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# Expression of the *Bacillus thuringiensis* Crystal Protein Gene in *Pseudomonas* Isolated from Rhizosphere Soil of Korean Crops

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# 국내 농작물의 근부토양에서 분리한 *Pseudomonas* 내에서의 *Bacillus thuringiensis* 독소단백질 유전자의 발현

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**Screening of *Pseudomonas* strains that can be used as hosts for expression of crystal protein gene of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 was carried out. From rhizosphere soil of 7 kinds of crops 35 fluorescent *Pseudomonas* strains were isolated. A hybrid plasmid, pKTC1, composed of the broad host range vector pKT230 and the crystal protein gene was constructed and used for transformation of the 35 *Pseudomonas* strains. As the result, the crystal protein gene could be introduced into 4 isolates. Several methods including bioassay and immunochemical detection indicated that the crystal protein gene was expressed in the *Pseudomonas* isolates.**

The genus *Pseudomonas* have received widespread attention due to its medical and phytopathogenic importance, its catabolic versatility, and its close correlation with plant. Application of the pseudomonads strains as hosts for recombinant DNA, particularly for *B. thuringiensis* (BT) crystal protein gene, which is toxic to the lepidopteran larvae, has been studied to generate root-colonizing microbial pesticides that produce the toxin protein constitutively (1, 2). This report describes the isolation of *Pseudomonas* from rhizosphere soil of Korean crops and the transfer of BT toxin protein gene to these strains by means of plasmid transformation.

## Materials and Methods

### Bacterial strains, plasmids and culture media

*E. coli* HB101 (*F*$^-$ *hsd*S20 *rec*A13 *pro*A2 *lac*Y1 *gal*K2) was used as a host for the recombinant plasmids. 35 strains of *Pseudomonas* isolated in our laboratory were used as preliminary hosts for the crystal protein gene expression. pKT 230 is a broad host range vector derived from RSF 1010 (3). Construction of pMK74 containing crystal protein gene has been described previously (4). *Pseudomonas* selective medium used here was King's B (2% proteose peptone, 1% glycerol, 0.15% $K_2HPO_4$, 0.15% $MgSO_4$, 1.5% agar pH 7.2) containing 0.03% cetrimide (5). L-broth (1% tryptone, 0.5% yeast extract, 1% NaCl) was used for culture of *E. coli* and *Pseudomonas* strains. Kanamycin was added when appropriate, to a final concentration of 100 $\mu$g/ml.

Plasmid DNA from *E. coli* and *Pseudomonas* was prepared by the rapid alkaline extraction method (6).

Key words: *Pseudomonas* isolation, crystal protein gene expression
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**Construction of pKTC1**

3.7 kb *Bam*HI fragment containing crystal protein gene of the *Bacillus thuringiensis* subsp. *kurstaki* HD73 was prepared from the plasmid pMK74 by agarose gel elution after *Bam*HI digestion. The DNA fragment was ligated into the pKT230 linearized with the same enzyme and dephosphorylated with CIP. This DNA ligation mixture was used for transformation of *E. coli* HB101 and kanamycin resistant transformants were screened. Construction of recombinant plasmid containing crystal protein gene was confirmed by agarose gel electrophoresis after digestion with restriction endonucleases *Bam*HI and *Hin*dIII.

**Field isolation and identification of *Pseudomonas***

Roots of crops were carefully removed from the field or from the greenhouse pot and the superfluous soil was dislodged by gentle agitation. The roots with adhering rhizosphere soil were placed in flasks containing 100 m*l* of sterile 0.9% NaCl solution and shaken for 1 hr at 120 rpm. Tenfold dilutions of each suspension were plated on King's B medium containing 0.03% cetrimide and the plates were incubated at 30°C for 48 hr. Colonies grown on the pseudomonads selective medium and fluoresced with an ultraviolet lamp were counted as fluorescent *Pseudomonas* and partially identified by Palleroni's method (7).

