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〈論 文〉

Cloning and Expression of the *Bacillus thuringiensis* var. *kurstaki* HD-1 Crystal Protein Gene in *Eschelichia coli*

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Abstract

The 44Md plasmid of *Bacillus thuringiensis* var. *kurstaki* HD-1(B. t k HD-1) was partially digested with Sau3AI and the fragments were cloned into *E. coli* HB101 on vector pBR322. Of 2,950 clones with a recombinant pBR322, only one clone KC1 was determined to have the gene for crystal toxic proteins from the 44Md plasmid of B. t k HD-1 at the BamHI site of pBR322. The recombinant pBR322 was named pKC1 and its molecular size was 12 kb. The KC1 produced a protein which was toxic to the the silkworm and antigenically similar to the crystal toxic protein of B. t k HD-1. Also, electrophoretic mobility of the KC1 protein was apparently the same as that of the crystal toxic protein of B. t k HD-1.

Key words : *Bacillus thuringiensis* var. *kurstaki*, *E. coli* HB101, crystal toxic protein

INTRODUCTION

Bacillus thuringiensis a gram-positive, endospore-forming bacteria, produces insecticidal δ-endotoxin which is highly toxic to wide variety of lepidoptran larvae (Angus, 1954; Dulmage *et al.*, 1971; Goldberg *et al.*, 1977). The δ-endotoxin is a glycoprotein (1.3×10^5 daltons) that appears during the sporulation phase of B. t as phase-reflectile and bipyramidal intracelluar crystalline inclusions (Krieg *et al.*, 1983; Kronstad *et al.*, 1983). Genes coding for the δ-endotoxin are usually located on large plasmids in several *B. thuringiensis* species (Whiteley *et al.*, 1981).

In our previous study (Kim *et al.*, 1986) the 29 and 44 Md plasmids of B. t k HD-1 were shown to have the δ-endotoxin encoded function (Battisti *et al.*, 1985). It was considered that isolation of the crystal protein gene(CP gene) should permit detailed studies of the mechanism regulating the expression of this gene (Wong *et al.*, 1983). Here we describe the cloning and expression of crystal toxic protein gene from B. t k HD-1 using *E. coli* HB101 and pBR322 as the cloning host and vector respectively.

MATERIALS AND METHODS

Bacterial strains *B. thuringiensis* var. *kurstaki* HD-1 and var. *thuringiensis* HD-2 were obtained from R. Faust (USDA, Beltsville, MD). *E. coli* HB 101, the cloning host, was obtained from H. Boyer (Univ. of California Medical School, San Francisco, CA). *B. thuringiensis* and *E. coli* were grown at 30℃ on glucose-yeast extract-salt medium, respectively.

1. Enzymes

All enzymes used in this study were purhased from Bethesda Research Laboratories, Inc. All enzymes and buffers were used as recommended by the manufacturer.

2. Purification of plasmids

Plasmids of B. t k HD-1 were isolated by a modified alkaline lysis method (Birnboim & Doly, 1979).

The 29 and 44Md plasmids were purified by fractionation after the 5~20% sucrose density gradient centrifugation for 6 hr at 23,000 rpm using a Hitachi ultracentrifuge (SCT55H) with SPR-28SA rotor. Fractions of large molecular plasmids were analyzed by agarose gel electrophoresis and the 29 and 44 Md plasmid bands were eluted from the agarose gel with an electroelution kit.

3. Construction of recombinant DNA

Purified 29 and 44 Md plasmids from B. t k HD-1 were partially digested with 5 units of Sau3AI at 37℃ for various time periods between 5~30 min. The partially-digested DNA fragments from each of the 29 and 44 Md plasmids were ligated to BamHI-digested, calf intestinal alkaline phosphatase-treated pBR322. 10 μg of DNA fragments of B. t k HD-1 plasmids and 0.5 μg of pBR322 DNA were mixed in 50 μl of the ligation buffer, ligated with T4 DNA ligase (Fig. 1), and transformed into *E. coli* HB101. After screened for ampicillin resistance on LB-agar medium with 100 μg/ml of ampicillin, colonies sensitive to tetracycline (25 μg/ml) were selected. The Amp^r/Tet^s colonies of *E. coli* HB101 were assumed to contain pBR322 with insertions of DNA fragments of the 29 or 44 Md plasmid from B. t k HD-1.

4. Preparation of antiserum

Crystalline toxic proteins were purified from sporulated cultures of B. t k HD-1, grown in GYS medium by centrifugation at 16,000 g for 90min in the bilayer cushion of Renograffin 76 (30 and 65%). The purified crystals were solubilized in alkaline solution (pH 11.5) and mixed with an equal volume of Freund's complete adjuvant. The mixture was injected into rabbit three times at 1 week intervals. Antiserum was drawn from the immunized rabbit 4 weeks after the last injection, and analyzed by double immunodiffusion precipitation.

