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Environmental distribution and diversity of insecticidal proteins of *Bacillus thuringiensis* Berliner

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ABSTRACT

Bacillus thuringiensis Berliner based biopesticides have been successfully used world over for the control of agricultural pests and vectors of human diseases. Currently there are more than 200 *B. thuringiensis* strains with differing insecticidal activities are available as biocontrol agents and for developing transgenic plants. However, two major disadvantages are the development of insect resistance and high target specificity (narrow host range). Globally there is a continuous search for new *B. thuringiensis* strains with novel insecticidal activities. The present study aims to study the environmental distribution of *B. thuringiensis* and their toxic potential against insect pests. Soil and grain samples were collected from different environments and were processed by a modified acetate selection method. Initially *B. thuringiensis* isolates were screened on the basis of colony morphology and phase contrast microscopy for the presence of parasporal crystal inclusions. The population dynamics showed that *B. thuringiensis* is abundant in sericulture environment compared to other niches. Relative abundance of *B. thuringiensis* strains in sericulture environment shows the persistent association of *B. thuringiensis* with *Bombyx mori* (silk worm) as insect pathogen. The protein profiles of the selected strains were studied by SDS-PAGE. The protein profiles of majority of *B. thuringiensis* isolates from grain storage facilities predominantly showing the 130 kDa and 68 kDa proteins, which is characteristics of lepidopteran active *B. thuringiensis*. However, one isolate BTRX-4 has 80-85 kDa protein, which is novel in that, this strain also exhibits antilepidopteran activity, which is normally presented by *B. thuringiensis* strains having 130 kDa and 68 kDa proteins. The protein profile of *B. thuringiensis* isolates from sericulture environment shows two different protein profiles. *B. thuringiensis* isolates BTRX-16 to BTRX-22 predominantly show 130 kDa protein, however, the *B. thuringiensis* isolates BTRX-23 to BTRX-30 have two distinct protein profiles corresponding to 130 kDa and 68 kDa. These results show that there may be more than one *B. thuringiensis* strain that can infect *Bombyx mori*. The preliminary bioassay against second instar larvae of *Heliothis armigera* showed varying mortality rate. In conclusion, despite the ubiquitous presence of *B. thuringiensis* strains in different environments, specifically the sericulture environment supports *B. thuringiensis* in a significant manner compared to other environments. Further the ICPs produced by different strains of *B. thuringiensis* are unique in terms of the protein profile and hence may also differ in their insecticidal activities.

Keywords: Bacillus thuringiensis, biopesticides, insecticidal crystal proteins

INTRODUCTION

Synthetic insecticides have long been regarded as a panacea for the control of pests and diseases in crop plants. However, their use is limited because of environmental concerns and health problems in human beings (Zimmermann *et al*., 2005). *Bacillus thuringiensis* Berliner is a gram positive soil bacterium that has been successfully used as a biocontrol agent for more than fifty years (Navon, 2000).The insecticidal crystal proteins (ICP) produced by *B. thuringiensis* are highly specific, safe to non-target organisms including humans, completely degradable and eco-friendly (Schnepf *et al*., 1998). These toxins have highly specific activity against the larval forms of the insects belonging to the insect orders Lepidoptera, Diptera, and Coleoptera (Feitelson *et al*., 1992). The horizon of insecticidal activity of *B. thuringiensis* is ever expanding, as new strains of *B. thuringiensis* have been demonstrated to be active against a wide range of insects including cockroaches (*Blatta orientalis*) (Porcar *et al*., 2006). The insecticidal crystal proteins are encoded by the *cry* genes normally present in the plasmids. To date, over 200 *cry* gene sequences have been determined and classified into 44 families and different subclasses (Crickmore *et al*., 2005). The mode of action of the Cry proteins involves solubilization of the crystal protein, processing of the protoxins by intestinal proteases, and recognition of a binding site on the midgut brush border membrane surface, followed by pore formation and cell lysis, leading ultimately to insect death (Vachon *et al*., 2002). Recent information suggests that the vegetative insecticidal proteins (Vip) are also toxic to insects. Vip proteins are secreted during vegetative growth and do not exhibit any similarity to Cry or Cyt toxins. Currently, all Vip-related sequences that have been described fall into three different families, Vip1, Vip2, and Vip3 (Crickmore *et al*., 2005). The Vip1 and Vip2 proteins are the two components of a binary toxin that exhibit toxicity to coleopterans (Han *et al*., 1999). Vip3 proteins have a different host range, which includes several major

