Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19 No. 1, 25-30 (1991)

Integration and Expression of *Bacillus thuringiensis* Crystal Protein Gene in Chromosomal DNA of *Pseudomonas* Strains Using Transposon Tn*5*

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Transposon Tn*5*에 의한 *Bacillus thuringiensis* 독소단백질 유전자의 *Pseudomonas* 내로의 도입 및 발현

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Abstract — The crystal protein gene (*cp*) of *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) HD73 was subcloned into *Bam*HI site of central region (Tn*5*-*cp*) or *Bgl*II site of IS*50L* region (IS*50L*-*cp*) in Tn*5*, and transposed into the chromosomal DNA of five strains of root-colonizing *Pseudomonas*. The expression of *cp* gene in *Pseudomonas* transconjugants was demonstrated by immunoblot analysis and bioassay against larvae of the *Hyphantria cunea*.

Tn*5* is a compound transposon that consists of the insertion sequences IS*50R* and IS*50L* in an inverted orientation relative to one another, flanking a region that contains three antibiotic resistance genes (1). The movement of Tn*5* elements to new sites in a genome requires transposase encoded by IS*50R* and also short sequences at its ends which constitute sites of transposase action (2). When Tn*5* transposes, the outer ends (OE) of the compound transposons are used. When the insertion sequence IS*50* transposes, one OE and one inner end (IE) of IS*50* are used as the substrates. Deletion mapping has shown that the first 19 bp at each end of IS*50* constitute the sites needed for transposition. These and other properites of Tn*5* and IS*50* have been recently reviewed (3).

Tn*5* transposes at a high frequency in a variety of gram-negative bacteria with a low insertional specificity and has been used for mutagenesis, mapping of genes (4, 5) and stable expression of foreign gene (6). The various suicidal vehicles that carry the Tn*5* and have a wide range of efficient conjugal transmission has been constructed (7).

This report describes the use of Tn*5* as a vector to integrate the *cp* gene of *B.t.k.* HD73 into the chromosome of two root-colonizing strains of *Pseudomonas*. We also repeated the integration of *cp* gene into the chromosome of three root-colonizing strains of *Pseudomonas* only using the insertion sequence IS*50L* from Tn*5*. The IS*50L*-*cp* element is transposase-deficient due to the presence of an ochre termination codon (8) which would minimize the horizontal gene transfer of the *cp* gene to other bacteria. These root-colonizing microbial pesticides would produce the crystal protein constitutively and can be expected to be used for controlling the lepidopteran pests without repeated applications.

Key words: *Bacillus thuringiensis*, insecticidal protein gene, Tn*5*, gene expression in *Pseudomonas*
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Materials and Methods

Bacterial strains

Bacterial strains and plasmids used are listed in

Table 1. Bacterial strains and plasmids

Strain/Plasmid	Relevant characteristics	Sources
E. coli		
LE392	*sup*E44 *sup*F58 *hsd*R514 *gal*K2 *gal*T22 *met*B1 *trp*R55 *lac*Y1	9
HB101	*sup*E44 *hsd*S20 (r_B m_B) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl*-1	10
S17-1	Pro Res Mod$^-$, integrated RP4-2-Tet::Mu-Kan::Tn7 (Tp^r Str^r)	7
Pseudomonas		
RN4	Rif^r, Nal^r	This laboratory
RN7	Rif^r, Nal^r	This laboratory
RN8	Rif^r, Nal^r	This laboratory
RN23	Rif^r, Nal^r	This laboratory
RN24	Rif^r, Nal^r	This laboratory
P. putida		
KCTC1644	Type strain	KCTC
Plasmid		
pSUP2021	Ap^r, Cm^r, Km^r pBR325::Tn5::Mob-site	7
pRK2013	Km^r	11
pSKP10	Ap^r, Cm^r, Km^r pBR325::Tn5::Mob-site, *cp*	This study
pSKP20	Ap^r, Cm^r, Km^s pBR325::Tn5::Mob-site	This study
pSKP30	Ap^r, Cm^r, Km^r pBR325::Tn5::Mob-site, *cp*	This study

Table 1. Wild type strains of *Pseudomonas* were isolated from the rhizosphere soil of cabbage and pea (12). Nalidixic acid-resistant and rifampicin-resistant mutants, named RN strains, were selected as spontaneous mutants from some of the wild type strains. *E. coli* S17-1 containing the parental suicide plasmid pSUP2021 was obtained from KCTC (Korean collection for type cultures). All plasmids were constructed and characterized in *E. coli* LE392 strains. LB medium was used for liquid culture and, with 1.5% agar, for solid medium. Antibiotics were used at the following concentrations: 50 μg/m*l* of ampicillin (Ap) and 25 μg/m*l* of kanamycin (Km) for strains of *E. coli* and 50 μg/m*l* of Km for strains of *Pseudomonas*. All antibiotics were purchased from Sigma chemical Co.

