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Toxicological and molecular characterization of local *Bacillus thuringiensis* isolates from soil and insects

M. Harba, I. Idris, H. Ismail

Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091 Damascus, Syria.

Abstract

Previous research has investigated *Bacillus thuringiensis* (*Bt*) isolates to control pests that destroy vital crops particularly in Syria. However, novel *Bt* strains are still needed to be isolated and characterized across the country. A collection of 25 isolates of *Bt* from Syrian soil and 40 isolates from infected insects exhibit height toxicity have been studied. The study focuses on the isolates that possess high levels of toxicity versus the reference strain *Bt. kurstaki* HD-1. In order to determine the most toxic isolates, five isolates from soil SSy60-b, SSy111-c, SSy125-c, SSy126-c, SSy141-c, and five isolates from infected insects SyG46, SyG41, SyG43, SyG30-a, SyE3-S were tested. The Lethal Concentration 50 (LC_{50}) of the spore-crystal mixture ranged from 2.7 mg. g^{-1} to more than 15.6 mg. g^{-1}. A PCR test with a specific primer that is recognizing regions of the *cry* genes was used to analyze the present of *cry* genes in the isolates with the most toxic effects. Among all the studied isolates, the *Bt* SYG46 has exhibited the highest level of toxicity, therefore, it seems to be promising to be implemented in pest control programs against main economical lepidoptera pest.

Keywords: *Bacillus Thuringiensis, Ephestia kuehniella,* cry gene, toxicity, pest control.

Introduction

Using pests biological control methods as an alternative to the currently used chemical insecticides is required in integrated pest management for the benefits of both the environment and the human.

Microbial bioinsecticides has shown to be a promising approach to achieve the aforementioned goals and the statistics show that 95% of microbial pesticides have been successfully used and economically have achieved over $100 million per annum [22, 8, 26, 1].Microbial strains have been used to control pests such as Bt, which cover 150 insect's species and the insecticides formulations that contained Bt have shown a powerful entomopathogenic activity against the targeted insects [29, 9]. Bt is a rod-shaped positive gram stain bacterium that are commonly presented in soil, plant surfaces, stored grains, and dead insects [25, 3]. The toxic effects of Bt are attributed to the ability Bt species to produce a range of insect-toxic protein during sporulation where it has been reported eight different toxins in some strains of Bt [27, 8].

Insect-toxicity mechanisms of Bt Cry-binding protein start when the inactive Bt protein is released into the gut of insects' larvae and activated by specific proteases from the hosting insects at pH ranged from 9.0 to 10.5 [30]. Once the activated protein binds to a specific receptor on the targeted insects it attacks the gut lining cells of the insect causing starvation and Bt spores will spread and germinate in the insect leading to its death within 5 days [28, 4].

For the current study, ten isolates selected from previous research - all of which were more toxic than Bt kurstaki HD-1- were evaluated for their toxicities effects in order to select the most toxic isolate for future research. A broad range of comparative indentation features such as lethal concentration 50 (LC50), electron micrograph images, H stereotyping, cry genes, and plasmid profiles of isolates were investigated.

Corresponding authors: ascientific@aec.org.sy

2.Material and method

Isolation of Bt bacteria from infected insects and soil

Collections of 65 *Bt* isolates (40 from infected larvae and 25 from soil samples) to be toxic against *Ephestia kuehniella* [1, 2]. *Bt* isolates was collected from both the infected larvae and Syrian soil samples from different areas that have never been treated with *Bt*-based microbial insecticides.

Larvae has been stored in dry clean glass containers at 4°C, followed by a process of sterilization in which the larvae were passed through 70 % ethanol for 2 seconds, followed by 5% sodium hypochlorite for 1 minute, and three time washing by sterile distilled water. In each tube, the larva was soaked with a plastic pestle before 1 ml of SDW was added. The suspension was subsequently heated at 80 °*C* in a thermo mixer for 10 minutes, and then diluted immediately [2].

