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ORIGINAL ARTICLE

cry Genes profiling and the toxicity of isolates of *Bacillus thuringiensis* from soil samples against American bollworm, *Helicoverpa armigera*

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Bacillus thuringiensis, bioinsecticides, cry proteins, crystal protein, δ-endotoxins, lepidoptera.

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Abstract

Aims: The aim of this study was to search for *Bacillus thuringiensis* (Bt) harbouring *cry1A* gene which could effectively control cotton pest, American bollworm, *Helicoverpa armigera*.

Methods and Results: *cry* gene profiling of 50 Bt isolates showed the presence of *cry1*, *cry2*, *cry3*, *cry4*, *cry7*, *cry8* and *cry9* genes. None of the isolates harboured *cry1* gene alone. It was always found in combination with *cry3*. There was no isolate positive for *cry10* gene. Considering isolates with single *cry* genes, the frequency of *cry4* was predominant (22%) followed *cry2* (6%), *cry3* (4%) and *cry8* (2%). Isolates having two *cry* genes in combination had 14% incidence for *cry2* + *cry4*, 12% for *cry3* + *cry4* and 10% for *cry1* + *cry3*. The most dominant three gene linkage was *cry1* + *cry3* + *cry4*. Further profiling of *cry1* gene showed that *cry1K* gene was abundantly present in all combinations such as *cry1A*, *cry1D*, *cry1F* and *cry1I*. However, *cry1C* existed independent of other subtypes. Finally, the Bt isolates with *cry1A* were analyzed for 16S rRNA gene, which showed two distinct groups of isolates on the basis of sequence homology. Bioassays of spore–crystal mixtures of SBS-Bt4, 8, 17, 21 and 26 harbouring *cry1* against neonate larvae of *H. armigera* showed LC_{50} 1288, 1202, 467·7, 524·8 and 108·5 μg ml^{-1}. The SBS-Bt26 showed fourfold higher toxicity than the *cry 1Ac* harbouring positive control, HD-73.

Conclusions: None of the isolates harboured single *cry* 1 gene. They were always in combination of two or three genes. A Bt isolate (Bt26) had fourfold higher toxicity against *H. armigera* larvae compared with the positive control HD 73 and hence can be commercially exploited to control insect pest.

Significance and Impact of the Study: The inter relationship between the *cry* genes content and the toxicity may allow better understanding of Bt ecology.

Introduction

The insecticidal properties of δ-endotoxin from the bacterium *Bacillus thuringiensis* (Bt) have been recognized commercially for over 40 years (Porcar and Juarez-Perez 2003). Bt has the ability to synthesize a crystalline protein (so called δ-endotoxins) during the sporulation process (Kumar and Bambawale 2000).

The diversity of habitats from which subspecies of Bt strains have been isolated indicates that the ecology of this bacterium is probably very complex. For example, Bt has been isolated from habitats as different as soil, which is thought to be the preferred habitat (DeLucca *et al.* 1981; Martin and Travers 1989; Chilcott and Wigley 1993), grain mills (DeLucca *et al.* 1982), stored products (Kaelin *et al.* 1994), rearing facilities (Dulmage 1970), the phylloplane (Smith and Couche 1991) and diseased insects (Feitelson *et al.* 1992; Wiwat *et al.* 2000). Recent studies on insecticidal crystal protein (ICP) genes are beginning to provide insight into the possible origin of the diversity of subspecies. ICP genes have been shown to be borne on conjugative plasmids and arranged in clusters

or in operons flanked by insertion sequences. This particular organization can facilitate recombination and exchange of plasmids, providing potentially important mechanisms for the generation of new specificities (Aronson 1993; Meadow 1993). Efficient recombination can be achieved when a sufficient cell density is reached, which occurs when spores germinate in a suitable medium made by a dead insect larva (Aronson 1993). Meadow (1993) suggested that Bt would normally not germinate in the soil but would wait until favourable conditions for growth occur.

Phenotypically and genotypically, Bt can be differentiated from *Bacillus cereus* by the presence of the crystal protein and plasmid-encoded *cry* genes (Travers *et al.* 1987). The sequence of 16S rRNA gene has been widely used as a molecular clock to estimate phylogenetic relationships among bacteria, but more recently it has also become important as a means to identify an unknown bacterium to the genus and species level. A previous study using the 16S rRNA gene for rapid identification of the *Bacillus* genus was undertaken by Goto *et al.* (2000). At this time, the use of a hyper variable region (nucleotides 70–344) of the gene was demonstrated adequate to discriminate between all the species except among *B. thuringiensis, B. cereus* and *Bacillus anthracis* and between *Bacillus mojavensis* and *Bacillus atrophaeus*.

Many crystalline proteins characterized by their entomopathogenic activity are highly specific for several insect orders. Nevertheless, for several crystal-producing Bt strains, no toxicity has been determined (Höfte and Whiteley 1989; Schnepf *et al.* 1998). Some Bt strains are widely used to control Lepidoptera, Diptera and Coleoptera pests, and those in other invertebrates like nematodes. There has been intense interest in recent years in collecting, analyzing and screening Bt strains isolated from the environmental samples.

In this article, we determine the reliability of 16S rRNA gene sequences for the purpose of identification of Bt and molecular characterization of Pakistanian Bt isolates and frequency of different *cry* genes such as *cry1, cry2, cry3, cry4, cry7, cry8, cry9* and *cry10* genes in the local isolates. Moreover, the toxicity of Bt harbouring *cry1A* gene has been determined against American bollworm, *Helicoverpa armigera*.

Materials and methods

Sample collection

Fifty soil samples were collected from various habitats in different areas of Pakistan such as Lahore, Sheikhupura and Faisalabad. Bt strains were isolated from the samples collected from the topsoil (0–5 cm soil depth) of cultivated and uncultivated areas that had no history of treatment with Bt products. Samples collected from the cultivated land were mostly the mixture of humus, pebbles and dried fine-powdered soil.

