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Construction of Recombinant *Xanthomonas campestris* Strain Producing Insecticidal Protein of *Bacillus thuringiensis*

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An insecticidal crystal protein gene, *cryIA(c)*, from *Bacillus thuringiensis* HD-73 was integrated into the chromosome of a xanthan-producing bacterium, *Xanthomonas campestris* XP92. The *cryIA(c)* gene expression cassette was constructed that placed the gene between the *trc* promoter and *rrnB* transcriptional terminator. The *lacI*^{q} gene was also included to prevent the expression of *cryIA(c)* gene in *X. campestris* cells. Southern blot analysis confirmed the integration of the *cryIA(c)* gene expression cassette in chromosome of *X. campestris* XP92 transconjugant. Expression of the insecticidal crystal protein was confirmed by Western blot analysis and bioassay against the larvae of *Hyphantria cunea* (Lepidoptera: Arctiidae) and *Plutella xylostella* (Lepidoptera: Plutellidae).

Bacillus thuringiensis is a gram-positive spore-forming bacterium that produces insecticidal crystal proteins (Cry proteins) during sporulation process (34). *B. thuringiensis* strains belonging to more than 30 recognized serotypes have been isolated and classified to date (2). Many strains of this bacterium are active against the larvae of certain members of lepidopteran, dipteran and coleopteran species (16). These biopesticides are considered to be environmentally safe, and have been most successfully used throughout the world for the control of insect pests (20). But several significant limitations have restricted further use of this biopesticide. For example, a problem related to commercial preparations of *B. thuringiensis* has limited its efficacy in the field, as the Cry protein is susceptible to biodegradation and inactivation by sunlight (20). One approach to increase the efficacy of Cry protein is through the expression of insecticidal crystal protein gene (*cry* gene) in bacterial strains that inhabit the same environment as targeted insects.

Xanthomonas campestris is an industrially important bacterium. This bacterium produces xanthan gum, a substance that has been used in the production of petroleum as well as agricultural processes and the food industry as stablizing, viscosifying, emulsifying, thickening and suspending agent (1, 25). The vast quantity of *X. campestris* biomass was produced by industrial fermentors to provide large amounts of pure xanthan (6). This waste biomass was regarded to be useful for the production of insecticidal protein of *B. thuringiensis*.

In this study, we integrated the *cry* gene of *B. thuringiensis* into the chromosome of *X. campestris* using Tn5- derivative. Expression of the *cry* gene was demonstrated by immunoblotting and bioassay against *Hyphantria cunea* and *Plutella xylostella*.

MATERIALS AND METHODS

Bacterial Strains

Xanthomonas campestris XP92 was wild strain isolated from phylloplane of a pine tree. *Escherichia coli* HB101 was used for routine cloning procedures. *E. coli* HB101 carrying the pRK2013 was used in triparental mating as a source of helper plasmid (11). LB medium was used as a general-purpose medium. Nutrient broth-yeast extract (NBY) agar (32) was used for expression of the *cry* gene in *X. campestris* XP92. *E. coli* and *X. campestris* were cultured at 37°C and 28°C, respectively. Antibiotics were used at concentrations of 50 μg/ml for ampicillin and also for kanamycin.

Chemicals, Enzymes, and Nucleic Acids.

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All chemicals used were of analytical grade and were purchased from Sigma Chemical Co., unless specified otherwise. Restriction endonucleases, DNA modifying enzymes, and chemicals used for DNA manipulations were purchased from Boehringer Mannheim Biochemical and were used according to the manufacturer's instructions. Mungbean nuclease was used for removal of single-stranded overhangs from DNA fragments to produce blunt ends. The *cryIA*(c) gene was isolated from pMK74 (21), and *lacI*^{q} gene was derived from a *placI*^{q} plasmid. Construction of *Bgl*II-*Xba*I restricition enzyme sites between *Pst*I-*Hin*dIII of a plasmid pKK233-2 was performed by using two oligonucleotides 5'-GAGATCT-TCTAGAA-3' and 5'-AGCTTTCTAGAAGATCTCTGCA-3'.

