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cry Gene Profile of Native Entomopathogenic *Bacillus thuringiensis* from Soil

A.S. Patel[1]*, H.N. Shelat[1] and H.K. Patel[2]

[1]Department of Agricultural Microbiology, BA College of Agriculture, [2]AICRP Weed Management, Anand Agricultural University, Anand (GJ), India
**Corresponding author*

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Bacillus thuringiensis produces different insecticidal crystal proteins which are encoded by *cry* genes. Isolation was carried out from 17 diverse locations' soil; nine bacterial isolates were separated and selected on the basis of presence of parasporal body. Isolate ABt 10 was screened for different *cry* genes with universal and gene specific primers. Results indicated the presence of *cry1Ac* and *cry1Ab, cry 4,* and *cry 1D* genes in native isolate ABt 10. All these clearly indicated the specificity of these isolates for Lepidopteran insect pests. The search and selection of native isolates will be useful in the generation of a transgenic plant with an indigenous *cry* gene that offers resistance to the serious local pests.

Introduction

B. thuringiensis comprises of gram-positive, big rod shaped, spore-forming bacterium. It contains novel properties regarding toxin genes and their expressions. They have unusual property of producing a parasporal protein crystal (δ-endotoxin or *Cry* protein) which is toxic for many insect pests (Sarker and Mahbub, 2012).

Number of strains have been isolated and used to control pests of agricultural importance because of the insecticidal activity. Efforts have been made in many countries to isolate new strains with increased potency against target pest insects having a wider host range. These toxins have not only shown activity against Lepidoptera, Diptera, Hymenoptera, Isoptera, Orthoptera and Coleoptera but also against nematodes, mites, lice, aphids and ants (Rosas-Garcia, 2009).

The commercial *B. thuringiensis* products are powder based mixture of dried spores and toxin crystals which applicable to leaves or other plant parts where the insect larvae feed. The toxin genes have also been genetically engineered into several crop plants (Brookes and Barfoot, 2013; James, 2013). Scientists have identified at least 29 different crystals and δ-endotoxin effective against specific insects. *B. thuringiensis* can produce one or more type of *Cry* proteins (Sanahuja *et al*., 2011).

Materials and Methods

Sample collection

Representative soil samples (approximately 100 g) were collected from 17 different locations of Anand, Gujarat in sterile HDPE bags, brought to the laboratory and stored at 4°C till further processing.

Isolation of *B. thuringiensis* from soil samples and preliminary toxicity test

Native *B. thuringiensis* were isolated following the modified method described by Patel *et al.,* (2013). The cultures were then transferred on Nutrient agar slants and stored at 4^0C for further studies.

Test insects belonging to Lepidopteran group *H. armigera* and *S. litura* were selected and efficacy of isolate was tested following food contamination technique (Navon *et al.,* 1990). Haemolymph of the dead larvae was observed under 400X in Phase-Contrast Microscope for the presence of parasporal body of *B. thuringiensis* by preparing wet mounts.

Identification and characterization of native *B. thuringiensis* isolates

All the isolates were identified based on their morphological, cultural and biochemical characteristics using 9^{th} edition of Bergey's Manual of Determinative Bacteriology and standard literature (Halt *et al.,* 1994).

Screening of *cry* genes by PCR analysis

Screening of *cry* gene of one native *B. thuringiensis* isolate was performed by PCR analysis. PCR was performed as per method given by Carozzi *et al.* (1991). Appendix shows sequences of the general and specific primers used to identify specific cry genes. The oligonucleotides were synthesized at MWG Eurofins Genomics India Pvt. Ltd. PCR products were subjected to submarine gel electrophoresis with marker DNA of known molecular weight, in 2% agarose gel at voltage of 6V/cm using 1X TBE buffer and Ethidium bromide (0.5µg/ml) staining. Gels were viewed under UV light and photographed using Gel Documentation system.

Results and Discussion

This study was undertaken in order to carry out isolation of potential native *B. thuringiensis* from soil; its *cry* gene characterization.

Isolation of native *B. thuringiensis* from soil

Total 109 colonies on MYP agar plate were obtained from 17 soil samples and only 9 colonies designated as ABt 2, 10, 17, 21, 33, 49, 54, 61 and 63 were confirmed as *B. thuringiensis* (Table 1).

Nine isolates showed the presence of bipyramidal parasporal crystal inclusion body and successfully proved Koch's postulates in the laboratory against *H. armigera* and *S. litura* as target insects. Isolate ABt 10 gave maximum mortality 80% and above (Table 2).