Plasmid and chromosomal DNA preparation

Plasmid DNA was isolated according to standard procedures (10). Chromosomal DNA from *Pseudomonas* was prepared by simplified Marmur method (13). The cells were grown in 1 m*l* of LB broth supplemented with the appropriate antibiotics. After centrifugation, the cells were suspended with 300 μ*l* of 10 mM Tris-Cl (pH 8.0), 50 mM glucose and 25 mM EDTA. And then 10 μ*l* of 10% SDS solution was added and the suspension was rotated at room temperature until lysis was achieved. The mixture was deproteinized by phenol-chloroform extraction several times. After addition of 6 μ*l* of 5 M NaCl and two volumes of absolute ethanol, the DNA was collected by spooling onto a glass rod.

Southern hybridization

Southern blot of DNA form agarose gels to nitrocellulose filters and DNA-DNA hybridizations were performed as essentially described by Maniatis *et al*. (10). Probe DNA was labeled with digoxigenin-

dUTP and DNA-DNA hybrids were detected by anti-digoxigenin alkaline phosphatase conjugate and subsequent enzyme-catalyzed colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) (Boehringer Mannheim) as a substrates.

Detection of crystal protein

Electrophoresis on 9% polyacrylamide gel was carried out as described by Laemmli (14). For immunological characterization the proteins were electrophoretically blotted onto a nitrocellulose filter and immunostained with the antibody. The polyclonal antibody used in the assay was prepared from rabbits immunized with toxin protein purified from *B.t.k.* HD1. Goat anti-rabbit immunoglobulin G conjugate to alkaline phosphatase was used as the second antibody. The bands were visualized with BCIP and NBT as a coloured substrates. Bioassay was done against larvae of *Hyphantria cunea* by an artificial diet assay (15). RN strains transposed with Tn*5-cp* or IS*50L-cp* were grown in 4 m*l* of LB broth. Later, the harvested cells were resuspended in 1 m*l* of 10 mM Tris-Cl (pH 8.0), 10 mM NaCl and 1 mM EDTA and were disrupted by sonication. 200 μ*l* of each samples were mixed with 4 g of artificial diet (developed in our Lab.) and asceptically dispensed into each petri dish of 9 cm diameter. Twenty larvae of *H. cunea* were placed on each diet and incubated at room temperature. Controls for each test included parental bacterial strains and untreated diet.

Results and Discussion

Subcloning of *cp* gene into Tn*5*

The 3.7 kb *Bam*HI DNA fragment containing the *cp* gene of *B.t.k.* HD73 was isolated from the recombinant plasmid pMK74 (16). The plasmid pSUP 2021 used as a parental plasmid in this study contains a wild-type Tn*5* DNA element and encodes nontransposable resistance to Ap and chloramphenicol (Cm). This plasmid can not replicate in nonenteric gram-negative bacteria (7). The 3.7 kb *Bam*HI fragment containing *cp* gene was subcloned into the *Bam*HI site of Tn*5* (Tn*5-cp*) giving plasmid pSKP10

