Journal of Microbiology and Biotechnology
Vol. 4, No. 4, 322-326, 1994

Characterization of *Bacillus thuringiensis* StrainBT-14 having Insecticidal Activity against *Plutella xylostella*

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Bacillus thuringiensis strain BT-14 was isolated from alfalfa dust in Korea. The strain BT-14 produced one bipyramidal crystal and one spore in the cell. The biochemical characteristics of the strain BT-14 were similar to those of *Bacillus thuringiensis* subsp. *kurstaki* HD-1. Examination of its antibiotic resistance revealed that while the strain BT-14 was less resistant than BTK HD-1 to ampicillin, gentamycin, neomycin and tobramycin, it was more resistant to amikacin than BTK HD-1. The δ-endotoxin crystal of strain BT-14 consisted of a single protein with a high molecular weight of ca 135 KD on a 10% SDS-PAGE. The strain BT-14 contained at least nine different plasmids with sizes of 2.9, 5.3, 5.8, 6.2, 9.4, 15.1, 18.1, 23.1 and 79 Kb. In insect bioassay, the isolated strain BT-14 showed lethality of 67% against *Plutella xylostella* larvae at dilution of 5×10^{-4} (5×10 to 3×10^{2} spores/ml), which is almost equivalent to that of BTK HD-1.

B. thuringiensis is a gram positive aerobic soil bacterium characterized by its ability to produce crystalline inclusion bodies during sporulation (16). These inclusion bodies consist of proteins exhibiting a highly specified insecticidal activity (9). Many *B. thuringiensis* strains with different insect host spectra have been identified (2). More than 30 different subspecies or varieties of *B. thuringiensis* have been identified, based on the agglutination reaction of bacteria to antisera (5). Most strains are active against the larvae of certain members of the Lepidoptera. Indeed commercial formulations derived from *B. thuringiensis* sub. *kurstaki*, which is active against more than 100 lepidopteran species, are available to control the larvae of the Lepidoptera and are offered as an alternative to chemical pesticides in Western Europe and the USA (13).

Therefore, in order to develop bioencapsulated BT formulation with a high toxicity against insect larvae, as an alternative to chemical pesticides, we isolated about 300 *B. thuringiensis* isolates from soils, grain dusts and commercial compost in Korea. To select *B. thuringiensis* with a high lethality against Diamond-back moth, harmful to Chinese cabbage, we examined the toxicity of 119 *B. thuringiensis* isolates against this larva and selected one *B. thuringiensis* strain having a relatively high lethality. This report describes the biochemical characterization, antibiotic susceptibilities test and toxicity against an insect larva of *B. thuringiensis* strain BT-14. The molecular weight of toxin protein on SDS-PAGE and the plasmid profile on agarose gel are also included.

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Key words: *Bacillus thuringiensis*, δ-endotoxin crystal

MATERIALS AND METHODS

Bacterial Strains and Media

Bacillus thuringiensis BT-14 was isolated from alfalfa dust in Korea, and cultured in SYG medium (2.0% soytone, 0.2% yeast extract, 0.5% soluble starch, 1.0% glucose, 0.05% $MgSO_4\cdot7H_2O$, 0.002% $FeSO_4\cdot7H_2O$, 0.002% $MnSO_4$ and 0.002% $ZnSO_4\cdot7H_2O$, 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 used in this study was isolated from a commercial product manufactured by Mi Sung Agrichemicals Co. Ltd., under the trade name of Thuricide.

Isolation of *B. thuringiensis* Strain

B. thuringiensis strain BT-14 was isolated as described

by Travers *et al.* (17) with some modifications.

To isolate *B. thuringiensis* BT-14 from alfalfa dust, 0.5 g of grain dust was added to 10 m*l* of L broth buffered with 0.25 M sodium acetate in a 125 m*l* flask and then cultured at 30°C for 4 hr with agitation on a rotary shaker. At the end of 4 hr, 1m*l* of the culture was taken, heated at 85°C for 10 min, plated on L agar and then incubated for 24 hr at 30°C. All colonies with growth characteristics similar to *B. thuringiensis* were streaked on T3 agar, and then incubated to sporulate for 24 to 48 hr at 30°C. The cultures were examined by phase contrast microscopy for the presence of spores and crytals.

