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Full Length Research Paper

Characterization of *Bacillus thuringiensis* strains from Jordan and their toxicity to the Lepidoptera, *Ephestia kuehniella* Zeller

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Bacillus thuringiensis **was investigated in four different habitats (grain dust, olive-cultivated soils, waste and industrial-byproducts contaminated soils, and animal byproducts-contaminated soils). The bacterium was highly abundant in soils contaminated with animal byproducts. Eight serotypes with** ***Bacillus thuringiensis israelensis*** **being the most common. Out of the twenty-six isolated strains, five strains (serotype:** ***kenyae, kurstaki, kurstaki*** **HD1 and** ***thuringiensis*****) that produced bipyramid crystal proteins were toxic to the lepidoptera larvae of** ***Ephestia kuehniella*** **Zeller. The SDS-PAGE protein profile analysis showed a relationship between the crystal protein shape and the toxicity to the larvae of the tested insect.**

Key words: *Bacillus thuringiensis* serotypes, *Ephestia kuehniella*, parasporal crystal proteins.

INTRODUCTION

Biological pesticides are becoming an important component in crop and forest protection and in insect vector control. These pesticides are natural, disease-causing microorganisms that infect or intoxicate specific pest groups (Carlton, 1988; Spear, 1987).

The greatest successes in microbial pesticides have come from the uses of *Bacillus thuringiensis* (*B. t.*). Commercial preparations of *B. t.* have been shown to be the most successful biological control products worldwide (Carlton, 1988). This bacterium is a Gram-positive, able to produce parasporal crystal proteins that exhibited a wide range of toxicity to different insect orders such as Diptera, Lepidoptera and Coleoptera (Feitelson, 1993; Schnepf et al., 1998). It is a widespread bacterium detected in different habitats including; soil, grain dust, diseased insect larvae, and sericulture environments (Dulmage and Aizawa, 1982; Obeidat et al., 2000; Smith and Couche, 1991).

The objectives of the study are to isolate *B. t.* strains from different habitats and to characterize these strains according to parasporal crystal protein production and toxicity against the larvae of *Ephestia kuehniella* Zeller.

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Abbreviations. Bp: Bipyramidal\; *B. t.*: *Bacillus thuringiensis*; C: Cuboidal; CFU: Colony forming unit; LC_{50}: Lethal concentration that kill 50%; S: Spherical; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Sample Collection

Twenty samples were collected from different habitats in Jordan including grain dust, olive-cultivated soils, waste and industrial byproducts-contaminated soils, and animal byproducts-contaminated soils (Table 1).

Isolation of Bacteria

Isolation of *B. t.* was conducted according to the method of Ohba and Aizawa (1986) and Travers et al. (1987). One gram of each sample was suspended in 10 ml sterile distilled water and pasteurized at 80°C for 30 min. *B. t.* was selected by adding 1 ml of

Table 1. Abundance of local *B. thuringiensis* strains isolated from four habitats.

Habitat	No. of samples	No. of different *B. thuringiensis* strains	Average viable count of *B. thuringiensis* (CFU x 10^7/g)	% of *B. thuringiensis*[a]
Grain dust	5	5	5.25±1.46	0.72±0.06
Olive-cultivated soils	4	6	3.61±0.79	0.95±0.09
Animal-contaminated soils	7	10	8.74±2.27	4.56±1.03
Waste and industrial byproducts-contaminated soils	4	5	1.99±0.52	0.33±0.03
Total	20	26		

[a]The % of *B.t.* was calculated out of the total viable bacterial count.

Table 2. Serotyping and parasporal crystal protein morphology of twenty six *B. thuringiensis* strains.

Habitat	Strain	*B. thuringiensis* Serotype	Parasporal Crystal Protein Morphology[a]
Grain dust	1	*kurstaki* HD1	BP + C
	2	*israelensis*	S
	3	*israelensis*	S
	4	*pakistani*	S
	5	*israelensis*	S
	6		
Olive-cultivated soils	7	*kurstaki*	BP + C
	8	*thuringiensis*	BP
	9	*kurstaki*	BP + C
	10	*israelensis*	S
	11	*israelensis*	S
		malaysiensis	S
Animal-contaminated soils	12	*kurstaki*	BP + C
	13	*israelensis*	S
	14	*kurstaki* HD1	BP + C
	15	*kurstaki*	BP + C
	16	*israelensis*	S
	17	*israelensis*	S
	18	*kumamotoensis*	S
	19	*kenyae*	BP
	20	*israelensis*	S
	21	*israelensis*	S
Waste and industrial byproducts-contaminated soils	22	*kurstaki*	BP + C
	23	*israelensis*	S
	24	*israelensis*	S
	25	*kurstaki*	BP + C
	26	*israelensis*	S

[a]C: Cuboidal, BP: Bipyramidal, S: Spherical.

each suspension to 10 ml of LB broth buffered with 0.25 M sodium acetate pH 6.8. The suspensions were incubated at 30°C for 4 h and then heated to 80°C for 3 min. Suspensions were diluted and plated on T3 medium (per liter: 3 g tryptone, 2 g tryptose, 1.5 g

Table 3. Toxity of *B. thuringiensis* serotypes to *Ephestia kuehniella* larvae.