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lepidopteran pests (Bhalla *et al*., 2005). Owing to the difference in the mode of action compared with Cry toxins, Vip proteins are good candidates for resistance management strategies involving stacking or rotation of proteins with different insecticidal mechanisms (Rang *et al*., 2005).

Intensive screening programs all over the world, have identified *B. thuringiensis* strains from soil samples, plant surfaces, dead insects, and stored grains. The search for new *B. thuringiensis* strains is a continuous process, since a significant number of pests are not controlled with the available Cry proteins. It is also important to provide alternative means of coping with the problem of insect resistance, especially with regard to the expression of *B. thuringiensis* genes encoding insecticidal proteins in transgenic plants (Van Rie, 1991). The present study aims to isolate native *B. thuringiensis* strains from different environments, to understand their environmental distribution, insecticidal potential in relation to the protein profile.

MATERIALS AND METHODS

Sample collection

Soil samples were collected primarily from diverse environments, including the agricultural fields in a sericulture (silkworm) environment. Samples were collected by scraping off surface material with a sterile spatula and then collecting a 5 g sample, 2 cm below the surface. These samples were stored in sterile plastic bags at ambient temperature. Samples were also collected from poultry feed mills and warehouses, where cereals and pulses were stored. Approximately 2 g of sample was collected by scooping the materials into sterile plastic vials. The samples included broken grain dust, dead insects, insect webbings and rodent excreta.

Isolation of *B. thuringiensis*

A modified acetate selection method was used to isolate *B. thuringiensis* from the samples (Travers *et al*., 1987). Five gram of the sample was added to 10 ml of Luria Bertani (LB) broth containing 0.25 M sodium acetate in 100 ml flask. Sodium acetate selectively inhibits the germination of *B. thuringiensis* spores. The broth was inoculated on a shaker (200 rpm) for 4 h at 30˚C. The broth was thoroughly mixed and then 1 ml aliquot was heated at 80˚C for 10 min (Akiba and Katoh, 1986) in a pre-warmed 6 ml glass test tube and serially diluted aliquots were plated on LB agar and incubated overnight at 30˚C. Based on *B. thuringiensis* colony morphology and phase contrast microscopy colonies were selected at random.

Polymerase chain reaction (PCR)

Total DNA extracted from suspected *B. thuringiensis* isolates were used in PCR to identify the *B. thuringiensis* strains harbouring *cry* genes using the primer Lep1A and Lep2B (Carozzi *et al*., 1991). A Perkin- Elmer Gene Amp PCR system 9600 thermal cycler was used for DNA amplification. Ten µL of crude lysate was added to 90 µL of PCR mix, containing 1X PCR buffer, 2 mM deoxy nucleotide triphosphates, primers, double distilled water and 2.5 U Taq polymerase. The contents were mixed by gentle pipetting and overlaid with 30 µl of mineral oil. The step cycle program in the thermal cycler was set to denature at 95 ºC for 1 min, anneals at 48 ºC for 1 min and extended at 72 ºC for 3 min for a total of 35 cycles. Following amplification, the PCR products were electrophorsed on 0.7% agarose gel with ethidium bromide (Sambrook *et al*., 1989).

