Malaysian Journal of Microbiology, Vol 10(4) 2014, pp. 255-259
http://dx.doi.org/10.21161/mjm.63614

Malaysian Journal of Microbiology
Published by Malaysian Society of Microbiology
(In SCOPUS since 2011)

Soil enrichment and dominant occurrence of *Bacillus thuringiensis* subsp. *israelensis* in a garden sprayed with *Bacillus thuringiensis* based biopesticide

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Received 10 April 2014; Received in revised form 23 August 2014; Accepted 13 September 2014

ABSTRACT

Aims: *Bacillus thuringiensis* (Bt) based biopesticides have been successfully used to control agricultural pests and vectors of human diseases. *B. thuringiensis* subsp. *israelensis* (Bti) based formulations have been used as anti-dipteran agents to control the mosquito larvae. The study was conducted to understand the occurrence and relative dominance of Bti in a botanical garden, a non-agricultural ecosystem previously sprayed with Bt based formulation.
Methodology and results: The preliminary phenotypic investigations revealed that all the isolates were Bt. The Coomassie Brilliant Blue staining of the isolates revealed the presence of spherical crystals, a striking characteristic of Bt. The SDS-PAGE analysis provided basic information on the similarity of all the isolates based on the protein profile. The similarity between native isolates and Bti was further confirmed by RAPD-PCR.
Conclusion, significance and impact study: Through phenotypic and molecular methods, this study has conclusively proved the occurrence and the dominance of Bt isolates similar to the Bti used for spraying. This study provided new insight into the possible mechanism exerting continued pressure on the target insects, natural cry gene transfer because of the dominant presence.

Keywords: *Bacillus thuringiensis*, persistence, characterization, biocontrol.

INTRODUCTION

Parasporal inclusion or insecticidal crystal proteins produced by the spore-forming soil bacterium *Bacillus thuringiensis* (Bt) has been successfully used to control insect pests of agricultural importance and vectors of human diseases. There have been a differing opinion on the persistence of toxins and spores after a period of time of the application of Bt based biopesticides. Despite seasonal application of Bti for more than ten years in wetland ecosystem, the spores could not be detected in the environment (De Respinis *et al.*, 2006). Nevertheless, soil has been considered as a natural habitat for Bt and it has been isolated from soils in sericulture environment, grain storage facilities (Xavier *et al.*, 2007), and phylloplanes (Smith and Couche, 1991). Bacterial spores are presumed to be viable for long period of time in the environment compared to the toxin or the insecticidal crystal proteins. The conducive natural environment may facilitate the germination of the spores, multiplication of the bacterium until the onset of unfavourable conditions at which point of time the bacterium enter into sporulation stage to tide over the adverse condition. When Bt based biopesticides are sprayed for crop protection and vector control, it is surmised that even though the toxins may degrade, the spores may be viable, particularly the spores that are present at subsurface level of 2-5 cm, a zone free of direct sunlight or UV rays. There has been much speculation on the environmental persistence of Bt spores in the soil. Hence the present study is aimed to evaluate the occurrence of *Bacillus thuringiensis* var. *israelensis* (Bti) in a previously sprayed area in Japanese gardens by phenotypic, biochemical and molecular characterization.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacillus thuringiensis var. *israelensis* H-14 was obtained from Institute for Medical Research, Kuala Lumpur, Malaysia. All other isolates used in this study were isolated from Penang Botanical Gardens, Penang, Malaysia. Nutrient broth and nutrient agar were used as the culture medium.

Soil samples collection

Fifty soil samples were collected randomly from Penang Botanical Gardens, Penang, Malaysia. After removing the surface soil, approximately 20-30 g of soil samples

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

residing 2-5 cm below the surface were collected using a sterile spatula into a sterile plastic bag. The soil samples were kept at ambient temperature (25±2 °C) in the laboratory until further processing.

