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The occurrence and insecticidal activity of *Bacillus thuringiensis* in the arid environments

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Abstract

Pest control in Saudi Arabia is entirely relied on the application of chemical agents. Little information is known about the natural presence of *Bacillus thuringiensis* species that possess insecticidal activity in the environment of Saudi Arabia. It would be of interest to search for native species of toxic Bt strains that can be used in pest control management. Thus the aim of this study was to investigate the natural presence of *Bacillus thuringiensis* species that are toxic to pests in the environment of Makkah Province, western Saudi Arabia. A total of 100 soil samples and five dead larvae of *Spodoptera littoralis* (Lepidoptera) were examined for the presence of *Bacillus thuringiensis*. The bacterium was isolated by acetate-selective enrichment and plating. Identification of isolates was performed by microscopic examination, analysis of parasporal inclusions protein profiles by SDS-PAGE, toxicity assay, analysis of 16S rDNA genes and DNA sequencing for PCR products. The confirmed *Bacillus thuringiensis* isolates, eight in total, were recovered from 5% of soil samples and from 60% of dead larvae. These isolates exhibited strong activity against 1st instar larvae of *S. littoralis*. Although *Bacillus thuringiensis* was not found to be abundant in soil habitats in Makkah Province, the results suggest that the bacterium is part of the indigenous microflora of the area we have explored. This is the first report of the natural presence of lepidopteran-toxic strains of *Bacillus thuringiensis* in the environment of western Saudi Arabia, particularly in Makkah Province.

Keywords: *Bacillus thuringiensis*; Environment; Makkah; PCR; Saudi Arabia, 16S rDNA genes.
Abbreviations: bp, Base Pair; Bt, *Bacillus thuringiensis*; EDTA, Ethylene diamine tetra acetic acid; LC_{50}, Lethal Concentration; KDa, Kilo Dalton; PCR, Polymerase Chain Reaction; ppm, Part Per Million; rpm, Round Per Minute; SDS-PAGE, Sodium Dodecyl Sulfate polyacrylamide Gel Electrophoresis.

Introduction

Bacillus thuringiensis is a gram-positive, spore-forming bacterium that produces parasporal inclusions during the sporulation phase. Theses inclusions are composed of proteins (Cry proteins) or δ-endotoxins which are highly toxic to a wide variety of insect pests and some invertebrates (Chattopadhyay et al., 2004; Vilas-Bôas et al., 2007). Due to their high specificity and their safety to most non-target organisms and to the environment in general, *Bacillus thuringiensis* crystal proteins are preferred and widely used as an alternative to chemical pesticides in pest management strategies against insect pests of agricultural crops (Roh et al., 2007; van Frankenhuyzen, 2009). Despite the wide spread use of the crystal protein in pest control it has led to the potential for development of resistance by target organisms to *Bacillus thuringiensis* toxins (Tabashnik et al., 2000; Herrero et al., 2001; Liu et al., 2001; Sayyed et al., 2004), this has necessitated the needs to isolate new and novel Cry proteins to overcome the serious problem of evolved resistance by insects to the pesticidal activity of Cry proteins. *Bacillus thuringiensis* strains are ubiquitous in the environment and is naturally found in soils (Martin and Travers, 1989), aquatic environments (Ishimastu et al., 2000), plants (Maduell et al., 2002), insects (Cavados et al., 2001) and animal faeces (Lee et al., 2003). The natural occurrence of *Bacillus thuringiensis* in the environment and their insecticidal activity has been reported from various countries in Europe (Quesada-Moraga et al., 2004; Apaydin et al., 2008), North America (Park et al., 2008), South America (Ibarra et al., 2003), Africa (Abdel-Hameed et al., 1994), and Asia (Yasutake et al., 2006; Gao et al., 2008). In Saudi Arabia, previous study showed that the genus *Bacillus* is abundant in soil samples from various locations in Makkah Province, Western Saudi Arabia (Khodair et al., 1991), however, information about the presence of *Bacillus thuringiensis* strains in the environment of Saudi Arabia, particularly in Makkah Province is sparse. This study was carried out to isolate and identify *Bacillus thuringiensis* that are naturally present in environmental samples collected from various locations in Makkah province, Saudi Arabia.

