COEXPRESSION OF CHITINASE AND THE *cry11Aa1* TOXIN GENES IN *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS*

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The aim of this study is to coexpress chitinase with the *cry11Aa1* toxin genes in order to improve its larvicidal activity. Various transcriptional fusion plasmids harbouring *p19* gene, *cry11Aa1* gene from *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*) and chitinase from *B. licheniformis* TP-1 were constructed using the PCR amplification technique and gene cloning by restriction enzyme digestion. All fusion genes were arranged in operon under control of *p19* spore promoter. Plasmid p19CHI and p1968CHI contained *p19* promoter with its *p19* protein encoding gene of *p19-cry11Aa1* operon and promoterless chitinase gene, respectively. While, plasmid p19CHI68 carried promoterless chitinase gene in the middle of *p19-cry11Aa1* operon.

The chitinase and *cry11Aa1* genes in transcriptionally fused constructs were coexpressed at the spore stage in *B.t.i.* 4Q2-72 and c4Q2-72 hosts. Cry11Aa1 toxin (68 kDa and 30 kDa) was synthesized at high level only in *B.t.i.* 4Q2-72 (p16-1968CHI) and its non-transformed strain. All transformed *B.t.i.* strains [*B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) and *B.t.i.* c4Q2-72 (p16-19CHI)] constitutively produced three forms of TP-1 chitinase (Chi68, Chi62 and Chi50) and also constitutively produced two forms of *B.t.i.* chitinase (40 and 36 kDa). Whereas, production of major forms of residental chitinase from *B.t.i.* was depended on colloidal chitin induction. The recombinant enzyme initially synthesized at low level at about 9 h of growth when a portion of the cells started to sporulate. Their chitinase activity increased thereafter and reached maximum levels to 5.5, 4.9 and 4.7 mU/ml at 48 h, for *B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) and *B.t.i.* c4Q2-72 (p16-19CHI), respectively. The crude chitinase alone (TP-1 chitinase) from *B.t.i.* c4Q2-72 (p16-19CHI) at 4.5 mU/ml caused 40% mortality in second instar *Aedes aegypti* larvae. Co-application of concentrated chitinase (to get final activity of 2.5mU/ml in each assayed cup) from the recombinant strain (c4Q2-72 harbouring p1619CHI) with cell suspensions of *B.t.i.* 4Q2-72 and its transformants could enhance 3-50-fold larvicidal activity. The LD_{50} for combination of chitinase with *B.t.i.* 4Q2-72, *B.t.i.* 4Q2-72 (p16-19CHI) and *B.t.i.* 4Q2-72 (p16-1968CHI) were $2.75 \times 10 \pm 0.93 \times 10$ cells, $1.78 \times 10^4 \pm 0.63 \times 10^4$ cells, and $1.58 \times 10^2 \pm 0.94 \times 10^2$ cells, respectively. However, the toxicity of the transformed strains containing chitinase gene alone (p16-19CHI) was lower than wild type, *B.t.i.* 4Q2-72 host. This lower toxicity correlated with poor sporulation in the transformants. Exceptionally, *B.t.i.* 4Q2-72 transformants expressing both the chitinase and the *cry11Aa1* toxin genes (p16-1968CHI) were only 4-fold less toxic ($LD_{50} = 5.6 \times 10^3 \pm 1.99 \times 10^3$) than the untransformed host even though their spore count was 300 times lower.

It is suggested that the higher larvicidal activity of *B.t.i.* 4Q2-72 (p16-1968CHI) should result from high gene dosage of *cry11Aa1* encoded on the plasmid. So, the improvement in sporulation ability of these genetically engineered strains should be further developed in order to increase their larvicidal activity for effective use in future insect control.

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จุดประสงค์ของการศึกษานี้คือ ทำให้ยีนไคติเนสแสดงออกร่วมกันกับยีนสารพิษ *cry11Aa1* เพื่อพัฒนาฤทธิ์ในการฆ่าลูกน้ำ โดยการสร้างพลาสมิดแบบ transcription fusion 3 ชนิด ซึ่งมีส่วนประกอบของยีน *p19* และ *cry11Aa1* จาก *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*) และยีนไคติเนสจาก *B. licheniformis* TP-1 โดยใช้เทคนิคพีซีอาร์(PCR) และการโคลนยีน โดยการ ตัดต่อด้วยเอนไซม์ตัดจำเพาะ ยีนfusion ทั้งหมดมีการเรียงตัวในลักษณะ operon ซึ่งอยู่ภายใต้การควบคุมของ *p19* spore promoter พลาสมิด p19CHI และ p1968CHI ประกอบด้วย *p19* promoter และยีนสำหรับโปรตีน *p19* ของ *p19-cry11Aa1* operon และ ยีนไคติเนสตามลำดับ ขณะที่พลาสมิด p19CHI68 มียีนไคติเนสอยู่ตรงกลางระหว่าง *p19-cry11Aa1* operon

พลาสมิดแบบ transcription fusion มีการแสดงออกของยีนไคติเนสและ/หรือ *cry11Aa1* ร่วมกันในระยะสปอร์ใน *B.t.i.*4Q2-72 และ c4Q2-72 ในการสร้างสารพิษ Cry11Aa1 (68 kDa and 30 kDa) มีการสร้างในปริมาณมากใน *B.t.i.* 4Q2-72 (p16-1968CHI) และ *B.t.i.* 4Q2-72 เท่านั้น *B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) และ *B.t.i.* c4Q2-72 (p16-19CHI) สร้างไคติเนสจากยีน TP-1 แบบ constitutive ทั้งหมด 3 ขนาด (Chi68, Chi62 และ Chi50) และ สร้างไคติเนสจากยีนของ *B.t.i.* เองแบบ constitutive อีก 2 ขนาด (40 and 36 kDa) *B.t.i.* ทรานสฟอร์มแมนท์สร้างรีคอมบิแนนท์ไคติเนสแบบ constitutive ขณะที่การสร้าง major forms ของไคติเนสจาก *B.t.i.* host ต้องเหนี่ยวนำด้วย colloidal chitin รีคอมบิแนนท์เอมไซม์เริ่มต้นสร้างใน ระดับต่ำๆ ที่เวลา 9 ชั่วโมงของการเจริญเติบโต ซึ่งเซลล์บาง ส่วนเริ่มที่จะสร้างสปอร์ หลังจากนั้น activity จะเพิ่มขึ้นจนถึงระดับสูงสุดคือ 5.5, 4.9 และ 4.7 mU/ml ที่ 48 ชั่งโมง สำหรับ *B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) และ *B.t.i.* c4Q2-72 (p16-19CHI) ตามลำดับ ปริมาณไคติเนสจากยีน TP-1 ที่4.5 mU/ml ที่ได้มาจาก *B.t.i.* c4Q2-72 (p16-19CHI) สามารถฆ่าลูกน้ำ ยุง *Aedes aegypti* ระยะที่ 2 ได้ถึง 40% การใช้ไคติเนสเข้มข้นที่ได้จากสายพันธ์รีคอมบิแนนท์(c4Q2-72 ที่มีพลาสมิด p16-19CHI) ให้มี activity สุดท้าย 2.5 mU/ml ในแต่ละassayed cup ร่วมกับเซลล์ของ *B.t.i.* 4Q2-72 หรือ ทรานสฟอร์มแมนท์สามารถเพิ่มฤทธิ์การฆ่าลูกน้ำได้ 3 ถึง 50 เท่า ค่า LD_{50} ของการใช้ไคติเนสร่วมกับ *B.t.i.* 4Q2-72, *B.t.i.* 4Q2-72 (p16-19CHI) และ *B.t.i.* 4Q2-72 (p16-1968CHI) เท่า กับ $2.75 \times 10 \pm 0.93 \times 10$ เซลล์, $1.78 \times 10^4 \pm 0.63 \times 10^4$ เซลล์ และ $1.58 \times 10^2 \pm 0.94 \times 10^2$ เซลล์ตามลำดับ อย่างไรก็ตามประสิทธิภาพการฆ่าลูกน้ำของสายพันธ์ทรานสฟอร์มที่มียีนไคติเนส อย่าง เดียว (p16-19CHI) ยังต่ำกว่าประสิทธิภาพการฆ่าลูกน้ำของ *B.t.i.* 4Q2-72 ซึ่งฤทธิ์การฆ่าลูกน้ำที่ต่ำกว่านี้สัมพันธ์กับความสามารถในการสร้างสปอร์ของทรานสฟอร์มแมนท์ ยกเว้นเฉพาะ *B.t.i.* ทรานสฟอร์มแมนท์ที่มีการแสดงออก ทั้งยีนไคติเนสและยีนสารพิษ *cry11Aa1* (p16-1968CHI) พบว่ามีประสิทธิภาพในการฆ่าลูกน้ำต่ำกว่าเซลล์เจ้าบ้านที่ไม่ถูกทรานสฟอร์ม ($LD_{50} = 5.6 \times 10^3 \pm 1.99 \times 10^3$) เพียง 4 เท่า เท่านั้นถึงแม้ปริมาณสปอร์จะต่ำกว่าถึง 300 เท่า

ซึ่งคาดว่าฤทธิ์ในการฆ่า ลูกน้ำที่สูงขึ้นของ *B.t.i.* 4Q2-72 (p16-1968CHI) น่าจะเป็นผลมาจากปริมาณยีน (gene dosage) *cry11Aa1* บนพลาสมิดที่มีจำนวนมากกว่าทำให้สร้าง Cry11Aa1 ได้มาก ดังนั้นการปรับปรุงความสามารถในการ สร้างสปอร์ของสายพันธ์พันธุวิศวกรรมเหล่านี้น่าจะสามารถช่วยเพิ่มฤทธิ์ในการฆ่าลูกน้ำให้สูงขึ้นได้ ซึ่งจะทำให้เพิ่มประสิทธิภาพ ในการใช้ควบคุมแมลงได้ในอนาคต

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LIST OF ABBREVIATIONS

B.	*Bacillus*
B.t.	*B. thuringiensis*
B.t.i.	*B.t.* subsp. *israëlensis*
bp	Base pair
Chb	Chitobiase
CBDs	Chitin binding domains
°C	Degree celcius
Cont.	Continued
cry	Crystal protein gene
Cry	Crystal protein
Cfu	Colony forming unit
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetracetic acid
et al.	Et. Alii (latin), other things
Fig.	Figure
Fn	Fibronectin
GlcNAc	N-acetylglucosamine
h	Hour
kDa	Kilodalton
kV	Kilovolt
LB	Luria-Bertani medium

LIST OF ABBREVIATIONS (continued)

LD_{50}	50% lethal dose
min	Minute
mRNA	Messenger RNA
µF	Microfarad
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Millitre
mM	Millimolar
mU	Milliunit
OD	Optical density
Ω	Ohme
PMs	Peritrophic matrix structure
rpm	revolution per minute
subsp.	Subspecies
UV	Ultraviolet
w/v	weight by volume

CHAPTER I

INTRODUCTION

Bacillus thuringiensis subsp. *israelensis* (*B.t.i.*) has been used as an effective biological control agent for mosquito and black fly vectors of several tropical diseases (Federici *et al*, 1990; Porter *et al*, 1993). Several reasons issued for the increased interest in *B.t.i.*. For example, emergence of insect populations resistant to many chemicals has been rapid and there is a fear that resistance of mosquito larvae to the widely used *B.t.i.* or *B. sphaericus* toxins may occur. So, a new potential biopesticide based on mixture between chitinase enzyme and biopesticide has been developed recently. Chitinases are enzymes which degrade chitin to low molecular weight, soluble, and insoluble oligosaccharide. The important thing is that chitin is a structural component of peritrophic membrane of insect midgut. Thus, it is an excellent target for selective pest control strategies.

The entomopathogenic properties of *B.t.i* are attributed to its crystal toxin synthesis during sporulation. The crystals are composed of five major polypeptides with molecular masses of approximately 134, 128, 78, 72 and 27-kDa (Federici *et al*, 1990; Porter *et al*, 1993) and referred to as Cry4Aa1, Cry4Ba1, Cry10Aa1, Cry11Aa1 and Cyt1Aa1, respectively (revised nomenclature, Crickmore *et al*, 1998). Data from amino acid sequence alignments among a number of toxins revealed five well-conserved blocks (block1-5) in the toxic core and three additional blocks lying in the carboxyl-terminal halves. The high insecticidal activity is due to the synergistic interaction of all crystal components (Crickmore *et al*, 1995; Ben-dov *et al*, 1995). The genes encoding these

proteins are located on a large plasmid of 125-kb (Ben-Dov *et al*, 1999). Most of the cloned *Bacillus thuringiensis* (*B.t.*) toxin genes are monocistronic, but the *cry2A*, *cry2C* and *cry11Aa1* genes are organized in separate operons (Brown, 1993; Widner and Whiteley, 1989; Wu *et al*, 1991). The *cry11Aa1* gene is located midway between the *p19* and *p20* genes. P19, P20 and Orf2 are proposed to act as chaperone proteins to initiate, facilitate, or stabilize crystal formation (Dervyn *et al*, 1995). P19 has been suggested to play a role in protein-protein interaction and may be necessary either to confer a particular lattice structure or to allow the assembly of Cry11Aa1 in the crystalline inclusion (Dervyn *et al*, 1995; Poncet *et al*, 1993).

The action of chitinase is primarily exerted on the peritrophic membrane of the larval midgut, which consists of a network of chitin embedded in a protein-carbohydrate matrix (Smirnoff, 1973). An addition, chitinase has been found to increase the efficacy and potency of *B.t.* Cry toxins in insect control. Traces of the enzyme chitinase in *B.t.* preparations administered to spruce budworm larvae accelerated development of septicemia and increased the mortality rate (Smirnoff, 1973). The co-application of commercial chitinase preparations (Smirnoff, 1974) or of chitinolytic bacteria extracted from insect guts (Sneh *et al*, 1983) with *B.t.i.* toxin enhances the insecticidal activity of *B.t.i.*. A combination of chitinase of *B. licheniformis* TP-1 and *B.t.* subsp. *aizawai* at a sublethal dose retarded growth and development of *Spodoptera exigua* (Tantimavanich *et al*, 1997). Enhanced toxicity towards *S. littoralis* also resulted when a combination of low concentrations of a truncated recombinant CryIC toxin and a bacterial endochitinase ChiAII were incorporated into a semisynthetic insect diet (Regev *et al*, 1996). The results suggested that chitinolytic enzymes could perforate the peritrophic membranes in the larval insect gut and increase accessibility of *B.t.* toxin molecules to the epithelial

membrane (Regev *et al*, 1996).

Chitinase in various bacteria has been induced by chemicals such as chitin (Monreal and Reese, 1969). In *B. licheniformis* TP-1, the enzyme was not produced if there was no colloidal chitin in culture media but the cloned TP-1 chitinase gene cloned from it was constitutively expressed in *E. coli* and *B.t.* subsp. *aizawai* (Tantimavanich *et al*, 1997).

In the present work, we investigated the products of *B.t.i.* harbouring trancriptional fusions of the chitinase gene from *B. licheniformis* TP-1 together with the regulatory protein *p19* alone or with *p19* and the toxin gene *cry11Aa1*. *B. licheniformis* TP-1 produces multiple chitinases (Chi68, Chi62 and Chi50) from a single gene (Tantimavanich *et al*,1998). Our study of the transformants included work on the kinetics of enzyme production at various stages of cell growth and on the larvicidal effect against *Aedes aegypti* larvae. The objective was to determine whether co-production of chitinase and toxin at the spore-stage would increase the efficacy of *B.t.i.* strains against mosquito larvae.

CHAPTER II

LITERATURE REVIEW

1. The classification and nomenclature of *Bacillus thuringiensis*

Classification of *B. thuringiensis* (*B.t.*) strains according to H-serotypes is still an efficient way of classification of *B.t.* strains on the basic of stable and specific characters. The differentiation of these strains into serovarieties was developed by de Barjac and Bonnefoi since 1962 and has still been used ever since (de Barjac and Frachon, 1990). The numbers of new serovars relative to the total number of isolates during past 10 years was rapidly increased. New H-serotypes are numbered and registered at the International Entomopathogenic Bacillus Center (IEBC) Collection (Burges *et al*, 1982) at Pasteur Institute, Paris, France. By the end of 1998, about 3500 strains of *B.t.* were isolated and 69 serotypes (or serovars), 13 sub-antigenic groups and 82 serovars were recorded and named. The present state of this classification was shown in Table 1 (Lecadet *et al*, 1999).

In 1989, Hofte and Whiteley proposed a classification scheme for *cry* genes into four classes and designed a systematic nomenclature for them (CryI-CryIV), which based on both the amino acid sequence similarity and insecticidal spectra of the encoded proteins. The four classes were those specific for Lepidoptera (CryI), Lepidoptera and Diptera (CryII), Coleoptera (CryIII) and Diptera (CryIV). Recently, CryV which is toxic to Lepidoptera and Coleoptera (Tailor *et al*, 1992; Shin *et al*, 1995) and CryVI which is toxic

Table 1. Classification of *Bacillus thuringiensis* strains according to the H serotype (From Lecadet *et al*, 1999).

H-antigen	Serovar	Abbreviation	First mention and/or first valid description
1	*thuringiensis*	THU	Berliner 1915; Heimpel and Angus 1958
2	*finitimus*	FIN	Heimpel and Angus 1958
3a, 3c	*alesti*	ALE	Toumanoff and Vago 1951; Heimpel and Angus 1958
3a, 3b, 3c	*kurstaki*	KUR	De Barjac and Lemille 1970
3a, 3d	*sumiyoshiensis*	SUM	Ohba and Aizawa 1989
3a, 3d, 3e	*fukuokaensis*	FUK	Ohba and Aizawa 1989
4a, 4b	*sotto*	SOT	Ishiwata 1905; Heimpel and Angus 1958
4a, 4c	*kenyae*	KEN	Bonnefoi and de Barjac 1963
5a, 5b	*galleriae*	GAL	Shvetsova 1959; de Barjac and Bonnefoi 1962
5a, 5c	*canadensis*	CAN	De Barjac and Bonnefoi 1972
6	*entomocidus*	ENT	Heimpel and Angus 1958
7	*aizawai*	AIZ	Bonnefoi and de Barjac 1963
8a, 8b	*morrisoni*	MOR	Bonnefoi and de Barjac 1963
8a, 8c	*ostriniae*	OST	Ren *et al.* 1975
8b, 8d	*nigeriensis*	NIG	Weiser and Prasertphon 1984
9	*tolworthi*	TOL	Norris 1964; de Barjac and Bonnefoi 1968
10a, 10b	*darmstadiensis*	DAR	Krieg de Barjac and Bonnefoi 1968
10a, 10c	*londrina*	LON	Arantes *et al.* (unpublished)
11a, 11b	*toumanoffi*	TOU	Krieg 1969
11a, 11c	*kyushuensis*	KYU	Ohba and Aizawa 1979
12	*thompsoni*	THO	De Barjac and Thompson 1970
13	*pakistani*	PAK	De Barjac, Cosmao Dumanoir, Shaik and Viviani 1977
14	*israelensis*	ISR	
15	*dakota*	DAK	De Barjac 1978
16	*indiana*	IND	De Lucca, Simonson and Larson 1979
17	*tohokuensis*	TOH	DeLucca, Simonson and Larson 1979
18a, 18b	*kumamotoensis*	KUM	Ohba, Aizawai and Shimizu 1981
18a, 18c	*yosoo*	YOS	Ohba, Ono, Aizawai and Iwanami 1981
19	*tochigiensis*	TOC	Lee, H.H. *et al.* 1995
20a, 20b	*yunnanensis*	YUN	Ohba, Ono, Aizawai and Iwanami 1981
20a, 20c	*pondicheriensis*	PON	Wan-Yu, Qi-Fang, Xue-Ping and You-Wei 1979
21	*colmeri*	COL	Rajagopalan *et al.* (unpublished)
22	*shandongiensis*	SHA	De Lucca, Palmgren and de Barjac 1984
23	*japonensis*	JAP	Wang Ying *et al.* 1986
24a, 24b	*neoleonensis*	NEO	Ohba and Aizawai 1986
24a, 24c	*novosibirsk*	NOV	Rodriguez-Padilla *et al.* 1988
25	*coreanensis*	COR	Burtseva, Kalmikova et al. 1995
26	*silo*	SIL	Lee, H.H. *et al.* 1994
27	*mexicanensis*	MEX	De Barjac and Lecadet (unpublished)
28a, 28b	*monterrey*	MON	Rodriguez-Padilla and Galan-Wong (unpublished)
28a, 28c	*jegathesan*	JEG	Rodriguez-Padilla *et al.* (unpublished)
29	*amagiensis*	AMA	Seleena, Lee, H. L. and Lecadet 1995
30	*medellin*	MED	Ohba (unpublished)
31	*toguchini*	TOG	Orduz, Rojas, Correa, Montoya and de Barjac 1992
32	*cameroun*	CAM	Hodirev (unpublished)
33	*leesis*	LEE	Jacquemard, 1990; Juarez-Perez *et al.* 1994
34	*konkukian*	KON	Lee H.H. *et al.* 1994
35	*seoulensis*	SEO	Lee H.H. *et al.* 1994
36	*malaysiensis*	MAL	Lee H.H. *et al.* 1995
37	*andaluciensis*	AND	Ho (unpublished)
38	*oswaldocruzi*	OSW	Aldebis, Vargas-Osuna and Santiago-Alvarez 1996
39	*erasiliensis*	BRA	Rabinovitch et al. 1995
40	*huazhongensis*	HUA	Rabinovitch et al. 1995 Dai jingvuan et al. 1996

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Table 1. Classification of *Bacillus thuringiensis* strains according to the H serotype (continued).

H-antigen	Serovar	Abbreviation	First mention and/or first valid description
41	*sooncheon*	SOO	Lee H.H. *et al.* 1995
42	*jinghongiensis*	JIN	Li Rong Sen *et al.* (in press)
43	*guiyangiensis*	GUI	Li Rong Sen *et al.* (in press)
44	*higo*	HIG	Ohba *et al.* 1995
45	*roskildiensis*	ROS	Hinrinschen, Hansen and Daamgaard (unpublished)
46	*chanpaisis*	CHA	Chanpaisaeng (unpublished)
47	*wratislaviensis*	WRA	Lonc et al. 1997
48	*balearica*	BAL	Caballero *et al.* (unpublished)
49	*muju*	MUJ	Seung Hwan Park et al. (unpublished)
50	*navarrensis*	NAV	Caballero *et al.* (unpublished)
51	*xiaguangiensis*	XIA	Jian Ping Yan (unpublished)
52	*kim*	KIM	Kim *et al.* (unpublished)
53	*asturiensis*	AST	Aldebis, Vargas-Osuna and Santiago-Alvarez 1996
54	*poloniensis*	POL	Damgaard *et al.* (unpublished)
55	*palmanyolensis*	PAL	Santiago-Alvarez *et al.* (unpublished)
56	*rongseni*	RON	Li Rong Sen (in press)
57	*pirenaica*	PIR	Caballero *et al.* (unpublished)
58	*argentinensis*	ARG	Campos-Dias et al. (unpublished)
59	*iberica*	IBE	Caballero *et al.* (unpublished)
60	*pingluonsis*	PIN	Li Rong Sen *et al.* (in press)
61	*sylvestriensis*	SYL	Damgaard (unpublished)
62	*zhaodongensis*	ZHA	Li Rong Sen (in press)
63	*bolivia*	BOL	Ferre-Manzanero et al. (unpublished)
64	*azorensis*	AZO	Santiago-Alvarez et al. (unpublished)
65	*pulsiensis*	PUL	Khalique F. and Khalique A. (unpublished)
66	*graciosensis*	GRA	Santiago-Alvarez et al. (unpublished)
67	*vazensis*	VAZ	Santiago-Alvarez et al. (unpublished)
68	*thailandensis*	THA	Chanpaisaeng et al. (unpublished)
69	*pahangi*	PAH	Seleena and Lee H. L. (unpublished)

to nematode had been found.

Due to the increasingly rapid characterization of new crystal proteins with new pesticidal properties, it resulted in a variety of sequences and activities that no longer fit the original nomenclature system. A new nomenclature, based on the hierarchical clustering using amino acid sequence identity is proposed (Crickmore *et al*, 1998). This revised nomenclature emerged the system of internationally recognized cytochrome P-450 superfamily (Nelson *et al*, 1996; White *et al*, 1998). The names of gene superfamilies are designated according to their degree of evolutionary divergence as estimated by phylogenetic tree algorithms (Fig. 1). The nomenclature format in such a system is designed to convey rich informational content about these relationships by appending to the mnemonic root a series of numerals and letters assigned in a hierarchical fashion to indicate degrees of phylogenetic divergence. This change from a function-based to a sequence-based nomenclature allows closely related toxins to be ranked together and removed the necessity for researchers to bioassay each new protein against a growing series of organisms before assigning it a name (Crickmore *et al*, 1998). Each toxin is assigned a unique name incorporating all four ranks which reflected significant evolutionary relationships. The minimum change from the old name system was also concerned. Most *cry* genes retained the old names of Hofte and Whiteley system by a substitution of Arabic for Roman numerals, upper and lower case letters, and another Arabic numeral, eg. Cry1Aa1. There are a few notable exceptions: CryIG becomes Cry9A, CryIIIC becomes Cry7Aa, CryIIID becomes Cry3C, CryIVC becomes Cry10A, CryIVD becomes Cry11A, CytA becomes Cyt1A, and CytB becomes Cyt2A. Under the revised system, the known Cry and Cyt proteins fall into 24 sets at the primary rank_Cyt1, Cyt2, and Cry1 through Cry22.

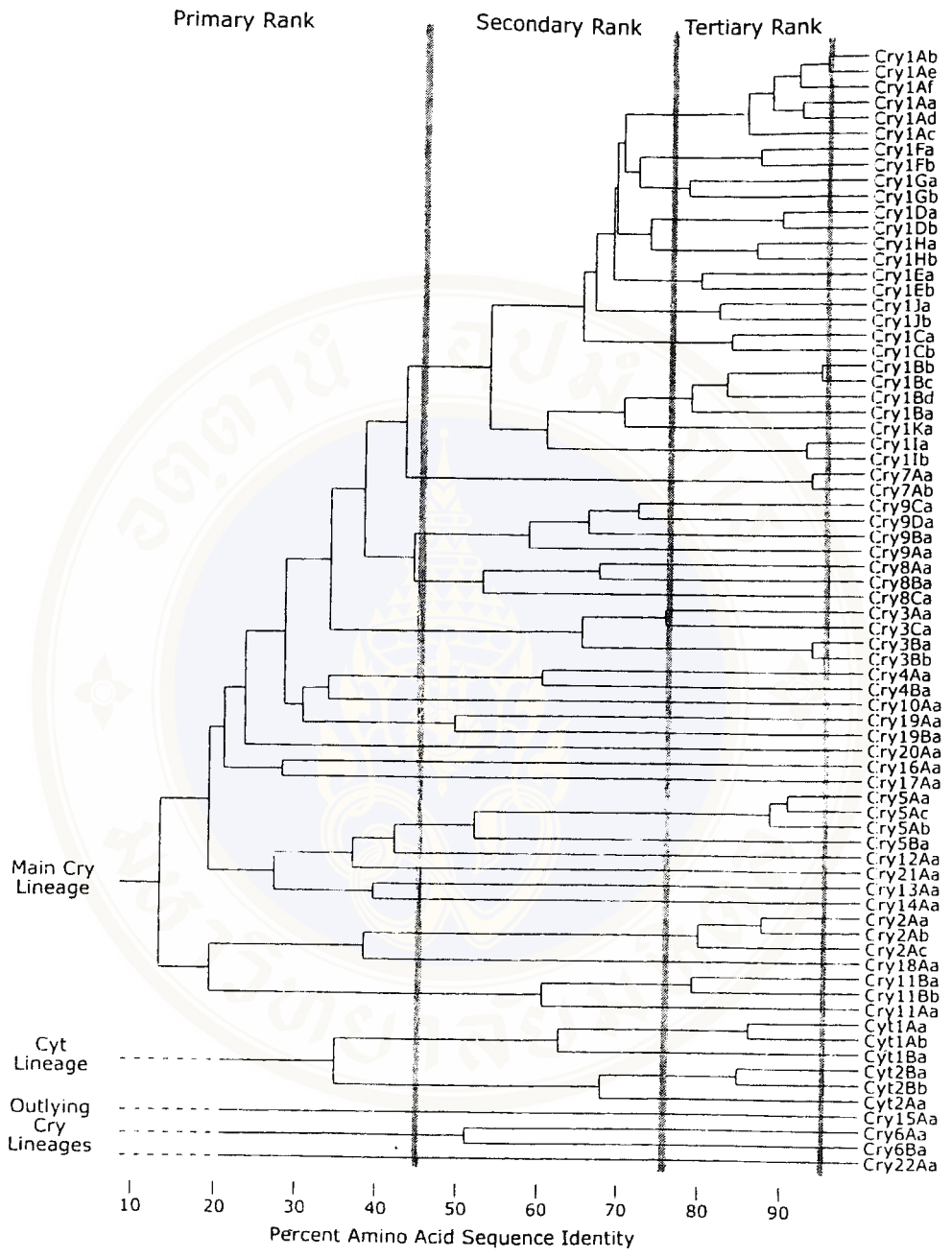

Fig. 1. Phylogram demonstrating amino acid sequence identity amino acid sequence identity among Cry and Cyt proteins. This phylogenetic tree is modified from a TREEVIEW visualization of NEIGHBER treatment of a CLUSTAL W multiple alignment and distance matrix of the full-length toxin sequences, as described in the text. The black vertical bars demarcate the four levels of nomenclature ranks Based on the low percentage of identical residues and the absence of any conserved sequence blocks in multiple-sequence alignments, the lower four lineages are not treated as part of the main toxin family, and their nodes have been replaced with dashed horizontal lines in this figure. (From Crickmore *et al*; 1998).

The revised nomenclature assignment is shown in Table 2. All protein sharing the first numeral in their names share at least 45% amino acid identity with other members of the group. Proteins sharing both the first numeral and the upper case letter share at least 75 % identity. Proteins sharing the same first numeral uppercase, and lower case letters share at least 95% identity (Crickmore *et al*, 1998).

Although, the old classification of Hofte and Whiteley could predict the insecticidal specificity against certain group of insect, some problems occur for this classification scheme, especially genes that were homologous to known genes but displayed different specificity and genes that had dual specificity. Moreover, it is difficult for a newly discovered Cry protein to predict which organisms might be susceptible to which Cry toxins. However, a revised nomenclature make a useful first approximation that proteins sharing a primary rank (CryI proteins, for example) are toxic to the same orders of insects or other invertebrates. Proteins sharing the same secondary rank (CryIA proteins, for example) are generally toxic to the same families. Finally, proteins sharing the same tertiary rank (such as the CryIAa proteins) typically are toxic to the same species. The Cry toxin specificity and the susceptible insects are shown in Table 3 (Zeigler, 1999).

2. Structure of *B. thuringiensis* toxin proteins and genes

The insecticidal crystal proteins are encoded by genes usually found on large plasmid (Ben-Dov *et al*, 1996; 1999). Most of the plasmids are of low copy number. Many of the plasmid encoded toxin genes are bordered by transposons and/or insertion sequences. Hofte and Whiteley (1989) had aligned the amino acid sequence among a number of toxins and found five well-conserved block (block1-5). Recently, comparison of the carboxyl-terminal halves of sequences with more than 1,000 residues suggested the

Table 2. Known *cry* and *cyt* gene sequences with revised nomenclature assignments. (From Crickmore *et al*; 1998).

Revised gene name	Original gene or protein name	Accession no.	Coding region[a]	Revised gene name	Original gene or protein name	Accession no.	2125-3990>
cry1Aa1	*cryIA (a)*	M11250	527-4054	*cry2Ab2*	*cryIIB*	X55416	874-2775
cry1Aa2	*cryIA (a)*	M10917	153->2955	*cry2Ac1*	*cryIIC*	X57252	2125-3990
cry1Aa3	*cryIA (a)*	D00348	73-3600	*cry3Aa1*	*cryIIIA*	M22472	25-1956
cry1Aa4	*cryIA (a)*	X13535	1-3528	*cry3Aa2*	*cryIIIA*	J02978	241-2172
cry1Aa5	*cryIA (a)*	D17518	81-3608	*cry3aa3*	*cryIIIA*	Y00420	566-2497
cry1Aa6	*cryIA (a)*	U43605	1->1860	*cry3Aa4*	*cryIIIA*	M30503	201-2132
cry1Ab1	*cryIA (b)*	M13898	142-3606	*cry3Aa5*	*cryIIIA*	M37207	569-2500
cry1Ab2	*cryIA (b)*	M12661	155-3622	*cry3Aa6*	*cryIIIA*	U10985	569-2500
cry1Ab3	*cryIA (b)*	M15271	156-3620	*cry3Ba1*	*cryIIIB2*	X17123	25->1977
cry1Ab4	*cryIA (b)*	D00117	163-3627	*cry3Ba2*	*cryIIIB*	A07234	342-2297
cry1Ab5	*cryIA (b)*	X04698	141-3605	*cry3Bb1*	*cryIIIBb*	M89794	202-2157
cry1Ab6	*cryIA (b)*	M37263	73-3537	*cryBb2*	*cryIIIC(b)*	U31633	144-2099
cry1Ab7	*cryIA (b)*	X13233	1-3465	*cry3Ca1*	*cryIIID*	X59797	232-2178
cry1Ab8	*cryIA (b)*	M16463	157-3621	*cry4Aa1*	*cryIVA*	Y00423	1-3540
cry1Ab9	*cryIA (b)*	X54939	73-3537	*cry4Aa2*	*cryIVA*	D00248	393-3935
cry1Ab10	*cryIA (b)*	A29125	*_b*	*cry4Ba1*	*cryIVB*	X07423	157-3564
cry1Ac1	*cryIA (c)*	M11068	388-3921	*cry4Ba2*	*cryIVB*	X07082	151-3558
cry1Ac2	*cryIA (c)*	M35524	239-3769	*cry4Ba3*	*cryIVB*	M20242	526-3930
cry1Ac3	*cryIA (c)*	X54159	339->2192	*cry4Ba4*	*cryIVB*	D00247	461-3865
cry1Ac4	*cryIA (c)*	M73249	1-3534	*cry5Aa1*	*cryVA(a)*	L07025	1->4155
cry1Ac5	*cryIA (c)*	M73248	1-3531	*cry5Ab1*	*cryVA(b)*	L07026	1->3867
cry1Ac6	*cryIA (c)*	U43606	1->1821	*cry5Ac1*		I34543	1->3660
cry1Ac7	*cryIA (c)*	U87793	976-4509	*cry5Ba1*	*PS86Q3*	U19725	1->3735
cry1Ac8	*cryIA (c)*	U87397	153-3686	*cry6Aa1*	*cryVIA*	L07022	1->1425
cry1Ac9	*cryIA (c)*	U89872	388-3921	*cry6Ba1*	*cryVIB*	L07024	1->1185
cry1Ac10		Aj002514	388-3921	*cry7Aa1*	*cryIIIC*	M64478	184-3597
cry1Ad1	*cryIA (c)*	M73250	1-3537	*cry7Ab1*	*cryIIIC(b)*	U04367	1->3414
cry1Ae1	*cryIA (e)*	M65252	81-3623	*cry7Ab2*	*cry3C(c)*	U04368	1->3414
cry1Af1	*icp*	U82003	172->2905	*cry8Aa1*	*cryIIIE*	U04364	1->3471
cry1Ba1	*cryIB*	X06711	1-3684	*cry8Ba1*	*cryIIIG*	U04365	1->3507
cry1Ba2		X95704	186-3869	*cry8Ca1*	*cryIIIF*	U04366	1-3447
cry1Bb1	*ET5*	L32020	67-3753	*cry9Aa1*	*cryIG*	X58120	5807-9274
cry1Bc1	*cryIB(c)*	Z46442	141-3839	*cry9Aa2*	*cryIG*	X58534	385->3837
cry1Bd1	*cryE1*	U70726		*cry9Ba1*	*cryX*	X75019	26-3488
cry1Ca1	*cryIC*	X07518	47-3613	*cry9Ca1*	*cryIH*	Z37527	2096-5569
cry1Ca2	*cryIC*	X13620	241->2711	*cry9Da1*	*N141*	D85560	47-3553
cry1Ca3	*cryIC*	M73251	1-3570	*cry9Da2*		AF042733	<1->1937
cry1Ca4	*cryIC*	A27642	234-3800	*cry10aa1*	*cryIVC*	M12662	941-2965
cry1Ca5	*cryIC*	X96682	1->2268	*cry11Aa1*	*cryIVD*	M31737	41-1969
cry1Ca6	*cryIC*	X96683	1->2268	*cry11Aa2*	*cryIVD*	M22860	<1-235
cry1Ca7	*cryIC*	X96684	1->2268	*cry11Ba1*	*jeg80*	X86902	64-2238
cry1Cb1	*cryIC (b)*	M97880	296-3823	*cry11Bb1*	*94 kDa*	AF017416	
cry1Da1	*cryID*	X54160	264-3758	*cry12Aa1*	*cryVB*	L07027	1->3771
cry1Db1	*prtB*	Z22511	241-3720	*cry13Aa1*	*cryVC*	L07023	1-2409
cry1Ea1	*cryIE*	X53985	130-3642	*cry14Aa1*	*cryVD*	U13955	1-3558
cry1Ea2	*cry1E*	X56144	1-3513	*cry15Aa1*	*34kDa*	M76442	1036-2055
cry1Ea3	*cryIE*	M73252	1-3513	*cry16Aa1*	*cbm-1*	M94146	158-1996
cry1Ea4		U94323	388-3900	*cry17Aa1*	*cbm-2*	M99478	12-1865
cry1Eb1	*cryIE (b)*	M73253	1-3522	*cry18Aa1*	*cryBP1*	M99049	743-2860
cry1Fa1	*cryIF*	M63897	478-3999	*cry19Aa1*	*jeg65*	Y07603	719-2662
cry1Fa2	*cryIF*	M73254	1-3525	*cry19Ba1*		D88381	
cry1Fb1	*prtD*	Z22512	483-4004	*cry20Aa1*	*86kDa*	U82518	60-2318
cry1Ga1	*prtA*	Z22510	67-3564	*cry21Aa1*		I32932	1-3501
cry1Ga2	*cryIM*	Y09326	692-4210	*cry22Aa1*		I34547	1-2169
cry1Gb1	*cryH2*	U70725					
cry1Ha1	*prtC*	Z22513	530-4045	*cyt1Aa1*	*cytA*	X03182	140-886
cry1Hb1		U35780	728-4195	*cyt1Aa2*	*cytA*	X04338	509-1255
cry1Ia1	*cryV*	X62821	355-2511	*cyt1Aa3*	*cytA*	Y00135	36-782
cry1Ia2	*cryV*	M98544	1-2157	*cyt1Aa4*	*cytA*	M35968	67-813
cry1Ia3	*cryV*	L36338	279-2435	*cyt1ab1*	*cytM*	X98793	28-777
cry1Ia4	*cryV*	L49391	61-2217	*cyt1Ba1*		U37196	1-795
cry1Ia5	*cryV159*	Y08920	524-2680	*cyt2Aa1*	*cytB*	Z14147	270-1046
cry1Ib1	*cryV465*	U07642	237-2393	*cyt2Ba1*	*"cytB"*	U52043	287-1204
cry1Ja1	*ETS*	L32019	99-3519	*cyt2Bb1*		U82519	416-1204
cry1Jb1	*ET1*	U31527	177-3686				
cry1Ka1		U28801	451-4098				
cry2Aa1	*CryIIA*	M31738	156-2054				
cry2Aa2	*cryIIA*	M23723	1840-3738				
cry2Aa3		D86064	2007-3911				
cry2Ab1	*cryIIB*	M23724	1-1899				

[a]The symbol< and > indicate that the coding region extends up- or downstream, respectively, from the known sequence data.

[b]Only the peptide sequence has been reported.

Table 3. Cry toxin specificities and susceptible insects (From Zeigler DR, 1999).

NAME	SOURCE STRAIN	KNOWN TOXICITY
Cry1Aa1	*B.t. kurstaki* HD-1; *B.t. aizawai* HD-68	*Heliothis virescens*, *Mamestra brassicae*, *Pseudoplusia includens* (Lepidoptera: Noctuidae; *Manduca Sexta* (Lepidoptera: Sphingidae); *Pieris Brassicae* (Lepidoptera: Pieridae); *Bombyx mori* (Lepidoptera: Bombycidae); Lepidoptera: Lymantriidae); *Sciropophaga incertulas*, *Chilo suppressalis*, *Ostrinia nubilalis* (Lepidoptera: Pyralidae); *Choristoneura fumiferana* (Lepidoptera: Tortricidae); *Hyphantria cunae* (Lepidoptera: Arctiidae); *Plutella xylostella* (Lepidoptera: Plutellidae)
Cry1Ab2	*B.t. kurstaki* HD-1	*Lymantria dispar* (Lepidoptera: Lymantridae); *Heliothis virescens*, *Trichoplusia ni* (Lepidopera: Noctuidae); *Manduca Sexta* (Lepidoptera: Sphingidae)
Cry1Ac1	*B.t. kurstaki* HD-73 *B.t. kurstaki* HD-244	*Bombyx mori* (Lepioptera: Bombycidae); *Agrotis segetum*, *Helicoverpa zea*, *Helicothis virescens*, *Mamestra brassicae*, *Trichoplusia ni*, *Spodoptera exigua* (Lepidoptera: Noctuidae); *Ephestia kuehniella*, *Sciropophaga incertulas*, *Chilo suppressalis*, *Ostrinia nubilalis* (Lepidoptera: Pyralidae); *Manduca sexta* (Lepidoptera: Sphigidae); *Lymantria dispar* (Lepidoptera: Lymantriidae); *Pieris brassicae* (Lepidoptera: Pieridae)
Cry1Ad1	*B.t. aizawai* PS81I	*Trichoplusia ni*, *Spodoptera exigua* (Lepidoptera: Noctuidae); *Choristoneura fumiferana* (Lepidoptera: Tortricidae); *Plutella xylostella* (Lepidoptera: Plutellidae)
Cry1Ae1	*B.t. alesti*	*Heliothis virescens*, *Trichoplusia ni* (Lepidoptera: Noctuidae)
Cry1Af1	*B. thuringiensis* NT0423	Reported dual activity against Diptera and Lepidoptera
Cry1Ba1	*B. thuringiensis* HD-290-1; *B. thuringiensis* HD2	*Chrysomela scripta* (Coleoptera: Chrysomelidae); *Manduca sexta* (Leidoptera: Sphigidae); *Artogeia rapae* (Lepidoptera: Pieridae)
Cry1Bb1	*B. thuringiensis* EG5847	*Spodoptera frugiperda*, *Pseudoplusia includens*, *Trichoplusia ni* (Lepidoptera: Noctuidae); *Plutella xylostella* (Lepidoptera: Plutellidae); *Lymantria dispar* (Lepidoptera: Lymantridae); *Ostrinia nubilalis* (Lepidoptera: Pyralidae)
Cry1Be1	*B. thuringiensis* 158C2	Strain of origin active against lepidopterans
Cry1Ca1	*B. t. entomocidus* 60.5 *B.t. aizawai* HD-229	*Sciropophaga incertulas*, *Chilo suppressalis* (Lepidoptera: Pyralidae); *Heliothis virescens*, *Spodoptera exigua*, *Spodoptera frugiperda*, *Trichoplusia ni* (Lepidoptera: Noctuidae); *Pieris brassicae* (Lepidoptera: Pieridae)
Cry1Cb1	*B.t. galleriae* HD-29	*Spodoptera exigua*, *Trichoplusia ni* (Lepidopera: Noctuidae)
Cry1Da1	*B.t. aizawai* HD-68	*Plutella xylostella* (Lepidoptera: Plutellidae); *Choristoneura fumiferana* (Lepidoptera: Tortricidae); *Bombyx mori* (Lepidoptea: Bombycidae); *Lymantria dispar*, *Orgyia leucostigma* (Lepidoptera: Lymantriidae); *Manduca sexta* (Lepidoptera: Sphingidae); *Malacosoma disstria* (Lepidoptera: Lasiocampidae); *Lambdina fiscellaria* (Lepidoptera: Geometridae); *Spodoptera frugiperda* (Lepidoptera: Noctuidae)
Cry1Ea1	*B.t. darmstadiensis* HD-146	*Spodoptera littoralis*, *Spodoptera exempta* (Lepidoptera: Noctuidae); *Manduca sexta* (Lepidoptera: Sphingidae)
Cry1Eb1	*B.t. aizawai*	Source strain is toxic to Trichoplusia ni, *Spodoptera exigua* (Lepidoptera: Noctuidae); *Plutella xylostella* (Lepidoptera: Plutellidae)
Cry1Fa1	*B.t. aizawai* EG6346	Plutella xylostella (Lepidoptera: Plutellidae); Heliothis virescens, Spodoptera exigua, Spodoptera littolos (Lepidoptera: Noctuidae); Ostrinia nubilalis (Lepidoptera: Pyralidae)
Cry1Ia1	*B.t. kurstaki* INA-02, 4835	*Spodoptera littoralis* (Lepidoptera: Noctuidae); *Bombyx mori* (Lepidoptera: Bombycidae); *Plutella xylostella* (Lepidoptera: Plutellidae); *Ostrinia nubilalis* (Lepidoptera: Pyralidae); *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)
Cry1Ib1	*B.t. entomocidus* BP465	*Plutella xylostella* (Lepidoptera: Plutellidae)
Cry1Ja1	*B. thuringiensis* EG5847	Helicoverpa zea, Helicothis virescens, Pseudoplusia includens, Spodoptera exigua, Spodoptera frugiperda, Trichoplusia ni (Lepidoptera: Noctuidae); Plutella xylostella (Lepidoptera: Plutellidae)
Cry1Jb1	*B. thuringiensis* EG5092	Pseudoplusia includens, Trichoplusia ni (Lepidoptera: Noctuidae); Ostrinia nubilalis (Lepidoptera: Pyralidae); Plutella xylostella (Lepidoptera: Plutellidae)

Table 3. Cry toxin specificities and susceptible insects. (Continued)

NAME	SOURCE STRAIN	KNOWN TOXICITY
Cry1Ka1	*B.t. morrisoni* BF190	*Artogeia rapae* (Lepidoptera: Pieridae)
Cry2Aa1	*B.t. kurstaki* HD-1, HD-263	*Sciropophaga incertulas*, *Chilo suppressalis*, *Ostrinia nubilalis* (Lepidoptera: Pyralidae); *Lymantria dispar* (Lepidoptera: Lymantriidae); *Helicoverpa armigera*, *Heliothis virescens*, *Trichoplusia ni* (Lepidoptera: Noctuidae); *Aedes aegypti* (Diptera: Cuclidae)
Cry2Ab1	*B.t. kurstaki* HD1	*Manduca sexta* (Lepidoptera: Sphingidae)
Cry2Ac1	*B. thuringiensis* S1	*Heliothis virescens*, *Trichoplusia ni* (Lepidoptera: Noctuidae); *Manduca sexta* (Lepidoptera: Sphingidae)
Cry3Aa1	*B.t. san diego*, B.t. tenebrionis	*Haltica tombacina*, *Leptinotarsa decemlineata*, *Pyrrhalta luteola* (Coleoptera: Chrysomelidae); *Hypera brunneipennis*, *Otiorhynchus sulcatus*, *Anthonomus grandis* (Coleoptera: Tenebrionidae)
Cry3Ba1	*B.t. tolworthi*	*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)
Cry3Bb1	EG2838	*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)
Cry3Ca1	*B.t. kumamotoensis* EG4961	*Pyrrhalta luteola* (Coleoptera: Chrysomeldae)
Cry4Aa1	*B.t. san diego*	*Anopheles stephensi*, *Aedes aegypti*, *Culex pipiens* (Diptera: Cuclidae)
Cry4Ba1	*B.t. isralensis* 4Q2-72	*Aedes aegypti* (Diptera: Cuclidae)
Cry5Aa1	*B.t. isralensis* 4Q2-72	*Caenorhabditis elegens*, *Pratylenchus* spp. (plant parasitic nematodes)
Cry5Ab1	*B. thuringiensis* PS17A	*Fasciola hepatica* (liver fluke); *Caenorhabditis elegans*, *Pratylenchus* spp. (plant parasitic nematodes)
Cry6Aa1	*B. thuringiensis* PS7	*Pratylenchus* spp. *Panagrellus redivivus* (plant pathogenic nematodes)
Cry7Aa1	*B. thuringiensis* PS52A1	*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)
Cry8Aa1	*B. thuringiensis* BTS137J	*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)
Cry8Ba1	*B.t. kumamotoensis* PS50C	*Cotinis* spp. (Coleoptera: Scarabaeidae)
Cry8Ca1	*B.t. kumamotoensis* PS50C	*Anomala cuprea* (Coleoptera: Scarabaeidae)
Cry9Aa1	*B.t. japonensis* strain Buibui	*Galleria mellonella* (Lepidoptera: Pyralidae)
Cry9Ca1	*B.t. galleriae* 11-67 *B.t. tolworthi* H9	*Agrotis segetum*, *Helicoverpa armigera*, *Heliothis virescens*, *Mamestra brassicae*, *Spodoptera exigua*, *Spodoptera littoralis* (Lepidoptera: Noctuidae); *Manduca sexta* (Lepidoptera: Sphingidae); *Ostrinia nubilalis* (Lepidoptera: Pyralidae); *Plutella xylostella* (Lepidoptera: Plutellidae); *Bombyx mori* (Lepidoptera: bombycidae); *Choristoneura fumiferana* (Lepidoptera: Tortricidae)
Cry10Aa1		*Aedes aegypti* (Diptera: Cuclidae)
Cry11Aa1	*B.t. israelensis* ONR60A	*Anopheles stephensi*, *Aedes aegypti*, *Culex pipiensis* (Diptera: Cuclidae)
Cry11Ba1	*B.t. israelensis* HD-567	*Anopheles stephensi*, *Aedes aegypti*, *Culex pipiensis* (Diptera: Cuclidae)
Cry11Bb1	*B.t. jegathesan* 367 *B.t. medellin*	Anopheles albimanus, Aedes aegypti, Culex quinquefasciatus (Diptera: Cuclidae)
Cry12Aa1		Pratylenchus spp. (plant pathogenic nematode)
Cry13Aa1	*B. thuringiensis* PS33F2	Nematodes
Cry14Aa1	*B. thuringiensis* PS63B	*Diabrotica* (Coleoptera:); nematodes
Cry15Aa1	*B.t. sotto* PS80JJ1	*Manduca sexta* (Lepidoptera: Sphingidae)
Cry16Aa1	*B.t. thompsoni* HD-542	*Anopheles stephensi*, *Aedes aegypti*, *Culex pipiensis* (Diptera: Cuclidae)
Cry19Aa1	Clostidium bifermentans malaysia CH18	*Anopheles stephensi*, *Culex pipiensis* (Diptera: Cuclidae)
Cry20Aa1	*B.t. jethesan* *B.t. fukuokaensis*	*Aedes aegypti* (Diptera: Cuclidae)

Table 3. Cry toxin specificities and susceptible insects. (Continued)

NAME	SOURCE STRAIN	KNOWN TOXICITY
Cry21Aa1	*B.t.higo*	Culex pipiens molestus (Diptera: Cuclidae)
Cry22Aa1		Hymenopterans *Tribolium castaneum*, (Coleoptera: Tenebrionidae); *Popillia japonica* (Coleoptera: Scarabaeidae)
Cyt1Aa1	*B.t. israelensis* IPS82	*Anopheles stephensi*, *Aedes aegypti*, *Culex pipiens* (Diptera: Culidae)
Cyt1Ab1	*B.t. medellin* 163-131	*Anopheles stephensi*, *Aedes aegypti*, *Culex pipiens* (Diptera: Culidae)
Cyt2Aa1	*B.t. kyushuensis*	*Anopheles stephensi*, *Aedes aegypti*, *Culex pipiens* (Diptera: Culidae)
Cyt2Bb1		*Aedes aegypti* (Diptera: Culidae)

presence of three additional blocks lying outside the active toxic core (Schnepf *et al*, 1998). All 8 conserved blocks with the amino acid sequences alignments among Cry proteins was shown in Fig. 2. The schematic diagram in Fig. 3 based on the sequence arrangement consistent with the conserved block distribution revealed 3 subgrouping of Cry and Cyt proteins (Schnepf *et al*, 1998). The first group consisting of Cry1, Cry3, Cry4, Cry7 to Cry10, Cry16, Cry17, Cry19 and Cry20 contains all five of the core blocks. A second group consisting of Cry5, Cry12 to Cry14 and Cry21 contains recognizable homologs of blocks 1, 2, 4 and 5. Block 1 shows more variability within this second group of sequences than within the first. The proteins within this second subgroup also possess a block 2 variant; block 2 sequences show greater sequence similarity within the two groups than between them (Fig. 2). Block 3 is completely absent from this second group of Cry proteins; an unrelated sequence, highly conserved within the second subgroup but absent from the first group, lies between blocks 2 and 4. For both groups when a protein possess the C-terminal extension, blocks 6, 7 and 8 are invariably present (Fig. 2). Members of a third sequences similarity group, composed of Cry2, Cry11, and Cry18, possess block 1 and a truncated variant of the block 2 core but lack convincing homologs of the other conserves blocks (Lereclus *et al*, 1989). An alternating arginine tract not otherwise homologous to block 4 is found near the C-terminus of Cry11 and Cry18. A weak homolog of block 5 may also be present among the proteins in this group, but its significance, if any is uncertain. The other proteins in the data set Cyt1, Cyt2 Cry6, Cry15 and Cry22 have no recognizable homologs to the conserved blocks seen in these three groups (Schnepf *et al*, 1998; Schnepf and Whiteley, 1981).

Block 1 encompasses helix 5 of domain I. The long hydrophobic and amphipathic helices of domain I suggest that this domain might be responsible for the membrane span-

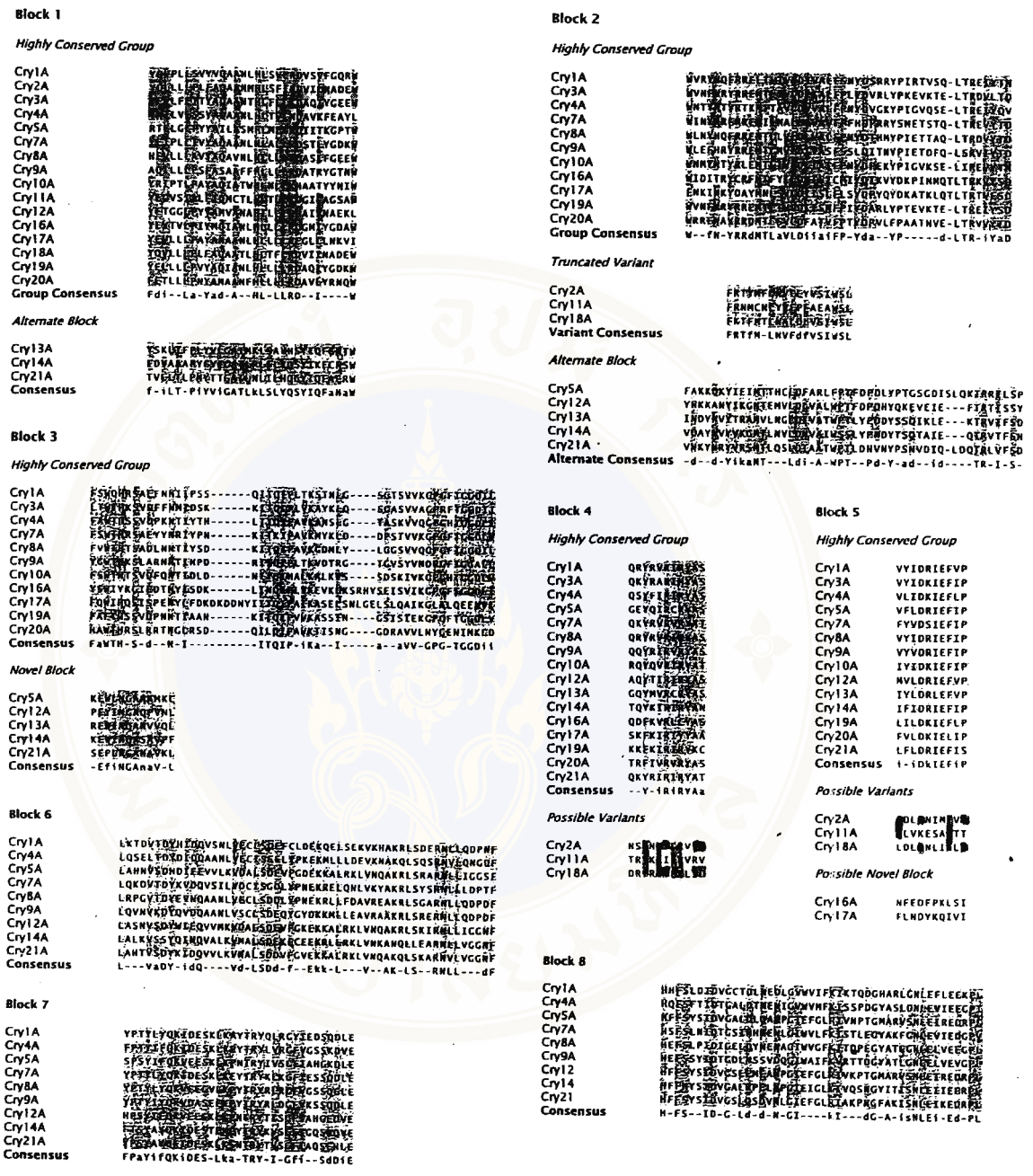

Fig. 2. Amino acid sequence blocks conserved among Cry proteins. For each block, the consensus sequence denotes the positions at which at least 75% of the aligned proteins in the group have an identical or conserved amino acid (indicated by shading). An uppercase letter within the consensus sequence indicates that at least 75% of the residues at that position are identical, while a lower case letter indicates that at least 75% of the residues are conserved. Conserved amino acids are those that fall into the following groups: a (A, G, S, T, or P): d (D, E, N, or Q); f(F, W,, or Y); I (I, L, M, or V): and k (K or R). Highly conserved sequences conform to the consensus sequence at 75% or more of its positions. Variant sequences conform to the consensus sequence of the highly conserved group at 50 to 75% of the positions. Alternate 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. Novel sequences have no discernible homology to a conserved block that occupies the same relative position within sequences in the conserved group. (From Schnepf *et al*; 1998).

