African Journal of Biotechnology Vol. 11(46), pp. 10504-10512, 7 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897/AJB11.2260
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Toxicity of *Bacillus thuringiensis* β-exotoxins and δ-endotoxins to *Drosophila melanogaster*, *Ephestia kuhniella* and human erythrocytes

Maher Obeidat[1], Hala Khyami-Horani[2]* and Fouad Al-Momani[3]

[1]Department of Biotechnology, Faculty of Agricultural Technology, Balqa' Applied University, 19117, Al-Salt, Jordan.
[2]Department of Biological Sciences, Faculty of Science, University of Jordan, Amman 11942, Jordan.
[3]Department of Biological Sciences, Jordan University of Science and Technology, P. O. Box 3030, Irbid, Jordan.

Accepted 10 May, 2012

A total of 73 *Bacillus thuringiensis* (Bt) strains were screened for the presence of non-hemolytic insecticidal β-exotoxin-free δ-endotoxins. Out of them, 45 Bt strains produced δ-endotoxins with specific insecticidal activity against *Drosophila melanogaster* and/or *Ephestia kuhniella* larvae. The thermostable β-exotoxin was observed only in 15 Bt strains and appeared to exhibit dual non-specific insecticidal activity against both *D. melanogaster* and *E. kuhniella* larvae and showed *in vitro* hemolysis for human erythrocytes. It was found that β-exotoxin was produced by Bt strains belonging to five serovars (*israelensis*, *kenyae*, *kurstaki*, *pakistani*, and *tohokuensis*) and two non-serotypable strains. This result suggests that β-exotoxin production is a strain-specific property rather than a serovar-specific property. To our knowledge, this is the first study that demonstrates β-exotoxins production association with Bt strains belonging to serovars *israelensis*, *pakistani*, and *tohokuensis*. The plasmid DNA profiles of some β-exotoxin producing Bt strains shared large plasmid patterns which may have the common β-exotoxin regulatory gene(s). It was found that 16 local Bt strains, 15 of which belonged to five serovars (*aizawai*, *israelensis*, *kurstaki*, *morrisoni*, and *pakistani*) and one was autoagglutinated strain, produced non-hemolytic insecticidal β-exotoxin-free δ-endotoxins. Based on random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), the genotypic relatedness among these 16 Bt strains was investigated. The strains were grouped into two clusters. Bt strains within serovars *israelensis* were grouped in two subclusters, Bt strains within serovars *aizawai* were genomically homogeneous and clustered together, while the other serovars were grouped together in one subcluster. The autoagglutinated strain was clustered within serovar *israelensis*. Thus, these δ-endotoxins can be developed for the use in Bt-based insecticidal preparations.

Key words: Endotoxin, exotoxin, hemolytic, serovar, *thuringiensis*.

INTRODUCTION

Bacillus thuringiensis (Bt) is an aerobic, spore-forming, Gram positive bacterium that produces several toxins, including insecticidal crystal proteins (Cry proteins), vegetative insecticidal proteins (Vips), and β-exotoxins (Schnepf et al., 1998). During sporulation, Bt is characterized by the capability to produce parasporal Cry proteins, also known as δ-endotoxins, toxic to different insect orders (Höfte and Witheley, 1989; Cannon, 1996; Pang et al., 1999). These insecticidal Cry proteins are encoded by plasmid *cry* genes (Crickmore et al., 1998; Schenpf et al., 1998; Pinto and Fiuza, 2002). The δ-endotoxin is known for its pathogenicity and specificity against insects. Therefore, some Bt-based formulations have been developed as biological insecticides in agriculture, forestry, and for the control of insect vectors of human diseases (Sanchis et al., 1996). Additionally, some Bt strains synthesize cytolytic proteins (Cyt) which

*Corresponding author. E-mail: horani-h@ju.edu.jo. Tel: +962779600008. Fax: +96265300435.

are toxic to dipterans and to a large number of eukaryotic cells (Ellar, 1997). During vegetative growth, some Bt strains secrete a thermostable low molecular weight (701 Da), adenine nucleotide analogue known as β-exotoxin or thuringiensin that interferes with RNA biosynthesis, resulting in a broad-spectrum of non-specific toxicity, including invertebrates and vertebrates (Lecadet and de Barjac, 1981). Two types of β-exotoxin have been separated by high performance liquid chromatography (HPLC) (Levinson et al., 1990; Gohar and Perchat, 2001). Type I β-exotoxin is plasmid encoded and composed of adenosine, glucose, and allaric acid (Levinson et al., 1990; Espinasse et al., 2002a, 2002b). Very few studies have dealt with type II β-exotoxin and its structure remains unknown. β-Exotoxin production is a strain-specific property rather than a serovar (subspecies)-specific property (Ohba et al., 1981; Hernández et al., 2001); highly toxic to a number of insect species from different orders. Dipteran larvae appear to be the most susceptible after ingestion of the β-exotoxin (Sebesta et al., 1981). The World Health Organization (WHO) has recommended that Bt strains that produce β-exotoxin should not be used for insect control, or at least that commercial Bt products should be free of β-exotoxin due to their toxicity to mammalians (WHO, 1999, 2007). Thus, detection of β-exotoxin is crucial in the development and production of Bt based biopesticides. Nevertheless, several β-exotoxin preparations have been used effectively to control fly larvae in piggeries, latrine, and compost toilets (Carlberg et al., 1985, 1986).

The current study aimed to screen for non-hemolytic β-exotoxin$^-$-δ-endotoxin$^+$ Bt strains to be used as biological control agents. This aim was achieved by assessment of the toxicity of Jordanian Bt strains against two insect models, *Drosophila melanogaster* and *Ephestia kuhniella* larvae, and against human erythrocytes.