In regard to soils sample, 100 g of soil was collected by scraping off the top layer of land with a sterile spatula, then samples from 2-5 cm below the surface were collected. Samples were then stored at 4 °C until further process. A modified protocol was used for to isolate spore-forming Bacillus from soil as describe by Ammouneh *et al*., 2011 [1]. Approximately 1 g of soil sample was added to 10 mL of Luria-Bertani broth and buffered with sodium acetate (0.25 M, pH 6.8), then the broth was shacked for 4 hours at 30 °C. one mL aliquots was heated at 80 °C for 15 minutes in Eppendorf thermomixer. Dilutions were then plated on nutrient agar (NA) and plates were incubated at 30 ºC for 24- 48 h. Bacteria-like colonies on new NA plates were sub cultured until pure cultures have been achieved and maintained at 4°C for further bioassay tests including spore-crystal mixture [20]. Professor Samir Jaoua from Centre for Biotechnology of Sfax, CBS, Tunisia has kindly provided the reference strains *Bt kurstaki* HD-1. Isolates that stored in 20% glycerol at -80°C.

Bioassay preparation: spore-crystal mixture

To grow parasporal isolates in suspension, a modified culture method by Ammouneh *et al*., (2011) [1] has been followed. A spike of bacteria culture was added to 50 mL of T3 medium and shacked at 200 rpm for five days at 30°C in 250-mL flasks. Based on microscopic monitoring, the majority of the population was in spores-crystals at the end of the incubation period.

In order to remove exotoxins, pellets that contains spores and parasporal crystal proteins were centrifuged at 10000 rpm for 10 minutes after being washed twice in sterile distilled water. The pellets were then dried in a freeze drier (-50°C, 0.1 mbar vacuum) for an extended period, the lyophilized pellets were stored for further bioassays**.**

Insect rearing and toxicity tests

Bioassays were performed on larvae obtained from the laboratory colony insects. *E. kuehniella* larvae have been reared in wheat meal without humidity control at 25 °C [21]. The toxicities were quantified by determining the lethal concentrations that kill 50% (LC50) of the *E. kuehniella* larvae. *Bt kurstaki* HD-1 was used as standard control since their toxicity against these insect species is well known [10], and sterile distilled water was used as a blank. Generally, all mixtures concentrations were adjusted to 1 mg. mL^{-1}, the concentration that induces 100% mortality by *Bt kurstaki* HD-1. Serial dilutions were prepared to achieve concentrations ranging from 200 to 0.2 µg spore crystal mixture (SCM) per gram wheat meal. Using 10 larvae in each replica, SCM was added with 500 mL of larval food to the petri dishes containing one gram of larval food per dose. Each isolate was tested with 500 larvae, and mortality after five days was recorded. The SOFTTOX computer program (WindowChemTM) was used to process and analyze results of the bioassays [12].

Cry genes Identification

Cry genes identification was performed via PCR as described by Ammouneh et al. (2011) [1]. Specific primer pairs presented in (Table 1) have been implemented to identify the major groups of cry genes. Ethidium bromide was added to a 1.5% agarose gel to separate the PCR products.

Sample preparation for Scanning electron microscope (SEM)

Bt SYG46 has been grown on LB at 30°C for 72 hours, after sporulation, a centrifuging at 10,000 rpm for 10 minutes at 4°C were performed and pellet was collected and resuspended in sterile distilled water. Sample that contains spore-crystal mixture was adhered to aluminum stubs fixed in 1% OsO4. Gold was subsequently sputter-coated on the sample in the IB-5 ion coater for 5 minutes. This SEM was taken at a VIGA II (XMU) digital scanning microscope.

