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Development of *Bacillus thuringiensis* var. *kurstaki* NRD 6-Stu 1 by Site-Directed Mutagenesis

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위치지정 변이기법을 이용한 *Bacillus thuringiensis* var. *kurstaki* NRD 6-Stu 1의 육성

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A cloned delta-endotoxin gene from *Bacillus thuringiensis* var. *kurstaki* NRD 6 was mutated at N-terminal toxic portion of the protoxin by site-directed mutagenesis. A Stu 1 restriction site was created in protoxin region of the mutant *B.t.kurstaki* NRD 6-Stu 1 which changed A to C in 177 region of the protoxin(silent mutation). Antigenic property of crystalline inclusions (delta-endotoxin) of the mutant was no difference to intact strain by the western blot analysis and its toxidity threshold value was also showed 0.015~0.030ng against *Choristoneura femiferana-1* insect, similar with 0.01~0.024ng of parent strain, but stronger than that of *B.t.kurstaki* NRD 5 (500~1000ng) and *B.t. kurstaki* NRD 4(none toxic).

위치지정 변이기법을 이용하여 *Bacillus thuringiensis* var. *kurstaki* NRD 6의 내독소 유전자의 N말단 독성부위를 변이시켜 *Bacillus thuringiensis* var. *kurstaki* NRD 6-Stu 1을 육종하였다. 내독소에 대한 염기서열 조사결과, 변이주의 내독소 유전자부위에 Stu 1 인식부위가 생성되었고 특히, 이 부위의 177번 염기 A가 C로 치환된 silent mutation이 일어났음을 확인하였다. 변이주의 내독소 유전자의 항원성질은 친주와 차이가 없었고 *Choristoneura femiferana-1*에 대한 독성도는 0.015~0.030ng으로 친주의 0.01~0.024ng과 유사하였으나 다른 친주계열인 *B.t.kurstaki* NRD 5의 500~1000ng과 *B.t. kurstaki* NRD 4(무독성)보다 강하였다.

Key words : *Bacillus thuringiensis* var. *kurstaki* NRD 6-Stu 1, Site-Directed Mutagenesis

For many years, formulation of *Bacillus thuringiensis (B. t.)*, sporulating gram positive bacterium, have been used worldwide to control a variety of foliage lepidopterans (moths and budworms) and of dipterans (mosquitoes and blackflies).[1,2]

It has been known that certain *B. thuringiensis* subspecies contain more than one endotoxin and its delta-endotoxin gene have been isolated, sequenced and expressed in *Escherichia coli*[3~9], *Bacillus subtilis*[10,11] and *Pseudomonas fluorescens*[12] and host ranges can also vary substantially.

Delta-endotoxin that is found as a parasporal crystalline inclusion body in *Bacillus thuringiensis* var. *kurstaki (B. t. kurstaki)* has potent insecticidal activity toward lepidopteran larve.[13]

The major component of the crystals is a protoxin protein of molecular mass approx. 130~140 KDa (P_1 toxin) and upon ingestion by insect larve, the crystals are subjected to the alkaline pH and enzymes of the insect gut and as a results are cleaved with the release of 68 KDa of a toxic peptide (P_2 toxin).

B. t. kurstaki HD1 produces bipyramidal delta-endotoxin (protoxin peptides) encoded by homologous crystal protein genes and cuboidal crystals containing a 66 KDa polypeptide which is toxic to both lepidopteran and dipteran larve.

The three cp genes of HindⅢ hybridizing fragments of 4.5 Kb, 5.3 Kb and 6.6 Kb (or cryA1, cryA2 and cryA3) have been identified in the *B. t. kurstaki* NRD 12 strain, a similar to the one found in the well-studied *B. t. kurstaki* HD-1 strain.[14],[15] Hefford et al.[16] reported on the cloning and nucleotide sequencing of a 5.3 type toxin gene of *B. t. kurstaki* NRD12.