**Transformation of *Pseudomonas***

We have adapted the procedure of Kushner for transformation of *Pseudomonas* strains (8). Transformation of *E. coli* was performed as described by Mandel and Higa (9).

**SDS – PAGE and densitogram**

Overnight cultures of each *Pseudomonas* transformant (in L-broth) were harvested and resuspended in one-tenth volume of 10 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA solution. After sonicated for 2 min., these were used as crude cell extracts for SDS-PAGE and bioassay. SDS polyacrylamide gel electrophoresis was carried out as usual procedure. Densitometer scans were obtained using Sebia System 2 Densitometer.

**Western blot analysis**

Antibody raised against crystal protein in the rabbit was used. For Western blotting, the proteins were electrophoretically blotted on a nitrocellulose filter (10) with electrophoretic transfer system (Trans-Blot cell, Bio-Rad) and immunostained with the antibody and goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma). The immunostain procedure was essentially that of Huynh *et al.* (11).

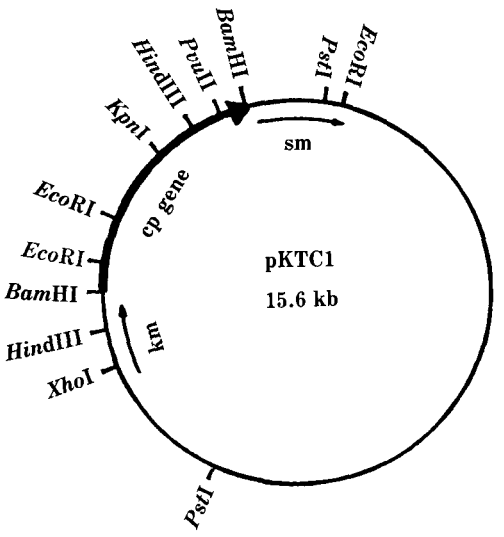


**Fig. 1. Restriction map of recombinant plasmid pKTC1. The heavy line represents the crystal protein gene and the arrowhead indicates the direction of the crystal protein gene.**

**Insect bioassay**

Each of the cell extract prepared as described above was added to artificial diets in petri dishes and ten of 3rd instar larvae of *Hyphantria cunea* or *Bombyx mori* per each diet were reared at 30°C for 7 days (12).

## Results

**Construction of recombinant plasmids containing the crystal protein gene**

Ligation mixture of the 3.7 kb *Bam*HI fragment containing crystal protein gene and pKT230 linearized with *Bam*HI was introduced into the competent cells of *E. coli* HB101 and transformants were selected for Kanamycin resistance. From some of these transformants, plasmids were isolated and analyzed by agarose-gel electrophoresis after digestion with restriction endonucleases *Bam*HI and *Hin*dIII. Mapping of the

**Table 1. Populations of fluorescent pseudomonads in rhizosphere soil of crops.**

| Crops* | pH of soil** | No. of fluorescent pseudomonads/gram of roots of crops |
|---|---|---|
| potato | 6.3 | $1.0\times10^5$ |
| radish | 6.5 | $4.3\times10^5$ |
| pepper | 6.4 | $2.4\times10^6$ |
| eggplant | 7.0 | $1.1\times10^6$ |
| cabbage | 5.8 | $4.7\times10^4$ |
| corn | 5.1 | $2.1\times10^5$ |
| pea | 6.2 | $4.0\times10^5$ |

* Crops were collected in Gyunggi Province on June, 1988.
** The pH was determined by resuspending the soil in an equal volume of distilled water.

plasmids indicated that the insert could be present in either orientation. One of them, designated as pKTC1, have the inserted crystal protein gene in the same orientation as that of kanamycin resistance gene. Restriction map of the pKTC1 was shown in Fig. 1.

