Université de Montréal

Bacillus thuringiensis Strain M15, a Novel Autoagglutinable, Non-Serotypeable Strain -Cloning and Characterization of a Novel *cry31A*-type Crystal Protein Gene, *cry31Aa2*, and Two New Insertion Sequences, IS*231*M and -N

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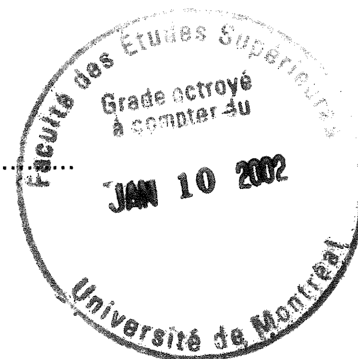

Commit your way to the LORD,

Trust also in Him, And He shall bring it to pass.

He shall bring forth your righteousness as the light,

And your justice as the noonday. (PSALM 37: 5-6)

RÉSUMÉ

L'utilisation à grande échelle d'insecticides organiques de synthèse pose un certain nombre de problèmes. La plupart de ces produits présentent une certaine toxicité pour l'homme et leur difficulté de biodégradabilité a favorisé leur accumulation dans l'environnement. Un autre problème très préoccupant est celui de l'apparition rapide d'insectes résistants à ces produits. Pour ces raisons, il est important de développer des agents de lutte biologique dans le but de contrôler les populations de ravageurs.

Bacillus thuringiensis est la bactérie entomopathogène la plus largement utilisée comme insecticide biologique. *B. thuringiensis* est une bactérie du sol, gram-positive et sporulante. Pendant la phase stationnaire, elle synthétise une inclusion parasporale ayant une activité insecticide contre certains insectes des genres Lepidoptera, Diptera et Coleoptera. Les inclusions parasporales sont de nature protéique. Ces protéines sont codées par des gènes de toxines. Notre projet de recherche consistait à isoler et caractériser une nouvelle souche de *B. thuringiensis* venant de la tétranyque à deux points (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae). Une nouvelle protéine cristalline a ensuite été clonée et séquencée. Elle a été désignée *cry31Aa2* par le *B. thuringiensis* Pesticide Crystal Protein Nomenclature Committee. Le gène a été exprimé dans une souche acristallifère de *B. thuringiensis*. L'inclusion parasporale protéique exprimée a été isolée et était composée d'un seul polypeptide majeur de 83-kDa (chapitre 1).

Les séquences d'insertions et les transposons ont été retrouvés à proximité des gènes *cry*. Nous avons cloné et caractérisé deux nouvelles séquences d'insertion de la famille IS*231*, IS*231*M (chapitre 2) et -N (chapitre 3). Bien que l'analyse de leurs

séquences d'ADN ait révélé une grande homologie à la famille IS*231*, leurs cartes structurales étaient différentes. IS*231*M présente deux cadres de lecture ouverts superposés, ORF1 et ORF2, qui peuvent coder pour des polypeptides de 334 et 143 acides aminés respectivement, alors que IS*231*N contient trois cadres de lecture ouverts (ORFs) qui peuvent coder pour des polypeptides de 329 (ORF1), 118 (ORF2) et 17 (ORF3) acides aminés respectivement. IS*231*N a montré 99% d'identité nucléotidique à IS*231*M, mais partage seulement 83% d'identité au niveau des acides aminés dû à des substitutions et des additions nucléotidiques. La possibilité que IS*231*M et -N soient des éléments de transposition fonctionnels restent à déterminer.

Mots-clés: *Bacillus thuringiensis*; Cry31Aa2; séquence d'insertion; IS*231*M; IS*231*N

ABSTRACT

The synthetic organic pesticides have caused considerable damage on the environment and human health due to their accumulation in the ecosystem and the contamination of the food chain in the environment. Several resistances to synthetic insecticides have appeared in some insect pest populations. For these reasons, there is a need for the development of biological control agents as an alternative for the management of insect pests.

Bacillus thuringiensis is the leading biocontrol agent and has been used as a biorational pesticide in insect pest control. *B. thuringiensis* is a Gram-positive, spore-forming bacterium that is commonly found in natural environments. During the stationary phase of its growth cycle, *B. thuringiensis* produces a parasporal inclusion that exhibits specific insecticidal activity against certain insect species among Lepidoptera, Diptera, and Coleoptera. The parasporal inclusions are made of proteins. These are encoded by toxin genes. Our research project was to isolate and characterize a new *B. thuringiensis* strain from the non-insect, two-spotted spider mite (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae). A novel crystal protein gene was cloned and sequenced. It was designated *cry31Aa2* by the *B. thuringiensis* Pesticide Crystal Protein Nomenclature Committee. It was expressed in an acrystalliferous *B. thuringiensis* strain. The parasporal inclusion protein expressed was isolated and confirmed to be composed of single major polypeptide of 83-kDa (Chapter 1).

Insertion sequences and transposons have been found in the vicinity of *cry* genes. We have cloned and characterized two new insertion sequences of the IS*231* family, IS*231*M (Chapter 2) and N (Chapter 3). Although analyses of their DNA

sequences revealed high homology to the IS*231* family, their structural maps were different. IS*231*M has two overlapping open reading frames, ORF1 and ORF2, that could encode polypeptides of 334 and 143 amino acids, respectively, while IS*231*N contains three open reading frames (ORFs) that could code for polypeptides of 329 (ORF1), 118 (ORF2) and 17 (ORF3) amino acids, respectively. IS*231*N shows 99% nucleotide identity to IS*231*M, but shares only 83% amino acid identity because of nucleotide substitutions and additions. Whether IS*231*M and -N are functional transposable elements remains to be determined.

Key words: Bacillus thuringiensis; Cry31Aa2; insertion sequence; IS*231*M; IS*231*N.

TABLE OF CONTENTS

RÉSUMÉ iv

ABSTRACT vi

TABLE OF CONTENTS viii

LIST OF TABLES xii

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xvi

ACKNOWLEDGMENTS xix

INTRODUCTION..1

1. *Bacillus thuringiensis* and Its Crystal Proteins 1

    1. 1. Historical background of *B. thuringiensis*......................................1

    1. 2. Crystal protein of *B. thuringiensis*..6

    1. 3. Mode of action of the crystal protein...7

2. *Bacillus thuringiensis* as a Biological Control Agent 10

    2. 1. The global market for *B. thuringiensis*-based biopesticides..........10

    2. 2. Delivery systems for crystal proteins...11

    2. 3. Resistance Management to *B. thuringiensis* toxins.....................11

3. The Transposable Elements of *B. thuringiensis* 12

4. Summary of this research project..17

5. REFERENCES 19

CHAPTER 1

Isolation and Characterization of a Novel *Bacillus thuringiensis* Strain expressing a Novel Crystal Protein

– Cloning, Sequencing, Characterization and Expression of *cry31Aa2,* a Novel Crystal Protein Gene 30

ABSTRACT 31

1. 1. INTRODUCTION 33

1. 2. MATERIALS AND METHODS 35

1. 2. 1. Bacterial strains and plasmids 35

1. 2. 2. Isolation of a new *B. thuringiensis* strain 35

1. 2. 3. Biochemical characterization of *B. thuringiensis* strain....36

1. 2. 4. Analyses of parasporal inclusion proteins by SDS-PAGE....36

1. 2. 5. Crystal protein N-terminal sequencing....37

1. 2. 6. Plasmid DNA preparation and Southern hybridization 38

1. 2. 7. Cloning and subcloning of the crystal protein gene....39

1. 2. 8. DNA sequencing 40

1. 2. 9. Expression of the crystal protein in the *B. thuringiensis* Cry^- B strain....40

1. 2. 10. Electron microscopy....41

1. 3. RESULTS 43

1. 3. 1. Morphological and biochemical characteristics....43

1. 3. 2. SDS-PAGE analysis and N-terminal sequencing of the parasporal inclusion protein 43

1. 3. 3. Southern hybridization and gene cloning 49

1. 3. 4. Characterization of a new crystal protein gene, *cry31Aa2* 55

1. 3. 5. The *cry31Aa2* gene expression in *B. thuringiensis* Cry$^-$ B strain..60

1. 4. DISCUSSION 72

1. 5. REFERENCES 75

1. 6. APPENDIX 79

CHAPTER 2

A New Insertion Sequence, IS*231*M, in an Autoagglutinable Isolate of *Bacillus thuringiensis* 80

Contribution of each of the co-authors 81

ABSTRACT 82

2. 1. INTRODUCTION 83

2. 2. MATERIALS AND METHODS..85

2. 2. 1. Bacterial strains and plasmids..85

2. 2. 2. Plasmid DNA preparation and Southern hybridization.............85

2. 2. 3. Cloning and DNA sequencing..87

2. 3. RESULTS 88

2. 3. 1. Cloning and characterization of IS*231*M 88

2. 3. 2. Comparison of IS*231*M with the iso-IS*231* elements 89

2. 4. DISCUSSION 97

2. 5. ACKNOWLEDGMENT 100

2. 6. REFERENCES 101

CHAPTER 3

Cloning and Nucleotide Sequence of a New Insertion Sequence, IS*231*N, from a Non-Serotypeable Strain of *Bacillus thuringiensis* 104

Contribution of each of the co-authors............105

ABSTRACT 106

3. 1. INTRODUCTION 107

3. 2. MATERIALS AND METHODS 108

3. 2. 1. Bacterial strains and plasmid 108

3. 2. 2. Plasmid DNA preparation............108

3. 2. 3. Probe DNA preparation and Southern hybridization 109

3. 2. 4. Cloning and DNA sequencing 110

3. 3. RESULTS 111

3. 3. 1. Cloning and characterization of a new insertion sequence IS*231*N............111

3. 3. 2. Comparison of IS*231*N with IS*231*M and other IS*231* elements 113

3. 4. DISCUSSION 121

3. 5. REFERENCES 125

4. GENERAL DISCUSSION 128

4. 1. REFERENCES 131

5. REFERENCES............132

LIST OF TABLES

INTRODUCTION

Table 1. Classification of *Bacillus thuringiensis* strains according to the H-serotype 3

Table 2. *Bacillus thuringiensis* transposable elements 13

CHAPTER 1

Isolation and Characterization of a Novel *Bacillus thuringiensis* Strain expressing a Novel Crystal Protein

- Cloning, Sequencing, Characterization and Expression of *cry31Aa2,* a Novel Crystal Protein Gene

Table 1. The biochemical profile of *B. thuringiensis* M15 and selected control strains 48

LIST OF FIGURES

INTRODUCTION

Fig. 1. Positions of conserved blocks among Cry proteins 8

CHAPTER 1

Isolation and Characterization of a Novel *Bacillus thuringiensis* Strain expressing a Novel Crystal Protein

- Cloning, Sequencing, Characterization and Expression of *cry31Aa2,* a Novel Crystal Protein Gene

Fig. 1. A) Phase-contrast micrograph of a lysed culture of *Bacillus thuringiensis* strain M15 44

B) Transmission electron micrograph of *Bacillus thuringiensis* strain M15 containing a spore and a tightly bound parasporal inclusion 46

Fig. 2. SDS-PAGE analysis of the parasporal inclusion protein(s) of *B. thuringiensis* strain M15 50

Fig. 3. Southern blot analyses of *B. thuringiensis* strain M15 ....53, 56

Fig. 4. The structural organization of the pYCH217 insert 58

Fig. 5. Nucleotide sequence and deduced amino acid sequence of the *cry31Aa2* gene 62

Fig. 6. Comparison of the deduced amino acid sequences of Cry31Aa2 and Cry31Aa1 64

Fig. 7. Restriction map of the recombinant plasmid pYCP31A2 containing the *cry31Aa2* gene 66

Fig. 8. Transmission electron micrograph of a *B. thuringiensis* Cry⁻B transformant expressing a novel crystal protein gene, the *cry31Aa2* gene....68

Fig. 9. SDS-PAGE analysis of the parasporal inclusion protein from a *B. thuringiensis* transformant expressing a novel crystal protein gene, the *cry31Aa2* gene....70

CHAPTER 2

A New Insertion Sequence, IS*231*M, in an Autoagglutinable Isolate of *Bacillus thuringiensis*

Fig. 1. Nucleotide sequence of IS*231*M 91

Fig. 2. Structural maps of IS*231*M and iso-IS*231*....93

Fig. 3. Nucleotide sequence of the stem-and-loop secondary structures of IS*231*M 95

CHAPTER 3

Cloning and Nucleotide Sequence of a New Insertion Sequence, IS*231*N, from a Non-Serotypeable Strain of *Bacillus thuringiensis*

Fig. 1. IS*231*N nucleotide sequence and alignment with IS*231*M 115

Fig. 2. Structural organization of IS*231*N and comparison with IS*231*M 117

Fig. 3. Conserved domains of amino acid sequences of IS*231*N and iso-IS*231* elements 119

Fig. 4. Dendrogram of IS*231*N and selected iso-IS*231* elements 123

LIST OF ABBREVIATIONS

°C	Celsius
ΔG	the calculated free energy of interaction (kcalorie)
Ω	ohm (resistance unit)
μF	microfarad (capacitance unit)
μg	microgram
Asn	Asparagine
Asp	Aspartic acid
ATCC	American Type Culture Collection
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
bp	basepair
cm	centimeter
Cry	crystal protein
cry	crystal protein gene
Da	dalton
DIG	digoxigenin
DNA	deoxyribonucleic acid
DR	direct repeat
EDTA	ethylenediaminetetraacetic acid
Lys	Lysine
g	gravity
Gln	Glutamine
Glu	Glutamic acid

HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hr	hour
H_2S	hydrogen sulfide
ICP	insecticidal crystal protein
IPTG	Isopropyl-β-D-thiogalactoside
IR	inverted repeat
IS	insertion sequence
kb	kilobase
kcal	kilocalorie
kDa	kilodalton
kg	kilogram
kV	kilovolt
LB	Luria-Bertani
LiCl	lithium chloride
M	mol(e)
Met	Methionine
mg	milligram
min	minute
ml	milliliter
mM	millimol(e)
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
N-terminal	amino-terminal
ORF	open reading frame

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	hydrogen ion activity
Phe	Phenylalanine
Pro	Proline
PVDF	polyvinylidene difluoride
RBS	ribosome binding site
rRNA	ribosomal ribonucleic acid
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
subsp	subspecies
TEM	transmission electron microscopy
Tn	transposon
TnpA	transposase
Tyr	Tyrosine
V/V	volume per volume
X–Gal	5'-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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INTRODUCTION

Over the last half-century, the intensive use of synthetic organic pesticides, although useful at controlling various pests, has not been without problems. Chemical pesticides have caused considerable environmental problems and they have even threatened human health (Gill *et al.*, 1992).

The biorational insecticide *Bacillus thuringiensis* is a useful alternative to chemical pesticides that has been developped for the control of certain insect pests. The biological insecticides based on *B. thuringiensis* have been valued for their environmental safety, their low development costs, and their specific activity against certain insect pests (Lambert and Peferoen, 1992).

1. *Bacillus thuringiensis* and Its Crystal Proteins

1. 1. Historical background of *B. thuringiensis*

Bacillus thuringiensis is a ubiquitous Gram-positive, spore-forming bacterium that produces parasporal inclusions during the stationary phase of its growth cycle. Many *B. thuringiensis* strains exhibit insecticidal activity against certain insect species among the orders Lepidoptera, Diptera, and Coleoptera (Höfte and Whiteley, 1989). Recently, some *B. thuringiensis* strains have also been shown to be active against other insect orders, such as Hymenoptera, Homoptera, Orthoptera, and Mallophaga (Schnepf *et al.*, 1998), and against a much broader range of invertebrate pests including nematodes, protozoa, and mites (Feitelson *et al.*, 1992).

In 1901, the Japanese biologist S. Ishiwata isolated a bacillus from dying silkworms, *Bombyx mori*, larvae (Ishiwata, 1901). About 10 years later, a similar bacillus was isolated by the German biologist E. Berliner in 1911 from the diseased larvae of the Mediterranean flour moth, *Anagasta (Ephestia) kuehniella*, in Thüringia, Thüringen, Germany. This Gram-positive bacterium was found to produce crystalline inclusions during sporulation, and to be highly toxic to many Lepidopteran larvae. It was named *Bacillus thuringiensis* and its use as an insecticide was suggested (Berliner, 1911; Beegle and Yamamoto, 1992).

Bacillus thuringiensis was applied for the first time in the late 1920s and early 1930s in the field to control the European corn borer, *Ostrinia nubilalis,* in south-eastern Europe. The first commercial formulation of *Bacillus thuringiensis* was marketed as Thuricide by Sandoz in 1957 (Peferoen, 1992; van Frankenhuyzen, 1993).

The discovery of *B. thuringiensis* subsp. *kurstaki* strain HD-1 by H. T. Dulmage (1970), which was up to 200 times more active against some major agricultural pests than other *B. thuringiensis* isolates used in commercial products, accelerated the commercialization of *B. thuringiensis*. This *B. thuringiensis* strain was adopted for commercial production and still forms today the basis for most formulations used in agriculture and forestry.

In the early 1960s, de Barjac and Bonnefoi (1962) developed a classification method for various *B. thuringiensis* strains, based on the agglutination reaction of vegetative bacterial cells with antisera. With this method several thousands of *B. thuringiensis* strains have been classified into at least 80 serovars (Table 1) (Lecadet *et al.*, 1998; Iriarte *et al.*, 2000).

In the late 1970s and early 80s, some new *B. thuringiensis* strains were isolated. They showed toxicity against mosquito and blackfly larvae among the order Diptera

Table 1. Classification of *Bacillus thuringiensis* strains according to the H-serotype

H ANTIGEN	SEROVAR	FIRST MENTION AND/OR FIRST VALID DESCRIPTION
1	*thuringiensis*	Berliner, 1915; Heimpel & Angus, 1958
2	*finitimus*	Heimpel & Angus, 1958
3a,3c	*alesti*	Toumanoff & Vago, 1951; Heimpel & Angus, 1958
3a, 3b, 3c	*kurstaki*	de Barjac & Lemille, 1970
3a, 3d	*sumiyoshiensis*	Ohba & Aizawa, 1989
3a, 3d, 3e	*fukuokaensis*	Ohba & Aizawa, 1989
4a, 4b	*sotto*	Ishiwata, 1905; Heimpel & Angus, 1958
4a, 4c	*kenyae*	Bonnefoi & de Barjac, 1963
5a, 5b	*galleriae*	Shvetsova, 1959; de Barjac & Bonnefoi, 1962
5a, 5c	*canadensis*	de Barjac & Bonnefoi, 1972
6	*entomocidus*	Heimpel & Angus, 1958
7	*aizawai*	Bonnefoi & de Barjac, 1963
8a, 8b	*morrisoni*	Bonnefoi & de Barjac, 1963
8a, 8c	*ostriniae*	Gaixin, Ketian, Minghua & Xingmin, 1975
8b, 8d	*nigeriensis*	Weiser & Prasertphon, 1984
9	*tolworthi*	Norris, 1964; de Barjac & Bonnefoi, 1968
10a, 10b	*darmstadiensis*	Krieg, de Barjac & Bonnefoi, 1968
10a, 10c	*londrina*	Arantes *et al.* (unpublished)
11a, 11b	*toumanoffi*	Krieg, 1969
11a, 11c	*kyushuensis*	Ohba & Aizawa, 1979
12	*thompsoni*	de Barjac & Thompson, 1970
13	*pakistani*	de Barjac, Cosmao Dumanoir, Shaik & Viviani, 1977
14	*israelensis*	de Barjac, 1978
15	*dakota*	De Lucca, Simonson & Larson, 1979
16	*indiana*	De Lucca, Simonson & Larson, 1979
17	*tohokuensis*	Ohba, Aizawa & Shimizu, 1981
18a, 18b	*kumamotoensis*	Ohba, Ono, Aizawa & Iwanami, 1981
18a, 18c	*yosoo*	Lee H. H. *et al.*, 1994
19	*tochigiensis*	Ohba, Ono, Aizawa & Iwanami, 1981
20a, 20b	*yunnanensis*	Wan-Yu, Qi-Fang, Xue-Ping & You-Wei, 1979
20a, 20c	*pondicheriensis*	Rajagopalan *et al.* (unpublished)
21	*colmeri*	De Lucca, Palmgren & de Barjac, 1984
22	*shandongiensis*	Ying, Jie & Xichang, 1986
23	*japonensis*	Ohba & Aizawa, 1986
24a, 24b	*neoleonensis*	Rodriguez-Padilla *et al.*, 1988
24a, 24c	*novosibirsk*	Burtseva, Kalmikova *et al.*, 1995
25	*coreanensis*	Lee H. H. *et al.*, 1994
26	*silo*	de barjac & Lecadet (unpublished)
27	*mexicanensis*	Rodriguez-Padilla & Galan-Wong, 1988
28a, 28b	*monterrey*	Rodriguez-Padilla *et al.* (to be published)
28a, 28c	*jegathesan*	Seleena, Lee H. H., and Lecadet, 1995
29	*amagiensis*	Ohba (unpublished)
30	*medellin*	Orduz, Rojas, Correa, Montoya and de Barjac, 1992
31	*toguchini*	Hodirev (unpublished)
32	*cameroun*	Jacquemard, 1990; Juarez-Perez *et al.*, 1994
33	*leesis*	Lee H. H. *et al.*, 1994
34	*konkukian*	Lee H. H. *et al.*, 1994
35	*seoulensis*	Shim, Lee H. H. *et al.*, 1995
36	*malaysiensis*	Ho (unpublished)
37	*andaluciensis*	Santiago-Alvarez *et al.*, 1996

38	*oswaldocruzi*	Rabinovitch *et al.*, 1995
39	*brasiliensis*	Rabinovitch *et al.*, 1995
40	*huazhongensis*	Yu Ziniu, Dai *et al.*, 1996
41	*sooncheon*	Lee H. H. *et al.*, 1995
42	*jinghongiensis*	Rong Sen Li *et al.*, 1995
43	*guiyangiensis*	Rong Sen Li *et al.*, 1995
44	*higo*	Ohba *et al.*, 1995
45	*roskildiensis*	Hinrinschen, Hansen and Daamgaard (unpublished)
46	*chanpaisis*	Chanpaisang (unpublished)
47	*wratislaviensis*	Lonc *et al.*, 1997
48	*balearica*	Caballero *et al.*, 2000
49	*muju*	Seung Hwan Park *et al.*, (unpublished)
50	*navarrensis*	Caballero *et al.*, 2000
51	*xiaguangiensis*	Jian Ping Yan (unpublished)
52	*kim*	Kim *et al.*, 1996
53	*asturiensis*	Santiago-Alvarez *et al.*, 1996
54	*poloniensis*	Damgaard *et al.* (unpublished)
55	*palmanyolensis*	Santiago-Alvarez *et al.* (unpublished)
56	*rongseni*	Li Rong Sen (unpublished)
57	*pirenaica*	Caballero *et al.* (to be published)
58	*argentinensis*	Campos-Dias *et al.* (unpublished)
59	*iberica*	Caballero *et al.* (to be published)
60	*pingluonsis*	Li Rong Sen (unpublished)
61	*sylvestriensis*	Damgaard *et al.* (unpublished)
62	*zhaodongensis*	Li Rong Sen (unpublished)
63	*bolivia*	Ferré-Manganero *et al.* (to be published)
64	*azorensis*	Santiago-Alvarez *et al.* (unpublished)
65	*pulsiensis*	Khalique F. and Khalique A. (unpublished)
66	*graciosensis*	Santiago-Alvarez *et al.* (unpublished)
67	*vazensis*	Santiago-Alvarez *et al.* (unpublished)

Adapted from Lecadet *et al.*, 1998 and Iriarte *et al.*, 2000.

References cited are to be found in the two above papers.

(Goldberg and Margalit, 1977), and against Colorado potato beetle and elm leaf beetle larvae among the order Coleoptera (Krieg *et al.*, 1983). In addition, in the late 1980s, newer *B. thuringiensis* strains toxic to nematodes were isolated (Edwards *et al.*, 1988).

In 1981, the first *B. thuringiensis* gene coding for the δ-endotoxin crystal protein toxic against certain lepidopteran larvae was isolated (Schnepf and Whiteley, 1981). This led to the cloning of additional toxin genes from other subspecies. In 1985, the first crystal protein gene sequences were published (Adang *et al.*, 1985; McLinden *et al.*, 1985; Schnepf *et al.*, 1985; Shibano *et al.*, 1985).

