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Transfer of Insecticidal Toxin Gene in Plants: Cloning of Insecticidal Protein Gene in *Bacillus thuringiensis*

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식물세포에 살충독소 유전자의 전이 : *Bacillus thuringiensis* 살충단백질 유전자의 클로닝

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The production of delta-endotoxin crystal and the cloning of endotoxin protein gene in *Bacillus thuringiensis* subsp. *kurstaki* HD1 strain were studied. The strain produced bipyramidal crystals (2.9 × 1.0 μm) in their cells during sporulation. The *B. thuringiensis* contained about 10 plasmid DNA elements ranging from 2.1 to 80 kilobases. The 73 kb plasmid DNA, the 29 kb *Bam*HI fragment and the 7.9 kb *Pst*I DNA fragment hybridized to the pHL probe. The 7.9 kb fragment was eluted and cloned in the *Pst*I site of pBR322 vector and transformed into *E. coli* HB101, which produced insecticidal proteins killing *Bombyx mori* larvae.

The spore-forming bacterium, *Bacillus thuringiensis* produces one or more delta-endotoxin crystals (crystalline protein inclusions) during sporulation (2), which are toxic to various insect larvae (1, 3, 9, 23). The endotoxin was encoded by a variously located gene on various plasmid DNAs (4, 10, 18, 21). The toxin is consisted of a polypeptide (9, 24). Recently by recombinant DNA techniques the single gene on certain plasmids from various strains of *B. thuringiensis* was manipulated; *B. thuringiensis* var *aizawai*(7), var. *kurstaki* (8), var. *berliner* (11), var. *kuristaki* (13, 15, 16-18) and var. *israelensis* (19, 22). The cloned insecticidal protein genes may be useful to transfer to plants to protect them from insects. Therefore we undertook the cloning of the gene coding the insecticidal protein.

Here we describe the cloning of the insecticidal protein gene from *B. thuringiensis* subsp. *kurstaki* HD1 and expression of the gene in *Escherichia coli.*

Key words: Endotoxin gene cloning, *B. thuringiensis* toxin gene
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Materials and Methods

Bacteria, phage and plasmids

Bacillus thuringiensis subsp. *kurstaki*HD1 and *Escherichia coli* HB101 carrying pBR322 were obtained from D.H. Dean, the Bacillus Stock Center, Ohio State University, USA. *E. coil* V517D was from R.M. Faust, USDA, Sam 7 λ phage was from H.J. Lee, Kyunghee University. Seoul, Korea. pHL-0.9 carrying about 0.85 kb DNA fragment from *B. thuringiensis* 3a3b in*Pvu*II site of pBR322 vector was prepared in this laboratory (13) and used for probing the endotoxin gene in *B. thuringiensis* plasmids.

Enzyme

Enzymes were purchased from Besthesda Research Laboratories Inc (Gaithersburg, MD, USA). All en-

zymes and buffers were used as recommended by the manufacturers. Other chemicals were purchased from Sigma Co (St. Louis, USA)

Preparation of endotoxin crystals from *B. thuringiensis*

One loop of *B. thuringiensis* was inoculated and cultured in 250 m*l* of GBY medium (glucose 2g, yeast extract 2g, bactopeptone 2g, $MgSO_4 \cdot H_2O$ 0.3g, $MnSO_4 \cdot H_2O$ 0.05g, $CaCl_2$ 0.08g, $ZnSO_4$ 0.005g, $CuSO_4 \cdot 7H_2O$ 0.005g, $(NH_4)_2SO_4$ 2g, K_2HPO_4 0.5g in one liter of distilled water, pH 7) at 28°C for 72 h with shaking at 180 rpm. The cultures were harvested at 17,000×g for 15 min and then the supernatant was decanted. The pellets were washed three times with distilled water. The pellets were resuspended in 5 m*l* of distilled water containing 1.0 M NaCl and 0.01% Triton X-100 and then sonificated for 15 times at 30 seconds pulses with the intervals of 30 seconds. The lysates were centrifuged on a 30 to 70% NaBr gradient at 20,000×g for 2 h at 4°C. After centrifugation, the bands formed were collected with pasteur pipettes, washed twice with distilled water and observed with a phase contrast microscope.