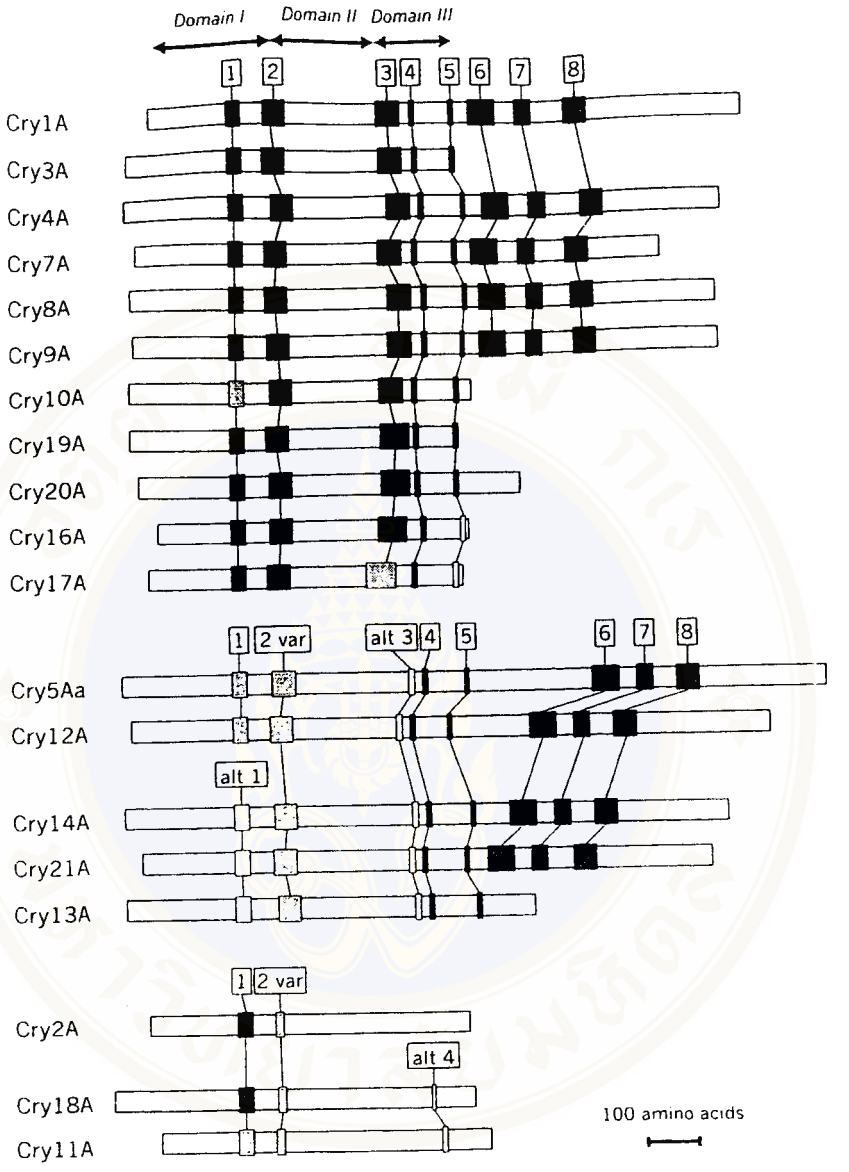

Fig. 3. Positions of conserved blocks among Cry proteins. The cartoon shows the sequence arrangement for each holotype toxin (e.g., Cry1Aa1) having at least one of the conserved blocks defined in the legend to Fig.2. Sequence blocks are shown as dark black, light black, or white to indicate high, moderate, or low degrees of homology, respectively, to the consensus sequence for each conserved block. Variant (var) alternate (alt) are as defined in the legend to Fig. 2. The lengths of each protein and the conserved blocks within them are drawn to scale. (From Schnepf *et al*; 1998).

ning, formation of lytic pores in the intestinal epithelium of the target organism and ion channel regulation. The central location of helix 5 within domain I also suggests an essential role in maintaining the structural integrity of the helix bundle. Domain I bears many striking similarities to the pore-forming or membrane-translocating domains of several other bacterial protoxins including colicin A, diptheria toxin and -to a lesser extent- *Pseudomonas* exotoxin A (Parker and Pettus, 1993).

Block 2 includes helix 7 of domain I and the first β-strand of domain II. The three antiparallel β-sheets which apparently play an essential role in toxin selectivity and is likely to be associated with the toxin receptor binding. These two structures comprise the region of contact between the two domains which acts as the salt bridge between domain I and II. This salt bridge is suggested to be responsible for maintaining the protein in a globular form during solubilization and activation (Schnepf *et al*, 1998).

Block 3, 4, and 5 each lie on one of the three buried strands within domain III. Domain III is a sandwich of antiparallel β-sheets in a "jelly-roll" configuration. Block 3 contains the last β-strand of domain II, a structure involved in interactions between domains I and III. Domain III functions in maintaining the structural integrity of the toxin molecule, perhaps by protecting it from proteolysis within the gut of the target organism (Li *et al*, 1991). The central two arginines of block 4 may be involved in intermolecular salt bridges affecting crystal or oligomeric aggregation (Grochulski *et al*, 1995; Li *et al*, 1991). The first and last arginines, which are solvent exposed (Grochuski *et al*, 1995), have been implicated in channel function (Chen *et al*, 1993; Schwartz, 1997; Wolfersberger *et al*, 1996).

3. The selectivity and toxicity of *B. thuringiensis* toxins.

The selectivity of *B.t.* toxins is determined both by the toxin structure and by factor inherited to the insect which are midgut structure and function, toxin diversity, synergistic interactions between toxins, toxin processing in the insect midgut, the identification of toxin receptors in susceptible insects and toxin pore formation in midgut cells (Gill, 1995).

Factors affecting *B.t.* toxicity are as followed.

1. Insect midgut structure and function.

An understanding of insect midgut ion regulation is crucial for understanding *B.t.* toxin mode of action because a major consequence of toxicity is the disruption of midgut cellular osmotic balance. In addition, the midgut plays a key role in insect nutrition, and enzymes involved in food digestion, such as trypsin, chymotrypsin, also plays a role in *B.t.* toxin processing. The midgut also plays an important role in detoxifying secondary plant product which might be affected by *B.t.* toxins. Finally, the midgut is an important site for insecticidal action. The site of *B.t.* toxin action is on the columnar cell at apical membrane of insect midgut, which affected the ion regulation, and lead to cell swelling, loss of osmotic balance and cell lysis (Singh, *et al*, 1986).

2. Selective toxicity of *B.t.* toxin.

The toxicity of each *B.t.* strain is dependent on the toxins that are present in that strain. The selective insecticidal activity of each toxin is dependent on the concentration levels used against particular insect species. These toxins are selective, but not strictly specific because although each toxin has its predominant activity towards a particular insect species, it usually also has very low insecticidal activity against other insects. Hence,

it is possible to obtain lepidopteran activity with toxin that is usually regarded as having only dipteran activity, and vice versa (Gill, 1995).

3. Toxin structure and toxin processing.

It is assumes that all *B.t.* Cry toxins have similar three-domain structures as mentioned previously in toxin structure section. It was suggested that the-toxicity-determining regions involving to the hypervariable regions of domain I. *B.t.* toxin processing is a key in the formation of an activated toxin, and can affect the selectivity of a toxin. For example, CryIIA toxin which is toxic to both dipteran and lepidopteran insects depended on the toxin C-terminal processing. If a dipteran enzyme is involved a toxin more active towards dipterans is obtained, and similarly if processing is performed by lepidopteran proteases the toxins become active towards lepidopterans (Widner and Whiteley, 1989).

4. Toxin receptor in susceptible insects.

A key feature in toxin selectivity is the interaction of toxins with receptor proteins in the insect midgut. There is a great diversity of toxin receptors. For example, with the CryIAc toxin a number of receptors have been observed in some insects while in others only one major protein appears to be involved. In *Manduca sexta* a single protein binds the toxin (Knight *et al*, 1993; Sangadala *et al*, 1994). While in *Heliothis virescens* at least two proteins are involved (Garczynski *et al*, 1991). Similarly, there is preliminary evidence that in *B.t.i.*, a number of different midgut proteins bind to different kinds of receptors (Hofmann, and Luthy, 1986). Consequently, the selective toxicity of different *B.t.* proteins is determined by the receptors that are presented in the insect midgut.

5. Synergism of each toxin.

Since a number of *B.t.* strains contain multiple toxins, these toxins do not act

alone. For example, the mosquitocidal toxicity of the intact crystal is greater than the sum of the toxicity of each individual toxins (Crickmore *et al*, 1995).

4. Mechanism of action of *B. thuringiensis* toxins

In general, the mechanism of action of the *B.t.* Cry proteins consists of three major steps: (1) solubilization and activation of the crystal in the insect midgut, (2) binding of the activated toxin to midgut receptors and (3) insertion of the activated toxin into the midgut apical membrane to create ion channels or pores. Most of the information was obtained from studies of lepidopteran insects. The details of each step are as followed (Rajamohan *et al*, 1998; Honee *et al*, 1993).

1. Solubization and activation.

Upon ingestion by the larvae, the crystal is solubilized in the alkaline environment of the insect midgut. The solubility is a factor codetermining crystal protein specificity. In addition to the midgut lumen composition, the total crystal composition influences the efficiency of the dissolving process and consequently crystal protein specificity (Aronson *et al*, 1991). The protoxin then are proteolytically converted releasing toxic fragments encompassing the N-terminal domain of the crystal proteins. Like solubility, proteolysis can influence the activity of the crystal proteins (Rajamohan *et al*, 1998; Honee *et al*, 1993).

2. Receptor binding.

The toxic fragments then bind in a competitive manner to a protein receptor on the columnar cell of the insect midgut. The selectivity observed with a number of *B. t.* toxins can in part be explained by the affinity of a toxin to midgut brush border membrane receptors of a particular insect. Consequently, when change in toxin affinity is observed in an

insect that is resistant to a particular toxin, a change in these binding constants is usually observed. The receptor binding of Cry toxins involves various steps shown in equation 1 (Liang *et al*, 1995).

$$R+T \underset{k-1}{\overset{k1}{\rightleftharpoons}} R^*T \xrightarrow{k2} R\text{-}T$$

This scheme shows that at early stage of binding there is a large saturable accumulation of reversible receptor (R) and toxin (T) complex (R*T) at a rate constant of k1, and then the formation of an irreversibly or tightly associated complex either with the receptor or membrane (R-T) at a rate constant of k2 (Liang *et al*, 1995). The differences in the rate of irreversible association can account for variations in toxicity. Mutation studies identify three types of mutation: (i) mutations in domain II that affect initial (reversible) binding affinity (Rajamohan *et al*,1996a; 1996b; Wu and Dean, 1996) ; (ii) mutations in domain II that show similar initial binding affinity but affect the dissociation rate of the toxin, assuming domain II is not involved in membrane insertion (Rajamohan *et al*, 1996a; 1996b); and (iii) mutations in domain I that affect insertion of toxin into the membrane but do not affect initial binding affinity (Wu and Aronson, 1992; Chen *et al*, 1995). Charged and neutral polar residues such as arginines and asparagines and aromatic hydrophilic residues have been implicated as particularly important for receptor binding. The current models proposed that the surface-exposed loops, connecting the β-sheets of domain II, recognize and tightly bind to specific receptors located in the insect midgut. The involvement of domain III in receptor binding and insect specificity has also been reported (Aronson *et al*, 1995).

3. Toxin insertion and oligomerization.

The α helix structure of domain I has been proposed as the primary candidate for promoting membrane penetration by analogy with the pore-forming structures of colicin A and diptheria toxin (Rajamohan *et al*, 1998; Lesieur *et al*, 1997). Once the toxins are inserted, oligomerization of the toxin molecules apparently occurs in the cell membrane lipid bilayer to form a pore. Whether oligomers form prior to binding to the receptor, on the receptor, or within the membrane after insertion remains to be determined. The pores are permeable to small ions and molecules (Knowles *et al*, 1989; Slatin *et al*, 1990; Schwatz *et al*, 1993). Most of the channels that were formed are voltage independent cation pores. A consequent of pore formation lead to a disruption of columnar cell osmotic balance, loss of cell function and ultimately results in cell lysis and insect death (Rajamohan *et al*, 1998). The ion channel function of Cry toxins affected by several factors such as pH dependent (Schwartz *et al*, 1993), voltage dependent (Montal, 1974) and insect midgut membrane receptor proteins (Martin and Wolfersberger, 1995; Sangadala *et al*, 1994). Several ion channel activities of Cry toxins were obtained from the artificial system such as voltage clamping (Harvey and Wolfersberger, 1979), brush border membrane vesicle (BBMV) swelling assays (Carroll and Ellar, 1993; Wolfersberger MG, 1996), insect tissue culture cells (Knowles and Ellar, 1987; Schwartz *et al*, 1991), phospholipid vesicles (Gazit and Shai, 1993; Haider and Ellar, 1989), and lipid bilayers (Lorence *et al*, 1995; Martin and Wolfersberger, 1995; Schwartz *et al*, 1993; Gazit and Shai, 1995). However, all these systems are not a real physiological conditions, they contain neither the membrane proteins (receptor) nor the ambient pH and ionic conditions of the insect midgut. Hence, the interpretation should be done with caution (Rajamohan *et al*, 1998). The use of primary midgut cells, maintained at higher pH can be one of the instructive tool in the study of the molecular mode of action (Masson *et al*, 1994). Al-

though domain I contains ample information to generate ion channels, such channels are significantly different than those formed by native toxins. Thus, the other domains may also play an indirect role in channel formation either by assisting in structural conformation, in oligomerization, or in regulation of channel activity (Rajamohan *et al*, 1998).

Numerous proposals have been made regarding the mode of action of the Cry toxins and most have included models. Knowles and Ellar (1987) used CF-1 cells and proposed a "colloidal osmotic lysis" theory as a model for the cytolytic toxicity of Cry toxins. According to this model, toxic fragments cause the appearance of small non-specific pores in the plasma membrane of the midgut epithelial cells resulting in a net inflow of ions and an accompanying influx of water. This water influx leads to cell swelling and causes the size and number of lesions in the membrane to increase which eventually results in cell lysis (Knowles and Ellar, 1987). Li *et al*, 1991 have proposed that α-helices 4 and 5 penetrate the membrane while the remainder of α-helices remain on the surface (the umbrella model). Hodgman and Ellar (1990) have proposed that α-helices 5 and 6 flip out of domain I and penetrate the membrane as a "penknife" (the penknife model). However, recent site-directed mutagenesis studies revealed that α-helices 4 and 5 affected penetration (Chen *et al*, 1995) but that the residues in the loop between α-helices 5 and 6 do not (Hussain *et al*, 1996). These results argue against the penknife model proposed by Hodgman and Ellar (1990). Disulfide bridges between α-helices 2 and 5 and between α-helices 5 and 7, do not disrupt penetration or toxicity, arguing against both penknife and umbrella models. Further research is needed to clarify the details of Cry toxin mode of action. A better understanding of the details of the mode of action is needed to allow rational design of better protein pesticides (Rajamohan *et al*, 1998).

5. Regulation of toxin synthesis in *B. thuringiensis*

The production of insecticidal crystal proteins in *B.t.* normally coincides with sporulation, resulting in the appearance of parasporal crystalline inclusions within the mother cell. The very high level of crystal protein synthesis in *B.t.* and its coordination with sporulation are controlled by a variety of mechanisms. In most instance, the temporal and spatial regulation of toxin genes expression is determined at the transcriptional level by mother-cell-specific sigma factors that share homology with σ^E and σ^K from *B. subtilis* (Haldenwang, 1995; Errington, 1993). Transcription alone, however, can not account for the impressive ability of this bacterium to accumulate insecticidal proteins. A variety of post-transcriptional and post-translational mechanisms also contribute to the efficient production of crystal toxin in *B.t.* (Baum and Malvar, 1995).

5.1 Transcriptional mechanism

The *cry* genes have long been considered typical examples of sporulation-specific genes. However, recent studies on the expression of the *cry3Aa* gene have revealed that this assumption is not always valid. It is therefore necessary to distinguish among the *cry* genes expressed during the stationary phase, those that are dependent on sporulation from those that are not (Agaisse and Lereclus, 1995).

5.1.1 Sporulation-dependent *cry* gene expression

At the transcriptional level, the development of sporulation is controlled by the successive activation of sigma factors, which bind the core RNA polymerase to direct the transcription from sporulation- specific promoters (Moran, 1993). The accumulation of crystal proteins coincides with sporulation in *B.t.*, sug-

gesting that the transcription is hold on until factors that are expressed during sporulation activate or derepress transcription. Extensive studies have shown that many of the proteins that regulate sporulation in *B. subtilis* are present and appear to function similarly in *B.t.*. These proteins are the primary sigma factor of vegetative cells, σ^A, and five factors called σ^H, σ^F, σ^E, σ^G and σ^K, which appear in that order in a temporally regulated fashion during development (Adams *et al*, 1991). The σ^A and σ^H factors are active in the predivisional cell, σ^E and σ^K are active in the mother cell, and σ^F and σ^G are active in the forespore (Agaisse and Lereclus, 1995; Wong *et al*, 1983).

The genes encoding σ^{35} and σ^{28} have been cloned and sequenced. Their deduced amino acid sequences show 88 and 85% identity with σ^E and σ^K of *B. subtilis*, respectively (Adam *et al*, 1991). Several *cry* gene promoters have been identified, and their sequences have been previously determined (Brizzard *et al*,1991; Brown, 1993; Dervyn *et al*, 1995; Yoshisue *et al*, 1993a; 1993b). Consensus sequences for promoters recognized by *B.t.* RNA polymerase containing σ^E or σ^K have been deduced from alignment of promoter regions of these genes (Agaisse and Lereclus, 1995; Baum and Malvar, 1995). The results are that, in addition to the transcription of *cry1Aa*, *cry1Ba* and *cry2Aa*, the transcription of many other *cry* genes (eg. *cry4Aa*, *cry4Ba*, *cry11Aa*, *cry15Aa*, etc) is likely to be σ^E or σ^K-dependent (Brown, 1993; Dervyn *et al*, 1995; Yoshisue *et al*, 1993a; 1993b). However, low-level transcription of the *cry4Aa*, *cry4Ba*, and *cry11Aa* genes in *B.t.* has been detected during the transition phase, beginning at about T-2 (where Tn is n hours after the end of the exponential phase) and lasting until the onset of sporulation (Poncet, 1997; Yoshisue *et al*, 1995). This expression may be due to the σ^H RNA polymerase, and it is suggested that SpoA repress this weak expression, specific to the transition phase, when the cells enter the sporulation phase (Poncet *et al*, 1997).

5.1.2 Sporulation-independent *cry* gene expression

The *cry3Aa* gene, isolated from the coleopteran-active *B.t.* var *tenebrionis*, was found to be expressed during vegetative growth, although at a lesser extent than during the stationary phase (De-Souza *et al*, 1993; Malvar *et al*, 1994; Sekar, 1988). The *cry3Aa* promoter, although located unusually far upstream of the start codon (position-558), resembles promoters recognized by the primary sigma factor of vegetative cells, σ^A (Agaisse and Lereclus, 1994a). A similar promoter was found 542 bp upstream of the start codon of the *cry3Bb* gene (Baum and Malvar, 1995). The expression of *cry3Aa* is not dependent on sporulation-specific sigma factors either in *B. subtilis* (Agaisse and Lereclus, 1994b) or in *B.t.* (Salamitou *et al*, 1996). It was found that *cry3Aa* expression is increased and prolonged in mutant strains unable to initiate sporulation (Agaisse and Lereclus, 1994b; Lereclus *et al*, 1995; Malvar and Baum, 1994; Salamitou *et al*, 1996). Thus, *cry3Aa* expression is activated by a non-sporulation-dependent mechanism arising during the transition from exponential growth to the stationary phase (Agaisse and Lereclus, 1994b; Salamitou *et al*, 1996).

5.1.3 Post-transcriptional mechanisms

The stability of mRNA is an important contributor to the high level of toxin production in *B.t.*. The half-life of *cry* mRNA, about 10 min, is at least fivefold greater than the half-life of an average bacterial mRNA (Glatron and Rapaport, 1972). Wong and Chang (1986) showed that the putative transcriptional terminator of the *cry1Aa* gene (a stem-loop structure) acts as a positive retroregulator. The fusion of a DNA fragment carrying this terminator with the 3' end of heterologous genes increases the half-life of their transcripts two- to three-fold, which in turn increases the expression of their gene

products. It has been demonstrated in other systems that the processive activities of 3'-5' exoribonucleases are impeded by 3' stem-loop structures (Nierlich and Murakawa, 1996). Therefore, it is likely that the *cry* terminator is involved in mRNA stability by protecting the *cry* mRNA from exonucleolytic degradation from the 3' end. Similar terminator sequences, potentially able to form stable stem-loop structures are found downstream from various *cry* genes and may contribute to their high-level expression by stabilizing the transcripts.

For *cry3Aa* gene, it was found that full expression requires the presence of a large DNA region extending up to 600 bp upstream of the translational start site. Structural and functional analysis using the transcriptional fusions to the *lacz* reporter gene has revealed that two distinct regions are involved in *cry3Aa* expression. The upstream region, located from positions -560 to 600, is involved in transcription and harbours the *cry3Aa* promoter. The downstream region at the translational start codon is a region involved at a posttranscriptional level with the accumulation of *cry3Aa* mRNA as a stable transcript with a 5' end corresponding to nucleotide positions -129. The determinant of stability appears to be a consensus Shine-Dalgano sequence (GAAAGGAGG) mapping at a position between -125 and -117; this sequence has been designated STAB-SD (Agaisse and Lereclus, 1996)(STAB stands for stable). The stability of the *cry3Aa* mRNA could result from an interaction between the 3' end of 16S rRNA and STAB-SD. Therefore, the binding of a 30S ribosomal subunit to this SD sequence may protect the mRNA against 5'-3' ribonuclease activity, resulting in a stable transcript. Potential STAB-SD is also present in similar positions upstream of the *cry3Ba*, *cry3Bb*, and *cry3Ca* genes (Donovan *et al*, 1992; Lambert *et al*, 1992).

5.2 Post-translational mechanisms

The proteolytic degradation of proteins is a common problem occuring in *Bacillus* species, especially during the stationary phase. The bacterium must therefore have mechanisms to prevent premature proteolysis of the toxins. The ability of the protoxins to crystallize may decrease their susceptibility to the protease action. However, the crystals have to be solubilized rapidly and efficiently in the gut of insect larvae to become biological active. The formation of the crystal structure and its solubility characteristics presumably depend on a variety of factors, including the secondary structure of the Cry proteins, the energy of the disulfide bonds, and the presence of additional *B.t.* specific-components (Schnepf *et al*, 1998).

The 130- to 140- kDa Cry1 protoxins can spontaneously form crystals, which might result from disulfide bonds formation of the cysteine-rich C-terminal half (Bietlot *et al*, 1990). A similar mechanism of protein self-assembly also found in other 130- to 140-kDa protoxins (e.g., Cry4, Cry5, and Cry7). For 73 kDa Cry3A protoxins, the cysteine-rich C-terminal region is absent and no need of specific host factor for the protein assembly (Bernhard, 1986). Analysis of three-dimensional structure of Cry3A toxin suggested that four intermolecular salt bridges might participate in the formation of the crystal inclusion (Li *et al*, 1991).

There is various evidences demonstrating that crystallization of Cry2A (71 kDa), Cry11Aa (72 kDa) and Cyt1A (27 kDa) requires the presence of accessory proteins (Agaisse and Lereclus, 1995; Dervyn *et al*, 1995; Baum and Malvar, 1995). The 20 kDa protein has been found to stabilize Cyt1A and Cry11Aa in *E. coli* (Wu and Federici, 1995; 1993). The 20 kDa protein forms a complex with Cyt1A which may act as a scaffold to facilitate (or stabilize) assembly of Cyt1A into protease-resistant oligomers. The

role of the 20 kDa protein in Cyt1A and Cry11Aa crystallization in *B.t.* is less clear. The results of two studies disagree on the importance of its role (Chang *et al*, 1993; Visik and Whiteley HR, 1991). However, the discrepancy between these results could arise from the presence or absence of the P19 protein, which may also involved in crystallization (Dervyn *et al*, 1995). These accessory proteins may act at a post-translational level to stabilize the nascent protoxin molecule and to facilitate crystallization. However, the precise mechanism of their role in crystal formation has not been determined (Dervyn *et al*, 1995; Poncet *et al*, 1993).

5.3 The *cry* gene copy number

The copy number, and gene amplification may influence the expression of a gene. In *B.t.*, the crystal synthesis is limited and reaches a maximum at a certain number of *cry* gene copies in the cell, above which there is no further increase in synthesis. The production of toxins is not strictly proportional to the copy number of the *cry* genes (Agaisse and Lereclus, 1995). Moreover, cloning *cry* genes by using high copy number plasmids frequently disturbs the physiological equilibrium of the cells and prevents their sporulation (Donovan *et al*, 1988a; 1988b).

6. Peritrophic matrix structure of insect gut

Traditionally, insect peritrophic matrix structures (PMs) are categorized as type I and type II according to their method of formation (Lehane, 1997). Type I PMs are produced by the entire midgut; this is believed to be the ancestral situation and is found in the majority of species today. Type II PMs are produced by a restricted zone of cells in the anterior midgut of Diptera, Dermaptera, Embiodea, and some families of Lepidoptera. Interestingly, many species produce different PM types at different life stages. For ex-

ample, larval mosquitoes produce a type II PM, whereas adult females produce a type I PM (Stamm *et al*, 1978).

A type I PM is formed by delamination from the general surface of the midgut epithelium. The different regions of the midgut may vary in the extent of their contribution to type I PM. The production of types I PM may be continuous, as in locusts, or in response to feeding, as in adult female mosquitoes. While type II PM is continuously produced as an unbroken sleeve (or group of concentric sleeves) lining the entire midgut. Type II PM formation occurs in a specialized fold of the anterior midgut and foregut; the entire organ is correctly termed the cardia but is more commonly called the preventriculus. Type II PM is more highly organized than type I. They may form as a single sleeve, as two concentric sleeves, or as three concentric sleeves. Each sleeve is often multilayered, with a layers formed sequentially in the cardia by successive zones of cells (Lehane, 1976; 1997).

The general consensus is that when the PM is created, as chitin microfibrils are embedded in a proteoglycan matrix that forms as secreted proteoglycan molecules fold and hydrate in the gut lumen (Lehane, 1997). Chitin forms between 3 and 13 % of PMs. The α and γ forms of chitin are most commonly found in PMs, although the β form has been found in the specialized beetle *Ptinus tectus*, which uses PMs to build cocoons. The form of chitin used in a particular situation may be related to function, but the significance of this for the PM is unknown. The remainder of the PM is formed of a mixture of proteins, glycoproteins, and proteoglycans. Protein forms 20-55% of the total mass of the PM in a range of insects. The size of proteins are range from 15-220 kDa, with most less than 100 kDa and with more glycosylated than nonglycosylated types present (Rupp and Spence, 1985; Stamm *et al*, 1978). Proteoglycans probably form the remainder of

the PM (Lehane, 1976; Stamm *et al*, 1978). Proteoglycan, longer and unbranched carbohydrate chains covalently attached to the protein core, are usually glycosaminoglycans (GAG). This is a hexosamine, uronic acid residue of either D-glucuronic acid or iduronic acid (Kjellen and Lindahl, 1991).

The function of PM is involved in the intestinal biology of the insect. The PM may protect the midgut epithelium from mechanical damage and insult from pathogens, toxins and other damaging chemicals. The type II PM presents an effective, permanent barrier between the midgut epithelial cells and the digested food (Lehane, 1997). The PM must act as a semipermeable membrane regulating passage of the molecules between the different midgut compartments, and the PM may partition the midgut lumen into different, physiologically significant compartments (Lehane, 1997). Section of fully formed PM of last-instar larva of *Aedes aegypti* was showed in Fig. 4.

7. General background of chitin

Chitin is a straight chain homopolymer composed of β-(1, 4)-linked *N*-acetylglucosamine (GlcNAc) with a three-dimensional α-helical configuration stabilized by intermolecular hydrogen bonding (Cohen, 1993). Chitin can vary by the arrangement of GlcNAc strands, degree of deacetylation, and presence of cross-linked structural components, such as proteins and glucans (Gooday, 1990). It is one of the most abundant polysaccharides in nature. X-ray diffraction analysis of chitin crystallites revealed two major polymorphs. α-chitin, in which the polysaccharide chains in the microfibril are arranged in an antiparallel pattern (Minke and Blackwell, 1978), is the most abundant crystallite polymorph in nature. A β-chitin polymorph, which was observed in limited cases, is characterized by a parallel arrangement of the chains (Gardner and Blackwell,

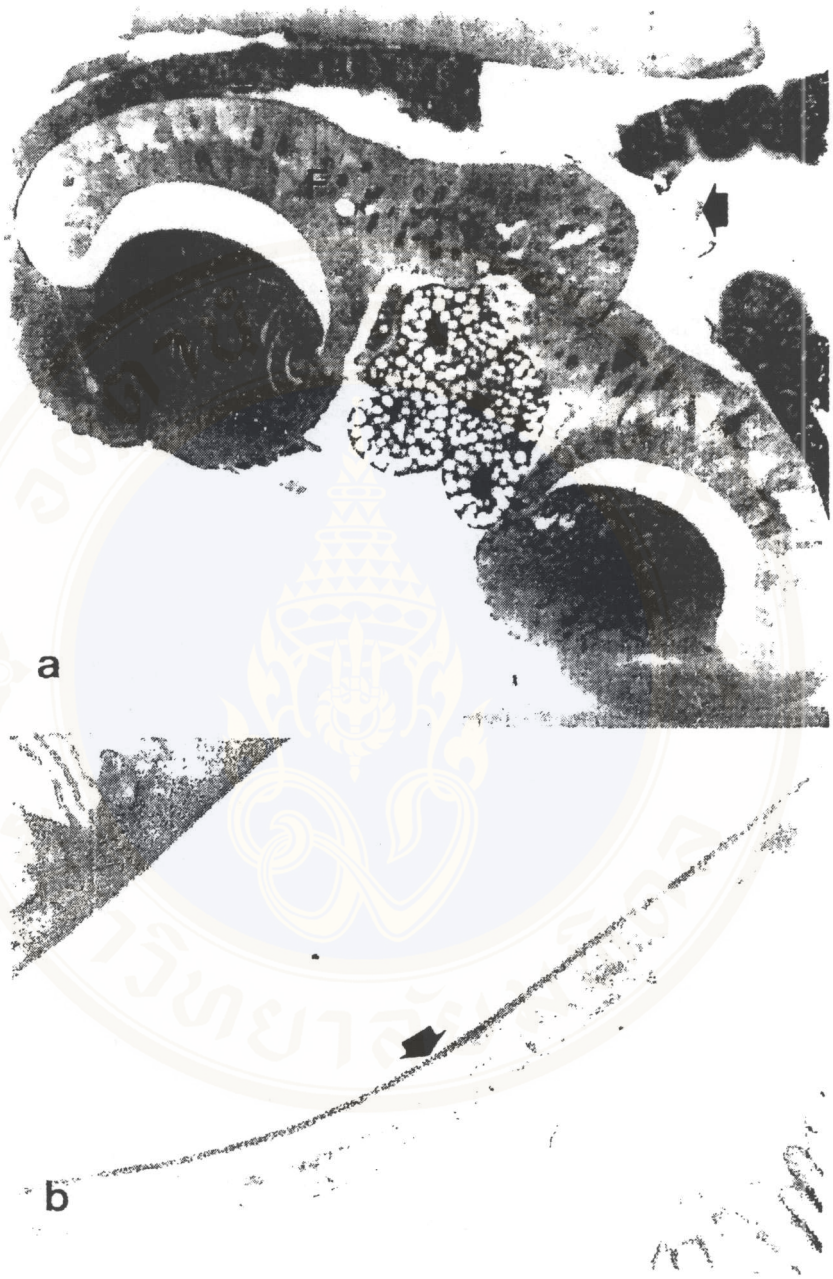

Fig. 4. (a) Longitudinal section through the cardia of adult *Stomoxys calcitrans* (X 400). F, foregut tissue; M, midgut tissue; arrow, the formed PM streaming back into the midgut. (b) Transverse section through the single PM sleeve of adult *S. calcitrans* (X 50,000). Note that the PM, although formed as a single tube, is multilayered; the endoperitrophic surface is indicated by the arrow (from Lehane, 1997).

1975). γ-chitin arranged in that for every three polymer chains two are parallel. The 3 forms are shown in Fig. 5. Chitin, in particular the α-polymorph, is chemically stable and is insoluble in water and most organic solvents. It is degradable in very strong acid solutions or in concentrated hot alkali (Cohen, 1993). Chitin has become an attractive target for pest control because it is a common constituent of insect exoskeletons, peritrophic membrane of insect midgut, shells of crustaceans and fungal cell walls (Shaikh and Deshpande, 1993). These chitin-containing organisms produce chitinases which hydrolyze chitin. Hydrolytic degradation of the chitin polymer is essential for hyphal growth, branching, and septum formation in fungal systems as well as for the normal molting of arthropods. Some other organisms, which do not contain chitin also, produce chitinases: for example, a wide variety of bacteria and higher plants. The latter develop several biochemical defense mechanisms in response to pathogens and abiotic stresses (Flach *et al*, 1992).

8. General background of chitinase

Chitinases are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive *N*-acetylglucosamines of chitin. Endochitinases, exochitinases (EC 3.2.1.14), β-*N*-acetylglucosaminidases and chitobiases (EC3.2.1.30) have been characterized (Henrissat, 1991). β-*N*-acetylglucosaminidase acts preferentially on a dimer, diacetylchitobiose and cleave GlcNAc units from the non-reducing ends of chitin chains releasing *N*-acetylglucosamine monomers. Endochitinase randomly hydrolyses GlcNAc polymers, eventually giving diacetylchitobiose as the major product together with some triacetylchitobiose. Exochitinase is an enzyme releasing chitobiose. Chitobiase hydrolyses chitobiose (Vorgiase *et al*, 1996; Tews *et al*, 1992). Some chitinases also display lysozyme

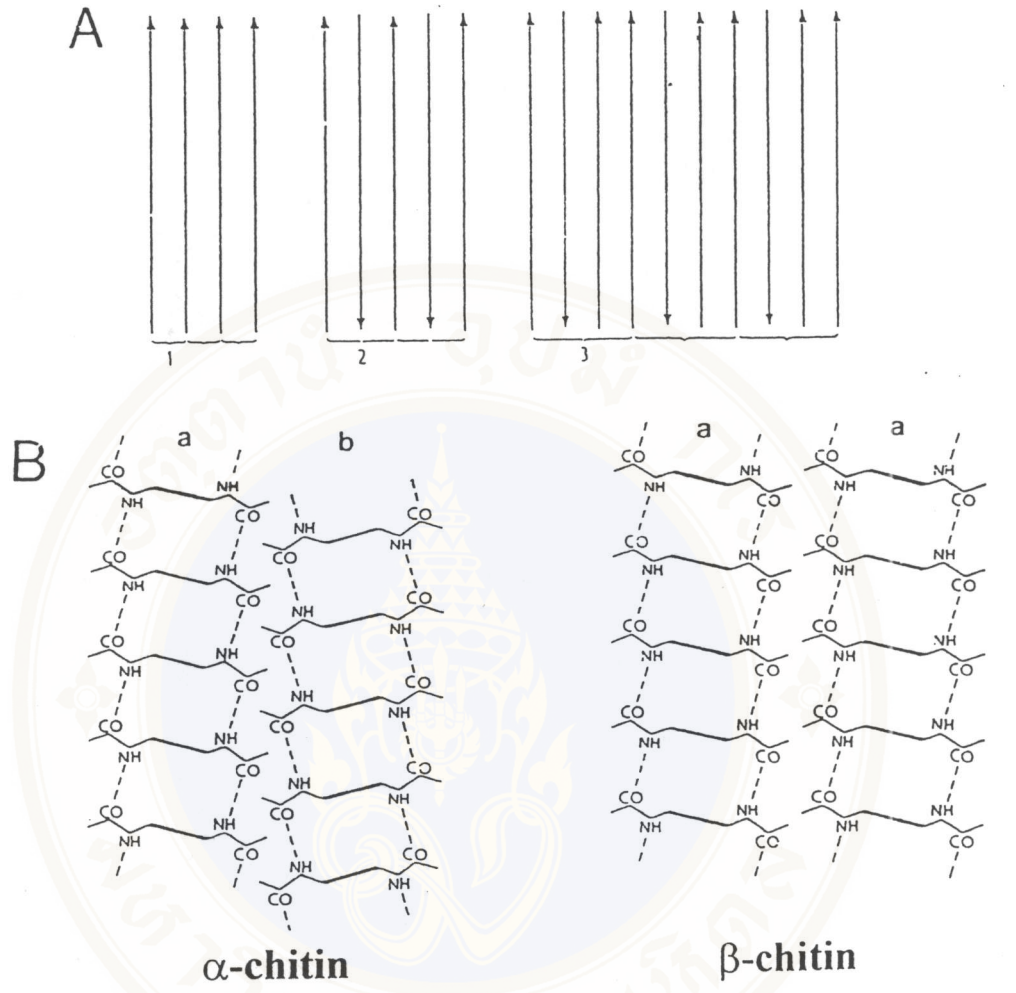

Fig. 5. Diagramatic illustration of three types of chitin with arrangements as parallel (β-chitin) (1), antiparallel (α-chitin) (2), and an alternative between two parallel and one antiparallel chains (γ-chitin) (3). (B) Structure of α-chitin and β-chitin showing two piles of chitin chains a (up), and b (down) linked to C=O…NH group. (From Rudall, 1963).

activity (EC 3.2.1.17) corresponding to the cleavage of a glycosidic bond between the C1 of *N*-acetylmuramic acid and the C4 of *N*-acetylglucosamine in the bacterial peptidoglycan (Monzingo *et al*, 1996).

Chitinase could produce from various organisms including plants, fungi, insects, crustaceans and bacteria which all play the most important ecological role in the degradation of chitin (Flach *et al*, 1992). The roles of these chitinases differ in the various hosts. In fungi, chitinase activity plays a physiological role in apical growth and morphogenesis of fungal hyphae (Flach *et al*, 1992). The production of chitinases in higher plants is considered part of their defense system against fungal interaction (Shin *et al*, 1987). Bacterial chitinases appear to have a nutritional or scavenging function in the decomposition of insoluble chitin and also the utilization of chitin as a carbon and energy sources (Flach *et al*, 1992).

The diversity in chitinases is not unexpected because the structure of chitin is very diverse due to variation in arrangement of strands, degree of deacetylation and cross-linking to other structural components. However, the enzymes from closely related bacteria are more similar than enzymes from distantly related bacteria (Chernin *et al*, 1997).

8.1 Glycosyl hydrolases families of chitinase

Glycosyl hydrolases (EC 3.2.1.x) are key enzymes of carbohydrate metabolism that are found in the three major kingdoms (archaebacterium, eubacteria and eukaryotes). The first three digits indicates enzymes hydrolyzing *O*-glycosyl linkages whereas the last number indicates the substrate and sometimes reflects the molecular mechanism. *O*-glycosyl hydrolases (EC 3.2.1.-) are a widespread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or

between a carbohydrate and a non-carbohydrate moiety. The classification of glycosyl hydrolases based on amino acid sequence similarities allowed their grouping into 35 families (Henrissat, 1991; 1996; Henrissat and Bairoch, 1993). The chitinases (EC 3.2.1.14) enzymes was categorized into glycosyl hydrolase family 18 and 19 (Henrissat, 1991). While, *N*-acetylglucosaminidases (Chitobiase, Chb) (EC 3 .2.1.30) and *N*-acetylgalactosaminidases (hexosaminidases) (EC 3.2.1.52) established the family 20 of glycosyl hydrolases. This classification is expected to reflect the structural features of these enzymes better than their sole substrate specificity, help to reveal the evolutionary relationships between these enzymes and provides a convenient tool to derive mechanistic information (Henrissat, 1991; Henrissat and Bairoch, 1993). Enzymatic hydrolysis of the glycosidic bond of glycosyl hydrolases takes place via general acid catalysis that requires two critical residues: a proton donor and a nucleophile/base. This hydrolysis occurs via two major mechanisms giving rise to either an overall retention, or an inversion, of anomeric configulation (Davies and Henrissat, 1995).

Many bacteria in the environments are able to hydrolyze chitin to *N*-acetylglucosamine (GlcNAc) (Monreal and Reese, 1969) by using two enzymes, chitinase and *N'*, *N'*-diacetylchitobiase (chitobiase). Chitin is first hydrolyzed by chitinase to low-molecular-weight multimers of GlcNAc, the dimer *N*, *N'*-diacetylchitobiose (chitobiose) being predominant. Chitinolytic enzymes from many microorganisms have been purified and characterized (Perrakis *et al*, 1993). The Chb is required to complete digestion of chitin to GlcNAc monomers used by bacteria as a nutrient source while recycling the billions of tons of chitin debris in the marine environment (ZoBell and Rittenberg, 1937).

The general protein folding motif of glycosyl hydrolases including chitinase is

the $(\beta/\alpha)_8$ TIM barrel which consists of a cylindrical, barrel-like framework made from eight internal parallel β-strands that are alternatively connected by eight exterior α-helices (Reardon and Farber, 1995). In the glycosidases this structure appropriately places in the substrate-binding groove between two critical acidic residues at the correct distance above and below the sissle glycosidic bond. Like all TIM-barrel enzymes, these two catalytic residues are located just beyond the carboxyl ends of two of eight β-strands making the $(\beta\text{-}\alpha)_8$ barrel (Aronson *et al*, 1997).

8.2 Homology modeling of family 18 chitinases

The chitinases so far sequenced are classified into two different families based on the amino acid sequence similarity of their catalytic domains. These forms families 18 and 19 in the family classification system of glycosyl hydrolase. Family 18 contains chitinases from bacteria, fungi, viruses and animals, and some plant chitinases. On the other hand, family 19 contains only plant chitinases and the recently identified *Streptomyces griseus* chitinase C (Ohno *et al*, 1996). The chitinases of the two different families do not share amino acid sequence similarity, have completely different three-dimensional (3D) structures (Coulson, 1994) and molecular mechanisms, and are therefore likely to have evolved from different ancestors (Perrakis *et al*, 1994; van Scheltinga *et al*, 1994; Hart *et al*; 1995).

Sequence homologies between family 18 chitinases (mostly chitodextrinases) and two other types of related proteins, chitobiase and chitinase-related/oviduct-specific proteins were shown in Table 4. Homology analysis indicates that a common progenitor chitinase likely gave rise to these groups of proteins. However, all members of these chitinase-related groups are not active chitinases because they no longer possess the critical

Table 4. Sequence homologies in (I) Family 18 chitinases; (II) Chitobiases; and (III) Chitinase-Related/Oviduct-specific proteins. (From Aronson *et al*; 1997).

ENZYME/PROTEIN	β3	β4	β5	β6	β7	β8	GenBank
I. Chitinases							
S. marcescens A	267KILPSIGGWT	308DGVDIDWE	358ELTSAISAG	383DHIFLMSYD	436KIVVGTAMYGRGWT	535GLFSW	Z36294
A. caviae	267KILPSVGGWT	308DGVDIDWE	358ELTSAISAG	383DHIFLMSYD	436KIVVGAAMYGRGWT	535GLFAW	U09139
A. sp. strain0-7	265KILPSVGGWT	306DGVDIDWE	357ELTSAIGAG	382DYIFAMTYD	451KLVMGVAMYGRGWE	564GLFGW	D13762
B. circulans	156KTIISVGGWT	197DGVDLDWE	247LLTIASGAS	272DWINIMTYD	330KLVLGVPFYGRGWD	429GAMFW	M57601
S. plicatus	334KILYSFGGWT	376DGIDLDWE	417LITAAVTAD	445DWYNVMTYD	499KLLIGIGFYGRGWT	586GAFFW	M82804
S. lividans	333KILYSFGGWT	376DGIDLDWE	417LITAAVTAD	445DWYNVMTYD	498KLLIGIGFYGRGWT	585GAFFW	D12647
S. thermoviolaceus	141KVLWSFGGWT	183DG--IDWE	220LVTAAVTAD	248DWYNVMTYD	302KLLLGIGFYGRGWT	389GAFVW	D14536
S. marcescens B	89RIMFSIGGWY	137DGVDIDWE	180QLTIAGAGG	207DYINLMTYD	284KIVMGVPFYGRAFK	399GVMFW	X15208
J. lividum	360KLFISLGGWS	417DGIDIDWE	467LLTAAVGAG	492DWINLMTYD	548KLLLGIPFYGRGWT	643GAFSW	U07025
R. oligosporus	105KVSLSIGGYT	146DGLDIDWE	191LLTVAVPCG	216DLFYLMAYD	262KLVVGMPLYGRGFC	351GAMFW	D87894
S. cerevisiae	175KVIMSIGGWS	216DGIDLDWE	263QLSIAAPAF	288DYWNMMTYD	336KLVLGMAAYGRSFH	442GGGFW	U28373
K. lactis	441KKIPSFGGWD	488DGIDLDWE	532TLSIAIPSS	557DYMVYMTYD	604KVFGGVANYGRSYK	703GTSLW	X07127
A. californica	257KILPSIGGWT	298DGVDIDWE	349ELTSAISAG	374GKIFLMSYD	427KIIVGVAMYGRGWT	524GLFAW	L22858
B. mori	113KILPSIGGWT	154DGVDVDWE	205ELTSAISAG	230DKIFLMTYD	283KIIVGVAMYGRGWT	374GLFAW	L33180
Phycodnaviridae PBCV-1	102NMHASIGGWS	145NSISLDWE	194MCTIAAPEK	215DEVHVMTYD	269KIFVGVAFYSRGFS	359GILVW	U42580
N. tabacum	92KTFLSIAGGR	134HGLDLDWE	176LLTAAVSYS	202NWINLMAYD	252KLVLGIPFYGYAWR	348GYFAW	X78325
C. thermocellum	115KTLISVGGWT	156DGVDIDWE	206LLTIAAPAG	231DFINIMTYD	288KINVGVPYYAAGWQ	381GIMIW	Z68924
B. malayi	99KVLLSYGGYN	141DGFDLDWE	181LLTAAVSAG	207DLLFLMSYD	261KIIIGIPMYAQGWT	358GAFIW	M73689
B. pahangi	35KVLLSYGGYN	77DGFDLDWE	117LLTAAVSAG	143DLLFLMSYD	197KIIIGIPMYAQGWT	294GAFIW	U59690
E. dispar	271KVLASIGGWN	318DGIDIDWE	365LLTIAAPAG	390DWINLMTYD	437KIMLGMAHYGRGWT	533GVMFW	U78318
E. histolytica	220KVLASIGGWN	267DGIDIDWE	314LLTIAAPAG	339DWINLMTYD	386KIMLGMAHYGRGWT	482GVMFW	U78319
E. invadens	226QVLASIGGWN	273DGIDIDWE	321LLTIAAPAG	346DWINLMTYD	393KMFVGMAHYGRGWT	489GAMIW	U78320
A. album	123KVMLSIGGWT	164DGIDIDWE	207LLSIAAPAG	232DYINLMAYD	285KIVLGMPIYGRSFQ	374GTMFW	X64104
T. harzianum	124KVMLSIGGWT	165DGIDVDWE	208LLSIAAPAG	233DYVNLMAYD	286KIVLGMPIYGRAFE	375GSMFW	L14614
T. hamatum	124KVMLSIGGWT	165DGIDVDWE	210LLSIAAPAG	235DYINLMAYD	290LIVLGMPIYWRSFE	379GSMFW	Z71415
A. viteae	98KILLSYGGYN	140DGFDLDWE	180LLTAAVSAG	206DLLFLMSYD	260KIIIGIPAYSRGWT	357GAFMW	L42010
A. nidulans	108KVLLSIGGWT	149DGIDIDWE	188QLTVAVPAG	213DFYNLMAYD	267KIILGMPIYGRAFQ	356GAMWW	D87063
O. volvulus	98KILLSTGGYN	140DGFDFDWE	180LLTAAVSAG	206DLLSLMSYD	260KIIIGIPTYGRGWT	357GAFIW	L42021
M. sexta	97KFMVAVGGWA	139DGLDLDWE	182ELTAAVPLA	208DAIHVMSYD	262KLVVGIPFYGRSFT	366GAMTW	P36362
Chelonus sp	96KIMVAVGGWN	138DGFDIDWE	177ILSAAVAAP	203DFINLMTYD	257KLVVGVPAYGKSFT	355GAMVW	U10422
K. zopfii	156KTIISVGGWT	197DGVDLDWE	247LLTIASGAS	272DWINIMTYD	330KLVLGVPFYGRGWD	429GAMFW	D63702
V. furnissii	267KILPSVGGWT	308DGVDIDWE	358ELTSAISAG	383DYIFLMSYD	436KVVVGAAMYGRGWT	535GLFAW	L42548
P. japonicus	116KTNIAVGGWA	158DGLDLDWE	201EITCAVPVA	227DAIHLMTYD	282KLVVGTPFYGRTYT	384GAMTW	D84250
C. elegans	102KLLYAVGGWE	143DGVDIDWE	191LISFAGAAG	217DFVNVMSYD	274KINMGVPFYGRFWK	374GVMIW	Z66524
Coccidioides immitis	123KTLLSIGGWT	164DGIDIDWE	207LLTIASPAG	232DFWNLMAYD	285KIFLGMPLYGRAFA	373GGGMW	L41663
Chitotriosidase (Human)	91KTLLAIGGWN	133DGLDLDWE	179LLSAAVOAG	205DFVNLMAYD	259KLILGMPTYGRSFT	354GAMVW	U29615
II. Chitobiase							
Human	105KGDVSLKDII	136DGINIDIE	175QVTFDVAWS	202DFLFVMSYD	246KLVMGVPWYGYDYT	350GIGMW	M95767
Rat	90KGDVALKDII	121DGINIDIE	160QVTFDVAWS	187DFLFVMSYD	231KLVMGIPWYGYDYI	335GIGMW	M95768
III. Chitinase-Related/Oviduct-Specific(OSG) Proteins							
Human gp39	91KTLLSVGGWN	133DGLDLAWL	173LLSGAVSAG	199DFISIMTYD	253KLVMGIPTFGRSFT	348GAMVW	M80927
Human YKL-39	90KILLSIGGYL	133DGLDVSWI	174LLTAGVSAG	200DFINLLSFD	256KVVMGIPTYGHSFT	351GAMIW	U49835
Mouse gp39	92KTLLAIGGWK	134DGLNLDWQ	180LLTSTGAGI	206DYIQVMTYD	260KLIVGFPAYGHTFI	357GAVVW	S27879
Mouse brp-39	92KTLLSVGGWK	134DGLDLAWL	174LLSAALSAG	200DFINLMTYD	254KLLMGIPTFGKSFT	349GAMVW	X93035
Mouse ECF-L	91KTLLAIGGWK	133DGLNLDWQ	179LLTSTGAGI	205DYIQVMTYD	259KLIVGFPAYGHTFI	356GAVVW	D87757
Pig gp38	91KTLLSVGGWN	133DGLDLAWI	173LLSGAVSAG	199DFISLLTYD	253KLVMGIPTFGRSFT	348GAMVW	U19900
Drosophila secreted gp	107KILLSVGGDK	158DGLDVAWQ	205QFTALLRDV	231NVNSSLFYD	301KINVGVATYGRPWK	395GIWSF	U13825
Human OSG	92KTLLSIGGWN	134DGLDLFFL	180LLSAAVSGV	206DFINVLSYD	255KLIMGIPTYGRTFR	351GAMVW	U09550
Baboon OSG	92KTLLSIGGWN	134DGLDLFFL	180LLSAAVSGV	206DFINVLSYD	255KLIMGIPTYGRTFR	351GAMVW	M59903
Mouse OSG	92KTLLSIGGWN	134DGLDLFFL	180LLSAAVSGI	206DFINVKSYD	255KLIMGFPTYGRNFY	351GAMVW	D32137
Hamster OSG	71KTLLSVGGWN	113DGLGLFFL	159LLSAAVSGI	185DFINVLSYD	234KLLMGFPAYGRTFH	330GAMVW	U15048
Bovine OSG	89KTLLSIGGWN	131DGLDLFFL	177LLSAAVSGD	203DFISVLSYD	252KLLMGLPTYGRTFH	348GAMVW	D16639
Ovine OSG	83KTLLSVGGWN	125DGLDLFFL	171LLSAAVSGD	197DFISVLSYD	246KLLMGLPTYGRTFH	342GAMVW	U17988

[a] The crystal structure of *S. marcescens* chitinase A has been determined to be a $(\beta/\alpha)_8$ barrel protein.[3] The sequences represent strands $\beta3-\beta8$ and a few adjacent amino acids. The sequences have been translated from GenBank (accession numbers indicated). The starting amino acid position of each protein segment is numbered based on the translational start methionine being residue 1.

glutamic acid H^+ donor at the carboxyl end of the fourth β-strand. The "active" site residues are D315/E319 [based on *S. marcescens* chitinase (SmChiA) numbering]. It has been proposed that residue D313 of SmChiA is the proton donor to residue E315 and is therefore necessary for catalytic activity (Aronson *et al*, 1997).

9. Bacterial chitinases

Various microbial chitinase genes have been cloned and extensively studied including bacterial chitinases genes (Table 5). A number of chitinase genes have been cloned from various bacteria and nucleotide sequences of several genes have been reported such as *Alteromonas sp* (Tsujibo *et al*, 1993), *Bacillus circulans* (Watanaba *et al*, 1990b), *Vibrio harveyi* (Svitil *et al*, 1997), *Vibrio vulnificus* (Wortman *et al*, 1986), *Serratia marcescens* (Gal *et al*, 1998; Suzuki *et al*, 1999) and *B. licheniformis* (Tantimavanich *et al*; 1997, 1998). Some of these organisms produce multiple chitinases from different genes (Harpster and Dunsmuir, 1989; Jones *et al*, 1986, Takayanaki *et al*, 1991; Tantimavanich *et al*; 1998; Watanabe *et al*, 1990a), and the efficient degradation of chitin is assumed to be achieved by the combined action of the multiple chitinases. Some chitinase isoforms derived from other chitinases. The presence of multiple, divergent, chitinases in a single chitinolytic bacterium is perhaps necessary for efficient synergistic degradation of chitin (Suzuki *et al*, 1999).

9.1 Classification of bacterial chitinases

All prokaryotic chitinases sequenced so far appeared to possess the regions sharing sequence similarities, in spite of the divergences of their overall primary structures. Prokaryotic chitinases can be divided into three groups based on their sequence similarities (Fig. 6). Group A chitinases contain regions (putative catalytic domains) obviously

Table 5. A brief summary of research done on the cloning of microbial chitinase gene in bacteria. (From Felse and Panda; 1999).

