COLEOPTERA-SPECIFIC (CRY3Aa) DELTA-ENDOTOXIN BIOSYNTHESIS BY A LOCAL ISOLATE OF BT SUBSP. TENEBRIONIS, GENE CLONING AND CHARACTERIZATION

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ABSTRACT

COLEOPTERA-SPECIFIC (CRY3Aa) DELTA-ENDOTOXIN BIOSYNTHESIS BY A LOCAL ISOLATE OF *BT* SUBSP. *TENEBRIONIS*, GENE CLONING AND CHARACTERIZATION

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Cry3Aa is a 73 kDa protoxin toxic to insect larvae of Coleoptera order. It is processed to form a stable 65 kDa δ-endotoxin by endogenous proteases. The first part of this study involved the determination of the patterns of biosynthesis of Coleoptera-specific δ-endotoxin by a local isolate of *Bacillus thuringiensis* subsp. *tenebrionis* (*Btt*) in relation to its growth and sporulation. Among four different media compared (DSM, GYS, HCT and C2) Cry3Aa production was the highest in DSM, especially at 72^{nd} h and 120^{th} h of incubation.

For improvement of Cry3Aa production, the effects of different carbon and nitrogen sources, inorganic phosphate and other mineral elements were tested. Increasing concentrations (5-10 $g.L^{-1}$) of glucose or sucrose decreased the toxin yield probably by suppressing sporulation. Inorganic phosphate was found to have the most striking effect on toxin biosynthesis. 200 mM inorganic phosphate concentration resulted in 5 fold increase in Cry3Aa yield. Cry3Aa production was greatly reduced when various combinations of organic and inorganic nitrogen sources, especially ammonium sulphate and Casamino acids were replaced with Nutrient broth in DSM. The highest Cry3Aa production was obtained in the media containing 10^{-5}-10^{-7} M $MnCl_2$, 10^{-5} M $FeSO_4$ and 5.10^{-4} M $MgSO_4$, corresponding to their original concentrations in DSM. Decrease of iron concentration or its omission from the medium decreased the toxin yield.

Toxin production capacity of our local isolate was compared with those of 30 different anti-Coleopteran *Bt* strains. Most of the strains producing this protein gave general protein banding patterns quite similar to that of our local isolate.

Lastly, the *cry*3Aa gene of the *Btt* local isolate was PCR-amplified and cloned into the *E. coli*/*Bacillus* shuttle vector pNW33N. The recombinant plasmid was amplified in *E. coli* and the sequence of the *cry*3Aa was determined. Amino acid sequence deduced was found to be 97.4 %-99.2 % identical to the *cry*3Aa sequences (GenBank) of 10 different quaternary ranks. In this respect, the gene has to represent the 11^{th} quaternary rank of the *cry*3Aa ones. The recombinant plasmid carrying *cry*3Aa gene was next used to transform *Bs* 168 as an intermediate host and low level of expression was seen.

Keywords: *Bacillus thuringiensis* subsp. *tenebrionis*; Cry3Aa δ-endotoxin biosynthesis; regulation; *cry*3Aa gene, gene cloning.

ÖZ

BT SUBSP. *TENEBRIONIS* ' İN YEREL BİR İZOLATINDA KINKANATLILARA KARŞI AKTİF (CRY3Aa) DELTA-ENDOTOKSİN BİYOSENTEZİ, GEN KLONLANMASI VE KARAKTERİZASYONU

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Cry3Aa Kınkanatlılar takımından böcek larvalarına karsı öldürücü etkiye sahip 73 kDa'lık bir protoksindir. Proteazlar tarafından daha kararlı bir form olan 65 kDa'lık δ-endotoksine dönüştürülür. Bu çalışmanın ilk bölümü, *Bacillus thuringiensis* subsp. *tenebrionis'*in (*Btt*) yerli bir izolatı tarafından Kınkanatlılara özgü δ-endotoksin biyosentezinin ve bunun organizmanın üremesi ve sporulasyonu ile olan ilişkisinin belirlenmesini içermiştir. Karşılaştırılan dört farklı besiyeri içinden (HCT, GYS, DSM ve C2) DSM besiyerinin diğer besiyerlerine kıyasla toksin üretimini daha iyi desteklediği, bu besiyerinde üretilen hücrelerde toksin üretiminin 72. ve 120. saatlerde en yüksek olduğu görülmüştür.

Toksin üretiminin artırılmasına yönelik çalışmalarda farklı karbon ve nitrojen kaynaklarının, inorganik fosfatın ve diğer minerallerin etkileri test edilmiştir. 5 $g.L^{-1}$ ile 10 $g.L^{-1}$ aralığında artan glukoz ve sükroz konsantrasyonları muhtemelen sporlanmayı baskılamak suretiyle toksin veriminin düşmesine neden olmuşlardır. İnorganik fosfatın toksin biyosentezi üzerinde en dikkat çekici etkiye sahip olduğu bulunmuştur. 200 mM'lık inorganik fosfat konsantrasyonun toksin üretimini 5 misli artırdığı görülmüştür. DSM'nin "Nutrient broth" içeriğinin organik ve inorganik azot kaynaklarının farklı kombinasyonlarıyla özellikle amonyum sülfat ve Kasamino asitler ile değiştirilmesi Cry3Aa sentezini inhibe etmiştir. $MnCl_2$, $FeSO_4$ ve $MgSO_4$'ın kontrol besiyerinde kullanılan konsantrasyonlarının (sırasıyla 10^{-5}-10^{-7} M, 10^{-6} M ve 5.10^{-4} M) denenen diğer konsantrasyonlara kıyasla daha yüksek toksin üretimini sağladığı bulunmuştur. $FeSO_4$'ın konsantrasyonunun düşürülmesi ya da besiyerine hiç ilave edilmemesi Cry3Aa biyosentezinde düşüşe neden olmuştur.

Yerli izolatın toksin üretim kapasitesi, Kınkanatlılar takımına mensup böceklere karşı aktif toksin üreten 30 farklı *Bt* suşunun toksin üretim kapasiteleri ile kıyaslanmıştır. Karşılaştırılan suşların çoğu, yerli izolata çok benzer protein profilleri vermiştir.

Çalışmanın son aşamasında, *Btt* izolatına ait *cry*3Aa geni PCR ile amplifiye edilmiş ve bir *E. coli*/*Bacillus* vektörü olan pNW33N vektörüne klonlanmıştır. Rekombinant plazmid *E. coli*'de çoğaltılmış ve *cry*3Aa geninin nükleotid dizisi analiz edilmiştir. Buna göre elde edilen amino asit dizilimi *cry*3Aa'nın bugüne dek bilinen 10 farklı kuaterner sınıfını temsil eden GenBank'da mevcut dizileri ile karşılaştırılmıştır. Yerli izolatın ürettiği Cry3Aa proteinin bu proteinlere 97.4 %- 99.2 % oranında özdeş olduğu bulunmuştur. Bu sonuca göre gen, *cry*3Aa geninin 11. kuaterner sınıfını temsil edecektir. *cry*3Aa genini içeren rekombinant plazmid daha sonra *Bs* 168'i transforme etmekte kullanılmış ve ara konakçı olan bu organizmada genin orijinal üreticiye göre düşük seviyede ifade edildiği görülmüştür.

Anahtar kelimeler: *Bacillus thuringiensis* subsp. *tenebrionis*; Cry3Aa δ-endotoksin biyosentezi; regülasyon; *cry*3Aa geni, gen klonlanması.

To My Parents

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Bt	*: Bacillus thuringiensis*
Btt	*: Bacillus thuringiensis* subspecies *tenebrionis*
Bti	*: Bacillus thuringiensis* subspecies *israelensis*
Btk	*: Bacillus thuringiensis* subspecies *kurstaki*
Bc	: *Bacillus cereus*
Bs	: *Bacillus subtilis*
Btg	: *Bacillus thuringiensis* subpecies *galleriae*
ICP	: Insecticidal Crystal Protein
kDa	: Kilodalton
mDa	: MegaDalton
DSM	: Difco's Sporulation Medium
SDS-PAGE	: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
U	: Unit
aa(s)	: Amino acid(s)
bp	: Base pairs
kb	: Kilobase
IUPAC	: International Union of Pure and Applied Chemistry
BGSC	: Bacillus Genetic Stock Center
NCBI	: National Center for Biotechnology Information

CHAPTER 1

INTRODUCTION

1.1. *Bacillus thuringiensis*: The Best Known Biological Control Agent

Phytophagous insects give rise to considerable amount of agricultural yield loss which constitutes 20-30% of the total production. The use of synthetic chemical insecticides has played a key role not only in the increase of agricultural productivity and protection of crops and forests, but also in the control of insect vectors of human diseases. Nevertheless, frequent and prolonged use of the chemical insecticides have resulted in contamination of water and food sources, poisoning of non-target fauna and flora, concentration in the food chain and selection of insect pest populations resistant to the chemical insecticides. Therefore, increased public concern of the potential adverse environmental effects about the extensive use of chemical pesticides, together with the rising cost of discovering new molecules, has stimulated interest in the development of environmentally safe and cheap biopesticides.

The method alternative to insect pest control by the use of chemicals is the use of microbial insecticides. Such insecticides consist of microorganisms or their by-products. Microbial insecticides are target-specific, thus, safe for human, non-target plants and animals as well as environmentally friendly for being biodegradable. Microbial insecticides are also known as biological pathogens and biological control agents. The most widely used microbial control agent is the bacterium *Bacillus thuringiensis* (*Bt*). The other entomopathogenic microorganisms used for microbial control include baculoviruses, protozoa, fungi, nematodes and some bacteria such as

Bacillus popilliae, Brevibacillus laterosporus, Clostridium bifermentans, Penibacillus spp. (Barloy *et al*., 1998; Park *et al*., 2000; Charles and Nielsen-LeRoux, 2000; De Maagd, *et al.*, 2001; Lacey *et al.*, 2001).

Bt was started to be used for insect pest control in the late 1920s and in the early 1930s, it was used against *Ostrinia nubulis*, the European corn borer. *Bt* became available as a commercial insecticide in France as spore based formulations called Sporeine to kill flour moths in 1938 (Weiser, 1986) and in the 1950s entered commercial use in the United States (Faust, 1974). Commercial production and worldwide use of *Bt* was well established by the early 1980s. In 1961, *Bt* subsp. *kurstaki* (*Btk*) was used as a biopesticide and *Btk*-based products for the control of susceptible lepidopteran pests in agriculture and forestry became predominant in markets (Lüthy *et al*., 1982). New markets were opened by the 1976 discovery of the *israelensis* subspecies, which is toxic to larval mosquitoes and black flies (Goldberg and Margalit, 1977) and the discovery of *Bt* subsp. *tenebrionis* which is toxic to several beetle species (Krieg *et al*., 1983).

Bt is a gram-positive, rod shaped, spore-forming soil bacterium. The most striking characteristic of *Bt* is the production of large, proteinaceous, crystalline inclusions (crystals) made of δ-endotoxins or crystal (Cry) proteins during sporulation (Tamez-Guerra *et al.*, 2004). The high specificity of δ-endotoxins could limit the widespread utilization of *Bt* bioinsecticides to control large varieties of agricultural pests (Zourai *et al.*, 2002). On the other hand, these 'crystal shaped proteins' can target not only the specific groups of insects (Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phthiraptera or Mallophaga and Acari), but also have a lethal effect on other organisms such as Nemathelminthes, Plathyhelminthes and Sarcomastigophora (Feitelson, 1993).

In addition to sporulation-linked expression of insecticidal toxins, some strains of *Bt* produce insecticidal proteins, unrelated to the Cry proteins, in the vegetative phase of growth which are termed as vegetative insecticidal proteins, VIP (Estruch *et al*., 1996). These VIPs do not form parasporal crystal proteins and are secreted from

the cell into the growth medium (Arora *et al*., 2003). In addition to VIPs, *Bt* also produces α-exotoxins, β-exotoxins, hemolysins, enterotoxins, chitinases and phospholipases (Hansen and Salamitou, 2000). Observations indicate that β-exotoxins interfere with mitotic cells. As they are deactivated only to some extent in the gut, predators feeding on insects containing toxin are adversely affected. There is a strict regulation in many countries to use β-exotoxin in controlling insect pests owing to the risk to non-target organisms and mammals (Glare and O-Callaghan, 2000). However, Tsuchiya *et al.* (2002) proposed to consider the possibility of practical application of β-exotoxin in suppressing difficult-to-control Coleopteran pests, including the cigarette beetle and the Colorado potato beetle, *Leptinotarsa decemlineata*.

Bt seems to be indigenous to many environments**.** *Bt* was first isolated by the Japanese bacteriologist S. Ishiwata from diseased silkworm *Bombyx mori* (L.) larvae in 1901. In 1911, E. Berliner (Germany) isolated a similar organism from diseased larvae of Mediteranean flour moth, *Anagasta küehniella*, and named it as *Bacillus thuringiensis.* In 1916, Aoki and Chigasaki found that its activity was due to a toxin present in sporulated cultures, but not in young cultures of vegetative cells (Beegle and Yamamoto, 1992). Many other strains have been isolated worldwide from different habitats, including different types of soil (Bernhard *et al*., 1997; Hossain *et al*., 1997; Bravo *et al.*, 1998), phylloplane of different plants (Daamgard *et al*., 1998; Hansen *et al*., 1998), insects and their habitats (Chilcot and Wigley, 1993; Apoloyo *et al*., 1995) and stored products (Meadows *et al*., 1992; Kaelin *et al*., 1994).

The characterization of new isolates relies on the basis of crystal serotype (Krywienxczyk, 1977), Southern blot hybridization to test new strains with known genes (Kronstad and Whiteley, 1986) or reactivity with various monoclonal antibodies (Hofte *et al*., 1988). The diversity in flagellar H-antigen agglutination reactions is one indication of the enormous genetic diversity among *Bt* isolates. Besides these methods, the use of PCR is an alternative procedure for rapid determination of the presence and absence of target DNA sequence and advantageous for small amount of DNA requirement for analysis, being highly

sensitive and allowing large numbers of samples to be processed in a relatively short time. The electrophoretic products of PCR are used for prediction of insecticidal activity of known and novel isolates (Carozzi *et al*., 1991; Ben-Dov *et al*., 1997). Several PCR and hybridization-based strategies have been used to screen *cry* genes having minor amino acid substitutions (Porcar and Juara-Perez, 2003).

1.2. Insecticidal Crystal Proteins (ICPs) of *Bt*

As mentioned earlier, the insecticidal activity of *Bt* is attributed to its ability to produce parasporal crystals (δ-endotoxins) which fall into two categories, Cry toxins and cytolysins (Cyt). In 1989, Hofte and Whiteley reviewed systematic nomenclature and classified crystal proteins into five major groups according to their insecticidal and molecular relationships (CryI, CryII, CryIII, CryIV and CryV, Cyt). The discovery of new strains having an activity spectrum that spans two or three insect orders (such as those producing CryIB protein that shows insecticidal activity against both Lepidoptera and Coleoptera) led the scientist to establish a new classification system based on amino acid sequence homology (Schnepf *et al*., 1998). Revised gene names used in this new classification system are listed in Table 1.1. New nomenclature have defined four hierarchical ranks. Romen numerals, which comes after the Cry or Cyt words, have been exchanged for Arabic numerals in the primary rank, it is followed by other three hierarchical rank consisting of numbers, capital letters, lower case letters and numbers (e.g. Cry23Aa1), depending on its place in a phylogenetic tree (Figure 1.1). Proteins with the same primary rank often affect the same order of insect; those with different secondary and tertiary ranks may have altered potency and targeting within an order. The quaternary rank was established to group “alleles” of genes coding for known toxins that differ only slightly, either because of a few mutational changes or an imprecision in sequencing. The proteins less than 45 % sequence identity differ in primary rank (Cry1, Cry2, etc.) and 78 % and 95 % identity constitute the borders for secondary and tertiary rank, respectively (Crickmore *et al*., 1998).

Table 1.1. Known *cry* and *cyt* gene sequences with revised nomenclature assignments (Crickmore *et al*., 1998).

Revised gene name	Original gene/protein name	Revised gene name	Original gene/protein name
cry1Aa1	*cryIA(a)*	*cry1Ca2*	*cryIC*
cry1Aa2	*cryIA(a)*	*cry1Ca3*	*cryIC*
cry1Aa3	*cryIA(a)*	*cry1Ca4*	*cryIC*
cry1Aa4	*cryIA(a)*	*cry1Ca5*	*cryIC*
cry1Aa5	*cryIA(a)*	*cry1Ca6*	*cryIC*
cry1Aa6	*cryIA(a)*	*cry1Ca7*	*cryIC*
cry1Ab1	*cryIA(b)*	*cry1Cb1*	*cryIC(b)*
cry1Ab2	*cryIA(b)*	*cry1Da1*	*cryID*
cry1Ab3	*cryIA(b)*	*cry1Db1*	*prtB*
cry1Ab4	*cryIA(b)*	*cry1Ea1*	*cryIE*
cry1Ab5	*cryIA(b)*	*cry1Ea2*	*cryIE*
cry1Ab6	*cryIA(b)*	*cry1Ea3*	*cryIE*
cry1Ab7	*cryIA(b)*	*cry1Ea4*	
cry1Ab8	*cryIA(b)*	*cry1Eb1*	*cryIE(b)*
cry1Ab9	*cryIA(b)*	*cry1Fa1*	*cryIF*
cry1Ab10	*cryIA(b)*	*cry1Fa2*	*cryIF*
cry1Ac1	*cryIA(c)*	*cry1Fb1*	*prtD*
cry1Ac2	*cryIA(c)*	*cry1Ga1*	*prtA*
cry1Ac3	*cryIA(c)*	*cry1Ga2*	*cryIM*
cry1Ac4	*cryIA(c)*	*cry1Gb1*	*cryH2*
cry1Ac5	*cryIA(c)*	*cry1Ha1*	*prtC*
cry1Ac6	*cryIA(c)*	*cry1Hb1*	
cry1Ac7	*cryIA(c)*	*cry1Ia1*	*cryV*
cry1Ac8	*cryIA(c)*	*cry1Ia2*	*cryV*
cry1Ac9	*cryIA(c)*	*cry1Ia3*	*cryV*
cry1Ac10		*cry1Ia4*	*cryV*
cry1Ad1	*cryIA(c)*	*cry1Ia5*	*cryV159*
cry1Ae1	*cryIA(e)*	*cry1Ib1*	*cryV465*
cry1Af1	*icp*	*cry1Ja1*	ET4
cry1Ba1	*cryIB*	*cry1Jb1*	ET1
cry1Ba2		*cry1Ka1*	
cry1Bb1	ET5	*cry2Aa1*	*cryIIA*
cry1Bc1	*cryIB(c)*	*cry2Aa2*	*cryIIA*
cry1Bd1	*cryE1*	*cry2Aa3*	
cry1Ca1	*cryIC*	*cry2Ab1*	*cryIIB*
cry1Ca2	*cryIC*		

Table 1.1. (continued)

Revised gene name	Original gene/protein name	Revised gene name	Original gene/protein name
cry2Ab2	*cryIIB*		
cry2Ac1	*cryIIC*	*cry9Ba1*	*cryX*
cry3Aa1	*cryIIIA*	*cry9Ca1*	*cryIH*
cry3Aa2	*cryIIIA*	*cry9Da1*	N141
cry3Aa3	*cryIIIA*	*cry9Da2*	
cry3Aa4	*cryIIIA*	*cry10Aa1*	*cryIVC*
cry3Aa5	*cryIIIA*	*cry11Aa1*	*cryIVD*
cry3Aa6	*cryIIIA*	*cry11Aa2*	*cryIVD*
cry3Ba1	*cryIIIB2*	*cry11Ba1*	Jeg80
cry3Ba2	*cryIIIB*	*cry11Bb1*	94 kDa
cry3Bb1	*cryIIIBb*	*cry12Aa1*	*cryVB*
cry3Bb2	*cryIIIC(b)*	*cry13Aa1*	*cryVC*
cry3Ca1	*cryIIID*	*cry14Aa1*	*cryVD*
cry4Aa1	*cryIVA*	*cry15Aa1*	34kDa
cry4Aa2	*cryIVA*	*cry16Aa1*	*cbm71*
cry4Ba1	*cryIVB*	*cry17Aa1*	*cbm72*
cry4Ba2	*cryIVB*	*cry18Aa1*	*cryBP1*
cry4Ba3	*cryIVB*	*cry19Aa1*	Jeg65
cry4Ba4	*cryIVB*	*cry19Ba1*	
cry5Aa1	*cryVA(a)*	*cry20Aa1*	86kDa
cry5Ab1	*cryVA(b)*	*cry21Aa1*	
cry5Ac1		*cry22Aa1*	
cry5Ba1	PS86Q3		
cry6Aa1	*cryVIA*	*cyt1Aa1*	*cytA*
cry6Ba1	*cryVIB*	*cyt1Aa2*	*cytA*
cry7Aa1	*cryIIIC*	*cyt1Aa3*	*cytA*
cry7Ab1	*cryIIIC(b)*	*cyt1Aa4*	*cytA*
cry7Ab2	*cryIIIC(c)*	*cyt1Ab1*	*cytM*
cry8Aa1	*cryIIIE*	*cyt1Ba1*	
cry8Ba1	*cryIIIG*	*cyt2Aa1*	*cytB*
cry8Ca1	*cryIIIF*	*cyt2Ba1*	*"cytB"*
cry9Aa1	*cryIG*	*cyt2Bb1*	
cry9Aa2	*cryIG*		

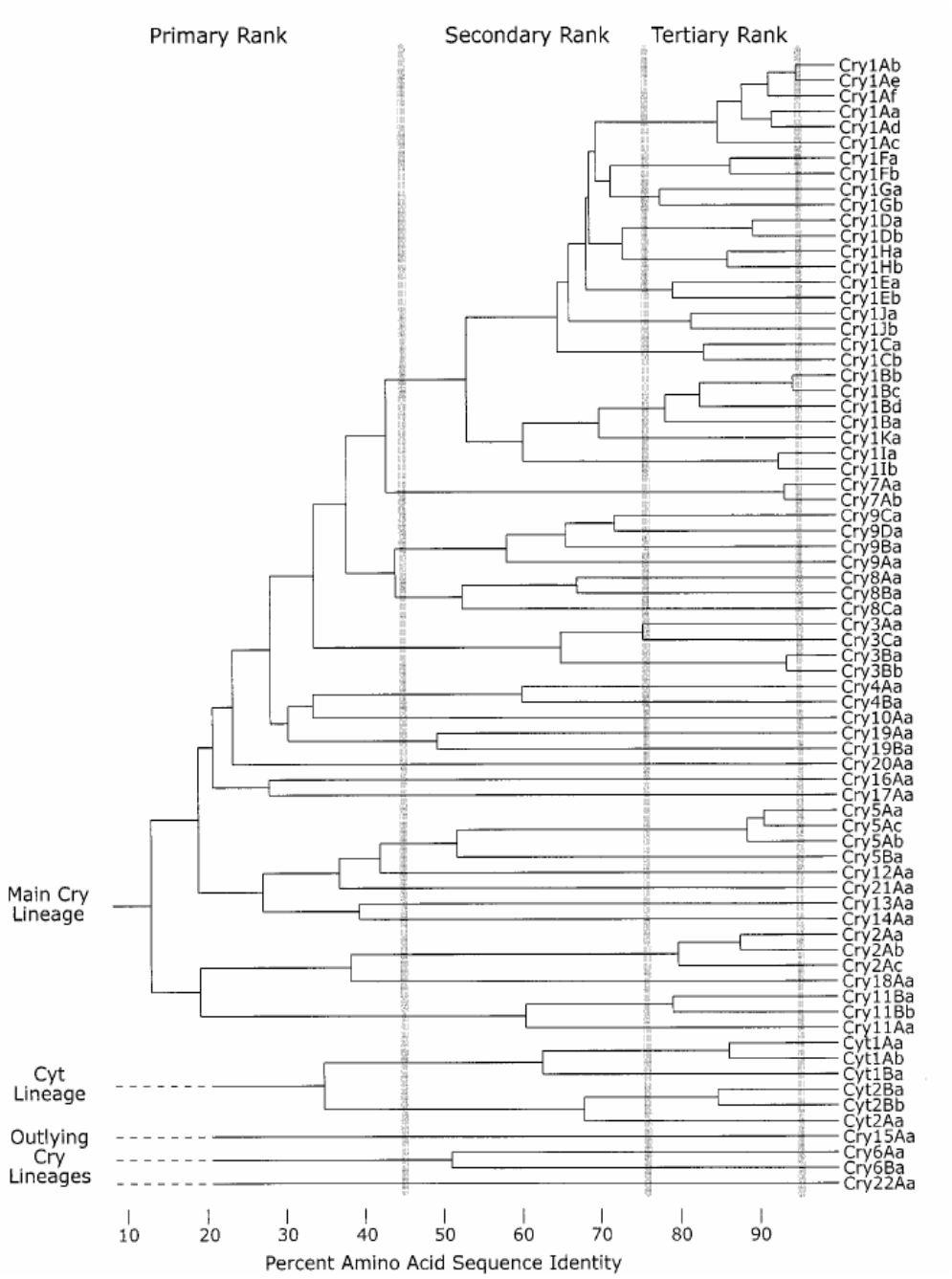


Figure 1.1. Phylogram demonstrating amino acid sequence identity among Cry and Cyt proteins. This phylogenetic tree is modified from a TREEVIEW visualization of NEIGHBOR treatment of a CLUSTAL W multiple alignment and distance matrix of the full-length toxin sequences. The gray vertical bars demarcate the four levels of nomenclature ranks. Based on the low percentage of identical residues and the absence of any conserved sequence blocks inmultiple-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 (Crickmore *et al*., 1998)

More than 3000 insect species within 16 orders were demonstrated to be susceptible to different ICPs. The search for new genes is an on-going effort worldwide. So far, the δ-endotoxins from the members of *Bt* comprise a group of 292 Cry and 22 Cyt proteins and are classified into Cry1 to Cry44 and Cyt1 to Cyt2 according to the degree of amino acid sequence homology (Stobdan *et al*., 2004; Huang *et al*., 2004) (Table 1.2).

Table 1.2. Types of crystal proteins and the insects and their orders to which they are active

Activity spectrum		**Crystal Protein**
Order	**Target Insect Species**	
Coleoptera	*Leptinotarsa decemlineata* *Diabrotica undecimpunctata* *Melolontha melolontha* *Agelastica alni* *Pyrrhalta luteola* *Lasioderma serricorne* *Anomola cuprea*	Cry3, Cry7, Cry8, Cry14, Cry 34, Cry 35, Cry 36, Cry 38
Diptera	*Psychoda alternata* *Dixa* spp. *Tipula paludosa* *Chironomus plumosus*	Cry4, Cry10, Cry 11, Cry 16, Cry 19, Cry 20, Cry 24, Cry 25, Cry 27, Cry 29, Cry 30, Cry 39, Cry 40
Lepidoptera	*Plutella xylostella* *Spodoptera exigua* *Malocosoma disstria* *Bombyx mori*	Cry1, Cry9, Cry15
Lepidoptera and Diptera	*Heliothis virescens* *Trichoplusia ni* *Manduca sexta*	Cry2
Hymenoptera	*Monomorium pharaonis*	Cry22
Nematoda	*Caenorhabtidtis elegans*	Cry5, Cry6, Cry12, Cry13, Cry21

cyt and *cry* genes do not share significant sequence homology; however, both types of toxins seem to work through pore formation that leads to cell lysis and irreversible damage of the insect midgut (Knowles *et al*., 1987; Knowles *et al*., 1989). While Cry toxins act via specific receptor recognition and binding (Gill *et al*., 1992), no specific receptors have been described for Cyt toxins, although they show specificity of action *in vivo* (Koni and Ella, 1994).

