academicJournals

Vol. 12(30), pp. 4827-4835, 24 July, 2013
DOI: 10.5897/AJB2013.12449
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

An extensive characterization study of different *Bacillus thuringiensis* strains collected from the Nashville Tennessee area

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Accepted 19 July, 2013

***Bacillus thuringiensis* (*Bt*) is a delta-endotoxins producing bacterium that inhibits the digestive process of many insect orders such as; lepidopteran, dipteran and coleopteran. A comparative study between sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and DNA hybridization was performed on 25 chosen different strains of *B. thuringiensis.* Based on patterns from these molecular tools, the 25 isolates where grouped based on insect killing capability. Soil samples from Tennessee were collected and analyzed for the presence of *B. thuringiensis.* Fifty six (56) of more than one hundred isolates collected were initially processed using the sodium acetate and a crystal protein staining procedure. Proteinaceous and genomic profiles were gathered for 25 isolates which were named *Bt1* thru *Bt25*. DNA was extracted, quantified, amplified, cloned and sequenced. Alignment studies were performed on the sequenced products. This sequencing data helped decipher which of the *Bt* isolates belonged to the resembling parent category. The spore/crystal mixtures produced during sporulation were harvested by centrifugation at 10,000 *g* at 4°C. The spores and crystals were then separated using a discontinuous sucrose gradient with ultracentrifugation. The separation was confirmed by polarized microscopy. The crystal proteins were quantified and then separated using sodium dodecyl sulphate-polyacrylamide gel electrophresis (SDS-PAGE). A correlation was achieved between genomic and proteomic profiles which directly helped in grouping the 25 isolates. The compilation of data suggest that 14 of the 25 isolates resembled *Bacillus thuringiensis* subspecies *kurstaki*, four of the 25 isolates resembled *B. thuringiensis* subspecies *israelensis* while just one of the 25 isolates resembled *B. thuringiensis* subspecies *tenebrionis.* The overwhelming majority of the *Bt* isolates collected in our study resembled *B. thuringiensis* subspecies *kurstaki*. This can be due to the over use of genetically modified *Bt* corn in the late 1980's which was subsequently dispersed into the environment.**

Key words: *Bacillus thuringiensis,* Nashville Tennessee area, bacterium, SDS-PAGE.

INTRODUCTION

Bacillus thuringiensis (*Bt*) is one of over 20 species of soil growing, spore-forming, Gram positive, aerobic *Bacilli* that exist. A few important species of the genera *Bacilli* are; *Bacillus subtilis*, a source of industrial enzymes; *B. thuringiensis*,a known insecticidal bacterium; *Bacillus cereus,* the cause of gastrointestinal discomfort and

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Abbreviations: IEBC, International Entomopathogenic Bacillus Centre; ***Btk*,** *B. thuringiensis* subspecies *kurstaki*; ***Bti*,** *B. thuringiensis* subspecies *israelensis;* Cry, crystal protein; Cyt, cytolytic proteins; **VIP,** vegetative insecticidal proteins; **PCR,** polymerase chain reaction; **NCBI,** National Center of Biotechnological Investigation; **GM,** genetically modified.

Bacillus anthracis, the causative agent of anthrax. Most *Bacilli* genera and species are found in the soil, whereas *B. thuringiensis* has been found in living and dead insects, insect feces, granaries, and on plant surfaces (Baig et al., 2010). The International Entomopathogenic Bacillus Centre (IEBC) in the early 1990's reported that there were 3,493 *B. thuringiensis* strains grouped into 69 serotypes based on their flagellar H antigens. Later reports grouped the strains into 82 serotypes (Lecadet and Frachon, 1999). The two most discussed of these serotypes are *B. thuringiensis* subspecies *kurstaki* (*Bt*k) and *B. thuringiensis* subspecies *israelensis* (*Bti*). These serotypes are important because they produce δ-endotoxins that have insecticidal capabilities. *Btk* is used for the control of lepidopteran larvae while *Bti* is used to control dipteran larvae.

Studies of crystal genes carried by *Bacillus thuringiensis* revealed that they are needed to be a systematic method for naming the genes (Höfte and Whiteley, 1989). In 1989 they defined four classes of crystal genes (c*ry*) and two classes of cytolytic genes (c*yt*) that actually produced the toxins that killed insects. This original classification scheme by Höfte and Whiteley (1989) worked very efficiently, while the number of δ-endotoxin genes remained low at around 14. When this number of δ-endotoxin genes approached 100, a revision of the nomenclature scheme was suggested and accepted (Crickmore et al., 2013). In this new system, the roman numerals were replaced with Arabic numbers and each toxin was given four ranks (example Cry72Aa1). The primary ranks (Cry72), represents the crystal and cytolytic gene families. The secondary ranks (A), represents major differences in the amino acids sequence, whereas tertiary rank (a) represents minor changes. The quaternary ranking (1) is an optional ranking that is used only to distinguish between toxins that are more than 95% identical. If the toxin is less than 95% identical the quaternary ranking will be different, that is, Cry72Aa1 to Cry72Aa2 (Crickmore et al*.,* 2013).