Source Organism	Transformed into	Through	Reported information
Actinomycetes			
Streptomyces plicatus	*Escherichia coli*	Shuttle vector containing a *cDNA* library	The signal peptide sequence of the wild-type, containing 30 amino acids, was removed in the recombinant enzyme, The cloned enzyme was secreted into the periplasmic space, but the wild-type enzyme wassecreted into the medium. Removal of 150-200 bp from the *Streptomyces* insert resulted in greater expression of the enzyme. Removal of more than 200 bp resulted in incomplete inactivation of the enzyme
	E. coli	Plasmid *pUC* vector	
Streptomyces lividans	*S. lividans*	Multi-copy-number plasmid *pIJ702*	The 2-kb chitinase gene consisted of an open reading frame of 1713 bp coding for a 571-amino acid protein. The region upstream of nucleotide 133 was essential for the expression of chitinase in *S. lividans*
Bacteria			
Serratia marcescens	*E. coli* and then into *P. fluorescens* and *P. putida*	Cosmid vectors	Resulted in four strains of *Pseudomonas* with enhanced chitinase activity
	E. coli and then into *S. marcescens*	High-copy-number plasmid vector	*E. coli* containing *S. marcescens* chitinase gene had high chitinase activity and was used as an effective biocontrol agent against fungal infection in plants
	E. coli	Broad-host-range cosmid *pLAFR1* containing a genomic library	Four independent clones were obtained and all were found to have a common *EcoR I* fragment containing a 57-kDa chitinase gene
	E. coli	Broad-host-range cosmid *pLAFR3* containing a genomic library of chromosomal DNA	Positive clones were identified on chitin medium and high levels of chitinase were obtained following chitin induction
	E. coli	Plasmid *pBR322* containing a *chiA DNA* fragment. This was further modified by including an operatory promoter of bacteriophage *λ, oLpL* and the resulting phagemid *pLCHIA* was introduced into *E. coli*	High levels of chitinase were observed following chitin induction
	Lactobacillus lactis subsp. *lactis* MG 1363 and *Lactobacilllus plantarum E196*	-	The chitinase gene was expressed in *L. lactis* and *L. plantarum* with 9-27-fold increase in chitinase activity
Serratia liquefaciens	*E. coli*	Plasmid *pBR329* with a chitinase gene insert	The transformed cells were capable of expressing the cloned chitinase gene
	E. coli	-	Increased chitinase activity was observed in the transformants
	E. coli	-	Among the three genes cloned *chiA* and *chiB* coded for chitinase while *chiC* coded for chitobiase. Removal of the repressor gene *chiD* led to increased production of chitinase
Serratia liquefaciens	Transposon-Tn5-mediated mutagenesis was used to obtain high-yielding mutants	-	A chitinase-overproducing mutant, which lacked a negative regulatory element; showed about 80% increase in chitinase activity over the wild type
Bacillus circulans	*E. coli*	Plasmid vector *pKK233-3* with chromosomal DNA fragments	The gene coding for chitinase activity contained an ORF of 2097 bp that codes for a precursor protein with signal sequence of 15 amino acids. The recombinant chitinase showed 33% homology with chitinase A of *S. liquefaciens*
	E. coli	Site-directed mutagenesis	Glu-204-Gln and Glu-204-Asp mutations decreased chitinase activity. Asp-200-Glu mutation did not affect chitinase activity
Aeromonas hydrophila	*E. coli*	Plasmid *pJP2512*	The chitinase gene from *Aeromonas hydrophila* consisted of an open reading frame of 2.6 kb and was translated on its own promoter to give a 96-kDa protein. Chitinase in *E. coli* accumulated in the periplasmic space.
	E. coli		The recombinant chitinase was similar to the wild-type chitinase with a molecular mass of 85 kDa and was subjected to catabolite repression
Vibrio vulnificus	*E. coli*	-	*V. vulnificus* chitinase was expressed in *E. coli*, but the enzyme accumulated in the periplasmic space, while in wild-type cells the enzyme was secreted into the medium.

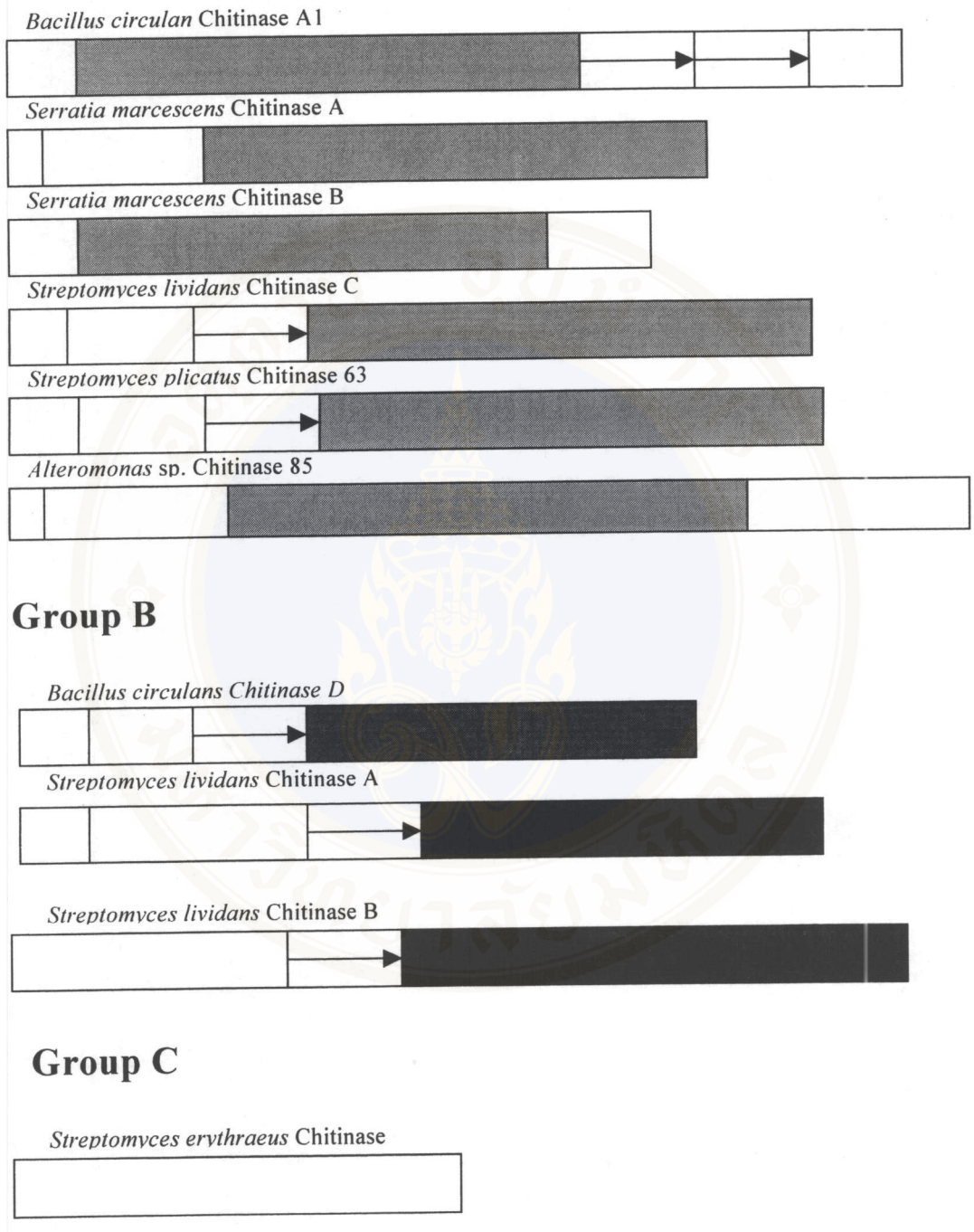

Fig. 6. Classification of the bacterial chitinases based on the amino acid sequence feature. Shadowed area indicates the homologous regions of chitinases to the catalytic domain of other chitinase A1 or D of *B. circulans*. Arrows indicate fibronectin type III-like domains. *Streptomyces livicidans* chitinase A and chitinase B are from unpublished results obtained by Miyashita K and Fujii T. (From Watanabe *et al*; 1993).

homologous to almost the entire catalytic domain of chitinase A1 of *B. circulans*. Group B chitinases contain the regions homologous to almost the entire catalytic domain of chitinase D of *B. circulans*. Group C chitinase does not show obvious sequence similarity with the catalytic domain of either chitinase A1 and chitinase D. Amino acid identities of the putative catalytic domains of chitinases classified into same groups are higher than 32% (Watanabe *et al*, 1993).

9.2 Structure of bacterial chitinases

Bacterial chitinases generally consist of multiple functional domains, such as chitin-binding domains (CBDs) and fibronectin type III-like domains (FnIII domains), linked to the catalytic domain. Two of the corresponding genes, *chiA* and *chiD* from *B. circulans*, have been cloned (Watanabe *et al*, 1990b; 1992). The predicted domain consist of a catalytic domain, a chitin binding domain and a region with similarity to the fibronectin type III (FnIII) module (Bork and Doolittle, 1992; Watanabe *et al*., 1994). The relative arrangements of the domains may vary in different proteins. The architecture of chitinase-63 (*chtA*) of *Streptomyces plicatus* (Robbins *et al*, 1988), *chiA*, *chiB* and *chiC* of *S. lividans* (Miyashita *et al*, 1991), exo-chiO1 of *S. olivaceoviridis* (Blaak and Schrempf, 1995) are very closely related to *chiD* of *B. circulans* which composed of three main regions, a C-terminally located catalytic region, one FnIII module and an N-terminal substrate binding domain. The chitin-binding domain of the *S. olivaceoviridis* exochitinase shares no significant similarity with the few chitin-binding domains from other chitinases or from cellulases identified in various organisms. The substrate binding region of the exo-ChiO1 enzyme has an especially high affinity to crystalline form of chitin (Blaak and Schrempf, 1995).

9.2.1 catalytic domain

Prokaryotic chitinases, class III plant chitinases, yeast chitinases and endo-β-*N*-acetylglucosaminidases share weak amino acid sequence similarities at the certain regions of each enzyme. These regions have been assumed to be important for catalytic activities of the enzymes. The catalytic mechanism of chitinases has been suggested to be similar to that of lysozyme (Verburg *et al*, 1992). The catalytic mechanism of lysozyme was proposed almost exclusively from the structural information. Glu-35 acts as a general acid catalyst, which donates proton to the glycosidic oxygen, and Asp-52 contributes to the lowering of the energy barrier of the reaction by stabilizing the transient carbonium ion intermediate electrostatically. Asp and Glu are the most conserve residues, among the similar sequence regions found in number of chitinases and related enzymes, provided a strong indication of the involvement of these residues in the catalytic mechanism of chitinase by analogy with Glu-35 and Asp-52 residues of lysozyme (Watanabe *et al*, 1993). The catalytic site for chitinases contains four residues, Ser, Gly, Asp, and Glu. Asp and Glu were suggested to involve in the catalytic mechanism (Watanabe *et al*, 1992). ChiA of *V. harveyi* contains three of the four residues in this conserved region and contain 29% similar to the cataytic domain of ChiD from *B. circulans* WL-12 (Svitil and Kirchman, 1998). Glu-204 and Asp-200 in chitinase A1 of *B. circulan* WL-12 were proved to be important for catalytic activity (Watanabe *et al*, 1993).

Using BESTFIT pairwise comparisons, the catalytic domains can be divided into at least four groups (Fig. 7). Group I (93% mean similarity) consists of 4 chitinases, all from g-proteobacteria (Gram negative). Members of group II (85% mean

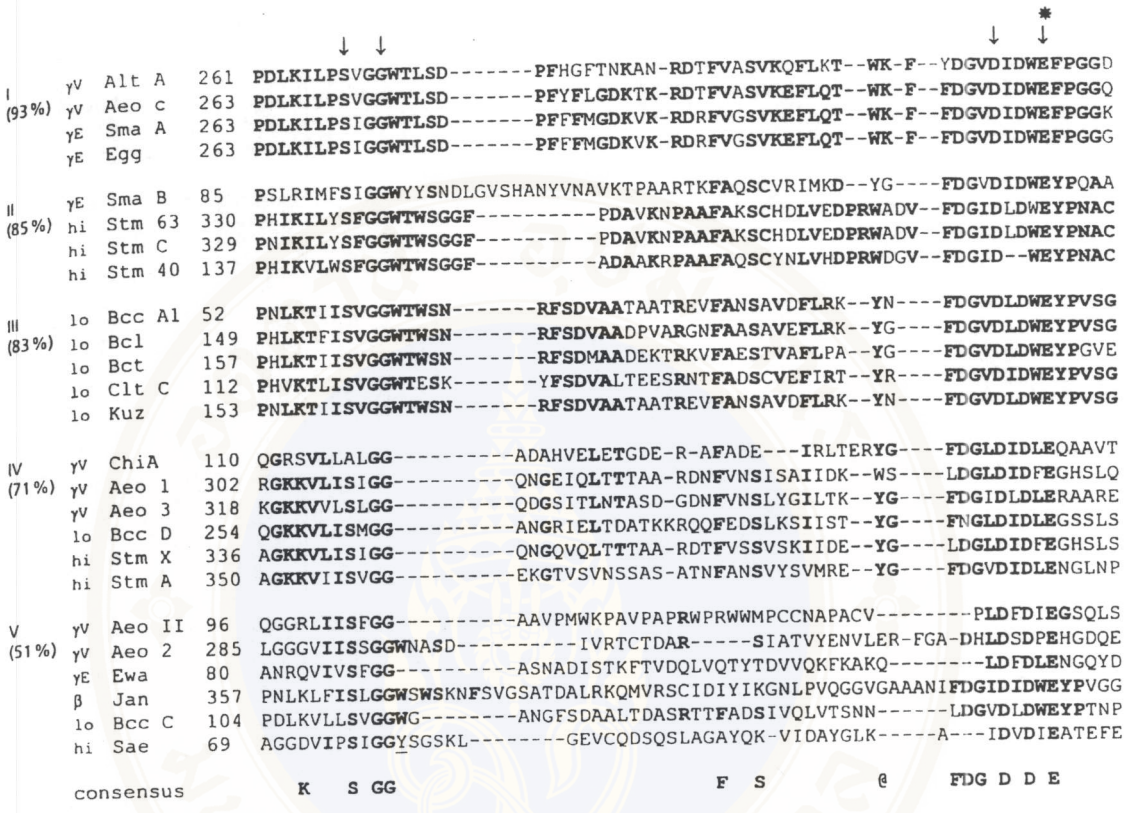

Fig. 7. Comparison of putative catalytic region in bacterial chitinases. Conserved residues are in bold. Arrows indicate the four residues identified by Watanabe *et al.* (1993) as being conserved among bacterial and plant class III chitinases; *, residue necessary for activity (Watanabe *et al.*, 1993); @, conserved aromatic residue; γE, Gram-negative γ-subdivision, family Enterobacteriaceae; V, Gram negative γ-subdivision, family Vibrionaceae; β, Gram-negative β-subdivision; lo, Gram-positive low-G+C-content; hi, Gram-positive high-G+C-content; Alt A, *Alteromonas sp.* strain O-7 chitinase (accession number D13762; Tsujibo *et al.*, 1993b); Aeo c, *Aeromonas caviae* chitinase (U09139; Sitrit *et al.*, 1995); Sma A, *Serratia marcescens* chitinase A (X03657; Jones *et al.*, 1986); Egg, *Enterobacter agglomerans* chitinase (U59304; Chernin *et al.*, 1997); Sma B, *S. marcescens ChiB* (X15208; Harpster & Dunsmuir, 1989); Stm 63, *Streptomyces plicatus* chitinase 63 (M82804); Robbins *et al.*, 1992); Stm C, *Streptomyces livicidans* chitinase C (D12647; Fujii & Miyashita, 1993); Stm 40, *Streptomyces thermoviolaceus* chitinase 40 (D14536; Tsujibo *et al.*, 1993a); Bcc A1, *Bacillus circulans* WL-12 chitinase chitinase A1 (JO5599; Watanabe *et al.*, 1990); Bcl, *Bacillus licheniformis* TP chitinase (U71214); Bct, *Bacillus thuringiensis* chitinase (U89796); Clt A, *Clostidium thermocellum* ChiA (Z68924); Kuz, *Kurthia zopfii* chitinase (D63702); ChiA, *Vibrio harveyi* BB7 chitinase A (this study); Aeo 1 and 3 , *Aeromonas sp.* NO. 10S-24 chitinases ORF 1 and 3, respectively (D63139, D63141; Shiro *et al.*, 1996) Bcc D, *B. circulans* WL-12 chitinase D (D90534; Watanabe *et al.*, 1992); StmX, *Streptomyces olivaceoviridis* exochitinase (X71080; Blaak *et al.*, 1993); Stm A, *St. lividans* 66 ChiA (D13775; Miyashita & Fujii, 1993); Aeo II, *Aeromonas sp.* NO. 10S-24 chitinase II (D31818; Ueda *et al.*, 1994); Aeo 2, *Aeromonas sp.* NO. 10S-24 chitinases ORF 2 (D63140; Shiro *et al.*, 1996); Ewa, *Ewingella americana* chitinase (X90562); Jan, *Janthinobacterium lividum* chitinase chitin-binding domain 2 (U07025; Gleave *et al.*, 1995); Bcc C, *B. circulans* WL-12 ChiC (D89568; Alam *et al.*, 1995); Sae, *Saccharopolyspora* (*Streptomyces*) *erythraeus* chitinase (P14529; Kamei *et al.*, 1989). (From Svitil and Kirchman, 1998).

similarity) include chitinase from a γ-proteobacterium and three chitinases from Gram-positive high-G+C-content bacteria. Group III (83% mean similarity) is comprised of five chitinases all from Gram-positive low-G+C- content bacteria. Group IV (71% mean similarity) contains three chitinases from *Vibrios* (including ChiA from *V. harveyi*) and three chitinases from Gram-positive bacteria. The domains which could not be placed into a group were assigned to group IV (51% mean similarity). From these comparisons indicated that the evolution of the catalytic domain does not follow bacterial phylogeny and that taxonomically related bacteria might have dissimilar catalytic domains (Svitil and Kirchman, 1998).

9.2.2 FnIII/cadherin-like domain

The region between the catalytic domain and the chitin binding domain was found to display sequence similarity to FnIII domains. The bacterial FnIII domain was first identified in chitinase A1 of *B. circulans* WL-12 (Watanabe *et al*, 1990b). The FnIII modules were also found within various other enzymes, which interact with macromolecules, including one cellulase, amylases and a polyhydroxybutyrate depolymerase (Little *et al*, 1994). The alignment of the FnIII domain of several bacterial enzymes were shown in Fig. 8. Among bacterial enzymes degrading insoluble substrates, the FnIII domains have been most frequently found in chitinases (Suzuki *et al*, 1999). The single FnIII domain was also found in *B.t.* subsp. *pakistani* (Thamthiankul *et al*, 2001). FnIII region is not essential for substrate binding but suggested to play an important role in maintaining the optimal distance and orientation between catalytic and chitin-binding domains (Watanabe *et al*, 1994). The frequent occurrence of this domain in chitinases may have a special importance for the degradation of the insoluble and crystalline polysaccharide chitin. The truncated form containing the FnIII module does not bind to both colloidal and crystalline

Fig. 8. Alignment of the FnIII domain of chitinase C1 with that of other bacterial enzymes. Smar C stands for *S. marcescens* 2170 chitinase C1; CbhA, *Cellulomonas fimi* cellobiohydrolase A; AmyA180, Unclassified alkalophilic Gram-positive bacteria DSM 5853 exo-maltopentaohydrolase (amylase); Ppic PHB, *Pseudomonas pickettii* poly93-hydroxybutyrate) depolymerase; CbhB, *Cellulomonas fimi* cellobiohydrolase A; Sliv C *Streptomyces lividans* chitinase C; Afae PHB, *Alcaligenes faecalis* poly(3-hydroxybutyrate) depolymerase; Smal A, *Stenotrophomonas maltophilia* chitinase A; CenD, *Cellulomonas fimi* endo-1, 4-β-D-glucanase (CenD); Bcir A1, *B. circulans* WL-12 chitinase A1; Kzop, *Kurthia zopfii* chitinase; Spli 63, *Streptomyces plicatus* chitinase 63; Bcir D, *B. circulans* WL-12 chitinase D1; Sliv A, *Streptomyces lividans* 66 chitinase A (From Suzuki *et al*, 1999).

chitin, hence the domain does not involve binding to this substrate (Suzuki *et al*, 1999). Among all chitinase of *S. marcescens*, only the chitinase C1 has an FnIII domain (Suzuki *et al*, 1999). Unlike the chitinases in *B. circulans* (Watanabe *et al*, 1990b, 1992) and *S. olivaceoviridis* (Blaak *et al*., 1993), ChiA from *V. harveyi* does not have a fibronectin type III domain. But it contains a pair of repeats which found to be cadherin-like domains (Svitil and Kirchman, 1998). *C. paraputrificum* ChiB also have no sequence homology with FnIII but some homology with cadherin domains of the cadherin-related tumor suppressor protein (Fat) from *D. melanogaster* (Morimoto *et al*, 1997).

On the basic of a systematic screen of a protein database, Bork and Doolittle (1992) concluded that bacterial FnIII modules were initially acquired from an animal source and are being spreaded further by horizontal transfers between distantly related. Fibronectin is a multifunctional extracellular matrix and plasma protein and plays a central role in cell adhesion bacteria (Doolittle and Bork, 1993; Little *et al*, 1994). Cadherins, all of which contain conserved cadherin domains of ca. 130 amino acids, cell membrane proteins of animal cells and play an essential role in Ca^{2+}-dependent cell adhesion in contrast to FnIII, which is involved in Ca^{2+}-independent cell adhesion. Cadherin-like domains also might act as a linker to maintain an optimal distance and orientation between the catalytic domain and CBD since cadherin-like domains by themselves did not have chitin-binding activity. Further studies are necessary to test the precise role of the FnIII/cadherin-like module (Morimoto *et al*, 1997).

9.2.3 Chitin binding domain (CBD)

The chitin binding domain was important for the insoluble substrate

chitin binding. Among all bacterial chitinases sequenced to date, there is no relationship between percentage similarity of catalytic domains and chitin-binding domains in pairwise comparisons, suggesting that these two domains have evolved separately (Svitil and Kirchman, 1998). The chitin binding domain appears to be evolutionarily conserved among many bacterial chitinase and is also somewhat similar to cellulose-binding domains found in microbial cellulases and xylanases (Svitil and Kirchman, 1998). The chitin binding domain of plant chitinases contain eight conserved cysteine residues (Graham and Sticklen, 1994). In contrast, the chitin-binding domain of chitinase A1 from *B. circulans* WL-12 contains only one cysteine and is not similar to that of plants (Watanabe *et al*, 1994). Several aromatic amino acids, principally tryptophan, are prominent among the residues conserved in the bacterial chitin-binding domain (Svitil and Kirchman, 1998). Tryptophans have been shown to be involved in the binding of a cellulase to cellulose (Poole *et al*, 1993). Some of the Gram-negative bacteria have another aromatic amino acid (phenylalanine or tyrosine) in place of the second or third tryptophan in the chitin-binding domain. Aromatic amino acids appear to be essential for hydrophobic binding of 1,4- β-glycanases to polysaccharides (Poole *et al*, 1993). A consensus binding sequence was found in the chitin-binding domains of several bacterial chitinase which consisted of 15 residues (17 including conserved aromatic residues) (Fig. 9) (Svitil and Kirchman, 1998). A search of *V. harveyi* ChiA revealed a region, which is 37% and 26% identical to *B. circulans* ChiA1 (CA) and ChiD (ND) binding regions, respectively. A truncated ChiA of the marine bacterium *V. harveyi* without the putative chitin-binding domain did not bind to chitin, but it could hydrolyse chitin, although not as well (Svitil and Kirchman, 1998). The removal of chitin-binding domain of a chitinase from *C. paraprutrificum* decreased hydrolysis of unprocessed chitin but

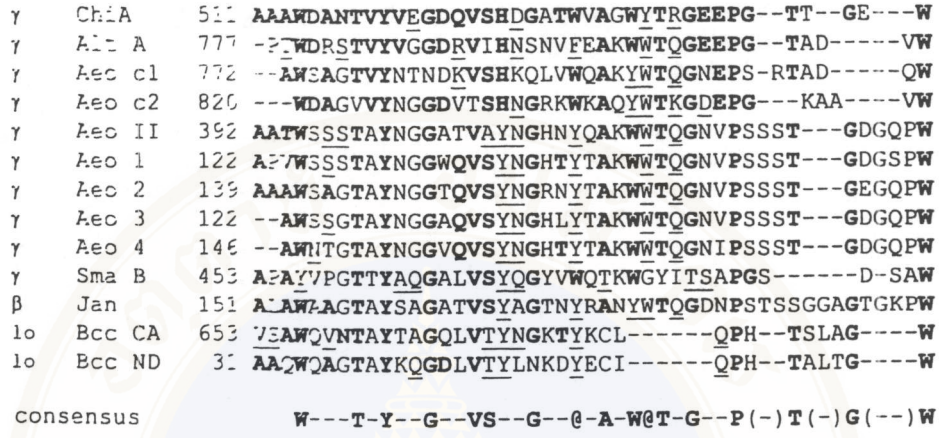

Fig. 9. Alignment of protein sequences to identify similarities in the putative bacterial chitin-binding domains. Conserved residues are in bold and closely related amino acids are underlined. Abbreviations and symbols are the same as in Figure 7, except that dashes indicate a variable number of intervening residues; Aeo c1 and c2, regions 1 and 2 in *Aeromonas caviae* chitinase (Sitrit *et al*, 1995) Aeo 4, *Aeromonas sp.* NO. 10S-24 ORF 4 (D63142; Shiro *et al*, 1996) Bcc CA, chitin-binding domain identified in *Bacillus circulans* WL-12 chitinase A1 (Watanabe *et al*., 1990); Bcc ND, chitin-binding domain identified in *B. circulans* WL-12 chitinase D (Watanabe *et al*, 1992). (From Svitil and Kirchman, 1998).

not of colloidal chitin (Morimoto *et al*, 1997). *Alteromonas sp.*, *Aeromonas sp.*, *Janthinobacterim lividasn* and *S. marcescens* also demonstrated the similar chitin-binding domains. The chitin-binding domains have evolved not only to aid in hydrolysis of different forms of chitin but also in response to factors that affect movement of the enzyme along the substrate and in different environments (Svitil and Kirchman, 1998).

9.3 Cloning of bacterial chitinase genes

9.3.1 Cloning of chitinase genes from *Serratia* species.

S. marcescens is an efficient degrader of chitin, and the *chiA* and *chiB* genes encoding chitinase A and B of five *S. marcescens* strains, QMB1466 (Harpster and Dunsmuir, 1989; Jones *et al*, 1986), BJL200 (Brurberg *et al*, 1995; 1994), KCTC 2172 (Gal *et al*, 1997) and 2170 (Watanabe *et al*, 1997), had been cloned and sequenced. The nucleotide sequence encoding the 52 kDa chitinase gene of *S. marcescens* KCTC 2172 has been reported recently (Gal *et al*, 1998). *S. marcescens* 2170 has been chosen as a model organism, since this strain produces higher levels of chitinase activity and is more amenable to genetic analysis than the more extensively studied strain QMB1466. Four chitinases, A, B, C1, C2 and a 21 kDa chitin-binding protein (CBP21) lacking chitinsae activity were detected in the culture supernatant of *S. marcescens* 2170 (Watanabe *et al*, 1997; Suzuki *et al*, 1998). Chitinase C2 is a derivative of chitinase C1 generated by removal of the FnIII domain and the C-terminal CBD. (Suzuki *et al*, 1999). The three dimensional structure of chitinase A from strain QMB1466 was described by Perrakis *et al*, 1994.

9.3.2 Cloning of chitinase gene from *Bacillus*

Various species of *Bacillus* had been shown to secrete chitinase, including *B. circulans* (Watanabe *et al*, 1990), *B.licheniformis* (Takayanaki *et al*, 1991; Tantimavanich *et al*, 1997), and *B. cereus* (Pleban *et al*, 1997). The chitinase genes have been predominantly characterized from *B. circulans*. At least six major chitinases, A1, A2, B1, B2, C, and D have been found in the culture supernatant of *B. circulans* WL-12 when induced with chitin (Watanabe *et al*, 1990a). A *chiA1* gene of *B. circulans* WL-12 was cloned in *E. coli* and sequenced. Cloned chitinase produced in *E. coli* had an 8-amino acid sequence that was not found in the mature chitinase A1. This chitinase A1 showed a 33% amino acid match with chitinase A of *S. marcescens*. The signal peptide of chitinase A1 in *E. coli* was cleaved between Ala-32 and Leu-33, whereas the signal peptide in *B. circulans* WL-12 was cleaved between Ala-40 and Ala-41. A cloned *B. licheniformis* chitinase gene in *E. coli* also had more 5-amino acid residues at N-terminus in addition to the mature chitinase (Tantimavanich *et al*, 1997). This difference in cleavage site probably reflects the different protein export mechanisms in these two bacteria (Watanabe *et al*, 1990b). Chitinase A1 was detected only in the culture supernatant of day1 and day 2 cultures. The disappearance of chitinase A1 was accompanied with the occurrence of chitinase A2. The conversion of Chitinase B1 and B2 also occur in the same fashion. Among these chitinase, A1 and A2 had the highest activity in hydrolyzing colloidal chitin. Chitinase A1 has a strong affinity for insoluble chitin, whereas other chitinases except chitinase D do not. Chitinase A1 released predominantly chitobiose $(GlcNAc)_2$ and a trace amount of *N*-acetylglucosamine (GlcNAc). The other chitinases are much less active. The close proximity of *chiA* and *chiD* genes and the amino acid sequence similarity revealed an intimate relationship for the two chitinases. The N-termi-

nal 55-amino-acid segment (ND) in chitinase D was found to be highly homologous to the C-terminal 52 amino acid segment (CA) of chitinase A1. The 95 amino acid segment (R-D) located immediately adjacent to ND in chitinase D was found to be a close homolog of the R-1 and the 95-residue type III-like tandem repeats located next to the CA segment of chitinase A1. The 73 amino-acid sequence in the C-terminal two-thirds of chitinase D and the 78 amino acid segment in the N terminal two-thirds of chitinase A1 contained a high similarity with a wide range of other chitinase especially in positions 59 to 71 (Fig. 10). The ND/CA segment, the type III-like segment(s), and the remaining region in chitinase D lie in a direction opposite to that of the segments in chitinase A1 (Fig. 10). It was suggested that gene duplication occurred in a fairly complicated way in the course of evolution, giving to diversification of these two chitinase genes (Watanabe *et al*, 1992).

9.3.3 Cloning of chitinase genes from marine bacteria

The marine environment is variable in nutrient composition but is generally nutrient depleted in that most marine waters contain relatively little phosphate and are limited in carbon and nitrogen. In such an environment, chitin, mainly from zooplankton, provides a carbon and nitrogen for marine organisms. And the marine chitinolytic bacteria are responsible for biological recycling of chitin and play a major role in the ecology of marine environments (Techkarnjanaruk *et al*, 1997). A cluster of three closely linked chitinase genes organized in the order *chiA*, *chiB* and *chiC*, with the same transcriptional direction, and two unlinked genes, *chiP* and *chiQ*, involved in chitin degradation in *Pseudoalteromonas sp.* strain S91 were cloned, sequenced and characterized. The deduced amino acid sequences revealed that ChiA, ChiB and ChiC exhibited similarities to chitinases belonging to family 18 of the glycosyl hydrolases while ChiP and ChiQ be-

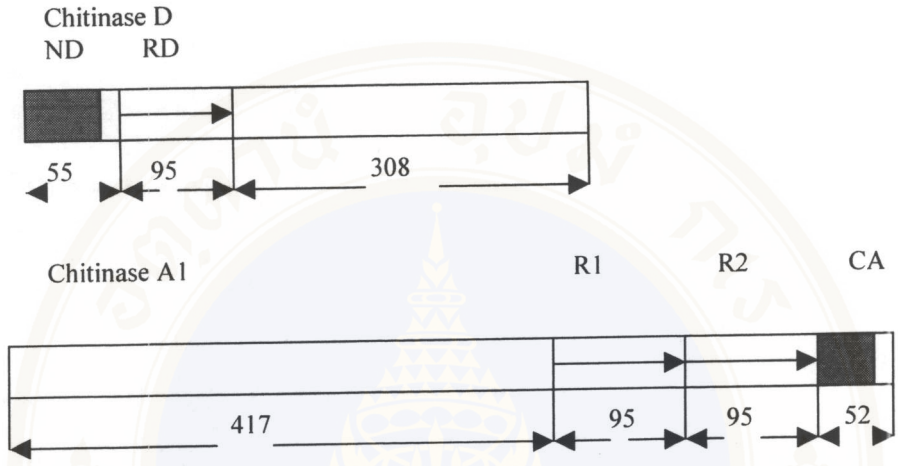

Fig. 10. Schematic representation of the domain structures deduced from the amino acid sequence features of the chitinase D and chitinase A1 mature proteins. Hatched boxes represent the N-terminal short segment (ND) of chitinase D and the C-terminal short segment (CA) of chitinase A1. Thick arrows indicate type III-like domains (R-1, R-2 and R-D). Numerals with arrows indicate the numbers of amino acid residues in the domains. (From Watanabe *et al*, 1992).

longed to family 20. ChiP and ChiQ showed different enzymatic activities against fluorescent chitin analogues, but neither was able to colloidal chitin (Techkarnjanaruk and Goodman, 1999).

The chitinolytic marine bacterium *Alteromonas sp.* strain O-7 produced at least three chitinases (ChiA, ChiB, and ChiC) and three different β-*N*-acetylglusaminidase (GlcNAcase) (GlcNAcaseA, GlcNAcaseB, and GlcNAcaseC) in the presence of chitin (Tsujibo *et al*, 1998; 1995; 1994; 1993; 1992). The genes encoding ChiA (Tsujibo *et al*, 1993), ChiC (Tsujibo *et al*, 1998), GlcNacaseB (Tsujibo *et al*, 1995), and GlcNAcaseC (Tsujibo *et al*, 1994) have been cloned and characterized.

V. harveyi contained six separate chitinase genes *chiA*, *chiB*, *chiC*, *chiD*, *chiE*, and *chiF* (Svitil *et al*, 1997) and two genes encoding chitin degradation enzymes have been at least partially sequenced (Jannatipor *et al*, 1987; Soto-Gil and Zyskind, 1989). The first gene cloned and sequenced encodes a chitobiase, which cleaves the bond joining the two *N*-acetylglucosamine units in chitobiase and was tentatively identified as the main chitinase of *V. harveyi* (Jannatipour *et al*, 1987). ChiA and ChiB are most likely endochitinases, which randomly cleave in the center of chitin and produce soluble oligosaccharides. The low activities were detected with ChiD, -E, and -F. It was suggested that these proteins are either chitodextrinases which catalyze hydrolysis of internal glycosidic bonds in soluble oligosaccharides or chitobiosidases (Svitil *et al*, 1997). It was found that this bacterium had a higher growth rate and produced more chitinase activity when grown on β-chitin than α-chitin. This probably because the parallel arrangement of strands in β-chitin permits easier access to the enzymes (Svitil *et al*, 1997). In a related marine bacterium, *V. furnissii*, two other enzymes (chitodextrinase and *N*-acetyl-β-glucosaminidase) involved in the complete degradation of chitin have been ex-

amined (Bassler *et al*, 1991).

9.3.4 Cloning of chitinase genes from *Aeromonas* species

The gene encoding extracellular chitinase from *Aeromonas hydrophila* had been cloned and expressed in *E. coli* using plasmid pJP2512 with a 3.9-kb chitinase-producing insert. The gene was transcribed from its own promoter, producing a protein of molecular mass 96 kDa. In this *E. coli* host, it was found that the chitinase enzyme could not traverse to the outer membrane but accumulated in the periplasmic space and cytoplasm. The region between *Cla* I and internal *Bam*H I site was responsible for the secretion of the protein across the cytoplasmic membrane (Chen *et al*, 1991). PCR technique was used to clone the gene encoding chitinase from *A. caviae* into *E. coli* BL21 (DE3) (Lin *et al*, 1997). Protein sequencing showed that the recombinant chitinase, as compared to the wild-type chitinase, contained additional 33 amino acids at its N-terminus and 13 amino acids at its C-terminus. The recombinant enzyme was secreted into the cytoplasm as well as into the culture medium (Chung-Saint *et al*, 1997). The gene encoding chitinase from *A. hydrophila* (pHYA1) and *Pseudomonas maltophila* (pHYB1, pHYB2 and pHYB3) were cloned into *E. coli* DH5α from the shuttle vector pHY300PLK (Wiwat *et al*, 1996).

10. Regulation of chitinase gene expression

Chitinase gene expression in microorganisms has been reported to be controlled by a repressor/inducer system in which chitin or other products of degradation act as inducers. The addition of carbohydrates, lipid, or proteins to chitin-grown cultures represses chitinase production (Felse and Panda, 1999). High levels of chitinases were found in cultures of *Metarhizium anisopilae* supplied with chitin but not with pectin,

xylan or cellulose. Chitinases and β-*N*-acetylglucosaminidases are secreted when the entomopathogens, *M. anisopliae*, *Beauveria bassiana*, *Verticilium lecanii*, and *Nomuraea rileyi*, are grown on insect cuticles (St Leger *et al*, 1986, Coudron *et al*, 1989). Chitobiase was always produced along with chitinase in all media and was not affected by catabolic repression. Chitobiase was very effective in degrading the dimers and trimers of *N*-acetylglucosamine thus contributing to the release of chitinase inducers (St Leger *et al*, 1986). In batch cultures of *S. marcescens*, a larger amount of chitinase was produced in the presence of chitin than in the presence of *N*-acetylglucosamine (Monreal and Reese, 1969; Young and Carroad, 1981). It was suggested that chitinase of *S. marcescens* is regulated by *N*-acetylglucosamine induction and catabolic repression in much the same way as the chitinase gene is regulated in *M. anisopilae*. But chitinase gene of *Aphanocladium album* E3 mutant was induced by *N*-acetylglucosamine while it was repressed by glucose. Ulhoa and Peberdy (1991) suggested that the signal for the induction of chitinase may result from physical contact between the cell surface and the insoluble substrate. This suggestion implied that the organisms producing chitinase should have a receptor on their surface specific to chitin. They also observed that induction of chitinase was inhibited by 8-hydroxyquinoline (a RNA inhibitor) and cyclohexamide (a protein synthesis inhibitor), thus establishing that the induction of chitinase was dependent on *de novo* transcription and translation of proteins (Ulhoa and Pederby, 1991). However, no experimental verification has proved the involvement of cell signaling in the expression of this enzyme. Repression of chitinase synthesis by glucose in almost all organisms indicated that catabolite repression may be involved in the regulation of microbial chitinase genes. The gene encoding for CHIT33 gene in *Trichoderma harzianum* CECT 2413 was strongly repressed by glucose and derepressed under starvation conditions or

its oligomers. It was suggested that post-transcriptional regulation might be involved. However, high proteolytic activity reported under similar conditions may also be the reason for low levels of chitinase (Limon *et al*, 1995).

Several chitinases can derive from a larger primary gene product by posttranslational proteolytic cleavage as in *Streptomyces olivaceoviridis* (Romaguera *et al*, 1992). In *Janthinobacterium lividum*, a 56-kDa extracellular chitinase was derived from a 69 kDa chitinase (Gleave *et al*, 1995). The multiplicity of chitinases in *B. circulans* is due to proteolytic modification of one or two precursor proteins (Watanabe *et al*, 1990a). In *S. marcescens* KTCT2172, a 35 kDa chitinase derived from 52-kDa chitinase by post-translational proteolytic modification (Gal *et al*, 1998). Chitinase A2 of *B. circulans* WL-12 was truncated form of chitinase A1 by proteolytically removal the C-terminal portion. It was suggested that multiple chitinases with different properties are necessary for an efficient synergistic hydrolysis of chitinous substrates, a situation analogous to that encountered in the degradation of cellulose by cellulases. In addition to the synergistic effects on a particular substrate, different chitinases may also be beneficial for the digestion of various types of chitin characterized by different degrees of acetylation, crystallinity, crystalline form, etc (Suzuki *et al*, 1999).

11. Chitinase as biopesticide

Insects themselves produce chitinase. The chitinase gene is not expressed by insect during feeding periods (Kramer *et al*, 1993) and the protein is detected only during a narrow period just prior to mouting (Zen *et al*, 1996). If the insect is continually exposed to chitinase, chitin-containing structures such as the peritrophic membrane might malfunction due to chitin degradation and loss of structural integrity.

Chitinase expression in the insect gut normally occurs only during moulting, where the chitin of the peritrophic membrane is presumably degraded. Thus, insects feeding on plants that constitutively express an insect chitinase gene might be adversely effected, owing to an inappropriately timed exposure to chitinase. Plant resistance to fungal pathogen could be enhanced by introducing of a chitinase transgene (Broglie *et al*, 1991; Vierheilig *et al*, 1993; Lin *et al*, 1995; Zhu *et al*, 1994; Jach *et al*, 1995). Hen egg white lysozyme, an enzyme with chitinolytic activity, was isolated from transgenic tobacco expressing it and shown to inhibit the growth of some bacterial and fungal plant pathogens (Trudel *et al*, 1995). If the expression level of the insect chitinase is properly managed it appears that the enzyme could be utilized to promote increased protection of transgenic plants toward insect and microbial pests (Kramer *et al*, 1996). *Manduca* chitinase transgene could enhance resistance to budworm neonates feeding on tobacco (Ding *et al*, 1998). However, the mechanism of resistance is still unknown. It was suggested that chitinase might directly toxic to larvae, enhance sensitivity to existing microbes or dietary components, or elevate or induce other defensive mechanisms in the plant, which are, in turn, toxic to the larvae. The truncated chitinase from transgenic tobacco is toxic to the merchant grain beetle, *Oryzaephilus mercator*, in an artificial diet (Wang *et al*, 1996).

Bacterial chitinases are generally ineffective in assays when some types of insects are fed diets containing the enzymes. No mortality of the nymphal stages of the rice brown plant hopper, *Nilaparvata lugens*, occurred when 0.09% (w/v) *S. griseus* chitinase was added to an artificial diet (Powell *et al*, 1993). Similarly, *Serratia* and *Streptomyces* chitinases at 1-2% levels in the diet of the merchant grain beetle, *Oryzaephilus mercator*, caused no mortality. The inactivity of many of these chitinase preparations can be explained by the presence of primarily exo- instead of endo-cleaving enzymes, the former

being substantially less effective than the latter in degrading chitin (Kramer and Muthukrishnan, 1997). However, chitinases purified from some bacteria can disrupt the peritrophic membrane of the insect gut (Regev *et al*, 1996). Chitinase from *Streptococcus grieseus* could elevate blood lysozyme in hemolymph of the greater wax moth (Jarosz *et al*, 1987). A potential site of action of chitinase is the peritrophic membrane, which is a thin, membranous sac that encloses the food in the midgut and hindgut of most insects (Richards and Richards, 1977).

12. Synergism between chitinases and *B. thuringiensis*

Chitin occurs in insects as a major component of the cuticle and of the peritrophic membrane, a protective sleeve lining the gut of many insects. Peritrophic membrane provides a physical barrier against mechanical damage and invasion of microorganisms (Brandt *et al*, 1978; Dow, 1986; Terra, 1990). Isolated peritrophic membranes were shown to limit penetration of dissolved δ-endotoxin in vitro (Yunovitz *et al*, 1986). Increasing the level of endochitinases in the larval midgut may elevate the larvicidal effect as a result of peritrophic membrane perforation and increase accessibility of the δ-endotoxin molecules to the epithelial membranes (Regev *et al*, 1996). Pathogens that infect through the gut must penetrate this chitin-rich barrier. Chitinase activities may play key roles in the virulence of some pathogens that infect insects via the peritrophic membrane, including the malarial parasite *Plasmodium gallinaceum* (Huber *et al*., 1991) the trypanosome, *Leishmania* (Schlein *et al*, 1991), the nematode *Brugia malayi* (Fuhrman *et al*, 1995), the nucleopolyhedrovirus *Autographa californica* (Hawtin *et al*, 1997) and *B.t.* (Sampson and Gooday, 1998).

Microbial chitinases have been used in mixing experiments to increase the po-

tency of entomopathogenic microorganisms. Synergistic effects among chitinolytic enzymes and microbial insecticides have been known to occur since the early 1970s. Microbial chitinases can kill or retard growth of certain insects when combined with a baculovirus (Shapiro *et al*, 1987), *B.t.* (Smirnoff, 1971; 1974; Morris 1976; Wiwat *et al*, 1996; Tantimavanich *et al*, 1997), or *B.t.* spore crystals (Sneh *et al*, 1983). Traces of the enzyme chitinase in *B.t.* preparations administered to spruce budworm larvae accelerated development of septicemia in insect and increased the mortality rate (Smirnoff, 1971). Co-application of *B.t.* (Sandoz-Wander preparation 26B or Dipel 36B) and chitinase significantly increased the insecticidal effect of *B.t.* on *Choristoneura fumiferana* larvae (Smirnoff, 1977). Similarly, application of low concentrations of a mixture of spore crystal suspension from *B.t.* subsp. *entomocidus* and chitinolytic bacteria resulted in a significant synergistic insecticidal effect against *Spodoptera littoralis* larvae (Sneh *et al*, 1983). An enhanced toxic effect towards *S. littoralis* also resulted when a combination of low concentrations of a truncated recombinant CryIC toxin and a bacterial endochitinase ChiAII were incorporated into a semisynthetic insect diet (Regev *et al*, 1996). Pore formation in the peritrophic membrane was observed even at a chitinase concentration as low as 0.1 μg/ml. The effective concentrations of CryIC and ChiA mixtures are within the expression range of a single transgene in a transgenic plant (Ely, 1993). It is very likely that cointroduction of *cry* and *chiA* genes into transgenic bacteria or plants could increase the insecticidal efficacy of low concentations of transgenic, self-folded δ-endotoxins which lack the protection against the larval midgut proteases provided by the natural folding in the crystalline structure (Regev *et al*, 1996). Crude chitinase preparations from *B. circulans* enhanced the toxicity of *B.t. kurstaki* towards diamondback moth larvae (Wiwat *et al*, 1996). It was suggested that a compromised peritrophic membrane barrier facili-

tated the *B.t.* toxin synergism with chitinases by enhancing contact of toxin molecules with their epithelial membrane receptors (Regev *et al*, 1996). Furthermore, the involvement of chitinolytic activities during pathogenesis in insects had been investigated with *B.t.i.* IPS78 against larvae of the midge *Culicoides nubeculosus*, and with *B.t.a.* HD133 against caterpillars of the cotton leafworm *S. littoralis*. Inhibition of chitinase activities from supernatant extracts of the bacterial strains by allosamidin varied depending on the substrate used. The IC_{50} values of both strains were about 50 μM with colloidal chitin and between 1 and 10 μM with 4-methylumbelliferyl-diacetylchitobioside and 4-methylumbelliferyl-triacetylchitotrioside, respectively. Differences might be due to *B.t.* producing a mixture of chitinase with different affinities for allosamidin. Allosamidin (100 mM) had a strong inhibitory effect on the toxicity of both *B.t.i.* against larvae of *C. nubeculosus* and *B.t.a.* HD133 against larvae of *S. littoralis*. The allosamidin itself had no discernible effect on the larval stages of growth of either *C. nuberculosus* or *S. littoralis*. Addition of *S. marcescens* chitinase enhanced the insecticidal activity and the killing rate of both bacterial strains against their susceptible larvae (Sampson and Gooday, 1998). These results reinforce observations that the addition of commercial chitinase preparations enhances insecticidal activity of *B.t.* (Smirnoff, 1974; Fast, 1978; Shapiro *et al*, 1987). The precise mechanism through which chitinase exertes its effect on insect pathogenesis is still unclear. One suggestion is that microbial chitinases partially digest the peritrophic membranes allowing more ready access of the bacterial toxins to the gut epithelia.

13. Background information about TP-1 chitinase gene and enzyme

B.licheniformis TP-1 was an aerobic, Gram positive bacilli which formed subterminal elipsoidal spore. It was classified in *Bacillus* group BII. This strain was a strong chitinase producer with the enzyme activity about (2.25 mU/ml). The enzyme production required chitin for induction. An enzyme could hydrolyse chitin at pH ranging from 9-11 (Tantimavanich, 1997). Young cultures (6-7 h) of *B.licheniformis* TP-1 gave 3 enzyme activity bands after renaturation in SDS-PAGE with estimated molecular masses of 68, 62 and 50 kDa (named Chi68, Chi62, and Chi50). But old cultures produced only a single chitinase band of 50 kDa. From N-terminal amino acid sequences data, indicated that all three chitinase processing forms derived from a single gene by deletion at the C-terminus. Chi68 contained chitin binding domain at the C-terminus, while it is absence in Chi62 and Chi50. This domain was found to play a role for complete hydrolytic activity towards colloidal chitin (Tantimavanich *et al*, 1998). A chitinase gene from TP-1 strain was cloned in *E. coli* (pCHIL3) and subsequently subcloned in *B.t.a.* (pCHIL3-16). The nucleotide sequence revealed a single open reading frame of 1,815 bp, which encoded 604 amino acids with a signal peptide of 35 amino acids. From the deduced amino acid sequence, TP-1 chitinase showed highest homology (53%) to chitinase A-1 of *B. circulans* WL-12 including conserved amino acids residues, Asp-196 and Glu-200 (Watanabe *et al*, 1990; 1993). The chitinase enzyme in both *E. coli* and *B.t.a.* was constitutively expressed. A combination of chitinase and *B.t.a* at a sub-lethal dose could retarded growth and development of *S. exigua*. The transformed *B.t.a* strain harbouring pCHIL3-16 slightly improved the larvicidal activity against *S. exigua* due to the low chitinase production. The larvicidal activity might be improved by further gene manipulation to overexpress the enzyme (Tantimavanich *et al*, 1997).

CHAPTER III

MATERIALS AND METHODS

1. Chemical and reagents.

All of chemicals and reagents used in this study were analytical grade or the pure grade available. Chemicals were purchased from Sigma and Merck (Germany). Agarose was obtained from Gibco BRL (USA). Bacterial culture media were purchased from Difco, Merck (Germany) and Gibco BRL (USA). Antibiotics were purchased from Sigma (USA). Restriction enzymes and other modifying enzymes were obtained from Gibco BRL (USA) and New England Biolab (USA). Taq polymerase and dNTP were obtained from Perkin Elmer (USA). Oligonucleotide primers were synthesized by Eppendorf (USA) and Bioservices Unit (BSU, Thailand). PCR cloning kit was derived from Invitrogen (USA). Plasmid extraction and Qiagen gel extraction kits were obtained from Qiagen (USA). Sequencing kit was purchased from USB (USA). Digoxigenin (Dig) detection kits were purchased from Boehringer Mannheim (Germany). Protein molecular weight marker and western blot kit were obtained from Biorad (USA). Radioisotope deoxyadenosine 5'-[α-35S]-thiotriphosphate (35S-dATP), 10 mCi/ml and deoxycytidine 5'-[α-32P]-triphosphate (32P-dCTP), 10 mCi/ml were purchased from Dupont NYR (USA).

2. Bacterial strains, culture condition and bacterial storage

All bacterial strains used in this study were listed as the following.

1. *E. coli* DH5α [*deo*R*end*A1*gyr*A96*hsd*R17($r^{k-}m^{k+}$)*rec*A1*sup*E44thi-1Δ (*lac*ZYA-*arg*FV169)ϕ80*lac*ZΔM15F-] was used as a host for cloning and subcloning experiments.

2. *B. thuringiensis* subsp. *israelensis* 4Q2-72 and c4Q2-72 was used as a host for final subcloning of transcriptional fusion constructs of *p19-cry11Aa1* and chitinase genes.

All *E. coli* strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C overnight with shaking. *B.t.i.* 4Q272 and c4Q272 were grown in nutrient broth supplemented with salt, NYSM (Li and Yousten, 1975) which composed of nutrient broth 8 g/l, 1 mM $MgCl_2$, 0.7 mM $CaCl_2$ and 0.5 mM $MnCl_2$. Agar was added to make 1.5% for solid medium. Antibiotics were supplemented as the following concentration; 50 μg/ml amplicillin and 15 μg/ml tetracycline.

For long time storage, 600 μl an overnight culture were transferred to sterile vial containing 300 μl 45% glycerol and kept at -70 °C.

3. Plasmid vectors

3.1 Plasmid pCR 2.1 vector (Invitrogen, USA) was used for cloning of PCR products in *E. coli.*

3.2 Plasmid pBluescriptSK(+) (Strategene, USA) was used as an alternative vector for subcloning of *p19* gene in *E. coli.*

3.3 Plasmid pBC16 (Bernhard *et al.*, 1978) was used as a vector for subcloning of transcriptional fusion plasmid in *B.t.i.* .

4. General procedures for DNA isolation

4.1 Plasmid preparation from *E.coli*

Small scale plasmid DNA from *E.coli* was prepared by rapid alkaline method as described by Birnboim and Doly (1979).

For high purity of large scale plasmid DNA preparation, Qiagen plasmid midi kit (Qiagen, USA) was used, the procedure was followed as indicated in the kit manual.

4.2 Plasmid preparation from *Bacillus*

Alkaline lysis method (Birnboim and Doly, 1975) was used for both small scale and large scale plasmid extraction from *Bacillus*. The procedure was done similarly to that described in *E.coli* except 2 μg/ml of lysozyme was added to solution I (50 mM glucose, 25 mM Tris pH 8.0 and 1 mM EDTA pH 8.0) and incubated for 1 h at 37°C. Phenol/Chloroform extraction was done to remove protein contamination. For large scale plasmid preparation, the volume of any reagent were increased proportionally to the volume of bacterial culture.

5. General DNA analysis procedures

5.1 Restriction endonuclease digestion

Digestion or modification of plasmids with various restriction and modifying enzymes were performed by using appropriate buffers provided with the enzyme. Restriction endonuclease digestion was accomplished by incubating the enzyme(s) with DNA in appropriate reaction condition as recommended by manufacturers.

5.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a horizontal submarine gel electrophoresis apparatus (Mupid, Japan). The DNA sample was mixed with equal volume of loading dye (0.25% bromophenol blue, 0.25% Xylene cyanol, 30% glycerol in H_2O). The concentration of agarose gel ranging from 0.7-1.2% was prepared and electrophoresed in 1xTBE buffer (0.045M Tris-borate, 0.001 M EDTA). After electrophoresis (at 50-100 volts), gel was stained in 0.5 μg/ml Ethidium bromide and visualized under UV transilluminator (WL 300-310). Photographs was taken using Polaroid camera and type 667 Polaroid film. Lambda DNA digested with *Hind* III or ϕX 174 digested with *Hae* III were used as standard markers to estimate the molecular size of the DNA fragments.

6. Transformation of recombinant plasmid into *E.coli* host

The competent *E. coli* was performed according to method developed by Hanahan (1983) and stored at -70°C until used. Transformation of plasmid into competent *E. coli* was performed according to the method described by Sambrook *et al* (1989).

7. Transformation of recombinant plasmid in to *Bacillus* host by electroporation

7.1 Preparation of electroporated cell

The electroporation method of Matsuno *et al* (1992) was used to transform DNA into *Bacillus* spp. A fresh single colony of *Bacillus* strain was precultured in 5 ml LB broth, grown overnight at 37 °C with shaking. A one ml of preculture was transferred to 100 ml LB broth containing in a 500 ml flask, and incubated at 37 °C for 3-4 h until exponential phase. Cells were pelleted by centrifugation at 8000 rpm, 5 min. The pellet

was washed in 10 ml of 1 mM HEPES (N-2-hydroxyethyl piperazine-N'-ethanesulfonic acid) pH 7.0, incubated on ice for 30 min, centrifuged at 3000 rpm, 10 min. The pellet was washed in another 10 ml of the same buffer. Subsequently, cells were washed once with 10 ml of PM buffer (10% polyethylene glycol or PEG 6000, 0.1M mannitol) and resuspended in 200-300 µl of PM buffer. Aliquote of 40 µl were transferred to steriled Eppendorf tubes and stored at -70 °C or freshly used for electroporation.

