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Crystal Morphology and Plasmids of Local *Bacillus thuringiensis* SY56.3 Isolate

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ABSTRACT

Bacillus thuringiensis (*Bt*) is an aerobic, Gram (+) and spore-forming entomopathogenic bacterium belonging to *Bacillus cereus* group together with *Bacillus anthracis. Bt* is the most widely and effectively used biological control agent against insect pests throughout the world. Following morphological and molecular characterization of the *Bt* SY56.3 isolate, scanning electron microscopy (SEM) analysis was performed to determine the morphology of spore and parasporal crystal proteins (Cry) in this study. A small amount of lyophilized spore-crystalline mixture of *Bt* SY56.3 was put on a microscope slide containing a drop of distilled water and homogenized thoroughly. Then, it was dried at room temperature while quickly passing the slide 20 times over burner flame. The slide was then fixed onto the electron microscope holder with the aid of SC7620 Mini-Sputter Coater device. The slide was made conductive by coating with Gold-Palladium (Au/Pd) for 30 seconds. The samples containing the Cry proteins in the range of 25-130 kDa were then placed in scanning electron microscope (LEO 440) and visualized at 20 kV. The size of crystal proteins ranged between 0.3-1 µm. It was observed that *Bt* SY56.3 isolate had cubic, bipyramidal, circular and irregular shaped Cry proteins. Present findings revealed that *Bt* SY56.3 could be used for biotechnological control applications because of different functions of these different Cry proteins.

Keywords: *Bacillus thuringiensis*, *Bt*, Cry protein, Bioinsecticide

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ÖZET

Bacillus thuringiensis (*Bt*), *Bacillus cereus* grubuna ait, aerobik, Gram (+) ve spor oluşturan bir entomopatojenik bakteridir. *Bt*, dünya çapında böcek zararlılarına karşı en yaygın ve etkili kullanılan biyolojik kontrol ajanıdır. Bu çalışmada, *Bt* SY56.3 izolatının morfolojik ve moleküler karakterizasyonunun ardından, spor ve parasporal kristal proteinlerinin (Cry) morfolojisini belirlemek için taramalı elektron mikroskobu (SEM) analizi yapılmıştır. Az miktarda liyofilize edilmiş *Bt* SY56.3 spor-kristal karışımı, bir damla saf su içeren bir mikroskop lamına konulmuş ve iyice homojenize edilmiştir. Daha sonra lam, oda sıcaklığında 20 kez hızlıca bek alevi üzerinden geçirilerek kurutulmuştur. Ardından, lam, SC7620 Mini-Sputter Coater cihazı yardımıyla elektron mikroskobu tutucusuna sabitlenmiştir. Lam, 30 saniye boyunca Altın Poladyum (Au/Pd) ile kaplanarak iletken hale getirilmiştir. 25-130 kDa aralığında Cry proteinleri içeren örnekler daha sonra taramalı elektron mikroskobuna (LEO 440) yerleştirilmiş ve 20 kV'de görüntülenmiştir. Kristal proteinlerin boyutu 0,3-1 µm arasında gözlenmiştir. *Bt* SY56.3 izolatının kübik, bipiramidal, dairesel ve düzensiz şekilli Cry proteinlerine sahip olduğu gözlemlenmiştir. Mevcut bulgular, bu farklı Cry proteinlerinin farklı işlevleri nedeniyle *Bt* SY56.3'ün biyoteknolojik kontrol uygulamalarında kullanılabileceğini ortaya koymaktadır.

Anahtar Kelimeler: *Bacillus thuringiensis*, *Bt*, Cry protein, Biyoinsektisit

1. Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive, rod-shaped bacterium that is commonly found in soil, water, and plant surfaces in nature. It is either aerobic or facultative anaerobic [1,2]. Taxonomically a member of the *Bacillus cereus* sensu lato group, the most distinctive feature that sets *Bt* apart from other closely related species is its ability to produce parasporal crystal inclusions intracellularly during the sporulation phase [3,4]. These protein crystals are primarily responsible for the bacterium's entomopathogenic effects on insects, nematodes, and some other invertebrates [3,5]. The vegetative insecticidal proteins (Vip) proteins secreted by the bacterium during the vegetative phase throughout its biological cycle, and the Cry and Cyt toxins accumulated during sporulation, form the basis of biopesticides and transgenic plants in modern agriculture [6-8].