1.2.1. Classes of Insecticidal Crystal Proteins

1.2.1.1. Lepidoptera–Specific Cry Proteins

Lepidopteran-specific δ-endotoxins are produced by different *Bt* subspecies such as *Bt* subsp. *kurstaki*, *berliner*, *entomoicius* 6.01, *aizawai* 7.29, *thompsoni*, *sotto*, *fukuokaensis, canadensis*, and *galleriae* strains. The first characterized antilepidopteran δ-endotoxin was Cry1 produced by *Btk* HD1 (Dulmage, 1970). In 2003, Huang and Guan isolated a novel *Bt* strain named as WB9 which is highly toxic to Lepidopteran *Plutella xylostella* and *Spodoptera exigua* species, contains *cry*1A, *cry*1B, *cr*y1Cb, *cry*1Fa, *cry*1Ga genes and a *vip*3A gene. Beside Cry1 type of toxins Cry2, Cry9 and Cry15 families are also toxic to Lepidoptera (Bravo, 1997). For examle, Cry15Aa1 and Cry9Da2 were isolated from *Bt thompsoni* and *Bt japonensis,* respectively (Brown and Whiteley, 1992; Wasano and Ohba, 1998). The encoded protoxins are 130-140 kDa molecular weight and are accumulated in the bipyramidal crystalline inclusions. However, Cry15 is found in crystals with a second apparently unrelated protein having a molecular weight of 40 kDa (De-Maagd *et al.*, 2003).

1.2.1.2. Diptera–Specific Cry Proteins

Antidipteran *Bt* strains commonly feature the presence of Cyt proteins with cytolytic and hemolytic activities (Guerchicoff *et al.,* 2001). The discovery of antidipteran *Bt israelensis* (*Bti*) in 1975 inagurated a new chapter in vector control (Goldberg and Margalit, 1977). The search for native strains with activity against dipteran

species could have an impact on the control of mosquitoes worldwide. Spherical shaped parasporal body of *Bti* comprises three types of Cry toxins; Cry4A (125 kDa), Cry4B (134 kDa) and Cry11A (67 kDa) and two types of Cyt toxins; Cyt1Aa (27 kDa) and Cyt2Ba (27-28 kDa) (Delecluse *et al*., 2000). Tests on single, solubilized and purified proteins showed that each of the crystal proteins are toxic to mosquitocidal larvae, but each one was not as toxic as the intact form alone (Becker and Margalit, 1993). Ibarra *et al*. (2003) characterized certain *Bt* isolates LBIT315, LBIT320, LBIT348, IB604 and 147-8906 from Latin America based on their insecticidal activities, scanning electron microscopy (SEM), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and plasmid profiles as well as PCR analysis using novel general and specific primers for *cry* and *cyt* genes and they observed higher mosquitocidal activity than that of *Bti*. Cry11Ba1, Cry10Aa2, Cry20Aa1 ,Cry19Ba1, Cry25Aa1, Cry27Aa1, Cry29Aa1, Cry39Aa1 and Cry40Aa1, Cry30Ba1 and Cry24Ba1 were the proteins found from *Btk* HD1, *Bt jegathesan* 367, *Bti* ONR-60A, *Bt fukuokaensis, Bt higo, Bt jegathesan, Bt higo, Bt medellin, Bt aizawai, Bt sotto, Bt entomocidus,* respectively (GenBank Accession numbers: X86902, E00614, U82518, D88381, U88189, AB023293, AJ251977, BAB722016, BAD00052 and BAD32657)

1.2.1.3. Coleoptera–Specific Cry Proteins

Bt BI256-82, the first identified strain as having activity against the larvae of Coleoptera order, was isolated from a dead pupa larvae of the yellow meal worm *Tenebrio molitor* by Huger in 1982 and the organism was described as *Bt* subsp. *tenebrionis* (*Btt*) by Krieg in 1983. The Coleopteran activity of *Btt* is related with the production of a flat, wafer shaped-rhomboidal crystal. To date, several other anti-Coleopteran *Bt* strains have been isolated and their toxicities have been evaluated. Hernstadt *et al* (1986) identified *Bt san diego* which was later found to be identical to *Btt*. EG2158 was isolated and characterized by Donovan *et al* (1988) to produce 73 kDa crystal protein toxic to Coleoptera larvae. EG2838 (*Bt* subsp. *tolworthi*) and EG4961 (*Bt* subsp. *kumamotoensis*) were isolated and characterized by Rupar *et al*. (1991). They produce a 74 kDa polypeptide of CryIIIB (Cry3B) and

Cry IIIB2 (Cry3Ba1), having a 94% sequence identity to each other (Donovan *et al*., 1992). 73 kDa protein of CryIIID (Cry7Aa) is produced by *Btk* strain BT109P and active against larvae of Coleoptera (Lambert *et al*., 1992). Cry7Aa has an amino acid identity of 74 %, 61 % and 33 % with Cry3A, Cry3B and Cry3C, respectively. *Bt* LM63 and LM79 strains were isolated from soil samples by Chaufaux *et al* (unpublished data) and identified as Coleoptera-active strains (Lecadet *et al*., 1992). Kaelin *et al.* (1994), analysed 88 samples which were mostly consisting of dried leaf *Lasioderma serricorne* (F.) from different countries. 59% of the isolates produced rhomboidal crystals and gave 65 kDa protein bands which were similar to those residues, processed tobacco or finished product and dead tobacco beetles produced by Coleoptera-specific *Btt*. Cry3A is highly toxic to *Leptinotarsa decemlineata*, one of the most important pest in the Coleoptera. This insect causes extensive damages on potato, tomatoes and eggplants. Other insect species belonging to Coleoptera order such as *Melolontha melolontha, Agelastica alni, Pyrrhalta luteola, Lasioderma serricorne* are also susceptible to anti-Coleopteran *Bt* species.

Ellis *et al.* (2002) found new families of insecticidal crystal proteins with molecular masses of ca. 14 and 44 kDa. produced by *Bt* isolates PS80JJ1, PS149B1, and PS167H2 that have cotton rootworm (genus *Diabrotica*) insecticidal activity. These binary Cry proteins designated as Cry34Aa1, Cry34Ab1 and Cry34Ac1 (the 14-kDa polypeptide components) and Cry35Aa1, Cry35Ab1 and Cry35Ac1 (the 44- kDa polypeptide components) were required for insecticidal activity.

A new *Bt galleriae* strain containing novel *cry*8 gene was discovered and shown to be highly toxic against scarab beetles such as *Anomola cuprea* (Asano *et al.,* 2003). Anti-Coleopteran Cry7Ab2 and Cry14Aa1 proteins were also isolated from *Bt kumamotoensis* 867 and *Bt sotto* PS80JJ1, respectively (GenBank Accession numbers: U04368 and U13955).

The *cry*3A gene specifies the synthesis of the 73 kDa protoxin in *Btt* (Hernstadt *et al.,* 1986; Hofte *et al*., 1987; Sekar *et al*., 1987). During sporulation, the 73 kDa protoxin of *Btt* is processed to 64-68 kDa polypeptide which predominate in the

released crystal protein. This process is performed by the removal of 55-57 amino acids from N-terminus by endogenous proteases (McPherson *et al*., 1988; Carroll *et al*., 1989). Cry3 proteins lack the large nontoxic C-terminus of Cry1 toxins. Thus, 64-68 kDa form is the stable form of Cry3A, lacks S-S bonds and obviates the requirement for reducing agents in solubilization (Slaney *et al.*, 1992; Koller *et al.*, 1992). Trypsin and some insect midgut extracts have also been shown to proteolyze soluble Cry3A producing a stable 55 kDa polypeptide.(Carroll *et al*., 1989). While the pH of larval midgut is alkaline in both Lepidoptera and Diptera, the larvae of beetle family Crysomelidae have a midgut pH range of 4.5-6.6 (Koller *et al*., 1992).

1.2.1.4. Nematoda–Specific Cry Proteins

The phylum Nematoda contains free-living species and parasitic species in addition to beneficial ones. Certain *Bt* crystal proteins have potential in controlling nematode pests that infect animals and plants (Wei *et al*., 2003; Huffman *et al*., 2004). Using *Bt* lysates, Cry5B, Cry6A, Cry14A and Cry21A were shown to be nematicidal towards *Caenorhabtidtis elegans* which is a model system to study host-pathogen interactions (Marroquin *et al*., 2000). Cry12Aa1, Cry13Aa1 and Cry5Aa1 were isolated from *Bt* PS33F2, *Bt* PS63B and *Bt darmstadiensis* PS17, respectively (GenBank Accession numbers: L07027, L07023 and L07025).

1.2.1.5. Hymenoptera–Specific Cry Proteins

Cry22 proteins were initially identified in *Bt* strains as active on ants (Order: Hymenoptera). The proteins have molecular weights of 75 to 86 kDa and Cry5 toxins were also reported to be active against the ant *Monomorium pharaonis* that belongs to the Hymenoptera (Payne *et al*., 1997). Cry5A toxin from *Bt* strain PS86Q3 was active against at least one hymenopteran species, *Diprion pini.* Strain PS86Q3 contains a long bipyramidal crystal composed of five proteins of 155, 135, 116, 97 and 58 kDa., respectively. The 155 kDa protein was proven to be Cry5B toxin by N-terminal sequencing, whereas the other proteins were Cry5A toxins. It is

possible that the 135, 116, 97 and 58 kDa proteins represent proteolytic fragments of the same protein, since PCR analysis demonstrated that this strain harbors only *cry*5Ac and *cry5*Ba genes. The treatment with proteases present in *D. pini* or *Cephalcia abietis* midgut content produced a single 75 kDa protein (Garcia-Robles *et al*., 2001).

1.2.2. Structural and Sequential Similarities Among Cry Toxins

When the structural and sequential similarities are considered, conserved amino acid sequences drew attention among most Cry toxins (Hofte and Whiteley, 1989). Alignment of the Cry toxins reveals the presence of five conserved sequence blocks common to a large majority of proteins as shown in Figure 1.2. The amino acid sequence which starts by the end of the conserved block 5 and continues down to C-terminal region is the cleavage part by the proteases during crystal protein activation. The function of block 5 is largely structural, rather than related to activity. Blocks 1-4 are involved in ion channel function or toxin stability.

Within different classes of toxins the number of common blocks can also differ. The 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 blocks 1, 2, 4, and 5. Hovewer, Cyt1, Cyt2, Cry6, Cry15, and Cry22 have no recognizable homologs to the conserved blocks (Schnepf, 1995; Schnepf *et al.*,1998; De-Maagd *et al.*, 2001).

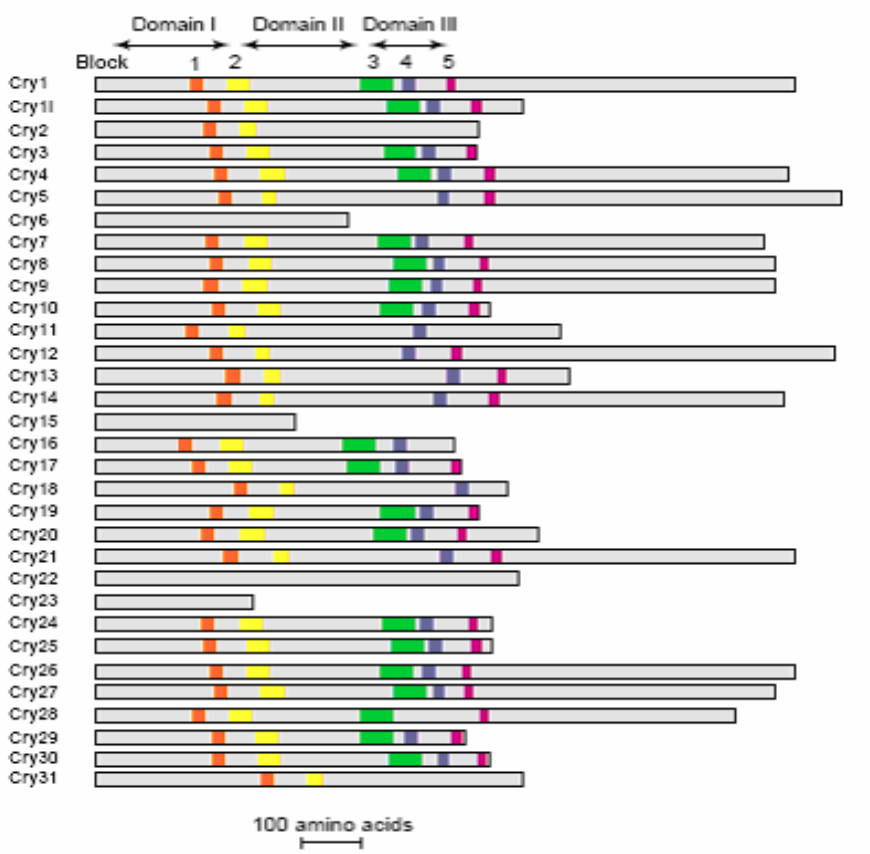


Figure 1.2. Five conserved sequences of insecticidal crystal proteins.

Cry3A and Cry1Aa, in contrast to Cyt2A, both possess three domains, as shown in Figure 1.2. Domain I consists of a cluster of seven antiparallel α-helices in which helix 5 is encircled by the remaining ones (Schnepf *et al.,* 1998). Domain II consists of three antiparallel β-sheets joined in a typical "Greek key" topology, arranged in a so-called β-prism fold (Sankaranarayanan *et al*., 1996; Shimizu and Morikawa, 1996). Domain III consists of two twisted, antiparallel β-sheets forming a β-sandwich with a "jelly roll" topology (Li *et al*., 1991).

Domain I starts from the N-terminal region and has a crucial role in membrane insertion and pore formation. Domain I has long, hydrophobic and amphipathic seven α helices and this structure bears many striking similarities to the pore-

forming or membrane-translocating domains of several other bacterial protein toxins, such as colicin A and diphtheria toxin (Parker and Pattus, 1993).

The surface-exposed loops at the apices of the three β-sheets of domain II show similarities to immunoglobin antigen-binding sites and are involved in receptor binding and specificity determination. This is further supported by the reports that domain II shared the same structural fold with three carbohydrate binding proteins: the vitelline membrane outer layer protein I from hen's eggs, the plant lectin jacalin and the *Maclura pomifera* agglutinin. (Knowles *et al.,* 1984; Schnepf *et al.,* 1998; Jenkins and Dean, 2000; De-Maagd *et al.,* 2003).

The β-sandwich structure of domain III could play a number of key roles in the biochemistry of the toxin molecule. Li *et al.* (1996) suggested that domain III functions in maintaining the structural integrity of the toxin molecule, by protecting it from proteolysis within the gut of the target organism, and also in receptor binding and in ion channel activity.

1.2.3. Mechanism of Action

The toxicity as well as specificity of *Bt* Cry toxins correlates directly with the binding of toxins to high-affinity receptors on the epithelial cells that line the midgut of the susceptible insects (Loseva *et al*., 2002). The mechanism of action of the *Bt* Cry proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry toxin to midgut receptors and insertion of the toxin into the apical membrane to create ion channels or pores. The specific pH and proteases in the insect midgut favour solubilization and proteolytic cleavage of the inclusions (Rausell *et al.*, 2004).

As mentioned earlier, crystals are comprised of protoxins. For the protoxins to become active, a susceptible insect must eat them (Schnepf *et al*., 1998). Solubilization of protoxins with an extended C-terminus depends on the highly alkaline gut pH of Lepidoptera and Diptera. Some of the toxins with potential

activity against Coleoptera are only toxic after *in vitro* solubilization which may be due to the property of the protoxins to be insoluble at the neutral to weakly acidic gut pH of Coleoptera; for example, Cry1Ba for some species (Bradely *et al*., 1995) and Cry7Aa (which additionally also requires *in vitro* activation) (Lambert *et al.,* 1992). Differences in the extent of solubilization sometimes explain differences in the degree of toxicity among Cry proteins (Aronson *et al*., 1991; Du *et al*., 1994). The main digestive proteases of Lepidoptera and Diptera are serine proteases, whereas those of Coleoptera are mainly cysteine and aspartic proteases (Lambert *et al.,* 1992; Terra and Ferreira, 1994).

The activated toxin binds to receptor in the midgut. In the midgut epithelium of certain insects, such as *Manduca sexta* and *Bombyx mori*, the Cry receptors are identified as specific cell adhesion molecules, cadherins. Cadherins represent a large familiy of calcium-dependent, transmembrane glycoproteins and are responsible for maintaining the integrity of cell to cell contacts in multicellular organisms (Nollet *et al*., 2000; Angst *et al*., 2001). In addition to cadherin receptors for Cry toxins, several 120-170 kDa Cry toxin-binding proteins, aminopeptidase N (APN), have also been identified in some lepidopteran insects. (Knight *et al.*, 1994; Sangadala *et al*., 1994; Valaitis *et al*., 1995; Gill *et al*., 1992; Oltean *et al*., 1999). Binding of crystal toxin to the brush border membrane vesicules (BBMV) of insect larvae occurs in two stages

involving reversible binding and irreversible binding (Baum and Malvar, 1995). In reversible binding, domain II binds tightly to the receptor and is necessary for further activity of toxin. Irreversible binding is exclusively associated with membrane insertion and occurs via the insertion of domain I. It was suggested that toxicity correlates with irreversible binding (Liang *et al*., 1995). Binding of δ-endotoxin to cadherine-like receptors in the midgut causes stress, consequently, disrupts the apical brush border membrane microvillae of midgut epithelium and destroys the entire midgut tissue. Nonspecific ion channels form, causing midgut cells to lose their membrane potential. If the rate of damage to the midgut exceeds the rate of repair, lesions form, bacteria invade the hemocoele and death results from septicemia (Wu *et al.*, 2000).

1.3. The *Bt* Genome and Plasmids

Bt, B. cereus (*Bc*) and *B. antrachis* (*Ba*) are genetically very closely related members of the genus Bacillus. *Ba* and *Bt* are readily distinguished from *Bc* by the presence of plasmid-borne specific toxins (*Ba* and *Bt*) and capsule (*Ba*) (Helgason *et al*., 2000; Ivanova *et al*., 2003). However, the transfer of ICP encoding plasmids from *Bt* to *Bc* makes *Bc* indistinguishable from *Bt* and vice versa (González *et al*., 1981; González *et al*., 1982). *Bt* strains have a genome size of 2.4 to 5.7 million bp and also physical maps have been constructed for two *Bt* strains (Carlson and Kolsto, 1993; Carlson *et al*., 1994; Carlson *et al*., 1996). *Bt konkukian* (*Bt* 97-27) is a human pathogen, therefore, pathogenic property is unlike most *Bt* isolates. It was shown by amplified fragment length polymorphism (AFLP) analysis that *Bt konkukian* was to be very closely related to *Ba* (Radnedge *et al*., 2003). The complete genome sequence of *Bt* 97-27 was submitted to NCBI and EBI-Proteome database; Data-search: NC005957, AE017355. *Bt* 97-27 has a genome size of 5.237 Mb with a 64.6 % A+T rich sequence and comprises 5.083 genes.ongoing *Bt* genome projects, on the other hand, include those of *Bt*, *Bt* NBT97 and *Bti* ATCC 35646 (http://www.genomesonline.org).

Most *Bt* isolates have several extrachromosomal elements, some of them are circular and others linear (Carlson *et al*., 1994). Most *cry* genes are found on large plasmids with low copy number, ranging form 35 to 200 mDa and most probably the genes encoding the crystal proteins are often clustered on transmissible plasmids with flanking transposable elements. Regarding the role of the transposable elements in *Bt*, it is postulated that they are involved in the amplification of *cry* genes in the bacterial cell (Schnepf *et al*., 1998; Kaur, 2000).

The genes for the four dipteran-active protoxins and a cytolytic factor in *Bti* are all on a 72 mDa plasmid (Aronson, 1993). The *cry*4A gene of the *israelensis* subspecies is flanked by two repeated sequences in opposite orientations designated IS240 (Bourgouin *et al*., 1988). Insertion sequences have been found upstream of the *cry*1Ca gene (Smith *et al.,* 1994) and downstream of a cryptic *cry*2Ab gene

(Hodgman *et al*., 1993). *Btk* HD1 contains 12 size classes of 'non-nuclear' DNA (Carlton and Gonzales, 1985) including a linear element and a transducing phage (Walter and Aronson, 1991). Unlike the *kurstaki* HD1 strain, which contains three related but significantly different crystal protein genes (Kronstad *et al*., 1983), subsp. *tenebrionis* contains only a single crystal protein gene. *Btt* was found to have at least 5 different plasmids with sizes of 105, 90, 58, 56, and 9.5 mDa (Sekar *et al*., 1987). The crystal protein gene of this organism was located on the 90 mDa one (Aronson *et al*., 1995).

1.4. *cry* Gene Expression

A common characteristic of the *cry* genes is their expression during the stationary phase. Their products generally accumulate in the mother cell compartment to form a crystal inclusion that can account for 20 to 30% of the dry weight of the sporulated cells (Sanchis *et al*., 1996). The very high level of crystal protein synthesis in *Bt* and its coordination with the stationary phase are controlled by a variety of mechanisms occurring at the transcriptional, posttranscriptional and posttranslational levels. The accumulation of protoxins begins at about Stage III of sporulation and continues through Stage V (Aronson, 1993). *cry*1Aa, the first cloned and sequenced *cry* gene, is expressed during sporulation of the producer *Bt* strain from two overlapping promoters known as BtI and BtII (Wong *et al*., 1983). The transcription from BtI is initiated by a form of RNA polymerase containing σ^{35} that shows 88% identitiy with a sporulation-specific σ^{E} of *Bacillus subtilis*. Later transcription is from an upstream promoter, BtII, which is dependent upon an RNA polymerase containing a sigma subunit, σ^{28} with 85% identity to other sporulation-specific σ^{K} of *B. subtilis* (Adams *et al*., 1991). BtI is active between about T_2 and T_6 of sporulation and BtII is active from about T_5 onwards (where Tn is n hours after the end of the exponential phase). In addition to *cry*1Aa, both the *cry*1Ba and *cry*2Aa genes contain either BtI alone or BtI with BtII (Brown and Whiteley, 1988). Beside this, the transcription of many other *cry* genes (e.g., *cry*4Aa, *cry*4Ba, *cry*11Aa, *cry*15Aa, etc.) is likely to be σ^{E}- or σ^{K} -dependent (Schnepf *et al*., 1998).

The *cry*3Aa gene, isolated from the Coleopteran-active *Btt*, was found to be expressed during vegetative growth at a lesser extent than during the stationary phase (Sekar, 1988; De Souza *et al*., 1993; Malvar *et al*., 1994). Activation of *cry*3A expression was independent of the key genes involved in the initiation of sporulation (key regulators or sigma factors). It differs in this respect from other *cry* genes The promoter that directs the transcription of anti-Coleopteran *cry*3A gene resembles σ^A vegetative stage promoters (Agaisse and Lereclus, 1994 a,b).

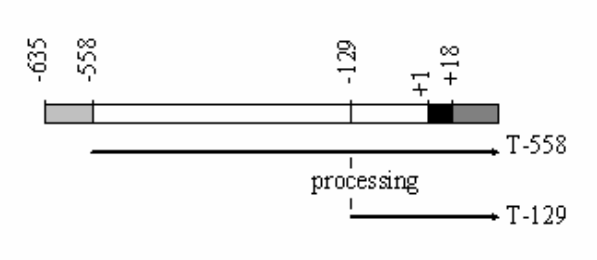


Figure 1.3. Model for the *cry*3A gene expression (Agaisse and Lereclus, 1994 a,b).

DNA sequences required for the full expression of *cry*3A gene are at the upstream region extending from nucleotides -635 to -553 involved in a transcriptional event, and the downstream region extending from nucleotides -367 to +18 involved in a post-transcriptional event. Transcription of *cry*3A is initiated at nucleotide position -558, giving the transcript T-558. This transcript is thought to be subsequently processed a stable form (T-129), which accumulates in the cells appearing as the major *cry*3A mRNA (Figure 1.3). The internal region between nucleotide position -558 and -129 harbours no potential protein-coding sequence and therefore not coding for a gene product involved in *cry*3A regulation. This indicates that this region may involve a specific sequence that have a role in negative regulation of *cry*3A expression, by acting as a binding site for a repressor at transcriptional level or as a secondary structure determinant at post-transcriptional level (Agaisse and Lereclus,

1994 b). The high level of crystal protein production may result partly from the stability of the corresponding RNA. Glatron and Rapoport (1972) showed that the crystal protein mRNAs have an average half-time of 10 min in *Bt* during sporulation versus 2-6 min for many spore-specific mRNAs. The 3′ terminal fragment of the *cry*1Aa gene from *Btk* strain HD1 is a positive regulator (Wong and Chang, 1986). The stability of the mRNA is enhanced by the formation of a stemloop structure during termination of transcription. This protects the mRNA from the activity of exonucleases present in the cell from degradation. 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 threefold, by increasing the expression level as well as maintaining the mRNA stability (Agaisse and Lereclus, 1996).

mRNA of *Bt cry*3Aa gene have a 5′ stabilizer that is not involved in the translation initiation. The stability is due to the presence of a consensus Shine-Dalgarno sequence (GAAAGGAGG) mapping at a position between -125 and -117 at 5′ UTR of the gene, which is designated as STAB-SD. The interaction with the 16S rRNA of the 30S subunit of the ribosome confers stability to the mRNA. The upstream of the *cry*3Ba, *cry*3Bb, and *cry*3Ca genes also have this sequence in similar positions (Donovan *et al*., 1992; Lambert *et al*., 1992).

At the posttranslational level, larvacidal proteins form crystalline inclusions in the mother cell compartment. Depending on the protoxin composition, the crystals have various forms. The ability to crystallize helps in protecting the protein itself from premature proteolytic degradation (Schnepf *et al*., 1998).

1.5. Strategies to Improve *Bt* and Toxin Yields

1.5.1. Optimization of Nutritional and Cultural Conditions

Bt is the most inexpensive of all microbial insecticides in terms of production costs. Still, high protein yields for optimization of *Bt* fermentations are required to make its production cost-competitive with synthetic insecticides. Systematic studies on the physiological and nutritional requirements of *Bt* especially those required for maximum yield of ICPs are still limited (Sachidanandham *et al*., 1997). Fermentation and media optimization for each strain put into commercial production is needed as different strains may have different responses to nutritional and cultural conditions (Kaur, 2000). The culture conditions of *Bt* are optimized to achieve not only high cell densities, but also high crystal protein concentration and high toxicity (Dulmage *et al*., 1990). Previously, it was believed to be important to end up with high spore counts however, Rossa and Mignone (1993) reported that a good spore count did not lead to high larvacidal potency in *Bti.* It was later shown that bacterial spore counts do not necessarily reflect the insecticidal activity of a *Bt* strain or *Bt* product because the number and amount of crystal protein per bacterial cell can vary (Enviromental Health Criteria, 1999; Paramatha, 2000). In some countries like Japan, products of *Bt* biopesticides with viable *Bt* spores are not authorized. Dissemination and persistence of *Bt* spores in locations where sericulture is an economic activity is a matter of concern due to toxicity to economically important insect, such as the toxicity of

*cry*1Aa gene carrying spores to silk worms. Thus, from a commercial perspective, *Bt* strains which produce crystals but not spores and thereby are nonpersisting in nature are desirable (Kaur, 2000).

For the fermentation of *Bt,* carbon source is very important. The presence of a carbon source in the growth medium can repress expression of certain genes and operons indirectly related with toxin formation (Brückner and Titgemeyer, 2002). The excessive glucose concentration may prolong the vegetative growth and also minimize the toxin yield. For example, Bhatnagar (1998) showed that Cry4A toxin of *Bti* was not synthesized when the medium contained >0.4 % glucose. In

the study of İçgen *et al.* (2002 b), it was found that the carbon sources such as sucrose, lactose and inulin supported the production of Lepidoptera-specific Cry1 and Cry2 toxins by *Bt* 81. Especially, the replacement of glucose with sucrose greatly increased the yields of Cry4Ba and Cry11A toxins (Özkan *et al*., 2003).

It was suggested that the depletion of nitrogen sources triggers sporulation and toxin expression. Inorganic nitrogen compounds, such as ammonium sulphate are not usually sufficient to support the growth of *Bt* and organic nitrogen sources such as meat peptone, fish meal and soybean flour are required for rapid growth (Yang and Wang, 2000). Among different organic and inorganic nitrogen compounds tested, peptone was found to be best source supporting the optimum Cry1 and Cry2 δ-endotoxin formation (Içgen *et al*., 2002 b).

Another important component for the production of crystal protein is potassium. Wakisaka *et al* (1982) detected an increased crystal protein production in the presence of potassium salts. However, when potassium salts were replaced with the same salts of sodium, the same effect was not seen. Besides this, Bhatnagar (1999) reported a corresponding increase in the Cry4A specific mRNA in the presence of inorganic phosphate which stimulated 135 kDa protoxin synthesis by *Bti* cells. Phosphate is considered to be essential for not only protein expression, but is also critical for cell growth and its concentration varies during the toxin-formation phase (Yang and Wang, 2000). In the studies of İçgen *et al*. (2002 b) and Özkan *et al*. (2003), high yields of antilepidopteran and antidipteran δ-endotoxins were obtained with 50-100 mM inorganic phosphate among the concentrations tested.

