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Characterization of *Bacillus thuringiensis* Isolates from Soil in Wonju Area

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Three strains (KW-1, KW-14, KW-15) of *Bacillus thuringiensis* were isolated from soil in Wonju area and characterized. The three strains produced parasporal inclusion bodies (crystals) and spores in their cells. The KW-1 strain produces spherical crystals. The crystals of strain KW-14 are bipyramidal crystal. The KW-15 strain harbors irregular crystals. Only minor biochemical characteristics of the three isolates were different and distinctive, however general characteristics were similar to the known serotypes of *B. thuringiensis*. Three strains were resistant to penicilin G, oxacillin and cephalothin. Three strains were highly toxic to *Bombyx mori* larvae, but not to the *Culex pipiens* larvae.

Key words: ***B. thuringiensis*, endotoxin, *Culex pipiens*, *Bombyx mori***

Bacillus thuringiensis produces entomocidal endotoxin crystals during its sporulating cycle (1). The crystals have a speicific lethality to certain insect larvae (1); therefore, the crystals and the bacteria are being developed as an important microbial insecticide (1). De Barjac and Bonnefoi(4) showed that subspecies of *B. thuringiensis* can be distinguished by serotypes or their flagellar (H) antigens. Thereafter about 50 serotypes of *B. thuringiensis* were found (1-6, 9-12, 15, 17, 18, 25-30). 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, 4, 14, 16-22, 24). Recently we isolated three strains of *B. thuringiensis* from soil in Wonju area, and we investigated their biochemical, microscopic characterizations, antibiotic resistant patterns and toxicity against insect larvae.

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℃ 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 g of polymyxin B sulfate and 4 μg of penicilin G per ml (Sigma) were added aseptically to the molten agar (45℃) before the plates were poured. The nutrient agar containing polymyxin and penicillin G was incubated for 48 h at 37℃. All colonies with growth characteristics simillar 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.

Confirmation of crystal formation

B. thuringiensis isolates were precultured in 20 ml of nutrient broth at 28℃ 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 sporu-

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lation at 28℃ by rotary agitation at 180 rpm for 20 to 30 h. 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 with a phase-contrast microscope and a transmission electron microscope (Hitachi H-5000) at an accelerating voltage of 75 kV(19).

Biochemical characterization of *B. thuringiensis* isolate

Biochemical characteristics of the isolates were examined by the procedures of Lennette *et al.* (23).

Antibiotic susceptibility test

The antibiotic sensitivity of *B. thuringiensis* was measured with a standardized filter paper disc(BBL Co) method (23).

Bioassay

One or two loops of pure-cultured isolates were inoculated in 10 ml of fresh nutrient broth and then cultured at 28℃ at 180 rpm overnight. 2.5 ml of the culture were transferred into 50 ml of UG medium and cultured again for 48 to 72 h. After pelleting the culture at 4000×g for 20 min, the supernatants were discared and the pellets were washed twice with sterilized saline by centrifugation at 4,000×g for 20 min. The pellets were suspended with 5 ml of saline. Then, 1.0 ml of the suspended spore-crystal complex (about 10^7 to 10^8 spores/ml) were added to 150 ml 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℃ and 48 h.

Results and Discussion

Characteristics of *Bacillus thuringiensis* isolates

B. thuringiensis strains were isolated in the wide ranges of soil samples. Three isolates containing parasporal inclusion bodies (crystals) were found (Fig. 1) and named KW-1, KW-14 and KW-15. There is no significant differences in the shape and size of the vegitative cells between the *B. thuringiensis* isolates and the knwon *B. thuringiensis* serotypes. The KW-15 strain was motile, however the KW-1 and KW-14 strains were immotile. The three strains were rods with dimensions of 1.3~1.4×3.7~4.1 μm and gram-positive. The formation of crystals was confirmed with electron and phase contrast microscopy. As shown in Fig. 1 the isolates showed the general features of *B. thuringiensis*. The crystal shape in the KW-1 strain was spherical (Fig. 1A(E and L)). The crystal shape of the KW-14 was bypiramidal (Fig. 1B(E and L). The crystal shape of the KW-15 was irregular or

