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# Spacing Effect of the Intervening Sequences between Ribosome Binding Site and the Initiation Codon on Expression of *Bacillus thuringiensis* δ-Endotoxin

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**To verify importance of the intervening sequence between the ribosome binding site (RBS) and the initiation codon for expression of *Bacillus thuringiensis* δ-endotoxin, the pProMu, containing *Sph*I and *Nco*I sites between RBS and the initiation codon of the *cry1Ac* gene, and the deletion derivatives of pProMu were constructed and transformed into the *B. thuringiensis* subsp. *kurstaki* Cry⁻B strain. The pProMu-Δ*Sph*I had identical six bases of intervening sequence to pProAc though the arrangement of sequence was different. Other mutants containing pProMu had 1 or 10 or 14 bases between RBS and the initiation codon. Among deletion mutants, only ProMu-Δ*Sph*I/CB only produced 130 kDa typical bipyramidal crystals like those seen for ProAc/CB. However, ProMu/CB, ProMu-Δ*Nco*I, and ProMu-Δ*Sph*I+*Nco*I did not produce Cry1Ac crystals. In conclusion, the results suggest that 6-base intervening sequence was important for expression of *cry1*-type class gene. Furthermore, spacing effect of the intervening sequences may play an important role in expression of individual crystal proteins in *B. thuringiensis* without doubt.**

**Key words**: *Bacillus thuringiensis*, δ-Endotoxin, Ribosome binding site, Initiation codon, Intervening sequences, Spacing effect

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## Introduction

Insecticidal crystal proteins from *Bacillus thuringiensis* have been used intensively as biopesticides for the several decades. A general feature of the *cry* genes is their expression during the sporulation period. Crystal proteins generally accumulate in the mother cell compartment to form a crystal inclusion that can account for 20 to 30% of the dry weight of a sporulated cell (Schnepf *et al.*, 1998). The very high level of crystal protein synthesis in *B. thuringiensis* and its coordination with the stationary phase are controlled by a variety of mechanisms occurring at the transcriptional, posttranscriptional, and posttranslational levels (Agaisse and Lereclus, 1995; Baum and Malvar, 1995).

Among these controls, the current knowledge concerning the process of translation in *B. thuringiensis* mainly comes from untranslated regions of the *cry* transcripts. Two specific regions of the *cry* gene mRNAs are involved in remarkable stability, which are found in the 3' and 5' untranslated region. The *cry1Aa* transcriptional terminator (3' positive retroregulator) increase the *cry* mRNA stability by protecting it from 3' to 5' exonuleolytic degradation by forming stable stem-loop structures (Wong and Chang, 1986). Also, the 5' untranslated region of the *cry3A* mRNA has all the characteristics of a 5' mRNA stabilizer (Agaisse and Lereclus, 1996).

Furthermore, this fact was generally known that spacing of intervening sequences between the ribosome binding site (RBS) and the initiation codon would affect the rate of the first-order rearrangement to an initiation complex with the start codon base paired to the anticodon of the tRNA (Vellanoweth, 1993). However, the study on spacing between the RBS sequence and the initiation codon in the

**Table 1.** Primers used to insert restriction enzyme sites, *Sph*I and *Nco*I between the ribosome binding site and the initiation codon of the *cry1Ac* gene

| Primer | Sequence[a] |
|---|---|
| M13R | 5'-AACAGCTATGACCATG-3' |
| ISR | 5'-CTTTTAGAGAGTGGGAAGCAGATCC-3' |
| 1Ac-MuF | 5'-GAGAT**GGAGG**CATGCCTTCC**ATG**GATAACAATCC-3' |
| 1Ac-MuR | 5'-GGATTGTTATC**CAT**GGAAGGCATG**CCTCC**ATCTC-3' |

*The ribosome binding site (GGAGG) and start codon (ATG) are in bold face type, and restriction enzyme sites for *Sph*I and *Nco*I are underlined.

translation of *B. thuringiensis* has not been reported even though the adaptable intervening sequences would be important to express individual δ-endotoxin gene. Therefore, in the present study, to verify the spacing effect of the intervening sequences, we constructed pProMu, containing two restriction enzyme sites (*Sph*I and *Nco*I) between RBS and the initiation codon the *cry1Ac* coding region under the control of the native promoter, and several mutants derived from pProMu. All mutant constructs were expressed in *B. thuringiensis* subsp. *kurstaki* Cry$^-$B strain and were compared with the non-mutational constructs, pProAc.