MATERIALS AND METHODS

Bacterial strains

A total of 78 previously isolated Bt strains from Jordanian habitats (Obeidat et al., 2000; Khyami-Horani, 2002, 2003; Al-Momani et al., 2004) were used in this study; 73 of which belonged to 14 serovars [*aizawai* (one strain), *entomocidus* (one strain), *higo* (one strain), *israelensis* (26 strains), *jordanica* (one strain), *kenyae* (seven strains), *kumamotoensis* (two strains), *kurstaki* (20 strains), *malaysiensis* (three strains), *morrisoni* (two strains), *pakistani* (five strains), *sooncheon* (one strain), *thuringiensis* (two strains), and *tohokuensis* (one strain)], one autoagglutinated, and four were nonserotypable (NSP). Three reference strains obtained from the International Entomopathogenic *Bacillus* Collection Center (IEBC), Institute Pasteur, Paris including T03A005 (serovar *kurstaki* HD1), T07001 (serovar *aizawai*) and T14001 (serovar *israelensis*) were used in this study.

Growth of strains for bioassay

Each Bt strain was inoculated into 40 ml of T_3 medium (0.3% tryptone, 0.2% tryptose, 0.15% yeast extract, 0.05 M sodium phosphate, and 0.005% $MnCl_2$) (Travers et al., 1987) and incubated at 37°C for 24 h. Spores and parasporal inclusion bodies were harvested by centrifugation at 4,000 rpm for 10 min at 25°C. The supernatants were autoclaved for 30 min at 121°C to be used for β-exotoxin bioassay (Carozzi et al., 1991). For δ-endotoxin bioassay, pellets (spores and parasporal inclusions) were solubilized in 5 ml of 50 mM Na_2CO_3 (pH 10) containing 10 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA) for 1 h at 37°C (Mizuki et al., 1999). After solubilization, 1 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich, USA-Germany) was added to stop solubilization. The pH of DTT-solubilized pellets was adjusted to 8 by 1 M HCl, and then passed through a 0.45-µM membrane filter (Mizuki et al., 1999, 2000). The protein concentration of the DTT-solubilized pellets was determined by the method of Bradford (1976) and adjusted to 10 µg/ml. Thereafter, five-fold serial dilutions were prepared for toxicity assay.

Insect bioassay

The toxicity of the 78 Bt strains was tested against *D. melanogaster* (Diptera) and *E. kuhniella* (Lepidoptera). The Bt serovars *israelensis* (IEBC No. T14001) and *kurstaki* HD1 (IEBC No. T03A005) were used in δ-endotoxin toxicity assay whereas, Bt serovar *aizawai* (IEBC no. T07001) was used in β-exotoxin toxicity assay. The toxicity assay of δ-endotoxin and β-exotoxin was performed according to the methods of Saadoun et al. (2001) and Cantwell et al. (1964), respectively. For *D. melanogaster* assay, ten 3rd instar larvae were placed into each well of 24-well plates, and 0.6 ml of δ-endotoxin (DTT-solubilized pellet) or β-exotoxin (autoclaved supernatant) was added to each well. The toxicity of each strain was assayed in triplicates. Plates were incubated at 25°C for 24 h. Mortality was scored in comparison with a parallel control of 0.6 ml δ-endotoxin vehicle components except the toxin and of 0.6 ml sterile T_3 media for β-exotoxin assay. For *E. kuhniella*, the toxicity assay was performed in triplicate according to Obeidat et al. (2004). The scored mortality was corrected according to Abbott's formula (Abbott, 1925) and the LC_{50} values of DTT-solubilized pellets were calculated by the regression analysis method (Luo et al., 1993).

Hemolytic activity assessment

Hemolytic activity of δ-endotoxin and β-exotoxin of Bt strains that exhibited larvicidal activity was investigated in this study to be tested on normal human erythrocytes by spectrophotometric method (Saitoh et al., 1998). Blanks for δ-endotoxin and β-exotoxin treatments were prepared by including the vehicle components (all added materials were included but not the toxins). Positive controls using hemolysing buffer (170 mM Tris base, pH 7.2, 0.83% NH_4Cl) were run in parallel. The results were observed and recorded as +++: high hemolysis ($A_{540} > 1.0$; > 30% hemolysis); ++: moderate hemolysis ($1.0 \geq A_{540} > 0.5$; 30% ≥ hemolysis > 15%); +: low hemolysis ($0.5 \geq A_{540} > 0.2$; 15% ≥ hemolysis > 5%), and -: non-hemolytic ($0.2 \geq A_{540}$; 5% ≥ hemolysis).

RAPD-PCR amplification and cluster analysis

All Bt strains that produced non-hemolytic insecticidal β-exotoxin-free δ-endotoxins, as well as the reference strains, were inoculated into 20 ml of Luria-Bertani (LB) broth and incubated overnight at 37°C in an orbital shaker until cultures reached late exponential phase. The bacterial cultures were centrifuged and the bacterial cells were used to isolate total genomic DNA by the Wizard® Genomic DNA Purification Kit (Promega, USA, part number A1120).

After the screening of 40 randomly prepared arbitrary primers, a total of 7 oligonucleotides were used (RP-1; 5′-CAGGCCCTTC-3′, RP-9; 5′-TCATCGCGCT-3′, RP-11; 5′-GCGATCCCCA-3′, RP-16; 5′-CAATCGCCGT-3′, RP-18; 5′-GTGAGGCGTC-3′, RP-24; 5′-TGGACCGGTG-3′, and RP-33; 5′-AAAGCTGCGG-3′). Amplification was carried out in DNA thermal cycler for 40 reaction cycles. Each reaction was carried out in 10 µL; 1 µL of template DNA (1 ng) was mixed with 1X reaction buffer (1 µL), 200 µM deoxynucleoside triphosphate (0.8 µL), 0.4 µM primer (0.4 µL), and 1 U of *Taq* DNA polymerase (1 µL). Template DNA was pre-denatured at 94°C for 2 min, then denatured (1 min at 94°C) and annealed to primers (1 min at 35°C); extensions of PCR products was achieved at 72°C for 2 min. After the 40 cycles were completed, an extra extension step for 6 min at 72°C was carried out. Products were analyzed by electrophoresis on a 1% agarose gel. A Hi-Lo DNA ladder marker (Minnesota Molecular Inc., USA) was used. Generated bands were screened and digitally photographed under ultraviolet (UV) light, and only bright and reproducible bands were scored for the analysis.