Results

Isolation of Bacillus thuringiensis *from soil and insects*

A total of 100 soil samples and five dead larvae of *Spodoptera littoralis* were collected from various areas in Makkah Province, Western Saudi Arabia, and were examined for the presence of *Bacillus thuringiensis*. For soil samples, a total of 75 *Bacillus* isolates were recovered from 100 soil samples. Of these 75 isolates, only five were confirmed as *Bacillus thuringiensis* on the basis of the production of parasporal inclusions (Table 2). Samples from Al-Jamoom

area were shown to harbor 60% of the total *Bacillus thuringiensis* isolates that were recovered from soil. On the other hand, soil samples from Al-Konfothah and Jizan Cities did not harbor *Bacillus thuringiensis* (Table 2). As shown in Table 2, the five dead larvae yielded 20 *Bacillus* isolate, only three isolates (15 %) were found to produce parasporal inclusions. The morphology of parasporal inclusions was found to be bipyramidal (90%), cubiodal (8%) and irregular (2%).

Bioassay

All eight isolates that were confirmed as *Bacillus thuringiensis* by microscopic examination, were tested for activity against 1st instar larvae of *Spodoptera littoralis* (Lepidoptera). Pesticidal activity of all eight isolates exhibited high activity with mortality percentage over 95% with LC_{50} equal to 1 ppm. In these tests the mortality rates of tested larvae in controls were less than 5%.

Analysis of crystals' protein profiles

The parasporal inclusion proteins of eight isolates that were toxic to larvae of cotton leaf worm were analyzed by SDS-PAGE, and were compared with broad range protein marker. The results of the crystal protein profiles of all eight isolates were found to have molecular mass of 130, 66 and 29 (Fig. 1).

Molecular analysis

PCR amplification of the 16S rDNA of all eight isolates was performed using various primer sets, primer F9 was used as general forward primer combined with different reverse primers (Table 1). The results showed that isolate number 3 (from soil sample of Al-Jamoom area) yielded an amplified fragment with 261, 527, 1106 and 793 bp in length when primer F9 was combined with primers 16S-2, R536, R1115 and R802 respectively (Fig. 2), which were the expected product size. Similarly, when primers F785 was combined with reverse primers R1115 and R1510, the ampilicon size of the 16S rDNA of isolate number 3 was as expected 330 and 725 bp in length respectively (Fig. 2). It is worth mentioning that all other isolates gave similar results as isolate number 3 with regards to PCR analysis. DNA sequencing of PCR products showed that all our eight isolates regardless of their origin were found to share 100%-identical new sequence which had been registered in GenBank under accession number (HM452382). This had a 99% identity with *Bacillus thuringiensis* IBL 200 sequence GenBank accession number (NK01000211). The PCR and sequencing results confirms that all our eight isolates that have parasporal inclusions and were shown to have insecticidal activity against *S. littoralis* 1st instar larva were *Bacillus thuringiensis*.