## Materials and Methods

### Bacterial strains and transformation

*B. thuringiensis* subsp. *kurstaki* Cry$^-$B (Cry$^-$B) was used as the host for the expression of the fusion protein. The Cry$^-$B transformant (ProAc/CB) transformed with pProAc was used as control strain (Roh *et al.*, 2000). *B. thuringiensis* was grown at 30°C with vigorous shaking in SPY medium for plasmid preparation and GYS medium for expression of crystal proteins.

Electroporation was performed according to the method of Lereclus *et al.* (1989), with a slight modification. *B. thuringiensis* cells were grown to an $OD_{600}$ of 1 in 100 ml of a Brain Heart Infusion (BHI, Difco Co., USA) with shaking at 30°C. The cells were harvested and washed once in 10 ml of cold distilled water. The pellet was then resuspended in 4 ml of cold sterile polyethyleneglycol (PEG) 6000 (40%, w/v). Thereafter, cell aliquots of 0.4 ml were mixed with the plasmid DNA in 0.2 cm electroporation cuvettes (Bio-Rad Co., USA) at 4°C. The Bio-Rad Gene Pulser apparatus was set at 25 μF and 2.5 kV, and the pulse controller was set to 400 Ω. The cuvette was placed in the safety chamber and the pulse was applied once. Following electroporation, the cells were diluted in 2 ml of a pre-warmed BHI medium and incubated for 2 h at 30°C. After this expression period, the cells were then plated on a nutrient agar medium (Difco Co., USA) supplemented with erythromycin (25 g/ml) for growth and sporulation.

### Oligonucleotides, PCR and plasmids

For construction of pProMu, pXV, pAmyAc and pProAc, were used from previous report (Roh *et al.*, 2000). To construct pProMu, about 550 bp Mu-fragment containing the *cry1Ac* promoter, a part of *cry1Ac* coding region and the restriction enzyme sites (*Sph*I and *Nco*I) between RBS and the initiation codon, was first amplified by 2-step PCR from pProAc as a template. Used primers are shown in Table 1. Polymerase chain reaction (PCR) amplification was performed, using Pyrobest™ DNA polymerase (Takara Co., Japan), for the amplification of the Mu-fragment for 30 cycles as follows: 98°C for 10 sec, 55°C for 30 sec and 72°C for 1 min.

For deletion derivatives of pProMu, after digestion with a restriction enzyme, pProMu was treated with the Klenow fragment or T4 DNA polymerase and ligated with T4 DNA ligase. All modified enzymes were used according to manufacturer's manuals (Takara Co., Japan).

### Phase contrast microscopy

The development stage of *B. thuringiensis,* parasporal inclusions in sporulated cell and autolysis were monitored with a phase contrast microscope Nikon Type 104 (Nikon Co., Japan) using a × 100 oil-immersion objective.

### SDS-PAGE

*B. thuringiensis* strains were grown in GYS medium and sampled at 72 hrs. The spore-crystal mixtures were harvested and washed 3 times with a washing solution (0.5 M NaCl, 2 mM EDTA). And sporulated 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 a 12% SDS-polyacrylamide separating gel with a 3% stacking gel. The gel was stained with Coomassie brilliant blue and

