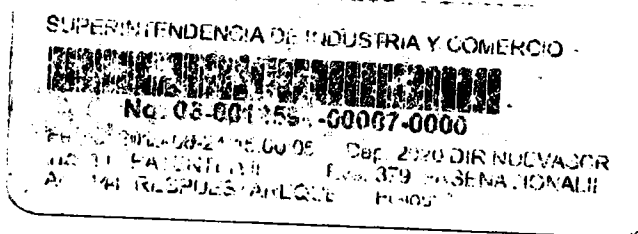


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E. S. D.

Re.: Expediente No. 06.001.259

Solicitud de Patente PCT Entrada en Fase Nacional, para: "PROTEINAS INSECTICIDAS DE BACILLUS THURINGIENSIS Y USOS DE LAS MISMAS"

Solicitante: MONSANTO TECHNOLOGY LLC

### **RESPUESTA AL EXAMEN DE BUSQUEDA DE ANTERIORIDADES**

MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, me permito presentar los documentos abajo relacionados, que fueron solicitados por su Despacho mediante el Oficio No.6452 bajo el artículo 46 de la Decisión 486, el día 14 de mayo de 2010.

- HWANG S-H; et al. "A novel class of mosquitocidal delta-endotoxin, cry 19B, encoded by a Bacillus thuringiensis serovar higo gene" Systematic and applied microbiology, 21, 179-184; ISSN: 0723-2020, 1998, XP008063276.

Cumplido así con lo dispuesto, atentamente solicito a Ud. se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

De la Señora Jefe,

  
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SYSTEMATIC AND  
APPLIED MICROBIOLOGY

## A Novel Class of Mosquitocidal $\delta$ -Endotoxin, Cry19B, Encoded by a *Bacillus thuringiensis* serovar *higo* Gene

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### Summary

Partially digested *Hinc*II fragments of DNA from a mosquitocidal strain of *Bacillus thuringiensis* serovar *higo* were cloned into pBluescript II SK(+) and propagated in *Escherichia coli*. Recombinant cells were screened immunologically for the production of parasporal inclusion antigens. One *E. coli* clone harboring a recombinant plasmid exhibited larvicidal activity to *Culex pipiens molestus*, but not to *Anopheles stephensi*. Hybridization experiments revealed that the gene of the toxin protein is located on a 110 kb plasmid of *B. thuringiensis* serovar *higo*. Sequence analysis detected an open reading frame of 2046 nucleotides encoding a polypeptide of 682 amino acid residues with a predicted molecular weight of 78,467. The gene encoded five block regions commonly conserved in the insecticidal protein genes of *B. thuringiensis*. Amino acid sequence of the 78 kDa protein shared 49% identity and 56% similarity with that of the Cry19A protein from *B. thuringiensis* serovar *jagathesan*. A new class of  $\delta$ -endotoxin protein, designated Cry19B, was established on the basis of this protein.

**Key words:** *Bacillus thuringiensis* - Cry19B protein - mosquitocidal  $\delta$ -endotoxin - *Culex pipiens molestus*

### Introduction

Among the medical and veterinary insect pests, most important are the dipterans because many of them are the vectors for a number of virus diseases, malaria, filariasis and onchocerciasis. *Bacillus thuringiensis* serovar *israelensis* is a promising biological control agent for mosquitoes and blackflies (DE BARJAC and SUTHERLAND, 1990). In fact, the products from this bacterium have been successfully applied to suppress the populations of dipteran vectors in Tropics. In particular, this agent has played a key role in the Onchocerciasis Control Programme for eradication of blackflies in West Africa (DROBNIEWSKI, 1993).

To date, no cases have been reported for the development of resistance to *B. thuringiensis* serovar *israelensis* in field populations of Diptera. However, recent studies have often reported the occurrence of field lepidopteran populations resistant to *B. thuringiensis* insecticides (TARASHNIK et al., 1990; IQBAL et al., 1996). This leads us to consider the possible occurrence of resistant dipterans in the future upon continuous field applications of *B. thuringiensis* serovar *israelensis*. In this respect, the search for novel mosquitocidal toxins, with different molecular structures and mode of action, would be of great value. This may provide the tactics to overcome the resistance problems.