In 1989, based on a collection of the nucleotide sequences of 38 toxin genes, Höfte and Whiteley suggested a nomenclature and classification scheme that resulted in the distinction of 14 subclasses of genes based on DNA sequence homology and toxicity spectra of the encoded proteins (Höfte and Whiteley, 1989). The rapidly expanding characterization of newer crystal protein genes has since resulted in a variety of DNA sequences and activities that are no longer amenable to the original nomenclature system proposed by Höfte and Whiteley. Accordingly, a new nomenclature system has been proposed and is based on hierarchical clustering using amino acid sequence indentities. Additionally, the initial Roman numerals have been replaced by Arabic numerals in the primary rank (e.g., Cry1Aa) for a better accommodation of the increasing number of newer crystal protein genes. The crystal protein genes have been classified as *cry1Aa* to *cry32Aa*, and *cyt1Aa* to *cyt2Bb* according to the degree of amino acid sequence homology (Crickmore *et al.*, 1998; 2001).

So far, the identification of *B. thuringiensis* strains has been based on serology using their flagellar H-antigen agglutinations, and on phenotypic characterization (de Barjac *et al.*, 1973; 1990). In addition, it has been shown that an

acrystalliferous *B. cereus* can cross-react serologically with some of the *B. thuringiensis* flagellar H-antigens at a frequency close to 30% (Ohba and Aizawa, 1986). Accordingly, a new molecular method using the polymerase chain reaction (PCR) has been developed for the identification of *cry*-type genes and the differentiation among *B. thuringiensis* strains and between *B. thuringiensis* and *B. cereus* (Carozzi *et al.*, 1991; Bourque *et al.*, 1993; Brousseau *et al.*, 1993; Ceron *et al.*, 1994; Chak *et al.*, 1994; Jung *et al.*, 1997).

1. 2. Crystal protein of *B. thuringiensis*

The parasporal crystalline inclusions produced by *B. thuringiensis* often appear microscopically as distinctively shaped crystals (Feitelson *et al.*, 1992). In some strains of *B. thuringiensis*, the crystal proteins can account for 20 to 30% of the dry weight of the sporulated cells. They comprise proteins, named δ-endotoxins, exhibiting a highly specific insecticidal activity against certain insect species (Lereclus *et al.*, 1989).

Most of the Cry toxins contain five conserved amino acid blocks. However, comparison of the carboxyl-terminal halves of the sequences with more than 1,000 amino acids shows that three additional conserved blocks are present outside the active toxic domain (Fig. 1). The amino acid block 1 includes helix 5 of domain I. The central location of helix 5 within domain I plays an essential role in maintaining the structural integrity of the helical bundle. The block 2 contains helix 7 of domain I and the first β-strand of domain II. These two structures encompass the region of contact between the two domains. The blocks 3, 4, and 5 each consist in one of the three buried strands within domain III. The block 3 includes the last β-strand of domain II. This structure is involved in interactions between domains I and III (Schnepf *et al.*, 1998).

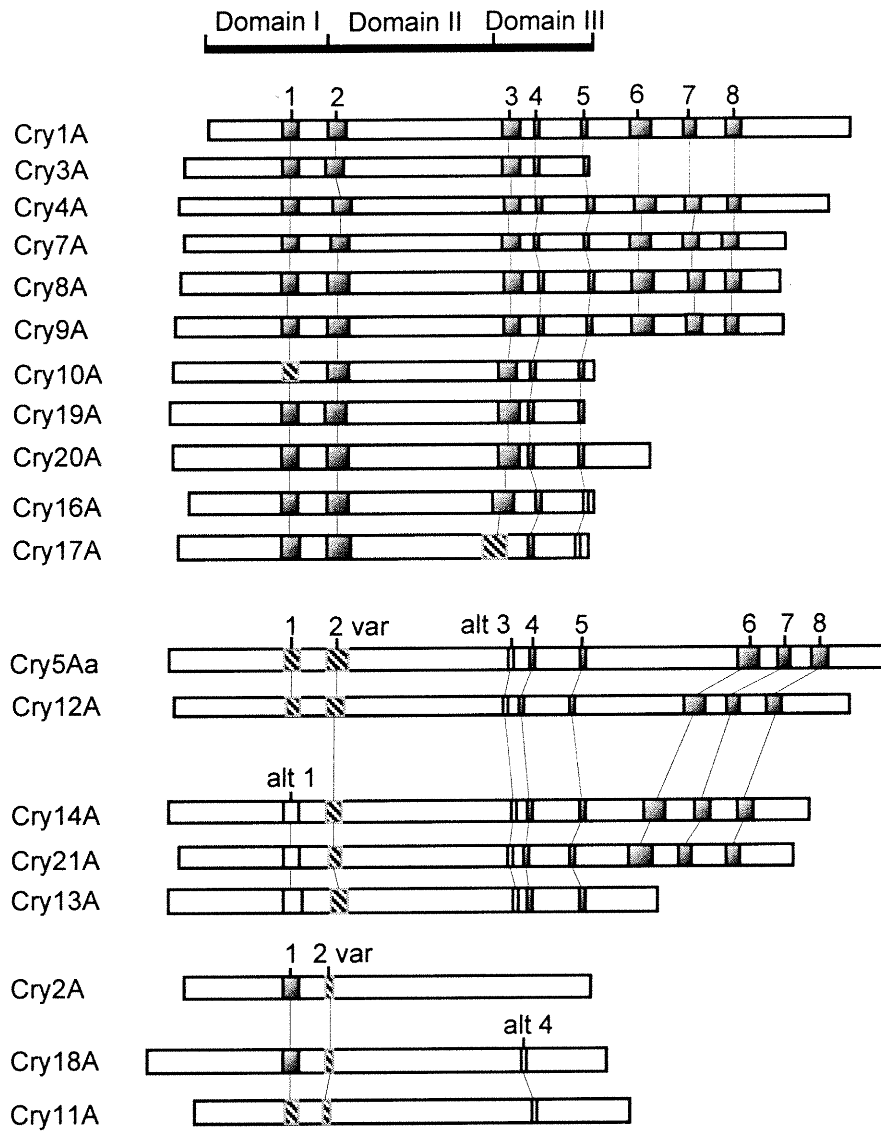
The domain I of the crystal protein contains long hydrophobic and amphipathic helices. This domain could be responsible for the formation of lytic pores in the intestinal epithelium of the target insect. The three β-sheets of domain II contain the surface-exposed loops at their apices. The surface-exposed loops are known to be involved in receptor binding (Schnepf *et al.*, 1998). The domain III plays a role in maintaining the structural integrity of the toxin molecule by protecting it from proteolysis within the midgut of the target insect (Li *et al.*, 1991).

1. 3. Mode of action of the crystal protein

The mode of action of the insecticidal crystal proteins involves solubilization of the crystal proteins in the midgut of target insect, proteolytic processing of the protoxin by proteases in the midgut, binding of the Cry toxin to midgut receptors, and insertion of the toxin into the apical membrane to form pores or ion channels (Schnepf *et al.*, 1998). The 130-kDa protoxins of the Cry1A proteins are digested to the active 65-kDa toxins by the action of insect midgut proteases. The major proteases of the midgut of lepidopteran insects are trypsin-like or chymotrypsin-like. This process starting at the C-terminus of the protoxin proceeds toward the 55- to 65-kDa toxic moieties. Accordingly, the toxic moiety has been identified as the N-terminal half of the protoxin molecule (Chestukhina *et al.*, 1982; Choma *et al.*, 1990). The active toxins of 30- to 35-kDa of *B. thuringiensis* subsp. *israelensis* are known to be produced by the activation of the 72-kDa proteins (Ibarra and Federici, 1986; Pfannestiel *et al.*, 1986). After the ingestion of crystal protein, the most obvious symptoms in lepidopteran larvae are paralysis of the gut and the mouth part, and change of the gut permeability. The larval midgut epithelial cells are the primary target of the toxins. The important damage comprises swelling and

Fig. 1. Positions of conserved blocks among Cry proteins. The figure shows the sequence arrangement for each holotype toxin (e.g., Cry1Aa1) having at least one of the conserved blocks. Sequence blocks are indicated by dark gray boxes, hatched boxes, or light gray boxes to show high, moderate, or low degrees of homology, respectively, to the consensus sequence for each conserved block. Variant (var) blocks show 50 to 75% identity to the consensus sequence of the highly conserved group, which has the consensus sequence of 75% or more. Alternate (alt) blocks are derived from groups of proteins having a consensus sequence over that sequence block that differs from the corresponding highly conserved sequence at more than half of its positions.

Adapted from Schnepf *et al.*, 1998, Fig. 3.


Domain I
Domain II
Domain III
1
2
3
4
5
6
7
8
Cry1A
Cry3A
Cry4A
Cry7A
Cry8A
Cry9A
Cry10A
Cry19A
Cry20A
Cry16A
Cry17A
1
2 var
alt 3
4
5
6
7
8
Cry5Aa
Cry12A
alt 1
Cry14A
Cry21A
Cry13A
1
2 var
Cry2A
alt 4
Cry18A
Cry11A

destruction of the microvilli, swelling of the cells, progressive disintegration of the cell organelles, and a general intracellular vacuolization (Percy and Fast , 1983). It has been proposed that after binding to specific membrane receptors of susceptible epithelial cells, the δ-endotoxin from the *B. thuringiensis* subspecies toxic to lepidopteran or dipteran larvae, could generate small pores in the plasma membrane, consequently leading to colloid osmotic lysis of the cell (Knowles and Ellar, 1987).

2. *Bacillus thuringiensis* as a Biological Control Agent

2. 1. The global market for *B. thuringiensis*-based biopesticides

B. thuringiensis is the most widely used biological agent for pest control. In 1991, worldwide insecticide sales were estimated at more than $6 billion, and the total sales of *Bacillus thuringiensis* was estimated at $105 million, which accounted for 90 to 95% of the insect biocontrol market (Rigby, 1991; Lambert and Peferoen, 1992; Peferoen, 1992). The annual amount of the world-widely distributed *B. thuringiensis* was reported to be 2.3 x 10^6 kg (Rowe *et al.*, 1987). Nearly 200 *Bacillus thuringiensis*-based pesticides were registered by the U. S. Environmental Protection Agency in 1997 (Schnepf *et al.*, 1998). The crystal proteins-based pesticides generally have low costs for development and registration. It has been determined that crystal proteins are not pathogenic to mammals, birds, amphibians, or reptiles, whereas they have high specificity against certain species of insects and invertebrate pests (Schnepf *et al.*, 1998).

2. 2. Delivery systems for crystal proteins

New delivery systems for crystal proteins have been developed to increase field persistence. Two different crystal protein genes have been inserted in genetically modified *B. thuringiensis* strains (Peferoen, 1992). To prolong the persistence of crystal proteins, the crystal protein genes have been introduced into live endophytic or epiphytic bacteria as hosts, which can propagate themselves at the site of feeding and continue to produce crystal proteins (Stock *et al.*, 1990; Tomasino *et al.*, 1995). Since the first introduction of a crystal protein gene into tobacco plants (Barton *et al.*, 1987), many major crops including tomato (Fischhoff *et al.*, 1987), potato (Adang *et al.*, 1993), cotton (Perlak *et al.*, 1990), corn (Koziel *et al.*, 1993), and rice (Fujimoto *et al.*, 1993) have been transformed. In 1996, new varieties of potato, cotton, and corn containing modified crystal protein genes began to be sold to growers. The plant delivery system using the crystal proteins expands the control range against the pests like sucking and boring insects, root-dwelling insects, and nematodes (Schnepf *et al.*, 1998).

2. 3. Resistance Management to *B. thuringiensis* toxins

Bacillus thuringiensis has been reported to be liable to resistance development (Lambert and Peferoen, 1992). In laboratory selection experiments, high levels of resistance to some *B. thuringiensis* have been observed in the Indian meal moth (*Plodia interpunctella*; McGaughey, 1985), the almond moth (*Cadra cautella*; McGaughey and Beeman, 1988), the tobacco budworm (*Heliothis virescens*; Stone, *et al.*, 1989), and the Colorado potato beetle (*Leptinotarsa decemlineata*; Whalon *et al.*, 1993). Resistance in the field to *B. thuringiensis*-based insecticides has been reported (Tabashnik *et al.*,

1990), and a 400-fold increase of resistance of diamondback moth (*Plutella xylostella*) has been observed in Florida fields that had been heavily treated with a *B. thuringiensis*-based insecticide (Lambert and Peferoen, 1992). In the absence of exposure to insecticidal crystal proteins, however, reversal of up to 2,800-fold resistance to *B. thuringiensis* toxin has been obtained (Tabashnik *et al.*, 1994).

Accordingly, Oberlander (1996) suggested the use of spatial refuges for the survival of susceptible insects in the populations, the use of mixtures of toxins or alternating different toxins, the expression of very high doses of *B. thuringiensis* toxin in the transgenic plants for destruction of all of the heterozygous insects carrying genes for *B. thuringiensis* resistance, or the production of low levels of *B. thuringiensis* toxin in the transgenic plants combined with the use of other insect control methods as part of an integrated pest management strategy and for proper management of resistance to *B. thuringiensis* toxin.

3. The Transposable Elements of *B. thuringiensis*

The multiple locations and genetic mobility of *cry* genes encoding insecticidal crystal proteins (ICP), or δ-endotoxins, have been attributed to their close association with both class I insertion sequences (IS) elements and class II transposons. Twenty-one insertion sequences and two class II transposons have been isolated and characterized (Table 2).

Thirteen iso-IS*231* transposable elements, IS*231*A, -B, and -C (Mahillon *et al.*, 1985, 1987), -D, -E, and -F (Rezsöhazy *et al.*, 1992), -G and -H (Ryan *et al.*, 1993), -V and -W (Rezsöhazy *et al.*, 1993), -M (Jung *et al.*, 2001a; this work, chapter 2), -N (Jung

Table 2. *Bacillus thuringiensis* transposable elements

Name	Synonyms	Family	Origin	Length (bp)	IR (bp)	DR (bp)
IS*231* A	IR1, IR1750	IS*4*	*B.thuringiensis* subsp. *thuringiensis* berliner 1715 (plasmid 65Kb)	1656	20	10, 11, 12
IS*231* B	IR1, IR1750	IS*4*	*B.thuringiensis* subsp. *thuringiensis* berliner 1715 (plasmid 65Kb)	1646	7/20	ND
IS*231* C	IR1, IR1750	IS*4*	*B.thuringiensis* subsp. *thuringiensis* berliner 1715 (plsamid 65Kb)	1655	19/20	11
IS*231* D	-	IS*4*	*B.thuringiensis* subsp. *finitimus*	1657	17/20	ND
IS*231* E	-	IS*4*	*B.thuringiensis* subsp. *finitimus*	1616	0 /20	ND
IS*231* F	-	IS*4*	*B.thuringiensis* subsp. *israelensis* (plasmid 112kb)	1655	19 /20	12
IS*231* G	-	IS*4*	*B.thuringiensis* subsp. *darmstadiensis*	1649	20	ND
IS*231* H	-	IS*4*	*B.thuringiensis* subsp. *darmstadiensis*	817	ND	ND
IS*231* V	-	IS*4*	*B.thuringiensis* subsp. *israelensis* (plasmid 112kb)	1964	21/22	ND
IS*231*M	-	-	*B.thuringiensis* M15 (this work)	1652	18/20	11
IS*231*N	-	-	*B.thuringiensis* M15 (this work)	1654	18/20	ND
IS*231*Y	-	-	ND	ND	ND	ND
IS*231* W	-	IS*4*	*B.thuringiensis* subsp. *israelensis* (plasmid 112kb)	1964	21/22	ND
IS*232* A	IR2, IR2150	IS*21*	*B.thuringiensis* subsp. *thuringiensis* berliner 1715 (plasmid 65Kb)	2184	28/37	ND
IS*232* B	IR2, IR2150	IS*21*	*B.thuringiensis* subsp. *kurstaki* HD-73 (plasmid 75Kb)	2200	28/37	ND
IS*232* C	IR2, IR2150	IS*21*	*B.thuringiensis* subsp. *kurstaki* HD-73 (plasmid 75Kb)	2200	28/37	ND
IS*240* A	IRA	IS*6*	*B.thuringiensis* subsp. *israelensis* (plasmid 112kb)	865	15/16	ND
IS*240* B	IRB	IS*6*	*B.thuringiensis* subsp. *israelensis* (plasmid 112kb)	865	16	ND
OrfA			*B.thuringiensis* subsp. *fukuokaensis*	802	16/17	ND
ISBT *1*	*orf* X	IS*3*	*B.thuringiensis* subsp. *aizawai* HD229	999	15/17	ND
ISBT *2*	-	IS*3*	*B.thuringiensis* YBT-226	187	ND	ND
Tn*4430*	Th	Tn*4430*	*B.thuringiensis* subsp. *kurstaki* Kto (plasmid 87kb)	4149	38	5
Tn*5401*	-	Tn*4430*	*B.thuringiensis* subsp. *morrisoni* EG2158	4837	53	5

Adapted from Mahillon *et al.*, 1994; Dunn and Ellar, 1997; Jung *et al.*, 2001a and b; Chen and Mahillon, unpublished data.

et al., 2001b; this work, chapter 3), and -Y (Chen and Mahillon, unpublished data) have been cloned and characterized. IS*231*A, -B, and -C were isolated from *B. thuringiensis* subsp. *thuringiensis.* IS*231*D and -E were isolated from *B. thuringiensis* subsp. *finitimus.* IS*231*F, -V and -W were isolated from *B. thuringiensis* subsp. *israelensis.* IS*231*G and -H were isolated from *B. thuringiensis* subsp. *darmstadiensis.* IS*231*A , -B, -C, -D, -E, and -F are 1655 to 1657-bp in length including 20-bp inverted repeat (IR) at their ends. They contain a single open reading frame (ORF) coding for a putative transposase of 477-478 amino acids in length, and are flanked by 11-bp direct repeat (DR). However, the last 13-bp of IS*231*B are missing due to the insertion of IS*232*A in its right IR. IS*231*E lacks its first 42 bp, including the left IR. Consequently, these two elements are structurally inactive. IS*231*H has only been partially characterized and IS*231*G contains several stop codons in its ORF (Ryan *et al.*, 1993). The most distantly related IS*231*V and -W are 1964-bp in length and are 307 to 309-bp longer than the other iso-IS*231*. Both IS contain two open reading frames (ORFs). These ORFs overlap by 8 and 27 codons in IS*231*V and IS*231*W, respectively (Rezsöhazy *et al.*, 1993). All IS*231* transposases contain five conserved domains, N1, N2, N3, C1, and C2, which share more than 60% nucleotide identity (Mahillon *et al.*, 1994). The transposition of IS*231*A has been demonstrated in *E. coli.* Its transposition results in 10- to 12-bp direct repeats (DRs) at the target site (Hallet *et al.*, 1991).

At least three iso-IS*232* transposable elements, IS*232*A, -B, and -C, have been identified (Menou *et al.*, 1990; Lereclus *et al.*, 1992). IS*232* is structurally associated with either Tn*4430* or iso-IS*231* (Mahillon *et al.*, 1994). IS*232*A is a 2,184-bp element and is delimited by two 37-bp imperfect inverted repeats with nine mismatches. IS*232*A contains two long open reading frames that code for two potential polypeptides of 50- and 30-kDa, respectively. Three copies of IS*232* are located on the 75-kb plasmid of *B.*

thuringiensis subsp. *kurstaki* HD-73 (Menou *et al.*, 1990), whereas there are two copies, IS*232*A and -B, on the 65-kb plasmid in *B. thuringiensis* subsp. *thuringiensis* berliner 1715 (Menou *et al.*, 1990). Moreover, a *cryIA(c)* crystal protein gene is flanked by IS*232*A and -C in *B. thuringiensis* subsp. *kurstaki* HD-73 (Menou *et al.*, 1990). IS*232* is shown to be distributed in only 7 of 61 *B. thuringiensis* serovars and is apparently exclusive to *B. thuringiensis* (Léonard *et al.*, 1997). The transposition of the IS*232*A was demonstrated in *E. coli* (Menou *et al.*, 1990).

The *cry4A* crystal protein gene is flanked by two repeated sequences in opposite orientations on a 112-kb plasmid of *B. thuringiensis* subsp. *israelensis* (Bourgouin *et al.*, 1988). These two repeat sequences, designated IS*240*, show characteristic features of insertion sequences. IS*240*A and IS*240*B are both 865-bp in length. IS*240*B has two perfect inverted repeats of 16-bp, while IS*240*A has two 16-bp imperfect inverted repeats with one mismatch. IS*240*A is 99% identical to IS*240*B due to a 6-bp differences (Delécluse *et al.*, 1989). IS*240* is widely distributed in *B. thuringiensis* and is invariably present in the known dipteran-active *B. thuringiensis* strains (Rosso and Delécluse, 1997). An IS*240*-related element has been found 500-bp upstream from the *cry11B* gene encoding an 80-kDa mosquitocidal toxin in the *B. thuringiensis* subsp. *jegathesan* (Delécluse *et al.*, 1995). An IS*240*-like element, OrfA, is located upstream of *orf1* encoding the 24-kDa Orf1 protein from *B. thuringiensis* subsp. *fukuokaensis* (Dunn and Ellar, 1997). This element is 802-bp in length and is delimited by two 17-bp imperfect inverted repeats with 1 mismatch.

ISBT*1* associated with the *cry1Ca* gene is located on the chromosomal DNA of *B. thuringiensis* subsp. *aizawai* and *entomocidus* (Smith *et al.*, 1994). This element contains an open reading frame encoding a 30-KDa polypeptide, and is located less than 200-bp upstream of the *cry1Ca* gene. ISBT*1* is also positioned downstream of the

cry1D. This element is 998-bp in length, and is flanked by two 17-bp imperfect inverted repeat. Another 187-bp element, ISBT*2*, is located downstream of the cryptic *cry2Ab* gene of *B. thuringiensis* YBT-226 (Hodgman *et al.*, 1993), and exists in the same 6.5-kb *Hin*dIII fragment in *B. thuringiensis* subsp. *galleriae* 916 (Ahmad *et al.*, 1989).

Tn*4430*, previously designated as the Th-sequence, found on an 87-kb plasmid of *B. thuringiensis* subsp. *kurstaki* is carried by both large and small plasmids, and is frequently associated with the *cryIA* type genes in various lepidopteran-active strains (Lereclus *et al.*, 1984; Sanchis *et al.*, 1988). Tn*4430* was originally isolated in the course of conjugation experiments dealing with a conjugative plasmid pAMβ1 from *Enterococcus faecalis* in *B. thuringiensis* (Lereclus *et al.*, 1983). Tn*4430* is a cryptic and 4,149-bp replicative transposon, and has identical inverted repeated sequences of 38-bp at each end. There is a direct repeat of 5-bp at the target site. The transposase (TnpA) of 987-amino acids in length is required for the transposition of Tn*4430*, and is functional in *E. coli* (Lereclus *et al.*, 1986).

The second known class II transposon, Tn*5401*, was isolated from a sporulation-deficient variant of *B. thuringiensis* subsp. *morrisoni* EG2158 following its insertion into a recombinant plasmid (Baum, 1994). This transposon shows distant homology to Tn *4430*, albeit it is also crytic and shares a structural organization similar to Tn*4430*. Tn*5401* and Tn*4430* are not known to coexist in any *B. thuringiensis* strain. Tn*5401* is a 4,837-bp transposon that is 688-bp longer than Tn*4430*. It contains four potential open reading frames and 53-bp perfect inverted repeats at the ends of Tn*5401*. The two large open reading frames code for putative polypeptides of 306 and 1,005 amino acids, respectively, in the same direction. Two small open reading frames encode putative proteins of 85 and 74 amino acids, respectively, that are oriented in opposite directions

to the two large coding regions. Tn*5401* is also located just downstream of the *cry3Aa* gene in *B. thuringiensis* subsp. *tenebrionis* NB176 (Adams *et al.*, 1994).

Bacillus thuringiensis has yielded several transposable elements. So far, at least four transposable elements, IS*231*A, IS*232*, Tn*4430* and Tn*5401*, have been shown to be active in *E. coli*, *B. subtilis* and *B. thuringiensis*. Most transposable elements are located in the vicinity of the crystal protein genes and could contribute to the translocation of the crystal protein genes (Mahillon *et al.*, 1994).

Although the role of the transposable elements in *B. thuringiensis* has not been clearly deciphered, two possibilities have been proposed. A first possible role is the involvement of the transposable elements in the amplification of the *cry* genes in *B. thuringiensis.* A second possibility is one of mediating the transfer of plasmids by a conduction process that is involved in the formation of co-integrate structures between self-conjugative plasmids and chromosomal DNA or non-conjugative plasmids. Accordingly, the horizontal dissemination of the genetic material may be a major proper function for the transposable elements (Schnepf *et al.*, 1998).