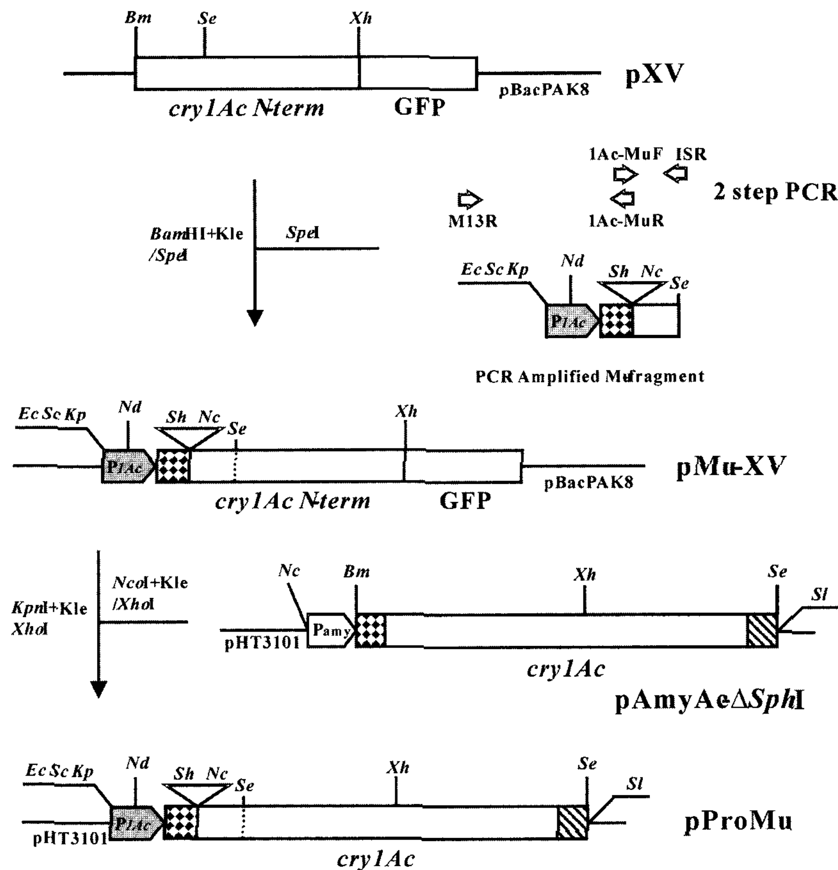


**Fig. 1.** Construction of pProMu vector. A 3.8 kb *cry1Ac* fragment in pProMu contained the *cry1Ac* coding region (open box), promoter region (dark box), ribosome binding site (RBS, dotted box), and the terminator (lined box). The open arrows in 2-step PCR indicate the designed primers for amplification of Mu-fragment. Restriction enzymes are abbreviated as follows. *Bm*, *Bam*HI; *Se*, *Spe*I; *Xh*, *Xho*I; *Ec*, *Eco*RI; *Sc*, *Sac*I; *Kp*, *Kpn*I; *Nd*, *Nde*I; *Sh*, *Sph*I; *Nc*, *Nco*I; *Sl*, *Sal*I; and Kle, Klenow fragment.

a 10 kDa protein ladder (Difco Co., USA) was used as the standard.

## Results and Discussion

To construct pProMu containing the restriction enzyme sites (*Sph*I and *Nco*I) between RBS and the initiation codon in pProAc, the Mu fragment containing *Sph*I and *Nco*I site between RBS and the initiation codon was amplified and cloned into *Bam*HI (fill in with Klenow) and *Spe*I (pMu-XV). The cloned *cry1Ac* N-terminal fragment in pMu-XV was transferred into the *Sph*I-deleted pAmyAc (pAmyAc-Δ*Sph*I). The pProMu was successfully constructed and confirmed by restriction enzyme patterns and nucleotide sequencing (Fig. 1).

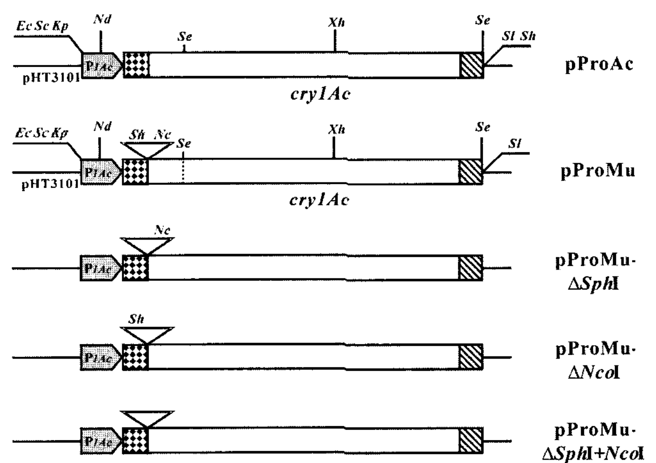


**Fig. 2.** Summary of pProMu deletion-variants.

The deletion derivatives of pProMu were constructed (Fig. 2) and transformed into the Cry⁻B strain for verification of importance on intervening sequence between RBS and the initiation codon. The intervening sequences of pProMu and its deletion variants were represented in Fig. 3. The pProMu-Δ*Sph*I had identical six bases of intervening sequence to pProAc though the arrangement of

