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Screening, Diversity and Partial Sequence Comparison of Vegetative Insecticidal Protein (*vip3A*) Genes in the Local Isolates of *Bacillus thuringiensis* Berliner

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Abstract Characterization, direct sequencing of the PCR amplicon and phylogenetic relationship was done to discover a novel Vip protein genes of the *Bt* isolates, to improve the prospects for insect control, more Vip proteins should be sought out and researched to predict their insecticidal activity. Characterization was based on direct sequencing of PCR amplicon using primers specific to *vip3A* gene was presented here. 12 out of 18 isolates screened were positive for *vip* gene-specific primers. Homology search for the partial sequences using BLAST showed that 11 isolates had high similarity to *vip*3Aa gene and only one fragment with *vip*3Ae gene (25–100% at nucleotide and amino acid level). Phylogenetic analysis showed that the gene sequences were responsible for geographic separation for divergence within *vip* genes, consistent with the evaluation of distinct bacterial population. Despite the geographical distances, strains harbouring *vip* genes have originated from common ancestors may significantly contribute to control resistant insect pests. Some strains have evolved to be quite distinct and others remain as members of closely related groups. The reported method is a powerful tool to find novel Vip3A proteins from large-scale *Bt* strains which is effective in terms of time and cost. Further the Vip proteins produced by different strains of *B. thuringiensis* are unique in terms of the sequence divergence and hence may also differ in their insecticidal activities.

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Introduction

The various crystal protein toxins (Cry) produced by *Bt* have been successfully used in pest management programmes both in agriculture and public health. In the recent years, there is a renewed interest in isolating potent *Bt* strains with increased host spectrum and also a source of isolation of novel genes. *Bt* has been isolated from different sources viz. fresh water, saw dust, cured tobacco leaves, rice bran, stored products, compost, phylloplane, marine sediments and ancient glacial ice [1–8]. The host range of *Bt* includes lepidoptera, coleopteran, diptera, acarina, protozoa, hymenoptera, trematode and nematodes [9]. Intensive, large-scale screening programmes have yielded new genes such as vegetative insecticidal proteins (*vip*3A) [10] are a group of insecticidal proteins and represent the second generation of insecticidal trans-genes that will complement the novel δ-endotoxins in future. Vip proteins have been reported to have insecticidal properties, providing a potent broad spectrum of insect control. Vip proteins fall into three families viz. Vip1, Vip2 and Vip3, where Vip1 and Vip2 proteins are the two components of a binary toxin that exhibits toxicity to coleopterans [11]. Host range of Vip3 proteins includes several major lepidopteran pests [12, 31].

*vip*3A gene homologues in approximately 15% of the *Bacillus* strains, which had shown activity against

lepidopteran insect larvae, including black cutworm (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), tobacco budworm (*Heliothis virescens*) and corm earworm (*Helicoverpa zea*) [11]. Histopathological observations showed that Vip3A ingestion by susceptible insects such as the black cutworm (*Agrotis ipsilon*) and fall armyworm (*Spodoptera frugiperda*) caused gut paralysis at concentrations as low as 4 ng/cm^2 of diet spread on leaves, resulting in larval death at concentrations above 40 ng/cm^2 [30]. The importance of Vip3A protein for the insecticidal activity of *B. thuringiensis* was determined by deleting the *vip*3A gene from strain HDl. Compared to HD1, the strain HD1Delta*vip*3A, with deleted *vip*3A showed only one-fourth toxicity to *Agrotis ipsilon* larvae and <10% toxicity to *Spodoptera exigua* larvae [29].

Unlike the crystal protein toxins (Cry) Vip3 proteins are secreted during vegetative growth and have no homology to Cry or cytolytic toxins (Cyt). Like Cry toxins, Vip3 proteins must be activated by proteases prior to recognition at the surface of the midgut epithelium of specific 80 and 100 kDa membrane proteins different from those recognized by Cry proteins [13]. Vip3 proteins are suitable candidates in *Bt* resistance management as these proteins recognize different receptors as compared to Cry proteins. However, the potential Vip proteins remains untapped to date, there are approximately 82 kinds of vegetative insecticidal protein genes that have been identified and cloned. These genes can be classified into three groups, eight subgroups, 25 classes and 82 subclasses according to the encoded amino acid sequence similarity (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html/). The search for novel *vip* sequences might prove to be useful for increasing the target range of future insecticidal transgenic plants while allowing resistance management by associating *vip* genes with the functionally different *cry* genes. As the environment is diverse, hence the insecticidal proteins are also diverse showing differential insecticidal activities. Therefore, it is necessary to screen more *Bt* isolates to clone and characterize *vip* genes and their variants. Hence, in this study the local isolates of *Bt* were screened with *vip*3A gene-specific primers to identify isolates harbouring these genes to further clone and characterize full length of the same.