7.2 Electroporation of *Bacillus* spp.

A 40 µl of electroporated cells was mixed with 0.5-1 µg of plasmid DNA which had been resuspended in 1-5 µl of steriled deionized water. An equal volume of 40% PEG 6000 was added mixed and incubated on ice for 5 min. Then 20 µl of the mixture was transferred to a steriled prechilled cuvett (inter-electrode distance 0.2 cm) and placed into sample chamber of Gene pulser apparatus (Gibco BRL, USA). The electroporation condition was setted at 2.5 kV, 400 Ω and 330 µF at a fast charge rate. Following the pulse, the electroporated cells were transferred immediately to 1 ml LB broth and incubated at 37 °C with shaking for 2 h. The transformants were selected by plating on LB agar supplemented with appropriated antibiotic and incubated overnight at 37 °C overnight.

8. DNA sequencing

The analysis of DNA sequence was performed using dideoxy chain termination method (Sanger, 1977) using Sequenase version 2.0 kit (Amersham, UK) and 35S-dATP (Amersham, UK) with T7 and RT sequencing primers.

8.1 Sequencing reaction

Plasmid DNA was denatured by adding 0.1 volumes of 2 M NaOH, 2 mM EDTA and incubated at 37°C for 30 min. The mixture was neutralized by adding 0.1 volumes of 3 M sodium acetate pH 4.5-5.5. Then, DNA was precipitated with 2.5 volumes of cold absolute ethanol, incubated at -70 °C, for 15 min. After washing the pelleted DNA with 70% ethanol and dried at room temperature, DNA was redissolved in 7 µl of steriled distilled water. Then, 2 µl of T7 Sequenase reaction buffer and 1 µl of primer were added and DNA was allowed to anneal by heating at 65 °C for 2 min and slowly cool down to < 35 °C.The annealed DNA mixture was added to labeling reaction containing 1 µl of 0.1 M DTT, 2 µl of 5 folds diluted labelling mix, 0.5 µl of α-35S dATP and 1 µl of diluted T7 Sequenase polymerase (1 µl of Sequenase polymerase was mixed with 0.5 µl of inorganic pyrophosphatase in 6.5 µl enzyme dilution buffer). The labeling reaction was incubated at room temperature for 2-5 min and transferred to 3.5 µl of each termination reaction (ddGTP, ddCTP, ddATP, ddTTP) which had been preincubated at 37 °C. The reactions were further incubated at 37 °C for 5 min and 4 µl of stop solution was added and kept at -20 °C for loading the gel.

8.2 Preparation of sequencing polyacrylamide gel

The 6% sequencing gel was prepared (5.7 g acrylamide, 0.3 g N, N'-methylene bis acrylamide, 42 g urea, 10 ml of 10xTBE buffer, adjusted the volume to 100 ml with distilled water), filtered through 0.45 µm membrane filter and de-gased. 0.5-0.1 g of ammonium persulfate and 25 µl of TEMED (N, N, N', N'- tetramethylethylenediamine) were subsequently added and mixed well before pouring the gel. The gel was allowed to polymerize at least 4 h prior to used.

8.3 Sequencing gel electrophoresis

The sequencing reactions were heated at 75 °C for 2 min, immediately chilled on ice and loaded to the pre-run gel. Gel was pre-run at 50 watts for 30 min with 1xTBE buffer. After the samples were loaded into the well, the gel was run under the same condition. Then gel was fixed with 10% methanol and 10% acetic acid for 15 min, blotted to 3MM Whatman paper, dried under vacuum and exposed to Kodak X-ray film. Then, film was developed in developing solution and fixing solution (Kodak) which had been prepared as recommended by the manufacturer.

9. Southern blot DNA hybridization

9.1 Southern transfer of DNA onto nylon membrane

After gel electrophoresis was completed, the gel was stained with ethidium bromide and photographed with a ruler laid alongside in order to subsequently identify the bands positions on the membrane. The gel was treated by soaking in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neatralized by soaking in neutralizing solution (0.5 M Tris pH 7.4, 1.5 M NaCl) for 30 min with gentle agitation. The DNA was blotted to a nylon membrane by upward capillary action as described by Sambrook *et al* (1989). Then, the blotted membrane was rinsed briefly in 2 x SSC and fixed by UV cross-linker.

9.2 Recovery of DNA fragment from agarose gel to be used as a probe

The QIAEXII gel extraction kit (Qiagen, USA) was used to recover DNA fragment from agarose gel. The procedure was followed as suggested by the kit handbook. The gel containing DNA band of interest was excised from the agarose gel and weighted. 3 volume of buffer QX1 was added to 1 volume of gel (for example, add 300 µl to each

100 mg of gel). The QIAEXII was resuspended by vortexing for 30 sec before adding to the sample (add 10 µl / 2 µg of DNA, 30 µl / 2-10 µg of DNA), incubated at 50°C for 10 min and mixed by vortexing every 2 min. The sample was spinned for 30 sec and discarded the supernatant. The pellet was washed with 500 µl of buffer QX1 and then washed with 500 µl of buffer PE. The pellet was air-dried for 10-15 min. DNA was eluted by adding 20 µl of TE buffer, vortexing and incubated at 50°C for 5 min. After centrifugation, the supernatant was transferred to a new tube and kept in the refrigerator for later used.

9.3 Southern blot DNA hybridization with radioactive probe

9.3.1 Radioactive labeling of DNA probe

The Random primed DNA labeling method was performed using Radprime labeling kit (Gibco BRL, USA). Plasmid containing the DNA fragment to be labeled was digested with the appropriate restriction endonuclease. The DNA fragment was excised, recoveries and purified from agarose gel as described previously. The probes for *p19* gene and *cry11Aa1* gene derived from 0.4 kb *Nsi* I fragment of *p19* gene and 1.4 kb *Sca* I fragment of *cry11Aa1* gene, respectively. For chitinase probe, it is derived from 0.9 kb *Pst* I/*Sac* I fragment of chitinase gene. These linear DNA molecules were denatured by boiling for 5 min, chilled briefly on ice and added to the reaction mixture containing 20 µl 2.5x Radprime buffer, 1 µl 500 µM dATP, dGTP, dTTP, 5 µl α-^{32}P dCTP (3000Ci/µmol), adjusted volume to 49 µl with steriled deionized distilled water. Then, 1 µl of klenow enzyme was added, mixed and incubated at 37 °C, 10 min. Finally, 5 µl the stop reaction buffer was added and kept on ice for used in the following hybridization steps.

9.3.2 Hybridization analysis of a DNA blot with a radiolabeled DNA probe

The blotted membrane was incubated in a prehybridization solution containing reagents that block non specific DNA binding sites on its surface (6xSSC, 0.1% ficoll, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin, 5 mM EDTA pH 8.0, 0.5% SDS, and 20 mM Tris pH 7.6). The prehybridization was carried out at 65 °C for 2 h with gentle agitation. Then, the prehybridization solution was discarded and replaced with a new solution. The labeled DNA was denatured by boiling for 5 min, immediately cool on ice and subsequently added. The hybridization was further carried out at 65 °C overnight with gentle agitation. Then, membrane was washed twice with 2xSSC, 0.1% SDS at 65 °C, 10 min. The membrane was wrap in a plastic saran wrap and exposed to X-ray film (Kodak, USA). The film was developed in a developer and a fixer solutions (Kodak, USA) according to manufacturer's recommendation.

For rehybridization of Southern blot, the membrane must not be completely dried after hybridizing with the first DNA probe. Hybridized probe DNA was stripped from the membrane by boiling in 0.02% SSC, 0.01% SDS for 15 min. Then, the membrane was ready for reprobing or kept dry between 3 MM Whatman paper and stored in dessicator for later used.

10. Partial purification of recombinant TP-1 chitinase enzyme

The partially purified and concentrated chitinase enzyme from all bacterial strains were analyzed for chitinase activity on SDS-PAGE and western blotting. The parental *B. licheniformis* TP-1 was precultured in 5 ml NYSM broth supplemented with 0.1% colloidal chitin for enzyme induction. The bacterial preculture was inoculated to obtained

2% inoculum into 20 ml of the same medium, incubated at 37°C with shaking for 48 h. For *E. coli* transformants, the bacterial strains were precultured on a rotary shaker at 37°C overnight in LB broth supplemented with 50 μg/ml amplicillin (without colloidal chitin induction). *B.t.i.* 4Q2-72, c4Q2-72 and their transformants were precultured on a rotary shaker at 37°C overnight in NYSM broth supplemented with 15 μg/ml tetracycline (without colloidal induction). A 0.4 ml of *E. coli* and *B.t.i.* bacterial precultured were transferred to 20 ml of the same medium and incubated at 37°C with shaking for 24 h and 48 h, respectively.

The culture supernatant containing chitinase was collected by centrifugation at 6,000 rpm for 20 min at 4°C using a Sorvall RC-5 superspeed refrigerated centrifuge (Dupont, USA). The supernatant was concentrated by ammonium sulfate precipitation. Solid ammonium sulfate (9.44 g) was slowly dissolved in 20 ml of culture supernatant in ice bath to get 70% saturation and kept standing at 4°C for overnight. The enzyme was collected as a precipitate after centrifugation at 8,000 rpm for 30 min. Then, the pellet was dissolved in steriled distilled water and subsequently dialyzed overnight against 3 changes of cold distilled water. The dialysate was centrifuged at 10,000 rpm for 20 min to remove undissolved portion. The enzyme volumes of all culture supernatant were finally adjusted to 1 ml with distilled water and kept at -20°C for later used.

11. Chitinase assay and detection

11.1 Detection of chitinase enzyme on colloidal chitin plate

The chitinase producing transformants were screened by conventional plate assay using colloidal chitin as substrate. The capability of chitinase in hydrolyzing insoluble chitin resulted in a clear halo zone formation around the bacterial colony. The

transformants was replicated on LB agar containing appropriate antibiotic which had been overlaid with 2.0% colloidal chitin and incubated at 37^{0}C. The clear halo zone was observed everyday for at least 2 days.

11.2 Quantitative assay of chitinase activity by colorimetric spectrophotometry

Chitinase activity was measured quantitatively by determining the amount of *N*-acetylglucosamine (GlcNAc), the product of the enzymatic reaction, using the method of Reissig *et al* (1955). Proper diluted chitinase enzyme was reacted with colloidal chitin substrate resulting in GlcNAc and chito-oligomers end product. The production of color resulted from two independent processes: the formation of an intermediate compound, possibly a glucoxazoline, formed by heating the acetylhexosamine with alkali, and the reaction of this intermediate with p-dimethyl aminobenzaldehyde (pDAB) during the development of color in an acid medium (Reissig *et al*, 1955).

The enzyme assay was performed as the following. A 200 µl of enzyme crude extract or culture supernatant was reacted with equal volume of 5% colloidal chitin in 200 mM sodium acetate pH 5.0, incubated at 37^{0}C for 30 min. The reaction mixture was centrifuged at 10,000 rpm, at 4^{0}C, for 10 min. A 200 µl of collected supernatant or GlcNAc standard solution were boiled in 40 µl of 0.8 M potassium tetraborate pH. 9.1, for 3 min, followed by reacting with 1.2 ml 1xpDAB solution (10xpDAB solution : 1 mg pDAB, 12.5 ml 10 N HCl, 87.5 ml glacial acetic acid, diluted to 1xpDAB with glacial acetic acid immediately before using) and further incubated at 37 ^{0}C for 20 min. The colour product was measured spectrophotometrically at 585 nm and calculated for the amount of GlcNAc-equivalent using a standard curve of GlcNAc. The standard curve of GlcNAC was plotted between concentrations of GlcNAc ranging from 0.05-0.5 µmoles

versus their corresponding absorbance measured at 585 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmole of GlcNAc or its quivalent per minute.

11.3 Detection of chitinase activity on SDS-PAGE

The chitinase activity was detected on SDS-PAGE based on the method of Trudel and Asselin (1989). A 10% denaturing PAGE was performed according to the method of Laemmli (1970). The sizes of chitinase activity bands were determined by comparing with the coomassie blue-G250 staining of standard protein molecular weight markers which were loaded in the same gel.

11.3.1 Gel preparation and electrophoresis operation

A 10% polyacrylamide gel was prepared and casted as a seperating gel by mixing 6.6 ml of solution A [(29.2% (w/v) acrylamide, 0.8% N, N'-methylene bis acrylamide)], 5.0 ml of solution B (1.5 M Tris-Cl pH 8.8, 0.4% SDS), 200 μl of freshly prepared 10% (w/v) ammonium persulfate, 8 μl of TEMED, 200 μl of 1% glycol chitin and adjusted the volume to 20 μl with water. Gel was allowed to completely polymerized for at least 30 min. Then, 4% of stacking gel was prepared by mixing 1.3 ml of solution A, 2.5 ml of solution D (0.5 M Tris-Cl pH 6.8, 0.4% SDS), 60 μl of 10% (w/v) ammonium persulfate, 10 μl of TEMED, adjusted the volume to 10 ml with distilled water and poured on top of seperating gel. After inserting the comb, the stacking gel was allowed to polymerize for 30 min. The comb was removed and all the wells were flushed several times with distilled water to eliminate unpolymerized acrylamide.

The slab gel was set with the electrophoresis apparatus (Hoeffer, USA), electrophoresed in 1x running buffer (0.025 M Tris-Cl pH 8.3, 0.192 M glycine, 0.1%

SDS) at constant current (25 mA) for 4-5 h until the tracking dye reached the bottom of the gel.

11.3.2 Sample preparation

For sample preparation, 10 µl of 20X concentrated enzyme from *B.t.i.* 4Q2-72 and *B.t.i.* c4Q2-72 and 1 µl of 20X concentrated enzyme from their transformants (mixed with 9 µl of distilled water) were mixed with 3.3 µl of 4 X sample buffer (125 mM Tris.Cl pH 6.7, 2.5% SDS, 2% β-mercaptoethanol, and 0.01% bromphenol blue). Ten µl of these mixtures were boiled for 5 min before loading the gel. Protein molecular weight markers (Biorad, USA) was used which has size ranging from 31.0 to 97.4 kDa.

11.3.3 Renaturation and detection of chitinase activity on SDS-PAGE

After complete electrophoresis, gel was renatured in 100 mM sodium acetate buffer pH 6.0 containing 1% (v/v) Triton X-100 and incubated in shaking waterbath at 37^{0}C, overnight. The gel was washed 2-3 times with distilled water, stained with brightener solution [0.01% (w/v) Calcofluor white M2R in 0.05 M Tris-Cl pH 8.9] and destained by shaking in distilled water several times. The chitinase activity bands were visualized for nonfluorescent band of digested glycol chitin on UV transluminator, photographed and compared to standard molecular weight markers.

12. Protein assay and detection

12.1 Quantitative determination of protein concentration

The amount of protein was determined with Bradford reagent (Biorad, USA).

The colour was measured spectrophotometrically at 595 nm. The protein concentration was calculated and expressed as mg/ml comparing with a standard curve of bovine serum albumin.

12.2 Determination of protein molecular weight on SDS-PAGE

Fifteen µl of 20X concentrated enzyme were mixed with 5 µl of 4 X sample buffer (125 mM Tris.Cl pH 6.7, 2.5% SDS, 2% β-mercaptoethanol, and 0.01% bromphenol blue). The mixtures were boiled for 5 min before loading the gel. After complete electrophoresis, gel was stained in staining solution [0.2% (w/v) coomassie brilliant blue G-250 in 50% (v/v) methanol, 10% (v/v) acetic acid] for a few hours or overnight. Then, gel was destained several times in destaining solution I [40% (v/v) methanol, 10% (v/v) acetic acid] until observing the protein bands. Gel was further destained in solution II [5% (v/v) methanol, 7% (v/v) acetic acid] and kept in 7% (v/v) acetic acid. The molecular weight of proteins were determined by comparison with the standard protein marker (Biorad, USA).

12.3 Western blot analysis

Western blot analysis was performed to confirm the production of chitinase enzyme and Cry11Aa1 toxin. The protein crude extract or culture supernatant was electrophoresed through 10% SDS-PAGE. Proteins were electro-transferred from the gel to PVDF membrane and reacted with first and second antibody. The colour reaction was developed with the substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

12.3.1 Sample preparation

Protein samples for detection of TP-1 chitinase were prepared as described in sample preparation for chitinase activity staining in SDS-PAGE. For detection of Cry11Aa1 toxin, 0.5 ml of a 48 h cell culture was centrifuged at 10,000 rpm, for 5 min and resuspended in 10 µl of deionized water. The bacterial suspension was mixed with 3.3 µl of 4XSDS sample buffer and boiled for 5 min before loading the gel.

12.3.2 Protein blotting to PVDF membrane

Ten µl of proteins mixtures were run through 10% SDS-PAGE and blotted to PVDF membrane (Sambrook *et al*, 1989). Six sheets of Whatman 3MM paper and a PVDF membrane were cut equally to the gel size. The membrane was briefly soaked in methanol for 20 sec and transferred the pretreated membrane and Whatman3MM papers into transferred buffer (25 mM Tris, 192 mM glycine pH 8.3, 20% methanol) and soaked for 30 min. After completely electrophoresis, gel was also soaked in the transferred buffer for 15-30 min. Three sheets of presoaked Whatman 3MM paper were placed on the anode side of protein transferred cassette, overlaid with PVDF membrane, gel and another 3 sheets of Whatman3 MM paper, respectively. The cassette was placed in the buffer tank. The buffer tank was placed in the ice chamber and applied constant current (60-70 mA/ 1 gel) for 2 h.

12.3.3 Detection of protein on PVDF membrane

After transferring the protein to PVDF membrane, proteins on membrane were detected using digoxigenin non-radioactive detection system (Boehringer Mannheim, Germany). The membrane was incubated in TBST solution (10 mM Tris-Cl

pH 8.0, 150 mM NaCl, 0.1% tween20) containing 5% BSA for 30 min with shaking at room temperature. The membrane was washed for 5 min each for three times in TBST solution. Then, the membrane was incubated for at least 1 h in the first antibody solution which was either rabbit anti-chitinase antibody (kindly provided by Dr. Srisurang Tantimavanich, Mahidol University) or rabbit anti-Cry11Aa1 toxin antibody (kindly provided by Dr. Chanan Angsuthanasombat, Institute of Molecular Genetics and Genetic Engineering, Mahidol University). Antibodies were diluted to 1: 3000 using TBST solution containing 1% BSA. Then, the membrane was again washed for 5 min, each for another three times in TBST solution. The membrane was subsequently incubated in second antibody which was anti rabbit IgG conjugated with alkaline phosphatase (Biorad, USA). The second antibody was also diluted to 1: 3000 with TBST solution containing 1% BSA. The membrane was washed as described in the previous step. The color reaction was developed by incubating the membrane in 10 ml Alkaline phosphatase (AP) buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl, 1 mM $MgCl_2$) containing 200 µl NBT/ BCIP solution, stop reaction with 20 mM Tris pH 8.0/ 5 mM EDTA pH 8.0.

13. PCR amplification of *p19*, *cry11Aa1* and chitinase gene

13.1 Primer design

Various primers were designed from the published nucleotide sequences with additional restriction site at the 5' end. The published nucleotide sequences of *B.t.i.*, *p19* and *cry11Aa1* genes derived from Genbank accession number S78174 and M31737 (Fig. 11A and 11B). While, the nucleotide sequence of *B. licheniformis*TP-1, chitinase gene was derived from Genbank accession number U71214 (Fig. 12). All oligonucleotide primers were in 5' to 3' direction. The forward oligonucleotide primers were derived

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ACCESSION   S78174
VERSION     S78174.1  GI:999362
SOURCE      Bacillus thuringiensis HD567 ssp. israelensis.
  ORGANISM  Bacillus thuringiensis
            Bacteria; Firmicutes; Bacillus/Clostridium group;
            Bacillus/Staphylococcus group; Bacillus; Bacillus cereus group.
REFERENCE   1  (bases 1 to 1806)
  AUTHORS   Dervyn,E., Poncet,S., Klier,A. and Rapoport,G.
  TITLE     Transcriptional regulation of the cryIVD gene operon from Bacillus
            thuringiensis subsp. israelensis
  JOURNAL   J. Bacteriol. 177 (9), 2283-2291 (1995)
  MEDLINE   95247658
  REMARK    GenBank staff at the National Library of Medicine created this
            entry [NCBI gibbsq 166957] from the original journal article.
            This sequence comes from Fig. 1B.

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                     /note="19 kda crystal protein CryIIA/CryIIC
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                     PIHECMTCSANPHKENIAVSCQDTVCVDQVLYCSVDCLPDCDINCDNVKICDVSIEPI

GDCDCHAGEN"

BASE COUNT      650 a    222 c    278 g    656 t
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       61 actccttaag ttaattagaa tagtggcctt tcaacattaa attatgattt taaacttgca
      121 attattaatg aaatataaag taacataata aattaaattg atagacttaa ttcacataat
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      481 ttcataataa actaaaacat ctctattacc tttctcaggc attcaatcat atttaagttt
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     1801 attgcg
```

Fig. 11A. The sequence of *p19* gene and intergenic region between *p19* and *cry11Aa1* genes from *B.thuringiensis* subsp. *israelensis* (from genbank accession number S78174, http://www.ncbi.nlm.nih.gov/entrez/)

Forward primer (19FbamHI) = 5'GCGGATCCATGATACGTTTGGATATCGCC3'

Reverse primer (19RsphI) = 5'GCGC ATGCTATTCATTTAATAATTAGGG3'

```
ACCESSION   M31737 J03510
VERSION     M31737.1  GI:142762
SOURCE      B.thuringiensis israelensis (strain HD-567, serotype 14) DNA,
            clones pEG21[4,6].
  ORGANISM  Bacillus thuringiensis
            Bacteria; Firmicutes; Bacillus/Clostridium group;
            Bacillus/Staphylococcus group; Bacillus; Bacillus cereus group.
REFERENCE   1  (bases 1 to 2100)
  AUTHORS   Donovan,W.P., Dankocsik,C. and Gilbert,M.P.
  TITLE     Molecular characterization of a gene encoding a 72-kilodalton
            mosquito-toxic crystal protein from Bacillus thuringiensis subsp.
            israelensis
  JOURNAL   J. Bacteriol. 170, 4732-4738 (1988)
  MEDLINE   89008093
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                     AIGKWAAKAAFSKVLSLIFPGSQPATMEKVRTEVETLINQKLSQDRVNILNAEYRGII
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                     LTLLKDGILAGSAWGFTQADVDSFIKLFNQKVLDYRTRLMRMYTEEFGRLCKVSLKDG
                     LTFRNMCNLYVFPFAEAWSLMRYEGLKLQSSLSLWDYVGVSIPVNYNEWGGLVYKLLM
                     GEVNQRLTTVKFNYSFTNEPADIPARENIRGVHPIYDPSSGLTGWIGNGRTNNFNFAD
                     NNGNEIMEVRTQTFYQNPNNEPIAPRDIINQILTAPAPADLFFKNADINVKFTQWFQS
                     TLYGWNIKLGTQTVLSSRTGTIPPNYLAYDGYYIRAISACPRGVSLAYNHDLTTLTYN
                     RIEYDSPTTENIIVGFAPDNTKDFYSKKSHYLSETNDSYVIPALQFAEVSDRSFLEDT
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                     SGNNRMLGSFTANANPEWVDFVTDAFTFNDLGITTSSTNALFSISSDSLNSGEEWYLS
                     QLFLVKESAFTTQINPLLK"
     BASE COUNT      746 a    316 c    378 g    660 t
ORIGIN      929 bp upstream of PvuII site.
        1 tttaaaataa aaaaattcaa taaaaggtgg aatgaattat atggaagata gttctttaga
       61 tactttaagt atagttaatg aaacagactt tccattatat aataattata ccgaacctac
      121 tattgcgcca gcattaatag cagtagctcc catcgcacaa tatcttgcaa cagctatagg
      181 gaaatgggcg gcaaaggcag cattttcaaa agtactatca cttatattcc caggttctca
      241 acctgctact atggaaaaag ttcgtacaga agtggaaaca cttataaatc aaaaattaag
      301 ccaagatcga gtcaatatat taaacgcaga atataggggg attattgagg ttagtgatgt
      361 atttgatgcg tatattaaac aaccaggttt tacccctgca acagccaagg gttattttct
      421 aaatctaagt ggtgctataa tacaacgatt acctcaattt gaggttcaaa catatgaagg
      481 agtatctata gcacttttta ctcaaatgtg tacacttcat ttaactttat taaaagacgg
      541 aatcctagca gggagtgcat ggggatttac tcaagctgat gtagattcat ttataaaatt
      601 atttaatcaa aaagtattag attacaggac cagattaatg agaatgtaca cagaagagtt
      661 cggaagattg tgtaaagtca gtcttaaaga tggattgacg ttccggaata tgtgtaattt
      721 atatgtgttt ccatttgctg aagcctggtc tttaatgaga tatgaaggat taaaattaca
      781 aagctctcta tcattatggg attatgttgg tgtctcaatt cctgtaaatt ataatgaatg
      841 gggaggacta gtttataagt tattaatggg ggaagttaat caaagattaa caactgttaa
      901 atttaattat tctttcacta atgaaccagc tgatatacca gcaagagaaa atattcgtgg
      961 cgtccatcct atatacgatc ctagttctgg gcttacagga tggataggaa acggaagaac
     1021 aaacaatttt aattttgctg ataacaatgg caatgaaatt atggaagtta gaacacaaac
     1081 tttttatcaa aatccaaata atgagcctat agcgcctaga gatattataa atcaaatttt
     1141 aactgcgcca gcaccagcag acctattttt taaaaatgca gatataaatg taaagttcac
     1201 acagtggttt cagtctactc tatatgggtg gaacattaaa ctcggtacac aaacggtttt
     1261 aagtagtaga accggaacaa taccaccaaa ttatttagca tatgatggat attatattcg
     1321 tgctatttca gcttgcccaa gaggagtctc acttgcatat aatcacgatc ttacaacact
     1381 aacatataat agaatagagt atgattcacc tactacagaa aatattattg tagggtttgc
     1441 accagataat actaaggact tttattctaa aaaatctcac tatttaagtg aaacgaatga
     1501 tagttatgta attcctgctc tgcaatttgc tgaagtttca gatagatcat ttttagaaga
     1561 tacgccagat caagcaacag acggcagtat taaatttgca cgtactttca ttagtaatga
     1621 agctaagtac tctattagac taaacaccgg gtttaatacg gcaactagat ataaattaat
     1681 tatcagggta agagtacctt atcgcttacc tgctggaata cgggtacaat ctcagaattc
     1741 gggaaataat agaatgctag gcagttttac tgcaaatgct aatccagaat gggtggattt
     1801 tgtcacagat gcatttacat ttaacgattt agggattaca acttcaagta caaatgcttt
     1861 atttagtatt tcttcagata gtttaaattc tggagaagag tggtatttat cgcagttgtt
     1921 tttagtaaaa gaatcggcct ttacgacgca aattaatccg ttactaaagt agaagtcatg
     1981 ttagcacaag aggagtgagt attgtggctc ctcttgtaat tttaatcgct aatatttcta
     2041 atagatataa attatatata atatttaaaa agttataatt atgtaattgt agaaaatcat
```

Fig. 11B. The sequence of *cry11Aa1* genes from *B.thuringiensis* subsp. *israelensis* (from genbank accession number M31737, http://www.ncbi.nlm.nih.gov/entrez/)

Forward primer (68FKpnI) = GCGGTACCATAAGGTCATAATTTATGAAT

Reverse primer (68RxbaI) = 5'GCTCTAGACGTATAC TCTGTTTCCTCGTC3'

Reverse primer (68RsphI) = 5'GCGCATGCCATGACTTCTACTTTAGTAAC3'

```
DEFINITION  Bacillus licheniformis TP chitinase (CHI) gene, complete cds.
ACCESSION   U71214
VERSION     U71214.1  GI:1845336
SOURCE      Bacillus licheniformis.
  ORGANISM  Bacillus licheniformis
            Bacteria; Firmicutes; Bacillus/Clostridium group;
            Bacillus/Staphylococcus group; Bacillus.
REFERENCE   1  (bases 1 to 2935)
  AUTHORS   Tantimavanich,S. and Panbangred,W.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-SEP-1996) Microbiology, Mahidol University, Rama VI
                    /translation="MNIVLVNKSKKFFVFSFIFVMLLSLSFVNGEVAKADSGKNYKII
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                    GVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGW
                    TWSNRFSDVAADPVARGNFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKR
                    NYTLLLQEVRKKLDAAEAKDGKEYLLTIASGASDRYVSNTELDKIAQTVDWINIMTYD
                    FNGGWQSISAHNAALFYDPKAKEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFY
                    EGAGAVVNPAATENIRSADRRKKGRGKMEYSTFQILKRTYVNQNGYKRYWNDQAKVPF
                    LYNAENGNFITYDDEQSFGHKTDFIKANGLSGAMFWDFSGDSNRTLLNKLAADLDFAP
                    DGGNPEPPSSAPVNVRVTGKTATSVSLAWDAASSGTNITEYVVSFESRSISVKETSAE
                    IGNLNRGTAYSFTVSAKDADGELHTGPTVKVTTNSDQACSYDEWKETNAYTGGERVAF
                    NGKVYEAKWWTKGDRRINPVNGAYGGWSEAANNRKSNG"
BASE COUNT      872 a    611 c    742 g    710 t
ORIGIN
        1 aagcttagca agagtatgac ctgccagtgc aaaggtcaat catgatcatc cttcactgtg
       61 gaagggtgta aggtcgaacg atgcttttga gctgaaagtc gatcgattga aggatctttc
      121 gagcatcctt cgtgattatc aagacgcagc agtgattgaa gtgatgtgcc atccgcttac
      181 gtcgataaat gtctgcttga aaaatcgtct ttccattatc ctcgggtata tgaactttca
      241 ctgctgacgg aaccttctct taaatcggct ttgcaacaat gtccggatat tcgtcttacg
      301 tcttacgcta gtctatgaag aggctagagg cgccgcttct attccttaaa tggagtagaa
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      421 gatttgtcat aacaaattca gcaacccttc gccattaaaa agtcttcttt tatacgcaaa
      481 tgtatagaca tctatacgtg tttcaccctc agcacaacca gcttttccaa acgaacacac
      541 ccgaaatttt aatgaaatca tgatgtcaat ctcatttatg taagcgtttt cccttgttgg
      601 cttctgtgca gctgcagcta ttagatgaca aggaaaaata taaaacaaac aaaaaaggcg
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      781 gttgctgagc ttgtcatttg taaatgggga agttgcaaag gccgattccg gaaaaaacta
      841 taaaatcatc ggctactatc catcatgggg tgcttacgga aggaattttc aagtttggga
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     1021 aaacggagtg atcgacgcgc caaatggaac aatcgtaatg ggcgatccct ggattgacgc
     1081 acaaaagtca aatcccgggg atgtctggga tgaaccgatc cgcggcaact ttaaacaatt
     1141 gctgaagctg aaaaagagcc accctcattt gaaaacgttc atatcggtcg gcggatggac
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     1261 ttcggccgtt gaatttttaa ggaaatacgg gtttgacggg gtcgatcttg actgggaata
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     1381 gctgctcctg caagaggtgc gtaaaaaact tgatgctgca gaagcaaagg acggcaagga
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     1621 cgttccaaat gctgagacct acaacattga aaacactgtg aaacgctata aggaagccgg
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     1741 gaatccggcg gccacggaga atatcagaag tgcggaccgg cgaaagaagg gacgtgggaa
     1801 aatggagtat tcgacttttc agatcttgaa aagaacctat gtaaatcaaa acggctataa
     1861 aaggtattgg aacgatcaag cgaaagtgcc gtttttgtat aacgcggaaa atggcaattt
     1921 catcacttat gatgatgaac aatcattcgg ccacaaaacg gattttatta aagcaaacgg
     1981 attaagcgga gcgatgttct gggatttcag cggcgattcc aatcggacgc ttctcaataa
     2041 attggcagcc gatttagatt ttgcaccgga cggaggcaat ccggagccgc cttcatcggc
     2101 acctgtgaat gtgcgtgtaa ccggaaaaac tgctacaagt gtcagcctgg cgtgggatgc
     2161 cgcgagcagc ggaacaaaca ttacggaata tgtcgtgtca tttgaaagcc ggtcgatatc
     2221 tgtaaaagaa acatcagcgg aaatcggcaa cttgaaccgg ggtacggcct actcatttac
     2281 agtttcagca aaggatgccg atggagagct ccataccgga ccaacagtaa aggtcacgac
```

Fig.12. The sequence of TP-1 chitinase gene from *B. lichenifoemis* (from genbank accession number U71214, http://www.ncbi.nlm.nih.gov/entrez/)