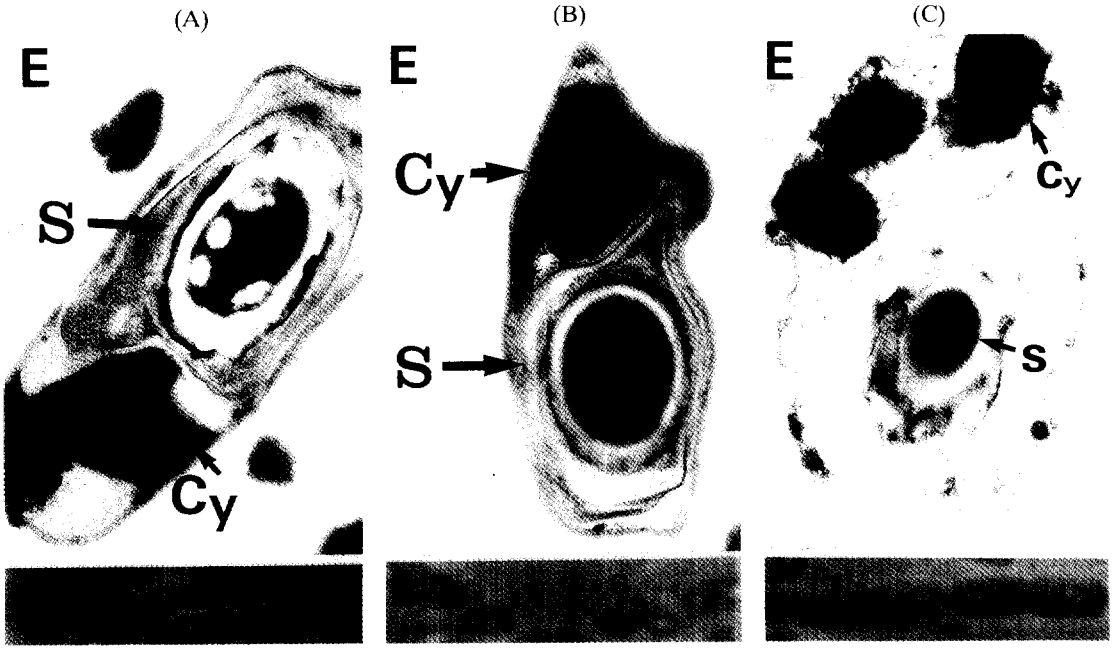

Fig. 1. Photographs of *B. thuringiensis* isolates. The shape of crystals and cells of the kW-1 strain(A) kW-14 strain(B) and kW-15 strain (C) was illustrated in both transmission electron(E) and lgiht(L) microscopy. Arrowheads in Cy represents crystals and S indicates refractile spores in cells. Magnificantions: ×15,000(E) and ×4,000(L).

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

Characteristics	Biochemical reactions of the isolates		
	KW1	KW2	KW15
Gram stain	+	+	+
Motility	-	-	+
Methyl-red reastion	+	+	+
Productions of			
indole	-	-	-
H_2S	-	-	-
hemolysin	β	β	β
catalase	+	+	+
phenylalanine deaminase	-	-	-
lysine decarboxylase	-	-	-
arginine decarboxylase	+	+	-
ornithine decarboxylase	-	-	-
oxidase	+	+	+
urease	+	+	+
Acid from glucose	+	+	+
Utilizations of			
arabinose	-	-	-
citrate	-	-	-
dulcitol	-	-	-
lactose	-	-	-
maltose	+	+	+
manitol	-	-	-
rhamnose	-	-	-
salicine	+	+	+
sorbitiol	-	-	-
sucrose	-	-	+
xylose	-	-	-

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

spherical (Fig. 1C(E and L). Generally the irregular shape of the crsytals was distinctive, however the round or bipyramidal shape is similar to the known serotypes of *B. thuringiensis* (1, 13, 14, 17, 19-22, 24, 25).

Biochemical characteristics of the three strains were examined(Table 1). They did not commonly produced H 2S, indole, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, gas from glucose and formation of pellicle; and did not commonly utilized arabinose, citrate, cellobiose, dulcitol, lactose, manitol, rhamnose, sorbitol and xylose.The three strains showed commonly positive on methyl red reaction, produced catalase, oxidase, urase and acid from glucose, β-hemolysin and utilized adonitol, glucose, maltose, raffinose, and salicin.

The KW-1 and KW-14 strains were positive in the nitrate reduction and arginine decarboxylase production, and utilization of esculin, but the KW-15 was not.

The KW-1 and KW-14 strains did not produced DNase and did not utilized sucrose, but the KW-15 strain did. The isolates had general biochemical characteristics as the known serotypes of *B. thuringiensis* (3,16).

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

Antibiotics	Antibiotic resistance of the isolates		
	KW1	KW14	KW15
Vancomycin	S	S	S
Penicilin G	R	R	R
Clindermycin	S	S	S
Erythromycin	S	S	S
Oxacillin	R	R	R
Tetracycline	I	S	S
Cephalothin	R	R	R
Chloramphenicol	S	S	S
Cotrimoxazole	S	S	S
Gentamicin	S	S	S
Ciplofloxacin	S	S	S
Teicoplanin	S	S	S

S; sensitive reaction, R; resistant reaction and I; intermediate reactions.

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

Isolates	No. of dead at 48 h	Mortality(%)
Control(BTK)	30	100
KW-1	30	100
KW-14	30	100
KW-15	16	58

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

Isolates	No. of dead at 48 h	Mortality(%)
Control(BT1)	20	100
KW-1, -14, -15	0	0

The antibiotic resistant patterns of the three isolates are in Table 2. The strains of KW-1, KW-14 and KW-15 were resistant to penicilin G, oxacillin and cephalothin, but against the other antibiotics they were susceptible.

The toxicities of the three isolates(KW-1, KW-14 and KW-15) against *Bombyx mori* and *Culex pipiens* larvae were examined (Table 3 and 4). The three strains were toxic to *B. mori* larvae (Table 3), however the strains of KW-1 and KW-14 were strongly toxic to *B. mori* larvae (Table 3), but not to mosquito larvae(Table 4).

Bacillus thuringiensis strains were differentiated and classified by H antigen of the cells(2,5,7). Therefore the strains should be further studied by the H antgen.

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