Isolation of *Bacillus* spp. from the soil sample

Each sample was thoroughly mixed and approximately 10 g of soil was mixed with 100 mL of sterile distilled water in a 200 mL conical flask. Then the conical flask containing the sample was kept on an orbital shaker for homogenization for 24 h at 120 rpm. An aliquot of 1 mL from the thoroughly mixed sample was subjected to heat shock at 80 °C for 15 min to eliminate all the vegetative forms of all microbes. Then the sample was serially diluted and plated onto nutrient agar. The plates were then incubated at 30 °C for 24 to 48 h. The bacterial isolates were selected based on the colonial morphology resembling *Bacillus* spp. that were white to off-white, rough, opaque, slightly raised with perfect margin.

Phenotypic characterization through sporulation, Gram staining and Coomassie Brilliant Blue (CBB) staining

The presumptive *Bacillus* isolates were examined for the vegetative cell morphology, showing a typical rod shaped appearance and for the presence of spores under light microscope (Olympus BX41-CCD fitted with Xcam-α colour video camera). Then the confirmed *Bacillus* isolates were subjected to Gram staining. All the Gram positive sporulating isolates were used for CBB staining to determine the presence of parasporal crystal inclusion, a characteristic feature that distinguishes Bt from other *Bacillus* spp. Briefly, a straight inoculating wire was used to transfer an aliquot of a sporulated culture onto a microscopic slide. Then the slide was heat fixed and stained (0.133% CBB stain in 50% acetic acid), rinsed in distilled water, dried and observed under light microscope using 100X oil immersion objective (Rampersad and Ammons, 2002). The presence of parasporal inclusions were clearly observed as dark blue stained bodies.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The bacterial isolates were grown until 90% of the cells were lysed. The culture was harvested by centrifugation at 9,000 rpm at 4 °C for 20 min (Universal 320, Hettich Zentrifugen). The spore-crystal mixture was thoroughly washed with 1 M NaCl thrice, followed by sterile distilled water twice to remove cell debris. Finally, the pellet was re-suspended in 300 µL of sterile distilled water. The total protein concentration was estimated by Bradford (1976) method. The samples were boiled for 10 min in 5X sample buffer and 30 µg of total protein loaded in each well of 10% sodium dodecyl sulfate-polyacrylamide gel. The electrophoresis was performed at room temperature at 100 V for 2 h. Upon completion, the gel was stained with CBB R-250 staining solution (Bio-Rad Laboratories) overnight. The gel was then destained with CBB R-250 1X destaining solution (Bio-Rad Laboratories) until the background of the gel was clear. The molecular masses of proteins were determined by comparison with protein standards. Bti was used as reference strain.

Random amplified polymorphic DNA (RAPD-PCR)

A single colony of bacterial isolates was transferred to a microcentrifuge tube containing 50 µL of sterile deionized water. The samples were boiled at 100 °C for 10 min and spun at 10,000 rpm for 1 min (Universal 320, Hettich Zentrifugen) and 2 µL of supernatant was used as the DNA template for PCR. The 20 µL PCR mixture included 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U Taq DNA polymerase (Fermentas) and 2 pmol Primer 18 (First Base, Malaysia) (Table 1). PCR was performed with initial denaturation at 94 °C for 2 min; 45 cycles of denaturation at 94 °C for 1 min, annealing at 32 °C for 1 min, and extension at 72 °C for 2 min; and final extension at 72 °C for 6 min. PCR products were analyzed by 1.5% agarose gel electrophoresis.

Table 1: Sequence of primer 18 used in RAPD PCR.

Primer	Sequence	Reference
RAPD 18	5’ GCGATCCCCA 3’	Sadder *et al*. (2006)

RESULTS

Phenotypic characterization

The bacterial isolates were found to be aerobic, Gram positive, showing typical *Bacillus* morphology; the colonies are round, white with regular margin. The presumptive *Bacillus* isolates were rod-shaped, either single or in short chains with phase bright spores (Figure 1). The CBB staining of lysed cells showed the darkly stained spherical parasporal crystal proteins (Figure 2). Based on the above morphological features, the isolates were confirmed to be Bt. The sampling site was previously sprayed with a biopesticide to manage the vector-borne diseases. Apart from Bt, it is understood that a strain of *Bacillus subtilis* also exhibited mosquitocidal activity against *Anopheles culicifacies*, the vector of malaria parasite (Gupta and Vyas, 1989) and third instar larvae of *Culex quinquefaciatu*s (Das and Mukherjee, 2006). The present study conclusively proved the dominant presence of Bt strains, as confirmed by the presence crystal inclusion.

