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Characteristics of Six New *Bacillus thuringiensis* Serovarieties: *B. thuringiensis* serovar. *coreanensis*, *leesis*, *konkukian*, *seoulensis*, *sooncheon*, and *yosoo*

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Abstract Six new serovarieties of *B. thuringiensis* carrying specific H-antigen have minor differences in biochemical characteristics and morphological characteristics of crystals, which are commonly resistant against four antibiotics. The *B. thuringiensis* serovar. *coreanensis* is nontoxic to silkworm larvae, but it is moderately toxic against the *Culex pipiens* larvae. The *B. thuringiensis* serovar. *konkukian* and *leesis* are nontoxic against mosquitos larvae, but are toxic against silkworm larvae. The *B. thuringiensis* serovar. *seoulensis*, *sooncheon*, and *yosoo* are highly toxic to *B. mori* larvae and moderately toxic to *C. pipiens* larvae. The six serovarieties harbor different plasmid DNA patterns. A 102-kDa protein is a major crystal protein in the four serovarieties and a 86-kDa protein is in one serovariety.

Key words: *B. thuringiensis* serovar. *coreanensis*, *konkukian*, *leesis*, *seoulensis*, *sooncheon*, and *yosoo*, endotoxin

Bacillus thuringiensis produces one or more proteinaceous parasporal crystals during its sporulating cycle, which kills certain insect larvae [10]. The crystals and microorganisms are important for the development of microbial insecticidal pesticides [10]. De Barjac and Bonnefoi [2, 3, 4] showed that strains of *B. thuringiensis* could be distinguished by serotypes based on their flagellar (H) antigens. Subsequently, more than 73 serotypes of *B. thuringiensis* were reported [1, 5, 6, 7, 8, 16, 18, 21, 22, 29]. Previously, we isolated and determined six serotypes of *B. thuringiensis* based on their flagellar antigens and named them as *B. thuringiensis* serovar. *coreanensis* (H25), *B. thuringiensis* serovar. *seoulensis* (H35), *B. thuringiensis* serovar. *konkukian* (H34), *B. thuringiensis* serovar. *leesis* (H33), *B. thuringiensis* serovar. *sooncheon* (H41), and *B. thuringiensis* serovar. *yosoo* (H18a18c) [18, 21, 22]. We are interested in comparing the properties of the six serovarieties. This report describes the biochemical characteristics, scanning microscopic observations, antibiotic resistance patterns, parasporal crystal proteins, plasmid patterns, and toxicity of the six subspecies of *B. thuringiensis* isolated from Korean soil.

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Materials and Methods

Bacterial Strains and Media

The six new serotypes of *Bacillus thuringiensis*: *B. thuringiensis* serovar. *coreanensis* (H25); *B. thuringiensis* serovar. *seoulensis* (H35); *B. thuringiensis* serovar. *konkukian* (H34); *B. thuringiensis* serovar. *leesis* (H33); *B. thuringiensis* serovar. *sooncheon* (H41); and *B. thuringiensis* serovar. *yosoo* (H18a18c) [18, 21, 22], were stored in this laboratory and used in this study. Bacterial cells for parasporal proteinaceous crystal analysis were cultured at 28°C in a basal medium (U. G. medium) [2]. Mueller-Hinton medium (Difco, Detroit, MI, U.S.A.) was used for the measurement of inhibition zones of antibiotics.

Insect Larvae

Bomyx mori larvae were obtained from Dr. S. P. Lee, National Institute of Agricultural Science and Technology, Suwon, Korea. *Culex pipiens* larvae were obtained from the National Institute of Health, Seoul, Korea.