Meanwhile, in our country, Lee et al.[17~19] reported on the cloning of *B. t. kurstaki* insecticidal protein gene, electrophoresis analysis and condition of delta-endotoxin production, and Oh et al.[20, 21] also reported on immunological analysis of endotoxin proteins produced by *B. t. kurstaki* HD1 and HD73, and entomocidal protein gene localization of *B. t. kurstaki* HD73. Recently, Park et al[22] reported construction of shuttle promoter-probe and expression vector for *E. coli* and *B. subtilis*, and expression of *B. t. kurstaki* HD73 crystal protein gene in the two species.

In this study, the cloned delta-endotoxin gene of *B. t. kurstaki* NRD 6 was mutated at a specific site near the N-terminal toxic portion of the protoxin in order to better understanding of the structure and function relationship of the *B. t. kurstaki* toxins and to investigate whether site specific mutation of the toxin gene has an effect on the gene expression and the toxidity threshold, and to introduce a cloning site for controlling expression of the gene in bacteria. For this purposes, Stu 1-specific mutagenesis, selection of mutants and DNA sequencing of protoxin gene, purification of inclusion body and following western blot analysis and bioassay for insect toxidity were performed.

Materials and methods

1. Bacterial strains

B. t. kurstaki NRD 6 was obtained from Natl. Res. Council Canada, Biotech. Res. Inst., which was cloned 6.6 Kb protoxin gene of *B. t. kurstaki* NRD12 in pUC18 and transformed into *E. coli* HB101. *E. coli* HB101 was used as host for cloning purpose.

2. Synthesis of oligonucleotides

The oligonucleotides containing Stu 1 recognition site was synthesized at the Biotech. Res. Inst. of Natl. Res. Council Canada and purified by electrophoresis on a 20% polyacrylamide gel. The product band visualized by UV-shadowing on a fluores-

cent thin layer chromatography plate was excised and the material was eluted by soaking.

3. Site-directed mutagenesis of the protoxin gene of *B. t. kurstaki* NRD 6

Plasmid of *B. t. kurstaki* NRD 6 was isolated by method of Birnboim et al.[23] and digested with Scal and purified. Meanwhile, after digestion of the plasmid with Nsi 1 and Spe 1, large fragment was also isolated electrophortically and purified. Scal fragment described above and large fragment of Nsil and Spel and sequence of the oligonucleotide 5'-TGGATTTGTGTTAGGCCTAGTTAA-3' (__; Stu 1 recognition site) were mixed by 1:10:1000 and boiled for 5 min, annealed and cooled slowly and transformed then into *E. coli* HB101. Plasmids of all transformanants were isolated and digested with Stu 1 and we selected mutant containing a Stu 1 recognition site (designed as *B. t. kurstaki* NRD 6-Stu 1) from investigation of its digestion pattern. (Fig. 1)