Cluster analysis was used to examine genotypic relationships among the tested strains. Amplification products were scored as either present [1] or absent [0]. A data matrix was prepared to determine similarities between each pair of genotypes. The Jaccard's function supported in the software package IBM SPSS statistics version 19 (2011) was used. The matrix was used to construct an unrooted tree using the unweighted pair group method with arithmetic mean (UPGMA). To support the UPGMA groupings on the tree, the data were bootstrapped 490 times using WINBOOT software (Yap and Nelson, 1996).

Plasmid DNA isolation

Bt strains that were found to produce the highest β-exotoxin activity representing different serovars were selected for plasmid DNA isolation. Eight Bt strains were inoculated into 10 ml LB broth and incubated for 12 h with agitation (150 rpm) at 37°C. Thereafter, cells were harvested by centrifugation at 14,000 rpm for 2 min. The plasmid DNA profiles were performed using Wizard® Plus SV Miniprep DNA purification kit (Promega, USA, part number TB225, Cat. No. A1340) according to the manufacturer's instructions. Plasmid DNA profiles were analyzed and compared by electrophoresis on 0.7% horizontal agarose gels. A GeneRuler™ DNA ladder mix (Fermentas, #SM0338, Germany) was used.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). For statistical evaluation of data for β-exotoxin mortality, one-way ANOVA (Tukey's studentized range) was applied using the program IBM SPSS statistics 19.0 for Windows. Significant differences were considered significant at $P < 0.05$.

RESULTS

The insecticidal and hemolytic activities of δ-endotoxins and β-exotoxins of 78 Bt strains were investigated in the current study. It was found that DTT-solubilized δ-endotoxins of 43 Bt strains (42 strains belonging to 12 serovars and one was autoagglutinated) exhibited larvicidal activity against the Diptera, *D. melanogaster*, with LC_{50} ranging from 0.17 to 5.96 ng/ml (Table 1). The highest toxicity was exhibited by 10 local Bt strains belonging to serovars *entomocidus* (J115), *israelensis* (J63 and J74), *kenyae* (J37 and J81), *kurstaki* (J26, J33, and J35) and *pakistani* (J52 and J139). Furthermore, several Bt strains exhibited higher larvicidal activity against *D. melanogaster* than the reference strains T14001 and T03A005. For the lepidopteran *E. kuhniella*, 12 Bt strains of six serovar types (*israelensis*, *kenyae*, *kurstaki*, *morrisoni*, *pakistani* and *thuringiensis*) exhibited larvicidal activity with LC_{50} values higher than the values recorded with *D. melanogaster* (Table 1). Strain J25 (serovar *kurstaki*) showed the highest toxicity (LC_{50} is 8.13 ng/ml). Additionally, two Bt strains (J29 and J69) exhibited larvicidal activity against *E. kuhniella*, but not against *D. melanogaster* (Table 1). Three local Bt strains (J25, J30, and J37) showed higher larvicidal activity against *E. kuhniella* than the reference strain T03A005.

Moreover, 45 Bt strains, which exhibited insecticidal activity to *D. melanogaster* and/or *E. kuhniella*, were screened for their hemolytic activity against human erythrocytes (Table 1). 16 strains [J44 (serovar *aizawai*), J40, J45, J56, J60, J62, J63, J70 and J78 (serovar *israelensis*), J26, J35, and J36 (serovar *kurstaki*), J29 (serovar *morrisoni*), J52 and J139 (serovar *pakistani*), and the autoagglutinated strain (J71)] produced non-hemolytic insecticidal δ-endotoxins. Thermostable β-exotoxins were observed only in 17 strains (Table 2). Interestingly, all β-exotoxin producing Bt strains were found to exhibit dual insecticidal activity against larvae of *D. melanogaster* and *E. kuhniella*. 15 Bt strains belonged to five serovars (*israelensis*, *kenyae*, *kurstaki*, *pakistani*, and *tohokuensis*) and two NSP strains produced thermostable β-exotoxin (Table 2). The corrected mortality against larvae of *D. melanogaster* and *E. kuhniella* ranged from 25.9 ± 6.4 to 100% and from 14.8 ± 6.4 to 100%, respectively. Strains J15, J81, and J28 caused 100% mortality against larvae of *D. melanogaster*. Strains J53, J81, and J32 caused 100% mortality against *E. kuhniella* larvae (Table 2). It was clearly observed that J81 (serovar *kenyae*) gave the highest significant δ-endotoxin and β-exotoxin mortality against *D. melanogaster* larvae, and the highest significant β-exotoxin mortality against *E. kuhniella* larvae (Tables 1 and 2). Furthermore, the 17 Bt strains which produced thermostable β-exotoxin were screened for hemolytic activity (Table 2). All β-exotoxin producers exhibited *in vitro* hemolysis for human erythrocytes ranging from low to high.

Based on RAPD-PCR amplification of Bt strains which produced non-hemolytic insecticidal β-exotoxin-free δ-endotoxins, a dendrogram was constructed to detect genotypic relatedness among the strains (Figure 1). The amplified strains of Bt were allocated to two RAPD clusters (cluster-I and cluster-II); cluster-I was subdivided into three subclusters (IA, IB, and IC). The generated dendrogram from RAPD-PCR patterns revealed that Bt strains were relatively heterogeneous in subcluster-IA, but homogenous within subcluster-IB, subcluster-IC, and

Table 1. Larvicidal and hemolytic activities of Bt δ-endotoxins.

