



## Molecular characterization of *Bacillus thuringiensis* strains with differential toxicity to the spotted stalk borer, *Chilo partellus*

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### ABSTRACT

**Objective:** Three *Bacillus thuringiensis* isolates designated 1M, K10-2 and V24-M with varying levels of toxicity to *Chilo partellus* (spotted stalk borer) were characterized to establish the basis for the differential toxicity and to identify any unique properties that may be used to screen other isolates.

**Methodology and results:** *Bt* isolate colonies were picked from plates and inoculated into LB medium and growth rate estimated by measurement of cell density at absorbance 600nm. Secreted proteins was quantified and analysed through SDS-PAGE. *Bt* plasmid DNA was isolated and restricted with different restriction enzymes and analysed by electrophoresis. Isolate 1M had a slightly elevated growth rate in LB medium at the lag and exponential phases of growth than both isolates K10-2 and V24-M, but at the plateau phase the growth rates were not significantly different for all the three isolates. All crystals were bi-pyramidal in shape suggesting that the *Bt* isolates are lepidopteran specific. The major protein bands for isolate K10-2 and V24-M had molecular weights of 28, 65, and 130kDa while main protein bands in isolate 1M were of molecular weight 28 and 65kDa. The three *Bt* isolates showed varying levels of plasmid DNA concentration with isolate 1M having the highest plasmid concentration suggesting greater plasmid copy number. Upon restriction digestion of the isolates plasmid DNA with *EcoRI*, *BamHI* and *HindIII*, one particular unique band of 7200 bp was evident in all the digests.

**Conclusion and application of findings:** *Bt* isolate, 1M which had the highest toxicity against *C. partellus* in a previous study showed a unique protein profile and higher plasmid copy number than the other isolates. The results suggest that these properties may be used for selective identification of *Bt* isolates that exhibit effective toxicity against *C. partellus*.

**Key words:** *Bacillus thuringiensis*,  $\delta$ -endotoxin, plasmid, differential toxicity, *Chilo partellus*.

### INTRODUCTION

*Bacillus thuringiensis* (*Bt*) is a ubiquitous Gram positive, aerobic, spore-forming bacterium that forms parasporal crystals during the stationary phase of its growth cycle (Ohba & Aizawa, 1986). Although *Bt* was initially used mainly against lepidopteran insect pests, a number of strains have

been shown to be toxic to a wide array of insects as well as nematodes and protozoans (Schnepf & Whiteley, 1998). Toxicity to the insects is attributed to the parasporal crystal protein with the type and number of different protoxins in the crystalline inclusions of *Bt* determining a particular strain

toxicity profile, hence the basis of *Bt* classification (Hofte & Whiteley, 1989).

*Bt* strains produce two types of toxins. The main type is the crystal (Cry) toxins, encoded by different *cry* genes, which are the basis for classification of *Bt* strains. The second type is the cytolytic (cyt) toxins, which can augment the Cry toxins, enhancing the effectiveness of insect control (Hofte & Whiteley, 1989). Earlier the Cry genes were classified into four; Cry I, Cry II, Cry III, Cry IV proteins based on their insecticidal activities and two classes of cytolytic genes (Cyt) that govern production of the toxins that kill the susceptible insects. Cry II and Cry I are active against lepidopterans, Cry III and Cry IV are active against dipterans and Cry III is active against coleopterans (Hofte & Whiteley, 1989, Schnepf et al., 1998). A number of *Bt* strains have since been cloned and sequenced leading to the adoption of a new nomenclature based on amino acid sequence homology (Crickmore et al., 1998).

The distinct advantage of *Bt* as a biopesticide is its well studied mechanism of action which precludes toxicity to non target organisms. The mode of action involves the ingestion of the protoxin by the susceptible insects followed by solubilization by the alkaline mid-gut environment. Proteolytic cleavage of the protoxin by the mid-gut proteases activates the toxin. This is followed by binding of the toxin to receptors within the mid-gut of susceptible insects. Toxicity arises from the formation of pore structures within the mid-gut membrane resulting in free flow of ions and water into the cells, eventually resulting in swelling, lysis and death of the host insect. Individual Cry toxins are usually toxic to only a few species within an order, and receptors on midgut epithelial cells have been shown to be critical determinants of Cry

specificity (Schnepf et al., 1998, Pigott & Ellar, 2007).