Visualization of crystals with electron microscope

The purified crystals were loaded on formvar-treated grids, dried, coated with platinum and observed (2) with a scanning electron microscope (Hitachi S-450) at 20 KV.

Preparation of plasmid DNAs from *B. thuringiensis*

Plasmid elements were purified by the revised procedure of Lee *at al.* (13). Cells were grown in 10 m*l* of brain heart infusion (BHI) broth at 28°C with shaking at 150 rpm for 16 h. The 5 m*l* of the seed culture was inoculated into 100 m*l* of BHI broth and cultured for about 16h until 20% of optical density is reached. The cells were pelleted at 3000×g for 20 min at 10°C and washed twice in TES buffer (40 mM tris-OH, 2 m*l* EDTA, 20% sucrose, pH 8.0) (17). The pellets were resuspended in 2 m*l* of TES buffer, and 1.0 m*l* of lysozyme solution (lysozyme 50 mg per m*l* in TES buffer) was added with gentle shaking (14). After 1 h incubation at 37°C, 6 m*l* of lysis buffer (3% SDS, 50 mM tris-OH, 20% surcrose, pH 11.7) was added. The mixture tubes were rapidly inverted 20 times to mix the cells and buffer, and then placed in a 60°C water bath for 30 min. The proteinase K (50 μg/m*l*) or pronase (0.2 mg/m*l*) and RNase A solution (500μ *l*/m*l*) (14) were added to the reaction mixture and incubated for 60 min at 30°C. The lysate was extracted with 2 volumes of phenol-choroform (1:1, vol/vol) of inverting the tubes 50 times. Then the emulsions were separated by centrifugation at 12000×g for 15 min at 15°C, and the supernatant layer was collected. After adding 2 volumes of 95% cold ethanol to the supernatants, the mixture solution was inverted 20 times and then stored at −70°C for 20 min or at −20°C overnight. The DNA precipitates were collected by centrifugation at 12,000×g for 20 min at 4°C and washed with 70% ethanol. The supernatant ethanol was decanted and the rest of the ethanol in the tube was completely dried with a vacuum pump for 20 min or in air for 2 h. Finally DNA pellets were dissoved in a proper volume with TE buffer (10 mM tris-OH, 1 mM EDTA, pH 8.0).

The DNAs from 500 m*l* culture were dissolved in 8 m*l* of TE buffer in ultracentrifuge tube and then 8g of CsSl (1g/m*l*) were added and dissloved by inverting slightly. Ethidium bromide stock solution (10 mg/m*l*) was added to be final concentration of 400 μg/m*l*. The tube was placed at 37°C for 10 min. After 10 min, the buoyant protein matters were discarded by a pasteur pipette. The tubes were filled with parafin oil on the top of the solution in the tube, capped, and then run at 100,000×g for 33 h at 20°C. The lower DNA bands that formed in the ultracentrifuge tube were collected by a syringe. Ethidium bromide was extracted in n-butanol and then dialyzed against TE buffer three times for 6 h each. The dialyzed DNA solution was stored at −20°C.

Restriction enzyme digestions and agarose gel electrophoresis of DNA

DNAs were digested with restriction enzymes by the procedures of manufactures and analyzed routinely on a horizontal 0.7% agarose (type III medium EEO, Sigma) gel in tris-borate buffer for 4 to 6h at 70 volts. Gels were stained with ethidium bromide (1 μg/m*l* in tris-borate buffer) (6). The probe pHL DNA was digested with *Pvu*II enzyme and used for hybridization.

Isolation of plasmids from *E. coli*

E. coli cells containing recombinant plasmids were cultured in LB broth (DIFCO) at 37°C. The plasmid

DNAs were purified by lysozyme-SDS lysis procedures (14).

