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Molecular Characterization of Entomopathogenic Bacteria, *Bacillus thuringiensis*

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ABSTRACT

The genetic variation of the new *Bacillus thuringiensis* isolate from soil and (Agrin) as a reference were evaluated by using random amplified polymorphic (RAPD-PCR) in order to distinguish between the isolates using random primers. It was noticed that; 1- Genetically, *B.t* .isolate is the closest to Agrin which is a commercial product of *B.t.*, 2- Estimating such genetic variations among and between different strains or isolates of *B.t* may be of practical value for designing integrated pest management (IPM) programs. It is hoped that the results obtained from this study may safely and effectively help in managing this serious insect pest of potato in an integrated pest management (IPM) program.

INTRODUCTION

The isolation of new *Bacillus thuringiensis* strains from soil or other environment received the attention of scientists worldwide according to the recommendation of UNEP (United Nations of Environmental Program, 1992) to isolate indigenous isolates for pest control. The present study aims to know the molecular characterization of the *B.t.* isolate in order to show the genetic variability this isolate and a commercial *B.t.* product (Agrin).

MATERIALS AND METHODS

1- Soil Samples:

Soil samples were collected randomly from different fields in El-Bahariya Oases, Surface materials of the soil was removed; and with a sterile spatula, about 100 gm sample of soil was taken from at least 5 cm in depth. The soil samples were preserved in sterile plastic bags and stored for 2 - 12 months at 4°C until analyzed. The collection sites had no history of treatment with *B.t.*

1- Isolation Technique, Culturing and enumeration of Bacteria:

Based on the acetate selective method described by (Smith *et al*., 1991), The germinated colonies were fixed to clean slides and stained according to (Smirnoff 1962) stain method, then examined microscopically.

For culturing the obtained isolates, the method Shake Flask Fermentation described by (Morris *et al*. 1996). The method of enumeration of Bacteria described by (Dulmage1971).

2- Preparation of Cell Lysates from *B. thuringiensis* Isolate:

This technique was carried out at specified laboratories, at the Faculty of Agriculture, Cairo University.

1- A new subculture from each of tested isolates was inoculated on nutrient agar plates for 12 hours at 30°C (the cells must be the vegetative stage).
2- A loop from single colony was transferred to a micro centrifuge tube containing 100µl diethyl pyro carbonate (DEPC) water and boiled for 5 minutes using water bath to allow burst of the cells.
3- The resulting cell lysates were spun for 5 minutes at 10.000rpm cooling centrifuge.
4- The supernatant was transferred into a clean micro centrifuge tube and stored at –20°C until used.

3- Random Rmplified Polymorphic DNA (RAPD):

An arbitrary primer polymerase chain reaction technology was applied to identify the selected strain of *B. thuringiensis* by using total DNAs extracted as templates. Primers shown in Table (1) were used. DNA amplification was performed in 50-1 reaction mixture as shown in Table (2).

Table 1. Sequences of the selected random Primers, which successfully gave reproductive amplification products with selected isolates.

No.	Primer	Sequence	Authers
1	0940-12	5'-ACGCGCCCT-3'	Brousseau *et al,* 1993
2	UAM 1	5'-GTTTCGCTCC-3'	
3	UAM 10	5'-GGACACCACT-3'	Hu *et al.,* 2004
4	UAM 13	5'-GCCAGGGACA-3'	

Table 2. Components of the mixture used in RAPD-PCR reaction

No.	Reagent	Concentration	volume
1	dNTPs (dATP, dCTP, dGTP, dTTP)	1mM	4.0µl
2	Buffer, containing $MgCl_2$	10 X	2.0µl
3	Template DNA	50-ng/ µl	2.0µl
4	Taq polymerase	5U/ µl	0.2µl
5	Primer	70 P mole	2.0µl
6	Distilled sterile water		9.8µl
	Total volume		20µl

a- Reagent and Solution:

1- PCR reaction buffer (10 X):

0.67 M Tris HC1; pH 8.8
0.067 M $MgC1_2$.
0.166 M $(NH_4)_2S0_4$.
0.100 M β- mercaptoethanol.

Note: The buffer was filtered by 0.22 µ sterilized filter

2- dNTPs Solution:

All four free dNTPs were mixed together to a final concentration of 1 mM each in a solution is equivalent to a 10X stock.

