Kor. J. Appl. Microbiol. Biotechnol.
Vol. 20, No. 4, 377-383 (1992)

Characterization of *Bacillus thuringiensis* Seven Isolates from Soil

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토양에서 분리한 *Bacillus thuringiensis* 7균주의 특성

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Abstract — Seven strains of *Bacillus thuringiensis* were isolated from soil in Korea and characterized. The isolates were named HL-8, 10, 12, 13, 14, 15 and 16 which produced parasporal crystals and endospores in their cells. The biochemical characteristics of the seven isolates were only minor different in specific chracteristics to the known serotypes of *Bacillus thuringiensis*. The number of the plasmid DNA elements from the isolates were studied. The computerized molecular weights of the six plasmid elements in the HL-8 and HL-10 strains were from 3.01 to 15.1 Md, four plasmid elements in the HL-12 were from 5.4 to 21.9 Md, four plasmid elements in HL-13 were from 5.1 to 20 Md, three plasmid elements in HL-15 were from 3.4 to 11.3 Md and three plasmid elements in the HL-16 were from 2.4 to 20.1 Md. The seven isolates showed resistances to ampicillin, bacitracin, cephalothin, methicillin and penicillin G. The strains of HL-8, HL-10, HL-12, HL-14, HL-15 and HL-16 showed lethalities against *Culex pipiens* 3rd instar larvae. The HL-8 and 14 strains showed 100% lethality to the larvae within 48 hours. HL-13 strain did not have toxicity against the larvae.

A rod shaped and spore-forming bacterium, *Bacillus thuringiensis* is uniquely characterized by the production of one or more proteinaceous parasporal crystals during its sporulating cycle (1). The crystals kill certain insect larvae (1); therfore, the crystals and the microorganisms are important for the development of microbial insecticidal pesticides (1). de Barjac and Bonnefoi (2) showed that strains of *B. thuringiensis* can be distinguished by serotypes based on their flagella (H) antigens. Thereafter about 35 serotypes of *B. thuringiensis* were reported (1, 3-19). Recently we isolated seven strains of *B. thuringiensis* from soil in Korea, then we undertook their characterization.

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

Key word: *B. thuringiensis*
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Materials and Methods

Bacterial strains and media

Bacillus thuringiensis strains were isolated from soil and cultured at 28℃ in UG medium (20). LB and Muller-Hinton media were used for plasmid isolation and the reading of inhibition zones of antibiotics, repectively.

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 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 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 m*l* (Sigma) were added aseptically to the molten agar (45℃) before the plates were poured. The nutrient agar containing polymyxin and penicillin was incubated for 48 hrs at 37℃. 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.

Reconfirmation of crystal formation

B. thuringiensis isolates were precultured in 20 m*l* of nutrient broth at 28℃ by rotary agitation at 180 rpm overnight, and 1.0 m*l* of the preculture was transferred into 20 m*l* of UG media. Then it was cultured until sporulation at 28℃ 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 suspended in saline. Formation of spores and parasporal crystals was 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.* (21).

Isolation of plasmid DNA

Plasmid DNAs in *B. thuringiensis* were isolated by the procedure of Lee *et al.* (22). The relative molecular weights of plasmids were computerized.

Antibiotic susceptibility test

Antibiotic sensitivity of *B. thuringiensis* was determined by a diffusion test of a standardized filter paper disc on Muller-Hinton agar (21).

Bioassays

One or two loops of pure-cultured isolates were inoculated in 10 m*l* of fresh nutrient broth and then cultured at 28℃ 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 4000×g for 20 min, the supernatants were discanted and the pellets were washed twice with sterillized 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 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 larvae. The motality was recorded at 28℃ for 48 h.

Results and Discussion

Identification of *B. thuringiensis* isolates

Wide ranges of soil samples were examined, and then *B. thuringiensis* strains were isolated. Seven isolates containing parasporal inclusion bodies (crystal) were found (Fig. 1) and named HL-8, HL-10, HL-12, HL-13, HL-14, HL-15 and HL-16. 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, HL-8, 10, 12, 13 and 15 were motile rods with dimensions of 1.3~1.4×3.7~4.1 μm and gram-positive, however the HL-14 and 16 were not motile. As shown in Fig. 1, the isolates showed the general features of *B. thuringiensis*. The crystal shape in the isolates, HL-8, 10 and 12 was spherical by a phase contrast microscope (Fig. 1.(1)~(3)), but those of HL-13, 14 and 15 were ovoidal (Fig. 1.(4)~(6)). The crystal shape of HL-16 isolate was rectangular and cells contained one cyrstal per cell (Fig. 1.(7)). The shapes of the other known strains are usually round or bipyramidal (1, 23, 24), but our findings were several different shapes accroding to the strains.

