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Presence of Multiple *cry* Genes in *Bacillus thuringiensis* Isolated from Dead Cotton Bollworm *Heliothis armigera*

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ABSTRACT

***Cry* genes encoding Cry proteins toxic to Lepidoptera, Coleoptera and Diptera species were studied in thirty seven *B. thuringiensis* strains isolated from twelve naturally infested *Heliothis armigera* larvae. To further confirm the isolates, two groups of species-indicative biochemical tests were applied while discriminative biochemical tests being employed to figure out the repetitive strains. A PCR experiment was performed using five sets of universal primers for *cry1, cry2, cry3, cry4, cry7/8* genes. All strains reacted appropriately, for *B. thuringiensis*, to the biochemical tests and while the reactions to the discriminative tests being varied. Based upon the results of the discriminative tests, twenty four non-repetitive strains were selected and employed in the PCR assay. Each of the selected strains presented one *cry* gene, at least; *cry1* being the most frequently detected one (91.7%), followed by *cry2* (87.6%), *cry3* (50%) and *cry4* (42%) but no isolate harbored a coleopteran-active *cry7/8* gene. All the strains presented combinations of two or more *cry* genes: 20% presenting *cry1+cry2*, 12.5% *cry1+cry3*, 4% *cry2+cry4*, 20% *cry1+cry2+cry3*, 20% *cry1+cry2+cry4*, 4% *cry1+cry3+cry4* and 12.5% carrying all the four *cry* genes studied and only one strain bearing a single *cry* gene. The *cry1-cry2* combination was common in many strains (72.5%). Genetic characterization of this collection provides an opportunity for selection of strains with improved and multiple insecticidal toxicity.**

Keywords: *Bacillus thuringiensis*, Biochemical characters, *Cry*, PCR.

INTRODUCTION

With more than 9000 species of insect pests Adversely Affecting the commercial crops in the world, the annual cost of chemical control agents has been estimated at 7,500 million dollars (Arrieta and Espinoza, 2006). Chemical agents are efficacious in most cases and easy to use, but are probable to lead to emergence of insect resistance and have a long residual effect along with toxicity to non-target organisms (Song *et al*., 2008). Therefore, such alternative biocontrol agents as *Bacillus thuringiensis*-based biopesticides are increasingly attracting interest. This bacterium produces parasporal crystalline inclusions (Cry proteins) which are toxic to many important agricultural pests. The Cry proteins are encoded by *cry* genes and so far, many *cry* genes have been identified in different *B. thuringiensis* strain collections and classified as *cry1* to *cry70* (http://www.lifesci.sussex.ac.uk/home/). Notwithstanding the diversity of *cry* genes described up to date, it is still necessary to search for more toxins, since a significant number of pests cannot be controlled through the presently available Cry proteins. It is also important to provide alternatives

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for coping with the problem of insect resistance, especially with regard to the expression of *B. thuringiensis* genes encoding insecticidal proteins in transgenic plants (Nester *et al*., 2002).

The need to identify *cry* genes has prompted development of molecular methods for quickly and easily characterizing *cry* genes present in *B. thuringiensis* isolates. This technique which was first introduced by Carozzi *et al.* (1991), is now widely being used for screening *cry* gene contents and particularly predicting the insecticidal activity of different *B. thuringiensis* strains (Bourque *et al*., 1993; Ben-Dov *et al.*, 1997, 1999, 2001; Juárez-Pérez *et al*., 1997; Bravo *et al*., 1998; Porcar and Juárez-Pérez, 2003; Carozzi *et al*., 1991).

Cotton is an important cash crop grown in many countries including in Iran and the cotton bollworm *Heliothis armigera* Hübner 1805 (Lepidoptera: Noctuidae) is one of the major pests of this crop worldwide. In his previous work (Keshavarzi, 1998), the author found many dead *H. armigera* larvae totally infested with *B. thuringiensis*. The observed 100% infestation of *H. armigera* to *B. thuringiensis* in natural conditions is a rare report that indicates naturally high anti-lepidopteran activities of the strains. Therefore, we became interested in further studying them with regard to *cry* gene contents potentially active against the three major classes of insect pests: Lepidoptera, Coleoptera and Diptera.