Transfer of Plasmid by Conjugation

The plasmid pBCON50 was transferred from *E. coli* to *X. campestris* by triparental mating (24) with the help of pRK2013 (11). Mid-log cultures of the three strains were mixed 1 : 1 : 1 and centrifuged in a microfuge for 1 min, and the bacterial pellets were resuspended in saline solution. The resuspension was spotted onto a nitrocellulose filter (0.45 μm) placed on LB agar and allowed to incubate overnight at 28°C. The bacteria on the filter were resuspended in 0.5 m*l* saline and plated on the Psedomonas Isolation Agar (Difco) containing 50 μg/m*l* of kanamycin.

Southern Hybridization

Total DNA was prepared from *X. campestris* as described by Stock *et al.* (27). Southern hybridization was carried out by separating the restricted DNAs by electrophoresis in 0.8% agarose gels and transferring them onto a nylon membrane by using a vacuum blotter, Trans-Vac TE80 (Hoefer Scientific). Hybridization and detection of hybridized DNA were done by using the Boehringer Nonradioactive DNA Labeling and Detection Kit according to the instuction manual.

Protein and Western Blot Analysis

Recombinant *X. campestris* cells were grown in NBY broth to an A_{600} of 0.5, and expression of the *cryIA*(c) gene was induced by the addition of isopropylthio-β-galactoside (IPTG) at a final concentration of 1 mM. The cells were harvested, washed once in saline solution and resuspended in 10 mM Tris·Cl (pH 8.0), 1 mM EDTA. The ultrasonic treatment was performed until all the cells were completely broken, and then this preparation was used as a sample for bioassay. Protein analysis was performed by electrophoresis on 8.0% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Separated polypeptides were blotted from the gel onto nylon membrane and immunostained using the antibody raised against crystal protein of *B. thuringiensis* subsp. *kurstaki* HD-1 in rabbit and goat anti-rabbit antiboby conjugated with alkaline phosphatase. The bands were visualized in 100 mM Tris·Cl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$ containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Insect Toxicity Assays

Insecticidal activity against *Hyphantria cunea* was measured by using an overlay technique in which the surface of the artificial diet (19) was covered with an aliquot lysate. Toxicity study on larvae of *Plutella xylostella* was done on fresh leaf discs (7 cm^2), onto which 50 μ*l* of diluted samples were applied. Ten third-instar larvae were each placed on a artificial diet or leaf disc, and larval death was monitored after 2 days in the case of *P. xylostella* and after 4 days in the case of *H. cunea*.

RESULTS

Construction of pBCON50

We designed a plasmid pBCON50 based on the broad-host-range plasmid pSUP2021 (26) which can not replicate in non-enteric, gram- negative bacteria and contains a wild-type Tn5 (Fig. 1). A *trc* promoter (17-bp spacing between the *trp* region and *lacUV5* region) and a *rrn*B T^1T^2 terminator of expression vector pKK233-