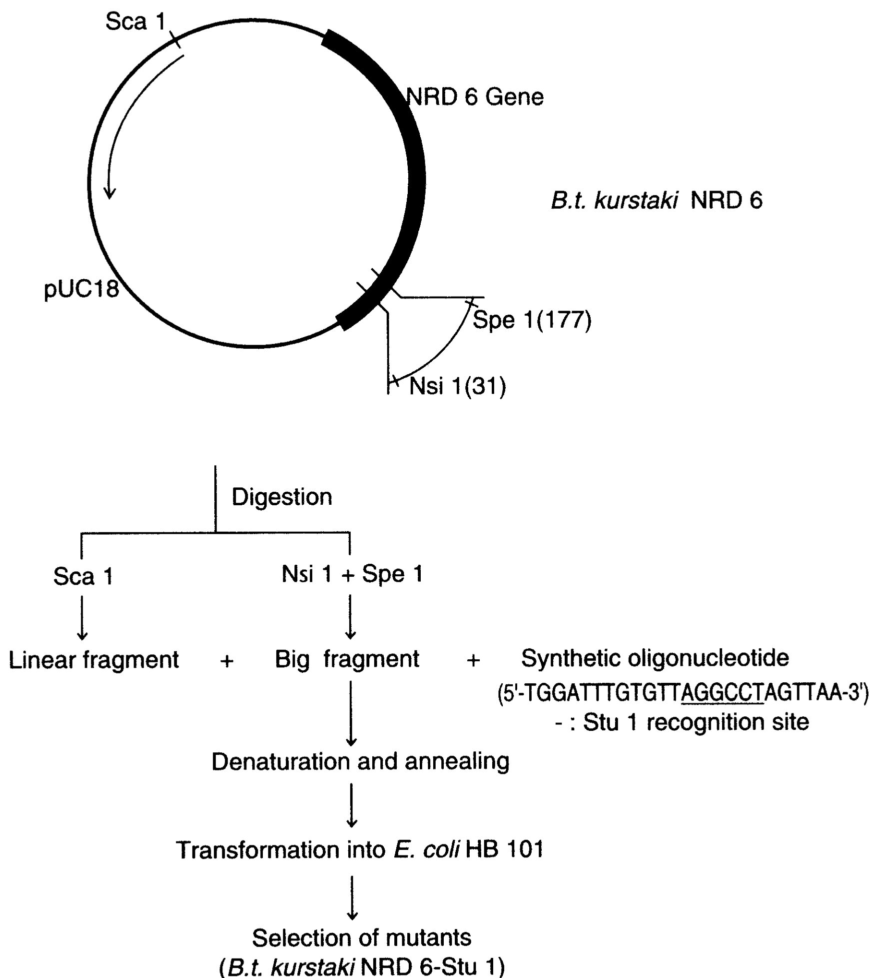

Fig. 1. Construction of B. t. kurstaki NRD6-Stu 1 by site-directed mutagenesis

4. Preparation of inclusion body (protoxin) from mutant *B. t. kurstaki* NRD 6-Stu 1

Proteinaceous crystalline inclusion body (protoxin) of the mutant was purified by Renograffin gradient centrifugation as Fig. 2 scheme.

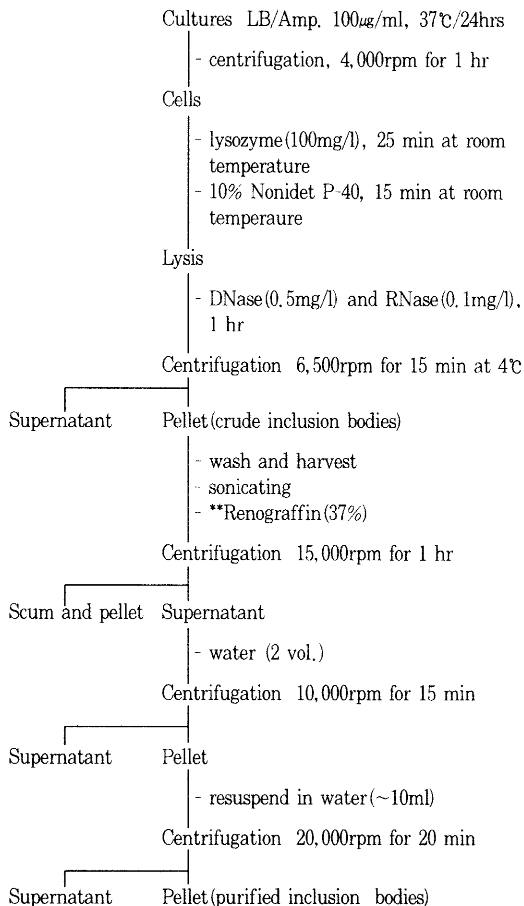

Fig. 2. Flowchart on the purification of inclusion body from *B. t. kurstaki* NRD6-Stu1.

5. Electrophoresis and western blot analysis

For detection of the purified inclusion body, electrophoresis on 7.5% SDS-polyacrylamide gel was carried out as described by Laemmli[24]. Protein bands was stained with 0.1% coomassie brilliant blue R-250 (Sigma Co.) in 10% acetic acid. The protein separated by SDS-PAGE were electrophoretically blotted onto nitrocellulose membrane and immunostained with an Anti NRD 12 and Protein A as primary and secondary antibody, conjugated with alkaline phosphatase (Sigma Co.). The band were colored in HRP color reagent.