Preparation of Crystals

B. thuringiensis serovarieties were precultured in 20 ml of LB broth (Difco) at 28°C by rotary agitation at 180 rpm overnight, and 1.0 ml of the preculture was transferred into

20 ml of U. G. media [2]. Then it was cultured at 28°C by rotary agitation at 180 rpm for 6 days until sporulation and lysis. Formation of spores and parasporal crystals were observed in a phase contrast microscope. The fully matured crystals and lysed cells were harvested and washed twice with sterilized H_2O by centrifugation at 12,000 ×*g* for 10 min. The crystal and spore mixtures were incubated in 0.01% Triton X-100 and 1.0 M of NaCl solution at 4°C overnight. Parasporal crystals of *B. thuringiensis* were separated from spores and cellular debris by a 30 to 70% NaBr gradient centrifugation at 17,000 ×*g* for 120 min [25]. After centrifugation, the crystal layer (upper layer) was harvested with pasteur pipette, dissolved in H_2O, and washed twice by centrifugation. Then the crystal pellets in H_2O were stored at - 20°C.

Scanning Electron Microscropy

Crystal suspensions of *B. thuringiensis* serovarieties were prefixed in 2.5% glutaraldehyde in 0.1 M of phosphate buffered saline (PBS, pH 7.3) for 20 min at 4°C and fixed with 1.33% osmium tetroxide in the same buffer for 10 min at 4°C. They were dehydrated with 50% to 100% ethanol. Samples were air dried on a collodion grid, coated with gold in an ion sputter (Hitachi Co., Tokyo, Japan) and then examined in a scanning electron microscope (Hitachi Co., Tokyo, Japan) at 20 kV.

Biochemical Characterization and Antibiotic Susceptibility

Biochemical characteristics and the antibiotic susceptibility of the serovarieties of *B. thuringiensis* were examined by following the procedures of Lennette *et al.* [27]. Antibiotic sensitivity of *B. thuringiensis* was determined by the diffusion test with a standardized filter paper disc on Mueller-Hinton agar (Difco) [27].

Bioassays

Bioassay for *Culex pipiens* or *Bombyx mori* larvae was carried out with the procedures described by de Barjac [1, 2], and Lee *et al.* [13, 14, 15, 23] with slight modifications. One or two loopfuls of pure-cultured cells were inoculated in 10 ml of fresh nutrient broth and then cultured at 28°C at 180 rpm overnight. A 2.5 ml of the culture was transferred into 50 ml of U. G. medium and cultured again for 48°C to 72 h. After pelleting the culture at 4,000 ×*g* for 20 min, the supernatant was discarded and the pellet was washed twice with sterilized saline. The pellet was suspended in 5 ml of saline. Then, 1.0 ml (10^8 spores/ml) of the suspended spore-crystal complex was added to 150 ml of distilled water in a disposable cup (72×80 mm) for bioassay by using either *C. pipiens* 3rd instar larvae or a lump (2×2×1.5 cm^3) of semisolid food in a petridish (2×20 cm) containing *B. mori* 3rd instar larvae. The mortality rate was checked after 48 h at 28°C.

Purification of Plasmids

Plasmids in *B. thuringiensis* cells were isolated and purified according to the procedure described by Lee *et al.* [25] and Hwang *et al.* [13].

Crystal Solubilization and SDS-PAGE Analysis

Parasporal crystals purified by NaBr gradient centrifugation [24] were solubilized in alkaline solution [30], and analyzed by 10% PAGE as described by Bollag *et al.* [9].

Results and Discussion

Morphology of Six *B. thuringiensis* Serovarieties

Six new serovarieties of *B. thuringiensis* isolated from Korean soil samples [18, 21, 22] were characterized for different spectra of biological activities and toxicity. The six new serovarieties having specific H-antigen [18] are as