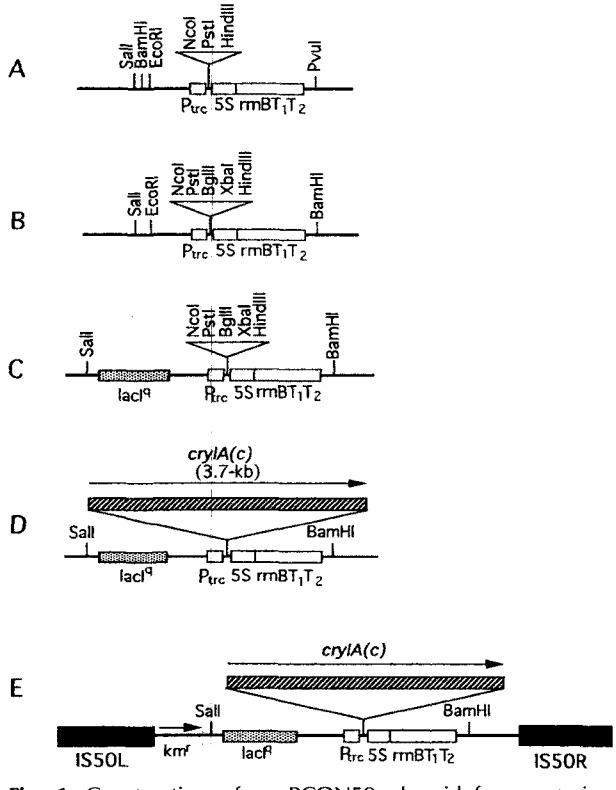

Fig. 1. Construction of a pBCON50 plasmid for expression of *cryIA*(c) gene in *X. campestris*.

Only the subcloned portion was shown. After insertion of *lacI*^{q} and *cryIA*(c) genes through several steps (A-D), a *lacI*^{q}-*trc*-*cryIA*(c)-T_1T_2 cassette was moved into the inner portion of Tn5 (E).

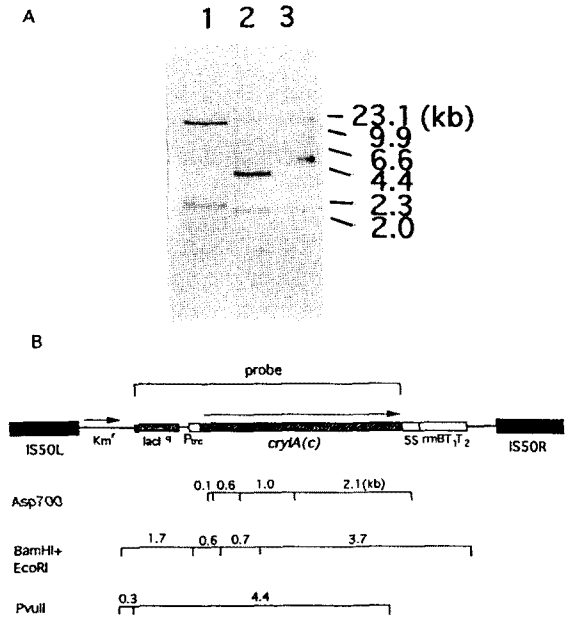

Fig. 2. Southern blot analysis of a putative *X. campestris* XP93::*cry* transconjugant.
(A) The chromosomal DNA was digested with various restriction enzymes. The 3.7-kb *cryIA(c)* gene and the 1.7-kb *lacI*^{q} gene fragments from pBCON50 plasmid were used as a probe. Lanes: 1, *Asp700* (10-kb ISL junction, 2.1, 1.0 and 0.6-kb); 2, *Bam*HI+*Eco*RI (3.7, 1.7, 0.7 and 0.6-kb); 3, *Pvu*II (4.4-kb and two *IS50R* junction). (B) Expected restriction enzyme fragments for the integrated *lacI*^{q}-*trc*-*cryIA(c)*-T_1T_2 cassette were also shown.