The seven isolates were examined on their biochemical characteristics as shown in Table 1. The seven isolates showed commonly negative reactions on the Voges-Proskauer reaction, the production of H_2S, indole, lysine decarboxylase, phenylalanine

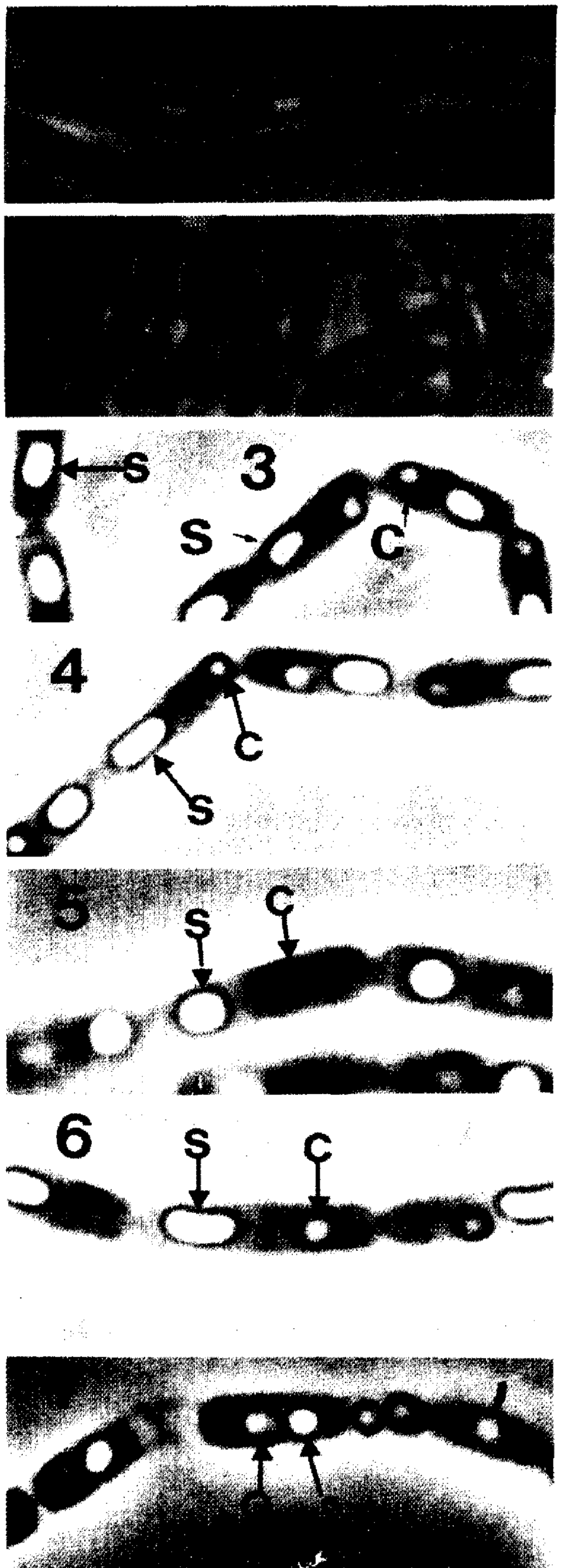

Fig. 1. Photograph of *B. thuringiensis* HL-8 isolate (1), *B. thuringiensis* HL-10 (2), *B. thuringiensis* HL-12 (3), *B. thuringiensis* HL-13 (4), *B. thuringiensis* HL-14, (5) *B. thuringiensis* HL-15 (6) and *B. thuringiensis* HL-16 (7).

deaminase, oxidase, ornithine decarboxylase and β-galactosidase, and utilization of adonitiol, arabinose, dulcitol, lactose, inositol, mannitol, sorbitol, raffinose, rhamnose, xylose and sucrose. The isolates showed commonly positive reactions on nitrate reduction and methyl-red reaction, utilization of glucose, maltose and gelatin, production of catalase, and production of acid and alkali from glucose.