In an earlier study (OHBA et al., 1995), we have obtained unique isolates belonging to *B. thuringiensis* serovar *higo* that exhibit mosquito larvicidal activity preferential for malaria-transmitting *Anopheles stephensi*. The objectives of this study were: (1) to isolate the mosquitocidal protein gene(s) of the *higo* strain and (2) to determine the nucleotide sequence to compare it with those of the established gene classes.

### Materials and Methods

**Bacterial strains, plasmid, and culture media:** The reference strains of *B. thuringiensis* serovar *higo* (OHBA et al., 1995) and *B. thuringiensis* serovar *israelensis* (DE BARJAC, 1978) were used in this study. *Escherichia coli* K12-Blue and pBluescript II SK(+) were used as a cloning host and a vector, respectively. An *E. coli* strain, harboring pBGH4 (SEN et al., 1988) as the source of the *cry4A* gene, was supplied by Dr. H. SAKAI, Kyoto University. *B. thuringiensis* was routinely grown in L broth containing 0.1% glucose and *E. coli* on LB medium.

**Abbreviations:** O.C., open circular form DNA; linear, linear and chromosomal DNA region.

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**Purification and solubilization of inclusions and antiserum preparation:** Parasporal inclusions were purified from sporulated cultures and solubilized by the method described by ISHII and OHBA (1993). Inclusions were partially purified from recombinant *E. coli* by the procedures of LIANG and DEAN (1994) and solubilized with the SDS-urea buffer (1% SDS, 2%  $\beta$ -mercaptoethanol, 6 M urea, 100 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , pH 7.2). Protein concentration of the purified inclusion was measured by the method of LOWRY et al. (1951) with bovine serum albumin as the standard. Antiserum was raised in rabbit against the solubilized inclusion proteins as previously described (ISHII and OHBA, 1993).

**DNA preparation and cloning of mosquitoicidal protein gene:** DNA was extracted from *E. coli* by the alkaline method (BUNNOM and DOLY, 1979). Extraction of DNA from *B. thuringiensis* was done according to HIMENO et al. (1985). The isolated DNA was purified by  $\text{CsCl}$ - $\text{EtBr}$  density gradient centrifugation and analyzed by electrophoresis on 0.7% agarose gels. DNA from *B. thuringiensis* serovar *higo* was digested with *HincII* (1 unit per  $\mu\text{g}$  DNA) for 1 h and mixed with *HincII*-digested pBluescript II SK(+) at a 1:10 molar ratio of vector to insert DNA (final DNA concentration: 50  $\mu\text{g}/\text{ml}$ ). After purification by phenol extraction and ethanol precipitation, the mixture was ligated by using T4 DNA ligase (Takara, Tokyo) and used to transform *E. coli* XL1-Blue to ampicillin resistant by the  $\text{CaCl}_2$  method (SAMBROOK et al., 1989). White colonies on X-Gal plates containing 100  $\mu\text{g}$  ampicillin/ml and 23.8  $\mu\text{g}$  IPTG/ml were collected and stored at  $-80^\circ\text{C}$ . About 1,000 cells were plated on the LB medium containing 100  $\mu\text{g}$  ampicillin/ml and cultured for 24 h at  $37^\circ\text{C}$ . Colonies were transferred onto nitrocellulose filter membranes (SCHLEICHER & SCHLELL; BA 85) and screened immunologically for the production of the inclusion proteins by using the antiserum described above. The screening was done by the method of HELFMAN and HUGHES (1987) and SAMBROOK et al. (1989) with some modifications. Finally, bound antibody was detected by using peroxidase-conjugated goat anti-rabbit IgG (Dako, Denmark) and was visualized by using 4-chloro-1-naphthol (Wako, Tokyo) as the color substrate.

