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Insecticidal Characterization of Thirteen *Bacillus thuringiensis* Isolates from Soil (III)

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Thirteen strains of *Bacillus thuringiensis* were isolated from soil in Korea and characterized. The all strains produced parasporal crystals and spores in their cells. Two strains had bipyramidal crystals, seven strains contained round ones and four strains had unregular ones. Only minor biochemical characteristics of the thirteen isolates were different and distinctive, however general characteristics were similar to the known serotypes of *B. thuringiensis*. Two strains were resistant to ampicillin. Three strains were resistant to bacitracin, six strains were resistant to cephalothin, two strains were resistant to colistin, HL-68 strain was resistant to gentamycin, HL-67 strain was resistant to kanamycin and HL-71 was resistant to teteracycline. Two strains were resistant to penicillin G. Four strains were toxic to *Bombyx mori* larvae and eleven strains were toxic to *Culex pipiens* larvae.

KEY WORDS □ ***B. thuringiensis*, endotoxin, *Culex pipiens*, *Bombyx mori*.**

An unique property of *Bacillus thuringiensis* is the production of entomocidal endotoxin crystals during its sporulating cycle (1). The crystals have a specific lethality to certain insect larvae (1); therefore, the crystals and the microorganisms are being developed as important microbial insecticidal pesticides (1). de Barjac and Bonnefoi (5) showed that subspecies of *B. thuringiensis* can be distinguished by serotypes or their flagellar (H) antigens. Thereafter about 35 serotypes of *B. thuringiensis* were found (1-6, 9-12, 14, 18-23). For identification of *B. thuringiensis* strains biochemical characteristics, serological test, microscopic observation of crystal formation, antibiotic resistant patterns and toxicity against insect larvae are usually investigated (1). Recently we isolated thirteen strains of *B. thuringiensis* from soil, and we tested their biochemical, microscopic characterizations and toxicity against insect larvae.

In view of such characterization this report describes the biochemical characteristics, microscopic observations, antibiotic resistant patterns and toxicity against insect larvae of the thirteen isolates of *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial strains and media

B. thuringiensis isolates were used in this study. Bacterial cells for parasporal proteinaceous crystals were cultured at 28°C in UG medium containing minerals, peptone and glucose (8). Muller-Hinton medium (Difco) was used for the reading of inhibition zones of antibiotics.

Isolation of *B. thuringiensis* from soil

Soils were sampled from various fields planted with several crops in virgin soil, in rocky soil and in forest areas in Korea. In all cases, to minimize the defects of surface contamination, soil samples were taken by first removing the top soil (2 to 3 cm) from the sampling areas and then transferring a small portion of the soil with a clean spoon to a sterile plastic bag. The plate count method was used for colony enumeration. Five μg of polymyxin B sulfate and 4 μg of penicilin G per ml (Sigma) were added aseptically to the molten agar (45°C) before the plates were poured. The nutrient agar containing polymyxin and penicillin was incubated for 48 hours at 37°C. All colonies with growth characteristics similar to *B. thuriengiensis* were picked and examined by phase contrast microscopy for the presence of spores and crystals. The presence of crystals in cells was taken as presumptive evidence that the culture was *B. thuriengiensis*. Isolates were subcultured onto UG medium and tested for furthur identification.

Reconfirmation of crystal formation

B. thuringiensis isolates were precultured in 20 ml of nutrient broth at 28°C by rotary agitation at 180 rpm overnight and 1.0 ml of the precultures was transferred into 20 ml UG media. Then it was

cultured until sporulation at 28°C by rotary agitation at 180 rpm for 20 to 30 hrs. The fully mature-unlysed cells were harvested and washed twice with sterilized saline by centrifugation at 3000×g for 20 min. For microscopic observation, the pellets were resuspened with saline. Formations of spores and parasporal crystals were observed by using a phase-contrast microscope.

Biochemical characterization of *B. thuringiensis* isolate

Biochemical characteristics of the isolates were examined by the procedures of Lennette *et al.* (16).