4. Summary of this research project

The chapter 1 reports the isolation and characterization of a novel auto-agglutinable, non-serotypeable *Bacillus thuringiensis* strain from dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae) collected in an apple orchard in Quebec, Canada. It also reports the cloning, sequencing, characterization and expression of a novel crystal protein gene, *cry31Aa2*, which codes for an 83-kDa crystal protein.

Additionally, in the course of cloning the novel crystal protein gene, *cry31Aa2*, two new insertion sequences have been cloned and sequenced. The chapters 2 and 3 report the cloning and characterization of IS*231*M and -N, respectively.

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CHAPTER 1

Isolation and Characterization of a Novel *Bacillus thuringiensis* Strain expressing a Novel Crystal Protein – Cloning, Sequencing, Characterization and Expression of *cry31Aa2*, a Novel Crystal Protein Gene

Manuscript in preparation

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ABSTRACT

A novel *Bacillus thuringiensis* strain, referred to as M15, was isolated from dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae). The *B. thuringiensis* strain M15 is autoagglutinable and hence non-serotypeable. Its biochemical profile is different from the ones of *B. thuringiensis* var. *kurstaki* HD-1, -var. *israelensis* HD-500 and -var. *higo* BT205.

Under phase-contrast microscopy, *B. thuringiensis* strain M15 produces a roughly spherical parasporal inclusion body tightly coupled to the spore. Further analysis by transmission electron microscopy (TEM), however, revealed that the parasporal inclusion body has an hexagonal shape.

The parasporal inclusion appears to be composed of at least two major polypeptides of 86- and 79-kDa as estimated on a 10% SDS-polyacrylamide gel. Both polypeptides share identical N-terminal amino acid sequences. An oligonucleotide derived from the amino acid sequence was synthesized and used as a probe in Southern hybridization and cloning experiments. Southern hybridization against M15 DNA revealed a single 8-kb *Hin*dIII fragment indicating that the corresponding crystal protein gene is probably present in only one copy.

The novel crystal protein gene, named *cry31Aa2*, was cloned. It is 2,226-bp in length and codes for a polypeptide of 742 amino acids with a predicted molecular mass of 83,068Da. The start codon is not ATG but GTG. The *cry31Aa2* gene has an open reading frame (ORF) that is 57-bp longer than *cry31Aa1* due to the addition of 19 codons at nucleotide positions 202-258.

The new crystal protein, Cry31Aa2, shares 94% amino acid identities with Cry31Aa1, the so-called parasporin protein. It contains the five conserved sequence

blocks also present in Cry31Aa1. All five conserved amino acid blocks of Cry31Aa2 are identical to those of Cry31Aa1 except for the substitution of a lysine residue in the second conserved block of Cry31Aa2.

The *cry31Aa2* gene was expressed in a *B. thuringiensis* Cry^{-} B strain. The transformant produces distinct, well separated, spore and crystal. The parasporal inclusion body is composed of the single major polypeptide of 83-kDa.

Key words: Bacillus thuringiensis; Cry31Aa2

1. 1. INTRODUCTION

Bacillus thuringiensis is a Gram-positive bacterium that produces parasporal crystalline protein inclusions during sporulation. The inclusions of several *B. thuringiensis* strains exhibit specific toxicity against a wide spectrum of insects including Lepidoptera, Diptera, and Coleoptera (Höfte and Whiteley, 1989) and a range of invertebrate pests including nematodes, protozoa, and mites (Feitelson *et al.*, 1992).

Most *B. thuringiensis* strains have been classified into 67 different H-serotypes based on their flagellar agglutinations (de Barjac and Frachon, 1990; Lecadet *et al.*, 1999). Recently, however, it has been reported that many non-insecticidal *B. thuringiensis* isolates are ubiquitous to natural environments and may be classified into a non-serotypeable group due to nonmotility or conspicuous autoagglutination (Ohba and Aizawa, 1986; Ohba, 1996; Roh *et al.*, 1996; Kim, 2000). Surprisingly, some of these non- insecticidal isolates exhibit unique cytolytic activities against various vertebrate cells including human leukaemia T cell (MOLT-4), human lung cancer cell (A549) and human uterus cervix cancer cell (HeLa) (Mizuki *et al.*, 1999; Lee *et al.*, 2000).

Since the first complete determination of the nucleotide sequence of a crystal gene, the *cry1Aa* gene from *B. thuringiensis* var. *kurstaki* HD-1-Dipel (Schnepf *et al.*, 1985), nearly 200 *B. thuringiensis* toxin genes have been cloned and sequenced (Crickmore, 2001). Taxonomy of these genes is based on the degree of the encoded amino acid sequence homology (Crickmore *et al.*, 1998).

We have cloned a novel *cry31A*-type gene, designated *cry31Aa2*, from a new *B. thuringiensis* strain that was isolated from dead twospotted spider mites. Surprisingly, the crystal protein cloned was found to have highly similarity to the parasporin that

exhibits strong human anti-cancer cell activity (Mizuki *et al.*, 2000). We report here the isolation and characterization of a novel *B. thuringiensis* strain expressing a novel crystal protein in addition to cloning, sequencing, characterization and expression of *cry31Aa2,* a novel crystal protein gene from a novel *B. thuringiensis* strain.

1. 2. MATERIALS AND METHODS

1. 2. 1. Bacterial strains and plasmids

The autoagglutinable, non-serotypeable strain of *Bacillus thuringiensis* used in this study, referred to as M15, was isolated from dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae). *B. thuringiensis* var. *kurstaki* HD-1 and -var. *israelensis* HD-500 were obtained from "Laboratoire des bactéries entomopathogènes", Institut Pasteur (Paris, France). *B. thuringiensis* var. *higo* BT205 was in our collection (Jung *et al.*, 1998). *E. coli* DH5α (Gibco BRL, Burlington, Ontario, Canada) was used as a bacterial host for the cloning vectors pUC18 (Amersham Pharmacia Biotech, Montréal, Québec, Canada) and pBluescript II KS(+) (Stratagene, La Jolla, California, USA). The *E. coli-B. thuringiensis* shuttle vector pHPS9 (Haima, *et al.*, 1990) was purchased from American Type Culture Collection, (Manassas, Virginia, USA). The acrystalliferous *B. thuringiensis* var. *kurstaki* HD-1 Cry^{-} B strain (Stahly *et al.*, 1978) was kindly provided by the *Bacillus* Genetic Stock Center, The Ohio State University (Columbus, Ohio, USA).

1. 2. 2. Isolation of a new *B. thuringiensis* strain

Dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae), parasitic on apple leaves, were collected in an apple orchard located in Frelighsburgh, Québec, Canada. They were homogenized in 3 ml of phosphate-buffered saline (PBS) (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g l^{-1}). The homogenized solution was incubated for 16 hr at room temperature and heated

at 78°C for 15 min. Afterwards, the homogenate was plated on 2YT agar medium (Bacto Tryptone 16 g, Bacto Yeast Extract 10 g, NaCl 5 g, Agar 18 g l^{-1}), and incubated for 24 hr at 30°C. All colonies with a morphology similar to *B. thuringiensis* were streaked on T3 agar medium (Bacto Tryptone 3 g, Bacto Tryptose 2 g, Bacto Yeast Extract 1.5 g, $MnCl_2$ 0.005 g, 0.05M Sodium phosphate, pH6.7, Agar 18 g l^{-1}) and incubated at 30°C for 48 hr. The cultures were examined by phase-contrast microscopy (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) for the presence of spores and crystals.

1. 2. 3. Biochemical characterization of *B. thuringiensis* strain

The *B. thuringiensis* strain M15 was biochemically characterized using the API 50CH and API 20E kits as recommended by the manufacturer (bioMérieux, St-Laurent, Québec, Canada). *B. thuringiensis* var. *kurstaki* HD-1, -var. *israelensis* HD-500, and -var. *higo* BT205 were used as controls.

1. 2. 4. Analyses of parasporal inclusion proteins by SDS-PAGE

The *B. thuringiensis* strain M15 was grown in T3 broth for 5 days at 30°C on a rotary shaker to allow crystal protein production. Spores and crystals were separated from each other in the tightly bound parasporal duplexes using an ultrasonic processor model VC130 (Sonics & Materials, Inc., Newtown, Connecticut, USA) and purified by sucrose density gradient centrifugation as described elsewhere (Thomas and Ellar, 1983). The *B. thuringiensis* Cry^-B transformant containing the *B. thuringiensis* M15 parasporal crystal protein gene was incubated in nutrient broth (Bacto Beef Extract 3 g,

Bacto Peptone 5 g l^{-1}) at 30°C for 3 days to allow expression of the toxin gene and crystal formation. Parasporal crystals were purified by sucrose density gradient centrifugation. Twenty microliters of the crystal suspension were added to 3 volumes of gel loading buffer (4% SDS, 20% glycerol, 125 mM Tris-HCl, 10% 2-mercaptoethanol, pH 6.8) in a 1.5-ml microtube, incubated at 90°C for 7 min and centrifuged for 2 min to remove unsolubilized materials. Thirty microliters of the supernatant were loaded on top of 10% SDS-polyacrylamide gels. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli and Favre (1973). The parasporal crystal of *B. thuringiensis* var. *kurstaki* HD-1 was used as a control.

1. 2. 5. Crystal protein N-terminal sequencing

The N-terminal amino acid sequence of the crystal protein from *B. thuringiensis* strain M15 was determined as follows. The purified parasporal crystal was added into 0.1N NaOH-3M HEPES solution and solubilized in 10 volumes of gel loading buffer by incubating in boiling water for 5 min. The crystal protein was separated on 10 % SDS - PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Mississauga, Ontario, Canada). The crystal protein band stained with Coomassie brilliant blue R-250 (Bio-Rad) was excised and subjected to a pulsed liquid phase sequencer model 473A (Applied Biosystems, Foster City, California, USA) at the Regional Sequencing Facility (Centre de recherche du Centre hospitalier de l'Université Laval, Québec, Canada).

1. 2. 6. Plasmid DNA preparation and Southern hybridization

B. thuringiensis strain M15 was grown in Luria-Bertani (LB) medium (Bacto Tryptone 10 g, Bacto Yeast Extract 5 g, NaCl 5 g l^{-1}) at 30°C for 16 hr on a rotary shaker. Plasmid DNA was isolated using the alkaline extraction method as described elsewhere (Birnboim and Doly, 1979) except that lysozyme (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) was added at a concentration of 2 $mg \cdot ml^{-1}$ and the cell suspension was incubated at 37°C for 1hr.

Recombinant plasmid DNAs from *E. coli* DH5α were isolated by the alkaline extraction method (Birnboim and Doly, 1979). The plasmid DNA for DNA sequencing was purified with Wizard® Plus SV minipreps DNA purification system following the manufacturer's recommendation (Promega, Nepean, Ontario, Canada).

An 18-mer degenerate oligonucleotide, named M15-M, was designed based on the N-terminal amino acid sequence of the crystal protein and used as a DNA probe for Southern hybridization. The M15-M oligonucleotide was labelled by the Digoxigenin (DIG) oligonucleotide 3'-end labelling kit containing DIG-11-ddUTP (Roche, Laval, Québec, Canada) as recommended by the manufacturer. The labelled oligonucleotide was precipitated with 0.1 volume of 4M LiCl and 2.5 volumes of ice-cold ethanol, and transferred at -70°C for 30 min. The reaction was centrifuged at 16,000g for 15 min at 4°C. The washed pellet was resuspended in nuclease-free water, and stored at -20°C until use.

Plasmid DNA from *B. thuringiensis* strain M15 was digested with *Hin*dIII, *Hin*dIII/*Eco*RI and *Eco*RI (Gibco BRL), respectively, electrophoresed on a 0.7% agarose gel and transferred onto a Nytran® nylon membrane (Schleicher & Schuell, Keene, New

Hampshire, USA) by the method of Southern (1975). Southern blot hybridization using the DIG-labelled oligonucleotides was done with the standard hybridization solution (5X SSC, 1% blocking reagent (Roche), 0.1% N-lauroylsarcosine, 0.02% SDS) for 13 hr at 39°C. After hybridization, the membrane was washed twice for 15 min each in 4X wash solution (4X SSC, 0.1% SDS) at 39 °C. Following the washes, detection of signals on the membrane was performed with the color-substrate solution containing NBT (4-Nitroblue tetrazolium chloride, Roche) and BCIP (5-Bromo-4-chloro-3-indolyl-phosphate, Roche) as recommended by the manufacturer.

1. 2. 7. Cloning and subcloning of the crystal protein gene

Plasmid DNA purified from *B. thuringiensis* strain M15 was digested with *Hin*dIII and ligated with *Hin*dIII-digested SAP (Shrimp Alkaline Phosphatase, Roche)-treated pBluescript II KS(+). After ligation, the recombinant DNA was transformed into *E. coli* DH5α. Preparation of *E. coli* DH5α competent cells and transformation were done as described elsewhere (Sambrook *et al.*, 1989). The transformants were grown on LB agar plates containing 100 μg ml^{-1} ampicillin (Sigma-Aldrich Canada Ltd.) and 40 μg ml^{-1} X–Gal (5'-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma-Aldrich Canada Ltd.) at 37°C. White colonies were toothpicks-transferred to 1 ml of fresh LB media supplemented with 100 μg ml^{-1} ampicillin, and incubated overnight at 37°C. The recombinant plasmid DNAs were isolated by the cracking procedure (Sambrook *et al.*, 1989) and electrophoresed on a 0.7% agarose gel. Based on inserts estimated sizes, three recombinant plasmid DNAs were selected, digested with *Hin*dIII, electrophoresed on a 0.7% agarose gel, transferred onto a Nytran® nylon membrane by the method of

Southern (1975) and probed with the M15-M oligonucleotide. The recombinant selected by Southern hybridization was further digested using a series of restriction enzymes [*Eco*RI, *Bgl*II (Gibco BRL), *Dra*I, *Sph*I (Amersham Pharmacia Biotech)] for subcloning purposes and ligated to either pUC18 or pBluescript II KS(+).

1. 2. 8. DNA sequencing

DNA sequencing was done with the near-infrared (NIR) fluorescence automated DNA sequencer (LI-COR Model 4200, LI-COR, Inc., Lincoln, Nebraska, USA). Nucleotide and protein sequences were analyzed with the DNASIS program (Hitachi Software Engineering Co., Scarborough, Ontario, Canada).

1. 2. 9. Expression of the crystal protein in the *B. thuringiensis* Cry$^-$ B strain

The 3.6-kb *Hin*dIII/*Sph*I fragment containing the crystal protein gene was excised from the recombinant plasmid pYCH217, and then cloned into the *E. coli-B. thuringiensis* shuttle vector pHPS9 doubly digested with *Hin*dIII/*Sph*I. The *B. thuringiensis* var. *kurstaki* HD-1 acrystalliferous Cry$^-$ B strain was transformed with the cloned *B. thuringiensis* M15 crystal protein gene by electroporation as described by Vehmaanperä (1989) with the following modifications. Bacterial cells cultured in 200 ml of LB supplemented with 0.25 M sucrose and 0.05 M potassium phosphate, pH7.0 (LBSP) to an optical density of 1.0 at 600 nm were centrifuged, washed three times with ice-cold SHMG buffer (250 mM sucrose, 1 mM HEPES, 1 mM $MgCl_2$, 10% (v/v) glycerol, pH 7.0), and then resuspended in 1 ml of ice-cold SHMG buffer. The cell suspension was mixed with plasmid DNA at a final DNA concentration of 10 $\mu g\ ml^{-1}$ in a 0.2-cm

electroporation cuvette (Bio-Rad), kept on ice for 30 min, and then electroporated by a Gene Pulser™ model 1652076 (Bio-Rad) at 25μF, 2.5kV and 400Ω with the pulse once. After electroporation, 3ml of LBSP supplemented with 10% (v/v) glycerol (LBSPG) were immediately added into the cuvette and incubated at 37°C for 2 hr with shaking. The *B. thuringiensis* transformants were selected on nutrient agar plates containing 5 μg ml^{-1} of erythromycin (Sigma-Aldrich Canada Ltd.) and 5 μg ml^{-1} of chloramphenicol (Sigma-Aldrich Canada Ltd.) at 37°C. The presence of parasporal inclusions was examined by phase-contrast microscopy.

The selected *B. thuringiensis* transformant was cultured in 250 ml of nutrient broth supplemented with 5 μg ml^{-1} of erythromycin and 5 μg ml^{-1} of chloramphenicol until cell autolysis was observed. The lysate was harvested and then washed twice with 10 mM EDTA (pH 8.0)-1 M NaCl-1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich Canada Ltd.). The parasporal inclusion from a *B. thuringiensis* transformant was purified by sucrose density gradient centrifugation as described previously (Thomas and Ellar, 1983). The expressed crystal protein was analysed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as reported previously (Laemmli and Favre, 1973).

1. 2. 10. Electron microscopy

The indigenous *B. thuringiensis* strain M15 was grown on a T3 agar plate for 48 hr at 30°C. The *B. thuringiensis* Cry^{-}B transformant harboring the expressed crystal protein was incubated on a nutrient agar plate supplemented with 5 μg ml^{-1} of erythromycin and 5 μg ml^{-1} of chloramphenicol for 48 hr at 30°C. For transmission

electron microscopy (TEM), the samples were ultra-thinly sectioned according to Beveridge *et al.* (1994).

1. 3. RESULTS

1. 3. 1. Morphological and biochemical characteristics

A *Bacillus thuringiensis* strain was isolated from dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae) and named M15. It is an autoagglutinable, non-serotypeable strain. The parasporal inclusion bodies produced by a sporulated culture of *B. thuringiensis* strain M15 appear roughly spherical when observed under phase-contrast microscopy (Fig. 1A) and are tightly coupled to the spores even in lysed cultures. Further analysis under the transmission electron microscope (TEM), however, reveals that the parasporal inclusion body has a polygonal shape (Fig. 1B).

The strain M15 was characterized for its ability to ferment specific carbon sources, and for the production, utilization and reduction of specific compounds (Table 1). The biochemical characteristics of *B. thuringiensis* strain M15 were different from those of three controls, *B. thuringiensis* var. *kurstaki* HD-1, -var. *israelensis* HD-500 and -var. *higo* BT205.

1. 3. 2. SDS-PAGE analysis and N-terminal sequencing of the parasporal inclusion protein

The *B. thuringiensis* strain M15 parasporal inclusion was purified by sucrose density gradient centrifugation and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). At least two major bands of approximately 86- and 79-kDa in size were revealed. They were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-

Fig. 1. A) Phase-contrast micrograph of a lysed culture of *Bacillus thuringiensis* strain M15.

B. thuringiensis strain M15 was incubated for 5 days at 30ºC in T3 broth. Arrows show the roughly spherical parasporal inclusions tightly bound to the white ovoid spores.

Magnification : 800 X.

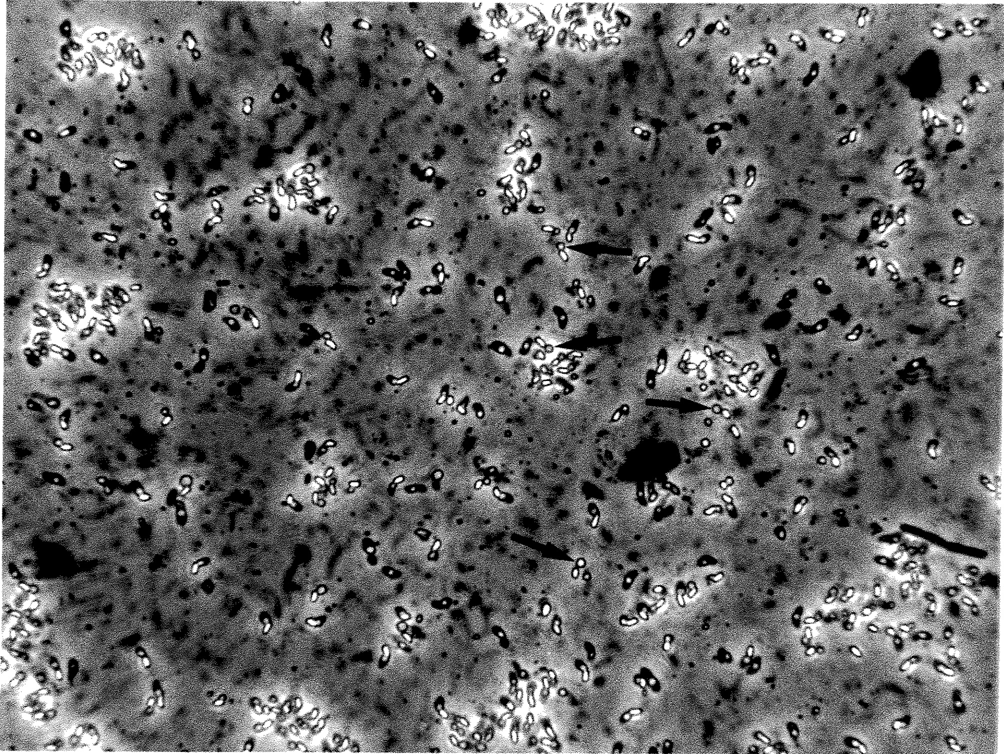


Fig. 1. B) Transmission electron micrograph of *Bacillus thuringiensis* strain M15 containing a spore and a tightly bound parasporal inclusion.

S: spore; P: parasporal inclusion.

Magnification : 25,000 X.

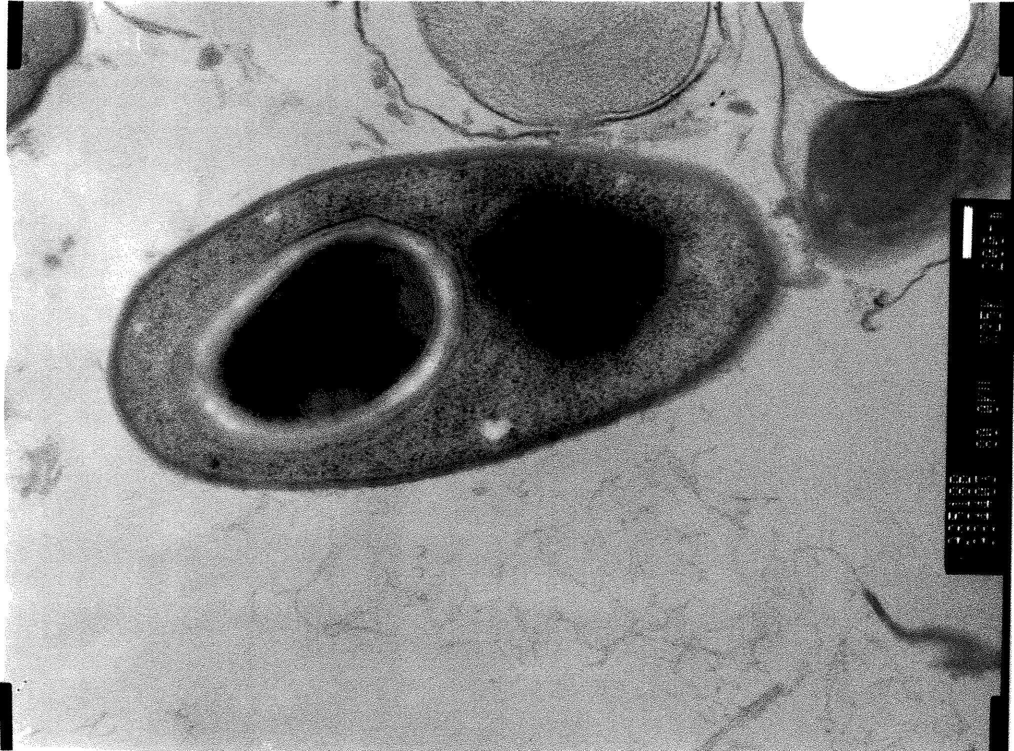


Table 1. The biochemical profile of *B. thuringiensis* M15 and selected control strains

Tests	*B. thuringiensis* var. *kurstaki* HD-1	*B. thuringiensis* var. *israelensis* HD-500	*B. thuringiensis* var. *higo* BT 205	*B. thuringiensis* M 15
Fermentation of				
Glycerol	+	+	+	±
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	-
Ribose	+	+	+	+
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
D-Galactose	-	-	-	-
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	-	-	-	-
L-Sorbose	-	-	-	-
Inositol	-	-	-	-
D-Mannitol	-	-	-	-
D-Sorbitol	-	-	-	-
N-Acetylglucosamine	+	+	+	+
Arbutin	+	+	+	-
Esculin	+	±	+	±
Salicin	+	-	+	+
D-Cellobiose	+	+	+	-
D-Maltose	+	+	+	+
Lactose	-	-	-	-
Melibiose	-	-	-	-
Sucrose	-	-	-	-
Trehalose	+	+	+	+
Starch	-	+	+	-
Glycogen	+	+	+	-
Gluconate	+	+	±	-
Production of				
β-Galactosidase	-	-	-	-
Arginine dihydrolase	+	+	+	-
Ornithine decarboxylase	-	-	-	-
Urease	+	-	+	+
Tryptophane deaminase	-	-	-	-
Gelatinase	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
H_2S	-	-	-	-
Indole	-	-	-	-
Acetoin	+	+	+	+
Citrate utilization	+	-	-	-
Nitrate reduction	+	-	-	-

+, -, and ± indicate positive, negative, and weak reactions, respectively.