Within the past 40 years, interest in *B. thuringiensis* has increased because of its insecticidal activities. Insecticidal capability is based on a variety of enzymes and other proteins released by *B. thuringiensis* during sporulation, such as: crystal (Cry) and cytolytic (Cyt) proteins, phospholipases (Zhang et al., 1993), β-exotoxins (Mac Innes and Bouwer, 2009), proteases (Lövgren et al., 1990), chitinases (Rosas-García et al., 2013), vegetative insecticidal proteins (VIP) (Sauka et al., 2012) and other heat-labile toxins (Schnepf and Whiteley, 1985). In addition to the previously stated proteins the bacterial spore if ingested can germinate into the vegetative state inside the insect destroying it from the inside out (Stephens, 1952; Heimpel, 1955; Himeno and Ihara, 1995).

The most observed of the above processes are the crystal (*Cry*) and cytolytic (*Cyt*) proteins. The crystal proteins have specific insecticidal activity against different insect orders such as, Lepidoptera, Diptera, Coleoptera, Homoptera, Orthoptera, Mallophaga and Hymenoptera. In addition to insects, these crystal proteins are also active against nematodes, mites and protozoa (Schnepf et al., 1998; Sanahuja et al., 2011). Certain crystal proteins are active against only one specific insect order whereas other crystal proteins have a more broad killing range. Some examples of such proteins and their origins are as followed: lepidopteran-specific Cry1 protein (*Bacillus thuringiensis kurstaki),* lepidopteran and dipteran specific Cry2 protein (*Bacillus thuringiensis israelensis*), coleopteran-specific Cry3 protein (*Bacillus thuringiensis tenebrionis)* and dipteran-specific Cry4 protein (*Bacillus thuringiensis israelensis)* (Sanahuja et al., 2011).

Companies like Monsanto's earned millions annually manufacturing and distributing transgenic plants containing genes like the ones described above. One of their most notable transgenic products is the soybean plant. This soybean is genetically altered to resist the herbicide Round up. Another such company called AgrEvo made the Starlink transgenic corn seed which contained the *cry9C* gene that was isolated from *B. thuringiensis*.

Since this corn seed contained a more stable crystal protein (Cry9C), the EPA only approved this product for animal consumption. Unfortunately for AgrEvo, the more stable the crystal protein product during animal digestion the more likely for the individual to develop a food allergen to it. So the less stable form (Cry1A) has been used in *Bt* corn designated for human consumption since the 1980's. The EPA approved *Bt* corn strain with *cry1A* and is currently found in corn products like corn-flour and corn-syrup.

In the late 1990's the parent company of AgrEvo called Aventis S.A. was informed by the EPA that they found traces of Cry9C protein in taco shells, tortilla and many corn based products. Apparently some egregious errors were made and crops were mislabeled and switched because Cry9C was not designated for human consumption. Subsequently in 2000 Aventis retrieved 90% of the *Bt* corn products that contained the Cry9C proteins.

With careful observations of many of these above mentioned variables, a hypothesis was developed. *Bt* isolates gathered from the middle Tennessee area should have a disproportional presence of *cry1A* genes in reference to other crystal and cytolytic genes. This increased maybe contributed to the increased use of these transgenic plants containing *cry1A* genes and dispersal factors such as wind, animals and insects.

MATERIALS AND METHODS

Isolation of *B. thuringiensis* subspecies from the soil

Soil samples were taken from the surface of uncultivated fields, granaries, feed stores and ponds that had no history of treatment with *B. thuringiensis* products in the Nashville, Tennessee area.

Soil samples were scooped from no more than 10 cm below the surface whereas water samples consisted of the layer on the surface and just below. These samples were processed by the sodium acetate selective method which only elucidates Bacillus organisms (Travers et al., 1987). One gram of the sample was incubated for 4 h at 30°C in a 250 rpm agitated baffled Erlenmeyer flask containing 20 ml of 0.30 M sodium acetate (pH 6.8). The sodium acetate specifically suppressed *B. thuringiensis* spores from germinating. Two milliliters of the sample were heated at 80°C for 10 min effectively killing everything that had germinated during the incubation period. The heat treated sample (300 μl) was cultured on an agar plate containing: 3 g tryptone; 2 g tryptose; 1.5 g yeast extract; 0.05 M sodium phosphate (pH 6.8); 0.005 g MnCl and 15 g agar per liter and incubated for 24 h. Developing Bacillus colonies were picked, grown on fresh medium agar slants at 30°C overnight and stored at 4°C until needed for further analysis.

