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Isolating, cloning, and sequencing of the *cry1C* gene encoding protein crystal toxic to Lepidoptera

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Abstract:

***Bacillus thuringiensis* (Bt) is a Gram-positive, spore-forming bacterium widely recognised for its ability to produce insecticidal crystal (Cry) proteins that are highly toxic to various insect orders. Among them, the *cry1C* gene encodes the Cry1C protein, which exhibits specific insecticidal activity against Lepidopteran larvae, a major group of agricultural pests responsible for significant crop losses worldwide. In this study, we aimed to isolate, clone, and sequence the *cry1C* gene from native Bt strains to support the development of environmentally friendly biological control agents. As concerns grow regarding the ecological and health impacts of synthetic chemical pesticides, Bt-based biopesticides have emerged as a sustainable alternative due to their host specificity and biodegradability. We utilised the polymerase chain reaction (PCR) technique with specific primers to identify Bt isolates harbouring the *cry1C* gene. A 288 bp fragment was successfully amplified from positive isolates and subsequently sequenced. Sequence analysis confirmed 100% homology with previously reported *cry1C* gene sequences in the GenBank database, verifying the presence and integrity of the target gene in selected Bt strains.**

Keywords: ***Bacillus thuringiensis*, *cry1C*, isolation, Lepidoptera, sequencing.**

Classification numbers: **3.4, 3.5, 5.3**

1. Introduction

Lepidoptera, the second largest order of insects with over 180,000 species [1], includes numerous pests that cause significant damage to crops such as cabbage, tea, and grains [2, 3]. Traditional chemical pesticides have long been the primary method of controlling these pests, but growing concerns over their environmental impact and resistance issues have led to increased interest in biological alternatives [4]. Among these, *Bacillus thuringiensis* (Bt) has proven effective, as it produces Cry proteins that are highly toxic to Lepidoptera species [5, 6]. Bt carries cry genes that determine the ability to produce different types of toxic crystals to destroy many types of insect pests, such as scaled insects, bivalves, and hardbirds [7]. Among the various Cry proteins produced by Bt, *cry1C*, encoded by the *cry1C* gene, is known for its specific activity against Lepidoptera species, which are notorious for causing extensive agricultural damage. This protein's unique bipyramidal and cubic crystal structure offers a targeted approach to pest control, making it an ideal candidate for developing environmentally friendly insecticides or genetically modified pest-resistant crops [8-10]. In this study, we aim to isolate and characterise *cry1C* gene-containing strains of Bt var. *aizawa* with the ultimate goal of utilising these strains to develop more effective, biologically-based pest control methods targeting Lepidoptera species.

2. Materials and methods

2.1. Materials

E. coli and strains of *Bacillus thuringiensis* (Bt) bacteria were obtained from the Institute of Biotechnology. The primer pairs used to amplify the *cry1C* gene follow the sequence of S. Asano (1996) [10].

The sequences of the primer pairs used to amplify the *cry1C* gene are:

TYIC 5'-CAACCTCTATTTGGTGCAGGTTC-3'.

TYIUNI 5'-TCACTGAGTCGCTTCGCATGTTTGACTTTCTC-3'.

The standard immune serum kit was provided by the Department of Molecular Microbiology, Institute of Biotechnology. The diamondback moths (*Plutella xylostella*) were obtained from the Plant Protection Research Institute.

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2.2. Methods

2.2.1. Cry1C gene isolation method

Isolation of *Bacillus thuringiensis* bacteria from soil samples was conducted using the improved Ohba and Aizawa method [11]. Soil samples were collected from agricultural fields and processed by suspending them in sterile distilled water. The suspension was heat-treated at 80°C for 10 minutes to select spore-forming bacteria, followed by serial dilution and plating on nutrient agar. Colonies exhibiting characteristic Bt morphology were selected for further analysis. From the Bt strains obtained, classification was performed using a serological method [12, 13]. To evaluate the ability of the study strains to kill scaled-winged insects, a test was conducted on silkworms using the method of I. Thiery, et al. (1997) [14].

