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Expression of a Recombinant *Bacillus thuringiensis* δ-Endotoxin Fused with Enhanced Green Fluorescent Protein in *Escherichia coli*

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The expression of a fusion protein comprised of the *B. thuringiensis* crystal protein, Cry1Ac, and enhanced green fluorescent protein (EGFP) in *Escherichia coli* XL1-blue was examined. Three recombinant plasmids were transformed into *E. coli* XL1-blue and named as ProAc/Ec, MuEGFP/Ec and ProMu-EGFP/Ec, respectively. All transformants were observed by light and fluorescence microscopy at mid-log phase. The expression in *E. coli* transformants, ProMu-EGFP/Ec and MuEGFP/Ec, exhibited bright enough fluorescence to be observed. Furthermore, ProMu-EGFP/Ec produced fluorescent inclusions, which may have been recombinant crystals between EGFP and Cry1Ac while MuEGFP/Ec expressed soluble EGFP in cell. In SDS-PAGE, ProAc/Ec had 130 kDa crystal protein band and MuEGFP/Ec had thick 27 kDa EGFP band. However, ProMu-EGFP/Ec had about 150 kDa fusion protein band. Accordingly, these results indicated that a fusion protein between the *B. thuringiensis* crystal protein and a foreign protein under the *lacZ* promoter was successfully expressed as granular structure in *E. coli*. It is suggested that the *E. coli* expression system by N-terminal fusion of *B. thuringiensis* crystal protein may be useful as excellent means for fusion expression and characterization of *B. thuringiensis* fusion crystal protein.

Key words: *Bacillus thuringiensis*, *E. coli* XL1-blue, Cry1Ac, EGFP, fusion expression

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Introduction

During sporulation, *Bacillus thuringiensis* synthesizes one or more proteinaceous crystalline inclusions that are toxic to certain insect larvae (Höfte and Whiteley, 1989). The many subspecies of *B. thuringiensis* that are toxic to lepidopteran larvae typically contain bipyramidal crystals composed of protoxins of 130 – 140 kDa encoded by one or more plasmid-borne genes (named as *cry* genes or ä-endotoxin genes). In *B. thuringiensis*, *cry* genes are expressed differentially by poorly understood control mechanisms. Thus, the specificity of *B. thuringiensis* against insect host varies, depending not only upon the presence of a particular *cry* gene, but also upon the level of expression of a *cry* gene. For instance, *cry1Ac* gene, although present, is believed to be poorly expressed in strain *B. thuringiensis* subsp. *kurstaki* HD-1 (Yamamoto *et al.*, 1988). Furthermore, Cry2A and Cry2B proteins also vary among different strains (Yamamoto and Iizuka, 1983).

Generally, *Escherichia coli* is an effective expression system for *cry* genes, because the parameters of the biosynthetic processes are better understood in this bacterium. There are a lot of reports in which the cloning and expression of novel *B. thuringiensis* toxin gene have been performed mainly to attempt their characterization (McPherson *et al.*, 1988; Shimizu *et al.*, 1988; Ge *et al.*, 1990; Brizzard *et al.*, 1991; Ben-Dov *et al.*, 1994). The use of *E. coli* as an expression host allows the selective production of Cry proteins with particular insecticidal specificity for their individual study. In addition, it can also be used as an effective vehicle for industrial production of crystal proteins, functionally improved by genetic and protein engineering (Caramori *et al.*, 1991; Bosch *et al.*, 1994; Aronson *et al.*, 1995; De Maagd *et al.*, 1996).

To understand expression mode of a *B. thuringiensis*

fusion gene, the *cry1Ac* gene was fused with enhanced green fluorescence protein (EGFP) gene. The EGFP gene encodes the GFPmut1 variant, which contains the double-amino acid substitution of Phe-64 to Leu and Ser-65 to Thr and, consequently has a single, red-shifted excitation peak that fluoresces about 35 times more intensely than native GFP (Chalfie *et al.*, 1994) when excited at 488 nm (Cormack *et al.*, 1996). In this study, experiments were performed to make an N-terminally fused recombinant crystal protein gene containing EGFP (EGFP-Cry1Ac fusion) and to characterize the fusion protein expressed in *E. coli* XL1-blue.