Crystal protein visualization by staining

Each Bacillus organism that grew after the sodium acetate procedure was grown for 72 h to enable sporulation to occur. If the Bacillus organism was indeed *B. thuringiensis,* during sporulation it would produce crystal proteins that would be visible under polarized microscopy. One inoculating loop of each cultured organism was smeared on microscope slides. After the smear air dried, the slide was heat fixed by passing it over a flame. Each slide was covered for 60 min with solution 1 containing: 1 part 0.2% Naphtole in 50% acetic acid, and four parts of a 5% malachite green solution. The slides were rinsed gently with water followed by a 60 s counter stain with 0.25% Safranin. The slides were then rinsed with water and observed under polarized microscopy for the presence of crystal proteins (Ejiofor and Johnson, 2002). Those organisms that had visible crystal proteins were cataloged as *Bt* isolates.

Plasmid DNA isolation and purification

Bacterial cells were taken from the stored slant tubes and placed in Luria-Bertani (LB) broth containing 1% glucose. The cells were allowed to grow in a 250 ml baffle flask for 8 h at 32°C. The flasks were shaken at 250 RPM on an Innova™ 2000 platform shaker by New Brunswick Scientific. Cells were harvested after 8 h and 50 ml of the cell suspension was centrifuged at 10,000 rpm for 2 min. The brief centrifugation separated the bacterial cell from the LB broth. The pelleted cells were washed twice by vortexing with 2 ml of solution I (TES/STE buffer: 0.01 M Tris (pH 8), 0.001 M EDTA, 1 M NaCl). The pellet was resuspended in 2 ml of lysis buffer (0.025 M Tris (pH 8), 0.01 M EDTA, 25% sucrose and 4 mg/ml lysozyme) and incubated at 37°C for 1 h. Two milliliter of solution II containing: 10 N NaOH, 10 ml of 10% SDS, and 88 ml of H_2O was then added. The cells were gently lysed by inverting the tube five times. The tube was placed on ice for 5 min after which 1 ml of 5 M NaCl was placed in the tube followed by a brief but gentle vortex. Then the mixture was stored on ice for 3 to 5 min, and the content was transferred to a polypropylene tube. This mixture was extracted with phenol/chloroform/isoamyl alcohol solution (25:24:1) and centrifuged at 10,000 RPM to pellet precipitated proteins. The upper phase was collected and the purified nucleic acids were precipitated by storing it overnight with double volume of ice cold alcohol (95% ethanol) at -20°C (Sambrook et al., 1989).

Primer design

The specific primers were designed using this website http://www.biols.susx.ac.uk/Home/Neil_Crickmore/*Bt*/index.html. It is important to note that these are not arbitrary primers. These specific primers were designed based on already having the crystal and cytolytic gene sequences beforehand. Ninety-eight (98) forward and reverse primers were used to target 47 crystal and two cytolytic gene sequences in this study. Nonspecific binding occurred, so to eliminate this from interpretation statistical analysis was performed based on previous work (Rolle et al., 2005). A detailed list of primers is shown in Table 1.

Prepare for PCR

The amplification process used a 200 μl reaction tube containing 100 μl of 200 ng DNA, 10 mM of dNTP mixture, 0.5 μM of each of the 49 primer pairs, 1X PCR buffer plus Mg^+, and 2.5 units of Taq DNA polymerase, which was run in a GeneAmp PCR System 2400 DNA thermal cycler (Perkin-Elmer, Norwalk, CT). There was an initial 4 min denaturing step of the double stranded DNA at 94°C, followed by 35 cycles of amplification. The 35 cycles included: a 1 min denaturing step at 94°C, a 45 s annealing step at 45°C, and a 2 min extension step at 72°C. The product was automatically held at 4°C until further analysis was performed.

Statistical analysis

The products of amplification were electrophoresed in Tris-acetate-EDTA buffer (TAE) in a 2% agarose gel at a 70 v and stained with ethidium bromide. The banding pattern of the amplified product was viewed using the imaging system Gel Doc 2000™ of BIO-RAD manufactured in Hercules, California. Base pair of each fragment was determined using Diversity Database™, which was one of four programs included in The Discovery Series™ Fingerprinting Software by BIO-RAD. Chi square values were calculated for all of the genes using the average of three separate runs. Values equal or below 3.84 were accepted as true amplified crystal and cytolytic gene product. Values above 3.84 were rejected as true product and accepted as artifact.