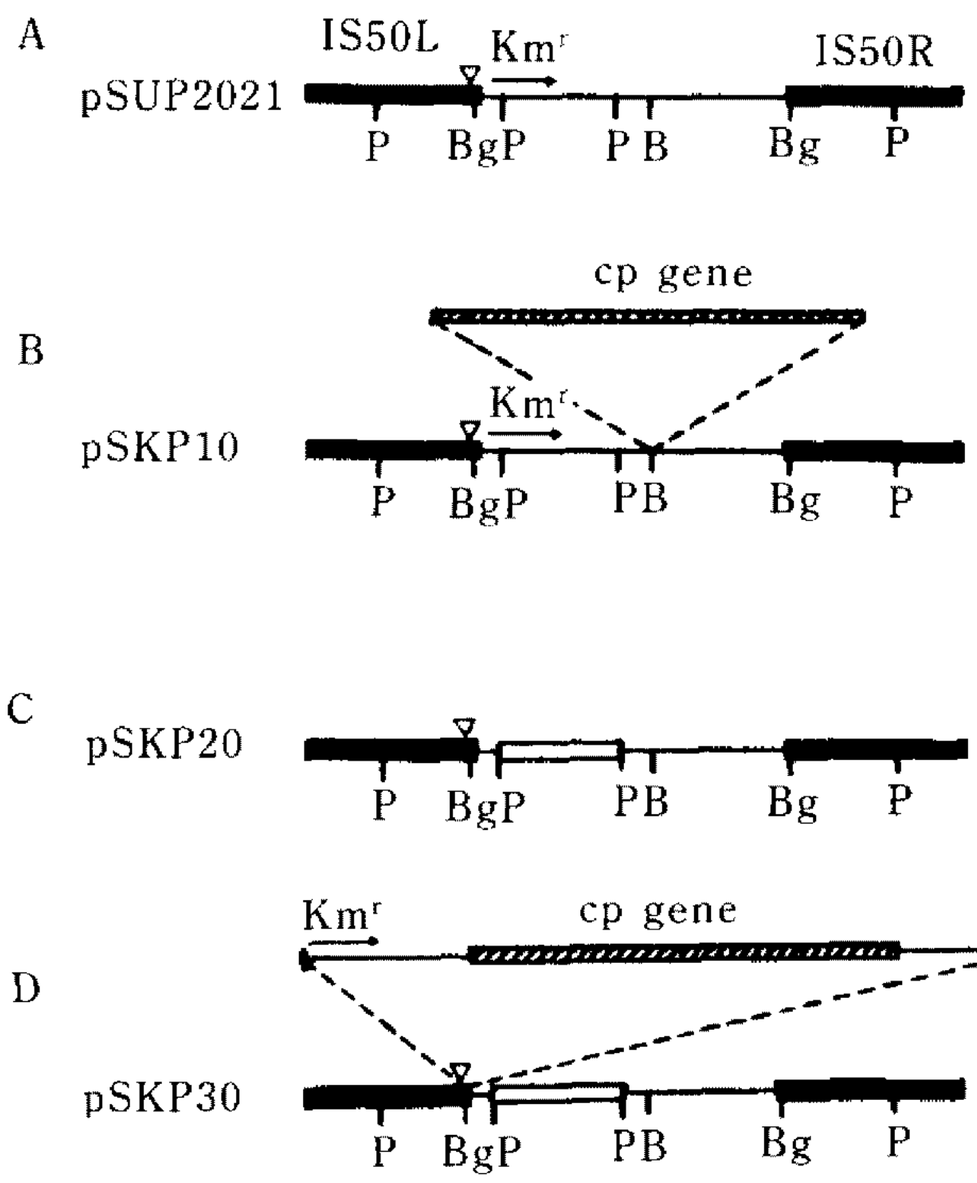

Fig. 1. Construction of recombinant plasmids pSKP10 and pSKP30 for transposition of *cp* gene using Tn*5* as a base. pSUP2021 containing a wild-type Tn*5* element was used as the parental plasmid. Only the Tn*5* portion is shown. Open triangle imply the NPTII promoter and open boxes of C and D imply the deleted portion.
Abbreviations: B, *Bam*HI; Bg, *Bgl*II, H, *Hin*dIII; P, *Pst*I.

(Fig. 1B). Since another *Bam*HI site is present on the vector, partial digestion of the plasmid and elution of single cutted DNA were done for the subcloning.

