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Protein profile diversity of *Bacillus thuringiensis* strains isolated from lepidopteran larvae

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Abstract
Bacillus thuringiensis (*Bt*) is an aerobic, gram positive, spore-forming, facultative bacteria that produces parasporal crystals containing one or more insecticidal crystal (Cry) proteins, which are selectively toxic to insects and widely distributed in the environment. In spite of the variability of Cry proteins and the range of susceptible organisms, a significant number of insects that cause great losses on crop production are not sensitive to the commercially available *Bt* toxins. In the present study seven *Bt* strains isolated from lepidopteran larvae and one reference *Bacillus thuringiensis* var. *Kurstaki* strains HD-1 were used for surface and crystal protein profile through sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). Protein profile revealed presence of 20 to >245 kDa in pre-solubilized form and 18-110 kDa in solubilized form. Based upon protein profile of pre-solubilized and solubilized form of *Bt* strains, major proteins are categorized into three main groups *viz*., groups I (18-60 kDa), group II (65-105 kDa) and group III (110->245 kDa).Variation in the molecular weight of crystal proteins showed the presence of a group of Cry toxins in *Bt* strains isolated from even same source. Low genetic similarity was observed when different *Bt* strains were clustered based on protein patterns using UPGMA. However to confirm the insecticidal activity of these native *Bt* strains, there is a need to examine their bio-activity against different insect groups.

Keywords: *Bacillus thuringiensis* (*Bt*), Cry toxins, SDS-PAGE, UPGMA

Introduction
Microbial insecticides are one of the promising and potential alternatives for chemicals but their use is limited as some microbes are species specific, others are order specific and most of them show a narrow spectrum of activity that enables them to kill only certain insect species. Moreover, they have low environmental persistence as they are sensitive and besides that, many of these pathogens are specific to young insect larval stages. Till date the most successful insect pathogen used for insect control is the *Bacillus thuringiensis*, which presently occupies 2.5% of the total insecticidal market. More than a century ago the era of *Bt* was begun, when a Japanese bacteriologist Shigetane Ishiwata isolated a bacterium from diseased silkworm *Bombyx mori* (L.) larvae in 1901, while he was examining the basis of the so called "sotto disease" (sudden-collapse disease) and named the bacterium as *Bacillus sotto*. After a decade in 1911, Ernst Berliner isolated from the diseased larvae of Mediterranean flour moth, *Anagasta kuehniella* collected from the German province of Thuringen and named it *B. thuringiensis* [1]. *Bt* action relies on insecticidal Cry toxins that are active during the pathogenic process and solely active against larval stages of different insect orders and kills the insect by disruption of the midgut tissue followed by septicemia caused not only by *Bt* but also by other bacterial species. *Bt* also produces an array of virulence factors that contribute to insect mortality [2, 3].

The present commercial strain of *B. thuringiensis* var. *kurstaki,* HD-1 was isolated from *Pectinophora gossypiella* [4]. *Bt* was considered to be toxic only to Lepidopterans until the activity of *Bt* var. *israelensis* against mosquitoes [5]. Since then, other isolates had been reported to be active against Coleoptera [6] and active against both Lepidoptera and Diptera [7]. Many subspecies were discovered and commercialized in subsequent years which were lethal to pests of Lepidoptera and Diptera [8], *B.thuringiensis* var. *sandiego* and *Bt tenebrionis* against beetles [9]. With the advent of genetic engineering, it became feasible to insert the *cry* gene that encodes the Cry toxin protein into a plant. The first genetically engineered plant, corn, was registered with the EPA in 1995. Since 1996, insect-resistant transgenic crops, known as *Bt* crops, have expanded around the globe and are proving to be quite efficient and helpful in reducing the use of chemical insecticides.