**SDS-PAGE and immunoblotting:** Solubilized inclusion proteins were separated in 10% separating and 4% stacking gel (LAEMMLI, 1970). After transfer to nitrocellulose filter membranes, proteins were probed with appropriately diluted antiserum, followed by goat anti-rabbit IgG-conjugated peroxidase (Dako, Denmark). Visualization was performed as described above.

**Southern blot hybridization:** DNA was separated by electrophoresis in horizontal 0.7% agarose gel and transferred to Hybond N<sup>+</sup> nylon membrane (Amersham, England) by using 0.25 M NaOH as a transfer reagent. *HincII*-digested 1.5 kb fragment of pHSMO2 (Fig. 1) and 1.9 kb *EcoRI* fragment of pBGH4 (SEV et al., 1988) were used as probes in hybridization experiments. Probes were labeled with peroxidase by using the

ECL nucleic acid labeling system (Amersham). Hybridization, washing, and detection procedures were followed as described by the manufacturer. Finally, the signal was visualized by using Hyperfilm-ECL (Amersham) exposed to the blot and reacted with the GBX developer (Kodak).

**DNA sequencing:** The nucleotide sequence was determined on an ABI sequencer Model 373S using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). The sequences obtained were analyzed by the DNASIS Program from Hitachi Software Engineering Co., Ltd., Japan.

**Toxicity test:** *B. thuringiensis* serovar *higo*, *B. thuringiensis* serovar *israelensis*, and the recombinant *E. coli* harboring pHSMO2 were examined for quantitative mosquitoicidal activity against two dipteran species. Twofold serial dilutions of purified inclusions were prepared in distilled water. Twenty larvae (4-day-old) of *Culex pipiens molestus* or *Anopheles stephensi* were introduced into wells containing 5 ml of each dilution. Mortality was scored after incubation at  $25^\circ\text{C}$  for 24 h. The  $\text{LC}_{50}$  values were determined by probit analysis (FINNEY, 1952).

## Results and Discussion

Two serologically positive *E. coli* colonies produced inclusions when observed with phase-contrast microscope and showed larvicidal activity against *C. pipiens molestus*. Unexpectedly, however, they were substantially not active on the larvae of *A. stephensi*. In the restriction analysis, these colonies proved to have the same recombinant plasmid (data not shown), pHSMO1, which contained partially digested 5.3 kb *HincII* insert in the same orientation. A newly constructed plasmid, pHSMO2, containing the 3.6 kb *HincII* insert (Fig. 1) from pHSMO1 also produced similar inclusions that exhibit the lethal toxicity to larvae of *C. pipiens molestus*. In mosquito larval  $\text{LC}_{50}$  values (Table 1), partially purified inclusions of the recombinant *E. coli* harboring pHSMO2 showed slightly stronger activity than the purified inclusions of *B. thuringiensis* serovar *higo* against larvae of *C. pipiens molestus*.

The result of SDS-PAGE revealed that the recombinant *E. coli* harboring pHSMO2 produced significant amount of 78 kDa protein (data not shown). In immunoblot analysis, this protein reacted with the antiserum against the total inclusion proteins of *B. thuringiensis* serovar *higo*.

As shown in Fig. 2c (lane 1), *B. thuringiensis* serovar *higo* had at least five plasmid DNAs ranging from 1.3 to

Table 1. Mosquitoicidal activity of inclusions from a recombinant *Escherichia coli* harboring pHSMO2 and two *Bacillus thuringiensis* strains.

| Source for inclusions                              | $\text{LC}_{50}$ ( $\mu\text{g}/\text{ml}$ ) <sup>a</sup> against |                               |
|----------------------------------------------------|-------------------------------------------------------------------|-------------------------------|
|                                                    | <i>Anopheles stephensi</i>                                        | <i>Culex pipiens molestus</i> |
| <i>E. coli</i> /pHSMO2                             | >400                                                              | 5.93 (5.47–6.65)              |
| <i>B. thuringiensis</i> serovar <i>higo</i>        | 0.17 (0.15–0.19)                                                  | 7.52 (7.18–8.62)              |
| <i>B. thuringiensis</i> serovar <i>israelensis</i> | 0.019 (0.019–0.022)                                               | 0.026 (0.024–0.028)           |

<sup>a</sup> Mortality was scored after 24 h for 4-d-old larvae. The confidence limits are 95% level is given in parentheses.