5. Preparation of cell extracts for protein analysis and bioassay

Bacterial cells of each strain grown in 100 ml of L-broth in the presence of 100 μg/ml ampicillin was washed once with PBS buffer and twice with TESP buffer. The cell pellet was resuspended in 10 ml of TESP buffer. After disruption of the cells by ultrasonicator using Soniprep 150 (MSE), centrifugation was done at 10,000 g for 5 min. Protein in the clear supernatant were analyzed by 7.5% SDS polyacrylamide gel electrophoresis. For bioassay, mixture of the clear supernatant and artificial diet was fed to the 4th instar of the silkworm larvae.

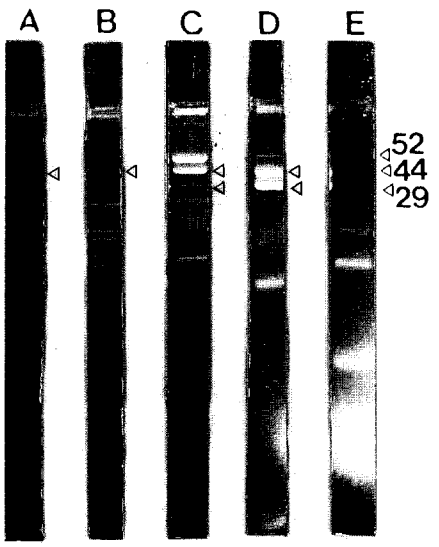

Fig. 1. *Agarose gel electrophoresis of B. thuringiensis var kurstaki HD-1 plasmid fractions obtained from the 5~20% sucrose density gradient centrifugation at 130,000 g for 6 hours. Fractions 12~13 (A); 16-17 (B); 20-22 (C); 23-25 (D); 29-30 (E).*

RESULT AND DISCUSSION

1. Cloning of CP gene from B. t k HD-1

Fragments of the 29 Md and 44 Md plasmid of B. t k HD-1 were cloned into *E. coli* HB101 on vector pBR322 as described in Materials and Methods(Fig. 1, Fig. 2). From 3,600 ampicillin-resistant clones, 2,950 were tetracycline-sensitive, indicating an insertion of a B. t k HD-1 plasmid DNA fragment onto the BamHI site of pBR322. When those

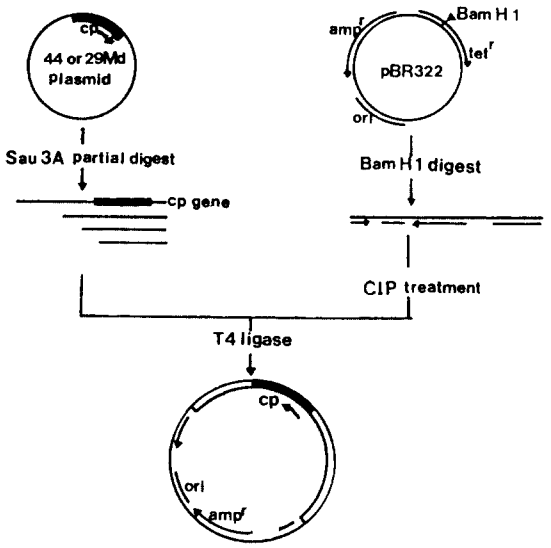

Fig. 2. *Construction of a recombinant plasmid. The crystal protein (CP) gene was ligated by T_4 ligase to BamHI digested, calf intestinal alkaline phosphatase (CIP) treated pBR322.*

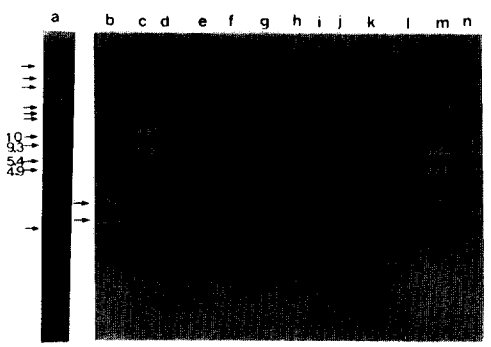

Fig. 3. *Rapid plasmid screening for a recombinant strain (lane c-n). Each lane shows plasmid purified from Amp^r/tet^s colony as described text. Lane c; pKCl plasmid, Lane 6; pBR322 vector DNA, Open arrow; open circular form plasmid, Closed arrow; CCC-form plasmid, Lane A; total B. t K HD-1 plasmids were used as the molecular weight marker.*

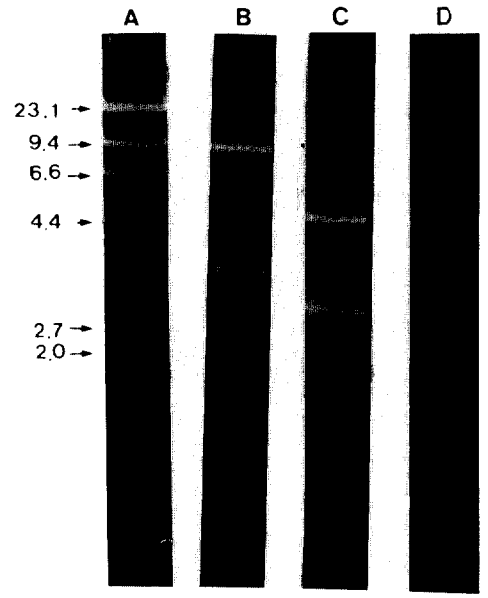

Fig. 4. *Restriction enzyme digestion pattern of pKC 1. Lane A; Hind III digested λ phage DNA, Lane B; Hind III digested pKC 1, Lane C; EcoRI digested pKC 1, Lane D; BamHI digested pBR 322.*