Labeling of probe DNA by nick translation

Probe DNA was labeled with [α-^{32}P-dCTP] (Amersham Co.) using a nick translation kit from Promega Co (U.S.A). The total mixture(50μl)was incubated for 2 h at 15°C. The reaction was stopped by the addition of 5 μl of 0.25 M EDTA (pH 8) and heated at 65°C for 30 min. To isolate the labeled DNA the reaction mixture was eluted through a Sephadex G-50 column (0.5 × 12 cm) with TE buffer. The eluate was fractionated such that 600 μl was collected in the first tube and 100 μl each for the next 10 tubes. cpm per tube was measured by liquid scintillation counter (LKB). Solutions over 10^7 cpm were pooled and used for Southern blotting.

Southern blot hybridization and autoradiography

DNA in the gel was transferred on the nitrocellulose (NC) filter and hybridization with [α-^{32}P-dCTP] labeled pHL-0.9 DNA by the procedrues of Southern (20) and Maniatis *et al.* (14). After electrophoresis on a 0.7% agarose gel at 4°C for 3h at 100 V. DNA was denatured, neutralized, and transferred onto NC filter. The filter was dried at 80°C in a vacuum dry oven for 2h. After hybridization, the filter was washed and exposed autoradiographically to X-ray film for 3h at −70°C. The film was manually developed following procedures suggested by Kodak Co.

Elution of DNA fragment from agarose gel

DNA fragment digested by restriction enzymes were fractionated on a 0.6% low melting temperature-agarose gel in tris-borate buffer with ethidium bromide. The bands were identified using long wavelength ultraviolet light (300 nm) and excised from the gel. The sliced gel was melted at 65°C, dilulted to 0.2% agarose in 100 mM tirs-OH (pH 8), cooled to 37°C and mixed with phenol for 25 min at 37°C. The organic and aqueous phases were separated using a microfuge and the aqueous layer was re-extracted briefly with phenol/chloroform (1:1) in TE buffer. After recentrifugation the DNA was precipitated with ethanol, pelleted, and redissolved in TE buffer.

Cloning and transformation

Cloning of the crystal protein gene was performed as follows: Two μg of *Pst*I digested plasmid DNA isolated from *B. thuringiensis* var. *kustaki* HD1 and one μg of *Pst*I digested vector pBR322 DNA were incubated for 16 h at 15°C in the presence of 0.5 unit of T4 DNA ligase (Sigma Co.). The ligation mixture was used to transform *E. coli* HB101 strain, as desctribed by Cohen *et al.* (5). Transformed cells (Ap^s, Tc^r) were selected on LB agar plates supplement with tetracycline. The selected transformants were confirmed on ampicillin LB agar medium.