Reconfirming my products authenticity by cloning and sequencing

The TOPO TA cloning kit from Invitrogen was utilized for the first 12 isolates. A Mach^{TM} –T1^{R} *E. coli* strain was the competent cell line used to mass produce the cloned pCR® 2.1-TOPO® plasmid vector. The remaining isolates 13 through 25 were cloned using a more cost efficient vector, the pSTBlue-1 Perfectly Blunt Giga kit by Novagen. A NovaBlue GigaSingles^{TM} strain was the competent cell line used to mass produce the AccepTor^{TM} plasmid vector. A blue/white screening test was performed after using both kits to visualize the transformed cells. The cloned colonies were selected and grown to the stationary phase before they were sent to the Genomic Sequencing Technical Facilities (GSTF) at Michigan State University for sequencing.

Alignment studies

The finished product was retrieved from the Finch server site supplied by Michigan State University (GSTF). The sequence was copied from the Finch site and paste into a Blast program by the National Center of Biotechnological Investigation (NCBI). This program performed the alignment studies by comparing the sequence to all known sequences in the NCBI database. The results confirmed the authenticity of the amplification study.

Crystal purification

A single inoculum was grown for 8 h in 100 ml of fresh LB media

Table 1. List of primers.

Target gene	Primer sequence 5' to 3'		Expected product length (bp)
Cry1A (U)	19-mer	CAAGATGGGCACGCAAGAC	522
Cry1A (L)	18-mer	ACGACCCGGACAGACACG	522
Cry1B (U)	20-mer	CCTATGGCCGCTTTCAGTTG	428
Cry1B (L)	24-mer	TTGTATCCGCTTGTAATCTATCAT	428
Cry1C (U)	23-mer	TTGTCTTGTTGGTAGGCGAGTGT	465
Cry1C (L)	24-mer	GAAAAATCCAAATCAGCAATCCAT	465
Cry1D (U)	21-mer	TCGTTGGCTCATTCACCTCT	426
Cry1D (L)	21-mer	TACTCCCTCGACAGCAACAGC	426
Cry1E (U)	19-mer	GTTGCGGCTATTTTTGACG	416
Cry1E (L)	19-mer	ACAGAACCGCACGCACACT	416
Cry1F (U)	22-mer	GGGGTGTGGGTATTTTCCTCTC	553
Cry1F (L)	21-mer	CATCTTCTCCCCGCTCTTCAC	553
Cry1G (U)	19-mer	AGGGGGAGCTTTCTTATTG	452
Cry1G (L)	19-mer	ACACCGCAGCACCACTTC	452
Cry1H (U)	23-mer	GGGCTTTTCTCCGTTATTCATTC	422
Cry1H (L)	18-mer	ACACTCCGCTCCGTCCAC	422
Cry1I (U)	20-mer	TTCGAAACCCGCATCTACTC	413
Cry1I (L)	22-mer	TTCATAATTTGGCTGTCCTGTT	413
Cry1J (U)	23-mer	CTCTTTTCCCGAACTATGATGGT	516
Cry1J (L)	21-mer	CCTGTGGCGGTATGCTGACTA	516
Cry1K (U)	18-mer	AAGCGCTGTCCCGTGTGA	508
Cry1K (L)	24-mer	CATCCCTCTCCATATCCCTCTTTA	508
Cry2A (U)	23-mer	CTGCAAGCGAATGTAGAAGAGTT	473
Cry2A (L)	24-mer	GCATATAAATTAGCGCCAGAAGAT	473
Cry3Aa (U)	20-mer	GGTCTGGCCGTCCGCTGTAT	416
Cry3Aa (L)	22-mer	TTGCCGCACTTCCATTTTCTGT	416
Cry3B (U)	24-mer	TTATTTCTCCGGTGGTGGTAGTGA	404
Cry3B (L)	23-mer	AAAAGACGCAATGGAATGACGAA	404
Cry3Ca (U)	18-mer	CCAAGGGCGCATAAGAGA	477
Cry3Ca (L)	23-mer	AAGGTTGTTCCATAGCCATTCAT	477
Cry4Aa (U)	21-mer	GAATCGGCTACGCTTGGTTGG	425
Cry4Aa (L)	22-mer	TGGGAATCCTGACATACGACAT	425
Cry5A (U)	22-mer	CCTAGTTGGAGAGCGGGACAGT	553
Cry5A (L)	24-mer	CATTCGCACCATTTAACCATTCTT	553
Cry5Ba (U)	20-mer	AGCGCGTAACCTCTTGATAG	465
Cry5Ba (L)	18-mer	CGTGCCATTCCAGTTCGT	465
Cry6 (U)	22-mer	CCTAGTTGGAGAGCGGGACAGT	553
Cry6 (L)	24-mer	CATTCGCACCATTTAACCATTCTT	553
Cry7a (U)	22-mer	CTTTGCTGCGCGGTAGATGAAT	414
Cry7a (L)	23-mer	AACCCCAATAACAGCCCCAACTT	414
Cry8Aa (U)	22-mer	TGAGACATATGAGGCGGAACAA	530