The use of *Bt* as a biopesticide has been exploited for a number of years with a dramatic increase in use following the introduction of Cry genes into plants. Transgenic crops expressing one or more Cry toxins have become agriculturally important. In 2007 the global area of genetically modified (GM) crops was 58.7 million hectares grown in 16 countries by six million farmers, of whom five million were small scale farmers in developing countries. Transgenic crops containing *Bt* genes was estimated to constitute 20% of the GM crops with global deployment of the Cry 1Ab gene in *Bt* maize having the potential to increase maize production by upto 35 million MT valued at \$ 3.7 billion per year (James, 2007).

Usually *Bt* strains isolated locally are more effective than imported strains due to higher specificity on target host, greater field persistence due to higher adaptation to the natural environment and toxicity at a higher temperature range (Brownbridge 1989). To derive full benefit from the *Bt* based biopesticides, there is need for continued isolation and characterization of local *Bt* strains that would be utilized as biopesticides for the indigenous crops.

Wangondu et al. (2003) isolated three *Bt* strains, namely 1M, K10-2 and V24-M, showing toxicity levels to *Chilo partellus* of 100, 91 and 77%, respectively in bioassay studies using 2<sup>nd</sup> instar larvae. This study was initiated to elucidate the underlying factors that form the basis for the differences observed in toxicity among the strains. The results of the study would contribute to the development of molecular based approaches for screening and identifying *Bt* isolates for formulating more effective biopesticides.

## MATERIALS AND METHODS

**Bacillus thuringiensis:** *Bt* isolates for this study were originally obtained from soils collected from two regions in Kenya, i.e. Machakos (isolates 1M and V24-M) and Kakamega (isolate K10-2) (Wangondu et al., 2003). From the previous study, the three isolates showed differential toxicities towards larval stages of *Chilo partellus* with isolate 1M exhibiting the highest toxicity of

100% larval mortality followed by isolate K10-2 (91%) and V24-M (77%). The *Bt* isolates were stored in glycerol stocks at -20°C For this study, the *Bt* isolates were inoculated onto LB agar plates and incubated overnight at 37 °C; single colonies were picked for subsequent studies.

**Growth rate of Bt isolates:** A single colony of each of the isolates was picked from the plates and inoculated separately into 150ml of the LB broth at 37°C, 160 rpm. At intervals of 6h (for 72h), aliquots of 1.5ml were obtained and the cell density estimated through measurement of absorbance at 600 nm. At each sampling the density of sporulation and crystal formation were also monitored using the Smirnov stain, viewed under a standard light microscope with an oil immersion objective (Smirnov, 1962).

**Analysis of secreted proteins:** In order to analyze the secreted proteins, the samples taken at 6 hourly intervals were subjected to centrifugation at 10,000 rpm for 5 minutes in order to separate the bacterial cells from the protein in solution. Protein content in the bacterial supernatant was determined using Lowry assay (Lowry, 1951) with bovine serum albumin (BSA) as the standard. The proteins were also analyzed through electrophoresis on 12% SDS-polyacrylamide gels as previously described by Laemmli (1970). Samples were mixed with an equal volume of 2x concentrated sample buffer and heated at 80°C for 10 min and loaded onto the gel immediately. Electrophoresis was carried out at 30mA until the tracker dye (Bromophenol blue) reached the bottom of the gel. Gels were stained for protein with Coomassie brilliant blue and destained with a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) methanol. The molecular weights were estimated using SDS-PAGE protein molecular weight markers (Sigma, London, UK).