SDS-PAGE protein profile

Bt isolates that produced crystal protein were analysed by SDS-PAGE to determine the protein profile. From the protein profile (Figure 3), all the five isolates showed the presence of Cry4 and Cry11 proteins at approximately 130 kDa and 63 kDa, respectively, and a protein with low

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

molecular mass of around 32 kDa. The 130 kDa protein representing Cry4 is one of the four major insecticidal proteins that are found in Bti (Barusrux *et al.*, 2003) while the 63 kDa Cry11 is another important anti-dipteran toxin. Besides, the 32 kDa protein might be related to the Bti cytolytic toxin (*cyt* gene) which has recognized activity and specificity against dipterans (Gobatto *et al.*, 2010). This protein, when synergize with the Cry toxins, can delay or prevent the development of insect resistance to Cry toxins (Ibarra *et al.*, 2003).

The size of Cry11 proteins and cytolytic toxin of Bti were slightly different. Hofte and Whiteley (1989) reported that the molecular mass of Cry11 (previously designated as CryIVD) type of protein was between 72 kDa while Cyt protein was between 25-28 kDa (Glare and O'Callaghan, 2000). This is probably due to the fact that two different Bt strains could exhibit different protein profile although they carry same type of *cry* genes. Other possible reasons for the differences could be that some environmental factors which can turn on or turn off expression of some cry genes or due to post transcriptional and post-translational regulation of cry gene proteins (Cetinkaya, 2002).

Random amplified polymorphic DNA (RAPD- PCR)

RAPD is a PCR based molecular technique for identifying genetic variation (Welsh and McClelland, 1990). This procedure detects nucleotide sequence polymorphisms in a DNA amplification based assay using only a single primer of arbitrary nucleotide sequence. For the genetic analysis primer 18 was selected in the present study because of its highest number of amplified polymorphic bands (Sadder *et al*., 2006). The results revealed that, the RAPD pattern of all the Bt isolates showed a similar banding pattern as that of the control strain Bti (Figure 4). This result confirms the molecular identity of the field isolates with that of Bti and conclusively prove the wide spread occurrence and long persistence of biopesticide derived Bt in this environment.

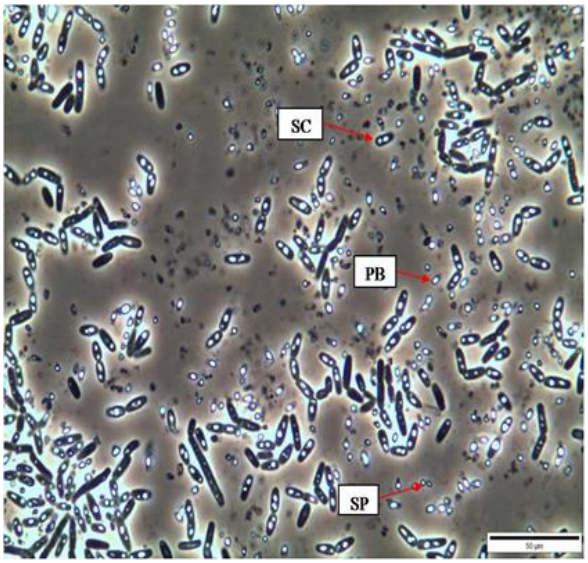

Figure 1: Rod-shaped, sporulating cells of Bt isolate S1 with phase bright spores. SC, sporulated cell; PB, parasporal body; SP, spore. Magnification: 100X. Scale bar: 50 µm

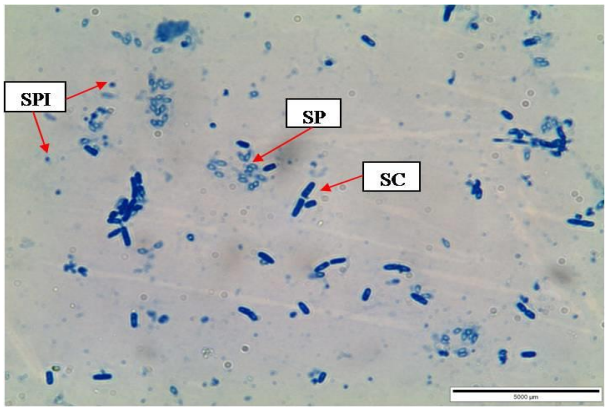

Figure 2: CBB stained cells of Bt isolate S1. SC, sporulated cell; SPI, spherical parasporal inclusions; SP, spore. Magnification: 1000X. Scale bar: 500 µm