Bacterial toxins possess many attributes of secondary metabolites and their yield is known to be greatly affected by trace metals (Rose, 1979). Mn is the most important key metal co-regulating secondary metabolism and differentiation in bacilli (Weinberg, 1977). Sikdar *et al.* (1991) reported on the mineral requirements of *Bti* for production of the mosquidocidal δ-endotoxin and found that Fe, Mn and Cu were required for the production of crystal protein while Mo had an inhibitory effect. They also obtained no direct relationship between cell growth and toxin production by comparing optimum levels of metals for both. In the study of İçgen *et al.*

(2002 a), the toxin yield was almost zero when Mg was omitted from the medium indicating that Mg was the most critical trace element for antilepidopteran Cry protein synthesis by *Bt* 81. The stimulatory effect of Cu was seen between the range of 10^{-6} to 10^{-7} M. Ca and Zn had no effect on toxin production. However, Ca favored and Zn, Fe and Cu negatively influenced the synthesis of antidipteran toxin production by *Bti* HD500 (Özkan *et al*., 2003).

Sachidanandham *et al.* (1997) identified the amino acid requirements for a stable and enhanced production of crystal proteins by *Bt* subsp. *galleriae*. In the study, several amino acids resulted in a better carbon utilization as well as improving the stability and volumetric productivity of biomass for *Btg* biomass.

Several studies were conducted to decrease the cost of fermentation by employing low-cost media components. For example, Vora and Snetha (1999) reported enhanced growth, sporulation and toxin yields by *Btk* in a medium containing defatted soybean and groundnut seed meal extracts with the supplementation of cysteine. Gruel and fish media were investigated for the growth of both Diptera- and their use to cultivate antidipteran species led to decreased toxin biosynthesis (Zourai *et al.,* 2002). In other studies, such as those of Lachhab *et al.* (2001) and Vidyarthi *et al.* (2002), wastewater sludge as a raw material for *Bt* fermentation was evaluated.

1.5.2. Molecular Approaches

Several techniques, such as genetic recombination and construction of engineered *Bt* strains have been used to improve the insecticidal properties of *Bt* strains (Sanchis *et al.*, 1996; Kaur, 2000).

Expression of *cry* genes can be enhanced by altering the elements regulating transcription and translation. Cry3Aa protein production was enhanced 2.5 fold in an asporogenous *Bt* strain suggesting sporulation-independent transcription of *cry*3Aa (Malvar *et al.*, 1994). Cry3A production is not only sporulation-independent but it can also be elevated in *spo*$^-$ backgrounds. The expression of cry3Aa was increased in sporulation-negative *spo*A *B. subtilis* mutants (Agaisse and Lereclus, 1994 a). Sanchis *et al.* (1996) reported the accumulation of Cry1C toxin in

large amounts by a *spo*0A mutant expressing the *cry*1C coding sequence from the sporulation-independent *cry*3A promoter.

20 kDa helper proteins encoded by the *cry*2A and *cry*11A operons enhanced synthesis and stability of *cry*2A by acting as molecular chaperons (Ge *et al*., 1998). Transcipt stability is another important factor for Cry synthesis. As substantial increase in 65 kDa Cry1 was obtained using the STAB-SD sequence of the *cry*3A gene (Agassie and Lereclus, 1996). The use of dual *cyt*1Aa promoter along with STAB-SD sequence resulted in several-fold increase in the expression of *cry*3Aa gene. Besides, it was shown that *cyt*1 promoters in combination with the *cry*3A STAB-SD enhanced Cry2A and Cry11A yields almost 5 and 1.3 folds, respectively (Park *et al*., 1998).

An increase in irreversible binding to receptors was correlated with increase in toxicity as well. Amino acid residues involved in receptor recognition, membrane insertion and toxicity can be substituted through in vitro mutagenesis (Smedley and Ellar, 1996). A combination of mutations in the a α8 loop and loop 2 in domain II of Cry1Ab resulted in a 32-fold increase in toxicity to gypsy moth (*Lymantaria dispar*) over the wild type protein (Rajamohan *et al*., 1996). Wu *et al.* (2000) used site-directed mutagenesis to modify *Bt cry*3A gene in amino acid residues 350-354 and showed a significantly improved toxicity against *Tenebrio molitor.*

Insecticidal activity of *Bt* strains can be further improved by elimination of activities that reduce Cry production. During sporulation, *Bt* synthesizes proteolytic enzymes for processing of non-toxic protoxins to smaller insect-active toxins. However, they may also process and degrade crystal protein resulting in yield reduction (Kaur, 2000; Tan and Donovan, 2000). Donovan *et al.* (1997) by using a gene deletion strategy, showed that neutral protease A, a sporulation-specific protease, contributed to degradation of crystal proteins.

1.6. Transgenic Organisms, Insect Resistance to *Bt* Toxins and Resistance Management, *Bt* Formulations and Field Applications

Insect-resistant transgenic plants are constructed by introducing toxin genes. The genetic code of plants differs slightly from that of bacteria. The relatively AT-rich *Bacillus* DNA contains a number of sequences that could provide signals deleterious to gene expression in plants, such as splice sites, poly(A) addition sites, ATTTA sequences, mRNA degradation signals, and transcription termination sites besides a codon usage different from that of plants. Thus, it is necessary to use synthetic genes whose nucleotide sequence is altered in such a way that it still encodes the desired bacterial amino acid sequence. Truncation of the unmodified genes to synthesize only the toxic portion of the protein typically results in much improved, but still comparatively low expression (Sanchis and Ellar, 1993; Tappeser *et al*., 1998). However, several *cry* genes have been introduced into plants, starting with tobacco (Barton *et al*., 1987; Vaeck *et al*., 1987) and now including many major crop species such as rice, potato, soybean, *Brassica* spp, tomato, chickpea, peanut, conifer, legume, corn, maize, cotton. Alfalfa was protected from the Coleopteran pest, clover root cuculio (*Sitona hispidulus*) and pea leaf weevil (*Sitona lineatus*) upon transfer of the *cry*3Aa gene from *Btt* to *Rhizobium melloti* and *R. leguminosarum* (Bezdicek *et al*., 1994). Giddings *et al.* (1997) reported a better nodule occupency by *R. leguminosarum* carrying the *cry*3Aa gene. In 1996, varieties of potato, cotton, corn and other plants containing modified *cry* genes were sold to growers and provided higher yields (Schnepf *et al*., 1998; Groot and Dicke, 2002). For example, the higher yields of rice have been obtained in Asia by the use of transgenic rice (Toenniessen *et al*., 2003).

Insecticide resistance develops due to genetic variation in large insect populations. When the insecticide is applied, unaffected individuals survive and pass their genes onto following generations. Over time, a large number of resistant insect populations become dominant in environment. Prolonged use of insecticides also increases the risk of resistance. Several insects gained resistance to certain *Bt* toxins in the laboratory and field. The first case of field-selected resistance to *Bt* was the populations of diamondback moth (*Plutella xylostella*) which showed

different levels of susceptibility to a formulated *Bt* product (Dipel) (Schnepf *et al*., 1998). On the other hand, a statistically significant resistance to *Bti* has been reported in mosquitoes *Culex quinquefaciatus* and *Aedes aegypti* (Georghiou *et al*., 1983; Goldman *et al.,* 1986). At least one strain of Colorado potato beetle *Leptinotarsa decemlineata* has been selected for resistance to a Coleoptera-active strain of *Btt* (Miller *et al*., 1990). The species including *Plodia interpunctella, Cadra cautella, Leptinotarsa decemlineata, Chrysomela scripta, Tricholplusia ni, Spodoptera littoralis, Spodoptera exigua, Heliothis virescens* and *Ostrinia nubilalis* (Schnepf *et al*., 1998) gained resistance one of either *Btk*, *Bti*, *Btt* or other *Bt* subsp.

Although little is known about the mechanism(s) of resistance to Cry proteins, the possible mechanisms can be categorized as a decrease in toxin binding to insect midgut (Van Rie *et al*., 1990; Fere *et al*., 1995; Sayyed *et al*., 2000) and proteases that interact with toxin in insect midgut (Keller *et al*., 1996; Oppert *et al*., 1997; Loseva *et al*., 2002). *Plodia interpunctella* appears to have a 50-fold decrease in Cry1A binding associated with a 100-fold decrease in toxicity (Van Rie *et al*., 1990). There did not seem to be fewer binding sites developing in *P. interpunctella*, but simply less binding affinity (Van Rie *et al.,* 1992). In addition to decreased affinity, resistance in *P. interpunctella* was linked with the absence of a major gut proteinase. Presumably this proteinase is associated with the proteolytic cleavage and activation of *Bt* protoxins (Oppert *et al*., 1997). Forcada *et al.* (1996) reported that proteases from a strain of tobacco mudworm *Heliothis virescens* resistant to *Bt kurstaki* HD-73 degraded the toxin faster than proteases from the susceptible strain. Resistance to the Cry1Ac toxin by *H. virescens* was linked to retrotransposon-mediated disruption of a specific cadherin gene (Gahan *et al.*, 2001). On the other hand, resistance of Colorado potato beetlte (CPB) to Cry3A toxin correlates with the presence of different forms of digestive ptoteases in the resistant strain as well as with a decreased binding affinity. Thus, changes in the activity or composition of gut proteases are involved in altering the susceptibility by insects to crystal toxins (Loseva *et al*., 2002).

Resistance management strategies try to prevent or diminish the selection of the rare individuals carrying resistance genes and to keep the frequency of resistance

genes sufficiently low for insect control (Schnepf *et al*., 1998). In transgenic plants, selection pressure could be reduced by restricting the expression of crystal protein genes to certain tissues of crop so that remaining parts of the plant are fully protected. It has been proposed that cotton lines in which *cry* gene expression is limited to the young bolls may not suffer dramatic yield loss from *Heliothis* larvae feeding on other plant structures (Gould, 1988). However, tissue-specific expression would not be a viable option for some pests which target nearly the entire plant, such as the European corn borer (Monsanto, 2000).

Another management option is the rotation of plants or sprays of particular *Bt* toxin with those having another toxin type binding to a different receptor. The second strategy has a potential value when is a fitness cost is associated with resistance. Such fitness cost has been reported in *P. xylostella* lines, in which resistant males have lower mating success than their non-resistant competitors (Groeters, 1993). The use of a patchwork of *Bt* and non-*Bt* aeras (mosaics) in resistant management is another strategy, but mosaics and rotations cannot be successful if the cross- resistant (resistance to one of them cannot be positively correlated with or helpful in developing resistance to the other) is present between insecticides (Tabashnik, 1994).

The larvae of western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), and related *Diabrotica* species are major coleopteran pests of corn. Crop rotation with soybeans is the most common pest management practice for corn rootworm control and is used in 80% of north central United States area (Drees *et al*., 1999; Ellis *et al*., 2002). Babu *et al.* (2002) compared the growth inhibition and mortality patterns of *Helicoverpa armigera* (H) treated with Cry1Ac and Cry2Aa proteins and found that deploying Cry1Ac and Cry2Aa in rotation would be a rational strategy for managing resistance development in cotton bollworm.

Other attractive management tactics include the combination of two or more insecticides on the same field, such as a *Bt* and non-*Bt* toxin and combinations of two or more different *Bt* Cry toxins, or a *Bt* Cry and a *Bt* Cyt toxin. A 1997 study investigated the toxic effects of a combination of *Bt* and the parasitoid

Cotesia plutella on *P. xylostella*. The interaction of the two did not significantly affect mortality of *P. xylostella* populations resistant to *Bt*, though *Bt*-susceptible populations were affected by the combination more than by either the parasitoid or *Bt* used alone (Chilcutt and Tabashnik, 1997). In comparison to *Btk* strains, there is a limited *Bti* insect resistance development in mosquitoes, mainly because of the presence of the multiple protein toxin genes (Becker and Margalit, 1993). Sublethal doses of CytA toxins, when combined with Cry4 toxins, appear to suppress and/or reduce up to 1000-fold resistance in Cry4-resistant populations of *Culex quinquefasciatus* (Wirth *et al*., 1997). Similarly, the use of *Bt* Cyt1Aa helped to overcome 5000-fold resistance to *Bt* Cry3Aa toxins in C*hrysomela scripta* (Frutos *et al*., 1999).

Natural enemies, instead of other toxins, can be used in combination with low doses of *Bt*. This strategy cannot be used with most insecticides, as they have broad-range effects and will kill natural enemies as well as pests (Chilcutt and Tabashnik, 1997). Besides this, the high dose strategy hopes to delay resistance by using a high enough dosage of toxin to kill heterozygous insects (with alleles for both resistance and susceptibility). In this strategy, the control of dose carefully is the key factor (Neppl, 2000).

The largest share of the bio-pesticide market currently goes to *Bt*. Estimates range from $75 million to $125 million for recent annual sales worldwide. The strain most commonly used against insects is *Btk* (Jerry, 2004). *Bt*-based products represent about the 95 % of biopesticides and 2% of the total global insecticide market (Kaur, 2000). As of early 1998, there were nearly 200 registered *B. thuringiensis* products in the United States. Although commercial applications of *Bt* have been directed mainly against Lepidopteran pests of agricultural and forest crops, strains active against Coleopteran pests have also been marketed in recent years. Strains of *Bti* active against dipteran vectors of parasitic disease organisms have been used in public health programmes as well.

Most commercial *Bt*-based products contain ICP and viable *Bt* spores, but the spores are inactivated in some *Bti* products. Commercial *Bt* formulations include

wettable powders, suspension concentrates, water dispersible granules, oil miscible suspensions, capsule suspensions and granules (Tomlin, 1997) and are applied after the application of a *Bt* subspecies to an ecosystem, the vegetative cells and insecticides to foliage, soil, water environments or food storage facilities. spores may persist at gradually decreasing concentrations for weeks, months or years as a component of the natural microflora. A drawback of spore-crystal formulations is their limited foliar persistence in the environment due to inactivation by ultraviolet light, heat, leaf exudates and pH (Kaur, 2000). Quality standards for *Bt* fermentation products have been accepted by International Union of Pure and Applied Chemistry (IUPAC) (Quinlan, 1990). The standards include limits on the concentration of microbial contaminants and metabolites.

1.7. Present Study

The present study focused on anti-Coleopteran delta-endotoxin production by a local isolate of *Btt* which we found to produce high yields of Cry3Aa toxin. Toxin levels and sporulation were next monitored in four different media with the aim of selecting an efficient starting medium. Toxin levels at various stages of fermentation of this isolate were next compared to those of 30 different Cry3Aa producer strains. This comparison gave the idea about the high performance of our isolate regarding Cry3Aa production. The effects of varying the concentrations of sugar, inorganic phosphate and minerals on toxin biosynthesis were determined and compared to their effects on growth and sporulation of the organism.

The second part of the work involved cloning in *E. coli* and characterization of *cry*3Aa gene that the isolate harbours and revealed its identity to those reported so far. *cry*3Aa gene of the isolate was next cloned in *B. subtilis* as an intermediate host and expressed at a low level in this organism from its own promoter.

CHAPTER 2

MATERIALS AND METHODS

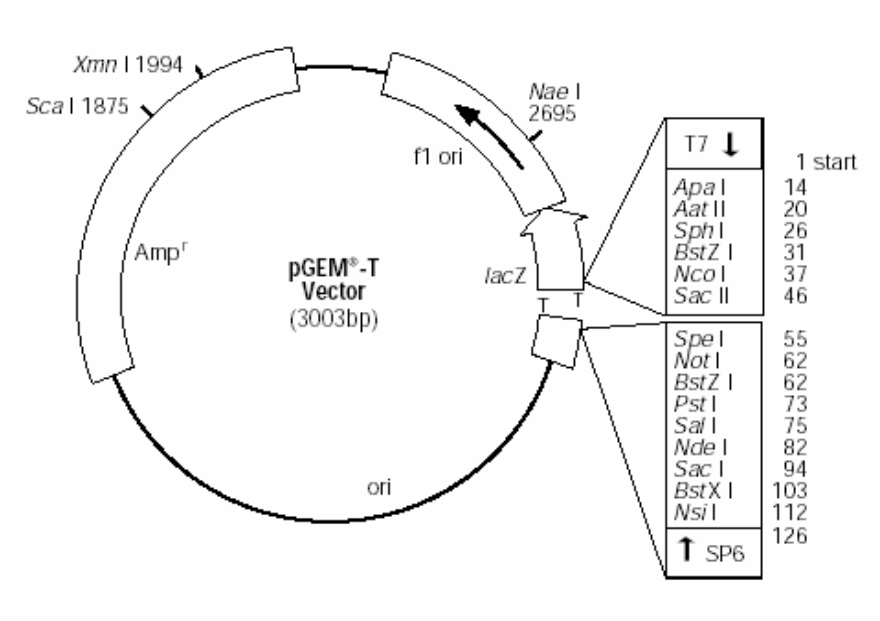
2.1. Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study and their sources are listed in Table 2.1. The strains were streaked onto LB agar (Appendix A), subcultured monthly and stored at + 4 ^{o}C. For long term maintenance of *Bacillus* strains, stock solutions were stored at - 80 ^{o}C in Luria broth covered with 20 % glycerol. Stock solutions of *E. coli* DH5α was also prepared in LB containing 15% glycerol and stored at – 80 ˚C.

Seed culture of *Btt* local isolate was prepared according to the procedure adopted from Stahly *et al* (1992). The *Btt* strain was grown to mid-log phase (ABS_{600}= 0.5). A 100 µL aliquot from this culture was transferred into a 100 mL DSM medium and left to grown until OD_{600} becomes 0.5. Then 100 µL of the culture was diluted 50 folds by adding 4.9 mL of 50 % glycerol (v/v). The diluted culture was divided in Eppendorf tubes as 100 µL aliquots and stored at – 80 ºC.

The plasmids, PGEM-T (cloning vector for PCR products) and pNW33N (*E. coli*/*Bacillus* shuttle vector), are shown in Figure 2.1.

a)



b)

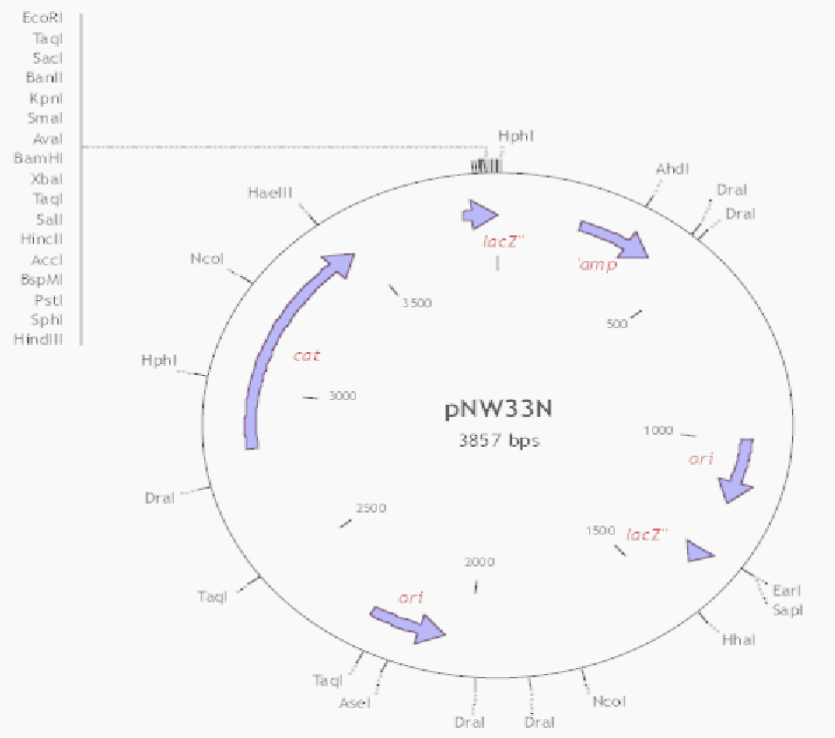


Figure 2.1. Structure of the plasmid vectors: (a) pGEM-T and (b) pNW33N.

Table 2.1. A list of bacterial strains and plamids used in the present study

No.	Strain	*Bt* serovar	Source and Reference
1	3273	ND*	Prof. Feruccio Gadani, Philip Morris Europe S.A., Neuchatel, Switzerland; Kaelin *et al.* (1994)
2	6003	*neoleonensis*	
3	5993	*neoleonensis*	
4	3123	*morrisoni*	
5	3283	*morrisoni*	
6	5903	*neoleonensis*	
7	3203	*morrisoni*	
8	3904	*morrisoni*	
9	3973	*morrisoni*	
10	3213	*morrisoni*	
11	7703	*morrisoni*	
12	2983	*morrisoni*	
13	3063	*morrisoni*	
14	3043	*morrisoni*	
15	3023	*morrisoni*	
16	1915	*morrisoni*	
17	1935	*morrisoni*	
18	1925	*morrisoni*	
19	3103	*neoleonensis*	
21	1401	*morrisoni*	
22	3003	*morrisoni*	
23	4AA1	*tenebrionis*	Dr. Daniel Zeigler, Bacillus Genetic Stock Center (BGSC); McPherson, *et al*. (1989)
24	4AB1	*san diego*	Dr. Daniel Zeigler, BGSC; Hernstadt *et al.* (1987)
25	4L1	*tolworthi*	Dr. Daniel Zeigler, BGSC; Burges HD, unpublished
26	4L2	*tolworthi*	Dr. Daniel Zeigler, BGSC; Burges HD, unpublished
27	4L3	t*olworthi*	Dr. Daniel Zeigler, BGSC; deBarjac H, unpublished
28	4W1	*kumamtoensis*	Dr. Daniel Zeigler, BGSC; Ohba *et al*. (1981)
29	4AT1	*japonensis*	Dr. Daniel Zeigler, BGSC; Ohba M., Aizaawa K. (1986).
30	4AU1	*higo*	Dr. Daniel Zeigler, BGSC; Ohba M., unpublished
31	*Btt* BI256-82	*tenebrionis*	Prof. Klaus Geider, Max Planck Institute, Germany; Rhim *et al*. (1990)

Btt local isolate		Local isolate, Prof. Dr. Zihni Demirbağ, Department of Biology, KTÜ.
E. coli DH5α	F'ϕd*lac*ZΔ(*lac*ZYA-argF)U169 *sup*E44λ⁻ *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	American Type Culture Collection; Hanahan (1983)
Bacillus subtilis 168; standard strain	*trp*C2, *thy*A1, *thy*B1	Dr. Daniel Zeigler; BGSC
Plasmids		
pGEM-T	Amp^r *lac*Z'	Promega
pNW33N	Cm^r	BGSC (*E. coli* strain ECE136)

*ND: Not Determined

2.2. Culture Media

The composition and the preparation of the culture media are given in Appendix A.

2.3. Buffers and Solutions

Buffers, solutions, their composition and preparation are listed in Appendix B.

2.4. Chemicals and Enzymes

The chemicals and the enzymes used and their suppliers are listed in Appendix C.

2.5. Growth Curves

0,5 mL of overnight culture was used to inoculate 50 mL of Difco's sporulating medium in a 250 mL Erlenmayer flask. The culture was incubated at 30 ℃ by shaking for 72 h at 200 rpm. Inoculation time was considered as time zero. Samples were taken from the culture at two hour intervals and used for quantitative determination of growth which was measured spectrophotometrically as absorbance 600 nm. Viable counts were also made and cell concentrations were determined

as ‘Colony Forming Units/mL’ (CFU/mL).

2.6. Determination of Sporulation Frequency

The samples were taken from the bacterial cultures grown in DSM by shaking at 30 °C. 1 mL aliquots of the cultures were serially diluted in tubes containing physiological saline solution. Colony forming units were determined after overnight incubation of inoculated Nutrient agar plates with prescribed volume of the diluted tubes at 30 °C. For spore count, the tubes were heated at 75 °C for 15 min in a waterbath, were cooled, dilutions were placed onto Nutrient agar plates and incubated at 30 °C for 16 h. Sporulation frequency was expressed in terms of the ratio of the numbers of heat-resistant spores per mL to the ratio of the number of viable cells per mL (Özcengiz and Alaeddinoğlu, 1991).

2.7. Protein Extraction

A slightly modified procedure of Armelle (1991) was used for protein extraction. 5-10 mL of 72 h cultures were centrifuged at 4 000 rpm for 20 min. The pellet was resuspended in 500 µL of 1M NaCl. This mixture was transferred into an Eppendorf tube and centrifuged at 7 000 rpm for 7 min. The pellet was resuspended in 250 µL of TE buffer (Appendix B) and centrifuged at 7 000 rpm for 7 min. The last step was repeated after which the pellet was resuspended in a 250 µL dH_2O to obtain a homogenous suspension. 150 µL of 10 $mg.mL^{-1}$ lysozyme solution in TE buffer was added and the suspension was incubated at 37 °C for 30 min. 25 µL of 10% SDS solution was added into the suspension and then the tube was vortexed for 30 sec. It was centrifuged at 6 000 rpm for 10 min and 100 µL of 0.2 % SDS solution was added to the pellet. For denaturation, 40 µL of the protein sample was mixed in an Eppendorf tube with 10 µL gel loading buffer (Appendix B). This sample was incubated at 90 °C for 7 min. for three times and placed onto ice until it cools. The final native and denatured samples

were stored at – 20 °C.

2.8. Determination of Protein Concentration

Protein concentrations were measured by the Bradford quantification method (1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 at 595 nm when binding to protein occurs. Assay reagent was made by dissolving 100 mg of Coomassie Blue G250 in 50 mL of 95 % ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Watman No. 1 filter paper. Bovine serum albumin (BSA) was used as the standard for preparation of protein calibration curve. Volumes of 10, 15, 20, 30 and 50 µL of 1 $mg.mL^{-1}$ standard protein, BSA were added to tubes and volumes were adjusted to 500 µL with water. 500 µL of distilled water was added into a tube as reagent blank. 4.5 mL of assay reagent was added to each tube and mixed gently, but thoroughly. A standard curve of absorbance versus micrograms protein was prepared and the amounts of proteins were determined from the curve.

2.9. SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by using a vertical polyacrylamide gel apparatus. Electrophresis was run at 20 mA at the beginning until the samples reached to the separating gel, then the current was increased to 40 mA and the electrophoresis was continued until the loading dye reached to the end of the gel.

The SDS-polyacrylamide gels were prepared as described below (Laemmli, 1970):

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel** 0.375 M Tris, pH 8.8
Monomer concentration	**4.5%**	**12%**
Acrylamide/bis	1.3 mL	4mL
dH_2O	6.1 mL	3.35 mL
1.5 M Tris-HCl, pH 8.8	-	2.5mL
0.5 M Tris-HCl, pH 6.8	2.5 mL	-
10% (w/v) SDS	100 µL	100 µL
10% Ammonium persulphate (fresh)	50 µL	50 µL
TEMED	10 µL	5 µL
TOTAL MONOMER	10 mL	10 mL

2.10. Staining of the SDS-Polyacrylamide Gel

2.10.1. Coomassie Blue R-250 Staining

Proteins were visualized by Coomassie Blue R-250 staining of the gels. After electrophoresis, the gel was soaked in 200 mL of freshly prepared Coomassie blue stain (Appendix B) for 1 h at room temperature. The gel was then destained by keeping it in destaining solution (Appendix B) for at least 24 h.

2.10.2. Silver Staining Method

Silver staining was performed as described below (Blum *et al*., 1987):

Step	Solution	Time of Treatment
Fixation	50 % Methanol 12 % Acetic Acid 0.05 % Formaldehyde	1 h
Washing	50 % Ethanol	3X 20 min
Pre-treatment	0.29 $g.L^{-1}$ $Na_2S_2O_3$	1 min
Rinsing	dH_2O	2X 20 sec
Impregnation	2 $g.L^{-1}AgNO_3$ 0.75 mL Formaldehyde	20 min
Rinsing	dH_2O	2X 20 sec
Development	60 $g.L^{-1}$ Na_2CO_3 0.5 mL Formaldehyde 4 $mg.L^{-1}$ $Na_2S_2O_3$	1- 10 min
Rinsing	dH_2O	2X 20 sec
Stop	50 % Methanol 12 % Acetic Acid	10 min
Storage	50 % Methanol	-

2.11. Protein Quantification

Gels were photographed by Vilber Lourmat Gel Imaging System and amount of the proteins were detected by Bio-Profile Image Analysis Software (Vilber Lourmat).