**Plasmid DNA isolation:** *Bt* cells were inoculated in 150ml LB medium respectively and incubated at 37 °C at 200rpm for 18h. Cells were harvested by centrifugation (5,000 rpm in a Microfuge for 5 min at 4 °C), then washed twice by vortexing with 2ml of solution I [STE/TES buffer: 0.01M Tris (pH 8), 0.01M EDTA, 1M NaCl]. Plasmid DNA was isolated using the modified alkali lysis method for mega size plasmid isolation (Rolle

et al., 2005). The pellet was re-suspended in 2ml of TE lysis buffer (0.025M Tris, pH 8, containing 0.01M EDTA, 25% sucrose, 4 mg/ml lysozyme) and incubated at 37°C for 1hr. To the above mixture, 2µl of solution II (0.2N NaOH and 1% SDS) was added and the tubes inverted gently about five times and placed on ice for 5 minutes to equilibrate. One milliliter of 5M NaCl was then added followed by a brief but gentle vortexing then equilibration on ice for 5 min. After centrifugation at 15,000 rpm for 5 min at 4°C in a microfuge, the supernatant was transferred into a fresh tube and 10 µl of RNase (500µg/ml) added and incubated at 37°C for 10 min. An equal volume of phenol: chloroform was added and mixed gently, then centrifuged at 15,000 rpm for 15 min at 4 °C in a microfuge. The supernatant was then transferred into a fresh tube. Plasmid DNA was then precipitated by addition of 2.5 volumes of ethanol followed by centrifugation at 12,000 rpm for 15 minutes. The pellet was washed using 70% ethanol and air-dried before reconstitution in 50µl of TE (Tris- EDTA) pH 8.0.

**Characterization of plasmid DNA:** In order to determine the plasmid yield, plasmid DNA quantification was carried out by measurement of absorbance at 260 and 280nm. The ratio of absorbance measured at 260 to 280 nm was used as an indicator of DNA purity. The isolated DNA was also analyzed through 0.7% agarose gel electrophoresis. Electrophoresis was carried out at 70V for 45min at room temperature (25°C) with λphage DNA cut with *HindIII* as size markers. Further analysis of the isolated DNA was carried out through restriction digestion using five restriction enzymes, i.e. *EcoRI*, *BamHI*, *Sall*, *KpnI* and *HindIII*. An aliquot of DNA sample (1µg) was digested in 20 µl reaction mixture with 2µl of enzyme (10 units/µl) at 37 °C overnight. The digest was then analysed in 1% agarose gel with λphage DNA cut with *HindIII* as size markers.

## RESULTS

**Growth rates of Bt isolates:** Isolate 1M grew more rapidly compared to isolates V24-M and K10-2 during the lag and exponential phases but there was no significant difference at the plateau phase (Fig. 1). All the strains produced both crystals and spores as evident

from almost black luster with lilac blue tint after Smirnov staining for the crystals while the spores were stained pink. Bacterial cells and their fragments had a light lilac tint (Fig 2).