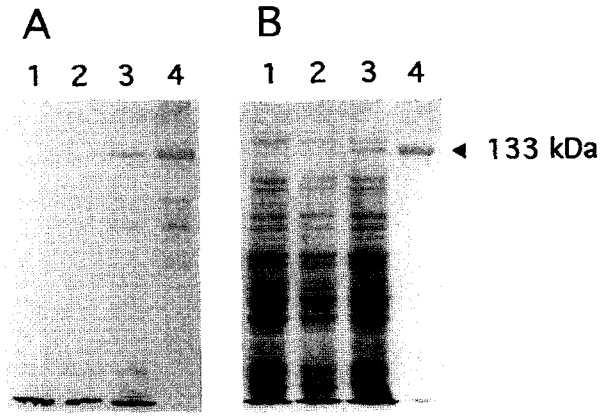

Fig. 3. Expression of insecticidal crystal protein in *X. campestris* XP93::*cry* strain.
(A) SDS-polyacrylamide gel stained with Coomassie brilliant blue. Lanes: 1, *X. campestris* XP93; 2, *X. campestris* XP93::*cry* (not induced); 3, *X. campestris* XP93::*cry* (induced with IPTG); 4, purified crystal protein of *B. thuringiensis* subsp. *kurstaki* HD-1. (B) Western blot of a SDS-polyacrylamide gel identical to that shown in panel A, reacted with polyclonal antibodies directed with crystal of HD-1.

2 (7) were used for expression of *cryIA(c)* gene (Fig. 1A). The 3.7-kb *cryIA(c)* gene fragment was inserted into the pKK233-2 in several steps. First, *Bgl*II-*Xba*I cloning sites were added into the multicloning site of the pKK 233-2 by using a *Pst*I-*Bgl*II-*Xba*I-*Hin*dIII oligonucleotide adaptor to facilitate the subcloning of the *cryIA(c)* gene (Fig. 1B). After *Bam*HI site was removed and a *Pvu*I site was changed to *Bam*HI site (Fig. 1B), a 1.7-kb *lacI*^{q} gene fragment was inserted into a *Eco*RI site (Fig. 1C), and then 3.7-kb *Bam*HI *cryIA(c)* gene fragment was inserted into the *Bgl*II site (Fig. 1D). The *lacI*^{q}-*trc*-*cryIA(c)*-T_1T_2 DNA fragment was eluted by *Sal*I and *Bam*HI site, and finally inserted into the corresponding internal sites of Tn*5* of pSUP2021, generating pBCON50 (Fig. 1E).

Transposition of *CryIA(c)* Gene

Standard triparental matings were performed between *E. coli* HB101 harboring pBCON50, HB101 harboring pRK2013, and *X. campestris* XP92, and kanamycin-resistant transconjugants were selected in Psedomonas Isolation Agar. As the plasmid cannot replicate in *X. campestris* XP92 stains, kanamycin selects for the recombinant cells that the *lacI*^{q}-*trc*-*cryIA(c)*-T_1T2 cassette was integrated into the chromosome. Southern blot analysis was carried out to confirm the presence of *lacI*^{q}-*trc*-*cryIA(c)*-T_1T_2 cassette in the chromosome of *X. campestris* XP92 stains (Fig. 2). Total DNAs prepared from expected *X. campestris* XP92 transconjugant were digested with *Asp* 700, *Bam*HI+*Eco*RI and *Pvu*II and probed with the 3.7-kb *cryIA(c)* gene fragment and the 1.7-kb *lacI*^{q} gene fragment from pBCON50. The probe could detect the expected fragments of expression cassette in the DNA isolated from a putative XP92 transconjugant (Fig. 2). These results confirmed the presence of the *cryIA(c)* expression cassette in XP92 strain and we designated one of these transconjugants as *X. campestris* XP93::*cry*.

Expression of the *CryIA(c)* gene in *X. campestris* XP93::*cry*

X. campestris XP93::cry was examined for expression of the *cryIA(c)* gene by SDS-polyacrylamide gel electrophoresis (Fig. 3A). A expected 133-kDa band correponding to the CryIA(c) protein appeared in lanes 3. As shown in Fig. 3, XP93::*cry* cells did not express the *cryIA (c)* gene until IPTG was added. To verify the expression of the CryIA(c) protein, a Western blot analysis was performed (Fig. 3B). Proteins reactive to polyclonal antibody were seen at 133-kDa from IPTG-induced protein samples. A 133-kDa cross-reactive protein band was also faintly observed in the uninduced protein samples indicating leaky expression of Cry protein.

