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<論 文>

Comparison of Characteristics between Insecticidal and Noninsecticidal *Bacillus thuringiensis* Strains belonging to Serotype H8a8b

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ABSTRACT

A noninsecticidal strain, *Bacillus thuringiensis* NTB-88, isolated from Korean soil, had a typical bipyramidal parasporal inclusion and its serotype is identical to *B. thuringiensis* subsp. *morrisoni* (H8a8b). To elucidate differences between insecticidal and noninsecticidal strains, we compared strain NTB-88 to other toxic *B. thuringiensis* subsp. *morrisoni* strains (HD-12 and PG-14). Restriction endonucleases digested plasmid DNA patterns showed that strain NTB-88 was different from lepidopteran-toxic strain, HD-12, but it was similar to dipteran-toxic strain, PG-14. The gene type of strain NTB-88 was different from those of other insecticidal strains. Furthermore, the NH_2-terminal amino acid sequence of crystal protein of strain NTB-88 had no relation to those of the previously known δ-endotoxins in other toxic strains as well as HD-12 and PG-14 strains. Therefore, the noninsecticidal crystal protein in strain NTB-88 is novel and its property is different from insecticidal ones.

Key words : noninsecticidal, *Bacillus thuringiensis, morrisoni*, NTB-88, HD-12, PG-14

INTRODUCTION

Bacillus thuringiensis produces insecticidal crystal proteins, δ-endotoxins, active against insect larvae during sporulation period. But, particularly, many of crystal-forming isolates (*ca.* 40% from soil samples and 55% from animal feed mills) have not yet been shown to be toxic to insects (Martin & Travers, 1989; Meadow *et al.*, 1992). Furthermore, it is generally believed that *B. thuringiensis* isolates that produce parasporal bodies without insecticidal activity are more extensively distributed than toxic ones (Ohba & Aizawa, 1986). Up to now, many noninsecticidal strains have been reported. Thus Ohba *et al.* (1981) isolated two subspecies, subsp. *kumamotoensis* (H18) and subsp. *tochigiensis* (H19). Parasporal inclusions of these strains were typically rhomboidal but noninsecticidal to lepidopterous and dipterous larvae tested. Subsp. *tohokuensis* (H17) is also non-toxic against six insect species. This strain has typically rhomboidal and usually multiple inclusions (two or three) were formed in a single sporangium (Ohba *et al.*, 1981). In addition, subsp. *colmeri* (H21), subsp. *yunnanensis* (H20a20b), subsp. *neoleonensis* (H24), subsp. *shandongiensis* (H22), and subsp. *novosibirsk* (H24a24c) appeared nonpathogenic to the tested insect species of two or four different insect orders.

Noninsecticdal *B. thuringiensis* strains have been widely isolated, however, little is known about the characteristics and mechanisms of their crystal proteins. We had reported some *B. thuringiensis* strains producing noninsecticial crystal proteins (Roh *et al.*, 1996; Park *et al.*, 1998). Interestingly, although strain NTB-88 produced noinsecticidal crystal protein, this strain had same serotype to subsp. *morrisoni* (H8a8b) as like lepidoptera-toxic strain HD-12 and diptera-toxic strain PG-14. In this study, to investigate characteristics of noninsecticidal

crystal protein, NTB-88 was compared with insecticidal strains, HD-12 and PG-14.

MATERIALS AND METHODS

1. Bacterial strains and growth media

B. thuringiensis NTB-88 strain was isolated from soil samples in Korea according to the method of Ohba and Aizawa (1978). *B. thuringiensis* subsp. *morrisoni* HD-12 and PG-14 were kindly provided by Dr. M. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Japan).

To culture *B. thuringiensis* strains for purification of crystal proteins and plasmid DNA, GYS and SPY media were used. LB medium was used as a primary culture medium for plasmid DNA preparation.

2. Preparation of plasmid DNA

Plasmid DNAs of *B. thuringiensis* strains was isolated by partially modified alkaline lysis method (Birnboim & Doly, 1979). *Eco*RI-, *Hin*dIII- and *Kpn*I-digested plasmid DNAs were analyzed on a 0.7% agarose gel.