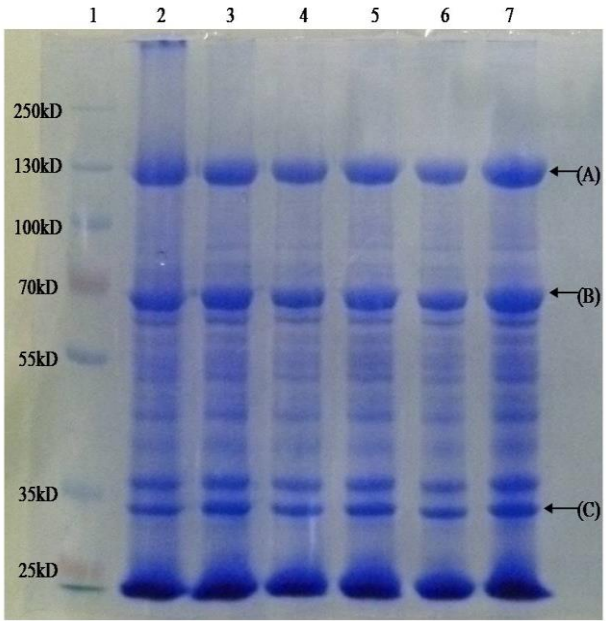

Figure 3: SDS-PAGE analysis of the Bt isolates. (A) Cry4 protein at 130 kDa, (B) Cry11 protein at 63 kDa, (C) Cyt protein at 32 kDa. Lane 1, molecular weight marker (Fermentas PageRuler Plus Prestained Protein Ladder); Lane 2, Bti; Lane 3, Isolate S1; Lane 4, Isolate S2; Lane 5, Isolate S3; Lane 6, Isolate S4; Lane 7, Isolate S5.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

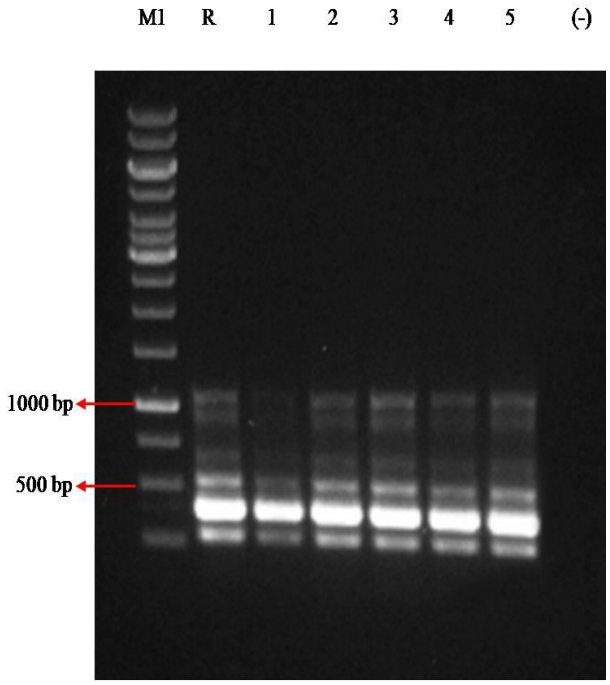

Figure 4: Agarose gel electrophoresis profile of the Primer 18 PCR products obtained from isolate S1, S2, S3, S4 and S5. Lane M1, 1 kb DNA ladder (Fermentas); Lane R, Bti; Lane 1, Isolate S1; Lane 2, Isolate S2; Lane 3, Isolate S3; Lane 4, Isolate S4; Lane 5, Isolate S5; Lane (-), negative control.