2.2.2. Cry1C gene amplification polymerase chain reaction method

The primer pair used for PCR amplification was designed based on the published *cry1C* sequence available in GenBank (Accession No. X96682.1) [15, 16]. The primer sequences were selected to amplify a 288 bp fragment specific to *cry1C*. PCR conditions were optimised with an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes.

2.2.3. Gene line separation method

The PCR product is directly attached to the pGEM-T Easy vector using the T4-DNA ligase enzyme at 4°C to create recombinant vectors [4] and transformed into bacterial cells *E. coli* and cultured on LB medium containing antibiotics Amp (100 µg/ml) and X-gal (80 µg/ml) at 37°C.

3. Results and discussion

3.1. Isolation and separation of gene lines cry1C

3.1.1. Crystal shape of Bacillus thuringiensis strains

Bacillus thuringiensis (Bt) can produce crystal forms: spherical, bipyramidal, cubic, and indeterminate shapes. Different strains can produce crystals of varying shapes. By preparing samples with fuchsin stain from Bt characteristic colonies and observing them directly under an optical microscope, we could classify different crystal forms of 30 Bt strains. The results are shown in Fig. 1. The isolated Bt strains are crystallising, with bipyramidal crystalline strains being predominant (50%). Additionally, some Bt strains produce two types of crystals (33.6%) and three types of crystals (3.3%). Therefore, Bt has a wide spectrum of insecticidal activity against scaly insects, hard-winged insects, bivalves, and plant nematodes.

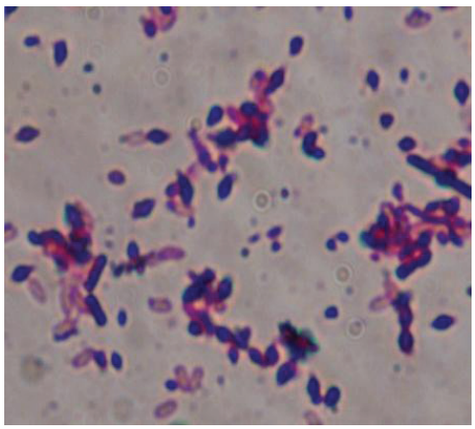

Fig. 1. The spore and crystal shape of strain 18.2 magnified 100 times under optical microscopy.

3.1.2. Classification of Bacillus thuringiensis by seroreaction

Subspecies classification was conducted using the serological classification method of Barjac and Bonnefoi for the study strains. Bt bacteria are divided into many different subspecies, each with unique characteristics. The species Bt subsp. *aizawa* has the ability to kill scale insects of the H7 serotype. To identify strains carrying the *cry1C* gene, we screened the isolated Bt strains using the H7 serotype for classification (Fig. 2). The results showed that all 30 studied strains were confirmed to belong to the *aizawa* species through agglutination reactions with the H7 serotype. Thus, it can be concluded that all 30 strains obtained for the study belong to the Bt subspecies *aizawa*.

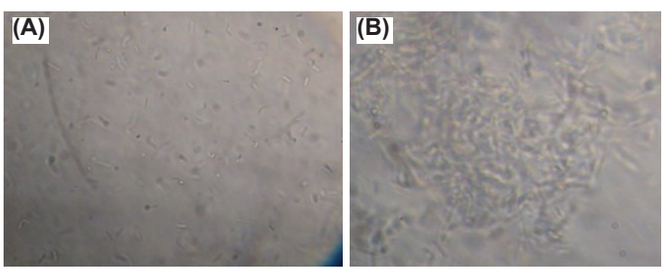

Fig. 2. The agglutination image of strain 18.2 isolated with the serotype under optical microscopy: (A) Before serum instillation; **(B)** After serum instillation.