Lepidopteran specific PCR Primers:
Lep1A: 5’ CCGGTGCTGGATTTGTGTTA3’
Lep2B: 5’ TACATGCCCTTTCACGTTCC3’

Protein estimation and SDS-PAGE

The protein estimation was carried out as per the protocol of Lowry *et al.* (1951). Proteins present in cell extracts were analyzed by SDS-PAGE according to the Laemmli method (1970). Electrophoresis was performed at room temperature at a constant current of 30 mA. On completion, the gel was stained with Coomassie Brilliant Blue and the excess stained was removed with destaining solution.

Bioassay

The isolates were cultured at 30 °C in 250 ml flasks in KTB medium (K-mineral salts medium 15 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.403 g KH_2PO_4, 2.96 g K_2HPO_4, distilled water 1000 ml supplemented with 25% tryptose broth and the final pH was 7.5) (Meenakshi and Jayaraman, 1979) at 250 rpm. During growth the cultures were periodically examined for sporulation. Cultures were harvested when at least 95% of the population had lysed releasing spores and crystals as determined by microscopic examination. To remove the exotoxin, the broths were centrifuged and pellets were resuspended in distilled water. This spore crystal mixture was used for bioassay. Preliminary studies on the larvicidal activity of *B. thuringiensis* isolates were tested against the second instar larvae of *Heliothis armigera* at a fixed dose of 300 µg/ml of spore crystal mixture, using surface contamination method (Beegle, 1989). The spore crystal suspension in sterile distilled water containing 0.02% Triton X-100 was smeared on the natural diet (Bhendi vegetable) and air dried at room temperature. The larvae were fed on the contaminated food at 25 °C and the mortality was recorded after 72 h.

RESULTS AND DISCUSSION

Population dynamics

B. thuringiensis has been found to occur naturally in diverse habitats. *B. thuringiensis* spores have been

recovered from nearly every kind of soil (Martin and Travers, 1989). Strains of *B. thuringiensis* have also been isolated from grain dust (Meadows, 1992) in sericulture environment (Ohba and Aizawa, 1986).The samples collected in this study were categorized into three major groups based on their source, such as grain storage facilities including animal feed mills, soil samples from sericulture based agricultural regions and soil samples from non-sericulture regions. It is evident that, among the three groups of samples subjected for screening those collected from the sericulture region were enriched with *B. thuringiensis* isolates compared to those collected from other environments (Table 1). Adverse environmental and climatic factors and the problem of adherence to soil particles may be the reasons for the lack of *B. thuringiensis* spores in soils from non–sericulture environment as observed by De Luca *et al* (1981). However, the high frequency of recovery of *B. thuringiensis* in sericulture environment shows that the persistent association of this insect pathogen with *Bombyx mori*, over a period of time. The results of this study showed that *B. thuringiensis* can be recovered from grain dust and soil samples, especially from sericulture-based agricultural regions**.**

Table 1: Population dynamics of *B. thuringiensis* isolates obtained from different environmental sources

	Total number of samples	**Number of spore forming isolates**	**Number of isolates resembling *B. thuringiensis* morphology (ICP)**	**Number of PCR positive isolates for *cry*I primers**	**Percentage *B. thuringiensis* strains in the population**
Grain storage facilities	100	62	28	15	15
Soil from sericulture environment	50	40	32	15	30
Soil from non-sericulture environment	50	21	nil	nil	nil