Biochemical Characterization of *B. thuringiensis* Strain

Biochemical characteristics of the strain were examined by the procedures of Cowan *et al.* (4), Gordon *et al.* (7) and Logan *et al.* (12).

Antibiotic Susceptibility of *B. thuringiensis* Strain

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 hr at 30°C.

Isolation of δ-Endotoxin Crystal

Isolation of δ-endotoxin crystal was performed as described by Thomas *et al.* (15) with some modifications. One or two loops of a pure-cultured isolate were inoculated in 10 m*l* of nutrient broth and precultured at 30°C with agitation on a rotary shaker overnight, and 6.0 m*l* of the preculture was transferred into 300 m*l* of SYG medium and cultured at 30°C for 72 hr until the autolysis was completed. Spores, crytals, and cell debris were harvested by centrifugation at 12,000×g at 4°C for 10 min in a Sorvall RC-5 refrigerated centrifuge using a SS-34 rotor. The pellet was washed three times with cold, sterilized distilled water and then suspended in cold, sterilized distilled water. The suspension was loaded onto a linear 30 to 82% (w/v) sucrose-density gradient, and centrifuged in a Beckman L8-70M ultracentrifuge using a SW 28 rotor at 77,000×g at 4°C for 1 hr. After the centrifugation, the band containing the crytal was collected with a Pasteur pippette, and then washed three times with cold, sterilized distilled water. After lyophilization, purified crystals were weighed and stored at −20°C.

Analysis of δ-Endotoxin Crystal by SDS-PAGE

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 hr at 28°C 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 (11).

Analysis of Plasmid DNA Profile

Plasmid DNAs in *B. thuringiensis* were isolated by the protoplast alkaline 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°C for 1 hr. Plasmid profiles were analysed on a 0.5% agarose gel in TBE buffer.

Insect Bioassay

To produce δ-endotoxin crystals of *B. thuringiensis*, one or two loopfuls of a pure-cultured strain were inoculated in 50 m*l* of SYG medium and then cultured for 5 days at 30°C. The sporulated cultures (10^6 to 10^7 spores/m*l*) were diluted with Triton X-100 (100 ppm) at dilutions of 1×10^{-3}, 5×10^{-3}, 1×10^{-4} and 5×10^{-4}. Leaf-disks (diameter 5 cm) were dipped into 500 m*l* of each diluted culture suspensions for 5 seconds and dried in the shade. A leaf-disk was then transferred to a petri dish covered with a filter paper. Ten larvae of *Plutella xylostella*, which is the second to third-instar larvae, were placed on a leaf-disk in a petri dish. Larval mortality was recorded at room temperature (25± 2°C) with a constant humidity of 40~60% for 72 hr. Bioassay was performed in triplicate (8, 14).

RESULTS AND DISCUSSION

Morphological and Biochemical Characteristics

B. thuringiesis BT-14 was isolated from alfalfa dust

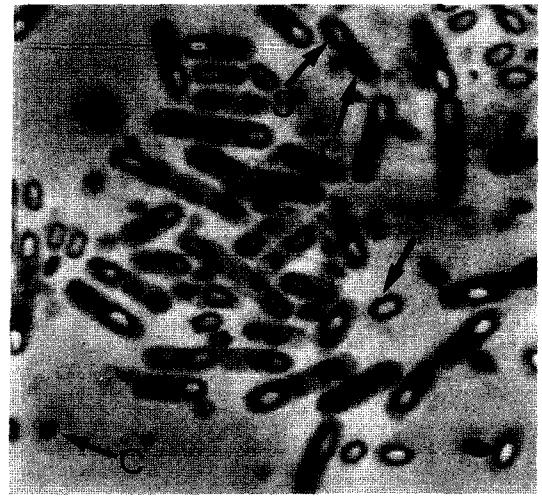

Fig. 1. Phase contrast micrograph of the spore and crystal produced by *B. thuringiensis* BT-14.
Symbols; C: δ-endotoxin crystal, S: spore.

in Korea. The B.t strain was identified by the presence of parasporal crystal and spore in a cell using a phase contrast microscopy (Fig. 1). As shown in Fig. 1., the strain was motile rods and gram positive. The crystal shape and the size of the spore in the strain were typical bipyramidal and 0.86×1.3~1.7 μm, respectively. There was no significant difference in the shape and size of BT-14 as compared with BTK HD-1.