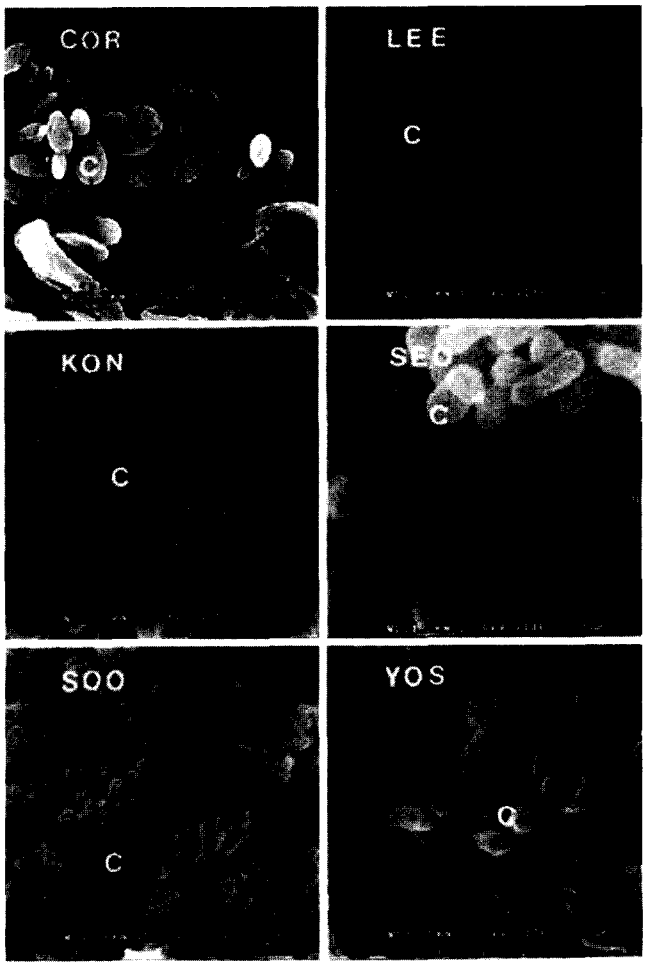

Fig. 1. Crystals of six *B. thuringiensis* (*Bt*) serovarieties.
The cells were grown for 6 days at 28°C and observed in SEM. Abbreviations: COR, *Bt.* ser. *coreanensis*; LEE, *Bt.* ser. *leesis*; KON, *Bt.* ser. *konkukian*; SEO, *Bt.* ser. *seoulensis*; SOO, *Bt.* ser. *sooncheon*, and YOS, *Bt.* ser. *yosoo*. C indicates crystals.

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

Characteristics examined	Biochemical reactions of the following strains						
	COR	LEE	KON	SEO	SOO	YOS	BTI
Gram stain/motility/methyl red/gelatin hydrolysis /β-hemolysis	+	+	+	+	+	+	+
VP reaction	-	+	+	+	+	+	+
Nitrate reduction	+	+	-	+	+	+	-
Productions of							
- indole/H_2S/phe deaminase/	-	-	-	-	-	-	-
β-galactosidase	-	-	-	-	-	-	-
- arginine dehydrolase	+	-	-	+	-	+	-
- lecithinase	+	-	+	+	+	+	+
- lys decarboxylase	-	+	-	-	-	-	-
- arg decarboxylase	-	-	-	-	+	+	+
- ornithine decarboxylase	-	-	-	-	-	-	+
- catalase	+	+	+	+	+	+	-
- oxidase	-	+	+	+	+	+	-
- urease		-	+	-	-	+	-
- gas from glucose	-	-	-	-	-	-	+
Utilizations of							
- adonitol/arabinose/erythritol/gentiobiose/inositol/lactose/manitol/mannose/raffinose/rhamnose/sorbitol/sorbose/xylose/ xylitol/	-	-	-	-	-	-	-
- cellobiose	-	-	-	+	+	+	+
- citrate	-	-	-	+	-	-	-
- dulcitol	-	-	-	-	-	-	+
- esculin/maltose	+	+	+	+	+	+	+
- ribose	+	+	+	+	+	+	+
- salicin	+	+	+	-	+	+	-
- sucrose	+	-	-	-	+	-	-