6. Nucleotides sequencing

Protoxin gene of plasmid DNA of the mutant was sequenced by the dideoxy chain termination procedure developed by Sanger et al.[25,15]

7. Insect toxidity assay

The insect cell (*Choristoneura femiferana*, IPRI-CF1 line) were grown in spinner flasks(250ml) up to a density of 10 cells/ml in grace media supplemented with 0.25% tryptose and 10% heat inactivated fetal calf serum. Final as molality of the media was adjusted to 330 mOSM/kg. These cells were washed two times in an physiological saline (PS ; MOPS buffered saline(g/l); KCl 6.61, NaCl 1.31, sucrose 26.68, glucose 0.70, $MgCl_2.6H_2O$ 0.228, $MgSO_4$ 0.136, $CaCl_2$ 0.100, MOPS 2.093, pH 7.0) and resuspended in 4ml of PS. This cell suspension was then mixed with an equal volume of 2% low-melting agarose at 37℃ and the mix was poured into the cover of a microtiter plate (96 wells) over 1% agarose in the same buffer.

Meanwhile, the purified inclusion body was resuspended in 1% gut juice/0.2% DTT/0,2M CAPS buffer(pH 10.5) and incubated for 24 hours and spum then the suspension and filtered. The toxin was diluted serially in a microtiter plate using a multi channel micropippette. 1㎕ of each

dilution was applied and hold the plate for 1 hour at room temperature after the last spot have been applied onto the overlay of insect cell.

The plates were dyed 10 min. with 10ml of 0.2% trypan blue in PS and then washed out with 1.34% KCl and destain 1 hour in PS followed by 12 hours destaining in KCl

A positive (toxic) effect on the cells is indicated by a blue spot where the sample was applied. The threshold was measured as the amount of protein present in the last visible spot of the dilution series.

Results and discussion

1. Modification of *B. t. kurstaki* NRD 6 toxin gene (6.6 Kb types)

The 6.6 Kb of *B. t. kurstaki* NRD6 toxin gene cloned in HindⅢ site of pUC18 was mutagenized with Stu 1 recognition site containing oligonucleotide at N-terminal of protoxin gene.

From investigation of Stu 1 digestion pattern of plasmid DNA of all mutants, A-4 mutant was selected, which was created a new Stu 1 recognition site in protoxin gene (Fig. 3).

DNA sequence analysis of the protoxin gene of A-4 mutant (designed as *B. t. kurstaki* NRD 6-Stu 1) revealed the nucleotide A of 177 was substituted by C to introduce a new Stu 1 recognition site, but it was not changed in amino acid (gly) (Fig. 4).

On the other hand, there were changed in pro(52) to asp in NRD6-Hpa1 mutant and pro-gly(52, 53) to arg-asp in NRD6-Nru1 which were obtained from further site-directed mutagenesis of *B. t. kurstaki* NRD6(J. S. Lee and L. Pelloquine et al. 1989. unpublished work).

2. Characteristics of purified inclusion body from *B. t. kurstaki* NRD 6-Stu 1

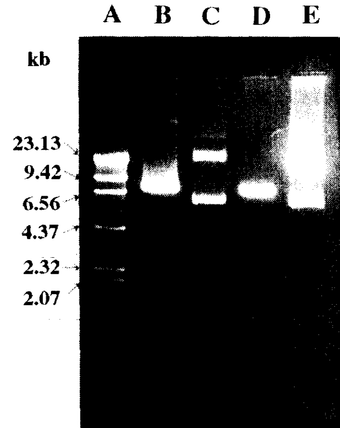

Fig. 3. Agarose gel electrophoresis of recombinant plasmid DNA of *B. t. kurstaki* NRD 6-Stu 1.