3- Taq polymerase (Promega):

4- Primers:

Five different primers namely (0940-12, PM 13, UAM 1, UAM 1o and UAM 13) were used (Table 1).

5-Tris-Acetate Buffer (50X): per liter

1- Tris Base 242g.
2- Glacial acetic acid 57.1 ml.
3- EDTA (0.5M – pH 8.0) 100 ml.

6- Ethidium bromide stock solution: 10 mg/ml of H_2O.

7- Loading buffer:

Bromophenol blue 0.25 g.
Glycerol (30%) 100 ml.
*This buffer was stored at -70 °C.

8- Marker:

1 Kbp DNA ladder (Gibco) and 100 bp DNA ladder (BIORON) were used.

9- Sample preparation:

PCR- product 15 µ1.
Loading buffer 3 µl.

10- Gel preparation :

Agarose 1 g.

TAE buffer (1X) 100 ml.
Ethiduium bromide (10 mg/ml.)

The buffer was added to the agarose then heated in a microwave till melting; cooled to 60 °C then the ethidium bromide was added.

b- Procedure:

All reaction components except of the DNA template and primer were mixed well. The primer and DNA template were added to the previous reaction mixture then spun down briefly. The reaction was assembled on ice. The PCR Eppendorf tubes containing the reaction mixture were quickly transferred into the PRC apparatus. Amplification is accomplished through a Thermal Cycler using first one incubation at 92°C for 3 minutes and the step cycle program set to denaturant at 92°C for 45 seconds, to anneal at 35°C for 45 seconds and extend at 72°C for 90 seconds for a total of 44 cycles; after that, an extra step of extension at 72°C for 10 minutes were performed (Table 3). Results were obtained from single experiment for each primer.

Table 3. Amplification program used in RAPD-PCR reaction

Order	Action	Temperature	Duration
1	1st Denaturation	92°C	3 sec.
2	Denaturation	92°C	45 sec.
3	Annealing primer	35°C	45 sec.
4	Extension	72°C	90 sec.
	Steps 2, 3 and 4 were repeated for 44 cycles.		
5	Final Extension*	72°C	10 min

***This step allows the complete extension of all DNA products.**

Characteristic DNA banding patterns obtained by resolving on 1 % agarose gel (containing 8 µl Of 10 mg/ml ethidium bromide) electrophoresis in 1X TAE buffer. The gel was run in the same buffer using power supply (Bio Rad, Germany) with constant voltage of 70 volts for 15 minutes. The migrated fluorescing DNA-ethidium bromide complex was visualized as bands under a UV-transilluminator (Foto Dyne, Incorporated Cat No. 5-5334) and then the gel was photographed with a Polaroid camera. The resulting bands were scored as present (1) and absent (0) using Labimage program. Also, the phylogenetic relationship tree among different isolates was detected using NET SYS program.

RESULTS AND DISCUSSION

Random Amplified Polymorphic DNA–Polymerase Chain Reaction:

During RAPD analysis on pooled DNA, four primers were used in order to identify the *B.t.* isolate. Amplified RAPD banding patterns made it possible to distinguish between commercial product (Agrin) and *B.t.* isolate. Each of these primers amplified on average 5 to 9 RAPD fragments; and one had to make use of two ladders because the range of the molecular weight was different. The First approximate size ranged from 100 to 3000bp and the second size ranged from 200 to 12000 bp. The banding pattern of a standard strain, As shown in Table (4) the total number of the bands obtained with 5 RAPD primers used were about 29. For the first primer (0940-12), there were 9 bands obtained; 4 bands were shared by the *B.t.* isolate and the stander strain Agrin. The numbers of the bands shared by strain differed for the subsequent primers. For UAM 1 primer, there were 4 bands, while for UAM10 primer there was one band; and for UAM13 primer there were 3 bands.

