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Isolation and Genetic Characterization of Native *Bacillus thuringiensis* Strains Toxic to *Spodoptera littoralis* and *Culex pipiens*

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ABSTRACT

Eight *Bacillus thuringiensis* strains (Sn-2, Gh-4, Ts-5, Is-8, Qa-2, Fa-7, As-3 and As-4) were isolated from soils of seven Egyptian governorates and identified according to their morphology, presence of parasporal crystals and a 1400 bp band PCR amplification of the 16S rRNA gene. A bioassay revealed that Ts-5 and As-3 were highly toxic to 2nd instar larvae of cotton leafworm (*Spodoptera littoralis*) with 100 and 90% mortality, respectively after 4 feeding days. After 7 days, Ts-5 and As-4 were toxic with similar mortality values, while toxicity caused by As-3 reached 85%. Two other isolates, Qa-2 and Fa-7, displayed high toxicity (75%) after 4 and 7 feeding days. Moreover, As-4 and Fa-7 showed high mortality (90 and 80%) against northern house mosquito (*Culex pipiens*) larvae after a two-day feeding period and most isolates showed more than 50% mortality at half of the original concentration (1.5×10^7 cells/ml). The eight isolates were resistant to six antibiotics and five of them showed variable patterns to gentamycin (Gm) and neomycin (Nm). A plasmid profile revealed divergent patterns in the number, molecular size and existence of plasmids, whereas Ts-5 and Sn-2 showed the highest number (7) and Qa-2 the lowest (3). A 3-kb plasmid was found in all isolates and the seven other plasmids varied noticeably among the eight isolates. SDS-PAGE analysis of the spore/crystal mixture individually characterized each of the eight isolates. Protein analysis revealed that each of the eight isolates possess a unique protein pattern either in their absence, presence or in the total numbers, even between isolates from the same location, such as As-3 and As-4, although they displayed a similar total variable 10 bands, but varied in the appearance among the bands.

Keywords: 16S rRNA gene, new native *Bt* isolates, plasmid and SDS-PAGE profiles, toxicity against *Culex pipiens* and cotton leafworm

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a naturally occurring soil-borne, Gram-positive bacterium that produces an insecticidal crystal protein toxin, δ-endotoxin, during sporulation (Schnepf *et al.* 1998). The increasingly rapid characterization of new crystal protein genes, triggered by efforts to discover proteins with new pesticidal properties, has resulted in a variety of sequences and activities (Crickmore *et al.* 1998).

In order to broaden the host range spectrum of the appropriate bacterial host for insect control, several studies have been carried out to construct novel *Bt* strains with different insecticidal activities. For instance, Tanapongpipat *et al.* (2003) constructed a recombinant plasmid p4BDA-5142 harboring *c*ry4A, *c*ry4B and *c*ry11A from *Bt israelensis* and binary toxin genes from *B. sphaericus*. Their recombinant *E. coli* strain harboring p4BDA-5142 exhibited broad range mosquito-larvicidal activity against all *Aedes*, *Culex* and *Anopheles* larvae.

Alternatively, worldwide screening efforts have been based on the possible existence of new strains with new pathogenic spectra or host ranges (Alberola *et al.* 1999). For instance, seven *Bt* strains were isolated from soils of seven Egyptian governorates and characterized by their higher toxicity against *Spodoptera littoralis* than the two *Bt* standards (HD-1 and HD-24) and a commercial product (Dipel 2x). The seven *Bt* isolates were uniquely different in resistance patterns, plasmid numbers and SDS-PAGE patterns (Nariman *et al.* 2003). In Senegal, screening programs were developed by Aïdara *et al.* (1998) to isolate new strains from various sites and insect samples, which led to the isolation of 194 *Bt* and 9 *B. sphaericus* strains that were toxic to two major malaria vectors, *Anopheles gambiae* and *A. arabiensis*. In Brazil, 280 *Bt* isolates were characterized by the presence of crystal protein genes detected by PCR and were selected according to their geographic origin for genetic characterization. The plasmid profiles of 95 isolates among them showed a remarkable diversity not observed in the 16S rRNA gene. These suggested that the genetic diversity of *Bt* species results from the influence of different ecological factors and spatial separation between strains generated by the conquest of different habitats (Vilas-Boas and Lemos 2004). In Mexico, Tamez-Guerra *et al.* (2004) isolated four *Bt* strains characterized by their high toxicity against Lepidopteran and Coleopteran pests. One *Bt* strain isolated from soil collected at mushroom houses showed high toxicity to mushroom flies, *Lycoriella mali* and *Coboldia fuscipes*. Although the plasmid and SDS-PAGE protein profiles of the isolated strain were similar to those of its reference strain, PCR analysis showed that it is a unique strain with respect to gene type (Choi *et al.* 2004). Another study by Chen *et al.* (2002) demonstrated that the wild type *Bt* strain 15A3 belonging to subspecies *colmeri* produced a broad spectrum with high toxicity against three Lepidopteran pests: *Heliothis armigera*, *Spodoptera exigua* and *Heliothis cunea*.