The minor different biochemical reactions appeared in the seven strains. The HL-8, 10, 12, 13 and 15 strains were motile, but HL-14 and 16 strains were not motile. HL-8 and 10 strains showed β-hemolysis. HL-8, 10, 12, 13 and 15 strains only produced arginine decarboxylase. HL-14 and 15 strains only produced lecithinase. The HL-8 strain utilized citrate. HL-8, 10, 14, 15 and 16 strains utilized salicine. These results indicate that the seven isolates had general biochemical characteristics as the already known serotypes of *B. thuringiensis* (4, 25). The HL-8 and HL-10 are similar in the biochemical characteristics, which were different in only one characteristic, but the flagellar antigenicity might be different.

The seven strains were commonly resistant to ampicillin, bacitracin, cephalothin, methicillin and penicillin G, and sensitive to amikacin, clindamycin, erythromycin, gentamycin, streptomycin, tetracycline and tobramycin (Table 2).

Plasmid DNA elements in the seven strains of *B. thuringiensis* were isolated (Fig. 2 and 3). The HL-8 and 10 had commonly 6 different plasmid DNA elements with ranges from 3.01 to 15.1 megadaltons, the HL-12 had 4 with ranges from 5.4 to 21.9 Md, HL-13 had 4 with ranges from 5.1 to 20 Md, HL-14 strain had 3 with ranges from 2.4 to 13.5 Md, HL-15 contained 3 with ranges from 3.4 to 11.3 Md and the HL-16 had 3 with ranges from 2.4 to 20.1 Md (Table 3). The plasmid patterns of the new isolates were distinguishable to those of the known serotypes in sizes and numbers. The HL-8 and HL-10 strains had similar plasmid patterns, but the flagellar antigenicity of the two strains might be different. Plasmid patterns of *B. thuringiensis* strains may be different according to the strains and also other reports (26) were similar to our findings.

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

Biochemical Characteristics	*B. thuringiensis* isolates						
	HL-8	10	12	13	14	15	16
Gram stain	+	+	+	+	+	+	+
Motility	+	+	+	+	−	+	−
Kligler's iron agar	K/A	K/A	K/A	K/A	K/A	K/A	K/A
Voges-Proskauer reaction	−	−	−	−	−	−	−
Methyl-red reaction	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+
Hemolysis	β	β	−	−	−	−	−
Productions of							
indole	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−
β-galactosidase	−	−	−	−	−	−	−
catalase	+	+	+	+	+	+	+
phenylalanine deaminase	−	−	−	−	−	−	−
lysine decarboxylase	−	−	−	−	−	−	−
arginine decarboxylase	+	+	+	+	−	+	−
ornithine decarboxylase	−	−	−	−	−	−	−
oxidase	−	−	−	−	−	−	−
lecithinase	−	−	−	−	+	+	−
urease	+	+	−	−	−	−	−
Acid from glucose	+	+	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+	+
Utilization of							
adonitol	−	−	−	−	−	−	−
arabinose	−	−	−	−	−	−	−
citrate	+	−	−	−	−	−	−
dulcitol	−	−	−	−	−	−	−
inositol	−	−	−	−	−	−	−
lactose	−	−	−	−	−	−	−
maltose	+	+	+	+	+	+	+
mannitol	−	−	−	−	−	−	−
raffinose	−	−	−	−	−	−	−
rhamnose	−	−	−	−	−	−	−
salicine	+	+	−	−	+	+	+
sorbitol	−	−	−	−	−	−	−
sucrose	−	−	−	−	−	−	−
xylose	−	−	−	−	−	−	−

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

By the toxicity test against insect larvae the HL-8, 10, 12, 14, 15 and 16 strains were toxic to *C. pipiens* larvae, however the HL-13 strain showed no toxicity against the mosquito larvae (Table 4). The seven strains were not toxic to *B. mori* larvae.