On the other hand, we constructed another recombinant plsmid pSKP30 containing the *cp* gene in IS*50L* region of Tn*5* (IS*50L-cp*) as shown in Fig. 1D. To obtain pSKP30, first, the Km gene of Tn*5* of pSUP2021 was inactivated by deleting a 1 Kb *Pst*I fragment as the following procedures to discriminate the IS*50L-cp* transposition from the Tn*5-cp* transposition. A 2.7 kb *Bgl*II fragment of Tn*5* containing antibiotic resistance genes was first eluted and digested with *Pst*II. Then the digests were ligated with large *Bgl*II fragment of pSUP2021 and screened for Km sensitive clones to give pSKP20

(Fig. 1C). Construcion of pSKP20 was confirmed by *Bgl*II digestion giving 11.7 and 1.7 kb fragments. As the next step a 6.4 kb *Bgl*II fragment of the pSKP10 containing the Km resistance gene and *cp* gene was isolated and inserted into the *Bgl*II site of the IS*50L* region of pSKP20 downstream from the neomycin phosphotransferase II (NPTII) promoter (17) giving the pSKP30 (Fig. 1D). In this step partial digestion of pSKP20 with *Bgl*II was required because it has two recognition sites, and so four different combinations were possible when the 6.4 kb *Bgl*II fragment of pSKP10 was ligated with the *Bgl*II-digested pSKP20. But only one recombinant plasmid was selected in the presence of Km, that is, the Km gene of the 6.4 kb fragment was juxtaposed with NPTII promoter of IS*50L* of pSKP20. During the process the IS*50L* IE recognition site of pSKP30 was kept intact and the transposition of IS*50L-cp* will be possible using transposase supplied in *cis* by a linked IS*50R* element (18).

Transposition of Tn*5-cp* or IS*50L-cp* into the chromosome of root-colonizing *Pseudomonas*

Plasmid pSKP10 and pSKP30 was used for delivery of Tn*5-cp* and IS*50L-cp*, respectively, into the chromosomes of five RN strains of root-colonizing *Pseudomonas*. *P. putida* KCTC1644 was also used as a host. Filter matings (18) were performed using *E. coli* LE392 (pSKP10 or pSKP30) as a donor, RN strains as recipients and *E. coli* HB101 (pRK2013) as a source of helper plasmid.

Two RN strains (RN23 and RN24) and *P. putida* KCTC1644 were transposed with Tn*5-cp* and three strains (RN4, RN7 and RN8) were transposed with IS*50L-cp*. Transposition of the Tn*5-cp* or IS*50L-cp* into the chromosome was confirmed by Southern hybridization using nick translated *Bam*HI fragments of pSKP10 DNA as a probe. Chromosomal DNAs of transconjugants which appeared to contain Tn*5-cp* or IS*50L-cp* were isolated and analyzed after digestion with *Hin*dIII or *Bam*HI. As shown in Fig. 2A in case of *Hin*dIII digests, lanes of RN23, RN24 and KCTC1644 show two hybridized fragments (4.7 kb and 2.3 kb) as are expected. Absence of the 2.3 kb fragment at the lanes of RN4, RN7 and RN8 indicates the IS*50L-cp* transposition events. Fig 2B shows all recombinant strains contained the 3.7 kb fragment carrying *cp* gene identically.