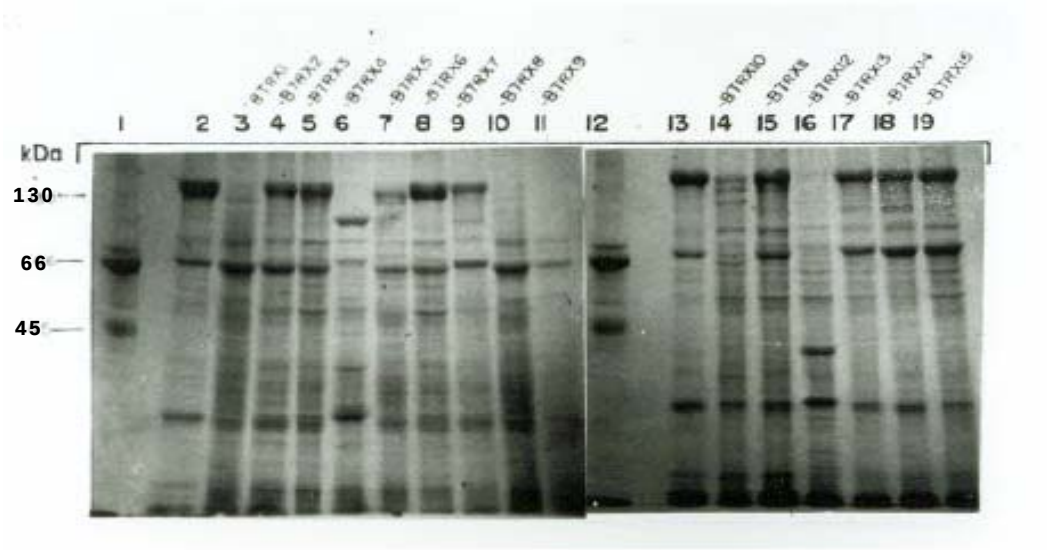

Figure 1. SDS-PAGE analysis of isolates of *B. thuringiensis* strains from stored grain samples
Lane 1. Molecular weight standard
Lane 2. *B. thuringiensis* subsp. *galleriae*
Lane 3-11. *B. thuringiensis* isolates from stored grain samples
Lane 12. Molecular weight standard
Lane 13. *B. thuringiensis* subsp. *galleriae*
Lane 14-19. *B. thuringiensis* isolates from stored grain samples

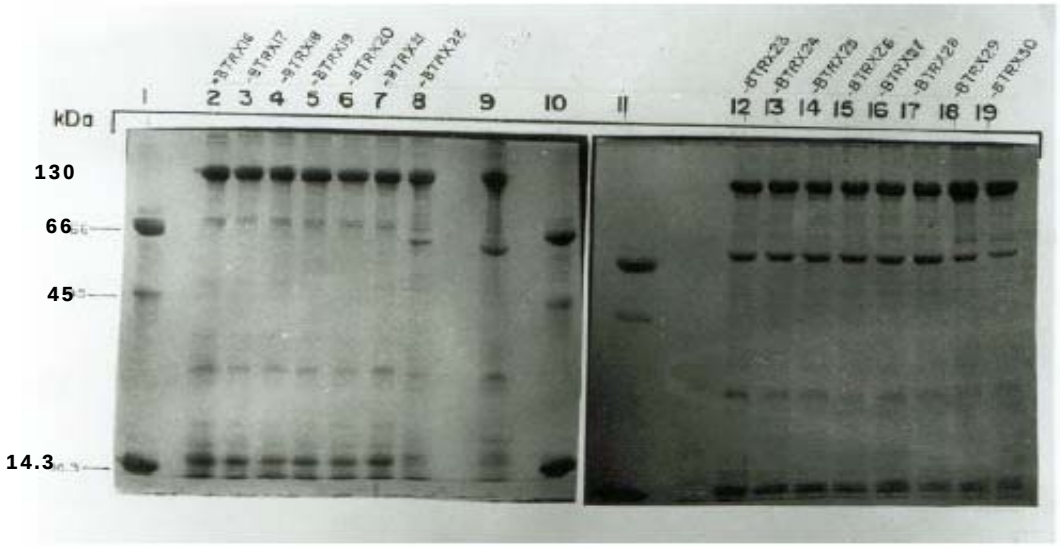

Figure 2. SDS-PAGE analysis of *B. thuringiensis* isolates from soil samples from sericulture environment
Lane 1. Molecular weight standard
Lane 2-8. *B. thuringiensis* isolates from soil samples from sericulture environment
Lane 9&13. *B. thuringiensis* subsp. *galleriae*
Lane 10-11. Molecular weight standard
Lane 12-19. *B. thuringiensis* isolates from soil samples from sericulture environment