SDS-PAGE has been used for protein profiling of *Bt* strains from last three decades. A toxin strain from a nontoxic HD-1 *Bt* var. *Kurstaki* differentiated by using SDS-PAGE profiles [10]. Two major proteins of HD-1 are 125 and 65 kDa, while non toxic isolate to Lepidoptera contained a major protein of 60 kDa and several other minor proteins. The isolates having 50, 48, 70, 65, and 43 kDa proteins were non toxic to Lepidoterans [11]. The varying amounts of 130 and 65 kDa proteins crystals were toxic against Lepidopterans. The isolates which are having 130, 68, and 28 kDa are active against the Dipterans. Some unusual Dipteran-active isolates only contained 50, 40 and 42 kDa. The isolates contained 68 kDa proteins were active against the Coleopteran. The 130 and 65 kDa proteins are active against some Lepidoptera and Diptera. The predicted size range of the Cry 1 protein was between 130 and 150 kDa, Cry 2 protein between 65-70 kDa, Cry 3 was 75 kDa, Cry 7 and Cry 8 was 130 kDa, Cry 9 was 130-140 kDa, Cry 22 was 76 kDa, Cry 34 and Cry 37 were 14 kDa, Cry 35 was 44 kDa and Cyt was 28-29 kDa reported by [12].

The intent of the present work was to predict the insecticidal activity of native *Bt* strains and to evaluate their potential utilityto promote research, leading to create a new bio-pesticide with a broader and higher spectrum of toxicity against insect pests than those already used. In the present paper SDS-PAGE was used to estimate protein profile of native *Bt* strains isolated from insect cadavers and to evaluate their potential role in further investigations.

Material and Methods

Bacterial strains

Seven *Bt* strains isolated from larvae of lepidopteran insects were used in this study. Three strains were isolated from *Spodoptera litura* larvae, two strains from *Plutella xylostella* and one each from *Leucinodes orbonalis* and *Earias vitella* (Table1). One reference strain, *Bacillus thuringiensis* var. *Kurstaki* strains HD-1 (Received from Pasteur Institute, Paris).

Isolation of native *Bacillus* species

Bacillus strains were isolated by using the sodium acetate selection procedure [13] and modified by [14]. The isolated *Bacillus* colonies were streaked on the plates individually and incubated for 24 h at 37°C. The colonies were examined for morphology after 24 h. Gram staining and the presence of parasporal crystals by phase-contrast microscopycarried out after 48 h. Acetone precipitated spore-crystal mixture of all the *Bt* strains was prepared [4]. All the prepared acetone powder were stored in airtight sterile glass vials at 4 °C for further use.

Table 1: Number of *Bacillus thuringiensis* strains isolated from larvae of lepidopteran insects and their ID

Name of Insect	No. of *Bt*strain per sample	*Bt*strain ID
1. *Spodoptera litura*	1	VKK-SL1
	2	VKK-SL2
	3	VKK-SL3
2. *Plutella xylostella*	1	VKK-PX1
	2	VKK-PX2
3.*Earias vitella*	1	VKK-EV
4. *Leucinodes orbonalis*	1	VKK-LO

Characterization of *Bt* toxin protein by Sodium-Dodecyl-Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein profiles of *Bt* spore-crystal toxins were studied by SDS-PAGE according to the discontinuous system [15]. For pre-solubilized protein profiling, 20 mg acetone precipitated spore-crystal complex of reference strains *Bt* var. *Kurustaki* (HD-1) and native *Bt* strains were dissolved in 200 µl of autoclaved distilled water. The mixture vortexed thoroughly for proper mixing of proteins after that they were used for estimation of protein concentration and characterization of proteins by SDS-PAGE. For solubilized form, in 200 µl of solubilization buffer (*i.e.* 50 mM sodium carbonate buffer, 10 mM dithiothreitol, pH 10.5), 20 mg of acetone precipitated spore-crystal complex of reference strains *Bt* var. *Kurustaki* (HD-1) as wells as seven native *Bt* strains was dissolved. They were sonicated twice at 0.5 cycle, 50% amplitude; timer on 2 min (UP 100H, Ultrasonic processor, Hielscher, Germany) and incubated at 37 °C for 3 to 4 hours at 50 rpm. Solubilized samples were centrifuged at 10,000 rpm for 10 minutes. The supernatant containing solubilized crystal protein were transferred in new autoclaved micro centrifuge tubes and stored at -20°C. They were used for estimation of protein concentration and characterization of proteins by SDS-PAGE. Total protein concentration of all the native *Bt* strains along with reference strain *Btk* (HD-1), was quantified by coomassie brilliant blue dye binding method using bovine serum albumin as a standard [16]. Quantification of protein was carried out before doing SDS-PAGE to ensure that equivalent amounts of protein for loading each sample. Then protein samples were analysed by 10%SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and de-stained. Alphaimager™ Documentation and analysis system was used for gel analysis. The molecular weights of different bands of proteins were calculated by comparing the relative mobility and log molecular weight of protein standard markers using Alpha Ease™stand alone Software computer programme.

