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Biochemical and Multiplex PCR Analysis of Toxic Crystal Proteins to Determine Genes in *Bacillus thuringiensis* Mutants

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***Abstract*—**The Egyptian *Bacillus thuringiensis* isolate (M5) produce crystal proteins that is toxic against insects was irradiated with UV light to induce mutants. Upon testing 10 of the resulting mutants for their toxicity against cotton leafworm larvae, the three mutants 62, 64 and 85 proved to be the most toxic ones. Upon testing these mutants along with their parental isolate by SDS-PAGE analysis of spores-crystals proteins as well as vegetative cells proteins, new induced bands appeared in the three mutants by UV radiation and also they showed disappearance of some other bands as compared with the wild type isolate. Multiplex PCR technique, with five sets of specific primers, was used to detect the three types of *cryI* genes *cryIAa*, *cryIAb* and *cryIAc*. Results showed that these three genes exist, as distinctive bands, in the wild type isolate (M5) as well as in mutants 62 and 85, while the mutant 64 had two distinctive bands of *cryIAb* and *cryIAc* genes, and a faint band of *cryI Aa* gene. Finally, these results revealed that mutant 62 is considered as the promising mutant since it is UV resistant, highly toxic against *Spodoptera littoralis* and active against a wide range of Lepidopteran insects.

Keywords*—Bacillus thuringiensis*, biological control, *cry1* genes, multiplex PC, SDS- PAGE analysis.

I. Introduction

SYNTHETIC insecticides have been widely replaced by safe biological insecticides due to the various problems associated with their application, such as pollution of soil and groundwater, and have harmful effects on a wide range of non target organisms (beneficial insects, economic plant varieties, birds, mammals, and humans). Moreover many of synthetic chemical insecticides are non biodegradable and others are degraded slowly and persist in the environment Chattopdhyay et al. [1]. Soil bacterium, which is produces unique crystalline cytoplasmic inclusion bodies during the process of sporulation. Both the spores and the crystalline inclusion bodies are released upon lyses of the parent vegetative cells at the end of sporulation. Spores and crystals act as biological poisons for insect pests. *Bacillus thuringiensis* is referred to as a stomach poison. The spores and crystals are active against Lepidopteran, Dipteran, Coleopteran Beegle, et al. [2] and Hymenopterans larvae Feitelson [3]. SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) assays have been widely used as a rapid and accurate test to identify and characterize proteins of different microorganisms [4-6]. This method was used to analyze proteins of *B. thuringiensis* as total cell proteins and as purified crystal insecticidal protein by Liang et al. [7] who reported that *Bacillus thuringiensis* strains harbored about one to three major proteins and exhibiting a wide range of insecticidal spectrum toxic to Lepidopteran and Depteran. Solubilization and proteolytic digestion of crystal protein using trypsin was reported by Kati et al [8]. Crystal purification was performed by different methods like density gradient centrifugation using sodium bromide gradients Carlabrese et al. and Jarrett [9], [10], sucrose gradient centrifugation Baum et al. [11] and column chromatography using gradient of sodium chloride and Sephadex G-200 column Milne et al. and Lynch et al. [12], [13].

Multiplex PCR is a rapid method for the detection and differentiation of *B.thuringiensis* subsp. *kurstaki* strains and allows better follow-up in commercial formulations and in the field. Bourque et al, Ceron et al. and Ben –Dov et al. [14]-[16] used PCR techniques where DNA amplification was carried out with specific primers to identify specific cry1 genes in *B. thuringiensis* isolates and for rapid screening of *B. thuringiensis* strains that harbor genes from different classes. Liang et al [7] applied the PCR restriction fragment length polymorphism (PCR-RFLP) to identify the type of cry2 genes of *B. thuringiensis* isolated from soil samples in China. Their results showed that 322 *B.thringiensis.* Strains harbored cry2 type genes and four RFLP patterns. The combination of cry2Aa, cry2Ab genes was the most frequent, followed by cry2Aa and cry2Ab as well as one novel type of cry2 gene was cloned from one isolate. The present study aimed to apply PCR technique with different specific primers to detect the existence of cry1Aa, cry1Ab and cry1Ac genes in the highly toxic *Bacillus thuringiensis* induced mutants and the wild type isolate. SDS-PAGE was used as a rapid and accurate test to identify and characterize these strains.