Discussion

Bacillus thuringiensis was found to be ubiquitous in diverse environments, particularly in soil habitats (Vilas-Bôas et al., 2007; Raymond et al., 2010). The geographical distribution of *Bacillus thuringiensis* in soil habitats is well documented. The bacterium was reported to be naturally exists in soil environments in South America (Martin and Travers, 1989; Ibarra et al., 2003; Armengol et al., 2007), North America (Martin and Travers, 1989; Park et al., 2008), Europe (Martin and Travers, 1989; Doroszkiewicz and Lonc, 1999; Quesada-Moraga et al., 2004), West and South Asia (Martin and Travers, 1989; Seifinejad et al., 2008; Bukhari and Shakoori, 2010), South East Asia (Martin and Travers, 1989; Hastowo et al., 1992; Yasutake et al., 2006), far East (China, Korea and Japan) (Martin and Travers, 1989; Ohba et al., 2000; Gao et al., 2008), Africa (Martin and Travers, 1989; Ogunijimi et al., 2000; Saadaoui et al., 2009) and Antarctica (Forsyth and Logan, 2000). In the Middle East, the natural occurrence of *Bacillus thuringiensis* in soil environments was reported from Egypt (Merdan and Labib, 2003) and Jordan (Sadder et al., 2006). In Saudi Arabia however, very little is known about the presence of the bacterium in soil habitats. Thus the primary aim of this study was to investigate whether or not *Bacillus thuringiensis* naturally exists in terrestrial environments of Makkah Province, western Saudi Arabia. In this study we collected 100 soil samples from different locations in three different cities (Makkah, Jizan and Al-Konfothah), and one small town (Al-Jamoom). The types of soil samples we examined were agricultural soils originated from Al-Jamoom and urban soils from Makkah, while the types of soils that were collected from Jizan and Al-Konfothah were desert sands and beach sands respectively. Our results showed that *Bacillus thuringiensis* isolates were found in agricultural and urban soil but not in beach and desert sand samples (Table 2). These results are in accordance with those previously reported that the recovery of the bacterium is more frequently from agricultural soils and to some degree from urban soils, however in beach soil and desert sands the incidence of the bacterium may be very low or absent (Martin and Travers, 1989; Vilas-Bôas and Lemos, 2004; Quesada-Moraga et al., 2004), this is probably explained by the variation of the biotic (other microorganisms) and abiotic (temperature, humidity, texture etc.) factors in these different soil habitats and their possible effects on the distribution of *Bacillus thuringiensis*. We found that agricultural soil samples from Al-Jamoom area yielded around 60% of soil-derived *Bacillus thuringiensis* isolates (Table 2). This is probably due to the presence of entomofauna associated with the crops being cultivated in this area such as tomatoes, sweet potatoes and other vegetables. Al-Jamoom is a small town that is known for its small scale farming practices, thus different types of entomofauna would most likely to be present in the soil habitats of this area. Indeed, we found dead larvae of *Spodoptera littoralis* (Lepidoptera) in Al-Jamoom area, the Egyptian cotton leaf worm (locally known as Alfalfa leaf worm) is considered to be amongst the entomofauna of economic importance in Saudi Arabia (Faraj-Allah and Al-Ghamdi, 2003). We managed to recover *Bacillus thuringiensis* from 60% of the dead larvae we collected (Table 2). It is important to mention that all samples analyzed in this study were collected from areas with no previous history of using *Bacillus thuringiensis* commercial products to control insect pests. Thus we suggest that soil-derived and dead insect-derived *Bacillus thuringiensis* isolates are part of the indigenous microflora of the areas we have explored. We managed to recover a total of 100 *Bacillus* isolates from soil and dead insects. Microscopic examinations showed that only 8% of these isolates were producing parasporal inclusions (Table 2), thus identified as *Bacillus thuringiensis*. A total of 90% of these parasporal inclusions were bipyramidal in shape. These eight isolates were further identified by analysis of protein profiles of parasporal inclusions by SDS-PAGE. The results showed that all eight

Table 1. Primers specific for 16S rDNA amplification and sequencing

Primer	Sequence	Product size bp
F9†	5'-GAGTTTGATCCTGGCTCAG-3'	261
16S-2	5'-CCCACTGCTGCCTCCCGTAGGAGT-3'	
F9†	5'-GAGTTTGATCCTGGCTCAG-3'	1501
R1510	5'-GGCTACCTTGTTACGA-3'	
F9†	5'-GAGTTTGATCCTGGCTCAG-3'	1106
R1115‡	5'-AGGGTTGCGCTCGTTG-3'	
F9†	5'-GAGTTTGATCCTGGCTCAG-3'	793
R802	5'-TACCAGGGTATCTAATCC-3'	
F9†	5'-GAGTTTGATCCTGGCTCAG-3'	527
R536	5'-GTATTACCGCGGCTGCTG-3'	
F9†	5'-GAGTTTGATCCTGGCTCAG-3'	776
F785	5'-GGATTAGATACCCTGGTAGTC-3'	