Cluster Analysis

Protein profiles having variable band sizes were obtained from eight *Bt* strains when subjected to SDS-PAGE analysis. The Protein patterns were scored as present (+) and absent (-). Protein patterns obtained from Pre-solubilized and solubilized form were analyzed by Jaccard's Coeffecient similarity index and dendrogram was constructed by unweighted pair-group method arithmetic average (UPGMA) cluster analysis based on the similarity matrix using http://genomes.urv.cat/UPGMA/.

Results

Characterization of *Bt* toxin protein by Sodium-Dodecyl-Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Surface protein profile characterization based on the SDS-PAGE gel in pre-solubilized form showed the banding patterns ranged from 20 to >245 kDa (Table. 2 and Fig.1). Reference strain *Btk* (HD-1) showed maximum of five bands, of which 230 kDa band was highest molecular weight and 20 kDa band was lowest molecular weight. However maximum seven bands were observed in *Bt* strains, VKK-PX2 and VKK-EV followed by five bands in case of VKK-SL1, VKK-SL3, VKK-PX1 and VKK-LO. Three protein bands of >245, 57 and 47 kDa molecular weight were found to be common in most of the strains. To certain extent there was variation in the protein composition among strains.

Protein profile of spore crystal complexes in solubilized form of *Bt* strains varied from 18 to 110kDa (Table. 2 and Fig.2). *Bt* strain VKK-SL1 showed maximum 9 bands followed by 7

bands in reference strain *Btk* HD-1 and VKK-PX2. Rest of the strains VKK-SL2, VKK-SL3, VKK-PX1, VKK-EV and VKK-LO showed five bands each. Although there is a variation in the protein molecular weights even in the native *Bt* strains isolated from the same insects host. However in all these strains certain common protein bands were observed, *viz.,* 110, 105, 70, 60 and 27 kDa. This combination is found to be very effective against insects of the order Lepidoptera and Hemiptera (Kalia *et al*., unpublished).

Table 2: Protein molecular weight of *Bacillus thuringiensis* strains isolated from lepidopteran larvae and reference strain *Bacillus thuringiensis* var. *Kurstaki* (HD-1)

	Molecular weight (kDa)	
***Bt*stains**	**Pre-solubilized**	**Solubilized**
VKK-SL1	>245,112,66,57,47	110, 105, 80, 72, 65,60,45,35,27
VKK-SL2	>245,69,57,47	70,60,48,35,27
VKK-SL3	>245,112,69,57,47	110,74,60,48,35
VKK-PX1	80,57, 47, 24, 20	98,70,60,38, 18
VKK-PX2	>245,112,98,57,47, 30, 20	110, 105, 88,70,60,27, 18
VKK-EV	>245,112,98,66,57,47, 30	110, 105, 91,74,24
VKK-LO	>245,112,66,47, 30	110, 105, 88,60,27
*Btk*HD-1	230,112, 94, 45,20	110,75,65,60,35,25,18