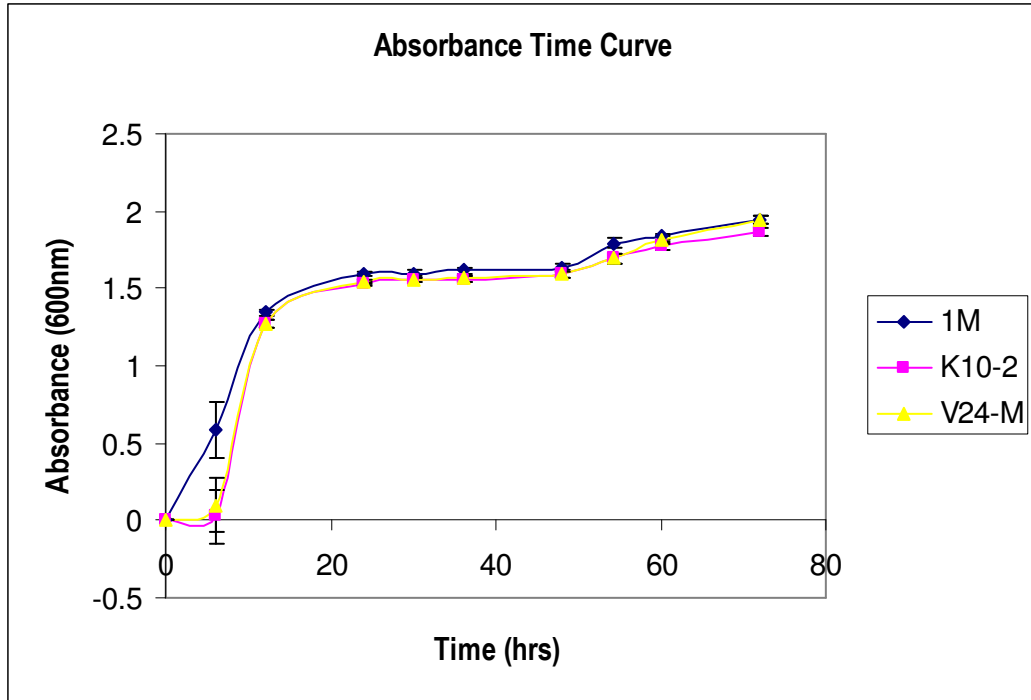
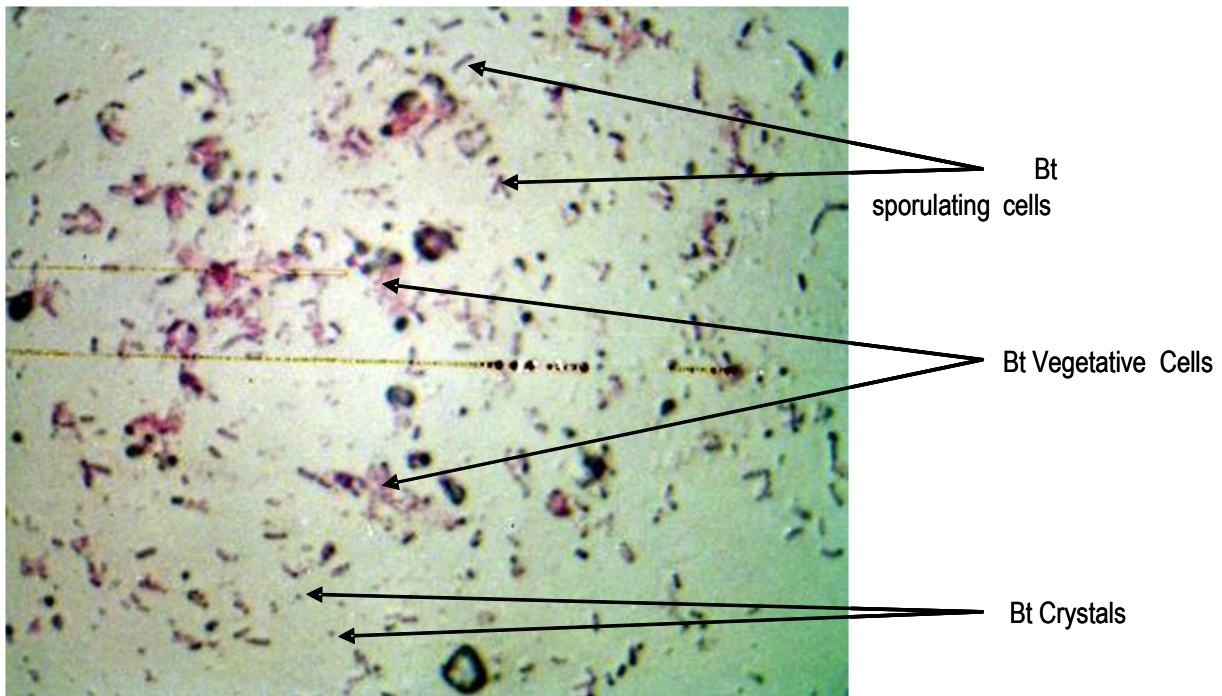


Figure 1: Growth patterns of three Kenyan *Bacillus thuringiensis* isolates (1M, K10-2, V24-M) with varying toxicity to *Chilo partellus*.