```
2341 gaattctgac caggcatgtt catatgacga atggaaagag acgaacgcat acacgggcgg
2401 agagcgggtt gcatttaacg gaaaagtgta tgaagcgaaa tggtggacga aaggcgaccg
2461 gcggatcaat ccggtgaatg gggcgtatgg cggctggtcg gaggctgcga ataacagaaa
2521 gtcaaatgga tagaaaacga taaagagaga tttgggaaaa agcttatgac gctggggctg
2581 accgctgtgc tcggatcgtc ggcagtgctg atccccctaa aaagcaacca tgctttagct
2641 tatgaagact tggagaaaga aaagagcgac gttcaaagca agaaatctga aaacgagtca
2701 aaactcgaga aaaagaaaca cccccagctt tcaaagcttt aaacataccg ccggctccat
2761 ttgtttcctg cccgattagg ccgtggtgag cggaattttt tcgtctaaag tctctcgcct
2821 tcagtccgcc gactctgatt tgaaccgtga cgaaatcggc accttgcaac gcttctgccc
2881 gatctattct atggctcagt tcgatgggaa ccccgctatg cctgatcatt cgctt
```

Fig.12. The sequence of TP-1 chitinase gene from *B. lichenifoemis* (from genbank accession number U71214, http://www.ncbi.nlm.nih.gov/entrez/) (continued)

Forward primer (chiFSphI) = GCGCATGCGCGGTGAGGAATAGGAGATTTC

Reverse primer (chiRXbaI) = GCTCTAGACTAATCGGGCAGGAAACAAA

Reverse primer (chiRKpnI) = GCGGTACCGTCAG CCCCAGCGTCATAAGC

from the sense strand while reverse oligonucleotide primers were derived from the complementary strand. Oligonucleotides were synthesized from Eppendorf company, USA or Bioservice Unit, Thailand as desalted lyophilized powder. The amount of each primers was at 0.15 μmol/12 O.D. scale.

13.2 PCR reaction

The total volume of all PCR reaction are 100 μl containing 50 pmol of each primer, 50-100 ng of template, 200 μM of dNTP, 10xPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% w/v gelatin), 2.5 M of $MgCl_2$, 2.5 U of Taq polymerase (Perkin-Elmer, USA).

The amplification cycle of *p19* gene are as the following 94 °C 5 min, 35 cycles of 94 °C 45 sec, 45 °C 45 sec, 72 °C 1 min, and 72 °C 5 min at final cycle. For PCR amplification of *cry11Aa1* gene are 94 °C 5 min, 35 cycles of 94 °C 1.45 min, 56 °C 1.45 min, 72 °C 3 min, 72 °C 5 min. And PCR amplification of chitinase gene are 94 °C 5 min, 35 cycles of 94 °C 1.45 min , 62 °C 1.45 min, 72 °C 3 min and 72 °C 5 min. PCR products were subjected to 1% agarose gel electrophoresis as previously described.

14. TA cloning

PCR product was cloned into plasmid pCR2.1 vector (Invitrogen, USA). This plasmid provided a quick, one-step cloning strategy for the direct insertion of a PCR product. For optimal ligation efficiencies, Invitrogen recommends using fresh (less than 1 day old) PCR products. The single 3' A-overhangs on the PCR products will be degraded over time, reducing ligation efficiency.

The total ligation volume was 10 μl. The amount of PCR product needed to

ligate with 50 ng (20 fmoles) of pCR2.1 vector was estimated using the formula below:

$$X \text{ ng PCR product} = \frac{(Y \text{ bp PCR product})(50 \text{ ng pCR2.1 vector})}{(\text{size in bp of the pCR2.1 vector:}\sim 3900)}$$

Where X ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 (vector:insert) molar ratio. In general, 0.5 to 1.0 µl of a typical PCR sample was enough. The volume of the PCR should be not more than 2-3 µl, because salts in the PCR sample may inhibit ligase. The reaction was incubated at 14°C for overnight or kept at -20°C until ready for transformation.

15. Study of chitinase enzyme production from *B. licheniformis* TP-1, *B.t.i.* 4Q2-72, c4Q2-72 and their transformants.

15.1 Enzyme production

15.1.1 Comparative study of enzyme production between culture with and without chitin induction of *B.t.i.* 4Q2-72 transformant

Whether the chitinase enzyme production of *B.t.i.* 4Q2-72 transfromant at various stages of cell growth depended on chitin induction or not, was determined. The *B.t.i.* 4Q2-72 transformant was precultured in 5 ml NYSM medium supplemented with 15 µg/ml tetracycline, incubated at 37 °C overnight on a rotary shaker. The preculture was transferred to 2 sets to obtained 0.1% final concentration of 100 ml of NYSM broth adding with the same antibiotic. The 0.1% colloidal chitin was added to one set of culture. The cultures were incubated at 37 °C on a rotary shaker. The 2 ml from each culture

was removed at 0, 6, 12, 24, and 48 h. The level of chitinase enzyme was quantitatively measured by colorimetric method as described previously. The viable cells and spore formation were determined by direct plate count technique as described in 15.2.

15.1.2 Study of enzyme production of *B.t.i.* 4Q2-72, c4Q2-72 and their transformants and *B. licheniformis* TP-1

B.t.i. 4Q2-72, c4Q2-72 and their transformants and *B. licheniformis* TP-1 were compared for the level of chitinase enzyme production at various stages of cell growth. The *B. licheniformis* TP-1 was precultured in 5 ml NYSM broth with additional of colloidal chitin (0.1%) for induction. The *B.t.i.* 4Q2-72, c4Q2-72 and their transformants were precultured in 5 ml NYSM broth without colloidal chitin and 15 µg/ml tetracycline was added for transformant cultures. All cultures were incubated at 37 °C overnight on a rotary shaker. The 2 ml of each precultured was transferred to 100 ml of the same medium and incubated under the same condition. A 2 ml of each culture was removed at 0, 3, 6, 9,, 12, 24, 48 and 72 h. The level of chitinase enzyme was quantitatively measured by colorimetric method. The number of viable cells and spores were counted.

15.2 Determination of bacterial viability and spore count

The bacterial viability was determined by standard plate count method. Bacterial cultures taken at various time intervals were serially ten-fold diluted in NYSM broth by using 100 µl of bacterial culture and 900 µl of broth. The 100 µl of three appropriate dilutions were spreaded on NYSM agar (15 µg/ml of tetracycline was added for transformants), incubated overnight at 37 °C. The numbers of colonies were recorded.

For spore count, the samples were heated at 80 °C for 10 min and subjected to serially ten-fold dilution and spreaded on NYSM agar as described in viable plate count method. The numbers of spores were recorded after overnight incubation at 37 °C.

16 Plasmid stability

B.t.i. harbouring the recombinant plasmid was precultured in 5 ml of NYSM broth supplemented with 15 μg/ml tetracycline at 37°C on rotary shaker. A 1 ml of this preculture was inoculated into each of 100 ml NYSM broth without and with tetracycline at concentration 15 and 30 μg/ml. The cultures were then daily transferred to corresponding medium using 1% inoculum. Plates counts and plasmid stability were determined every day for one week. Plate counts was performed by ten-folds serially diluting the bacterial culture in NYSM broth and spreading 100 μl of three appropriate dilution on NYSM agar. Plasmid stability was determined by replicating 100 colonies from NYSM agar on 3% colloidal chitin overlaid NYSM agar containing 15 μg/ml tetracycline and 3% colloidal chitin overlaid NYSM agar, respectively. Number of colonies with and without clear halo zones was recorded every day.

17 Mosquitocidal bioassay

17.1 Larvicidal assay of recombinant TP-1 chitinase enzyme from *B.t.i.* c4Q2-72 transformant against *Aedes aegypti* larvae

The effect of chitinase alone on mosquito larvicidal activity was studied. The chitinase enzyme was obtained from cultured supernatant of *B.t.i.* c4Q2-72 transformant. The bacterial strain was precultured in 5 ml NYSM broth supplemented with 15 μg/ml tetracycline without colloidal chitin at 37°C overnight with shaking. A 1 ml of preculture was transferred to 100 ml of the same

medium, incubated at 37°C with shaking for 48 h. The culture was centrifuged at 6,000 rpm for 20 min at 4°C using a Sorvall RC-5 super speed refrigerated centrifuge. In order to obtain pure supernatant without bacterial cells and spores, the culture supernatant was filtered through 0.2 μm filter (Millipore, USA) and checked the presence of bacterial cells and spore count by plating 200 μl of the filtrate on NYSM agar. The filtrated enzyme was used directly in testing for toxicity against second instar *Aedes aegypti* larvae by diluting with distilled water. The total volume of each dilution is 50 ml and ratio of enzyme: distilled water is 1:5, 1:2, 1:0, respectively. Twenty mosquito larvae were added to each cups. The assays were performed in duplicate and mortalities were recorded after 2 days. The percent mortality was calculated.

17.2 Larvicidal assay of combination between TP-1 chitinase and *B.t.i.* 4Q2-72, c4Q2-72 and their transformants

The insect bioassays using *B.t.i.* 4Q2-72, c4Q2-72 and their transformants in combination with recombinant TP-1 chitinase against second instar *Aedes aegypti* larvae were performed. The toxicity of each *B.t.i.* strains and their transformants were compared individually. Also, the toxicity of each *B.t.i.* strains and their transformants in combination with fix amount of chitinase enzyme were determined and compared.

The bacterial strain was precultured in 5 ml NYSM broth supplemented with 15 μg/ml tetracycline without colloidal chitin at 37°C overnight with shaking. A 1 ml of precultured was transferred to 100 ml of the same medium, incubated at 37°C with shaking for 48 h. The bacterial cultures were subjected to serially ten-fold dilution in distilled water (total volume of 50 ml). Twenty mosquito larvae were added to each cup. Another sets of chitinase and bacterial cell combinations were performed by adding fix amount of chitinase enzyme (2.5 mU) to each serially ten-fold bacterial dilutions (total volume of 50 ml). Then, twenty mos-

quito larvae were added to each cup. All bioassays were performed in duplicate and mortalities were recorded after 2 days. The lethal concentration required to kill 50% of the larvae (LC_{50}) was determined by log-probit analysis (Finney, 1952).

CHAPTER IV

RESULTS

1. PCR amplification of *p19*, *cry11Aa1*, and chitinase genes

1.1 Primer designation

In order to obtain the complete gene of *p19* gene, *cry11Aa1* gene, and chitinase gene, PCR amplification reaction was used. Although, restriction enzyme digestion technique is more convenience, but no suitable restriction sites were available for each gene. Various primers were designed from the published sequence of *p19* gene and *cry11Aa1* gene of *B. thuringiensis* subsp. *israelensis* (*B.t.i.*) (Fig. 11A and 11B in Materials and Methods) and chitinase gene of *B. licheniformis* TP-1 (Fig. 12 in Materials and Methods). Each primer with additional restriction site at the 5' end was designed and summarized in Table 6. These restriction sites were used for joining each gene together after PCR cloning.

The *cry11Aa1* operon composes of three genes which are arranged sequentially as the following: *p19*, *cry11Aa1* and *p20* gene (Dervyn *et al*, 1995; Adam *et al*, 1989). The schematic diagram and restriction map of *cry11Aa1* operon was shown in Fig. 13. Primers for *p19* gene was designed from nucleotide sequence at position 720-1650. The *p19* forward primer (19FBamHI) was located upstream from *cry11Aa1* gene promoter. The *p19* reverse primer (19RSphI) was located between intergenic region of *p19* and *cry11Aa1* gene (between *p19* gene stop codon and *cry11Aa1* gene start codon).

Table 6. The nucleotide sequence of PCR primer for amplification of *p19*, *cry11Aa1* and TP-1 chitinase gene

Gene	Primer name	Primer sequence	From nt...to nt...	Size of PCR product (bp)
19 kDa	19FBamHI	GCGGATCCATGATACGTTTGGATATCGCC	720-1650	930
	19RsphI	GCGC ATGCTATTCATTTAATAATTAGGG		
cry11Aa1	68FKpnI	GCGGTACCATAAGGTCATAATTTATGAAT	1650-3820	2170
	68RXbaI	GCTCTAGACGTATAC TCTGTTTCCTCGTC		
	68RSphI	GCGCATGCCATGACTTCTACTTTAGTAAC	1650-3660	2010
Chitinase TP-1	chiFSphI	GCGCATGCGCGGTGAGGAATAGGAGATTTC	660-2790	2130
	chiRXbaI	GCTCTAGACTAATCGGGCAGGAAACAAA		
	chiRKpnI	GCGGTACCGTCAG CCCCAGCGTCATAAGC	660-2590	1930

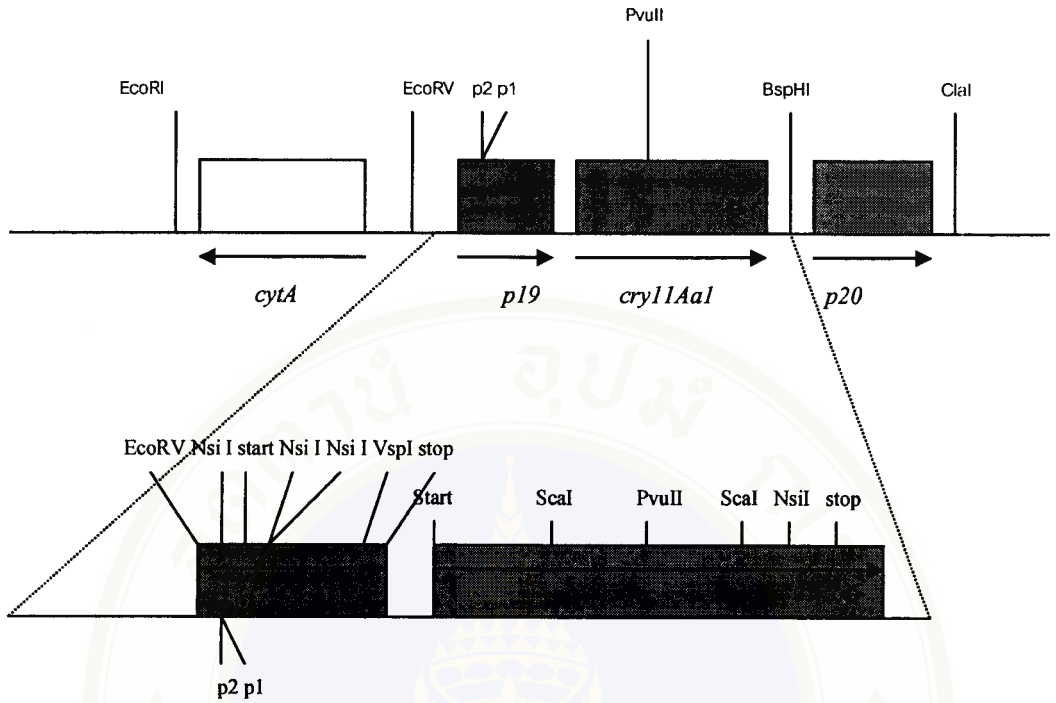


Fig. 13. Schematic diagram illustration of p19-*cry11Aa* operon, *p20* gene and *cytA* gene and fine restiction mapping of p19-*cry11Aa* operon. The abbreviation are as followed: start, start codon; stop, stop codon; p1, p2, promoter; ⇔, terminator; Arrow, direction of transcription.

The schematic diagram of *p19* gene and location of *p19* primers were shown in Fig. 14A. The nucleotide sequences of *p19* gene, 19FBamHI and 19RSphI primers were shown in Fig. 14B.

Primers for *cry11Aa1* gene were designed from nucleotide sequence at position 1650-3660 and 1650-3820 to obtain *cry11Aa1* gene without or with terminator, respectively. The *cry11Aa1* forward primer (68FKpnI) was located between intergenic region of *p19* and *cry11Aa1* gene. The first *cry11Aa1* gene reverse primer (68RSphI) was located between *cry11Aa1* stop codon and its terminator. Another *cry11Aa1* reverse primer (68RXbaI) was located downstream from its terminator. The schematic diagram of *cry11Aa1* gene and location of *cry11Aa1* primers were shown in Fig. 15A. The primer sequences of 68FKpnI 68RXbaI, and 68RSphI were shown in Fig. 15B and 15C, respectively.

The primers for chitinase gene were designed from nucleotide sequences 660-2590 and 660-2790 to obtain chitinase gene without or with its terminator, respectively. The forward primer (chiFSphI) was located between chitinase promoter and the start codon (61 bp downstream of start codon). The first chitinase reverse primer (chiRKpnI) was located between its stop codon and terminator. Another reverse primer (chiRXbaI) was located downstream from chitinase terminator. The schematic diagram of TP-1 chitinase gene and location of chitinase primers were shown in Fig. 16A. The primer sequences of chiFSphI, chiRXbaI and chiRKpnI were shown in Fig. 16B and 16C, respectively.

1.2 PCR amplification of *p19*, *cry11Aa1* and chitinase gene

PCR amplification was performed as described in Materials and Methods. Plas-

A.

B.

BamHI

720 atgata cgtttg gatatcgcca aaatgaaatc taatttaaga gtatgtattt tcataaaata

19FBamHI

gtttcgtatg agattttatt attttgaaac aatatttctt atttttatat acataagtag

catttattca gagaagactt atccatcgga agatttttca aatggctata tgatatagta

tcttgttttt ttagtcaaag atacattttt gtttatacat gcatcgtttt tatacaagta

acatatattt gttatgtaac aggagaatag tgaaattggt actaaatttt taagaaagat

attttgaaat cttatttgaa taagtttttt aaaattgcat agaagggaga gaagataaat

start

atgaatatga attttgattt cgaggatcat gaaaataaga atttatctgt gcaggaggaa

catcaccatt gtagtgaagg aggggaacat aaaatagcat tttgttgtgt agtctcaatt

ccaaaaggtt ttaaatatgt tgcccattgt gatccgaaat ttgtatataa ccttgattgt

ctatccgttt caaaagaaaa atgccgtaag gttgttccta tagaaggatg tggatgtgca

gaggtagatt tacatgtatt aaaggtaaag ggatgcatct catttgtatc gaatatagaa

atagaaccta ttcatgaatg catgacctgc tcagcaaatc cacataaaga aaacattgct

gtgagttgcc aagatactgt ctgcgtagat caagttttgt attgcagtgt agattgtttg

ccagattgtg atattaattg tgataatgta aaaatttgcg atgtgagcat tgaaccaatt

stop

ggagattgtg attgtcacgc cggtgaaaat taaagggaaa ttttcacttc actataaata

SphI

aaaaat ccct aattattaaa tgaata gcatgc 1650

19RsphI

Fig. 14. A) The schematic diagram illustration of *P19* gene. The arrow inidicated the primers. B) The nucleotide sequence of *p19* gene. Underlines are the primer sequence of 19FBamHI and 19RSphI, respectively. The abbreviation are as followed: start, start codon; stop, stop codon; ⇔, terminator.

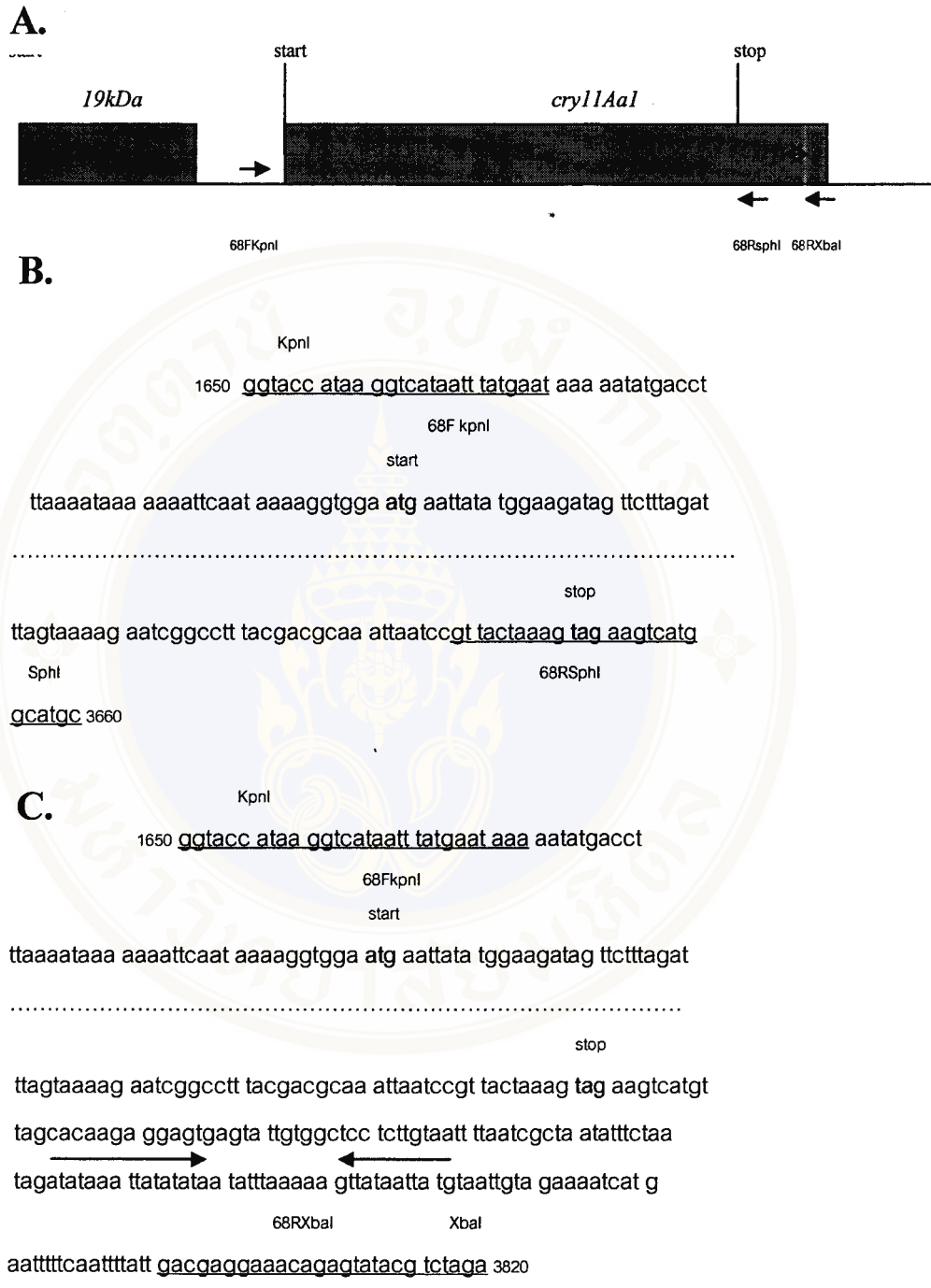
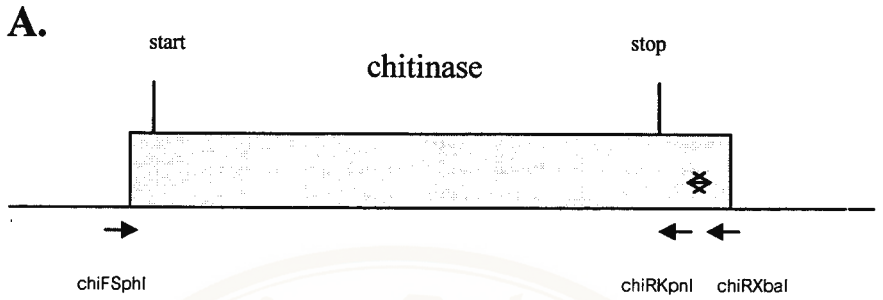


Fig. 15. A) The schematic diagram illustration of *cry11Aa1* gene. The arrow inidicated the primers. B) Nucleotide sequences around start and stop codon of *cry11Aa1*.The primer sequence of cry11Aa1FKpnI and cry11Aa1RXbaI are underlined. C) Nucleotide sequences around start and terminator of *cry11Aa1*. The primer sequence of cry11Aa1FKpnI and cry11Aa1RSphI are underlined. The abbreviation are as followed: start, start codon; stop, stop codon; ⇔, terminator.



B.

SphI chiFSphI

660 GCATGC GCGGTGAGGAATAG GAGATTTC TA GTTTCATGGT TTGTCAAAAA AATT GGATGA

start

AAAGGAG ATG AACATCGTGT TGGTCAACAA AAGCAAAAAG TTTTTCGTTT TTTCTTTCAT

.....

stop

TAACAGAAAG TCAAATGGA T AG AAAACGAT AAAGAGAGAT TTGGGAAAAAG CTTATGACG

CTGGGGCTGA C GGTACC 2590

ChiRKpnI KpnI

C.

SphI chiFSphI

660 GCATGC GCGGTGAGGAATAG GAGATTTC TA GTTTCATGGT TTGTCAAAAA AATT GGATGA

start

AAAGGAG ATG AACATCGTGT TGGTCAACAA AAGCAAAAAG TTTTTCGTTT TTTCTTTCAT

.....

stop

TAACAGAAAG TCAAATGGA T AG AAAACGAT AAAGAGAGAT TTGGGAAAAAGCTTATGACG

CTGGGGCTGA CCGCTGTGCT CGGATCGTCG GCAGTGCTGA TCCCCCTAAA AAGCAACCAT

GCTTTA GCTTATGAAGACTT GGAGAAAGAA AAGAGCGACG TTCAAAGCAA GAAATCTGAA

AACGAGTCAA AACTCGAGAA AAAGAAACAC CCCCAGCTTT CAAAGCTTTA AACATACCGC

chi RXbaI XbaI

CGGCTCCATT TG TTTCCTGC CCGATTAG TCTAGA 2790

Fig. 16. A) The schematic diagram illustration of chitinase gene. The arrow inidicated the primers. B) Nucleotide sequence around start and stop codon of chitinase gene. The primer sequence of chiFSphI and chiRKpnI are underlined. C) Nucleotide sequence around start and terminator of chitinase gene. The primer sequence of chiFSphI and chiRXbaI are underlined. The abbreviation are as followed: start, start codon; stop, stop codon; ⇔, terminator.

mid pBTC68A harbouring *cry11Aa1* operon of *B.t.i.* toxin gene was used as template for PCR amplification of *p19* gene and *cry11Aa1* gene. Plasmid pBTC68A was extracted from *E. coli* DH5α host. The 0.9 kb of *p19* gene was obtained using primer 19FBamHI and 19RSphI. This 19 kDa fragment contained the spore promoter, SD sequence and complete *p19* gene. While, 2.0 kb and 2.2 kb *cry11Aa1* gene were obtained using primers, 68FKpnI-68RSphI and 68FKpnI-68XbaI, respectively. The 2.0 kb fragment carried complete gene of *cry11Aa1* gene and its SD sequence. Whereas, the 2.2 kb fragment carried the SD sequence, *cry11Aa1* complete structural gene and its terminator. PCR products of 0.9 kb of *p19* gene, 2.2 and 2.0 kb of *cry11Aa1* fragments were resolved by 1% agarose gel electrophoresis (Fig. 17, lane 1, 4, and 5).

PCR amplification of chitinase gene was used plasmid pCHIL1 as template. Plasmid pCHIL1 harbouring chitinase gene of *B. licheniformis* TP-1 was extracted from *E. coli* DH5α host. The 1.9 kb fragment harbouring the SD sequence of TP-1 chitinase and its complete structural gene, was obtained using primer chiFSphI and chiRKpnI. The 2.1 kb fragment containing the same fragment as 1.9 kb with additional of terminator region at the 3' end, was obtained using primer chiFSphI and chiRXbaI. PCR products of 2.1 and 1.9 kb of chitinase gene were detected by 1% agarose gel electrophoresis (Fig. 17, lane2, and 3).

2. Cloning of PCR fragment into TA vector

All PCR products were firstly cloned into TA vector, pCR2.1 plasmid (Invitrogen, USA) for further convenient subcloning and joining of each gene. The commercial plasmid, pCR2.1 vector, is a 2.9 kb, linear DNA fragment containing thymine (T) overhang at the 3' end. The protruding T was perfectly matched with the adenine (A) overhang of

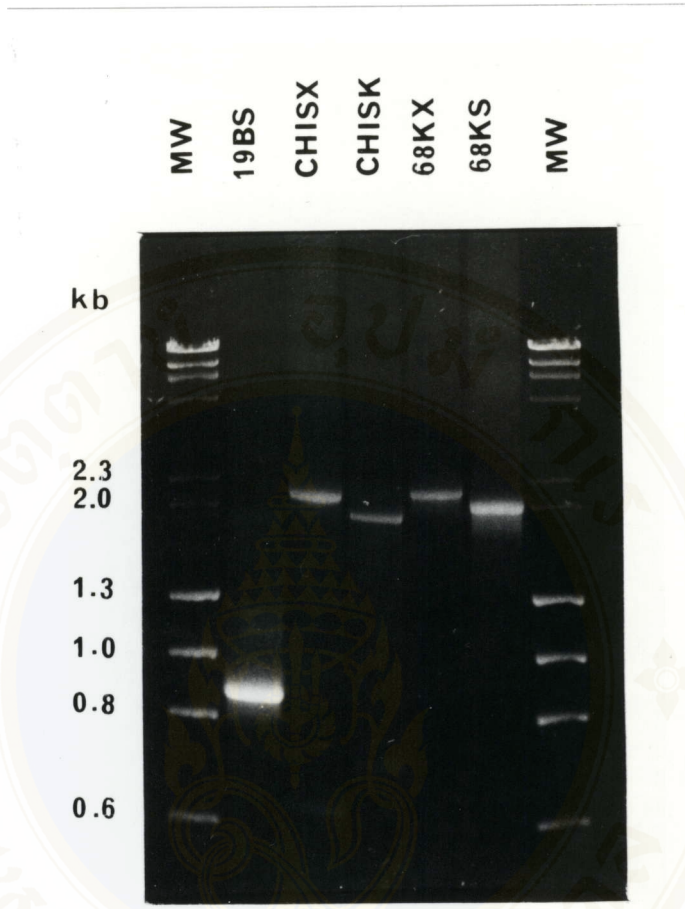


Fig. 17. Agarose gel electrophoresis of PCR amplified products of *p19*, chitinase and *cry11Aa1* gene

Lane 1	λ *Hind* III molecular weight marker
Lane2	*p19* gene
Lane 3	chitinase gene with its terminator
Lane 4	chitinase gene without terminator
Lane 5	*cry11Aa1* gene with its terminator
Lane 6	*cry11Aa1* gene without terminator
Lane 7	λ *Hind*III molecular weight marker

PCR product. So the PCR fragment was easily cloned in this vector. Physical map of plasmid pCR2.1 was shown in Fig. 18. Each PCR fragment (which was 0.9 kb of *p19* gene, 2.0 and 2.2 kb of *cry11Aa1* gene, 1.9 and 2.1 kb of TP-1 chitinase gene, respectively) was ligated to pCR2.1 vector as described in Materials and Methods. The ligated products were transformed into *E. coli* DH5α host to allow replication of recombinant plasmids. The transformants were selected on LB agar containing 50 μg/ml ampicillin which had been previously spreaded with 50 μl X-gal (2 μg/ml). The white colonies which presumably contained the inserted fragment were randomly selected and subjected to plasmid extraction using alkaline lysis method. One colony, each from each transformation with larger molecular size than that of plasmid pCR2.1 were selected, purified and used further for restriction enzyme analysis in order to confirm the presence of each PCR fragment. Clones which harboured 0.9 kb of *p19* gene, 2.0 and 2.2 kb of *cry11Aa1* gene, 1.9 and 2.1 kb of TP-1 chitinase gene were designated as *E. coli* DH5α (TA19/5), *E. coli* DH5α (TA68S/9), *E. coli* DH5α (TA68X/14), *E. coli* DH5α (TACHISK9), and *E. coli* DH5α (TACHI15), respectively. The schematic restriction map of all TA plasmids were illustrated in Fig. 19 (TA19/5), Fig. 20 (TA68S/9), Fig. 21 (TA68X/14), Fig. 22 (TACHISK9), and Fig. 23 (TACHI15).

2.1 Restriction analysis of plasmid TA19/5, TA68S/9, TA68X/14, TACHISK9 and TACHI15

All TA plasmids harbouring *p19*, *cry11Aa1* and chitinase genes were subjected to various restriction enzyme digestion. The restriction pattern of each gene was analyzed by agarose gel electrophoresis comparing with standard molecular weight marker.

Plasmid TA19/5 was digested with *Nsi* I, *Ssp* I/*Sph* I, *Eco*R V and *Vsp* I/*Sac* I and electrophoresed through 1.5% agarose gel (Fig. 24, lane 2-5). The *Eco*R V restricted

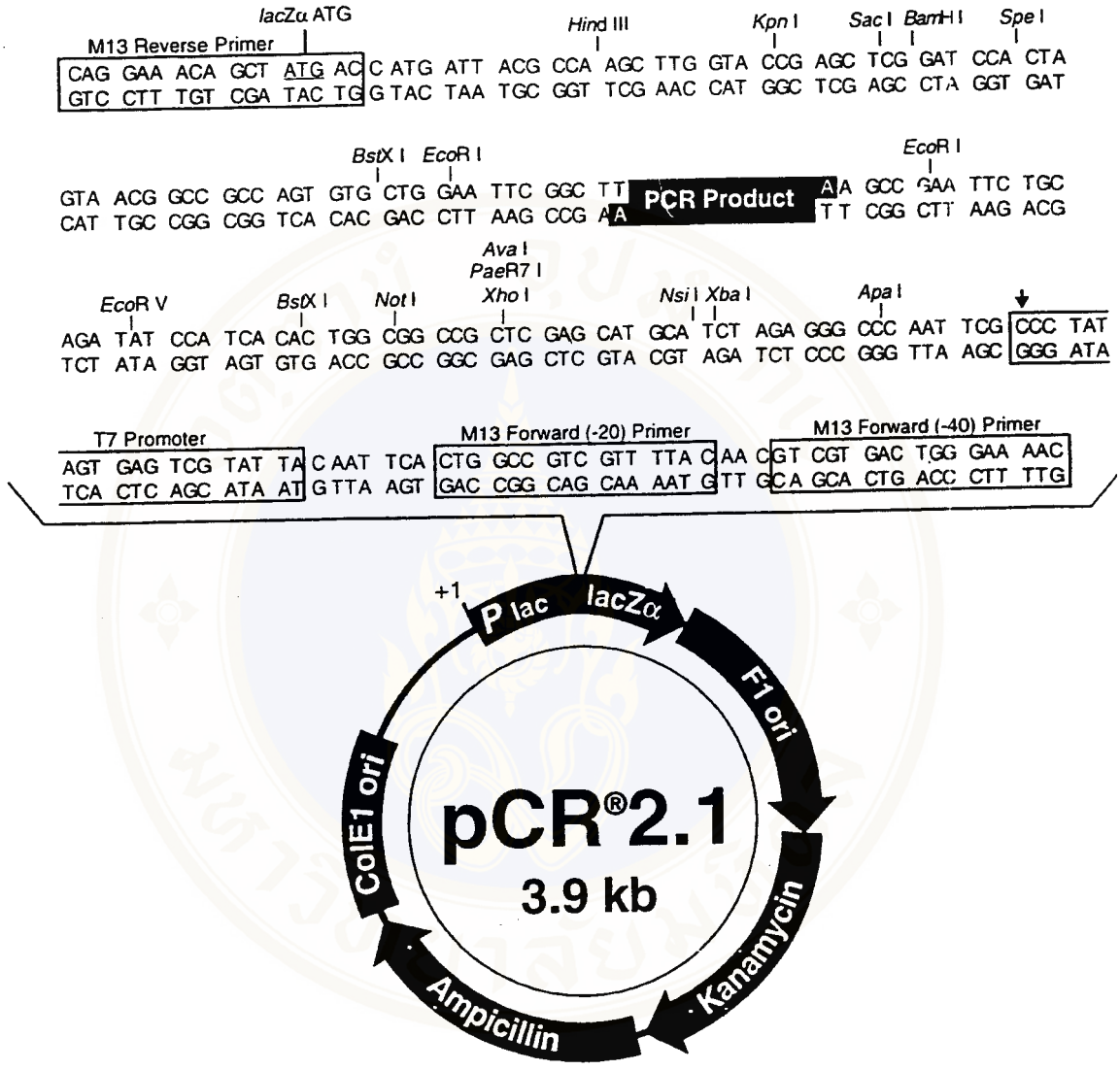


Fig. 18. The map of linearized vector, pCR2.1. The sequence of the multiple cloning site is shown with an inserted PCR product. The arrow indicated the start of transcription for the T7 RNA polymerase.

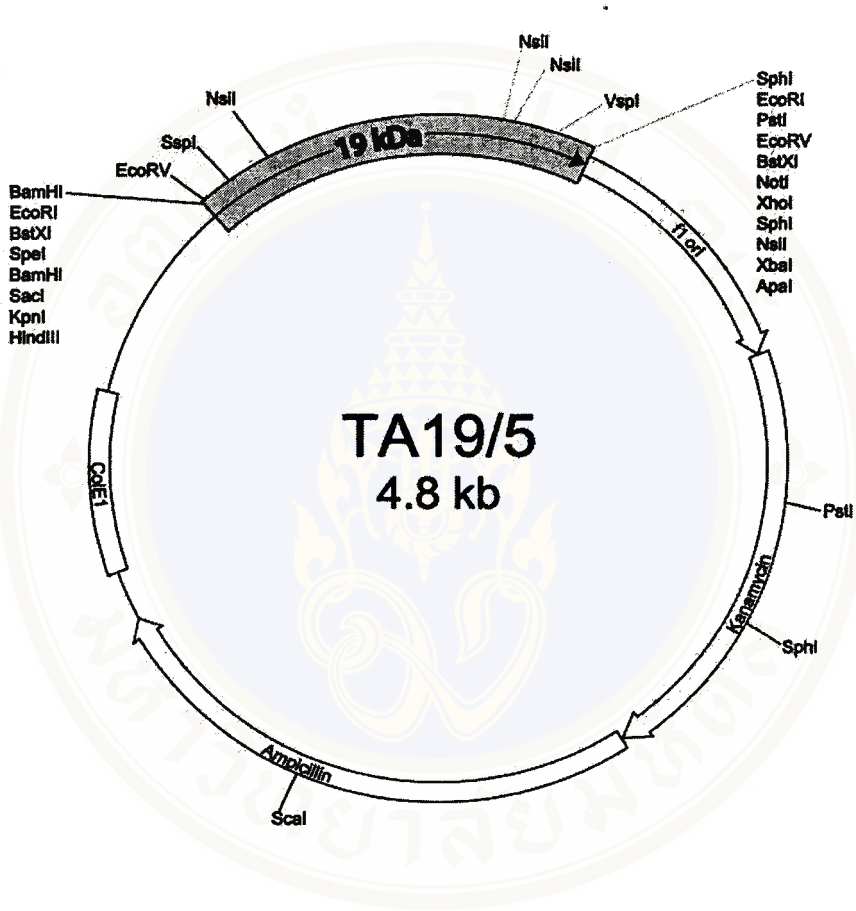


Fig. 19. A schematic restriction map of TA19/5. PCR product carrying *p19* gene from *B.t.i.* was inserted into pCR 2.1 vector to obtain plasmid TA19/5. Arrows indicate direction of transcription.

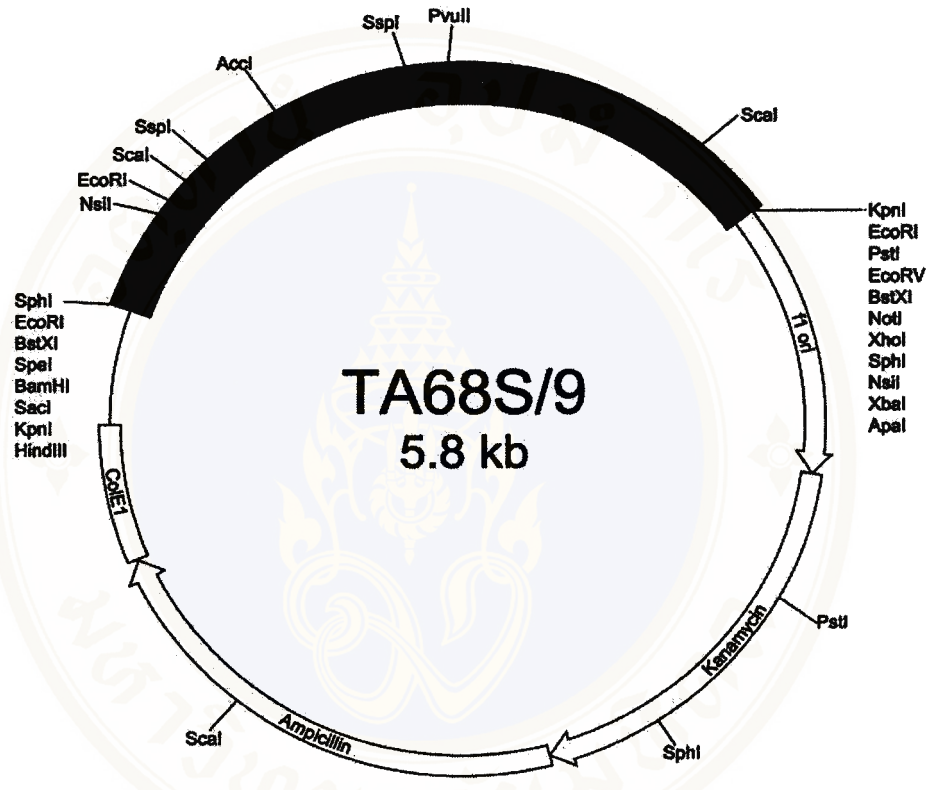


Fig. 20. A schematic restriction map of TA68S/9. PCR product carrying *cry11Aa1* gene without terminator was inserted into pCR2.1 vector to obtain plasmid TA68S/9. Arrows indicate direction of transcription.

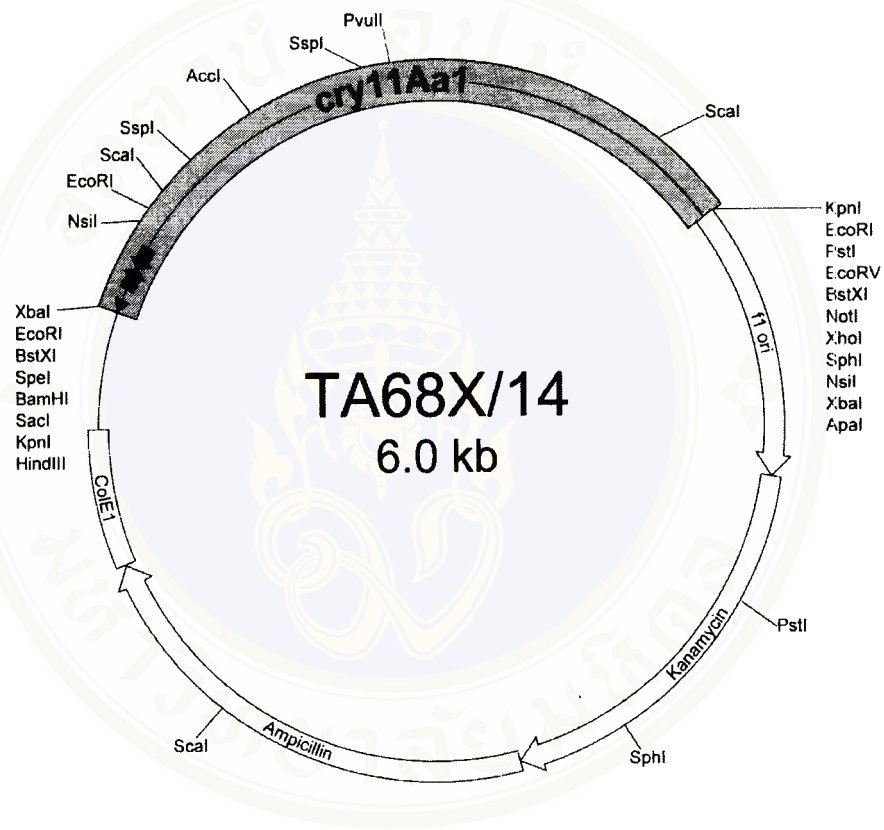


Fig. 21. A schematic restriction map of TA68X/14. PCR product carrying *cry11Aa1* gene with terminator was inserted into pCR2.1 vector to obtain plasmid TA68X/14. The symbol ➡◀ is terminator. Arrows indicate direction of transcription.

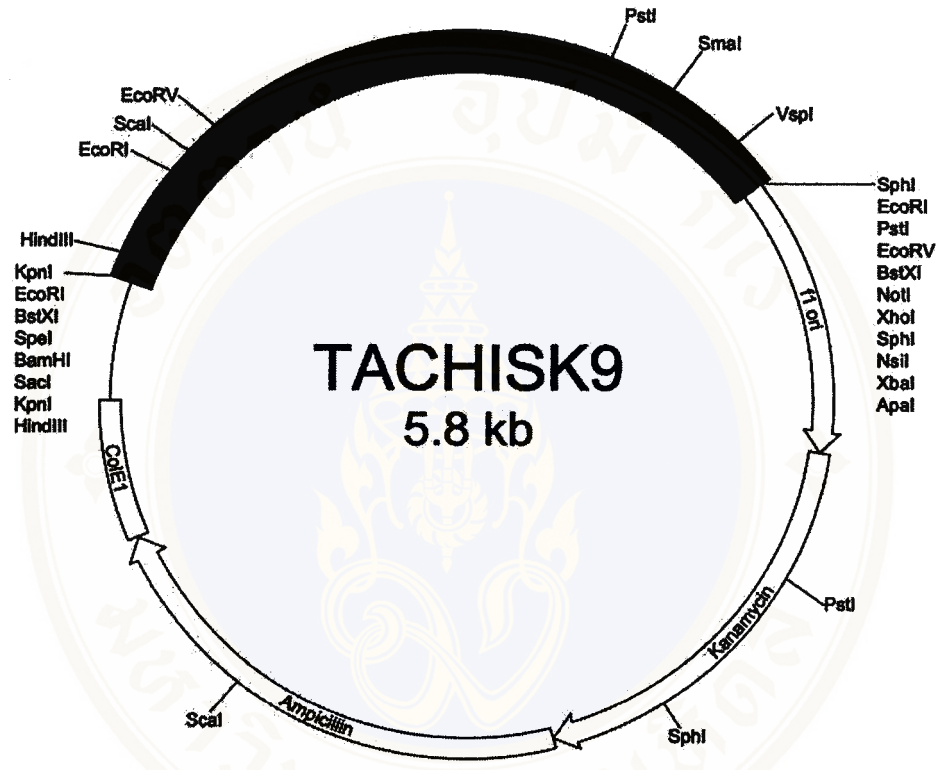


Fig. 22. A schematic restriction map of TACHISK9. PCR product carrying chitinase gene without terminator was inserted into pCR2.1 vector to obtain plasmid TACHISK9. Arrows indicate direction of transcription.

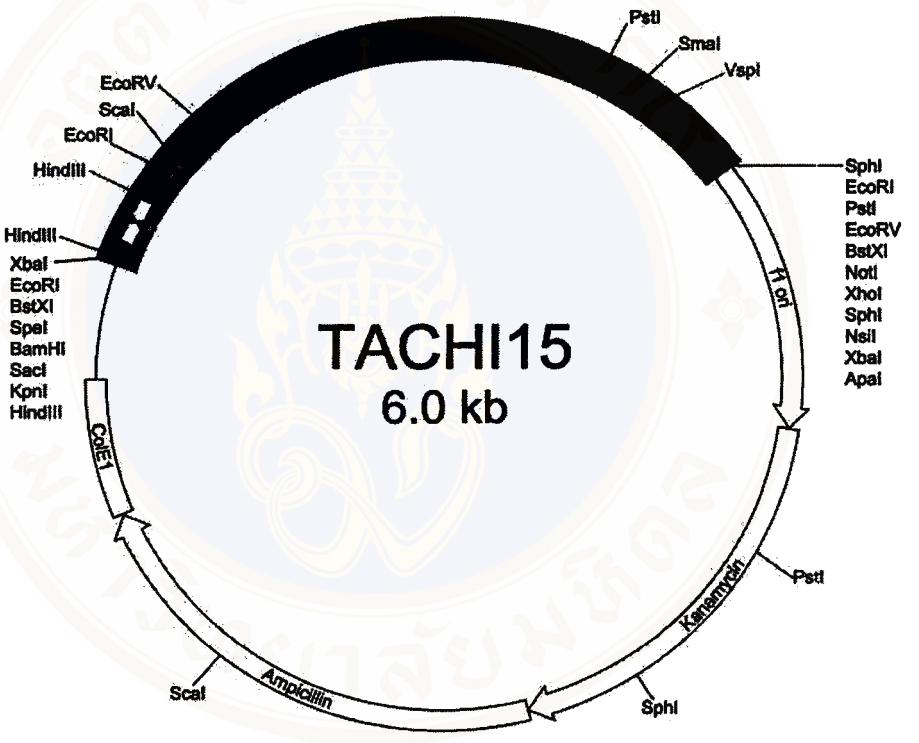


Fig. 23. A schematic restriction map of TACHI15. PCR product carrying chitinase gene with terminator was inserted into pCR2.1 vector to obtain plasmid TACHI15. The symbol ⊏X⊐ is terminator. Arrows indicate direction of transcription.

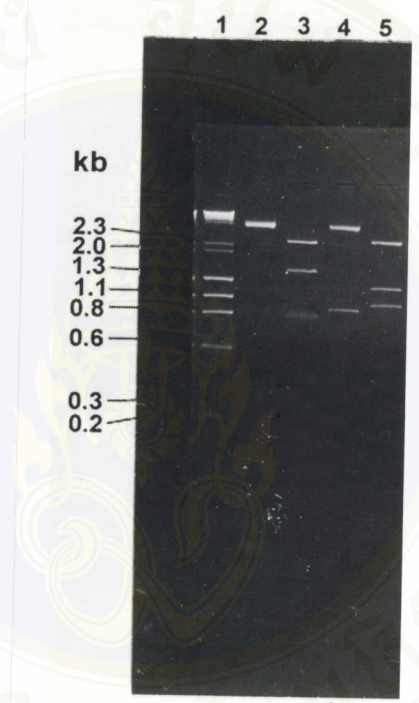


Fig. 24. Agarose gel electrophoresis of plasmid TA19/5 cut with various restriction enzymes.

Lane 1 λ DNA cut *Hind*III and ϕX174 cut with *Hae*III

Lane 2 TA19/5 cut with *Nsi*I

Lane 3 TA19/5 cut with *Ssp*I/*Sph*I

Lane 4 TA19/5 cut with *Eco*RV

Lane 5 TA19/5 cut with *Vsp*I/*Sac*I

TA19/5 (Fig. 24, lane 4) showed two DNA bands, a 3.9 kb and 0.9 kb fragments. The 3.9 kb bands was corresponded to the size of pCR2.1 vector. The additional band of 0.9 kb was the inserted PCR fragment of *p19* gene. Plasmid TA19/5 digested with various combinations of the restriction enzymes such as *Nsi* I, *Ssp* I/*Sph* I and *Vsp* I/*Sac* I indicated that the inserted fragment was 0.9 kb PCR fragment of *p19* gene. The *Nsi* I digested TA19/5 (Fig. 24, lane 2) lead to the presence of three DNA fragments of sizes 4.1, 0.4 and 0.3 kb. Double digestion of TA19/5 with *Ssp* I and *Sph* I (Fig. 24, lane 3) showed three DNA bands of 2.5, 1.5 and 0.8 kb fragments. Double digestion of TA19/5 with *Vsp* I and *Sac* I (Fig. 24, lane 5) also resulted in three DNA fragments of 2.75, 1.1 and 0.95 kb. The presence of additional 0.9 kb band and the restriction patterns of TA19/5, demonstrated that the TA19/5 plasmid from transformed *E. coli* DH5α possessed the 0.9 kb inserted *Bam*H I-*Sph* I PCR fragment of *p19* gene.

Plasmid TA68S/9 and TA68X/14 were digested with *Sca* I, *Nsi* I, *Kpn* I and *Eco*R I and electrophoresed through 1.2% agarose gel (Fig. 25, lane 2-9). The *Kpn* I digested TA68S/9 and TA68X/14 (Fig. 25, lane 4 and 8) showed two DNA bands, 3.9, 2.0 kb and 3.9, 2.2 kb, respectively. The 3.9 kb fragment corresponded to the size of pCR2.1 vector. While 2.0 and 2.2 kb were the inserted PCR fragments of *cry11Aa1* gene which were different in additional part of *cry11Aa1* terminator in TA68X/14. The plasmid patterns of TA68S/9 and TA68X/14 digested with various other combinations of the restriction enzymes such as *Sca* I, *Nsi* I, and *Eco*R I indicated that the inserted fragment was 2.0 and 2.2 kb PCR fragment of *cry11Aa1* gene. The *Sca* I digested TA68S/9 and TA68X/14 (Fig. 25, lane 2 and 6) shown three DNA fragments of sizes, 2.5, 2.1, 1.4 kb and 2.5, 2.3, 1.4 kb. Two DNA bands of 4.2, 1.8 kb (Fig. 25, lane 3) and 4.4, 1.8 kb (Fig. 25, lane 7) appeared when plasmid TA68S/9 and TA68X/14 were digested with *Nsi* I. The *Eco*R I digested

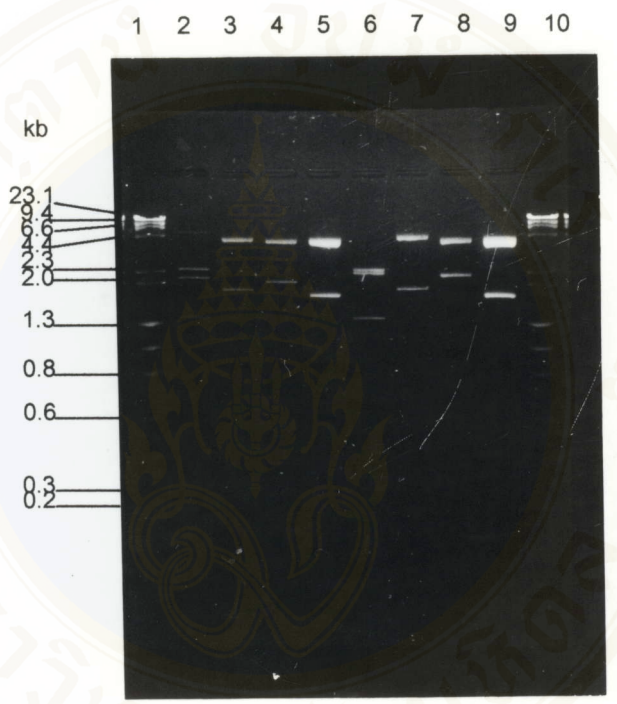


Fig. 25. Agarose gel electrophoresis of plasmid TA68S/9 and TA68X/14 cut with various restriction enzymes.

Lane 1, 10 λ DNA cut *Hind*III and ϕX174 cut with *Hae*III

Lane 2, 6 TA68S/9 and TA68X/14 cut with *Sca*I, respectively

Lane 3, 7 TA68S/9 and TA68X/14 cut with *Nsi*I, respectively

Lane 4, 8 TA68S/9 and TA68X/14 cut with *Kpn*I, respectively

Lane 5, 9 TA68S/9 and TA68X/14 cut with *Eco*RI, respectively

TA68S/9 and TA68X/14 (Fig. 25, lane 5 and 9) lead to the presence of three DNA fragments of sizes, 4.15, 1.7, 0.15 and 4.15, 1.7, 0.35. Thus, from the restriction patterns of TA68S/9 and TA68X/14, it showed that TA68S/9 and TA68X/14 from transformed *E. coli* DH5α possessed the inserted PCR fragments which presumably to be the 2.0 and 2.2 kb inserted *Kpn* I-*Sph* I and *Kpn* I and *Xba* I PCR fragments of *cry11Aa1* gene.

Plasmid TACHI15 and TACHISK9 were digested with *Pst I*, *Eco*R I, *Eco*R V and *Vsp* I/ *Sac* I and electrophoresed through 1.5% agarose gel (Fig. 26, lane 2-9). The *Eco*R I digested TACHI15 and TACHISK9 (Fig. 26, lane 3 and 7) resulted in three DNA fragments of sizes 3.9, 1.7, 0.4 kb and 3.9, 1.7, 0.2 kb. The 3.9 kb fragments of both TACHI15 and TACHISK9 corresponded to the size of plasmid pCR2.1 vector. The 1.7 and 0.4 kb in *Eco*R I digested TACHIX/15 and 1.7 and 0.2 in *Eco*R I digested TACHISK9 were derived from 2.1 and 1.9 kb PCR inserted fragment of TP-1 chitinase gene. Plasmid TACHI15 was larger than TACHISK9 because it contained an additional terminator region at the 3'end. Restriction fragments obtained from TACHI15 and TACHISK9 digested with various restriction enzymes such as *Pst* I, *Eco*R V, *Vsp* I/ *Sac* I indicated that both plasmids contained the 2.1 and 1.9 kb inserted PCR fragments of TP-1 chitinase gene, respectively. The *Pst* I digested TACHI15 and TACHISK9 (Fig. 26, lane 2 and 6) generated three DNA bands of 4.1, 1.2, 0.7 kb and 3.9, 1.2 and 0.7 kb. Two DNA bands with molecular weight of 4.2, 1.6 kb and 4.4, 1.6 kb (Fig. 26, lane 4 and 9) appeared when TACHI15 and TACHISK9 were digested with *Eco*R V. Double digestion of TACHIX/15 and TACHIK/9 (Fig. 26, lane 5 and 8) with *Vsp* I and *Sac* I led to the presence of five DNA fragments, 3.0, 1.35, 1.15, 0.5, 1.4 kb and 3.0, 1.35, 1.15, 0.3 and 0.14 kb. From restriction patterns and sizes of inserted DNA fragments, it indicated that TACHI15 and TACHISK9 plasmids from transformed *E. coli* DH5α possessed the

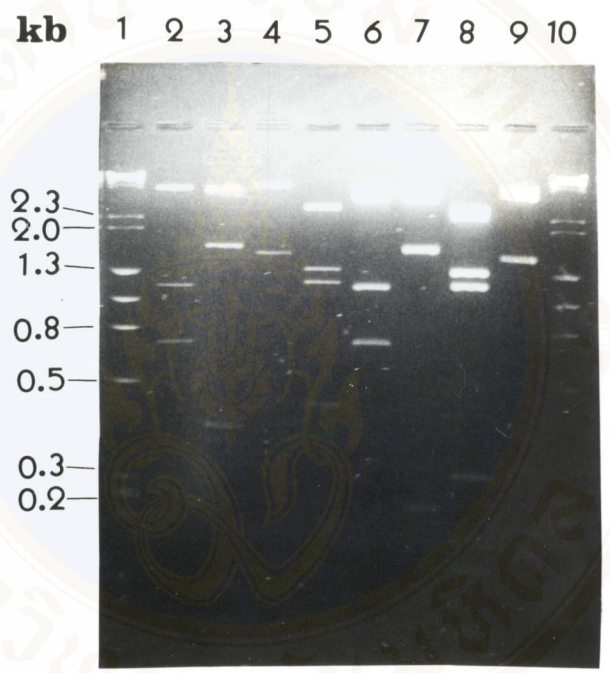


Fig. 26. Agarose gel electrophoresis of plasmid TACHI15 and TACHISK9 cut with various restriction enzymes.

Lane 1, 10 λ DNA cut *Hind*III and ϕX174 cut with *Hae*III

Lane 2, 6 TAchiX/15 and TAchiK/9 cut with *Pst*I, respectively

Lane 3, 7 TAchiX/15 and TAchiK/9cut with *Eco*RI, respectively

Lane 4, 9 TAchiX/15 and TAchiK/9 cut with *Eco*RV, respectively

Lane 5, 8 TAchiX/15 and TAchiK/9 cut with *Vsp*I/*Sac*I, respectively

inserted 2.1 and 1.9 kb, *Sph* I-*Xba* I and *Sph* I-*Kpn* I, PCR fragments of TP-1 chitinase gene.

Thus, by restriction analysis all TA plasmids harbouring each of PCR amplified genes (*p19*, *cry11Aa1* and chitinase genes) carried the genes of interest. DNA sequence analysis had been done to confirm the nucleotide sequence at the ends of all genes in *E. coli* TA clones.

2.2 DNA sequence analysis of all *E. coli* TA clones

DNA sequence analysis around joining region of each plasmid from all TA clones was performed using T7 and RT primer as described in Materials and Methods. Normally, sequencing was able to proceed only 350-400 bases from primer. So, not all the complete gene could be sequenced with T7 and RT primers. However, at both 3' and 5' ends of each gene contained many important regions such as the joining sites at the primers of each gene, the promoter region, SD sequence, start and stop codon, terminator region. The nucleotide sequences of these parts must be correct.

Data of nucleotide sequences at both 3' and 5' ends of each gene were obtained and compared with the published nucleotide sequences (Fig. 11A, 11B and 12). The result from RT primer showed that plasmid TA19/5 had *Bam*H I site at 5' end, *p19* promoter, its SD sequence, and start codon. The nucleotide sequence of TA19/5 obtained from T7 primer revealed *p19* stop codon, part of DNA sequence between *p19* gene and *cry11Aa1* gene and *Sph* I site at 3'end.

The nucleotide sequences of *cry11Aa1* gene in TA68S/9 and TA68X/14 obtained from T7 primer revealed *Kpn* I site, part of DNA sequence between *p19* gene and *cry11Aa1* gene, and its start codon. The nucleotide sequence of TA68S/9 resulted from RT primer

showed the *cry11Aa1* stop codon and *Sph* I site. While, the nucleotide sequences of TA68X/14 using RT primer showed the *cry11Aa1* stop codon, its terminator, and *Xba* I site.

The nucleotide sequences of TP-1 chitinase gene in TACHISK9 and TACHI15 obtained from T7 primer revealed *Sph* I site, chitinase SD sequence, and start codon. The nucleotide sequence of TACHISK9 result from RT primer showed chitinase stop codon, and *Kpn* I site. While, the nucleotide sequences of TACHI15 using RT primer revealed chitinase stop codon, its terminator and *Xba* I site.

The data of sequence analysis confirmed that all TA plasmids contained the correct nucleotide sequence with presence of restriction sites at joining boundary and presence of the promoter region, SD sequence, start codon, stop codon, and terminator region of each gene (*p19*, *cry11Aa1* and TP-1 chitinase genes).

3. Subcloning of *p19* gene from TA vector into pBluescriptSK+ vector

Because the cloning sites in TA plasmid were not appropriated for fusion of all genes (*p19*, *cry11Aa1* and chitinase genes) in the same transcriptional direction. Plasmid pBluescriptSK+ vector was used to create those suitable cloning sites. The restriction map of pBluescriptSK+ vector was illustrated in Fig. 27. Firstly, the *p19* gene in TA19/5 was subcloned in pBluescriptSK+ vector. The new recombinant plasmid was used for subsequent fusion of the other genes, *cry11Aa1* and chitinase genes. The constructions of various plasmids were carried out in *E. coli* host prior to transform into *B.t.i.*.

Plasmid TA19/5 was doubly digested with *Pst* I and *Hind* III. A 0.9 kb fragment was recovered and purified from 0.7% agarose gel using QIAEXII gel extraction kit

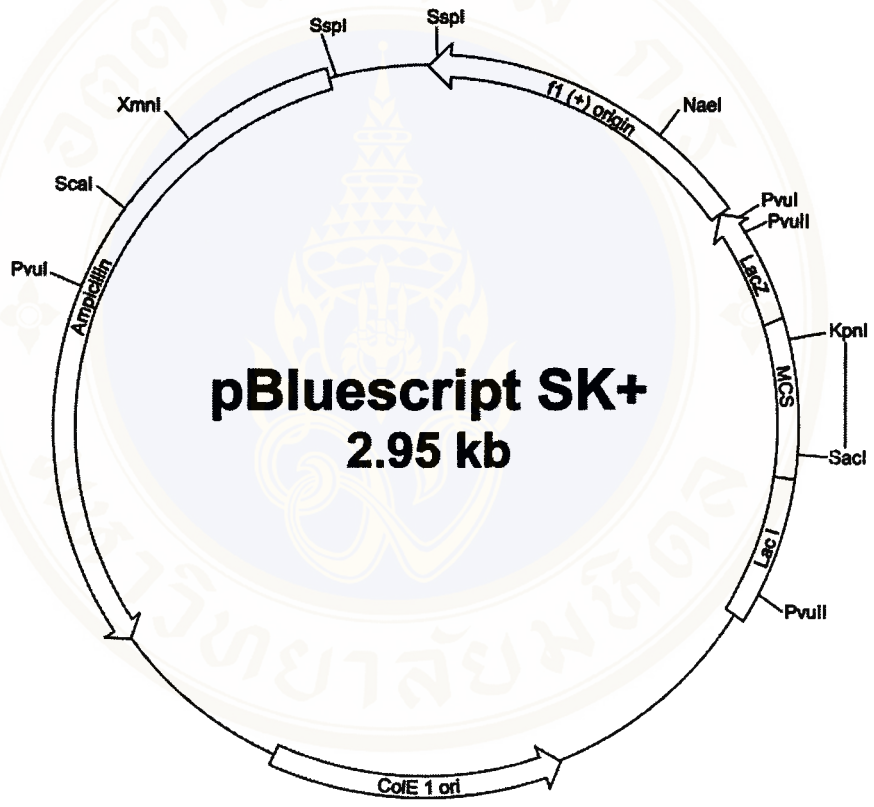


Fig. 27. The schematic restriction map of pBluescript SK+ vector. The sequence of the multiple cloning site is shown below the plasmid map.

(QIAGEN, USA). The recovered 0.9 kb fragment was ligated into *E. coli* vector, pBluescriptiptSK+ vector, at *Pst* I-*Hind* III sites. Thus, the *p19* gene was subcloned in this new vector with the suitable fusion sites downstream. The new recombinant plasmid was designated as pSK19/5 and the schematic diagram for construction of this hybrid plasmid was outlined in Fig. 28. The size of pSK19/5 was 3.9 kb.

After overnight ligation at 15 °C, the plasmid pSK19/5 was introduced into *E. coli* DH5α by competent cell transformation technique. The transformants were selected on LB agar containing 50 μg/ml ampicillin which had been spreaded with 50 μl X-gal (2 μg/ml). The white colonies which presumably contained the inserted fragment were randomly selected and subjected to plasmid extraction using alkaline lysis method. One of those clones with smaller molecular weight than that of plasmid TA19/5 were selected, purified and further used for restriction enzyme analysis in order to confirm the presence of *p19* gene. This clone was designated as *E. coli* DH5α (pSK19/5).

3.1 Analysis of plasmid pSK19/5 in *E. coli* DH5α (SK19/5) transformants

For determination of restriction analysis patterns of plasmid pSK19/5 in *E. coli* DH5α (pSK19/5), the plasmid was extracted, purified, digested with appropriated restriction enzymes and subjected to 1.5% agarose gel electrophoresis. As shown in Fig. 29, a single band of 3.9 kb fragment (lane 8) of *Eco*RV digested SK19/5 was detected. The 3.9 kb corresponded to the sum of molecular weight between 3.0 kb of pBluescriptSK+ and 0.9 kb of the inserted *Pst* I-*Hind* III fragment of TA19/5. The *Nsi* I restricted pSK19/5 (lane 6) led to two DNA bands corresponding to 3.5 kb and 0.4 kb fragments. The 0.4 kb fragment comigrated with 0.4 kb fragment of plasmid TA19/5 (lane 2) which also digested with *Nsi* I. The doubly digested pSK19/5 with *Ssp* I and *Sph*

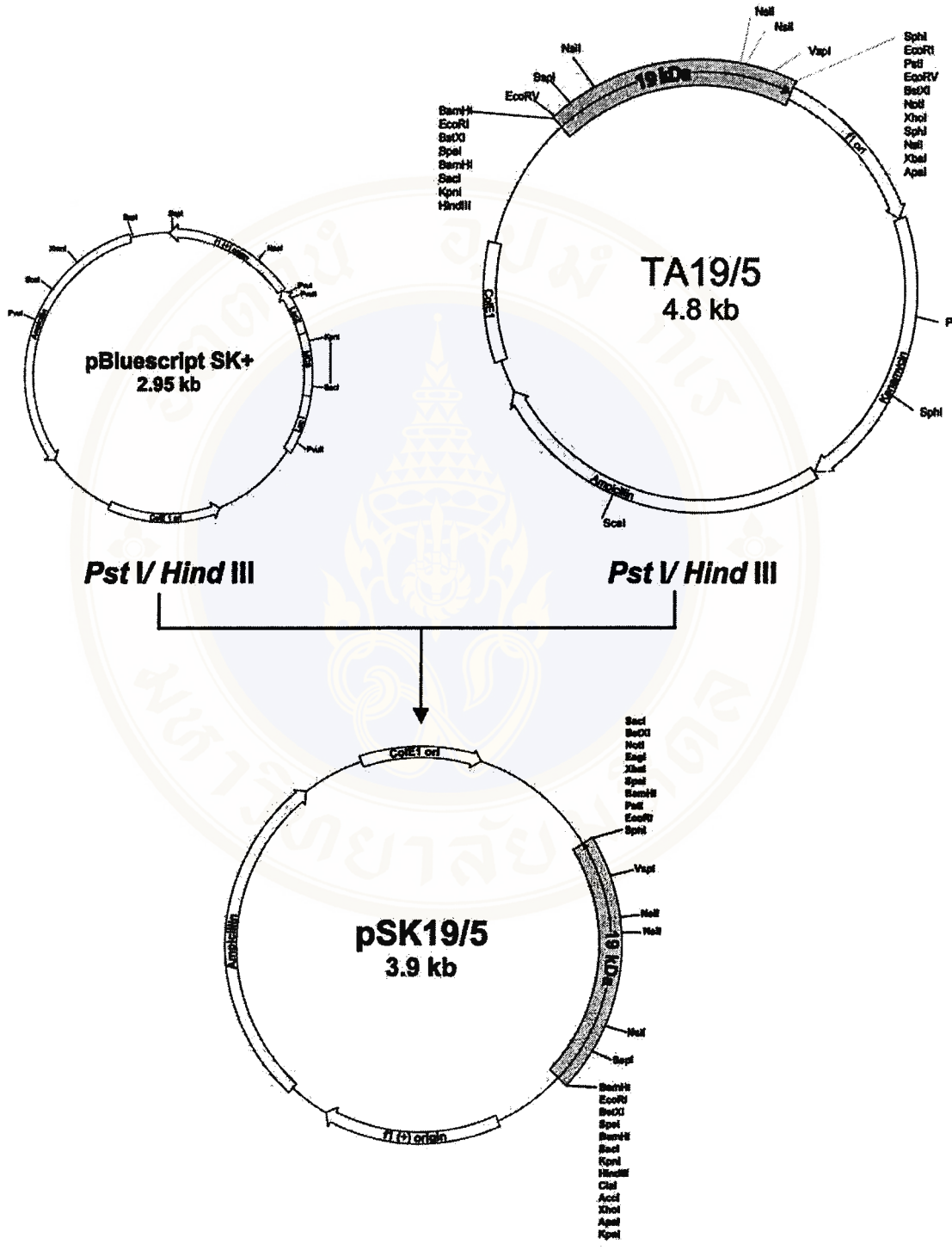


Fig 28. A schematic diagram of pSK19/5 plasmid construction. Plasmid TA19/5 from *E. coli* DH5α (TA19/5) contained PCR product of *p19* gene from *B.t.i.* was doubly digested with *Pst* I and *Hind* III. The *Pst* I-*Hind* III fragment harboring *p19* gene was inserted into *Pst* I and *Hind* III sites of pBluescriptSK+ vector.

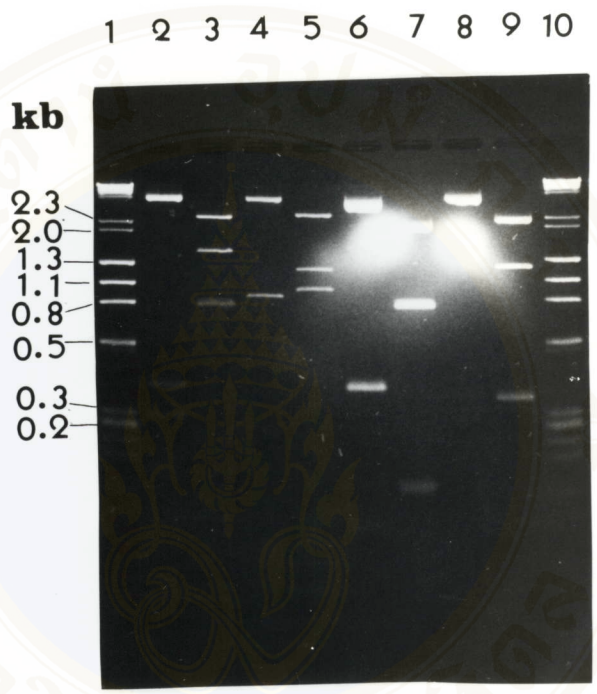


Fig. 29. Agarose gel electrophoresis of plasmid pSK19/5 from *E.coli* DH5α cut with various restriction enzymes.

Lane 1, 10 λ DNA cut *Hind*III and ϕX174 cut with *Hae*III

Lane 2, 6 TA19/5 and pSK19/5 cut with *Nsi*I

Lane 3, 7 TA19/5 and pSK19/5 cut with *Ssp*I/*Sph*I

Lane 4, 8 TA19/5 and pSK19/5 cut with *Eco*RV

Lane 5, 9 TA19/5 and pSK19/5 cut with *Vsp*I/*Sac*I

I showed three DNA bands of 2.2, 0.8 and 0.1 kb (lane 7). Two 0.8 kb fragments of *Ssp* I-*Sph* I digested pSK19/5 appeared which comigrated with 0.8 kb fragment of plasmid TA19/5 (lane 3) also digested with the same enzymes. The doubly digested *Vsp* I and *Sac* I generated three DNA bands of 2.3, 1.2 and 0.4 kb (lane 9). Thus, it appeared that the pSK19/5 plasmid possessed the inserted fragment which presumably was the 0.9 kb of *p19* gene from plasmid TA19/5 ligated to a 3.0 kb from plasmid pBluescriptSK+. The pSK19/5 plasmid was subsequently used for fusion of *cry11Aa1* gene and chitinase gene.

4. Construction of transcriptional fusion harbouring *p19* gene and chitinase gene

In order to study the expression of chitinase gene under spore promoter of *cry11Aa1* gene from *B.t.i.*, transcriptional fusion plasmid of *p19* and chitinase genes was constructed. The *cry11Aa1* gene arranged in operon which composed of *p19*, *cry11Aa1*, and *p20* gene, respectively. All three genes used the same spore promoter, *p19* promoter, which located upstream from *p19* gene. Thus, the chitinase structural gene with its terminator was fused downstream to *p19* gene.

Plasmid pSK 19/5 was digested with *Sph* I and *Xba* I. The *Sph* I site was derived from 19RSph I primer as described in primer design. The *Xba* I site was in the pBluescriptSK+ multicloning sites. Plasmid TACHI15 digested with *Sph* I and *Xba* I released the 2.1 kb fragment. Both *Sph* I and *Xba* I in TACHI15 derived from primer design, which was chiFSph I and chiRXba I primers. This 2.1 kb *Sph* I-*Xba* I fragment of TACHI15 harbouring complete chitinase gene with its terminator was inserted into pSK19/5 at the same restriction sites. Thus, the chitinase gene arranged in operon downstream to *p19* gene which was control under the same *p19* spore promoter. The size of new

hybrid plasmid was 6.0 kb which was designated as p19CHI. The schematic diagram for construction of this plasmid was shown in Fig. 30.

After overnight ligation at 15 °C, the plasmid p19CHI was introduced into *E. coli* DH5α by competent cell transformation technique. The transformants were selected on LB agar containing 50 μg/ml ampicillin. Subsequently, the transformants were screened for the chitinase producing clones by replicating on LB agar containing 50 μg/ml ampicillin which had been overlaid with 2% colloidal chitin. The transformants which generated the clear halo zones around colonies were selected, subjected to plasmid extraction and further used for restriction enzyme analysis. One of those clones which had the correct restriction patterns was selected and designated as *E. coli* DH5α (p19CHI). Southern blot analysis was subsequently performed to confirm the presence of both *p19* and chitinase genes in p19CHI.

5. Construction of transcriptional fusion plasmid harbouring *p19, cry11Aa1* and chitinase genes

In order to enhance the mosquito larvicidal effect of Cry11Aa1 toxin, a novel prospect was proposed using chitinase enzyme. The *cry11Aa1* was coexpressed with chitinase genes at the spore stage. Two transcriptional fusion plasmids comprising of *p19*, *cry11Aa1* and chitinase genes, was constructed. The first construct, *cry11Aa1* gene located in the middle between *p19* gene and chitinase gene. While, the second construct, chitinase gene was in the middle flanking with *p19* gene and *cry11Aa1* gene. The series of each gene in the operon normally affects their expression. If gene locates near the promoter, it would strongly express. Thus, the production of Cry11Aa1 toxin and chitinase enzyme was compared between both constructs.

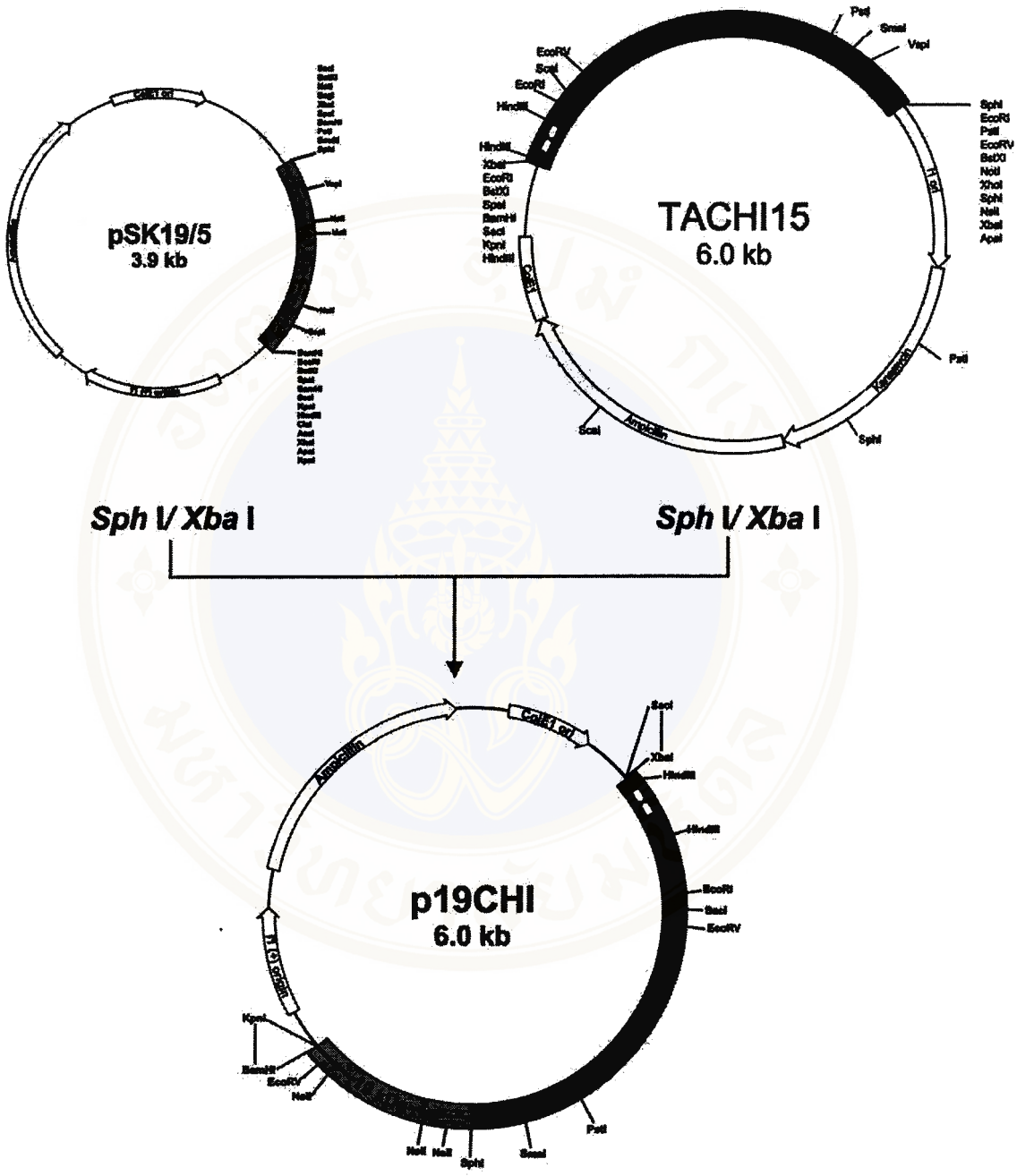


Fig. 30. A schematic diagram of p19CHI plasmid construction. Plasmid TACHI15 from *E. coli* DH5α (TACHI15) with chitinase gene from *B. licheniformis* TP-1 was doubly digested with *Sph* I and *Xba* I. the *Sph* I-*Xba* I fragment harbouring chitinase gene was inserted into *Sph* I and *Xba* I sites of pSK19/5 to obtain plasmid p19CHI.

5.1 Construction of transcriptional fusion plasmid harbouring *p19*, *cry11Aa1* and chitinase genes, respectively

For the first construct, the *cry11Aa1* gene from plasmid TA68S/9 was inserted in the middle of *p19* and chitinase genes in plasmid pSK19/5. The detail of construction was as followed. Firstly, plasmid p19CHI was digested with *Sph* I and the linearized fragment was then treated with enzyme alkaline phosphatase to protect self-ligation. Plasmid TA68S/9 was doubly digested with *Sph* I and *Pvu* II. The 2.0 kb *Sph* I fragment harbouring *cry11Aa1* gene was inserted into *Sph* I site of p19CHI. Thus, a new recombinant plasmid contained *p19*, *cry11Aa1*, and chitinase genes, respectively, which was designated as p1968CHI. The size of p1968CHI was 8.0 kb. Thus, all three genes arranged in the operon and regulated by a *p19* spore-specific promoter located upstream of *cry11Aa1* and chitinase genes. The schematic diagram illustrating the construction of p1968CHI was shown in Fig. 31.