containing 1% glucose. A 1% transfer was done to fresh medium where it grew for an additional 72 h. Bacterial cells were pelleted at 1,000 *g* (3800 rpm) for 5 min and the supernatant which contained the crystal and spores were kept. Supernatant was centrifuged at 10,000g (11,500 rpm) for 10 min to form a pellet. The pellet was washed with ice-cold deionized water six times to remove the sporangial debris. The crystals were re-suspended by the addition of 5 ml of 50mM Tris HCL (pH 7.5). The crystals then were separated from the residual spores by using differential ultra centrifugation using a discontinuous sucrose density gradient. The gradient components consisted of: 10 ml of 30% sucrose, 10 ml of 50% sucrose, and 10 ml of 79% sucrose, all mixed in 50 mM Tris-HCl (pH7.5) containing 10 mM KCl. The crystal/spore mixture was layered on top and ultra centrifugation at 80,000 *g* (26,000 rpm) for 14 h at 4°C. The crystals were removed from the middle 50% sucrose layer with an 18 gauge syringe. The crystal/sucrose mixture was then washed three times in ice-cold 50 mM Tris-HCl (pH7.5) by centrifugation at 14,500 rpm for 5 min at 4°C to remove

the sucrose. The final pellet which contained the crystals were placed in deionized water and stored frozen at -20°C (Thomas and Ellar, 1983).

Quantification of protein

Biuret method of quantifying protein was utilized. A standard curve was made using Biuret reagent with the wavelength of 540 nm and different protein standard concentrations. The proteins isolated were also measured with the Biuret reagent and the values were checked against the standard curve to determine the unknown concentration.

Native crystal proteins

Native crystals were the original crystals that were washed immediately after ultracentrifugation in the discontinuous sucrose gradient. These crystals were subjected to SDS PAGE analysis.

SDS PAGE

This method of separating molecules based on size was modified from the original work of Laemmli (1970). A 10% separating gel was poured which consisted of; 1.25 ml lower gel buffer; 2.45 ml water; 1.25 ml 40% acrylamide; 25 μl ammonium persulfate (APS); and 2.5 μl N,N,N´,N´-tetramethylethylenediamine (TEMED). APS and TEMED were added just prior to pouring because they promote the polymerization of acrylamide. Deionized water was added on top of the separating gel so it would not dry out during polymerization. After polymerization, the stacking gel was added which consist of; 1.25 ml upper gel buffer; 3.3 ml water; 0.4 ml 40% acrylamide; 50 μl APS; and 5 μl TEMED. A 10 well comb was immediately placed into the stacking gel before polymerization took place.

Sample preparation

Equal volume of 2X loading buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol was added to the sample. SDS coats the protein giving it a uniform negative charge. This anionic detergent binds tightly to most proteins at the rate of about 1.4 mg SDS/1 mg of protein. The sample/buffer mixture was incubated at 95°C for 2 to 5 min which helps to facilitate the reaction. To each well 20 μl of the solubilized mixture was added, except for the marker lanes. Only 10 μl of the Midrange molecular weight markers were added to the marker lanes. A current of 100 V was applied until the proteins stacked up in the stacking gel. This was followed by a current of 150V until the green dye ran off the bottom of the gel.

Staining gel

The gel was stained with the addition of 10 ml of Coomassie Blue stain reagent containing; 50% v/v methanol, 10% v/v acetic acid, and 0.25% w/v Coomassie® Blue R-250 for 1 h which detects protein quantities as small as 50 ng. The gel was then soaked in destaining solution containing; 10% v/v methanol, 5% v/v acetic acid. Broad range protein molecular marker from Promega was used to determine the size of our proteins. This marker contained nine bands: 225, 150, 100, 75, 50, 35, 25, 15, 10 kDa. Each protein band was present at the concentration of 0.1 μg/μl, except for the 50 kDa protein, which was 0.3 μg/μl.

RESULTS

Each amplicon was statistically analyzed and those accepted as real bands and not artifacts are depicted in Table 2. These accepted amplicons were then sequenced to confirm the authenticity (data not shown). A blast and alignment analysis was performed on these sequences using the National Center for Biotechnological Information database. This data was then placed into its respective categories based on which isolate of *Bt* the amplicon came from and where that *Bt* isolate was collected from. This data is also illustrated in Table 2.