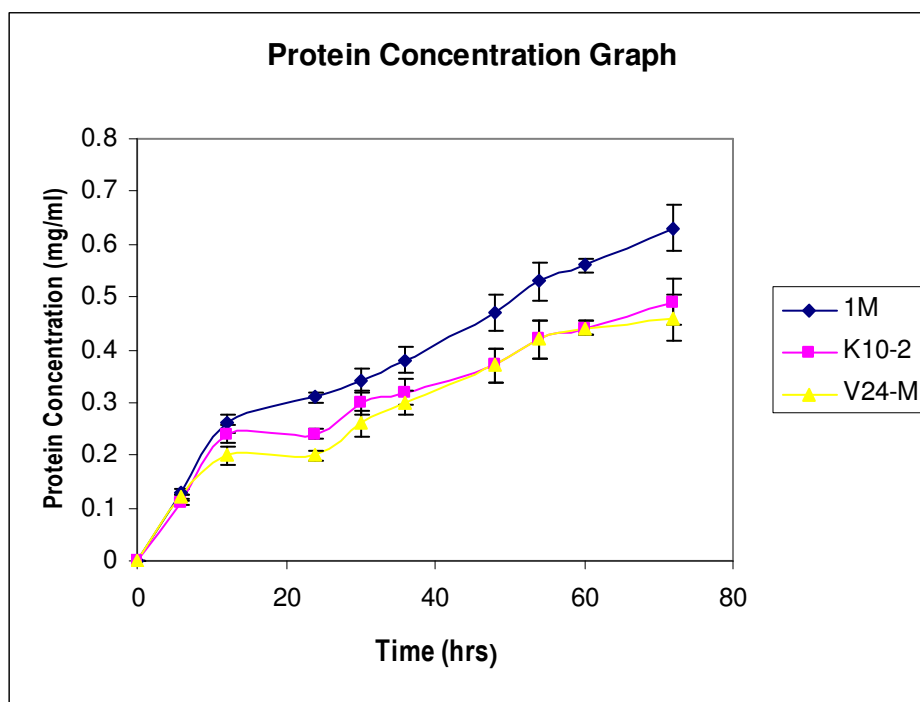


Magnification X 600

Figure 2: *Bt* isolate 1M cells and spores after 72 hr incubation at 37°C, 160 rpm.

**Proteins secreted by Bt isolates 1M, V24-M and K10-2:** Proteins secreted by the *Bt* isolates exhibited varying concentration over the 72 h study period, with isolate 1M recording a markedly rapid increase in the proteins secreted as compared to isolates V24-M and K10-2 (Fig. 3). The first 12 h was marked by an increase in the protein concentration for all the *Bt* isolates with 1M having significantly greater protein concentration than isolates V24-M and K10-2 thereafter. Electrophoretic analysis of proteins (crystal/spore complex) of the