3. Polymerase Chain Reaction

The PCR analyses were used to identify the gene types of strain NTB-88 using 19 oligonucleotide primers which were especially detected *cryl, cry2, cry3, cry4* and *cryl1*-type genes (Sasaki *et al.*, 1996).

The reaction was conducted for 250 ng of sample DNA with 2.5 U of *Taq* DNA polymerase (Promega Co.), 200 nM each deoxynucleotide triphosphate, 100 pM each primer and 3 mM $MgCl_2$ in a final volume of 50 μ*l*. Amplification was accomplished with the DNA Thermal Cycler (Perkin Elmer Cetus) by using the step-cycle program set. Identification of *cry* genes in plasmid DNA samples was based on a unique-size DNA fragment amplified by PCR on each *cry* gene.

4. NH_2-terminal amino acid analyses

Crystal protein in strain NTB-88 was purified by slightly modified method of Thomas and Ellar (1983). *B. thuringiensis* strains were cultured in GYS media for 5 days at 30°C to ensure sporulation and complete autolysis. The spore-parasporal inclusion mixtures were thoroughly washed with 1 M NaCl, 0.01% Triton X-100 and sedimented by centrifugation at 15,000 g for 10 min. The pellets were resuspended in DW, sonicated three times (22, 000 cycle/sec for 30 sec), loaded onto a discontinuous 30 to 67% Renograffin-67 (Squibb Diagnostics Co.) step gradient and lastly centrifuged at 80,000 g for 2 h.

For amino terminal sequencing of full- and trypsinized-crystal proteins of strain NTB-88, SDS-PAGE was performed according to Laemmli's method (1970) on a 12.5% polyacrylamide gel. After electrophoresis, electrotransfer to ProBlot PVDF (polyvinylidene difluoride)-type membranes (Perkin-Elmer Co.) was performed at 0.25 A for 3 h (Lauriere, 1993). The membranes were stained with amido black 10B in 50% methanol for 5 min, destained in distilled water and air-dried. The protein bands were excised and subjected to pulse-liquid amino terminal protein sequencing (Applied Biosystems Model 475A) by manufacture's manual.

RESULTS AND DISCUSSION

We reported that *B. thuringiensis* NTB-88, isolated from Korean soil samples, had no insecticidal activities against 21 insect species of three orders but had typical bipyramidal crystal protein. The size of this protein was 138 kDa and tryptic resistant core was 68 kDa. HD-12 and PG-14 strains were selected for comparison because their serotypes was same to subsp. *morrisoni* (H8a8b) and had bipyramidal crystal proteins which are composed of high molecular mass (*ca.* 130 kDa) similar to strain NTB-88. Western blot analyses were performed with the crystals of strains HD-12 and PG-14, using antisera of strain NTB-88 crystal proteins. There was no significant positive reaction between any crystal components and antisera of strain NTB-88 (data not shown). Therefore, there was no correlation at all between serotype classification and crystal similarity.

Preliminary characteristics of *B. thuringiensis* subsp. *morrisoni* strains used in this study were summarized in Table 1. In the points of crystal size and shape, strain NTB-88 was similar to strain HD-12 but their total plasmid profiles showed distinctive differences.

Table 1. *Characterization of B. thuringiensis subsp. morrisoni strains used in this study*

Strain	Plasmid No. (size range, MDa)	Crystal proteins (kDa)	Crystal shape	References
NTB-88	6(4-160)	138	bipyramidal	This study
HD-12	5(10-160)	136	bipyramidal	Kronstad *et al.* (1983)
PG-14	6(4-72)	27, 72, 128, 135	spherical, rectangular, bipyramidal	Ibarra & Federici (1986)