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II. Materials and Methods

The (M5) isolate of *B. thuringiensis* maintained at the Microbial control lab. At the National Research Centre (Cairo-Egypt) was used as the wild type strain in the present study. This isolate proved to produce the insecticidal crystal protein that is efficiently toxic against larvae of cotton leafworm. Upon exposing this isolate to UV irradiation, several mutants were induced; ten of them were selected and tested for their toxicity against the larvae. The most toxic mutants 62, 64 and 85 were chosen along with their parental isolate for SDS-PAGE analysis profile of total proteins of spore-crystals and vegetative cells extracts according to a patent of Von-Tersch and Gonzalez [17].

A. SDS-PAGE Electrophoresis

SDS Poly acrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [4] and modified according to Hames [18].

1. Gel Preparation

Vertical slab (20x21cm) gel electrophoresis apparatus was used. All glass plates were washed with distilled water, and then the glass surface was washed with ethanol 70%. One gel was poured simultaneously to a height of 1.5cm below the bottom of the comb. Gel was overlaid with isopropanol and left to polymerize for at least 15min. Isopropanol was removed and gel was washed with water then dried with filter paper before the stacking gel was poured. This stacking gel was quickly poured over the two resolving gels, and 15 well-combs were used. Gels were left to polymerize for 20-30min before gel was run.

2. Application of Samples

A 25µl of 2x buffer and 3µl of mercaptoethanol were added to 50µl volume of each sample. This mixture was digested for 10min at 100°C in a water bath. A 10µl of bromophenol blue were added to each sample then inoculated into the gel.

3. Gel Running and Staining

Four liters of the run buffer were poured in the running tank and pre-cooled in Frigidaire (4°C). After polymerization and removing the comb gently, a small volume of the running buffer was added to the upper tank just before loading the samples. Gel was run at 100V till samples moved from stacking layer to resolving layer. Then the voltage reduced to 60V and the gel left overnight until the dye reach to the edge of the gel. Gel was removed from the apparatus and placed in plastic tanks, then covered with staining solution overnight. After removing the staining solution, gel was covered with a distaining solution. Gel was agitated gently for one hour. After removing the distaining solution, a new one added. This step was repeated several times until gel background becomes clear to be photographed.

4. Multiplex PCR

Multiplex PCR was used with the wild type strain (M5) and the selected mutants (62, 64 and 85) to examine the existence of *cry1* gene's types (*cry1 Aa*, *cry1 Ab* and *cry1 Ac*) using 5 different primers (Table I) that were described by [14]. The reaction mixture of PCR was carried out using lyophilized PCR beads (master mix tubes (Biron), each tube contains: dNTPs, $MgCl_2$ and Taq DNA polymerase) for 25µl total volume. Lyophilized primers were diluted to 100µM concentration. Primers were SB-1, U3-18c, U8-15c, and SB-2 at 1µl of each, 2µl of DNA and 19µl of distilled water were added to the lyophilized PCR beads.

Amplification was performed, using a DNA thermal cycler (MWG. Biotech Primus model of Whatman Biometra, Germany), is for a single denaturation step (5min at 94°C) for a 10-reaction cycle. Parameters for amplification consisted of 1min at 94°C (denaturation), 2min at 45°C (primer annealing), and 3min at 72°C (primer extension-polymerization). Subsequently, primer RB-19 (1µl) was added to the reaction mixture and PCR was continued for 25 cycles. Then, a final extension step (72°C for 5min) was also used. A total of 15µl of each PCR product was electrophoresed on 1.2% agarose gel in 1x of TBE buffer at 100V for 60-90min, stained with ethidium bromide and visualized under UV light.

III. Results and Discussions

A. Biochemical Characterization of B. thuringiensis Mutants

SDS-PAGE profile of total proteins of spore-crystals and vegetative cells extracted from the wild type isolate, that wasn't exposed to UV radiation, and the three highly toxic mutants' number 62, 65 and 85, that were revealed after UV radiation is presented in Fig. 1.

B. SDS-PAGE Profile of Total Proteins of Spore-Crystals

SDS-PAGE analysis of spore-crystal proteins revealed 36 protein bands with different molecular weights ranged from 221 to 11 KDs as shown in Table II Among such protein bands, six were varied in the wild type isolate and its mutants (numbers 62, 64 and 85), while the other 30 protein bands were commonly detected among the four samples. These six variable protein bands were obviously affected by UV exposure either by disappearance or induction according to their appearance on the gel (Fig. 1 and Table I).