Antibiotic susceptibility test

A standardized filter paper disc on agar diffusion test was to determine the antibiotic sensitivity of *B. thuringiensis* (Lennette *et al.*, 1975).

Bioassay

One or two loops of pure-cultured isolates were inoculated in 10 m*l* of fresh nutrient broth and then cultured at 28°C at 180 rpm overnight. 2.5 m*l* of the culture were transferred into 50 m*l* of UG medium and cultured again for 48 to 72 hours. After pelleting the culture at 4000×g for 20 min, the supernatants were discanted and the pellets were washed twice with sterilized saline by centrifugation at 4,000×g for 20 min. The pellets were suspended with 5 m*l* of saline. Then, 1.0 m*l* of the suspended spore-crystal complex (about 10^7 to 10^8 spores/m*l*) were added to 150 m*l* of distilled water in the disposable cup (72×80 mm) for *Culex pipiens* (*Dipetera*) larvicidal test or a lump (2 cm^3) of semisolid food in a petri dish (2×20 cm) for *Bombyx mori* (*Lepidoptera*) larvicidal test containing insect 3rd instar larvae. The lethality was observed at 28°C for 48 hrs.

RESULTS AND DISCUSSION

Characteristics of *Bacillus thuringiensis* isolates

Wide ranges of soil samples were examined, and then *B. thuringiensis* strains were isolated. Thirteen isolates containing parasporal inclusion bodies (crystal) were found (Fig. 1~13) and named HL-44, 45, 55, 63, 64, 66, 67, 68, 70, 71, 74, 75 and 76. There is no significant differences in the shape and size of the vegitative cells of the *B. thuringiensis* isolates to the reference strains. The isolates were motile rods with dimensions of 1.3~1.4×3.7~4.1 μm and gram-positive. The formation of crystals were confirmed by phase contrast microscopy. As shown in Fig. 1, the isolates showed the general features of *B. thuringiensis*. The crystal shape in the HL-44 isolate was spherical by phase contrast microscopy (Fig. 1). The crystal shapes of the HL-45 and 55 were bypiramidal by phase contrast microscopy (Fig. 2 and 3).The crystal shapes of HL-63, 64, 66 and 67 were round by phase contrast microscopy (Fig. 4~7). The HL-68, 70, 71 and 74 had unregular and round shapes of the crystal (Fig. 8~11). The HL-74, 75 and 76 had round crystals in their cells (Fig. 11~13). Generally the unregular shape of the crsytals was distinctive, however the round or bipyramidal shape are similar to the known serotypes of *B. thuringiensis* (1, 13, 15, 17).

The thirteen isolates were examined on their biochemical characteristics as shown in Table 1. The thirteen isolates showed commonly negative reactions on the productions of H_2S, indole, lysine decarboxylase, phenylalanine deaminase and arginine decarboxylase; and utilizations of arabinose, dulcitol, inositol, lactose, manitol, rhamnose, salicine, sorbitol, sucrose and xylose.

The isolates showed commonly positive reactions on motility; productions of alkali and acid from glucose, catalase, oxidase, acid from glucose; Voges-Proskauer reaction; β-hemolysis; utilization of glucose and maltose.

The methyl-red reaction of HL-55 was negative, however other strains were positive.

The HL-44 and 45 strains were negative in the production of ornithine decarboxylase, but other strains were positive. The strains of HL-68, 70, 71 and 74 produced lecithinase, but others were not tested. The HL-55 produced urease, but the HL-63, 64, 66 and 67 strains were not produced. The HL-55 utilized adonitol, but other strains did not. The strains of 64, 66, 67, 68, 70, 75 and 76 utilized citrate, but other strains did not. The strains of 44, 45 and 55 utilized galactose, but other strains were not examined. The isolates had general biochemical characteristics as the known serotypes of *B. thuringiensis* (3, 15). Some of the isolates show similar phenotypes in the biochemical characteristics, however the isolates cannot be classified in the same strain because their flagellar antigenecities could be different which is the most important characteristics in the classification of the *B. thuringiensis* strains.