Toxic Isolate		LC_{50}[a]
No.	Serotype	
2	*B. t. thuringiensis*	6.31
7	B. t. kurstaki HD1	4.61
12	*B. t. kurstaki*	5.83
15	*B. t. kurstaki*	7.25
19	*B. t. kenyae*	6.97

[a]LC_{50} = log (spore concentration/ml) assayed in triplicate.

yeast extract, 0.05 M sodium phosphate pH 6.8, and 0.005 g of $MnCl_2$) and incubated at 30°C for 24 h. Smears were examined under the light microscope to observe the parasporal bodies of the bacterium.

Serology

Serotyping of *B. t.* strains was according to the method of Laurent et al. (1996). 90 µl of each bacterial suspension which taken from a 5-8 h culture was placed in each well of a 96-well microplate (U-bottom). 10 µl of two antisera dilutions (1: 10 and 1: 20) were used. The microplate was incubated for 75 min at 37°C. The agglutination was determined by observation under compound microscope. Positive reactions were visible as floccular sediment at the bottom of the well and a clear supernatant.

SDS-PAGE Analysis of parasporal inclusion proteins

Parasporal crystal protein analysis was performed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970).

Bioassay

Isolates with parasporal bodies were cultured in 100 m1 of T3 medium and incubated for 7 days at 30°C with continuous shaking at 250 rpm (Meadows et al., 1992; Travers et al., 1987). Samples were centrifuged at 5000 rpm for 15 min. Pellets (spores and parasporal protein crystals) were washed in 20 ml sterile distilled water and centrifuged at 5000 rpm for 5 min. Washing was repeated twice. The pellets were resuspended in 20 ml of sterile distilled water and kept at 4°C (Carozzi et al., 1991).

The suspensions of *B. t.* strains were examined for their toxicity to the third instar larvae of *E. kuehniella*. Diet for larvae was prepared by soaking one gram of peanut pieces in 10 ml of each bacterial suspension for 5 min using three fold serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}). The diet was then dried and placed in a vial where 10 larvae were placed. The toxicity of each strain was assayed in triplicate for either the original toxin-spore suspension or the diluted ones. Vials were incubated at 25°C for 72 h. Mortality was scored in comparison with parallel control in which peanut pieces soaked in sterile distilled water instead of bacterial suspension. The LC_{50} values were determined by probit analysis on log-probit paper (Bourgouin et al., 1990).

RESULTS

The viable count of *B. t.* was scored after acetate selection. A total of 26 *B. t.* strains were selected according to the colonial morphology differences (Table 1). Results showed that these strains were highly abundant (~8.74×10^7 CFU/g) in soils contaminated with animal byproducts. Whereas soils contaminated with waste and industrial byproducts found to contain the lowest viable count (~1.99×10^7 CFU/g).

The twenty-six *B. t.* strains were found belonging to eight different serotypes (*israelensis, kenyae, kumamotoensis, kurstaki, kurstaki* HD1*, malaysiensis, pakistani,* and *thuringiensis*) (Table 2). The serotype *B. t. israelensis* that represents 14 strains was the predominant in the four habitats. Out of the 26 *B. thuringiensis* strains, sixteen (serotypes: *israelensis, kumamotoensis, malaysiensis,* and *pakistani*) were characterized by the production of spherical parasporal crystals. Eight strains (serotypes: *kurstaki* and *kurstaki* HD1) were characterized by the production of both bipyramid and cuboidal parasporal crystal proteins. The remaining two strains (serotypes: *kenyae* and *thuringiensis*) produced only bipyramid parasporal crystal proteins (Table 2).

Bioassay showed that five strains were toxic to *E. kuehniella* larvae (Table 3). Two of these were isolated from animal byproducts-contaminated soils were belonged to serotype *kurstaki*, one (*kurstaki* HD1) was isolated from grain dust, and another (*kenyae*) was isolated from animal byproducts-contaminated soils. The fifth was isolated from olive-cultivated soils. *B. t. kurstaki* HD1 was the most toxic.

The SDS-PAGE protein component of the 5 toxic strains was also examined. The crystal protein profile of the reference strain of serotype *israelensis* was used as a marker (lane 1) (Figure 1). SDS-PAGE profile shows that the examined strains contain heterogeneous multiple protein components, with molecular masses ranging from 20 to 140 kDa. The protein profiles of the strains that produced both bipyramid and cuboidal crystal proteins were identical (lanes 2, 3 and 5). Furthermore, strains that produced bipyramid crystals were also identical (lane 4 and 6).