The spore-crystal protein bands of the wild type isolate before UV, as a control, and the protein bands of the three mutants after UV were varied in number, Where the wild type isolate revealed 35 band followed by mutants (62 and 85) with 33 bands and mutant (64) with 32 bands. The analysis of the four *B. thuringiensis* samples showed some unique and distinctive protein bands. The wild type isolate revealed the highest total variable bands with five, while two of the three mutants (62 and 85) displayed three and the mutant 64 showed two protein bands. One induced band with 151 KD was newly induced after UV irradiation, while the other residual bands were existed in the wild type isolate that not exposed to UV irradiation. The three mutants showed variable existence of such bands, for instance, two bands with 221 and 73 KD were merely existed in mutant 85, while they disappeared in the

other two mutants. The band with 183 KD was existed in mutant 62 only. While, the band with 60 KD is appeared in the two mutants (62 and 64) and disappeared in the mutant 85 (Table I). On the other hand, the band with 39 KD disappeared in the three mutants after UV treatment. It can be concluded that the appearance of the new band with 151 KD and the disappearance of the band with 39 KD in the three UV mutants may increased their ability to produce melanin by which their UV resistance increase.

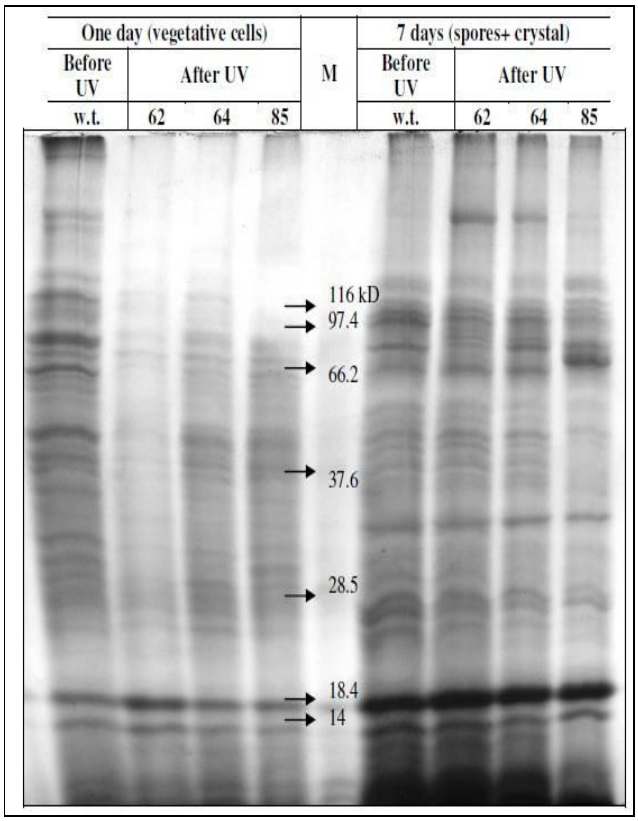

Fig. 1 SDS-PAGE profile of protein extracted from wild type isolate of *B.thuringiensis* and the highest three mutants with bioassay, after a day (vegetative cells) and after 7 days (spores and crystals)

TABLE I
CHARACTERISTICS OF *CRY1* GENE TYPES (*CRY1AA, CRY1AB* AND *CRY1AC*) USED IN MULTIPLEX PCR

Cry1 genes	Primers	Sequence	Positions	PCR Product Size(bp)
Cry1 Aa	SB-1	5'-TGCATAGAGGCTTTAAT-3'	925-941	1500
	U8-15c	5'-CAGGATTCCATTCAAGG-3'	2408-2424	
Cry1 Ab	SB-2	5'-TCGGAAAATGTGCCCAT-3'	2375-2391	858
	U3-18c	5'-AATTGCTTTCATAGGCT-3'	3215-3231	
Cry1 Ac	RB-19	5'-GGGACTGCAGGAGTGAT-3'	1779-1795	653
	U8-15c	5'-CAGGATTCCATTCAAGG-3'	2408-2424	

C. SDS-PAGE Profile of Total Proteins of Vegetative Cells

SDS-PAGE analysis of vegetative cells total proteins revealed 38 protein bands with different molecular weights ranged from 215 to 10 KDs as shown in Table III. Among such protein bands, ten ones were varied in the wild type isolate and its mutants (numbers 62, 64 and 85), while the other 28 protein bands were commonly detected among the four samples. These ten variable protein bands were obviously affected by UV exposure either by disappearance or induction according to their appearance on the gel (Fig. 1 and Table III). The vegetative cells protein bands of the wild type isolate and the three mutants were varied in numbers, where the wild type isolate revealed 36 bands, whereas, mutant (62) had 29 bands, mutant (64) with 30 bands and mutant (85) with 35 bands.

