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Isolation and Characterization of *Bacillus thuringiensis* Strain BT-209 producing Cuboidal δ-endotoxin crystals

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Bacillus thuringiensis strain BT-209 was isolated from a soybean grain dust sample in Korea. The strain BT-209 produced two different sizes of cuboidal crystals and one spore in the cell. In the biochemical characterization, the strain BT-209 showed negative reactions on the production of urease, and the utilization of citrate and sucrose. Examination of its antibiotic resistance revealed that while the strain BT-209 showed higher sensitivity than *B. thuringiensis* subsp. *kurstaki* HD-1 to ampicillin, bacitracin, chlortetracycline, gentamycin, neomycin, penicillin G, tetracycline and tobramycin, it was more resistant to methicillin than *B. thuringiensis* subsp. *kurstaki* HD-1. The δ-endotoxin crystal of strain BT-209 consisted of three proteins with apparent molecular weights of appoximately 148, 135 and 62 kDa on a 10% SDS-PAGE. The strain BT-209 had at least eight different plasmids with sizes of 4.1, 5.2, 6.3, 8.6, 14.6, 24.5, 67.6 and 77.6 Kb. The strain BT-209 showed strong lethalities of 70% and 87% against *Bombyx mori* and *Hyphantria cunea* larvae at 72 h, respectively.

B. thuringiensis is a gram positive aerobic soil bacterium characterized by its ability to produce crystalline inclusion bodies during stage III to V of sporulation, and is the most widely used bacterial insect pathogen among commercial bioinsecticide preparations (16). More than 30 different subspecies or varieties of *B. thuringiensis* have been identified to date, based on the agglutination reaction of bacteria to antisera (5). Most *B. thuringiensis* strains can synthesize more than one crystal, which may itself be formed by different δ-endotoxins. These δ-endotoxin crystals consist of proteins exhibiting a highly specified insecticidal activity. Depending on their δ-endotoxin composition, the crystals have various forms and a particular correlation between structure and protein composition of the crystals has been established (12). Many *B. thuringiensis* strains are active against the larvae of certain members of the Lepidoptera, but some show toxicity against dipteran or coleopteran species (8). At the present time, commercial bioinsecticide formulations derived from *B. thuringiensis* are widely used for the control of insect pests and are offered as an alternative to chemical pesticides (14).

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In order to develop a bioencapsulated BT formulation with a high toxicity against insect larvae to be used as an alternative to chemical pesticides, we isolated about 300 *B. thuringiensis* isolates from soils, grain dusts, and commercial compost in Korea. In the process of this study, we isolated one *B. thuringiensis* isolate BT-209 producing unique δ-endotoxin crystals of a cuboidal form. Most *B. thuringiensis* strains like *B. thuringiensis* subsp. *kurstaki*, which is active against lepidopteran species, have been known to produce typical bipyramidal crystals. There have been no previous reports of *B. thuringiensis* strains like the *B. thuringiensis* strain BT-209 in Korea. Here, we report the characterization of the *B. thuringiensis* strain BT-209 producing unique δ-endotoxin crystals toxic to lepidopteran larvae.

MATERIALS AND METHODS

Bacterial Strains and Media

Bacillus thuringiensis strain BT-209 was isolated from a soybean grain dust sample in Korea, and cultured in SYG medium (2.0% soytone, 0.2% yeast extract, 0.5% soluble starch, 1.0% glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002 % $FeSO_4 \cdot 7H_2O$, 0.002% $MnSO_4$, and 0.002% $ZnSO_4 \cdot 7H_2O$, pH 7.0) at 30°C for 3 to 5 days on a rotary shaker

for crystal formation and insect bioassay. LB and Muller-Hinton media were used for plasmid isolation and the MIC test of antibiotics, respectively.

Bacillus thuringiensis sub. *kurstaki* HD-1 was used for comparison of crystal morphology and characterization with strain BT-209.