COR, *B. thuringiensis* (*Bt*) ser. *coreanensis*; LEE, *Bt.* ser. *leesis*; KON, *Bt.* ser. *konkukian*; SEO, *Bt.* ser. *seoulensis*; SOO, *Bt.* ser. *sooncheon*; YOS, *Bt.* ser. *yosoo*. BTI, *B. thuringiensis* serovar. *israelensis*.
(+), positive reaction; (-), negative reaction.

follows; *B. thuringiensis* serovar. *coreanensis* (COR); *B. thuringiensis* serovar. *seoulensis* (SEO); *B. thuringiensis* serovar. *konkukian* (KON); *B. thuringiensis* serovar. *leesis* (LEE); *B. thuringiensis* serovar. *sooncheon* (SOO); and *B. thuringiensis* serovar. *yosoo* (YOS). There were no significant differences in shapes and sizes of vegetative cells (1.3-1.4 μm×3.7-4.1 μm) between the six serovarieties and the known 73 *B. thuringiensis* serotypes [1, 7, 11, 17, 19, 20, 23]. The shapes of crystals observed in a scanning electron microscopy are illustrated in Fig. 1. The COR, LEE, KON, and SEO serovarieties had ovoidal crystals with various dimensions. The SOO and YOS serovarieties had spherical crystals with different sizes. The SEO serovariety had larger crystals than those of the others. The sizes ranged from 0.42-0.8 μm×0.26-0.52 μm. These results demonstrated that the six serovarieties consisted of the general shape of delta-endotoxin crystals like the already known serotypes of *B. thuringiensis* [2, 17].

Biochemical Characteristics

Biochemical characteristics of the six serovarieties were examined as shown in Table 2. Minor differences in biochemical reactions appeared among the subspecies. The six serovarieties were Gram-positive rods and motile (Table 1). Only the COR was negative in the methyl-red test and oxidase production, the LEE was negative in the lecithinase production, and the KON was negative in the nitrate reduction. The other strains were positive. The six serovarieties showed positive reactions in methyl red test,

Table 2. Toxicity of *B. thuringiensis* serovar. against *B. mori* 3rd instar larvae.

Bt subsp.	No. of larvae tested	No. of dead larvae at h (s)						Lethality (%)
		1	2	3	4	8	12	
H_2O	20	0	0	0	0	0	0	0
BTK	20	14	3	3	-	-	-	100
COR	20	0	0	0	0	0	0	0
KON	20	10	1	1	1	1	0	100
LEE	20	10	2	1	1	0	0	100
SEO	20	0	0	8	2	1	1	85
SOO	20	0	0	8	2	1	1	85
YOS	20	0	0	8	2	2	0	85

Abbreviations of *Bt.* serovar. are the same as in Table 1.

gelatin hydrolysis, β-hemolysis, catalase production, and utilization of esculin, maltose, and ribose. VP reaction was only negative in the COR strain. Lysine decarboxylase production was positive only in the LEE strain. Urease production was positive in the KON and YOS serovarieties and the other strains were negative. Arginine decarboxylase production was positive in the SOO and YOS serovarieties and the other strains were negative. Arginine dehydrolase production was positive in the COR, SEO, and YOS serovarieties and the other three serovarieties were negative. Cellobiose utilization was positive in the SEO, SOO, and YOS serovarieties and negative in the COR, LEE, and KON serovar. Salicin utilization was negative in the SEO serovar. except the other five strains. Sucrose utilization was positive in the COR and SOO strains only. Utilization of 15 carbon sources were negative in the 6 serovarieties (Table 1). These biochemical characteristics revealed that the 6 serovarieties had general properties similar to the already known serotypes of *B. thuringiensis* [2, 26]; however, each serovariety showed unique characteristics.