Bt Serovar[a]	Bt Strain	LC_{50}[b] (ng/ml)		Degree of erythrocytes hemolysis[c]
		D. melanogaster	*E. kuhniella*	
aizawai	J44	3.70 (3.06-4.14)	0	-
entomocidus	J115	0.29 (0.11-0.41)	0	++
higo	J77	5.19 (4.54-5.96)	0	+++
	J19	1.85 (1.21-2.49)	0	+++
	J21	1.11 (0.47-1.96)	0	+++
	J40	3.39 (2.03-4.93)	0	-
	J45	3.70 (3.06-4.81)	0	-
	J50	3.33 (1.93-4.36)	0	++
	J56	1.48 (0.52-2.02)	0	-
	J59	5.93 (4.29-7.57)	0	+
israelensis	J60	5.20 (4.55-7.13)	0	-
	J62	2.22 (1.58-3.07)	0	-
	J63	0.17 (0.14-0.29)	0	-
	J66	1.48 (0.84-2.27)	0	+++
	J67	4.44 (3.32-5.55)	0	+++
	J70	4.07 (2.96-5.68)	0	-
	J74	0.27 (0.13-0.39)	51.8 (40.6-65.2)	+
	J78	4.81 (4.16-5.63)	0	-
jordanica	J112	1.13 (0.46-1.78)	0	++
	J13	4.47 (3.32-5.56)	37.1 (24.7-51.3)	+++
	J15	1.11 (0.53-1.76)	0	++
kenyae	J30	5.96 (5.29-7.19)	25.9 (19.4-33.2)	+++
	J37	0.19 (0.14-0.33)	22.2 (15.8-31.6)	+++
	J81	0.26 (0.18-0.43)	0	++
kumamotoensis	J51	4.81 (4.16-5.65)	0	+++
	J06	1.85 (1.22-2.75)	0	+
	J17	4.83 (4.13-5.66)	43.4 (21.8-59.1)	+++
	J25	5.56 (4.92-6.68)	8.13 (1.7-14.5)	+++
	J26	0.18 (0.13-0.31)	0	-
kurstaki	J33	0.23 (0.13-0.38)	0	++
	J34	4.81 (4.16-5.45)	33.3 (17.8-49.6)	+++
	J35	0.27 (0.17-0.41)	44.4 (34.8-52.8)	-
	J36	4.81 (4.16-5.92)	0	-
	J41	5.19 (4.54-6.32)	0	++
	J49	5.90 (5.27-7.55)	0	+
malaysiensis	J20	3.33 (2.13-4.53)	0	+
	J80	4.81 (4.19-5.45)	0	++
morrisoni	J29	0	48.1 (35.7-62.5)	-
	J28	5.19 (4.52-6.79)	0	+++
pakistani	J52	0.19 (0.10-0.39)	0	-
	J139	0.30 (0.23-0.43)	59.3 (43.1-78.0)	-

Table 1. Contd.

sooncheon	J18	2.22 (1.57-3.33)	0	+++
thuringiensis	J23	5.89 (5.29-7.53)	25.7 (18.8-37.3)	+
	J69	0	24.6 (16.6-36.2)	+
Autoagglutinated	J71	0.74 (0.47-1.38)	0	-
Bti	T14001	5.48 (4.84-6.12)	0	++
Btk HD1	T03A005	3.62 (3.28-4.57)	42.3 (34.2-53.4)	-

[a] *Bti* is the reference strain *B. thuringiensis* serovar *israelensis* IEBC No. T14001. *Btk* HD1 is the reference strain *B. thuringiensis* serovar *kurstaki* HD1 IEBC No. T03A005. [b] Numbers between parentheses are 95% fiducial limits. [c]The degree of hemolysis was graded as +++ (high), ++ (moderate), + (low), and – (non-hemolytic).

Table 2. Larvicidal and hemolytic activities of Bt β-exotoxins.

Bt Serovar[a]	Bt Strain	Mortality[b] % of β-exotoxins against		Degree of erythrocytes hemolysis[c]
		D. melanogaster	*E. kuhniella*	
israelensis	J21	77.8±11.1d^{ef}	22.2±11.1^{bc}	+++
	J38	74.1±12.8^{def}	63.0±6.4^{e}	++
	J39	66.7±0.0^{de}	25.9±6.4^{bc}	+++
	J50	74.1±12.8^{def}	88.9±0.0^{f}	+++
	J53	88.9±11.1^{ef}	100±0.0^{g}	++
	J82	74.1±12.9^{def}	63.0±6.4^{e}	+
kenyae	J15	100±0.0^{f}	88.9±11.1^{f}	++
	JN23	55.6±11.2^{cd}	18.5±6.4^{b}	+
	J81	100±0.0^{f}	100±0.0^{g}	++
kurstaki	J31	88.9±0.0^{ef}	66.7±11.1^{e}	+++
	J32	85.2±17.0^{ef}	100±0.0^{g}	++
	J33	63.0±6.4^{cde}	18.5±6.4^{b}	+
	J49	51.9±6.5^{bcd}	25.9±6.4^{bc}	++
pakistani	J28	100±0.0^{f}	88.9±11.1^{f}	+
tohokuensis	J72	25.9±6.4^{b}	40.7±6.4^{d}	+++
Nonserotypable	J43	40.7±6.4^{bc}	55.6±11.2^{de}	+
	JN71	74.1±12.8^{def}	25.9±6.4^{bc}	++
Bta	T07001	77.8±11.1^{def}	66.7±0.0^{e}	++
Control	C	0.0^{a}	0.0^{a}	-

[a]*Bta* is the reference strain *B. thuringiensis* serovar *aizawai* IEBC No. T07001. [b] The mortality was corrected according to Abbott's formula and represented as means ± SD. Means ± SD within column followed by the same letter are not significantly different (Tukey's studentized range test: $\alpha = 0.05$). [c]The degree of hemolysis was graded as +++ (high), ++ (moderate), + (low), and – (non-hemolytic).