MATERIALS AND METHODS

Bacterial Strains

Thirty seven *B. thuringiensis* strains isolated from twelve dead *H. armigera* insects collected from a cotton field in Torogh, Khorassan Razavi Province of Iran were experimented (Keshavarzi, 2008). The field had no history of *B. thuringiensis* spray. *B. thuringiensis* strains 4D1, 4J3, 4AA1 and 4Q2 (supplied by Dr. Zeigler, *Bacillus* Genetic Stock Center, Ohio) were used as standards for *cry 1,2, cry 1,2,7/8, cry3,* and *cry4* genes, respectively. The strains were routinely cultured on Nutrient Agar (NA) plates and maintained over 30% glycerol in -80ºC deep freeze.

Biochemical Characters

To narrow the number of strains isolated from one dead larvae, biochemical methods were applied. Three groups of biochemical tests are routinely used for *B. thuringiensis* classification (de Barjac and Frachon, 1990). The first group consists of characters positive for all the strains, the second, groups together those characters that are generally negative. The third category contains characters which are taxonomically useful because they can discriminate between isolates. The main factors in the third group include the presence of Arginine Dihydrolase (ADH), enzymes for the reduction of nitrates, urease as well and the ability to ferment sucrose, mannose, cellobiose or salicin. Therefore, all the local strains were biochemically confirmed using the first and second groups and as based on the third one, repetitive (twin) strains, coming from the same larva, were omitted with only the non-repetitive strains being used in the PCR assay.

Detection of *Cry* Genes

PCR analysis was performed to identify *cry1, cry2, cry3, cry4* and *cry 7/8* genes in the non-repetitive isolates (based on discriminative tests). The PCR conditions were according to Ben-Dov *et al.* (1997) but using bacterial cell lysate as the template DNA. The strains were grown for 12 hours on NA plates. A loop of cells was transferred to 100 µl water in a 100ºC bath for 5 minutes and the resulting cell lysate was briefly centrifuged for 10 seconds at 10,000 rpm with 5 µl of supernatant being used in the PCR. PCR reactions were carried

out in 20 µl reaction mixture containing 5 µl template DNA, 150 mM dNTPs, 20 pM of each primer (Table 1) and 0.5U of *Taq* DNA polymerase. Amplification was carried out in a DNA thermocycler with the program: one denaturing cycle at 94ºC for 4 minutes, 35 cycles containing: 94ºC for 45 seconds, annealing at 48-55ºC for 45 seconds and 1 minute at 72ºC and then the reaction being terminated by a for 4 minutes one at 72ºC. The optimal appealing temperature for each primer set was estimated as based on its nucleotide content and examined in standard before running main procedure in local isolates. The *cry* gene banding patterns were visualized through agarose gel electrophoresis. A 15 µl aliquot of each amplification product was loaded onto 1.2% agarose gel and run in TAE buffer (40mM Tris-Acetate, 1 mM EDTA) at 100 V for 1 hour. The gels were stained with ethidium bromide and documented with a 1-kb molecular weight marker (Synagenta).

RESULTS AND DISCUSSION

Biochemical Characters

Frequently many *B. thuringiensis* isolates from a single sample might be identical. Therefore, biochemical tests were employed to not only further confirm the nature of the isolates (categories 1 and 2), but also to identify the repetitive isolates from one larval sample (category 3). Based on the results, all the 37 *B. thuringiensis* strains reacted positively to the positive tests and negatively to the negative ones. There existed variations in reactions to the discriminative tests of ADH, urease, and to the fermentation of sugars (sucrose, mannose, cellobiose) but not for nitrate reduction, and VP (Tables 2 and 3). Accordingly, thirteen isolates (37%) exhibiting similar reactions to the discriminative tests were considered as repetitive (twin strains) and omitted from PCR analysis. Omission of twin strains has already been reported by Uribe *et al.* (2003) and Sauka *et al.* (2006). They used SDS-PAGE and PCR to identify and discard strains of similar insecticidal properties and in the meantime not to overestimate the *cry* gene distribution frequencies.

Cry Gene Content

The optimal annealing temperature for *cry 1* and *cry7/8* genes was determined as: 48°C, and for *cry2, cry3* and *cry4*, it amounted to 55°C. PCR amplification results showed that the expected fragments of 270, 700, 590, 440 and 300 bp corresponding to *cry1, cry2, cry3, cry4* and *cry7/8* genes were obtained in tests with five sets of universal primers in the standard isolates. The most frequent *cry* gene identified was *cry1* (91.7%) followed by *cry2* (87.6%), *cry3* (50%) and *cry4* (42%)

Table 1. Primers utilized in PCR assay[a].