Fig. 1. Schematic diagram of the 7.6 kb protein-coding region of the *Bacillus thuringiensis* serovar *higo* protease gene. The gene contains a 1.5 kb fragment which was used in hybridization experiments. The open 2046 nucleotide polypeptide acid residue coding molecule (78,467.6) is shown in the box and sequence (position) is marked by rows under

## B. thuringiensis Cry19B protein 181

[illegible]

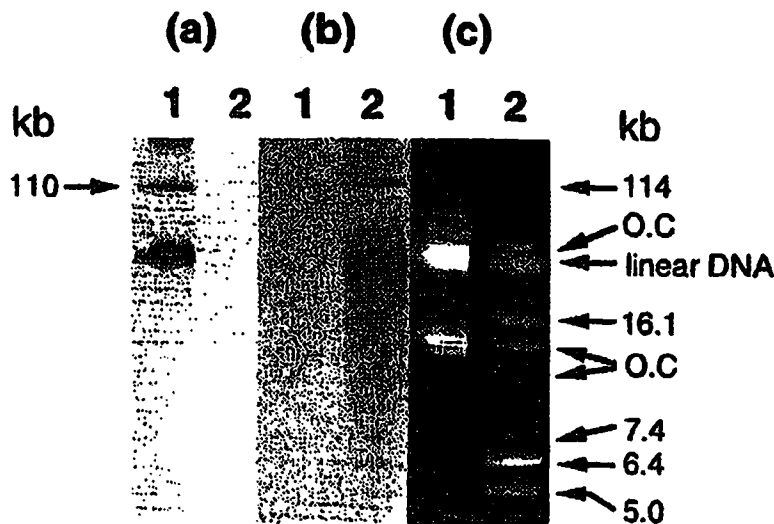


Fig. 2. Agarose gel electrophoresis and Southern blotting analysis. DNAs of *Bacillus thuringiensis* serovar *higo* and *B. thuringiensis* serovar *israelensis* were separated on 0.7% agarose gel (c). To determine the size of the plasmids the *B. thuringiensis* serovar *israelensis* plasmids (GONZÁLEZ and CARLTON, 1984) were used as the standard. After transferring to a nylon membrane, each DNA was probed with (a) a 1.5 kb *Hind*III fragment of pHSO2 and (b) a 1.9 kb *Eco*RI fragment of *cry*4A gene (SEN et al., 1988). Lanes: 1, total DNA of *B. thuringiensis* serovar *higo*; 2, total DNA of *B. thuringiensis* serovar *israelensis*. The position of the large sized plasmid that contains the mosquitocidal protein gene was nearly the same in the DNA of the two *B. thuringiensis* strains. Plasmid DNA sizes of *B. thuringiensis* serovar *israelensis* are marked on the right margin.

110 kb. When 1.5 kb *Hind*III fragment (Fig. 3) of pHSO2 was used as a probe DNA, it hybridized to 110 kb plasmid DNA and linear DNA region of the *higo* strain, but not to the DNA of *B. thuringiensis* serovar *israelensis* (Fig. 2a). When 1.9 kb *Eco*RI fragment of *cry*4A gene (SEN et al., 1988) was used as a probe DNA, it hybridized to the 114 kb plasmid DNA and linear DNA region of *B. thuringiensis* serovar *israelensis*, but hybridized only very weakly to the DNA of *B. thuringiensis* serovar *higo* (Fig. 2b).