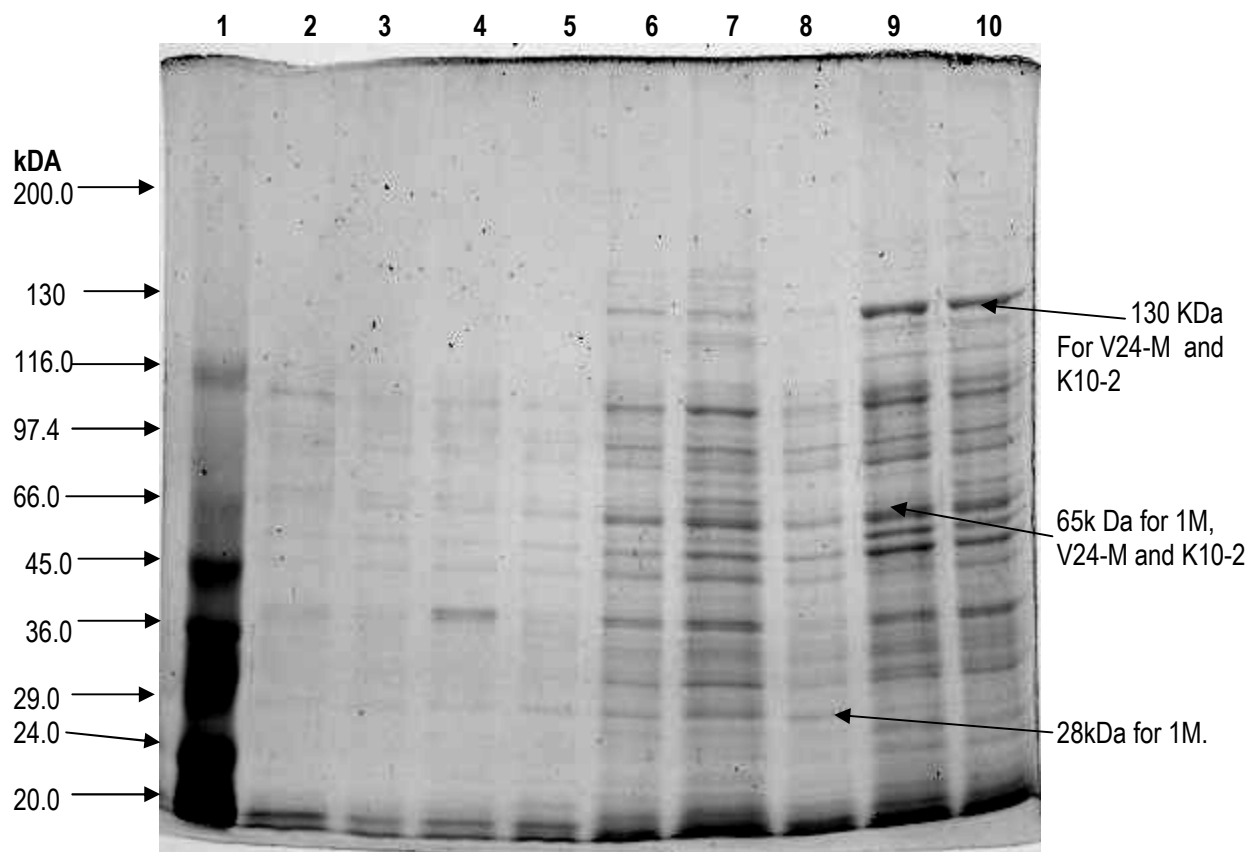
different *Bt* isolates revealed three major protein subunits of molecular weight 28kDa, 65kDa protein bands and 130kDa for all the isolates (Fig. 4). It was notable that at 72 h post inoculation isolate 1M had a prominent 28kDa protein band that was absent in both isolates K10-2 and V24-M. Conversely, it was noted that the supernatant of isolate 1M cultures did not show the 130kDa protein that was present in both isolates V24-M and K10-2.



**Figure 3:** Protein secretion trends of three Kenyan *Bacillus thuringiensis* isolates cultured over 72 h.

**Characterization of Plasmid DNA:** Plasmid DNA was successfully isolated from the three *Bt* isolates using the modified alkaline lysis method as evident from  $OD_{260/280}$  ratio of 1.7 to 2.0 with no contaminating bacterial genomic DNA on agarose gels. After 18h of culture, the amounts of plasmid DNA in 150 ml culture volume was 2.40, 1.63 and 1.58  $\mu$ g for isolates 1M, V24-M and K10-

2, respectively. The restriction profiles for the three *Bt* isolates using *EcoRI* and *HindIII* were also compared on a single gel with  $\lambda$ phage DNA cut with *HindIII* as size markers. Restricted plasmids revealed a unique band of 7200 bp for all the *Bt* isolates cut with both restriction enzymes *EcoRI* and *Hind III* (Fig. 5).