Primer	Sequence (5'-3')	*cry* genes	Predicted product size (bp)
Un1(r)	*TTG TGA CAC TTCTGC TTC CCA TT*	*Cry1*	277, 274
Un1(d)	*CATGATTCATGCGGCAGATAAAC*		
Un2(r)	*CGG ATA AAA TAA TCTGGG AAA TAG T*	*Cry2*	701,689
Un2(d)	*GTT ATT CTT AATGCA GAT GAA TGG G*		
Un3(r)	*CAT CTG TTG TTTCTG GAG GCA AT*	*Cry3*	589, 595, 604
Un3(d)	*CGT TAT CGC AGAGAG ATG ACA TTA AC*		
Un4(d)	*GCATATGATGTAGCGAAACAAGCC*	*Cry4*	439
Un4(r)	*GCG TGA CAT ACCCAT TTC CAGGTC C*		
Un7,8(d)	*AAG CAG TGA ATG CCTTGT TTA C*	*Cry7/8*	423
Un7,8(r)	*CTT CTA AAC CTT GAC TAC TT*		

[a] Ben Dov *et al*. (1997).

Table 2. Reactions of 37 *B. thuringiensis* isolated from 12 dead *Heliothis armigera* to three categories of biochemical characters.

Characters	Positive reaction (%)	Characters	No. of positive strains
Positive		Discriminant	
Hydrolysis of:		ADH[a]	2
Starch	100	Nitrate reduction	37
Gelatin	100	Urease	9
Fermentation of:		VP [b]	0
Trehalose	100	Fermentation of:	
Glucose	100	Sucrose	17
Negative		Mannose	13
Indole production	0	Cellobiose	14
H_2S production	0	Salicin	33

[a]Arginine Dihydrolase, [b] Voges Proskauer

Table 3. Results of discriminative biochemical tests in 24 local *B. thuringiensis* strains isolated from 12 dead *Heliothis armigera*.

Larva No.	Strain No.	Fermentation of:					
		Sucrose	Salicin	Cellobiose	Mannose	ADH	Urease
1	68	+	+	-	+	-	-
2	2	+	+	+	-	+	+
″	86	+	+	-	-	-	-
″	92	-	+	-	+	-	-
3	56	-	+	-	+	+	-
″	57	+	+	-	-	-	+
4	83	+	+	+	-	-	-
5	79	-	+	-	-	-	-
7	45	-	+	-	+	-	+
″	60	-	-	-	+	-	-
8	17	+	+	+	-	-	-
9	81	-	+	-	-	-	-
15	85	-	+	-	-	+	+
″	30	+	+	-	-	-	-
16	62	+	-	-	+	-	-
18	11	+	+	+	-	-	-
20	55	+	-	+	-	-	-
21	63	-	-	-	+	-	+
22	73	-	+	-	-	-	-
23	46	+	-	+	-	-	-
″	29	-	+	-	-	-	-
24	53	-	+	+	+	-	-
″	31	+	+	+	-	-	+
″	54	-	+	-	-	-	+

but no isolate was found to harbor coleopteran-active *cry7/8* gene (Tables 4 and 5, Figure 1). A high frequency of *cry1* genes seems to be common to many *B. thuringiensis* strain collections analyzed so far (Hernandez *et al*., 2005; Cinar *et al*., 2008; Sahukhal *et al*., 2008; Song *et al.*, 2003). Uribe *et al.* (2003) analyzed *cry* gene content after removal of twin strains and found the highest frequency (73%) for *cry1* gene. Other than Uribe *et al*.'s (2003), other studies have also been conducted on collections with no emphasis on the removal of twin strains. Bravo *et al.* (1998) found