Bt has been isolated from a range of environments, including insects, soil, dust from stored grain and leaves (Bernhard *et al*. 1997). However, it has been suggested that the normal habitats for this organism are soil (Martin and Travers 1989) and phylloplanes (Mizuki *et al*. 1999). As

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there are problems of approval and acceptance for genetically engineered organisms (Hilder and Boulter 1999), the long-established approach of isolating naturally existing strains with novel toxicities is an attractive alternative that has been very successful (Feitelson *et al*. 1992). Endospore-forming 124 *Bt*-like strains were isolated from 223 samples collected from agricultural and non-cultivated soils in Tamilnadu, India, water and dead insects and SDS-PAGE of spore-crystal mixtures revealed a diverse genetic background; strains were differentiated into 16 distinct protein profiles (Mahalakshmi *et al.* 2005). Accordingly, the authors isolated one naturally occurring novel *Bt* among seven that was toxic to both *Culex quinquefasciatus* and *Aedes aegypti*. Novel insecticidal bacteria, with an extended target spectrum are an environmentally safe biocontrol practice that can lead to increased food production and postharvest protection (Van Frankenhuyzen 1993).

The present work aimed to identify new native *Bt* strains isolated from soils of different Egyptian governorates with more toxic potency toward a Lepidopteran insect pest (*Spodoptera littoralis*) and a Dipteran mosquito (*Culex pipiens*), than any other *Bt* strains currently used. In addition, we genetically characterized such isolate strains using different techniques: antibiotic resistant patterns, 16S rRNA gene, plasmid profile and SDS-PAGE protein analysis.

MATERIALS AND METHODS

Isolation and identification of *Bt* from soil

Soil samples were collected from the surface to a depth of 10 cm of different fields belonging to seven governorates: Sinai, Gharbyia, Toshkey, Ismailyia, Qaluobyia, El-Fayioum and Aswan (**Table 1**). *Bt* strains were isolated by selective sample enrichment (Travers *et al*. 1987) and subsequent plating on nutrient agar medium for five days at 30°C. *Bt*-like colonies were checked for the presence of parasporal crystals (Rampersad and Ammons 2005) by a phase contrast microscope using admidoschwartz stain (Smirnoff 1962). The eight *Bt* isolate strains were grown at 30°C overnight in Luria broth medium containing 2% tryptone, 1% yeast extract, and 1% NaCl with shaking.

Bioassay of *Bt* isolates against *Spodoptera littoralis* and *Culex pipiens* larvae

Eight Egyptian *Bt* isolates were bioassayed against 2nd larval instar of *S. littoralis* and *C. pipiens* that were maintained in the lab of the Pests and Plant Protection Department. Three concentrations (I, II and III) of the eight *Bt* cultures were used: I = 1.5×10^7 cells/ml, II = dilution to half of the original concentration (I/2) and III = dilution to half of II's concentration (II/2). Each of three newly moulted 2nd larval instars of *S. littoralis* were assigned as one replicate for each concentration (10 larvae/concentration/isolate), while the fourth group was used as a check (control); the experiment was replicated three times. Castor oil (*Ricinus communis*) leaves were used for larval feeding and leaves were treated using a dipping technique according to Salama *et al.* (1991), while the check group was dipped in distilled water only. Treated leaves were left at room temperature until dry before being offered to the larvae. After 4 days the treated leaves were substituted with fresh untreated castor oil leaves. The percentage mortality was calculated after 4 and 7 days post treatment. A bioassay against *C. pipiens* followed the method of Goldberg and Margalit (1977) in which the larvae were hatched and raised in ddH_2O at room temperature for 10 to 12 days and fed a diet of ground fish food (TetraMin) purchased from Aquatic Superstore, Birmingham, UK. The percentage mortality was calculated after one and two days post treatment.