DISCUSSION

Though Bt is a ubiquitous spore-forming organism, soil has been one of the principal sources of novel Bt isolates. Insecticidal formulations based on different strains of Bt have been widely used as biopesticides against a variety of insects including medically important vectors. Commercial interest in biocontrol of agricultural pests and vectors of human diseases intensified the worldwide search for new Bt isolates with novel biological activities.

Commercial formulation based on Bti is widely used as an anti-dipteran biopesticide, resulting in the increased spread of Bti in the environment worldwide. Natural epizootics by Bt could be associated with the simultaneous occurrence of high population density of the target pest and the corresponding Bt strains in that particular environment. The present study has provided a novel insight into the dominant occurrence of a specific strain of Bt. The sampling area generally will be sprayed with a Bt based biopesticide to manage the mosquito population. Significant dominance and persistence in the environment poses several questions such as, (i) Will this prolonged persistence would be beneficial or detrimental? (ii) Will Bti have a competitive advantage over other microbes in the sprayed area? (iii) Will this persistence be advantageous to maintain the vector population under check?

The prolonged persistence of Bt in soil environment may be beneficial in terms of vector control from the perspective of possibility of natural epizootics. If the soil environment is associated with crop cultivation, the prolonged and dominant presence of a particular bacterium may deprive the crop from the beneficial activity of other soil microbes.

The present study, a combination of phenotypic and molecular studies allowed us to conclusively prove the persistence and dominant occurrence of a particular type of Bt, in the sprayed area. The phenotypic characterization confirmed the isolates are Bt, as indicated by the presence of spores and parasporal crystal inclusion. The light microscopic study of CBB stained Bt isolates, showed spherical crystals, thus indicating the presence of Cry4 proteins, which are active against the dipteran larvae (Schnepf *et al*., 1998). The *cry* genes have been organized into six different groups on the basis of their sequence similarities and range of specificity. The CryI, CryII, CryIII, CryIV, and CryV proteins are toxic to lepidopteran, lepidopteran and dipteran, coleopteran, dipteran, and nematode larvae, respectively (Feitelson *et al*., 1992). The revised classification of Cry proteins by Crickmore *et al*. (1998) delineated that the proteins toxic for lepidopteran insect belongs to the Cry1, Cry9, and Cry2 groups; toxins active against coleopteran insects are the Cry3, Cry7, and Cry8 proteins as well as the Cry1B and Cry11 proteins, which have dual activity. The Cry5, Cry12, Cry13 and Cry14 proteins are nematicidal, and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19 and Cyt proteins are toxic for dipteran insects. The SDS-PAGE analysis of protein pattern showed that the banding pattern of all the isolates were similar including the control strain Bti, thus proving the occurrence of the one type of Bt strain. Further, the protein profile revealed the presence of Cry4, Cry11 and Cyt proteins, thus indicating the possible anti-dipteran activity. Further, SDS-PAGE of whole–cell protein profiling provides valuable information on the similarity of bacteria at the species and subspecies level and has the advantage of being fairly simple and fast (Vandamme *et al*., 1996). Thus SDS-PAGE in this study provided a basic evidence of occurrence of a dominant species of Bt. The RAPD technology is very simple to perform, fast and informative on the diversity of group of isolates. From similarity of RAPD-PCR profile, it is evident that all the isolates may belong to one genotype, thus providing molecular evidence on the occurrence and dominant distribution of a particular Bt strain in the sprayed environment. The *cry* genes are confined to few larger plasmids in Bt (Aronson, 1993) and are self-transmissible through conjugation-like mechanism (Chapman and Carlton, 1985). Further such natural plasmid transfer may result in new combination of toxin genes through recombination between *cry* genes, thus producing novel biological activity. Tilquin *et al*. (2008) speculated that the long lasting persistence of Bti may lengthen the exposure time of insects to bioinsecticides, thereby increasing the risk of resistance acquisition in target insects, and of a negative impact on non-target insects. However, dynamic

ISSN (print): 1823-8262, ISSN (online): 2231-7538

environmental condition, including the seasonal variation, rain fall, wind and water erosion of soil, microbial interactions may help in maintaining the biotic balance.

ACKNOWLEDGEMENT

We sincerely thank Universiti Sains Malaysia for the financial support.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538