cluster-II. In subcluster-IA, Bt strains of serovar *pakistani* (J52 and J139) were clustered together with 99.0% similarity matrix. This high similarity was strongly supported by bootstrap value of 95.6% for the cluster node that grouped them. Moreover, J29 strain (serovar *morrisoni*) clustered with Bt strains belonging to serovar *pakistani* supported by a good bootstrap value (83.7%). Interestingly, all Bt strains of serovar type *kurstaki*, including the reference strain (IEBC No. T03A005) and the local strains (J26, J35, and J36) clustered in subcluster-IA. The Bt strains of serovar type *israelensis* clustered together in subcluster-IB with a high bootstrap value (98.2%) and subcluster-IC with 87.9% bootstrap value (Figure 1). The reference strain *Bt israelensis* (IEBC No. T14001) clustered in subcluster-IC with the local Bt strains of the same serovar type and showed

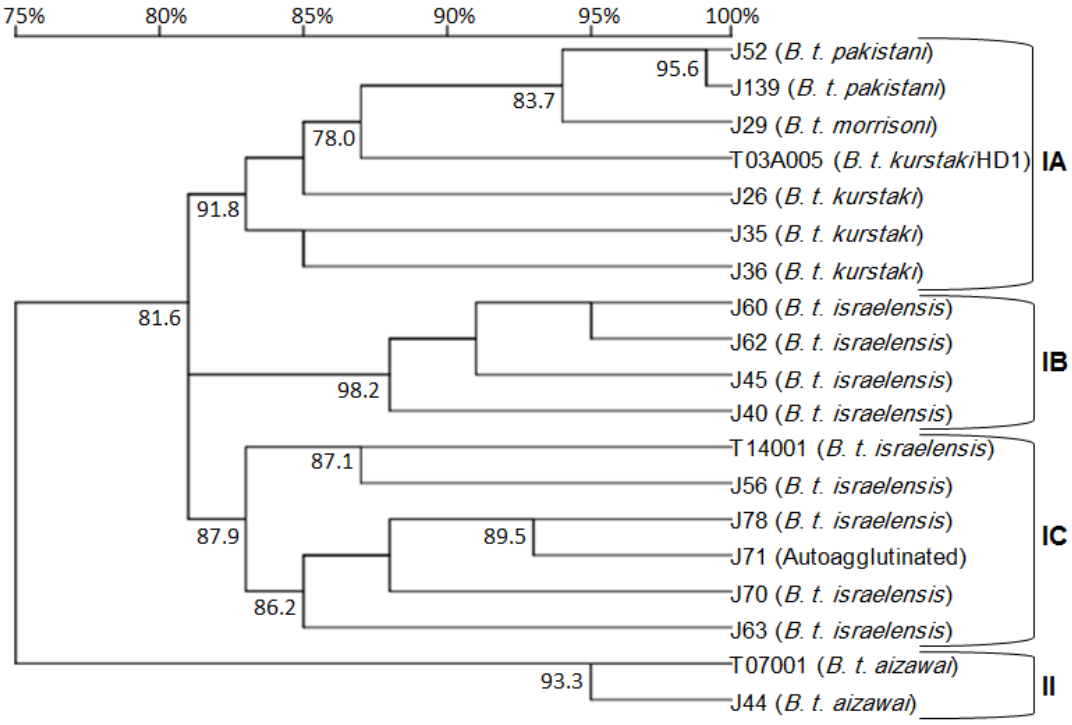

Figure 1. Dendrogram based on RAPD-PCR patterns of tested *B. thuringiensis* strains using the UPGMA clustering algorithm. The numbers at the nodes are bootstrap values, and are expressed as percentages of 490 bootstrap replications.

87.0% similarity matrix to the local strain J56. The autoagglutinated strain was also clustered with serovar *israelensis* and had 93.1% similarity to the local strain J78. The reference strain IEBC No. T07001 and the local strain J44 which belonged to serovar *aizawai* were clustered together in cluster-II with 94.9% similarity and supported by a high bootstrap values (93.3%). Moreover, the plasmid DNA was isolated from eight Bt strains that exhibited the highest β-exotoxin activity (Figure 2). Different profile patterns were observed: three common monomorphic large bands (>10 kbp) were produced by the investigated strains.

DISCUSSION

The main goal of the current study was to determine the distribution of insecticidal δ-endotoxins and β-exotoxins among 78 Jordanian Bt strains in an attempt to develop a non-hemolytic β-exotoxin$^-$-δ-endotoxin$^+$ insecticide. In this study, more than 50% (45 strains) of the total Bt strains were found to produce toxic δ-endotoxins to *D. melanogaster* and/or *E. kuhniella* larvae. 44 of them belonged to 13 serovars (*aizawai*, *entomocidus*, *higo*, *israelensis*, *jordanica*, *kenyae*, *kumamotoensis*, *kurstaki*, *malaysiensis*, *morrisoni*, *pakistani*, *sooncheon* and *thuringiensis*), and one strain was autoagglutinated. It was observed that larvae of *E. kuhniella* were intoxicated by 12 Bt strains, whereas *D. melanogaster* larvae were intoxicated by 43 Bt strains with lower LC_{50} values, suggesting that *D. melanogaster* larvae are more susceptible to Bt δ-endotoxins than *E. kuhniella* larvae. This difference in the susceptibility levels could be correlated to the fact that δ-endotoxin is host specific (Sanchis et al., 1996).