Table 1 Location of Egyptian *Bt* isolates used in this study.

Bacterial isolates	Source of isolation
Sn-2	Sinai
Gh-4	Gharbyia
Ts-5	Toshkey
Is-8	Ismailyia
Qa-2	Qaluobyia
Fa-7	El-Fayioum
As-3	Aswan
As-4	Aswan

DNA extraction and PCR amplification of 16S rRNA gene

DNA was extracted from the eight isolates following the method of Ben-Dov (1999). Genomic DNA was prepared from an exponential phase overnight in Luria-Bertani medium. Aliquots of 10 ml of bacterial culture were harvested by centrifugation at 12,000 rpm for 15 min and washed once in sterile distilled water. the pellets were resuspended in 400 µl of lysis buffer containing 2% glucose, 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 3 mg/ml lysozyme and 200 mg/ml RNase. The cell suspension was incubated for 1 h at 37°C. Further DNA extraction was performed as described by Sambrook *et al.* (1989).

The PCR products were separated in a 1.2% agarose gel containing 0.5 mg/ml of ethidium bromide and were visualized using Gel Doc XR System (Bio-Rad Laboratories, Inc., Cali, USA). In order to rapidly identify the *Bt* isolates from other closely related spore-forming *Bacillus* species existing in the soil, the 16S rRNA gene was amplified using two accessions (AM292029 and EU702408) containing the sequences of the *Bt*-16S rRNA gene. They were obtained from the NCBI GenBank and multalin to design a pair of primers: Forward (5′-agagtttgatcctggctcag-3′) and reverse (5′- TACGGCTACCTTGTTACGACTT-3′) with a final product size of 1400 bp.

Amplification was performed according to Xu and Côté (2003) in a thermal cycler, GeneAmp 9600 Perkin Elmer (Martinsburg, West Virginia, USA) in a total volume of 25 µl containing 50 ng DNA, 1 mM of each primer, 200 mM dNTP, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Promega, Madison, USA). PCR was performed under the following conditions: 5 min at 95°C and then 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C and a final extension step at 72°C for 5 min.

Antimicrobial susceptibility and plasmid isolation

Eleven antibiotics (Sigma, St. Louis, Missouri, USA) were used with final concentrations in (µg/ml) according to Dionisio *et al.* (2002) as follows: rifampicin (Rif) 100, ampicillin (Amp) 100, amikacine (Amk) 30, streptomycin (Sm) 200, kanamycin (km) 40, tetracycline (Tc) 15, chloramephincol (cm) 35, gentamycin (Gm) 15, polymyxin (Pmx) 50, neomycin (Nm) 40 and erythromycin (Erm) 20. The Kirby-Bauer disc diffusion method for antimicrobial susceptibility test was used (NCCLS 1999). Plasmids of the eight *Bt* isolates were isolated using the mini-prep of Rodriguez and Tait (1983). Electrophoresis was performed using a 0.7% agarose gel.

Preparation and analysis of spore/crystal mixture

The eight *Bt* isolates were grown in suspension following the method of Alberola *et al.* (1999). 100 ml of nutrient broth was inoculated into 500 ml flasks with one loop of bacteria and shaken for 3 days at 30°C (220 rpm). At the end of this incubation, the majority of the population was in the form of free spores and crystals (less than 5% vegetative cells). The suspension was centrifuged for 10 min at 10,000 rpm at 4°C; the pellet was washed twice in water and resuspended in 4 ml of water. This suspension was adjusted with water to give $Abs_{600\ nm} = 15$. The supernatants of toxic samples were autoclaved (121°C for 10 min). Colonies were resuspended into 1 ml of ice-cold 0.5 M NaCl. The cells were centrifuged at 13,000 rpm for 5 min and the pellet was resuspended in 1% SDS, 0.01% β-mercaptoethanol, boiled for 10 min, and recentrifuged at 13,000 rpm for 10 min. The supernatant was removed and analyzed by 15% SDS-PAGE according to Von Tersch and Gonzalez (1994).