Lane A : λ DNA digested with Hind Ⅲ (size marker)
B : *B. t. k* NRD 6 (parent strain) digested with BamH I
C : *B. t. k* NRD 6-Stu 1 rDNA (mutant)
D : *B. t. k* NRD 6-Stu 1 rDNA (mutant) digested with Stu 1
E : *B. t. k* NRD 6 rDNA (parent strain) digested with Stu 1

1) Solubility

After purified the inclusion body of *B. t. kurstaki* NRD 6-Stu 1 as Fig. 2, solubility on some solvents were determined by assay of protein in each solvent suspension which were shaked for 1 hour.

As shown in Table 1, the purified inclusion body was highly soluble in 6.0M guanidine -50mM tris-Cl solution at pH 8.0, but showed very low solubility in 8.0M urea-tris-acetate solution and insolubility in water. In general, the fusion proteins[26] can be solubilized in a strong denaturant such as 6-8M urea solution(IFN-γ etc), 5-8M guanidine solution(insuline A and B chain etc) and some organic solvents so on. But inclusion body containing some DNA binding protein are difficult to solubilize. Therefore, the effectiveness of a particular solvent is likely to differ between proteins expressed; being dependent on the nature of the polypeptides themselves.

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      3        9        15       21       27       33       39       45
      |        |        |        |        |        |        |        |
  1 ATG GAT AAC AAT CCG AAC ATC AAT GAA TGC ATT CCT TAT AAT TGT
    TAC CTA TTG TTA GGC TTG TAG TTA CTT ACG TAA GGA ATA TTA ACA

 46 TTA AGT AAC CCT GAA GTA GAA GTA TTA GGT GGA GAA AGA ATA GAA
    AAT TCA TTG GGA CTT CAT CTT CAT AAT CCA CCT CTT TCT TAT CTT

 91 ACT GGT TAC ACC CCA ATC GAT ATT TCC TTG TCG CTA ACG CAA TTT
    TGA CCA ATG TGG GGT TAG CTA TAA AGG AAC AGC GAT TGC GTT AAA
                                                        177
136 CTT TTG AGT GAA TTT GTT CCC GGT GCT GGA TTT GTG TTA GGC CTA
    GAA AAC TCA CTT AAA CAA GGG CCA CGA CCT AAA CAC AAT CCG GAT
                                                        Stu 1
181 GTT GAT ATA ATA TGG GGA ATT TTT GGT CCC TCT CAA TGG GAC GCA
    CAA CTA TAT TAT ACC CCT TAA AAA CCA GGG AGA GTT ACC CTG CGT

226 TTT CTT GTA CAA ATT GAA CAG TTA ATT AAC CAA AGA ATA GAA GAA
    AAA GAA CAT GTT TAA CTT GTC AAT TAA TTG GTT TCT TAT CTT CTT

271 TTC GCT AGG AAC CAA GCC ATT TCT AGA TTA GAA GGA CTA AGC AAT
    AAG CGA TCC TTG GTT CGG TAA AGA TCT AAT CTT CCT GAT TCG TTA

316 CTT TAT CAA ATT TAC GCA GAA TCT TTT AGA GAG TGG GAA GCA GAT
    GAA ATA GTT TAA ATG CGT CTT AGA AAA TCT CTC ACC CTT CGT CTA

361 CCT ACT AAT CCA GCA TTA AGA GAA GAG ATG CGT ATT CAA TTC AAT
    GGA TGA TTA GGT CGT AAT TCT CTT CTC TAC GCA TAA GTT AAG TTA

406 GAC ATG AAC AGT GCC CTT ACA ACC GCT ATT CCT CTT TTT GCA GTT
    CTG TAC TTG TCA CGG GAA TGT TGG CGA TAA GGA GAA AAA CGT CAA

451 CAA AAT TAT CAA GTT CCT CTT TTA TCA GTA TAT GTT CAA GCT GCA
    GTT TTA ATA GTT CAA GGA GAA AAT AGT CAT ATA CAA GTT CGA CGT