**Figure 4:** Profile of proteins secreted by three Kenyan *Bacillus thuringiensis* isolates expressed on 12% SDS-PAGE.

Lane 1, Molecular weight markers; lane 2, Isolate 1M -54hrs, 3, Isolate V24-M -54 hrs; 4, Isolate K10-2 -54hrs; 5, Isolate 1M- 60 hrs; 6, Isolate V24-M -60 hrs; 7, Isolate K10-2 -60 hrs; 8, Isolate 1M- 72 hrs; 9, V24-M -72 hrs; 10, Isolate K10-2 -72 hrs.

## DISCUSSION

Characterization of *Bt* isolates has largely been based on bioassays on susceptible insects. However, this is time consuming and it has additionally been shown, e.g. in bioassays against *Helicoverpa armigera* larvae, that a wide variation exists in the insecticidal potency even among strains sharing the same set of *Cry* genes and within the same serotype (Martinez *et al.*, 2005). A number of molecular techniques including DNA hybridization and immunological techniques using antibodies have also been used to identify *Bt* toxins.

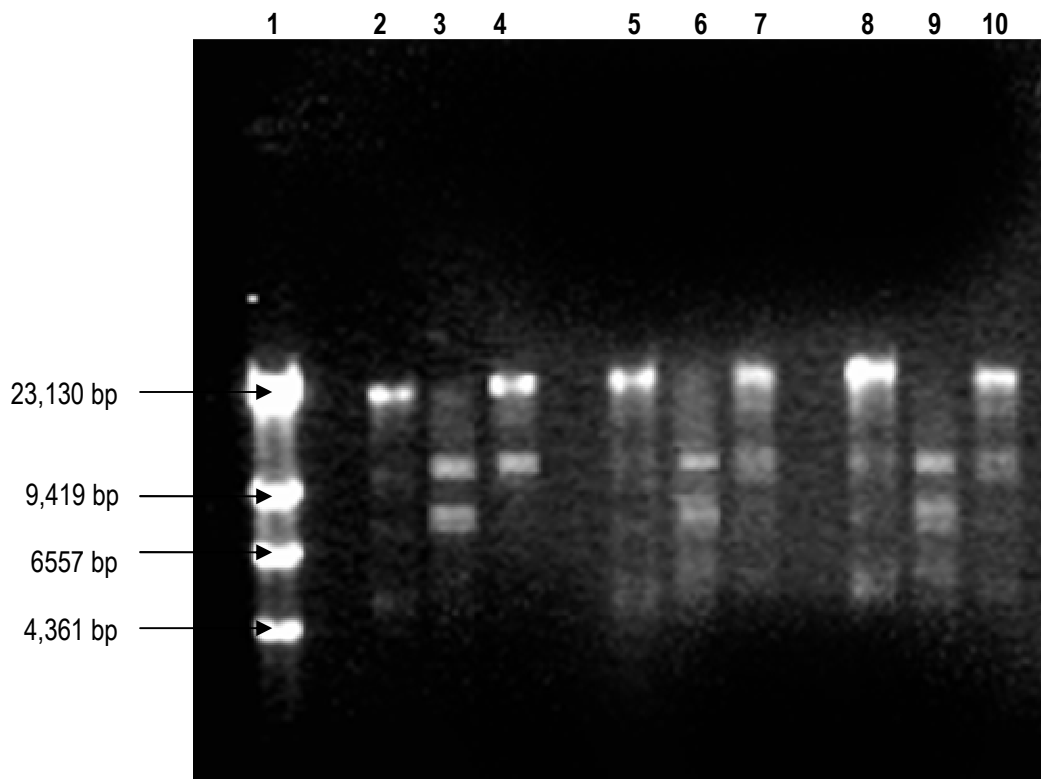
A milestone in the analysis of *Bt* collections using PCR was first described by Carozzi *et al.* (1991). The method was viewed as accurate and fast for the identification of novel genes and the prediction of insecticidal activity of new isolates. However, normal PCR procedures do not accurately predict the insecticidal activity of a strain as other factors such as the expression level of the *Cry* genes present influence

the insecticidal potency of each strain. This study was initiated to establish the basis of differential toxicity (100 – 77%) expressed by Kenyan *Bt* isolates against *Chilo partellus* (Wangondu *et al.*, 2003).