Materials and Methods

Bacterial Strains and DNA Preparations

Eighteen native lepidopteron active *Bt* isolates were cultured on Luria agar at 30°C for 72 h and stored at 4°C until further use. Single colony of the above *Bt* isolates was cultured in Luria broth at 30°C for 24 h and centrifuged at 9,000×*g*, 10 min at 4°C. The pellet was washed with double distilled water at 9,000×*g*, 10 min at 4°C and total DNA was isolated and used for PCR analysis [Published data 14].

Oligonucleotide Primers and PCR Analysis

For the detection of *vip* genes primers specific to *vip*3A gene viz. *vip*3A forward 5′-GCC CAT GGA CAA GAA TAA T-3′ and *vip*3A reverse 5′-GAA CTA GTT TCT GTA GCA A-3′ [15] with an expected product size of 584 bp was used. PCR was carried out in 25 µL total reaction volume, contained the following components template DNA—2 µl, Tris–HCl 10 mM pH 8.3; KCl—50 mM; $MgCl_2$—2.5 mM—2.5 µl, dNTP mix—2.5 mM each *Taq* polymerase—0.5 U (5 U/µl) (Fermentas Life Sciences), forward and reverse primers—20 pmol of each and rest of the volume was made up with DNAase and RNAase-free water (Eppendrof). Amplification was done in a applied biosystem thermal cycler under the following conditions: initial denaturation −94°C for 3 min, followed by 40 cycles of denaturation—94°C for 30 s, annealing—47°C for 35 s, extension—72°C for 1 min and a final extension—72°C for 20 min. The PCR amplified products were analysed by 1.5% agarose gel in TBE buffer for 150 V/h and stained with ethidium bromide (10 µg/ml) and visualized in a transilluminator at 365 nm.

Sequencing and Analysis of *vip* Gene

The PCR amplified fragments were eluted using Perfect Prep® gel clean up kit (Eppendorf) according to the manufacture's protocol. Direct sequencing of the above fragments was carried out using the forward primer of the gene-specific primers used in this study in an automated sequencer (ABI PRISM 310). Sequencing was repeated five times for each fragment and the sequences were aligned in a sequence alignment editor, 'Bioedit' to look for any introduced errors while sequencing, if any. Finally, one sequence was selected for each *Bt* isolate and homology search was carried out using BLAST X version 2.2.6 (http://www.ncbi.nlm.nih.gov) and were deposited with NCBI. As direct sequencing of PCR amplified fragments resulted in unequal lengths of partial sequences they were made of uniform length and compared with the first published *vip*3Aa sequence, L48811 [9] based on which *vip*3A gene-specific primers used in this study were designed. The sequence analysis was done after converting into FASTA format on internet (website: http://www.ncbi.nlm.nih.gov). Multiple alignments of nucleotide and deduced amino acid sequences were done using CLUSTAL W software (version 1.8). Both percent nucleotide identity and amino acid residue substitutions were studied on comparison with

those different *vip*-like genes from various geographical regions. Phylogenetic analysis was performed using MEGA 4.1 (Beta 3) software. A rooted neighbour joining tree was constructed using the partial nucleotide and deduced amino acid sequences of the putative *Bt* isolates with other *vip*-like genes from different parts of the world. The other GenBank accession numbers used to construct tree AY074707, AY074708, AF399667, L48811, AY489126, AY743436, AY945939, L48812, EU294496, AJ971413, AF500478, DQ016969, EU107765, AY295778, DQ016968, AY547270, DQ241674, DQ250256, AY466020, AF399668, AY466018, AY466016, AY466015, AY466019, AY466017 and AF442384.