3.1.3. Testing the activity of Bacillus thuringiensis subsp. aizawa strains against silkworms

Bacillus thuringiensis strains were diluted to 10.5 and 10.7 spores/ml concentrations with sterile distilled water. Testing was conducted on silkworms according to Abbott's formula. The mortality rate was monitored for 3 days. The test results (Fig. 3) show that the insecticidal activity of the isolated Bt strains is relatively high. After 3 days of testing, most of the Bt subsp. *Aizawa* strains exhibited

a worm mortality rate greater than 50%. Notably, strains AV11.8, 18.2, HT4.2.1, TC1.3, 51.1, 62.1, 25.1, and 34.1 demonstrated the highest insecticidal activity. Some strains showed high insecticidal activity after just 1 day of testing (582.7, 62.1, 25.1, 34.1), indicating that certain Bt strains can kill insects in a short time.

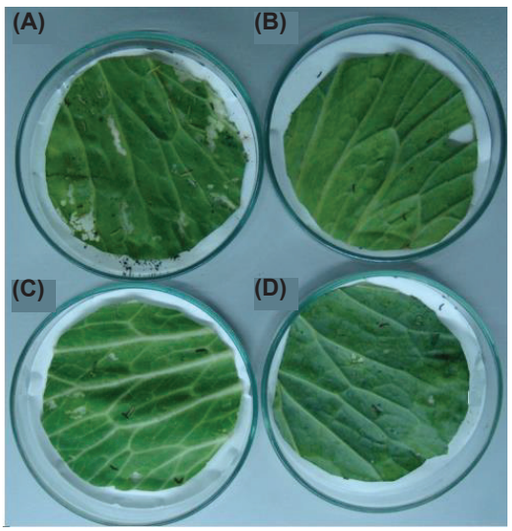

Fig. 3. Experimental image of silkworm killing activity of *Bacillus thuringiensis* strains studied: (A) Negative control; **(B)** 51.1; **(C)** Positive control 4J4; **(D)** 18.2.

3.1.4. Detection of cry1C gene from Bacillus thuringiensis strains

According to theoretical calculations, the *cry1C* gene fragment, after being synthesised with a specific pair of primers, should yield a product of 288 bp. The results indicate that the PCR product (Fig. 4) is a single band, nearly 300 bp in size, as expected. Thus, preliminary observations suggest that strains belonging to the subspecies *Bacillus thuringiensis* subsp. *Aizawa* all carry the *cry1C* gene. To confirm whether these strains indeed carry the *cry1C*, from genetically carrying strains *cry1C* identified after electrophoresis product inspection PCR, we randomly selected 2 strains, 18.2 and 51.1, to separate the gene lines and sequence it.

3.2. Gene fragment separation cry1C

The transformed product was cultured on laked blood agar (LBA) containing the antibiotics ampicillin (100 µg/ml) and X-gal (80 µg/ml) at 37°C. After 14-16 hours, blue and white colonies appeared on the Petri dish (Fig. 5). The plasmid separation results were tested by electrophoresis on a 1% agarose gel (Fig. 6). The plasmid DNA from the white colonies was then selected for testing

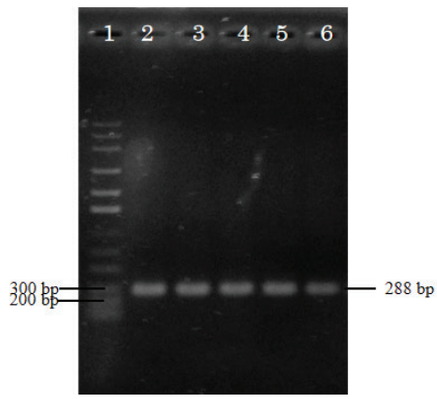

Fig. 4. Electrophoresis of polymerase chain reaction products on agarose gel 1%. Well 1 is the standard DNA bar and wells 2-6 are strains 582.7, 18.2, 51.1, 62.1, and 34.1, respectively.

with a restriction enzyme. Since the multiple cloning site (MCS) region of the vector contains the cleavage site for the EcoRI restriction enzyme, we performed plasmid DNA cleavage with EcoRI to check for the presence of the *cry1C* gene. The cleavage product was electrophoresed on a 1% agarose gel (Fig. 7). The results showed that the plasmid DNA from the white colonies, after being cut with EcoRI, produced two bands: the larger band was the pGEM-T Easy vector, approximately 3015 bp in size, and the smaller band was nearly 300 bp, corresponding to the PCR product of the *cry1C* gene. In contrast, the product from the blue colonies yielded a single band about 3000 bp in size, corresponding to the vector. Thus, it can be concluded that two lines have been successfully transformed with the pGEM-T Easy vector, indicating that the gene of interest, *cry1C*, has been inserted.