Antibiotic Resistance

The six serovarieties were tested against eleven antibiotics (data not shown). The six serovarieties had common resistances to ampicillin, cephalothin, colistin, and penicillin G. The COR serovariety showed resistances against bacitracin and methicillin. Six serovarieties were sensitive to other antibiotics (amikacin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, neomycin, streptomycin, tetracycline, tobramycin, vancomycin). These results indicated that the isolates had antibiotic resistance similar to the already known serotypes of *B. thuringiensis* [19, 20, 23].

Insect Toxicity

By bioassay against insect larvae, the five new serovarieties of *B. thuringiensis* showed high toxicity to *B. mori* larvae (Table 2) except for the COR strain, and low toxicity to *C. pipiens* larvae (Table 3). There were 85-100% mortalities of *B. mori* larvae in 12 h at a dose of 10^8 spores per a lump (6 cm^3) of food except for the COR serovariety. There were 17-97% mortalities of *C. pipiens* 3rd instar larvae against the COR, SEO, SOO, and YOS serovarieties in 48 h at a dose of 10^8 spores per 150 ml. The COR serovariety was not toxic against *B. mori* 3rd instar larvae, but the other *B. thuringiensis* strains showed very high toxicity against the larvae. In one hour feeding process, 50-90% mortality of the *B. mori larvae* was shown. The SEO serovariety showed 97% toxicity against the *C. pipiens* larvae. The KON and LEE serovarieties did not show any toxicity against *C. pipiens* larvae. However, the other strains were moderately toxic to the *C. pipiens* larvae in 48 h. Considering the data shown in Tables 2 and 3, we could not predict toxicity from serotypes or crystal morphology [11]. The variable toxicity of *B. thuringiensis* to different taxonomic groups of insect larvae has been interpreted by using different mechanisms that lead to the activation of the crystal and by different gut conditions of insects.

Table 3. Toxicity of *B. thuringiensis* serovar. against *C. pipiens* larvae.

Bt subsp.	No. of larvae tested	No. of dead larvae at 48 h	Mortality (%)
H_2O	30	0	0
BTK	30	30	100
COR	30	9	30
KON	30	0	0
LEE	30	0	0
SEO	30	29	97
SOO	30	5	17
YOS	30	8	27

Abbreviations of *Bt.* serovar. are the same as in Table 1.

Plasmid Patterns

The six serovarieties of *B. thuringiensis* were examined for the presence of plasmid DNA elements because the crystal genes are usually located in a plasmid [12, 25]. Vegetable cells of the six serovarieties appeared to have one to ten plasmids (Fig. 2). Figure 2 showed the typical plasmid bands. The plasmids in the *B. thuringiensis* serovar. *kurstaki* strain were used as a standard molecular weight marker [12, 24]. The number and range in mega dalton of plasmid elements for each strain were as follows (data not shown): COR strain had ten plasmid DNA elements

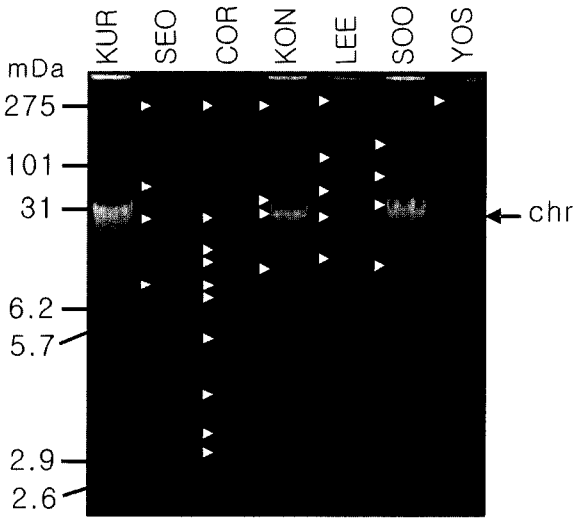

Fig. 2. Plasmid patterns of *B. thuringiensis* (*Bt*) serovarieties. The plasmids of *Bt.* serovarieties grown for 16 h at 28°C were isolated and analyzed on 0.7% agarose gel. The abbreviations of serovarieties are the same as in Fig. 1. Lanes SEO, COR, KON, LEE, SOO, and YOS. Numbers at the left side refer to the molecular weight of plasmids in mega daltons of *Bt.* subsp. *kurstaki* (KUR) strain.