Isolation of *Bacillus thuringiensis*

The soil samples were processed for isolation of Bt by sodium acetate method (Ejiofor and Johnson 2002; Tzeng *et al.* 2002). The soil samples (1 g) in 10 ml of 0·3 mol l^{-1} sodium acetate (pH 6·8) were incubated at 30°C for 4 h in 100 ml flask with 250 rev min^{-1} agitation. The sodium acetate solution suppresses Bt spores from germinating. Two millilitres of the incubated sample was heated at 80°C for 10 min to effectively kill micro-organisms that germinated, diluted tenfold and 100-fold in sodium acetate buffer (pH 6·8), and then 300 μl of each was serially spread on nutrient-rich agar plates (3 g tryptone, 2 g tryptose, 1·5 g yeast extract, 6·9 g sodium dihydrogen phosphate, 8·9 g disodium hydrogen phosphate, 0·005 g $MnCl_2$ and 15 g agar per litre) and incubated at 37°C for 24 h. A measurable number of colonies (more than 10) were routinely obtained on 10^2–10^3 dilution plates after 24–48 h of incubation at 37°C. Well-isolated off-white, dry, round with wavy margin representing rich growth of colonies representing *Bacillus*-like morphology were picked at random, purified by restreaking on agar plates of the same medium and screened for rod-shaped Gram-positive bacteria by Gram staining, and endospores were stained with 5% malachite green and 0·5% safranine.

The Gram-positive spore-forming rods were further characterized by various biochemical tests, the diagnostic scheme and protocols of which were followed from Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994).

The Bt index was calculated as follows:

$$\text{Bt index} = \frac{\text{Total number of Bt isolates}}{\text{Total number of isolates with } \textit{Bacillus} \text{ morphology}}$$

Identification of crystals

Sporulated cultures of Bt isolates were observed under electron microscope (JEOL, JM-1010) for the presence of crystals. The spore–crystal mixture was loaded on carbon-coated copper grid (1 mm diam.) and observed at different magnifications.

Identification of *cry* genes

All Bt strains were grown for 16–18 h on LB agar plate. A loopful culture was resuspended in the autoclaved distilled water and the mixture was frozen at −70°C for

10 min and then transferred to the boiling water for 10 min. This heat shock step was repeated twice to lyse the cells. The resulting cell lysate was centrifuged at 12 406 ***g*** for 5 min (Minispin plus Eppendorf AG centrifuge 22331, Vernon Hills, IL), and freshly prepared supernatant was used as DNA sample in the PCR (Bobrowski *et al.* 2001). For detection of eight different *cry* genes, specific primers (Table 1) were used (Ben-Dov *et al.* 1997; Alberola *et al.* 1999). The PCRs were carried out using 0·2 μg of DNA template in a reaction mixture (total volume 50 μl) containing each dNTP at a concentration of 400 μmol l^{-1}, each primer at a concentration of 1 μmol l^{-1}, and 0·5 U of *Taq* DNA polymerase (Fermentas Life Sciences, Glen Burnie, MD) dissolved in the corresponding reaction buffer. Amplifications were performed with a thermocycler (Applied Biosystem 2720, Carlsbad, CA) under the following conditions: 5 min of denaturation at 94°C, followed by 35 cycles each of denaturation for 2 min at 94°C, annealing for 1 min at 50–55°C (depending on the Tm of primer pairs) and extension for 2 min at 72°C. An extra extension step consisting of 7 min at 72°C was added after completion of 35 cycles.

Cloning and sequencing of 16SrRNA gene

For ribotyping, rDNA-F (5′-TGAAAACTGAACGAAA-CAAAC-3′) and rDNA-R (5′-CTCTCAAAACTGAACA-AAACGAAA-3′) were used as forward and reverse primers, respectively, to amplify a full-length 1554-bp fragment of

Table 1 Primers for the detection of different *cry* genes in the local isolates of *Bacillus thuringiensis*