2.12. DNA Techniques and Manipulations

2.12.1. Total DNA Isolation

Procedure of Cutting and Horn (1990) was used for total DNA isolation. 1.5 mL of an overnight culture was centrifuged at 13 000 rpm for 5 min, the pellet was resuspended in 567 µL TE buffer by pipetting. 30 µL of proteinase K (20 $mg.mL^{-1}$), 6 µL of RNase (10 $mg.mL^{-1}$), 24 µL of lysozyme (100 $mg.mL^{-1}$) and 30 µL of 10 % SDS were added and this mixture was incubated at 37 °C for 1 h. 100 µL of 5 M NaCl was added and the tube contents were mixed throughly. Then 800 µL of CTAB/NaCl solution was added and the mixture was incubated at 65 °C for 10 min. The sample was extracted with the same volume of phenol/chloroform/isoamylalcohol (25/24/1) and centrifuged at 13 000 rpm for 10 min. The upper phase was transferred to a new Eppendorf tube to which 0.7 volume of isopropanol was added, mixed and centrifuged for 15 min. The resulting pellet was washed with 1 mL of 70 % ethanol by centrifugation for 5 min. Then it was dried at 37 ° C for 1 h and dissolved in 20 µL of TE buffer.

2.12.2. Plasmid Isolation from *Bacillus* spp.

Plasmid DNA was isolated by using the method which was described by Birnboim and Doly (1979). Bacterial strains were grown in Luria broth at 30° C by shaking for 16 h. 1 mL of such an overnight culture was added to 20 mL LB and incubated by shaking at 30° C until OD_{600} becomes 1.0. 5 mL portions of the resulting culture are centrifuged at 4 000 rpm for 10 min. Supernatant is removed and 200 µL of solution I (TGE buffer, pH 8, lysozyme 10 $mg.mL^{-1}$, RNase 10 $mg.mL^{-1}$) was added to the pellet which was kept at room temperature for 30 min. For *E. coli* V517, final lysozyme concentration was 2 $mg.mL^{-1}$ in Solution I. The mixture was transferred to an Eppendorf tube after which 200 µL of Solution II (0.1 N NaOH + 1 % SDS) was added. The tube was kept on ice for 7 min followed by the addition of 155 µL of Solution III (K-acetate pH 4.8-5). After incubation for 20 min on ice, the mixture

was centrifuged at 13 000 rpm for 15 min and the supernatant was transferred into a new Eppendorf tube. 400 µL of phenol/chloroform/isoamylalcohol (in a ratio of 25/24/1, respectively) was added, mixed gently and the tube contents were centrifuged at 13 000 rpm for 12 min. The upper phase was collected and phenol/chloroform/ isoamylalcohol extraction was repeated. 2 volumes of 99 % ethanol and 1/ 10 volume of K-acetate added to the resulting aqueous phase which was then mixed gently for a min and incubated at -20 °C for an hour. After centrifuging at 13 000 rpm for 30 min, the supernatant was discarded and 1 mL of 80 % ethanol was added to the pellet and the solution was centrifuged at 13 000 rpm for 30 min. The pellet was allowed to dry in a laminar hood and the pellet was dissolved in 20 µL TE buffer. 12 µL of dH_2O and 4 µL of loading buffer were added before agarose gel electrophoresis. When plasmid DNA of more than one strains are to be isolated, the same concentration of DNA were applied to the gel. DNA concentrations of different samples were equalized before applying to the gel. The following formula was used to determine DNA concentration:

$A_{260} \times$ Dilution Factor $\times$ 50 = DNA concentration ($\mu g.mL^{-1}$)

2.12.3. Plasmid Isolation from *E. coli*

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were used for isolation of *E. coli* plasmid DNA as specified by the manufacturers. *E. coli* plasmid DNA was also prepared by the plasmid miniprep method described by Hopwood *et al*. (1985). Each strain was grown as a patch on selective medium, LB agar containing 100 $\mu g.mL^{-1}$ ampicillin. About 1 square cm of cell mass was scraped with a sterile toothpick and put into Eppendorf tube containing 100 µL cold STE solution containing 2 $mg.mL^{-1}$ lysozyme (Appendix B). Each tube was mixed by vortexing to disperse the cells and the toothpick was discarded. Then, the tubes were incubated on ice for 20 minutes. 3/5 volume of lysis solution (Appendix B) was added to each tube and vortexed immediately. The mixture was incubated at room temperature for 10 minutes to lyse the cells and then at 70 ˚C for 10 minutes to denature DNA. Then, tubes were cooled rapidly in cold water. An equal amount of phenol-chloroform (water saturated, Appendix B) was added, vortexed hard until

homogeneous and milky white mixture was obtained. Finally, the samples were spinned for 5 minutes at 13 000 rpm to separate phases. 10 μL of supernatant was loaded directly on an agarose gel for electrophoresis.

2.12.4. Primer Design

For amplification of *cry*3Aa gene of the *Btt* local isolate, the primers were designed according to the nucleotide sequence of *cry*3Aa gene of *Btt* strain NB176 and *Bt* LM79 (the Gene Bank acsession numbers U10985 and L03393, respectively).

The primer design was made to include the promoter region for further expression of the gene. The regions for which the primers were designed are shown in Figure 2.2. The nucleotide sequences of the primers are shown in Table 2.2. Restriction enzyme sites, *Sac*I and *Kpn*I, for c3AKF and c3AKR respectively, were included in the 5′ region of the primers for cloning into pNW33N (underlined bases in Table 2.2). The primers were synthesized by the Iontek Company (İstanbul, Turkey).

Table 2.2. Primers used for PCR amplification and sequencing of *cry*3Aa gene of the *Btt* local isolate.

Primer designation	Nucleotide sequence of the primer	Expected product size (bp)
c3AKF	5'GAGCTCTCGAAACGTAAGATGAAACCTT3'	2589
c3AKR	5' CGGTACCGTTAATTTAATTCACTGGAA 3'	
c3AK1F	5' GAAGCACTAGATAGCTCTACAAC 3'	890
c3AK2F	5' GCATTGATGGATCAGAAAATAG 3'	886
c3AK3R	5'GCTATTTTCTGATCCATCAATGC 3'	894
c3AK4F	5' GATAAATTAAGAGGTTCATC 3'	903
c5AK5F	5' CACATCTCCATTCTATGG 3'	909

Figure 2.2. Nucleotide sequence of *cry*3Aa6 gene of *Btt* NB176. Arrows show the regions where the primers bind and the bold letters show start and stop codons. The 67 bases in box corresponds to the promoter of *Bt* LM79 and was included to ensure gene expression.

2.12.5. Polymerase Chain Reaction (PCR)

PCR reaction mixtures contained 28 µL of dH_2O, 5 µL 10 x PCR Mg^{2+} buffer, 50 pmols of each primers, 5 µL of 2 mM dNTP, 0.5 µg genomic DNA and 3 unit (1µL) *Taq* polymerase. PCR for the amplification of *cry3Aa* gene was carried out in 35 cycles: 1 min at 94 °C, 1 min at 63 °C and 3 min at 72 °C. The initial denaturation was carried out at 94 ° C for 10 minutes. The final extension was performed as 10 min at 72 °C.

Reaction mixtures were run in a 0.8 % agarose gel. PCR products were extracted from the gel as mentioned in Section 2.12.8**.**

2.12.6. Agarose Gel Electrophoresis

A horizontal apparatus was used for agarose gel electrophoresis. Agarose gels were prepared at concentrations of 0.6-1 % in 1X TAE buffer. The gels containing ethidium bromide at a final concentration of 0.5 $\mu g.mL^{-1}$ were run for 1-2 h at 90 Volt. The DNA bands were visualized on a UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. *Pst*I digested/λ DNA marker (Appendix C) was used to determine the molecular weights of DNA bands. Molecular weights of the plasmids were determined by using the plasmids of *E. coli* V517 as the standards.

2.12.7. Restriction Endonuclease Digestion

Restriction enzymes (*Sac*I and *Kpn*I) were added in a suitable buffer to the DNA samples to introduce 1 Unit of each enzyme per µg of DNA. The mixture was incubated at 37 °C for 4-5 h.

2.12.8. Extraction of Digested DNA Fragments/PCR Products from Agarose Gels

After separation of DNA fragments/PCR products usually in agarose gels, the desired fragments were extracted from the gel by using a Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was excised from the gel and weighed. DNA in such gel slices was recovered according to the Qiagen's instructions. After recovery, an aliquot was run on an agarose gel to assess the yield.

2.12.9. Ligation Reaction

Ligation of PCR products with pGEM-T vector was performed as follows: 5 µL 2× ligase buffer, 0.5 µL (50 ng/µL) pGEM-T vector, 400 ng insert DNA, 1 µL T4 DNA Ligase (3 unit/µL) were mixed. Sterile dH_2O was added to the mixture to complete total volume up to 10 µL. Before the addition of t4DNA ligase, the ligase buffer, ligase vector and insert DNA mixture were incubated at 45 ˚C for 10 min in a waterbath followed by incubation on ice for 5 min. Lastly, the mixture was incubated overnight at 16 ˚C. for ligation. The same ligation reaction procedure was used for ligation of *cry*3Aa gene to pNW33N by using an appropriate vector insert ratio.

2.13. Transformation

2.13.1 Preparation of *E. coli* Competent Cells

E. coli competent cells were prepared according to the protocol described by Sambrook *et al.* (1989) with slight modifications. In a 250 mL flask, 5- 50 mL of LB broth (Appendix A) was inoculated with *E. coli* from a fresh LB agar plate and incubated overnight with shaking at 37 ° C to obtain a stationary phase culture. 300 µL from this seed culture was inoculated into a fresh flask containing 50 mL LB broth. The culture was incubated for 2- 2.5 h at 37 ° C with vigorous shaking (300 rpm) in an orbital shaker to obtain an exponentially growing culture. Then the culture was split into two sterile pre-chilled 40 mL screwcap centrifuge tubes asepticaly and

stored on ice for 10 minutes. After centrifuging at 4000 rpm for 10 minutes at 4 °C, supernatants were decanted and each pellet was resuspended in 5 mL of ice-cold 10 mM $CaCl_2$ by vortexing. The cells were spun down at 3000 rpm for 10 minutes at 4 °C. Finally, supernatants were decanted and each pellet was resuspended gently in 1 mL ice-cold 75 mM $CaCl_2$. The competent cells were stored at – 80 °C.

2.13.2. Transformation of *E. coli* Competent Cells

A slightly modified procedure of Sambrook *et al*. (1989) was used for transformation of *E.coli* competent cells. 200 µL aliquots of *E. coli* competent cells were taken from -80 °C and kept on ice for 15 min. 100 µL of this was transferred to a prechilled Eppendorf tube. Appropriate amount of DNA (1- 50 ng) in a maximum volume of 10 µL was added and mixed gently by pipetting. The tubes were left on ice for 30 minutes. Then, the tubes were placed in a 42 °C waterbath and heatpulsed for 90 seconds. Next, the tubes were immediately put on ice for 5 minutes. 900 µL of LB broth was added to each tube and the cultures were incubated for 80 min at 37 °C with gentle shaking (180-200 rpm). The incubated cultures were microfuged at 4 000-5 000 rpm for 10 min to collect the cells. Supernatant was decanted and the pellet was resuspended 100 µL of sterile LB. Finally, the suspension containing 100 µL of transformed competent cells were spread onto selective LB agar plate and incubated at 37 °C for overnight.

2.13.3. Preparation of *B. subtilis* Competent Cells and Transformation

Transformation of *B. subtilis* competent cells were performed as described previously (Klein *et al.,*1992). 3 mL of overnight culture was prepared in HS medium (Appendix A) by incubation at 37 °C and shaking at 350 rpm. 0.5 mL of it was then transferred into 20 mL of LS medium (Appendix A) and incubated at 30 °C with shaking at 100 rpm until OD_{600} becomes 0.55. Next, 1 mL of competent cells was transferred into 2 mL of Eppendorf tube and 1 µg of DNA was added. Cells were then incubated at 37 °C for 2 h with shaking at 350 rpm and were collected by centrifugation. Cells were resuspended in 100 µL of sterile saline solution and plated out onto selective LB agar plates and incubated at 37 °C for 16 h.

2.14. Sequencing Reactions

DNA sequencing was carried out at İontek Company (İstanbul, Turkey) by using the chain termination method with dye-labelled dideoxy terminators of Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham).

Forward and reverse primers that were designed for sequencing of *cry*3Aa gene of the *Btt* local isolate were listed in Table 2.2. Deduced nucleotide and amino acid sequence data were compared with the National Center for Biotechnology Information (NCBI) database using the BLAST search at the web site (http://www.ncbi.nlm.nih.gov/BLAST).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Growth, Sporulation and Toxin Yield of the Local Isolate of *Bacillus thuringiensis* subsp. *tenebrionis* in C2 medium

As a preliminary study, the growth curve of the *Btt* local isolate was obtained by incubating the cells (30 °C, 200 rpm) for three days. C2 medium which was used for cultivation of *Btt* in most of the previous studies, (Donovan *et al*., 1988; Slaney *et al*., 1992; Malvar *et al.*, 1994; Von Tersch *et al*., 1994; Carroll *et al*., 1997) was preferred for the cultivation of the organism in our preliminary experiments.

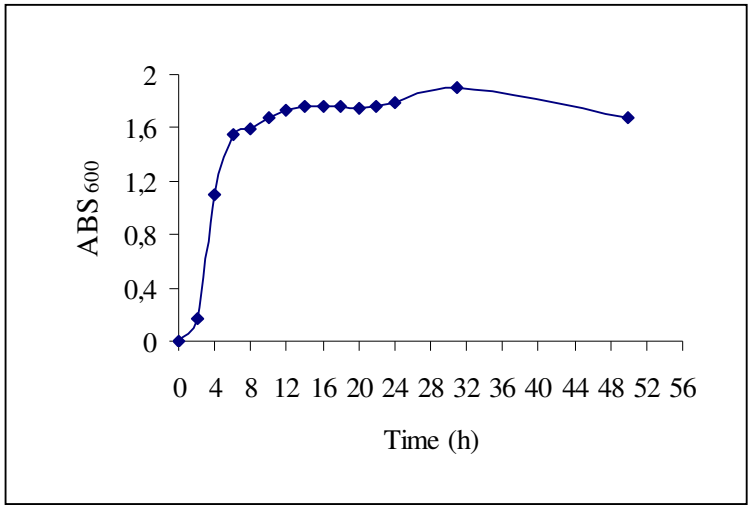


Figure 3.1. Growth of the *Btt* local isolate in C2 medium.

As shown in Figure 3.1, the culture of the isolate entered stationary phase after 8 h of exponential growth, indicating that the t_0 of the sporulation corresponds to the 8th h of incubation. Cry3Aa is 64-68 kDa polypeptide formed after proteolytic cleavage of 55-57 amino acids from N-terminus of 73 kDa protoxin by endogenous proteases during sporulation (Hernstadt *et al.,* 1986; McPherson *et al*., 1988). Time-dependent protein profile of the isolate on an SDS-PAGE gel is shown in Figure 3.2. It was observed that the amount of a 65 kDa protein corresponding to Cry3Aa increased with time, reaching to its highest level at 72nd h of incubation.

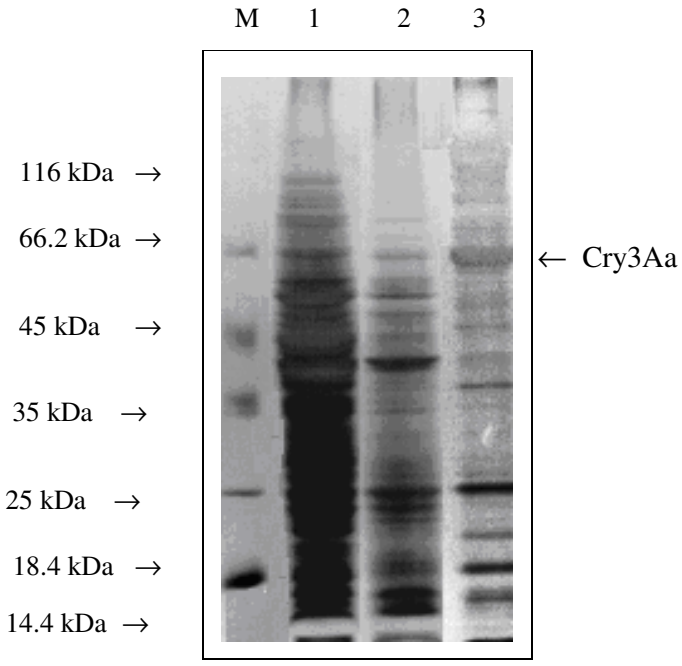


Figure 3.2. Silver-stained SDS-PAGE showing the protein profile of the isolate when grown in C2 medium for three days. Lane 1: Cry3Aa production at 24th h of incubation, Lane 2: Cry3Aa production at 48th h of incubation, Lane 3: Cry3Aa production at 72nd h of incubation.

3.2. Selection of the Most Appropriate Growth Medium

Choosing a standard growth medium that favors toxin biosynthesis by the *Btt* isolate was necessary for playing with medium ingredients for a better production. For this purpose, the organism was grown in four different standard media, namely C2, DSM,

GYS and HCT for five days and samples were taken from each culture at 24 h intervals. Proteins were extracted and protein profiles were determined by SDS-PAGE analysis. Prescribed incubation times were 48 h for HCT (Lecadet *et al*., 1980; Arantes and Lereclus, 1991), approximately five days for DSM and GYS (Donovan *et al*., 1992; Park *et al*., 1998) and three to four days for C2 (Donovan *et al*., 1988; Von Tersch *et al*., 1994), respectively. Yet, the incubation time was kept as five days (120 h) for all types of media. It was found that both the time of appearance of Cry3Aa protein and its production level greatly varied in these media (Figure 3.3).

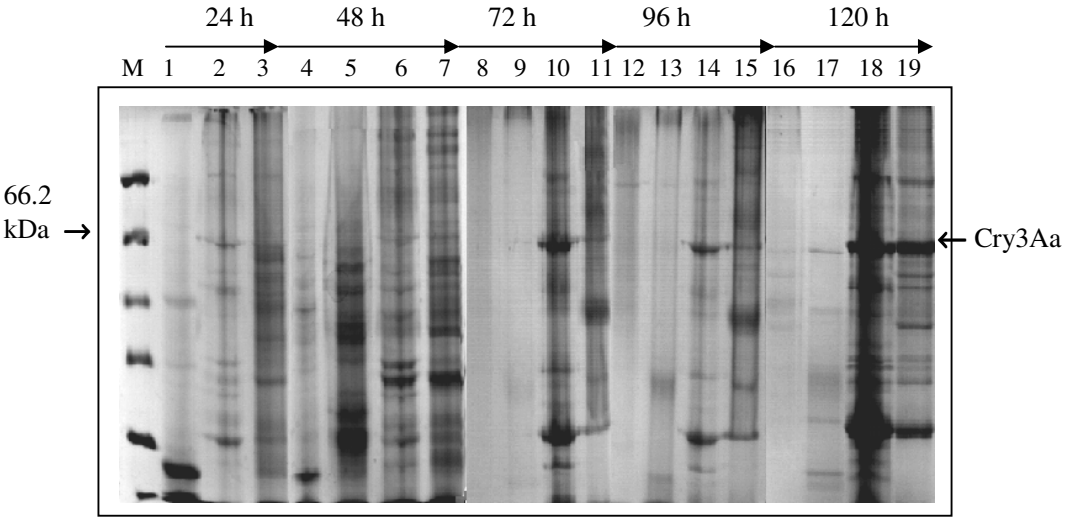


Figure 3.3. Cry3A production by the *Btt* isolate when grown in different media. Lane M: Protein molecular weight marker, Lanes 1, 4, 8, 12, 16: HCT medium. Lanes 5, 9, 13, 17: GYS medium, Lanes 2, 6, 10, 14, 18: DSM, Lanes 3, 11, 15, 19: C2 medium.

HCT and GYS media were not suitable at all for Cry3Aa production. GYS medium is the poorest one in its nutrient content, containing only 0.1% glucose and 0.2% yeast extract+0.02% ammonium sulphate as the carbon and nitrogen sources and it even did not permit any growth within 24 h. In this medium, Cry3Aa protein appeared as a very thin band only at the end of 120 h fermentation. With its 0.7% casein hydrolysate and 0.3% glucose content, HCT medium supported growth much better than GYS medium, yet toxin protein could not be detected during 120 h incubation in this medium. DSM appeared to be the best one for an early onset of Cry3Aa biosynthesis as well as its high yields. The organism produced some toxin in this medium even at 24^{th} h of incubation, its biosythetic capacity increased with time and lasted throughout the course of incubation. When grown in C2 medium, toxin yields were much lower than that in DSM except for 120^{th} h when the amounts of toxins produced in two media appeared almost equal.

When we compare the ingredients of DSM and C2, the latter contains at least 2 times more nutrients which might have accounted for less toxin yields by causing some kind of repression. The growth and sporulation patterns in these two media were next compared. Growth was monitored both by performing viable cell count and OD measurements at 600 nm (Figure 3.4 a,b). While the rate and extent of growth of the organism in these media did not much differ, it sporulated more effectively in DSM especially between 12 to 48 h (Figure 3.4 c,d). Cry3Aa levels as a function of time in these media were compared in another set of experiment to test the reproducibilty of the results shown in Figure 3.3. The results confirmed that DSM supports toxin synthesis much better (Figure 3.5). Therefore, for the rest of the study, DSM and 72 h were chosen as the standard medium and incubation time, respectively.

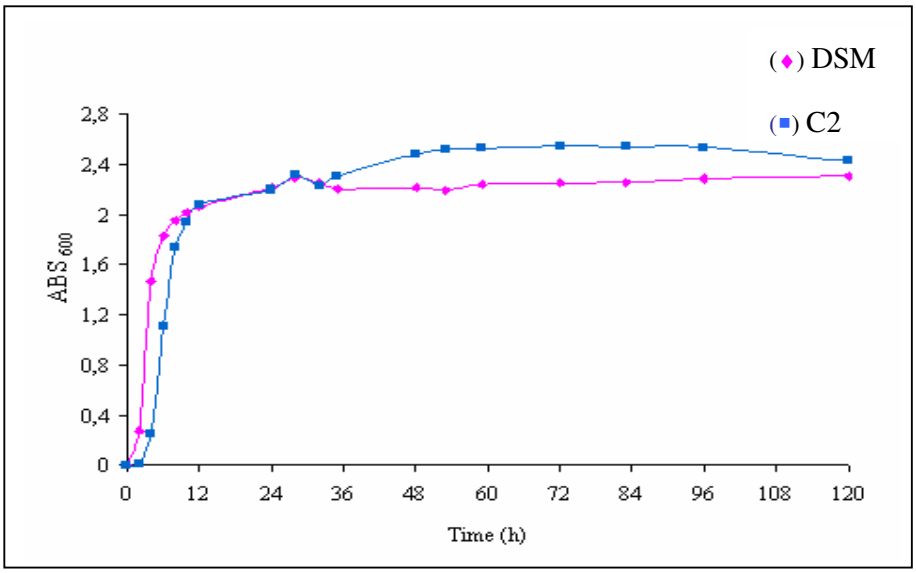


Figure 3.4 a Growth of the *Btt* isolate in DSM and C2 media.

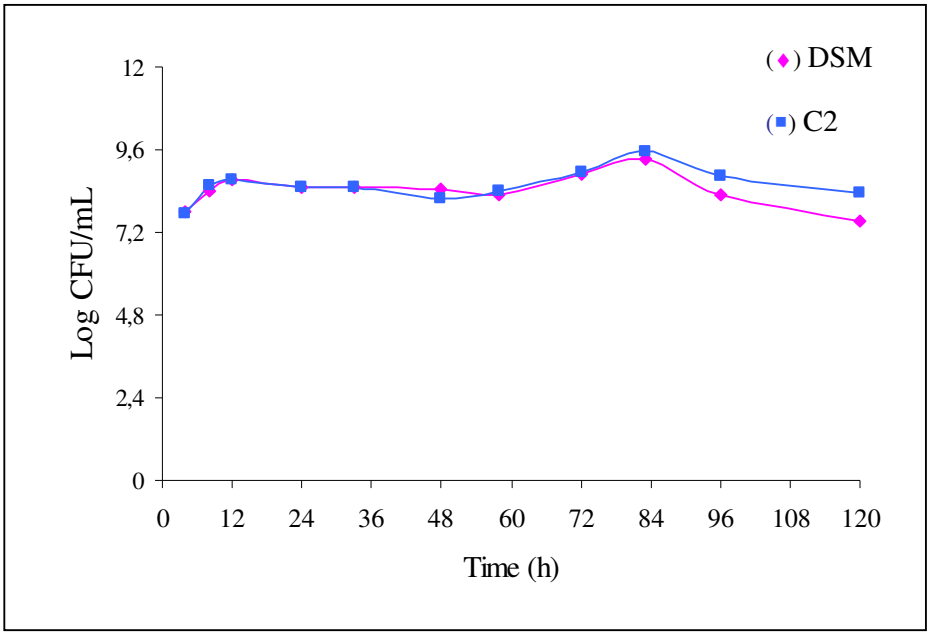


Figure 3.4 b Viable cell count of the *Btt* isolate in DSM and C2 media.

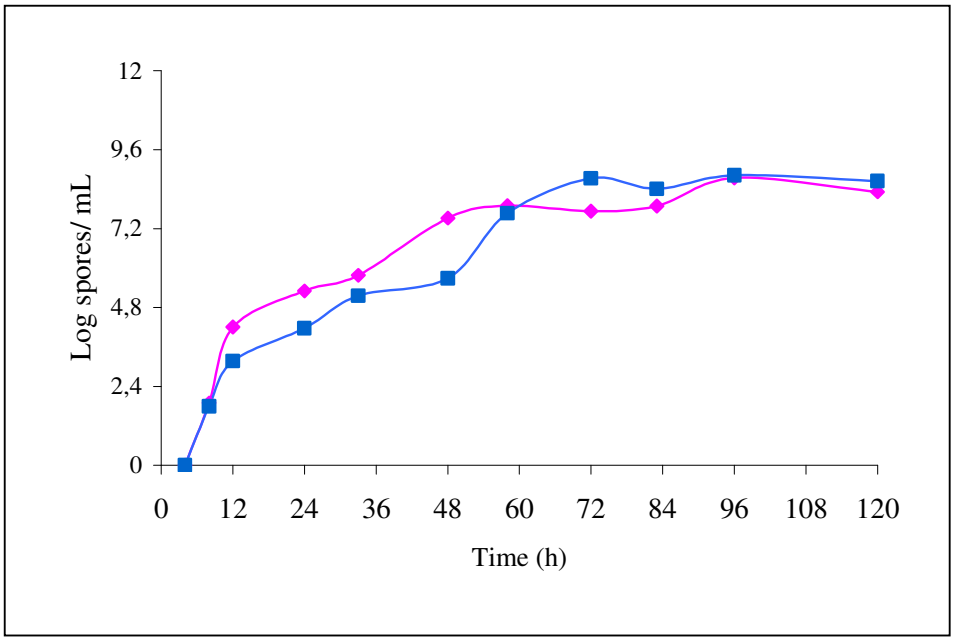


Figure 3.4 c Spore count of the *Btt* isolate in DSM and C2 media.

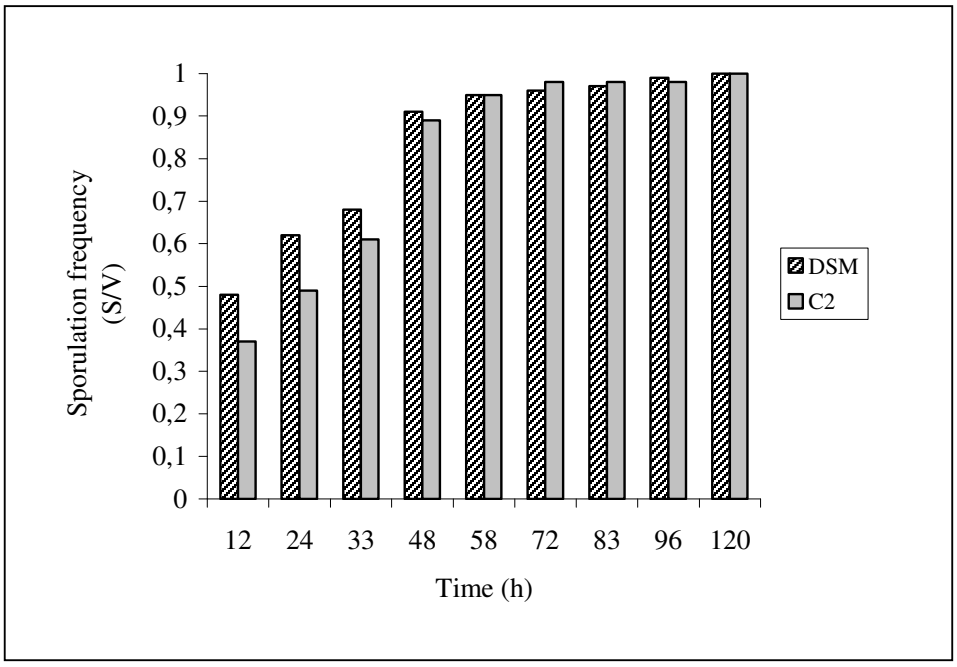


Figure 3.4 d Sporulation frequency of the *Btt* isolate in DSM and C2 media.

