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Characterization of Microbial Pathogen *Bacillus thuringiensis* Isolates from Soil Against Mosquito and Silkworm Larvae (II)

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Eight strains of *Bacillus thuringiensis* were isolated from soil in Korea and characterized. The isolates were named HL-24, HL-25, HL-33, HL-34, HL-35, HL-38, HL-39 and HL-40. Strains HL-24 and HL-25 produced irregular parasporal crystals, HL-33 and HL-35 produced bipyramidal crystals, and others were round form in their cells. The biochemical characteristics of the eight isolates were only minor different in specific chracteristics to the known serotypes of *Bacillus thuringiensis*. The HL-25, HL-33 and HL-34 strains showed resistances to cephalothin, colistin and penicillin G, and HL-39 and HL-40 strains were resistant to penicillin G. The strains of HL-24, HL-25, HL-33 and HL-34 were toxic to *Bombyx mori* larvae and HL-24, HL-25, HL-38, HL-39 and HL-40 strains killed *Culex pipiens* 3rd instar larvae. The HL-24 and 25 strains showed lethal activity against two kinds of the larvae, however lethality against mosquito larvae was low.

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

Bacillus thuringiensis is a spore-forming bacterium and uniquely characterized by the production of one or more proteinaceous parasporal crystals upon sporulation (1). The crystals kill certain insect larvae (1); therfore, the crystals and the microorganisms are important for the development of microbial insecticide (1). de Barjac and Bonnefoi (2) showed that strains of *B. thuringiensis* can be distinguished by serotypes based on their flagellar (H) antigens. Thereafter about 35 serotypes of *B. thuringiensis* were reported (1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19). Recently we isolated eight different strains of *B. thuringiensis* from soil in Korea, and then we undertook for their characterization.

This report describes the biochemical characteristics, microscopic observations, toxicity to insect larvae and antibiotic resistance patterns of the eight isolates of *B. thuringiensis*.

MATERIALS AND METHODS

1. Bacterial strains and media

Bacillus thuringiensis strains were isolated from soil and cultured at 28°C in UG medium (20). Muller-Hinton media was used for the reading of inhibition zones of antibiotics.

2. Isolation of *B. thuringiensis* from soil

Soils were sampled from various fields, planted with several different crops in virgin soil, in rocky soil, and in forest areas. In all cases, to minimize the defects of surface contamination, soil samples were taken first by removing the top soil (2 to 3 cm) from the sampling areas and then transferred 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 penicillin 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 at 37°C for 48 hrs. All colonies with the morphological characteristics similar to those of known *B. thuringiensis* 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. thuringiensis*. Isolates were subcultured onto UG agar and tested for further identification.

3. 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 preculture was transferred into 20 ml of UG media. Then it was cultured until sporulation by rotary agitation at 180 rpm at 28°C for 20 to 30 hrs. The

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fully mature-unlysed cells were harvested and washed twice with sterilized saline by centrifugation at 3000g for 20 min. For microscopic observation, the pellets were suspended in saline. Formation of spores and parasporal crystals was observed by using a phase-contrast microscope.

4. Biochemical characterization of *B. thuringiensis* isolates

B. thuringiensis isolates were cultured in nutrient broth at 28°C for 12 h, which were transferred into the several biochemical test broths and media by the procedures of Lennette *et al.* (21), and then the results were examined according to the procedures.

5. Antibiotic susceptibility test

B. thuringiensis isolates were cultured in nutrient broth at 28°C for 12 h and their antibiotic sensitivities were determined by a diffusion test of a standardized filter paper disc on Muller-Hinton agar (21).