The specificity of δ-endotoxins might vary due to the difference in the target insect species which differ in their susceptibility to the mode of action of the δ-endotoxins, or due to the difference in their ability to repair the injured cells of the gut, or even due to the insect gut conditions (Karamanlidou et al., 1991). On the other hand, several local Bt strains showed higher larvicidal activities to *D. melanogaster* and *E. kuhniella* than the tested reference Bt strains. It was suggested by Jaquet et al. (1987) that the difference in strains could play a role in their toxins specificity. Therefore, the difference at strain level between local and reference Bt could be responsible for the high toxicity effect of some local strains in comparison to the reference strains. Out of the 45 toxic Bt strains (Table 1), 29 produced hemolytic δ-endotoxins [7 of them (J21, J50, J15, J81, J33, J49, and J28) produced hemolytic β-exotoxins]. The hemolytic activity of δ-endotoxins might be correlated to the presence of Cyt proteins, which is known for their cytolytic activity toward erythrocytes (Chow et al., 1989). It was observed that 16 Bt strains produced non-hemolytic insecticidal δ-

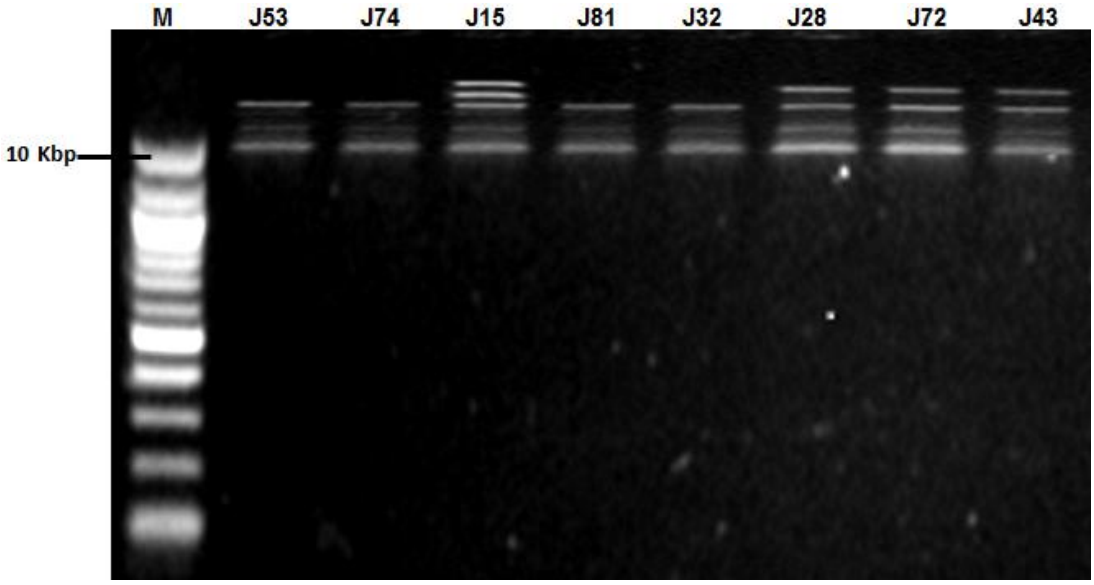

Figure 2. Plasmid DNA profiles of Bt strains belonging to five serovars that exhibited the highest β-exotoxin activity. M, GeneRuler™ DNA ladder mix; J53 and J74 (serovar *israelensis*), J15 and J81 (serovar *kenyae*), J32 (serovar *kurstaki*), J28 (serovar *pakistani*), J72 (serovar *tohokuensis*) and J43 (nonserotypable).

endotoxins and were free from β-exotoxins (Tables 1 and 2). Thus, biological products of these strains can be used in Bt-based formulations for biocontrol of insects. Furthermore, the current study demonstrates that Jordan soils are rich sources for isolation of Bt strains that produce insecticidal non-hemolytic β-exotoxin$^-$-δ-endotoxin$^+$. Additionally, β-exotoxin can be added in low amounts to δ-endotoxin-based bio-insecticides to give synergistic insecticidal effect against target insects.

Although *D. melanogaster* and *E. kuhniella* belonged to different insect orders, both of them were susceptible to β-exotoxin. Moreover, β-exotoxin showed hemolytic activity against human erythrocytes, indicating that β-exotoxin is non-specific and exhibit wide spectrum of toxicity against invertebrates and vertebrates. This finding is in agreement with findings of Lecadet and de Barjac (1981). The broad spectrum of β-exotoxin toxicity is contributed to β-exotoxin inhibition for RNA polymerases by acting competitively with ATP (Faust, 1973; Farkaš et al., 1977). Since RNA synthesis is a vital process in all life, β-exotoxin exerts its toxicity for almost all forms of life. Therefore, detection of β-exotoxin before development and production of Bt-based biopesticides is required. Studies relating β-exotoxin production from several Bt strains belonging to different Bt serovar types are very limited. Therefore, the present work shed some light on the possible relationship between β-exotoxin producing strains type and the type of serovars. The results indicate that Bt strains that produced β-exotoxin belonged to serovars *israelensis*, *kenyae*, *kurstaki*, *pakistani*, and *tohokuensis*. In addition, two NSP strains produced β-exotoxin, whereas none of the Bt strains belonging to serovars *aizawai*, *entomocidus*, *higo*, *jordanica*, *kumamotoensis*, *malaysiensis*, *morrisoni*, *sooncheon* and *thuringiensis* produced β-exotoxin. Several other studies (Cantwell et al., 1964; Mohd-Salleh et al., 1980; Ohba et al., 1981; Levinson et al., 1990; Hernández et al., 2001) showed that β-exotoxin was associated with certain Bt serovars, including; *aizawai*, *alesti*, *darmstadiensis*, *galleriae*, *kenyae*, *kurstaki*, *morrisoni*, *sotto*, *tenebrionis*, *tolworthi*, and *thuringiensis*. However, Ohba et al. (1981) and Hernández et al. (2001, 2003) demonstrated that β-exotoxin production is a strain-specific property rather than a serovar-specific property. This is in agreement with the present study.