Primer pair	Sequence (5′ 3′)	Genes	Temperature (°C)	Product size (bp)	Source
Un1(d)	5′-CATGATTCATGCGGCAGATAAAC-3′	*Cry1*	55·2	276	Ben-Dov *et al.* (1997)
Un1(r)	5′-TTGTGACACTTCTGCTTCCCATT-3′	*Cry2*	54·5	700	Ben-Dov *et al.* (1997)
Un2(d)	5′-GTTATTCTTAATGCAGATGAATGGG-3′	*Cry3*	54·5	589	Ben-Dov *et al.* (1997)
Un2(r)	5′-CGGATAAAATAATCTGGGAAATAGT-3′	*Cry4*	50·2	439	Ben-Dov *et al.* (1997)
3F	5′-CGTTATCGCAGAGATGACATTAAC-3′	*Cry7*	52·2	264	Alberola *et al.* (1999)
3R	5′-CATCTGTTGTTTCTGGAGGCAAT-3′	*Cry8*	50·2	430	Alberola *et al.* (1999)
Un4(d)	5′-GCATATGATGTAGCGAAACAAGCC-3′	*Cry9*	55·4	354	Ben-Dov *et al.* (1997)
Un4(r)	5′-GCGTGACATACCCATTTCCAGGTCC-3′	*Cry10*	58·2	623	Alberola *et al.* (1999)
7AbF	5′-TGATCCAGCAACTATAACACGAGTGATAGAT-3′	*Cry1A*	61·4	272	Ben-Dov *et al.* (1997)
7AbR	5′-CACTTAACGCAATGGTTAGAATATTCAGAGATA-3′	*Cry1C*	60·4	670	Alberola *et al.* (1999)
8F	5′-AACGTAATTCGTCCGCCACACTT-3′	*Cry1D*	58·6	802	Alberola *et al.* (1999)
8R	5′-TTTCAGCTACCGGTACAGTTCTATCTA-3′	*Cry1F*	54·3	245	Alberola *et al.* (1999)
Un9(d)	5′-CGGTGTTACTATTAGCGAGGGCGG-3′	*Cry1G*	60·2	419	Alberola *et al.* (1999)
Un9(r)	5′-GTTTGAGCCGCTTCACAGCAATCC-3′	*Cry1H*	58·2	731	Alberola *et al.* (1999)
10F	5′-TATTGTTGGAGTTAGTGCAGGTATTATTGTAG-3′	*Cry1I*	57·1	621	Alberola *et al.* (1999)
10R	5′-TATTCCATGTTGCGTTAGTATTAGTTC-3′	*Cry1K*	52·6	246	Alberola *et al.* (1999)
1AF	5′-CGTAGAAGAGGAAATCTATCCA-3′	*Cry1*	50·7	276	Ben-Dov *et al.* (1997)
1AR	5′-TATCGGTTTCTGGGAAGTA-3′	*Cry2*	48·4	700	Ben-Dov *et al.* (1997)
1CF	5′-ATTGGGGAGGACATCGAGTAATATCTAGCCTTA-3′	*Cry3*	63·8	589	Ben-Dov *et al.* (1997)
1CR	5′-ACTCCTGTGGATGCCGCTCCTGTTAATACTAT-3′	*Cry4*	65·3	439	Ben-Dov *et al.* (1997)
1DF	5′-GTCGCTAAACGCTCTAACATAGACCTT-3′	*Cry7*	56·9	264	Alberola *et al.* (1999)
1DR	5′-GTTTAAGTAATCCTGATGCGATATTACTAGATGC-3′	*Cry8*	59·6	430	Alberola *et al.* (1999)
1FF	5′-GCTTATACTATTGTTAATATAAATGCGC-3′	*Cry9*	52·8	354	Ben-Dov *et al.* (1997)
1FR	5′-GTATCAGCACCTAVTGTGAAACTACTCT-3′	*Cry10*	53·0	623	Alberola *et al.* (1999)
1GF	5′-TTGCAGGTAATATAAATTTTGGTTTAAGTATAGC-3′	*Cry1A*	57·9	272	Ben-Dov *et al.* (1997)
1GR	5′-TCCCTCAATTGGAATTTCATCAATAGTATC-3′	*Cry1C*	59·0	670	Alberola *et al.* (1999)
1HF	5′-CACGCGCTTTCTTTTGGAGTCACTTCTC-3′	*Cry1D*	64·9	802	Alberola *et al.* (1999)
1HR	5′-GTTCTCTTAATATATTCTGTAAATTCGCATTCAA-3′	*Cry1F*	58·5	245	Alberola *et al.* (1999)
1IF	5′-TTGAAAATGTCTGAGTATGAAAATGTAG-3′	*Cry1G*	52·5	419	Alberola *et al.* (1999)
1IR	5′-GTTCGACTTGACGGTTATAAAATGTTG-3′	*Cry1H*	56·4	731	Alberola *et al.* (1999)
1KF	5′-AAAACGCAATTATTCGACAACCTCAC-3′	*Cry1I*	58·6	621	Alberola *et al.* (1999)
1KR	5′-GCCCACCAGCCTAATGATACAGTTCG-3′	*Cry1K*	59·8	246	Alberola *et al.* (1999)

16S rRNA gene. PCR mixture was prepared as previously described by Sacchi *et al.* (2002) using Fermentas Life Sciences *Taq* DNA polymerase. The thermal profile for amplification reaction included single denaturation step of 5 min at 95°C, a step cycle program set for 35 cycles with each cycle consisting of denaturation at 94°C for 1 min and 30 s, annealing temperature from 48 to 55°C for 45 s, and extension at 72°C for 1 min 30 s, and an extra extension step for 7 min at 72°C. The amplified product was cloned in pTZ57 cloning vector. The competent cells of *Escherichia coli* DH5α were transformed with the recombinant plasmid carrying 16S rRNA gene using method given in Sambrook *et al.* (1989).

The gene was sequenced on Genetic Analyzer CEQ8000 (Beckman-Coulter, Brea, CA; using GenomeLab DTCS-Quick Start kit (catalogue no. 608120). The sequences of 16S rRNA genes were aligned and compared using the multiprocessor of ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Full-length 16S rRNA gene sequence data were obtained from GenBank (accession numbers AY138290, AY138289, AY 138288, AY138287, AY138286, AY138285, AY138284, AY138283, AY138282, AY138281, AY138280) to determine the sequence homology. The aligned sequences were analyzed using the Molecular Evolutionary Genetics Analysis package ver. 4 (31; http://www.megasoftware.net). Rooted phylogenetic tree was built with neighbour-joining method. A close neighbour-interchange search was performed to examine the neighbourhood of the neighbour-joining tree to find a potential minimum evolution tree. Bootstrap confidence values were generated using 1000 permutations of the data set to derive the nucleotide sequence similarities.

Bioassays of Bt isolates with Cry1A protein against target insect

To evaluate the toxicity of *cry1A* gene product, bioassays were conducted against American bollworm, *H. armigera.*

Preparation of artificial diet

The American bollworms, *H. armigera*, were reared on artificial diet, prepared with several modifications as described by Shao *et al.* (2001). Bioassay diet included 30% of chick pea powder, 3%(w/v) of baking yeast powder, 0·075% (w/v) formaldehyde, 0·3% (w/v) choline chloride, 3% (w/v) sucrose, 0·3% (v/v) wheat germ oil, 0·6% (w/v) ascorbic acid, 0·02% (w/v) thiamin hydrochloride, 0·02% (w/v) folic acid, 0·224% (w/v) sorbic acid, 0·12% (w/v) cholestrin, 0·374% (w/v) methyle-4-hydroxy benzoate and 3% agar. Freshly prepared microbial diet was poured into the glass vials (5·5 cm long and 2·0 cm diameter) and stored at 4°C.

Preparation of bacterial dose

Nutrient broth (100 ml) was inoculated with a single isolated Bt colony, incubated at 32°C and 100 rev min^{-1} for 72–96 h to sporulate the culture. At the end of incubation, the majority of the population was in the form of spores and crystals (<5% vegetative cells). Bacterial cells were harvested by centrifugation at 5514 ***g*** for 10 min at 4°C. The bacterial pellets were washed three times with chilled 0·5 mol l^{-1} NaCl and four times with cold distilled autoclaved H_2O. The wet and dry weight of final pellet (mixture of crystal and spores) was determined and stored at −20°C. Final pellet was resuspended in 20 ml distilled autoclaved H_2O at the time of bioassays. The density of bacterial doses was optimized on *cry1Ac* harbouring positive control strain of Bt HD-73 (*B. thuringiensis* U89872 *cry1Ac* delta endotoxin gene) obtained from Dan Zeigler of Bacillus Genetics Stock Center, Columbus, OH, USA.