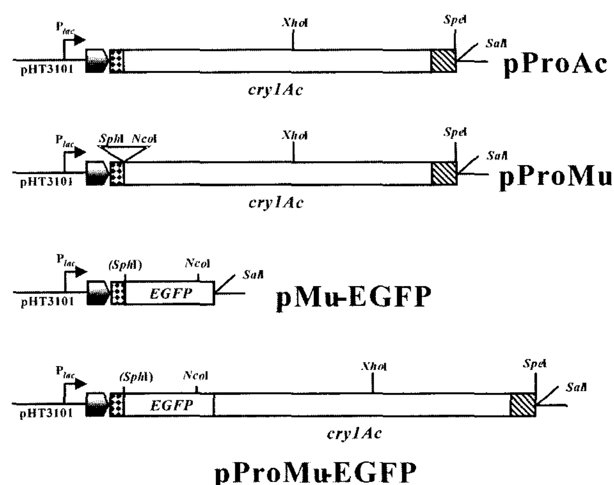

Fig. 1. Summary of used recombinant plasmids for fusion expression between Cry1Ac and EGFP. A 3.8 kb *cry1Ac* fragment contained the *cry1Ac* coding region (open box), promoter region (dark box), ribosome binding site (RBS, dotted box), and the terminator (lined box). P_{lac} and arrow indicate the promoter of *lacZ* gene and its direction of transcription.

Materials and Methods

Bacterial strains and genes

The *E. coli* strain used as the host for the transformation and expression of the recombinant plasmids was *E. coli* XL-1 blue [*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi*-1 *relA*1 *lac*⁻ F' (*proAB*⁺ *lacI*^{q} *lacZ*△M15 Tn*10*-*tet*^{r})]. *E. coli* was grown at 37°C with vigorous shaking in LB medium for expression of fusion protein.

The *cry1Ac* gene (Genbank Accession No. M11068, Adang *et al.*, 1985) was cloned from *B. thuringiensis* subsp. *kurstaki* HD-73. The EGFP gene was amplified from the plasmid pEGFP (Clontech Co., USA).

Plasmids and transformation

The plasmid used to express the *cry1Ac* in *E. coli* strain was the *E. coli-B. thuringiensis* shuttle vector, pHT3101 (Lereclus *et al.*, 1989). This vector had promoter (P_{lac}) and the amino-terminal fragment of the *lacZ* gene (*β-galactosidase* gene) originated from pUC18 as well as genetic markers, *β*-lactamase gene (Amp^r) and erythromycin resistance gene (Em^r), and origins of *E. coli* and *B. thuringiensis*. The recombinant construct, pProAc was composed of the *cry1Ac* gene including ribosome binding site and terminator region under the control of the native promoter in pHT3101 (Roh *et al.*, 2000) and pProMu had identical structure with pProAc except for two restriction enzyme sites, *Sph*I and *Nco*I, between ribosome binding site and the initiation codon of pProAc. pProMu was just used for construction of pMuEGFP and pProMu-EGFP. The PCR-amplified EGFP fragment was transferred to the restriction sites, *Sph*I (fill in with Klenow) and *Sal*I, of pProMu (named as pMuEGFP). Finally, the *cry1Ac* full coding region containing the terminator region of pProMu was ligated with *Nco*I and *Sal*I sites of pMuEGFP (named as pProMu-EGFP). Schematic structures of recombinant plasmids were shown in Fig. 1.

The preparation and transformation of competent *E. coli* cell and transformation was performed according to simply modified Cohens method (1972).