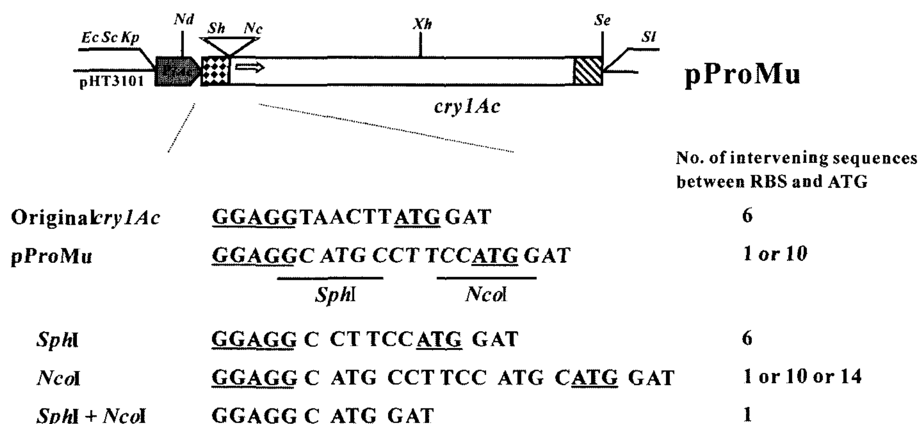


**Fig. 3.** Nucleotide sequences between the ribosome binding site and the initiation codon of the pProMu deletion-variants. The original *cry1Ac* sequence means that of pProAc.

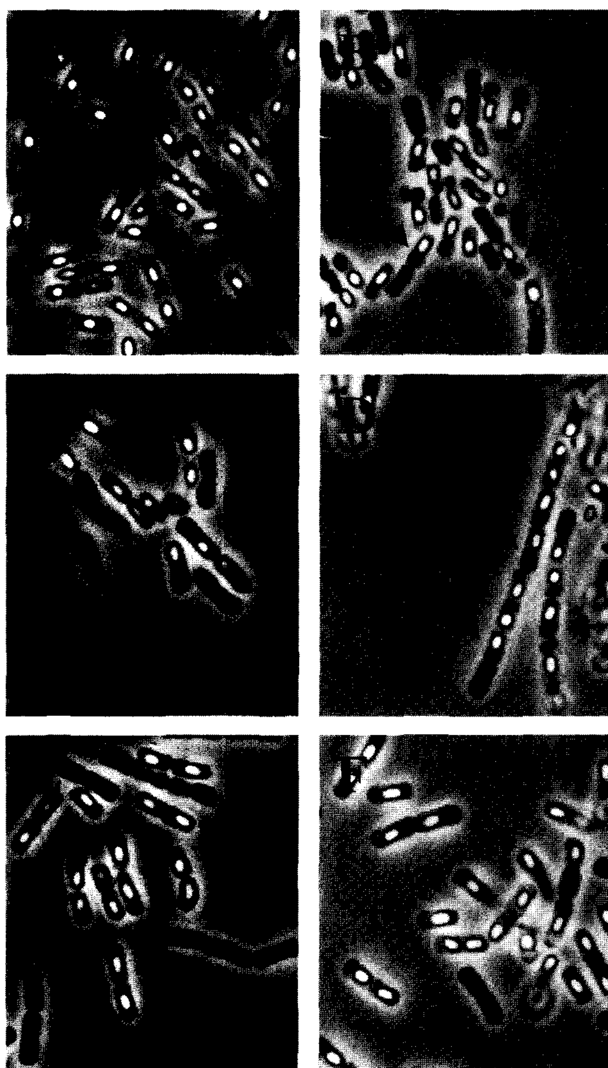


**Fig. 4.** Phase contrast micrographs of the wild-type Cry⁻B strain (A), and the Cry⁻B transformants (B: ProAc/CB; C: ProMu/CB; D: ProMu-ρ*Sph*I/CB; E: ProMu-Δ*Nco*I/CB; F: ProMu-Δ*Sph*I +*Nco*I/CB). Arrows indicate parasporal inclusions.