Antibiotic resistant patterns of *B. thuringiensis* isolates

The antibiotic resistant patterns of the thirteen isolates were various (Table 2). The strains of HL-68, 70, 71, 74, were resistant to ampicillin, the six strains of HL-44, 45, 55, 66, 67 and 71 were resistant to cephalothin. The two strains, HL-44 and 45 were resistant to colistin. The HL-68 strain was resistant to gentamycin. The HL-67 strain was resistant to kanamycin. The strains of 44 and 71 were resistant to peniclin G. The strain of HL-70 was resistant to tetracycline. Some of the isolates show similar phenotypes in the antibiotic resistant characteristics, however the isolates cannot be classified in the same strain because their plasmid patterns and their flagellar antigenecities could be different which is the most important characteristics in the classification of the *B. thuringiensis* strains.

Toxicity of *B. thuringiensis* isolates

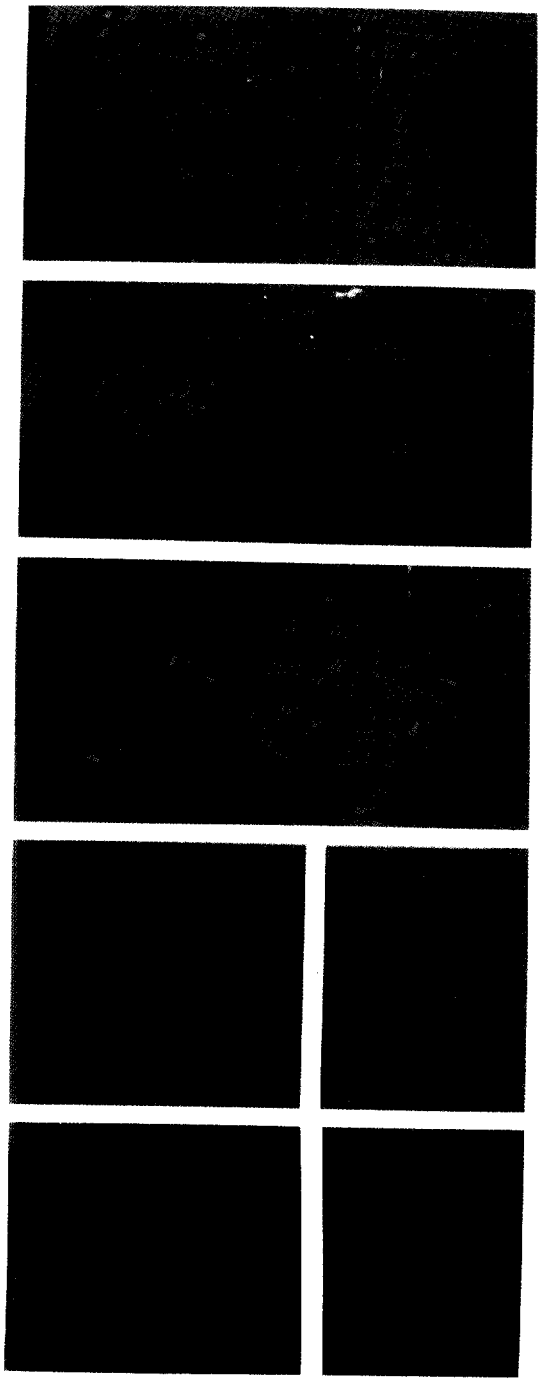

Fig. 1. *B. thuringiensis* isolate. 1, HL-44; 2, HL-45; 3, HL-55; 4, HL-63; 5, HL-64; 6, HL-66 and 7, HL-67. C is crystal and S is spore.