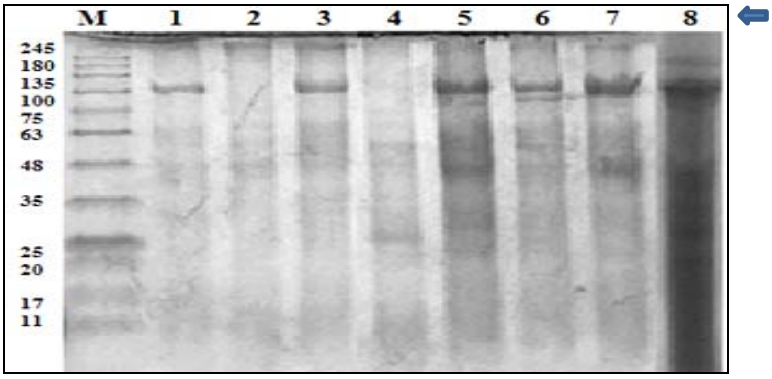

Fig 1: Profile of Cry proteins of *Bacillus thuringiensis* strains of pre-solubilized form.Lane M-Marker, Lane1-VKK-SL1, Lane2-VKK-SL2, Lane3-VKK-SL3, Lane4-VKK-PX1, Lane5-VKK-PX2, Lane6- VKK-EV, Lane7-VKK-LO, Lane8- HD-1.The arrows indicate the protoxins.

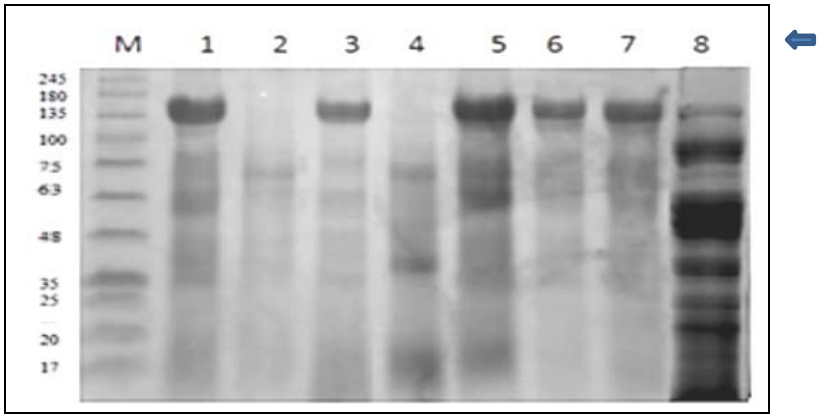

Fig 2: Profile of Cry proteins of *Bacillus thuringiensis* strains of solubilized form.Lane M-Marker, Lane1-VKK-SL1, Lane2-VKK-SL2, Lane3-VKK-SL3, Lane4-VKK-PX1, Lane5-VKK-PX2, Lane6- VKK-EV, Lane7-VKK-LO, Lane8- HD-1.

Cluster Analysis

Protein profile similarity among the eight *Bt* strains were performed by Jaccard's Similarity index based on protein bands (Table.2) for both pre-solubilized and solubilized protein forms. Dendrogram constructed using unweighted pair-group method arithmetical average (UPGMA) cluster analysis based on the similarity matrix in protein patterns of pre-solubilized form, depicted the relationship among the different *Bt* strains and grouped isolates into two major cluster (Fig.3). The dendrogram clearly indicated that HD-1 is different from rest of the strains and formed major cluster showing three unique bands of 45 kDa, 94 kDa & 230 kDa which are not shared by rest of the isolates, hence it is more diverse. The second cluster includes remaining *Bt* isolates. The second cluster further grouped in to two where in VKK-PX1 formed major cluster as it contains two unique bands of 24 kDa & 80 kDa and other cluster sub divided forming VKK-SL2, VKK-SL3 as major cluster sharing common bands and second branch again categorised into two *viz.,* VKK-SL1&VKK-LO, VKK-EV & VKK-PX2, by showing dissimilarity with VKK-SL2& VKK-SL3 due to presence of one or two extra bands.