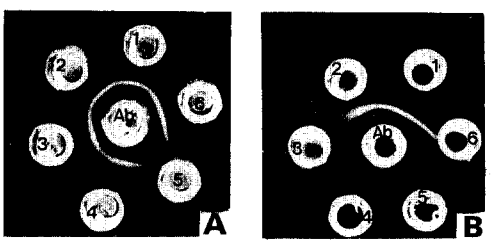

Fig. 5. *Double immunodiffusion assay of proteins extracted from B. thuringiensis var kurstaki HD-1, E. coli KCl, and E. coli HB101.*

transformed colonies were screened for the production of crystal toxin protein by insect toxicity test and double immunodiffusion assay. Only one clone harbouring pBR322 with a Sau3AI fragment of the 44 Md plasmid of B. t k HD-1 showed pathogenic clone in Fig. 3 indicates that the pathogenic clone harbours a recombinant plasmid pBR322 with the target DNA insertion (Fig. 3). The pathogenic strain and its recombinant plasmid were named KC1, respectively. The molecular size of pKC1 was determined to be 12 kb (Fig. 4).

2. Evidence for expression of pKC1 in *E. coli*

The double immunodiffusion assay showed that

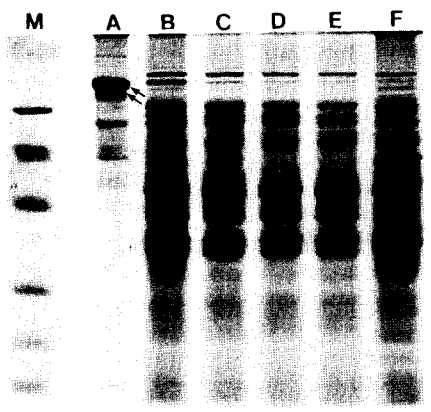

Fig. 6. *SDS-PAGE of the dissolved crystal toxic protein of B. thuringiensis var kurstaki HD-1. (A): proteins (25 μg) extracted from E. coli KCl (B); from E. coli HB 101 with pBR 322 (C-E); from E. coli HB 101 without pBR 322 (F); and the molecular weight marker proteins (M). Lanes C, D, and E were loaded with 25, 50, and 100 μg, respectively, of pretein extract.*

Table 1. *Assays for toxicity of preparations purified from B.t k HD-1 and recombinant E. coli strains*

Strain	Tested Larvae
	B. mori
HD-1	50 / 50
KC1	39 / 50
E. coli HB101	0 / 50

*No. Dead Individuals/No. Tested Individuals

the antigen made from KC1 strongly reacted with antiserum raised against the purified crystal toxic protein of B. t k HD-1 (Fig. 5). Since there was no interference between the two immuno-precipitine lines of KC1 and B. t k HD-1, it was apparent that an antigenically similar protein was expressed by KC1. Also, electrophoretic mobility of polypeptide antigen from the cell extract of KC1 on 7.5% SDS-polyacrylamide gel was apparently the same as that of the dissolved B. t k HD-1 crystal toxic protein (Fig. 6). This polypeptide band was missing in the cell extract of HB101 (Fig. 6, lane F).

3. Bioassay for insect toxicity

The cell extract of the recombinant strain KC1 was toxic to the silkworm. Only 500 μg/ml of the cell extract of KC1 was sufficient to achieve about 80% mortality of the larvae in 48 hours after treatment (Table 1). But the minimal amount of the cell extract of the KC1 required to kill the larvae has not yet been determined. Larvae exposed to an equal amount of the cell extract of pBR322 harbouring HB101 did not affected the larvae growth and development through at least the 5th instar compared with control.

摘 要

Bacillus thuringiensis var. *kurstaki* HD-1의 內毒素 단백질 유전자의 發現機作을 규명하기 위하여 이 菌으로 부터 내독소 단백질 遺傳子가 존재하는 것으로 확인된 29Md와 44Md plasmid를 분리한 후, Sau3AI 制限酵素로 部分切斷하고, pBR322 BamHI site에 ligation하여, *E. coli* HB101 strain에 transformation 시켜, 3,000여개의 Amp^r/Ter^s한 colony를 얻어 免疫學的 방법과 殺蟲力 검정을 통해 再組合 菌株 KC1을 얻었다. pKC1 plasmid DNA는 vector DNA를 포함하여 약 12 kb 정도의 크기를 가지며, B. t k HD-1 내독소 단백질과 移動度가 같거나 일치하는 132 kd, 117 kd의 KC1 specific band 2개를 얻었다. KC1 cell extract를 첨식한 결과 약 80% 치사율을 보였다.

사 사

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