The results showed that most of *B. thuringiensis* isolates produced a protein profile similar to that of Lepidopteran – specific *B. thuringiensis* strains with 130 kDa and 68 kDa proteins. However, a few isolates showed varying protein pattern. Particularly, isolate BTRX-4 from grain samples (Fig 1, lane 6) showed a characteristic protein profile with the protein of approximately 80-85 kDa. In contrast, the protein profile of all the *B. thuringiensis* isolates from sericulture environment showed a typical Lepidopteran pattern having approximately 130 kDa and 68 kDa proteins (Fig 2). A deeper insight into these protein profiles of *B. thuringiensis* isolates from sericulture environment reveals that isolates BTRX-16 to BTRX-22 (Fig 2, Lanes 2 to 8), the expression of the 68 kDa protein is comparatively lesser to that of the isolates BTRX-23 to BTRX-30 (Fig 2, Lanes 12 to 19). This differential expression indicates that there may be more than one *B. thuringiensis* strain in that particular environment. Further, *B. thuringiensis* strains isolated from grain samples also exhibited a high diversity in terms of protein profile (Fig 1). The possible reason for this may be the emergence of diverse populations of *B. thuringiensis* brought about by a natural process of plasmid transfer in stored product environment (Meadows *et al*., 1992).

Bioassay

The results showed that all the thirty *B. thuringiensis* isolates were toxic to the second instar larvae of *Heliothis armigera* and the mortality ranged from 40% to 100 % (Table 2).

Table 2: Larvicidal activity of *B. thuringiensis* isolates against second instar larvae of *Heliothis armigera*

***B. thuringiensis* isolates**	**% Mortality* (Mean of three replications)**
BTRX1	40
BTRX 2,4,5,6,8,9,10,12	60
BTRX 3,7,11,13,14,15,16,19,20,21,22,25,26,29,30	80
BTRX17,18,23,27	90
BTRX 24,28	100

*After 72 hours of exposure.

The objective of this preliminary bioassay is to understand the insecticidal potential of the diverse ICP in nature and to identify the entomocidally potent *B. thuringiensis* isolates. It is interesting to note that the majority of the *B. thuringiensis* isolates that are exhibiting higher mortality had the 130kDa protein, which is characteristic of antilepidopteran *B. thuringiensis* strains (Yamamoto and Powell, 1993). The less active ones especially BTRX 1,4 ,8,9,10 and 12 are expressing proteins other than 130 kDa proteins. However, insecticidal activity of these may be attributed to other confounding factors such as the proteases, phospholipases, chitinases. The *B. thuringiensis* isolates with elevated level of insecticidal activity are from the sericulture environment. As these isolates are naturally toxic to *Bombyx mori*, a member of the insect order Lepidoptera, they could also be toxic to other lepidopteran larvae, however with differing toxicity. The *B. thuringiensis* isolates from soils from non sericulture environment and grain storage facilities were relatively less toxic to *Heliothis armigera*, as these environments are less preferred by lepidopteran larvae. From this study it may be hypothesized that the environmental distribution and the insecticidal specificity of *B. thuringiensis* strains are primarily based on the prevalence and abundance of the target insects in a particular environment and the *cry* gene content of the *B. thuringiensis* strains.

CONCLUSIONS

Though *B. thuringiensis* is a soil bacterium, it has been isolated from different environments. As the environment is diverse, hence the insecticidal proteins are also diverse showing differential insecticidal activities. The present study demonstrated how the *B. thuringiensis* isolates from diverse habitats differ in their protein profile, which is reflected in varying levels of insecticidal activity. Continuous efforts to isolate novel *B. thuringiensis* strains from different environments and genetic manipulation of such strains may be helpful in solving the problems such as insect resistance and narrow host range.

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