† = Used as general forward primer, ‡ Used for DNA sequencing

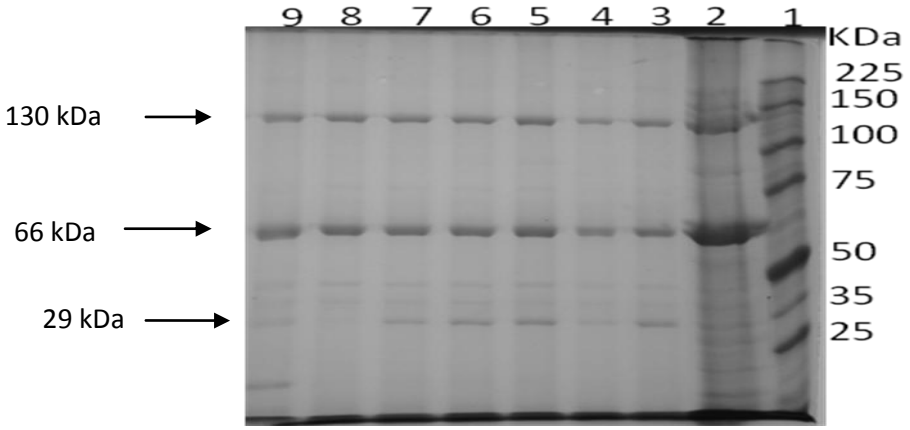

Fig 1. SDS-PAGE analysis of parasporal inclusions protein profiles of *Bacillus thuringiensis* isolates recovered in this study. Lane 1: broad range proteins marker; lane 2 to lane 6 represent the soil isolates, and lane 7 to lane 9 represent the isolates that were recovered from dead larvae.

isolates synthesized crystal proteins with molecular masses of 130, 66, 28 and 14 kDa respectively (Fig. 1).The higher molecular weight band with 130 kDa could probably correspond to the Cry1 complex of proteins, while the additional 66 kDa band observed may correspond to a degradation of the 130 kDa proteins or to a Cry2 protein (Arango et al., 2002; Armengol et al., 2007; Seifinejad et al., 2008; Bukhari and Shakoori, 2010). The smaller band of 29 kDa that was observed may correspond to the cytolytic (Cyt) protein toxin (Ohgushi et al., 2003; Gough et al., 2005). Due to difficulty in obtaining larvae for toxicity assay, we were unable to examine the activity of these isolates against other insect orders (Diptera and Coleoptera), however the observation of a protein band with 29 kDa suggests that our isolates may have activity against Diptera. The results of microscopic examination and protein profile analysis confirmed that all eight isolates were *Bacillus thuringiensis*. Previous studies reported that many soil-derived *Bacillus thuringiensis* isolates could have low or no toxic activities against insect pests (Hastowo et al., 1992; Roh et al., 1996; Yasutake et al., 2006), this is interesting enough to investigate the bioactivity of our soil isolates. We examined all eight isolates (those recovered from soil and from dead insects) for their bioactivity against 1st instar larvae of *Spodoptera littoralis* (Lepidoptera). The results showed that all eight isolates exhibited strong toxicity to *S. littoralis* larvae. These results further confirm the results of microscopic examination and protein profiles analysis, and further suggest that *Bacillus thuringiensis* strains that produce bipyramidal parasporal inclusions, with protein profiles between 130 and 66 kDa may exhibit strong toxicity to Lepidoptera (Arango et al., 2002; Uribe et al., 2003; Obeidat et al., 2004; Hernandez et al., 2005; Seifinejad et al., 2008). Members of the *Bacillus cereus* group are very closely related organisms (Vilas-Bôas et al., 2007). Analysis of 16S rDNA hypervariant region (approximately 275 bp close to the 5' end region), followed by DNA sequencing was reported to be a useful tool in the discrimination between the species in the *Bacillus cereus* group (Mohamed et al., 2006). We amplified and sequenced the 16S rDNA genes of the eight isolates that showed to form parasporal inclusions and used in the toxicity assay (Fig. 2). The primer selections we have used have been successfully used to distinguish between members of the *Bacillus cereus* group (Mohamed et al., 2006). DNA sequencing of PCR products further confirmed that the eight environmental-derived isolates that formed parasporal inclusions and exhibited strong activity against *Lepidoptera* were novel isolates of *Bacillus thuringiensis*.