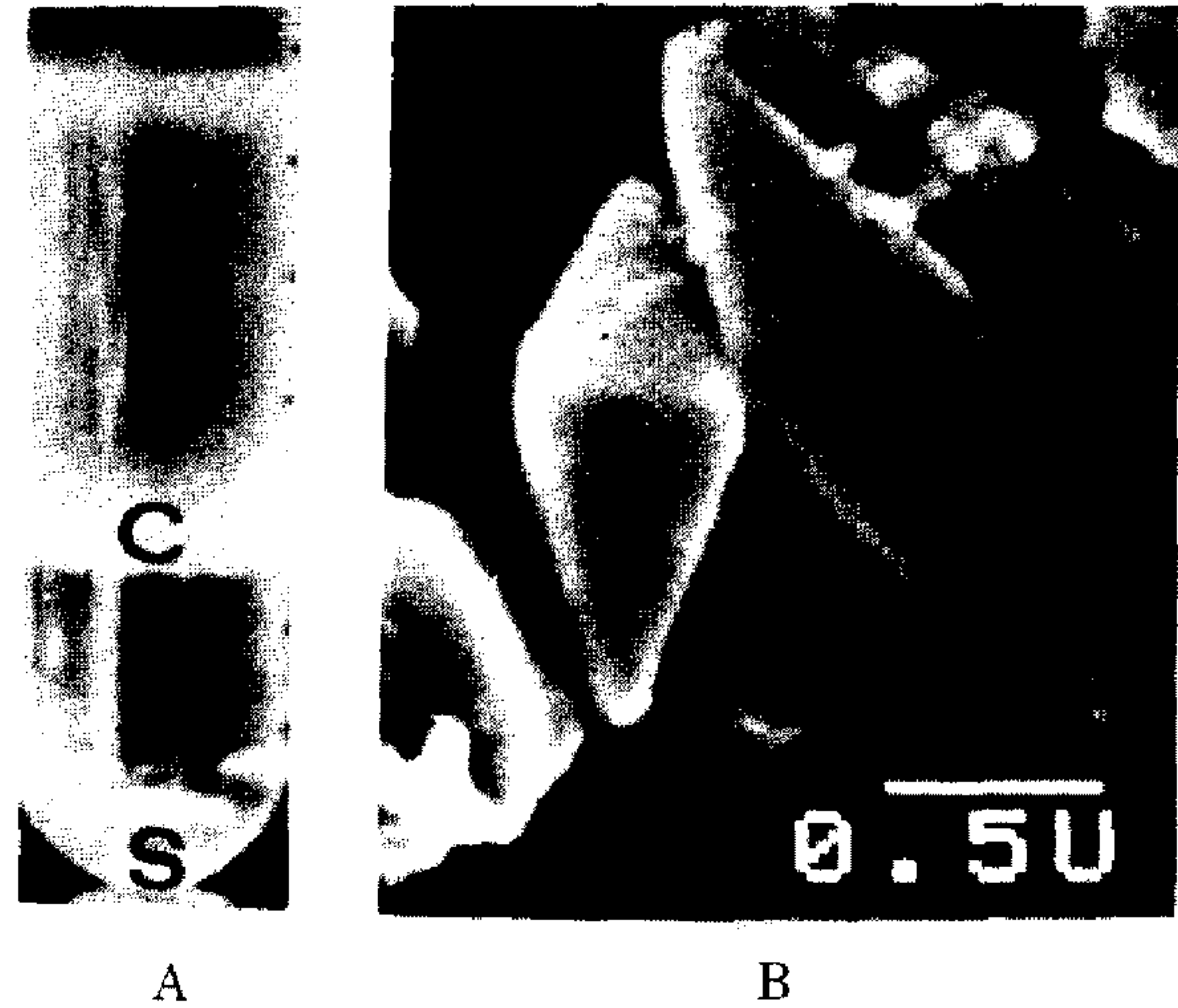

Fig. 1. Purification (A) and scanning electron micrograph (B) of endotoxin crystals in *B. thuringiensis* subsp. *kurstaki* HD1 by 30 to 70% NaBr gradient centrifugation.
C represents crystals

Bioassay on *Bombyx mori* larvae

E. coli clones were cultured in 200 ml of LB broth overnight at 37°C with shaking with 150 rpm, collected by centrifugation at 4000 × g for 15 min, resuspended with 5 ml of TES buffer, sonification 10 times at 30 seceonds pulses with the intervals of 30 seconds and then fed to *Bombyx. mori* larvae. About 1 ml was spread on food lumps (20 × 20 nm) and fed to the larvae. Dead larvae were scored after 48 h of free feeding at room temperature.

Results and Discussion

Bacillus thuringiensis subsp. *kurstaki* HD1 produced insecticidal toxin crystals. The crystals were purified by NaBr gradient centrifugation (Fig. 1A) and visualized by a scanning electron microscope (Fig. 1B). After centrifugation, the crystals were formed a band at the center of the gradient (Fig. 1A), and the shape