Rad), excised and subjected to a pulsed liquid phase sequencer for determination of N-terminal amino acid sequence. The N-terminal sequence analysis revealed that both polypeptides shared identical 20-amino acids residues. These were Met, Asp, Pro, Phe, Ser, Asn, Tyr, Ser, Glu, Gln, Lys, Tyr, Pro, Asp, Ser, Asn, Asn, Asn, Gln and Glu.

1. 3. 3. Southern hybridization and gene cloning

An 18-mer oligonucleotide sequence, referred to as M15-M, deduced from part of the N-terminal amino acid sequence (Glu, Gln, Lys, Tyr, Pro, Asp) of the 86-kDa protein was synthesized with the following sequence: 5'-GARCARAARTAYCCNGAY-3'. Three samples of purified *B. thuringiensis* M15 plasmid DNA were digested with *Hin*dIII, *Hin*dIII/*Eco*RI and *Eco*RI, respectively, separated by molecular weight on agarose gel (Fig. 3A panel a), transferred to a nylon membrane and probed with the DIG-labelled 18-mer M15-M oligonucleotide (Fig. 3A panel b). As shown in Fig. 3A panel b, after hybridization and post-hybridization washes at 39°C, the M15-M probe strongly hybridized to an 8-kb *Hin*dIII, a 2.6-kb *Hin*dIII/*Eco*RI, and a 2.6-kb *Eco*RI fragment. The purified *B. thuringiensis* M15 plasmid DNA was digested with *Hin*dIII and ligated with the *Hin*dIII-digested pBluescript II KS(+) vector. After ligation, the recombinant plasmid DNAs transformed into *E. coli* DH5α were isolated by the cracking procedure, electrophoresed on agarose gel to assess the size of the undigested recombinant plasmids. The three recombinant plasmids with the highest molecular weight were selected and digested with *Hin*dIII. They were designated pYCH27, pYCH40 and pYCH217, respectively. All three plasmids contained an 8-kb *Hin*dIII insert. In addition, pYCH27 and pYCH40 also contained a 0.75-kb and a 1.9-kb *Hin*dIII fragment, respectively (Fig. 3B panel a). The M15-M probe hybridized to the 8-kb *Hin*dIII

Fig. 2. SDS-PAGE analysis of the parasporal inclusion protein(s) of *B. thuringiensis* strain M15.

The *B. thuringiensis* strain M15 parasporal inclusion purified by sucrose density gradient centrifugation was subjected to a 10% SDS-PAGE electrophoresis (lane 4). Crude extracts of the fully lysed *B. thuringiensis* var. *kurstaki* HD-1 were also subjected to electrophoresis on a 10% SDS-polyacrylamide gel (lane 3) as a control. High molecular (lane 1) and low molecular masses (lane 2) of standard protein markers are indicated on the left.

kDa 1 2 3 4

200

116

97.4

86 kDa

79

66

45

31

21.5

fragments in pYCH27, pYCH40 and pYCH217 as revealed by Southern blot hybridization (Fig. 3B panel b).

The 8-kb *Hin*dIII fragments from pYCH27, pYCH40 and pYCH217 were doubly digested with *Hin*dIII/*Eco*RI, electrophoresed on agarose gel, Southern transferred, and hybridized with the M15-M probe. For each of the three recombinant plasmids, a single 2.6-kb fragment was detected (data not shown). This confirms that this 2.6-kb fragment is the same as the one in the *Eco*RI-digested plasmid DNA of strain M15 (Fig. 3A panel b lane 4).

The 8-kb *Hin*dIII insert was excised from recombinant plasmid pYCH217, digested with various restriction enzymes, and a restriction map constructed (Fig. 4). As shown in Fig. 4, the 8-kb *Hin*dIII fragment contains a 3.4-kb *Hin*dIII/*Eco*RI, a 2.6-kb *Eco*RI/*Eco*RI, a 1.4-kb *Eco*RI/*Eco*RI and a 0.6-kb *Eco*RI/*Hin*dIII fragment.

To identify the region homologous to the M15-M probe, the recombinant plasmid pYCH217 was doubly digested with *Hin*dIII/*Eco*RI, and the resulting fragments were subcloned into *Eco*RI-digested pBluescript II KS(+). After ligation, four subclones were obtained to give the recombinant plasmids pYC12S, pYC22S, pYC30S, and pYC31S. Plasmids pYC12S and pYC30S contained a 1.4-kb (Fig. 3C panel a lane 3) and a 2.6-kb (Fig. 3C panel a lane 5) insert, respectively, while pYC22S and pYC31S both harbored a 2.6-kb insert along with a 0.6-kb (Fig. 3C panel a lane 4) and a 1.4-kb (Fig. 3C panel a lane 6) fragment, respectively. Only the 2.6-kb *Eco*RI/*Eco*RI fragment from subclones, pYC22S, pYC30S and pYC31S hybridized with the M15-M probe (Fig. 3C panel b). To further localize the region of hybridization of the M15-M probe in the 2.6-kb *Eco*RI/*Eco*RI fragment, the recombinant plasmid pYC30S (Fig. 3C panel a lane 5) was digested with *Eco*RI (Fig. 3D panel a lane 2), *Eco*RI/*Dra*I (Fig. 3D panel a lane 3), *Eco*RI/*Sph*I (Fig. 3D panel a lane 4), and *Eco*RI/*Bgl*II (Fig. 3D panel a lane 5),

Fig. 3. Southern blot analyses of *B. thuringiensis* strain M15.

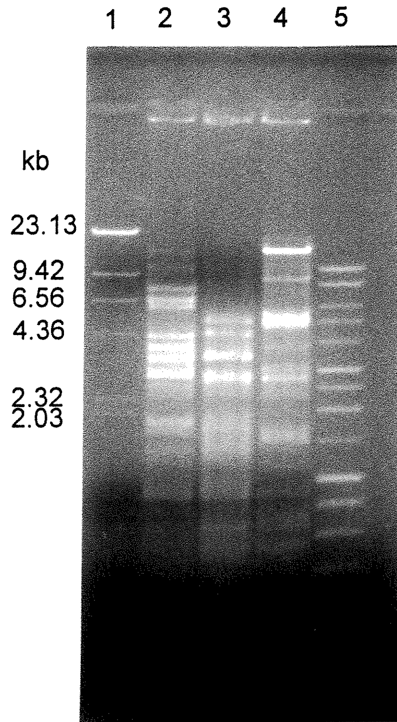
A. Panel a) Three purified plasmid DNA samples of *B. thuringiensis* strain M15 were digested with *Hin*dIII (lane 2), *Hin*dIII/*Eco*RI (lane 3), and *Eco*RI (lane 4), respectively, and separated by molecular weight on a 0.7% agarose gel. Molecular masses of lambda DNA digested with *Hin*dIII (lane 1) and 1kb DNA ladder (lane 5) are indicated on the left- and right-hand sides, respectively.

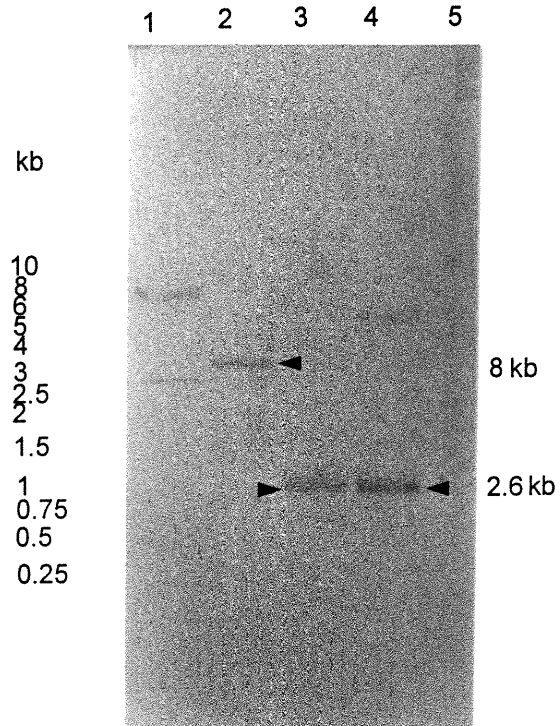
Panel b) Southern hybridization of three purified plasmid DNA samples of *B. thuringiensis* strain M15 digested with *Hin*dIII (lane 2), *Hin*dIII/*Eco*RI (lane 3), and *Eco*RI (lane 4), respectively. The DIG-labelled 18-mer M15-M oligonucleotide hybridized with a 8-kb *Hin*dIII (lane 2), a 2.6-kb *Hin*dIII/*Eco*RI (lane 3), and a 2.6-kb *Eco*RI (lane 4) fragments. These are indicated by arrowheads, respectively. Molecular masses of each fragment detected by the M15-M probe are indicated on the right-hand side. Two fragments, 23.13- and 6.56-kb, of lambda DNA digested with *Hin*dIII (lane 1) hybridized faintly with the M15-M oligonucleotide probe.

B. Panel a) *B. thuringiensis* strain M15 plasmid DNA (lane 2) and three recombinant plasmids, pYCH217 (lane 3), pYCH40 (lane 4) and pYCH27 (lane 5), containing each an 8-kb *Hin*dIII insert were digested with *Hin*dIII and separated by molecular weight on a 0.7% agarose gel. Molecular masses of lambda DNA digested with *Hin*dIII (lane 1) and 1kb DNA ladder (lane 6) are indicated on the left- and right-hand sides, respectively.

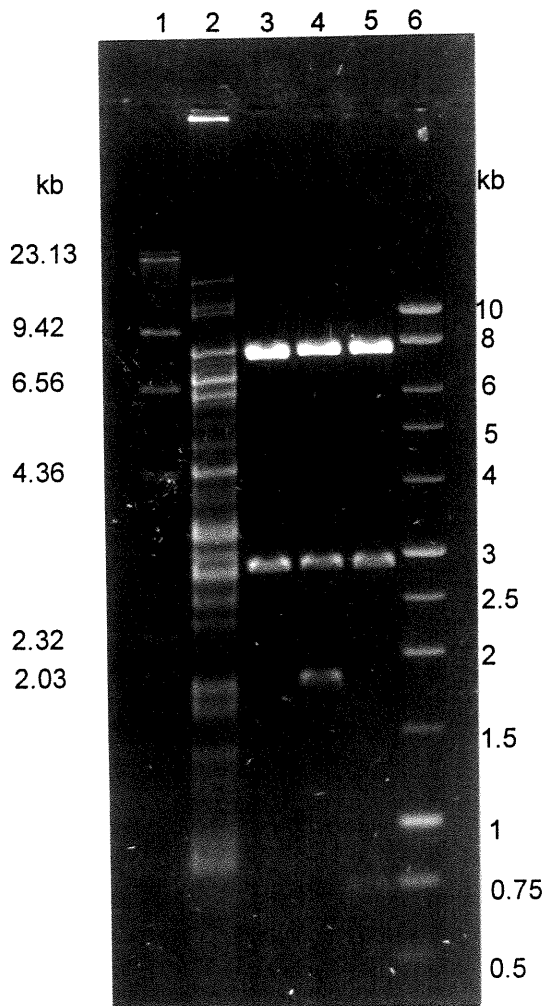
Panel b) Southern hybridization of *B. thuringiensis* strain M15 plasmid DNA digested with *Hin*dIII and of three recombinant plasmids, pYCH217 (lane 3), pYCH40 (lane 4) and pYCH27 (lane 5), containing each an 8-kb *Hin*dIII insert. An 8-kb *Hin*dIII fragment from *B. thuringiensis* strain M15 plasmid DNA and from each of the three recombinant plasmids, pYCH217, pYCH40 and pYCH27, hybridized with the M15-M oligonucleotide probe. This is indicated by the arrowhead. Molecular mass of the fragment is indicated on the right-hand side.

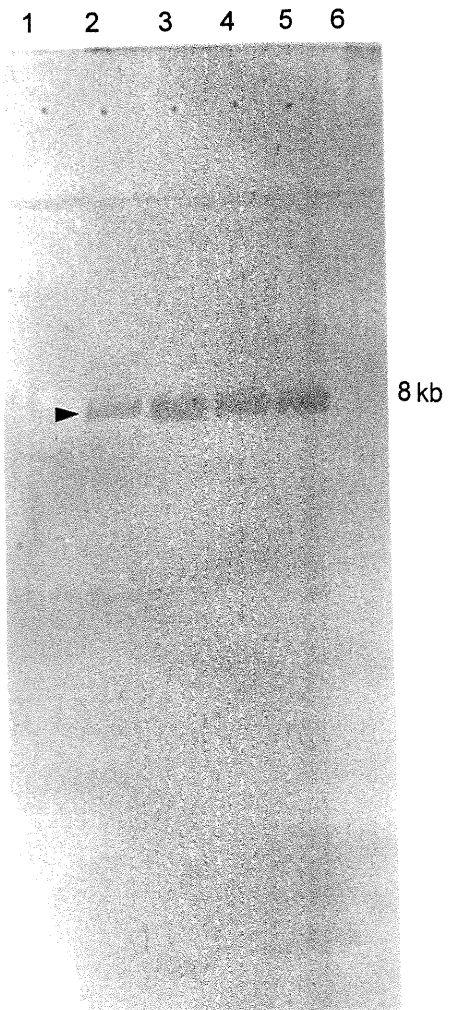
A a) b)





B a) b)





respectively, and then hybridized with the M15-M probe (Fig. 3D panel b). The M15-M probe detected a 2.6-kb *Eco*RI, a 0.6-kb *Dra*I, a 1.6-kb *Eco*RI/*Sph*I, and a 0.85-kb *Eco*RI/*Bgl*II fragment, respectively (Fig. 3D panel b lanes 2, 3, 4 and 5, respectively). It became evident that the region of hybridization of the M15-M probe lied between the *Bgl*II and *Dra*I sites within the 2.6-kb *Eco*RI fragment (Fig. 4)

1. 3. 4. Characterization of a new crystal protein gene, *cry31Aa2*

The nucleotide sequences of the 2.6-kb *Eco*RI/*Eco*RI, 1.4-kb *Eco*RI/*Eco*RI and 0.6-kb *Eco*RI/*Hin*dIII fragments were determined. An open reading frame (ORF) of 2,226-bp in length that codes for a polypeptide of 742 amino acids with a predicted molecular mass of 83,068Da (Fig. 5) was found. The start codon is not ATG but GTG. One potential promoter-like sequence in the 5' non-coding region (Lereclus *et al.*, 1989; Baum and Malvar, 1995) shows a 13-bp spacing between the putative -10 and -35 sequences located 138-bp upstream from the start codon (GTG). The potential ribosome binding site (RBS) (GAAAGGTGG) is located 7-bp upstream of the start codon (GTG) and is partially complementary to the 3' end (UCUUUCCUCC) of *B. subtilis* 16S rRNA (McLaughlin *et al.*, 1981; Moran *et al.*, 1982). The calculated free energy of interaction (ΔG, 25°C) between the *B. subtilis* 16S rRNA and the putative ribosome binding site is -14.8 kcal·mol^{-1} (Tinoco *et al.*, 1973). An inverted repeat that could form a stem-and-loop secondary structure with a calculated energy (ΔG, 25°C) of -12.2 kcal·mol^{-1} (Tinoco *et al.*, 1973) is located 112-bp downstream from the stop codon (TAA) and may function as a transcription terminator.

Fig. 3. Southern blot analyses of *B. thuringiensis* strain M15.

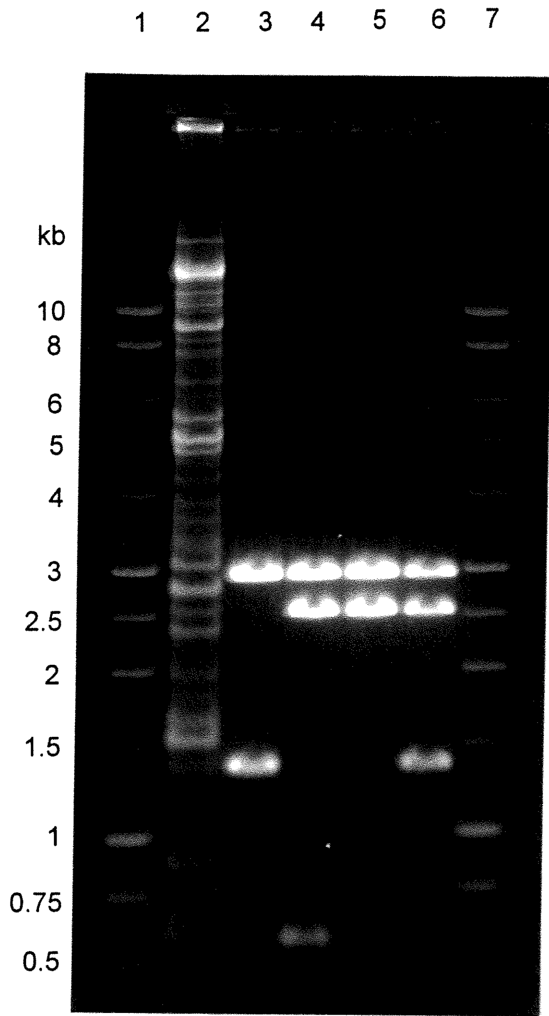
C. Panel a) *B. thuringiensis* strain M15 plasmid DNA (lane 2) and four subclones, pYC12S (lane 3), pYC22S (lane 4), pYC30S (lane 5) and pYC31S (lane 6), from an 8-kb *Hin*dIII fragment of the pYCH217 were digested with *Eco*RI and separated on a 0.7% agarose gel. Molecular masses of 1kb DNA ladders (lanes 1 and 7) are indicated on the left-hand side.

Panel b) Southern hybridization of *B. thuringiensis* strain M15 plasmid DNA (lane2) and four subclones, pYC12S (lane 3), pYC22S (lane 4), pYC30S (lane 5) and pYC31S (lane 6), from an 8-kb *Hin*dIII fragment of the pYCH217 digested with *Eco*RI. A 2.6-kb *Eco*RI fragment from *B. thuringiensis* strain M15 plasmid DNA and from each of three subclones, pYC22S, pYC30S, and pYC31S, hybridized with the M15-M oligonucleotide probe. This is indicated by the arrowhead. Molecular masses of the fragment is indicated on the right-hand side.

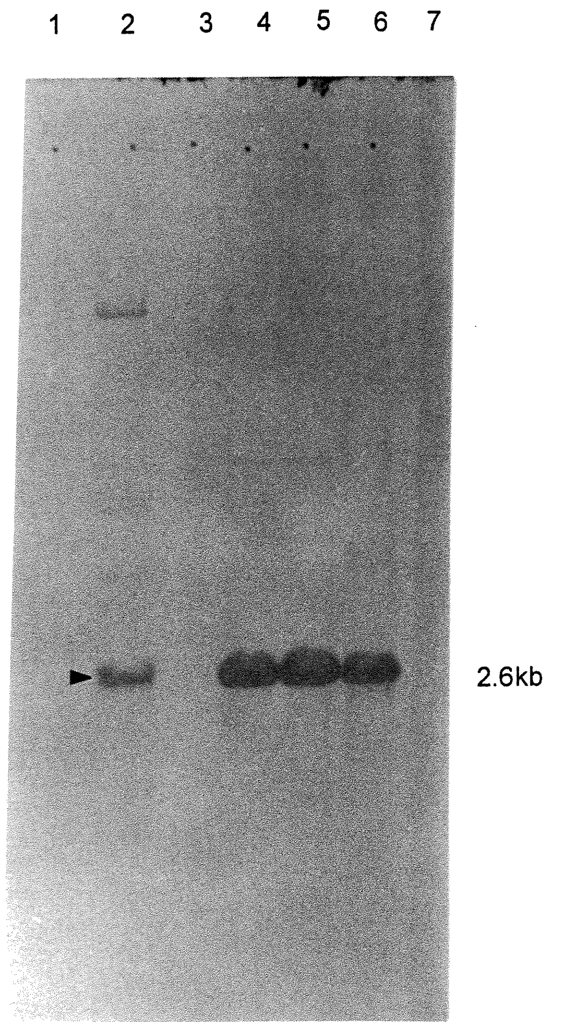
D. Panel a) Four purified plasmid DNAs of the subclone pYC30S were digested with *Eco*RI (lane 2), *Eco*RI/*Dra*I (lane 3), *Eco*RI/*Sph*I (lane 4), and *Eco*RI/*Bgl*II (lane 5), respectively, and separated by molecular weight on a 0.7% agarose gel. Molecular masses of 1kb DNA ladder (lane 1) are indicated on the left-hand side.

Panel b) Southern hybridization of four purified plasmid DNAs of the subclone pYC30S digested with *Eco*RI (lane 2), *Eco*RI/*Dra*I (lane 3), *Eco*RI/*Sph*I (lane 4), and *Eco*RI/*Bgl*II (lane 5), respectively. The M15-M oligonucleotide hybridized with a 2.6-kb *Eco*RI (lane 2), a 0.6-kb *Dra*I (lane 3), a 1.6-kb *Eco*RI/*Sph*I (lane 4), and a 0.85-kb *Eco*RI/*Bgl*II (lane 5) fragments. These are indicated by arrowheads. Molecular masses of each fragment detected by the M15-M probe are indicated on the right-hand side.

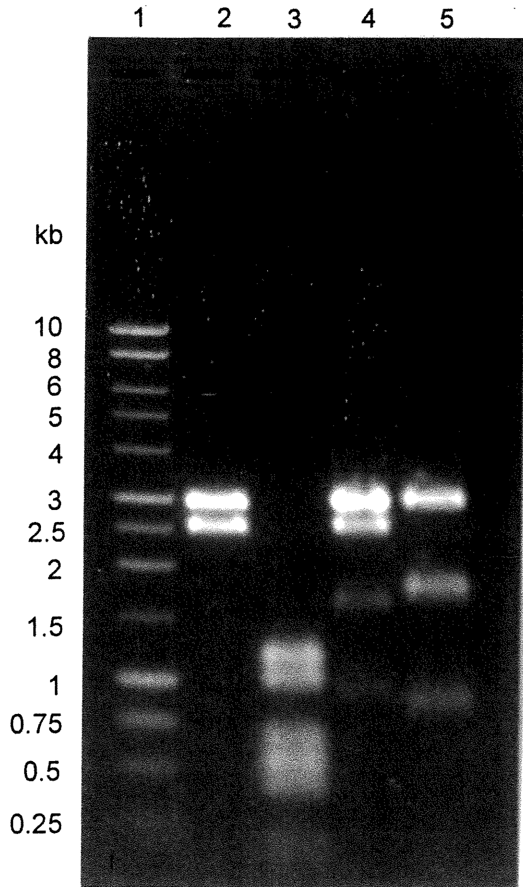
C a)



b)



D a)



b)

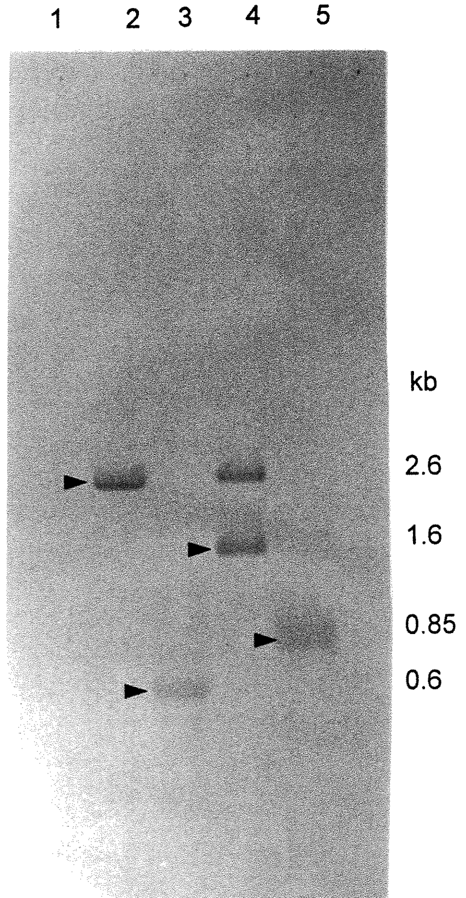
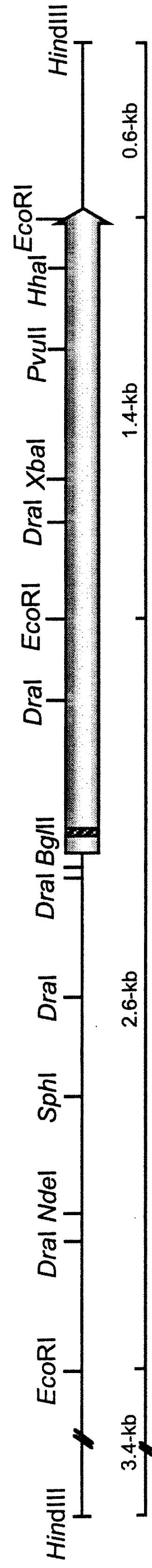


Fig. 4. The structural organization of the pYCH217 insert.