Consequently the seven isolates had minor different biochemical characteristics and antibiotic resistances when compared to the known serotypes, so they were classified as *B. thuringiensis* new strains. However flagellar antigenicity of the strains

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

Antibiotics	Antibiotics resistance of isolates						
	HL-8	10	12	13	14	15	16
Amikacin (30 μg)	S	S	S	S	S	S	S
Ampicillin (10 μg)	R	R	R	R	R	R	R
Bacitracin (10 units)	R	R	R	R	R	R	R
Cephalothin (30 μg)	R	R	R	R	R	R	R
Clindamycin (2 μg)	S	S	S	S	S	S	S
Erythromycin (15 μg)	S	S	I	I	S	S	S
Gentamycin (10 μg)	S	S	S	S	S	S	S
Kanamycin (30 μg)	S	S	S	S	S	S	S
Methicillin (5 μg)	R	R	R	R	R	R	R
Penicillin G (10 units)	R	R	R	R	R	R	R
Streptomycin (10 μg)	S	S	S	S	S	S	S
Tetracycline (30 μg)	S	S	S	S	S	S	S
Tobramycin (10 μg)	S	S	S	S	S	S	S

S: sensitive, R: resistant, I: intermediate reactions.

Table 3. Relative molecular weights of plasmid DNA elements in new *B. thuringiensis* isolates

Isolates	Relative molecular weights ($\times 10^6$) of plasmid element DNAs
HL-8	15.1, 10.4, 6.45, 5.75, 3.98, 3.01
HL-10	15.1, 10.4, 6.45, 5.75, 3.98, 3.01
HL-12	21.9, 10.5, 6.6, 5.4
HL-13	20, 10.5, 6.1, 5.1
HL-14	13.5, 4.0, 2.4
HL-15	11.3, 8.5, 3.4
HL-16	20.1, 3.9, 2.4

Table 4. Toxicity bioassay of *B. thuringiensis* isolates against 3rd instar larvae of *Culex pipiens*

Isolates tested	No. of larvae tested	No. of the dead for 48h	Ratio of lethality(%)
Control	20	0	0
HL-8	20	20	100
HL-10	20	14	70
HL-12	20	4	20
HL-13	20	0	0
HL-14	20	20	100
HL-15	20	2	10
HL-16	20	6	30

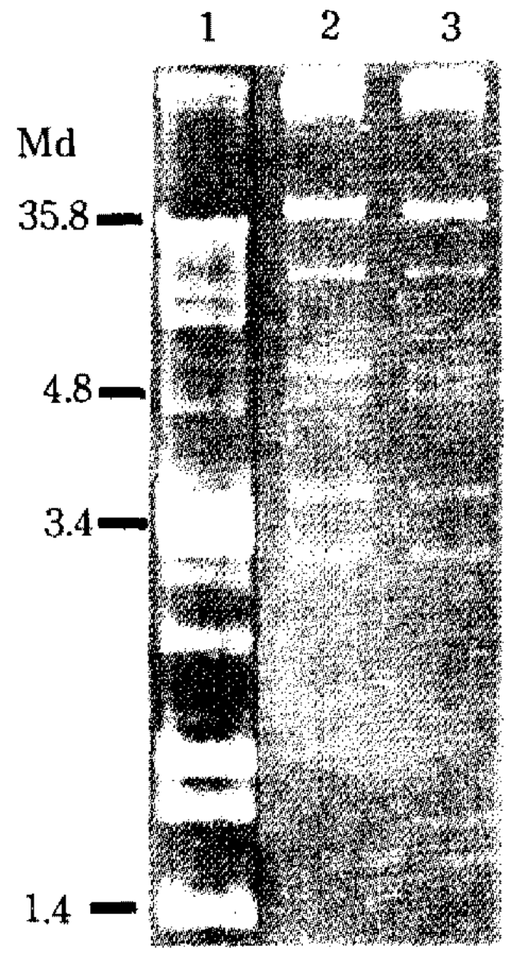

Fig. 2. Plasmid DNA element patterns of the new *B. thuringiensis* isolates on 0.7% agarose gel.
Lanes: 1, standard molecular weight plasmids in *E. coli* V517D; 2, plasmid DNA bands of *B. thuringiensis* isolate, HL-8; 3, those of the HL-10. The right side numericals are molecular weights of DNA which are in megadalton.