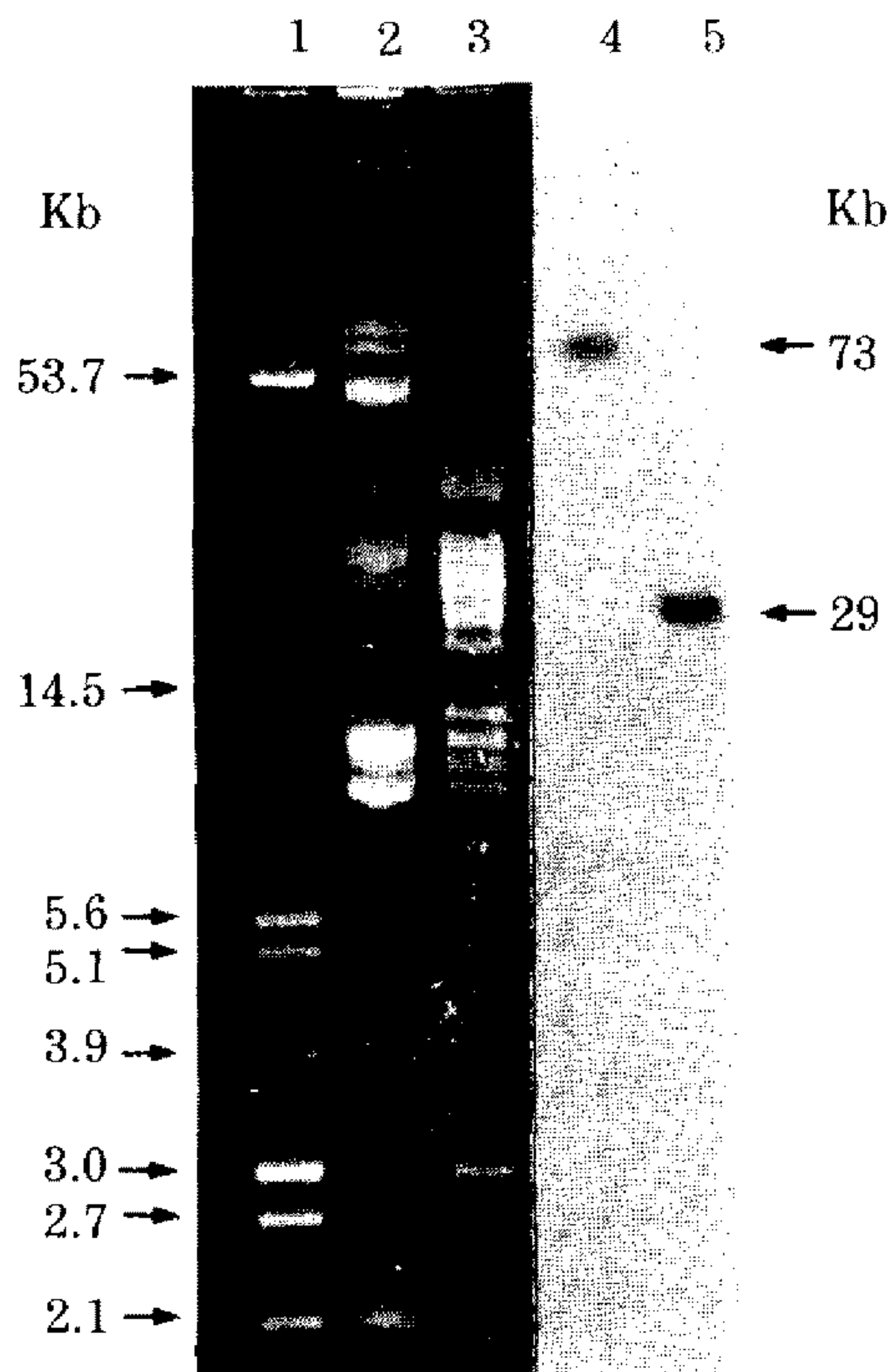

Fig. 2. Agarose gel and Southern blotting analyses of endotoxin plasmid DNAs.
Lanes 1, *E. coli* V517D; 2, total plasmid DNAs from *B. thuringiensis* subsp. *kurstaki* HD1; 3, *Bam*HI-digested total plasmid DNAs; 4 and 5, Southern blot hybridization with probe ^{32}P-labeled pHL-0.9 DNA to lanes 2 and 3. The molecular weights are in kilobase.