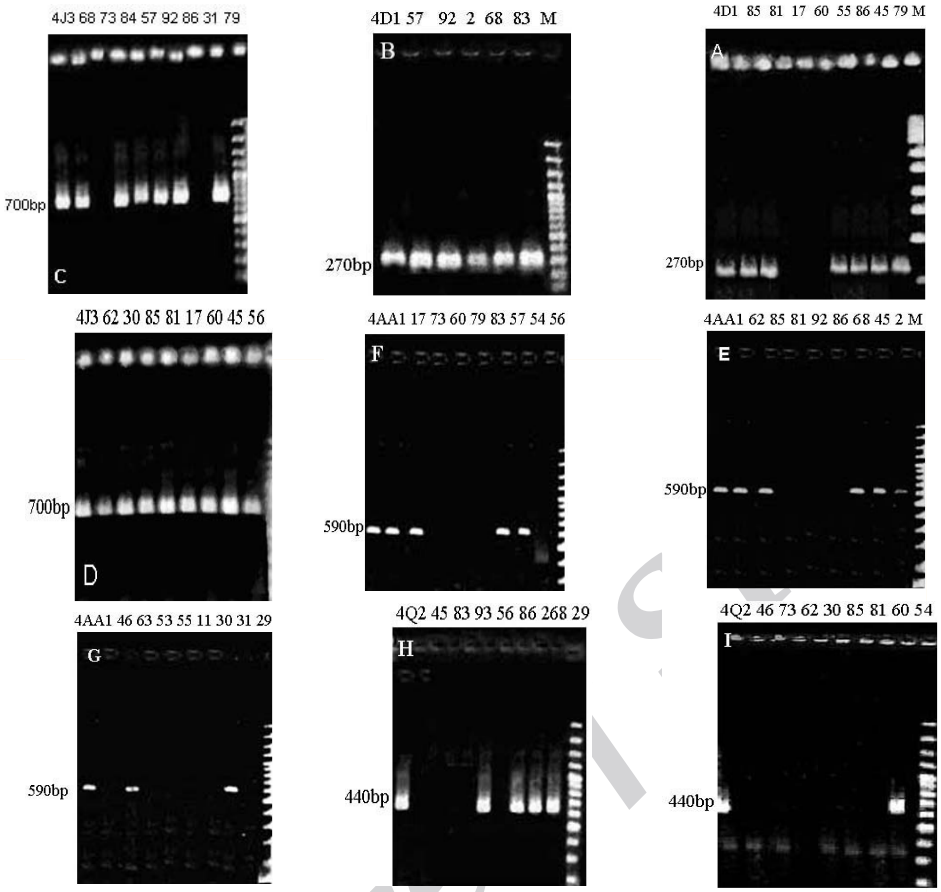

Figure 1. Agarose gel electrophoresis of PCR products obtained with universal primers for *cry1* (A, B), *cry2* (C, D), *cry3* (E, F, G), *cry4* (H, I) genes in a number of *B. thuringiensis* strains originated from dead *H. armigera* larvae. Lane numbers correspond to the number of isolates. M: Marker (1 kb plus ladder).

49% frequency of *cry1* gene. Wang *et al.* (2003) detected 76.5% *cry1* and 70% *cry2* genes. Thammasittirong and Attathom (2008) reported *cry1* at the same frequency of *cry2* (81.3% and 80.6%, respectively). The observed high frequency of *cry1* genes might be because *cry1* gene-containing strains are possibly more abundant in nature. In the present study, the high frequency of *cry1* and *cry2* might be related to the origin of the isolates, the lepidopteran *H. armigera*. Toxicity of Cry1 and Cry2 proteins to *H. armigera* has already been reported by Padidam (1992) and cited by Porcar and Caballero (2000). Seifinejad *et al.* (2007) also found high frequency of *cry2* and *cry1* (56.5 and 49%, respectively) in 70 *B. thuringiensis* strains isolated from diverse locations in Iran.

To date, there are very few reports on *cry* genes of naturally infested insects (Xie *et al*., 2010; López-Pazos *et al*., 2009; Porcar and Caballero, 2000). Xie *et al*. (2010) isolated only six *B. thuringiensis* strains from 100 diapausing silkworm larvae (6%) but a high (100%) infestation of the target insect (*H. armigera*) to *B. thuringiensis* was observed in the present study which might indicate a high natural toxicity of the isolates. In strains isolated from dead larvae, concordance between *cry* gene and toxicity is usually observed. For example, *cry2* was the highest in Andean weevil (*Premnotrypes vorax*, Coleoptera), a pest of potato in the USA (López-Pazos *et al*., 2009), while *cry1*

Table 4. Distribution of four main classes of *cry* genes among 24 *B. thuringie*nsis originated from 12 dead *Heliothis armigera*.