Nucleotide Sequence Accession Numbers

The sequence of the gene encoding the Vip3A protein has been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

Results and Discussion

In contrast to insecticidal Cry proteins that have been extensively described and widely used in insect management programs for over 100 years [25], studies on the diversity and of Vip proteins are still in the early stage of discovery and application. Many instances of Bt with the *cry* gene have been reported, whereas only a few instances of Bt with *vip* genes had been found. The molecular and biological properties of VIPs established them as distinct class of insecticidal toxins that are different from *Bt* δ-endotoxin family. They represent second generation of insecticidal toxins that can be used to target important insect pests that are not susceptible to *Bt* δ-endotoxins [19]. To date, several PCR-based methods for cry gene identification have been developed [20–23], including specific primer PCR, multiplex PCR, exclusive PCR and PCR-RFLP. These methods directly detect known *cry* genes from *B. thuringiensis* [20, 21] as well as identify novel cry genes [23]. The NCBI accession numbers for the above sequences are EF679794 (IIHR-1), EF679795 (IIHR-2), EF679796 (IIHR-3), EF679797 (IIHR-4), EF679798 (IIHR-5), EF679800 (IIHR-7), EF679801 (IIHR-8), EF679802 (IIHR-9), EF679803 (IIHR-10), EF679805 (IIHR-12), EF679807 (IIHR-14) and EF679808 (IIHR-15). Sequence comparison of the above sequences with the reference L48811 showed that EF679803 had variations in 35 positions and EF679807 had a single nucleotide variation at 106th position (Fig. 1 and Supplementary data). Doss et al. [10] isolated *vip*3V gene employing primers used in this study, which corresponds to the coding region of the *vip*3Aa gene. Similarly by screening a large collection of *Bacillus* strains, *vip*3Ba1 identified using *vip*3A specific primers, which were initially designed to amplify the conserved regions in *vip*3A genes [14]. In this study, even though new gene was not detected it has yield *vip*3Aa and a related gene *vip*3Ae, potential of which could be studied after the isolation of full length of the same. The first report on the isolation of *vip*3A gene was by Estruch et al. [12, 13] and only a decade later *vip*3Ae gene was identified by Van Rie in the year 2005 (NCBI accession AJ872072) and Hernandez-Rodrignez et al. [16]. Recently, some of the *vip*3 genes have been employed to develop insect resistant transgenic plants. The number and diversity of *vip* genes available is abysmally low as compared to about 300 *cry* genes that have been cloned and characterized. Many of the known Vip toxins have insecticidal potential similar to that of Vip3Aa1. Therefore, enriching the diversity of available *vip* genes could broaden the spectrum of activity of the Vip3 family of proteins and facilitate the application for insect pest management [12]. Identification of new vip gene also increase the possibility of developing a broad spectrum vip genes, as it has been shown in the case of chimeric Vip3AcAa [17]. Hence, there is a potential of isolation and characterization of novel *vip* genes with improved toxicity and enhanced host spectrum. Further studies are needed to isolated full length of the different *vip* genes that are identified in this study in order to determine their toxicity and an effective tool for the management of *Bt* resistance as the mode of action of Vip proteins are found to be different to that of *cry*1Ab [18]. Finally, the results shows that the strategy used in this study can lead to the classification of known *vip* genes as well as the identification of novel *vip* genes from large scale of *B. thuringiensis* strains. The *vip3Aa* and *vip3Ae* gene proteins may be used to control insect pest or resistant insect pests by constructing genetically engineered strains or transgenic plants. The presence of vip3Aa1-insecticidal gene homologues in 15% of *Bacillus* strains analysed [11]. In a similar study, the presence of vip3Aa1-like genes in two out of 11 *Bacillus thuringiensis* kurstaki strains and absence in *Bacillus thuringiensis* israelensis strains [17]. Twenty-four serovars of *Bacillus thuringiensis* (Bt) were screened by polymerase chain reaction to detect the presence of vegetative insecticidal protein gene (vip)-like sequences by using *vip3Aa1*-specific primers. *vip*-like gene sequences were identified in eight serovars. These genes were cloned and sequenced. The deduced amino acid sequence of the *vip3Aa14* gene from *Bacillus thuringiensis* tolworthi showed considerable differences as compared to those of *Vip*s reported so far [32].