Isolation of *B. thuringiensis* Strain BT-209

B. thuringiensis strain BT-209 was isolated as described by Travers et al. (17) with some modifications.

To isolate *B. thuringiensis* BT-209 from a soybean grain dust sample, 0.5 g of grain dust was added to 10 ml of L broth buffered with 0.25 M sodium acetate in a 125 ml flask and then cultured at 30℃ for 4 h with agitation on a rotary shaker. At the end of 4 h, 1 ml of the culture was taken, heated at 85℃ for 10 min, plated on L agar, and then incubated for 24 h at 30℃. All colonies with growth characteristics similar to *B. thuringiensis* were streaked on T3 agar, and then incubated to sporulate for 24 to 48 h at 30℃. The cultures were examined by phase contrast microscopy for the presence of spores and crytals.

Biochemical Characterization of *B. thuringiensis* Strain BT-209

Biochemical characteristics of the strain were examined by the procedures of Cowan et al. (4), Gordon et al. (7), and Logan et al. (13).

Antibiotic Susceptibility of *B. thuringiensis* Strain BT-209

The test was performed with the use of serial 2-fold dilutions of each antibiotics as described by Cleeland et al. (3). The resistance of *B. thuringiensis* against antibiotics was examined after cultivation for 18 h at 30℃.

Analysis of δ-endotoxin crystal by SDS-PAGE

To analyse δ-endotoxin crystal by SDS-PAGE, isolation of δ-endotoxin crystal from *B. thuringiensis* BT-209 was performed as described by Thomas et al. (15) with some modifications. To solubilize the dry crystals, 0.1%(w/v) crystals were solubilized by incubating in 1%(w/v)sodium dodecyl sulfate(SDS), 2%(v/v)β-mercaptoethanol, 6 M urea, and an equimolar (0.01 M) ratio of NaH_2PO_4 and Na_2HPO_4(pH 7.2) for 1 h at 28℃ as described by Tyrell et al. (18).

Twenty microliters of the solution were transferred to a boiling water bath for 2 min and loaded on top of 10% SDS-polyacrylamide gel. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli and Favre(10).

Analysis of Plasmid DNA Profile

Plasmid DNAs in *B. thuringiensis* were isolated by the protoplastalkaline lysis (PAL) procedure as described by Voskuil and Chambliss (19) except that the bacterial pellet was incubated with 10 mg ml^{-1} of lysozyme at 37℃ for 1 h. Plasmid profiles were analysed on a 0.4% agarose gel in TBE buffer.

Insect Bioassay

Insecticidal activity of *B. thuringiensis* against *Bombyx mori* and *Hyphantria cunea* larvae was examined as described by Lee et al. (11) with some modifications. To produce δ-endotoxin crystals of *B. thuringiensis*, one or two loopfuls of a pure-cultured strain were inoculated in 5 ml of fresh nutrient broth and then cultured for 15 h at 30℃. Two ml of the culture were transferred into 100 ml of SYG medium and then cultured for 5 days at 30℃. The sporulated cultures (10^7 to 10^8 spores/ml) were diluted with sterilized saline at dilutions of 1×10^{-1} and 1×10^{-2}. Then, 1.0 ml of the diluted sample was added to the surface of the artificial diet (2.0 cm^3) in a petri dish covered with a filter paper and dried in the shade. Ten larvae of *Bombyx mori* or *Hyphantria cunea*, which is the third-instar larvae, were placed on the artificial diet in a petri dish. Larval mortality was recorded at room temperature(25± 2℃) with a constant humidity of 40~60% for 72 h. Bioassay was performed in triplicate.

RESULTS AND DISCUSSION

Morphological and Biochemical Characteristics of *B. thuringiensis* Strain BT-209

B. thuringiensis strain BT-209 was isolated from a soybean grain dust sample in Korea. The strain was identified by the presence of parasporal crystals and a spore in the cell using phase contrast microscopy (Fig. 1). As shown in Fig. 1, the strain exhibited motile rods and was gram positive. From observation under a phase

