Isolation and Characterization of *Bacillus thuringiensis* Isolated

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Six isolates of *Bacillus thuringiensis* named BTA1, BTA2, BTA3, BTA4, BTA5 and BTA6 were adopted from ten isolates obtained from 15 different agriculture habitat samples, using special spore-enhancement method. It was found that these isolates produce very large-sized crystalline protein, which showing differences in shape under phase-contrast microscopy. Total genomic DNA of such isolates showed presence of mega plasmids, ringing from 70 to 125 MD, on fractionation by conventional agarose electrophoreses. The insecticidal activity (Bioassay) of these isolates against *Ephestia larvae* showed the isolates BTA1, BTA4, BTA5 and BTA6 have no pathogenic effects, and no hemothytic activity against human and sheep erythrocyte.

عزل وتوصيف سلالات من بكتيريا الباسلس ثرنجنسس

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تم اعتماد ستة عزلات من بكتيريا *Bacillus thuringiensis*:BTA1, BTA2, BTA3, BTA4, BTA5، BTA6 من أصل عشرة عزلات عزلت من عينات جمعت من 15 منطقة زراعية، باستخدام طريقة خاصة لتعزيز نمو السبورات. وجد ان هذه العزلات تنتج بلورات بروتينية كبيرة الحجم مختلفة الأشكال عند فحصها باستخدام مكيروسكوب الطور المتباين. تم عزل الدنا المجيني الكلي للعزلات تحت البحث حيث أظهرت النتائج احتوائها على بلازميدات كبيرة يتراوح حجمها بين 70- 125 ميكادالتون عند فحصها بطريقة الترحيل الكهربائي بالأكاروز.
جرى اختبار فعالية هذه العزلات في قتل يرقات حشرة عثة التمور *Ephestia* حيث وجد ان العزلات BTA1, BTA4 و BTA6 ليس لديها القدرة على قتل اليرقات ضمن التراكيز المستخدمة كما انها غير قابلة على تحليل كريات الدم الحمراء للانسان والحيوان باستثناء العزلة BTA4 حيث اظهرت فعالية ضعيفة جداً.

- Introduction:

Bacillus thuringiensis is a gram-positive, spore forming bacterium. It seems indigenous to many environments (1), and isolated worldwide from many habitats including soil, insects and stored-product dust (2 , 3). There are number of subspecies which synthesize insecticidal toxin that vary in insect host specificity. the toxic activity is primarily associated with a parasporal crystalline toxin produced during the sporulation cycle(4). The entomocidal activity is associated with glycoprotein subunit of the crystal that controlled by single or small number of genetic loci and consequently should be relatively easy to genetically engineer compared to more complex multigene systems(5). The genome size of *B. thuringiensis* strains is 2.4 to 5.7 milion bp (6). Physical maps had been constructed for two strains of *B. thuringiensis* and compared with that of *B.cereus*, suggests that all of these chromosomes have similar half near replication origin while display great variability in the terminal half (6). A feature common to *B. thuringiensis* subspecies is the presence of plasmids, the number and size (1.4 to 180 MD) vary considerably between strains (7, 8) but independent of serotype and pathotype. Most plasmids are cryptic, the main function assigned to them is the production of entzomocidal toxin. Evidence for a correlation between crystal protein and the presence of a given plasmid was provided by curving experiment leading to loose of large plasmids(9). Farther investigation demonstratated the transfer of such plasmids between *B. thuringiensis* strains through conjugation-like process (10). The aim of this work is to isolate *B. thuringiensis* strains from different sources, characterization of isolates and comparison of their bioactivity (parsporal proteins) against Ephestia larvae.