Analysis of insecticidal virulence factors in the Polish *Bt* collection, indicated that 57% of the vip3A-positive isolates (8 out of 14) also carried genes encoding insecticidal d-endotoxins potentially active against lepidopteran larvae,

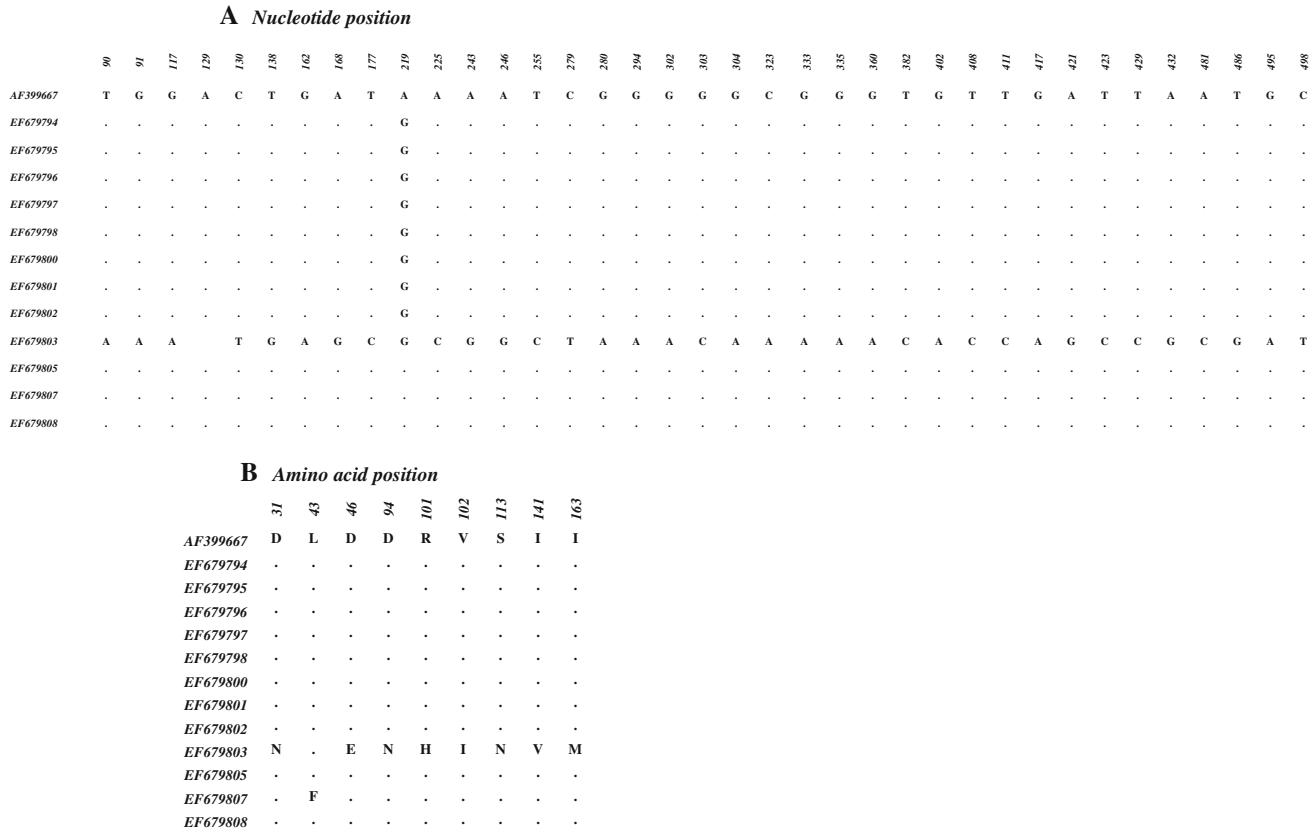

Fig. 1 Positional differences in **a** nucleotide and **b** amino acids sequences of HD1 (vip3A) gene homologues and predicted Vip3A proteins in environmental isolates of *Bacillus thuringiensis*. The sequences of the vip3A homologues present in EF679794, EF679795, EF679796, EF679797, EF679798, EF679800, EF679801, EF679802, EF679803, EF679805, EF679807 and EF679808 and in *Bacillus thuringiensis* HD-1 in GenBank under accession numbers AF399667, respectively. The nucleotide and amino acid substitutions in the vip3A gene in those strains suggests the possibility that the observed mutations could be environmentally linked, though a much larger sampling size is required to support this hypothesis. The vip3A homologues of environmental isolates showed identities from 25 to 100% known vip3A genes at nucleotide and amino acid level

such as Cry1, Cry2 and/or Cry9. The same frequency of *Bt* isolates with both insecticidal toxins genes, *vip3A* and *cry1I* [24] in 125 strains surveyed. On one hand, these results may suggest a genetic linkage (e.g. carried by the same plasmid) between genes for insecticidal virulence factors, *vip3A* and *cry1*, in natural *Bt* collections. However, on the other hand, we cannot exclude the possibility that the co-occurrence of these genes in *Bt* is coincidental, due to a natural high frequency of cry1 than other genes encoding δ-endotoxins in *Bt* populations [2, 20, 26, 28]. Nevertheless, it is plausible that the co-occurrence of *vip3A* and *cry* toxin gene combinations significantly contributes to the successful occupation of new ecological niches and perhaps expanding the insecticidal host range and propagation of such *Bt* strains [27].