To test the insecticidal activity of recombinant XP93::*cry* cells, we performed bioassays against larvae of lepidopteran insects (Table 1). Both uninduced and induced XP93::cry cells were toxic towards the larvae of *H. cunea* and *P. xylostella*. The XP93 parental strain was not toxic. The results show that integrated *cryIA(c)* gene cassette was capable of expression of Cry protein in *X. campestris*.

Table 1. Insecticidal activity of *X. campestris* XP93::*cry* transconjugant

	P. xylostella	*H. cunea*
X. campestris XP93	−	−
X. campestris XP93::*cry* (not induced)	++[a]	+
X. campestris XP93::*cry* (induced)	+++	++

[a] Levels of mortality: +, 10~30% mortality; ++, 30~70% mortality; +++, 90~100% mortality.

DISCUSSION

To improve the effectiveness of Cry proteins of *B. thuringiensis*, various groups have investigated the expression of selected *cry* genes in bacteria that inhabit the same environment as targeted insects (13). Efforts to express the *cry* genes in other bacterial systems were based on two different approaches: expression using *cry* gene-carrying plasmid, or using chromosomally integrated *cry* gene. Generally, recombinant plasmids were unstable as the accumulation of generation times (30) and antibiotics were required to prevent the dilution of plasmids. Moreover, horizontal transfer of *cry* gene to other natural bacteria could occur when carried on a mobilizable plasmid, as most of broad-host-range plasmids are. Other researchers have integrated the *cry* genes into chromosomes of the host bacteria using Tn5-mediated system (23), or using homologous recombination between indigenous sequences (33). However, expressions of the Cry protein by integrated *cry* gene tended to be low because of only single copy of *cry* gene present in the chromosome. We integrated the *cry* gene into the chromosome along with a strong *trc* promoter and a T_1T_2 transcriptional terminator. For efficient induction of the *cry* gene expression, *lacI*^{q} repressor gene was also transposed with trc-*cryIA*(c)-T_1T_2 expression cassette. There was also a reason that the *trc* promoter induction system was used for the expression of the *cry* gene. For the efficient production of xanthan by *X. campestris* XP92::*cry* strain, transcription of the *cry* gene must be repressed until enough amount of xanthan was produced. Detectable decrease of growth rate was observed when *cry* gene was expressed continuously (data not shown). Interestingly, many researchers noted that *tac* or *lac* promoter upstream of *cry* gene was not repressed by *lacI*^{q} gene and consequently Cry protein was produced continuously in *E. coli* expression system (12, 22, 31). It is apparent that *lac* repressor gene or its product was not functional in these cases. In this study, although leaky expression of the Cry protein was detected by immunoblot analysis, both *trc* promotor and *lacI*^{q} gene products were functional in *X. campestris* XP93::*cry*.

Steps in xanthan biosynthesis identified are very similar to those in the biosynthesis of exopolysaccharide of other gram negative bacteria (28) and a number of genes involved in xanthan biosynthesis are clustered together (15, 29). These gene cluster regions are suggested to be favored targets for insertional sequence elements (17) and thus instability in exopolysaccharide production has been reported for several organisms, including *Pseudomonas aeruginosa* (9, 14), *Pseudomonas atlantica* (3, 4), *Zoogloea ramigera* (10) and *X. campestris* (18). Tn5 transposes at a high frequency in a variety of gram-negative bacteria with a low insertional specificity (5) and it is possible that our Tn5-based *cry* gene expression cassette can inactivate the xanthan biosynthesis function of wild strain. In fact, many clones of recombinant *X. campestris* XP92 cells did not seem to produce xanthan during the cultivation (data not shown). Therefore, a number of transconjugants should be tested for normal production of xanthan.

X. campestris is the phytopathogenic bacterium causing black rot in crucifers (8), so cells must be killed before release of preparations. Xanthan is usually recovered from cultures by precipitation with isopropanol or methanol, and cells are killed during this step.

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