Materials and Methods

- Isolation of *Bacillus thuringiensis*:

According to the procedure of (11), sixty five samples from 15 agricultural sites in Baghdad, Wasit and Al-Qadesyia governorates were collected and used for isolation of *B. thuringiensis*. Soil sample (2cm below the surface) collected from grain stores and agricultural field. One gram of each sample was suspended in 10 ml sterile distilled water and pasteurized at 80C˚for 30 minutes. One ml of suspension transferred to 9ml of LB broth-sodium acetate medium and incubated at 30C˚ with shaking (150rpm) for 4hrs. Small volume (2ml)from incubated samples were incubated then at 60C˚for1hr. Aliquots of serial dilution were prepared and then subcultured on petri-dishes containing T3 medium for three days at 28c. Colonies with more than 2mm in diameter were picked for subsequent biochemical lecithenase and catalase tests. Colonies that are positive for both tests were selected and then checked by phase contrast for the presence of crystals.

- DNA analysis of BTA isolates:

Each isolates was subcultured in 20ml modified SCGY medium overnight at 30 C˚ with shaking (200rpm). Grown culture was transferred to one little flask containing 25ml SCGY medium and

incubated at the same conditions. The culture was monitored for lag-phase optimization at O.D.600 every hour, until 0.7-0.9 reading was obtained. The culture was centrifuged for 15 min. at 5000rpm with SW-15 rotor. The pellet was washed with TE buffer and centerfugeed again, then resuspended in 6 ml TE25S containing 1mg/ml lysozyme freshly prepared (100µL lysozyme solution), and incubated at 37C˚ for 30min. with gentle agitation, followed by adding 0.18 mg/ml proteinase K (50µL proteins K) to the mixture, then 300µL SDS was added and inqupated for 60min. at 55C˚, one ml. of 5M NaCl was added to the mixture and mixed by inversion followed by adding 0.65ml. CTAB. This mixture has been cooled to 37C˚and mixed with 5ml. Chloroform/isoamyl alcohol by inversion for 30min. and centrifuged at 10000 rpm in SW-60i rotor for 15min. at -20C˚. The supernatant was transferred to a new tube, DNA precipitation was performed by adding 0.6 volume cold isopropanol, mixed by inversion and kept in ice for 30min., precipitated DNA were spooled on to Pasteur pipette, rinsed in 70٪ ethanol, air dried then dissolved in 1ml. TE buffer at 55C˚, and stored at -20C˚. Total genomic DNA analysis of *B. thuringiensis* isolates were performed according to Kiesr, 1984(12).

- **Bioassay against *Ephestia Larvae*:**

Serial dilutions of collected spores for each isolate were prepared and assayed against *Ephestia larvae* as recommended by WHO scientific group on biological control (13).

Third to fourth instars larvae were tested using series of dilutions of NaBr purified spores and cell suspension of each isolate. Bioassay was conducted at 25C˚ (room temperature). Topically applying 100µL of each dilution upon each cube of the agar base artificial diet in the plastic feeding cup. One larva was placed in each cup. Four replicates were made per concentration; mortality was recorded after 24 hours.

Results and discussion

Of the 65 collected samples 10 (15٪) showed positive test for *Bacillus thuringiensis* (table 1), with an average of 6.5 isolates per sample. The richest inhabitant was grain dust from grain stores, which yield 2.5 isolates per positive sample, followed by vegetable-cultivated soil, garden soil and animal contaminated soil.

The viable count of *B. thuringiensis* was scored after acetate selection. A total of 10 *B. thuringiensis* isolates were selected according to the colonial morphology differences (table1). These isolates were highly abundance (8.32 ± 0.27 CFU/g) in soil cultivated with vegetable. Whereas, animal contaminated soil has (0.97 ± 2.57 CFU/g). Numerous studies have found that grain dust and soils are suitable environmental habitats for *B. thuringiensis*(14,15), 70٪ of soil samples collected from 30 countries were found positive in *B. thuringiensis* (16).

Six isolates out of ten were adopted for farther experiment according to large or different shape-crystal exists. All isolates were grow at almost the same rate, sporulation was completed (beginning of lysis) after 10 hours, parasporal-crystals formed at late stage of sporulation, the PH of the culture medium during exponential growth was decreased from 7 to 6.6 at the beginning of sporulation, and then increased to 8 and remained constant during the subsequent stages of sporulation. Crystalline protein, spores and sporangium of the six isolates were shown in the figures (1.1 - 1.10).