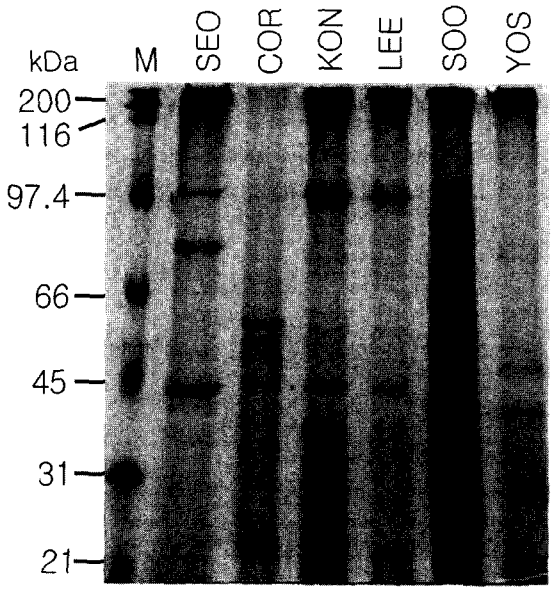

Fig. 3. SDS-PAGE analysis of crystal proteins from *B. thuringiensis* (*Bt*) serovarieties.
The crystals of *Bt* grown for 6 days at 28°C were collected, solubilized, and analyzed on 10% polyacrylamide gel. The abbreviations of serovarieties are the same in Fig. 1. Lanes SEO, COR, KON, LEE, SOO, and YOS. Numbers at the left side refer to standard molecular weight markers in kDa.

ranged from 3.1 to 275; KON, four of 11.5 to 275; LEE, five of 6.8 to 280; SEO, four of 9 to 275; SOO, four of 11.5 to 102; YOS, one of 280. These results indicated that *B. thuringiensis* serovarieties contained various plasmid DNA elements [12, 24].

Polyacrylamide Gel Electrophoresis of Crystal Proteins

The crystals of six serovarieties of *B. thuringiensis* were solubilized in denaturants such as SDS, urea, and β-mercaptoethanol to render them soluble [30]. SDS-PAGE analysis of the solubilized crystal proteins are illustrated in Fig. 3. Major bands were detected with molecular weights of approximately 102, 80, and 45 kDa in the SEO serovariety, four bands in the COR, four bands in the KON and LEE serovarieties, eight bands in the SOO serovariety, and two bands in the YOS strain. SEO, COR, KON, and LEE serovarieties shared a common 102 kDa protein, which might be protoxin protein of the four serovarieties and the 80 to 37 kDa bands might be different to the other *B. thuringiensis* serovarieties. The SOO strain had a 86 kDa protoxin protein and 70 to 27 kDa bands. The YOS strain had 51 and 41 kDa bands, which are typical patterns among the five other serovarieties.

Consequently, the six *B. thuringiensis* serovarieties had minor differences in biochemical characteristics, four antibiotic resistances, and ovoidal and/or spherical shape of crystals. The COR serovariety was nontoxic to silk worm, but moderately toxic against the *C. pipiens* larvae. The KON and LEE serovarieties were nontoxic against mosquito larvae. However, they were toxic against silkworm larvae. The SEO, SOO, and YOS strains were highly toxic to *B. mori* larvae and moderately toxic to *C. pipiens* larvae. The six serovarieties contained three to ten plasmid DNA elements. The major crystal proteins in the four serovarieties were 102 kDa and the major crystal proteins were 86 kDa in one serovar. Each of the 6 serovarieties of *B. thuringiensis* showed unique characteristics.

Acknowledgment

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