Bioassay procedure

Five concentrations of bacterial dose *viz,* 0·3, 0·6, 1, 1·3 and 1·6 mg ml^{-1} were selected. Each dose was thoroughly mixed in 3ml of artificial diet. Thirty neonate larvae of *H. armigera* of almost same weight were used in each dose experiment. These experiments were replicated thrice for each dose. The experimental glass vials were kept in insect culture room at 28 ± 2°C, 60 ± 5% humidity and 12L : 12D photoperiod for 3 days. Larval mortality was recorded after 72 h. Mortality of the control larvae reared on toxin-free diet and under the same conditions was also recorded. Abbot's Formula (Abbott 1925) was used to correct the test mortality. Regression curve was plotted between percentage mortality of neonate larvae and log dose of spore–crystal suspension mixed in artificial diet. LC_{50} of local Bt isolates was calculated using linear line equation of regression curve (Moar *et al.* 1989).

Results

Characterization of Bt isolates

Of 470 *Bacillus*-like colonies, 250 isolates (53%) were stained as purple rods with Gram stain, and had green spores, and deep pink protein crystals and vegetative cells after staining with malachite green and safranine.

Most of the Bt isolates formed off-white dry colonies with smooth, entire wrinkled or wavy margins. On biochemical analysis, these isolates were found to be motile and showed positive catalase and Voges–Proskauer tests, utilized citrate, utilized different sugars (xylose, arabinose, mannitol) and produced no acid, reduced nitrates, decomposed tyrosine, hydolysed casein and starch, grew on Sabouraud Dextrose agar and media containing 7% NaCl

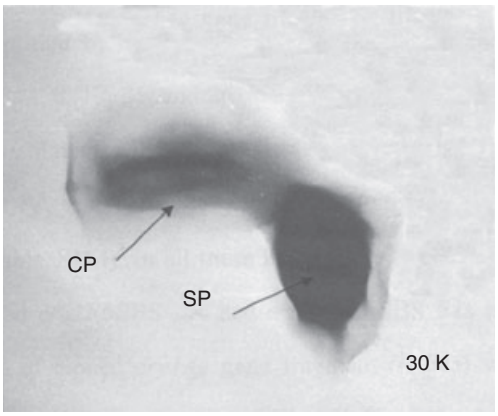

Figure 1 Electron micrograph of sporulation phase (96 h) of one of the Bt isolates reported in this study. Sporulated (SP) cell and the characteristic crystal protein (CP) are distinctly visible.

and 0·001% lysozyme, but did not grow at 65°C. On the basis of above biochemical tests, fifty isolates of 250 *Bacillus*-like colonies were identified as *B. thuringiensis* (Bt) and designated as School of Biological Sciences (SBS) Bt1–50.

The sporulated cells of Bt isolates were visualized under high-resolution transmission electron microscope. The cells of HD-73 appeared to be elongated and larger in size than the Bt isolates. The Bt isolates were found to contain crystal proteins as parasporal inclusions, mostly intact with the sporulated cells, and showed different stages of crystal protein synthesis – a pattern distinctly different from that of HD-73. A complete bipyramidal shaped crystal formation was observed only in SBS Bt21. The crystal protein of HD-73 remained intact within the spore until 72 h after incubation and was released after 96 h, whereas in most of the Bt isolates the crystal toxins were released after 72 h.

Figure 1 shows the electron micrograph of Bt isolate during sporulation phase. A characteristic crystal protein is visible.

Bt index

Soil samples collected from agricultural and nonagricultural fields gave different Bt indices. Seventy-five per cent of the Bt isolates came from agricultural soil, 18% from the wheat grain dust and 12% from cattle waste. The Bt index for soil sample was 1·296, for cattle waste 0·6 and for wheat grain dust 0·69, with an average of 0·86.

cry genes and their combinations in Bt isolates

The typing of primary rank of *cry* genes (*cry 1*, *2*, *3*, *4*, *7*, *8*, *9* and *10*) of Bt isolates was performed by the PCR amplification using specific primers given in Table 1. Fifty isolates were found to be positive for the presence of *cry* genes, of which 17 (34%) carried single *cry* gene alone, whereas 25 (50%) isolates had combinations of two *cry* genes, and eight (16%) had combination of three *cry* genes. *cry10* was not detected in any of the isolates.

The isolates in this investigation were classified into three large groups (Table 2). The first group of isolates contained exclusively only one *cry* gene namely *cry1, cry2, cry3, cry4, cry7, cry8, cry9* and *cry10*, as indicated by their specific product size of 276, 700, 586, 439, 264, 430, 300 and 400 bp, respectively (Fig. 2). *cry7*, *cry9* and cry1 were not found in this group of isolates. Both these genes were present in combination with other *cry* genes. The isolates carrying single gene showed dominance of *cry4* (22%), followed by *cry2* (6%), *cry3* (4%) and *cry8* (2%). Within the group, *cry4* constituted 65% and *cry2* 18% of the total single gene harbouring isolates (Table 2). In second group, seven combinations were found (Table 2). The most dominant combination was that of *cry2* + *cry4* (14%), followed by *cry3* + *cry4* (12%), *cry1* + *cry3 (10%), cry4* + *cry9* (6%), *cry2* + *cry3* (4%), *cry3* + *cry7* (2%) and *cry4* + *cry8* (2%). Within the group, *cry3* gene was found in combination with *cry4* gene (24%), *cry1* (20%), *cry2* (8%) and *cry7* (4%). On the other hand, *cry 4* gene was found in combination with *cry2* (28%), *cry9* (12%) and *cry8* (4%) (Table 2). The third group of eight isolates had three combinations *viz.*, *cry1* + *cry3* + *cry4* (50%), *cry2* + *cry3* + *cry4* (25%) and *cry4* + *cry7* + *cry9* (25%) (Table 2). *Cry4* is common in all combinations in this group, whereas *cry3* is present in the first two combinations along with *cry1* in one and *cry2* in the other combination.