Table 1. List of specific Primers implemented in *cry* genes amplification

Primer pair	Gene(s) recognized	Annealing temperature (°C)	Sequence	Reference
CJ1 CJ2	*cry1Aa*	52	5'TTATACTTGGTTCAGGCCC (d) 5'TTGGAGCTCTCAAGGTGTAA (r)	Ceron et al. (1994)
SB-2 U3-18c	*cry1Ab*	42	5`TCGGAAAATGTGCCCAT (d) 5`AATTGCTTTCATAGGCT (r)	Bourque et al. 1993
CJ6 CJ7	*cry1Ac*	50	5'GTTAGATTAAATAGTAGTGG (d) 5`TGTAGCTGGTACTGTATTG (r)	Ceron et al. (1994)
EE-2Aa	*cry2Aa*	60 (with UN2d)	5'GAGATTAGTCGCCCCTATGAG (r)	Ben-Dov et al. (1997)
EE-2Ab	*cry2Ab*	60 (with UN2d)	5'TGGCGTTAACAATGGGGGGAGAAAT (r)	Ben-Dov et al. (1997)
EE-2Ac	*cry2Ac*	60 (with UN2d)	5'GCGTTGCTAATAGTCCCAACAACA (r)	Ben-Dov et al. (1997)
Cry1I	*cryI1*	48	5`ATGAAACTAAAGAATCCAGA(d) 5`AGGATCCTTGTGTTGAGATA (r)	Masson et al. 1998

H stereotyping

Thirty-five reference H antisera obtained from Wuhan Institute of Virology, China, were used to identify the local *Bt* isolates. Agglutination tests were performed using diluted antisera to determine the serotype, as described by Lecadet *et al*., (1999) [16]. Tests were performed twice to ensure accuracy.

Plasmid extraction

A Qiagen Plasmid MINI Kit was used to extract and purify plasmid DNA with some modifications, the manufacturer's protocol was followed with the addition of lysozyme in the first stage. DNA – plasmid products were separated on a 0.7% agarose gel for 5-6 hours and 30-40 v to which ethidium bromide was added, and photographed under UV light.

3.Results and Discussion

Forty *Bt* strains have been previously isolated in our laboratories from *Galleria mellonella*, *Helicoverpa armigera*, and *E. kuehniella* larvae, 25 *Bt* isolates from soil with high toxicity to the larvae of *E. kuehniella*, *Phthorimaea operculella* Zeller, and *Cydia pomonella* L. (Lepidoptera) have been also isolated [1, 2]. The LC50 for the first 40 isolates trialed on *E. kuehniella* larvae ranged from 3 to 200 µg g-1. However, the LC50 ranged from 8.4 to 97.6 µg g-1 in regards to the 25 stains that have been isolated from soil [1, 2]. In comparison with the reference strain *Bt kurstaki* HD-1, LC50 values of some of the tested strains may be as toxic or more toxic than the *Bt kurstaki* HD-1.

Despite the fact that *Bt* is ubiquitous in soil, the isolates from the collected insects showed significant high toxicity against *E. kuehniella* larvae.

It is important to note that *Bt* toxins lethality is strongly depends on gut pH of target insect, proteolytic enzyme activity, and microvillar receptors existence. As toxins enter the midgut lumen, pH and proteases convert them from insoluble protoxins to a poisonous active form that is resistant to further proteolysis in susceptible species [31, 24]. Due to differences in physiology, some insects are in susceptible to *Bt* toxins while others are not [11]. Generally, the toxicity of *Bt* strains depends on the types and subgroups of cry genes present. Based on *Cry* genes identified as predictive of insecticidal activity for isolates in Table 2, *Cry* genes were distributed among *Bt* isolates. However, there was a wide range of LC_{50} values ranged from 2.7 to 15.6 µg g^{-1} among the tested isolates against *E. Kuehniella* larvae (Figure 2). All of the isolates showed higher toxicity comparing to the Bt. *kurstaki* HD-1 strain that has LC_{50} 22.8 µg g^{-1}. SyG46, SyG30-a, and SSy141-c isolates had LC50 values ranging from 2.7 to 4.7 µg g^{-1}, and were more toxic than the rest of isolates (Figure 2).