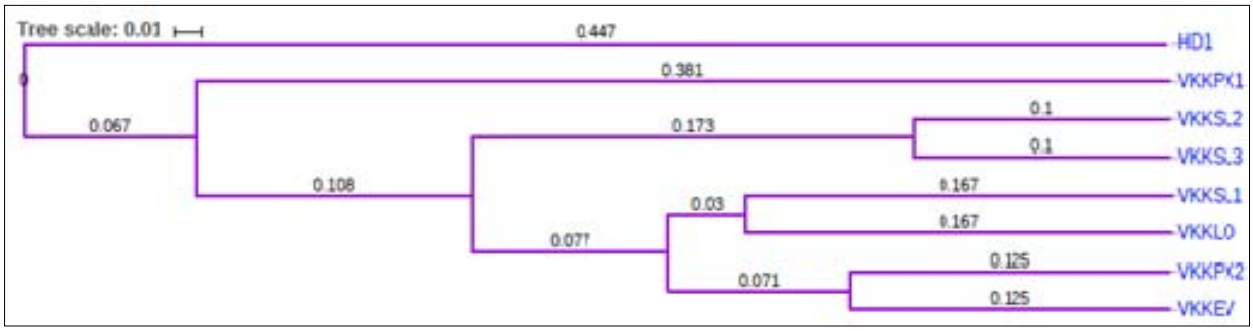

Fig 3: Dendrogram representing the relationship of different *Bacillus thuringiensis* strains using UPGMA based on pre- solubilized protein assay

In case of solubilized form, dendrogram obtained by UPGMA showed different grouping as compared to pre-solubilized form except the strains VKK-SL2 &VKK-SL3 which are showing much similarity, so grouped together and VKK-PX1 which formed separate cluster alone in both the cases. The *Bt* strains are grouped in to two major clusters with similarity coefficient of 0.43 (Fig.4). The strain VKK-EV is much diverse than other strains due to presence of unique bands of 24 kDa and 91 kDa. The second cluster further grouped into two sub clusters by forming major cluster with VKK-PX1which have unique bands of 38 kDa, 98 kDa and another sub cluster further categorized into two sub branches *viz*., VKK-SL2 &VKK-SL3 as major and HD-1 as sub cluster which is dissimilar due to presence of three unique bands. Strains VKK-PX2&VKK-LO were formed a separate group with VKK-SLI as not sharing three bands.

These results clearly indicate that strains VKK-SL2& VKK-SL3 and VKK-SL1, VKK-PX2 & VKK-LO showed least diversity among themselves in their banding pattern in both pre-solubilized and solubilized form, so grouped under same cluster in both forms. Whereas, VKK-PX1 formed separate cluster in both pre solubilized and solubilized forms due to presence of unique protein pattern in both forms. These dendrogram showed that all strains are diverse when compared to reference strain.

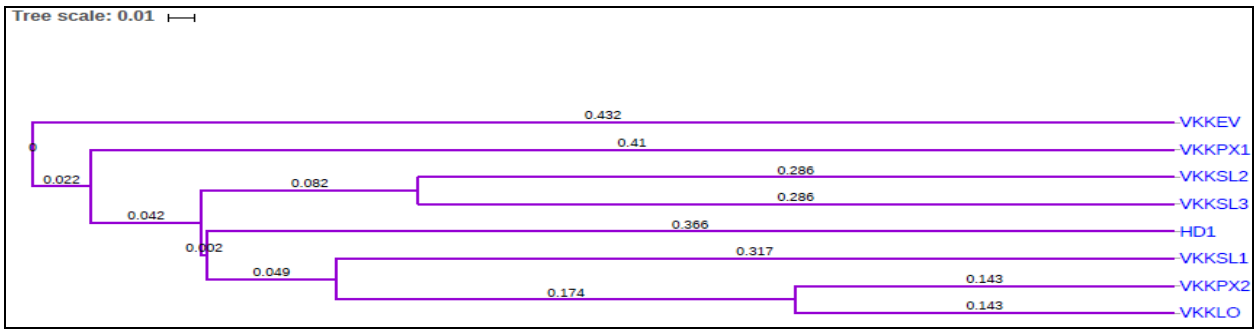

Fig 4: Dendrogram representing the relationship of different *Bacillus thuringiensis* strains using UPGMA based on solubilized protein assay