Profile of secondary rank of *cry1* type gene combinations

Several sets of primers (Table 1) for detecting the secondary rank of *cry1*-type genes were used to further characterize the local isolates containing the *cry1* genes. The amplification of *cry1* gene have shown different subtypes with varied sizes *e.g.* the *cry1A* 272 bp, *cry1C* 670 bp, *cry1D* 802 bp, *cry1F* 245 bp, *cry1G* 419 bp, *cry1H* 731 bp, *cry1I* 621 bp and *cry1K* 246 bp (Fig. 3). No *cry1* subtype was exclusively found in any of the isolates. Of 50 *cry*-positive isolates, only 10 had *cry1*, all in combinations except for *cry1C*. Five of these isolates had combination of two genes, and the remaining four had combination of three genes. *cry1K* was the most dominant constituting 28% of the *cry1*-positive isolates, followed by *cry1A* (20%), *cry1D* (16%), *cry1I* (16%), *cry1F* (12%) and *cry1C* (8%) (Table 3).

All *cry1* subtypes were found in combination with *cry3* gene (Table 2). *Cry1A* was always found in combination with *cry1K*, which appears to be universally present in all

Table 2 Incidence of different *cry* genes existing alone or in combinations in various local Bt isolates from Pakistan

	Group I (one *cry* gene)			Group II (two *cry* genes in combination)			Group III (three *cry* genes in combination)		
		% incidence out of			% incidence out of			% incidence out of	
No.	*cry* gene (in Bt isolates)	50-cry-positive isolates	17 Bt isolates with single cry gene	Combination of two *cry* genes (in Bt isolates)	50-cry-positive isolates	25 Bt isolates with two cry genes	Combination of three *cry* genes (in Bt isolates	50-cry-positive isolates	8 Bt isolates with two cry genes
1	*Cry1* (0)	0	0	*cry1* + *cry3* (SBS Bt4, 8, 17, 21, 24)	10	20	*cry1* + 3 + *4* (SBS Bt1, 6, 26, 44)	8	50
2	*Cry2* (SBS Bt12, 22, 28)	6	18	*cry2* + *cry3* (SBS Bt20, 33)	4	8	*Cry2* + 3 + *4* (SBS Bt34, 37)	4	25
3	*Cry3* (SBS Bt3, 10)	4	12	*cry3* + *cry4* (SBS Bt9, 11, 13, 14, 19, 35)	12	24	*Cry4* + *7* + *9* (SBS Bt46, 48)	4	25
4	*Cry4* (SBS Bt5, 10, 15, 16, 25, 27, 29–31, 36, 44)	22	65	*cry3* + *cry7* (SBS Bt39)	2	4			
5	*Cry7* (0)	0	0	*cry2* + *cry4* (SBS Bt7, 18, 23, 32, 38, 40–42)	14	28			
6	*Cry8* (SBS Bt2)	2	6	*Cry4* + *cry9* (SBS Bt45, 47, 49)	6	12			
7	*Cry9* (0)	0	0	*Cry4* + *cry8* (SBS Bt50)	2	4			
8	*Cry10* (0)	0	0						

cry1 subtype combinations. *cry1K* is present in all *cry1*-positive isolates except SBS Bt3, 6 and 19 (Table 4). *Cry1F* gene was found in the SBS Bt-1, 17 and 21, while *cry1C* gene was detected only in the SBS Bt-3 and 19. Likewise, *cry1D* was detected in SBS Bt6 and 26 and *cry1I* gene was identified in SBS Bt1, whereas *cry1D* and *cry1I* were found together in SBS Bt4 and 34. Two *cry1* subtype genes, *cry1G* and *cry1H* were not detected in any Bt isolate with the sets of primers used in this study.

Table 4 shows the multiple combinations of *cry1* subtypes in different Bt isolates. The two isolates which showed combination of two *cry1A* subtypes had *cry1K* in association with *cry1A* (SBS Bt8) and another with *cry1D* (SBS Bt6). The isolates which have combination of these subtypes had *cry1A* and *cry1K* common to all, with additional *cry1D* (SBS Bt26), *cry1F* (SBS Bt17 and 21) and *cry1I* (SBS Bt4). The isolate SBS Bt43 had *cry1D* + *cry1K* + *cry1I* combination. Two isolates SBS Bt3 and 19 had only one subtype (*cry*1C) without any linkage with other subtypes.

Diversity in isolates inferred from 16srRNA analysis

The 1554 -bp nucleotide sequences of the entire 16S rRNA gene of the Pakistanian Bt isolates were amplified, cloned in the cloning vector pTZ57 and sequenced. The sequences of 16S rRNA gene for different strains have been submitted to EMBL under accession numbers AM292317, AM292316, AM292314, AM292315, AM293342 and AM292318 for SBS Bt4, 8, 17, 21, 26 and HD-73, respectively. The sequences of the 16S rRNA gene were aligned with the reported full-length 16S rRNA Bt sequences obtained from the GenBank (Accession numbers AY138290, AY138289, AY138288, AY138287, AY138286, AY138285, AY138284, AY138283, AY138282, AY138281, AY138280) and compared using the multiprocessor of ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). It was found that our submitted sequences showed 99% homology with the reported sequences. Multiple alignments of these sequences revealed the presence of two characteristic regions with majority of the positional sequence differences: positions 560–686 and 1101–1146. Table 5 shows the most common positional differences such as T → C at 192 and 560, C → T at 342, A → C at 462, 1015 and 1121, T → A at 686 and 1101, and A → T at 1146. SBS Bt*17* had four variations (at positions 192, 342, 1015, 1101), SBS Bt*8* and 21 had three variations (at positions 192, 342, 1015 and 462, 686, 1121, respectively), SBS Bt*4* and 26 had two variations (at positions 462, 1121 and 462 and 560, respectively), and HD-73 has only one variation at position 1015 from the standard reported sequence of Bt (AY138283 *B. thuringiensis*