Microscopy

Microscopy of *E. coli* transformants with fusion plasmids was performed using a light and fluorescent microscope (Axiophot Universal Microscope, Zeiss, Germany). For observation, *E. coli* cultures were collected at 16 hrs after inoculation.

SDS-PAGE

E. coli transformants were grown in LB medium and sampled at 16 hrs after inoculation. The cultures were harvested and washed 3 times with a washing solution with PBS (phosphate buffered saline, pH 7.4). And cells were sonicated three times (22,000 cycle/sec for 1 min) and then mixed with an equal volume of an SDS sample buffer (4% SDS, 4% mercaptoethanol, 100 mM Tris-HCl, pH 8). After boiling for 5 min, each sample was loaded onto an SDS-12% PAGE gel. The SDS-PAGE was performed on a 12% polyacrylamide separating gel with a 3% stacking gel, as described by Laemmli (1970). The gel was stained with Coomassie brilliant blue and a 10 kDa protein ladder (Difco Co., USA) was used as the standard.

Results and Discussion

The structure of pProMu was almost similar with of pProAc since it had two restriction enzyme sites, *Sph*I and

*Nco*I (Fig. 1). Thus, except for pProMu, three recombinant plasmids were transformed into *E. coli* XL1-blue and named as ProAc/Ec, MuEGFP/Ec and ProMu-EGFP/Ec, respectively. The presence of a recombinant plasmid in each *E. coli* transformant was confirmed by a PCR with a set of oligonucleotide primers to detect the presence of the EGFP gene and *cry1Ac* gene (data not shown).

The action mechanism of *B. thuringiensis* Cry proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry toxin to midgut receptor, and insertion of the toxin into the apical membrane to create ion channels or pores (Schnepf *et al.,* 1998). The mature Cry1Ac protein, at least, is cleaved at Lys623 on the carboxy-terminal end (Bietlot *et al.,* 1989). Also, Cry1A toxin is cleaved at Arg28 at the amino-terminal end (Nagamatsu *et al.,* 1984). Accordingly, EGFP gene was inserted in front of N-terminus of *cry1Ac* by translation fusion manner because N-terminal fusion would not affect the inherent insecticidal activity of Cry1Ac.

All transformants were observed by light and fluorescence microscopy at mid-log phase. The expression in *E. coli* transformants, ProMu-EGFP/Ec and MuEGFP/Ec,

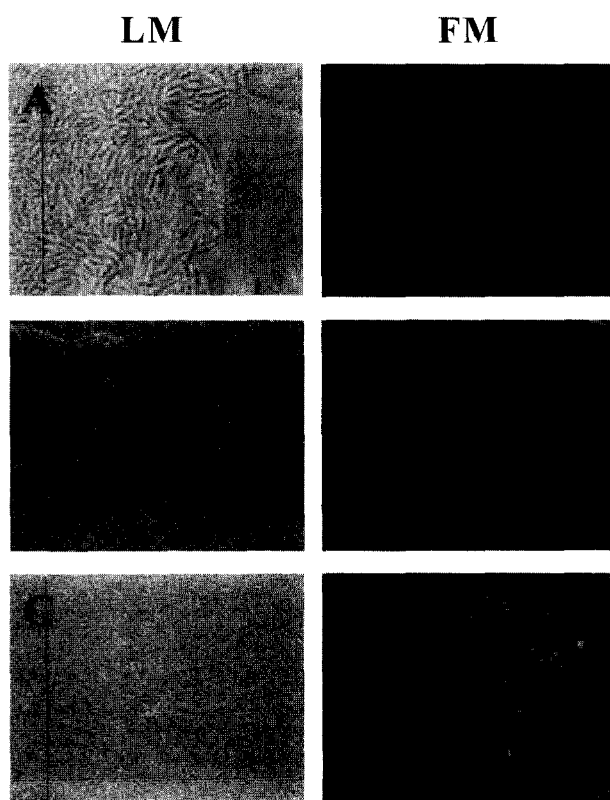

Fig. 2. Light (LM) and fluorescent (FM) micrographs of *E. coli* XL-1 blue strains transformed with pProAc (A), pMuEGFP (B), and pProMu-EGFP (C).