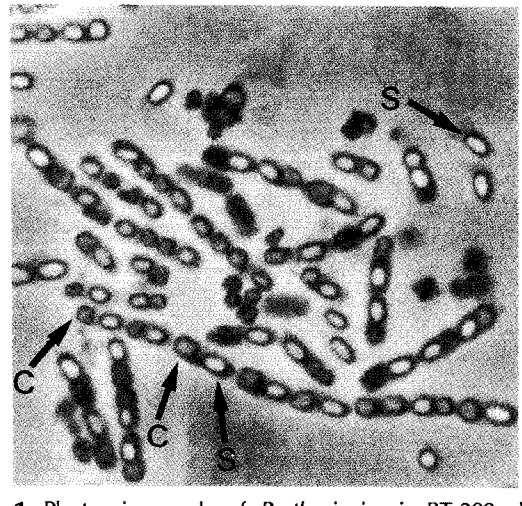

Fig. 1. Photomicrograph of *B. thuringiensis* BT-209 shows spores and δ-endotoxin crystals.
Symbols; C: δ-endotoxin crystal, S: spore.

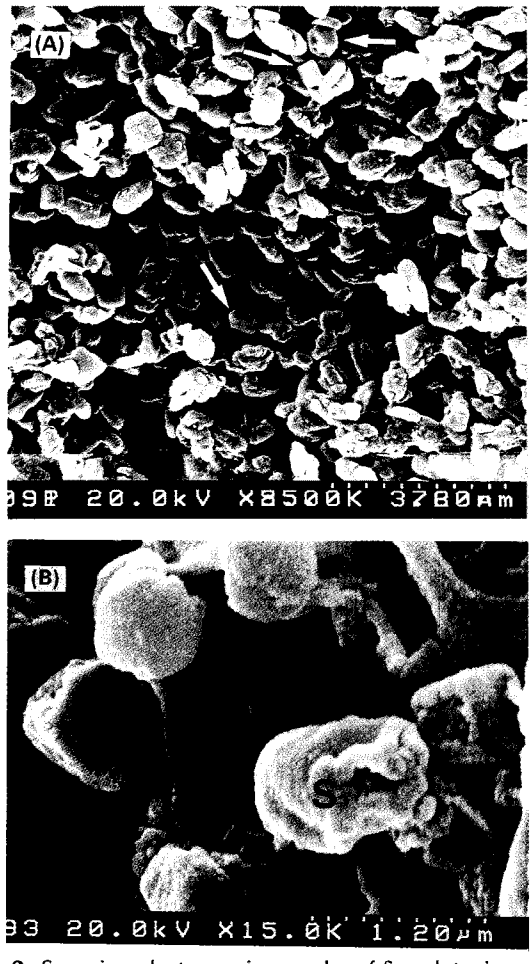

Fig. 2. Scanning electron micrographs of δ-endotoxin crystal produced by *B. thuringiensis* BT-209.
A: δ-endotoxin crystal purified by 30 to 82%(w/v) sucrose density gradient centrifugation. Arrows indicate two different sizes of cuboidal crystals, B: The spore and δ-endotoxin crystal produced by *B. thuringiensis* BT-209, Symbols; C: δ-endotoxin crystal, S: spore.

contrast microscope, the crystal shape produced by the strain was generally a square form. However, to determine whether the crystals were flat or cuboidal, δ-endotoxin crystals of the strain BT-209 were examined by scanning electron microscopy. The electron micrograph in Fig. 2 shows the three-dimensional structure of δ-endotoxin crystals of strain BT-209 with two different sizes of cuboidal rather than flat forms. Yet, there was no significant difference in the cell morphology and size of *B. thuringiensis* BT-209 as compared with *B. thuringiensis* subsp. *kurstaki* HD-1.

Biochemical characteristics of *B. thuringiensis* BT-209 were shown in Table 1. The strain BT-209 showed general biochemical characteristics similar to those of *B. thuringiensis* subsp. *kurstaki* HD-1. However, the strain BT-209 exhibited negative reactions on the production of urease, and the utilization of citrate and sucrose.

Table 1. Biochemical characteristics of *B. thuringiensis* BT-209.