Statistical analyses

Bioassay data were subjected to analysis of variance (ANOVA) using SPSS v. 10. Means of each treatment were compared using Duncan's Multiple Range analysis (Duncan 1955).

RESULTS

PCR amplification of 16S rRNA in *Bt* isolates

PCR amplification of the eight *Bt* isolates revealed one fragment with 1400 bp that represented the 16S rRNA gene (**Fig. 1**).

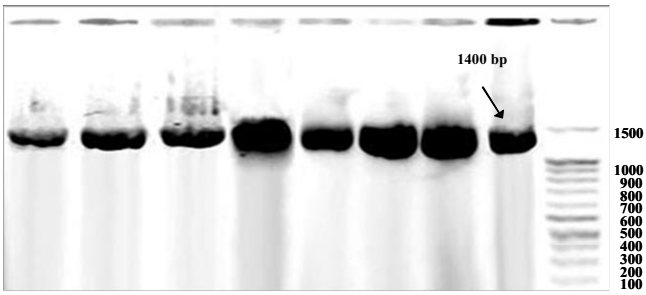

Fig. 1 PCR amplified the 16S rRNA gene with a fragment of 1400 bp in eight Egyptian *Bt* isolates using 0.7% agarose gel electrophoresis.

Toxicity bioassay against *Spodoptera littoralis* and *Culex pipiens* larvae

Results of the bioassay against *S. littoralis* presented in **Table 2** show that the original *Bt* concentration I was highly significant toxic to three isolates (Ts-5, As-3 and As-4) after four days feeding, whereas Ts-5 and As-3 reached 100% mortality and As-4 90% mortality. The three isolates steadily displayed high mortality at concentrations II and III, but at lower percentages than conc. I. After seven days feeding, Ts-5 and As 4 were significantly toxic with similar mortality percentages as showed for conc. I after four days, while mortality percentage of As-3 was reduced to 85%. After seven days, the three isolates maintained their high mortality percentages at concs. II and III, but with lower values compared with mortality percentages after a four-day feeding period. Moreover, another two isolates (Qa-2 and Fa-7) displayed highly significant toxicity with lower percentage values compared with the three former isolates, where Qa-2 displayed 75 and 70% mortality at the original conc. I after four and seven feeding days, respectively. Fa-7 showed 60 and 75% mortality at conc. I after 4 and 7 feeding days, respectively. However, mortalities of Qa-2 and Fa-7 were dramatically reduced to lower percentages at conc. III with 25 and 30% after 4 days and 20 and 45% after 7 days, respectively. The three other isolates (Sn-2, Gh-4 and Is-8) revealed fluctuating mortality and mostly inconsequential percentage values, as shown in **Table 2**.

On the other hand, the bioassay of the eight native *Bt* isolates against *C. pipiens* larvae showed mortality percentages that ranged from 10 to 60% after one day feeding period among the three tested concentrations (I, II and III). Whereas Fa-7 reached the highest significant mortality with 60%, isolates Sn-2, Is-8 and As-4 scored 50% mortality at all three concentrations. As-3 showed the lowest mortality at all three concentrations (II, I and III) with 10, 20 and 20% mortality, respectively compared with the seven other *Bt* isolates. However, mortality percentages of the eight isolates increased noticeably after two days feeding and ranged from 40% at conc. I and III to 90% at conc. I. As-4 and Fa-7 had the highest mortality values (90 and 80%), compared with the six other isolates. Among the eight isolates, the percentage toxicity varied greatly at each of the three concentrations used. For example, at conc. I, Sn-2 showed 40% mortality, Gh-4 and Fa-7 displayed 50% mortality while the other four isolates displayed 60% mortality. At conc. II, Qa-2 and As-3 showed 50% mortality, followed by Is-8 with 60% and the other five isolates showed similar mortality, 70%. At conc. III, Is-8 recorded 40% and Qa-2 50% mortality while the five other isolates showed 60% mortality.

Genetic characterization of *Bt* isolates based on their antibiotic resistant patterns

The antibiotic resistant patterns of the eight *Bt* isolates presented in **Table 3** shows that all *Bt* isolates were highly resistant to four antibiotics (Amp^r, Sm^r, Pmx^r and Er^r), although they were highly sensitive to four other antibiotics: Rif, Amk, Km, Tc and Cm. Moreover, six isolates were resistant to Gm^r, while Ts-5 and Fa-7 isolates were sensitive. Four isolates (Sn-2, Is-8, Qa-2 and Ts-5) were resistant to Nm^r and the four residual isolates were sensitive. Therefore, it is clear that the eight *Bt* isolates were similar in their resistant patterns to six antibiotics and five of them showed variable patterns to two antibiotics, Gm and Nm.