Cry proteins, also known as β-endotoxins, are the largest group of insecticidal proteins produced by *Bt* strains [9]. To date, over 1,600 Cry protein sequences have been identified based on their amino acid sequences, and these have been categorized under approximately 80 different holotype classes [10,11]. These proteins exhibit extremely high specific toxicity against specific insect orders such as Lepidoptera (butterflies and moths), Coleoptera (beetles), and Diptera (flies and mosquitoes), while being considered safe for humans, other vertebrates, and beneficial insects [3,12]. Cry proteins are generally synthesized as inactive protoxins, typically weighing approximately 65 kDa (short form) or 130-140 kDa (long form) [12,13].

The structural architecture of Cry proteins is directly related to their biological functionality. Active toxins generally consist of three main structural domains: Domain I, which is responsible for pore formation and consists of seven helices, Domain II, which manages receptor recognition and binding, and Domain III, which contributes to toxin stability through receptor interaction [13-15]. Long form protoxins (130-140 kDa), in addition to this three-domain core structure, contain a C-terminal prodomain (DIV-DVII domains) of approximately 500 amino acids that enable *in-vivo* crystallization and crystal stability [12,16]. The durability and stability of the crystal structure against environmental conditions is maintained thanks to the cross-linked networks provided by intermolecular disulfide bonds and cysteine residues between proteins. For example, while these bonds play a critical role in crystal packing in cysteine-rich toxins such as Cry1Aa and Cry1Ca18, it has been observed that non-covalent interactions are more dominant in the stability of low-cysteine toxins such as Cry8Ba2. In addition, the

presence of metal ions such as Zn, Ca and Mg in the crystal lattice not only supports crystal packing but also affects the activity and pH-dependent solubility of the toxin [16, 17].

The mechanism of action of Cry proteins involves a multi-step process that begins with the insect ingesting the crystals. The crystals dissolved in the alkaline pH environment in the midgut of the insect are broken down by intestinal proteases (such as trypsin or chymotrypsin) and converted into active toxin nuclei. Activated toxins bind with high affinity to specific receptors such as cadherin, aminopeptidase-N (APN), alkaline phosphatase (ALP) and ABC transporters on intestinal epithelial cells [18,19]. This binding process triggers the toxins to oligomerize and settle in the cell membrane, forming pores; the pores formed disrupt the osmotic balance of the cell (colloid-osmotic lysis), causing cell disintegration and ultimately death of the insect due to septicemia [20-22].

Today, *cry* genes encoding Cry proteins have been transferred to strategic crops such as corn and cotton, enabling the development of transgenic *Bt* plants that are resistant to pests. *Bt* crops increase agricultural productivity and protect environmental health by reducing the use of chemical insecticides. However, the evolving field resistance to these toxins in some insect populations constitutes a dynamic field of research that necessitates the continued discovery of new toxins with a broader spectrum of action and different binding mechanisms.

This study was planned to contribute to solving the global insecticide problem by determining the plasmid profile and Cry morphology of the local Bt SY56.3 isolate and making recommendations for its agricultural use.

2. Material and Method

2.1. Scanning Electron Microscopy

Bacillus thuringiensis SY56.3 isolate was incubated on T3 sporulation medium (pH 6.8±2, 3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.005 g manganese chloride ($MnCl_2$), 6 g monobasic sodium phosphate (NaH_2PO_4), 7.1 g dibasic sodium phosphate (Na_2HPO_4) in 1 liter of distilled water was prepared and autoclaved) at 200 rpm and 30 °C for 4-7 days. Then the spore crystal mixture was obtained after centrifugation at 15,000 x g for 10 min at 4 °C and then the pellet was lyophilized for long term storage. A small amount of lyophilized spore-crystalline mixture of *Bt* SY56.3 was put on a microscope slide containing a drop of distilled water and homogenized thoroughly. It was dried at room temperature while quickly passing the slide 20 times over burner flame. The slide was then fixed onto the electron microscope holder with the aid of SC7620 Mini-Sputter Coater device. The slide was made conductive by coating with Gold Palladium (Au/Pd) for 30 seconds [23]. The samples containing the Cry proteins in the range of 25-130 kDa were then placed in scanning electron microscope (LEO 440) and visualized at 20 kV.