Characteristics	*B. thuringiensis* strains	
	B.t.kurstaki HD-1	*B.t.*BT-209
Gram stain	+	+
Anaerobic growth	+	+
Motility	+	+
Methyl-red reaction	+	+
Nitrate reduction	+	+
Hemolysis	+	+
Voges-Proskauer reaction	+	+
Lysozyme resistance	+	+
Productions of		
indole	−	−
H_2S	−	−
β-galactosidase	−	−
catalase	+	+
phenylalanine deaminase	−	−
tryptophane deaminase	−	−
lysine decarboxylase	−	−
arginine dihydrolase	+	+
ornithine decarboxylase	+	+
oxidase	+	+
urease	+	−
gelatinase	+	+
Gas from glucose	−	−
Utilizations of		
adonitol	−	−
arabinose	−	−
casein	+	+
citrate	+	−
dulcitol	−	−
esculine	+	+
glucose	+	+
inositol	−	−
lactose	−	−
maltose	+	+
mannitol	−	−
raffinose	−	−
rhamnose	−	−
salicine	−	−
sorbitol	−	−
starch	+	+
sucrose	+	−
xylose	−	−

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

Antibiotic Susceptibility

The antibiotic susceptibilities of *B.thuringiensis* BT-209 were different from those of *B. thuringiensis* subsp. *kurstaki* HD-1. As shown in Table 2, the two strains showed similar resistance to amikacin, cephalothin, chloramphenicol, colistin, erythromycin, kanamycin, novobiocin, polymyxin B sulfate, rifampicin and streptomycin. However, the strain BT-209 showed higher sensitivity than *B. thuringiensis* subsp. *kurstaki* HD-1 to ampicillin, bacitracin, chlortetracycline, gentamycin, neomycin, penicillin G, tetracycline and tobramycin.

In case of methicillin, the strain BT-209 exhibited more

Table 2. Determination of minimum inhibitory concentration of various antibiotics against *B. thuringiensis* BT-209.

Antibiotics	MIC (ug/ml)	
	BTK HD-1	BT-209
Amikacin	3.125	3.125
Ampicillin	100	25
Bacitracin	>100	50
Cephalothin	100	100
Chloramphenicol	3.125	3.125
Chlortetracycline	3.125	<1.56
Colistin	>100	>100
Erythromycin	<1.56	<1.56
Gentamycin	3.125	<1.56
Kanamycin	12.5	12.5
Methicillin	50	100
Neomycin	3.125	<1.56
Novobiocin	<1.56	<1.56
Penicillin G	>100	100
Polymyxin B sulfate	>100	>100
Rifampicin	<1.56	<1.56
Streptomycin	12.5	12.5
Tetracycline	6.25	3.125
Tobramycin	6.25	3.125

>, greater than ; <, less than.

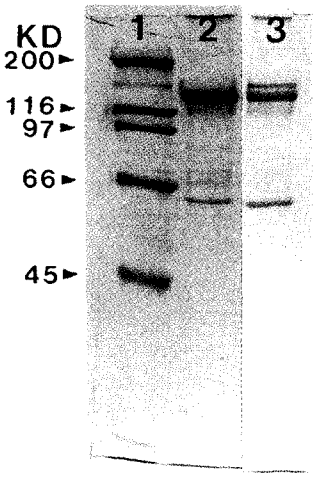

Fig. 3. SDS-PAGE analysis of δ-endotoxin crystal.
Lanes; 1: standard molecular weight, 2: *B. thuringiensis* subsp. *kurstaki* HD-1, 3: *B. thuringiensis* BT-209.

resistance than *B. thuringiensis* subsp. *kurstaki* HD-1.