The structural map of an 8-kb *Hin*dIII fragment was constructed. The open reading frame of a crystal protein gene, *cry31Aa2*, is indicated by a grey arrow. The hatched box indicates the region homologous to the DIG-labelled 18-mer M15-M oligonucleotide probe. Selected subfragment sizes are indicated below the structural map.



HindIII
EcoRI
DraI
NdeI
SphI
DraI
DraI
BglII
DraI
EcoRI
DraI
XbaI
PvuII
HhaI
EcoRI
HindIII
3.4-kb
2.6-kb
1.4-kb
0.6-kb

The 18-mer M15-M oligonucleotide sequence based on the N-terminal amino acid sequence (Glu, Gln, Lys, Tyr, Pro, Asp) of the crystal protein is homologous to a region located 24-bp downstream from the start codon (GTG) (Fig. 5).

The 83-kDa protein exhibits 94% amino acid sequence identities with the Cry31Aa1 protein, also called the 81-kDa protein or parasporin (Mizuki *et al.*, 2000). The 83-kDa protein was designated Cry31Aa2. It contains the five conserved sequence blocks (Fig. 6) also found in the Cry31Aa1 parasporin protein.

1. 3. 5. The *cry31Aa2* gene expression in *B. thuringiensis* Cry$^-$ B strain

To express the cloned *cry31Aa2* crystal protein gene in the acrystalliferous *B. thuringiensis* strain Cry$^-$ B, the 3.6-kb *Hin*dIII/*Sph*I fragment containing the entire crystal protein gene was cloned into the *Hin*dIII/*Sph*I doubly-digested *E. coli-B. thuringiensis* shuttle vector pHPS9 to yield recombinant plasmid pYCP31A2 (Fig. 7). When observed under a phase-contrast microscope, the *B. thuringiensis* transformants expressing the *cry31Aa2* gene contained, in addition to the spore, a roughly spherical inclusion, whereas no inclusions were found in the *B. thuringiensis* transformant harboring the non-recombinant shuttle vector pHPS9 alone (data not shown). Under the transmission electron microscope (TEM), however, the parasporal inclusion body has a nearly perfect hexagonal shape (Fig. 8). Both inclusions in the transformant, spore and crystal, are separated from each other as opposed to what is found in *B. thuringiensis* strain M15 where they are tightly bound to each other.

The parasporal inclusion from the *B. thuringiensis* transformant was isolated by sucrose density gradient centrifugation and analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The parasporal inclusion

protein in the *B. thuringiensis* transformant is composed of a single major polypeptide of 83-kDa (Fig. 9).

Fig. 5. Nucleotide sequence and deduced amino acid sequence of the *cry31Aa2* gene.

The *cry31Aa2* is 2,226-bp in length and codes for a polypeptide of 742 amino acids. The potential -35 and -10 boxes and a putative ribosome-binding site are underlined. The stop codon is marked with asterisks. The sequence of the DIG-labelled 18-mer oligonucleotide (M15-M) probe is indicated in bold capital letters. The terminal inverted repeat is indicated by arrows.

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AAGAGAATATTCCTGTAGATATTATATTTAAATATAGTCTCACTCCTCTTGCTTATCATACCGTTGATACCAATCATGTAAAACTCAAACATATTGGATTAG  102
TCCCTTTTTCTTCTTCCGATCCTAATCTATACAGCATACAAGGTGAATTTCAATTTTTTATGAATAAACAATACTTATGAAAAAACTATTTATAAGTATAT  204
TAAAGGACAACAAAGTGAGCATAATGATGGTTTTGATGGGAAAGAATAATAGGCTTTAGTCAATAGTGGTTCAGTTAATTATTGATATATTTTGATATTTAT  306

AATACAAACATTTCTCAAAAATTCTCCTTGCTTATGTCCATTTATACCCAAAAAAGCGAGGACAATGTATATATTTCTCTATCTATCATAGAGTAAATATAG  408
                                                                  -35                  -10

ACTGTATACATTTTTAGTCTTATCTTTGAGTTTTTATATATTTTAAAGTTTGTTTGATAAATTTTCAGGAAAAAAAGATCTCAACGACTTTTGTATGTCGGT  510

                                                          GARCARAARTAYCCNGAY
TGTTTACTATGTGAAAGGTGGAGATATTGTGGACCCATTTTCTAATTATTCTGAACAAAAATACCCAGATTCAAATAATAACCAAGAACTAATTACAGAATC  612
              RBS             M  D  P  F  S  N  Y  S  E  Q  K  Y  P  D  S  N  N  N  Q  E  L  I  T  E  S
CTCTTCATTTTATTCGGATACTACTAATGAAAATATGAAAACTTACCATCCAATTGAACAAGATATTCTCAAATTTGCAAATCAAGAATTTCCCGATAATTA  714
 S  S  F  Y  S  D  T  T  N  E  N  M  K  T  Y  H  P  I  E  Q  D  I  L  K  F  A  N  Q  E  F  P  D  N  Y
TTATCAACATTCCGATGTTTCTAATTCATATCAAAATATGAAAACAGAAATCGTAAATACAGATTTACCCTATAATACAAATAATATAAATAGTATGCGAAA  816
 Y  Q  H  S  D  V  S  N  S  Y  Q  N  M  K  T  E  I  V  N  T  D  L  P  Y  N  T  N  N  I  N  S  M  R  N
TACTCTATGCAGAGATTTACCTCCCGAGACTAACATGAGCATTTATGATAATTTACGATCTACTGTTACTGTTCCTTCATTTTCTAATCAATTTGATCCTAT  918
 T  L  C  R  D  L  P  P  E  T  N  M  S  I  Y  D  N  L  R  S  T  V  T  V  P  S  F  S  N  Q  F  D  P  I
AAAATTTCTTCACGATATTGAAATTGCTATAGAAACTGGATCATTTTCTGCATTAACGCAATCTAACATGAATCAAGGTGGTACTGATATTGCTCCAATGTT  1020
 K  F  L  H  D  I  E  I  A  I  E  T  G  S  F  S  A  L  T  Q  S  N  M  N  Q  G  G  T  D  I  A  P  M  L
AATCTCTACATTTTTTAAAGTTGCAGGTAGTTTACTTCCATTTCCTCTATCATCATTAGGTGCTTTGGCTTCCTTTTATGTTACAGATTCACAAACAGGCGC  1122
 I  S  T  F  F  K  V  A  G  S  L  L  P  F  P  L  S  S  L  G  A  L  A  S  F  Y  V  T  D  S  Q  T  G  A
TATGGCAAATTTATGGAGACAAATGGTAGATTATGTTGAAAAAAGAATTGATTCTAAAATATTAGATTATCATAATTTTATTATGGGAGCAGAACTCGCAGC  1224
 M  A  N  L  W  R  Q  M  V  D  Y  V  E  K  R  I  D  S  K  I  L  D  Y  H  N  F  I  M  G  A  E  L  A  A
ATTAAATGCAAGTTTAAAAGAATACGCACGAGTAGTTAAAATTTTTGAAAATGATATGAACAGAATAGCTGAACCACCTTCAACTGGAGTTATCACTCAATT  1326
 L  N  A  S  L  K  E  Y  A  R  V  V  K  I  F  E  N  D  M  N  R  I  A  E  P  P  S  T  G  V  I  T  Q  F
CAGAATTCTTAATGATAATTTCATTAAATATATTGCAAAATTACAATTCTCAACAAATCAATCAGATTTACAATATCCTGTCCTAACTTTACCATTACGTGC  1428
 R  I  L  N  D  N  F  I  K  Y  I  A  K  L  Q  F  S  T  N  Q  S  D  L  Q  Y  P  V  L  T  L  P  L  R  A
ACAAGCATGTGTAATGCATTTAATGTTATTAAAAGATGCAACGACTTCTGTGTGGGGACAACAAATAGACTCGCAACAATTAAATGGGTATAAAGCAGAATT  1530
 Q  A  C  V  M  H  L  M  L  L  K  D  A  T  T  S  V  W  G  Q  Q  I  D  S  Q  Q  L  N  G  Y  K  A  E  L
AATACGTTTAATAAAAGTATATACTAATGATGTAAACACAACGTATAATCAAGGGCTAGAGCTAGAAAAAGCTAAACCACTAAATTATTCTGATCCTGAAGA  1632
 I  R  L  I  K  V  Y  T  N  D  V  N  T  T  Y  N  Q  G  L  E  L  E  K  A  K  P  L  N  Y  S  D  P  E  E
ATATTTACAAGCAGGACGTCCAGATATATCTGTATTACGCAGTAACTTTAAAGAGGTTATGAAGTGGAATAAAGTAGCGAAATATAAACGTGGAATGGCTAT  1734
 Y  L  Q  A  G  R  P  D  I  S  V  L  R  S  N  F  K  E  V  M  K  W  N  K  V  A  K  Y  K  R  G  M  A  M
GAGTGCTTTATCATTAGCTGCATTATTTCCAACTTTCGGACCAAATTATCCAAAACAAGCATTAAAAGTTGTGCAATCTAGACAAATTTTTGCACCTGTAAT  1836
 S  A  L  S  L  A  A  L  F  P  T  F  G  P  N  Y  P  K  Q  A  L  K  V  V  Q  S  R  Q  I  F  A  P  V  I
TGGAATACCAGGCGGTATAACAAGTCAAGATAGTGGTCCCACTTTTGGTAGTATGAGATTTGATGTAAAAACTTATGATCAAATTGATGCGTTACGACAACT  1938
 G  I  P  G  G  I  T  S  Q  D  S  G  P  T  F  G  S  M  R  F  D  V  K  T  Y  D  Q  I  D  A  L  R  Q  L
AATGGAATTATATATTCAACCTTTAAAATCTGCTTACTTTTGGATATATGAATCGGATTGGAAAGTTCGTGCAACTTATGTCAATGATTATATTGGTAAAAG  2040
 M  E  L  Y  I  Q  P  L  K  S  A  Y  F  W  I  Y  E  S  D  W  K  V  R  A  T  Y  V  N  D  Y  I  G  K  R
AGGGTCAAATACAGGTGCTGCTTGGCACATGTGGTCAAGTGATCCTTCAGCCATATACACTTCTGCACTAGGAGCAGCAGGATACGCTCCTAACGTTGTTGG  2142
 G  S  N  T  G  A  A  W  H  M  W  S  S  D  P  S  A  I  Y  T  S  A  L  G  A  A  G  Y  A  P  N  V  V  G
TGTAAGATATTCACATGGGGGTAGTTACACAAAAGGTATGGCACCCGCAAATACTAATGCGTATGCTCCATTTGAATTTAAATATCCTGGTTATAAACTACA  2244
 V  R  Y  S  H  G  G  S  Y  T  K  G  M  A  P  A  N  T  N  A  Y  A  P  F  E  F  K  Y  P  G  Y  K  L  H
CAGTGTTAGTGCTTATGGATTAAGTAAAGCACCTGATGCAGCTGATTCTGTTATGTTTGGATTTAGACCTGTATTGTTAGAAAATGAAGCAAATCAATTATT  2346
 S  V  S  A  Y  G  L  S  K  A  P  D  A  A  D  S  V  M  F  G  F  R  P  V  L  L  E  N  E  A  N  Q  L  L
AACAGATACAGCATTGCAAATTCCAGCAGAAATAGGAATAACAGATGTCGTACCTGCATTTGGTAGAACAGAAGAACCTATTAATGGTCAAGATGCAATAAG  2448
 T  D  T  A  L  Q  I  P  A  E  I  G  I  T  D  V  V  P  A  F  G  R  T  E  E  P  I  N  G  Q  D  A  I  R
AATATGGGAAAGTTTTACAAGTGGATTTGGCTTTACTTATACTGTTGATTCTCCACAAAAACAAAAATATAAAATCATTTATAGAATTGCAAATAACTTAAG  2550
 I  W  E  S  F  T  S  G  F  G  F  T  Y  T  V  D  S  P  Q  K  Q  K  Y  K  I  I  Y  R  I  A  N  N  L  S
CGCTTCTACAGTTTCTTTAACCTATAATAATCAAACATTTTTCACTGATATTTTAAATACTTCATTAGATCCAAATGGAGTAAGAGGAAATTATGGTTCTTA  2652
 A  S  T  V  S  L  T  Y  N  N  Q  T  F  F  T  D  I  L  N  T  S  L  D  P  N  G  V  R  G  N  Y  G  S  Y
TACACTTGTAGAAGGTCCTATTATTGAATTTTCTCAAGGAACTAATATCTTTAAACTAGGATCACAAAAAGGAGAATTCGCTATAGATTCCATTATTTTTAG  2754
 T  L  V  E  G  P  I  I  E  F  S  Q  G  T  N  I  F  K  L  G  S  Q  K  G  E  F  A  I  D  S  I  I  F  S
TCCTGTTGTTTAATAGTGTAGTACCATTAGACCCAGACCCATGGTTTCCAGTCCAGAATATTCCCCAGATTTCATAGTATGCTTCGATCCCGCATGTTTTAT  2856
 P  V  V ***
GTACAAACACATCCTTTTTAGATAGCATTCCAATTATAGGGATGCTCTTTTTTTGATTTCTGGCCTATCCTTCTCATTTCATAGATTTTTAATTAGTACCCT  2958

TTACAAAAAGTAAACCCACCATCTTCGAACAAATCTTTGATTTCTATTTTTAAGAATAATCAATCTGTTGAACAATTTATAATTCTTTTGAAGAGAATTTCA  3060
TTTTATTTGTTCGCTTAAGTTGATAGGCATGTGGTTCTACCCCTAATAAGTGTCACAGAACACTAATTCTAAGACATTTATCGTAAAAAAATAGTAAATTCA  3162
TACAATACAGTTAAACTTTCCTCAGTAGCTCACGTTTTTCGATTTCGGGTGTTTTTACTCATTTCCCCCTTTGTTTTAGGAGAGAGTGCTGGCTGGGGGTT  3264
TGGGGGCTAGCCCCCAAGAACTTAACGTAACTGAATATGGAATAAGCTT  3313
```

Fig. 6. Comparison of the deduced amino acid sequences of Cry31Aa2 and Cry31Aa1.

The capital letters and dotted lines under the amino acid sequence of Cry31Aa2 correspond to the difference and alignment gaps between the Cry31Aa2 and Cry31Aa1 proteins. The asterisks under the Cry31Aa2 sequence indicate the identities between Cry31Aa2 and Cry31Aa1. The bold lines above the Cry31Aa2 sequence correspond to the five conserved amino acid blocks found in the amino acid sequence of Cry31Aa1. Highly conserved residues in the five conserved amino acid blocks of the known *B. thuringiensis* Cry and Cyt proteins are marked with the bold dotted lines under the Cry31Aa2 sequence.

```
Cry31Aa2:  1    MDPFSNYSEQKYPDSNNNQELITESSSFYSDTTNENMKTYHPIEQDILKFANQEFPDNYY  60
Cry31Aa1:  1    ***********************K************A*N***********T****S**H*  60

Cry31Aa2:  61   QHSDVSNSYQNMKTEIVNTDLPYNTNNINSMRNTLCRDLPPETNMSIYDNLRSTVTVPSF  120
Cry31Aa1:  61   *******-------------------D*********K***********************  101

Cry31Aa2:  121  SNQFDPIKFLHDIEIAIETGSFSALTQSNMNQGGTDIAPMLISTFFKVAGSLLPFPLSSL  180
Cry31Aa1:  102  *****************Q*******************N***********S**********  161

Cry31Aa2:  181  GALASFYVTDSQTGAMANLWRQMVDYVEKRIDSKILDYHNFIMGAELAALNASLKEYARV  240
Cry31Aa1:  162  ************************************************************  221

                                                                  Block 1
Cry31Aa2:  241  VKIFENDMNRIAEPPSTGVITQFRILNDNFIKYIAKLQFSTNQSDLQYPVLTLPLRAQAC  300
Cry31Aa1:  222  **********M*************************************************  281

                Block 1 (Continued)
Cry31Aa2:  301  VMHLMLLKDATTSVWGQQIDSQQLNGYKAELIRLIKVYTNDVNTTYNQGLELEKAKPLNY  360
Cry31Aa1:  282  ************************************************************  341

                                                Block 2
Cry31Aa2:  361  SDPEEYLQAGRPDISVLRSNFKEVMKWNKVAKYKRGMAMSALSLAALFPTFGPNYPKQAL  420
Cry31Aa1:  342  ****************************R*******************************  401

                Block 2 (Continued)
Cry31Aa2:  421  KVVQSRQIFAPVIGIPGGITSQDSGPTFGSMRFDVKTYDQIDALRQLMELYIQPLKSAYF  480
Cry31Aa1:  402  ***********************HSG*******************R**************  461

Cry31Aa2:  481  WIYESDWKVRATYVNDYIGKRGSNTGAAWHMWSSDPSAIYTSALGAAGYAPNVVGVRYSH  540
Cry31Aa1:  462  Y*************************L**G*******V**********************  521

                                                                     Block 3
Cry31Aa2:  541  GGSYTKGMAPANTNAYAPFEFKYPGYKLHSVSAYGLSKAPDAADSVMFGFRPVLLENEAN  600
Cry31Aa1:  522  **********P******************************T******************  581

                          Block 3 (Continued)                         Block 4
Cry31Aa2:  601  QLLTDTALQIPAEIGITDVVPAFGRTEEPINGQDAIRIWESFTSGFGFTYTVDSPQKQKY  660
Cry31Aa1:  582  ************************************I***********************  641

               Block 4 (Continued)
Cry31Aa2:  661  KIIYRIANNLSASTVSLTYNNQTFFTDILNTSLDPNGVRGNYGSYTLVEGPIIEFSQGTN  720
Cry31Aa1:  642  ************************************************************  701