Discussion and conclusion

SDS-PAGE analysis is generally used to compare protein profiles of *B. thuringiensis* strains. Protein profile analysis of pre-solubilized *Bt* strains showed the banding patterns of 20 - >245 kDa. In absence of reducing agents dissolved crystals give 230 kDa molecular weight of protein under controlled conditions [17]. The *Bt* strains isolated from same source also showed variation in the molecular weight, three *Bt* strains were isolated from the *S. litura* all are showing variable protein profile in pre-solubilized and solubilized form. The total protein content varied among *Bt* strains irrespective of the source from which they were isolated may be the one of the reason for variation in banding pattern.These results were positively correlated with the earlier reports [18]. They reported that SDS-PAGE analysis of surface proteins of *B. thuringiensis* var. *Kurstaki* HD 73 consisted of 60 kDa, 68 kDa, 70 kDa, 75 kDa, 130 kDa, 140 kDa proteins; *B. thuringiensis* var. *sotto* consisted 10 kDa, 25 kDa, 60 kDa, 68 kDa, 70 kDa, 75 kDa, 90 kDa, 130 kDa, 140 kDa proteins; and *B. thuringiensis* var. *japonensis* gave rise to 25 kDa, 60 kDa, 68 kDa, 70 kDa, 75 kDa, 90 kDa, 130 kDa, 140 kDa proteins. A toxin strain was differentiated from a non toxic HD-1 *Bt* var. *kurstaki* by using SDS-PAGE profiles [10]. Two major proteins of HD-1 are 125 and 65 kDa, while non toxic isolate to Lepidoptera contained a major protein of 60 kDa and several other minor proteins. The isolates having 50, 48, 70, 65, and 43 kDa proteins were non toxic to Lepidotera [11]. In the present study *Bt* strains yielded 60, 65 and 70 kDa proteins in solubilized form. This molecular weight range is found to be very effective against insects of the order Hemiptera [19].

The predicted size range of the Cry 1 protein was between 130 and 150 kDa, Cry 2 protein between 65-70 kDa, Cry 3 was 75 kDa, Cry 7 and Cry 8 was 130 kDa, Cry 9 was 130-140 kDa, Cry 22 was 76 kDa, Cry 34 and Cry 37 were 14 kDa, Cry 35 was 44 show insecticidal activity against Coleopterans [12] and Cyt proteins show toxicity against Diptera [20]. Cry1, Cry2, and Cry9 proteins are known to exhibit the strongest toxicity to Lepidopterans [21]. Proteins belonging to Cry4 and Cry11 classes are specifically toxic to Dipterans. Accordingly, *Bt* strains *viz*., VKK-SL1, VKK-SL2, VKK-PX2 and VKK-LO having 28 kDa protein showed the presence of cyt toxins whereas presence of 70 kDa protein in *Bt* strains VKK-SL2, VKK-PX1 and VKK-PX2 depicts the Cry2 protein. Thus the strains having Cyt toxins and Cry2 will be active against Dipteran and Lepidopteran respectively. Similarly, Colepteran-active isolates contained a 68 kDa protein was reported [11]. The protein profiles of *Bt* strains

were distinguished into three main protein groups *viz*., group I (28 -58 kDa), group II (60- 80 kDa) and group III (125-150 kDa) [22]. However, in the present study protein profiles of *Bt* strains based upon pre-solubilized and solubilized form is categorized into three main groups *viz*., groups I (18-60 kDa), group II (65-105 kDa) and group III (110 ->245 kDa). The protein profile of these strains ranges from 133-150kDa which corresponds to the Cry proteins, and 18 to 27 kDa proteins which correspond to the Cyt proteins. Similar findings were also reported [23-25]. Likewise in the present study in most of the strains18-28 kDa band was also present. The presence of 97, 90, 50 and 85 kDa proteins implies the presence of cry27, cry6, cry13 and cry9 genes which are responsible for the toxicity in Diptera and nematodes [26].

Protein profile analysis indicates that genetic similarity percentages are extremely low between HD-1, VKK-EV and other seven isolates in both pre-solubilized and solubilized form, respectively when calculated using UPGMA. So there is a variation among the seven strains. Similar results were found in the case of four *Bt* isolates of *Xenorhabdus* and *Photorhabdus* which showed low genetic similarity when calculated using UPGMA and RAPD markers [27]. By protein profiling of native *Bt* strains, insecticidal activity against Lepidopteran, Dipteran, Coleopteran and Homopteran insect pests was predicted in the light of available literature. However to confirm the insecticidal activity of these native *Bt* strains, there is a need to examine their bioactivity against different insect groups. Protein analysis alone is not sufficient for discriminating among *Bt* strains. Moreover, gene profiling of potential *Bt* strains need to be studied to fish out the new cry genes which canbe exploited as one of the components in pest management aspects.

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