To determine whether an isolate possessed a determined gene, the PCR profiles of all PCR products were compared to those of *Bt kurstaki* HD-1, and amplification products of the expected size were achieved. Local *Bt* isolates had several *cry1* and *cry2* genes in common, according to an analysis of the tested isolates (Table 2). The cry1Aa/cry1Ac primers (CJ1, CJ2/CJ6, CJ7) produced PCR products with the sizes of 246/180 bp respectively. In all cases, the isolates that were PCR-amplified with cry1Ab primers (SB-2, U3-18c) produced the PCR products 855 bp. All of the isolates produced the PCR products with sizes of 498/546bp when cry2Aa/cry2Ab primers (EE-2Aa, EE-2Ab) were used. Cry1I primer produced the PCR products of cryI1 with 1137 bp size for all tested isolates. When using EE-2Ac primer none of the isolates produced cry2Ac.

Table 2. Characterization of local *Bacillus thuringiensis* isolates by genetic and serotyping approaches.

Strain	cry 1Aa	cry 1Ab	cry 1Ac	cry 1I	cry 2Aa	cry 2Ab	cry 2Ac	Serovar
KHD1								K
SSy60-b	√	√	√	√	√	√	-	K
SSy111-c	√	√	√	√	√	√	-	K
SSy125-c	√	√	√	√	√	√	-	K
SSy126-c	√	√	√	√	√	√	-	K
SSy141-c	√	√	√	√	√	√	-	K
SyG30-a	√	√	√	√	√	√	-	K
SyG41	√	√	√	√	√	√	-	K
SyG43	√	√	√	√	√	√	-	K
SyG46	√	√	√	√	√	√	-	K
SyE3-s	√	√	√	√	√	√	-	K

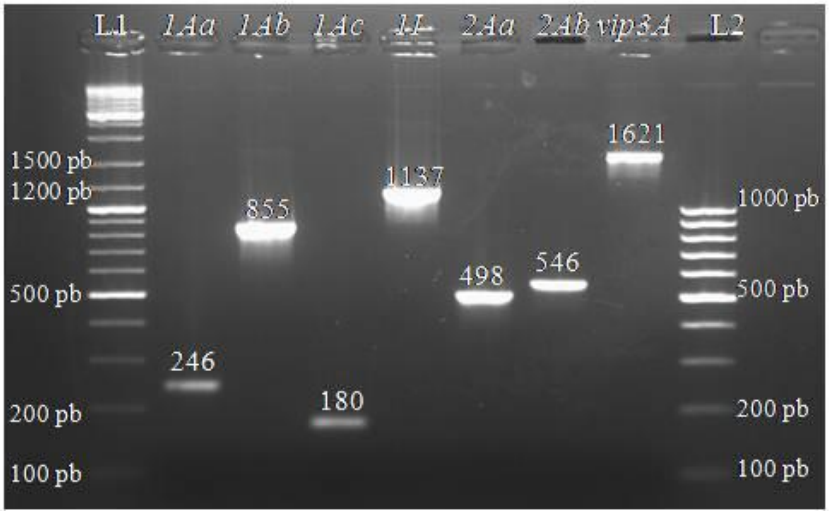

Figure 1. Detection of *Bacillus thuringiensis* isolates of SyG46 insecticidal genes with: *cry1Aa* specific primers (CJ1, CJ2); *cry1Ab* specific primers (SB-2, U3-18c); cry*1Ac* specific primers (CJ6, CJ7); *cry1I* general primers (cry1I); *cry2Aa* specific primers (EE-2Aa); and *cry2Ab* specific primers (EE-2Ab).