p1968CHI was introduced into *E. coli* DH5α and screened in the same way as described in section 4. Southern blot analysis was subsequently performed to confirm the presence of *p19*, *cry11Aa1* and chitinase genes in p1968CHI.

5.2 Construction of transcriptional fusion plasmid harbouring *p19*, chitinase and *cry11Aa1* genes, respectively

5.2.1 Construction of transcriptional fusion plasmid harbouring chitinase and *cry11Aa1* genes with *cry11Aa1* terminator

The *cry11Aa1* gene with its terminator from plasmid TA68X/14 was firstly ligated downstream from chitinase gene in plasmid TACHISK9. Plasmid TA68X/14

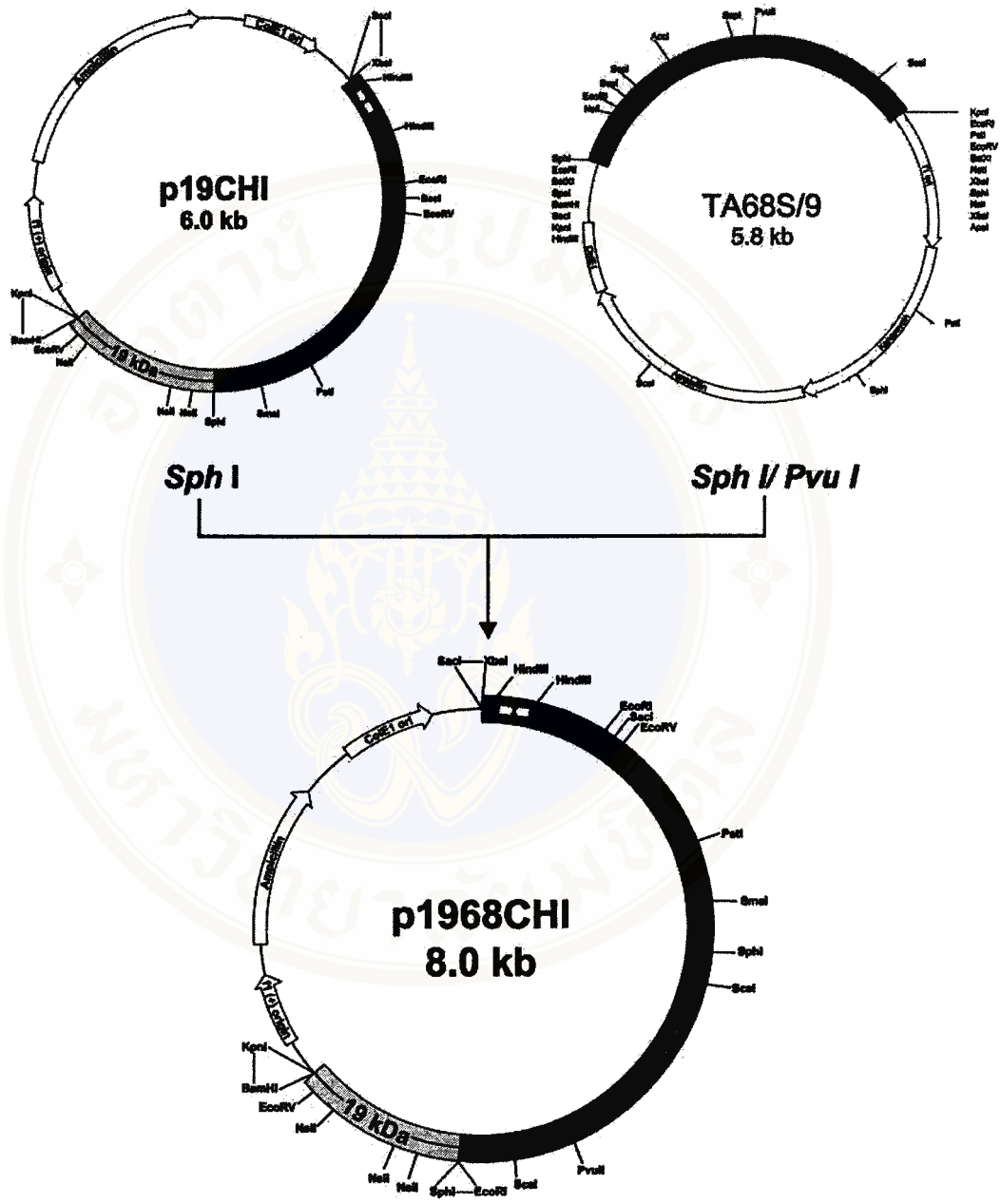


Fig. 31. A schematic diagram of p1968CHI plasmid construction. Plasmid TA68S/9 from *E. coli* DH5α (TA68S/9) with *cry11Aa1* gene from *B.t.i.* was doubly digested with *Sph*I and *Pvu*I. The *Sph*I-*Pvu*I fragment harbouring *cry11Aa1* gene was inserted into *Sph*I site of p19CHI to obtain plasmid p1968CHI.

was doubly digested with *Kpn* I and *Pvu* I. The 2.2 kb *Kpn* I fragment harbouring *cry11Aa1* gene with its terminator was inserted into *Kpn* I site of plasmid TACHISK9. The new recombinant plasmid was obtained which designated as pCHI68. The size of new plasmid was 8.0 kb. The schematic diagram illustrating the construction of pCHI68 was shown in Fig. 32.

Plasmid pCHI68 was introduced into *E. coli* DH5α and screened in the same way as described in section 4. Southern blot analysis was subsequently performed to confirm the presence of both chitinase and *cry11Aa1* genes. The transcriptional fusion of chitinase and *cry11Aa1* genes in pCHI68 was further subcloned into pSK19/5.

5.2.2 Construction of transcriptional fusion plasmid harbouring *p19*, chitinase and *cry11Aa1* genes with *cry11Aa1* terminator

Plasmid pCHI68 was doubly digested with *Sph* I and *Xba* I. The 4.1 kb *Sph* I-*Xba* I fragment carrying chitinase gene, *cry11Aa1* gene with its terminator, was inserted into *Sph* I-*Xba* I sites of plasmid pSK 19/5. Thus, *p19* gene, chitinase gene, *cry11Aa1* gene and *cry11Aa1* terminator was arranged serially. This new recombinant plasmid was designated as p19CHI68 with the molecular weight of 8.0 kb. The schematic diagram for construction of p19CHI68 was illustrated in Fig. 32.

Plasmid p19CHI68 was introduced into *E. coli* DH5α and screened in the same way as described in section 4. Southern blot analysis was subsequently performed to confirm the presence of *p19*, chitinase and *cry11Aa1* genes.

6. Southern blot analysis

Because each gene in all transcriptional fusion *E. coli* DH5α clones which were

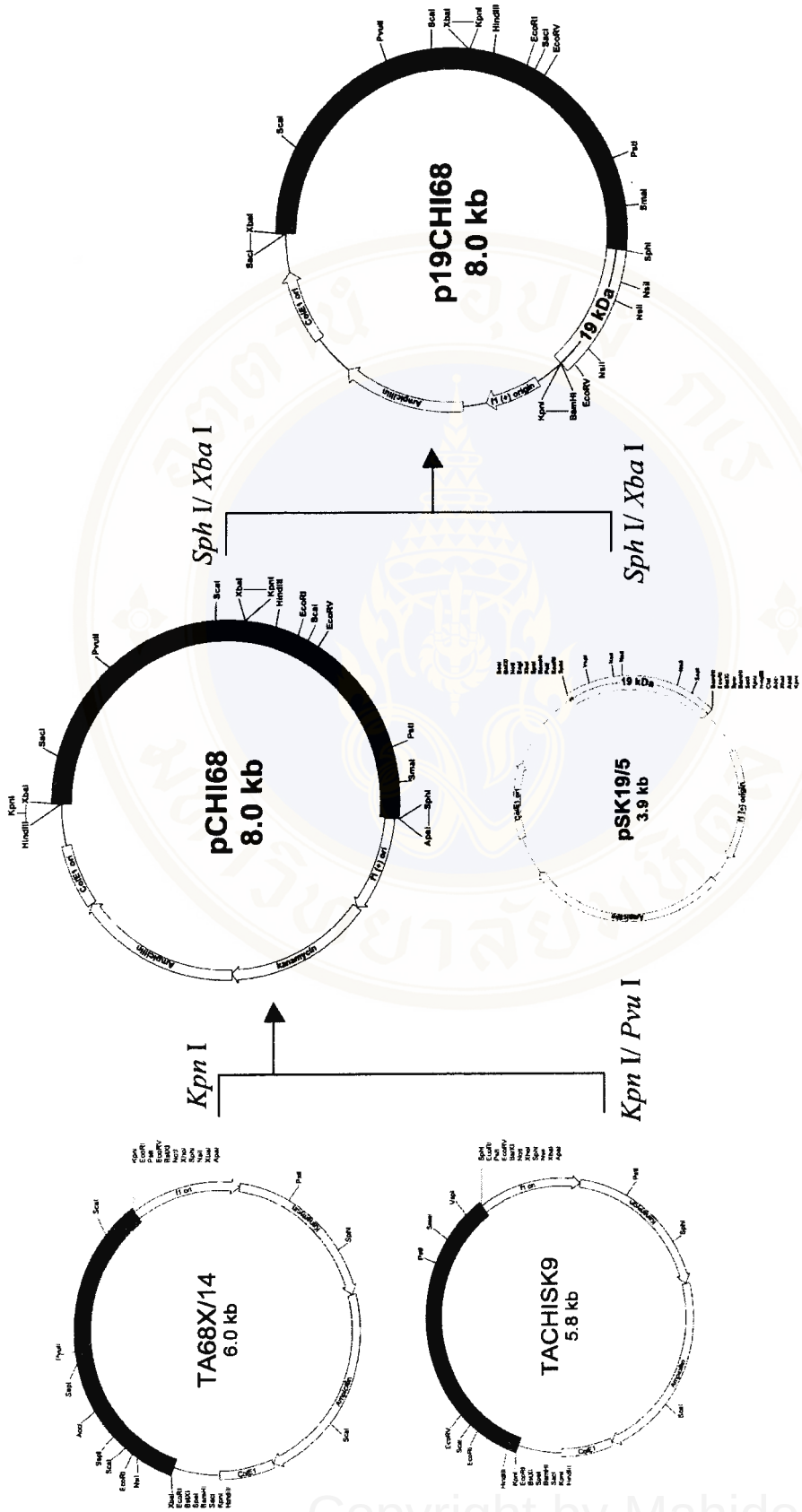


Fig. 32. A schematic diagram shows the construction of recombinant plasmid pCHI68 harbouring chitinase and *cry11Aa1* genes with its terminator and p19CHI68 harbouring *p19*, chitinase and *cry11Aa1* genes with *cry11Aa1* terminator. Plasmid TA68X/14 was doubly digested with *Kpn* I and *Pvu* I. The 2.2 kb *Kpn* I fragment was inserted into *Kpn* I site of plasmid TACHISK9 resulted in plasmid pCHI68. The 4.1 kb *Sph* I-*Xba* I fragment of pCHI68 was subsequently inserted into *Sph* I-*Xba* I sites of plasmid pSK 19/5. The new recombinant plasmid was designated as p19CHI68.

E. coli DH5α (p19CHI), *E. coli* DH5α (p1968CHI), *E. coli* DH5α (pCHI68) and *E. coli* DH5α (p19CHI68) were generated from PCR amplificaton technique. To prove that each gene was derived from corresponding genes, southern blot analysis with specific probe was performed. The 0.4 kb *Nsi* I fragment and 1.4 kb *Sca* I fragment from plasmid pBTC68A which contained the original *p19* and *cry11Aa1* genes from *B.t.i.* were used as probes to detect the relevant fragments in plasmid p19CHI, p1968CHI and p19CHI68. Also, the 0.9 kb *Pst* I-*Sac* I fragment from plasmid pCHIL1 which contained the original TP-1 chitinase gene from *B. licheniformis* TP-1 was used as a probe to detect the TP-1 chitinase gene in all transcriptional fusion plasmids.

Plasmids from *E. coli* DH5α (p19CHI), *E. coli* DH5α (p1968CHI), and *E. coli* DH5α (p19CHI68) were extracted, purified, digested with appropriate enzymes and subjected to agarose gel electrophoresis (Fig. 33, lane 2-13). Restriction enzyme digestion of plasmid p19CHI, pCHI68, p19CHI68, and p1968CHI were as followed, digested with *Eco*R I (Fig. 33, lane 2, 5, 8, and 11), *Nsi* I (Fig. 33, lane 3, 6, 9 , and 12), and *Pst* I (Fig. 33, lane 4, 7, 10, and 13). After electrophoresis, the DNA bands in agarose gel were transferred to nylon membrane by upward capillary action and subjected to hybridization with α-P^{32}dCTP labeled probe as described in details in the Materials and Methods section.

The results in Fig. 34A, showed that all three transcriptional fusion plasmids (p19CHI, p19CHI68 and p1968CHI) when hybridized with a *p19* probe conferred positive bands. The result indicated the existence of *p19* gene. While, no bands could be detected when pCHI68 hybridized with *p19* probe. In addition, the sizes of the positive bands in all transcriptional fusion plasmids were correlated to their restriction mapping. The 3.5 kb, 5.6 and 0.4 kb, 6.0 kb derived from digestion of p19CHI with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig.34A, lane 2-4) could hybridize with 0.4 kb *Nsi* I probe of

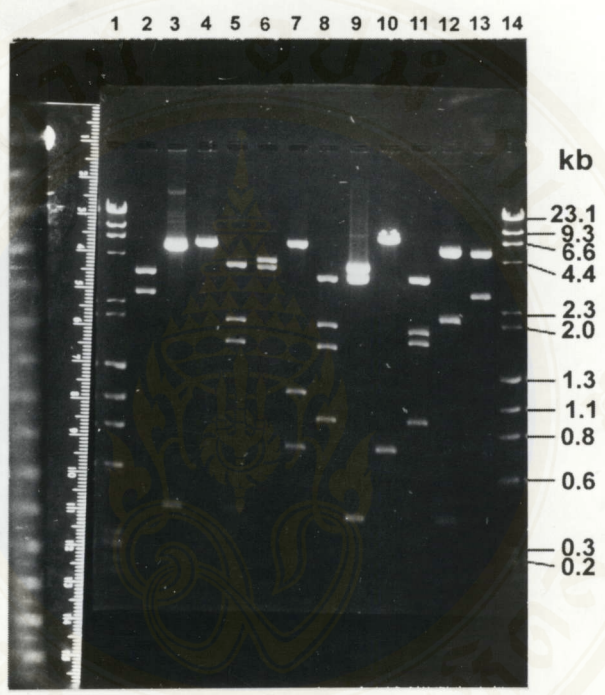


Fig. 33. Agarose gel electrophoresis showing the restriction fragments of p19CHI, pCHI68, p19CHI68 and p1968CHI digested with various enzymes.

Lane 1 and 14	λ DNA cut *Hind*III and ϕX174 cut with *Hae*III
Lane 2, 3, and 4	p19CHI cut with *Eco*R I (2), *Nsi* I (3), and *Pst* I (4)
Lane 5, 6, and 7	pCHI68 cut with *Eco*R I (5), *Nsi* I (6), and *Pst* I (7)
Lane 8, 9, and 10	p19CHI68 cut with *Eco*R I (8), *Nsi* I (9), and *Pst* I (10)
Lane 11, 12, and 13	p1968CHI cut with *Eco*R I (11), *Nsi* I (12), and *Pst* I (13)

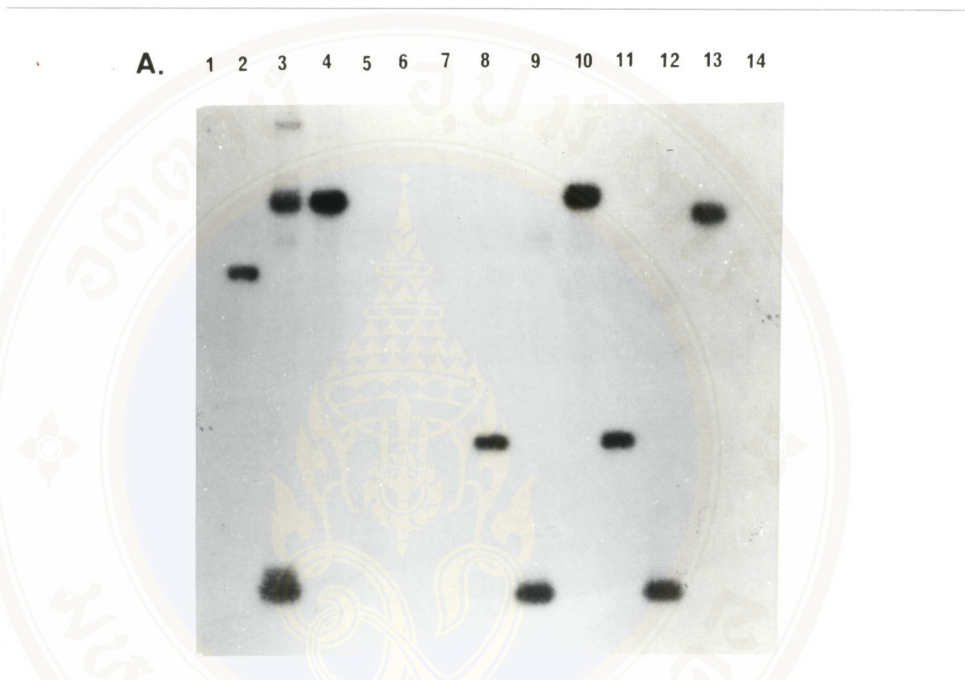


Fig. 34A. Southern hybridization of plasmid p19CHI, pCHI68, p19CHI68 and p1968CHI with α-p^{32}dCTP labeled 0.4 kb *Nsi* I fragment of *p19* gene probe. All lanes were the same as described in Fig. 33.

p19 gene. Similarly, the 1.5 kb, 0.4 kb, and 4.0 kb of p19CHI68 digested with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig.34A, lane 8-10) could positively reacted with the same *p19* gene probe. Also, the 1.7 kb, 0.4 kb, and 5.5 kb from digestion of p1968CHI hybridized with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig.34A, lane 11-13) could positively hybridized with the same probe. Thus, it was cleared that the three transcriptional fusion plasmids, p19CHI, p19CHI68, and p1968CHI, in *E. coli* DH5α harboured *p19* gene from *B.t.i.*.

The results shown in Fig. 34B, indicated that all three transcriptional fusion plasmids (pCHI68, p19CHI68 and p1968CHI) conferred positive bands when hybridized with *cry11Aa1* probe. While, no bands could be detected when p19CHI hybridized with *cry11Aa1* probe. Also, the sizes of the positive bands in all transcriptional fusion plasmids were correlated to their restriction mapping. The 2.0 kb, 4.0 kb, and 6.1 kb obtained from digestion of pCHI68 with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig.34B, lane 5-7) could positively reacted with 1.4 kb *Sca* I probe of *cry11Aa1* gene. Similarly, the 2.0 kb, 4.0 kb, and 7.5 kb from p19CHI68 digested with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig.34B, lane 8-10) could also react with *cry11Aa1* probe. Also, the 1.9 kb, 2.1 kb, and 2.5 kb from p1968CHI digested with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig.34B, lane 11-13) generated hybridized bands with the same *cry11Aa1* probe. Thus, it obviously confirmed that pCHI68, p19CHI68 and p1968CHI in *E. coli* DH5α host harboured *cry11Aa1* gene from *B.t.i.*.

The existence of TP-1 chitinase in all four transcriptional fusion plasmids (p19CHI, pCHI68, p19CHI68, and p1968CHI) was also confirmed by Southern hybridization as shown in Fig. 34C. The 3.5 kb, 5.6 kb, and 6.0 kb from p19CHI digested with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig.34C, lane 2-4) reacted with 0.9 kb *Pst* I-*Sac* I

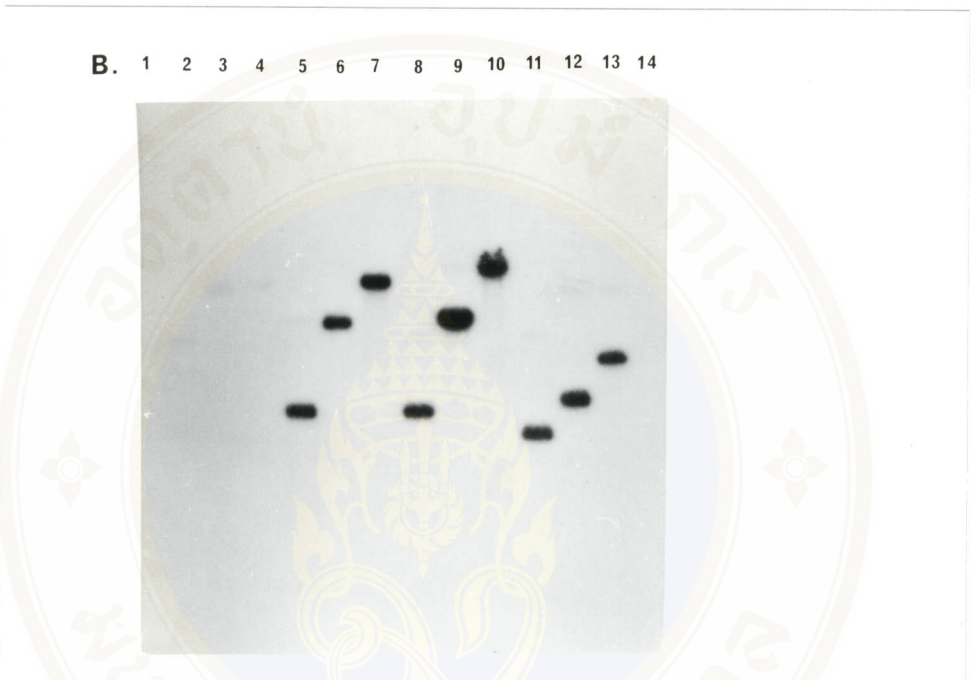


Fig. 34B. Southern hybridization of plasmid p19CHI, pCHI68, p19CHI68 and p1968CHI with α-p^{32}dCTP labeled 1.4 kb *Sca* I fragment of *cry11Aa1* gene probe. All lanes were the same as described in Fig. 33.

Fig. 34C. Southern hybridization of plasmid p19CHI, pCHI68, p19CHI68 and p1968CHI with α-p^{32}dCTP labeled 0.9 kb *Pst* I-*Sac* I fragment of chitinase gene probe. All lanes were the same as described in Fig. 33.

probe of TP-1 chitinase gene. The 1.5 kb, 4.0 kb, and 6.1 kb from digestion pCHI68 with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig. 34C, lane 5-7) could positively react with chitinase probe. The 1.5 kb, 4.0 kb, and 7.5 kb obtained from p19CHI68 digested with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig. 34C, lane 8-10) could react with the same chitinase probe. The 1.9 kb, 5.5 kb, and 5.5 kb obtained from digestion p1968CHI digested with *Eco*R I, *Nsi* I, and *Pst* I, respectively, (Fig. 34C, lane 11-13) could also hybridize with chitinase probe. Thus, it was clear that the four transcriptional fusion plasmids in *E. coli* DH5α host harboured the chitinase gene from *B. licheniformis* TP-1.

7. Determination of chitinase activity of *E. coli* clones

The activity of chitinase enzyme in *E. coli* with transcriptional fusion genes was quantitatively determined by colorimetric spectrophotometry method. Data from previous study (Tantimavanich , 1997) indicated that expression of chitinase gene in *E. coli* was constitutive. *E. coli* harbouring a chitinase gene grown under either induced (LB+Amp+chitin) or uninduced (LB+Amp) conditions produced nearly the same level of chitinase activity. A comparable chitinase activity under both conditions was also preliminary observed in all *E. coli* clones (p19CHI, p1968CHI and p19CHI68). The result also indicated that the chitinase activity of all fusion plasmids in *E. coli* host was constitutive (data not shown). Thus, the specific chitinase activity of all *E. coli* transformants were compared with the *E. coli* (pCHIL1) under uninduced condition (LB+Amp).

All *E. coli* strains were grown in 20 ml LB without colloidal chitin induction and incubated at 37°C overnight with shaking at 200 rpm. The chitinase activity and total protein was measured as described in Materials and Methods section. The chitinase

specific activity was calculated and compared in Table 7. The level of chitinase specific activity from *E. coli* (pCHIL1), *E. coli* (p19CHI), *E. coli* (p1968CHI), *E. coli* (p19CHI68) were 5.05, 5.7, 5.2 and 5.5 mU/mg, respectively. No chitinase activity was detected in *E. coli* DH5α host and *E. coli* (pBTC68).

8. Subcloning of transcriptional fusion genes into *Bacillus* vector, plasmid pBC16

8.1 Construction of shuttle plasmids containing transcriptional fusion genes and transformed into *E. coli* DH5α host

In order to characterize the expression of *p19*, *cry11Aa1* and chitinase genes in *B.t.i.* host, all transcriptional fusion constructs were subcloned into plasmid pBC16 *Bacillus* vector. The linearized 6.0 kb, 8.0 kb and 8.0 kb fragments of plasmid p19CHI, p1968CHI and p19CHI68, respectively, were cleaved with *Bam*H I digestion and inserted into *Bam*H I site of plasmid pBC16. These new hybrid plasmids were firstly transformed into *E. coli* DH5α and subsequently introduced into *B.t.i.* The new recombinant plasmids were designated as p16-19CHI, p16-1968CHI and p16-19CHI68 and the molecular size of these plasmids were 10.4 kb, 12.4 kb and 12.4 kb respectively. The restriction map of p16-19CHI, p16-1968CHI and p16-19CHI68 were outlined in Fig. 35, 36 and 37, respectively.

8.2 Restriction analysis of p16-19CHI, p16-1968CHI and p16-19CHI68 in *E. coli* (p16-19CHI), *E. coli* (p16-1968CHI) and *E. coli* (p16-19CHI68) transformants

E. coli (p16-19CHI), *E. coli* (p16-1968CHI) and *E. coli* (p16-19CHI68)

Table7. Comparison of specific chitinase activity from *E. coli* clones grown in LB broth without colloidal chitin

Bacterial strains	Specific chitinase activity (mU/mg)
E. coli DH5α host	0
E. coli DH5α host (pBTC68A)	0
E. coli DH5α host (pCHIL1)	5.05
E. coli DH5α host (p19CHI)	5.7
E. coli DH5α host (p1968CHI)	5.2
E. coli DH5α host (p19CHI68)	5.5

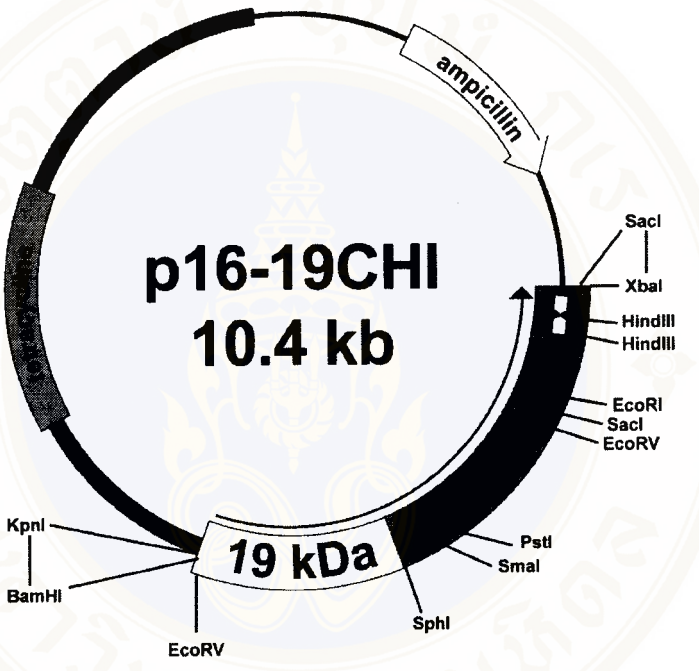


Fig. 35. A restriction map of p16-19CHI. Plasmid p19CHI from *E. coli* DH5α (p19CHI) harbouring transcriptional fusion gene of *p19* and chitinase genes was digested with *Bam*H I and subcloned into *Bam*H I site of pBC16 to obtain p16-19CHI. The symbol ⊏⋈⊐ is chitinase terminator.

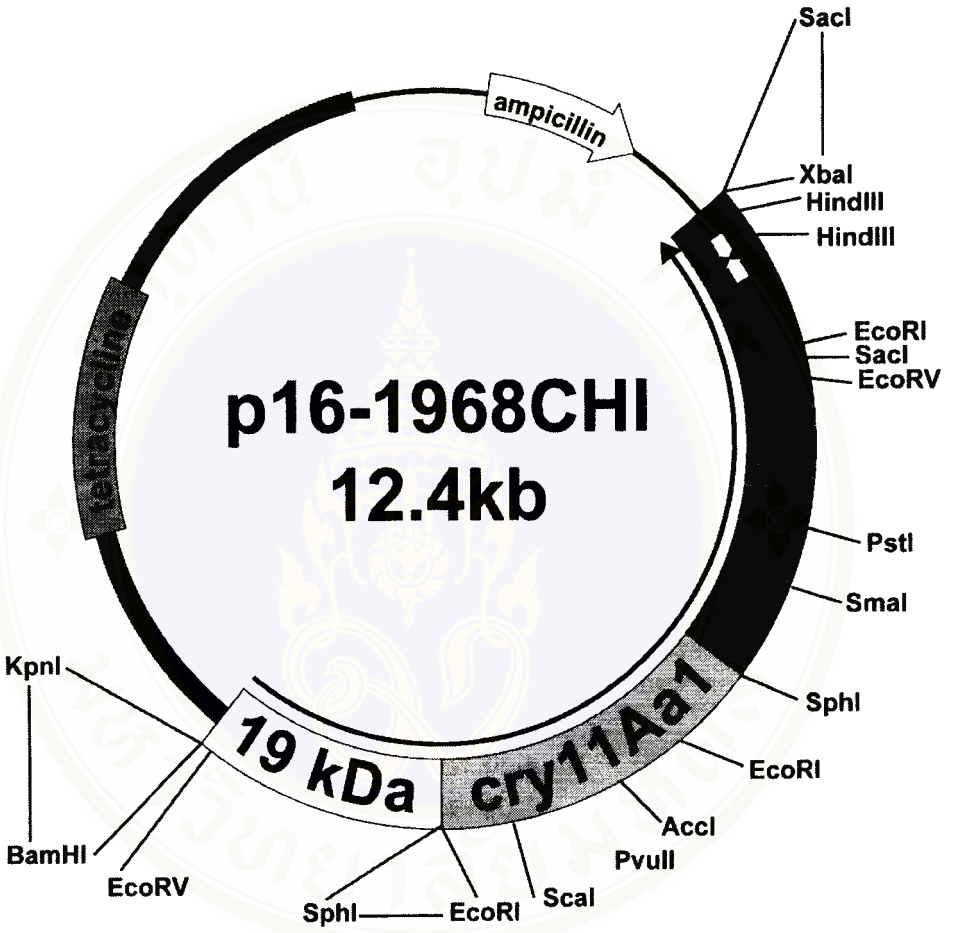


Fig. 36. A restriction map of p16-1968CHI. Plasmid p1968CHI from *E. coli* DH5α (p1968CHI) harbouring transcriptional fusion gene of *p19*, *cry11Aa1* and chitinase genes was digested with *Bam*H I and subcloned into *Bam*H I site of pBC16 to obtain p16-1968CHI. The symbol ⊏◇⊐ is chitinase terminator.

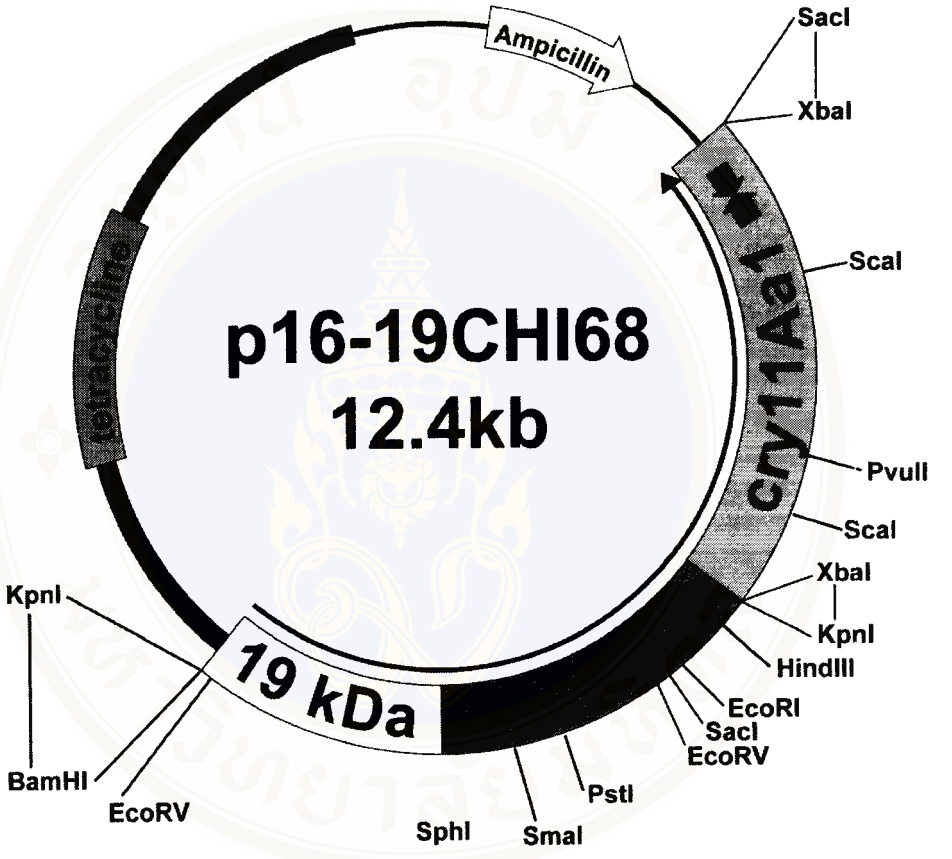


Fig. 37. A restriction map of p16-19CHI68. Plasmid p19CHI68 from *E. coli* DH5α (P19CHI68) harbouring transcriptional fusion genes of *p19*, chitinase and *cry11Aa1* genes was digested with *Bam*H I and subcloned into *Bam*H I site of pBC16 to obtain p16-19CHI68. The symbol ⬅➡ is *cry11Aa1* terminator.

transformants were subjected to plasmid extraction, digested with *Bam*H I and *Xba* I-*Pst* I and analysed in 0.7% agarose gel electrophoresis. Data as shown in Fig. 38, revealed two DNA fragments of either 4.4 kb and 6.0 kb (lane 2), 4.4 kb and 8.0 kb (lane 3) or 4.4 kb and 8.0 kb (lane 4) were obtained from for *Bam*H I digestion of p16-19CHI, p16-1968CHI and p16-19CHI68, respectively. The 4.4 kb fragment of all shuttle plasmids were pBC16 digested with *Bam*H I. While, another *Bam*H I fragments which were 6.0 kb from p16-19CHI, and 8.0 kb from both p16-1968CHI and p16-19CHI68 were corresponded to the size of p19CHI, p1968CHI and p19CHI68, respectively. The *Xba* I-*Pst* I digested p16-19CHI, p16-1968CHI and p16-19CHI68 showed three DNA bands with molecular weight of 1.6, 1.9, and 6.9 kb; 1.6, 3.5, and 7.3 kb; 0.9, 4.1, and 5.8 kb, respectively. Thus, it appeared that all transcriptional fusion plasmids were successfully ligated with plasmid pBC16.

9. Subcloning of shuttle plasmid p16-19CHI, p16-1968CHI and p16-19CHI68 from *E. coli* into *B.t.i.* 4Q2-72 and c4Q2-72 host via electroporation technique

Two strains which were *B.t.i.* 4Q2-72 and c4Q2-72 were used as hosts in this study. *B.t.i.* 4Q2-72 was the strain which contained the intact crystal toxin genes on a 130 kDa plasmid (*cry4Aa1*, *cry4Ba1*, *cry10Aa1*, *cry11Aa1* and *cytAa1*). While, a large plasmid of intact toxin genes was cured in *B.t.i.* c4Q2-72. The intact shuttle plasmids were extracted from *E. coli* (p16-19CHI), *E. coli* (p16-1968CHI) and *E. coli* (p16-19CHI68) and introduced into *B.t.i.* 4Q2-72 and c4Q2-72 host by electroporation technique as described in Materials and Methods.

The transformants were selected on LB agar containing 15 µg/ml tetracycline.

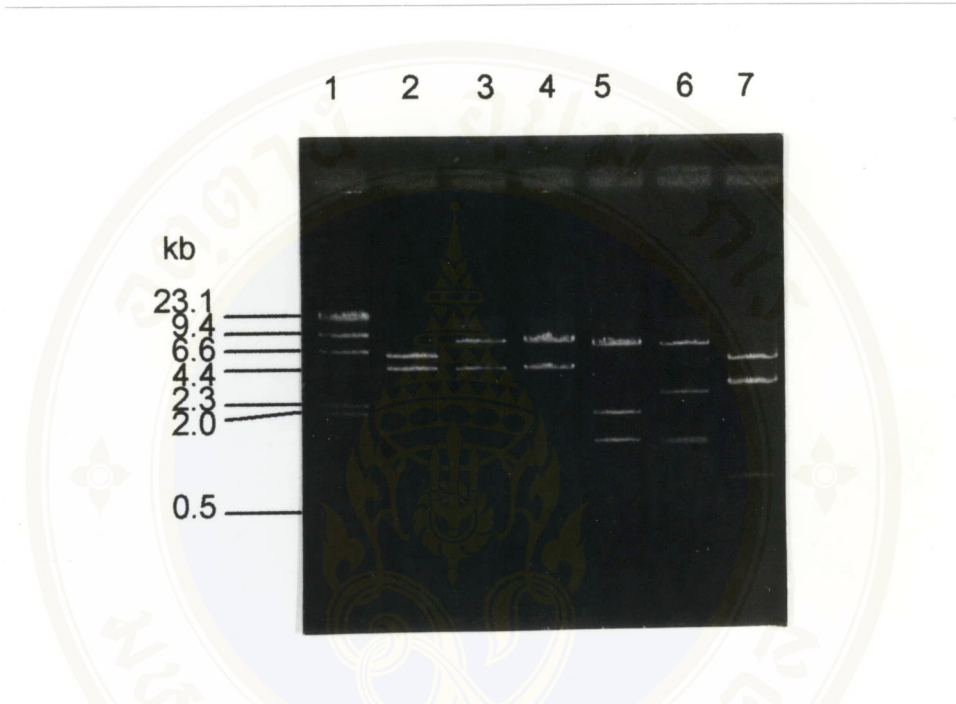


Fig. 38. Agarose gel electrophorsis of plasmid p16-19CHI, p16-1968CHI, and p16-19CHI from *E. coli* DH5α cut with *Bam*H I and *Xba* I-*Pst* I.

Lane 1	λ DNA cut *Hind*III
Lane 2	p16-19CHI cut with *Bam*H I
Lane 3	p16-1968CHI cut with *Bam*H I
Lane 4	p16-19CHI68 cut with *Bam*H I
Lane 5	p16-19CHI cut with *Xba* I-*Pst* I
Lane 6	p16-1968CHI cut with *Xba* I-*Pst* I
Lane 7	p16-19CHI68 cut with *Xba* I-*Pst* I

Only *B.t.i.* 4Q2-72 transformant harbouring p16-19CHI and p16-1968CHI and *B.t.i.* c4Q2-72 transformants harbouring p16-19CHI could be obtained. None of *B.t.i.* 4Q2-72 transformants harbouring p16-19CHI68 and *B.t.i.* c4Q2-72 transformants harbouring p16-1968CHI and p16-19CHI68 could be obtained though various conditions were performed. Those transformants were randomly picked up and replica on LB agar containing 15 μg/ml tetracycline which had been overlaid with 2% colloidal chitin to screen for chitinase producing clones. Colonies which produced clear halo zones were purified and further used for restriction analysis. One of those clones were selected, purified and designated as *B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) and *B.t.i.* c4Q2-72 (p16-19CHI), respectively.

10. Restriction analysis of plasmid p16-19CHI from *B.t.i.* 4Q2-72 and *B.t.i.* c4Q2-72 and plasmid p16-1968CHI from *B.t.i.* 4Q2-72

For determination of restriction analysis patterns of plasmid p16-19CHI and p16-1968CHI from *B.t.i.* transformants, the plasmids were extracted, purified, digested with appropriated restriction enzymes and subjected to 1.0% agarose gel electrophoresis. As shown in Fig. 39, the *Xba* I-*Pst* I digested p16-19CHI from *B.t.i.* 4Q2-72, c4Q2-72 and *E. coli* clones showed three DNA bands with molecular weight of 1.6, 1.9, and 6.9 kb (lane 9, and 10, and 5, respectively). Also, the *Xba* I-*Pst* I digested p16-1968CHI from *B.t.i.* 4Q2-72 released three DNA fragments of 1.6, 3.5, and 7.3 kb (lane 11) which comigrated with *Xba* I-*Pst* I digested p16-1968CHI from *E. coli* DH5α (lane 6). Thus, it was clearly shown that plasmid p16-19CHI and p16-1968CHI from *B.t.i.* 4Q2-72 and *B.t.i.* c4Q2-72 were the same plasmid obtained from *E. coli* DH5α Western analysis was subsequently performed to characterize the expression of *cry11Aa1* and chitinase

Fig.39. Agarose gel electrophorsis of plasmid p16-19CHI, and p16-1968CHI from *E. coli* DH5α, *B.t.i.* 4Q2-72 and c4Q2-72 digested with *Bam*H I and *Xba* I-*Pst* I.

Lane 1, 8, 12	λ DNA cut *Hind* III
Lane 2, 3, 4	p16-19CHI, p16-1968CHI, and p16-19CHI68 from *E. coli* DH5α cut with *Bam*H I
Lane 5, 6, 7	p16-19CHI, p16-1968CHI, and p16-19CHI68 from *E. coli* DH5α cut with *Xba* I-*Pst* I
Lane 9	p16-19CHI from *B.t.i.* 4Q2-72 cut with *Xba* I-*Pst* I
Lane 10	p16-19CHI from *B.t.i.* 4Q2-72cut with *Xba* I-*Pst* I
Lane 11	p16-1968CHI from *B.t.i.* c4Q2-72cut with *Xba* I-*Pst* I

genes from *B.t.i.* transformants.

11. Western analysis

11.1 Western analysis of chitinase enzyme

In order to distinguish that the chitinase enzyme from *B.t.i.* host from that of the cloned TP-1 chitinase in *B.t.i.* 4Q2-72 (16-19CHI), *B.t.i.* 4Q2-72 (16-1968CHI), and *B.t.i.* c4Q2-72 (16-19CHI), western blot analysis with TP-1 chitinase antibody was performed. Due to all transcriptional fusion genes were regulated by spore promoter of *p19* gene, characterization of gene expression in *Bacillus* host was done at spore stage of cell-growth. So all *Bacillus* strains were cultured for 48 h and subjected to partial purification and concentrated by 70% (final concentration) ammonium sulfate precipitation. Ten microlitres of the 20X concentrated culture supernatant was electrophoresed through 10% SDS-PAGE and electro-transferred from the gel to PVDF membrane. Proteins on membrane were reacted with TP-1 chitinase antibody and detected as described in Materials and Methods.

By protein staining, three proteins bands with molecular weight of 68, 62 and 50 were observed in all *B.t.i.* 4Q2-72 and c4Q2-72 transformants (Fig. 40A, lanes 1-3) whereas only 50 kDa was found in *B. licheniformis* TP-1 (Fig. 40A, lanes 4). All of these bands were tentative chitinase enzyme from TP-1 as judged by molecular weight. No protein bands were found in *B.t.i.* 4Q2-72 (Fig. 40A, lane 5) and c4Q2-72 (Fig. 40A, lane 6). By chitinase activity staining as shown in Fig. 40B, lane 2-4, revealed three positive bands of chitinase enzyme which were corresponded to Chi68, Chi62 and Chi50 of *B. licheniformis* TP-1. The Chi68 was found to be the major processing forms in *B.t.i.* transformants. Another faint bands (Fig. 40B, lanes 1-3) were also detected which were

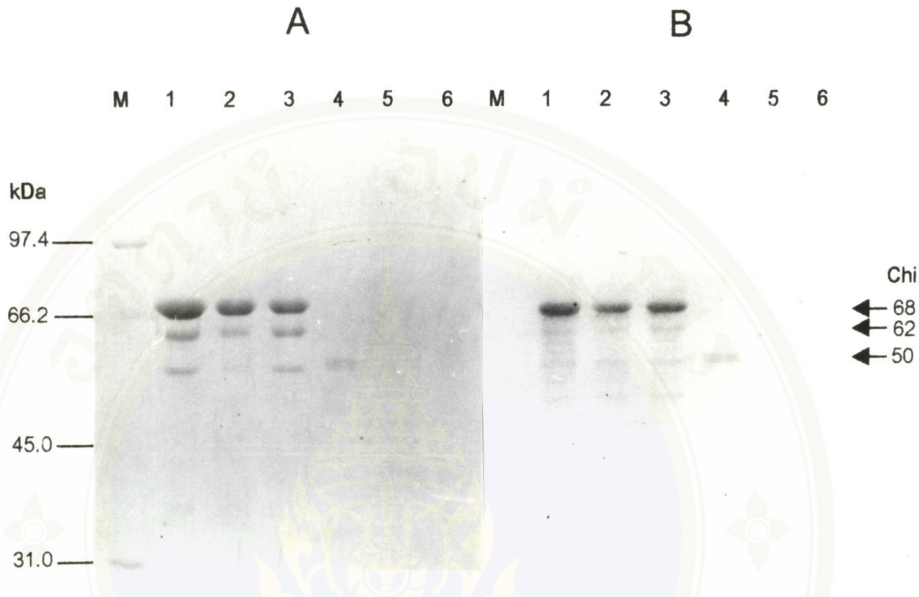


Fig. 40. Analysis of chitinase enzyme synthesis from *B.t.i.* strains 4Q2-72 and c4Q2-72 and their transformants. (A) Coomassie brilliant blue staining. (B) Western blot analysis with anti-TP-1 chitinase antibody. The locations of Chi68, Chi62 and Chi50 are indicated by arrows.

Lane M	Protein molecular mass markers ranging from 31.0 to 97.4 kDa
Lane 1	*B.t.i.* 4Q2-72 (p16-19CHI)
Lane 2	*B.t.i.* 4Q2-72 (p16-1968CHI)
Lane 3	*B.t.i.* c4Q2-72 (p16-19CHI)
Lane 4	*B. licheniformis* TP-1 (48 h culture supernatant)
Lane 5	*B.t.i.* 4Q2-72 host
Lane 6	*B.t.i.* c4Q2-72 host

probably resulted from improper processing or degradation of TP-1 chitinase proteins in the *B.t.i.* host. The 48 h culture supernatant of *B. licheniformis* TP-1 could produce only a single form of chitinase enzyme (50 kDa) which was used as positive control. While, no positive bands could be observed from culture supernatant of *B.t.i.* 4Q2-72 (Fig. 40B, lane 5) and c4Q2-72 (Fig. 40B, lane 6). Thus, it was cleared that *B.t.i.* 4Q2-72 (16-19CHI), *B.t.i.* 4Q2-72 (16-1968CHI), and *B.t.i.* c4Q2-72 (16-19CHI) under uninduced condition produced only chitinase enzyme from the cloned TP-1 gene. Three processing forms of chitinase enzymes (Chi68, Chi62 and Chi50) were produced in *B.t.i.* 4Q2-72 and c4Q2-72 transformants at the spore stage of cell growth with Chi68 as a major form. While only a single form of Chi50 was produced in *B. licheniformis* TP-1.

11.2 Western analysis of Cry11Aa1 toxin

To detect the Cry11Aa1 toxin in *B.t.i.* 4Q2-72 and c4Q2-72 transformants, western analysis was performed with Cry11Aa1 antibody as described in Materials and Methods.

Result from protein staining of crude culture from *B.t.i.* 4Q2-72 and c4Q2-72 host and their transformants was shown in Fig. 41A, lanes 1-5. While, 48 h culture supernatant of *B. licheniformis* TP-1 was shown in lane 6. When these protein bands reacted with Cry11Aa1 antibody, the positive band representing the Cry11Aa1 toxin were detected in *B.t.i.* 4Q2-72 host and their transformants (Fig. 41B, lanes 1-3). The 68-kDa Cry11Aa1 toxin was produced from *B.t.i.* 4Q2-72 transformants harboured p16-19CHI and p16-1968CHI. *B.t.i.* 4Q272 harboring p16-1968CHI (Fig. 41B, lane 3) produced higher amounts of the Cry11Aa1 protein than the non-transformed host (Fig. 41B, lane 1) while *B.t.i.* 4Q2-72 (p16-19CHI) produced less (Fig. 41B, lane 2). *B.t.i.* c4Q2-72

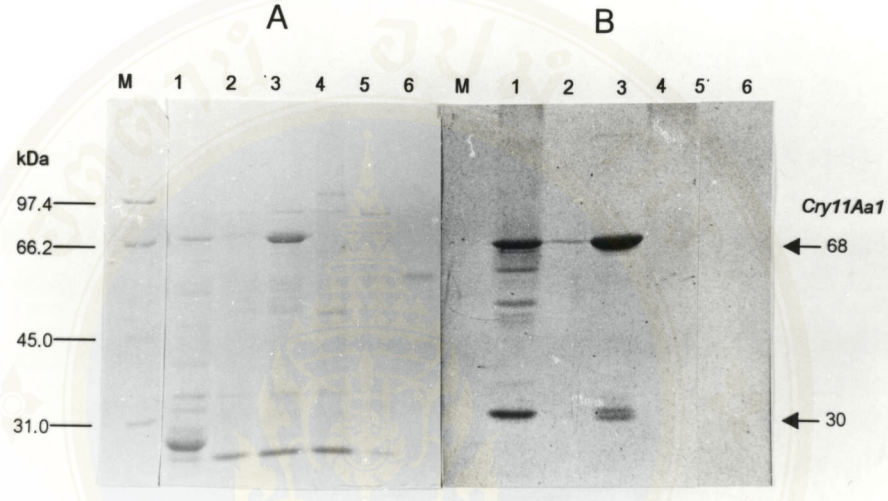


Fig.41. Analysis of Cry11Aa1 toxin synthesis from *B.t.i.* strains 4Q2-72 and c4Q2-72 and their transformants (48 h cell culture). (A) Coomassie brilliant blue staining. (B) Western blot analysis with anti-Cry11Aa1 antibody. The location of Cry11Aa1 is indicated by an arrow.

Lane M	Protein molecular mass markers ranging from 31.0 to 97.4 kDa
Lane 1	*B.t.i.* 4Q2-72;
Lane 2	*B.t.i.* 4Q2-72 (p16-19CHI);
Lane 3	*B.t.i.* 4Q2-72 (p16-1968CHI);
Lane 4	*B.t.i.* c4Q2-72;
Lane 5	*B.t.i.* c4Q2-72 (p16-19CHI);
Lane 6	*B. licheniformis* TP-1 (48 h culture supernatant)

(Fig.41B, lane 4) and *B.t.i.* c4Q2-72 harboring p16-19CHI (Fig. 41B, lane 5) did not produce Cry11Aa1 toxin. The dense bands at 30-kDa observed in Fig. 41, lanes 1 and 3 were processed forms of Cry11Aa1 (Chilcott and Ellar, 1988; Pfannenstiel *et al.*, 1986). The other bands seen in Fig. 41B, lane 1 were probably degraded forms of the 68-kDa protein.

12. Analysis of chitinase produced by *B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) and *B.t.i.* c4Q2-72 (p16-19CHI) in SDS-PAGE

In order to determine activity pattern of chitinases produced by *B.t.i.* 4Q2-72 and c4Q2-72 host strains and their transformants (*B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) and *B.t.i.* c4Q2-72 (p16-19CHI)), activity staining was performed according to the method of Trudel and Asselin, 1989. Coomassie blue protein staining of SDS-PAGE gel was shown in Fig. 42A. It revealed four proteins bands with estimated molecular weight of 68, 62, 58 and 50 kDa from *B.t.i.* 4Q2-72 (p16-19CHI) (lane 3), *B.t.i.* 4Q2-72 (p16-1968CHI) (lane 4) and *B.t.i.* c4Q2-72 (p16-19CHI) (lane 6) culture supernatant, respectively. A 58 kDa band could obviously seen in *B.t.i.* c4Q2-72 (p16-19CHI) (Fig 42A, lane 6) while very faint band was observed in *B.t.i.* 4Q2-72 transformants (Fig 42A, lanes 3 and 4). A single band of 50 kDa was detected in *B. licheniformis* TP-1 (Fig 42A, lane 1). No protein bands were found in *B.t.i.* 4Q2-72 (Fig. 40A, lane 2) and c4Q2-72 (Fig. 42A, lane 5), although same volume (10 µl) of 20X concentrated supernatant was loaded in to the gel.

Three multiple chitinase activity bands of 68, 62, and 50 kDa were detected in all *B.t.i.* 4Q2-72 and c4Q2-72 transformants (Fig. 42B, lanes 3, 4 and 6) which matched those expected from the cloned chitinase gene of *B. licheniformis* TP-1 (Chi 68, 62 and 50). Those

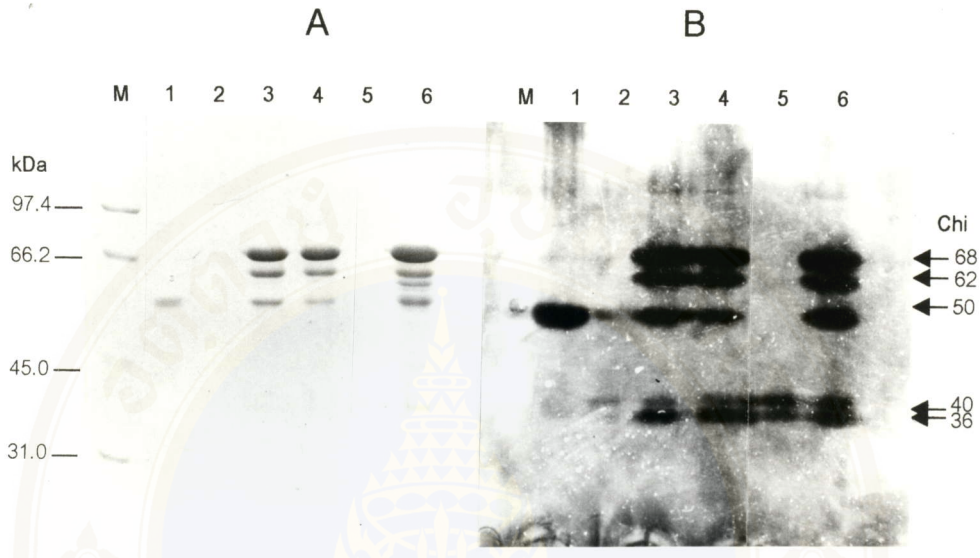


Fig. 42. SDS PAGE analysis for chitinase activity detection from *B.t.i.* strain 4Q2-72 and c4Q2-72 and their transformants. (A) Coomassie brilliant blue staining. (B) Chitinase activity determination. The locations of protein bands with chitinase activity, Chi68, Chi62, Chi50, 40-kDa and 36-kDa proteins are indicated by arrows.

Lane M	Protein molecular mass markers ranging from 31.0 to 97.4 kDa
Lane 1	*B. licheniformis* TP-1 (48 h culture supernatant);
Lane 2	*B.t.i.* 4Q2-72;
Lane 3	*B.t.i.* 4Q2-72 (p16-19CHI);
Lane 4	*B.t.i.* 4Q2-72 (p16-1968CHI);
Lane 5	*B.t.i.* c4Q2-72;
Lane 6	*B.t.i.* c4Q2-72 (p16-19CHI)

three activity bands were clearly seen for transformed *B.t.i.* 4Q2-72 harbouring p16-19CHI (Fig. 42B, lane 3) or p16-1968CHI (Fig. 42B, lane 4), and for transformed *B.t.i.* c4Q2-72 harbouring p16-19CHI (Fig. 42B, lane 6). Two other chitinase activity bands of low molecular weight (40 and 36-kDa) were also found and matched those of the non-transformed hosts *B.t.i.* 4Q2-72 and *B.t.i.* c4Q2-72 (Fig. 42B, lanes 2 and 5, respectively). These 2 chitinases were not the processed forms of TP-1 chitinase because they were found in the non-transformed *B.t.i.* strains. Data from western analysis with TP-1 chitinase antibody in Fig. 40B revealed that these two chitinase bands could not react with the antibody. Thus, it was suggested that these two chitinase activity bands should belong to *B.t.i.* host and they were produced at low level in the absence of colloidal chitin. A single processed chitinase protein band of 50-kDa was found in 48 h culture of *B. licheniformis* TP-1 (Fig. 42B, lane 1). An extra protein band of about 58-kDa which present in all transformants contained no chitinase activity (Fig. 42B, lanes 3, 4 and 6). Thus, it appeared that culture from uninduced condition of *B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI) and *B.t.i.* c4Q2-72 (p16-19CHI) produced three processing forms of TP-1 chitinase (Chi68, Chi62, and Chi50) together with low level of *B.t.i.* chitinase enzyme (40 and 36 kDa).

13. Kinetic study of chitinase enzyme production in *B.t.i.* host

13.1 Comparison of chitinase enzyme production in *B.t.i.* 4Q2-72 host and *B.t.i.* 4Q2-72 (p16-19CHI) under induced and uninduced condition

The synthesis of chitinase enzyme under chitinase vegetative promoter was constitutive in both *E. coli* (pCHIL3) and *B.t.* subsp *aizawai* (pCHIL3) (Tantimavanich,1997). In *E. coli* transformants harbouring chitinase gene under spore promoter (p19CHI, p1968CHI, p19CHI68), chitinase production did not require colloidal chitin induction (data from this

study). In order to determine whether the synthesis of chitinase enzyme under spore promoter in *B.t.i.* depended on colloidal chitin induction or not, kinetic study of chitinase enzyme production from *B.t.i.* 4Q2-72 host and *B.t.i.* 4Q2-72 (p16-19CHI) was examined at various time intervals. The level of chitinase enzyme was compared between induced (NYSM + 0.2% colloidal chitin) and uninduced (NYSM) condition. The culture broth was taken at 0, 6, 12, 24, 48, and 72 h for determining viable and spore count. Its supernatant was used for chitinase activity determination.

Data in Table 8 and Fig. 43. demonstrated that growth, and sporulation ability of both *B.t.i.* 4Q2-72 host and *B.t.i.* 4Q2-72 (p16-19CHI) were not different between the medium with or without addition of colloidal chitin(Fig. 43A, B). The growth curve of *B.t.i.* 4Q2-72 host and *B.t.i.* 4Q2-72 (p16-19CHI) were similar. The maximum growth rate reached at 24 h and declined afterward. Thus, it appeared that colloidal chitin did not affect the growth and sporulation ability of both *B.t.i.* 4Q2-72 host and its transformants. However, it was found that the *B.t.i.* 4Q2-72 (p16-19CHI) sporulated very poorly ability and enter sporulation phase slowly when compared to *B.t.i.* 4Q2-72 host. *B.t.i.* 4Q2-72 (p16-19CHI) start sporulation at 48 and 72 h, the spore count was 7.0x10, $1.42x10^2$ Cfu/ml (induced) and 3.0x10, $1.2x10^2$ Cfu/ml (uninduced), respectively. While, *B.t.i.* 4Q2-72 host start sporulation at 12 h and reach maximum at 48 h. Its spore count at 12, 24, 48, and 72 h was $4.7x10^3$, $5.1x10^6$, $2.28x10^7$, and $9.5x10^6$ Cfu/ml (induced) and $2.85x10^3$, $4.0x10^6$, $1.2x10^7$, and $6.0x10^6$ Cfu/ml (uninduced), respectively. It was suggested that the poor sporulation of *B.t.i.* 4Q2-72 transformant might resulted from the high copy number of electroporated plasmid (p16-19CHI). It has been found that *Bacillus* species harboring high-copy-number plasmids frequently show disturbed physiological equilibrium that precludes sporulation (Agaisse and Lereclus, 1995). Other

Table 8. Comparison of growth, spore, and chitinase production in *B.t.i.* 4Q2-72 and *B.t.i.* 4Q2-72 (p16-19CHI) at various time intervals (0, 6, 12, 24, 48, 72 h) between induced (NYSM + 0.2% colloidal chitin) and uninduced (NYSM) condition.

Bacterial strains	Culture time (h)	Viable counts (Cfu/ml)		Spore counts (Cfu/ml)		Chitinase activity (mU/ml)	
		induced	uninduced	induced	uninduced	induced	uninduced
B.t.i. 4Q2-72	0	$1.48x10^7$	$1.14x10^7$	0	0	0	0
B.t.i. 4Q2-72 (p16-19CHI)	0	$2.67x10^7$	$3.31x10^7$	0	0	0	0
B.t.i. 4Q2-72	6	$3.7x10^8$	$3.2x10^8$	0	0	0.05	0
B.t.i. 4Q2-72 (p16-19CHI)	6	$3.4x10^8$	$5.1x10^8$	0	0	0.2	0
B.t.i. 4Q2-72	12	$9.2x10^{11}$	$5.2x10^{10}$	$4.7x10^3$	$2.85x10^3$	0.9	0
B.t.i. 4Q2-72 (p16-19CHI)	12	$1.2x10^{10}$	$2.5x10^{10}$	0	0	2.3	2.2
B.t.i. 4Q2-72	24	$1.1x10^{10}$	$1.04x10^{12}$	$5.1x10^6$	$4.0x10^6$	1.14	0
B.t.i. 4Q2-72 (p16-19CHI)	24	$8.0x10^8$	$4.6x10^8$	0	0	5.47	5.44
B.t.i. 4Q2-72	48	$5.2x10^8$	$2.9x10^8$	$2.28x10^7$	$1.2x10^7$	0.96	0
B.t.i. 4Q2-72 (p16-19CHI)	48	$3.7x10^7$	$3.0x10^6$	$7.0x10^1$	$3.2x10^1$	7.46	6.29
B.t.i. 4Q2-72	72	$3.5x10^7$	$4.6x10^7$	$9.5x10^6$	$6.0x10^6$	0.83	0
B.t.i. 4Q2-72 (p16-19CHI)	72	$5.4x10^6$	$1.2x10^6$	$1.42x10^2$	$1.2x10^2$	7.24	5.73

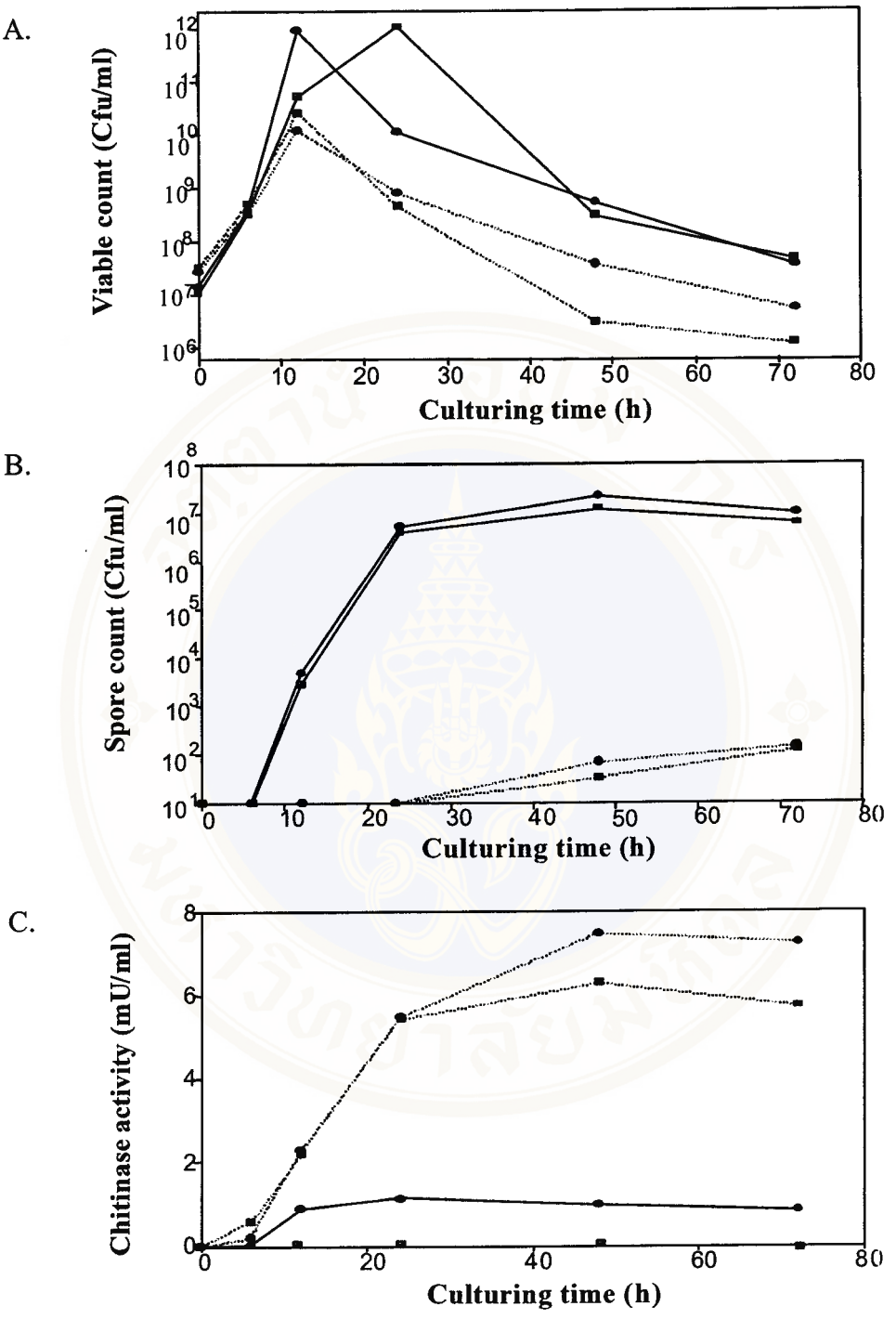


Fig. 43. Total viable and spore counts and kinetics of chitinase production by *B.t.i.* 4Q2-72 and *B.t.i.* 4Q2-72 (p16-19CHI) in NYSM medium and NYSM medium added with 0.2% colloidal chitin. (A) Growth curve (viable count). (B) Spore count. (C) Chitinase activity. Bacterial culture supernatants were collected at various time intervals from 0 to 72 h and assayed for chitinase activity, as described in Materials and Methods. The symbols are as follow:

- *B.t.i.* 4Q2-72 host (induced with 0.2% colloidal chitin)
- *B.t.i.* 4Q2-72 (uninduced)
- *B.t.i.* 4Q2-72 (p16-19CHI) (induced with 0.2% colloidal chitin)
- *B.t.i.* 4Q2-71 (p16-19CHI) (uninduced)

factors also should be studied further such as the electroporation condition, the effect of higher amount of transcriptional fusion products (P19, Cry11Aa1, chitinase), etc.

Relationship between growth and chitinase enzyme production of *B.t.i.* 4Q2-72 host and *B.t.i.* 4Q2-72 (p1619CHI) was shown in Fig. 43. The chitinase activity of *B.t.i.* 4Q2-72 host was high when colloidal chitin was added. The production of chitinase enzyme when induced with colloidal chitin start at 6 h (0.05 mU/ml) and rapidly increased at 12 h (0.9 mU/ml). The highest enzyme activity was observed at 24 h (1.14 mU/ml) and slightly decreased at 48 h (0.96mU/ml) and 72 h (0.83 mU/ml). While, the level of chitinase enzyme activity from culture with no colloidal chitin induction was 0.2, 0.74, 0.3, 0.4, and 0.3 mU/ml at 6, 12, 24, 48, and 72 h, respectively. This low chitinase activity was an indigenous uninduced chitinase of the host enzyme. Thus, the chitinase synthesis from *B.t.i.* 4Q2-72 host itself was depend on colloidal chitin induction. However, it was found that the chitinase activity of *B.t.i.* 4Q2-72 host grown in the medium with or without colloidal chitin was much lower than the chitinase activity of *B.t.i.* 4Q2-72 (p1619CHI). This higher enzyme activity in *B.t.i.* 4Q2-72 (p1619CHI) resulted from the chitinase enzyme encoded from the plasmid.

The kinetic of chitinase enzyme production from *B.t.i.* 4Q2-72 (p1619CHI) was slightly different between colloidal chitin induced and uninduced condition (Fig. 43C.). The enzyme production of induced culture was higher when growth was prolonged. The low level of chitinase enzyme started to produce at 6 h (0.2 mU/ml, induced and 0.58 mU/ml, uninduced) and rapidly increased at 12 h (2.3 mU/ml, induced and 2.2 mU/ml, uninduced) and 24 h (5.47 mU/ml, induced and 5.44 mU/ml, uninduced). The maximum enzyme activity was detected at 48 h which was 7.46 (induced) and 6.29 mU/ml (uninduced) and steadily maintained at 72 h which was 7.24 (induced) and 5.73 mU/ml

(uninduced). Thus, the expression of TP-1 chitinase from plasmid encoding gene in *B.t.i.* transformants was constitutive. Although, the major chitinase synthesis from *B.t.i.* host depended on colloidal chitin induction, the amount of enzyme was very low. So it did not affect the total level of chitinase enzyme in *B.t.i.* transformants. *B.t.i.* 4Q2-72 and c4Q2-72 hosts produced very low amount of chitinase under induced condition.

13.2 Comparison of growth, spore, and chitinase production in culture without colloidal chitin induction of *B. licheniformis* TP-1, *B.t.i.* 4Q2-72, *B.t.i.* c4Q2-72 and their transformants at various time intervals

Due to overall level of chitinase enzyme in *B.t.i.* transformants did not depend on colloidal chitin induction, the kinetic study of chitinase enzyme production from *B.t.i.* 4Q2-72, *B.t.i.* c4Q2-72 and their transformants was determined at various time intervals (0, 3, 6, 9, 12, 24, 48, and 72 h) in culture without colloidal chitin. So the interference of chitinase from *B.t.i.* host was very low and could be omitted. The chitinase from *B. licheniformis* TP-1, was also compared in this study. Only the culture of *B. licheniformis* TP-1 was induced with 0.2% colloidal chitin.

The level of chitinase at various stages of growth was determined and compared with the total cell number and spore count. Data was shown in Table 9 and Fig. 44. Patterns of cell growth of the transformed and non-transformed *B.t.i.* 4Q2-72 and c4Q2-72 strains were similar (Fig. 44A). They reached maximum growth at 12 h and rapidly decreased thereafter. However, total cell counts and especially spore counts of transformed *B.t.i.* 4Q2-72 were much lower (Fig.44A and 44B). *B.t.i.* c4Q2-72 produced comparatively lower spore numbers than did *B.t.i.* 4Q2-72 (Fig. 44B). Transformed *B.t.i.* c4Q2-72 harbouring p16-19CHI did not produce any spores. *B.t.i.* 4Q2-72 host started

Table 9. Comparison of growth, spore, and chitinase production in culture without colloidal chitin induction of *B. licheniformis* TP-1, *B.t.i.* 4Q2-72, *B.t.i.* c4Q2-72 and their transformants at various time intervals (0, 3, 6, 9, 12, 24, 48, 72 h)

Culturing time (h)	Bacterial strains	Viable count (Cfu/ml)	Spore count (Cfu/ml)	Chitinase activity (mU/ml)
0	*B. licheniformis* TP-1	$2.47x10^6$	1.6x10	0
	B.t.i. 4Q2-72	$3.20x10^6$	0	0
	B.t.i. c4Q2-72	$2.62x10^6$	0	0
	B.t.i. 4Q2-72 (p16-19CHI)	$8.9x10^5$	0	0
	B.t.i. 4Q2-72 (p16-1968CHI)	$2.33x10^6$	0	0
	B.t.i. c4Q2-72 (p16-19CHI)	$9.0x10^5$	0	0
3	*B. licheniformis* TP-1	$7.5x10^7$	6.3x10	0
	B.t.i. 4Q2-72	$1.15x10^9$	0	0
	B.t.i. c4Q2-72	$1.7x10^6$	0	0
	B.t.i. 4Q2-72 (p16-19CHI)	$2.05x10^7$	0	0
	B.t.i. 4Q2-72 (p16-1968CHI)	$8.9x10^6$	0	0
	B.t.i. c4Q2-72 (p16-19CHI)	$1.83x10^6$	0	0
6	*B. licheniformis* TP-1	$3.92x10^8$	$3.4x10^3$	0
	B.t.i. 4Q2-72	$1.02x10^9$	0	0
	B.t.i. c4Q2-72	$3.0x10^8$	0	0
	B.t.i. 4Q2-72 (p16-19CHI)	$3.28x10^8$	0	0
	B.t.i. 4Q2-72 (p16-1968CHI)	$1.48x10^8$	0	0
	B.t.i. c4Q2-72 (p16-19CHI)	$4.4x10^7$	0	0

Table 9. Comparison of growth, spore, and chitinase production in culture without colloidal chitin induction of *B. licheniformis* TP-1, *B.t.i.* 4Q2-72, *B.t.i.* c4Q2-72 and their transformants at various time intervals (0, 3, 6, 9, 12, 24, 48, 72 h). (continued)

Culturing time (h)	Bacterial strains	Viable count (Cfu/ml)	Spore count (Cfu/ml)	Chitinase activity (mU/ml)
9	*B. licheniformis* TP-1	$7.6x10^8$	$5.0x10^3$	0
	B.t.i. 4Q2-72	$2.6x10^{10}$	0	0
	B.t.i. c4Q2-72	$4.3x10^8$	0	0
	B.t.i. 4Q2-72 (p16-19CHI)	$2.5x10^9$	0	0.67
	B.t.i. 4Q2-72 (p16-1968CHI)	$2.8x10^9$	0	0.21
	B.t.i. c4Q2-72 (p16-19CHI)	$1.7x10^8$	0	0.13
12	*B. licheniformis* TP-1	$4.3x10^8$	$2.65x10^8$	2.59
	B.t.i. 4Q2-72	$6.6x10^{10}$	7.0x10	0
	B.t.i. c4Q2-72	$1.6x10^9$	0	0
	B.t.i. 4Q2-72 (p16-19CHI)	$2.97x10^{10}$	0	1.15
	B.t.i. 4Q2-72 (p16-1968CHI)	$6.7x10^9$	0	1.2
	B.t.i. c4Q2-72 (p16-19CHI)	$1.18x10^{10}$	0	0.99
24	*B. licheniformis* TP-1	$6.8x10^8$	$1.2x10^9$	2.67
	B.t.i. 4Q2-72	$1.6x10^{10}$	$4.2x10^{10}$	0
	B.t.i. c4Q2-72	$9.9x10^7$	3.0x10	0
	B.t.i. 4Q2-72 (p16-19CHI)	$4.4x10^8$	0	5.25
	B.t.i. 4Q2-72 (p16-1968CHI)	$1.03x10^9$	0	2.99
	B.t.i. c4Q2-72 (p16-19CHI)	$3.48x10^8$	0	4.29

Table 9. Comparison of growth, spore, and chitinase production in culture without colloidal chitin induction of *B. licheniformis* TP-1, *B.t.i.* 4Q2-72, *B.t.i.* c4Q2-72 and their transformants at various time intervals (0, 3, 6, 9, 12, 24, 48, 72 h).(continued)

Culturing time (h)	Bacterial strains	Viable count (Cfu/ml)	Spore count (Cfu/ml)	Chitinase activity (mU/ml)
48	*B. licheniformis* TP-1	$3.0x10^9$	$1.49x10^9$	1.92
	B.t.i. 4Q2-72	$1.72x10^7$	$2.09x10^6$	0
	B.t.i. c4Q2-72	$8.8x10^4$	$3.65x10^3$	0
	B.t.i. 4Q2-72 (p16-19CHI)	$2.9x10^7$	3.0x10	5.5
	B.t.i. 4Q2-72 (p16-1968CHI)	$4.75x10^7$	9.0x10	4.9
	B.t.i. c4Q2-72 (p16-19CHI)	$2.04x10^6$	0	4.7
72	*B. licheniformis* TP-1	$2.1x10^9$	$1.53x10^9$	1.57
	B.t.i. 4Q2-72	$4.2x10^6$	$2.91x10^6$	0
	B.t.i. c4Q2-72	$3.0x10^4$	$8.7x10^2$	0
	B.t.i. 4Q2-72 (p16-19CHI)	$1.56x10^6$	$1.6x10^2$	4.37
	B.t.i. 4Q2-72 (p16-1968CHI)	$1.29x10^6$	$4.5x10^2$	3.97
	B.t.i. c4Q2-72 (p16-19CHI)	$6.3x10^4$	0	3.92

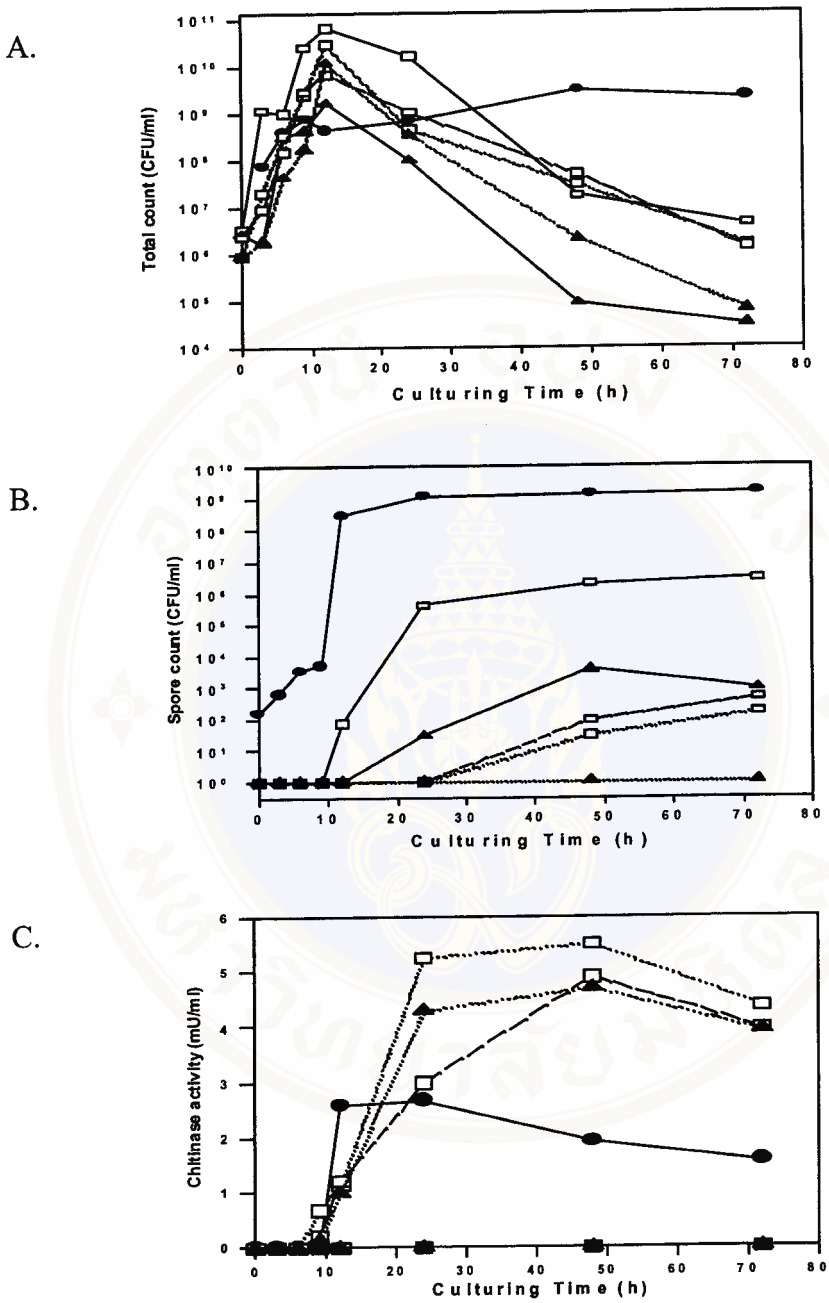


Fig.44. Total viable and spore counts and kinetics of chitinase production by *B. licheniformis* TP-1, *B.t.i.* 4Q2-72 and c4Q2-72 and its transformants. (A) Growth curve (viable count). (B) Spore count. (C) Chitinase activity. Bacterial culture supernatants were collected at various time intervals from 0 to 72 h and assayed for chitinase activity, as described in Materials and Methods. The symbols are as follow:

B. licheniformis TP-1
B.t.i. 4Q2-72 host
B.t.i. c4Q2-72 host
B.t.i. 4Q2-72 (p16-19CHI)
B.t.i. 4Q2-72 (p1619-68CHI)
B.t.i. c4Q2-72 (p16-19CHI)

sporulation at 12 h (7.0x10 Cfu/ml). While *B.t.i.* c4Q2-72 host, *B.t.i.* 4Q2-72 transformants start sporulation at 24 h (3.0x10 Cfu/ml) and 48 h, respectively, [*B.t.i.* 4Q2-72 (p16-19CHI), 3.0x10 Cfu/ml and *B.t.i.* 4Q2-72 (p16-1968CHI), 9.0x10 Cfu/ml]. For *B. licheniformis* TP-1, its growth and sporulation pattern was completely different from *B.t.i.* strains. It could grow rapidly and well sporulated.

