination was performed by double digestion and PCR (Figure 2). The recombinant strain was designated as B. thuringiensis Mm2AKC3.

Next, the growth of parental and recombinant B. thuringiensis strains was monitored both by performed DCW and OD measurements at 600 nm (Figure 3).

The Cry3Aa11 production capacities of parental B. thuringiensis Mm2 and recombinant B. thuringiensis Mm2AKC3 strains were also compared. They were grown in DSM for 60 h. Samples from cultures harvested at different time intervals were used for protein extraction to perform SDS-PAGE. In our previous study, it was shown that increasing

Figure 2. Verification of cloning of the cry3Aa11 gene into pHT315 by a) double digestion with SacI and KpnI restriction enzymes [lane 1: digestion products, lane 2: marker (Lambda DNA/PstI)] and b) PCR amplification [lane 1: negative control (no template), lane 2: 2589 bp PCR product of cry3Aa11].
of a 73-kDa protoxin. After 31 h of incubation, at T_{31}, it was replaced by the 65-kDa toxin band (Figure 4). Kurt et al. (26) also reported that the 73-kDa protoxin predominated in the culture instead of the 65-kDa toxin for 24-48 h of incubation, indicating that proteolysis was not completed until then in DSM. Carroll et al. (27) found predominance of protoxin in nutrient rich broths and explained this as a manifestation of lower production of bacterial proteases in such conditions.

As shown in Table 2, B. thuringiensis Mm2AKC3 produced more than 2-fold (from 0.3 μg to 0.8 μg) and 3-fold (from 0.8 μg to 2.77 μg) Cry3Aa11 toxins at T_{5} in the presence of 50 and 200 mM Pi, respectively.

However, the most striking increase in the toxin yield of B. thuringiensis Mm2AKC3 was apparent at T_{12}, when the Pi concentration was 50 mM in DSM (from 0.4 μg to 1.6 μg), as shown in Table 2. The difference in the production level of Cry3Aa11 between the strains, both in the presence of 50 mM and 200 mM Pi, gradually decreased after T_{12}. These results indicated that a higher Pi concentration did not have a drastic effect on toxin production by the recombinant B. thuringiensis Mm2AKC3, especially during the early hours of incubation. The growth of both strains in DSM containing 200 mM Pi was higher, especially after T_{12}. However, when the results of growth and toxin levels were compared, it was shown that better growth did not correspond to higher toxin production for parental and recombinant B. thuringiensis Mm2 strains.
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High-level expression of cry3Aa11 in the recombinant strain was also verified by the western blot analysis using anti-Cry3Aa11 polyclonal antibodies, in which a major signal was seen as a thicker band in the T_{12} cultures of B. thuringiensis Mm2AKC3 (Figure 5).

The recombinant B. thuringiensis Mm2AKC3 began to produce a higher amount of Cry3Aa11 delta-endotoxin at the logarithmic phase of fermentation, while the parental strain exerted its highest toxin production capacity during the stationary phase (Figures 3 and 4). A higher level of toxin production at an earlier time of incubation by recombinant Mm2AKC3, in comparison to the parental strain Mm2, was most probably due to the presence of multicy copy pHTAKC3 carrying the cry3Aa11 gene in the recombinant strain.

Recombinant DNA technology facilitates the introduction and expression of cry genes for the construction of novel B. thuringiensis strains with desired insecticidal activities (25). The Cry3Aa11 production by recombinant B. thuringiensis at high levels from the cultures harvested during the early hours of fermentation seems to be promising for its use as an impressive bioinsecticide against the larvae of Coleoptera. Thus, the toxicity of Cry3Aa11 expressed by B. thuringiensis Mm2AKC3, having the cry3Aa11 gene at a high copy number, will be evaluated in further bioassay experiments.
Figure 5. Western blot of analysis of total cellular proteins from parental *B. thuringiensis* Mm2 (lanes 1, 3, 5, and 9) and recombinant *B. thuringiensis* Mm2AKC3 (lanes 2, 4, 6, 7, and 8); M: protein molecular weight marker.

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