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Cloning and expression of *cry2Aa* from native *Bacillus thuringiensis* strain SY49-1 and its insecticidal activity against *Culex pipiens* (Diptera: Culicidae)

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ABSTRACT

Bacillus thuringiensis (Berliner) (*Bt*) is well known for having toxicity against pest insects because of their ability to form endospores and broad-range activity of their parasporal inclusions. In this study, a new member of *cry2A* gene from previously characterized native *B. thuringiensis* SY49-1 strain was cloned, expressed and used for its activity against *Culex pipiens* (Diptera: Culicidae) larvae. The sequence analysis of the cloned *cry2A* gene revealed that it encodes a polypeptide of 633 aa residues with 99% identity to Cry2Aa protein with expected molecular weight of 70.7 kDa. *Bacillus thuringiensis* delta-endotoxin nomenclature committee designed our sequence as Cry2Aa18 being a new member of *Bt* toxins. Bioassays against last instar larvae of *C. pipiens* indicated that Cry2Aa18 has considerable toxicity with LC_{50} of 630 μg ml^{-1}. In order to prevent the spread of infectious diseases mediated by *C. pipiens*, this newly characterized *cry2Aa18* gene could constitute as an important biological control tool for controlling mosquito larvae living in freshwater systems and can be used as a good alternative for minimizing the use of chemicals.

1. Introduction

Bacillus thuringiensis insecticidal crystal proteins are active against various insect orders and have been widely used for pest management programs worldwide. *B. thuringiensis* products are more desirable due to their target specificity compared to broad-spectrum chemical insecticides in terms of environmental and health concerns. Cry2 proteins, classified based on amino acid similarity, are an unusual subset with small size and unique mode of action possessing broad specificity and exhibiting toxicity against lepidopteran and dipteran species [1,2]. It is important to characterize new toxins from bacterial strains with broad spectrum of activity to overcome insecticide resistance for a sustainable agriculture and habitable environment. Insect resistance to Cry toxins [3] is attributed to decreasing affinity for receptor binding on midgut epithelial cell membrane after proteolytic digestion [4]. Thus, investigating novel *Bt* strains harboring new *cry* genes should be taken into consideration for preventing resistance development in pests [5–7]. Although remarkable sequence similarity exists among *cry* genes, individual proteins exhibit specific toxicity spectrum against various groups of insects [8]. Thus, evaluating the effectiveness of these proteins through cloning studies should be considered for developing a new product. As stated by many researchers Cry2Aa exhibit various toxicity levels on different insect pests depending on its concentration, conformation and solubility [9–12]. In the present study *cry2Aa18* gene from native *Bt* strain SY49-1, characterized by its insecticidal activity against *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), *Plodia interpunctella*

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(Hübner) (Lepidoptera: Pyralidae), *Thaumetopoea wilkinsoni* Tams (Lepidoptera: Thaumotepoeidae), and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) larvae [13,14], was cloned and expressed in *Escherichia coli* to determine its individual activity on medically important disease vector *C. pipiens* larvae in laboratory conditions.

2. Materials and methods

2.1. Strains and plasmid vectors

As experimental materials *Bacillus thuringiensis* SY49-1, *Escherichia coli* DH5α (supplied by molecular biology laboratory of METU, Turkey), pGEMT-Easy and pET28a (+) expression vector system (Promega corporation, Madison, USA) and *Escherichia coli* BL21 (DE3) were used. *Bt* and *E. coli* were respectively cultured at 30 and 37 °C in LB medium (10 g L^{-1} Triptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl).

2.2. PCR analysis

DNA extraction of *Bt* SY49-1 was performed using the method of Bravo et al. [15] and used as template for PCR analysis. Primer pairs F-5′-**GAA TTC** GGA TCC ATA TGA ATA GTG TAT TGA AT-3′ and R-5′ **GTC GAC** GTA CGG ATC CTA CTC AAA CCT TAA TAA-3′ were used for amplifying coding region of *cry2Aa* gene [16]. Recognition sequences of *Eco*RI for forward and *Sal*I for reverse primers were adapted at their 5′ ends. Reagents for PCR reactions included 2.3 mM $MgCl_2$, 1× Taq buffer, 0.2 mM dNTP mix, 0.3 pmol primers of each, 0.5 U MaxTaq DNA polymerase (Vivantis, PL2201), and 30 ng template DNA. The PCR program was adjusted as initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 2 min, 50 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 10 min [16] and 3′ end of PCR products were adenilated after amplification for T/A cloning [17].