Comparison of chitinase activity from *B. licheniformis* TP-1, *B.t.i.* 4Q2-72, *B.t.i.* c4Q2-72 and their transformants at various time intervals was illustrated in Fig. 44C. *B. licheniformis* TP-1 produced high enzyme activity at an early stage of growth (2.59 mU/ml at 12 h) and it increased only slightly thereafter up to 24 h (2.67 mU/ml) before declining at 48 h (1.92 mU/ml) and 72 h (1.57 mU/ml). By contrast, *B.t.i.* 4Q2-72 and *B.t.i.* c4Q2-72 harbouring p16-19CHI and *B.t.i.* 4Q2-72 (p16-1968CHI) did not produce high chitinase activity until 24 h of culture, reaching a maximum level at 48 h. The activity in all the transformed strains then decreased slightly by 72 h. All the transformants produced higher amounts of enzyme than *B. licheniformis* TP-1. *B.t.i.* 4Q2-72 (p16-19CHI) produced higher enzyme activity than *B.t.i.* 4Q2-72 (p16-1968CHI) and *B.t.i.* c4Q2-72 (p16-19CHI). The chitinase activity of *B.licheniformis* TP-1, *B.t.i.* 4Q2-72 (p16-19CHI), *B.t.i.* 4Q2-72 (p16-1968CHI), and *B.t.i.* c4Q2-72 (p16-19CHI) were 2.67, 5.25, 2.99, 4.29 mU/ml at 24 h, 1.92, 5.5, 4.9, 4.7 mU/ml at 48 h, and 1.57, 4.37, 3.97, 3.92 mU/ml at 72 h, respectively. Thus, it appeared that the synthesis of chitinase enzyme in *B.t.i.* transformants correlated to the sporulation. The high chitinase activity was examined when cell enter sporulation phase. This also confirmed that the chitinase gene in all transcriptional fusion plasmids were under control of *p19* spore promoter. In contrast, *B.t.i.* 4Q2-72 and c4Q2-72 hosts produced very low amount of chitinase under uninduced condition (less than 1 mU/ml).

14. Stability of transcriptional fusion plasmid in *B.t.i.*

The recombinant plasmids introduced into *Bacillus* host in some cases were not stable and has gene rearrangement (Jone *et al.*, 1982). This problem also had been reported in *B.t.* (Aronson, 1993) and *B. sphaericus* host (Seyler *et al.*, 1991). In this study, the size of the transformed transcriptional fusion plasmid in *B.t.i.* host was very large (about 10-12 kb) and has a high copy number. These also increase the possibility of plasmid loss. So the plasmid stability of the transcriptional fusion constructs in *B.t.i.* host was determined. One of the transcriptional fusions clone, *B.t.i.* 4Q2-72 (p16-19CHI) was studied for its plasmid stability under selective (NYSM+15 and 30 µg/ml tetracycline) and non-selective (NYSM) condition. Number of viable count of each culture was determined by plate count technique on NYSM agar. Subsequently, about 100 colonies obtained from each culture were randomly picked up and replicated on NYSM agar containing 15 µg/ ml tetracycline which had been overlaid with 3% colloidal chitin and NYSM agar. The number of replicated colonied per plate was about 50 colonies and most of them could utilize chitin so the percent of substrate (colloidal chitin) was increased for clearly observed the zones. The number of colonies that could grow on both NYSM and could also hydrolyze chitin on NYSM containing 15 µg/ml tetracycline was recorded and calculated for the percentage of plasmid stability.

As shown in Table 10, the transformants grown in non-selective condition (NYSM without tetracycline) started losing the plasmid at culture day 4. The percentage of retained plasmid was rapidly decreased in day 5 and remained nearly the same throughout the period of growth till 7 days. The percentage of plasmid stability was 92.5, 85.06, 84.9 and 84.3 at day 4, 5, 6, and 7, respectively. While 100% of the transformant grown in NYSM containing 15 and 30 µg/ml tetracycline could retain the plasmid until day 7.

Table 10. Stability of the plasmid p16-19CHI in *B.t.i.* 4Q2-72 host

Day	Medium[a]					
	NYSM		NYSM + 15 μg/ml Tc		NYSM + 30 μg/ml Tc	
	Viable count (Cfu/ml)	% of plasmid stability	Viable count (Cfu/ml)	% of plasmid stability	Viable count (Cfu/ml)	% of plasmid stability
1	$1.83x10^9$	100	$1.79x10^9$	100	$1.34x10^9$	100
2	$2.36x10^8$	100	$2.5x10^9$	100	$1.47x10^9$	100
3	$3.5x10^8$	100	$1.25x10^9$	100	$2.9x10^9$	100
4	$7.1x10^8$	92.5	$1.95x10^9$	100	$1.29x10^9$	100
5	$8.8x10^8$	85.06	$1.36x10^9$	100	$1.59x10^9$	100
6	$2.8x10^8$	84.9	$7.3x10^8$	100	$2.0x10^9$	100
7	$1.8x10^8$	84.3	$1.62x10^9$	100	$1.45x10^9$	100

[a]*B.t.i.* 4Q2-72 (p16-19CHI) was grown and daily subcultured under non-selective (NYSM) and selective (NYSM+15 μg/ml Tc and NYSM+30 μg/ml Tc) condition for 1 week. The total cells count and the percentage of tetracycline resistance colonies which could produce clear halo zone on 3% colloidal chitin plate were determined for each culture everyday. The random colonies from NYSM agar were picked and replicated on NYSM agar containing 15 μg /ml tetracycline which had been overlaid with 3% colloidal chitin and NYSM agar, respectively.

Thus, the construct p16-19CHI seemed to be relatively stably maintained in *B.t.i.* 4Q2-72 host grown under selective condition. While it was unstable when transformants were grown under non-selective pressure. Although, the size and restriction pattern of plasmid were not analyzed, the presence of both tetracycline resistance marker and chitinase activity could indicate the existence of the intact plasmid in the transformants. The large clear zone (0.3-0.6 mm from the edge of colonies) was observed within 2 days. Hence, Tc resistance colonies with large clear zone were regarded as the *B.t.i.* transformants harbouring the completed recombinant chitinase gene. The *B.t.i.* host itself could also produce the low level of chitinase under induction condition, but the duration of clear zone detection was delayed (more than 3 days) and the clear zone was very small (only 1-2 mm from the edge of colonies).

15. Mosquito larvicidal bioassay against *Aedes aegypti* larvae

15.1 Larvicidal activity of chitinase enzyme from *B.t.i.* c4Q2-72 (p16-19CHI)

A preliminary bioassay was conducted to examine the insecticidal effect and to establish the lethal dose of chitinase against *Aedes aegypti* larvae. The effect of chitinase alone was examined using crude chitinase from the *B.t.i.* c4Q2-72 (p16-19CHI) culture supernatant and it was shown (Table 11) to kill the larvae. The undiluted culture supernatant (4.5 mU/ml) resulted in 40% mortality while 2-fold and 5-fold dilutions (2.25 mU/ml, 0.9 mU/ml) gave 10% and 0% mortality, respectively. No viable cells were detected when plating 200 µl of filtered supernatant on NYSM agar plates (data not shown).

Table 11. Mosquitocidal activity of chitinase from the filtered culture supernatant of *B. thuringiensis* subsp. *israelensis* c4Q2-72 (p16-19CHI) against second instar *Aedes aegypti* larvae.

Filtered supernatant (ml) : distillled water (ml)	Chitinase activity[a] (mU/ml)	% mortality
50 : 0	4.5	40 %
25 : 25	2.25	10 %
10 : 40	0.9	0 %

[a] Final activity in the assay cup. The major chitinase activity was derived from cloned TP-1 chitinase (see test for detail).

15.2 Combined insecticidal effect of *B.t.i.* 4Q2-72 and chitinase

The LD_{50} of *B.t.i.* 4Q2-72 and c4Q2-72 hosts and their transformants were determined against second instar *Aedes aegypti* larvae as shown in Table 12. Co-application of chitinase (a fixed amount of 2.5 mU/ml/cup) with each of the *B.t.i.* 4Q2-72 strains was examined for combined larvicidal effect. The level of chitinase synthesized from the transformed plasmids was very low because of the ten-fold serial dilution of bacterial cultures (<0.45mU/ml/cup). Viable cell counts of 48 h cultures of *B.t.i.* 4Q2-72 and its transformants were not much different (6.4×10^7-1.68×10^8), while viable counts of *B.t.i.* c4Q2-72 strains were lower (1.0×10^6-2.04×10^6). The spore counts of *B.t.i.* 4Q2-72 transformants harboring p16-19CHI and p16-1968CHI were 3.3×10^3 and 4.2×10^2, respectively. This was approximately 30 and 300-fold, respectively, lower than the count for *B.t.i.* 4Q2-72 the untransformed host (1.2×10^5). Spore formation by *B.t.i.* c4Q2-72 was lower than that by *B.t.i.* 4Q2-72 and no spore formation was detected by *B.t.i.* c4Q2-72 (p16-19CHI). The LD_{50} of both *B.t.i.* 4Q2-72 harboring p16-19CHI and p16-1968CHI were 5.6×10^4 and 5.6×10^3, respectively. This was 40 and 4-fold, respectively, lower than that of the untransformed host (1.4×10^3). *B.t.i.* c4Q2-72 and its transformant did not kill mosquito larvae.

Coapplication of chitinase with all *B.t.i.* 4Q2-72 strains resulted in increased LD_{50} values. When chitinase from *B.t.i.* c4Q2-72 (p16-19CHI) was added to give final concentration of 2.5 mU/ml, the LD_{50} of untransformed *B.t.i.* 4Q2-72 and transformed *B.t.i.* 4Q2-72 (p16-1968CHI) increased 51 and 35-fold, respectively, whereas the LD_{50} for *B.t.i.* 4Q2-72 (p16-19CHI) with added chitinase increased only 3-fold.

Table 12. Mosquitocidal activity of *B. thuringiensis* subsp. *israelensis* strains 4Q2-72, c4Q2-72 and their transformants against second instar *Aedes aegypti* larvae.

Bacterial strains	Added chitinase (mU/ml)	Viable count (cfu/ml)	Spore count (cfu/ml)	LD_{50} (±SD) [b] (cfu/ml)	Folds of LD_{50} [d]
B.t.i. 4Q2-72 host	0	$6.4x10^7$	$1.2x10^5$	$1.40x10^3$ ($\pm 0.19x10^3$) [c]	
	2.5 [a]			$2.75x10$ ($\pm 0.93x10$) [c]	51
B.t.i. 4Q2-72 (p16-19CHI)	0	$1.68x10^8$	$3.3x10^3$	$5.6x10^4$ ($\pm 0.7x10^4$) [c]	
	2.5 [a]			$1.78x10^4$ ($\pm 0.63X10^4$) [c]	3
B.t.i. 4Q2-72 (p16-1968CHI)	0	$1.05x10^8$	$4.2x10^2$	$5.6x10^3$ ($\pm 1.99x10^3$) [c]	
	2.5 [a]			$1.58x10^2$ ($\pm 0.94x10^2$) [c]	35
B.t.i. c4Q2-72	0	$1.0X10^6$	$3.65x10^3$	$> 1.0x10^6$	
B.t.i. c4Q2-72 (p16-19CHI)	0	$2.04x10^6$	0	$>2.04x10^6$	

[a] Final activity in every cup

[b] LD_{50} was calculated based on total viable count, numbers in parentheses are standard deviation

[c] Significant difference ($P < 0.05$) between cultures with and without addition of chitinase

[d] Time (fold) of increase in LD_{50} when chitinase was added to spore suspensions

CHAPTER V

DISCUSSION

The bacterium, *B.t.i.*, was the only one biocontrol agent which was considered an operational success for control of both mosquito and blackfly larvae. The reasons for the success of *B.t.i.* are its cost-effectiveness and relative ease of use, which are due, respectively, to the ability of *B.t.i.* to be grown in artificial media and the development of formulations that can be applied using conventional insecticide application technology (Federeci, 1995). The advances of genetic engineering technologies also offer an approach to develop genetically altered *B.t.i.* strains with increased activities, and broader spectra (Nakamura, 1996; Porter *et al*, 1993). A consequence is that insect resistance to the toxin genes will be delayed or prevented (Porter, 1996). One strategy is to coexpress the chitinase gene with the toxin gene for the purpose of synergistic action.

B. licheniformis TP-1 was one of the high chitinase producers among other *Bacillus* species (Tantimavanich, 1997). Moreover, the chitinase enzyme from TP-1 strain could hydrolyse chitin in alkaline condition. Therefore, this enzyme is useful to function with *B.t.i.* toxin in the alkaline environment of insect midgut. The protein Cry11Aa1 is one of the *B.t.i.* toxins which plays an important role in toxicity to mosquito larvae. The mutant *B.t.i.* 4Q2-72 (*cry11Aa1*:: *erm*) caused a 45% decrease of toxicity to *Aedes aegypti* larvae and a 55% decrease of toxicity to *Culex pipiens* larvae (Poncet *et al.*, 1993). While the mosquitocidal activity of crystal containing both Cry4A (CryIVA) and Cry4B (CryIVB) proteins represented only about 10% of the overall toxicity of the wild type crystal (Delecluse *et al*, 1993). The transcription of *cry11Aa1* operon is initiated from

two sporulation-specific promoters, σ^{35} and σ^{28}, that are located upstream of *p19*, the first gene in the toxin operon (Dervyn *et al*, 1995). Up to date, no report on another fusion construct under control of *p19*. Various transcriptional fusion of TP-1 chitinase with *p19* gene and *cry11Aa1* gene had been constructed in this study. Those are p16-19CHI, p16-1968CHI and p16-19CHI68. Each gene in all constructs was arranged sequentially in an operon under control of the *p19* spore promoter. Two fusion constructs (p16-19CHI and p16-1968CHI) were successfully transferred to *B.t.i.* 4Q2-72 host, whereas p16-19CHI68 was not. The reason for this is still unclear. Whether or not, the location of chitinase gene between *p19-cry11Aa1* operon affecting the plasmid stability requires further study. Plasmid p16-19CHI was stable in *B.t.i.* grown in culture supplemented with 15 and 30 μg/ml tetracycline till 7 day. The loss of this plasmid in *B.t.i.* transformants (about 20%) occurred when cells were cultured under non-selective pressure.

The plasmid-encoded *cry* genes are transcribed throughout much of sporulation by forms of RNA polymerase, but there are variations in the types of promoters, as well as in the times of transcription of certain classes of *cry* genes (Agaisse and Lereclus, 1995; Baum and Malvar, 1995). It was found that the regulation of *cry* genes also involve regions upstream of the promoters which are unique to each *cry* genes. For example, mutations in the upstream region of the *cry1Ab* gene resulted in enhanced expression in *B.t.* subsp. *aizawai*. No differences were found when the *lacz* fusions contained the *cry1Ab* promoters but no upstream sequences (Cheng *et al*, 1999). The amount of Cry protein relates to the insect toxicity. The higher the yield, the higher the potential toxicity per unit weight. In the study of Cry3A, several strategies have been used to increase Cry3A synthesis, including expression of *cry3* genes in asporogeneous mutants

(Lereclus *et al*, 1995; Malvar *et al*, 1994) and *cry3A* gene amplification (Adams *et al*, 1994). Recently, the synthesis of Cry3A protein was increased by using the dual sporulation-dependent *cyt1Aa* promoters and STAB-SD (Stability SD) sequence (Park *et al*, 1998).

In this study, *p19* promoter not only could increase chitinase production but also expresses the gene at spore stage. The expressions of the cloned chitinase gene under *p19* spore promoter in both *E. coli* and *B.t.i.* transformants were constitutive. The synthesis of TP-1 chitinase under its own vegetative promoter in *E. coli* and *B.t.a* was also constitutive. It was found that the level of chitinase acticity of *B.t.i.* transformants (4.7-5.5 mU/ml, 48 h) was about 3 times higher than the activity of *B. licheniformis* TP-1 (1.92 mU/ml, 48 h). The level of chitinase expression by *B.t.a* (pCHIL3-16) harbouring the same TP-1 chitinase gene was 4 times higher than the wild type, *B. licheniformis* TP-1 (Tantimavanich, 1997). Although, the of chitinase gene was located far from the *p19* promoter (second and third gene in p16-19CHI and p16-1968CHI, respectively), it still could strongly express. This result indicated that *p19* promoter was a strong spore promoter. Comparison of mosquitocidal activities of individual toxins from *B.t.i.* showed that Cry11Aa1 is the most toxic with an LC_{50} value of 224 ng/ml. While the LC_{50} value of Cry4A, Cry4B, CytA were 1120 ng/ml, 470 ng/ml and 1210 ng/ml, respectively (Crickmore *et al*, 1995). The *p19*, *cry11Aa1* and chitinase gene were transcriptionally fused in operon without terminator sequence in the intervening region of each gene. RNA polymerase could readthrough from *p19* promoter until the end of the operon in the fused constructs. The synthesis of chitinase by *B. licheniformis* and *B.t.i.* host required induction by chitin. Chitinase gene expression in most microorganisms had been reported to be controlled by a repressor/inducer system in which chitin or other products

of degradation act as inducers (Felse and Panda, 1999). It was suggested that the constitutive expression of the cloned chitinase gene in heterologous hosts might resulted from difference in regulation machinery. Since only promoterless chitinase gene was used in each construct, therefore the specific TP-1 chitinase regulatory sequence should be absence in the transcriptional fusion plasmids.

In *B.t.i.* host, harbouring the transcriptional fusions of *cry11Aa1* and TP-1 chitinase gene, it was clearly shown that both proteins produced at the spore stage. Three processed chitinase forms corresponding to Chi68, Chi62 and Chi50 were secreted extracellularly with the full length Chi68 as the predominant form. These forms were stably maintained throughout the stationary phase in the *B.t.i.* host. The processing pattern of the chitinase from the fusion clones was different from that of the wild type *B. licheniformis* TP-1 (Tantimavanich *et al.*, 1998), where Chi50 is the only form that appears after 9 h of cultivation. The Chi50 resulted from post-translational processing at the C-terminus of Chi68, a chitinase precursor (Tantimavanich, 1997). The difference in chitinase processing patterns might be due to differences in bacterial physiology, e.g., proteolytic enzymes in the culture broth of one but not another. Since the larger forms of chitinase hydrolyzed insoluble chitin better than Chi50, it is possible that Chi50 lacks a chitin binding domain (Tantimavanich *et al.*, 1998). If so, *B.t.i.* producing Chi68 as the major enzyme in this study might have better potential in insect control.

Two extra chitinase activity bands of 40 and 36 kDa belong to *B.t.i.* host also produced at low level in the absence of colloidal chitin. However, no cross reaction occurred between TP-1 antibody and *B.t.i.* chitinase. The major forms of chitinase production from *B.t.i.* host depended on the colloidal chitin induction. Higher level of enzyme was produced in the presence of colloidal chitin and the maximum level was de-

tected at 24 h (1.14mU/ml), whereas no activity was detected by enzymatic assay without chitin induction. The synthesis of major chitinase enzyme from *B.t.i.* host should be controlled by a repressor/inducer system. The chitinase from *B.t.i.* was found to be rather stable. Slightly decrease of enzyme activity was observed for long period of culture (48 and 72 h).

While, *B.t.i.* 4Q2-72 harbouring fusion construct produced the 68 kDa Cry11Aa1 toxin with the 30 kDa processing form. However, the amount of Cry11Aa1 protein in *B.t.i.* 4Q2-72 (p16-1968CHI) was high whereas that of *B.t.i.* 4Q2-72 (p16-19CHI) was less when compared to the non-transformed host. This might be due to the defect in sporulation ability of *B.t.i.* 4Q2-72 transformants. It was known that the toxin synthesis is sporulation-dependent (Agaisse and Lereclus, 1995). Poor sporulation reduced the level of crystal synthesis. It has been found that *Bacillus* species harbouring high-copy-number plasmids frequently show disturbed physiological equilibrium that precludes sporulation (Agaisse and Lereclus, 1995). The *B.t.i.* 4Q2-72 host normally contains a low-copy-number, large plasmid encoding for the whole toxin complex that is regulated by a sporulation-specific promoter (Baum and Malvar, 1995; Schnepf *et al*, 1998). Thus, introduction of a high-copy-number plasmid into this host probably resulted in a reduction in sporulation that in turn affected toxin synthesis. The amount of Cry11Aa1 toxin synthesized from the wild type plasmid in *B.t.i.* 4Q2-72 transformants harbouring p16-19CHI was markedly reduced. Other Cry toxins and CytA were suggested to be drastically lowered in transformed *B.t.i.* 4Q2-72 strains. This correlated with poor larvicidal toxicity of *B.t.i.* 4Q2-72 (p16-19CHI). The higher amount of Cry11Aa1 toxin in *B.t.i.* 4Q2-72 (p16-1968CHI) is compensated for by an increased gene dosage of *cry11Aa1* gene encoded on the recombinant plasmid. Thus, in this strain, the high amount of

Cry11Aa1 toxin seemed to compensate for poor sporulation ability which probably affected other *cry* and *cytA* synthesis and mosquito larvicidal activity was comparable to that of *B.t.i.* 4Q2-72. The toxicity effect of each transformant will be discussed later.

Additive larvicidal effects for chitinolytic enzymes and microbial insecticides has been known to occur since the early 1970's (Smirnoff, 1973, 1974; Lysenko, 1976; Morris, 1976). Most experiments were done by mixing microbial chitinases with entomopathogenic microorganisms (*B.t.*). However a common characteristic of the crystal toxin genes is that they are expressed under a sporulation-specific promoter during the stationary phase (Baum and Malvar, 1995). In this study, the chitinase gene from *B. licheniformis* TP-1 was regulated under the *cry11Aa1* spore-promoter and coexpressed in the *B.t.i.* host. The recombinant chitinase was primarily synthesized in the spore stage and stably maintained throughout the stationary phase of cell growth. However, in order to kill 10 and 40% of second instar *Aedes aegypti* larvae, a certain amount of chitinase must be applied (not below 2.25 and 4.5 mU/ml, respectively). Although the transformed *B.t.i.* cells produced high chitinase constitutively, the enzyme was diluted to less than 0.05-0.1 mU/ml when cells were diluted for toxicity assays. Thus, the chitinase produced by recombinant plasmids in all the transformed *B.t.i.* 4Q2-72 in this study was not sufficient to result in an increase in larvicidal activity.

Poor sporulation also resulted in lower toxicity for the transformed strains when compared to their respective untransformed hosts. Enhancement of larvicidal activity by chitinase with different *B.t.i.* strains seemed to depend on the amount of whole toxin. With added chitinase (2.5 mU/ml for final concentration) the LD_{50} of untransformed *B.t.i.* increased 51-fold, whereas the same amount of enzyme with *B.t.i.* 4Q2-72 (p16-19CHI) and (p16-1968CHI) increased the LD_{50} by only 3-and 35-fold, respectively. The

low mosquitocidal enhancement of chitinase to *B.t.i.* 4Q2-72 (p16-19CHI) was unexpected. It might be due to very poor sporulation and poor crystal protein production. Result from western blot confirmed that at least Cry11Aa1 was synthesized at very low level compared to that of *B.t.i.* host. Further investigation is required to explain this difference. LD_{50} of *B.t.i.* 4Q2-72 (p16-1968CHI) was only 4 fold lower than that of *B.t.i.* host ($5.6x10^3 \pm 1.99x10^3$ V.S. $1.4x10^3 \pm 0.19x10^3$ Cfu/ml, resectively). High amount of Cry11Aa1 protein was detected in *B.t.i.* transformant harbouring p16-196CHI by western blot. The protein (Cry11Aa1) from the construct might play an important role in toxicity. When chitinase (2.5 mU/ml for final concentration) was added, this transformant was more toxic than the *B.t.i.* host about 10 fold. LD_{50} of *B.t.i.* 4Q2-72 (p16-1968CHI) with added chitinase was reduced to be only $1.58x10^2 \pm 0.94x10^2$ Cfu/ml.

Application of a spore-crystal suspension of all *B.t.i.* 4Q2-72 strains together with filtered culture supernatant containing chitinase resulted in enhanced insecticidal activity. Hence, chitinase plays some role in larvicidal toxicity. It is proposed that chitinolytic enzymes cause perforation in the peritrophic membrane barrier and enhance contact of the *B.t.* toxin molecules with their epithelial membrane receptors (Regev *et al*, 1996). For example, the recombinant endochitinase ChiAII in *E. coli* host encoded by *S. marcescens* chitinase gene could perforate the larval midgut peritrophic membrane even at a chitinase concentration as low as 0.1 μg/ml (Regev *et al*, 1996). A combination of CryIC and ChiAII synergized the toxic effect on neonate *S. littolaris* larvae (Regev *et al*,1996). *B. licheniformis* TP-1 chitinase when mixed with *B.t.* subsp. *aizawai* slightly improved larvicidal acitivity against *S. exigua* (Tantimavanich *et al*, 1997).

The P19 polypeptide has been suggested to play a role as a chaperone protein involved in toxin crystallization (Dervyn *et al*, 1995). This protein is well conserved and

similar with the *orf1* genes of the two *B.t.* subsp. *kurstaki cry2A* and *cry2C* operons (Wu *et al*, 1991; Kalman *et al*, 1993). The amino acid composition of the P19 protein is unusual: about 11.7% of the amino acids of P19 are cysteine residues. The C-terminus halves of most of the 130-kDa δ-endotoxins are also rich in cysteine residues and are involved in the crystallization properties (Dervyn *et al*., 1995; Hofte and Whiteley, 1989). The other well characterized 20-kDa protein was also act as another chaperone protein, stabilizing the Cry11Aa1 protein during and/or immediately after synthesis (Wu and Federeci, 1995). A similar role has been previously suggested for the 20-kDa protein in the case of CytA1 protein synthesis and inclusion formation (Wu and Federeci, 1993; Chang *et al*, 1993; Wu and Federeci, 1993). The 20-kDa protein could facilitate the net synthesis of the CytA1 (Wu and Federeci, 1993; Wu and Federeci, 1993), Cry4A proteins (Yoshisue *et al*, 1992) and the Cry11Aa1 proteins (Wu and Federeci, 1995). Moreover it also affects the size of these inclusions (Wu and Federeci, 1995). Although, the amino acid sequence and the biochemical properties of CytA and Cry proteins are markedly different, the 20-kDa proteins still could promote their synthesis. Thus, it may be useful in stabilizing other proteins (Wu and Federeci, 1995). Whether the role of the P19 proteins like the 20-kDa proteins or not remained to be determined. In this study, fusion of chitinase with *p19* or *p19-cry11Aa1* genes did not result in assembly of the enzyme into the crystal inclusion body, since it was soluble and remained in the filtered supernatant. Though the chitinase enzyme was produced at spore-stage in a high amount. The amino acid sequence and the biochemical properties of chitinase enzyme and Cry11Aa1 proteins are markedly different. Both 20-kDa and P19 proteins may require specific structure similar to Cry11Aa1 to form crystal inclusion. The problem of poor sporulation of transformed *B.t.i.* harboring the chitinase gene must also be overcome in order to improve the larvicidal activity of transformed *B.t.i.* 4Q2-72 strains.

CONCLUSION

Investigation of coexpression of chitinase gene from *B. licheniformis* TP-1 and *p19* or *p19-cry11Aa1* from *B.t.i.* was studied. Three transcriptional fusion plasmids were constructed (p16-19CHI, p16-1968CHI and p16-19CHI68) and introduced into *E. coli* DH5α and *B.t.i.* hosts. The gene arrangement in all fusion constructs was in an operon fashion under control of *p19* spore promoter. The constructs were stably maintained in *B.t.i.* host under the selective condition. Expression of the recombinant chitinase was constitutive in both *E. coli* and *B.t.i.* hosts. The synthesis of chitinase enzyme in *B.t.i.* host correlated with the sporulation timing which started about 9 h of growth and reached the maximum level at 48 h. The level of enzyme activity obtained from *B.t.i.* transformants was about 3 times higher than that of *B. licheniformis* TP-1, a gene donor host. SDS-PAGE analysis with chitinase activity staining revealed three TP-1 chitinase processing forms (Chi68, Chi62 and Chi50) and two *B.t.i.* chitinase (40 and 36 kDa). Kinetic of chitinase production under induced and uninduced condition showed that the synthesis of major forms of residental *B.t.i.* chitinase depended on colloidal chitin induction and the enzyme activity was 5-7 times lower than the recombinant chitinase.

For the Cry11Aa1 toxin synthesis, *B.t.i.* 4Q2-72 and its transformants could produce the 68-kDa Cry11Aa1 toxins with the 30-kDa as the major processing forms. The high gene dosage encoded on the plasmid was suggested to confer the high amount of Cry11Aa1 toxin production in *B.t.i.* 4Q2-72 (p16-1968CHI). However, it was found that the poor sporulation occurred in all *B.t.i.* transformants. It resulted in the less production of Cry11Aa1 toxin in *B.t.i.* 4Q2-72 (p16-19CHI) and caused poor larvicidal activity.

The crude TP-1 chitinase alone from *B.t.i.* c4Q2-72 (p16-19CHI) could kill second instar *Aedes aegypti* larvae when a certain amount of enzyme was used. For example, chitinase at 4.5 and 2.25 mU/ml caused 40% and 10% mortality, respectively. While the co-application of TP-1 chitinase (at final activity of 2.5 mU/ml in each assayed cup) with the cell suspension of *B.t.i.* 4Q2-72 and its transformants could increase the larvicidal activity about 3-50-fold. However, the poor sporulation resulted in the lower toxicity of the transformed strains when compared to the untransformed host. Thus, the chitinase enzyme actually could enhance the larvicidal activity. However, the problem of poor sporulation of the transformed strains should be solved which could make these genetic engineered strains to be more effective in biocontrol.

A cloned chitinase gene in all *B.t.i.* hosts produced and secreted chitinase enzyme into the culture supernatant. Thus, the enzyme could not co-crystallize with Cry11Aa1 although the P19 protein with its known function as chaperone, was present. It was suggested that interaction of P19 protein with other proteins might require specific protein structure in order to form crystal.

BIBLIOGRAPHY

Adam LF, Brown KL, and Whiteley HR. Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. J Bacteriol 1991; 173: 3846-3854.

Adams LF, Mathewes S, O'Hara P, Petersen A, and Gurtler H. Elucidation of the mechanism of CryIIIA overproduction in a mutagenized strain of *Bacillus thuringiensis* var *tenebrionis*. Mol Microbiol 1994; 14: 381-389.

Agaisse H, and Lereclus D. Expression in *Bacillus subtilis* of the *Bacillus thuringiensis* CryIIIA toxin gene is not dependent on a sporulation-specific sigma factor and is increased in a *spoOA* mutant. J Bacteriol 1994b; 176: 4734-4741.

Agaisse H, and Lereclus D. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? J Bacteriol 1995; 177: 6027-6032.

Agaisse H, and Lereclus D. STAB-SD: a Shine-Dalgarno sequence in the 5' untranslated region is a determinant of mRNA stability. Mol Microbiol 1996; 20: 633-643.

Agaisse H, and Lereclus D. Structural and functional analysis of the promoter region involved in full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis*. Mol Microbiol 1994a; 13: 97-107.

Aronson AI, Han ES, Mc Gaughey W, and Johnson D. The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. Appl Environ Microbiol 1991; 57: 981-986.

Aronson AI. Microreview: The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. Mol Microbiol 1993; 7: 489-496.

Aronson AI, Wu D, and Zhang C. Mutagenesis of specificity and toxicity regions of a *Bacillus thuringiensis* protoxin gene. J Bacteriol 1995; 177: 4059-4065.

Aronson NN, Blanchard CJ, and Madura JD. Homology modeling of glycosyl hydrolases family 18 enzymes and proteins. J Chem Inf Comput Sci 1997; 37: 999-1005.

Bassler BL, Yu C, Lee YC, and Roseman S. Chitin utilization by marine bacteria: Degradation and catabolism of chitin oligosaccharides by *Vibrio furnissii*. J Biol Chem 1991; 266: 24276-24286.

Baum JA, and Malvar T. Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. Mol Microbiol 1995; 18: 1-12.

Ben-dov E, Boussiba S, and Zaritsky A.. Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. J Bacteriol 1995; 177: 2851-2857.

Ben-Dov E, Einav M, Peleg N, Boussiba S, and Zaritsky A. Restriction map of the 125-kilobase plasmid of *Bacillus thuringiensis* subsp. *israelensis* carrying the genes that encode delta-endotoxins active against mosquito larvae. Appl Env Microbiol 1996; 62: 3140-3145.

Ben-Dov E, Nissan G, Pelleg N, Manasherob R, Boussiba S, and Zaritsky A. Refined, circular restriction map of the *Bacillus thuringiensis* subsp. *israelensis* plasmid carrying the mosquito larvicidal genes. Plasmid 1999; 42: 186-191.

Bernhard K. Studies on the delta-endotoxin of *Bacillus thuringiensis* var *tenebrionis*. FEMS Microbiol Lett 1986; 33: 261-265.

Bernhard K, Schrempf H, and Goebel W. Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. J Bacteriol 1978; 133: 897-903.

Bietlot HP, Vishnubhatla I, Carey PR, Pozsgay M, and Kaplan H. Characterization of the cystein residues and disulfide linkages in the protein crystal of *Bacillus thuringiensis*. Biochem J 1990; 267: 309-316.

Birnboim HC, and Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Necleic Acid Res 1979; 7: 1513-1523.

Blaak H, Schnellmann J, Walter S, Henrissat B, and Schrempf H. Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. Eur J Biochem 1993; 214: 659-669.

Blaak H, and Schrempf H. Binding and substrate specificities of a *Streptomyces olivaceoviridis* chitinase in comparison with its proteolytically processed form. Eur J Biochem 1995; 229: 132-139.

Bork P and Doolittle R. Proposed acquisition of an animal protein domain by bacteria. Proc Natl Acad Sci USA 1992; 89: 8990-8994.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976; 72: 248-254.

Brandt CR, Adang MJ, and Spence KD. The peritrophic membrane: Ultrastructural analysis and function as a mechanical barrier to microbial infection in *Orgyia pseudotsugata*. J Invertebr Pathol 1978; 32: 12-24.

Brizzard BL, Schnepf HE and Kronstad JW. Expression of the *crylB* crystal protein gene of *Bacillus thuringiensis*. Mol Gen Genet 1991; 231: 59-64.

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais J, and Broglier R. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 1991; 254: 1194-1197.

Brown KL. Transcriptional regulation of the *Bacillus thuringiensis* subsp. *thompsoni* crystal protein gene operon. J Bacteriol 1993; 175: 7951-7957.

Brurberg MB, Eijsink VG, and Nes IF. Characterization of a chitinase gene (*chiA*) from *Serratia marcescens* BJL200 and one-step purification of the gene product. FEMS Microbiol Lett 1994; 124: 399-404.

Brurberg MB, Eijsink VG, Haandrikman AJ, Venma G, and Nes IF. Chitinase B from *Serratia marcescens* BJL200 is exported to the periplasm without processing. Microbiology 1995; 141: 123-131.

Burges HD, Aizawa A, Dulmage HT, and de Barjac H. Numbering of the H-serotypes of *Bacillus thuringiensis*. J Invertebr Patho 1982; 40: 419.

Carroll J, and Ellar DJ. An analysis of *Bacillus thuringiensis* delta-endotoxin action on insect-midgut-membrane permeability using a light-scattering assay. Biochemistry 1993; 214: 771-778.

Chang C, Yu YM, Dai SM, Law SK, Gill SS. High-level *cryIVD* and *cytA* gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. Appl Environ Microbiol 1993; 59: 815-821.

Chen JP, Nagayama F, and Chang MC. Cloning and expression of a chitinase gene from *Aeromonas hydrophila* in *Escherichia coli*. Appl Environ Microbiol 1991; 57: 2426-2428.

Chen XJ, Curtiss A, Alcantara E, and Dean DH. Mutations in domain I of *Bacillus thuringiensis* delta-endotoxin CryIAb reduce the irreversible binding of toxin to *Manduca sexta* brush border membrane vesicles. J Biol Chem 1995; 270: 6412-6419.

Chen XJ, Lee MK, and Dean DH. Site-directed mutations in a highly conserved region of *Bacillus thuringiensis* δ-endotoxin affect inhibition of short circuit across *Bombyx mori* mid guts. Proc Natl Acad Sci USA 1993; 90: 9041-9045.

Cheng P, Wu L, Ziniu Y, and Aronson A. Subspecies-dependent regulation of *Bacillus thuringiensis* protoxin genes. Appl Environ Microbiol 1999; 65: 1849-1853.

Chernin LS, de al Fuente L, Sobolev V, Haran S, Vorgias CE, Oppenheim AB, and Chet I. Molecular cloning, structural analysis and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. Appl Environ Microbiol 1997; 63: 834-839.

Chilcott C, and Ellar DJ. Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal protein in vivo and in vitro. J Gen Microbiol 1988; 134: 2551-2558.

Chung-Saint L, Hsing-Chen C, and Fu-pang L. Expression and characterization of the recombinant gene encoding chitinase from *Aeromonas caviae*. Enzyme Microbiol Technol 1997; 21: 472-478.

Cohen E. Chitin synthesis and degradation as targets for pesticide action. Arch Insect Biochem Physiol 1993; 22: 245-261.

Coudron TA, Kroha MJ, and Ignoffo CM. Levels of chitinolytic activity during development of three entomopathogenic fungi. Comp Biochem Physiol 1989; 79B: 339-348.

Coulson AF. A proposed structure for family 18 chitinases: A possible function for nabonin. FEBS Lett 1994; 354: 41-44.

Crickmore N, Bone EJ, Williams JA, and Ellar DJ. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol Lett 1995; 131: 249-254.

Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Rie JV, Lereclus D, Baum J, and Dean DH. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 1998; 62: 807-813.

Davies G, and Henrissat B. Structures and mechanisms of glycosyl hydrolases. Structure 1995; 3: 853-859.

De barjac H, and Bonnefoi A. Essai de classification biochimique et serologique de 24 souches de *Bacillus* du type *B. thuringiensis*. Entomophaga 1962; 7: 5-31.

De Barjac H, and Frachon E. Classification of *Bacillus thuringiensis* strains. Entomophaga 1990; 35: 233-240.

Delecluse A, Poncet S, Klier A, and Rapaport G. Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl Environ Microbiol 1993; 59: 3922-3927.

Dervyn E, Poncet S, Klier A, and Rapaport G. Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J Bacteriol 1995; 177: 2283-2291.

De-Souza MT, Lecadet MM, and Lereclus D. Full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis* requires a distant upstream DNA sequence affecting transcription. J Bacteriol 1993; 175: 2952-2960.

Ding X, Gopalakrishnan B, Johnson LB, White FF, Wang X, Morgan TD, Kramer KJ, and Muthukrishnan S. Insect resistance of transgenic tobacco expressing an insect chitinase gene. Trans Res 1998; 7: 77-84.

Donovan WP, Dankocsik C, Gilbert MP. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. J Bacteriol 1988a; 170: 4732-4738.

Donovan WP, Gonzalez JM Jr, Gilbert MP, and Dankocsik C. Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the toxin gene. Mol Gen Genet 1988; 214: 365-372.

Donovan WP, Rupar MJ Slaney CA , Malvar T, Gawron-Burke MC, and Johnson TB. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to Coleoptera species. Appl Environ Microbiol 1992; 58: 3921-3927.

Doolittle RF and Bork P. Evolutionarily mobile modules in proteins. Sci Am 1993; 269: 50-56.

Dow JAT. Insect midgut function. Adv Insect Physiol 1986; 19: 187-290.

Ely S. The engineering of plants to express *Bacillus thuringiensis* δ-endotoxins. In Entwistle PF, Cory JS, Baily MJ, and Higgs S, editors. *Bacillus thuringiensis*, an environmental biopesticide: Theory and practice. John Wiley & Sons, New York 1993: 105-124.

Errington J. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol Rev. 1993; 57: 1-33.

Fast PG. Laboratory bioassays of mixtures of *Bacillus thuringiensis* and chitinase. Can Entomol 1978; 110: 201-203.

Federici BA. The future of microbial insecticides as vector control agents. J Am Mosq Control Assoc 1995; 11: 260-268.

Federici BA, Luthy P, and Ibarra JE. Parasporal body of *Bacillus thuringiensis israelensis*; structure, protein composition and toxicity. In: "Bacterial control of mosquitoes & black flies: biochemistry, genetics & applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*" (Barjac HD and Sutherland DJ., Eds), Rutgers University Press, New Brunswick. 1990: 16-44.

Felse PA and Panda T. Regulation and cloning of microbial chitinase genes. Appl Microbiol Biotechnol 1999; 51: 141-151.