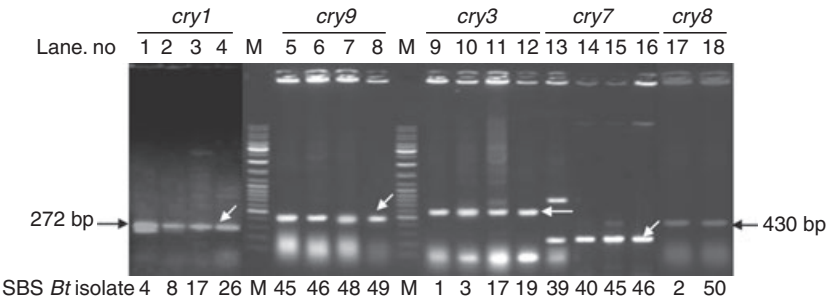

Figure 2 PCR analysis of crystal (Cry) protein genes of Bt isolates. PCR products of some of the *cry* genes of a few isolates have been shown. For example, *cry1*, lanes 1–4; *cry*9, lanes 5–8; *cry3*, lanes 9–12; *cry7*, lanes 13–16 and *cry8*, lanes 17 and 18. Lane M: 1kb DNA MW marker. The isolate numbers are given at the bottom of the gel against each lane.

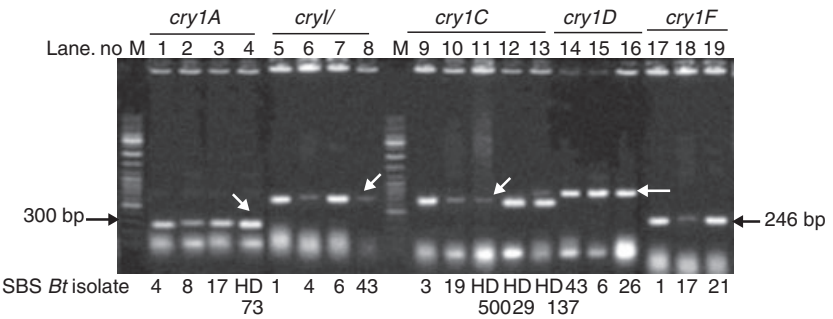

Figure 3 Agarose gel (1%) electrophoresis of PCR products of subtypes of *cry1* genes amplified from local Bt isolates. Lanes 1–4, *cry1A* (272 bp), lanes5–8, *cry1I* (621 bp), lanes 9–13, *cry1C* (670 bp), lanes 14–17, *cry1D* (802 bp), lanes 17–19, *cry1F* (246 bp). Lane M: 1 kb DNA MW marker. The isolate numbers are given at the bottom of the gel against each lane.

Table 3 Incidence of different subtypes of *cry1* genes from local Bt isolates

No	*Cry1* subtype genes	Per cent incidence of *Cry1* subtypes out of	
		50 *cry*-positive isolates	25 *cry1*-positive isolates
1	*cry1A*	10	20
2	*cry1C*	4	8
3	*cry1D*	8	16
4	*cry1F*	6	12
5	*cry1G*	0	0
6	*cry1H*	0	0
7	*cry1I*	8	16
8	*cry1K*	14	28

Table 4 Incidence of *cry1* subtypes gene combinations in local Bt isolates

No.	Combinations of *cry1* subtype	Bt isolates	Percentage incidences of different subtypes combinations in cry1-positive isolates
1	*cry1A* + *cry1K*	SBS Bt-8	10
2	*cry1A* + *cry1D* + *cry1K*	SBS Bt-26	10
3	*cry1A* + *cry1F* + *cry1K*	SBS Bt-17, SBS Bt-21	20
4	*cry1A* + *cry1F* + *cry1I* + *cry1K*	SBS Bt-1	10
5	*cry1D* + *cry1I* + *cry1K*	SBS Bt-43	10
6	*cry1A* + *cry1I* + *cry1K*	SBS Bt-4	10
7	*cry1D* + *cry1K*	SBS Bt-6	10
8	*cry1C*	SBS Bt-3, SBS Bt-19	20

strain 2002007400 16S ribosomal RNA gene, partial sequence).

In addition to already reported positional differences, a number of other strain-specific differences were noticed, but as the cloned PCR product was amplified with *Taq* polymerase without proof reading activity these variations were not considered in this study.

Bioassays with *cry1A*-positive Bt isolates

The results of toxicity bioassays revealed that the growth of neonate larvae of *H. armigera* was inhibited during the first 48 h of releasing them in crystal/spore mixed artificial diet. The LC_{50} of SBS Bt4, 8, 17, 21 and *26* were 1288, 1202, 467·7, 524 and 108·6 μg ml^{-1}, respectively. The LC_{50} of HD-73 was 480·5 μg ml^{-1}. There was no mortality recorded in the negative control assays. Although the LC_{50} of SBS Bt17 and SBS Bt21 was comparable to positive control HD-73, SBS Bt26 strain was found to be potentially the most toxic (Fig. 4).

The relative toxicity of all isolates was variable considering control HD-73 as standard. SBS Bt*4* and *8* had highest lethal concentrations 0·372 and 0·4, respectively and appeared relatively less toxic. Two strains SBS Bt17

Table 5 Already reported general position differences in full-length 16S rRNA gene sequences of Bt isolates

	Nucleotide position numbers								
Strain no.	192	342	462	560	686	1015	1101	1121	1146
AY138283	T	C	A	T	T	A	T	A	A
HD-73	T	C	A	T	T	C	T	A	T
SBS–Bt4	T	C	C	T	T	A	T	C	A
SBS–Bt8	C	T	A	T	T	C	T	A	A
SBS–Bt17	C	T	A	T	T	C	A	A	T
SBS–Bt21	T	C	C	T	A	A	T	C	A
SBS–Bt26	T	C	C	C	T	A	T	A	A

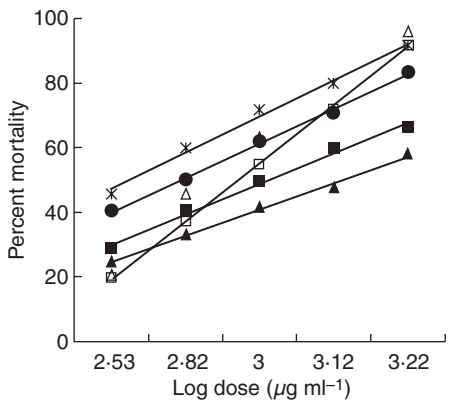

Figure 4 Toxicity of Bt isolates SBS Bt4 (□), SBS Bt8 (■), SBS Bt17 (▲), SBS Bt21 (●), SBS Bt26 (Δ) and Control HD-73 (⨯) against larvae of *Helicoverpa armigera*.

and 21 were equally toxic (1·02 and 0·916, respectively), while the SBS Bt26 strain was relatively most toxic (4·427) when compared with positive control and other locally isolated strains.