Table 2 Toxicity of the eight *Bt* isolates against 2nd instar larvae of *Spodoptera littoralis* and *Culex pipiens* after different feeding periods (days).

Insect larvae	Feeding time (day)	Conc.	Toxicity percentages								F-value
			Sn-2	Gh-4	Ts-5	Is-8	Qa-2	Fa-7	As-3	As-4	
Spodoptera littoralis	Four	Control	10 c	10 c	10 c	10 c	10 c	10 d	10 d	10 c	
		I	60 aD	65 aD	100 aA	46 a E	75 aC	60 aD	100 aA	90 aB	11.23**
		II	40 bF	64 aD	85 bA	23 bG	70 aC	45 bE	80 bB	70 bC	184.79**
		III	15 cG	24 bF	93 abA	47 aD	25 bF	30 cE	70 cC	75 bB	313.14**
		F-value	44.97**	67.76**	180.62**	26.27**	106.78**	47.20**	173.08**	111.84**	
	Seven	Control	10 c	10 c	10 c	10 c	10 b	10 d	10 c	10 c	
		I	66 aDE	63 aE	100 aA	54 bF	70 aCD	75 aC	85 aB	90 aB	81.48**
		II	45 bE	66 aC	77 bA	67 aC	60 aD	70 aBC	75 bAB	80 abA	31.54**
		III	10 cF	33 bD	80 bA	43 bC	20 bE	45 bC	70 bB	75 bAB	145.67**
		F-value	81.81**	66.71**	107.76**	51.37**	83.87**	69.28**	124.32**	115.04**	
	One	Control	0 c	0 c	0 b	0 c	0 c	0 d	0 c	0 d	
Culex pipiens		I	30 bC	40 aB	40 aB	40 bB	30 bC	40 bB	20 aD	50 aA	16.51**
		II	30 bB	30 bB	30 aB	50 aA	40 aB	30 cB	10 bC	40 bB	14.35**
		III	50 aB	40 aC	40 aC	40 bC	30 b D	60 aA	20 aE	30 cD	28.68**
		F-value	67.11**	91.49**	26.06**	120.41**	59.02**	144.23**	122.22**	280.00**	
	Two	Control	0 d	0 d	0 c	0 c	0 c	0 d	0 c	0 d	
		I	40 cD	50 cC	60 bB	60 aB	60 aB	50 cC	60 aB	90 aA	61.45**
		II	70 aA	70 aA	70 aA	60 aB	50 bC	70 bA	50 bC	70 bA	21.43**
		III	60 bB	60 bB	60 bB	40 bD	50 bC	80 aA	60 aB	60 cB	25.15**
		F-value	194.92**	527.27**	723.53**	188.24**	488.89**	190.00**	660.00**	500.00**	

I= The original concentration (1.5 x 10^7) cells/ml, II = Dilution to half of the original concentration (I/2), III = Dilution to half of II concentration (II/2).
**= Highly significant.
Means in a row/column followed with the same capital/small letter(s) are not significantly different at P = 0.05.

Table 3 Antibiotic resistant patterns of eight Egyptian *Bt* isolate strains.

Isolate no.	Antibiotics resistant patterns										
	Amp	Sm	Pmx	Er	Gm	Nm	Rif	Amk	Km	Tc	Cm
Sn-2	r	r	r	r	r	r	s	s	s	s	s
Is-8	r	r	r	r	r	r	s	s	s	s	s
Qa-2	r	r	r	r	r	r	s	s	s	s	s
Gh-4	r	r	r	r	r	s	s	s	s	s	s
As-3	r	r	r	r	r	s	s	s	s	s	s
As-4	r	r	r	r	r	s	s	s	s	s	s
Ts-5	r	r	r	r	s	r	s	s	s	s	s
Fa-7	r	r	r	r	s	s	s	s	s	s	s