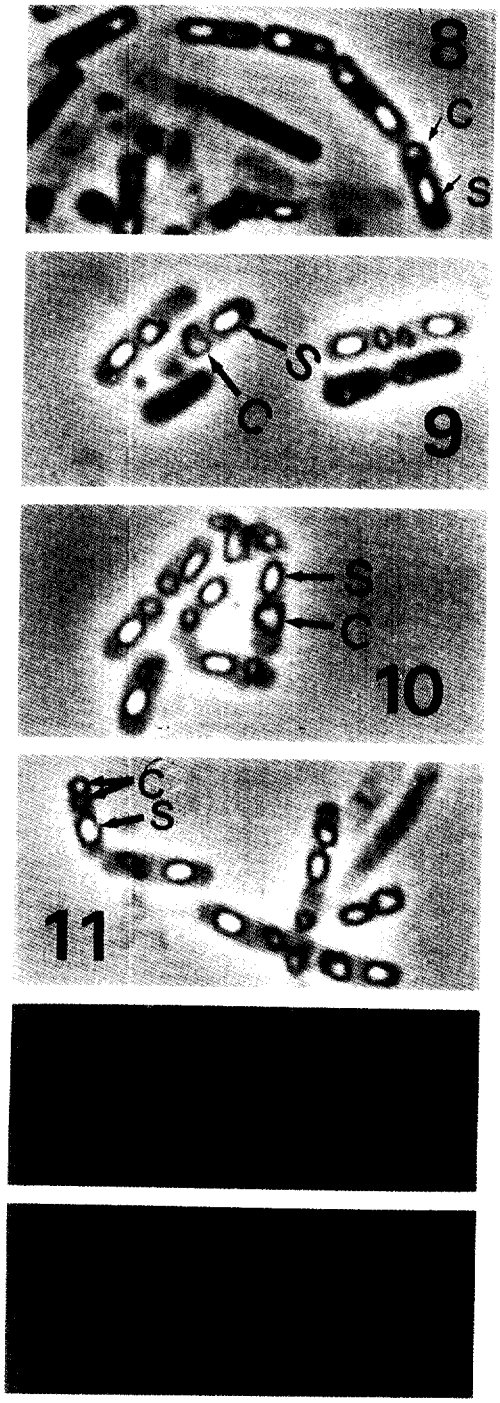

Fig. 2. *B. thuringiensis* isolate. 8, HL-68; 9, HL-70; 10, HL-71; 11, HL-74; 12, HL-75 and 13, HL-76. C is crystal and S is spore.

Table 1. *Biochemical characteristics of B. thuringiensis isolates*

Characteristics	Biochemical reactions of the Isolates												
	HL-44	45	55	63	64	66	67	68	70	71	74	75	76
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
Kligler's iron agar	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
Voges-Proskauer reaction	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-red reaction	+	+	+	+	+	+	+	+	+	+	+	+	+
Productions of indole	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+
hemolysin	β	β	β	β	β	β	β	β	β	β	β	β	β
catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
phenylalanine deaminase	+	+	+	+	+	+	+	+	+	+	+	+	+
lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+
arginine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+
ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+
oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
lecithinase	N	N	N	N	N	N	N	+	+	+	+	N	N
urease	N	N	+	+	+	+	+	N	N	N	N	+	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Utilizations of adonitol	+	+	+	+	+	+	+	+	+	+	+	+	+
arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
citrate	+	+	+	+	+	+	+	+	+	+	+	+	+
dulcitol	+	+	+	+	+	+	+	+	+	+	+	+	+
galactose	+	+	+	N	N	N	N	N	N	N	N	N	N
inositol	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
manitol	+	+	+	+	+	+	+	+	+	+	+	+	+
rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+
salicine	+	+	+	+	+	+	+	+	+	+	+	+	+
sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
xylose	+	+	+	+	+	+	+	+	+	+	+	+	+