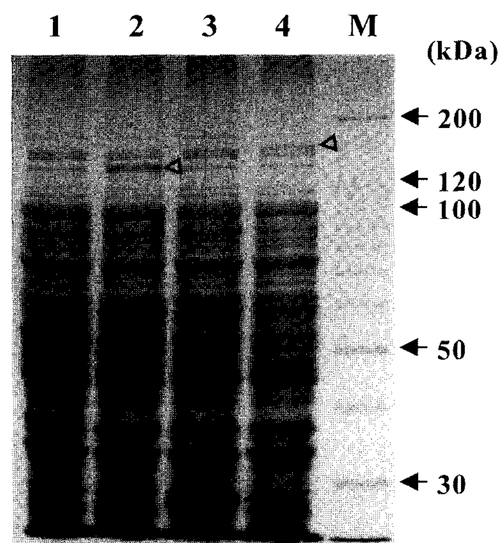

Fig. 3. SDS-PAGE of the *E. coli* transformants. Lane 1: *E. coli* XL1 blue; 2: *E. coli* transformed with pProAc; 3: pMuEGFP; 4: pProMu-EGFP. M indicates 10 kDa protein ladder. Arrow head indicates the expressed proteins in *E. coli* transformants.

exhibited bright enough fluorescence to be observed (Fig. 2). MuEGFP/Ec produced soluble EGFP protein in whole cell (Fig. 2B). However, ProMu-EGFP/Ec produced fluorescent inclusions, which may have been recombinant crystals between EGFP and Cry1Ac while ProAc/Ec could not find any particular inclusions (Fig. 2). Moreover, the cell precipitate of ProMu-EGFP/Ec after sonication had bright fluorescence on UV light while the supernatant of MuEGFP/Ec had fluorescence.

SDS-PAGE of cell extracts provided for the evidence of EGFP fusion protein expression in *E. coli* cells (Fig. 3). ProAc/Ec had 130 kDa crystal protein band and MuEGFP/Ec had thick 27 kDa EGFP band. Expectedly, ProMu-EGFP/Ec had over 150 kDa fusion protein band. Accordingly, these results indicated that a fusion protein between the *B. thuringiensis* crystal protein and a foreign protein under the *lacZ* promoter was successfully expressed as granular structure in *E. coli.*

Despite the advantages of the biodegradability, selectivity and safety for humans and the environment, the use of *B. thuringiensis*-based insecticides has several limitations. These include short-time field persistence, mainly because the susceptibility of the toxin to environmental conditions such as ultraviolet radiation from sunlight and in some cases, the inaccessibility of the target pests inside plant stems and leaves to the toxin (stem borer, leaf mining). As a solution, heterologous expression of *cry* genes has been studied in many microorganisms (Prieto-Samsónov *et al.,* 1997). Therefore, an effective expression system for *cry* genes in *Escherichia coli,* where the

parameters of expression are better understood, will allow selective expression of *cry* genes of particular host specificity and facilitate the study of their gene product. Furthermore, it can also serve as an effective vehicle for industrial production to deliver genetically modified and functionally improved crystal proteins as a result of protein engineering.

In conclusion, our results clearly demonstrated that the fusion protein with Cry1Ac and EGFP, had its own fluorescence activity and made granular structure in *E. coli*. It is suggested that the *E. coli* expression system was excellent tools for expression of the fusion *B. thuringiensis* crystal protein with a foreign protein having different mode of action of insecticidal activity. The *E. coli* expression system by N-terminal fusion of *B. thuringiensis* crystal protein may be useful as excellent means for fusion expression and characterization of *B. thuringiensis* fusion crystal protein.

Acknowledgments

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