Discussion

Diversity of habitat

In this study, Bt was isolated from wheat field areas and cattle waste–related habitats, where no Bt products have been applied before. Bt occurrence in all soil samples collected from agricultural field was found to be relatively higher compared with other soil samples. These agricultural fields were being used for growing vegetables and wheat crops.

The Bt index serves as measurement of success in isolating Bt. The average Bt index in the present study was 0·86. This index was 0·6 for cattle waste and 0·69 for wheat grain dust. Bravo *et al.* (1998) reported Bt index of about 0·24 in cultivated fields of Mexico, whereas Martin and Travers (1989) reported a value of 0·85 in the soil samples collected from different locations in Asia. On the contrary, Hongyu *et al.* (2000) and Bernhard *et al.* (1997) have reported abundant occurrence of Bt in the stored product environments than in the soil in China. Obeidat *et al.* (2004) collected samples from different habitats in Jordan and reported grain dust as the richest Bt source with relative index of 1·0 compared with 0·8 of industrial bye-products, 0·7 of animal faeces and 0·6 of cultivated soil.

Frequency of different *cry* genes

The identification of Bt *cry* genes by PCR has proven to be a very useful method for strain characterization and selection (Porcar and Juarez-Perez 2003). In addition, Carozzi *et al.* (1991) found correspondence of toxicity with the amplification of particular *cry* gene profiles, introducing PCR as a tool to predict Bt insecticidal activity. The identification of known *cry* genes in the Bt is important, as the specificity of action is known for many of the Cry toxin.

In this study, *cry10* was not detected in any of the isolates, whereas *cry1* and *cry7* were never detected alone – they were always found in combination with other *cry* genes. The isolates which had only one gene had prevalence of *cry* genes in the order *cry4* (65%) >*cry2* (18%) >*cry3* (12%) >*cry8* (6%). However, Bravo *et al.* (1998) have found *cry1* gene the most frequently occurring (49·5%) than the *cry3* gene (21·7%) and *cry9* gene the least abundant (2·6%). In contrast, Pinto and Fiuza (2003) reported that *cry9* gene was the most frequent (47·8%), whereas *cry1* gene was identified in a few isolates (6·5%). They reported *cry3* gene abundant after *cry9* gene. The differences among the frequencies obtained in this study and those described by Ben-Dov *et al.* (1997) become interesting when compared to each other because of the fact that the primers are identical. This data suggest a difference in the diversity of Bt when geographically compared. The results show how different geographic regions affect the diversity of *cry* gene contents of Bt strains.

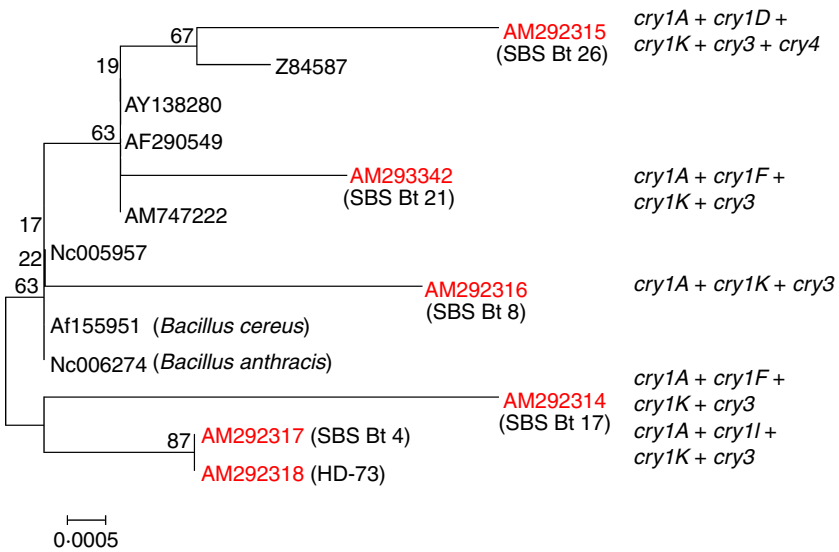

Figure 5 The evolutionary history of *cry1*-positive isolates was inferred using the Neighbour-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0·02414325 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown above the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set (Complete deletion option). There were a total of 998 positions in the final data set. Phylogenetic analyses were conducted in Mega4 (Tamura *et al.* 2007).

In the present studies, some strains containing combinations of *cry* genes, such as lepidopteran active *cry1* genes and coleopteran active *cry3*, less frequent combinations including *cry3* genes and *cry7* genes, and *cry3* genes and *cry9*, were identified. These strains are good candidates in the search for biocontrol agents with a wider spectrum of action. Other groups have reported the presence of *cry3* genes and *cry7* or *cry9* genes in the same Bt strains (Aronson 1993; Ben-Dov *et al.* 1997), suggesting that strains with dual activity are also present in other regions. In our Bt strains, *cry3* gene is present in combination with *cry1* and *cry7*, while *cry9* gene is also present along with *cry7*. This indicates that many of these isolates contain more than one *cry* genes, suggesting that Bt have high frequency of genetic information exchange.