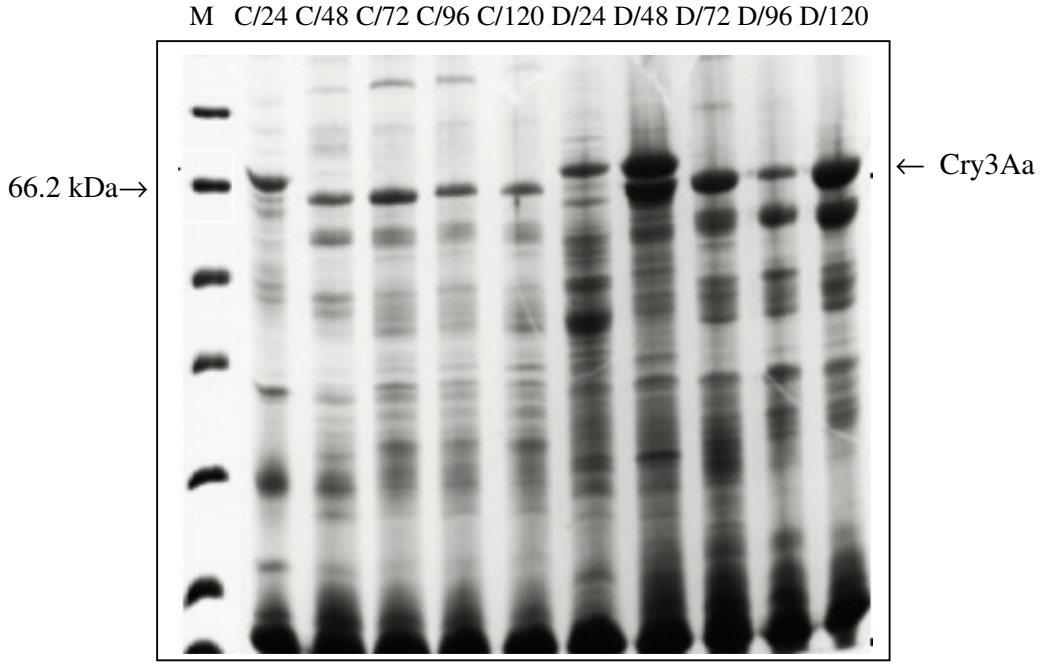


Figure 3.5. Another comparison between Cry3Aa levels as the function of time in DSM and C2 media C: C2 medium D: DSM. The numbers refer to incubation time in h.

3.3. Optimization Studies

Environmental factors play a critical role in modulating differentiation pattern and synthesis of toxins which form a distinct group of secondary metabolites and thus their synthesis has considerably narrower tolerances for concentrations of specific trace metals and inorganic phosphate (Weingberg, 1974; Bhatnagar, 1999). Also, the generality of repression or inhibition of secondary metabolism by catabolism of rapidly utilized carbon sources, especially glucose (carbon catabolite regulation) and nitrogen sources, especially ammonium, (nitrogen catabolite regulation) is well illustrated (Demain, 1995). In general, conditions for the culture of *Bt* are optimized to achieve both high cell densities and high sporulation rates. In this attempt, a careful balance of substrates must be provided to avoid high cell densities with little or no sporulation (Bernhard and Utz, 1993). Nevertheless, it has also been reported

that a high spore count is not sufficient to ensure good toxicity (Rossa and Mignone, 1993).

In striking contrast with commercial and scientific interest focused on anti-Coleopteran subspecies of *Bt* is the lack of adequate information on various aspects of Cry3Aa toxin production by the organism in the published literature. It was this focal point that led us to investigate the most crucial cultural parameters for a typical *Btt* batch fermentation to achieve high toxin titers.

3.3.1. Effects of Carbon Sources

DSM, the selected standard medium for optimization studies, contains glucose as its sugar complement. In our previous studies, the replacement of glucose with sucrose better supported the formation of Cry1, Cry4Ba and Cry11Aa toxins (İçgen *et al*., 2002b; Özkan *et al*., 2003). To reveal if sucrose had a positive effect also on Cry3Aa synthesis, the local *Btt* isolate was grown in DSM media containing different concentrations of glucose and sucrose (5 $g.L^{-1}$; 7.5 $g.L^{-1}$; 10 $g.L^{-1}$). As expected, increased concentrations of both sugars increased growth (Figure 3.6 a,b) and decreased sporulation (Figure 3.6 c,d).

Nevertheless, at the end of the 60 h incubation, the spore counts were almost equalized in all cultures. Another observation was that the cultivation on 5 $g.L^{-1}$ concentrations of either glucose or sucrose yielded more spores and a higher sporulation frequency was obtained in sucrose containing medium especially at 24^{th} h of incubation.

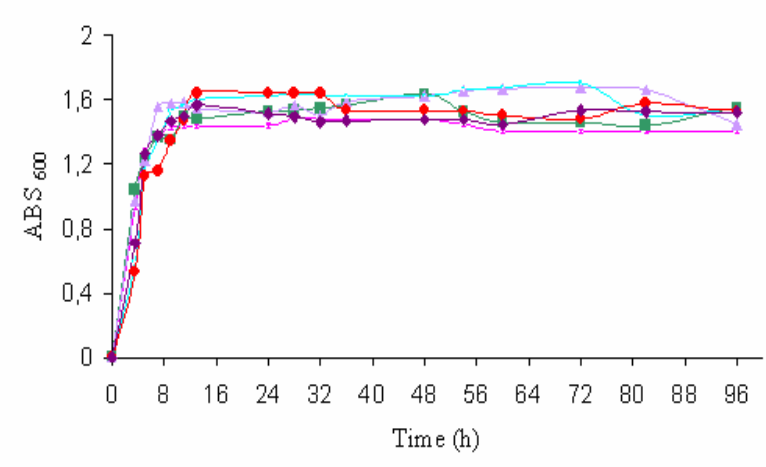


Figure 3.6 a Growth of the isolate on different glucose and sucrose concentrations. (*) Glucose, 5 g.L^{-1}. (▪) Glucose, 7.5 g.L^{-1} (▲) Glucose, 10 g.L^{-1} (▪) Sucrose, 5 g.L^{-1} (●) Sucrose 7.5 g.L^{-1} (◆) Sucrose 10 g.L^{-1} .

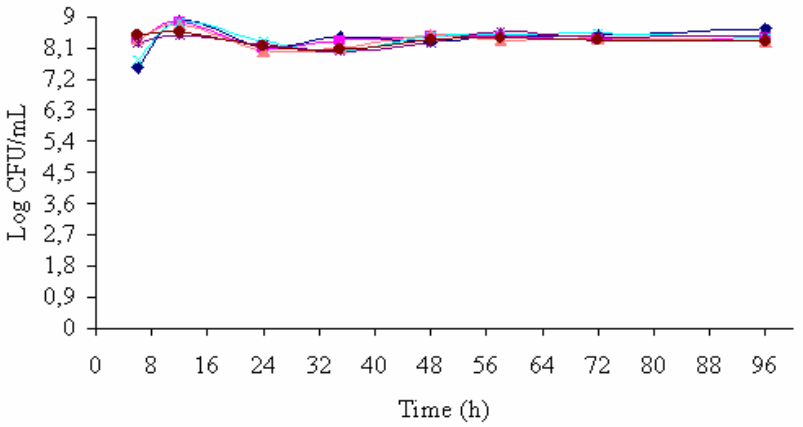


Figure 3.6 b Viable cell count when grown on different glucose and sucrose concentrations. (◆) Glucose, 5 g.L^{-1} (▪) Glucose, 7.5 g.L^{-1} (▲) Glucose, 10 g.L^{-1} (*) Sucrose, 5 g.L^{-1} (*) Sucrose 7.5 g.L^{-1} (●) Sucrose 10 g.L^{-1}.

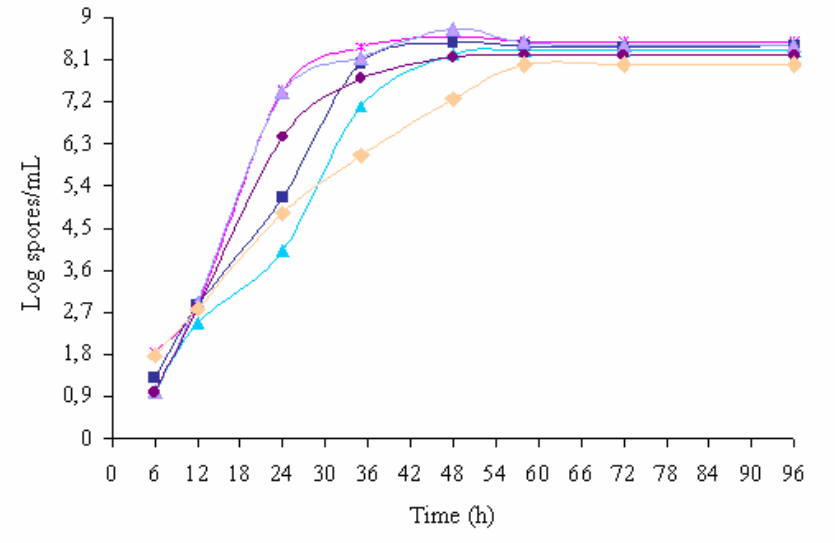


Figure 3.6 c Effects of different glucose and sucrose concentrations on spore counts. (*) Glucose, 5 g.L^{-1} (▪) Glucose, 7.5 g.L^{-1} (▲) Glucose, 10 g.L^{-1} (▲) Sucrose, 5 g.L^{-1} (●) Sucrose, 7.5 g.L^{-1} (◆) Sucrose, 10 g.L^{-1}.

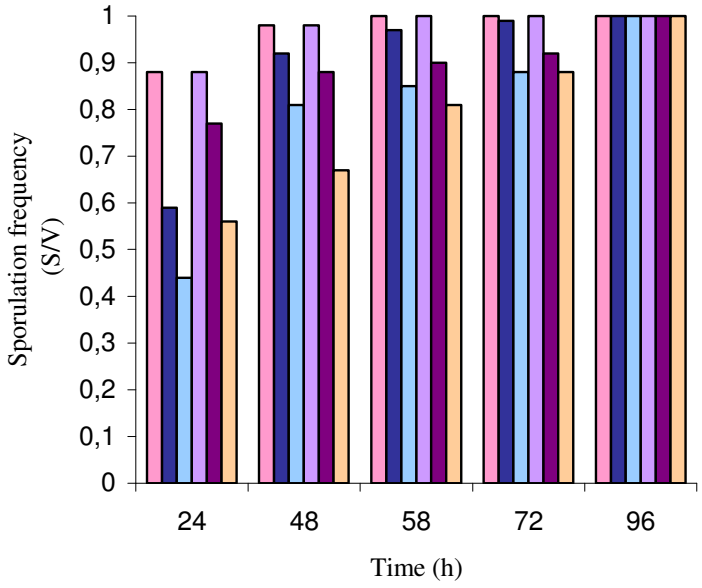


Figure 3.6 d Sporulation frequency of the isolate when grown on different concentrations of glucose and sucrose. (■) 5 g.L^{-1} glucose, (■) 7.5 g.L^{-1} glucose, (■) 10 g.L^{-1} glucose, (■) 5 g.L^{-1} sucrose, (■) 7.5 g.L^{-1} sucrose, (■) 10 g.L^{-1} sucrose.

It was seen that increasing concentrations of both sugars suppressed toxin biosynthesis and the highest Cry3Aa production was observed in the medium containing glucose or sucrose at a concentration of 5 $g.L^{-1}$ (Figure 3.6 e). Such a reduction in toxin yield with increasing concentration of sugars suggests the phenomenon of carbon catabolite repression which is typical for secondary metabolism (Demain, 1995). Since crystal protein synthesis is concomitant with sporulation, another explanation for reduced toxin yield could be the reduced amount of spores at higher sugar concentrations.

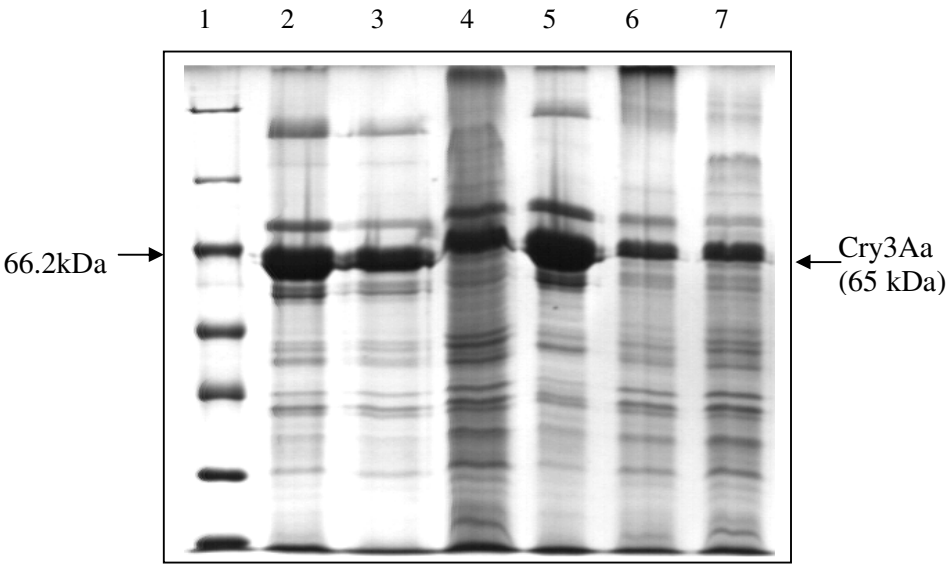


Figure 3.6 e Coomassie-stained SDS–PAGE showing the effect of different concentrations of glucose and sucrose on Cry3Aa biosynthesis. Lane 1: Protein molecular weight marker, Lane 2: 5 $g.L^{-1}$ glucose, Lane 3: 7.5 $g.L^{-1}$ glucose, Lane 4: 10 $g.L^{-1}$ glucose, Lane 5: 5 $g.L^{-1}$ sucrose, Lane 6: 7.5 $g.L^{-1}$ sucrose, Lane 7: 10 $g.L^{-1}$ sucrose.

3.3.2. Effects of Inorganic Phosphate and Different Nitrogen Sources

Phosphate is considered to be essential for protein expression and also critical for cell growth, thus its concentration varies during the toxin-formation phase (Yang and Wang, 2000). Inorganic nitrogen compounds, such as ammonium sulphate are not usually sufficient to support the growth of *Bt* (Bulla *et al*., 1980; Arcas *et al*., 1984) and organic nitrogen sources such as meat peptone, fish meal and soybean flour are required for rapid growth (Yang and Wang, 2000). Besides this, the depletion of nitrogen sources induces the formation of δ-endotoxins (Yang and Wang, 1998). Zourai *et al.* (2002) investigated the production of δ- endotoxins of several *Bt* strains in gruel-based media and found that the toxin production varied a lot depending on the *Bt* strains, however a general increase was seen in toxin production.

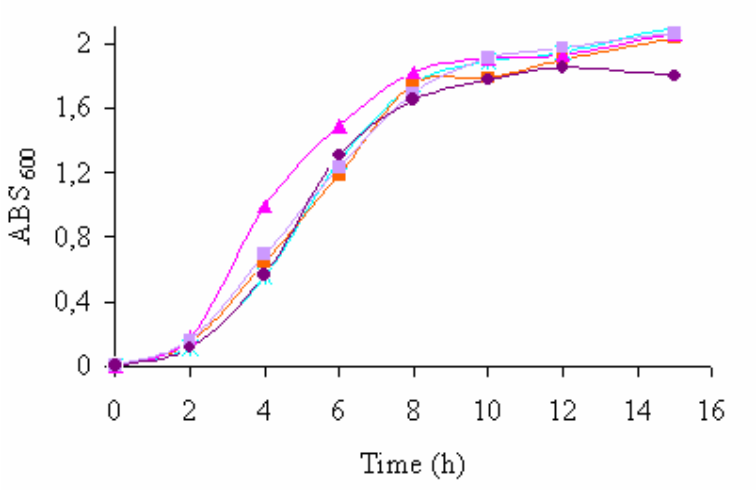


Figure 3.7. Growth of the *Btt* isolate in DSM with different inorganic phosphate concentrations. (▪) 5 mM, (▲) 20 mM, (*) 50 mM [control], (▪) 100 mM, (●) 200 mM.

To screen the effects of inorganic phosphate on growth, sporulation and Cry3Aa biosynthesis, our *Btt* isolate was grown in DSM containing different concentrations

of inorganic phosphate ranging from 5 mM to 200 mM. As shown in Figure 3.7, growth was the best at 20 mM and slightly decreased at a high inorganic phosphate concentration of 200 mM.

Nutrient broth in DSM (0.4 $g.L^{-1}$) was replaced with urea plus soybean flour, urea plus casamino acids, ammonium sulphate plus soybean flour and ammonium sulphate plus casamino acids (each 0.2 $g.L^{-1}$) to determine the effects of different inorganic and organic nitrogen sources on growth, sporulation and Cry3Aa production. However, except for ammonium sulphate plus casamino acids combination, each combination of nitrogen sources resulted in a poorer growth as compared with the growth in control medium (Figure 3.8).

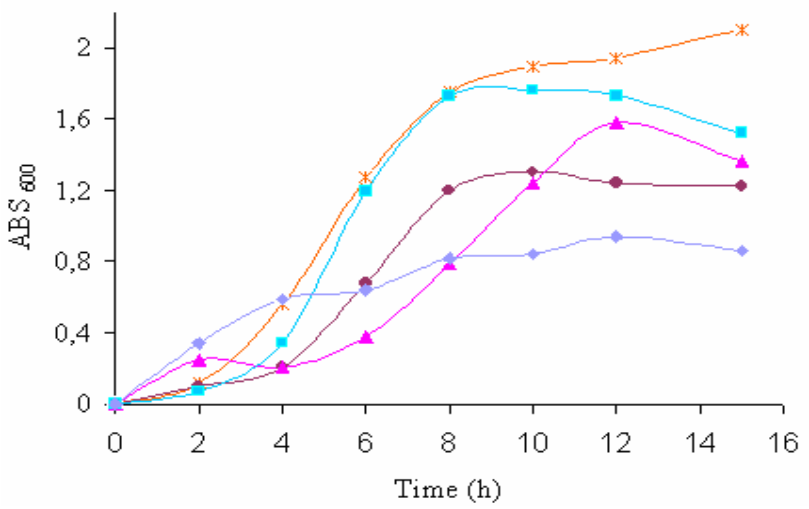


Figure 3.8. Growth of *Btt* strain with different combinations of inorganic nitrogen sources. (*) Nutrient Broth in DSM [Control], (•) Urea + Casamino acids, (▲) Urea + Soybean flour, (▪)Ammonium sulphate + Casamino acids, (▪) Ammonium sulphate + Soybean flour.

Our results showed that Cry3Aa production significantly increased with increasing concentrations of inorganic phosphate. As can be seen in Figures 3.9 and 3.10, the

toxin yield and the sporulation frequency were the highest at 200 mM (100 mM K_2HPO_4+100 mM KH_2PO_4) inorganic phosphate concentrations. In this respect, sporulation and toxin formation behaved quite consistently. SDS-PAGE analysis indicated that inorganic phosphate was a crucial nutrient affecting anti-Coleopteran Cry3Aa production. The positive effect of high inorganic phosphate concentrations on crystal protein production was also documented for other subspecies of *Bt* in our previous reports. Nonetheless, optimum production was generally obtained with 50-100 mM inorganic phosphate for anti-Lepidopteran and anti-Dipteran δ-endotoxins (İçgen *et al*., 2002 b; Özkan *et al*., 2003) and was never as high as 200 mM.

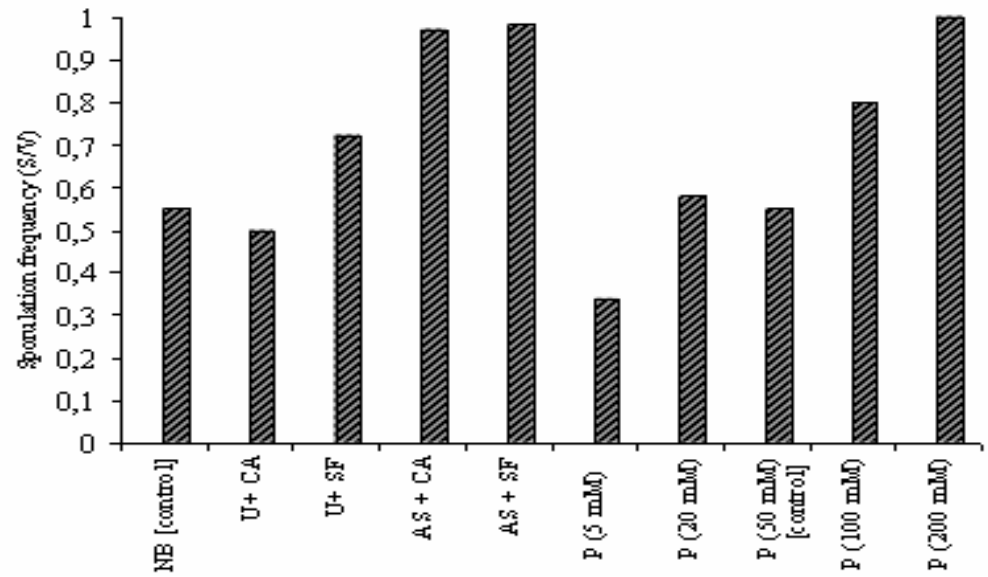


Figure 3.9. The effects of nitrogen sources and inorganic phosphate on sporulation. NB: Nutrient Broth in DSM [Control], AS: Ammonium sulphate, CA: Casamino acids, SF: Soybean flour, U: Urea, P: Inorganic phosphate.

Cry3Aa production was greatly reduced when different combinations of nitrogen sources were replaced Nutrient broth in DSM. The most negative effect was exerted by ammonium sulphate plus Casamino acids combination (Figure 3.10). Indeed, both

of the components were responsible for the negative effect seen since caused an adverse effect when combined with eachother. On the other hand, sporulation was better when the cells were grown in the modified media containing ammonium sulphate (Figure 3.9).

Thus, there was no consistency between the levels of sporulation and toxin formation for the nitrogen sources tested. For example, one of the most efficient sporulation was achieved in the medium containing ammonium sulphate plus soybean flour as nitrogen sources, however this did not lead to a good Cry3Aa protein production. This finding provides further support to the claim of Rossa and Mignone (1993) and Paramatha (2000) in that a high spore count is not always sufficient to ensure good toxicity.

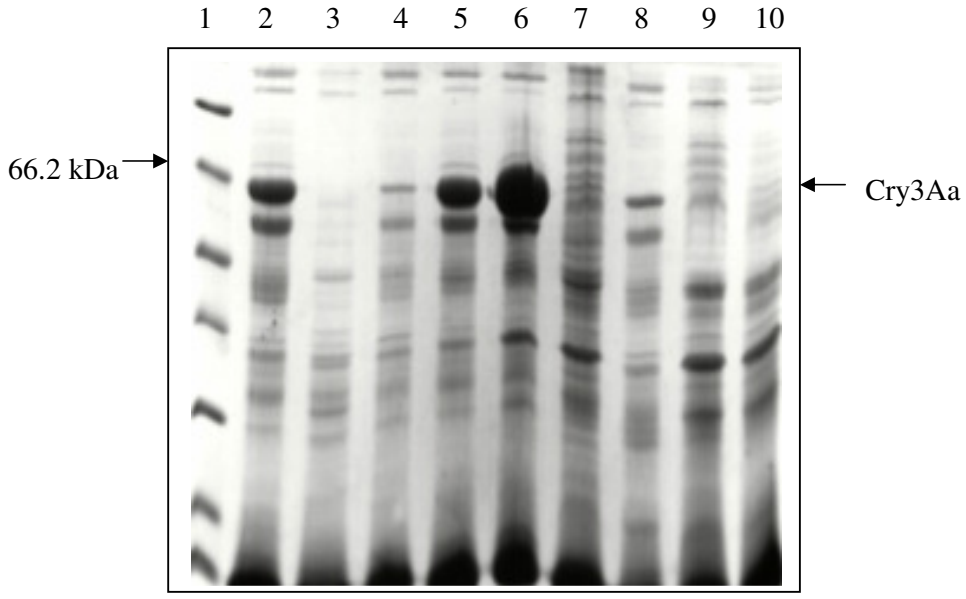


Figure 3.10. Cry3Aa biosynthesis on different nitrogen sources and different concentrations of inorganic phosphate. Lane 1: Protein molecular weight marker, Lane 2: 50 mM inorganic phosphate in DSM/Nutrient Broth in DSM [Control], Lane 3: 5 mM inorganic phosphate, Lane 4: 20 mM inorganic phosphate, Lane 5: 100 mM Inorganic phosphate, Lane 6: 200 mM inorganic phosphate, Lane 7: Urea + Casamino acids, Lane 8: Urea + Soybean flour, Lane 9: Ammonium sulphate + Casamino acids, Lane 10: Ammonium sulphate + Soybean flour.

3.3.3. Effects of Different Concentrations of Mineral Elements

Faloci *et al* (1986) found that an adequate formulation of media was mainly related with the content of Mn, Mg and Ca which were essential for obtaining high yields of spore plus crystal production by *Bacillus thuringiensis* HD-1 strain. It is known that the yields of δ-endotoxins are greatly influenced by trace metals and other minerals, and in most cases it is not known whether the effect is a direct one or simply a manifestation of sporulation (Özkan *et al*., 2003). The effects of three mineral elements ($FeSO_4$, $MgSO_4$ and $MnCl_2$) on growth, sporulation and Cry3Aa production were next examined by varying their concentrations in the medium.

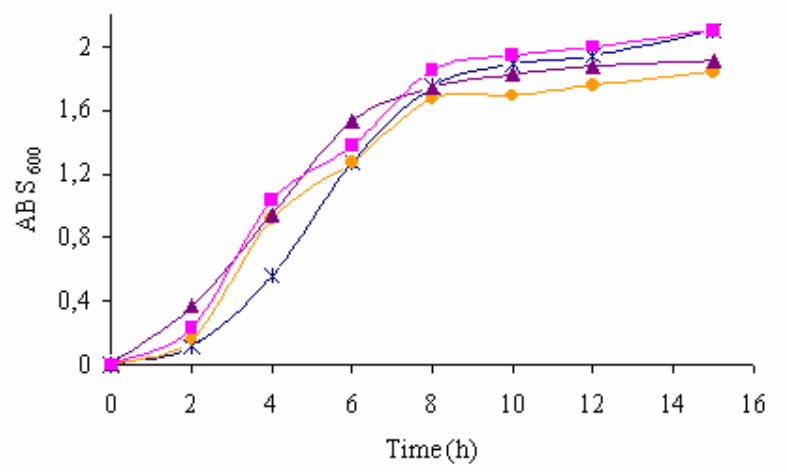


Figure 3.11. Growth of the *Btt* isolate in DSM with different $MnCl_2$ concentrations of (▪) 10^{-4} M, (∗) 10^{-5} M [control], (▲) 10^{-6} M and (●) 10^{-7} M.