2.2. Plasmid DNA isolation

Plasmid DNA isolation was performed using the alkaline lysis method [24,25]. The bacteria were incubated in 5 ml LB medium at 200 rpm at 30 ºC overnight and centrifuged at 10,000 x g for 2 minutes in 2 ml centrifuge tubes. The resulting pellet was redissolved in 100 µl of TE (40 mM Tris-HCl, 2 mM EDTA, pH 7.9) buffer, and then 200 µl of lysis solution [3% (w/v) SDS, 15% (w/v) sucrose, 50 mM Tris-hydroxide, pH 12.5] was added. After the resulting mixture was incubated at 60 ºC for 30 minutes, 2 µl Proteinase-K (20 mg/ml) was added to each tube. Then, the resulting mixture was shaken slightly and incubated at 41 ºC for 90 minutes. Then, 1 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added into the mixture and mixed by inverting 15-20 times. The obtained samples were centrifuged at 6,000 x g for 7 minutes and the supernatant was transferred to a new tube. Then, 500 µl of chloroform: isoamyl alcohol (24:1) mixture was added and centrifuged again at 6.000xg for 7 minutes. The supernatant containing plasmid DNA was taken and stored at -20 ºC. 40 µl of each sample was taken,

mixed with 6 µl of gel loading dye, and run on a 0.5% agarose gel prepared in 1X TBE buffer. During the preparation of the gel, 16 µl ethidium bromide was added to 400 ml TBE buffer. The resulting plasmids were visualized by electrophoresis. Plasmid DNA samples were visualized on a 0.5% agarose gel after running at 65 Volts at 5 ºC for 8 hours. Plasmid DNA sizes were determined by comparison with λ-pUC mix-Marker-4.

3. Results and Discussion

After incubation of the *Bt* SY56.3 isolate, plasmid isolation and Cry protein isolation were performed. Then, the plasmids were run on agarose gel (0.5%) and determined that the isolate carried plasmids of three different sizes with two of them larger than 19.3 kb and the other was around 4.5 kb (Figure 1). The genes encoding Cry proteins and many other metabolites utilized in different aspects of life of humans and animals, industry, agriculture, and foodstuffs are usually found on plasmids. Also, plasmids are quite usable to investigate all potential metabolites they code for and produce. Our findings are in compliance with literature, showing that *Bt* strains can have 1-17 plasmids in various sizes [26]. *Bt* SY56.3 isolate having these three plasmids (Figure 1) encode crystal proteins with cubic, spherical, irregular-spherical, and bipyramidal shaped inclusion bodies (Figure 2). Therefore, it stands out as an important *Bt* isolate that encodes three to four different Cry proteins ranging from 0.3 to 1 µm in size. It is known that *Bt* inclusion bodies exhibit insecticidal activity against orders such as Lepidopteran, Coleopteran and Dipteran, as well as other invertebrates [27]. The bipyramidal Cry proteins (~130 kDa) encoded by *Bt* strains exhibit toxicity against Lepidoptera, the cuboidal Cry proteins (~70 kDa) against Lepidoptera and Diptera, and the spherical Cry proteins (~65 kDa) against Lepidoptera, Coleoptera, and Diptera. This information shows that *Bt* SY56.3 isolate can efficiently be used in biopesticidal toxin formulations.