Biochemical characteristics of BT-14 were examined as shown in Table 1. BT-14 showed in general biochemical characteristics similar to those of BTK HD-1, however, BT-14 was negative in citrate and Voges-Proskauer reactions.

Table 1. Biochemical characteristics of *B. thuringiensis* BT-14

Characteristics	Biochemical reactions of *B. thuringiensis*	
	BTK HD-1	BT-14
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 BT-14 were somewhat different from those of BTK HD-1. As shown in Table 2, the two strains showed similar resistance to bacitracin, cephalothin, chloramphenicol, chlortetracycline, colistin, erythromycin, kanamycin, methicillin, novobiocin, penicillin G, polymyxin B sulfate, rifampicin, streptomycin and tetracycline. However, BT-14 showed less resistance than BTK HD-1 to ampicillin,gentamycin, neomycin,and tobramycin. Noticeably BT-14 exhibited a higher resistance to amikacin than BTK HD-1.

SDS-PAGE Analysis of δ-Endotoxin Crystal

The molecular weight of the δ-endotoxin crystal produced by BT-14 was confirmed by the SDS-PAGE analysis (Fig. 2). On the basis of its migration onto a 10% SDS-PAGE, crystal proteins from BT-14 appeared to be composed of only one high molecular weight of ca. 135 KD. On the other hand, it has been reported that BTK HD-1 produces two kinds of crystal in one cell, a bipyramidal crystal made of 135 KD proteins and a cuboidal crystal of 65 KD proteins (1, 6, 20). Furthermore, Yamamoto *et al.* (21) has reported that the mos-

Table 2. Determination of minimum inhibitory concentration of various antibiotics against *B. thuringiensis* BT-14 & BTK HD-1

Antibiotics (ug/ml)	MIC of *B. thuringiensis*	
	BTK HD-1	BT-14
Amikacin	3.125	6.25
Ampicillin	100	50
Bacitracin	>100	>100
Cephalothin	100	100
Chloramphenicol	3.125	3.125
Chlortetracycline	3.125	3.125
Colistin	>100	>100
Erythromycin	<1.56	<1.56
Gentamycin	3.125	<1.56
Kanamycin	12.5	12.5
Methicillin	50	50
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	6.25
Tobramycin	6.25	3.125

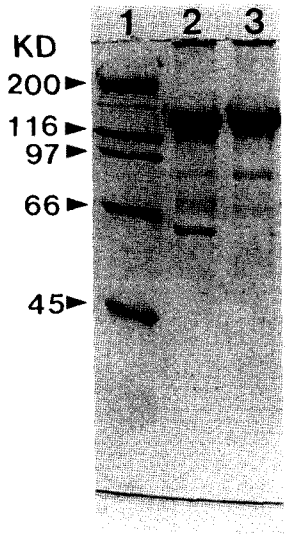

Fig. 2. SDS-PAGE analysis of δ-endotoxin crystal.
Lanes; 1: standard molecular weight, 2: BTK HD-1, 3: *B. thuringiensis* BT-14.

quitocidal activity of HD-1 is attributed to the 65 KD protein. However, the 65 KD protein from BT-14 was not observed on a 10% SDS-PAGE. A report that supports our findings was done by Huber *et al.* (10) who found that most B.t crystals toxic to lepidopteran species are made of proteins having molecular weights around 130 KD. Based on these findings, it appear that the 135 KD protein from BT-14 may be responsible for the significant toxicity to *Plutella xylostella* larvae, observed in this study.

Analysis of Plasmid DNA Profile

Plasmid DNAs in *B. thuringiensis* BT-14 were isolated (Fig. 3). The strain BT-14 contained at least nine different plasmids with sizes of 2.9, 5.3, 5.8, 6.2, 9.4, 15.1, 18.1, 23.1 and 79 Kb. The plasmid pattern of strain BT-14 was distinguishable from that of BTK HD-1.