Flach J, Pilet PE, and Jolles P. What's new in chitinase research? Experimentia 1992; 48: 701-716.

Fuhrman JA, Lee J, and Dalamagas D. Structure and function of a family of chitinase isozymes from *Brugian microfilariae*. Exp Parasitol 1995; 80: 672-680.

Gal SW, Choi JY, Kim CY, Cheong YH, Choi YJ, Bahk JD, Lee SY, and Cho MJ. Isolation and characterization of 54 kDa and 22 kDa chitinase genes of *Serratia marcescens* KCTC 2172. FEMS Microbiol Lett 1997; 151: 197-204.

Gal SW, Choi JY, Kim CY, Cheong YH, Choi YJ, Lee SY, Bahk JD, and Cho MJ. Cloning of the 52 kDa chitinase gene from *Serratia marcescens* KCTC2172 and its proteolytic cleavage into an active 35-kDa enzyme. FEMS Microbiol Letts 1998; 160: 151-158.

Garczynski SF, Crim JW, and Adang MJ. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl Environ Microbiol 1991; 57: 2816-2820.

Gardner KH, and Blackwell J. Refinement of the structure of β-chitin. Biopolymers 1975; 14: 1581-1595.

Gazit E, and Shai Y. Structural and functional characterization of the alpha 5 segment of *Bacillus thuringiensis* delta-endotoxin. Biochemistry 1993; 32: 3429-3436.

Gazit E, and Shai Y. The assembly and organization of the alpha 5 and alpha 7 helices from the pore-forming domain of *Bacillus thuringiensis* delta-endotoxin. Relevance to a functional model. J Biol Chem 1995; 270: 2571-2578.

Gill SS. Mechanism of action of *Bacillus thuringiensis* toxins. Mem Inst Oswaldo Cruz, Rio de Janeiro 1995; 90: 69-74.

Glatron MF, and Rapaport G. Biosynthesis of the parasporal inclusion of *Bacillus*

thuringiensis: Half-life of its corresponding messenger RNA. Biochimie 1972; 54: 1291-1301.

Gleave AP, Taylor RK, Morris BAM, and Greenwood DR. Cloning and sequencing of a gene encoding the 69-kDa extracellular chitinase of *Janthinobacterium lividum*. FEMS Microbiol Lett 1995; 131: 279-288.

Gooday GW. The ecology of chitin degradation. Adv Microbiol Ecol 1990; 11: 387-430.

Graham LS and Sticklen MS. Plant chitinases. Can J Bot 1994; 72: 1057-1083.

Grochulski P, Masson L, Borisova S, Pusztai-Carey M, Schwartz JL, Brousseau R, and Cygler M. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: Crystal structure and channel formation. J Mol Biol 1995; 254: 447-464.

Haider MZ, and Ellar DJ. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin: interaction with phospholipid vesicles. Biochim Biophys Acta 1989; 978: 216-222.

Haldenwang WG. The sigma factors of *Bacillus subtilis*. Microbiol Rev 1995; 59: 1-30.

Harpster MH, and Dunsmuir P. Nucleotide sequence of the chitinase B gene of *Serratia marcescens* QMB1466. Nucleic Acids Res 1989; 17: 5395.

Hart PJ, Pfluger HD, Monzingo AF, Hollis T and Robertus JD. The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 A resolution. J Mol Biol 1995; 248: 402-413.

Harvey WR, and Wolfersberger MG. Mechanism of inhibition of active potassium transport in isolated midgut of *Manduca sexta* by *Bacillus thuringiensis* endotoxin. J Exp Biol 1979; 83: 293-304.

Hawtin RE, Zarkowska T, Arnold K, Thomas CA, Gooday GW, King LA, Kuzio JA, and Possee RD. Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the intregrity of virus-encoded chitinase and cathepsin genes. Virology 1997; 238: 243-253.

Henrissat B, and Bairoch A. New families in the classification of glycosyl hydrolases based on amino-acid sequence similarities. Biochem J 1993; 293: 781-788.

Henrissat B. A classification of glycosyl hydrolases based on amino-acid sequence similarities. Biochem J 1991; 280: 309-316.

Henrissat B. Updating the sequence-based classification of glycosyl hydrolases. Biochem J 1996;316: 695-696.

Hodgman and Ellar. Models for the structure and function of the *Bacillus thuringiensis* delta-endotoxins determined by compilational analysis. DNA Seq 1990; 1: 97-106.

Hofmann C, and Luthy P. Binding and activity of *Bacillus thuringiensis* delta-endotoxin to invertebrate cells. Arch Microbiol 1986; 146: 7-11.

Hofte H, and Whiteley HR. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 1989; 53: 242-255.

Honee G, and Visser B. The mode of action of *Bacillus thuringiensis* crystal proteins. Entomol Exp Appl 1993; 69: 145-155.

Huber M, Cabib E, and Mieller L. Malarial parasite chitinase and penetration of the mosquito peritrophic membrane. Proc Natl Acad Sci USA 1991; 88: 2807-2810.

Hussain SR, Aronson AI, and Dean DH. Substitution of residues on the proximal side of

Cry1A *Bacillus thuringiensis* delta-endotoxins affects irreversible binding to *Manduca sexta* midgut membrane. Biochem Biophys Res Commun 1996; 226: 8-14.

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, and Maas C. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J 1995; 8: 97-109.

Jannatipour M, Soto-Gil RW, Ghilders LC, Zyskind JW. Translocation of *Vibrio harveyi* N, N'-diacetylchitobiase to the outer membrane of *Escherichia coli*. J Bacteriol 1987; 169: 3785-3791.

Jarosz J, Glinski Z, and Bartyzel M. Increased activity of blood lysozyme in the greater wax moth larvae affected personally by bacterial invaders with chitinase added. Comp Biochem Physiol 1987; 87A: 189-192.

Jone IM, Primrose SB, Ehrlich SD. Recombination between short direct repeats in a recA host. Mol Gen Genet 1982; 188: 486-489.

Jones JDG, Grady KL, Suslow TV, and Bedbrook JR. Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO J 1986; 5: 467-473.

Kalman S, Kiehne KL, Libs JL, and Yamamoto T. Cloning of a novel *cryIC*-type gene from a strain of *Bacilus thuringiensis* subsp. *galleriae*. Appl Environ Microbiol 1993; 59: 1131-1137.

Kjellen L, and Lindahl U. Proteoglycans: Structures and interactions. Annu Rev Biochem

1991; 60: 443-475.

Knight PJK, Crickmore N, Ellar DJ. The receptor for *Bacillus thuringiensis* CryIAc δ-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* in an aminopeptidase N. Mol Microbiol 1993; 11: 429-436.

Knowles BH, and Ellar DJ. Colloid-osmotic lysis ia a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. Biochim BioPhys Acta 1987; 924: 509-518.

Knowles BH, Blatt MR, Tester M, Horsnell JM, Carroll J, Menestrina G, Ellar DJ. A cytolytic delta-endotoxin from *Bacillus thuringiensis* var *israelensis* forms cation-selective channels in planar lipid bilayers. FEBS Lett 1989; 244: 259-262.

Kramer KJ, Corpuz L, Choi HK, and Muthukrishnan S. Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*. Insect Biochem Mol Biol 1993; 23: 691-701.

Kramer KJ, and Muthukrishnan S. Insect chitinases: Molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 1997; 27: 887-900.

Kramer KJ, Muthukrishnan S, Johnson L, and White F. Chitinases for insect control. In Carozzi N, and Koziel M, editors. Advanced in Insect Control: Transgenic Plants for the Control of Insect Pests. Washington DC, USA: Taylor and Francis Publishers; 1996: 185-193.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) 1970; 227: 680-685.

Lambert B, Theumis W, Agouda R, Van Audenhove K, Jansens DCS, Seurinck J, and Peferoen M. Nucleotide sequence of gene *cryIIID* encoding a novel coleopteran-active crystal protein from strain BTI109P of *Bacillus thuringiensis* subsp. *kurstaki*. Gene 1992; 110: 131-132.

Lecadet MM, Frachon E, Dumanoir VC, Ripouteau H, Hamon S, Laurent P, and Thiery I. Updating the H antigen classification of *Bacillus thuringiensis*. J Appl Microbiol 1999; 86: 660-672.

Lehane MJ. Peritrophic matrix structure and function. Ann Rev Entomol 1997; 42: 525-550.

Lehane MJ. The formation and histochemistry of the peritrophic membrane in the stable fly, *Stomoxys calcitrans*. J Insect Physiol 1976; 22: 1551-1557.

Lereclus D, Agaisse H, Gominet M, and Chaufaux J. Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis spoOA* mutant Bio/Technology 1995; 13: 67-71.

Lereclus D, Bourgouin C, Lecadet CC, Klier A, and Rapaport G. Role, structure, and molecular organization of the genes coding for parasporal δ-endotoxins of *Bacillus thuringiensis*. In Smith I, Slepecky RA, and Setlow P, editors. Regulation of procaryotic development: structural and functional analysis of bacterial sporulation and germination. American Society for Microbiology, Washinton, D.C.; 1989

Lesieur C, Vecsey-Semjen B, Abrami L, Fivaz M, and Gisou van der Goot F. Membrane insertion: The strategies of toxins (Review). Mol Memb Biol 1997; 14: 45-64.

Li E, and Yousten AA.. Metalloprotease from *Bacillus thuringiensis*. Appl Microbiol 1975; 30: 354-361.

Li J, Carroll J, and Ellar DJ. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 o A resolution. Nature 1991; 353: 815-821.

Liang Y, Patel SS, and Dean DH. Irreversible binding kinetics of *Bacillus thuringiensis* CryIA delta-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J Biol Chem 1995; 270: 24719-24724.

Limon MC, Lora JM, Garcia I dela Cruz J, Llobell A, Benitez T, Pinter-Toro JA. Primary structure and expression pattern of the 33-kDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet 1995; 28: 478-483.

Lin CS, Chen HC, and Lin FP. Characterization of the recombinant gene encoding chitinase from *Aeromonas caviae*. Enzyme Microbiol Technol 1997; 21: 472-478.

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, and Datta SK. Genetic engineering of rice for resistance to sheath blight. Bio/Technology 1995; 13: 686-691.

Little E, Bork P and Doolittle RF. Tracing the spread of fibronectin type III domains in bacterial glycohydrolases. J Mol Evol 1994; 39: 631-643.

Lorence A, Darszon A, Diaz C, Lievano A, Quintero R, and Bravo A. Endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar bilayers. FEBS Lett 1995; 360: 217-222.

Lysenko O. Chitinase of *Serratia marcescens* and its toxicity to insects. J Invertebr Pathol 1976; 27: 385-386.

Malvar T, and Baum JA. Tn5401 disruption of the *spoOF* gene, identified by direct chromo-

somal sequencing, results in CryIIIA overproduction in *Bacillus thuringiensis*. J Bacteriol 1994; 176: 4750-4753.

Malvar T, Gawron-Burke C, Baum JA. Overexpression of *Bacillus thuringiensis* HknA, a histidine protein kinase homolog, bypasses early Spo⁻ mutations that result in CryIIIA overproduction. J Bacteriol 1994; 176: 4742-4749.

Martin GG, and Wolfersberger MG. *Bacillus thuringiensis* delta-endotoxin and larval *Manduca sexta* midgut brush-border membrane vesicles act synergistically to cause very large increases in the conductance of planar lipid bilayers. J Exp Biol 1995; 198: 91-96.

Masson L, Mazza A, Gringorten L, Baines D, Aneliunas V, and Brousseau R. Specificity domain localization of *Bacillus thuringiensis* insecticidal toxins is highly dependent on the bioassay system. Mol Miceobiol 1994; 14: 851-860.

Matsuno Y, Ano T, and Shoda M. High-efficiency transformation of *Bacillus subtilis* NB22, an antifungal antibiotic iturin producer, by electroporation. J Ferm Bioeng 1992; 73: 261-264.

Minke R, and Blackwell J. The structure of α-chitin. J Mol Biol 1978; 120: 167-181.

Miyashita K, Fujii T, and Sawada Y. Molecular cloning and characterization of chitinase genes from *Streptomyces lividans* 66. J Gen Microbiol 1991; 137: 2065-2072.

Molano J, Duran A, and Cabib E. A rapid and sensitive assay for chitinase using tritiated chitin. Anal Biochem 1977; 83: 648-656.

Monreal J, and Reese ET. The chitinase of *Serratia marcescens*. Can J Microbiol 1969; 15: 689-696.

Montal MC. Formation of bimolecular membranes from lipid monolayers. Methods Enzymol 1974; 32: 545-554.

Monzingo AF, Marcotte M, Hart PJ, and Robertus JD. Chitinases, chitosanases, and lysozymes can be divided into procaryotic and eucaryotic families sharing a conserved core. Nat Struc Biol 1996; 3: 133-140.

Moran CP. RNA polymerase and transcription factors. In Sonenshein AL, Hoch JA, and Losick R, editors. *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, DC. 1993: 653-667

Morimoto K, Karita S, Kimura T, Sakka K, and Ohmiya K. Cloning, sequencing, and expression of the gene encoding *Clostridium paraputrificum* chitinase ChiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain. J Bacteriol 1997; 179: 7306-7314.

Morris ON. A 2-year study of the efficacy of *Baicllus thuringiensis*-chitinase combinations in spuce budworm (*Choristoneura fumiferana*) control. Can Entomol 1976; 108: 225-233.

Nakamura K. Genetic engineering on *Bacillus thuringiensis* insecticidal proteins. Bioprocess Technol 1994; 19: 665-682.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, and Nebert DW. P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 1996; 6:1-42.

Nierlich DP, and Murakawa GJ. The decay of bacterial mesenger RNA. Prog Nucl Acid Res Mol Biol 1996; 52: 153-216.

Ohno T, Armand S, Hata T, Nikaidou N, Henrissat B, Mitsutomii M and Watanabe T. A modular family 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT 6037. J Bacteriol 1996; 178: 5065-5070.

Park HW, Ge B, Bauer LS, and Federeci BA. Optimization of Cry3A Yields in *Bacillus thuringiensis* by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl Environ Microbiol 1998; 64: 3932-3938.

Parker MW, and Pattus F. Rendering a membrane protein soluble in water: A common packing motif in bacterial protein toxins. Trends Biochem Sci 1993; 18: 391-395.

Perrakis A, Tews I, Dauter Z, Oppenheim AB, Chet I, Wilson KS, and Vorgias CE. Crystal structure of a bacterial chitinase at 2.3 A resolution. Structure 1994; 2: 1169-1180.

Pfannenstiel MA, Couche GA, Ross EJ, and Nickerson KW. Immunological relationships among proteins making up the *Bacillus thuringiensis* subsp. *israelensis* crystalline toxin. Appl Environ Microbiol 1986; 52: 644-649.

Pleban S, Chernin L, Chet I. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. Lett Appl Microbiol 1997; 25: 284-288.

Poncet S, Anello G, Delecluse A, Klier A and Rapaport G. Role of the CryIVD polypeptide in the overall toxicity of *Bacillus thuringiensis* subsp. *israelensis*. Appl Environ Microbiol 1993; 59:2928-2930.

Poncet S, Dervyn E, Klier A, and Rapaport G. SpoA represses transcription of the *cry* toxin genes in *Bacillus thuringiensis*. Microbiology 1997; 143: 2743-2751.

Poole DM, Hazelwood GP, Huskisson NS, Virden R, and Gilbert HJ. The role of conserved tryptophan residues in the interaction of a bacterial cellulose binding domain with its ligand. FEMS Microbiol Lett 1993; 106: 77-84.

Porter AG. Mosquitocidal toxins, genes and bacteria: the hit squad. Parasitology Today 1996; 12: 175-179.

Porter AG, Davidson EW, and Liu JW. Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiol Rev 1993; 57: 838-861.

Powell KS, Gatehouse AMR, Hilder VA, and Gatehouse JA. Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, *Nilaparvata lugens* and *Nephotettix cinciteps*. Entomol Exp Appl 1993; 66: 119-126.

Rajamohan F, Cotrill JA, Gould F, and Dean DH. Role of domain II, loop 2 residues of *Bacillus thuringiensis* CryIAb delta-endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. J Biol Chem 1996a; 271: 2390-2396.

Rajamohan F, Hussain SRA, Cotrill JA, Gould F, Dean DH. Mutations at domain II, loop 3, of *Bacillus thuringiensis* CryIAa and CryIAb delta-endotoxins suggest loop 3 is involved in initial binding to lepidopteran midguts. J Biol Chem 1996b; 271: 25220-25226.

Rajamohan F, Lee MK, and Dean DH. *Bacillus thuringiensis* insecticidal proteins: Molecular mode of action. Prog Nucle acid Res Mol Biol 1998; 60: 1-27.

Reardon D, and Farber GK. The structure and evolution of α/β Barrel proteins. FASEB J 1995; 9: 497-503.

Regev A, keller M, Strizhov N, Sneh B, Prudovsky E, Chet I, Ginzberg I, Koncz-Kalman Z, Koncz C, Schell J, and Zilberstein A. Synergistic activity of a *Bacillus thuringiensis* delta endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Appl Environ Microbiol 1996; 62: 3581-3586.

Reissig JL, Strominger JL, and Leloir LF. A modification colorimetric method for the estimation of N-acetylamino sugars. J Biol Chem 1955; 27: 959-966.

Richards AG, and Richards PA. The peritrophic membranes of insects. Annu Rev Entomol 1977; 22: 219-240.

Robbins PW, Albright C, and Benfield B. Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. J Biol Chem 1988; 263: 443-447.

Romaguera A, Menge U, Breves R and Diekmann H. Chitinases of *Streptomyces olivaceoviridis* and significance of processing for multiplicity. J Bacteriol 1992; 174: 3450-3454.

Rudall RM. The chitin/protein complexs of insect cuticles. In : Beament JWL, Treherne JE, and Wigglesworth VB, editors. Advances in Insect Physiology. London, New York: Academic press, 1963 : 257-313.

Rupp RA, and Spence KD. Protein alterations in *Manduca sexta* midgut and haemolymph following treatment with a sublethal dose of *Bacillus thuringiensis* endotoxin. Insect Biochem 1985; 15: 147-154.

Salamitou S, Agaisse H, Bravo A, and Lereclus D. Genetic analysis of *cryIIIA* gene expression in *Bacillus thuringiensis*. Microbiology 1996; 142: 2049-2055.

Sambrook J, Fritsch EF, and Manatis T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, New York 1989.

Sampson MN, and Gooday GW. Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. Microbiology 1998; 144: 2189-2194.

Sangadala S, Walter FS, English LH, and Adang MJ. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIAc toxin binding and $86Rb^{+}$-K^{+} efflux in vitro. J Biol Chem 1994; 269: 10088-10092

Sanger S, Nicklen S, and Coulsen AR. DNA sequencing with chain determination inhibitors. Proc Natl Acad Sci USA 1977; 74: 5463-5467.

Schlein Y, Raymond LJ, and Shlomai J. Chitinase secreted by *Leishmania* functions in sandfly vector. Proc R Soc Lond Ser B 1991; 245: 121-126.

Schnepf HE, and Whiteley HR. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proc Natl Acad Sci USA 1981; 78: 2893-2897.

Schnepf E, Crickmore N, Rie JV, Lereclus D, Baum J, Feitelson J, Zeigler DR, and Dean DH. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 1998; 62: 775-806.

Schwartz JL, Garneau L, Masson L, and Brousseau R. Early response of cultured lepidopteran cells to exposure to delta-endotoxin from *Bacillus thuringiensis*: in-

volvement of calcium and anionic channels. Biochim Biophys Acta 1991; 1065: 250-260.

Schwartz JL, Garneau L, Savaria D, Masson L, Brousseau R, Rosseau E. Lepidopteran-specific crystal toxins from *Bacillus thuringiensis* form cation- and anion-selective channels in planar lipid bilayers. J Membr Biol 1993; 132: 53-62.

Schwartz JL, Potvin L, Chen XJ, Brousseau R, Laprade R, and Dean DH. Single-site mutations in the conserved alternating-arginine region affect ionic channels formed by CryIAa, a *Bacillus thuringiensis* toxin . Appl Environ Microbiol 1997; 63: 3978-3984.

Sekar V. The Insecticidal Crystal protein is expressed in vegetative cells of *Bacillus thuringiensis* var. *tenebrionis*. Curr Microbiol 1988; 17: 347-349.

Seyler RW jr, Yousten AA, and Burke WF Jr. Plasmid stability in *Bacillus sphaericus* 2362 during recycling in mosquito larvae. J Invetebr Pathol 1991; 58: 363-366.

Shaikh SA, and Deshpande MV. Chitinolytic enzymes: Their contribution to basic and applied research. World J Microbiol Biotech 1993; 9: 468-475.

Shapiro M, Preisler HK, and Robertson JL. Enhancement of baculovirus activity on gypsy moth (Lepidoptera: *Lymantriidae*) by chitinase. Econ Entomol 1987; 80: 1113-1116.

Shin BS, Park SH, Choi SK, Koo BT, Lee ST, Kim JI. Distribution of *cryV*-type insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV*-type genes from *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus thuringiensis* subsp. *entomocidus*. Appl Environ Microbiol 1995; 61: 2402-2407.

Shin H, Mohnen D, and Meins F. Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Natl Acad Sci USA 1987; 84: 89-93.

Singh GJP, Schouest Jr LP, and Gill SS. The toxic action of *Bacillus thuringiensis* var *israelensis* in *Aedes aegypti* in vivo. I. The relevance of midgut lesions to its poisoning syndrome. Pestic Biochem Physiol 1986; 26: 36-46.

Sitrit Y, Vorgias CE, Chet I, and Oppenheim AB. Cloning and primary structure of the *chiA* gene from *Aeromonas caviae*. J Bacteriol 1995; 177: 417-4189.

Slatin SL, Abrams CK, English LH. Delta-endooxin form cation-selective channels in planar lipid bilayers. Biochem Biophys Res Commun 1990; 169: 765-772.

Smirnoff WA. Confirmation experimentales du potential du complexe *Bacillus thuringiensis* chitinase pour la repression d ela tordeuse des bourgeons de espirette *Choristoneura fuminerana*. Can Entomol 1977; 109: 351-358.

Smirnoff WA. Effect of chitinase on the action of *Bacillus thuringiensis*. Can Entomologist 1971; 103: 1829-1831.

Smirnoff WA. Results of test with *B. thuringiensis* and chitinase on larvae of spruce bud worm. J Invertrbr Pathol 1973; 21: 116-118.

Smirnoff WA. Three years of aerial field experiments with *Bacillus thuringiensis* plus chitinase formulation against the spuce budworm. J Invert Pathol 1974; 24: 344-348.

Sneh B, Schuster S, and Gross S. Improvement of the insecticidal activity of *Bacillus thuringiensis* var *entomocidus* on larvae of *Spodoptera littoralis* (lepidoptera,

Noctuidae) by addition of chitinolytic bacteria, a phagostimulant and a UV-protectant. Z Angew Entomol 1983; 96: 77-83.

Soto-Gil RW, and Zyskind JW. N, N' diacetylchitobiase of *Vibrio harveyi*: Primary structure, processing, and evolutionary relationships. J Biol Chem 1989; 264: 14778-14783.

St Leger RJ, Cooper RM, and Charnley AK. Cuticle degrading enzymes of entomopathogenic fungi: Regulation of production of chitinoytic enzymes. J Gen Microbiol 1986; 132: 1509-1517.

Stamm B, D'Haese J, Peters W. SDS gel electrophoresis of proteins and glycoproteins from peritrophic membranes of some Diptera. J Insect Physiol 1978; 24: 1-8.

Suzuki K, Suzuki M, Taiyoji M, Nikaidou N, and Watanabe T. Chitin binding protein (CBP21) in the culture supernatant of *Serratia marcescens* 2170. Biosci Biotechnol Biochem 1998; 62: 128-135.

Suzuki K, Taiyoji M, Sugawara N, Nikaidou N, Henrissat B, and Watanabe T. The third chitinase gene (*chiC*) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. Biochem J 1999; 343: 587-596.

Svitil AL, Ni Chadhain SM, Moore JA, an Kirchman DL. Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Appl Environ Microbiol 1997; 63: 408-413.

Svitil AL, and Kirchman DL. A chitin-binding domain in a marine bacterial chitinase and other microbial chitinases: Implications for the ecology and evolution of 1,4-β-glycanases. Microbiology 1998; 144: 1299-1308.

Tailor R, Tippett J, Gibb G, Pells S, Pike D, Jordan L, and Ely S. Identification and character-

ization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. Mol Microbiol 1992; 6: 1211-1217.

Takayanaki T, Ajisaka K, Takiguchi Y, and Shimahara K. Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. Biochim Biophys Acta 1991; 1078: 404-410.

Tantimavanich S. Cloning of chitinase gene from *Bacillus licheniformis* TP-1 in *Bacillus thuringiensis* subsp. *aizawai* and study of its effect towards *Spodoptera exigua*. The degree of doctor philosophy (Microbiology). Faculty of Science Mahidol University; 1997.

Tantimavanich S,Pantuwatana S,Bhumiratana A, Panbangred, W. Cloning of a chitinase gene into *Bacillus thuringiensis* subsp. *aizawai* for enhanced insecticidal activity. J Gen Appl Microbiol 1997; 43: 341-347.

Tantimavanich S, Pantuwatana S, Bhumiratana A, Panbangred W. Multiple chitinase enzymes from a single gene of *Bacillus licheniformis* TP-1. J Ferm Bioeng 1998; 85: 259-265.

Techkarnjanaruk S, Pongpattanakitshote S, and Goodman AE. Use of a promoterless *lacZ* gene insertion to investigate chitinase gene expression in the marine bacterium *Pseudoalteromonas sp.* strain S9. Appl Environ Microbiol 1997; 63: 2989-2996.

Techkarnjanaruk S, and Goodman AE. Multiple genes involved in chitin degradation from the marine bacterium *Pseudoalteromonas sp.* strain S91. Microbiology 1999; 145: 925-934.

Terra WR. Evolution of digestive systems of insects. Annu Rev Entomol 1990; 35:181-200.

Terwissona van Scheltinga AC, Kalk KH, Beintema JJ, and Dijkstra BW. Crystal structures of hevamine, a plant defense protein with chitinase and lysozyme activity, and its complex with an inhibitor. Structure 1994; 2: 1181-1189.

Tews T, Dauter Z, Oppehheim AB, and Vorgiase CE. Crystallization of recombinant chitobiase from *Serratia marcescens*. J Mol Biol. 1992; 228: 696-697.

Thamthiankul S, Suan-Ngay S, Tantimavanich S, and Panbangred W. Chitinase from *Bacillus thuringiensis* subsp. *pakistani*. Appl Microbiol Biotechnol (in press).

Trudel J, and Asselin A. Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal Biochem 1989; 178: 326-366.

Trudel J, Potvin C, and Asselin A. Secreted hen lysozyme in transgenic tobacco: recovery of bound enzyme and in vitro growth inhibition of plant pathogens. Plant Sci 1995; 106: 55-62.

Tsujibo H, Orikoshi H, Tanno H, Fujimoto K, Miyamoto K, Imada C, Okami Y, Inamori Y. Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas sp.* strain O-7. J Bacteriol 1993;175: 176-181.

Tsujibo H, Fujimoto K, Tanno H, Miyamoto K, Imada C, Okami Y, and Inmori Y. Gene sequence, purification and characterization of N-acetyl-β-glucosaminidase from a marine bacterium, *Alteromonas sp.* strain O-7. Gene 1994; 146: 111-115.

Tsujibo H, Fujimoto K, Tanno H, Miyamoto K, Kimura Y, Imada C, Okami Y and Inamori Y. Molecular cloning of the gene which encodes β-N-acetylglucosaminidase from a marine bacterium, *Alteromonas sp.* strain O-7. Appl Environ Microbiol

1995; 61: 804-806.

Tsujibo H, Orikoshi H, Shiotani K, Hayashi M, Umeda J, Miyamoto K, Imada C, Okami Y and Inamori Y. Characterization of chitinase C from a marine bacterium, *Alteromonas sp.* strain O-7, and its corresponding gene and domain structure. Appl Environ Microbiol 1998; 64: 472-478.

Tsujibo H, Yoshida K, Miyamoto K, Imada C, Okami Y, and Inamori Y. Purification, properties, and partial amino acid sequence of chitinase from a marine *Alteromonas sp.* strain O-7. Can J Microbiol 1992; 38: 891-897.

Ulhoa CJ, and Peberdy JF. Regulation of chitinase synthesis in *Trichoderma harzianum*. J Gen Microbiol 1991; 137: 2163-2169.

Verburg JG, Smith CE, Lisek CA, and Huynh QK. Identification of an essential tyrosine residue in the catalytic site of a chitinase isolated from *Zea mays* that is selectively modified during inactivation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. J Biol Chem 1992; 267: 3886-3893.

Vierheilig H, Alt M, Neuhaus JM, Boller T, and Wiemken A. Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Mol Plant-Microbe Interact 1993; 6: 261-264.

Visick JE, and Whiteley HR. Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* is on production of the CytA protein by *Escherichia coli*. J Bacteriol 1991; 173: 1748-1756.

Vorgiase CE, Perrakis A, and Tews L. Structure-function studies on the chitinolytic enzymes of *Serratia marcescens* chitinase and chitobiase. Ann NY Acad Sci 1996; 799: 190-192.

Wang X, Ding X, Gopalakrishnan B, Morgan T, Johnson L, White FF, Muthukrishnan S, and Kramer KJ. Characterization of a 46 kDa insect chitinase from transgenic tobacco. Insect Biochem Mol Biol 1996; 26: 1055-1064.

Watanabe T, Ito Y, Yamada T, Hashimoto M, Sekine S, and tanaka H. The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. J Bacteriol 1994; 176: 4465-4472.

Watanabe T, Kimura K, Sumiya T, Nikaidou N, Suzuki K, Suzuki M, Taiyoji M, Ferrer S and Regue M. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. J Bacterol 1997; 179: 7111-7117.

Watanabe T, Kobori K, Miyashita K, Fujii T, Sakai H, Uchida M, and Tanaka H. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. J Biol Chem 1993; 268: 18567-18572.

Watanabe T, Oyanagi W, Suzuki K, and Tanaka H. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. J Bacteriol 1990a; 172: 4017-4022.

Watanabe T, Oyanaki W, Suzuki K, Ohnishi K, and Tanaka H. Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. J Bacteriol 1992; 174:

408-414.

Watanabe T, Suzuki K, Uoyanaki W, Ohnishi K, and Tanaka H. Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectrin. J Biol Chem 1990b; 265: 15659-15665.

Whistler RS, and BeMiller JN. Chitin. J Org chem. 1962; 27: 1161-1163.

White JA, Maltais LJ, and Nebert DW. An increasingly urgent need for standardized gene nomenclature. See: http://genetics.nature.com/web_specials /nomen/ nomen_article.html 1998.

Widner Wr, and Whiteley HR. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* posses different host range specificities. J Bacteriol 1989; 171: 965-974.

Wiwat C, Lertcanawanichakul M, Siwayapran P, Pantuwatana S and Bhumiratana A. Expression of chitinase-encoding genes from *Pseudomonas maltophila* in *Bacillus thuringiensis* subsp. *israelensis*. Gene 1996; 179: 119-126.

Wolfersberger MG, Chen XJ, and Dean DH. Site-directed mutations in the third domain of δ-endotoxin δ-endotoxin CryIAa affect its ability to increase the permeability of *Bombyx mori* midgut brush border membrane vesicles. Appl Environ Microbiol 1996; 62: 279-282.

Wong HC, and Chang S. Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc Natl Acad Sci USA 1986; 83: 3233-3237.

Wong HC, Schnepf HE, and Whiteley HR. Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. J Biol Chem 1983; 258: 1960-1967.

Wortman AT, Somerville CC, and Colwell RR. Chitinase determinants of *Vibrio vulnificus*: Gene cloning and applications of a chitinase probe. Appl Environ Microbiol 1986; 52: 142-145.

Wu D, Cao XL, Bay YY, and Aronson AI. Sequence of an operon containing a novel δ-endotoxin gene from *Bacillus thuringiensis*. FEMS Microbiol Lett 1991; 81: 31-36.

Wu D, and Aronson AI. Localized mutagenesis defines regions of the *Bacillus thuringiensis* delta-endotoxin involved in toxicity and specificity. J Biol Chem 1992; 267: 2311-2317.

Wu D, and Federici BA. A 20-kilodalton protein preserves cell viability and promoters CytA crystal formation during sporulation in *Bacillus thuringiensis*. J Bacteriol 1993; 175:5276-5280.

Wu D, and Federici BA. Improved production of the insecticidal CryIVD protein in *Bacillus thuringiensis* using *cryIAc* promoters to express the gene for an associated 20-kDa protein. Appl Microbiol Biotechnol 1995; 42: 697-702.

Wu SJ, and Dean DH. Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* CryIIIA delta-endotoxins. J Mol Biol 1996; 255: 628-640.

Yoshisue H, Yoshida K, Sen K, Sakai H, Komano T. Effects of *Bacillus thuringiensis* var. *israelensis* 20-kDa protein on production of the *B.t.i.* 130-kDa crystal protein in *Escherichia coli*. Biophys Biotechnol Biochem 1992; 56: 1429-1433.

Yoshisue H, Fukada T, Yoshida KI, Sen K, Kurosawa SI, Sakai H, and Komano T. Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis* mosquito larvicidal crystal protein *cryIVA*. J Bacteriol 1993a; 175: 2750-2753.

Yoshisue H, Ihara K, Nishimoto T, Saki H, and Komano T. Expression of the genes for insecticidal crystal proteins in *Bacillus thuringiensis*: *cryIVA*, not *cryIVB*, is transcribed by RNA polymerase containing Sigma H and that containing Sigma E. FEMS Microbiol Lett 1995; 127: 65-72.

Yoshisue H, Nishimoto T, and Komano T. Identification of a promoter for the crystal protein-encoding gene *cryIVB* from *Bacillus thuringiensis* subsp. *israelensis*. Gene 1993; 137: 247-251.

Young ME and Carroad PA. Dependence of extracellular chitinase activity of *Serratia marcescens* QMB 1466 on continuous culture dilution rate. Can J Microbiol 1981; 27: 142-144.

Yunovitz H, Sneh B, and Yawetz A. A new sensitive method for determining the toxicity of a highly purified fraction from δ-endotoxin produced by *Bacillus thuringiensis* var *entomocidus* on isolated larval midgut of *Spodoptera littoralis* (Lepidoptera, *Noctuidae*). J Invertebr Pathol 1986; 48: 223-231.

Zeigler DR. *Bacillus thuringiensis & Bacillus cereus*: Pesticidal activity of Cry and Cyt proteins. *Bacillus* genetic stock center catalog of strains. Seventh edition. 1999; 2: 48-50.

Zen KC, Choi HK, Krishnamachari N, Muthukrishnan M, and Kramer K. Cloning, expression, and hormonal regulation of an insect β-N-acetylglucosaminidase gene. Insect Biochem Mol Biol 1996; 26: 435-444.

Zhu Q, Maher EA, Masoud S, Dixon RA, and Lamb CJ. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/Technology 1994; 12: 807-812.

ZoBell CE, and Rittenberg SC. The occurrence and characteristics of chitinoclastic bacteria in the sea. J Bacteriol 1937; 35: 275-287.

APPENDIX

1. Calculation of LD_{50} by probit analysis method

Probit analysis is a statistical procedure used in analysis of bioassays.

Example

Table 13. Data used in calculation of LD_{50} of *B.t.i.* 4Q2-72 (p16-1968CHI) mixed with 2.5 mU/ml chitinase enzyme against *Aedes aegypti* second instar larvae.

Bacterial dilution	Bacterial concentration (Cfu/ml) x	r	%kill	Empirical	logx	Expected y
$0.2x10^{-3}$	$8x10^{3}$	19	95	6.64	3.9	6.6
$0.1x10^{-3}$	$4x10^{3}$	19	95	6.64	3.6	6.3
$0.5x10^{-4}$	$2x10^{3}$	15	75	5.67	3.3	6.0
$0.25x10^{-4}$	$1x10^{2}$	11	55	5.13	2.0	4.8

r = Number of killing larvae

x = Bacterial concentration (Cfu/ml)

Empirical = The probit trasformation (read from the Table of probit transformation)

Expected Y = Expected probits calculated from the equation: Y= 5 + b (x-m)

m = Log concentration (mg/ml) at probit value of 5.0

APPENDIX (continued)

(from graph in Fig.45.)

= 2.2

b = The estimated regression coefficient of probit on dose,

or the rate of increase of probit per unit increase in x

calculated from equation: b = y/ x = 0.93

Y = 5 + 0.93 (x-2.2)

LD_{50} = $10^m = 102.2$

Table 14. Probit transformation

% Kill	0	1	2	3	4	5	6	7	8	9
00	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.95	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.9	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.92	5.99	6.04	6.08	6.13	6.18	6.23
90	6.25	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

APPENDIX (continued)

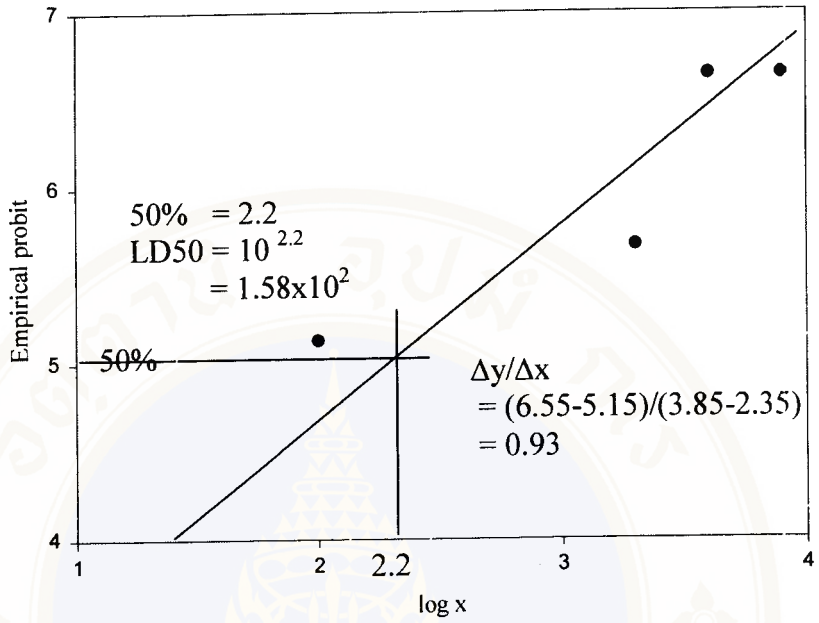


Fig.45. Relationship between probit of kill of *Aedes aeqypti* larvae and bacterial concentration, showing probit regression line.

2. Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Whistler and BeMiller (1962) with some modification. A 20 grams of coarse flake crab shell chitin (Sigma) was hydrolysed by adding with 300 ml of conc. HCl (12 M) and kept for 24 h at 4°C with continuous stirring. The chitin hydrolysate was deproteinated by pouring into 2,000 ml of chilled 95% ethanol followed by immediately shaking rapidly and vigorously. The mixture was kept standing overnight at -20 °C. The colloidal chitin was collected as paste-fine granule after centrifugation at 6,000 rpm for 10 min. The collected material was washed repeatedly with distilled water until the pH became neutral. The colloidal chitin was resuspended in steriled distilled water containing 0.02% (w/v) sodium azide and kept at 4°C. The solution can be kept at this condition for at least 2 years.

APPENDIX (continued)

3. Preparation of glycol chitin

Glycol chitin was prepared by acetylation of glycol chitosan based on a modification method of Molano et al (1977) which was described by Trudel and Asselin (1989). Five grams of glycol chitin was ground in 100 ml of acetic acid using a motar. The resulting solution was kept standing overnight at 22°C. Then the solution was slowly added with 400 ml of methanol and vacuum filtered through a Whatmann No. 4 filter paper. A 7.5 ml of acetic acid anhydride was subsequently added to the filtrate while keeping stirring. The resulting gel was kept standing for further 30 min at room temperature before it was cut into small pieces. Gel pieces was collected while the liquid portion was discarded. The gel pieces was homogenized in about 1 volume of methanol using a Waring Blender at top speed for 4 min. The gelatinous pellet was collected by centrifugation for 15 min at 27,000 g and 4°C. It was homogenized and centrifuged as described in the preceeding step. The pellet was resuspended in 500 ml of distilled water containing 0.02% (w/v) of sodium azide and homogenized for 4°C. This was the final 1% (w/v) of glycol chitin solution.

4. Construction of standard curve for GlcNAc

The standard curve of GlcNAc was constructed using a solution of GlcNAc at concentration of 0.05, 0.1, 0.2, 0.3, and 0.4 mole. The procedure was described in Materials and Methods section 11.2 using 200 µl of different concentration of GlcNAc. The standard curve was made by plotting between different concentration of standard GlcNAc against their absorbance value as shown in Table 15 and Fig. 46. The chitinase

APPENDIX (continued)

activity of enzyme solution was determined by calculation from the standard curve. One unit of chitinase enzyme was defined as the amount of enzyme which releases 1 μmole of GlcNAc end product or its equivalent per minute at 37°C. The equation for calculation is :

$$mU/ml = C \times A585 \times 2 \times 1/t \times 1000 \times \text{dilution factor}$$

When: C = concentration of GlcNAc at $OD_{585} = 1$

A_{585} = absorbance at 585 nm

T = reaction time between enzyme and chitin substrate

Table 15. The correlation between final concentration of standard GlcNAc and their absorbance value at 585 nm.

GlcNAc (μmole)	0.05	0.1	0.2	0.3	0.4
A_{585}	0.14	0.266	0.514	0.691	0.966

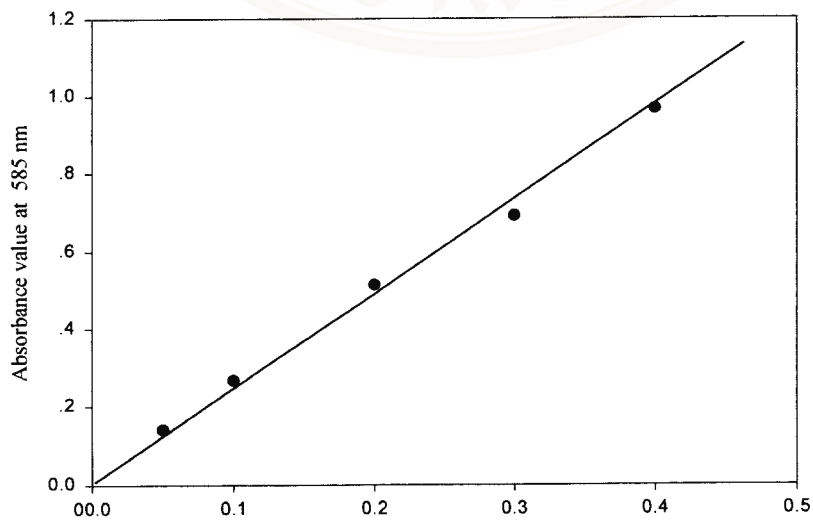


Fig. 46. The correlation between final concentration of standard GlcNAc (μmole/ml) and their absorbance value at 585 nm.

APPENDIX (continued)

5. Construction a standard curve of BSA

The standard curve of BSA was made using a solution of BSA at concentration of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml. The method of Bradford (1976) was used by performing as recommended in the kit's manual (Biorad, USA). A standard cuve was made by plotting between each BSA concentration and its corresponding absorbance value as shown in Table 16 and Fig. 47. The concentration of protein in the unknown sample was determined by comparison to this standard curve.

Table 16. The correlation between the final concentration at standard protein (BSA) and its corresponding absorbance value at 595 nm.

BSA (mg/ml)	0.05	0.1	0.2	0.3	0.4
A595	0.053	0.12	0.23	0.35	0.45

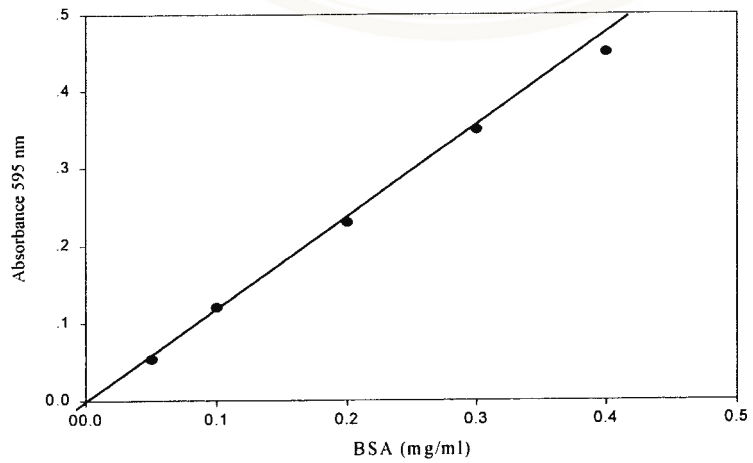


Fig. 47. The correlation between final concentration (mg/ml) of standard Bovine serum albumin (BSA) and their absorbance value at 595 nm

APPENDIX (continued)

Table 17. Summary of plamids, bacterial host strains and their descriptions.

Plasmid	Antibiotic resistance markers	Bacterial hosts	Description
pCR2.1	Am^r, Km^r	*E. coli* DH5α	PCR cloning vector (Invitrogen, USA)
TA19/5			*p19* gene
TA68S/9			*cry11Aa1* gene without terminator
TA68X/14			*cry11Aa1* gene with its terminator
TACHISK9			TP-1 chitinase gene without terminator
TACHI15			TP-1 chitinase gene with its terminator
pBluescript SK+	Am^r	*E. coli* DH5α	*E. coli* cloning vector (Strategene, USA)
pSK19/5			*p19* gene
p19CHI			Fusion of *p19* gene upstream from TP-1 chitinase gene
p1968CHI			Fusion of *p19* gene, *cry11Aa1* gene, and TP-1 chitinase gene
p19CHI68			Fusion of *p19* gene, TP-1 chitinase gene, and *cry11Aa1* gene

APPENDIX (continued)

Table 17. (Cont.)

Plasmid	Antibiotic resistance marker	Bacterial hosts	Description
PBC16	Tc^r	*Bacillus*	*Bacillus* cloning vector
p16-19CHI	Am^r, Tc^r	*E. coli* DH5α	Fusion of *p19* gene upstream from TP-1 chitinase gene
		B.t.i. 4Q2-72	
		B.t.i. c4Q2-72	
p16-1968CHI	Am^r, Tc^r	*E. coli* DH5α	Fusion of *p19* gene, *cry11Aa1* gene, and TP-1 chitinase gene
		B.t.i. 4Q2-72	
p16-19CHI68	Am^r, Tc^r	*E. coli* DH5α	Fusion of *p19* gene, TP-1 chitinase gene, and *cry11Aa1* gene

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