r = resistant, s = sensitive

Genetic characterization of *Bt* isolates based on their plasmid profiles

The plasmid profiles of the eight *Bt* isolates revealed diverse patterns, as shown in **Fig. 2**. Eight plasmids were detected among the eight isolates that were not completely existent in any isolate. Among the *Bt* isolates, plasmids varied in number, molecular size and existence (**Table 4**). Two isolates, Ts-5 and Sn-2, showed the highest total number (7) and the others had one less plasmid in four isolates and two less plasmids in Gh-4 compared with Ts-5 and Sn-2, while Qa-2 had the lowest number (3). Moreover, one plasmid with 1.28 kb existed in all isolates and the existence of the other seven plasmids varied noticeably among the eight isolates. For example, two plasmids with 3.30 and 0.90 kb existed in six isolates but not in Gh-4 and Qa-2, one plasmid with 0.45 kb existed in seven isolates but not in Qa-2. In addition, two plasmids with 0.72 and 0.61 kb were detected in four different isolates and not present in the other four isolates.

Genetic characterization of *Bt* isolates based on their total proteins

SDS-PAGE protein analysis of the eight *Bt* isolates revealed 43 bands with different molecular weights ranging from 203 to 7 kD (**Fig. 3**). Among the bands, 15 showed high variability among the isolates. Genetic discrimination of the eight isolates was based upon the absence or presence of band(s), as shown in **Table 5**.

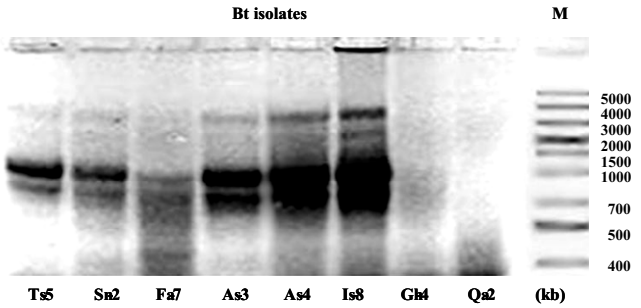

Fig. 2 DNA plasmid profiles of eight Egyptian *Bt* isolates using agarose gel electrophoresis. M = 1 kb DNA ladder (Fermentas, Germany).

Table 4 Plasmid numbers and corresponding sizes in eight Egyptian *Bt* isolates.

Plasmid No.	Ms (kb)	*Bt* isolates							
		Ts-5	Sn-2	Fa-7	As-3	As-4	Is-8	Gh-4	Qa-2
1	3.30	+	+	+	+	+	+		
2	2.34	+	+	+		+		+	+
3	1.28	+	+	+	+	+	+	+	+
4	0.90	+	+	+	+	+	+		
5	0.72	+			+		+	+	
6	0.61	+	+	+		+			
7	0.45	+	+	+	+	+	+	+	
8	0.40		+		+		+	+	+
Total number		7	7	6	6	6	6	5	3

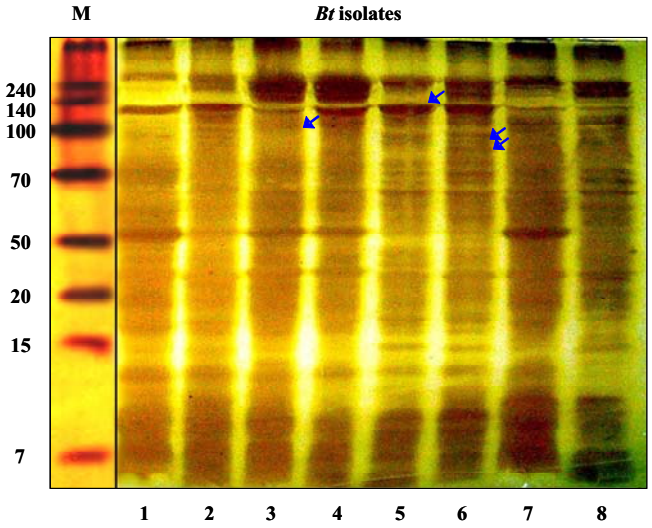

Fig. 3 SDS-PAGE protein profiles of eight Egyptian *Bt* isolates. M = Protein marker with molecular weights (kD). * The four arrows represented the toxic proteins according to the references. They are 80, 88, 100 and 135 kD.