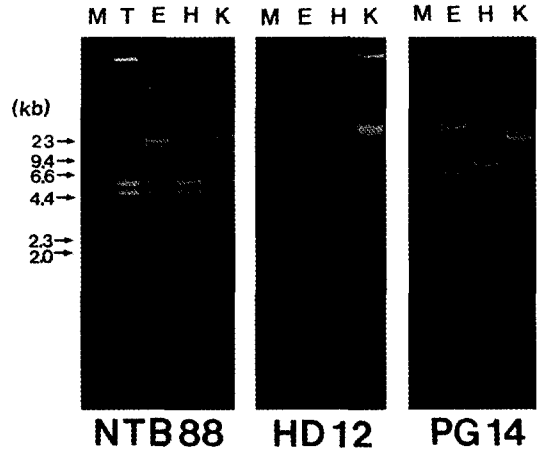

Fig. 1. *Plasmid DNA profiles digested with restriction endonucleases of B. thuringiensis NTB-88, subsp. morrisoni HD-12 and PG-14. Lane T, undigested; lane E, EcoRI-digested; lane H, HindIII-digested; lane K, KpnI-digested; lane M, Lambda-HindIII marker.*

Figure 1 shows the restriction endonuclease-digested plasmid profiles of strain NTB-88 along with the reference strains, HD-12 and PG-14. Strain NTB-88 was almost similar to strain PG-14 but significantly different from strain HD-12. This results illustrated characteristics of NTB-88 was partly related with those of HD-12 and PG-14 strains.

Table 2. *PCR analysis of B. thuringiensis NTB-88 using cry-specific oligonucleotides*

Gene type	HD-12	NTB-88	PG-14
cry1Aa	+	–	–
cry1C	+	–	–
cry1F	+	–	–
cry4A	–	–	+
cry4B	–	–	+
cry11A	–	–	+

To detect the crystal protein genes of strain NTB-88, PCR analyses were carried out with *cry* gene specific oligonucleotide sets. Strain HD-12 had *cry 1Aa, cry1C, cry1F* and strain PG-14 had *cry4A, cry 4B, cry11A*-type genes (Table 2). And other primer sets produced no product with template of HD-12 and PG-14 strains. However, any predicted PCR product is not detected in strain NTB-88. Interestingly, restriction enzyme-digested plasmid profile of strain NTB-88 showed similar to that of strain PG-14 but gene type analyses using PCR showed gene type of strain NTB-88 is different from that of strain PG-14. In addition, strain NTB-88 had no reported *cry* gene. The fact that template DNA of strain NTB-88 was entirely not reacted with any gene

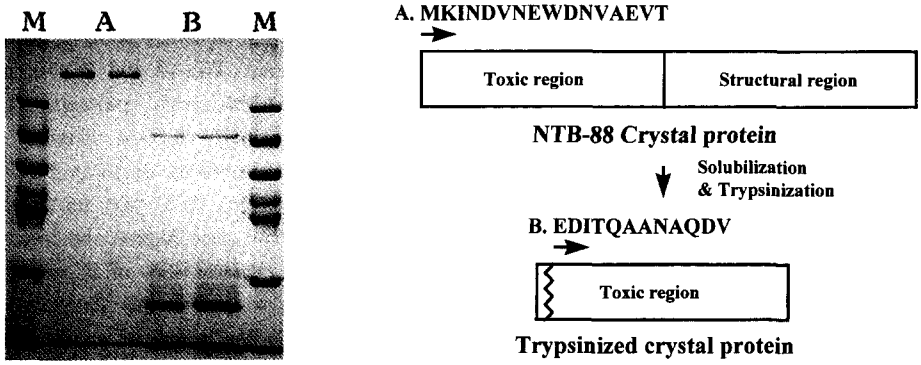

Fig. 2. *NH_2-terminal amino acid analyses of full (A) and trypsinized (B) crystal protein of NTB-88. M is midrange molecular marker (Promega Co.)*

Table 3. *Comparison of the N-terminal sequences of the noninsecticidal B. thuringiensis crystal proteins with those of known B. thuringiensis crystal proteins*