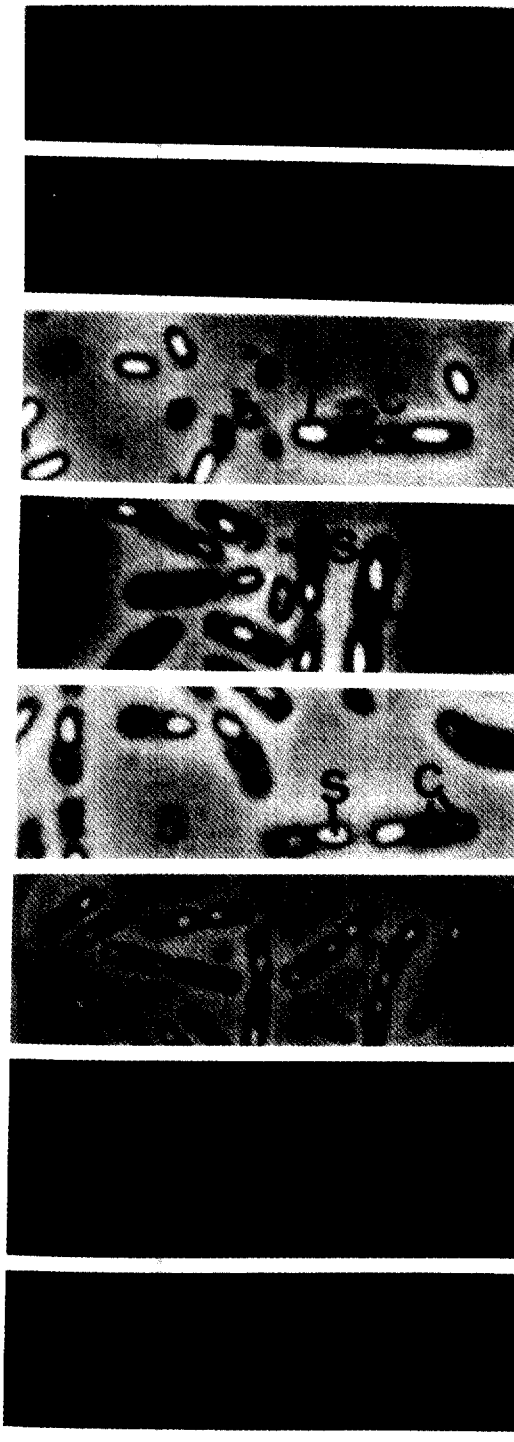
6. Bioassays

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 h. After pelleting the culture at 4000g for 20 min, the supernatants were decanted and the pellets were washed twice with sterilized saline by centrifugation at 4,000g 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 a disposable cup (72×80 mm) for bioassay of *Culex pipiens* 3rd instar larvae and a lump (2 cm^3) of semisolid food in a petri dish (2×20 cm) containing *Bombyx mori* 3rd instar. The mortality was recorded at 28°C for 48 h.

RESULTS AND DISCUSSION

1. Characteristics of *B. thuringiensis* isolates

Wide ranges of soil samples were examined, and then *B. thuringiensis* strains were isolated. Eight isolates containing parasporal inclusion bodies (crystal) were found (Figs. 1, 2, 3, 4, 5, 6, 7 and 8) and named HL-24, HL-25, HL-33, HL-34, HL-35, HL-38, HL-39 and HL-40. There is no significant difference in the shape and size of the vegetative cells of *B. thuringiensis* isolates to the known *B. thuringiensis* serotypes. The isolates were motile rods with dimensions of 1.3−1.4×3.7−4.1 μm and gram-positive. As shown in Figs. 1, 2, 3, 4, 5, 6, 7 and 8, the isolates showed the general features of *B. thuringiensis*. The crystal shapes in the isolates, HL-24 and 25, were irregular by a phase contrast microscope and cells contained two or more crystals (Figs. 1 and 2), but those of HL-33 and 35 were bipyramidal (Figs. 3 and 4). The crystal shapes of HL-34, 36, 38, 39



Figs. 1-8. *Photographs of B. thuringiensis isolates by a phase-contrast microscope.*
1, HL-24; 2, HL-25; 3, HL-33; 4, HL-34; 5, HL-35; 6, HL-38; 7, HL-39; 8, HL-40. C is crystal and S is spore.

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

Characteristics	Biochemical reactions of the strains							
	HL-24	25	33	34	35	38	39	40
Gram stain	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+
Kligler's iron agar	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
Voges-Proskauer reaction	−	−	−	−	−	−	−	−
Methyl-red reaction	+	+	+	+	+	+	+	+
Hemolysis	β	β	β	β	β	β	β	β
Productions of								
indole	−	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−	−
catalase	+	+	+	+	+	+	+	+
phenylalanine deaminase	−	−	+	+	+	−	−	−
lysine decarboxylase	−	−	+	+	+	−	−	−
arginine decarboxylase	−	−	+	+	+	+	+	+
ornithine decarboxylase	−	−	+	+	+	+	+	+
oxidase	−	−	+	+	+	+	+	+
lecithinase	+	−	+	+	+	N	N	N
urease	−	−	−	−	−	−	−	−
Acid from glucose	+	+	+	+	+	+	+	+
Utilizations of								
adonitol	−	−	−	−	−	−	−	−
arabinose	−	−	−	−	−	−	−	−
citrate	−	−	+	+	+	−	−	−
dulcitol	−	−	−	−	−	−	−	−
inositol	−	−	−	−	−	−	−	−
lactose	−	−	−	−	−	−	−	−
maltose	+	+	+	+	+	+	+	+
mannitol	−	−	−	−	−	−	−	−
raffinose	−	−	−	−	−	−	−	−
rhamnose	−	−	−	−	−	−	−	−
saccharose	−	−	−	−	−	−	−	−
salicine	−	−	+	+	+	−	−	−
sorbitol	−	−	−	−	−	−	−	−
sucrose	−	−	−	−	−	−	−	−
xylose	−	−	−	−	−	−	−	−

(+), positive reaction; (−), negative reaction; N, not done.

and 40 isolates were round and usually one crystal per cell was contained (Figs. 5, 6, 7 and 8). The crystal shapes of the other known strains are usually round or bipyramidal (1, 22, 23), but our findings were several different shapes as mentioned above accroding to the strains.

The eight isolates were examined on their biochemical characteristics as shown in Table 1. The eight isolates showed commonly negative reactions on the Voges-Proskauer reaction, the productions of H_2S and indole; and utilization of adonitol, arabinose, dulcitol, lactose, inositol, mannitol, sorbitol, raffinose, rhamnose, xylose, saccharose and sucrose. The isolates showed commonly positive reactions on motility, β-hemolysis and methyl-red reaction; utilization of glucose and maltose; production of catalase; and production of acid and alkali from glucose.