### Isolation and identification of *Pseudomonas*

Roots of crops were collected in Gyunggi Province on June, and fluorescent pseudomonads were isolated and counted as described in Materials and Methods (Table 1). From the fluorecent colonies isolated, approximately 450 colonies were selected randomly. Partial identification and characterization made it possible to classify those isolates into 35 groups and identify with fluorescent *Pseudomonas* strains different from one another (Table 2). Representative 35 isolates selected from each group were used as preliminary hosts for crystal protein gene expression.

### Introduction and expression of the crystal protein gene in *Pseudomonas* isolates

According to the Kushner's transformation method, we tried to transfer the pKTC1 into 35 *Pseudomonas* isolates. As a result of transformation, 4 strains could be introduced with pKTC1. Fig. 2 shows a Western blot analysis of the proteins separated on SDS-PAGE. The proteins from *Pseudomonas* transformants containing the crystal protein gene are shown in lane 4 and 5. Lane 5 indicates that the plasmid pKTC1 in *Pseudomonas* specify a 135 kda protein that reacts specifically with the antibody of BT crystal protein. Fig. 3 illustrates SDS-PAGE analysis of total cell protein from *Pseudomonas* transformants containing pKTC1. All 4 *Pseudomonas* isolates transformed with pKTC1 produce toxin protein which constitutes as much as 3% of the total cell protein when traced with a densitometer (Fig. 4).

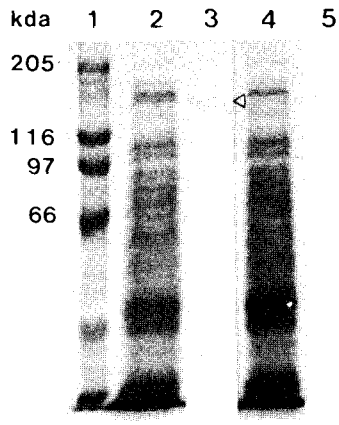


**Fig. 2. Western blot analysis of crude extracts of *Pseudomonas* transformed with pKTC1 and pKT230.**
Lanes: 1, molecular weight marker; 2, transformant of pKTC1; 3, immunostained filter strip of pKTC1 transformant; 4, transformant of pKT230; 5, immunostained filter strip of pKT230 transformant. The arrowhead indicates the immunostained band.

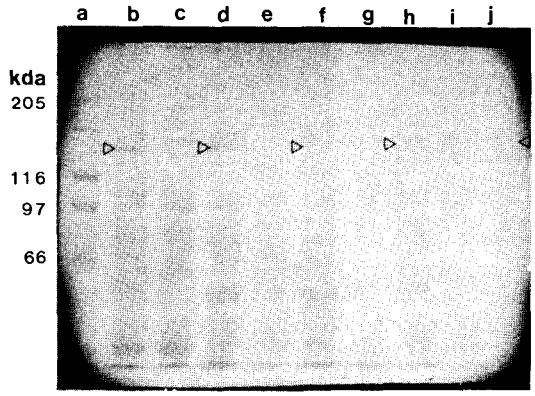


**Fig. 3. SDS-PAGE of crude extracts of *Pseudomonas* transformed with pKT230 and pKTC1.**
Lanes: a, molecular weight marker; b,d,f,h, pKTC1 transformants of *Pseudomonas* No. 5,7,8,24 respectively; c,e,g,i, pKT230 transformants of *Pseudomonas* No. 5,7,8,24, respectively.; The arrowhead points to the crystal protein band.