DISCUSSION

The present study provides an understanding of the relationships between protein profiles and crystal morphology as well as toxicity to lepidoptera.

B. t. was found in all selected habitats, suggesting that *B. t.* strains were highly abundant in these habitats. This is in agreement with our previous study (Obeidat et al.,

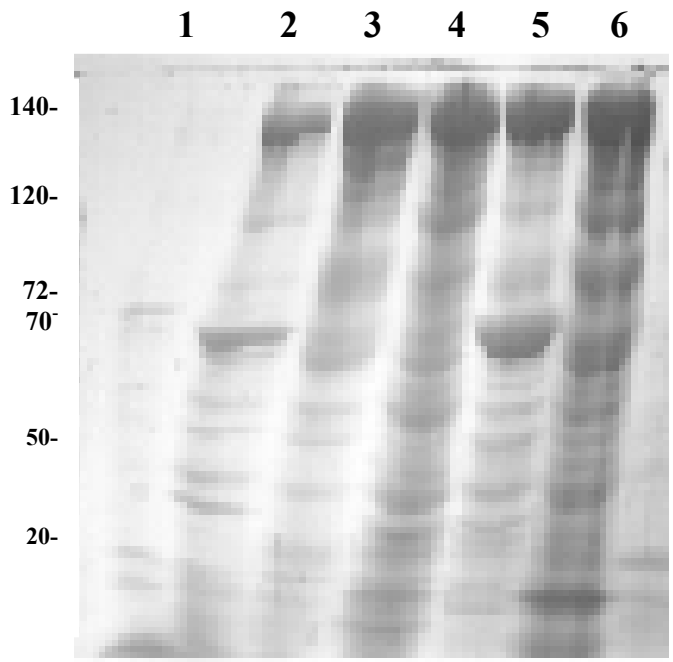

Figure 1. Comparative SDS-PAGE Analysis. Lane 1: Reference strain, *B. t. israelensis*. Lane 2: Strain No. 2, *B. t. thuringiensis*. Lane *3*: Strain No. 7, *B. t. kurstaki HD1*. Lane 4: Strain No. 15, *B. t. kurstaki*. Lane 5: Strain No. 19, *B. t. kenyae*. Lane 6: Strain No. 12, *B. t. kurstaki*.

2000) which showed that Jordanian soils are very rich in *B. t.* specially soils contaminated with animal byproducts. This abundance may be due to the presence of many insects. The increased potential for plasmid transfer among bacterial strains in such habitats may explain this abundance. On the contrary, the lowest *B. t.* counts were found in industrial byproduct and waste habitats. This may be due to the presence of disinfectants and waste chemicals.

Data showed that the most common serotype in the tested habitats is *B. t. israelensis*. This serotype was found in all tested habitats. Martin and Travers (1989) also isolated *B. t.* from several locations in Eastern Asia. They found that strains of *B. t. kurstaki* and strains of *B. t. israelensis* were the most common. Middle East habitats including Jordan was not been included in their study. The differences in the distribution of the serotypes might be related to the sample location, the habitat of isolation and genetic variation.

E. kuehniella larvae were chosen for the toxicity bioassay because *B. t.* is not normally toxic to insect larvae that live in the soil (Martin and Travers, 1989), but toxic to insects that have aerial or water-borne larvae (Wu and Chang, 1985). In this study, five of the bioassayed *B. t.* strains were found to be toxic to *E. kuehniella* larvae, three of which produced both bipyramid and cuboidal crystal proteins and the remaining two strains produced bipyramid crystal proteins. The results are in agreement with Ohba and Aizawa (1986) who suggested a possible relationship between the shape of the parasporal bodies and the toxicity; strains of bipyramid crystals being the most toxic. Most reports showed that strains that produced bipyramid crystal proteins exhibited only toxicity to lepidoptera (Karamanlidou et al., 1991; Martin and Travers, 1989; Meadows et al., 1992).

To date, over 50 *cry* gene sequences have been determined and classified into 15 families (Bravo, 1997). Type I genes encode 130-kDa proteins (bipyramidal crystal proteins) that are normally active only against lepidoptera species. Type II genes encode 70-kDa proteins (cuboidal crystal proteins) that also maintain activity against lepidoptera (Yamamoto and Powell, 1993). The profiles of SDS-PAGE analysis of this study revealed that strains producing bipyramid and cuboidal crystal proteins showed similar protein profiles. The strains producing bipyramid crystal proteins only showed similar protein profiles. This may be due to genetic similarity among them. The toxicity of the tested strains to *E. kuehniella* was attributed to their high molecular mass proteins (130 kDa and 70 kDa) recorded by Yamamoto and Powell (1993).

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