Proteomic analysis is the study of the function, structure and interaction of proteins. Only the proteins produced during sporulation of the 25 *Bt* isolates were analyzed. Of the three aspects of proteomics, only the structural aspect was further studied. Since identification of the bacteria was the ultimate goal, only the molecular weight analysis was needed to compare to the genomic sequencing results. These isolates were placed in similar growth conditions and allowed to grow through the stationary phase of their growth cycle. The crystal proteins were separated from the spores by ultra-centrifugation. The proteins collected from the *Bt* isolates yielded a distinct banding pattern that was unique only to the isolate itself. The *Bt* isolates were then placed into 2 groups based on their protein banding pattern. The first group had two major polypeptide fragments, the first ranged between 100 and 150 kDa and the second ranged between 55 and 65 kDa. The second group had two major polypeptide fragments; one at 63 kDa and the other at 35 kDa. It is suggested that a polypeptide fragments around 125 to 135 kDa indicate possible gene products from the *cry1* gene family (Ibrahim et al., 2010). The genetic sequencing data suggest that *Bt*1, *Bt*8, *Bt*10, *Bt*13, *Bt*15, *Bt*16, *Bt*17, *Bt*19, *Bt*22, *Bt*23 and *Bt*24 all had this *cry1* gene family. All of the above mentioned isolates except for *Bt*1 had a large polypeptide fragment which ranged between 100 to 150 kDa. Several protein profiles of the mentioned isolates are listed in Figure 1. DNA agarose studies, crystal gene sequence analysis and protein molecular weight detection all collectively further confirmed the presence of gene family *cry1* in all of the above listed isolates.

Polypeptide fragments around 70 kDa belong to the *cry1I, cry2, cry3, cry10 and cry11* gene family (Ibrahim et al., 2010). *Bt*1, *Bt*3, *Bt*6, *Bt*7, *Bt*8, *Bt*10, *Bt*13, *Bt*15, *Bt*16, *Bt*17, *Bt*19, *Bt*22, *Bt*23 and *Bt*24 all had polypeptide fragments around 65-70 kDa suggesting that they indeed had the *cry1I, cry2, or* both *cry1I/cry2* gene family (image not shown). Only one isolate, *Bt*3 had inconsistent data. This isolates had a 70kDa protein fragment but did not have the genetic template to make it.

DISCUSSION

Genomics

Most of the crystal genes discovered have been linked to movable plasmids, which are extra-chromosomal DNA

Table 2. *Bt* isolate location and gene presence.

Habitat	Isolate	Crystal and cytolytic gene presence
	1	*1A, 1I* and *2A*
	2	*1A, 1I, 1K, 2A, 4A, 8Aa, 19, 25* and *cyt2*
Site 1 Soil sample	3	*1A* and *cyt1*
Pond at Eaton Street /Sulfur Creek Road	4	*1A, 10Aa, 11* and *cyt2*
Nashville, Tennessee	5	*1A, 1D, 1E, 1I, 2A, 4Aa, 5Ba, 10Aa,20Aa, 30Aa*, and *cyt2*
	6	*1A, 2A and 25Aa*
	10	*1A, 1I, 2A* and *25Aa*
Site 2 Soil sample	7	*1A, 2A*, and *21Aa*
Aquaculture	8	*1A*, and *1I*
Huntsville, Alabama	9	*1F* and *5A*
Site 3 Soil sample	11	*1A, 1I, 2A, 4Aa, 10Aa, 11, 30Aa* and c*yt1*
Lydle Creek	12	*cyt2*
Bedford County, Tennessee	13	*1A, 1I, 1K, 2A, 8Aa* and *25Aa*
Site 4 Soil sample	14	*3Aa* and *19*
Parchcorn Creek	15	*1A, 1I, 2A* and *25A*
Bedford County, Tennessee		
Site 5 Soil sample	16	*1A, 1I, 1K,* and *2A*.
Wooded area	17	*1A, 1I* and *2A*.
Dickson County, Tennessee	18	*4Aa, 10Aa, 11, 19, 25Aa, cyt1*, and *cyt2*.
Site 6 Termite dust from tree stump	19	*1A, 1I, 2A* and *25Aa*.
Wooded area		
Dickson County, Tennessee		
Site 7 Soil sample	20	*1A, 2A, 4Aa,* and *11*
Wooded area	21	*1A, 2A* and *4A*
Dickson County, Tennessee	22	*1A, 1I, 2A, and 25Aa*
Site 8 Grain dust from granary	23	*1A, 1I, 2A* and *25Aa*
Dickerson Road	24	*1A, 1I, 2A, 5A, 6* and *25Aa*
Nashville, Tennessee	25	*1A* and *1I*

(Sanahuja et al., 2011). Plasmid profiles of 25 *Bt* isolates were analyzed using PCR analysis. Many genes in *Bt* have insecticidal capability but only 47 crystal and 2 cytolytic genes were observed. From these 25 analyzed isolates, 88% had genes capable of killing Dipteran insect larvae; 12% had genes capable of killing Coleopteran insect larvae; 92% had genes capable of killing lepidopterans larvae; and 12% had genes capable of killing nematodes (round worm). The overlapping numbers suggest that some *Bt* isolates have multiple insecticidal capabilities.