**Table 2. Characters of isolated strains.**

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| • isolate number | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
| • fluorescence in King's A | | | + | + | + | − | + | + | + | + | + | − | − | − | + | − | − | − | − | − | + | + | + | + | + | + | + | − | + | + | + | − | + | − | + | + | + |
| • fluorescence in King's B | | | + | + | + | + | + | + | + | + | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| • oxidase test | | | + | + | + | + | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| • catalase test | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| • arginine dihydrolase | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| • O-F | open | | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a |
| test | closed | | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | k | |
| • gelatinase | | | + | + | + | + | + | + | + | + | − | − | − | − | + | − | − | − | − | + | − | − | + | − | + | + | − | − | + | + | + | + | − | − | + | + | + |
| • hydrolysis of Tween 80 | | | + | + | + | − | − | − | − | + | − | − | − | − | + | − | − | − | − | + | − | − | − | − | + | + | − | − | + | + | + | − | − | − | + | − | − |
| • growth at 42 °C | | | + | − | − | − | − | + | − | − | + | − | − | − | − | − | − | − | − | − | − | + | − | − | − | + | + | − | + | + | − | + | − | − | − | − | − |
| • growth at 4 °C | | | + | + | + | + | + | + | + | + | + | − | − | − | + | − | − | − | − | + | − | − | + | − | + | + | − | − | + | + | + | + | − | − | + | + | + |
| • zone type | opaque | | − | − | − | − | − | − | + | + | + | − | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − | + | − | − | − | − | − | + | + |
| on egg york | indescent | | + | + | + | + | + | + | + | − | + | − | − | − | + | − | − | − | − | + | − | − | − | − | + | + | − | − | + | + | + | + | − | − | + | + | + |
| agar medium | elevation | | C | C | C | C | C | R | R | R | R | R | R | R | C | R | R | R | R | R | R | R | C | R | R | C | R | R | C | C | C | R | R | R | C | R | C |
| • bluegreen pigment | | | + | + | + | − | − | + | + | − | − | − | − | − | − | + | − | + | − | − | − | + | − | − | + | + | + | + | + | − | + | − | − | + | − | − | + |
| • mucoid colony morphology | | | + | + | + | + | + | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | + | − | + | + | − | − | + | + | + | − | − | + | − | − | − |
| • colony | elevation | | C | C | C | C | C | R | R | R | R | R | R | R | C | R | R | R | R | R | R | R | C | R | C | C | R | R | C | C | C | R | R | R | C | R | R |
| morphology | color | | G | G | G | G | G | W | W | G | G | B | B | B | G | W | W | G | B | Y | T | B | W | G | W | W | G | B | W | G | W | B | G | W | G | B | B |
| • source | | | e | r | p | c | c | c | c | c | c | p | p | p | t | t | t | e | e | p | p | p | p | p | p | o | o | o | o | n | n | c | c | c | c | p | p |

Abbreviations: a; acid, k; alkaline, C; convex, R; raised, F; flat, G; green, W: white, B; brown, Y; yellow, T; transparent, e; eggplant, r; radish, p; pepper, t; potato, c; cabbage, n; corn, o; pea