The nucleotide sequence of the 3.6 kb fragment of pHSO2 was determined on both strands (Fig. 1). There was one open reading frame encoding a polypeptide of 682 amino acid residues with a predicted molecular weight of 78,467. A putative ribosome binding site (GGAGG) was found 7 bases upstream from the initiation codon. In the downstream from the stop codon, there was an inverted repeat sequence that may probably act as a transcriptional terminator (position 2772 to 2803). As shown in Fig. 4, the deduced amino acid sequence contained five blocks conserved commonly in all types of the existing insecticidal  $\delta$ -endotoxin proteins (HÖFTE and WHITELEY, 1989; ROSSO and DELÉCLUSE, 1997). We compared the nucleotide and deduced amino acid sequence of the 78 kDa protein encoding gene with 83552 nucleotide sequences deposited in DDBJ and 136374 amino acid sequences in PIR-Prot Database, re-

spectively. Among them, only the sequence of *cry*4A gene (SEN et al., 1988) of *B. thuringiensis* serovar *israelensis* proved to be related to some extent to the sequence of the 78 kDa protein encoding gene. However, the homology between the two genes was 28% and only present in 150 N-terminal bases. This is in accordance with the results of Southern hybridization. The *Hind*III-digested 1.5 kb fragment of pHSO2, lacking the N-terminal region, showed no homology to the DNA of *B. thuringiensis* serovar *israelensis* that contains *cry*4A gene (Fig. 2A). *Eco*RI-digested 1.9 kb fragment of *cry*4A gene (SEN et al., 1988), having the N-terminal region, showed weak signals to the DNA of *B. thuringiensis* serovar *higo* (Fig. 2B). We also compared the relatedness between the 78 kDa *higo* protein and the established Cry proteins in an updated classification scheme. It appeared from the result that the *higo* protein is most related to the 74.7 kDa protein of *B. thuringiensis* serovar *jegathesan* (ROSSO and DELÉCLUSE, 1997), now called Cry19A. The homology in amino acid sequence was 49% in identity and 56% in similarity between the two proteins. Thus, the 78 kDa protein of *higo* can be allocated to a new class of the Cry protein, designated Cry19B by the *Bacillus thuringiensis*  $\delta$ -Endotoxin Nomenclature Committee.

It is clear from our findings that the 78 kDa protein obtained in this study is not responsible for the unique larvicidal activity, preferential for *Anopheles* mosquitoes

**Block1**

Cry1Aa 153 YQVPLLSVTVQAANLHLSVLEDSVVFQQRW  
 Cry3A 197 YEVFLTTTAAQAANTLFLLEDAQITGEW  
 Cry4A 202 YNILLVLSSTAAQAANLHLSVLEDSVVFQQRW  
 Cry4B 170 YELLLLPITAAQAANLHLSVLEDSVVFQQRW  
 Cry10A 195 YRIPTLPAYAAQIATWELNLLKHAATTYNTW  
 Cry19A 195 YELLLLPITAAQIAHLHLLLLADAQITGEW  
 78 kDa 184 YETVLLPSTAAQAANLHLSVLEDSVVFQQRW

**Block2**

Cry1Aa 226 YTDYAVRWNTGL.....ERVWGPDSDRWVRYNQFRELTLTVLDIVLPSNYDSRRYP.....IRTVSQLTREIYTNFV  
 Cry3A 270 YTDHCVKNTFVGL.....DKLRGSSYESWVNFNRYKRETLTVLDLIALFLYDVRLYP.....KEVKYELTADVLTDPI  
 Cry4A 289 YTHCVITVTKKGLMLKRTTPDSNLGHNNTYNTTETQITAVLDVVALFNYDVCKYP.....IQVQSELTRFIYQVLN  
 Cry4B 243 YIAHSITWYKKGK.....DVLNRKSNQGWITFNDYKRETLTVLDLIALFASYPDRYPADKIDNTKLSRTSEFTREIYTNFV  
 Cry10A 275 YTDYCIQTYNAGL.....THRTYTNATWNTYNTTETQITAVLDVVALFNYDVCKYP.....IQVQSELTRFIYQVLN  
 Cry19A 246 YTEYCIHWYKKGK.....NDPRTAGQWVNFNRYKRETLTVLDLIALFASYPDRYPADKIDNTKLSRTSEFTREIYTNFV  
 78 kDa 251 YSYCVETVTKKGL.....ESKKQIGNSDFNRYKRETLTVLDLIALFASYPDRYPADKIDNTKLSRTSEFTREIYTNFV