The 15 polymorphic bands were arranged according to their presence in some distinctive isolates and absence in others, where they existed in three to seven isolates but were absent in some unique isolates and existent in others (**Table 5**). For instance, three bands with 65, 41 and 22 kD disappeared in three isolates (As-3, Is-8 and Qa-2) and presented in the others, two protein bands with 80 and 19 kD were detected in four different isolates but in Gh-4 were absent. By a similar approach, three bands with 190, 47 and 24 kD appeared in five isolates and both of Sn-2 and Gh-4 did not have the three bands. Five bands appeared in different six isolates, Fa-7 displayed all and each of the five isolates did not have one of the five bands, while two isolates (Sn-2 and Qa-2) did not have two and three bands, respectively. Two other bands with 135 and 77 kD were detected in seven isolates, whereas a band of lower molecular weight disappeared in Gh-4 and the higher MW band did not exist in Is-8. On the other hand, the total number of variable bands in each isolate was highest in Ts-5 (14) and fewest in Gh-4 (6). In general, each of the eight isolates revealed a unique protein pattern either by the absence of some distinctive protein bands or by the appearance of different total band numbers, even between isolates from the same location, such as As-3 and As-4. Although they displayed similar total number (10), but As-3 showed four bands (80, 190, 47 and 31) that not existed in As-4. In contrast, As-4 displayed four bands (22, 65, 19 and 158) that disappeared in As-3.

DISCUSSION

The use of 16S rRNA gene to study the phylogeny and taxonomy of *Bacillus thuringiensis* has been by far the most common housekeeping genetic marker used for a number of reasons (Soufiane and Côté 2009). These reasons include (i)

Table 5 SDS-PAGE analysis of polymorphic protein bands of the eight Egyptian *Bt* isolates.

Existence in isolates	Band No.	MW (kD)	*Bt* isolates							
			Ts-5	Fa-7	As-3	As-4	Is-8	Sn-2	Qa-2	Gh-4
Three	36	22	+	+		+				
	28	41	+	+				+		
	21	65	+			+				+
Four	17	80	+	+	+			+		
	38	19	+			+	+		+	
Five	2	190	+	+	+		+		+	
	26	47	+	+	+		+		+	
	34	24	+	+	+	+	+			
Six	4	158	+	+		+	+	+		+
	20	70	+	+	+	+	+		+	
	31	31	+	+	+		+	+		+
	32	28	+	+	+	+	+			+
	41	14		+	+	+		+	+	+
Seven	7	135	+	+	+	+		+	+	+
	18	77	+	+	+	+	+	+	+	
Eight		*	+	+	+	+	+	+	+	+
Total variable number			14	13	10	10	9	7	7	6

*All the residual 28 proteins bands were commonly detected in the eight isolates, a 88-kDa band was included

its presence in almost all bacteria, often existing as a multi-gene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene designed in the present study with 1400 bp is large enough for informatics purposes.

In the present study, the eight identified *Bt* strains under study isolated from the soil of seven Egyptian governorates revealed high toxicity against the larvae of *Spodoptera littoralis* (Lepidoptera) and *Culex pipiens* (Diptera). A bioassay against *S. littoralis* revealed that three isolates (Ts-5, As-3 and As-4) were highly toxic with 100, 100 and 90% mortality, respectively while two other isolates (Qa-2 and Fa-7) displayed high toxicity (75%). Such results demonstrate that five of the eight *Bt* isolates (Ts-5, As-3, As-4, Qa-2, Fa-7) possibly harboring different types of *cry1* genes coding for toxic proteins against *S. littoralis*. Several reports reviewed *Bt* isolates that harbor different *cry* genes with high toxicity against insect pests. For example, Nariman (2007) characterized five Egyptian *Bt* isolates harboring a combination of different *cry* genes: Gh-3 containing (lepidoptera-active *cry1Aa* and *cry1Ac*, lepidoptera-diptera active *cry2* gene and coleoptera-active *cry3* gene), Sn-4 and Ts-1 harboring (*cry1Ac* and *cry2*), As-2 have (*cry1Aa* and *cry1Ac*) and Gh-5 have (*cry1Aa* and *cry1I*). Other reports showed a high frequency of combinations of *cry1* genes, for example the linkage of the *cry1C* and *cry1D* genes (Hongyu *et al.* 2000). Letowski *et al*. (2005) confirmed that a single *Bt* strain can harbor numerous different insecticidal crystal protein (*cry*) genes from 46 known classes or primary ranks and the *cry1* primary rank is the best known and contains the highest number of *cry* genes which currently totals over 130. On the other hand, Chen *et al.* (2004) indicated that the distribution of *cry* gene combinations of *Bt* isolates are geographically related.