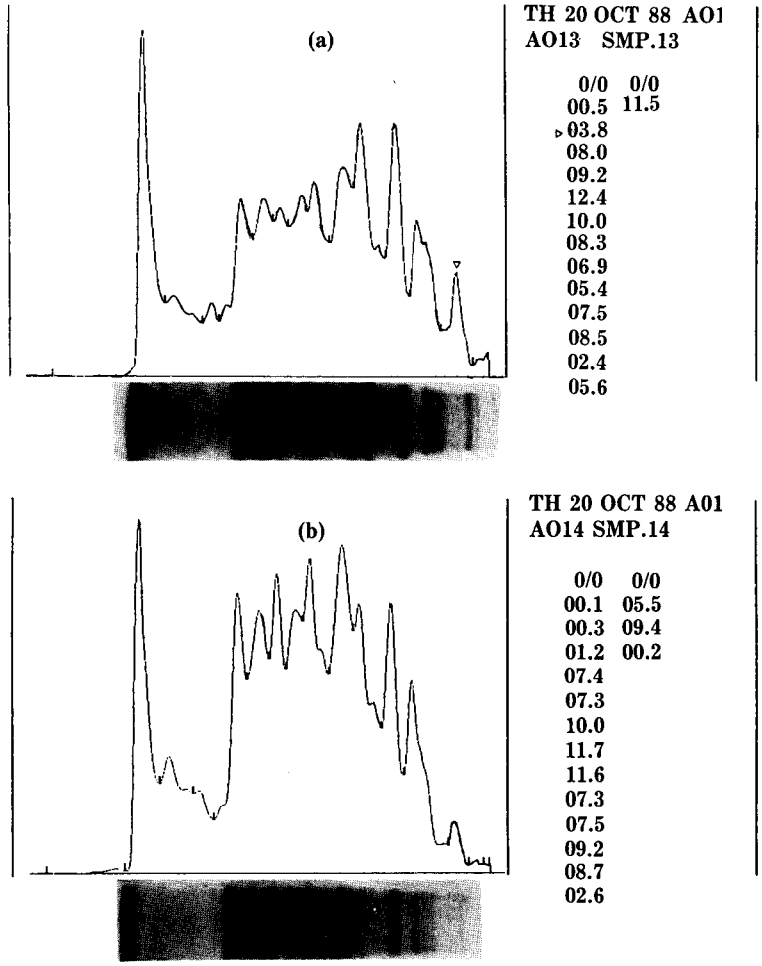


**Fig. 4. Densitograms of SDS-PAGE protein band of *Pseudomonas* transformant.**
Crude cell extracts from transformants of *Pseudomonas* No. 24 harboring pKTC1 (a) and pKT230 (b) were analysed by SDS-PAGE densitometer scans. The arrowheads point to the crystal protein bands.


**Fig.5. Bioassay for *Pseudomonas* transformants to *H. cunea*. *Pseudomonas* No. 24 and its transformants harboring pKTC1 and pKT230 were tested for bioassay to the fall webworm, *H. cunea*.**

**Insect biossay**

Four strains of *Pseudomonas* transformants harboring pKTC1 plasmid DNA were examined on their toxicities aginst larvae of *H. cunea* (Fig. 5) and *B. mori* as described in Materials and Methods. Concentrated cell extracts of 4 transformants showed similar larvicidal activity 70 and 80% scored after 7 days for *H. cunea* and *B. mori,* respectively. But that of the host cells or that of transformant having pKT230 had no larvicidal effect.

## Discussion

The potentiality of crystal toxin protein of various *B. thurigiensis* strain as clean insecticides seems to be enlarged by genetic manipulations such as cloning of the crystal protein gene and expression of the gene in other organisms. In this report we described the isolation of *Pseudomonas* from rhizosphere soil of crops abundant in Korean field and the introduction of the crystal protein gene into those isolates using a broad host range vector pKT230, a derivative of RSF1010. Kushner's transformation method made it possible to introduce the pKTC1 having the crystal protein gene into 4 among 35 isolates though the transformation efficiency is low. The pKTC1 in which the crystal protein gene has the same direction of transcription as kanamycin resistance gene of pKT230 revealed the production of crystal protein approximately to 3% of the total protein in those isolates. But a construct in which the direction is opposite to pKTC1, no apparant crystal protein band was observed (data not shown). These results indicate that the expression of the crystal protein gene in *Pseudomonas* might be controlled not by its own promoter but by other regulatory region present in pKT230. The improvement of the crystal protein gene expression in *Pseudomonas* is now under investigation, using various genetic manipulations.


## 요 약

*B. thuringiensis*가 생산하는 살충성 독소 단백질의 생태학적 응용방법을 개발하기 위한 목적으로 우선 독소 단백질 유전자를 옮겨 발현시키기에 적합한 숙주 미생물의 분리작업을 수행하였다. 국내 주요 농산물인 고추, 감자, 무우 등 7가지 농작물의 뿌리 부근에 군락을 형성하는 35종의 형광성 *Pseudomonas*들을 분리하였고 독소 단백질 유전자를 함유하는 재조합 plasmid에 대한 숙주로서의 이용가능성을 검토해 보기 위하여 분리균주 35주에 대한 형질전환을 실시한 결과 4주에 독소 단백질 유전자의 도입이 가능하였고 생물검정과 면역학적인 방법 등에 의한 결과 BT 독소 유전자의 발현을 확인하였다.


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