                          Block 5
Cry31Aa2:  721  IFKLGSQKGEFAIDSIIFSPVV                                        742
Cry31Aa1:  702  ****R****************S                                        723
```

Fig. 7. Restriction map of the recombinant plasmid pYCP31A2 containing the *cry31Aa2* gene.

The 3.6-kb *Hin*dIII/*Sph*I fragment containing the entire crystal protein gene was cloned into the *Hin*dIII/*Sph*I doubly-digested *E. coli-B. thuringiensis* shuttle vector pHPS9 to yield recombinant plasmid pYCP31A2.

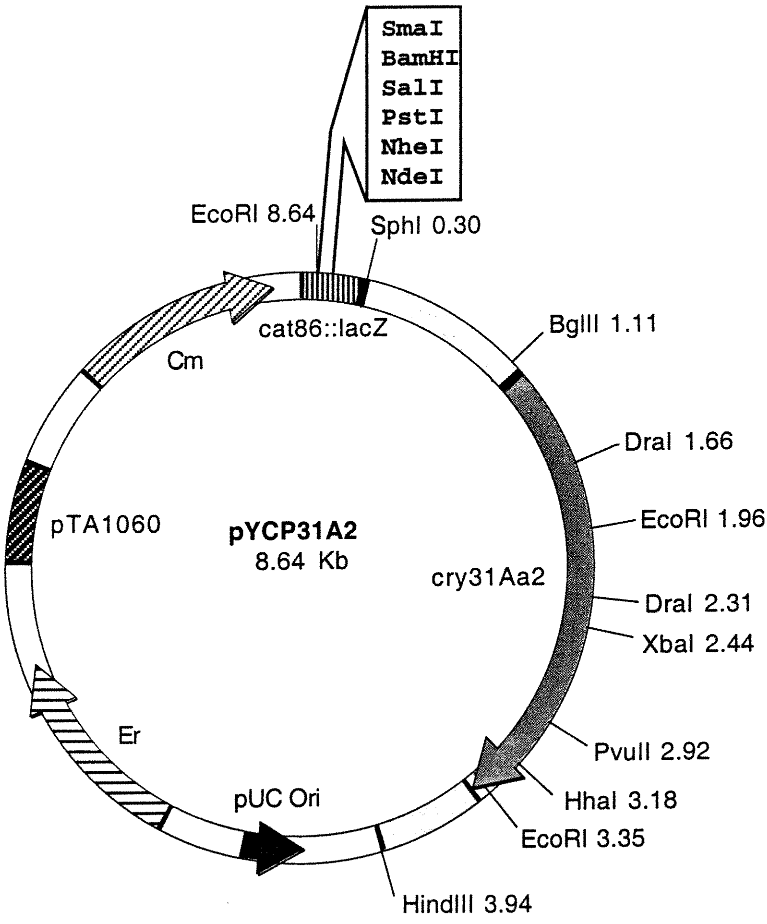

SmaI
BamHI
SalI
PstI
NheI
NdeI
EcoRI 8.64
SphI 0.30
BglII 1.11
DraI 1.66
EcoRI 1.96
DraI 2.31
XbaI 2.44
PvuII 2.92
HhaI 3.18
EcoRI 3.35
HindIII 3.94
cat86::lacZ
Cm
pTA1060
pYCP31A2
8.64 Kb
cry31Aa2
Er
pUC Ori

Fig. 8. Transmission electron micrograph of a *B. thuringiensis* Cry^- B transformant expressing a novel crystal protein gene, the *cry31Aa2* gene.

S: spore; P: parasporal inclusion.

Magnification : 20,000 X.

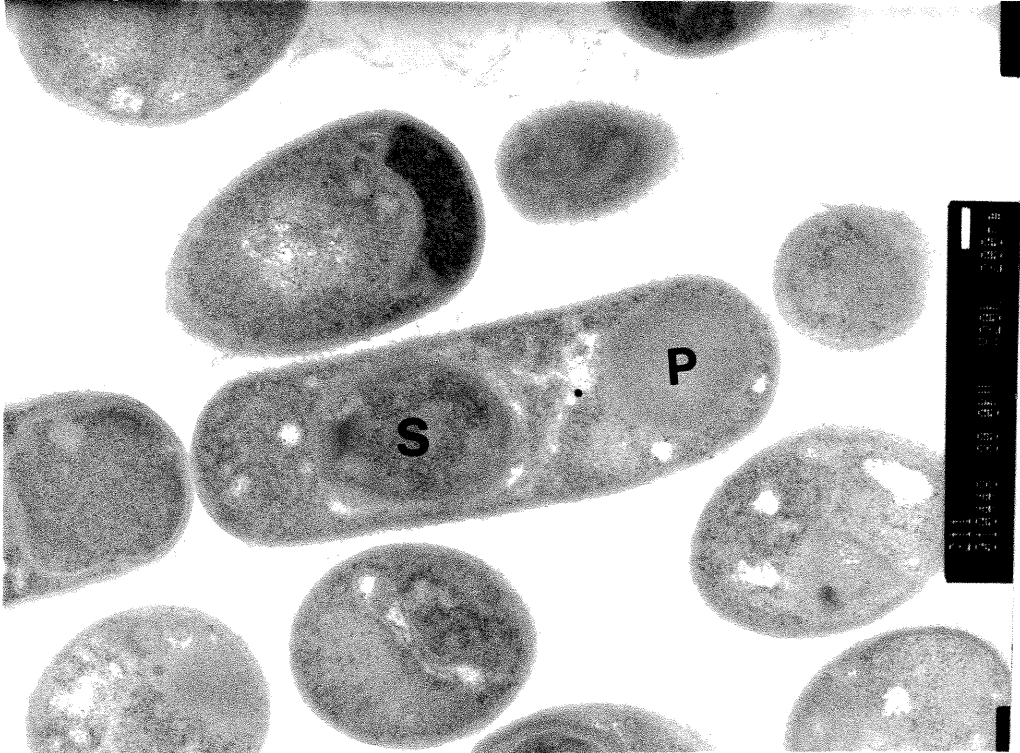
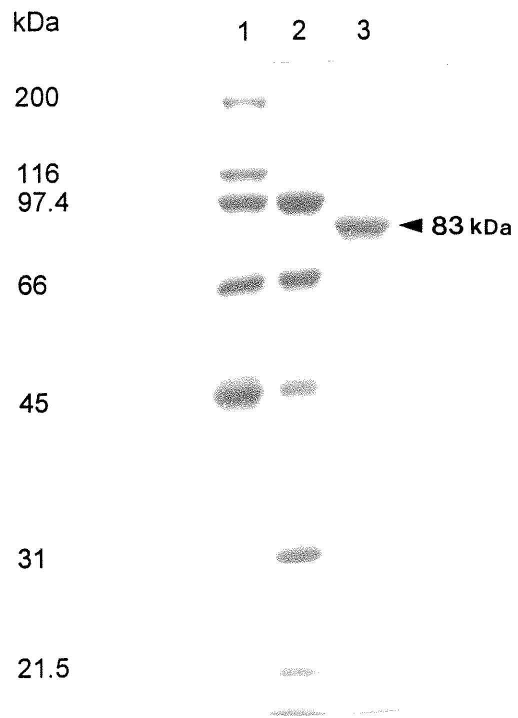

S
P

Fig. 9. SDS-PAGE analysis of the parasporal inclusion protein from a *B. thuringiensis* transformant expressing a novel crystal protein gene, the *cry31Aa2* gene.

The parasporal inclusion purified by sucrose density gradient centrifugation was subjected to a 10% discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (lane 3). High molecular (lane 1) and low molecular masses (lane 2) of standard protein markers are indicated on the left.


kDa
1
2
3
200
116
97.4
◄ 83 kDa
66
45
31
21.5

1. 4. DISCUSSION

A novel *Bacillus thuringiensis* strain, named M15, was isolated from dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae). It autoagglutinates and hence is non-serotypeable. It has been reported that many *B. thuringiensis* isolates occurring in natural environments were classified as non-serotypeable strains because of non-motility or apparent autoagglutination (Ohba and Aizawa, 1986; Ohba, 1996). Interestingly, many non-serotypeable *B. thuringiensis* isolates were also non-insecticidal (Ohba and Aizawa, 1986; Mizuki *et al.*, 1999).

The biochemical characteristics of strain M15 were rather different than those of known strains such as *B. thuringiensis* var. *kurstaki* HD-1, -var. *israelensis* HD-500, and -var. *higo* BT205 (Jung *et al.*, 1998). Although at present time, biochemical tests are not a key tool for the classification of *B. thuringiensis* strains (de Barjac and Frachon, 1990), they are clearly useful for the differentiation between *B. thuringiensis* strains.

Most *B. thuringiensis* insecticidal crystal proteins have molecular weights in the range of 130-140kDa for Cry1, Cry4A and Cry4B, and 65-80kDa for Cry2A, Cry3A, Cry10A and Cry11A (Aronson, 1993; Baum and Malvar, 1995). The protein profile of parasporal inclusions of *B. thuringiensis* strain M15 appeared to be composed of at least two major polypeptides of approximately 86- and 79-kDa as revealed on a 10% SDS-polyacrylamide gel. However, further analysis of the N-terminal sequences of both polypeptides revealed that the first 20 amino acids are identical. At least two hypotheses could be considered: they could be different proteins harboring identical N-terminal sequences or, alternatively, the 79-kDa polypeptide could be a truncated form of the 86-kDa protein. The latter is more likely. When the M15 plasmid DNA was probed with the 18-mer M15-M oligonucleotide, derived from the N-terminal amino acid

sequence, a single 8-kb *Hin*dIII fragment was revealed (Fig. 3A panel b lane 2). When the same probe was used against *Eco*RI/*Hin*dIII doubly-digested M15 plasmid DNA, a single 2.6-kb fragment was revealed (Fig. 3A panel b lane 3).

The *B. thuringiensis* M15 crystal protein gene was cloned and its nucleotide sequence determined. It encodes a polypeptide with a predicted molecular mass of 83,068Da. The 83-kDa crystal protein was novel enough to be assigned a new name and was designated Cry31Aa2 by the *Bacillus thuringiensis* Pesticide Crystal Protein Nomenclature Committee (Appendix). It shares high homology with Cry31Aa1, also called the parasporin protein or the 81-kDa protein, with some differences (Mizuki *et al.*, 2000). The cloned *cry31Aa2* gene contains an open reading frame (ORF) of 2,226-bp in length that is 57-bp longer than *cry31Aa1*. Consequently, the Cry31Aa2 protein has 742 amino acids and is 19 amino acids longer than Cry31Aa1 (Fig. 6). Both proteins share 94% amino acid identity. Interestingly, although they are both Cry proteins, they both show very low amino acid sequence homology to the known *B. thuringiensis* Cry and Cyt proteins (Mizuki *et al.*, 2000).

The Cry31Aa2 protein contains the same five conserved amino acid blocks found in the 81-kDa parasporin protein. Interestingly enough, the latter is capable of killing specific cancer cells (Mizuki *et al.*, 2000). The five conserved blocks have been found within the active toxin moiety of the protoxin cleaved by proteases (Gill *et al.*, 1992; Baum and Malvar, 1995). In addition, the cytocidal activity of the parasporin was due to the cleavage by proteinase K and trypsin albeit the toxic moiety is still unknown (Mizuki *et al.*, 2000). The amino acid sequence of the Cry31Aa2 protein shares extensive homology with the parasporin except for the substitution of 25 amino acid residues and the addition of 19 contiguous codons in Cry31Aa2. Suprisingly, the five conserved amino acid blocks of the Cry31Aa2 protein were especially identical to those

of the parasporin except for the substitution of a single lysine residue in the second conserved block of Cry31Aa2. It is possible that the Cry31Aa2 protein, like the parasporin protein, may exhibit cytocidal activity against specific human cancer cells. This avenue of research remains to be pursued.

That an autoagglutinable, non-serotypeable *B. thuringiensis* strain expressing a crystal protein, Cry31Aa1, with proven cytocidal activity was isolated from a soil sample in Hiroshima Prefecture, Japan and that a similar autoagglutinable, non-serotypeable *B. thuringiensis* strain expressing a highly homologous crystal protein, Cry31Aa2, was isolated from two-spotted spider mites in an apple orchard in Quebec, Canada is at the very least puzzling and certainly very exciting. Whether Cry31Aa2 shares similar cytocidal activity remains to be determined.

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1. 6. APPENDIX

Exp. : "Daniel R. Zeigler, Ph.D." <zeigler.1@osu.edu>
Dest. : "Jean-Charles_Côté_(Jean-Char_les_Côté)" <cotejc@E...
Date : Jeu, Nov 16, 2000 12:55 pm
Objet : RE: Name for New Bt Toxin Gene

Dear Dr. Côté:

Thank you again for submitting your sequence to the Cry Nomenclature Committee for analysis. The sequence enters the phylogenetic tree at a distance <5% from Cry31Aa1. Although there are distinct differences between these sequences, as you pointed out in your e-mail, we feel that the overall similarity makes the name Cry31Aa2 a logical choice. Once again, we appreciate your sharing these data with us, and we wish you the best of success in your ongoing research efforts.

On behalf of the committee,

Daniel R. Zeigler, Ph.D.
Director, Bacillus Genetic Stock Center
http://bacillus.biosci.ohio-state.edu/

CHAPTER 2

A New Insertion Sequence, IS*231*M, in an Autoagglutinable Isolate of *Bacillus thuringiensis*

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ABSTRACT

An insertion sequence was isolated from an autoagglutinable strain of *Bacillus thuringiensis*. Analysis of its DNA sequence revealed high homology to the IS*231* family. The name IS*231*M is proposed for this new insertion sequence. IS*231*M is 1652-bp long and is delimited by two imperfect 20-bp inverted repeat sequences with two mismatches, which are flanked by two perfect 11-bp direct repeats (DRs). The region upstream of the open reading frame, presumed to be able to form a stable hairpin structure, is particularly well conserved in IS*231*M. Based on primary nucleotide sequences, IS*231*M is most homologous to IS*231*F and IS*231*G and most distant from IS*231*V and IS*231*W. However, as opposed to the single transposase A ORF found in IS*231*A, -B, -C, -D, -F, and -G, IS*231*M has two overlapping open reading frames, ORF1 and ORF2, that could code for polypeptides of 334 and 143 amino acids, respectively. Whether IS*231*M is a functional transposable element remains to be determined.

Key words: Bacillus thuringiensis; insertion sequence; IS*231*M.

2. 1. INTRODUCTION

Bacillus thuringiensis is a Gram-positive soil bacterium that produces parasporal crystalline inclusions during sporulation. These inclusions exhibit specific insecticidal activities against the larvae of Lepidopteran, Dipteran, and Coleopteran insects. *B. thuringiensis* is the most widely used bacterial insect pathogen in commercial bioinsecticide preparations (Höfte and Whiteley, 1989).

The multiple locations and genetic mobility of *cry* genes encoding insecticidal crystal proteins (ICP), or δ-endotoxins, have been attributed to their close association with both class I insertion sequence (IS) elements and class II transposons.

Eleven iso-IS*231* transposable elements, IS*231*A, B, and C (Mahillon *et al.*, 1985, 1987), D, E, and F (Rezsöhazy *et al.*, 1992), G and H (Ryan *et al.*, 1993), V and W (Rezsöhazy *et al.*, 1993), and Y (Chen and Mahillon, unpublished data) have been cloned and characterized.

IS*231*A , -B, -C, -D, -E, and -F are 1655 to 1657-bp in length. They contain a single open reading frame (ORF) coding for a putative transposase of 477-478 amino acids in length. IS*231*H has only been partially characterized and IS*231*G contains several stop codons in its ORF. IS*231*V and W are 1964-bp in length and are 307 to 309-bp longer than the other iso-IS*231.* Both IS contain two open reading frames (ORFs). These ORFs overlap by 8 and 27 codons in IS*231*V and IS*231*W, respectively (Rezsöhazy *et al.*, 1993). All IS*231* transposases contain five conserved domains, N1, N2, N3, C1, and C2, which share more than 60% nucleotide identity (Mahillon *et al.*, 1994).

The transposition of IS*231*A has been demonstrated in *E. coli.* Its transposition results in 10- to 12- bp direct repeats (DRs) at the target site (Hallet *et al.*, 1991).

In the course of cloning experiments of crystal protein genes from an autoagglutinable *B. thuringiensis* isolate, we have cloned and sequenced a new IS element. Nucleotide sequence analysis of this new element indicates that it belongs to the IS*231* family and it was named IS*231*M. We report here the primary characterization of this new IS element.

2. 2. MATERIALS AND METHODS

2. 2. 1. Bacterial strains and plasmids

An autoagglutinable, hence non-serotypeable, *B. thuringiensis* strain, referred to as M15, was isolated from dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae). This bacterial strain was the source for the isolation of a new insertion sequence (IS). *E. coli* strains JM109 and XL1-Blue were used as bacterial hosts for the cloning vectors pUC18 (Pharmacia Biotech) and pBluescript II KS(+) (Stratagene).

The recombinant plasmid pGEM-5Zf(+) harbors a 4-kb *Nde* I restriction fragment which contains the *cry1A(b)* gene (Côté *et al.*, 1992) from the OZ-3 mutant of *B. thuringiensis* var. *kurstaki* HD-1 (Rivard *et al.*, 1989). The 4-kb *Nde*I insert was used as a probe for Southern hybridization.

2. 2. 2. Plasmid DNA preparation and Southern hybridization

B. thuringiensis strain M15 was grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) at 30°C for 16 h and transferred at 37°C for 4 h. Plasmid DNAs were isolated using the alkaline extraction method, as described by Birnboim and Doly (1979), with the following modifications. Lysozyme was added at a concentration of 2 mg ml^{-1} and the cell suspension was incubated at 37°C for 1 h.

E. coli strains were incubated in LB medium at 37°C. Recombinant plasmid DNAs from *E. coli* were isolated as described by Birnboim and Doly (1979). The

purification of plasmid DNA for DNA sequencing was performed with a Wizard Plus SV miniprep DNA purification system following the manufacturer's instructions (Promega).

The 4-kb *Nde*I restriction fragment containing the *cry1A(b)* gene was excised from the recombinant plasmid pGEM-5Zf (+) with *Nde*I, separated by electrophoresis on an agarose gel, and eluted with a Sephaglas Bandprep Kit (Pharmacia Biotech) as specified in the instruction manual. This 4-kb *cryIA(b)* gene was labeled with digoxigenin (DIG)-11-dUTP (Boehringer Mannheim Biochemicals) using the random-primed method as recommended by the manufacturer. The labeled DNA was precipitated with 0.1 vol of 4 M LiCl and 2.5 vol of ice-cold ethanol and transferred at -70°C for 30 min. The reaction was centrifuged at 13,000g for 15 min at 4°C. The washed pellet was resuspended in nuclease-free water and stored at -20°C until used.

B. thuringiensis plasmid DNA was digested with *Hin*dIII and the sample electrophoresed on a 0.7 % agarose gel and transferred onto a nylon membrane (Schleicher & Schuell) by the method of Southern (1975). Southern blot hybridization using the DIG-labeled 4-kb *cryIA(b)* gene was done with standard hybridization solution (5X SSC, 1% blocking reagent (Boehringer Mannheim Biochemicals), 0.1% N-lauroylsarcosine, 0.02% SDS) for 18 h at 66°C. After hybridization, the membrane was washed three times for 15 min each in 4X wash solution (4X SSC, 0.1% SDS) at room temperature. Following the low-stringency washes, detection of hybridization signals on the membrane was performed with the color-substrate solution containing 4-Nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer Mannheim Biochemicals) according to the manufacturer's recommendation.

2. 2. 3. Cloning and DNA sequencing

DNA fragments were eluted with the Sephaglas Bandprep Kit (Pharmacia Biotech) and ligated overnight at 10°C to *Hin*dIII-digested, alkaline phosphatase-treated pUC18 or pBluescript II KS (+), respectively. *E. coli* JM109 or XL1-Blue competent cells were transformed with the ligated mixture, plated on LB agar plates containing 100 μg ml^{-1} ampicillin, 0.5 mM IPTG, and 40 μg ml^{-1} X-Gal, and incubated overnight at 37°C. White colonies were transferred with toothpicks to 2 ml of fresh LB media supplemented with 100 μg ml^{-1} ampicillin and incubated overnight at 37°C. The recombinant plasmid DNAs from the resultant transformants were isolated using the alkaline extraction method as described by Birnboim and Doly (1979). The recombinant plasmid DNAs digested with *Hin*dIII were electrophoresed on a 0.7 % agarose gel and transferred onto nylon membranes (Schleicher & Schuell) by the method of Southern (1975). The positive clones were screened by Southern blot hybridization using the DIG-labeled 4-kb *cryIA(b)* gene, under the conditions described above.

The dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using the near-infrared (NIR) fluorescence automated DNA sequencer (LI-COR Model 4200, LI-COR, Inc.) was used to sequence the 1.9- and 2.5-kb *Hin*dIII fragments. Nucleotide and protein sequences were analyzed with the DNASIS program (Hitachi Software Engineering Co.)

2. 3. RESULTS

2. 3. 1. Cloning and characterization of IS*231*M.

B. thuringiensis strain M15 plasmid DNA digested with *Hin*dIII and Southern hybridized under low-stringency conditions with the *B. thuringiensis* 4-kb *Nde*I *cry1A(b)* probe revealed two *Hin*dIII fragments of 1.9- and 2.5-kb. These two fragments were cloned and hybridization results further confirmed by Southern hybridization using the same probe.

The complete nucleotide sequences were determined for both *Hin*dIII fragments. Analysis of the nucleotide sequences revealed that the 1.9- and 2.5-kb *Hin*dIII fragments were highly homologous to the 5' and 3' ends of IS*231*F, respectively, and closely related to the other iso-IS*231* elements. Consequently, the present sequence was named IS*231*M. The Nucleotide sequence of IS*231*M is presented in Fig. 1. A structural map of IS*231*M is presented in Fig. 2.

IS*231*M is 1652-bp in length and is delimited by two imperfect 20-bp inverted repeat (IR_L and IR_R) sequences with two mismatches, which are flanked by two perfect 11-bp direct repeats (DR_L and DR_R). One potential promoter region containing a 22-bp spacer between the -10 and -35 sequences is also found at a position similar to that of the other iso-IS*231* elements. The -10 sequence, TATTCT (Fig. 1), is identical to that of most IS*231* variants. A potential ribosome binding site (RBS1) of 11-bp sequence is located 8-bp upstream of the start codon (ATG) of ORF 1. Its sequence is identical to that of the potential RBS from IS*231*F. The 5' noncoding neighboring sequence is able to form a stem-and-loop secondary structure (Fig. 3). The calculated energy (ΔG, 25 °C) of its secondary structure is -18.2 kcal/mol (Tinoco *et al.*, 1973). The stem domain

is 1-bp longer than the one of IS*231*A. The loop domain is 2-bp shorter than that of IS*231*A (Fig. 3).

The RBS1 sequence in IS*231*M is only partially homologous to the *B. subtilis* AAGGGG RBS consensus sequence (Médique et al., 1995). Thus, it appears to be a presumably weak RBS, which is frequent for IS elements. Surprisingly, unlike most IS*231* elements and like IS*231*V and -W, IS*231*M contains two open reading frames, ORF1 and ORF2, that could code for polypeptides of 334 and 143 amino acids, respectively. A first start codon ATG is present at nucleotide 92 and a stop codon TGA located at nucleotide 1094 terminates the first ORF. A second start codon is present at nucleotide 1093 and the second stop codon is located at position 1522. ORF2 begins in a -1 frame with regard to ORF1. A search for a possible RBS upstream from ORF2 revealed a short sequence, TGGGAGTG, called RBS2, which also exhibits low homology to the *B. subtilis* RBS consensus sequence. Possible synthesis of a fusion product between ORF1 and ORF2 would yield a 56-kDa protein of 477 amino acids.

2. 3. 2. Comparison of IS*231*M with the iso-IS*231* elements

Comparison of nucleotide sequence alignments between IS*231*M and the other iso-IS*231* elements encoding the open reading frame shows that IS*231*M is more closely related to IS*231*F (87%) and IS*231*G (79%) than to IS*231*A (75%) or IS*231*V and -W (65%). A comparison of the IS*231*M ORF1-ORF2 putative fusion product to IS231F and IS*231*A transposases and to IS231W fusion protein reveals 83, 71, and 50% amino acid identities, respectively.

All IS*231* ORFs encoding transposase (TnpA) contain five conserved domains, N1, N2, N3, C1, and C2, having more than 60% identity. Amino acid alignments of the

open reading frames from IS*231*M, -A, -F, and -W reveal that the positions of these five domains are highly conserved in IS*231*M (Fig. 2). The functional role of these domains is still unclear. Interestingly, the minus 1-bp frame-shift between ORF1 and ORF2 in IS*231*M does not change the amino acids sequences in neighboring N3 and C1 domains (Fig. 2).

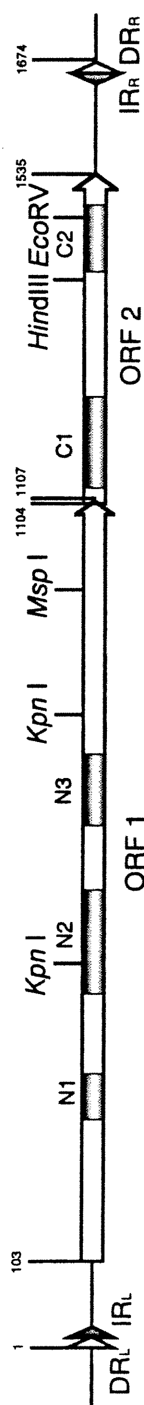
Fig. 1. Nucleotide sequence of IS*231*M.

The complete nucleotide sequence of IS*231*M is presented. IS*231*M is 1652-bp long containing two open reading frames that could encode polypeptides of 334 (ORF1) and 143 (ORF2) amino acids respectively. It is delimited by two imperfect 20-bp inverted repeats with two mismatches, which are flanked by two perfect 11-bp direct repeats. Boxed sequences show the inverted repeats. Nucleotides underlined by arrows show the direct repeat sequences and the two start codons (ATG). The stop codons are indicated. Underlined sequences indicate the –35, the -10 regions and the two potential ribosome-binding sites (RBS1 and RBS2). The GenBank Accession Number for IS*231*M is AF124259.

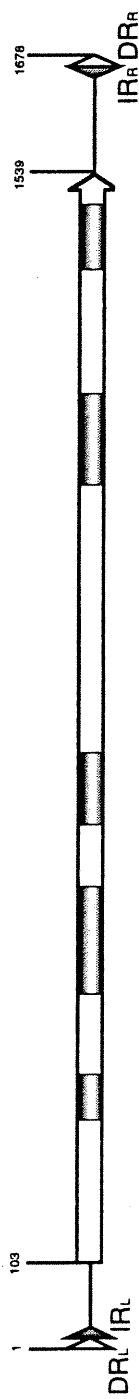
-11	1				49
GGGTAATACC	CCATGCCCAT	CAACTTAAGG	TTTTTTACTA	CCCCCAAGTA	CAAAAAAACG
DR_L		IR_L	-35		109
TTATTCTTTC	TTAATAACCT	ATGAGAAAGG	GTGACGTTTT	TTATGAATCT	TTCGATTCAA
-10		RBS 1		START	169
GAGGAGTTAC	ATTCATTTGC	AGAAGAATTG	CAGCGCTATA	TCACACCTGA	ATTTTTGGAA
				229
GAACTAGCAA	GAGAGGTTAA	ATTTGTAAAG	AGAAAACGCA	AATTTTCAGG	ATCAGATTTA
				289
GCTACCATTT	GTATTTGGAT	TAGTCAAAGA	GTGGCCAGTG	ATCCTTTAGT	CCGATTATGT
				349
AGTAGGCTTC	ATGCAGTTAC	AGGTACTGTA	CTTAGTCCGG	AGGGCTTAAA	TAAACGATTT
				409
AATGAAAAAT	CAGTGCTGTT	TTTAAAACAT	ATTTTTTCAT	TGTTATTAAA	ACAAAAAATA
				469
TGTGAGCAAA	CTCACATATC	TAACCAACTT	TTTACTCATT	TTAAACGTAT	TCGCATCATG
				529
GATGCCACGA	TGTTTCAGGT	ACCTGATACT	TTGGAGCATG	TATATCCTGG	TTCAGGTGGT
				589
TGCGCGCAAA	CAGCTGGAAT	CAAAATTCAA	TTAGAATATG	ATTTACATAG	TGGACAATTT
				649
CTTAATTTCC	AAGTGGGTCC	TGGAAAAAAT	AATGATAAAA	CATTTGGAAC	AAAATGTTTA
				709
GATACATTAC	GACCAGGCGA	TTTATGTATT	CGTGATTTAG	GATATTTTTC	ATTAGAGGAT
				769
TTAGATCAAA	TGGATCAACG	GGAGACATAT	TTTATTTCAC	GCCTAAAGTT	AAATACAAAC
				829
GTATATATCA	AGAATCCTAA	TCCAGCATTT	TTTCATAATG	GTACCATCAA	GAAACAAACA
				889
GAATATATAA	AAATCAATTT	ACAAATGATG	ATAGAGCGCT	TATTACCTGG	TGAAACTTAT
				949
GAAGTAGGAA	ATGTTTATGT	TGGGGATCAC	AAGGTTTTGT	TTGCGCGACT	CGTTCTGTAT
				1009
CGTCTAACAG	AAAAACAATT	ACGAGAACGC	CGGAAAAAAC	AAGAGGAAAA	CGAAAAGAAA
				1069
AAAGGGAAAT	CGTATTCGGA	GAAAAGTAAA	ATATTATCTG	GGCTGAATAT	ATATGTAACA
	STOP			1129
AACCTCCGTG	GGAGTGGGTT	CCGATGAAAC	AAGTCCATGA	ACTATACTCT	TTACGCTGGC
RBS 2	START			1189
AGATCGAAAT	TGTTTTTAAA	ACGTGGAAAT	CATTGTTTGA	TATAGATCAT	TGTCGCACTG
				1249
TCAAACAAGA	AAGAATAGAG	TGCCATTTAT	ACGGGAAACT	GATTGCTATT	TTCTTATGTT
				1309
CTTCTACGAT	GTTTAAAATG	CGCCAACTTT	TGTTACAGAA	GAAGCAAAAA	GAATTAAGTG
				1369
AATATAAAGC	CATTGGAATG	ATTCAAGACC	ACCTATTTCT	CCTCTATCAA	GCCATACAAA
	*Hin*dIII			1429
ACACCCAAGA	GATAACAAAG	CTTTTAATCC	GCCTGTTCCA	CCTTCTAGAG	AAAAACGGAC
				1489
GGAAATCTCA	CAGATATGAA	GAGAAAACAG	TCTTTGATAT	CATGGGTGTT	CATTATGAGT
		STOP		1549
ATAGTATAGC	TAGGGAACAA	AAGAAAGCTG	TATAATTTTA	AAAATAAAAC	TCGTTAGAGT
				1609
TTATTTAGCA	TGCGGATTTT	TAAGAAGAAT	CAATCTGTTG	AACAGTTTGT	AACCCTTTTG
AAGGGAATTT	CATTTTATTT	GTTCTCTTAA	GTTGATGGGC	ATGGGGTAAT	ACCC
		IR_R	1652	DR_R

Fig. 2. Structural maps of IS*231*M and iso-IS*231*.

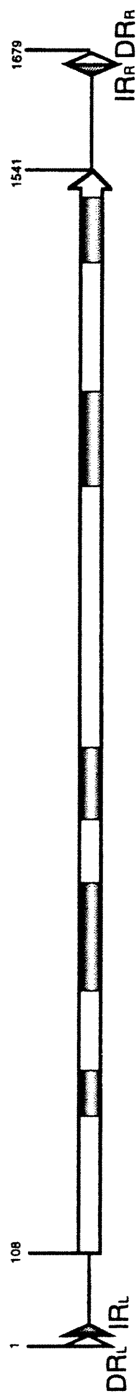
IS*231*M contains two open reading frames, ORF1 and ORF2, indicated by arrows. The terminal inverted repeats (IR_L and IR_R) and direct repeats (DR_L and DR_R) are designated by grey and white arrowheads respectively. The five conserved boxes N1, N2, N3, C1 and C2 are indicated by grey boxes. Is231A, -F and -W are indicated for comparison purposes. Selected enzyme restriction sites are shown. The figure is drawn to scale and the IS*231* elements aligned.



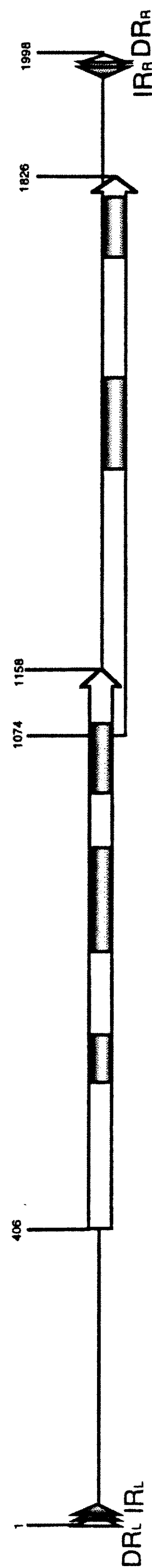
DR$_L$
IR$_L$
1
103
N1
Kpn I
N2
ORF 1
N3
Kpn I
Msp I
1104
1107
C1
ORF 2
*Hin*dIII
C2
*Eco*RV
1535
1674
IR$_R$
DR$_R$
IS*231* M



DR$_L$
IR$_L$
1
103
1539
1678
IR$_R$
DR$_R$
IS*231* A



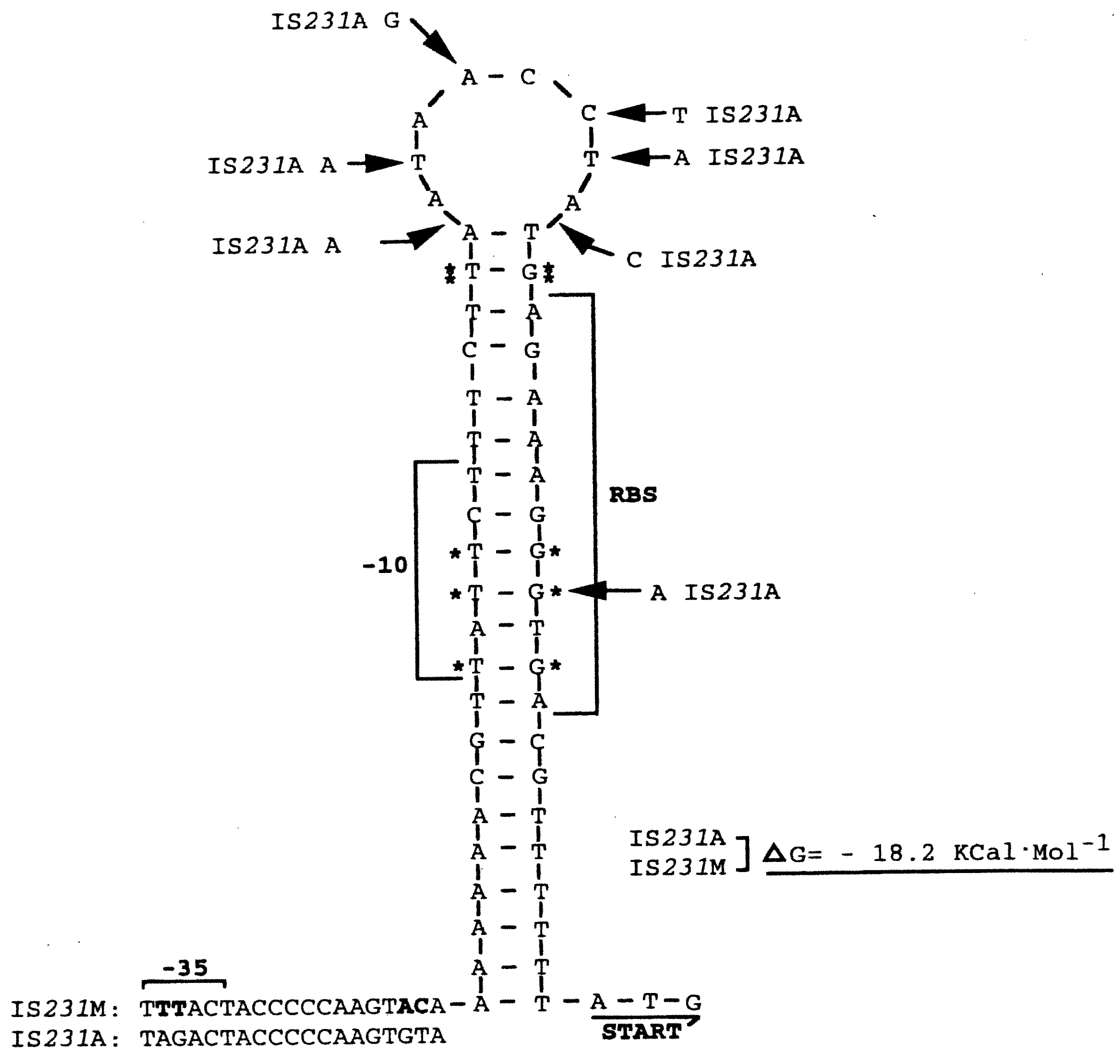
DR$_L$
IR$_L$
1
108
1541
1679
IR$_R$
DR$_R$
IS*231* F



DR$_L$
IR$_L$
1
406
1074
1158
1826
1998
IR$_R$
DR$_R$
IS *231* W

Fig. 3. Nucleotide sequence of the stem-and-loop secondary structures of IS*231*M.

The sequence of a stem-and-loop secondary structure from IS*231*A is added for comparison. The -35, -10 boxes and the putative Ribosome Binding Site (RBS) are indicated by half-boxes. The wobble T-G base pairs within the stem of the secondary structure in IS*231*M are indicated by asterisks, single or double. The extra nucleotides in IS*231*M are indicated by double-asterisks. Nucleotide substitutions or additions in IS*231*A are indicated by arrows. The start codon is underlined with an arrow. The calculated free energies (ΔG) of the secondary structure conformations of IS*231*M and IS*231*A are $-18.2 KCal \cdot Mol^{-1}$.



IS231A G
IS231A A
IS231A A
T IS231A
A IS231A
C IS231A
RBS
-10
A IS231A
IS231A
IS231M
ΔG= - 18.2 KCal·Mol-1
-35
IS231M: TTTACTACCCCCAAGTACA-A - T - A-T-G
IS231A: TAGACTACCCCCAAGTGTA
START

2. 4. DISCUSSION

We have isolated an autoagglutinable, non-serotypeable *B. thuringiensis* strain from dead two-spotted spider mites. In the course of cloning the crystal toxin gene, a labeled 4-kb *Nde*I fragment containing the *crylA(b)* gene from *B. thuringiensis* var. *kurstaki* strain HD-1 (Côté *et al.,* 1992) was used as a probe and detected, under low-stringency conditions, 1.9- and 2.5-kb *Hin*dIII fragments. Surprisingly, analysis of their complete nucleotide sequences revealed not a novel crystal protein gene but rather a new IS, which we named IS*231*M because of homologies with other iso-IS*231* elements. Comparison of nucleotide sequences between IS*231*M and the 4-kb *Nde*I fragment containing the *cry1A(b)* gene revealed a region of extensive homology. Nucleotides -11 to 135 in IS*231*M share 82% identities with nucleotides 3651 to 3793 in the 4-kb *Nde*I fragment containing the *cry1A(b)* gene. In IS*231*M, this region contains DR_L, IR_L, the -35 and -10 boxes, and RBS1 along with the first 45 nucleotides of ORF1. In *cry1A(b)*, the homologous region is located in the 3' end noncoding region, 183 nucleotides downstream from the TAA stop codon. It would be interesting to further analyze this region in *B. thuringiensis* var. *kurstaki* strain HD-1 for the possible presence of yet another IS*231* element. Clearly, the homology was high enough along this 146-nucleotide-long sequence to allow for hybridization between a 1.9-kb *Hin*dIII fragment containing the 5' end of IS*231*M and the *cry1A(b)* probe under the low-stringency conditions used. Likewise, nucleotides 1634 to 1664 in IS*231*M share 85% identities with nucleotides 3680 to 3650 in the complementary strand of the 4-kb *Nde*I fragment. In IS*231*M, this region contains IR_R and DR_R. In *cry1A(b)*, the homologous region is located in the 3' end noncoding region, 182 nucleotides downstream from the TAA stop codon.

Eleven IS*231* relatives have now been characterized since the discovery of the first, IS*231*A, in 1985. Several of the IS*231* elements are associated with crystal genes on large plasmids of *B. thuringiensis* (Lereclus *et al.*, 1989 ; Mahillon *et al.*, 1994). IS*231*A, -B, and -C were originally found in *B. thuringiensis* subsp. *thuringiensis* strain berliner 1715 (Mahillon *et al.*, 1985, 1987) ; IS*231*D and -E isolated from *B. thuringiensis* subsp. *finitimus* ; IS*231*F from the 112-kb plasmid of *B. thuringiensis* subsp. *israelensis* (Rezöhazy *et al.*, 1992) ; IS*231*G and -H from *B. thuringiensis* subsp. *darmstadiensis* (Ryan *et al.*, 1993) ; IS*231*V and -W from the 112-kb plasmid of *B. thuringiensis* subsp. *israelensis* (Rezöhazy *et al.*, 1993). IS*231*M was isolated from an autoagglutinable, non-serotypeable *B. thuringiensis* strain (this work). A 12th IS*231* element, -Y, has been isolated but has not been characterized further (Chen and Mahillon, unpublished). Our own hybridization results using a DNA fragment from *B. thuringiensis* var. *kurstaki* as a probe indicate that IS*231* homologous sequences are present in the latter. IS*231* sequences apprear to be not only widely distributed in *B. thuringiensis* but also in phylogenetically related *Bacillus* species such as *B. cereus* (Léonard *et al.*, 1998). IS*231* sequences can be found in different variations of structure, as exemplified by the single or double ORFs found in IS*231*A-F or IS*231*M, -V, and -W, respectively. However, based on primary sequences, all IS*231* elements can potentially encode a single 56- kDa, transposase A-like enzyme.

IS*231*M contains two overlapping open reading frames and thus appears at first sight structurally related to IS*231*V and -W, as opposed to IS*231*A, -B, -C, -D, -F, and -G, which contain a single TnpA ORF. Closer analysis at the nucleotide sequence level, however, reveals that it is most similar to IS*231*F, with which it shares 87% nucleotide identity and least similar to IS*231*V and -W, with which it shares 65% homology. This is further confirmed by analysis of the aminoacid sequence. Again, when both open

reading frames in IS*231*M are combined to produce a single fusion protein amino acid sequence, IS*231*M shares 83% amino acid identity with IS231F and 50% homology with IS231W.

The transposition of IS*231*A has been demonstrated to be functional in *E. coli* (Hallet *et al.,* 1991). It is yet unknown whether IS*231*M is indeed a functional transposable element in *B. thuringiensis* strain M15, albeit high sequence homology to IS*231*A strongly suggests that.

Using IS*231*M as a probe, we are pursuing the analysis of its distribution in *B. thuringiensis* strain M15 to determine the number of copies present and possible different insertion site sequences.

2. 5. ACKNOWLEDGMENT

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CHAPTER 3

Cloning and Nucleotide Sequence of a New Insertion Sequence, IS*231*N, from a Non-Serotypable Strain of *Bacillus thuringiensis*

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ABSTRACT

A new IS*231* variant, IS*231*N, has been isolated from an autoagglutinable, non-serotypable strain of *B. thuringiensis*. IS*231*N is 1654-bp in length and is delimited by two incomplete 20-bp inverted repeats (IR_L and IR_R) with two mismatches. No direct repeats (DRs) were found at the right and left borders of IS*231*N. Surprisingly, IS*231*N contains three open reading frames (ORFs) that could code for polypeptides of 329 (ORF1), 118 (ORF2) and 17 (ORF3) amino acids, respectively. IS*231*N lacks the 5th conserved amino acid domain, called C2, owing to the addition of an adenine residue at nucleotide 1319. IS*231*N shows the highest nucleotide identity (99%) with IS*231*M, another insertion sequence previously isolated from the same bacterial strain. IS*231*N, however, shares only 83% amino acid identity with IS*231*M because of nucleotide substitutions and additions. The ORF1 of IS*231*N has five fewer amino acids than ORF1 of IS*231*M. Furthermore, the ORF2-3 putative fusion product in IS*231*N contains eight fewer amino acids than ORF2 in IS*231*M.

The dendrogram showing the evolutionary relationship between members of the IS*231* family and IS*231*N indicates that IS*231*N is phylogenetically more closely related to IS*231*M (83%), followed by IS*231*F(74%), and is more distant to IS*231*V and W(46%).

Key words: Bacillus thuringiensis; insertion sequence; IS*231*N.

3. 1. INTRODUCTION

Bacillus thuringiensis is a Gram-positive soil bacterium that produces a parasporal inclusion body during sporulation. This inclusion is made of proteins, the δ-endotoxins, which exhibit specific toxicities against the larvae of lepidopteran, dipteran, and coleopteran insects (Höfte and Whiteley, 1989).

The δ-endotoxins are encoded by *cry* genes. Several *cry* genes have been shown to be adjacent to specific insertion sequences, IS*231* (Kronstadt and Whiteley, 1984), IS*232* (Kronstadt and Whiteley, 1984; Menou *et al.*, 1990), IS*240* (Bourgouin *et al.*, 1988; Delécluse *et al.*, 1989), ISBT*1* (Smith et al., 1994), ISBT*2* (Ahmad *et al.*, 1989; Hodgman *et al.*, 1993) and transposons, Tn*4430* (Lereclus *et al.*, 1984), Tn*5401* (Adams *et al.*, 1994). They are believed to play a role in the multiple locations and genetic mobility of the *cry* genes.

So far, 12 iso-IS*231* insertion sequences have been cloned from various *B. thuringiensis* varieties and characterized (Mahillon *et al.*, 1985, 1987, 1994; Rezsöhazy *et al.*, 1992, 1993; Ryan *et al.*, 1993; Jung *et al.*, 2001; Chen and Mahillon, unpublished data). Members of the IS*231* family could be divided into two groups depending on whether they contain one or two open reading frames (ORFs). Based on primary sequences of the ORFs, however, all IS*231* elements can potentially encode a single 56-kDa, transposase A-like enzyme. All IS*231* amino acid sequences contain five conserved domains called N1, N2, N3, C1 and C2 (Mahillon *et al.*, 1994)

We reported recently the isolation of IS*231*M, a new member of the IS*231* family, which contains two slightly orverlapping ORFs (Jung *et al.*, 2001). We report here the characterization of yet another member of the IS*231* family, IS*231*N.

3. 2. MATERIALS AND METHODS

3. 2. 1. Bacterial strains and plasmids

An autoagglutinable, non-serotypable *B. thuringiensis* strain M15 was isolated from dead two-spotted spider mites (Tetranychus urticae Koch; Arthropoda: Arachnida: Tetranychidae) and was the source for the isolation of new insertion sequences (IS).

Escherichia coli strain XL1-Blue was used as a bacterial host for pBluescript II KS(+) (Stratagene) for cloning purposes.

A 4-kb *Nde*I fragment containing a *cryIA(b)* gene (Côté *et al.*, 1992), isolated from the OZ-3 mutant of *B. thuringiensis* var. *kurstaki* HD-1 (Rivard *et al.*, 1989), was used as a probe in Southern hybridization. The recombinant plasmid pYC90 harbors a 2.5-kb *Hin*dIII insert which contains the 3' region of IS*231*M (Jung *et al.*, 2001). A 174-bp *Hin*dIII-*Sph*I sub-fragment was cloned and used as a probe.

3. 2. 2. Plasmid DNA preparation

Plasmid DNA from *B. thuringiensis* strain was isolated with a modified alkaline lysis method as described by Birnboim and Doly (1979). The bacterial strain was cultured in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) overnight at 30°C with vigorous agitation. Lysozyme was added at a concentration of 2 mg/ml, and the cell suspension was incubated at 37°C for 1 hr.

Recombinant plasmid DNAs from *E. coli* strain were isolated as described by Birnboim and Doly (1979). The plasmid DNA for DNA sequencing was purified with

Wizard Plus SV minipreps DNA purification system according to the manufacturer's recommendation (Promega).

3. 2. 3. Probe DNA preparation and Southern hybridization

The 4-kb *Nde*I fragment and 174-bp *Hin*dIII-*Sph*I fragment were labeled with Digoxigenin (DIG)-11-dUTP (Boehringer Mannheim Biochemicals) using the random primed method as recommended by the manufacturer. The labeled DNA fragments were precipitated with 0.1 volume of 4 M LiCl and 2.5 volumes of ice-cold ethanol, and transferred at -70°C for 30 min. The reaction was centrifuged at 16,000g for 15 min at 4 °C. The washed pellet was resuspended in nuclease-free water, and stored at -20°C until used.

Plasmid DNA from *B. thuringiensis* was digested with *Hin*dIII, electrophoresed, and transferred onto a nylon membrane (Schleicher & Schuell) by the method of Southern (1975). Southern blot hybridization using the DIG-labeled 4-kb *Nde*I and the 174-bp *Hin*dIII-*Sph*I fragments was done with standard hybridization solution (5X SSC, 1% blocking reagent (Boehringer Mannheim Biochemicals), 0.1% *N*-lauroylsarcosine, 0.02% SDS) for 18 hr at 66°C and 68°C, respectively. After hybridization, the membrane probed with a 4-kb *Nde*I fragment was washed three times for 15 min each in 4X washing solution (4X SSC, 0.1% SDS) at room temperature. The hybridization membrane probed with the 174-bp *Hin*dIII-*Sph*I fragment was rinced twice for 5 min each in 2X washing solution (2X SSC, 0.1% SDS) at room temperature, and then washed twice for 15 min each in 0.1X washing solution (0.1X SSC, 0.1% SDS) at 68°C. Following the washes, detection on the membrane was performed with the color-substrate solution containing NBT (4-Nitroblue tetrazolium chloride; Boehringer

Mannheim Biochemicals) and BCIP (5-Bromo-4-chloro-3-indolyl-phosphate; Boehringer Mannheim Biochemicals) as recommended by the manufacturer.

3. 2. 4. Cloning and DNA sequencing

DNA fragments detected by Southern hybridization were eluted with Sephaglas Bandprep Kit (Pharmacia Biotech) and ligated overnight at 16°C to *Hin*dIII-digested, SAP (shrimp alkaline phosphatase, Boehringer Mannheim Biochemicals)-treated pBluescript II KS (+). *E. coli* XL1-Blue competent cells were transformed with the ligated mixture, plated on LB agar plates containing ampicillin (100 μg/ml), 0.5 mM IPTG (Isopropyl-β-D-thiogalactoside) and X-Gal (5'-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 40 μg/ ml), and incubated overnight at 37°C.

DNA sequencing was done with the near-infrared (NIR) fluorescence automated DNA sequencer (LI-COR Model 4200, LI-COR, Inc.). Nucleotide and protein sequences were analyzed with the DNASIS program (Hitachi Software Engineering Co.).

3. 3. RESULTS

3. 3. 1. Cloning and characterization of a new insertion sequence IS*231*N

Plasmid DNA purified from *B. thuringiensis* strain M15 was digested with *Hin*dIII and hybridized with the 4-kb *cryIA(b)* and the 174-bp *Hin*dIII-*Sph*I probes, respectively. Southern blot hybridizations showed a 6.6- and a 3.5-kb *Hin*dIII fragment detected by the 4-kb *cryIA(b)* and the 174-bp *Hin*dIII-*Sph*I probes, respectively (data not shown). The 6.6- and the 3.5-kb *Hin*dIII fragments were cloned into *Hin*dIII-digested pBluescript II KS(+) to give two recombinant plasmids, pYC86 and pYC94, respectively.

The 6.6-kb insert from plasmid pYC86 was digested with *Eco*RI, and a 3-kb *Eco*RI-*Hin*dIII fragment was subcloned into an *Eco*RI/*Hin*dIII doubly digested pBluescriptIIKS(+). Complete nucleotide sequences were determined for the 3-kb *Eco*RI-*Hin*dIII and the 3.5-kb *Hin*dIII fragments. Comparison with the IS*231*M sequence (Jung *et al*., 2001) revealed extensive homologies, indicating that the two fragments contained a new member of the IS*231* family which we named IS*231*N.

IS*231*N is 1654-bp in length and is delimited by two incomplete 20-bp inverted repeats (IR_L and IR_R) with two mismatches (Fig. 1). No direct repeats (DRs) flanking either sides of IS*231*N could be revealed. IS*231*N contains three open reading frames, ORF1, -2 and -3, at nucleotide positions 92-1081, 1094-1450 and 1447-1500, respectively. They could code for polypeptides of 329, 118 and 17 amino acids of predicted molecular masses of 38-, 14- and 2-kDa, respectively.

The first start codon, ATG, is located at nucleotide position 92. The second open reading frame (ORF2) begins 13 nucleotides downstream from the TAG stop

codon of ORF1. The third ATG start codon is located 1 bp upstream from the TGA stop codon of ORF2.

One potential promoter-like sequence in the 5' non-coding region shows a 22-bp spacing between the -10 and -35 sequences that are located at positions similar to those of the other iso-IS*231* elements. The -10 sequence (TATTCT) is the same as that of most other iso-IS*231* elements with the exceptions of IS*231*V, -W and -H whereas the -35 sequence shows nucleotide substitutions like those found in the other IS*231* variants.

Three potential ribosome binding sites (RBS) with sequences partially complementary to the 3' end (UCUUUCCUCC) of *B. subtilis* 16S rRNA (McLaughlin *et al.*, 1981) are located 7-13bp upstream of the start codon (ATG) of the three ORFs. The calculated free energies of interaction (ΔG, 25 °C) between the *B. subtilis* 16S rRNA and these putative ribosome binding sites are -12 kcal.mol^{-1} for RBS1, -8.6 kcal.mol^{-1} for RBS2, and -13 kcal.mol^{-1} for RBS3 (Tinoco *et al.*, 1973). The sequence of RBS1 is identical to that of the putative RBS and RBS1 from IS*231*F (Rezsöhazy *et al.*, 1992) and -M (Jung *et al.*, 2001), respectively. The RBS2 in IS*231*N shares seven identical nucleotides out of eight with RBS2 from IS*231*M. Surprisingly, the 8-bp sequence of RBS3, AAACGGACG, reveals extensive homology with RBS0 , AAATGGAGG,from IS*231*V and -W. Should two cytosine residues in the RBS3 sequence be substituted by a thymine and a guanine, the homology would be perfect.

A possible ORF2-ORF3 fusion product would yield a protein of 135 aminoacids with predicted molecular mass of 16 KDa. If fused to ORF1, it would yield a transposase A-like enzyme of 464 aminoacids and 54 KDa.

The 5' non-coding region upstream of ORF1 containing the potential RBS1 can form a stem-and-loop secondary structure with a calculated energy (ΔG, 25 °C) of -18.2 kcal/mol (Tinoco *et al.*, 1973).

3. 3. 2. Comparison of IS*231*N with IS*231*M and other IS*231* elements

A comparison of the nucleotide sequences reveals that IS*231*N shares 99% identity with IS*231*M (Fig. 1). IS*231*N is 1654 bp long and is 2 bp longer than IS*231*M (Fig. 1, 2). The two extra nucleotides in IS*231*N are added at nucleotide positions 1073 and 1319. These additional nucleotides cause some major changes in the open reading frame structure of IS*231*N. The ORF1 in IS*231*N has five fewer amino acids than ORF1 in IS*231*M. Furthermore, the putative ORF2-3 fusion product in IS*231*N contains eight fewer amino acids than ORF2 in IS*231*M. Moreover, the amino acid sequence in the carboxy-terminus of IS*231*N shares almost no homology with its corresponding sequence in IS*231*M (Fig. 3). Consequently, in spite of the high nucleotide sequence identity, IS*231*N shows 83% amino acids identity with IS*231*M. A pairwise comparison between IS*231* putative transposase amino acid sequences reveals that IS*231*N is still most homologous to IS*231*M and least homologous to IS*231*G with which it shares 42% identity.

All IS*231* ORFs encoding transposases (TnpA) contain five conserved domains, N1, N2, N3, C1 and C2, having more than 60% identity. Amino acid sequences of these five domains were compared with IS*231*N (this work), -M (Jung *et al.*, 2001), and the single ORF in IS*231*A (Mahillon *et al.*, 1985, 1987) and the two-ORFs in IS*231*V (Rezsöhazy *et al.*, 1993) (Fig. 3). The N1, N2, N3 and C1 domains are highly conserved among all four IS*231* elements. The C2 domain, however, is very divergent

in IS*231*N. Interestingly, the nucleotide insertion at position 1073 did not change the amino acid sequence in the neighboring C1 domain in IS*231*N. The nucleotide insertion at position 1319, however, did change the entire amino acid sequence in the C2 domain. The functional role of these domains is still unclear.

Fig. 1. IS*231*N nucleotide sequence and alignment with IS*231*M.

The complete nucleotide sequences of IS*231*N aligned with IS*231*M are presented. IS*231*N is 1654 bp in length and is delimited by two incomplete 20-bp inverted repeats (IR_L and IR_R) with two mismatches. The two inverted repeats are indicated by half-boxes. Direct repeats (DR_L and DR_R) in IS*231*M are underlined. No direct repeats were found flanking IS*231*N. The -35 and the -10 boxes, the unique *Hin*dIII restriction site, and the three potential ribosome binding sites, (RBS1, RBS2 and RBS3) are underlined. IS*231*N contains three open reading frames, ORF1, -2 and -3, that code for 329, 118 and 17 amino acids, respectively. The sequences lined with arrows indicate the three start codons (ATG). Stop codons are also indicated. Hyphens indicate alignment gaps. The asterisks above the nucleotides at positions 1073 and 1319 indicate additions in IS*231*N. The small characters and dots under the IS*231*N sequence correspond to the difference and identity between IS*231*N and -M, respectively. The GenBank Accession Number for IS*231*N is AF138876