Characterization and identification of native *B. thuringiensis* isolates

All the nine isolates were found gram positive, straight, thick, sporulating rods, occurring in long chains. On Nutrient agar the colonies were round, medium sized, elevated with irregular margins and the color was creamish white, which later on showed dark center. All the test isolates were confirmed *B. thuringiensis* by biochemical characters (Table 3).

Table.1 Isolation of *B. thuringiensis*

Sr. No	Villages	Total soil samples collected	Total colonies on MYP agar plate	*Bt* like colonies	Confirmed *Bt*
1	Anand	1	09	06	01
2	Bakrol	1	05	03	00
3	Bedva	1	08	05	00
4	Chikhodara	1	03	01	00
5	Khambholaj	1	07	03	01
6	Navli	1	12	09	02
7	Anklav	1	03	02	00
8	Bhetasi	1	06	04	01
9	Kahanwadi	1	04	01	00
10	Umeta	1	07	05	00
11	Kantharia	1	10	06	00
12	Napa	1	08	05	00
13	Dali	1	04	03	01
14	Palol	1	02	01	01
15	Kanawada	1	05	03	01
16	Rhinza	1	07	04	00
17	Tarapur	1	09	06	01
Total		**17**	**109**	**67**	**09**

Table.2 Bioactive compounds Efficacy of isolates against *H. armigera* and *S. litura*

Sr. No.	Isolate No.	Mortality	
		H. armigera	*S. litura*
1	ABt 2	++	++
2	ABt 10	++++	++++
3	ABt 17	++	++
4	ABt 21	+	+
5	ABt 33	+++	+++
6	ABt 49	+	+
7	ABt 54	++	++
8	ABt 61	++	++
9	ABt 63	+	+

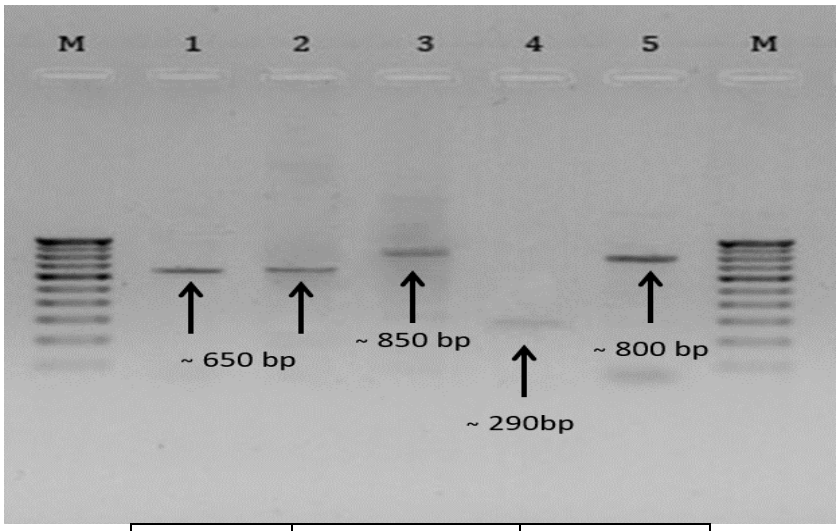
Note:+ = Poor effect, ++ = Moderate effect, +++ = Good effect, ++++ = Excellent effect.

Table.3 Biochemical characterization of native isolates

Biochemical test	**Isolate No.**								
	ABt 2	**ABt 10**	**ABt 17**	**ABt 21**	**ABt 33**	**ABt 49**	**ABt 54**	**ABt 61**	**ABt 63**
Malonate	-	-	-	-	-	-	-	-	-
Voges Proskaur's	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+
ONPG	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+

Note: + positive reaction; - negative reaction

Fig.1 PCR Amplification of native isolate ABt 10 produced by *cry* gene primers



Lane No.	**Isolate No.**	***cry* Primer**
M	100 bp Ladder	
1	HD 73 (Std.)	*cry 1 Ac*
2	ABt 10	*cry 1 Ac*
3	ABt 10	*cry 1 Ab*
4	ABt 10	*cry 1 D*
5	ABt 10	*cry 4*
M	100 bp Ladder	