In order to show the difference between the six isolates, total genomic DNA was studied , *B. thuringiensis* possesses a chromosomal DNA sized of

2.4 - 5.7 million bp(17), and numerous plasmid from 1.4 to 180Md., with 2 to17 plasmid in each isolate, depending upon subspecies(7).

Two procedures have been adapted to isolate total genomic DNA content CTAB (cetyl trimethyl ammonium bromide) and salting out. Total genomic DNA of BTA isolates by CTAB method is show in Fig.2. All BTA isolates have large plasmid, which differ greatly in size, ranged from 70MD (in BTA2 isolate) to 125 MD (in BTA6 isolate). Molecular weight estimated by using photo Capt-Bio profile program. Most of *B. thuringiensis* plasmids are cryptic; the main function that has been assigned to them is the production of crystalline protein. Evidence for these correlations was provided by curing experiments leading to loss the large plasmid (9). There are many studies on plasmid-curing experiments; showed loss of crystal production was associated with loss of 75MD plasmid in *B. thuringiensis* subspecies *thuringiensis* HD-2, 29MD plasmid in subspecies *alsti* HD-4, and130 MD plasmid in subspecies *galleria* HD-8. These results indicates that only single, large plasmid (size depend on subspecies) are involved in crystalline protein production(18).

Biological activities of six BTA isolates have been summarized in Table (2). The isolates BTA1, BTA5 and BTA6 have no ability to lyse blood cells (human or sheep).wile the rest isolates show hemolytic activity with different extent, from weak (+) to strong (+++), this phenomenon was studded previously, the hemolytic activity due to various virulence factors that are secreted into the culture medium, many of these factors could be the lyses agent like exotoxin (20), or production phospholipids(21), blood lyses can not be assigned to crystalline toxin only when Cry toxin in soluble phase. Also *B. thuringiensis* isolates can produce Cyt toxin, which has the ability to lyses erythrocytes under solublization conditions of such toxin(22).

The second activity of BTA isolates was assayed against insects larvae, results revealed showed the four isolates BTA1, BTA4, BTA5 and BTA6 have no pathogenic effect against *Ephestia larvae* (Table2), it was reported previously that the noninsecticidal *B. thuringiensis* widely distributed in nature than the insecticidal once(3,14). Also the crystalline protein of noninsecticidal strains and producing has another application as anti-tumor. It was reported that the protein associated with parasporal inclusions named “parasporin” has anti-tumor activity against human leukemic cell(24,25).Therefore activated parasporal proteins produced by these isolates will be examined for cytopathic effect on CLL and HeP2 cells in next studies.

Table 1: Abundance of local *Bacillus thuringiensis* isolates obtained from five habitats

Habitat	No. of samples	No. of B. thuringiensis isolates	Average viable count of B. thuringiensis (CFU×107/g)
Grain dust	5	2	4.65 ± 1.32.
Palm cultivated soil	15	1	5.61 ± .98.
Vegetable cultivated soil	15	3	8.32 ± 0.27
Animal contaminated soil	10	1	0.97 ± 2.57
Garden soil	20	3	7.53 ± 1.03
Total	65	10	

Table 2: Bioactivities of *Bacillus thuringiensis* isolates on blood erythrocyte and *Ephestia* larvae

B. thuringiensis isolates	a Hemolytic activity		b Activity against Ephestia larvae
	Sheep erythrocyte	Human erythrocyte	
BTA1	-	-	-
BTA2	++	++	+
BTA3	+	+++	+
BTA4	+	+	-
BTA5	-	-	-
BTA6	-	-	-

a- Hemolytic activity carried out on blood agar base plates.

b- Bioassay carried out against lepidopteran third instar larvae of *Ephestia*.

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