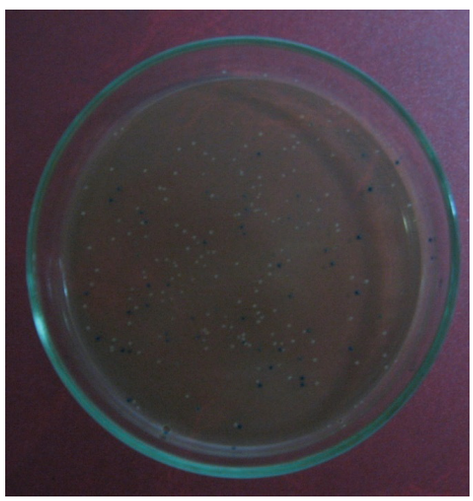

Fig. 5. Image of blue and white colonies on laked blood agar plates after 14 hours of culture.

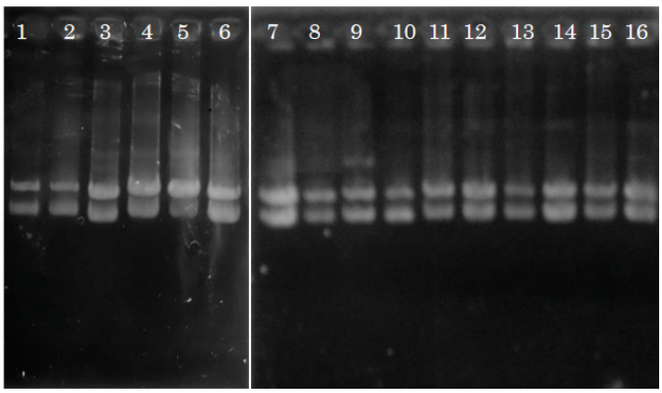

Fig. 6. Results of plasmid DNA extraction of blue and white colonies. Wells 1, 2, and 4-16 are the plasmid DNA of white colonies. Well 3 is the plasmid DNA of blue colonies.

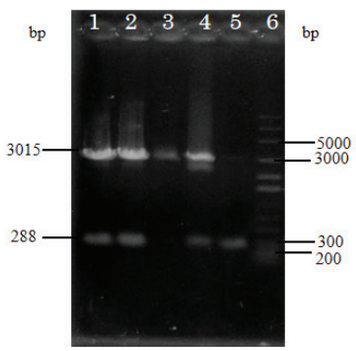

Fig. 7. Electrophoresis of pGEM-T Easy plasmid DNA cutting product - *cry1C* - 18.2 extracted from blue and white colony lines on 1% agarose. Wells 1, 2, and 4 are white colony line plasmid cutting product. Well 3 is blue colony line plasmid cutting product. Well 5 is PCR products from strain 18.2. Well 6 is the standard DNA ladder.

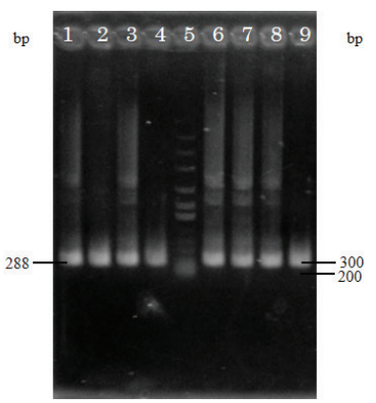

Fig. 8. Polymerase chain reaction results of pGEM-T Easy - *cry1C* - 18.2, pGEM-T Easy - *cry1C* - 51.1 white colonies. Wells 1-3 are DNA plasmid of pGEM-T Easy - *cry1C* - 18.2. Well 4 is PCR products from strain 18.2. Well 5 is the standard DNA scale. Wells 6-8 are the DNA plasmid pGEM-T Easy - *cry1C* - 51.1. Well 9 is the PCR products from strain 51.1.