Toxicity of *B. thuringiensis*

To select *B. thuringiensis* with relatively high toxicity to *Plutella xylostella* larvae, 119 isolates were examined for their lethality against insect larvae at dilutions of 1×10^{-3} (1×10^{3} to 1×10^{4} spores/ml) and 5×10^{-3} (2×10^{2} to 2×10^{3} spores/ml). Twenty two isolates, which showed toxicity of 100% at dilutions of 1×10^{-3} and 5×10^{-3}, were examined for the lethality against *Plutella xylostella* larvae at dilution of 1×10^{-4} (1×10^{2} to 1×10^{3} spores/ml) (Table 3). *B. thuringiensis* BT-14, which showed toxicity of 100% at dilution of 1×10^{-4}, was examined for the lethality against the insect larvae with BTK HD-1 at dilution of 5×10^{-4} (5×10 to 3×10^{2} spores/ml) (Table 4). When the lethality against *Plutella xylostella* larvae was observed at 72 hr, BT-14 and BTK HD-1 showed toxicities of 67% and 70%, respectively. From this result, it may be considered that BT-14 has almost an equivalent toxicity level to BTK HD-1 being used

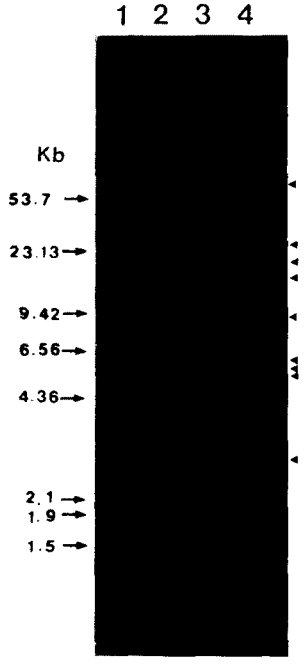

Fig. 3. Plasmid DNA patterns of *B. thuringiensis* on a 0.5% agarose gel.
Lanes; 1: *E. coli* V517D, 2: lambda DNA digested with *Hin*dIII, 3: BTK HD-1, 4: *B. thuringiensis* BT-14.

Table 3. Toxicities of 22 *B. thuringiensis* isolates against *Plutella xylostella* larvae at dilutions of 1×10^{-3}, 5×10^{-3} and 1×10^{-4} at 72 hr.

Isolate	Percent mortality		
	1 : 1,000	1 : 5,000	1 : 10,000
BT-3	100	100	99
BT-6	100	100	87
BT-7	100	100	99
BT-11	100	100	99
BT-12	100	100	100
BT-14	100	100	100
BT-27	100	100	83
BT-47	100	100	93
BT-51	100	100	99
BT-52	100	100	100
BT-58	100	100	93
BT-67	100	100	87
BT-73	100	100	99
BT-91	100	100	100
BT-99	100	100	100
BT-169	100	100	80
BT-209	100	100	99
BT-213	100	100	83
BT-228	100	100	93
BT-239	100	100	67
BT-240	100	100	80
BT-285	100	100	90

Table 4. Toxicity of *B. thuringiensis* BT-14 & BTK HD-1 against *Plutella xylostella* larvae at dilution of 5×10^{-4} at 72 hr.

B. thuringiensis	No. of larvae tested	No. of the dead	Mortality (%)
Control	30	0	0
BTK HD-1	30	21	70
BT-14	30	20	67

as a commercial formulation. On the other hand, BTK HD-1 showed the lethality against *Bombyx mori* larvae, whereas BT-14 did not (data not shown).

As a result of these investigations, *B. thuringiensis* BT-14 may be considered as a new strain having somewhat different characteristics from those of BTK HD-1. To classify *B. thuringiensis* BT-14 as a new *B. thuringiensis* strain, however, flagellar antigenicity and its insect host spectra should be further investigated. In addition, the gene encoding the lepidopteran-toxic protein of the strain BT-14 should also be further examined in detail.

Acknolwedgement

We thank Prof. Lee, Jun-Ho, Dept. of Agrobiology, Seoul National University, for bioassay of *B. thuringiensis* isolates against *P. xylostella* larvae. We also appreciate Dr. Jean-Charles Cote, Agriculture Canada, QUE, CANADA, for his assistance in this work.

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(Received August 19, 1994)