There were nine gene families used to determine that 88% of the isolates killed Dipterans. The gene families consisted of: *cry2, cry4, cry10, cry11, cry16, cry17, cry19, cyt1*, and *cyt2.* Of the total population studied for this insect order 68% had *cry2*, 24% had *cry4*, 16% had *cry10*, 20% had *cry11*, 12% had *cyt1* and 20% had *cyt2.* Two of the nine genes tested, *cry16* and *cry17* were not observed in any of the 25 *Bt* isolates tested.

Twelve percent of the original isolates were able to kill Coleopterans based on using 5 major gene families; *cry3, cry7, cry8, cry1B* and *cry1I*. The latter two must exist together in order to give their insecticidal affect. Of the isolates studied, 8% had *cry3,* 60% had *cry1I* and none had *cry1B*.

Ninety-two percent of the isolates collected from these

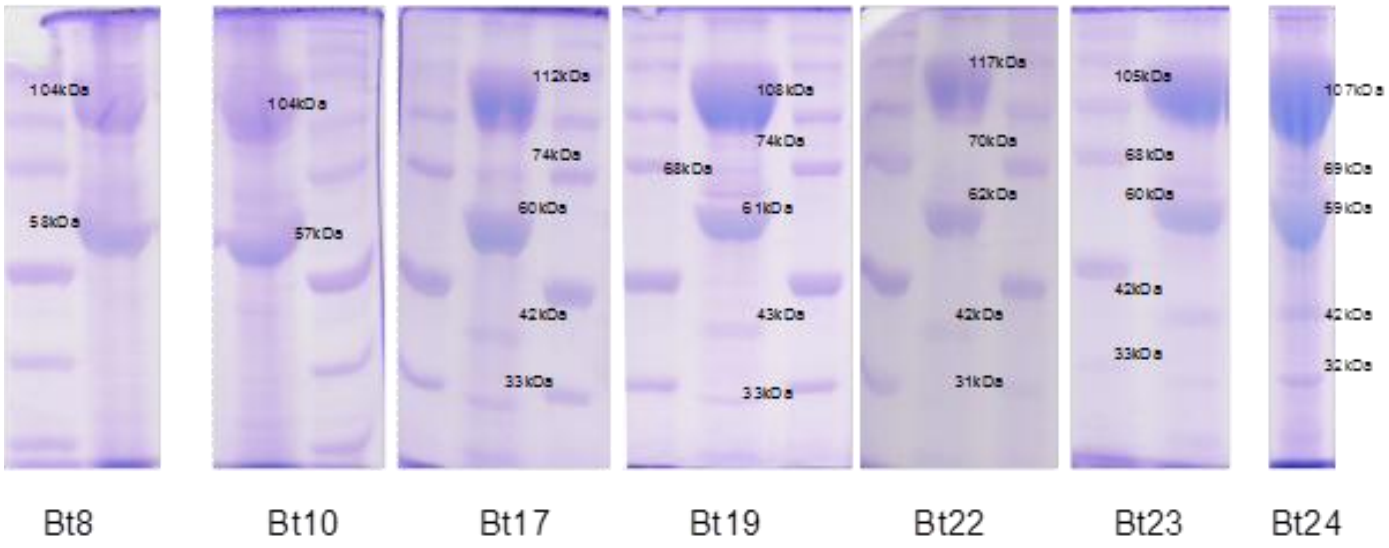

Figure 1. 7 of the 25 isolates. The marker used in these images contained nine bands: 225, 150, 100, 75, **50**, 35, 25, 15, 10 kDa. Each protein band was present at the concentration of 0.1 μg/μl, except for the 50 kDa protein, which was 0.3 μg/μl.

sites killed lepidopteran larvae base on the presence of three gene families: *cry1, cry2* and *cry9*. From the sample population tested 92% had *cry1,* 68% had *cry2* while *cry9* was not observed. The predictable susceptibility was extrapolated based on previous results from Sanahuja et al. (2011).

Geographical diversity

The geographical diversity of the sample sites was analyzed and several types of insecticidal crystal genes were o*bt*ained from the isolates of *Bacillus thuringiensis*. The first analyzed site had several different mosquito genera as well as butterflies and beetles. The *Bt* isolates collected from that site carried a variety of *cry* and *cyt* genes. Based on the gene profile 100% of the *Bt* isolates collected from this area would kill Lepidoteran and Dipteran whereas only 14% would kill Coleopteran and Nematode. The genetic diversity of the *Bt* isolates indeed correlated with the indigenous insects in that area.

Site 2 was predominantly a forestry experimental station and would consequently be a habitat for forestry associated insects. Some of the most common insect infestations found in forest areas are the southern pine beetle, gypsy moth, pales weevils and biting and stinging insects such as mosquitoes and black flies. Although beetles are typically seen in forest infestation, the results yielded no *Bt* isolates that could destroy Coleopteran. Only 33% of the *Bt* isolates at this site killed Dipteran and Nematode whereas 100% of the *Bt* isolates would kill Lepidoteran. This data was inconsistent with what was expected.