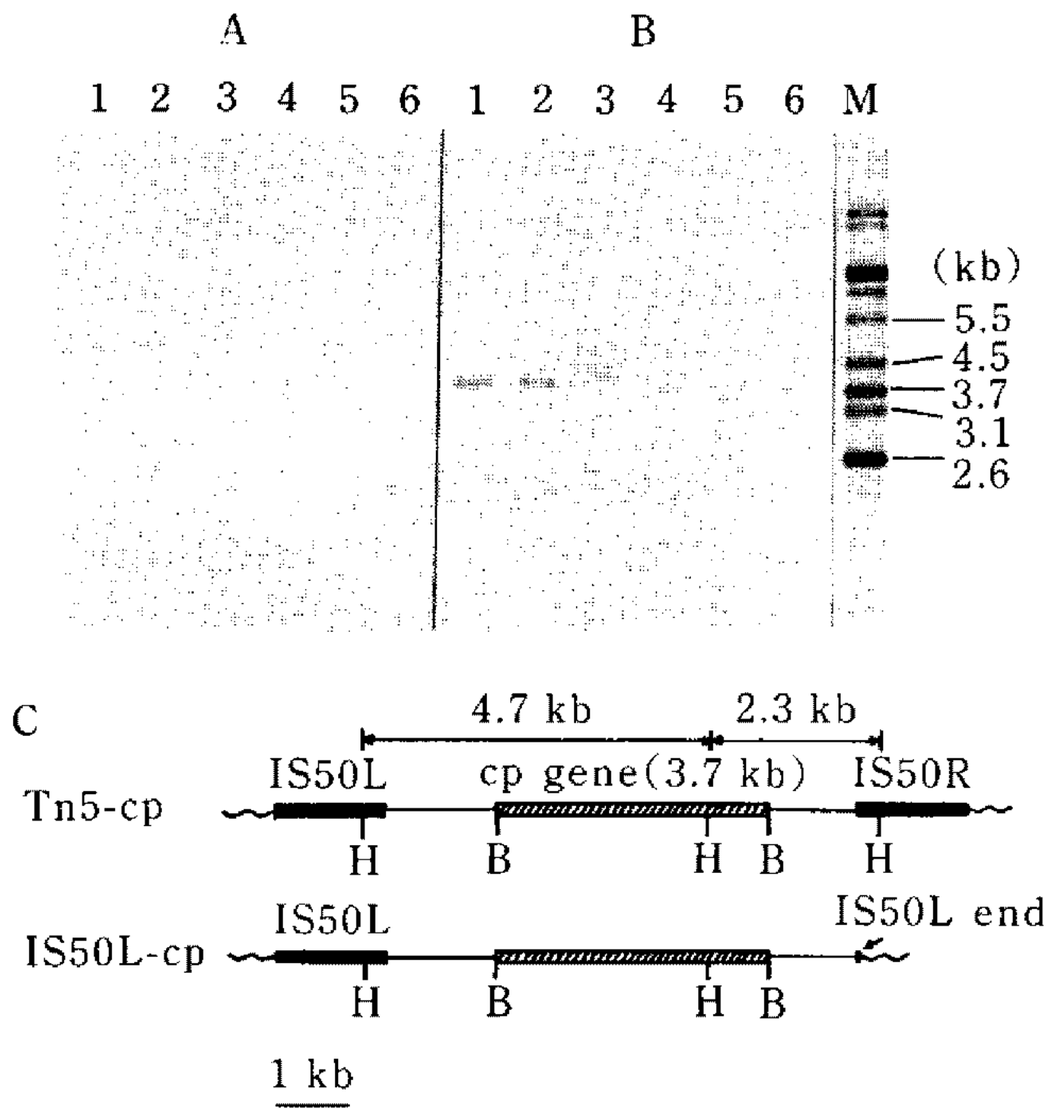

Fig. 2. DNA-DNA hybridization of chromosomal DNAs from the putative Tn*5-cp* and IS*50L-cp* recombinants of RN strains with nick-translated pSKP10 DNA. *Hin*dIII (A) and *Bam*HI (B) digests were performed.
Lanes: 1, RN24 (Tn*5-cp*); 2, RN23 (Tn*5-cp*); 3, (IS*50L-cp*); 4, RN7 (IS*50L-cp*); 5, RN4 (IS*50L-cp*); 6, KCTC 1644 (Tn*5-cp*). Expective restriction enzyme fragment sizes for the Tn*5-cp* and IS*50L-cp* are also shown (C). Abbreviations are as in Fig. 1.

There were also some possible problems with Tn*5* transposition. Tn*5* can promote replicon fusions (20). In the case of the our plasmid this event would result in integration of the whole Tn*5-cp*-carrier plasmid into the recipient genome. The Tn*5-cp* and IS*50L-cp* donor vehicle pSUP2021 can not allow a simple test for such events, since its Ap and Cm resistance can not be easily monitered in *Pseudomonas* strains (2). Insertion of vector DNA also can be occurred by inverse transposition of IE of IS*50*'s (22). A series of hybridizations was needed to check these possibilities.

The junction fragments of each transposition events were hardly detected in comparison with Tn*5-cp* and IS*50L-cp* internal fragment indicating that the chromosomes have more than single copy of Tn*5-cp* or IS*50L-cp*, or have random integration sites.