A good genotypic relationship among local strains and with the reference strains was demonstrated by the constructed dendrogram for Bt strains which produced non-hemolytic insecticidal β-exotoxin-free δ-endotoxins. Most Bt strains of the same serovar type were clustered together. The autoagglutinated strain could not be typed by serotyping, but was successfully amplified by RAPD-PCR and clustered with serovar *israelensis*, suggesting that RAPD-PCR amplification is a reliable tool for classification of Bt serovars. Furthermore, development of this simple molecular approach could be very informative and beneficial, particularly with respect to screening procedures such as the H-classification method. This is in accordance with the reports of Hansen et al. (1998) and Gaviria Rivera and Priest (2003). Bt strains of serovars *israelensis* and *aizawai* were genomically heterogeneous (Figure 1). Vilas-Bôas et al. (1998) and de Maagd et al. (2001) suggested that this genomically heterogeneous probably occurred through natural plasmid DNA exchange that has been implicated

as the source of the remarkable diversity of crystal protein genes in these bacteria.

Previous studies (Ozawa and Iwahana, 1986; Levinson et al., 1990) also demonstrate that production of β-exotoxin is a plasmid-related property. The involvement of a large plasmid in β-exotoxin production has been proved by curing and conjugation-like transformation experiments (Ozawa and Iwahana, 1986; Levinson et al., 1990). Recently, it was proposed that the genetic determinants responsible for β-exotoxin production, found in Cry-dependent plasmids, are regulatory elements (Espinasse et al., 2002b). Thus, any Bt strain (β-exotoxins$^-$) from any serovar type has a possibility for gaining plasmid(s) encoding β-exotoxin regulatory elements from another Bt strain (β-exotoxins$^+$) by natural conjugation or conjugation-like processes in the environment. This may explain why β-exotoxin, in this study, was produced from Bt strains belonging to serovar types comprising *israelensis*, *pakistani*, and *tohokuensis* serovars. Moreover, plasmid DNA profiles of Bt strains that produced β-exotoxin shared three large bands (>10 kbp), suggesting that the genes regulating synthesis of β-exotoxin or part of its structure could be encoded by common plasmid(s) bands.

REFERENCES

Abbott WS (1925). A method of computing the effectiveness of insecticides. J. Econ. Entomol., 18: 265-267.

Al-Momani F, Obeidat M, Saadoun I, Meqdam M (2004). Serotyping of *Bacillus thuringiensis* isolates, their distribution in different Jordanian habitats and pathogenicity in *Drosphila melanogaster*. W. J. Microbiol. Biotechnol., 20: 749-753.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Cannon RJC (1996). *Bacillus thuringiensis* use in agriculture: a molecular perspective. Biol. Rev. Camb. Phil. Soc., 71: 561-636.

Cantwell GE, Heimpel AM, Thompson MJ (1964). The production of an exotoxin by various crystal-forming bacteria related to *Bacillus thuringiensis* var. *thuringiensis* Berliner. J. Insect Pathol., 6: 466-480.

Carlberg G (1986). *Bacillus thuringiensis* and microbial control of flies. MIRCEN J., 2: 267-274.

Carlberg G, Kihamia CM, Minjas J (1985). Microbial control of flies in latrines in Dares Salaam with a *Bacillus thuringiensis* (serotype 1) preparation, Muscabac. MIRCEN J. Appl. Microbiol. Biotechnol., 1: 33-44.

Carozzi N, Kramer V, Warren G, Evola S, Koziel M (1991). Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol., 57(11): 3057-3061.

Chow E, Singh G, Gill S (1989). Binding and aggregation of the 25-kilodalton toxin of *Bacillus thuringiensis* subsp. *israelensis* to cell membranes and alteration by monoclonal antibodies and amino acid modifiers. Appl. Environ. Microbiol., 55(11): 2779-2788.

Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev., 62: 807-813.

de Maagd R, Bravo A, Crickmore N (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet., 17: 193-199.

Ellar DJ (1997). The structure and function of *Bacillus thuringiensis* δ-endotoxins and prospects for biopesticide improvement. BCPC Symp. Proc., 68: 83-100.

Espinasse S, Gohar M, Chaufaux J, Buisson C, Perchat S, Sanchis V (2002a). Correspondence of high levels of β-exotoxin I and the presence of cry1B in *Bacillus thuringiensis*. Appl. Environ. Microbiol., 68: 4182-4186.

Espinasse S, Gohar M, Lereclus D, Sanchis V (2002b). An ABC transporter from *Bacillus thuringiensis* is essential for β-exotoxin I production. J. Bacteriol., 184: 5848-5854.

Farkaš J, Šebesta K, Horská K, Samek Z, Dolejš L, Šorm F (1977). Structure of thuringiensin, the thermostable exotoxin from *Bacillus thuringiensis*. Coll. Czech. Chem. Commun., 42: 909-929.

Faust RM (1973). The *Bacillus thuringiensis* β-exotoxin: Current status. Bull. Entomol. Soc. Am., 19: 153-156.

Gaviria RAM, Priest FG (2003). Molecular typing of *Bacillus thuringiensis* serovars by RAPD-PCR. Syst. Appl. Microbiol., 26: 254-261.

Gohar M, Perchat S (2001). Sample preparation for beta-exotoxin determination in *Bacillus thuringiensis* cultures by reversed-phase high-performance liquid chromatography. Anal. Biochem., 298: 112-117.

Hansen B, Damgaard P, Eilenberg J, Pedersen J (1998). Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated from leaves and insects. J. Invert. Pathol., 71: 106-114.

Hernández CS, Ferré J, Larget-Thiéry I (2001). Update on the detection of β-exotoxin in *Bacillus thuringiensis* strains by HPLC analysis. J. Appl. Microbiol., 90: 643-647.

Hernández CS, Martínez C, Porcar M, Caballero P, Ferré J (2003). Correlation between serovars of *Bacillus thuringiensis* and type I β-exotoxin production. J. Invertebr. Pathol., 82: 57-62.

Höfte H, Whitheley HR (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev., 53: 242-255.