Table 2. Isolation of *Bacillus thuringiensis* in soil and dead larvae samples from Makkah Province, Western Saudi Arabia

Location	No. soil samples	No. soil samples with Bt	No. *Bacillus* colonies isolated	No. of Bt isolates	Bt index†
Makkah City	50	2	40	2	0.05
Al-Jamoom area	40	3	30	3	0.1
Al-Konfotha City	5	0	3	0	0.0
Jizan City	5	0	2	0	0.0
Total	100	5	75	5	0.066
Location	No. of dead larvae samples	No. of dead larvae samples with Bt	No. of *Bacillus* colonies isolated	No. of Bt isolates	Bt index†
Al-Jamoom	5	3	20	3	0.15

† The *Bacillus thuringiensis* index was calculated as a number of *Bacillus thuringiensis* isolates recovered divided by the total number of *Bacillus* isolates examined.

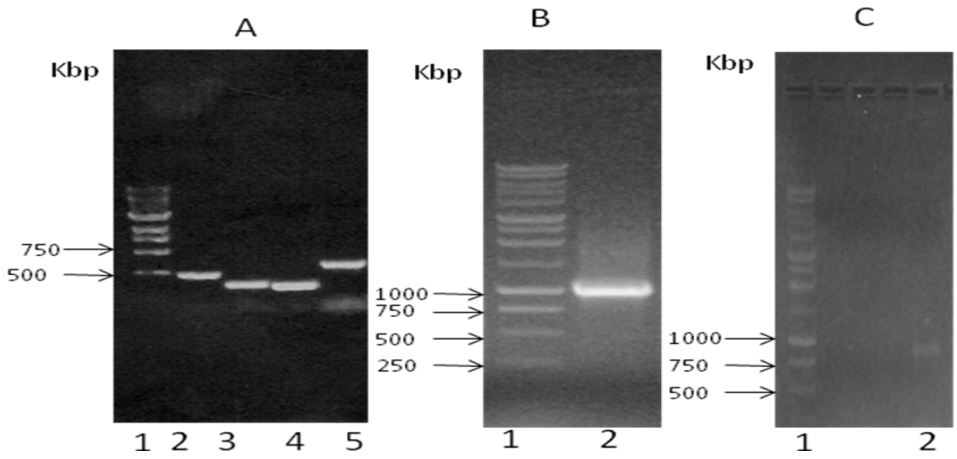

Fig 2. Agarose gel electrophoresis of PCR products of the 16S rDNA fragments for isolate number 3. Panel A: lane 1: 1Kb DNA ladder; lane 2: primers F9 and R536; lane 3: primers F785 and R1115; lane 4: primers F9 and 16S-2; lane 5: primers F785 and R1510. Panel B: lane 1: 1 Kb DNA ladder; lane 2: primers F9 and R1115. Panel C: lane 1: 1Kb DNA ladder; lane 2: primers F9 and R802.

To our best knowledge, this is the first published account that reports the natural presence of very active *Bacillus thuringiensis* strains from the environment of Makkah Province, western Saudi Arabia. Further genotypic characterization of these isolates is required to investigate their bioactivity spectrum and ecological distribution.