SDS-PAGE Analysis of δ-endotoxin Crystal

The molecular weight of the δ-endotoxin crystal produced by *B. thuringiensis* BT-209 was confirmed by the SDS-PAGE analysis (Fig. 3). Electrophoresis of the solubilized crystals produced two major bands on a 10% SDS-PAGE with apparent molecular weights of approximately 148 kDa and 135 kDa. Crystal proteins from the strain BT-209 also appeared to include the smallest protein with an apparent molecular weight of approximately 62 kDa. The molecular weights of crystal proteins

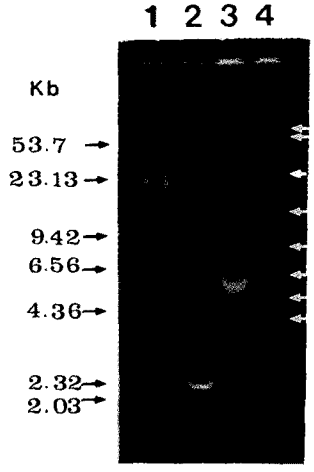

Fig. 4. Plasmid DNA patterns of *B. thuringiensis*.
Lanes; 1: Lambda DNA digested with *Hind III*, 2: *E. coli* V517D 3: *B. thuringiensis* subsp. *kurstaki* HD-1, 4: *B. thuringiensis* BT-209.

from the strain BT-209 were very distinct from those of *B. thuringiensis* subsp. *kurstaki* HD-1. On the other hand, on the basis of the SDS-PAGE data reported by Tyrell et al. (18), it is considered that the electrophoretic profile on SDS-PAGE of crystal proteins from the strain BT-209 may be similar to that of *B. thuringiensis* subsp. *berliner*. However, Tyrell et al. (18) have reported that the closely related lepidopteran-toxic *B. thuringiensis* subsp. *kurstaki* and *berliner* synthesize bipyramidal crystals, which are similar structually, biochemically, immunologically, and functionally. Therefore, it is considered that the strain BT-209 may be a different strain from the known strain, *B. thuringiensis* subsp. *berliner* due to the morphological aspect of its δ-endotoxin crystal.

Analysis of Plasmid DNA Profile

The strain BT-209 contained at least eight different plasmids with sizes of 4.1, 5.2, 6.3, 8.6, 14.6, 24.5, 67.6 and 77.6 kb (Fig. 4). The plasmid pattern of strain BT-209 was distinguishable from that of *B. thuringiensis* subsp. *kurstaki* HD-1.

Toxicity of *B. thuringiensis* BT-209

The strain BT-209 was examined for its lethality against insect larvae at dilutions of 1×10^{-2}(1×10^{5} to 1×10^{6} spores/ml). The toxicities of the strain BT-209 against *Bombyx mori* and *Hyphantria cunea* larvae were various(Table 3 and 4). When the lethality against two different kinds of insect larvae was observed at 72 h, the strain BT-209 showed a strong lethality of 70% and 87% against *Bombyx mori* and *Hyphantria cunea* larvae, respectively. It has been reported that the strain BT-209 also showed a strong lethality of 99% against *Plutella xylostella* larvae (9). From this result, it appears that the strain BT-209, which has many properties distinct from *B. thuringiensis* subsp. *kurstaki* HD-1, exhibits toxicity

Table 3. Toxicity of *B. thuringiensis* BT-209 against *Bombyx mori* larvae.

B. thuringiensis strains	No. of larvae tested	No. of the dead	Mortality (%)
Control	30	0	0
B.t.kurstaki HD-1	30	25	83
B.t. BT-209	30	21	70

Larval mortality was determined after incubation at room temperature (25± 2℃) for 72 h using 10^5 to 10^6 spores/ml.

Table 4. Toxicity of *B. thuringiensis* BT-209 against *Hyphantria cunea* larvae.

B. thuringiensis strains	No. of larvae tested	No. of the dead	Mortality (%)
Control	30	0	0
B.t.kurstaki HD-1	30	27	90
B.t. BT-209	30	26	87

Larval mortality was determined after incubation at room temperature (25± 2℃) for 72 h using 10^5 to 10^6 spores/ml.

equivalent to *B. thuringiensis* subsp. *kurstaki* HD-1 currently being used as a commercial strain.

Based on these findings, *B. thuringiensis* BT-209 may be considered as a new strain. However, the flagellar antigenicity and the insect host spectra of *B. thuringiensis* BT-209 should be further investigated. In addition, the gene encoding the lepidopteran-toxic protein of the strain BT-209 should also be further examined in detail.

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