Crystal protein type	Residue position														Reference
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
NTB-88	Met	Lys	Ile	Asn	Asp	Val	Asn	Glu	Trp	Asp	Asn	Val	Ala	Glu	This work
Cry1Aa	Met	Asp	Asn	Asn	Pro	Asn	Ile	Asn	Glu	Cys	Ile	pro	Tyr	Asn	Schnepf *et al.* (1985)
Cry1C	Met	Glu	Glu	Asn	Asn	Glu	Asn	Glu	Cys	Ile	pro	Tyr	Asn	Cys	Honée *et al.* (1988)
Cry1F	Met	Glu	Asn	Asn	Ile	Gln	Asn	Gln	Cys	Val	Pro	Tyr	Asn	Cys	Feitelson, J. S. (1991, GB)
Cry4A	Met	Asn	Pro	Tyr	Gln	Asn	Lys	Asn	Glu	Tyr	Gln	Thr	Leu	Asn	Ward & Ellar (1987)
Cry4B	Met	Asn	Ser	Gly	Tyr	Pro	Leu	Ala	Asn	Asp	Leu	Gln	Gly	Ser	Chungjatupornchai *et al.* (1988)
Cry11A	Met	Glu	Asp	Ser	Ser	Leu	Asp	Thr	Leu	Ser	Ile	Val	Asn	Glu	Donovan *et al.* (1988)

*GB : GenBank submitted data

specific oligonucleotide sets indicates the presence of novel crystal protein gene in strain NTB-88 being possible. Therefore, PCR analyses suggest that δ-endotoxin gene of NTB-88 has different gene structure from previously reported genes.

To confirm differences with insecticidal crystal proteins, NH2-terminal amino acid analyses of full (138 kDa) and trypsinized (68 kDa) crystal proteins of strain NTB-88 was performed (Fig. 2). The amino-terminal sequence of 138 kDa protein of NTB-88 had little in common with the previously known those of HD-1 and PG-14 strains-Cry1Aa, Cry1C, Cry1F, Cry4A, Cry4B and Cry11A-type proteins (Table 3). Only asparagine in the fourth and seventh position appeared to be conserved since it is also present in the Cry1C and Cry1F of strain HD-12. According to comparison of amino terminal sequence, maximum 4 residues (Cry1C) among 14 residues were identical. Furthermore, this sequence was not similar to any other insecticidal strains and NH_2-terminal sequence of trypsinized core is also different (data not shown). Thus crystal protein of strain NTB-88 had no relationship with those of other *morrisoni* strains and insecticidal strains.

In conclusion, crystal protein of *B. thuringiensis* NTB-88 strain was novel one not identical to those of other subsp. *morrisoni* strains and the corresponding gene structure was also different. We are now concentrate on two possibilities that the crystal protein gene of strain NTB-88 locate on genomic DNA or noninsecticidal property of crystal protein of strain NTB-88 is caused different gene structure from insecticidal ones.

摘 要

한국의 토양에서 분리한 무독성균주인 *B. thuringiensis* NTB-88균주의 무독성 내독소단백질의 특성을 분석하기 위하여, 동일한 *B. thuringiensis* subsp. *morrisoni*속에 속하는 나비목 독성균주 HD-12균주와 파리목 독성균주 PG-14균주를 비교균주로 하여 그 특성을 비교하였다. NTB-88균주는 내독소단백질의 모양에 있어서는 HD-12균주와 동일한 bipyramidal 형태를 보유하고 있었으며, 플라스미드패턴에 있어서는 PG-14균주와 유사한 특성을 가지고 있었다. 그러나, NTB-88균주의 유전자형을 결정하기 위하여 *cry* 유전자 특이 primers sets를 이용하여 PCR을 수행한 결과 HD-12균주가 *cry1Aa, cry1C, cry1F*를, PG-14균주가 *cry4A, cry4B, cry11A* 유전자를 갖는 반면 NTB-88균주에서는 기존에 알려진 *cry* 유전자는 존재하지 않음을 확인하였으며, NTB-88균주의 무독성 내독소단백질의 N-말단의 아미노산 잔기서열을 이미 보고된 독성 내독소단백질과 비교한 결과 역시 상동성을 나타내지 않았다. 이상의 결과를 종합하면 NTB-88균주가 형성하는 무독성 138 kDa 단백질은 독성 내독소단백질과 그 모양은 같으나 유전자 구조가 전혀 다른 새로운 내독소단백질임을 확인할 수 있었다.

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