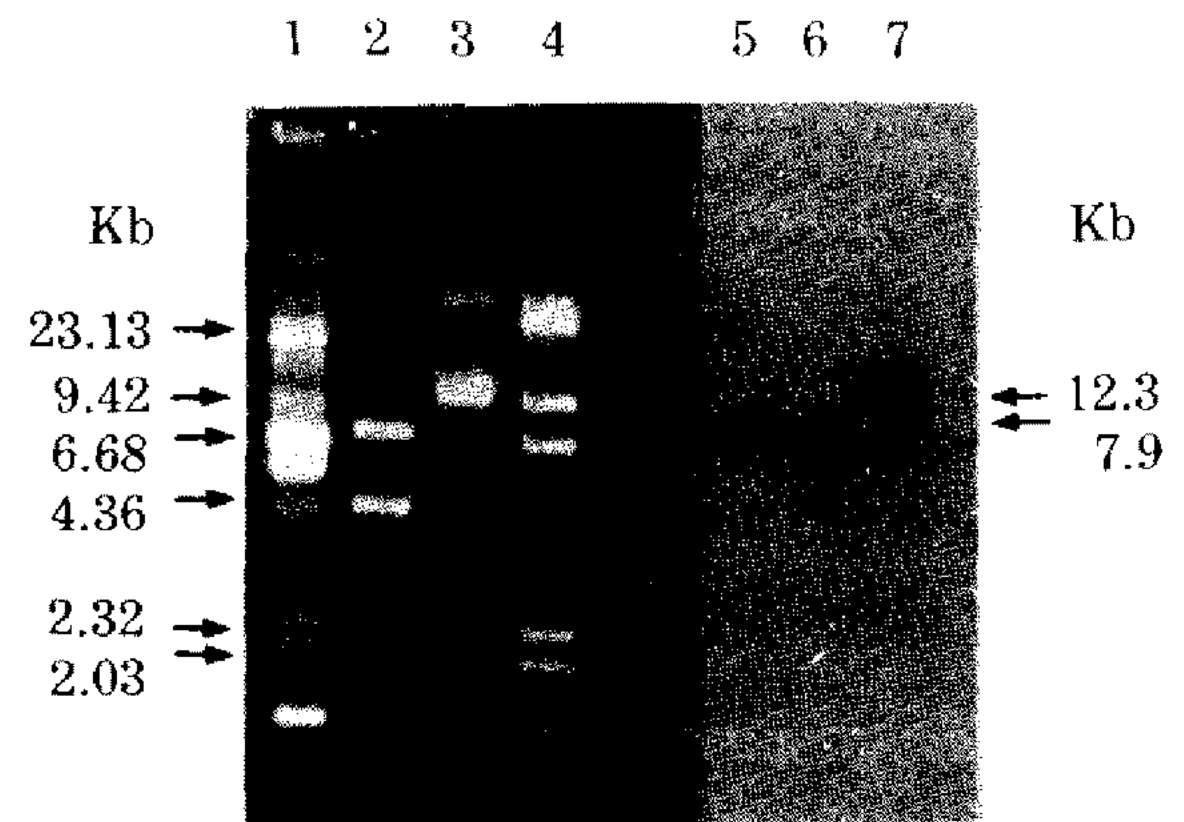

Fig. 3. Agarose gel and Southern blotting analyses of endotoxin plasmid DNAs.
Lanes 1, *Pst*I-digested total plasmid DNAs from *B. thuringiensis;* 2, pKL-20-1 DNA digested with *Pst*I; 3, pKL-20-1; 4, λ DNA digested with *Hind*III; 5, 6 and 7, Southern blot hybridization with the probe to lanes 1, 2, and 3

Table 1. Bioassay of pKL-20-1 clone and *B. thuringiensis* endotoxin lysates against *Bombyx mori* larvae

Lysated Cell	No. of tested larvae	No. of the dead at 48 h
Control (food only)	20	0
*E. coli*HB101	20	0
B. thuringiensis HD1	20	20
Clone pKL-20-1	20	8

of the crystals was bipyramidal with the size of 2.9×1.0 μm (Fig. 1B). The crystals killed *Bombyx mori* larvae (Table 1).

Plasmids in *B. thuringiensis* were isolated and their molecular weights were measured. The bacterium contained about 10 plasmid elements ranging from 2.1 to 80 kb (Fig. 2, lane 2) when compared with the *E. coli* V517D standard plasmid (Fig. 2, lane 1). The plasmid DNAs were hybridized with the probe DNA by the method of Southern (20) and Maniatis *et al.* (14). The results showed that the 73 kb plasmid was hybridized with the probe DNA (Fig. 2, lane 4). This means that the plasmid has homologous sequences of the insecticidal protein gene. Several publications have reported cloning of the insecticidal protein genes harbouring in plasmids of *B. thuringiensis* subsp. *kurstaki* HD1 (8, 15, 17, 18). These publications support our results that the toxin genes are harbouring on plasmids. McLinden *et al.*, (15) and Oh *et al.*, (17) found the toxin gene in 73 kb plasmid, and Held *et al.*, (8) found in 40 and 70kb plasmids.