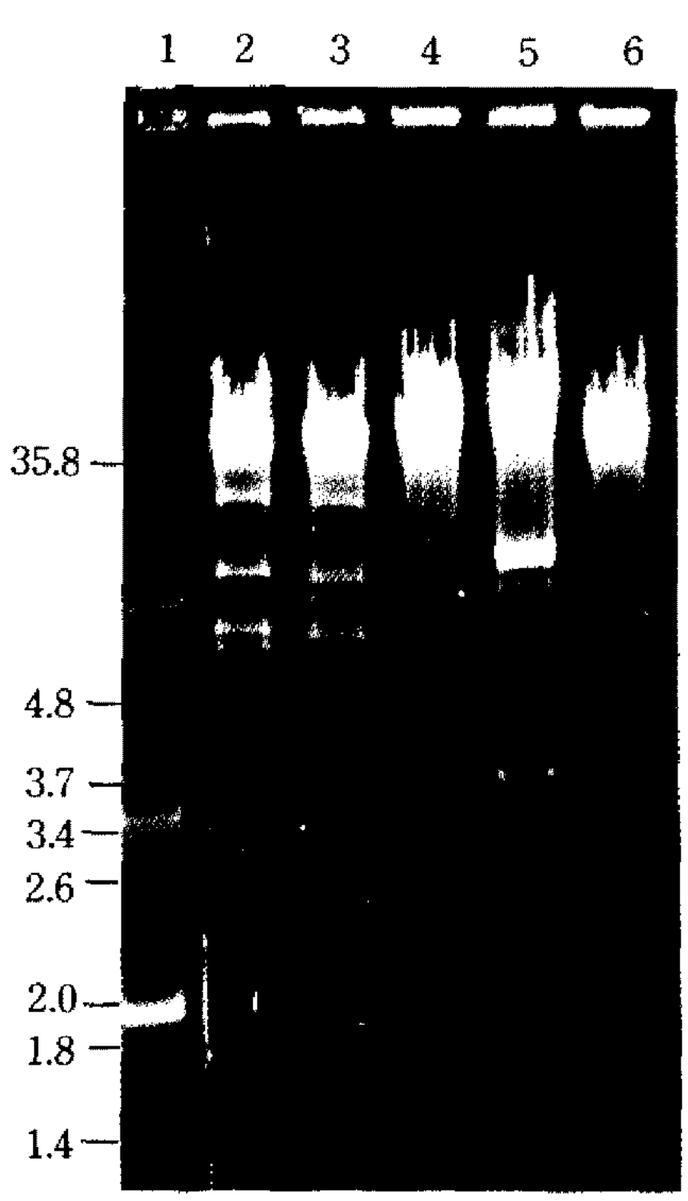

Fig. 3. Plasmid DNA element patterns of the new *B. thuringiensis* isolates on 0.7% agarose gel.
Lanes: 1, standard molecular weight plasmids in *E. coli* V517D; 2, plasmid DNA bands of *B. thuringiensis* isolate, HL-12; 3, those of the HL-13; 4, those of the HL-14; 5, those of the HL-15 and 6, those of the HL-16. The right side numericals are molecular weights of DNA which are in megadalton.

should be studied further to classify the strains in serotypes.

요 약

Bacillus thuringiensis 7균주를 한국의 토양에서 분리하여 특성을 연구했다. 상기의 7균주를 HL-8, 10, 12, 13, 14, 15와 16으로 명명했으며, 이들 균주들은 세포내에 다양한 형태의 parasproal endotoxin crystal과 내생포자를 형성한 것을 위상차현미경으로 관찰했다. 분리균들의 생화학적인 특성은 이미 알려진 균주들과 유사했으나, 특이한 차이점을 가지고 있었다. 각 분리균이 가지고 있는 플라스미드를 분리하여 분자량과 수를 조사한 결과, HL-8과 HL-10은 6종류의 플라스미드를 가지고 있었으며, 분자량의 범위는 3.01~15.1 Md이었고, HL-12균주는 4종류의 플라스미드를 가지고 있었으며, 분자량의 범위는 5.4~21.9 Md이었고, HL-13균주도 4종류의 플라스미드를 가지고 있었으나 분자량은 차이가 있었다. HL-14균주는 3종류의 플라스미드를 가지고 있었으며, 분자량의 범위는 2.4~13.5 Md이었고, HL-15균주도 3종류의 플라스미드를 가지고 있었으며, 분자량의 범위는 3.4~11.4 Md이었고, HL-16도 3종류의 플라스미드를 가지고 있었으며, 분자량의 범위는 2.4~20.1 Md이었다. 상기 분리균는 공히 ampicillin, bacitracin, cephalothin, methicillin과 penicillin G에 저항성을 나타냈다. HL-8과 15는 48시간내에 *Culex pipiens* 모기 3령 유충에 100%의 살충성을 나타냈고, 다른 균주는 이보다 낮았다. 특히 HL-13은 살충성이 없었다.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (87-0511).

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(Received June 18, 1992)