Sites 3 and 4 were both creek sites that experience high volumes of livestock development as well as human activity. Parts of the creeks were breeding habitats for insects of both agricultural and public health importance such as Dipteran. As previously discussed, these insects were susceptible to a variety of genes that were characterized in this project. Both sites 3 and 4 experienced a decline of genes that destroyed Lepidoteran, 66 and 50%, respectively. Dipteran specific genes in the isolates where seen at 100% for both sites. Finally, Coleopteran specific genes were found in 33% of isolates at site 3 and 50% at site 4. This data was consistent to what was expected.

Sites 5, 6 and 7 were all taken from areas that had observable presence of termite infested and decaying trees as well as scattered ponds. Consequently, *B. thuringiensis* toxins associated with forest pests would be expected to be associated with this area. At site 6 and 7 one hundred percent of the *Bt* isolated collected would destroy Lepidoteran whereas at site 5 only 66% would do the same. At sites 5, 6 and 7 the *Bt* isolates collected would all destroy Dipteran based on gene profiles whereas none of them would destroy Coleopteran or Nematode. This data was inconsistent from what was expected.

Site 8 was a granary site that was expected to contain δ-endotoxin genes responsible for Coleopteran destruct-tion. Weevils, which belong to the order Coleo-pteran, have been a nuisance to the granary industry for decades and one would expect to see the appearance of *Bacillus thuringiensis* isolates that would destroy Coleo-pteran. Unfortunately none of the *Bt* isolates collected at this site would destroy Coleopteran. This site was inconsistent with what was previously expected but 100% of the *Bt* isolates from this site would kill Lepidoteran and 66%

would kill Dipteran.

Although most of the sites did not produce the predictable outcome based on insect population it did agree with our original hypothesis. Based on the data from the sample sites in Middle Tennessee, 92% of the *Bt* isolates contained *cry1A* which suggests that this is either the natural predominant indigenous subspecies or horizontal transfer of genetic material from transgenic corn crops had occurred.

It is well documented that crops that are adjacent to genetically modified crops sometimes exhibit genetic transfer. To reduce this phenomenon in these refuge crops, farmers typically separate crops by large distances. Pollen drift occurs in moderate levels at distances under 31 m. This genetic material has been seen in moderate levels in kernels of non-*Bt* maize refuge plants which were planted adjacent to *BT* maize plants (Burkness and Hutchison, 2012). This phenomenon occurs by the natural mating process of the corn plant. The tassel of genetically modified (GM) maize containing the anther, will release the sex cells referred to as pollen. This pollen or pollen grain consists of vegetative cells containing Cry1A proteins and a generative cell containing 2 nuclei with the *cry1A* gene. Cross fertilization will occur between GM maize and non-GM maize yielding low levels of the Cry1A protein in the kernels. This abundant genetic material from the maize plant can sometimes be re-introduced into *Bacillus* species like *B. subtilis* and *B. licheniformis* due to a predisposition to become competent. Six factors must be meet in order for transformation to occur; release of DNA from the cell, dispersal of the DNA, persistence of DNA in the environment, development of competence, interaction of cells with DNA and expression of acquired traits (Lorenz and Wackernagel, 1994). The first variable, the release of DNA, is the most critical but once it occurs, the *Bacillus* is destined to transform. Once *B. subtilis* has been transformed then bacterial conjugation can occur with other *Bacillus* species.

Some of the first work done on the sequence of *cry1A* revealed two flanking sets of inverted repeats which shows how this gene can move. These inverted repeats were designated IS231 and IS232, based on their nucleotide sequence (Wang et al., 2010). This sequence usually defines the boundaries of transposable elements or sometimes referred to as, a movable element. This movable element that contains the *cry1A* gene allows the gene to be present on small 50kbp plasmids of a *Bt* isolate and on DNA larger than 1944 kbp within that same isolate (Rolle et al., 2005). The gene presence in both locations, further confirm the ability of *cry1A* to move. For this reason, we feel confident that this jumping gene has the ability to jump between the genomes of pollen from maize to soil growing *Bacillus* species.

Literature showed a clear process to achieve genetic transfer from (GM) maize to non (GM) maize plant, but the literature is still inconclusive in reference to indigenous *B. thuringiensis* picking up DNA remnants from the GM maize plant. So based on the finding in this project we conclude that most of the isolates found in the Middle Tennessee Area just naturally produce the Cry1A protein. Further studies need to be performed to test whether DNA remnants of *cry1A* can indeed be picked up through horizontal transfer to *B. thuringiensis* isolates.

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