Varying $MnCl_2$ concentration in DSM medium between 10^{-4} and 10^{-7} M did not affect the growth of the cells remarkably (Figure 3.11). Toxin yield decreased noticeably in the presence of 10^{-4} M $MnCl_2$ and the highest Cry3Aa production was observed with 10^{-5} to 10^{-7} M $MnCl_2$ (Figure 3.15). In our previous study, 10^{-6} M $MnCl_2$ specifically favored Cry4Ba production by *Bti* (Özkan *et al*., 2003). This concentration was also favorable for Cry3Aa production, but the effect was not as

remarkable as was found for Cry4Ba. It was previously reported that Mn is the most important key metal co-regulating secondary metabolism and differentiation in bacilli (Weinberg, 1977). The highest sporulation frequency was obtained with 10^{-5} M $MnCl_2$, the concentration which is originally contained in DSM (Figure 3.14).

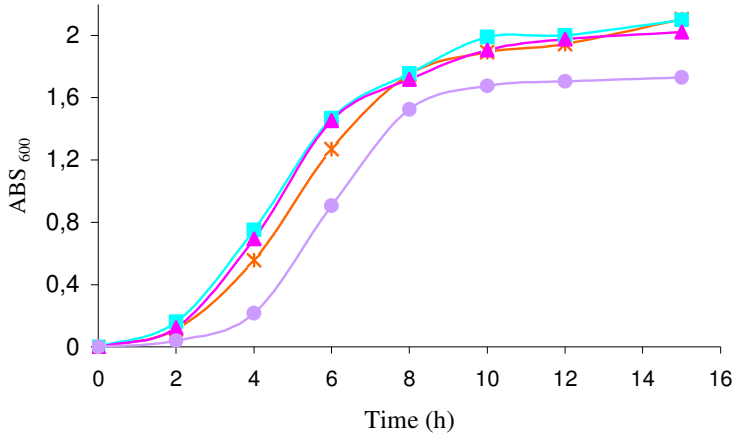


Figure 3.12. Growth of the *Btt* isolate in DSM with different $MgSO_4$ concentrations of (•) 5.10^{-2} M, (▲) 5.10^{-3} M, (*) 5.10^{-4} M [control] and (■) 5.10^{-5} M.

Our results showed that Cry3Aa production slightly decreased with increasing concentrations of $MgSO_4$. The highest toxin production was detected with 5×10^{-4} M $MgSO_4$, the amount originally found in DSM, and increasing or decreasing concentrations adversely affected the production (Figure 3.15). The growth was adversely affected by 10^{-2} M $MgSO_4$ and sporulation frequency was also found to decrease with increasing $MgSO_4$ concentrations (Figures 3.12 and 3.15). In the study of İçgen *et al.* (2002 a), high concentrations of $MgSO_4$ decreased Lepidoptera-specific Cry1 and Cry2 toxin productions by *Bt* 81. In contrast, increasing $MgSO_4$ concentrations caused an increase in Cry 4Ba and Cry 11Aa production by *Bt israelensis* (Özkan *et al.*, 2003). It thus appears that, high $MgSO_4$ levels affect toxin production in a different way, depending on the subspecies and hence the type of delta-endotoxin.

The onset of growth of our *Btt* isolate was delayed in a medium containing no $FeSO_4$ (Figure 3.13). In such a condition, the cells must have used up only the iron provided by the Nutrient broth complement of the medium. The concentration in the control medium (10^{-5}) was the optimal for sporulation and Cry3Aa biosynthesis (Figure 3.14 and 3.15) and as this concentration decreased or iron was omitted from the medium, the levels of toxin also decreased.

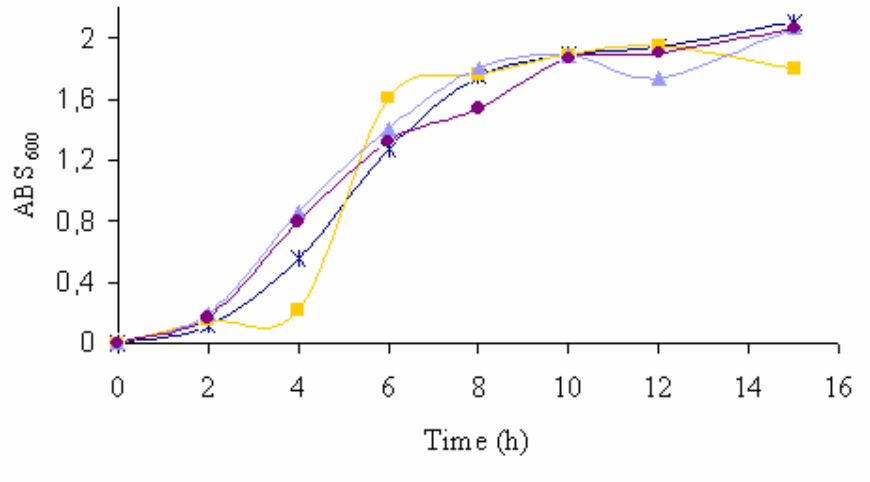
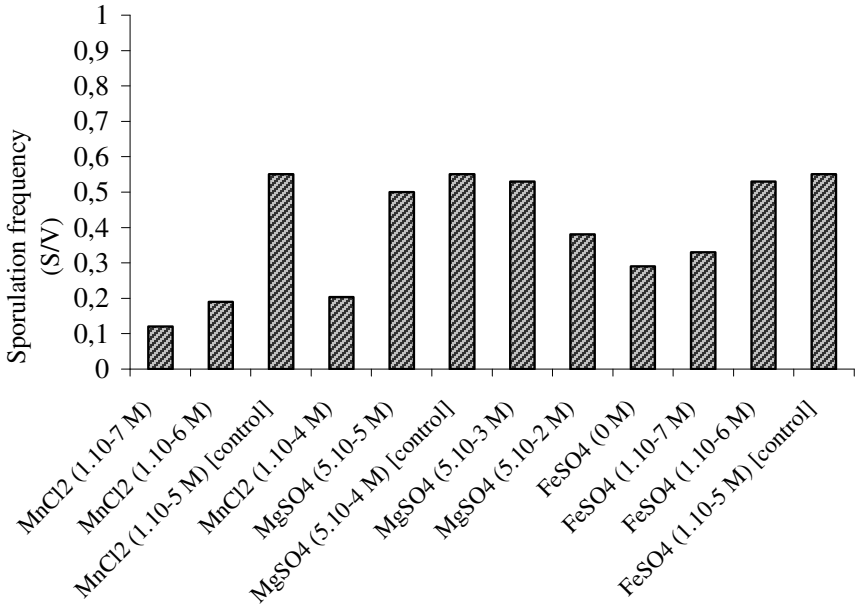


Figure 3.13. Growth of the *Btt* isolate in DSM with different $FeSO_4$ concentrations of (▪) 0 M, (∗) 10^{-5} M [control], (●) 10^{-6} M and (▲) 10^{-7} M.



Figur 3.14. Effects of different concentrations of mineral elements on sporulation.

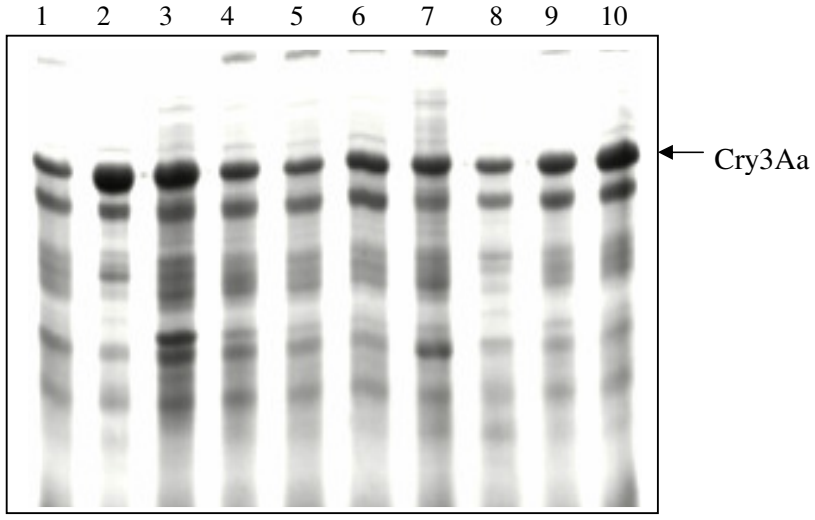


Figure 3.15. Effects of mineral ions on Cry3Aa biosynthesis at 72nd h of incubation. Lane 1: 1.10^{-4} M $MnCl_2$, Lane 2: 10^{-6} M $MnCl_2$, Lane 3: 10^{-7} M $MnCl_2$, Lane 4: 10^{-} $FeSO_4$, Lane 5: 10^{-7} M $FeSO_4$, Lane 6: 0 M $FeSO_4$, Lane 7: 5.10^{-2} M $MgSO_4$, Lane 8: 5.10^{-3} M $MgSO_4$, Lane 9: 5.10^{-5} M $MgSO_4$, Lane 10: Control (5.10^{-4} M $MgSO_4$, 10^{-5} M $FeSO_4$, 10^{-5} M $MnCl_2$.

According to our results, there was no direct correlation between toxin production and efficiency of sporulation. High toxin yield paralleled high sporulation frequency for Mg, Fe, sugar and inorganic phosphate levels, but not for Mn levels and the sources of nitrogen. The results were similar to those obtained in previous studies (Yang and Wang, 1998; Yang and Wang, 2000; Içgen *et al*., 2002 a,b; Zouari *et al*., 2002; Özkan *et al*., 2003) in that the high toxin yield is not always accompanied by high sporulation frequency.

3.3.4. Verification of Inorganic Phosphate Effect and Quantitive Assessment of Productivity

In view of our findings, the DSM was modified to contain 200 mM inorganic phosphate. A culture grown in iron-omitted DSM and another culture grown in DSM containing a lowered amount of inorganic phosphate (100 mM) were also included in this set of experiment in order to be able to cross check and verify the reproducibility of our findings about the crucial effect of inorganic phosphate. In modified DSM with 200 mM inorganic phosphate, Cry3Aa toxin production increased from 3 $\mu g.mL^{-1}$ to 15.6 $\mu g.mL^{-1}$, corresponding to a 5 fold increase in productivity as compared to the control culture (Figure 3.16). When $FeSO_4$ was omitted from the medium, toxin production decreased from 15.6 $\mu g.mL^{-1}$ to 8.99 $\mu g.mL^{-1}$ (a 1.7 fold decrease). When inorganic phosphate concentration was lowered to 100 mM in the presence of iron, the toxin level decreased to 5 $\mu g\ mL^{-1}$ which corresponded to a 3 fold decrease. Taken together, the beneficial effect of inorganic phosphate in a concentration range of 100 to 200 mM appeared to be much greater than that of iron inclusion in *Btt* fermentation.

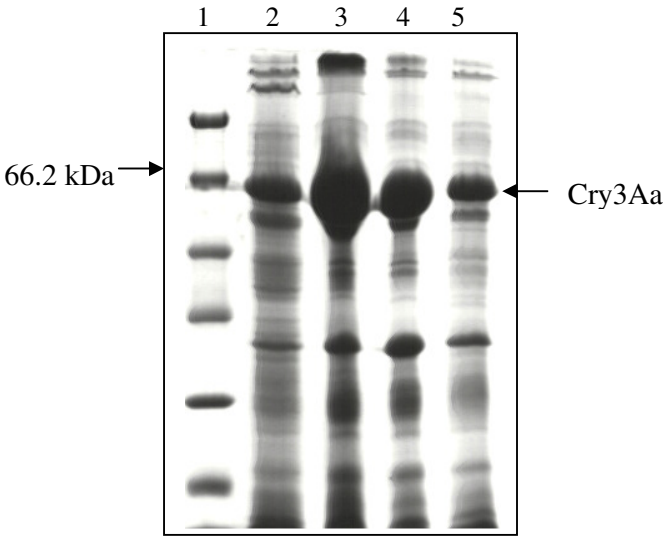


Figure 3.16. Cry3Aa biosynthesis in the modified DSM and the iron effect on product yield. Lane 1: Protein molecular weight marker, Lane 2: Control (50 mM inorganic phosphate), Lane 3: Modified DSM (200 mM inorganic phosphate), Lane 4: Modified DSM without $FeSO_4$, Lane 5: Modified DSM containing 100 mM inorganic phosphate instead of 200 mM.

3.4. Comparison of the Local Isolate to Various Anti-Coleopteran *Bt* Strains in Terms of Its Total Protein Profile and Biosynthetic Capacity

For this part of the study, we collected 30 different *Bt* strains reported to produce Coleoptera-specific δ-endotoxin (Table 2.1). The 21 of these strains have been mentioned in Kaelin *et al.* (1994) as being the isolates producing 65 kDa anti-Coleopteran toxin and having immunological homology with *Btt.* The strains including our local isolate were compared in terms of toxin production levels after they were grown in DSM for three days. Coomassie-stained SDS-PAGE gels showed that all the strains except *Bt* 5993, *Bt* 3973 and *Bt* 7703 produced the 65 kDa protein. Most of the strains producing this protein gave general banding patterns quite similar to that of our local isolate (for example *Bt* 3123, *Bt* 3203, *Bt* 3213, *Bt* 3023, *Bt* 1925, *Bt* 3103, *Bt* 1401 and 1935 at 48^{th} h); however protein profiles of certain strains such as *Bt* 3063, *Bt* 3043, 4AA1, 4L1 were quite different (Figures 3.17, 3.18 and 3.19).

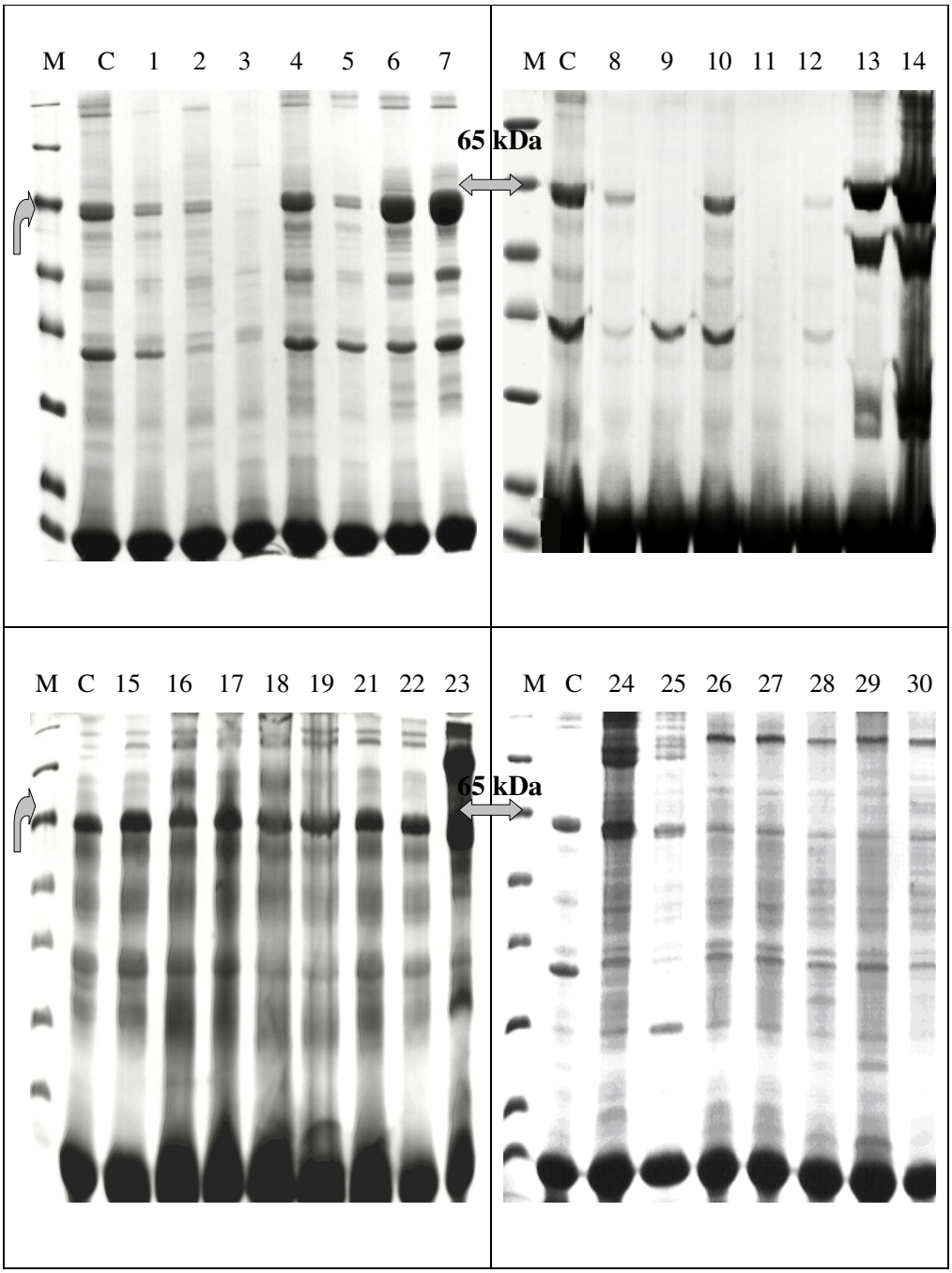


Figure 3.17. Protein profiles of different *Bt* strains at 48th h of incubation. M: Protein molecular weight marker; C [Control]: Our *Btt* isolate. 1: *Bt* 3273, 2: *Bt* 6003, 3: *Bt* 5993, 4: *Bt* 3123, 5: *Bt* 3283.,6: *Bt* 5903, 7: *Bt* 3203, 8: *Bt* 3904, 9: *Bt* 3973, 10: *Bt* 3213, 11: *Bt* 7703, 12: *Bt* 2983, 13: *Bt* 3063, 14: *Bt* 3043, 15: *Bt* 3023, 16: *Bt* 1915, 17: *Bt* 1935, 18: *Bt* 1925, 19: *Bt* 3103, 21: 1401, 22: *Bt* 3003, 23: 4AA1, 24: 4AB1, 25: 4L1, 26: 4L2, 27: 4L3, 28: 4W1, 29: 4AT1, 30: 4AU1.

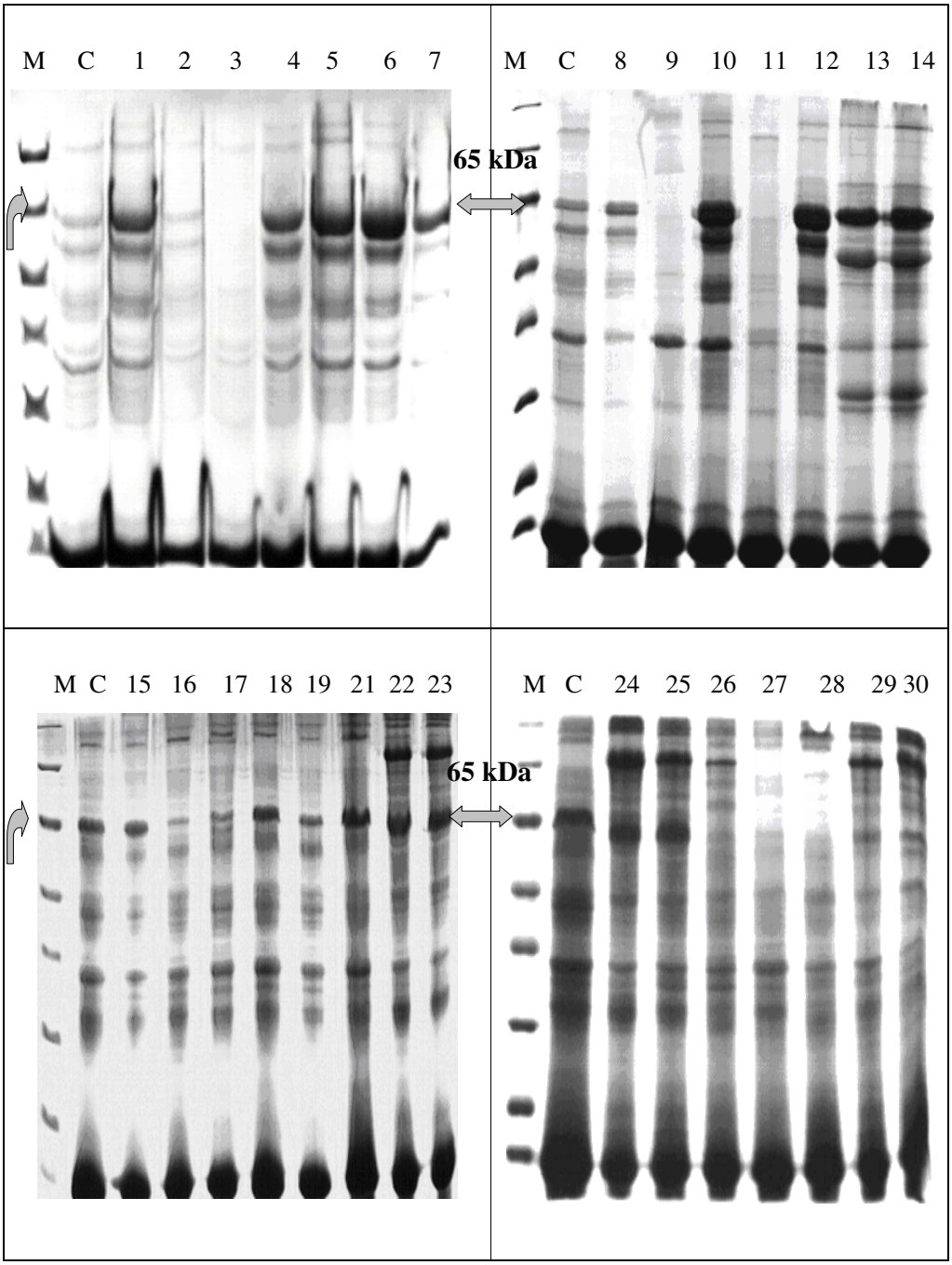


Figure 3.18. Protein profiles of different *Bt* strains at 72nd h of incubation. M: Protein molecular weight marker; C [Control]: Our *Btt* isolate, 1: *Bt* 3273, 2: *Bt* 6003, 3: *Bt* 5993, 4: *Bt* 3123, 5: *Bt* 3283, 6: *Bt* 5903, 7: *Bt* 3203, 8: *Bt* 3904, 9: *Bt* 3973, 10: *Bt* 3213, 11: *Bt* 7703, 12: *Bt* 2983, 13: *Bt* 3063, 14: *Bt* 3043, 15: *Bt* 3023, 16: *Bt* 1915, 17: *Bt* 1935, 18: *Bt* 1925, 19: *Bt* 3103, 21: *Bt* 1401, 22: *Bt* 3003, 23: 4AA1, 24: 4AB1, 25: 4L1, 26: 4L2, 27: 4L3, 28: 4W1, 29: 4AT1, 30: 4AU1.

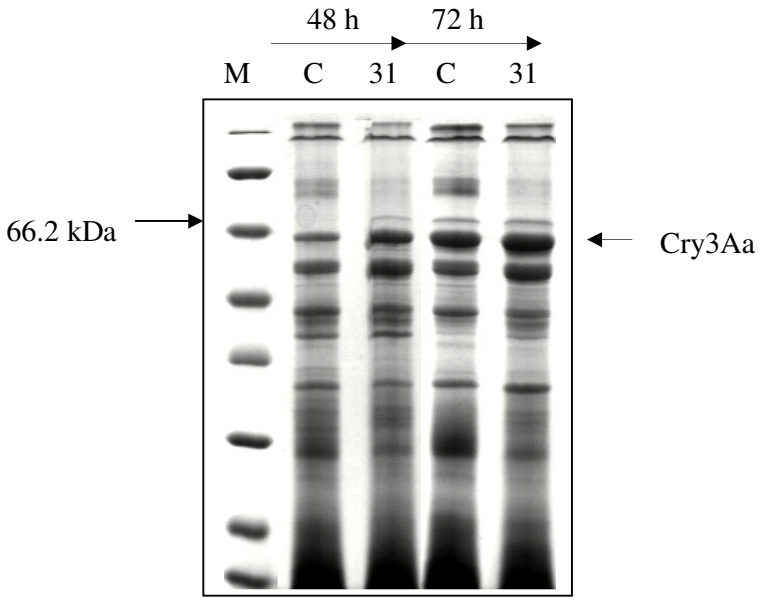


Figure 3.19. Comparison of protein profiles of the *Btt* isolate with that of the strain BI256-82 (31). C (Control): Our *Btt* isolate and *Btt* BI256-82 (31) at 48th and 72nd h of cultivation, respectively.

At 48th h, the strains *Bt* 5903, *Bt* 3203, *Bt* 3043, *Bt* 3063, 4AB1 and *Btt* BI256-82 and at 72nd h, the same strains plus *Bt* 3273, *Bt* 3123, *Bt* 3283, *Bt* 3213, *Bt* 2983, *Bt* 3003, *Bt* 1401, 4AA1 and 4L1 produced more Cry3Aa toxin than our *Btt* local isolate. These results suggested that 15 of the 30 isolates compared to the local isolate performed much better by producing the same toxin much earlier and at higher levels. The rest of the strains produced either comparable or much lesser amounts of toxin. The cell pellets were weighed in order to see if there was any relation between the toxin yields and the cell masses of the strains (Table 3.1). We could not find a direct correlation between these two parameters. For example, at 48th h, the highest cell masses of 77, 69 and 70 mg.mL^{-1} were obtained for the strains *Bt* 3123, 4L1 and *Bt* 6003 producing relatively less amounts of toxin, respectively. Similarly, the strains 3063 and 1935 producing higher amounts of toxin had lowest amounts of cells masses of 0.5 and 2.4 mg.mL^{-1}, respectively.

Table 3.1. Comparison of cell masses (wet weights) from the 48th and 72nd h cultures of different Cry3A producers.

Cell weight ($mg.mL^{-1}$)			
	Bt strains	48th h	72nd h
Control	The local isolate of *Btt*	2.6	19.2
1	*Bt* 3273	9.5	3.6
2	*Bt* 6003	70	14
3	*Bt* 5993	14	12
4	*Bt* 3123	77	29
5	*Bt* 3283	16	81
6	*Bt* 5903	8.3	29
7	*Bt* 3203	23	13
8	*Bt* 3904	14	13
9	*Bt* 3973	2.2	2.3
10	*Bt* 3213	20	96
11	*Bt* 7703	16	74
12	*Bt* 2983	20	13
13	*Bt* 3063	0.5	12
14	*Bt* 3043	16	19
15	*Bt* 3023	12	5.7
16	*Bt* 1915	15	19
17	*Bt* 1935	2.4	65
18	*Bt* 1925	17	20
19	*Bt* 3103	16	64
21	*Bt* 1401	20	17
22	*Bt* 3003	7.6	5
23	4AA1	69	8.4
24	4AB1	25	0.4
25	4L1	69	14
26	4L2	16	15.4
27	4L3	18	86
28	4W1	25	18
29	4AT1	5.4	83
30	4AU1	2.6	15
31	*Btt* BI256-82	19	10

With the aim of obtaining a clue about the relatedness/identity of Cry3A producing strains having similar protein profiles (the local isolate of *Btt*, *Bt* 3123, *Bt* 3203, *Bt* 3213, *Bt* 3023, *Bt* 1935, *Bt* 1925, *Bt* 3103 and *Bt* 1401), their plasmid patterns were also compared. As can be seen in Figure 3.20, *Btt* local isolate had two large plasmids of 135 and 61 kb and two small plasmids of 1.8 and 1.5 kb. Except for *Bt* 3023 which appeared plasmidless, all the strains harbored one large (135 kb) and both of the small plasmids. Four strains, namely *Bt* 1935, *Bt* 1925, *Bt* 3103 and *Bt* 1401 displayed the same plasmid profile by harboring an additinal large plasmid of 34 kb. Our local isolate and *Bt* 3123 had the same plasmid complements as they harboured another different large plasmid of 61 kb. The identity of these two strains remains to be determined via futher molecular studies such as RFLP analyses with total DNA.

Although there may be some *cry* genes that are found on chromosomal DNA (Aronson *et al*., 1986), crystal proteins are mostly encoded by the genes on large plasmids (Gonzales *et al*., 1981). The largest plasmid with a molecular size of 135 kb might be the one on which the *cry*3Aa gene of *Btt* local isolate is located though the possibility of a chromosomal location must not be disregarded. The low copy number of this plasmid did not allow us to excise it from the gel for being used as a template in PCR. Instead, Southern blot hybridization will be taken as the approach to investigate a possible plasmid location of *cry*3Aa gene(s) in the isolate.