Table 4. Number of bands obtained by using 5 RAPD primers

Primer	No. of bands	No. of Polymorphic bands
0940-12	9	4
UAM 1	7	4
UAM 10	4	1
UAM 13	5	3
total	29	12

1- First Primer: 0940-12

There were nine bands that ranged from 3000 to 100 bp. Seven bands were shared by *B.t* isolate. (track 3) was the most similar to the Commercial product, Agrin (track 2) sharing the same banding pattern except in lacking two band at about 1500 bp and 250 bp, but shared band at about 150bp. (Figure, 1).

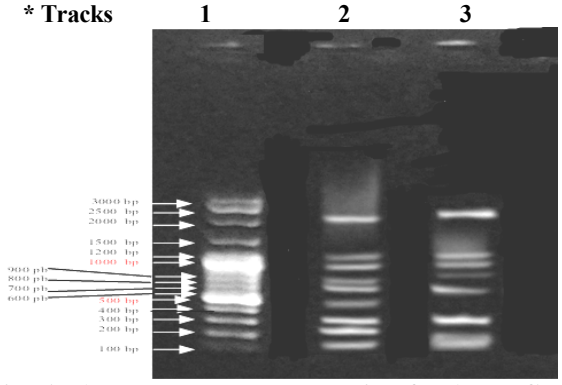

Fig. 1. Agarose gel electrophorasis of RAPD-PCR product of *Bacillus thuringiensis* isolate by using 0940-12 primer

*** Track 1 is a DNA ladder, Track 2 is commercial product (Agrin), Track 3 is *B.t* isolate**

2- Second Primer: UAM 1

There were seven bands that ranged from 200 to 12000 bp. There were four bands shared by *B.t.* isolate (track 3) was the most similar to the commercial *B.t.* (track 2) sharing the same banding pattern. *B.t.* isolate had a specific band between 1650 and 850 bp that did not appear in commercial *B.t.* (Figure. 2).

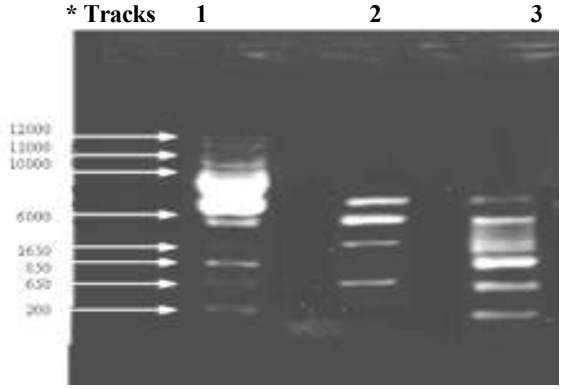

Fig. 2. Agarose gel electrophorasis of RAPD-PCR product of *Bacillus thuringiensis* isolate by using UAM 1 primer

3- Primer: UAM 10

There were four bands that ranged from 2600 to 100bp. Two bands were shared *B.t.* Isolate (track 3) was the most similar to the commercial *B.t.* (track 2) sharing the same banding pattern. *B.t.* Isolate (track 3) had specific band at 2600 bp, that did not appear in Agrin (Figure, 3).

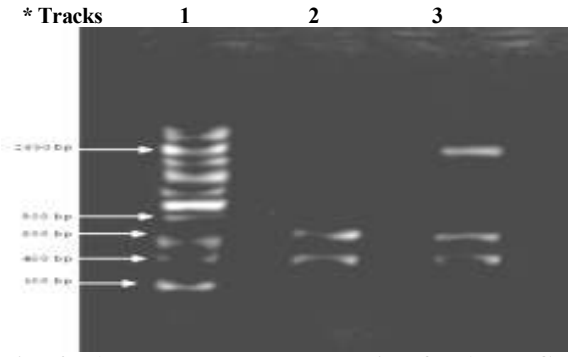

Fig. 3. Agarose gel electrophorasis of RAPD-PCR product of *Bacillus thuringiensis* isolate by using UAM 10 primer.