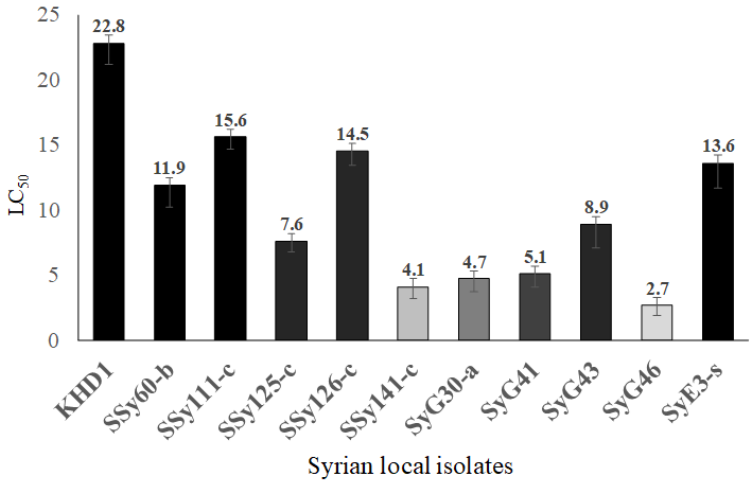

Figure 2. LC_{50} values (µg g^{-1}) of 10 local isolates of *Bacillus thuringeinsis* against *Ephestia kuehniella* larvae. HD-1: *B. thuringiensis kurstaki* HD-1 (controls), SSy and Sy: local isolates collected from Syrian soil and larvae samples; bars represent the 95% upper and lower fiducial limits.

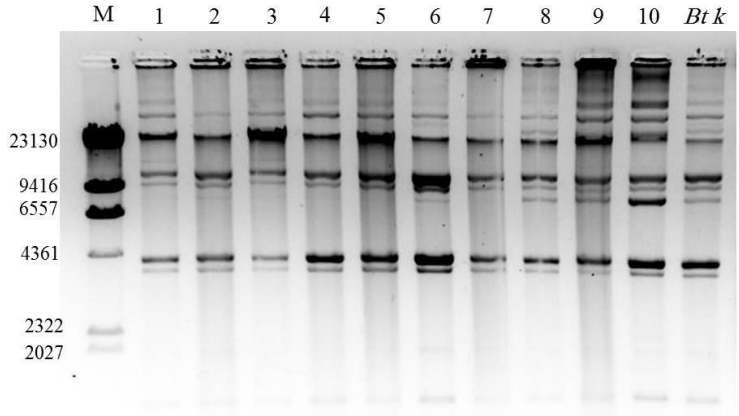

Figure 3. Plasmid patterns of local strains of *Bacillus thuringiensis* in 0.1% agarose gel. Lanes: 1, SSy60; 2, SSy111-c; 3, SSy126-c; 4, SSy125-c; 5, SyG30-a;6, SyG41;7, SyG43; 8, SyE3-s; 9, SSY141-c; 10, SyG46; *Btk*, KHD1.

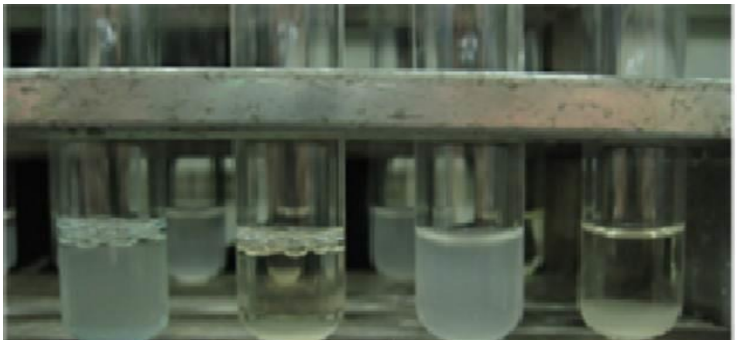

Figure 4. Stereotyping images for local strains and reference strains of *Bt kHD-1*