The analysis of the four *B. thuringiensis* samples showed some unique and distinctive protein bands. The wild type isolate revealed the highest total variable bands with eight, followed by mutant 85 with seven bands, mutant 64 with 2 bands and mutant 62 with one protein band. Two induced bands with 30 and 58 KD were newly synthesized due to UV irradiation in mutant 85 and one of them with 58 KD induced in mutant 64. The mutant 85 showed four bands with 215, 201, 134 and 12 KD after exposure to UV radiation that was existed in the wild type isolate, while such bands were not existed in the other mutants (62 and 64). The band with 71 KD was existed in mutant 62 and another band with 89 KD was existed in the other mutants (65 and 85). On the other hand, two bands with 167 and 36 KD were lost after the exposures to UV irradiation in the three mutants though existed in the wild type isolate that not exposed to UV radiation (Table III). From the results of analysis of vegetative cells total protein, it was observed that mutant 85 was the nearest one to the wild type isolate, as they were similar with 35 bands and were different only in 3 bands, although mutant 85 showed the lowest toxicity when compared with the other mutants (62 and 64). Our

results are in agreement with those reported by Liang et al. [7] who added that *B. t*. strains harboring Cry2–type genes produced arose, bipyramidal, square and spherical crystal inclusions under the phase contrast microscopy.

Finally, the analysis of spore crystal and vegetative cells total protein revealed that exposure to UV radiation induced some protein bands (like the band with 151 KD, in spore crystal proteins) to be appeared, with the mutants and didn't' exist in the wild type isolate.

Meantime, UV irradiation inhibited some other bands (like the band with 39 KD in spore crystal proteins and the bands with 167 and 36 KD in vegetative cell proteins), as they were already existed in the wild type isolate but disappeared after UV exposure in the three mutants. The appearance of the band with 151 KD, that was induced in spore crystal proteins, and the disappearance of the band with 167 and 36 KD, in vegetative cells protein, may be involved with the production of melanin pigment. The results indicated that the mutants revealed an outstanding difference after the exposure to UV radiation and could be used as molecular markers for any *B. thuringiensis* mutants. This was agreed with Saxenaet al. and Ruanet al. [19], [20].

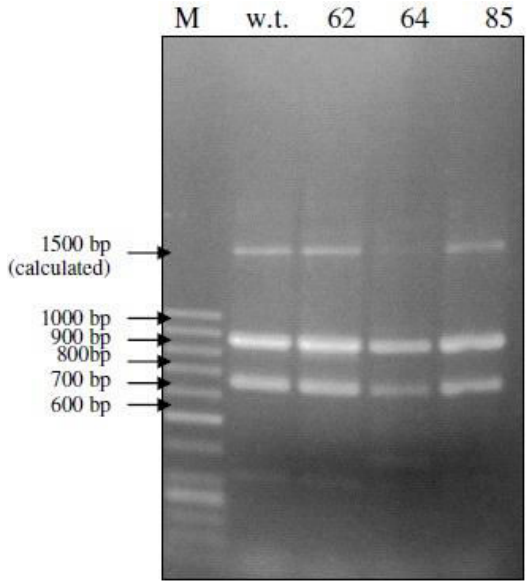

Fig. 2 Agarose gel (1.2%) electrophoresis analysis of multiplex PCR products obtained by using five sets of primers with wild type isolate and the three UV mutants

TABLE II
SDS-PAGE ANALYSIS OF SPORE CRYSTAL PROTEINS PRESENTING BAND NUMBER, MOLECULAR WEIGHT AND EXISTENCE (+) OR INEXISTENCE (-) OF DIFFERENT BANDS