Phylogenetic analysis of this study showed that all *vip* gene isolates formed one distinct grouping (Fig. 2). Based on percent nucleotide identity of partial gene sequence and grouping in phylogenetic tree between *vip*-like genes, it can be inferred that the our isolates is closely related to its corresponding isolates suggested that comparison of gene sequence was responsible for geographic separation for divergence within *vip*-like genes/serotypes, consistent with the evaluation of distinct *Bt* isolates. Some isolates have evolved to be quite distinct and others remain members of closely related groups. These strains are candidates for application as commercialized biocontrol agent, therefore research on optimization of fermentation and formulation, greenhouse and field trials, and the bioassay of the selected isolates against other pests will be continued. Although clearly different, these genes will be submitted for consideration in the *B. thuringiensis cry* gene nomenclature only after cloning, full-length sequencing, and expression in a recombinant host strain to confirm the toxicity of the protein. Applying of molecular adaptation to nucleotide sequence data can help investigators identify specific amino acid substitutions for further experiments. In conclusions the identified, and characterized novel *vip* gene from *Bt* isolates, which can be used either alone or in gene pyramiding with other insecticidal protein genes for

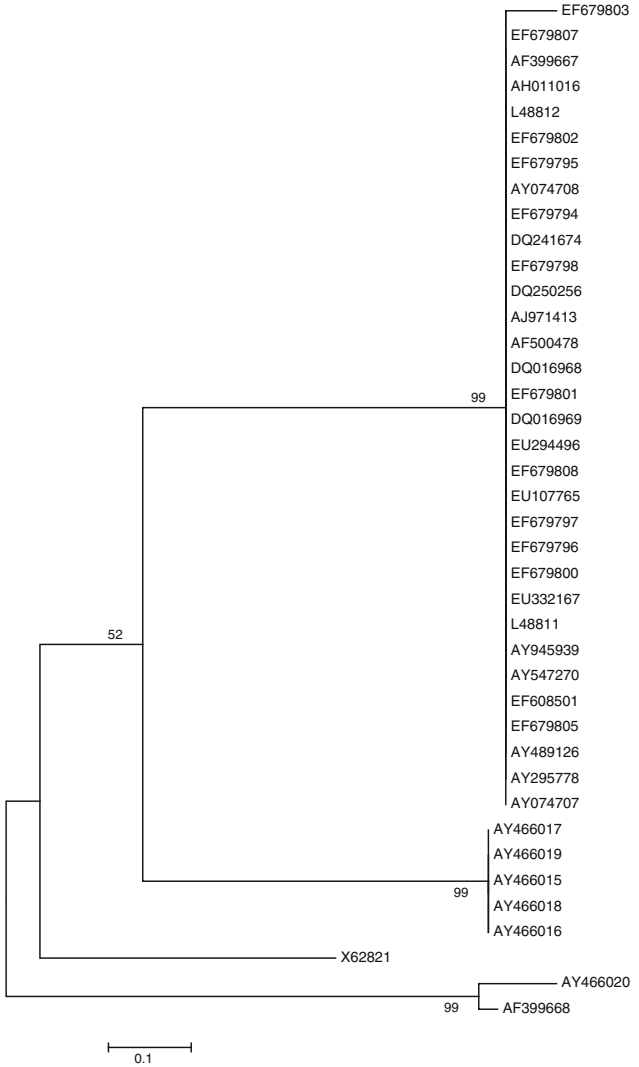

Fig. 2 Neighbour-joining tree showing phylogenetic relationship amongst different *Bacillus thuringiensis vip*-like genes based on nucleotides. The NJ tree was constructed using CLUSTAL W with default parameters are indicated at the nodes. Sequences were obtained from the databases of the National Center for Biotechnology Information. The gene sequences were responsible for geographic separation for divergence within *vip* serotypes, consistent with the evaluation of distinct bacterial population. Despite the geographical distances, *vip* strains have originated from common ancestors. Some strains have evolved to be quite distinct and others remain as members of closely related groups

durable resistance against lepidopteran insect pests. Further studies on the mechanism of the Vip protein against its host may help us develop new effective insecticidal proteins and delay the insects' resistance.

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