This study shows linkage of *cry3* with three other *cry* genes (*cry1, cry2, cry4*). The most dominant of these is with *cry4* followed by *cry 1* and *cry2*. Likewise, *cry4* is found in combination with *cry 2, 8* and *9*, of which the most dominant is with *cry2* followed by *cry* 9 and 8. Thirty-two different *cry* gene groups have been defined in the literature (Schnepf *et al.* 1998). The most common *cry* genes found in nature are those within the *cry1* subfamily (Porcar and Juarez-Perez 2003). The most abundant gene found in Costa Rica was *cry2*, and the second most abundant was the *cry1* gene family (Arrieta *et al.* 2004).

Linkage of *cry1* subtypes

Several reports show a high frequency of certain *cry1* gene combinations, for example the linkage *cry1C–cry1D* (Bravo *et al.* 1998; Ferrandis *et al.* 1999), which may be explained by their common location on the same replicon (Sanchis *et al.* 1989). However, Arrieta *et al.* (2004) found *cry1D* gene alone at a relatively high frequency (24 strains), and only seven strains presented such linkage. The absence of *cry1C* may be the result of a deletion or a negative selection of *cry1C* from an ancestral *cry1C–cry1D* linkage. One interesting observation was the high frequency of *cry1D* in combination with those of the subfamily *cry1*A. The opposite case was observed with the *cry1E* and *cry1F* genes, as only eight strains contained these genes (Ferrandis *et al.* 1999). In this study, *cry1K* was the most dominant (28%) and universally present along with *cry1A* in most of the isolates (62%). In isolates having linkage of three *cry1* subtypes, *Cry1A+cry1K* are linked with *cry1D, cry1F* and *cry1I*. SBS Bt1 has the unique combination *cry1A + cry 1F + cry1I and cry 1K*.

Diversity of Bt isolates based on ribotyping

Analysis of 16S rRNA sequences is a simple, commonly used method for the identification of micro-organisms (Amann *et al.* 1995; Obeidat *et al.* 2004). However, early studies performed with a limited number of isolates from

B. cereus group revealed that the 16S rRNA sequences of Bt isolates in this group had as high as a 99–100% homology. Although conserved in overall sequence, the 16S rRNAs actually exhibited great variation in some regions. These differences in 16S rRNA sequences provide the basis for designing nucleic acid probes of various specificities, ranging from probes targeting all living organisms to group- and species-specific probes. Another advantage of using the rRNAs as a target is the fact that these molecules are naturally amplified within the cell. In general, rRNA represents about 80% of the total nucleic acids in microbial cells and, thus, is present in many hundreds of thousands of copies per cell. This natural amplification allows for direct detection of rRNA sequences without the need for intermediate amplification (Amann *et al.* 1995).

Based on 16S rRNA, the *cry1A*-positive Bt isolates seem to fall into two major categories – three isolates *viz.*, SBS Bt26, SBS Bt21 and SBS Bt18 in one category, and SBS Bt17 and SBS Bt4 along with the control HD 73 in the second category (Fig. 5). The distances show the diversity of these isolates. *Bacillus cereus* (Nc006274 *B. cereus* E33L, complete genome) and *B. anthracis* (Af155951 *B. anthracis* strain Delta Ames 16S ribosomal RNA gene, partial sequence*)* occupy intermediate position. The SBS Bt26 and SBS Bt21 have three subtypes of *cry1* gene, *viz.*, *cry1A* + *cry1D* + *cry1K* in SBS Bt26 and *cry1A* + *cry1F* + *cry1K* in SBS Bt21, whereas Bt8 has two subtypes of *cry1* gene, SBS Bt17 and SBS Bt4 have three subtypes of *cry1*. *cry1A, cry1K* and *cry3* are common denominators in all these isolates. SBS Bt26 has an additional *cry4* gene, which might explain its high toxicity against American bollworm larvae.

The above conclusions were drawn irrespective of several random variations observed in 16rDNA sequences of the Bt isolates, which were ignored because of amplification of the gene with *Taq* polymerase with no proof reading ability.

Toxicity of *cry1*-positive isolates and their potential as bioinsecticide

The crystal protein has fairly broad spectrum of activity against lepidopteran insects, including members of the family Pyralidae, Plutillidae, Sphingidae and Noctudiae (Lambert *et al.* 1996). The toxicities of Bt subsp. *kurstaki* HD-1 and Bt ssp. *kurstaki* wap-2 were determined against diamondback moth larvae, resulting in LC_{50} of $4{\cdot}93 \times 10^4$ and $1{\cdot}32 \times 10^5$ spores ml^{-1}, respectively (Wiwat *et al.* 2000). American bollworm, *Heliothis virescens* in North America and *H. armigera* in Asian region show great behavioural variations in artificial diet bioassays, either because of food avoidance or resorting to canabalism.

The effect of some pesticides was studied on Bt strain HD-1, HD-29 and HD-241 against the three instars of *H. armigera* (Hub). The LC_{50} of these three strains were $7{\cdot}56 \times 10^8$, $1{\cdot}12 \times 10^8$ and $14{\cdot}86 \times 10^8$ spore ml^{-1}, respectively (Reddy *et al.* 1987). Two other strains HD-73 and HD-263 also proved promising biopesticides with LC_{50} 0·1 mg ml^{-1} spore–crystal mixture. The susceptibility of *Chilo partellus* larvae declined with the age; younger larvae (up to 56 h) exhibited high susceptibility; however, sharp decline in mortality was observed with ageing of larvae beyond 72 h. The toxicity of HD1-9 and HD-73 was compared against gypsy moth and HD1-9 strain was found to be more potent than HD-73 (Wolfersberger 1990). Later the toxicity of recombinant Cry1Ac ICP was reported to be highly toxic against the larvae of *Plutella xylostella* and *Artogeia rapa* compared to Xantari, Delfin and HD-73 (Tzeng *et al.* 2002).

The mean estimated LC_{50} of the ICPs from these five local Bt isolates containing *cry1Ac* along with *cry1K* and *cry3* genes based on 3 days' bioassays ranged between 108·6 and 1288 μg ml^{-1} artificial diet. The toxicity levels among these isolates were greatly different from one another. The isolate SBS Bt26 with LC_{50} 108·6 μg ml^{-1} has great potential to be used as bioinsecticide against cotton pest, *H. armigera.*

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