(+); positive reaction, (−); negative reaction

Table 2. *Antibiotic resistance of B. thuringiensis isolates*

Antibiotics	Antibiotic resistance of the isolates										
	HL-44	45	55	63-64	66	67	68	70	71	74	75-76
amikacin (30 μg)	S	S	S	S	S	S	S	S	S	S	S
ampicillin (10 μg)	R	R	S	S	S	S	S	S	S	S	S
bacitracin (10 μg)	N	N	N	N	N	N	R	R	R	R	N
cephalothin (30 μg)	R	R	R	S	R	R	S	S	R	S	S
chloramphenicol (30 μg)	S	S	S	S	S	S	S	S	S	S	S
colistin (10 μg)	R	R	S	S	S	S	S	S	S	S	S
erythromycin (15 μg)	S	S	S	S	S	S	S	S	S	S	S
gentamycin (10 μg)	S	S	S	S	S	S	R	S	S	S	S
kanamycin (30 μg)	S	S	S	S	S	R	S	S	S	S	S
neomycin (39 μg)	S	S	S	S	S	S	S	S	S	S	S
penicillin G (19 units)	R	S	S	S	S	S	S	S	R	S	S
streptomycin (10 μg)	S	S	S	S	S	S	S	S	S	S	S
tetracycline (30 μg)	S	S	S	S	S	S	S	R	S	S	S

S; sensitive reaction, R; resistant reaction and N, not done.

Table 3. *Toxicities of B. thuringiensis isolates against Bobyx mori larvae*

Isolates	No. of larvae tested	No. of the dead for 72 h	Ratio of lethality(%)
Control (food)	20	0	0
HL-44, 45	20	18	64
HL-55	20	20	100
HL-63,64,66,67 68,70,71,74	20	0	0
HL-75	20	10	50
HL-76	20	15	75

Table 4. *Toxicities of B. thuringiensis isolates against Culex pipiens larvae*

Isolates	No. of larvae tested	No. of the dead			Ratio of lethality(%)
		1h	2h	12h	
Control	20	–	–	0	0
HL-44,45	20	–	–	0	0
HL-55	20	–	–	6	30
HL-63,64	20	–	–	–	100
HL-66	20	–	18	2	100
HL-67	20	–	20	–	100
HL-68,70,71,74	20	20	–	–	100
HL-75	20	10	10	–	100
HL-76	20	12	8	–	100

The toxicities of the thirteen isolates against *Bombyx mori* and *Culex pipiens* larvae were various (Table 3 and 4). Five strains were toxic to *B. mori* larvae (Table 3). The strain of HL-55 was strongly toxic to *B. mori* larvae, but not to mosquito larvae. Eleven strains of the isolates killed mosquito larvae (Table 4). The four strains, HL-68, 70, 71 and 74 showed strong toxicity, they completely killed mosquito larvae within 1 hour. The known strains were toxic to various insect larvae (1). We found the strains toxic to various insect larvae. The HL-55 killed *B. mori* and *C. pipiens* larvae either, but it was stronger to *B. mori* larvae, also the HL-75 and 76 strains killed both *B. mori* and *C. pipiens* larvae, but they were stronger to mosquito larvae (Table 3 and 4).

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초 록: 토양에서 분리한 13 *Bacillus thuringiensis* 균주의 살충성 특성

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Bacillus thurigiensis 13균주를 토양에서 분리하여 특성을 조사했다. 분리균를 HL-44, 45, 55, 63, 64, 66, 67, 68, 70, 71, 74, 75와 76으로 명명했다. 분리균은 세포내에 내독소결정체를 형성했다. 두 균주는 이중피라미드형의 내독소, 4균주는 부정형결정 그리고 7균주는 둥근형의 결정체를 형성했다. 13균주의 생화학적인 특성은 전체적으로 기존의 *B. thuringiensis*의 특성과 유사했으나, 몇가지 특성은 차이가 컸다. 항생물질에 대한 저항성을 보면, HL-44와 45는 ampicillin에 내성을 가졌고, HL-70, 71과 74균주는 bacitracin에, 7균주는 cephalothin에, HL-44와 45균주는 colistin에, HL-70은 tetracycline에, HL-44와 71균주는 penicillin G에 내성을 나타냈다. 4균주는 누에유충에 치사성을 나타냈고, 11균주는 모기유충을 치사시켰다.