496 AAT TTA CAT TTA TCA GTT TTG AGA GAT GTT TCA GTG TTT GGA CAA
    TTA AAT GTA AAT AGT CAA AAC TCT CTA CAA AGT CAC AAA CCT GTT

541 AGG TGG GGA TTT GAT GCC GCG ACT ATC AAT AGT CGT TAT AAT GAT
    TCC ACC CCT AAA CTA CGG CGC TGA TAG TTA TCA GCA ATA TTA CTA

586 TTA ACT AGG CTT ATT GGC AAC TAT ACA GAT TAT GCT GTA CGC TGG
    AAT TGA TCC GAA TAA CCG TTG ATA TGT CTA ATA CGA CAT GCG ACC
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Fig. 4. DNA sequence analysis of the protoxin gene of *B. t. k* NRD 6-Stu 1 (mutant).
〔 〕 indicated the TM1 site of protoxin gene

Table 1. Solubility of purified inclusion bodies(protoxin) from B. t. kurstaki NRD 6-Stu 1 mutant for some solvents.

Solvents	Solubility* (μg protein/ml)
H_2O	0
8.0M urea/25mM tris-acetate(pH8.0)/1mM EDTA	170
6.0M guanidine/50mM tris-Cl(pH 8.0)	820
50mM Na_2CO_3	610

* 1mg of inclusion body was suspended in 1ml of each solvent and shaked for 30min. at room temperature. Solubility was determined as protein content in each solvent.

2) Electrophoresis and western blot analysis

Inclusion body (protoxin) of *B. t. kurstaki* NRD 6-Stu 1 was excellantly purified by Renograffin reagent. To investigate antigenic property of the purified inclusion body, western blotting was performed by using anti NRD 12 and protein A. (Fig. 5).

The protoxin of *B. t. kurstaki* NRD 6-Stu 1 was well reacted with these antibodies.

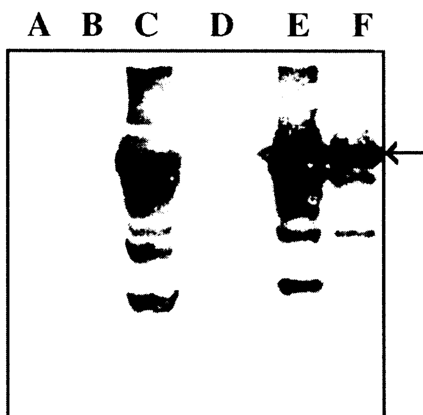

Fig. 5. Western blotting chromatogram of toxin during the production and purification of inclusion bodies from *B. t. kurstaki* NRD 6-Stu 1.
A: Standard protein
B: Supernatant of cell lysis
C: Pellet of cell lysis
D: Supernatant of DNase treatment
E: Pellet of DNase treatment
F: Purified inclusion bodies

3) Toxidity threshold

Toxidity threshold of purified inclusion body of *B. t. kurstaki* NRD 6-Stu 1 against to *Choristoneura femiferana-1* was 0.015~0.030ng as shown in Table 2.

It was similar with 0.01~0.24ng of *B. t. kurstaki* NRD(parent strain), but it was stronger than that of 500~1000ng of *B. t. kurstaki* NRD5.

In conclusion, I obtained *B. t. kurstaki* NRD6-Stul which was created new Stul recognition site by site-directed mutagenesis on *B. t. kurstaki* NRD6. Even though toxidity of the mutant was similar with parent strain, these data should be helpful to development useful strain by site-directed mutagenesis and further to understand a relationship between structure and gene function of *B. T. kurstaki* strains.

Table 2. Threshold values of *B. t. kurstaki* NRD 6-Stu 1 (mutant) and *B. t. kurstaki* NRD series strain toxin against *Choristoneura femiferena-1* insect.

Toxins	Toxidity threshold (ng)
B. t. kurstaki NRD 6-Stu 1 (mutant)	0.015~0.030
B. t. kurstaki NRD 4	not toxic
B. t. kurstaki NRD 5	500~1,000
B. t. kurstaki NRD 6 (parent)	0.010~0.024

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