The minor different biochemical reactions appeared in the eight strains. The HL-33, HL-34 and HL-35 strains produced phenylalanine deaminase, lysine decarboxylase, arginine decarboxylase and ornithine decarboxylase. The HL-38, HL-39 and HL-40 strains produced arginine decarboxylase and ornithine decarboxylase. Six strains, HL-33, HL-34, HL-35, HL-38, HL-39 and HL-40 produced oxidase. HL-24, HL-33, HL-34 and HL-35 produced lecithinase. The HL-33, HL-34 and HL-35 strains utilized citrate and salicine. These results indicated that the eight isolates had general biochemical characteristics as the already known serotypes of *B. thuringiensis* (4, 24), but they were different only in several biochemical characteristics.

Table 2. *Resistance of B. thuringiensis isolates to antibiotics.*

Antibiotics	Antibiotic resistances of the strains							
	HL-24	25	33	34	35	38	39	40
amikacin(30 μg)	S	S	S	S	S	S	S	S
cephalothin(30 μg)	S	R	R	R	S	S	S	S
chloramphenicol(30 μg)	S	S	R	S	R	S	S	S
colistin(10 μg)	S	R	R	R	S	S	S	S
erythromycin(15 μg)	S	S	S	S	S	S	S	S
gentamycin(10 μg)	S	S	S	S	S	S	S	S
kanamycin(30 μg)	S	S	S	S	S	S	S	S
neomycin(39 μg)	S	S	S	S	S	S	S	S
penicillin G(10 units)	S	R	R	R	S	S	R	R
streptomycin(10 μg)	S	S	S	S	S	S	S	S
tetracycline(30 μg)	S	S	S	S	S	S	S	S

S, sensitive; R, resistant.

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

Isolates tested	No. of larvae tested	No. of the dead at 48 h	% of Motality
Control	20	0	0
HL-24	20	20	100
HL-25	20	17	85
HL-33	20	11	55
HL-34	20	14	70
HL-35,38,39,40	20	0	0

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

Isolates tested	No. of larvae tested	No. of the dead at 48 h	% of Motality
Control	20	0	0
HL-24	20	2	10
HL-25	20	4	20
HL-33, 34, 35	20	0	0
HL-38	20	20	100
HL-39	20	20	100
HL-40	20	18	90

Antibiotic resistance patterns were shown in Table 2. HL-25, HL-33 and HL-34 were resistant to cephalothin, colistin and penicillin G. HL-33 and HL-35 strains were resistant to penicillin G.

By the toxicity test against insect larvae, the HL-24, HL-25, HL-33 and HL-34 strains were toxic to *Bombyx mori* larvae (Table 3) and HL-24, HL-25, HL-38, HL-39 and HL-40 were toxic to *C. pipiens* larvae (Table 4). The HL-24, HL-25 were toxic to the two species of insect larvae tested (Table 3, 4). The HL-24 and 25 strains were highly toxic to the *B. mori* larvae, but not highly toxic to *C. pipiens* larvae. The HL-38 and 39 strains showed high toxicity against the mosquito larvae (Table 4). The four strains, HL-35, 38, 39 and 40 were not toxic to *B. mori* larvae, and HL-33, HL-34 and HL-35 strains not toxic to *C. pipiens* larvae.

Consequently the eight isolates were different in the biochemical characteristics, antibiotic resistances and toxicity against insect larvae when compared with the known serotypes, so they could be classified as *B. thuringiensis* new strains.

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초 록: 토양에서 분리한 살충성 *Bacillus thuringiensis*의 모기와 누에 유충에 대한 독성효과 (II)
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*Bacillus thuringiensis*를 한국의 토양에서 분리하여 특성을 연구했다. 8균주를 분리하여 HL-24, HL-25, HL-33, HL-34, HL-35, HL-38, HL-39와 HL-40으로 명명했으며, HL-24와 HL-25는 부정형의 crystals를 가졌고, HL-33과 HL-35는 이중피라미드형, HL-34, HL-38, HL-39와 HL-40은 둥근형의 crystal를 가진 것을 위상차현미경으로 관찰했다. 분리균들의 생화학적인 특성은 이미 알려진 균주들과 유사했으나, 특이한 차이점을 가지고 있었다. HL-25, HL-33과 HL-34는 cephalothin, colistin과 penicillin G에 내성을, HL-33과 HL-35균주는 chloramphenicol에 내성을, HL-39와 HL-40은 penicillin G에 내성을 나타냈다. 균주HL-24, HL-25, HL-33과 HL-34는 누에 유충에 대한 치사성은 100, 85, 55, 및 70%를 각각 나타냈으며, HL-24, HL-25, HL-38, HL-39와 HL-40의 모기 유충에 대한 치사성은 10, 20, 100, 100 및 90%였다. HL-24와 HL-25균주는 누에와 모기 유충에 모두 치사성을 나타냈으나, 모기에는 살충효과가 10~20%로 매우 낮았다.