```
                                                      -35                                       -10
        -11                                                                                                        68
IS231N GGGTAGA-AC CACATGCCCA TCAACTTAAG GTTTTTAACT ACCCCCAAGT ACAAAAAAAC GTTATTCTTT CTTAATAACC
IS231M ·····-·t·· ·c········ ·········· ··]···t··· ·········· ·········· ·········· ··········
          DRL                    IRL
                               START                                                                              148
IS231N TATGAGAAAG GGTGACGTTT TTTATGAATC TTTCGATTCA AGAGGAGTTA CATTCATTTG CAGAAGAATT GCAGCGCTAT
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
             RBS1
                                                                                                                  228
IS231N ATCACACCTG AATTTTTGGA AGAACTAGCA AGAGAGGTTA AATTTGTAAA GAGAAAACGC AAATTTTCAG GATCAGATTT
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  308
IS231N AGCTACCATT TGTATTTGGA TTAGTCAAAG AGTGGCCAGT GATTCTTTAG TCCGATTATG TAGTAGGCTT CATGCAGTTA
IS231M ·········· ·········· ·········· ·········· ···c······ ·········· ·········· ··········
                                                                                                                  388
IS231N CAGGTACTGT ACTTAGTCCG GAGGGCTTAA ATAAACGATT TAATGAAAAA TCAGTGCTGT TTTTAAAACA TATTTTTTCA
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  468
IS231N TTGTTATTAA AACAAAAAAT ATGTGAGCAA ACTCACATAT CTAACCAACT TTTTACTCAT TTTAAACGTA TTCGCATCAT
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  548
IS231N GGATGCCACG ATGTTTCAGG TACCTGATAC TTTGGAGCAT GTATATCCTG GTTCAGGTGG TTGCGCGCAA ACAGCTGGAA
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  628
IS231N TCAAAATTCA ATTAGAATAT GATTTACATA GTGGACAATT TCTTAATTTC CAAGTGGGTC CTGGAAAAAA TAATGATAAA
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  708
IS231N ACATTTGGAA CAAAATGTTT AGATACATTA CGACCAGGCG ATTTATGTAT TCGTGATTTA GGATATTTTT CATTAGAGGA
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  788
IS231N TTTAGATCAA ATGGATCAAC GGGGGACATA TTTTATTTCA CGCCTAAAGT TAAATACAAA CGTATATATC AAGAATCCTA
IS231M ·········· ·········· ···a······ ·········· ·········· ·········· ·········· ··········
                                                                                                                  868
IS231N ATCCAGCATT TTTTTATAAT GGTACCATCA AGAAACAAAC AGAATATATA AAAATCAATT TACAAATGAT GATAGAGCGC
IS231M ·········· ····c····· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  948
IS231N TTATTACCTG GTGAAACTTA TGAAGTAGGA AATGTTTATG TTGGGGATCA CAAGGTTTTG TTTGCGCGAC TCGTTCTGTA
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  1028
IS231N TCGTCTAACA GAAAAACAAT TACGAGAACG CCGGAAAAAA CAAGAGGAAA ACGAAAAGAA AAAAGGGAAA TCGTATTCGG
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                            STOP            START                                 1108
IS231N AGAAAAGTAA AATATTATCT GGGCTGAATA TATATGTAAC AAACACTCCG TAGGAGTGGG TTCCGATGAA ACAAGTCCAT
IS231M ·········· ·········· ·········· ·········· ····_····· ·g········ ·········· ··········
                                                                      RBS2
                                                                                                                  1188
IS231N GAACTATACT CTTTACGCTG GCAGATCGAA ATTGTTTTTA AAACGTGGAA ATCATTGTTT GATATAGATC ATTGTCGCAC
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  1268
IS231N TGTCAAACAA GAAAGAATAG AGTGCCATTT ATACGGGAAA CTGATTGCTA TTTTCTTATG TTCTTCTACG ATGTTTAAAA
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                                                                  1348
IS231N TGCGCCAACT TTTGTTACAG AAGAAGCAAA AAGAATTAAG TGAATATAAA AGCCATTGGA ATGATTCAAG ACCACCTATT
IS231M ·········· ·········· ·········· ·········· ·········· _········· ·········· ··········
                                                 HindIII                                                          1428
IS231N TCTCCTCTAT CAAGCCATAC AAAACACCCA AGAGATAACA AAGCTTTTAA TCCGCCTGTT CCACCTTCTA GAGAAAAACG
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                           STOP                                                        STOP                       1508
IS231N GACGGAAATC TCACAGATAT GAAGAGAAAA CAGTCTTTGA TATCATGGGT GTTCATTATG AGTATAGTAT AGCTAGGGAA
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
        RBS3               START
                                                                                                                  1588
IS231N CAAAAGAAAG CTGTATAATT TTAAAAATAA AACTCGTTAG AGTTTATTTA GCATGCGGAT TTTTAAGAAG AATCAATCTG
IS231M ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
                                                                               1654
IS231N TTGAACAGTT TGTAACCCTT TTGAAGGGAA TTTCATTTTA TTTGTTCTCT TAAGTTGATG GGCATGCGTT TCTTCCC
IS231M ·········· ·········· ·········· ·········· ······[··· ·········· ·····g·g· aa·a···
                                                                 IRR          1652     DRR
```

Fig. 2. Structural organization of IS*231*N and comparison with IS*231*M.

The structural map of IS*231*N is represented with the one from IS*231*M. The three open reading frames in IS*231*N and the two open reading frames in IS*231*M are indicated by open arrows. The grey arrowheads correspond to the inverted repeats (IR_L and IR_R). The white arrowheads in IS*231*M correspond to the direct repeats (DR_L and DR_R). No direct repeats were found in IS*231*N. The conserved domains, N1, N2, N3, C1, and C2, are indicated by grey boxes. No C2 domain was found in IS*231*N. Selected enzyme restriction sites are shown. Nucleotide numbers of specific features, beginning and end of the IS*231* elements, start and stop codons, are indicated. The figure is drawn to scale, and both maps are aligned.

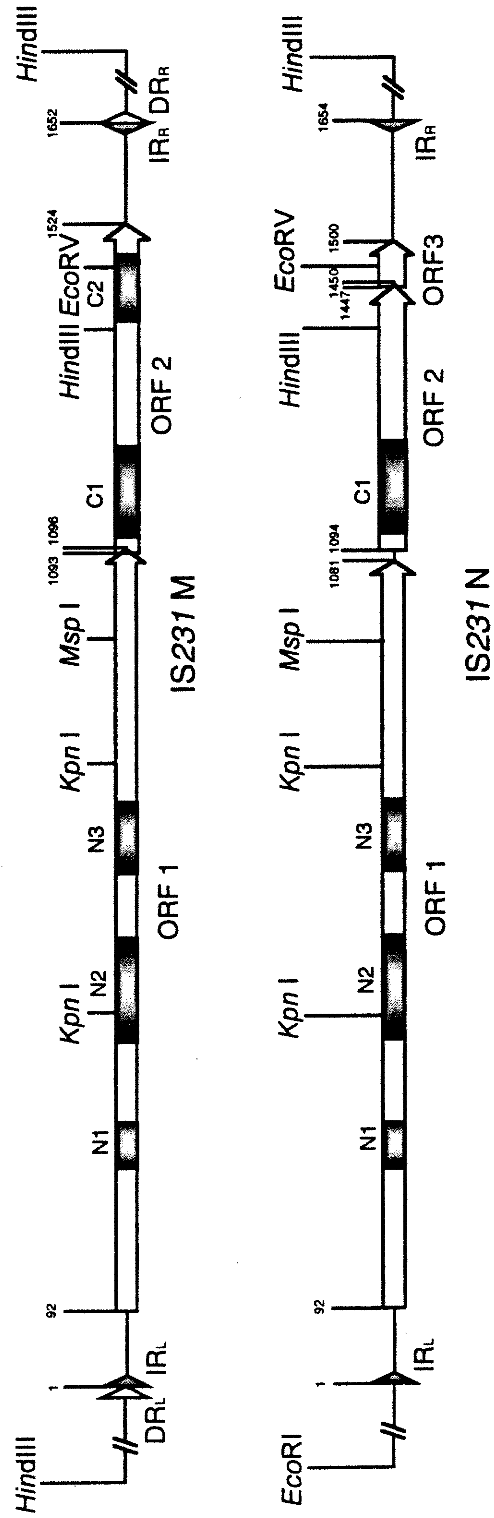

HindIII
DRL
IRL
1
92
N1
Kpn I
N2
N3
ORF 1
Kpn I
Msp I
1093
1096
C1
ORF 2
HindIII
EcoRV
C2
1524
1652
IRR
DRR
HindIII
IS231 M
EcoRI
1
IRL
92
N1
Kpn I
N2
N3
ORF 1
Kpn I
Msp I
1081
1094
C1
ORF 2
HindIII
1447
1450
EcoRV
1500
ORF3
1654
IRR
HindIII
IS231 N

Fig. 3. Conserved domains of amino acid sequences of IS*231*N and iso-IS*231* elements.

The amino acid sequences of the five conserved domains, N1, N2, N3, C1, and C2, are compared among IS*231*N, -A, -V, and -M. Characters in bold indicate conserved amino acids.