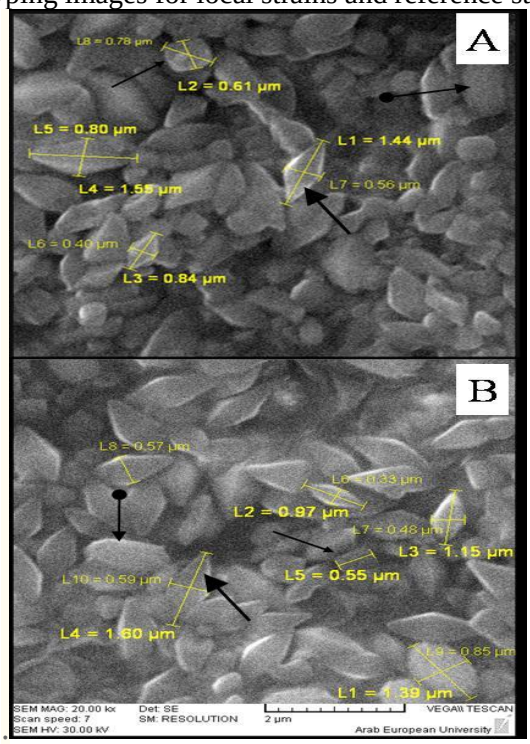

Figure 5. SEM image of protein crystals (bipyramidal and cuboidal shaped crystals) mixed with spores of A-*Bt* local strain SyG46, and B- reverence strain *Bt HD-1*. Crystallized bipyramidal protein is indicated by the large arrow, crystallized cubic protein by the small arrow, spores by the arrow with the point, and micrometer (µm) scale by the straight line.

SEM examination of the studied isolates showed bipyramidal and cuboidal shaped crystals (Figure 5). Depending on their protein composition and parasporal, crystals can take a variety of shapes including bipyramidal, cuboidal, flat, rhomboid, round, and amorphous [13, 18, 7]. The insecticidal properties and molecular relationships of *Cry* δ-endotoxins indicate that these proteins are active against Lepidoptera for *Cry*1 and Lepidoptera and Diptera for *Cry*2 [17]. Many reports revealed that strains constructing bipyramidal crystal proteins exhibited toxicity only on Lepidoptera and were associated with Cry1 toxins. In addition, the cuboidal crystal proteins exhibited toxicity on Lepidoptera and Diptera and were most probably associated with Cry2 toxin [10, 2].

The results of viral serotyping revealed that ten local isolates SSy 60b, SSy111-c, SSy125-c, SSy126-c, SSy141-c, SyG46, SyG41, SyG43, SyG30-a, SyE3-S had positive responses (Figure 4).

On agarose gel electrophoresis, the native plasmid profiles of all ten local strains showed a similar pattern, though substantial differences were evident in polymorphism and size of cryptic plasmid regions (Figure 3). The distribution of plasmid profiles in *Bt* has been shown to be affected by the composition of the growth medium and stress conditions, which also affects the stability of plasmids and their transmission to daughter cells [15].

Figure 3 demonstrated that the plasmid profiles of local strains that are most toxic against *E. kuehniella* (e.g. SSy141-c, SyG46, SyG30a) differ in number and size compared to other local strains. There has been some evidence reported previously that the functional significant differences between some species of *Bacillus sp* (e.g., *B. cereus*, *B. thuringiensis*, and *B. anthracis*) may be due to the presence of plasmids of varying numbers and sizes [15, 6, 23]. However, in several species of *B. thuringiensis*, loss or suppression of mega plasmid replication has been attributed to decreased crystal protein formation [19]. The results demonstrated that the plasmid profiles of local *Btk* strains can provide valuable information which can be used in direct identification of these strains in order to assess genomic relatedness between them. These results are consistent with previously reported findings, and this method makes it possible to quickly identify toxic isolates to insects from Lepidoptera or Lepidoptera and Diptera [15, 14, 32, 5].

Conclusion

A nationwide initiative is being conducted to find novel *Bt* isolates from Syria that are more effective against some critical lepidopteran pests. Ten isolates (SSy60b, SSy111-c, SSy125-c, SSy126-c, SSy141-c, SyG46, SyG41, SyG43, SyG30-a, and SyE3-S) were taken from prior studies in order to determine the most toxic isolate against *E. kuehniella*. *Bt* G46 isolate has high level of toxicity comparing to the other tested local isolates as determined by LC_{50}, H stereotyping, cry genes, SEM, and plasmid profiles. Therefore, the *Bt* G46 isolate maybe implemented in future pest control programs against main lepidoptera pests of economic importance on fruit and vegetables crops.

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Compliance with Ethics Requirements: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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