Materials and methods

Isolation of Bacillus thuringiensis from soil

A total of 100 soil samples were collected from Makkah City (urban soil), Al-Jamoom area, 40 km eastern outskirts of Makkah (agricultural soil), Al-Konfodhah City, 350 km south-west of Makkah City (beach sand) and from Jizan City, 685 km south-west of Makkah City (desert sand). Samples were collected from 2 to 5 cm below the surface with a shovel. Each soil sample was placed in a plastic bag at ambient temperature. *Bacillus thuringiensis* strains were isolated from soil samples using L broth and T3 agar following the method described by Travers et al. (1987).

Isolation of Bacillus thuringiensis from dead larvae

Dead larvae of Egyptian cotton leaf worm (also locally known as Alfalfa leaf worm) *Spodoptera littoralis* (Lepidoptera: Noctuidae), five in total were collected from Al-Jamoom area. Each dead larva was collected using sterile forceps and placed in a sterile plastic screw-top bottle. Each dead larva of *Spodoptera littoralis* was crushed in sterile crucible, and added to a tube containing 9 mL sterile phosphate buffered saline (PBS). The suspension was heated at 80 °C for 3 min then spun for 5 min at 8000 rpm. An aliquot of 50 µL of the supernatant were spread on L agar, plates and incubated overnight at 30 °C.

Confirmation of the isolates

After incubation period cells from *Bacillus* colonies were examined for morphological features of vegetative cells, spores, and the presence of parasporal inclusions, under light microscope (Leitz Wetzlar, Germany) using oil immersion at 1000× magnification. Further confirmatory tests were used, including; insecticidal activity test, analysis of crystal

proteins and molecular identification by PCR and sequencing of 16S rDNA.

Insect toxicity assays

All cultures that exhibited the presence of parasporal inclusions that had been previously stored as stock cultures on T3 agar were grown at 30 °C in 3 mL of T3 broth for 72 h. Concentrated suspensions of sporulated cultures (10^7 spore/mL) were prepared in T3 broth for qualitative oral toxicity test against 1st instar larvae of *Spodoptera littoralis*. The larvae were provided by the insectery of the Agricultural Genetic Engineering Research Institute in Giza, Egypt. The semi-artificial diet was prepared according to Levinson and Navon (1969), and the bioassay procedure was performed according to the method described by Loutfy (1973) and Ibarra and Federici (1987). The LC_{50} values were determined from probit analysis plot according to Finny (1962).

SDS-PAGE of parasporal inclusions protein analysis

Parasporal inclusions protein profiles of all eight isolates that showed pesticidal activity against larvae of *Spodoptera littoralis* were determined using SDS-PAGE. Isolates were grown in T3 broth for 72 h at 30 °C, then used to prepare pellets by centrifugation at 12000 rpm at 4 °C for 5 min, then washed once with distilled water followed by washing with 1 mL of 1 mM/L NaCl containing 5 mM/L EDTA. Whole cell proteins were separated on the basis of molecular weight. The procedure was performed according to the method of Laemmli (1970).

PCR of 16S rDNA genes sequencing

The eight isolates of *Bacillus thuringiensis* that had previously tested for pesticidal activity were used for PCR of 16S rDNA genes (Mohamed et al., 2006), followed by sequencing as molecular confirmatory test. Genomic DNA was isolated according to Wang et al. (2001). Synthesizing of primers (Bioneer, Alameda, USA) for PCR was according to Mohamed et al. (2006), primers sequence is shown in Table 1. The PCR reaction mixture (50 µL total volume) contained 200 µM of each dNTP, 0.5 µM primers, 10 mM tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 U Taq polymerase (ABgene, Surry, UK) and 100 ng of template DNA. Amplification of DNA using primers F9 and R802, 1510 was performed at the following temperature cycle: denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 60 s, 45 °C for 60 s, and 72 °C for 60 s, final extension at 72 °C for 7 min. A total of 20 µL of PCR products were analysed by 1% agaros gel (Bioline, London, UK) electrophoresis and made visible by ethidium bromide (0.5 mg mL^{-1}) staining and UV transillumination. Sequencing of PCR products was performed by the research team of Center of Excellence in Genomic Medicine Research, Jeddah, Saudi Arabia following the procedure described by Sanger et al. (1977).

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