**Block3**

Cry1Aa 452 FSWQHRBAEFNNTIPSSQITQIPLTKSTHLSGTSVVRGPGFTGGDIL  
 Cry3A 499 LITWTKRSDVDFNMIDSKITQLPLVAYKLQSGASVAVGPRFTGGDII  
 Cry4A 520 FAWTSSVDPKNTIYTHLTQIPAVKANSLGTSKVVQPGFTGGDIL  
 Cry4B 462 FAWTKEIVDFNNTIYTHLTQIPAVKANSLGTSKVVQPGFTGGDIL  
 Cry10A 492 FSWTSSVDPKNTIYTHLTQIPAVKANSLGTSKVVQPGFTGGDIL  
 Cry19A 494 FAWTSSVDPKNTIYTHLTQIPAVKANSLGTSKVVQPGFTGGDIL  
 78 kDa 488 FAWTSSVDPKNTIYTHLTQIPAVKANSLGTSKVVQPGFTGGDIL

**Block4**

Cry1Aa 519 SQRYVRIRYAST  
 Cry3A 566 SQRYVRIRYAST  
 Cry4A 583 QCSYFIRIRYASN  
 Cry4B 514 TRSYGLRIRYASN  
 Cry10A 556 SRQYQVIRYATN  
 Cry19A 559 DQRYVRIRYKCN  
 78 kDa 550 SMYFIRIRYASN

**Block5**

Cry1Aa 596 VYIDRIEIPVPE  
 Cry3A 641 VYIDRIEIPVPE  
 Cry4A 667 VLIDRIEIPVPE  
 Cry4B 623 VYIDRIEIPVPE  
 Cry10A 639 VYIDRIEIPVPE  
 Cry19A 637 LVIDRIEIPVPE  
 78 kDa 627 VYIDRIEIPVPE

Fig. 3. Comparison of the conserved block regions. Five block regions in the deduced amino acid sequences of the 78 kDa protein encoding gene were compared with the block regions of the established insecticidal  $\delta$ -endotoxins. Bold letters indicate the identical amino acid residues to the deduced amino acid residues of 78 kDa protein encoding gene.

(OHBA et al., 1995), of the *higo* strain. Cloning and identification of *Anopheles*-active protein gene(s) from this strain will be the subject in our future works. Recent worldwide efforts to screen *B. thuringiensis* effective for control of medically important dipteran pests have provided many mosquitocidal isolates belonging to various H serovars (ISHII and OHBA, 1993; RAGNI et al., 1996; SARON et al., 1996). It would be of interest to know the frequency of the present gene among naturally occurring mosquito-toxic *B. thuringiensis* populations.

**Acknowledgements**

The nucleotide sequence of the gene *cry19B* has been deposited in the databanks (EMBL, GenBank, and DDBJ) under the accession number: D88381. The authors thank Dr. N. CRICKMORE, the *Bacillus thuringiensis*  $\delta$ -Endotoxin Nomenclature Committee, for invaluable advice on the classification and numbering of the Cry protein classes. They also thank Dr. T. KAWARABATA for critical reading of the manuscript. This work was supported by the Science and Technology Agency of Japan and by the Grant-in-Aid (No. 0845639) from the Ministry of Education, Science and Culture of Japan.

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## Intro

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