Two isolates (As-4 and Fa-7) showed high mortality against *C. pipiens* (90 and 80%, respectively) and most isolates showed more than 50% mortality. Similarly, the eight isolates may harbor different *cry*2 genes that highly toxic to Dipteran and many other closely related pests (*C. quinquefasciatus*, *Aedes* and *Anopheles*). This finding is in agreement with that of Ibarra *et al.* (2003), who reported that the search for native strains with activity against Dipteran species could have an impact on the control of mosquitoes worldwide, such as *C. pipiens* that transmits West Nile fever, lymphatic filariasis (elephantiasis) and St. Louis encephalitis larvae. the new native strains should be noticeably more potent and act much faster than any other strain of *Bt* currently used in commercial vector control products. Thereby, the number of known *Bt* strains active on Lepidoptera and Diptera is growing and the living collection currently numbers 13,000 *Bt* strains and isolates obtained from samples from around the world (Martin 1996). The results obtained demonstrated that the foremost toxicity effect to *C. pipiens* larvae is due to the specific types of *cry* genes whose toxic proteins existed in each *Bt* isolate, and not merely to the high concentrations of *Bt* growth. However, another point of view reported by El-Husseini *et al*. (2000) indicates that in some cases higher concentrations might reveal negligible or low potency because they might induce an anti-feeding effect, which results in diminishing the ingested bacterial dose.

In addition, the antibiotic resistant markers, plasmid profiles and protein patterns were genetically characterized the eight *Bt* isolates uniquely. Such strategy was applied in other studies, for instance Nariman *et al.* (2006) used such techniques to characterize their new *Bt* isolates: *E. coli* hybrid strains that are more toxicity against *Spodoptera littoralis* larvae than the commercial *Bt* strains. Yoo *et al.* (1996) characterized their three Korea *Bt* isolated using antibiotic susceptibility test and other biochemical characters and found that they were highly toxic to *Bombyx mori* and not to *C. pipiens*.

On the other hand, plasmid profile of the eight isolates revealed variations in pattern (numbers and molecular masses). Such divergence in plasmid profiles of *Bt* isolates was reported, for instance by López-Meza and Ibarra (1996), who found an unusual set of plasmids (including small and large plasmids) in one *Bt* strain (LBIT-113). In another study, the plasmid pattern of one isolated *Bt* strain (K1) from a Korean soil was different from that of the reference strain, kurstaki HD-1 (Li *et al.* 2002). Soliman *et al.* (2003) confirmed the role of plasmid profile divergence on alkaline protease production in some local *Bacillus* isolated strains.

Moreover, insecticidal activity of the eight *Bt* isolates can be predicted from the analysis of SDS-PAGE protein, for instance the 80 kD protein was observed in (Ts-5, Fa-7, As-3 and Sn-2) and the 135 kD displayed in seven isolates. Such proteins were found in the study of Zhang *et al.* (2000) in 25 *Bt* isolates containing *cry1* type gene and 16 of them contain *cry1I* gene, which codes 80 kD protein and is larvicidal to both lepidopteran and coleopteran species. The patent of Warren *et al.* (1994) showed that the *vip1A* gene encodes a 100-kDa protein that is apparently processed from its N terminus to yield an 80-kDa protein upon secretion. The 80 kDa Vip1A protein is toxic to western corn rootworm larvae in conjunction with the Vip2A protein, whose coding region is located immediately upstream. In addition, an 88-kDa protein that is produced during vegetative growth but is not processed upon secretion is reported to exhibit toxicity towards a wide variety of lepidopteran insect pests, including *Agrotis ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua*, and *Helicoverpa zea* (Estruch *et al.* 1996).

In conclusion, the eight Egyptian *Bt* isolates with high toxicity and broad spectrum activity against *Spodoptera littoralis* (Lepidoptera) and *Culex pipiens* (Diptera) require further investigation in order to detect, isolate and sequence the *cry*-type genes, which will provide valuable applications with regard to the field performance and these isolates could be used as new commercial insecticidal bacteria.

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