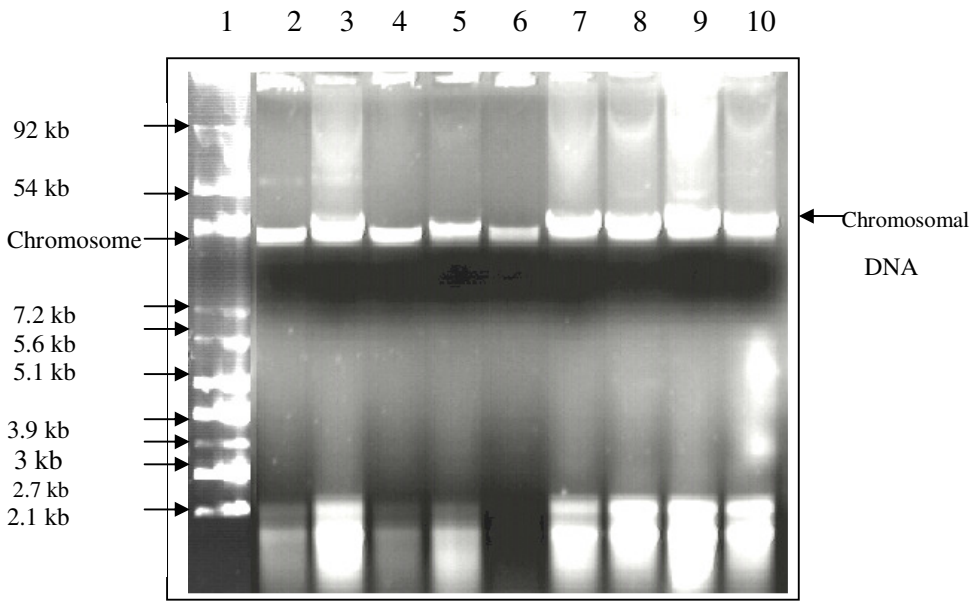


Figure 3.20. Comparison of plasmid profiles of strains having protein profiles similar to that of the local isolate of *Btt.* Lane 1: *E. coli* V517 plasmids as size markers, Lane 2: The *Btt* isolate. Lane 3: *Bt* 3123, Lane 4: *Bt* 3203, Lane 5: *Bt* 3213. Lane 6: *Bt* 3023, Lane 7: *Bt* 1935, Lane 8: *Bt* 1925, Lane 9: *Bt* 3103, Lane 10: *Bt* 1401.

In order to check the generality of inorganic phosphate effect together with iron effect on Cry3Aa production, two anti-Coleopteran *Bt* species, *Bt* 1925 and *Bt* 3123, were grown in modified DSM with and without $FeSO_4$ and their toxin production capacities were compared with that of the control. As shown in Figure 3.21, both *Bt* strains produced significantly increased amounts of Cry3Aa in modified DSM containing 200 mM phosphate. However, unlike our local isolate, they were not affected by the removal of $FeSO_4$ from the medium (Figure 3.20). This finding suggested that iron effect is rather unique to our isolate whereas inorganic phosphate effect can be common for the biosynthesis of Cry3Aa-type toxin.

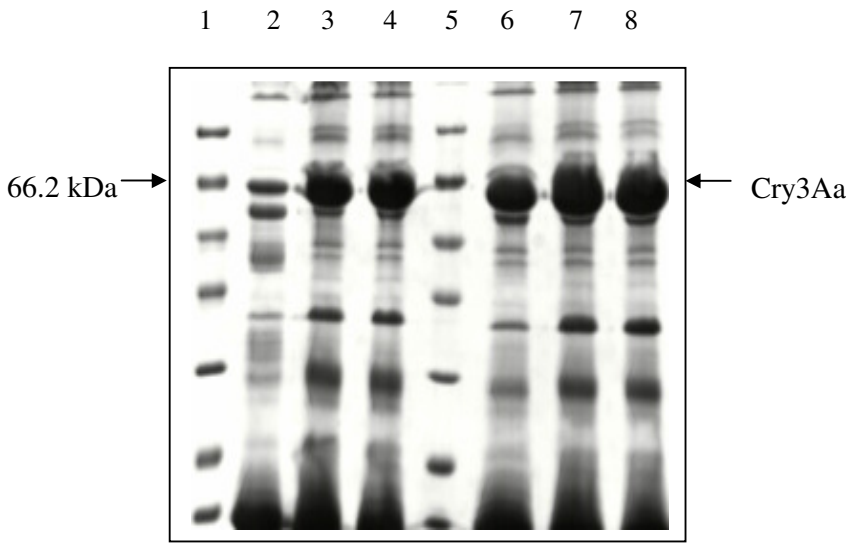


Figure 3.21. Cry3Aa production by *Bt* 3123 and *Bt* 1925 strains in different combinations of modified DSM. Lane 1, 5: Protein molecular weight marker, Lane 2: *Bt* 3123 in DSM [Control], Lane 3: *Bt* 3123 in modified DSM, Lane 4: *Bt* 3123 in modified DSM without $FeSO_4$, Lane 6: *Bt* 1925 in DSM [Control], Lane 7: *Bt* 1925 in modified DSM, Lane 8: *Bt* 1925 in modified DSM without $FeSO_4$.

3.5. Cloning of *cry*3Aa Gene into pNW33N and Expression in *Bacillus subtilis*

3.5.1. Cloning of *cry*3Aa Gene into pNW33N

For the cloning of *cry*3Aa gene into the *E. coli-Bacillus* shuttle vector, the gene was first amplified from the total DNA of the *Btt* local isolate by using the primers, c3AKF and c3AKR (Table 2.2). c3AKF and c3AKR contained *Sac*I and *Kpn*I restriction enzyme sites for cloning, respectively. 2589 bp of PCR product with an expected size was obtained from PCR (Figure 3.22). It was purified from the gel and ligated to pGEM-T vector. After transformation of *E. coli* DH5α cells with the ligation product, white colonies containing recombinant pGEM-T were selected on X-gal plus ampicillin-containing agar plates. pGEM-T carrying 2576 bp insert were isolated from four recombinant colonies (Figure 3.23) and digested with *Sac*I and *Kpn*I to release the insert DNA (Figure 3.24 a). Cloning to pGEM-T vector was also

verified by PCR amplification of the gene using putative recombinant plasmids as the template (Figure 3.24 b).

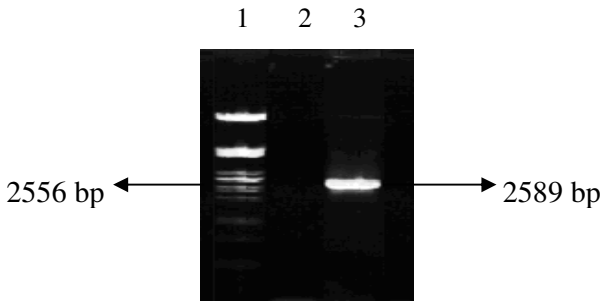


Figure 3.22. PCR product obtained with primers c3AKF and c3AKR designed for *cry*3Aa gene and its promoter. Lane 1: Lambda DNA/*Pst*I size marker (Appendix C), Lane 2: Negative control (no template DNA), Lane 3: *cry*3Aa gene with its promoter (2589 bp).

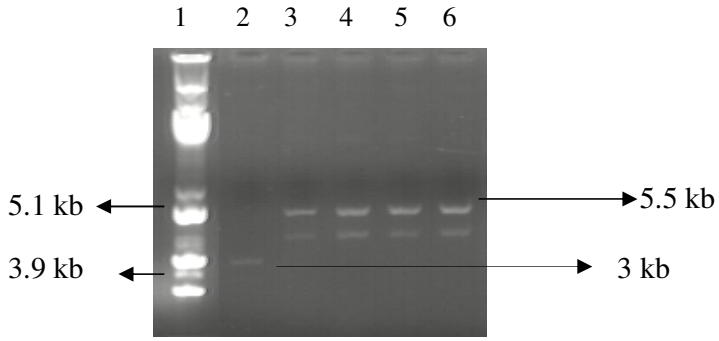


Figure 3.23. Putative recombinant pGEM-T plasmids carrying total *cry*3Aa gene. Lane 1: Marker (*E. coli* V517), Lane 2: Negative control (pGEM-T vector), Lane 3-6: Recombinant pGEM-T plasmids.

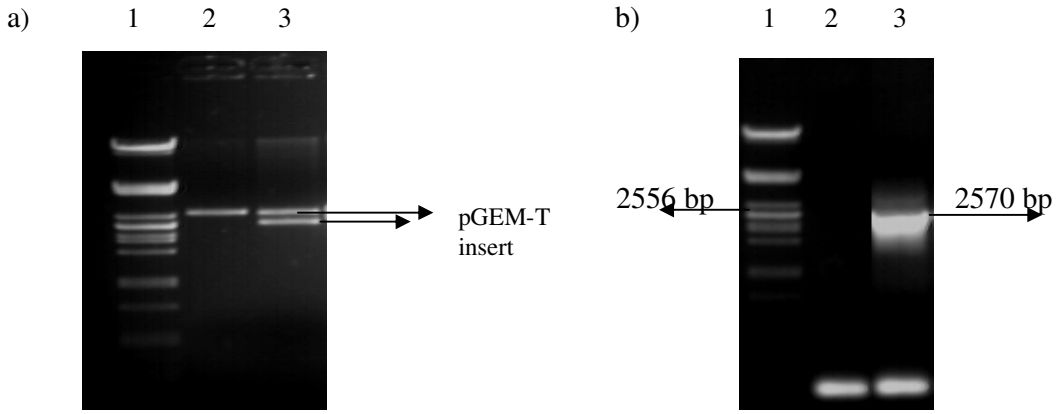


Figure 3.24 a) Double-digested recombinant pGEM-T plasmid carrying 2589 bp PCR product. Lane 1: Marker (Lambda DNA/*Pst*I), Lane 2: Control (pGEM-T vector), Lane 3: Recombinant pGEM-T digested with *Sac*I and *Kpn*I restriction endonucleases, b) PCR product using recombinant pGEM-T as a template. Lane 1: Marker (Lambda DNA/*Pst*I), Lane 2: Negative control (no template), Lane 3: 2589 bp PCR product of *cry*3Aa gene.

Recombinant pGEM-T and pNW33N were isolated from *E. coli* cells for cloning of *cry*3Aa into the shuttle vector pNW33N. Both were digested with *Sac*I and *Kpn*I restriction endonucleases and the digests were run in agarose gel. Linearized pNW33N and *cry*3Aa released from pGEM-T vector were extracted from the gel and the ligation was carried out. The ligation product was used to transform *E. coli* DH5α cells and putative recombinant colonies were selected on 25 $\mu g.mL^{-1}$ chloramphenicol-containing agar plates. The recombinant plasmid of an expected size (6.4 kb) was isolated from one of the transformant colonies (Figure 3. 25). The expected 2576 bp insert was obtained after double digestion of the plasmid with *Sac*I and *Kpn*I (Figure 3.26). Cloning was also verified by PCR amplification (Figure 3.28).

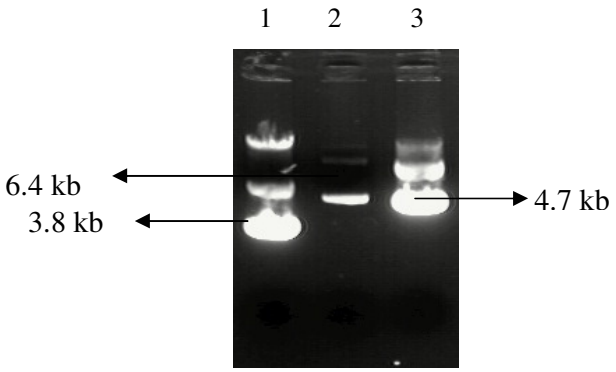


Figure 3.25. pNW33N carrying the *cry3*Aa gene. Lane 1: pNW33N vector, Lane 2: Recombinant pNW33N carrying *cry*3Aa gene, Lane 3: A 4.7 kb closed ccc plasmid for size control.

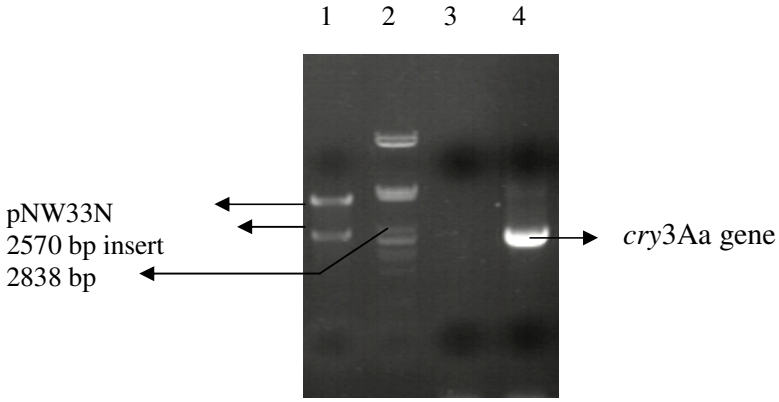


Figure 3.26. Verification of cloning of the *cry*3Aa gene into pNW33N by double digestion with *Sac*I and *Kpn*I restriction enzymes and PCR amplification. Lane 1: Digestion products, Lane 2: Marker (Lambda DNA/*Pst*I), Lane 3: Negative control (no template), Lane 4: 2589 bp PCR product.

3.5.2. Expression of the *cry*3Aa Gene in *Bacillus subtilis* 168

To date, different *cry* genes encoding the insecticidal crystal proteins have been cloned and expressed in various hosts such as *E. coli* (Jahn *et al*., 1989; Gomez *et al.*, 2000), *Bt* (Lereclus *et al.,* 1989), *Pseudomonas cerea* (Stock *et al*., 1990),

Cyanobacteria (Murphy and Stevens, 1992) and *Rhizobium* (Skot *et al*., 1990). Recently, Theoduloz *et al.* (2003) cloned and expressed *cry*1Aa gene in *B. subtilis* and *B. licheniformis* and obtained an expression potency comparable to the source organism.

Our ultimate goal is to introduce the *cry*3Aa gene cloned on multi-copy pNW33N to *Btt* with the aim of obtaining much higher toxin yields or to different subspecies of *Bt* to obtain recombinants with larger activity spectrum, i.e. producing more than one type of δ-endotoxin. In order to reveal if the gene cloned in this study was being expressed in a Gram-positive background, *B. subtilis* was used as a relatively easily transformable intermediate host to receive the recombinant pNW33N carrying the *cry*3Aa. After transformation, putative recombinant colonies were selected on chloramphenicol-containing agar plates. Plasmids isolated from recombinants were used as the templates for a PCR check. Figure 3.27 shows the PCR product of expected size obtained from such a template.

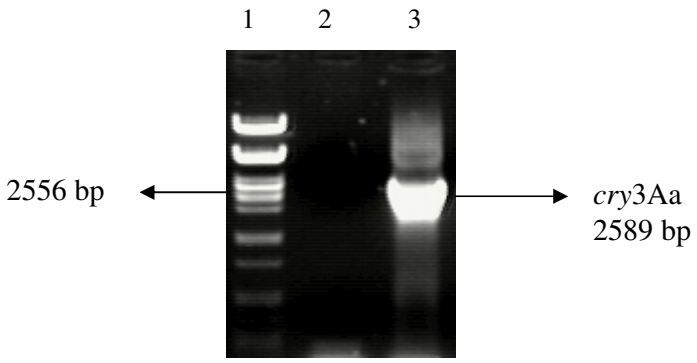


Figure 3.27. PCR product using recombinant *B. subtilis* carrying pNW33N as a template. Lane 1: Marker (Lambda DNA/*Pst*I), Lane 2: Negative control (no template), Lane 3: PCR product (*cry*3Aa gene).

Recombinant and non-recombinant *B. subtilis* 168 and *Btt* local isolate were grown in the modified DSM for three days at 30 °C and 200 rpm. Protein profile of the cells are shown in Figure 3.28. Although the expression level was not as high as that present in the original organism, expression at some degree of *cry*3Aa gene in *B. subtilis* 168 cells seemed to be achieved. Still, heterologous expression of the gene in *B. subtilis* remains to be proven by Northern or Western blots via further experiments. The lower expression level might suggest a less efficient recognition of *cry*3Aa gene promoter in a different *Bacillus* species, but it has to be noted that neither recombinant, nor non-recombinant *B. subtilis* could exhibit a good growth in DSM. Problems in recombinant expression were reported by Ogunjimi *et al.* (2002), with particular emphasis on transient expression, poor functionality and insolubility in heterologous hosts. The very first step in our trouble-shooting will involve a test of alterations of the culture medium for the propagation of the recombinant.

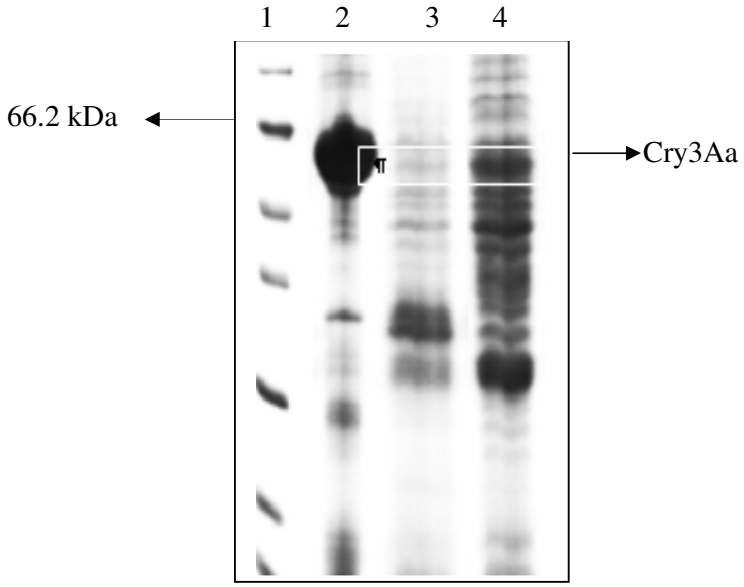


Figure 3.28. Cry3Aa biosynthesis by recombinant *B. subtilis* 168 in DSM after 72 h of incubation. Lane 1: Protein molecular weight marker, Lane 2: Cry3Aa production by the *Btt* local isolate, Lane 3: *B. subtilis* (*Bs*) 168 without cloned *cry*3Aa gene, Lane 4: Recombinant *Bs* 168.

3.5.3. *cry*3Aa Gene Sequence of the Local Isolate

DNA sequence analysis of *cry*3Aa of the local isolate was next performed (Figure 3.29) and the resulting sequence was compared to other Coleoptera-specific *cry* gene sequences in BLAST. The gene starts with 'atg' codon and terminates at position 1935 with a 'taa' stop codon (Figure 3.29). Computer-aided analysis indicated that *cry*3Aa gene product is a 645 residue protein with a calculated molecular mass of 73.125 kDa. The mean A+T content of the gene is 64.24 % which is quite close to the value 64.6% for ORFs recently reported for *Bt* 97-27 genome (GenBank, unpublished).

DNA sequence of the *cry*3Aa gene of *Btt* local isolate was compared with the *cry*3Aa sequences of 10 different quaternary ranks known so far and obtained from *Bt* YM-03, *Bt* UD-001, *Bt* 22, *Bt* 886, *Bt* var. *tenebrionis*, *Bt san diego*(BGSC No. 4AB1), *Btt* (BGSC No. 4AA1), *Btt* NB176, *Bt morrisoni* EG2158 and and an undesignated *Bt* strain, respectively. Its identity to these sequences was found as 99.6 %, 98.7 %, 99.7 %, 99.4 %, 99.7 %, 99.7 %, 99.7 %, 99.7 %, 99.7 % and 99.7 %, respectively. An alignment of our Cry3Aa δ-endotoxin sequence with the sequences of these Cry3Aa proteins is shown in Figure 3.30. Amino acid sequence of Cry3Aa of the local isolate showed 99.1 %, 97.4 %, 99.2 %, 98.6 %, 99.2 %, 99.2 %, 99.1 %, 99.2 %, 99.2 % and 99.2 % identity to the proteins encoded by these genes, respectively (Table 3.2 a). Alignment of Cry3Aa protein of the *Btt* local isolate revealed that the differences in amino acid sequence did not correspond to the five conserved blocks found in Cry3A protein (Figure 3.29). Of the strains used in comparison, *Btt* (BGSC No. 4AA1) and *Bt san diego* (BGSC No. 4AB1) were among the strains compared for their protein profiles to the local isolate (Section 3.4). The total protein profile of this strain was not quite similar to that of our isolate in spite of 99.1 % and 99.2 % identities found in the structure of Cry3Aa from these organisms (Figure 3.20). Table 3.2. b shows amino acids differed in Cry3A protein of the *Btt* local isolate and their positions.

As mentioned earlier (Chapter 1), in choosing locations for rank boundaries for cry genes, Crickmore *et al.* (1998) attempted to construct a nomenclature reflecting

ATGAATCCGAACAATCGAAGTGAACATGATACAATAAAAACTACTGAAAATAATGAGGTGCCAACTAACCATGTTCAATATCCT
M N P N N R S E H D T I K T T E N N E V P T N H V Q Y P

TTAGCGGAAACTCCAAATCCAACACTAGAAGATTTAAATTATAAAGAGTTTTTAAGAATGACTGCAGATAATAATACGGAAGCA
L A E T P N P T L E D L N Y K E F L R M T A D N N T E A

CTAGATAGCTCTACAACAAAAGATGTCATTCAAAAAGGCATTTCCGTAGTAGGTGATCTCCTAGGCGTAGTAGGTTTCCCGTTT
L D S S T T K D V I Q K G I S V V G D L L G V V G F P F

GGTGGAGCGCTTGTTTCGTTTTATACAAACTTTTTAAATACTAGTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTATGGAACAA
G G A L V S F Y T N F L N T S W P S E D P W K A F M E Q

GTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTCTTGCAGAGTTACAGGGCCTTCAAAATAATGTC
V E A L M D Q K I A D Y A K N K A L A E L Q G L Q N N V

GAAGATTATGTGAGTGCATTGAGTTCATGGCAAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAG
E D Y V S A L S S W Q K N P V S S R N P H S Q G R I R E

CTGTTTTCTCAAGCAGAAAGTTATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACGAGGTTCTATTTCTAACAACA
L F S Q A E S Y F R N S M P S T A I S G Y E V L F L T T

TATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAGACGCTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGAT
Y A Q A A N T H L F L L K D A Q I Y G E E W G Y E K E D

ATTGCTGAATTTTATAAAAGACAACTAAAACTTACGCAAGAATATACTGACCATTGTGTCAAATGGTATAATGTTGGGTTAGAT
I A E F Y K R Q L K L T Q E Y T D H C V K W Y N V G L D

AAATTAAGAGGTTCATCTTATGAATCTTGGGTAAACTTTAACCGTTATCGCAGAGAGATGACATTAACAGTATTAGATTTAATT
K L R G S S Y E S W V N F N R Y R R E M T L T V L D L I

GCACTATTTCCATTGTATGATGTTCGGCTATACCCAAAAGAAGTTAAAACCGAATTAACAAGAGACGTTTTAACAGATCCAATT
A L F P L Y D V R L Y P K E V K T E L T R D V L T D P I

GTCGGAGTCAACAACCTTAGGGGCTATGGAACAACCTTCTCTAATATAGAAAATTATATTCGAAAACCACATCTATTTAACTAT
V G V N N L R G Y G T T F S N I E N Y I R K P H L F N Y

CTGC**G**TAGAATTCAATTTCACACGCGGTTCCAACCAGGATATTATGGAAATGACTCTTTCAATTATTGGTCCGGTAATTATGTT
L R R I Q F H T R F Q P G Y Y G N D S F N Y W S G N Y V

TCAACTAGACCAAGCATAGGATCAAATGATATAATCACATCTCCATTCTATGGAAATAAATCCAGTGAACCTGTACAAAATTTA
S T R P S I G S N D I I T S P F Y G N K S S E P V Q N L

GAATTTAATGGAGAAAAAGTCTATAGAGCCGTAGCAAATACAAATCTTGCGGTCTGGCCGTCCGCTGTATATTCAGGTGTTACA
E F N G E K V Y R A V A N T N L A V W P S A V Y S G V T

AAAGTGGAATTTAGCCAATATAATGATCAAACAGATGAAGCAAGTACACAAACGTACGACTCAAAAAGAAATGTTGGCGCGGTC
K V E F S Q Y N D Q T D E A S T Q T Y D S K R N V G A V

AGCTGGGATTCTATCGATCAATTGCCTCCAGAAACAACAGATGAACCTCTAGAAAAGGGATATAGCCATCAACTCAATTATGTA
S W D S I D Q L P P E T T D E P L E K G Y S H Q L N Y V

ATGTGCTTTTTAATGCAGGGTAGTAGAGGAACAATCCCAGTGTTAACTTGGACACATAAAAGTGTAGACTTTTTTAACATGATT
M C F L M Q G S R G T I P V L T W T H K S V D F F N M I

GATTCGAAAAAAATTACACAACTTCCGTTAGTAAAGGCATATAAGTTACAATCTGGTGCTTCCGTTGTCGCAGGTCCTAGGTTT
D S K K I T Q L P L V K A Y K L Q S G A S V V A G P R F

ACAGGAGGAGATATCATTCAATGCACAGAAAATGGAAGTGCGGCAACTATTTACGTTACACCGGATGTGTCGTACTCTCAAAAA
T G G D I I Q C T E N G S A A T I Y V T P D V S Y S Q K

TATCGAGCTAGAATTCATTATGCTTCTACATCTCAGATAACATTTACACTCAGTTTAGACGGGGCACCATTTAATCAATACTAT
Y R A R I H Y A S T S Q I T F T L S L D G A P F N Q Y Y

TTCGATAAAACGATAAATAAAGGAGACACATTAACGTATAATTCATTTAATTTAGCAAGTTTCAGCACACCATTCGAATTATCA
F D K T I N K G D T L T Y N S F N L A S F S T P F E L S

GGGAATAACTTACAAATAGGCGTCACAGGATTAAGTGCTGGAGATAAAGTTTATATAGACAAAATTGAATTTATTCCAGTGAAT
G N N L Q I G V T G L S A G D K V Y I D K I E F I P V N

TAA
.

Figure 3.29. Nucleotide and deduced amino acid sequence of *cry*3Aa gene of the local isolate. Start and stop codons are in bold face. onserved blocks are underlined.

significant evolutionary relationships while at the same time minimizing changes from the gene names assigned under the old system. In the resulting system, proteins with a common primary rank are similar enough that the percent identity can be defined with some confidence. Proteins with the same primary rank often affect the same order of insect; those with different secondary and tertiary ranks may have altered potency and targeting within an order. At the tertiary rank, differences can be due to the accumulation of dispersed point mutations, but often they appear to have resulted from ancestral recombination events between genes differing at a lower rank level. The quaternary rank was established to group "alleles" of genes coding for known toxins that differ only slightly, either because of a few mutational changes or an imprecision in sequencing. Any *cry* gene specified with a quaternary rank is a natural isolate. No assumption about functionality is implied by the presence of this rank number in the gene name. In contrast, an allele number would be assumed, unless parenthetical or subscripted information indicated otherwise, to denote a nonfunctional mutant form of a wild-type gene found at a discrete genetic locus. Currently the boundaries represent approximately 95, 78, and 45% sequence identity.

A *B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee, consisting of the authors of above-mentioned paper, has been the standing committee of the *Bacillus* Genetic Stock Center (BGSC) to assist workers in the field of *B. thuringiensis* genetics in assigning names to new Cry and Cyt toxins. The corresponding gene or protein sequences are first deposited into a publicly accessible database (GenBank, EMBL, or PIR) and released by the repository for electronic publication in the database so that the scientific community may conduct an independent analysis. Researchers should submit new sequences directly to the BGSC director (D. R. Zeigler) who will analyze the amino acid sequence and suggest the appropriate name, subject to the approval of the committee. The committee periodically review the literature of the Cry and Cyt toxins and publish a comprehensive list. This list, alongside other relevant information, will also be available via the Internet at the following URL: http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/. The quaternary ranks are

assigned in the order that the gene sequences were discovered in the literature or submitted to the committee. Genes assigned the quaternary rank 1 represent holotype sequences.

Accordingly, as a new toxin, the sequence of Cry3Aa of our local isolate was submitted to both GenBank (Accession number AY882576) and BGSC by our laboratory. The protein will take its exact designation after an appropriate quaternary rank will be approved by the committee.