Variations were noted in the protein and plasmid profiles. Isolate 1M with the highest toxicity against *C. partellus* showed a unique protein profile and higher plasmid copy number than the other two isolates studied. Good correlation between whole cell protein profiles and patterns of DNA fragmentation of *Bacillus* spp has previously been reported (Vandamme *et al.*, 1996). Comparison of the electrophoretic whole cell pattern has also been reported to be useful in the evaluation of the relationship between *Bt* isolates (Swiecicka *et al.*, 2002). A previous study revealed that most lepidopteran-active crystals contain 130- and/or 65 kDa proteins (Dulmage, 1993). The major protein bands detected in this study were of molecular weights, 28, 65

and 130kDA for isolates V24-M and K10-2 while isolate 1M showed only two protein bands of molecular weight 28 and 65 Kda. The protein pattern of the crystals

observed in this study was thus similar to those of other strains ctive against lepidopteran larvae (Dulmage, 1993



**Figure 5:** Restriction profiles of plasmids from *Bacillus thuringiensis* isolates 1M, V24.M and K10.2.

Lane 1, DNA Size markers; Lane 2, 1M unrestricted; Lane 3, 1M(*Eco*RI); Lane 4, 1M (*Hind*III); Lane 5, V24-M (unrestricted); Lane 6, V24-M (*Eco*RI); Lane 7, V24-M (*Hind*III); Lane 8, K10-2 (unrestricted); Lane 9, K10-2 (*Eco*RI); Lane 10; K10-2, (*Hind*III).

*Bacillus thuringiensis* has been shown to harbour several extrachromosomal plasmids varying in size from 2 to 1000 kB with the Cry proteins being encoded for by the large plasmids of more than 30kb (Andrup et al., 2003; Rolle et al., 2005). The role of the smaller plasmids remains unknown. In this study the large plasmids (more than 30 kB) were observed though the method used could not separate out the various sizes of the plasmids (if more than one) or determine their accurate molecular sizes. However, the results obtained show that the isolate with the highest toxicity had the highest copy number. Plasmid copy number may determine toxicity as it determines the gene dosage accessible for expression and hence productivity. On restriction digestion, two isolates V24M and K10-2 showed similar patterns which were different from isolate 1M, although a

common band (7200 bp) was observed in all three isolates

As was discussed by Porcar and Juarez-Perez (2003), strains sharing the same *Cry/Cyt* genes content very often differ greatly in their insecticidal potency. Lack of correspondence between *Cry/Cyt* genotype and biological activity (bioassay) may be attributed to gene identity, toxin expression level, interaction of the proteins as well as other virulence factors. While Crystal proteins are synthesized in large amounts during stationary phase and accumulate in one or several parasporal crystals, the expression level of individual cry genes present in any one strain can vary greatly (Mason et al., 1998; Martinez et al., 2005). The poor expression may be attributed to weak promoter and/ or secretion of certain proteins during the early phase of sporulation. Furthermore, toxicity of *Bt* parasporal crystals depends



not only on the activity of the individual components but also on the interactions between such proteins and other extracellular compounds synthesized by Bt (Lee et al., 1996; Johnson and McGaughey, 1996).

Based on the present study, it is concluded that factors that may contribute to the selective toxicity of the Bt strains include differential growth patterns and

secretions of proteins, the plasmid copy number or the unique protein patterns observed. Further work is therefore necessary in order to determine the extent to which each of these factors contribute and how such differences may be exploited in future to complement bioassays in assessment of new isolates.

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