2.3. Cloning and full length sequencing of cry2Aa

Amplified product of approximately 1902 bp was purified and ligated into pGEM-T Easy vector system to obtain pGEM-*cry2Aa* according to the protocol of manufacturer (Promega, vector system I). The construct pGEM-*cry2Aa*T/A was transformed into *E. coli* DH5α rendered competent with $CaCl_2$. Positive clones were selected by blue white screening. Existence of the cloned *cry2Aa* was verified in positive clones through PCR analysis and restriction digestion with *Sal*I and *Eco*RI. *cry2Aa* gene was sequenced with Nextera XT DNA Sample Preparation Kit (Illumina1, San Diego, CA) using Illumina MiSeq in Genome and Stem Cell Center at Erciyes University. MiSeqTM platform (Illumina) together with the kit enabled a practical protocol for sequencing. Genius 6.1 software was used for data analysis. The full length sequence of *cry2Aa* gene was submitted to the *B. thuringiensis* delta-endotoxin nomenclature committee which assigned name to the isolated sequence from *B. thuringiensis* strain SY49-1 [18]. Sequence was submitted in GenBank and accession number was obtained.

2.4. Expression of cry2Aa gene and SDS-PAGE analysis

pET-*cry2Aa* and pET28a(+) vector system were digested with *Eco*RI and *Sal*I. *Eco*RI and *Sal*I treated *cry2Aa* PCR amplicon and pET28a (+) vector were ligated according to the protocol of manufacturer. The pET-*cry2Aa* construct was transferred into *E. coli* BL21 (DE3) cells rendered competent with $CaCl_2$. The positive clones, determined with colony PCR, were incubated in LB (100 ml) medium containing 30 μg ml^{-1} kanamycin at 37 °C until obtaining $OD_{600} = 0.5–1$. Half of the culture was incubated for 4 h by inducing with 1 mM IPTG.

For protein extraction, induced and non-induced cultures were centrifuged separately at 3242 × g for 5 min at 4 °C, and pellets were homogenized in 12 ml of Tris-HCl (20 mM, pH 7.5) and recentrifuged as described above. The samples were incubated for 15 min at 30 °C after solubilizing in 5 ml of Na_2CO_3 (50 mM, pH 10.2) containing 50 μl of lysozyme (10 mg ml^{-1}) and β-mercaptoethanol (1%). Following sonication for a minute to release proteins, samples were centrifuged at 9079 × g for 10 min at 4 °C [19,27]. After resuspending the pellet in 2 ml of Tris-HCl (20 mM, pH 7.5), 30 μl of sample was electrophoresed on SDS-PAGE gel [20] using 10% running and 4% stacking gel. Molecular weights of proteins were identified by comparing with Fermantas SM0661 marker. The proteins of *E. coli* BL21 (DE3) and *Bt* SY49-1 were obtained with the same procedure described as above.

2.5. Cry2Aa protein quantification

The SDS-PAGE image of approximately 70 kDa Cry2Aa band was used for determining the quantities. Cry protein concentrations were calculated by the following formula: Cry protein concentration (μg ml^{-1})=(μg ml^{-1} total protein) × (% proportion of Cry protein to total protein) [21]. The proportion of Cry2Aa to total protein was determined using Biorad Chemi Doc MP Imaging System Image Lab version 5.1 (Biorad Laboratories, Hemel Hempstead, UK). Total protein quantitation was determined according to the method of Bradford [22].

2.6. Bioassay

C. pipiens culture (kindly supplied by Dr. Mehmet Fatih ŞİMŞEK, Aydın, Turkey) was maintained in containers with tap water in rearing chamber (SANYO Electric Co., Ltd, Osaka, Japan) (27 ± 1 °C, 65± 5% relative humidity and 14L: 10D h photoperiod) and fed with flake fish food up to pupation on a daily basis. Pupated individuals were then transferred to veil cages for adult emergence. Emerging adults, fed with 10% sugar solution, were allowed to release their eggs into small containers with 10 cm diameter. The eggs were transferred to larval rearing containers (40 × 25 × 10 cm) with fresh water. After larval emergence, last instar larvae were used for bioassay trials.

The activity of lyophilized spore/δ-endotoxin mixture and Cry2Aa18 from *Bt* SY49-1, and plasmid-free *E. coli* BL21 (DE3) proteins were tested on 10 last instar larvae of *C. pipiens* at 50, 100, 250, and 500 μg ml^{-1} doses as three replicates. Lyophilized proteins with determined concentrations were separately solubilized in glass jars (300 ml) containing 25 ml of sterile tap water and the larvae were gently transferred to the containers. For the control, only sterile tap water was used.

Results were recorded for ten days by removing dead larvae. Data were subjected to analysis of variance (ANOVA) and means were separated at the 5% significance level by using the Tukey HSD posthoc test. Total mortality was corrected using Abbott's formula. The LC_{50} values were estimated using probit analysis with 95% confidential limit [10,23].

3. Results

3.1. Amplification and cloning of cry2Aa gene

PCR amplification revealed the estimated length of 1.9 kb *cry2Aa* from total DNA of *Bt* SY49-1 strain. Full length of *cry2Aa* (1902 bp) was purified and inserted into pGEM-T Easy vector system according to the manufacturer's protocol. pGEM-*cry2Aa* was then