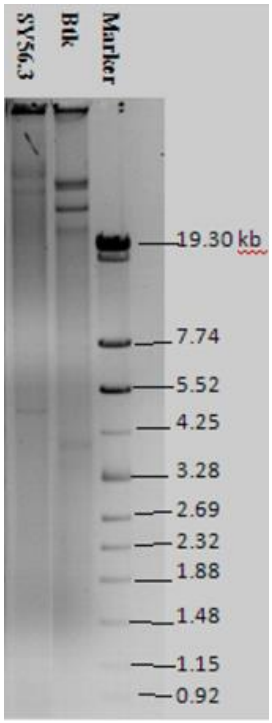


Figure 1. Plasmid profile of *Bt* SY56.3 [28]

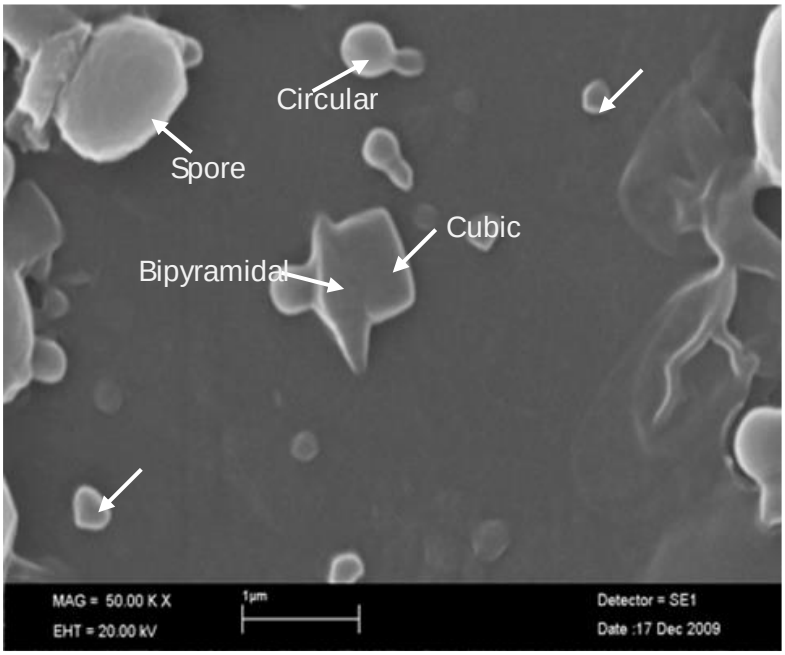


Figure 2. Scanning electron microscopy (SEM) view of *Bt* SY56.3 [28]

4. Conclusion

Bt SY56.3 plasmids encode crystal proteins having cubic, spherical, irregular-spherical, and bipyramidal shaped inclusion bodies. They are especially used in controlling; Pests through parasporal proteins such as Crys, Vips, Bins, Fungi through fungicides such as Zwittermycin, Surfactin, Iturin, Bacteria through antibiotics such as bacteriocins, ampicillin. In conclusion, the comprehensive investigation of *Bt* plasmids and the spectrum of their potential Cry proteins at the genomic and proteomic levels is of great importance for the development of next-generation bioinsecticide formulations, the optimization of transgenic plant technologies, and the creation of sustainable strategies in agricultural pest control. *Bacillus thuringiensis* SY56.3 can be used for bacterial originated biocontrol product formulations due to its unique plasmids and Cry proteins.

5. Declaration

5.1. Competing Interests

Authors declare that there is no conflict of interest in this study.

5.2. Authors' Contributions

Define the contribution of each researcher named in the paper to the paper.

Corresponding Author Semih YILMAZ: Developing ideas for the research and article, planning the materials and methods to reach the results, taking responsibility for the experiments, organizing and reporting the data, taking responsibility for the explanation and presentation of the results, taking responsibility for the literature review during the research, taking responsibility for the creation of the entire manuscript.

Abdurrahman AYVAZ: Developing ideas or hypotheses for the research, taking responsibility for the experiments, organizing and reporting the data, taking responsibility for the creation of the entire manuscript or the main part, reworking not only in terms of spelling and grammar but also intellectual content or other contributions.

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