Strain	*Cry1*	*Cry2*	*Cry3*	*Cry4*
68	+	+	-	-
2	+	+	+	-
86	-	+	-	+
92	+	+	-	+
56	-	+	-	-
57	+	+	-	-
83	+	+	-	+
79	+	+	-	-
45	+	+	+	+
60	+	+	-	-
17	+	+	-	+
81	+	+	+	-
85	+	+	+	-
30	+	+	-	-
62	+	+	+	-
11	+	+	-	+
55	+	-	+	-
63	+	+	+	+
73	+	-	+	-
46	+	+	-	+
29	+	+	+	-
53	+	+	+	+
31	+	-	+	+
54	+	+	+	-

and *cry2* were detected in a dead larva of the lepidopteran *Mythimna loreyi* collected from a corn crop in Spain during a natural epizootic (Porcar and Caballero, 2000).

It is important to be noted that all the isolates, except isolate number 56, presented combinations of two or more *cry* genes; 20% contained *cry1+cry2*, 12.5%, *cry1+cry3*, 4% *cry2+cry4*, 20% *cry1+cry2+cry3*, 20% *cry1+cry2+cry4*, 4% *cry1+cry3+cry4* and finally 12.5% contained all the four *cry* genes studied. The presence of different *cry* genes in the same *B. thuringiensis* strain has frequently been reported. For example, Aronson (1994) and Ben-Dov *et al.* (1997) reported the presence of *cry1, cry3*, *cry8* or *cry7*, and Bravo *et al.* (1998) *cry1*, *cry3* and *cry7* genes in the same strain. López-Pazos *et al*. (2009) demonstrated that 49% of their isolates carried combinations of *cry* genes. Combinations of multiple *cry* genes in one strain could be due to genetic information exchange between different strains. Such isolates could show simultaneous toxicities towards different insect families and are good candidates in the search for biocontrol agents covering a wider spectrum of action. The genetic characterization of the collection in the present study has provided opportunity for the selection of strains to be tested in bioassays against *H. armigera* and against other insect pests of agricultural importance and as well for a design Cry proteins of improved and multiple insecticidal toxicities.

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حضور توام ژن های گوناگون *cry* در *Bacillus thuringiensis* منشا گرفته از کرم غوزه پنبه *Heliothis armigera*

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چکیده

در این تحقیق، ژن های *cry* موثر بر گونه های بالپولک داران، سخت بالپوشان و دوبالان در سی و هفت سویه *B. thuringiensis* منشا گرفته از کرم غوزه پنبه *Heliothis armigera* بررسی شد. برای تائید بیشتر سویه ها، از دو گروه آزمون بیوشیمیایی بر اساس خصوصیات مثبت و منفی و برای حذف جدایه های تکراری، از آزمون های بیوشیمیایی افتراقی استفاده شد. ازمون پی سی آر با استفاده از ۵ جفت آغازگر عمومی برای ژن های *cry1, cry2, cry3, cry4, cry7/8* انجام شد. بر اساس نتایج، کلیه سویه ها واکنش های قابل انتظاری به واکنش های بیوشیمیایی نشان دادند. با توجه به نتایج آزمون های افتراقی، سی و پنج جدایه انتخاب و در پی سی آر بکار برده شدند. کلیه سویه دارای یک یا بیش از یک ژن *cry* بودند، *cry1* فراوان ترین (۹۱/۷٪) و سپس *cry2* (۸۷/۶٪)، *cry3* (۵۰٪) و *cry4* (۴۲٪) بودند اما ژن *cry7/8* موثر بر سخت بالپوشان در هیچ جدایه ای وجود نداشت. کلیه سویه ها حاوی ژن های متعدد *cry* بودند: ۲۰٪ دارای *cry1+cry2*، ۱۲/۵ *cry1+cry3*، ۴٪ دارای *cry2+cry4*، ۲۰٪ دارای *cry1+cry2+cry3* ، ۲۰٪ *cry1+cry2+cry4*، ۴٪ *cry1+cry3+cry4* و ۱۲/۵٪ دارای کلیه ژن های مطالعه شده بودند و فقط یک سویه تنها یک ژن داشت. حضور توام ژن های *cry1* و *cry2* در بسیاری از سویه ها (۷۲/۵٪) مشاهده شد. تعیین مشخصات ژنتیک این کلکسیون فرصتی مناسب برای انتخاب سویه هایی با خصوصیات برتر و خواص متعدد آفت کشی ایجاد می کند.