Appendix

General Primers for *cry* and *cyt* gene detection

No.	Gene	Sequence (5'→ 3')			
		Direct	Mer	Reverse	Mer
	cry1	TGT AGA AGA GGA AGT CTA TCC A	22	TAT CGG TTT CTG GGA AGT A	19
	cry 3,7,8	TTA ACC GTT TTC GCA GAG A	19	TCC GCA CTT CTA TGT GTC CAA G	22
	cry4	CAA GCC GCA AAT CTT GTG GA	20	ATG GCT TGT TTC GCT ACA TC	20
	cry 5,12,14,21	TTA CGT AAA TTG GTC AAT CAA GCA AA	23	AAG ACC AAA TTC AAT ACC AGG GTT	24
	cry 9	CGG TGT TAC TAT TAG CGA GGG CGG	24	GTT TGA GCC GCT TCA CAG CAA TCC	24
	cry11	TTA GAA GAT ACG CCA GAT CAA GC	23	CAT TTG TAC TTG AAG TTG TAA TCC C	25
	cry13	CTT TGA TTA TTT AGG TTT AGT TCA A	25	TTG TAG TAC AGG CTT GTG ATT C	22
	cyt1	AAC CCC TCA ATC AAC AGC AAG G	22	GGT ACA CAA TAC ATA ACG CCA CC	23

Specific Primers for *cry 1* group of genes

No.	Gene	Sequence (5'→ 3')			
		Direct	Mer	Reverse	Mer
	cry1A	CCG GTG CTG GAT TTG TGT TA	20	AAT CCC GTA TTG TAC CAG CG	20
	cry1B	CTT CAT CAC GAT GGA GTA A	19	CAT AAT TTG GTC GTT CTG TT	20
	cry1C	AAA GAT CTG GAA CAC CTT T	19	CAA ACT CTA AAT CCT TTC AC	20
	cry1D	CTG CAG CAA GCT ATC CAA	18	ATT TGA ATT GTC AAG GCC TG	20
	cry1Aa	TGC ATA GAG GCT TTA AT	17	CAG GAT TCC ATT CAA GG	17
	cry1Ab	TCG GAA AAT GTG CCC AT	17	AAT TGC TTT CAT AGG CT	17
	cry1Ac	GGG ACT GCA GGA GTG AT	17	CAG GAT TCC ATT CAA GG	17
	cry1Ad	CAG CCG ATT TAC CTT CTA	18	TTG GAG CTC TCA AGG TGT AA	20

Screening *of cry* genes by PCR analysis

The development of molecular tools has paved the way for rapid and specific identification of specific gene or markers (Carozzi *et al.*, 1991), thus PCR was run to predict insecticidal activities to identify *cry* gene, determine their distribution and detect new *cry* genes in *B. thuringiensis* strains. Native isolate ABt 10 was analyzed for

presence of different *cry* genes using general primers in the present study. *B. thuringiensis* spp. *kurstaki* HD-73 (standard) gave amplified product of approximately 655 bp size using specific primer of *cry 1Ac*. Similarly, isolate ABt 10 was positive for *cry 1Ac* gene (Bourque *et al.*, 1993; Morris *et al*., 1998 and Purani, 2005). Primers for *cry 1Ab, cry 1 D* and *cry 4*specific gene produced amplicon of ~ 858 bp, ~ 290 bp and 797 bp, respectively. Isolate ABt 10 gave same desired product. So, it was proved that isolate ABt 10 possessed *cry 1Ab*, *cry 1 D* and *cry 4* gene, which shows Lepidopteran specific insecticidal activity. Similar findings were also reported by Bourque *et al*., (1993), Ceron *et al.,* (1994) and Ben-Dov *et al*., (1999) (Figure 1).

PCR analysis was also carried out for general primers of *cry 3, 5, 7, 8, 11, 12, 13, 14, 21* and *cyt 1*. But none of the isolate found positive for presence of the above genes; so it was concluded that these genes are absent in native population.

In this study, we have focused only on preliminary *cry* gene characterization through PCR. Isolate ABt 10, which shown best performance during primary toxicity test in laboratory condition. This isolate can be tested in field for its potency and can be developed commercially as a formulation. The comparison of *cry* gene with standard strain and of our new isolate may lead to a better candidate for cloning and developing transgenic crop. The research work presented here opens the door for future research on developing a novel *B. thuringiensis* of Middle Gujarat for exploitation by farmers for pest control.

In conclusion, based on the results obtained from present investigations, it is concluded that modified method applied for isolation of *B. thuringiensis* from soil revealed good results and can be very useful in future. PCR is found suitable technique for rapid and specific detection of *cry* genes from *B. thuringiensis.* Even though, in the present study few selected primers for *cry* genes have been used, screening of more number of primers are essential for identification of different genes from this and other collections.

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