Jaquet F, Hutter R, Luthy P (1987). Specificity of *Bacillus thuringiensis* delta-endotoxin. Appl. Environ. Microbiol., 53(3): 500-504.

Karamanlidou G, Lambropoulos A, Koliais S, Manousis T, Ellar D, Kastritsis C (1991). Toxicity of *Bacillus thuringiensis* to laboratory populations of the olive fruit fly (*Dacus oleae*). Appl. Environ. Microbiol., 57(8): 2277-2282.

Khyami-Horani H (2002). Toxicity of *Bacillus thuringiensis* and *B. sphaericus* to laboratory populations of *Drosophila melanogaster* (Diptera: Drosophilidae). J. Basic Microbiol., 42: 105-110.

Khyami-Horani H, Hajaij M, Charles JF (2003). Characterization of *Bacillus thuringiensis* ser. *jordanica* (serotype H 71), a novel serovariety isolated in Jordan. Curr. Microbiol., 47: 26-31.

Lecadet MM, de Barjac H (1981). *Bacillus thuringiensis* beta-exotoxin. In: Davidson EW (ed) Pathogenesis of Invertebrate Microbiol Diseases, Allanheld O and Co., Totowa, NJ, USA, pp. 293-316.

Levinson BL, Kasyan KJ, Chiu SS, Currier TC, González Jr JM (1990). Identification of β-exotoxin production, plasmids encoding β-exotoxin, and a new exotoxin in *Bacillus thuringiensis* by using high-performance liquid chromatography. J. Bacteriol., 172: 3172-3179.

Luo X, Gu B, Jiang H, Shen J, Yu Z (1993). Assay of toxicity and standardization of *Bacillus thuringiensis* products. In: Yu Z (ed) Production and Application of *Bacillus thuringiensis* Preparations, Agricultural Pub. House, Beijing, pp. 167-202.

Mizuki E, Ohba M, Akao T, Yamashita S, Saitoh H, Park YS (1999). Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell-killing action on human cancer cells. J. Appl. Microbiol., 86: 477-486.

Mizuki E, Park YS, Saitoh H, Yamashita S, Akao T, Higuchi K, Ohba M (2000). Parasporin, human leukemic cell-recognizing parasporal protein of *Bacillus thuringiensis*. Clin. Diagn. Lab. Immunol., 7(4): 625-634.

Mohd-Salleh MB, Beegle CC, Lewis LC (1980). Fermentation media and production of exotoxin by three varieties of *Bacillus thuringiensis*. J. Invertebr. Pathol. 35: 75-83.

Obeidat M, Al-Momani F, Saadoun I (2000). Diversity of *Bacillus thuringiensis* in different habitats of nothern Jordan. J. Basic Microbiol., 40(5-6): 385-388.

Obeidat M, Hassawi D, Ghabeish I (2004). Characterization of *Bacillus thuringiensis* strains from Jordan and their toxicity to the Lepidoptera, *Ephestia kuehniella* Zeller. Afr. J. Biotechnol., 3(11): 622-626.

Ohba M, Tantichodok A, Aizawa K (1981). Production of heatstable

exotoxin by *Bacillus thuringiensis* and related bacteria. J. Invertebr. Pathol., 38: 26-32.
Ozawa K, Iwahana H (1986). Involvement of a transmissible plasmid in heat-stable exotoxin and δ-endotoxin production in *Bacillus thuringiensis* subsp. *darmstadiensis*. Curr. Microbiol., 13: 337-340.
Pang ASD, Gringorten JL, Bai C (1999). Activation and fragmentation of *Bacillus thuringiensis* δ-endotoxin by high concentrations of proteolytic enzymes. Can. J. Microbiol., 45: 816-825.
Pinto LMN, Fiuza LM (2002). Genes Cry de *Bacillus thuringiensis*: uma alternative biotecnológica aplicada ao manejo de insetos. Biociências, 10(2): 3-13.
Saadoun I, Al-Momani F, Obeidat M, Meqdam M, Elbetieha A (2001). Assessment of toxic potential of local Jordanian *Bacillus thuringiensis* isolates on *Drosophila melanogaster* and *Culex*. J. Appl. Microbiol. 90: 1-7.
Saitoh H, Higuchi K, Mizuki E, Hwang S-H, Ohba M (1998). Characterization of mosquito larvicidal parasporal inclusions of a *Bacillus thuringiensis* serovar *higo* strain. J. Appl. Microbiol., 84: 883-888.
Sanchis V, Chaufaux J, Lereclus D (1996). Amélioration biotechnologique de *Bacillus thuringiensis:* les enjeux et les risques. Ann. Inst. Pasteur/Actualités, 7: 271-284.
Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev., 62: 775-806.
Sebesta K, Farkas J, Horska K, Vankova J (1981). Thuringiensin, the beta-exotoxin of *Bacillus thuringiensis*, In: Burgess HD (ed), Microbial Control of Plant Pests and Diseases 1970-1980, Academic, London, pp. 249-277.
Travers R, Martin P, Reichelderfer C (1987). Selective process for efficient isolation of soils *Bacillus* spp. Appl. Environ. Microbiol., 53: 1263-1266.
Vilas-Bôas G, Vilas-Bôas L, Lereclus D, Arantes O (1998). *Bacillus thuringiensis* conjugation under environmental conditions. FEMS Microbiol. Ecol., 25: 369-374.
WHO (1999). Guideline Specification for Bacterial Larvicides for Public Health Use. WHO Document WHO/CDS/CPC/WHOPES/99.2. World Health Organization, Geneva.
WHO (2007). WHO Specifications and Evaluations for Public Health Pesticides: *Bacillus thuringiensis* Subspecies *israelensis* Strain AM65-52. World Health Organization, Geneva.
Yap IV, Nelson RJ (1996). WINBOOT: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms IRRI Discussion paper Series No. 14, International Rice Research Institute, Manila, Philippines.