7-Day (vegetative cells)					
Band No.	MW KD	Before UV	After UV		
		w.t.	62	64	85
1	215	+	-	-	+
2	201	+	-	-	+
3	167	+	-	-	-
4	155	+	+	+	+
5	134	+	-	-	+
6	118	+	+	+	+
7	105	+	+	+	+
8	100	+	+	+	+
9	95	+	+	-	+
10	89	+	-	+	+
11	81	+	+	+	+
12	77	+	+	+	+
13	75	+	+	+	+
14	71	+	+	-	-
15	68	+	+	+	+
16	63	+	+	+	+
17	58	-	-	-	+
18	56	+	+	+	+
19	48	+	+	+	+
20	45	+	+	+	+
21	42	+	+	+	+
22	39	+	+	+	+
23	37	+	+	+	+
24	36	+	-	-	-
25	34	+	+	+	+
26	30	-	-	+	+
27	28	+	+	+	+
28	26	+	+	+	+
29	24	+	+	+	+
30	22	+	+	+	+
31	20	+	+	+	+
32	18	+	+	+	+
33	16	+	+	+	+
34	14	+	+	+	+
35	13	+	+	+	+
36	12	+	-	-	+
37	11	+	+	+	+
38	10	+	+	+	+

TABLE III
SDS-PAGE ANALYSIS OF VEGETATIVE CELL PROTEINS PRESENTING BAND NUMBER, MOLECULAR WEIGHT AND EXISTENCE (+) OR INEXISTENCE (-) OF DIFFERENT BANDS

1-Day (vegetative cells)					
Band No.	MW KD	Before UV	After UV		
		w.t.	62	64	85
1	215	+	-	-	+
2	201	+	+	+	+
3	167	+	+	-	-
4	155	+	+	+	+
5	134	+	+	+	+
6	118	+	+	+	+
7	105	+	+	+	+
8	100	+	+	+	+
9	95	+	+	+	+
10	89	+	+	+	+
11	81	+	+	+	+
12	77	+	+	+	+
13	75	+	+	+	+
14	71	+	+	+	+
15	68	+	+	+	+
16	63	+	-	-	+
17	58	-	+	+	+
18	56	+	+	+	-
19	48	+	+	+	+
20	45	+	+	+	+
21	42	+	+	+	+
22	39	+	+	+	+
23	37	+	+	+	+
24	36	+	-	-	-
25	34	+	+	+	+
26	30	-	+	+	+
27	28	+	+	+	+
28	26	+	+	+	+
29	24	+	+	+	+
30	22	+	+	+	+
31	20	+	+	+	+
32	18	+	+	+	+
33	16	+	+	+	+
34	14	+	+	+	+
35	13	+	+	+	+
36	12	+	+	+	+
37	11	+	+	+	+
38	10	+	+	+	+

D.Multiplex PCR

Using the multiplex PCR technique, with 5 different sets of specific primers, the three types of *Cry 1* gene were detected in the wild type isolate and the three revealed UV mutants. Where, primers SB-1 and U8-15c from the *Cry 1(a)* gene gave a 1500bp PCR product. Primers SB- 2 and U3-18c selected from the *cry 1A(b)* gene gave an 858bp PCR product, and primers RB-19 and U8-15c from the *cry 1A(c)* gene gave a 653bp product. The revealed results (Fig. 1) confirmed the presence of three distinct bands easily visible in agarose gel with the wild type isolate (M5) of *B. thuringiensis*, 62 and 85 mutants while the 65 mutant had two distinct bands of the *cry 1A(b)* gene and the *cry 1A(c)* gene while it had a faint band of *cry 1A(a)* gene. Existence of these three types of *cry1* genes illustrates that these three UV mutants, in addition to the wild type isolate, have toxicity against wide range of Lepidoptera insects and that multiplex PCR method can be applied to rapidly detect the subgroups of Cry1 proteins that correspond with toxicity to various Lepidoptera insects [14], [15]. Liang et al. [7] applied (PCR-RFLP) to identify the type of cry2 genes resources of *B.t.* strains. Meantime, Kumar et al. [21] demonstrated that the rapid Multiplex PCR assay is simple and easy to identify and differentiate *Brucella spp.* isolates.

Finally, from this study, we can conclude that the revealed mutant number 62 of *B thuringiensis* is an ideal mutant. Whereas, this mutant has *cry1* gene so it is able to produce the insecticidal crystal protein with bipyramidial shape that is toxic against insects of order Lepidoptera. Mutant 62 has the ability to produce a dark brown pigment to resist the UV radiation damage. Bioassay results revealed that this mutant is highly toxic to cotton leaf worm, *S. littoralis*, and that only 52 µg/ml concentration of this mutant is able to kill 50% of the target insects. Also, mutant 62 has three types of *cry I* gene. Subsequently, this mutant has wide range spectra of toxicity against insects of order Lepidoptera. So, mutant number 62 is a promising mutant to be used as an applied biocontrol agent at field against Lepidoptera pests.

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