The plasmid DNAs from the bacterium were digested with *Bam*HI or *Pst*I, run on agarose gel, blotted onto nitrocellulose membrane and hybridized with the probe DNA (Fig. 2 and 3). The probe hybridized with the 29 kb *Bam*HI-digested DNA fragment (Fig. 2 lane 5) and 7.9 kb *Pst*I-digested DNA fragment (Fig. 3 lane 5) when compared with lambda DNA digested with *Hind*III. The *Bam*HI 29 kb an *Pst*I 7.9 kb DNA fragments were hybridized with the probe DNA, which means that the two DNA fragments contain the insecticidal gene. The 7.9 kb DNA was eluted for cloning in pBR322 vector.

The 7.9 kb *Pst*I DNA fragment was mixed with similarly digested pBR322 plasmid DNA. Following ligation with T4 DNA ligase the mixture was added to competent cell of *E. coli* HB101. The recombinant plasmid DNAs were transformed in the *E. coli*. The colonies were selected and cultured, and then the recombinant plasmids (12.3 kb) were reisolated from the cells and redigested with *Pst*I enzyme (Fig. 3 lanes

2 and 3). The clone which contained the 7.9 kb DNA fragment was named pKL-20-1. The recombinant plasmid, pKL-20-1 was examined by the method of Southern (20). The plasmid pKL-20-1 and the redigested 7.9 kb DNA fragements were specifically hybridized with the probe DNA (Fig. 3, lanes 6 and 7). So the recombinant DNA integrates the insecticidal protein gene derived from the *B. thuringiensis* subsp. *kurstaki* HD1. The recombinant plasmid, pKL-20-1 showed strong homology to the probe. McLinden *et al.* (15) and Oh *et al.* (17) cloned Sau 3A fragment and Held *et al.,* (8) cloned *Eco*RI fragment. Cloned fragments of the plasmid were of various kinds according to investigators because bacterial plasmid DNA sequences may be frequently changed a little by recombinations or there may be certain changes between plasmids within cell.

Bioassay with *Bombyx mori* larvae was used to demonstrate expression of the insecticidal protein gene in *E. coli* (Table 1). Crude lysates of the clone pKL-20-1 were fed to third instar of *B. mori* larvae. The percent mortality of the lysate of the clone was about 40% when compared to that of the *B. thuringiensis.* The *E. coli* clone consistently demonstrated all the toxicity symptoms of *B. thuringiensis.*

The results of our Southern blot hybridization experiments using the probe are completely coincident in that the insecticidal protein gene is located on various plasmids (10, 12). The cloned gene was expressed in the *E. coli,* however the larvicidal activity was lower than that of the *B. thuringiensis.* These results are similar to other cloned crystal genes (7, 11, 15-19).

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요 약

Bacillus thuringiensis subsp. *kurstaki* HD1 의 내독소생산과 내독소단백질 유전자의 클로닝에 관한 연구를 하였다. 상기 균주는 아포생성기간 중에 이중피라미드형의 내독소를 생산하였고, 크기는 약 2.9×1.0 μm 이었다. 상기 균주는 약 10 개의 플라스미드 DNA 를 가지고 있었으며, 플라스미드의 분자량의 범위는 2.1 에서 80 kilobases 였다. 플라스미드 73 Kb, *Bam*HI 절단 29 Kb DNA 단편과 *Pst*I 절단 7.9 Kb DNA 는 Probe DNA 와 혼성화되었다. *Pst*I 7.9 Kb DNA 를 추출하여 운반체인 pBR322 운반체의 *Pst*I 절단부위에 삽입하여 클로닝한 후에 *E. coli* HB101 균주에 형질전환하였으며, 이 클로운을 pKL-20-1 로 명명했고, 이 형질전환체는 *Bombyx mori* 유충을 치사시키는 독소물질을 생산하였다.

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