To determine the presence of the *cry1C* gene in white colonies, we conducted PCR on the separated plasmid DNA. The electrophoresis results shown in Fig. 8 indicate a single band corresponding to the size of the PCR product from strains 18.2 and 51.1. However, to accurately conclude whether the separated gene is the *cry1C* gene or not, we read the sequences of these 2 gene segments and compare them with the gene sequences registered in the GenBank using specialised software such as BioEdit, BLAST, and FastPCR™.

Our results confirm that *Bacillus thuringiensis* var. *aizawa* strains are effective in producing the Cry1C protein, which is known to target Lepidoptera pests. Previous studies by S. Asano, et al. (1996) [10] and [16] P.R. Queiroz, et al. (2023) [16] have similarly reported the insecticidal activity of *cry1C*-containing Bt strains. The high insecticidal activity observed in strains 18.2 and 51.1 suggests their potential use in biological pest control strategies. Additionally, the successful cloning and sequencing of the *cry1C* gene provides a foundation for future genetic engineering efforts aimed at developing Bt-based transgenic crops.

3.3. Sequencing the cry1C gene fragment

The *cry1C* gene sequences of 2 strains, 18.2 and 51.1, were compared with 2 sequences coded AF362020.1 and X96682.1 on the International GenBank (Fig. 9). The 287 bp gene fragment of strains 18.2 and 51.1 shows 100% similarity to the *cry1C* gene sequences of *Bacillus thuringiensis aizawa* 7.29 (X96682.1) and Bt strains C002 (AF362020.1) published in the International GenBank. Of

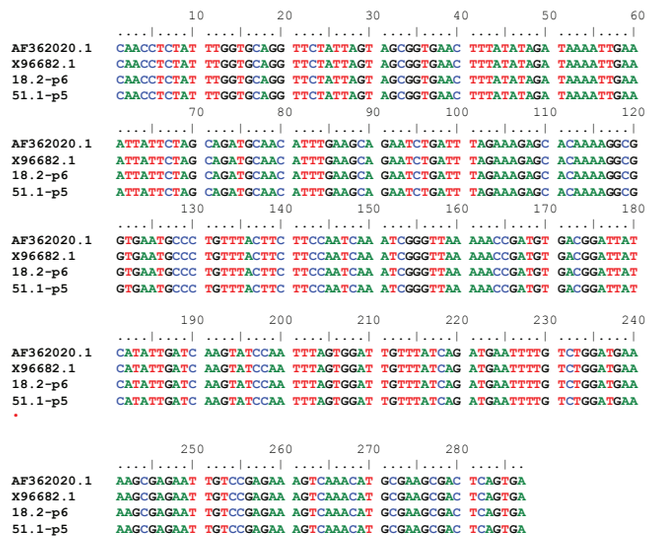

Fig. 9. Compare the *cry1C* gene sequences of strains 18.2 and 51.1 with the two sequences AF362020.1 and X96682.1 from the international GenBank.

these sequences, the AF362020.1 sequence corresponds to the *cry1Ca* gene encoding the cry1Ca protein. Thus, it can be concluded that the *cry1C* gene sequence of the two separated strains belongs to the cry1Ca subgroup.

4. Conclusions

In this study, we successfully isolated and identified *Bacillus thuringiensis* var. *aizawa* strains containing the *cry1C* gene, which encodes a protein crystal toxic to Lepidoptera pests. PCR amplification and sequencing confirmed that the *cry1C* gene fragment was 288 bp in size and showed 100% homology with known sequences from GenBank. Strains 18.2 and 51.1 demonstrated particularly high insecticidal activity against *Plutella xylostella*, indicating their potential for biological pest control.

CRediT author statement

Van Tien Tran: Conceptualisation, Methodology, Investigation, Data curation, Writing original draft; Thi Hue Nguyen: Formal analysis, Validation, Writing, Reviewing, Editing, Supervision.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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