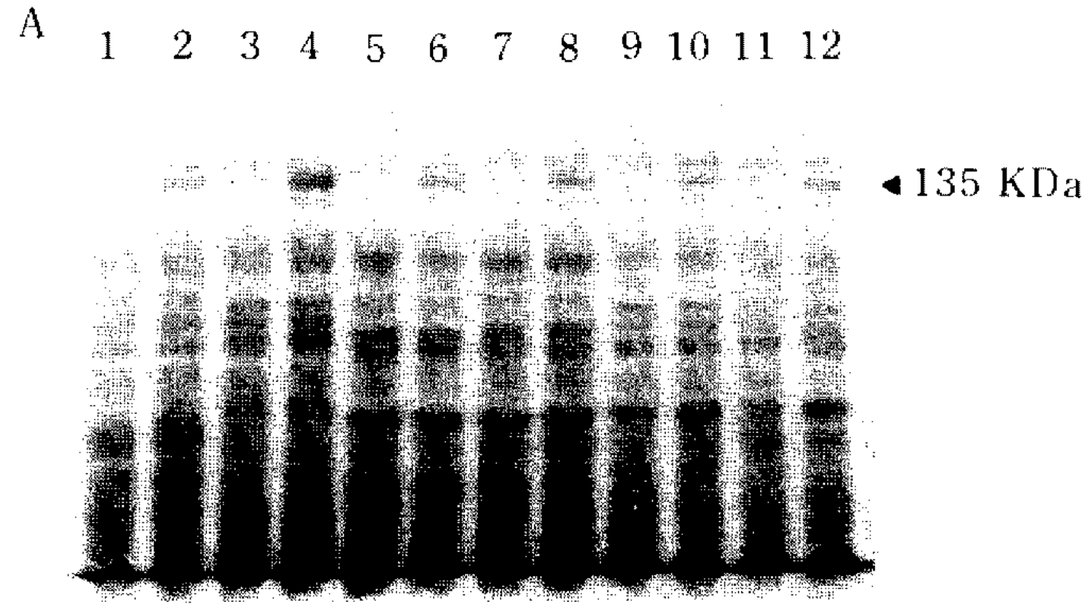

Fig. 3. Expression of *cp* in RN strains of *Pseudomonas*. SDS-polyacrylamide gel electrophoresis pattern (A) and corresponding protein immunoblot (B) are shown.
Lanes: 1, KCTC1644; 2, KCTC1644 (Tn*5*-*cp*); 3, RN4; 4, RN4 (IS*50L*-*cp*); 5, RN7; 6, RN7 (IS*50L*-*cp*); 7, RN8; 8, RN8 (IS*50L*-*cp*); 9, RN23; 10, RN23 (Tn*5*-*cp*); 11, RN24; 12, RN24 (Tn*5*-*cp*). Bands smaller than 135 Kdal are degradation products of the toxin protein (B).

Expression of *cp* in *Pseudomonas* strains

The expression of *cp* gene transposed into the chromosome of RN strains was analyzed by SDS-polyacrylamide gel electrophoresis of the total cell extracts. As shown in Fig. 3A, all of the recombinant strains produced the 135 Kdal crystal protein. Immunoblot analysis was also done to confirm the expression of toxin protein (Fig. 3B). One major band could be visulaized in the lanes of transconjugants of RN strains while it was absent at parental strains.

It seems that the NPTII promoter just upstream from the Km resistance gene was responsible for the transcription of *cp* gene. To test this possibility we also constructed a plasmid pSKP11 of which *cp* gene was reversed relative to the NPTII promoter. There was no detectable protein band at 135 kdal region (data not shown). Any DNA fragment inserted within internal region of Tn*5* seems to be effected by the NPTII promoter because three antibiotic resistant genes of Tn*5* belong to an common operon controlled by the NPTII promoter located just within the *Bgl*II site of IS*50L* (23).

The biological activity of product was also determined. All Tn*5*-*cp* and IS*50L*-*cp* recombinants of RN strains show more than 90% mortality within seven days against larvae of *H. cunea*. The parental strains containing Tn*5* caused no mortality.

요 약

국내농작물의 근부토양으로 분리한 *Pseudomonas*의 염색체 DNA에 Tn*5*를 사용하여 *Bacillus thuringiensis* subsp. *kurstaki* HD73의 독소유전자(*cp*)를 도입하였다. Tn*5*의 중심부위에 있는 *Bam*HI 위치(Tn*5*-*cp*)와 IS*50L*의 *Bgl*II 위치 (IS*50L*-*cp*)에 각각 독소유전자를 도입하였으며 두 종류의 *Pseudomonas* 균주에는 Tn*5*-*cp*로써 그리고 다른 세 종류의 *Pseudomonas* 균주에는 IS*50L*-*cp*로써 transposition하였다. 면역학적 방법과 흰불나방 애벌레에 대한 살충성 검정으로서 독소유전자의 도입과 발현을 확인하였다.

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(Received November 10, 1990)