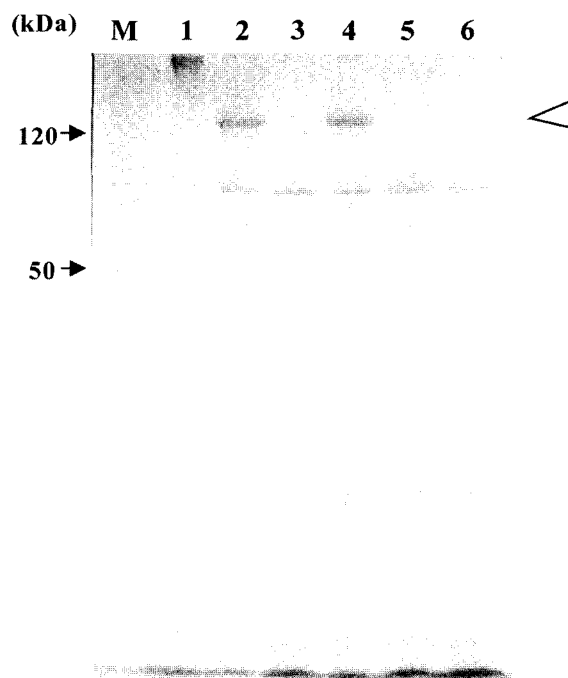


**Fig. 5.** SDS-PAGE analysis of the Cry⁻B strains transformed with pProMu deletion-variants. Lane 1: Cry⁻B; 2: ProAc/CB; 3: ProMu/CB; 4: ProMu-Δ*Sph*I/CB; 5: ProMu-Δ*Nco*I/CB; 6: ProMu-Δ*Sph*I+*Nco*I/CB; M: 10 kDa ladder. Arrowhead indicates Cry1Ac protein.

sequence was different. Other mutants containing pProMu had 1 or 10 or 14 bases between RBS and the initiation codon (Fig. 3).

Among deletion mutants, only ProMu-Δ*Sph*I/CB only produced 130 kDa typical bipyramidal crystals like those seen for ProAc/CB (Fig. 4 and 5). ProMu/CB, ProMu-ρ*Nco*I, and ProMu-Δ*Sph*I+*Nco*I did not produce Cry1Ac crystals. Though pProMu and three deletion mutants had the entire promoter, a full coding region, and the termi-

nator of the *cry1Ac*, they could not express the Cry1Ac protein except for the pProMu-Δ*Sph*I mutant (Fig. 4 and 5). Similarly, spacing effects by intervening sequences between RBS or Shine-Dalgarno (SD) sequence and the initiation codon, have been tested experimentally in *E. coli* (Thomas *et al.*, 1982; Wood *et al.*, 1984). Remarkably, Hartz *et al.* (1991) found that in *E. coli*, spacing in the range of 6 to 10 nucleotides had a minor effect on translational yield. In *B. subtilis*, taxonomically close to *B. thuringiensis*, the optimum spacing was around 7 to 9 bases, which is consistent with the average spacing found in the sequenced genes (Vellanoweth and Rabinowitz, 1992).

In the δ-endotoxin genes, the lengths of intervening sequences between putative ribosomal binding site (GGAGG) and initiation codon (AUG) were various. Most genes of *cry1* class had 6 base intervening sequences. But *cry2* and *cry9* classes had 8 bases and, *cry3* and *cry4* had 7 bases. Interestingly, *cry11* and *cyt* class had more 10 bases putative intervening sequence. However, 6-base intervening sequence was relatively conserved in *cry1*-type gene class except for *cry1B*, *cry1I* and *cry1K,* having 7 bases (Genbank accession No. X95704, X62821 and U28801), regardless of the base types. Especially, the first and second bases, TA, and the fifth and sixth, TT, among 6-bases intervening sequence, were highly conserved in *cry1* class. In this consideration, the intervening sequence would be changeable according to individual *cry* gene but at least, 6-base intervening sequence was important for expression of *cry1*-type class gene. Therefore, in *B. thuringiensis*, spacing effect of the intervening sequences may be very important to expression level of individual crystal proteins without doubt and should be investigate prior to expression under other promoter.

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