4- Fourth Primer: UAM 13

There were five bands that ranged from 200 to 12000bp. the bands were shared by *B.t*. Isolate (track 3). which similar to the commercial *B.t.* (track 2) sharing the same banding pattern (Figure, 4).

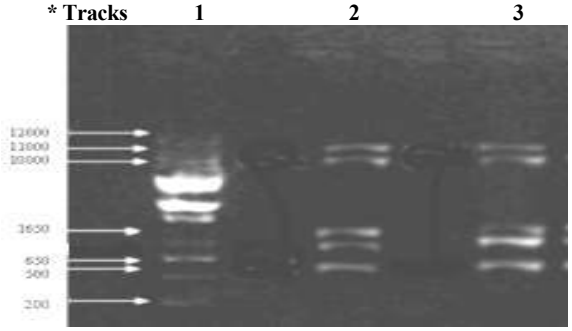

Fig. 4. Agarose gel electrophorasis of RAPD-PCR product of *Bacillus thuringiensis* isolate by using UAM 13 primer

Dendrogram

As shown in Figure (5) *B.t.* Isolate and Commercial product, Agrin had closer genetic distance. i.e. *B.t*. Isolate represented by hypothetical common ancestor who in turn had farther distance with respect to the standard strain.

In the present study, molecular analyses approaches have been used to characterize the *B. thuringiensis* soil isolate. Accordingly, molecular characterization of the potent isolate of *B. thuringiensis* has been carried out for characterization and explaining the variability in the potency of strain. Earlier, many traditional methods have been used for the characterization and identification of the entomopathogenic bacterium, *B. thuringiensis*. These methods include crystal morphology, bioassay with insects, biochemical reactions and serological test by flagellar (H) antigens. Although this method has been established to expedite the characterization of *B. thuringiensis*, there are some disadvantages, of being time-consuming, expensive, laborious and inaccurate (Ohba and Aizawa, 1986). Accordingly, these traditional methods seem to be inappropriate for screening of large collections of *B. thuringiensis* strain.

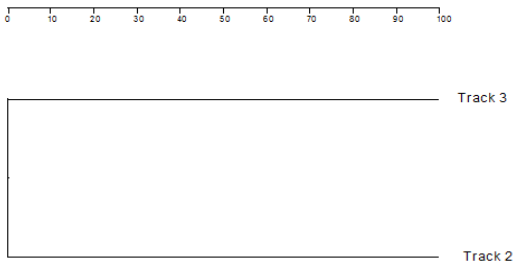

Fig. 5. Phylogenetic trees based on RAPD-PCR of *Bacillus thuringiensis* isolate by using 0940-12 Primer

Track 2: Commercial product, Agrin, Track 3: *B.t.* Isolate

Form the results obtained in the current study, Genetically, *B. thuringiensis* isolate strain is the closest to Agrin which is a commercial product used in the control process since 1980s and revealed success in many control programs (Dabi *et al.* 1988, Gadallah *et al*. 1990).

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التوصيف الجزيئي لاحدي العزلات البكتريا الممرضه للحشرات (باسيلس ثيرونجينسيس)

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تم تقييم الأختلافات الجينية للعزلات البكتيرية الجديدة ومقارنتها بمبيد بكتيري تجاري (أجرين) بأستخدام (RAPD-PCR) وذلك للتفرقة بينها بأستخدام عدة بوادئ عشوائية . وقد لوحظ الآتي : 1- كانت العزلة البكترية قريبه وراثيا للمبيد البكتيرى أجرين وهو مركب بكتيري فعال . 2- ان تقييم الأختلافات الجينية بين السلالات أو العزلات البكتيرية المختلفة قد يكون ذا أهمية عملية في وضع سياسات للمكافحة المتكاملة للآفات .