```
IS231A RLCSQLHAATGTLMSPEGLN
IS231V RLCGVLESETGVLISPEGLN
IS231M RLCSRLHAVTGTVLSPEGLN
IS231N RLCSRLHAVTGTVLSPEGLN

IS231A FQRIRILDATIFQIPKHLASIYPGSGGCAQTAGIKIQLEYDLHSGQF
IS231V FRRIRILDATTFQISDQLAAVYPGSGGSGKASGVKIQLEYDLLSGQF
IS231M FKRIRIMDATMFQVPDTLEHVYPGSGGCAQTAGIKIQLEYDLHSGQF
IS231N FKRIRIMDATMFQVPDTLEHVYPGSGGCAQTAGIKIQLEYDLHSGQF

IS231A LCIRDLGYFSLEDLDQMDQRGAYYISRLKLN
IS231V LCIRDLGYFSLIDLDGIQQKGAYYLSRLKMN
IS231M LCIRDLGYFSLEDLDQMDQRETYFISRLKLN
IS231N LCIRDLGYFSLEDLDQMDQRGTYFISRLKLN

IS231A YSLRWQIEIIFKTWKSLFQIHHWQNIKQERLECHVYGRLIA
IS231V YSLRWQIEIIFKTWKSIFRIHSNTNVKKERLECHIYGKLIA
IS231M YSLRWQIEIVFKTWKSLFDIDHCRTVKQERIECHLYGKLIA
IS231N YSLRWQIEIVFKTWKSLFDIDHCRTVKQERIECHLYGKLIA

IS231A RLFDLLQKNGRKSHRYEKKTIFDIMGVVYE
IS231V RLFHLLDKNGRKSHRYRKKTVFDILGTAYE
IS231M RLFHLLEKNGRKSHRYEEKTVFDIMGVHYE
IS231N PPVPPSREKRTEISQIMKRKQSLISWVFIM
```

3. 4. DISCUSSION

At least twelve IS*231* relatives have now been characterized. IS*231*A, -B, and -C were originally found in *B. thuringiensis* subsp. *thuringiensis* strain berliner 1715 (Mahillon *et al.*, 1985, 1987); IS*231*D and -E were isolated from *B. thuringiensis* subsp. *finitimus*; IS*231*F from the 112-kb plasmid of *B. thuringiensis* subsp. *israelensis* (Rezsöhazy *et al.*, 1992); IS*231*G and -H from *B. thuringiensis* subsp. *darmstadiensis* (Ryan *et al.*, 1993); IS*231*V and -W from the 112-kb plasmid of *B. thuringiensis* subsp. *israelensis* (Rezsöhazy *et al.*, 1993), and IS*231*M from the autoagglutinable, non-serotypable *B. thuringiensis* M15 strain (Jung *et al.*, 2001). IS*231*N was also isolated from the M15 strain (this work). A thirteenth IS*231* element, -Y, has been isolated but has not been characterized further (Chen and Mahillon, unpublished data). IS*231*A, -B, -C, -D, -E, -F, -G and -H, all contain a single ORF. IS*231*V and -W both contain two overlapping ORFs and are considered distant members of the IS*231* family (Rezsöhazy *et al.*, 1993). IS*231*M and -N contain 2 and 3 ORFs, respectively. A dendrogram was built using the alignments of the putative transposases A amino acids sequences to reveal possible phylogenetic relationships (Fig. 4). As could be expected, most closely related IS*231* elements were isolated from same bacterial hosts. One notable exception, IS*231*F, does not cluster with IS*231*V and -W although all three were isolated from *B. thuringiensis* var. *israelensis.* Again, IS*231*F contains a single ORF, whereas IS*231*V and -W contain two, and the structure of these three IS is quite different. It appears that *B. thuringiensis* var. *israelensis* contains two distinct "sub-groups" of IS*231* elements. IS*231*G and -H were not included in the dendrogram because the former contains several stop codons and is likely to be nonfunctional, whereas the latter has been only partially sequenced (Ryan *et al.*, 1993).

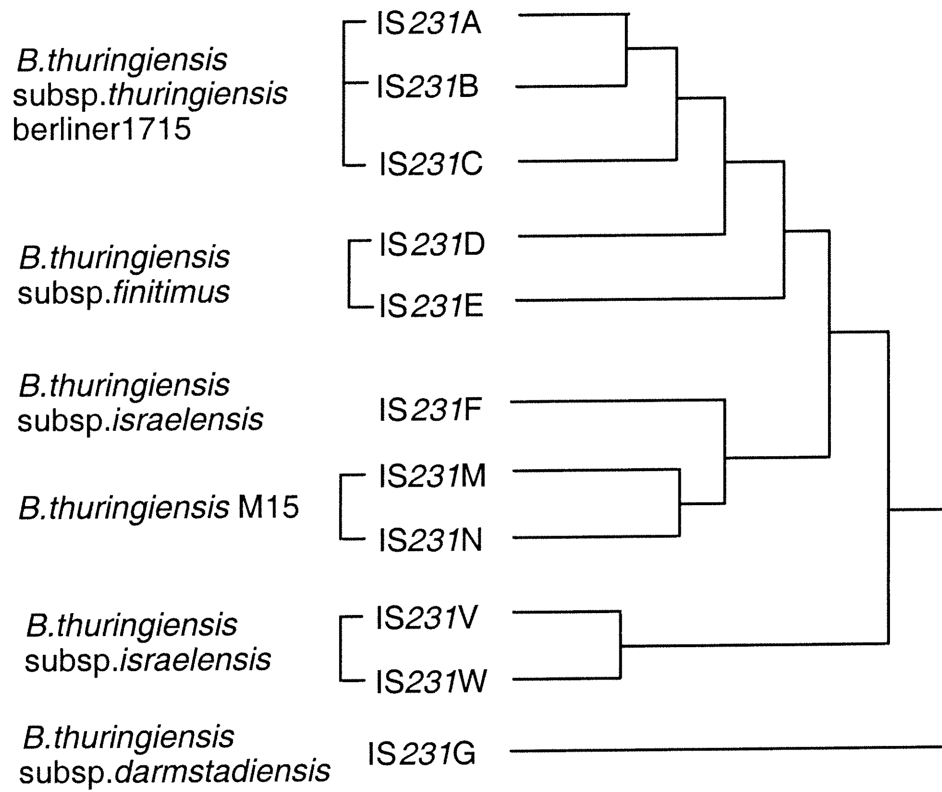
IS*231*N is a rather unusual IS*231* element in that it lacks the two direct repeats usually found flanking two inverted repeats. Also, it contains three ORFs and lacks the 5th conserved domain, called C2. Interestingly enough, although a nucleotide insertion at position 1073 in IS*231*N did not have an impact on the amino acid sequence of the downstream C1 domain, the nucleotide insertion at position 1319 did change the entire downstream amino acid sequence, thus eliminating the C2 domain. Presence of a stop codon at position 1081 and a start codon at 1094 has reduced the impact of the insertion at nucleotide position 1073 on the downstream amino acid sequence. It is interesting to wonder whether multiple ORFs, by dividing a key sequence into smaller domains, may serve as a mechanism to protect key sequences against mutations that otherwise could be lethal.

The transposition of IS*231*A has been demonstrated to be functional in *E. coli* (Hallet *et al.*, 1991). It is yet unknown whether or not transposition of IS*231*N is functional. Certainly, high sequence homology to IS*231*A strongly suggests that.

Using IS*231*M and -N as probes, we are pursuing the analysis of the distribution of IS*231* elements in *B. thuringiensis* strain M15.

Fig. 4. Dendrogram of IS*231*N and selected iso-IS*231* elements.

The dendrogram was constructed on the basis of the amino acid sequence alignments between the putative transposases (Tnp A) encoded by the known IS*231* family. The phylogenetic relationship between IS*231*N and selected iso-IS*231* elements is represented.



B.thuringiensis
subsp.thuringiensis
berliner1715
IS231A
IS231B
IS231C
B.thuringiensis
subsp.finitimus
IS231D
IS231E
B.thuringiensis
subsp.israelensis
IS231F
B.thuringiensis M15
IS231M
IS231N
B.thuringiensis
subsp.israelensis
IS231V
IS231W
B.thuringiensis
subsp.darmstadiensis
IS231G

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4. GENERAL DISCUSSION

We have isolated a new *Bacillus thuringiensis* strain from dead two-spotted spider mite (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae). This *B. thuringiensis* strain M15 is unusual in that it is autoagglutinable and non-serotypeable. It also shows rather different biochemical characteristics in comparison with those of known strains such as *B. thuringiensis* var. *kurstaki* HD-1, -var. *israelensis* HD-500, and -var. *higo* BT205 (Jung *et al.*, 1998). When observed under phase-contrast microscopy, it produces a roughly spherical parasporal inclusion body tightly bound to the spore, another unusual feature. Transmission electron microscopy (TEM) revealed that the parasporal inclusion body has a polygonal shape. The protein profile of the parasporal inclusion appeared to be composed of at least two major polypeptides of approximately 86- and 79-kDa as revealed on a 10% SDS-polyacrylamide gel. The finding of crystal proteins in the 79 - 86-kDa range was another unexpected finding since most insecticidal crystal proteins of *B. thuringiensis* strains have been known to have molecular weights in the range of 130 - 140-kDa and 65 - 80-kDa (Schnepf *et al.*, 1998).

The first 20 amino acids of the N-terminal sequence of the two major polypeptides of 86- and 79-kDa were determined and found to be identical. An 18-mer oligonucleotide (M15-M) sequence deduced from the N-terminal amino acid sequence of the 86-kDa protein was synthesized and used as a probe. The probe revealed a single 8-kb *Hin*dIII fragment when used against the plasmid DNA of *B. thuringiensis* strain M15. These two findings together suggested the presence of a single crystal protein gene. The crystal gene was cloned and its nucleotide sequence determined. Further comparison of its nucleotide sequence against other crystal protein genes indicated that we had cloned a novel one. It was designated *cry31Aa2* by the *Bacillus*

thuringiensis Pesticide Crystal Protein Nomenclature Committee. The overall amino acid sequence of the Cry31Aa2 protein shares 94% identity with Cry31Aa1, the 81-kDa protein, also called parasporin. The latter shows strong cytocidal activity against human leukemic T cells (MOLT-4) and human uterus cervix cancer cells (HeLa) (Mizuki *et al.*, 2000). We found that the five amino acid sequence blocks in the Cry31Aa2 protein were identical to those of the parasporin except for the substitution of a single lysine residue. This series of finding strongly suggests that the Cry31Aa2 protein may also exhibit a cytocidal activity against specific human cancer cells.

The *cry31Aa2* gene was expressed in an acrystalliferous *Bacillus thuringiensis* strain. The transformant produces well separated spore and crystal. This crystal is nearly perfectly hexagonal in shape. The crystal protein isolated from the transformant is composed of a single major polypeptide of 83-kDa. It is likely that the 79-kDa protein detected in the wild type strain M15 is a degradation product of the 83-kDa protein.

In the course of cloning the *cry31Aa2* crystal protein gene from *B. thuringiensis* M15, we have isolated two new insertion sequences, IS*231*M and -N. The insertion sequences in *B. thuringiensis* are believed to play a role in the regulation of the expression of the crystal protein gene. IS*231* is one of the most widely distributed transposable elements in *Bacillus thuringiensis*. IS*231*M has two overlapping open reading frames, ORF1 and ORF2, that could code for polypeptides of 334 and 143 amino acids, respectively. The IS*231*M ORF1-ORF2 putative fusion protein showed 83, 71, and 50% amino acid identities to the IS231F and IS*231*A transposases and to the IS231W fusion protein, respectively.

Nucleotide sequence analysis revealed that IS*231*N, was not flanked by the two direct repeats (DRs) found in other iso-IS*231* elements. Moreover, unlike the known iso-IS*231* transposable elements, IS*231*N was found to contain three open reading

frames (ORFs) that could code for polypeptides of 329 (ORF1), 118 (ORF2) and 17 (ORF3) amino acids, respectively. Nucleotide sequence analysis also revealed that although IS*231*N shared 99% nucleotide identity with IS*231*M, IS*231*N lacked the 5th conserved amino acid sequence block due to the addition of an adenine residue at nucleotide 1319. A phylogenetic tree showing the evolutionary relationship between members of the IS*231* family revealed that IS*231*N is more closely related to IS*231*M (83%) and is more distant to IS*231*V and W(46%).

We are following up this work by testing the cytocidal activity of the activated Cry31Aa2 protein against several human cancer cells. Moreover, using IS*231*M as a probe, we are planning to analyse the copy number, distribution, expression and role of the IS*231* elements in *B. thuringiensis* strain M15.

Tens of thousands of strains of *B. thuringiensis* strains are kept in collections around the world. These have been classified in more than 67 H-serotypes. Newer *B. thuringiensis* strains expressing novel features, novel crystal protein, and novel insertion sequences, remain to be discovered as exemplified here with *B. thuringiensis* strain M15. Some of these novel strains may express novel insecticidal activities while others may express novel pesticidal activities. Yet even greater benefit for mankind may lie in unusual strains expressing unusual biological activities.

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