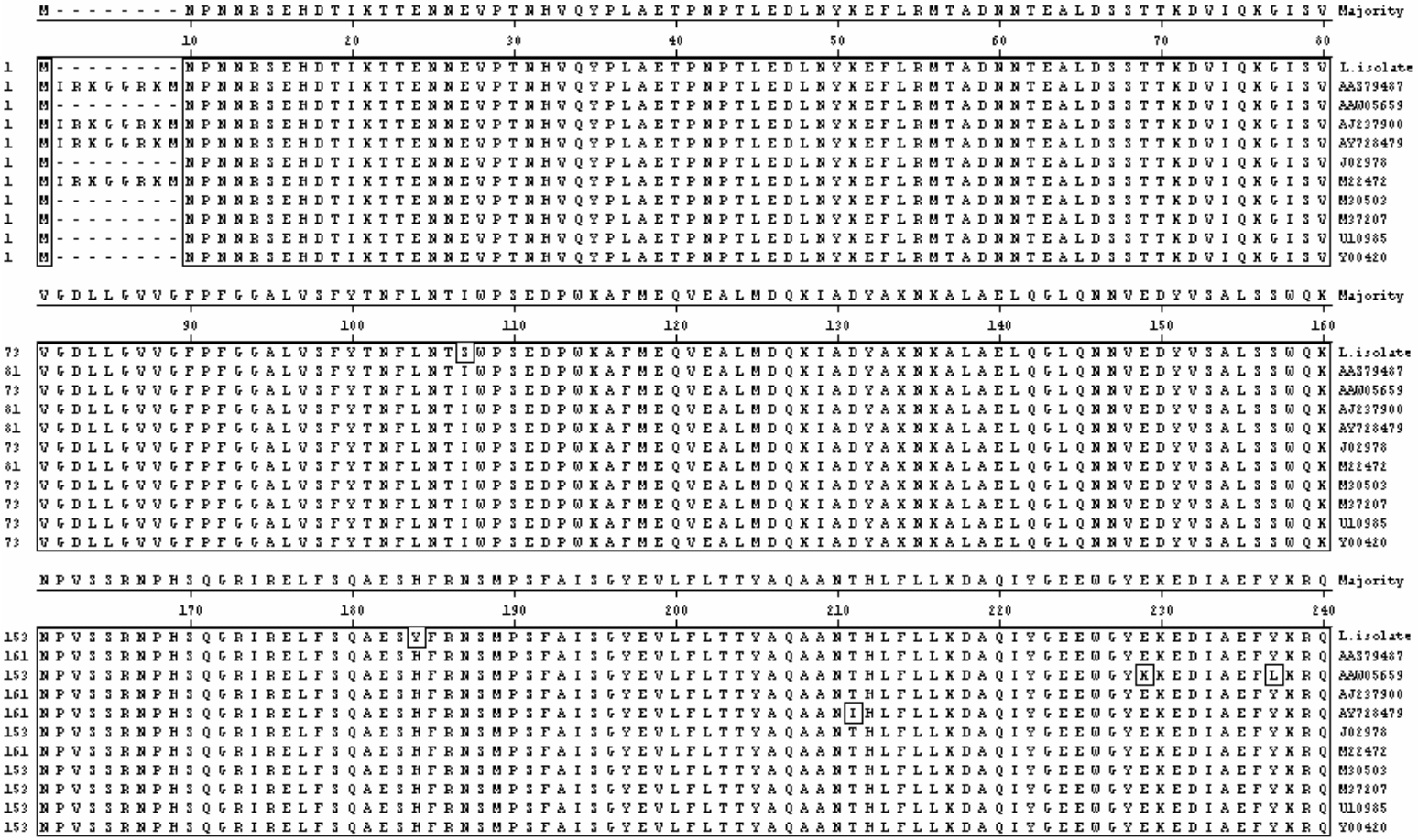


Figure 3.30. Alignment of Cry3Aa δ-endotoxin sequence with those of other Coleoptera-active *Bt* isolates.

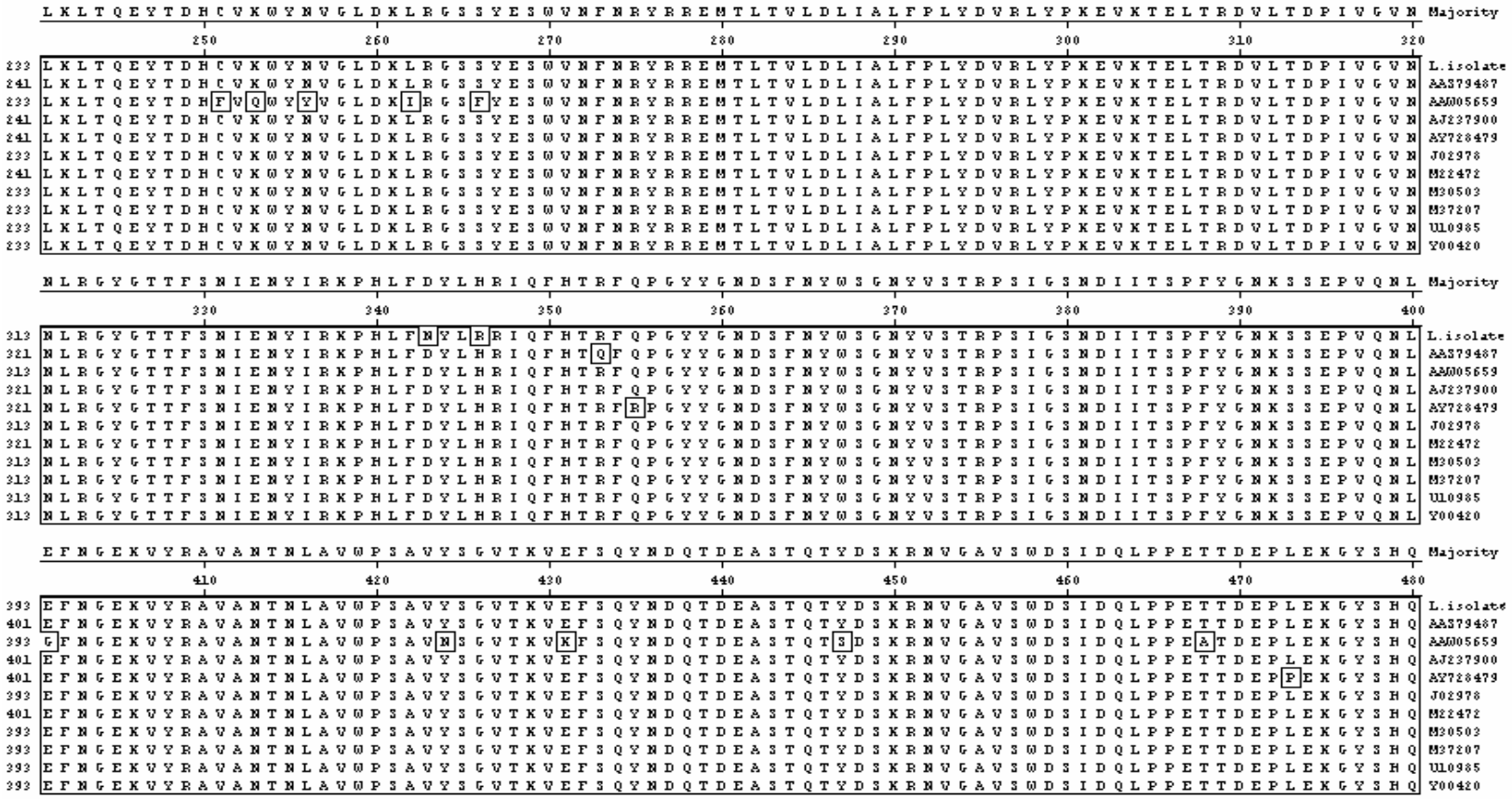


Figure 3.30. (continued)

Figure 3.30. (continued)

Table 3.2 a Percent similarity and divergence of anti-Coleopteran Cry3Aa δ-endotoxin of *Btt* local isolate from other Coleoptera-specific Cry3Aa producer *Bt* strains, 1. L. isolate: The local isolate of *Btt*, Cry3Aa producer. 2. AAS79487: *Bt* YM-03, Cry3Aa8 producer. 3. AAW05659: *Bt* UD-001, Cry3Aa9 producer. 4. AJ237900: *Bt* 22, Cry3Aa7 producer. 5. AY728479: *Bt* 886, Cry3Aa10 producer. 6. J02978: *Bt* var. *tenebrionis*, Cry3Aa2 producer. 7. M22472: *Bt san diego* (BGSC No. 4AB1), Cry3Aa1 producer. 8. M30503: *Btt* (BGSC No. 4AA1), Cry3Aa4 producer. 9. M37207: *Bt morrisoni* EG2158, Cry3Aa5 producer. 10. U10985: *Btt* NB176, Cry3A6 producer. 11. Y00420: *Bt*, Cry3Aa3 producer. **b** Amino acid (aa) replacements in Cry proteins of *Bt* strains

a)

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		99.1	97.4	99.2	98.6	99.2	99.2	99.1	99.2	99.2	99.2	1	L.isolate
2	0.8		97.8	99.7	99.1	99.7	99.7	99.5	99.7	99.7	99.7	2	AAS79487
3	2.5	2.0		98.0	97.4	98.0	98.0	97.8	98.0	98.0	98.0	3	AAW05659
4	0.6	0.2	1.9		99.2	99.8	99.8	99.7	99.8	99.8	99.8	4	AJ237900
5	1.3	0.8	2.5	0.6		99.2	99.2	99.1	99.2	99.2	99.2	5	AY728479
6	0.6	0.2	1.9	0.0	0.6		99.8	99.7	99.8	99.8	99.8	6	J02978
7	0.6	0.2	1.9	0.0	0.6	0.0		99.7	99.8	99.8	99.8	7	M22472
8	0.8	0.3	2.0	0.2	0.8	0.2	0.2		99.7	99.7	99.7	8	M30503
9	0.6	0.2	1.9	0.0	0.6	0.0	0.0	0.2		99.8	99.8	9	M37207
10	0.6	0.2	1.9	0.0	0.6	0.0	0.0	0.2	0.0		99.8	10	U10985
11	0.6	0.2	1.9	0.0	0.6	0.0	0.0	0.2	0.0	0.0		11	Y00420
	1	2	3	4	5	6	7	8	9	10	11		

b) Amino acid (aa) replacements in Cry proteins of *Bt* strains.

Aa position (Local isolate → *Bt* YM-3)	Aa position (Local isolate → *Bt* UD-001)	Aa position (Local isolate → *Bt* 22)	Aa position (Local isolate → *Bt* 886	Aa position (Local isolate → *Bt* var. *tenebrionis*)
2 (-→I) 3 (-→R) 4 (-→K) 5 (-→G) 6 (-→G) 7 (-→R) 8 (-→K) 9 (-→M) 107 (S→I) 184 (Y→H) 343 (N→D) 346 (R→H) 353 (R→Q)	107 (S→I) 184 (Y→H) 229 (E→K) 237 (Y→L) 251 (C→F) 253 (K→Q) 256 (N→Y) 262 (L→I) 266 (S→F) 343 (N→D) 346 (R→H) 401 (E→G) 424 (Y→N) 431 (E→K) 447 (Y→S) 468 (T→A)	2 (-→I) 3 (-→R) 4 (-→K) 5 (-→G) 6 (-→G) 7 (-→R) 8 (-→K) 9 (-→M) 107 (S→I) 184 (Y→H)	2 (-→I) 3 (-→R) 4 (-→K) 5 (-→G) 6 (-→G) 7 (-→R) 8 (-→K) 9 (-→M) 107 (S→I) 184 (Y→H) 211 (T→I) 343 (N→D) 346 (R→H) 355 (Q→R) 473 (L→P) 569 (Y→H)	107 (S→I) 184 (Y→H) 343 (N→D) 346 (R→H)
Aa position (Local isolate → *Bt san diego*)	Aa position (Local isolate → *Btt*, BGSC No.4AA1)	Aa position (Local isolate → *Bt morrisoni* EG2158	Aa position (Local isolate → *Bt tenebrionis* NB176)	Aa position (Local isolate → *Bt*)
2 (-→I) 3 (-→R) 4 (-→K) 5 (-→G) 6 (-→G) 7 (-→R) 8 (-→K) 9 (-→M) 107 (S→I) 184 (Y→H) 343 (N→D) 346 (R→H)	107 (S→I) 184 (Y→H) 343 (N→D) 346 (R→H) 552 (G→A)	107 (S→I) 184 (Y→H) 343 (N→D) 346 (R→H)	107 (S→I) 184 (Y→H) 343 (N→D) 346 (R→H)	107 (S→I) 184 (Y→H) 343 (N→D) 346 (R→H)

CHAPTER 4

CONCLUSION

- Both the time of appearance of Cry3Aa protein and its production level greatly varied in different media. HCT and GYS media were not suitable at all for Cry3Aa production. C2 medium was fairly good and DSM appeared to be the best one for an early onset of Cry3Aa biosynthesis as well as its high yields. The organism produced some toxin in this medium even at 24^{th} h of incubation, its biosythetic capacity increased with time and lasted throughout the course of incubation. When grown in C2 medium, toxin yields were much lower than that in DSM except for 120^{th} h when the amounts of toxins produced in two media appeared almost equal. For the rest of the study, DSM and 72 h were chosen as the standard medium and incubation time, respectively.

- When tested at 5 $g.L^{-1}$; 7.5 $g.L^{-1}$; 10 $g.L^{-1}$ concentrations, increased concentrations of both sugars increased growth and decreased sporulation. The increasing concentrations of both sugars suppressed toxin biosynthesis and the highest Cry3Aa production and also high sporulation frequency were observed in the medium containing glucose or sucrose at a concentration of 5 $g.L^{-1}$.

- Inorganic phosphate was the most crucial nutrient affecting anti-Coleopteran Cry3Aa production. When grown in DSM containing different concentrations of inorganic phosphate ranging from 5 mM to 200 mM, growth was the best at 20 mM and slightly decreased at a high inorganic phosphate concentration of 200 mM. Both sporulation and Cry3Aa production significantly increased

with this highest concentration of inorganic phosphate.

- Except for ammonium sulphate plus Casamino acids combination, each combination of nitrogen sources used to replace nutrient broth contained in DSM resulted in a poorer growth. Cry3Aa production, on the other hand, was greatly reduced with all different combinations. There was no consistency between the levels of sporulation and toxin formation for the nitrogen sources tested.

- Varying $MnCl_2$ concentration in the medium between 10^{-4} and 10^{-7} M did not affect the growth of the cells remarkably. On the other hand, toxin yield decreased noticeably in the presence of 10^{-4} M $MnCl_2$ and the highest Cry3Aa production was observed with 10^{-5} to 10^{-7} M $MnCl_2$. The highest sporulation frequency was obtained with 10^{-5} M $MnCl_2$, the concentration originally contained in growth medium.

- Cry3Aa production slightly decreased with increasing concentrations of $MgSO_4$. The highest toxin production was detected with 5×10^{-4} M $MgSO_4$, the amount originally found in DSM, and increasing or decreasing concentrations adversely affected the production. Growth was adversely affected by a high concentration of 10^{-2} M $MgSO_4$ and sporulation frequency was also found to decrease with increasing $MgSO_4$ concentrations.

- The onset of growth of our *Btt* isolate was delayed in a medium containing no $FeSO_4$. The concentration in the control medium (10^{-5}) was the optimal for sporulation and Cry3Aa biosynthesis and as this concentration decreased or iron was omitted from the medium, the levels of toxin also decreased.

- As revealed by optimization studies, there was no direct correlation between toxin production and efficiency of sporulation. High toxin yield paralleled high sporulation frequency for Mg, Fe, sugar and inorganic phosphate levels,

but not for Mn levels and the sources of nitrogen.

- In modified DSM containing 200 mM inorganic phosphate, Cry3Aa toxin production increased from 3 $\mu g.mL^{-1}$ to 15.6 $\mu g.mL^{-1}$, corresponding to a 5 fold increase in productivity as compared to the control culture. When $FeSO_4$ was omitted from the medium, toxin production decreased from 15.6 $\mu g.mL^{-1}$ to 8.99 $\mu g.mL^{-1}$ (a 1.7 fold decrease). When inorganic phosphate concentration was lowered to 100 mM in the presence of iron, the toxin level decreased to 5 $\mu g\ mL^{-1}$ which corresponded to a 3 fold decrease, further confirming the significant role of inorganic phosphate.

- 15 of the strains performed much better than our *Btt* local isolate when 31 different Coleoptera-toxic strains were compared in terms of Cry3Aa toxin production. Most of the strains producing this protein gave protein banding patterns quite similar to that of our local isolate, however protein profiles of certain strains were quite different. There was no relation between the toxin yields and the cell masses of the strains examined.

- The *Btt* local isolate have two large plasmids of 135 and 61 kb and two small plasmids of 1.8 and 1.5 kb. However, an additional large plasmid of 34 kb is harbored by some of the other anti-Coleopteran *Bt* strains that had similar protein profiles.

- Two other Coleoptera-toxic strains gave the same kind of responses to inorganic phosphate, but not to the omission of iron, suggesting that iron effect is rather unique to our isolate whereas inorganic phosphate effect can be common to the biosynthesis of Cry3Aa type toxin.

- Cloning, sequencing and computer-aided analysis of *cry*3Aa gene indicated that its product is a 645 residue protein with a calculated molecular mass of 73.125 kDa. The mean A+T content of the gene is 64.24 % which is quite close to the value 64.6 % for ORFs recently reported for *Bt* 97-27 genome.

DNA sequence of the *cry*3Aa gene of *Btt* local isolate was compared with the *cry*3Aa sequences of 10 different quaternary ranks known so far and obtained from *Bt* YM-03, *Bt* UD-001, *Bt* 22, *Bt* 886, *Bt* var. *tenebrionis*, *Bt san diego*(BGSC No. 4AB1), *Btt* (BGSC No. 4AA1), *Btt* NB176, *Bt morrisoni* EG2158 and and an undesignated *Bt* strain, respectively. Its identity to these sequences was found as 99.6 %, 98.7 %, 99.7 %, 99.4 %, 99.7 %, 99.7 %, 99.7 %, 99.7 %, 99.7 % and 99.7 %, respectively. In the toxin by our local isolate, the amino acid variation ranged between 4 to 16.

- The extent of amino acid variation present in the toxin of the isolate is sufficient to put it into a new quaternary rank. As a new toxin, we submitted the sequence of Cry3Aa of our local isolate to both GenBank (Accession number AY882576) and BGSC. The protein will take its exact designation after an appropriate quaternary rank will be approved by the "*B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee".

- The cloned *cry*3Aa gene can be used with the aim of toxin overproduction or to obtain recombinants with larger activity spectrum, after its expression at a high level is achieved in bacilli.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

C2 Medium

	$g.L^{-1}$
Glucose	10
Peptone	2
NZ amine (a casein hydrolysate)	5
Yeast extract	2
$(NH_4)_2SO_4$	1.98 (15 mM)
KH_2PO_4	3.13 (23 mM)
K_2HPO_4	3.48 (27 mM)
$MgSO_4.7H_2O$	0.25 (1 mM)
$CaCl_2$	0.088 (600 µM)
$MnCl_2$	0.0404 (250 µM)
$ZnSO_4.7H_2O$	0.00489 (17 µM)
$CuSO_4.5H_2O$	0.00425 (17 µM)
$FeSO_4.7H_2O$	0.00056 (2 µM)

The pH was adjusted to 7.2 and the medium was sterilized at 121 ° C for 15 minutes.

DSM

	$g.L^{-1}$
Nutrient Broth	4
Glucose	5

K_2HPO_4	4.3545 (25 mM)
KH_2PO_4	3.4045 (25 mM)
$Ca(NO_3)_2$	0.118 (0.5 mM)
$MgSO_4$	0.0602 (0.5 mM)
$FeSO_4$	0.00278 (10 µM)
$MnCl_2$	0.00162 (10 µM)

The pH was adjusted to 7.2 and the medium was sterilized at 121 °C for 15 minutes.

GYS Medium

	$g.L^{-1}$
Glucose	1
Yeast extract	2
K_2HPO_4	0.05
$(NH_4)_2SO_4$	0.2
$MgSO_4$	0.02
$MnSO_4$	0.05
$CaCl_2$	0.08

The pH was adjusted to 7.2 and the medium was sterilized at 121 °C for 15 minutes.

HCT Medium

	$g.L^{-1}$
Casein hydrolysate (vitamin free)	7
Glucose	3
KH_2PO_4	6,8
$MgSO_4.7H_2O$	0,12
$MnSO_4.7H_2O$	0,0022
$ZnSO_4.7H_2O$	0,014
$Fe_2(SO_4)_3$	0,02
$CaCl_2.4H_2O$	0,18

The pH was adjusted to 7.2 and the medium was sterilized at 121 °C for 15 minutes.

10X-S-Base

$(NH_4)_2.SO_4$	20 g
$K_2HPO_4.3H_2O$	140 g
KH_2PO_4	60 g
Na_3citrate, $2H_2O$	10 g
dH_2O	1 L

They were autoclaved together and allowed to cool to 50 °C and then supplemented with 1 mL of sterile 1 M $MgSO_4$.

HS-Medium

10X-S-Base	10 mL
Glucose, 50 % (w/v)	1 mL
Yeast extract, 10 % (w/v)	1 mL
Casamino acid, 2 % (w/v)	1 mL
Arginine 8 % (w/v) + Histidine 0.4 % (w/v)	10 mL
Tryptophane 0.5 % (w/v)	1 mL
Phenylalanine 0.3 % (w/v)	1.5 mL

Total volume was completed to 100 mL with sterile water.

LS-Medium

10X-S-Base	2 mL
Glucose, 50 % (w/v)	0.2 mL
Tryptophane, 0.5 % (w/v)	20µL
Phenylalanine, 0.3 % (w/v)	30 µL
Casamino acid, 2 % (w/v)	0.1 mL
Yeast exract, 10 % (w/v)	0.2 mL
50 mM Spermidine	0.2 mL

1 M $MgCl_2$,	50 µL

Total volume was made up to 20 mL with sterile water.

Luria Broth

	$g.L^{-1}$
Luria Broth	25

Sterilized at 121 ℃ for 15 minutes

Nutrient Agar

	$g.L^{-1}$
Nutrient Broth	8
Agar	10

Sterilized at 121 ° C for 15 minutes

Luria Broth Agar

	g.L-1
Luria Broth	25
Agar	15

Sterilized at 121 ° C for 15 minutes

APPENDIX B

SOLUTIONS AND BUFFERS

Acrylamide/Bis

Acrylamide	146 g
N.N'-Methylene-bis Acrylamide	4 g

Distilled water to 500 mL. Filtered and stored at 4 °C. Protected form light.

Tris HCl (1.5 M)

Tris base	54.45 g
dH_2O	150 mL

pH is adjusted to 8.8 with HCl, distilled water to 300 mL and stored at 4 °C.

Tris HCl (0.5 M)

Tris base	6 g
dH_2O	60 mL

pH is adjusted to 6.8 with HCl, distilled water to 100 mL and stored at 4 °C.

Tris-EDTA Buffer (TE) (Maniatis, 1989)

Tris	10 mM
EDTA	1 mM

pH is adjusted to 8.0 with HCl.

Sample Buffer

dH_2O	3 mL
Tris HCl (0.5 M)	1 mL
Glycerol	1.6 mL
SDS (10%)	0.4 mL
β- mercaptoethanol	0.4 mL
Bromophenol blue (0.5%, w/v) (in water)	0.4 mL

Running Buffer (5X)

Tris base	15 g
Glycine	72 g
SDS	5 g

Distilled water to 1 L. Stored at 4 °C.

Phenol-Chloroform Solution (water-saturated, Hintermann, 1981)

Phenol	500 g
Chloroform	500 mL
Distilled water	400 mL

The solution was stored at room temperature, protected from light.

Tris-Acetate-EDTA Buffer (TAE) (50 X)

Tris Base	242 g
Glacial Acetic Acid	57.1 mL
EDTA (0.4 M, pH 8.0)	125 mL

Distilled water added to 100 mL

Tris-EDTA Buffer (TE)

Tris-HCl (1 M, pH 8.0)	10 mL
EDTA (0.4 M, pH 8.0)	5 mL
Distilled water added to 1000 mL	

TBE Buffer (Maniatis, 1989)

Tris base	0.089 M
Boric acid	0.89 M
EDTA	2 mM
pH 8.0-8.3	

Total DNA Isolation

Solution I

TGE buffer, pH 8.0	
Lysozyme	10 $mg.mL^{-1}$
RNase	10 $mg.mL^{-1}$

Solution II

NaOH	1 N
SDS	1 %

Solution III

Potassium acetate	5 M
pH 4.55	

CTAB/NaCl Solution (10 % CTAB/ 0.7 M NaCl)

4.1 g of NaCl was dissolved in 80 mL of dH_2O. Then, 10 g of CTAB (hexadecyltrimethyl ammonium bromide) was added and dissolved with vigorously shaking and gentle heating up to 65 ° C. Final volume was made up to 100 mL with dH_2O.

X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside)

X-Gal	20 mg
Dimethylformamide	1 mL

The solution was stored at –20 ° C protected from light.

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
Distilled water	1 mL

The solution was filter sterilized and stored at –20 ° C.

Coomassie Blue R-250 Staining

Coomassie blue R-250	0.25 g
Methanol	125 mL
Glacial Acetic acid	25 mL
dH_2O	100 mL

Destaining Solution

Methanol	100 mL
Glacial Acetic acid	100 mL
dH_2O	800 mL

APPENDIX C

CHEMICALS AND THEIR SUPPLIERS

Chemicals

Acrylamide	Merck
Agarose	Prona
$AgNO_3$	Merck
Ammonium persulphate	AppliChem
Ampicillin	Sigma
Bovine Serum Albumin	Sigma
Bromophenol blue	Sigma
$CaCl_2.2H_2O$	Merck
$Ca(NO_3)_2.4\ H_2O$	Merck
Casamino acid	AppliChem
Casein hydrolysate (vitamin free)	Fluka
Chloramphenicol	Sigma
Chloroform	Merck
Coomassie Brillant Blue G-250	Merck
Coomassie Brillant Blue R-250	Sigma
CTAB	Sigma
Dimethylformamide	Merck
EDTA	AppliChem
Ethanol	Botafarma
Ethidium bromide	Sigma

$FeSO_4.7H_2O$	Sigma
$Fe_2(SO_4)_3$	Sigma
Formaldehyde	Merck
Glacial Acetic acid	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
HCl	Merck
IPTG	Sigma
Isoamyl alocohol	Merck
Isopropanol	Merck
K-Acetate	Merck
KH_2PO_4	Merck
K_2HPO_4	Merck
L-amino acids	Sigma
Luria Broth	Q-Biogene
Mercaptoethanol	Sigma
Methanol	Merck
$MgSO_4$	Sigma
$MgSO_4.7H_2O$	Merck
$MnSO_4.7H_2O$	Carlo Erba
$MnCl_2.2\ H_2O$	Merck
NaCl	Merck
NaOH	Merck
Na_2CO_3	Merck
Na_3citrate, $2H_2O$	Merck
N, N'-Methylene bis-acrylamide	Sigma
Nutrient broth	Merck
Nutrient Agar	Merck
Peptone	Sigma
Phenol-chloroform-isoamylalcohol	Sigma

Phenol	Merck
Spermidine	Sigma
Sodium thiosulphate	Carlo Erba
Sucrose	Merck
SDS	Merck
TEMED	Sigma
Tris Base	Merck
Tris-HCl	Merck
X-Gal	Sigma
Yeast extract	Difco
$ZnSO_4.7H_2O$	Sigma

Enzymes

*Kpn*I	MIB Fermantas
L buffer	Roche
Lysozyme	Q-Biogene
Proteinase K	Sigma
RNase A	Sigma
*Sac*I	MIB Fermantas
Taq DNA polymerase	MIB Fermentas

Size Markers

***Pst*I digested /Lambda DNA** MBI Fermentas

The DNA Marker yields the following 29 discrete fragments (in base pairs): 11501, 5077, 4749, 4507, 2838, 2556, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 82, 15.

Protein molecular weight marker MIB Fermentas

β-galactosidase (116.2 kDa), bovine serum albumin (66.2kDa), ovalbumin (45 kDa), lactate dehydrogenase (35kDa), restriction endonuclease *Bsp*981 (25 kDa),

β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) were used as the size markers.

Kits

DNA Extraction Kit	Qiagen
Plasmid Isolation Kit	Qiagen
p-GEM T Vector	Promega