

**Isolation of a *Bacillus thuringiensis* strain  
from South African soils and the  
characterization of its *cry* gene sequence**

**Neil Laridon**

**Supervised by Mrs Jean Mollett and Dr Vincent Gray**

School of Molecular and Cell Biology, University of the Witwatersrand.

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Masters of Science.

Johannesburg 2006

# Abstract

NEIL LARIDON, JEAN MOLLETT and VINCENT GRAY

The objectives of this project were to isolate and characterize a *Bacillus thuringiensis* strain from South African soils, determine its *cry* gene sequence, clone this gene sequence and determine its toxicity. Forty four putative *Bacillus thuringiensis* strains were extracted from soil samples taken from the Muldersdrift mountain range, the Roodekrans botanical gardens, Southbroom in Kwazulu Natal and Nelspruit in eastern Mpumalanga province. The bacterial populations of these soil samples were isolated and classified using different microbial, and biochemical techniques including sodium acetate tests to isolate putative *B. thuringiensis* spores. These spores were cultured and further characterized through colony shape and colour as well as the presence of  $\delta$ -endotoxin crystals. Once characterized, DNA was extracted from the isolates using an array of techniques to obtain high quality template DNA. This DNA was then screened via PCR using truncated versions of the *cryIA* specific primers TYIAA (f) and TYIUN12 (r). The insecticidal protein CRY1A was selected for this study since it is specific and highly toxic to lepidopteron insects. Homology to the *cryIA* gene was detected in six of the *Bacillus* strains analyzed, namely S4, S9, S10 n1, n3 and n5. PCR products were cloned into the pTZ57R/T cloning vector and transformed into JM109 competent cells. DNA from the six isolates was also characterized at the 16S rDNA level with the use of PCR. Primers capable of amplifying nearly full-length 16S ribosomal DNA (approximately 1,500-bp) fragments from many bacterial genera confirmed that the isolates were indeed *Bacillus thuringiensis*, showing evidence of lineage according to the signature sequences within the conserved regions. Spore/ $\delta$ -endotoxin mixtures of the randomly selected isolate S10 were used in a bioassay to test their toxicity against the lepidopteron insect *Galleria mellonella*. No significant mortalities were reported, but sensitivity to the S10  $\delta$ -endotoxin was evident when compared to results using known *B. thuringiensis*  $\delta$ -endotoxins at the same concentrations.

# Declaration

This research is being submitted to the University of the Witwatersrand in partial fulfillment of the degree of B. Sc. (Masters), 2006. I hereby declare that this is my own original, unaided work and has not been presented for any other degree, examination or research purpose.

---

Neil Edward Laridon

December 2006

# Acknowledgements

My sincere appreciation and gratitude goes to the people who have contributed to this research:

Mrs. Jean Mollett for her time, support and guidance.

Special thanks to Dr. Vince Gray for his invaluable advice, guidance and inspiration.

All the members of the Nematode lab, for their kindness, support and assistance.

Finally to my family and friends, for their love support and advice.

# Table of contents

<b>ABSTRACT</b> .....	<b>2</b>
<b>DECLARATION</b> .....	<b>3</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>4</b>
<b>TABLE OF CONTENTS</b> .....	<b>5</b>
<b>LIST OF FIGURES</b> .....	<b>8</b>
<b>LIST OF TABLES</b> .....	<b>10</b>
<b>LIST OF ABBREVIATIONS</b> .....	<b>11</b>
<b>A LITERATURE REVIEW</b> .....	<b>12</b>
1.1 <i>BACILLUS THURINGIENSIS</i> : NATURE’S DESIGN FOR AN INDUSTRIAL POWERHOUSE .....	12
1.2 <i>BACILLUS THURINGIENSIS</i> IN NATURE.....	14
1.3 THE $\Delta$ -ENDOTOXIN MODE OF ACTION .....	19
1.4 THE INSECTICIDAL CRYSTAL PROTEINS (ICP).....	21
1.5 THE <i>CRY</i> GENE FAMILY .....	29
1.6 THE <i>CRYI</i> GENES.....	29
1.7 COMMERCIAL APPLICATION OF CRY PROTEINS FOR THE CONTROL OF PEST INSECTS AND CROP PROTECTION .....	30
1.8 THE DEVELOPMENT OF CRY BASED PESTICIDES .....	32
1.9 THE USE OF <i>CRY</i> GENES IN GENETICALLY MODIFIED CROPS .....	32
<b>MOTIVATION FOR RESEARCH</b> .....	<b>34</b>
AIMS AND OBJECTIVES.....	34
<b>ISOLATION OF <i>BACILLUS THURINGIENSIS</i> FROM SOUTH AFRICAN SOIL</b> .....	<b>35</b>
2.1 INTRODUCTION .....	35
2.2 METHODOLOGY .....	39
2.2.1 <i>Bacillus thuringiensis</i> enrichment .....	39
2.2.2 Culture of bacteria.....	40
2.2.2.1 Sodium acetate selection test .....	41
2.2.3 Biochemical characterization of <i>Bacillus thuringiensis</i> . .....	42
2.2.3.1 The Gram stain test .....	42
2.2.3.2 Endospore stain (Schaeffer-Fulton staining method) .....	42
2.2.3.3 Catalase test .....	43
2.2.3.4 Growth above 45 °C.....	43
2.2.3.5 Presence of parasporal bodies ( $\delta$ -endotoxins).....	44
2.2.3.6 Total DNA extraction using a sodium dodecyl sulfate (SDS) mediated reaction.....	44
2.2.4 PCR amplification the <i>cryIA</i> gene sequence.....	45
2.2.5 Gel Electrophoresis .....	46
2.2.6 Automated DNA sequencing.....	46

2.2.7 Sequence alignment.....	47
2.3 RESULTS.....	48
2.3.1 <i>Bacillus thuringiensis</i> enrichment.....	48
2.3.2 Sodium Acetate selection test.....	49
2.3.3.1 Gram stain.....	50
2.3.3.2 Endospore stain.....	50
2.3.3.3 Catalase test.....	52
2.3.3.4 Growth above 45 °C.....	52
2.3.3.5 Presence of parasporal bodies ( $\delta$ -endotoxins).....	53
2.4 PILOT STUDY TO OBTAIN THE CRYIA GENE SEQUENCE THROUGH POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION.....	56
2.5 DISCUSSION.....	57

## **MOLECULAR CHARACTERIZATION OF UNKNOWN *BACILLUS***

### ***THURINGIENSIS* SPECIES ISOLATED FROM SOUTH AFRICAN SOILS ..... 59**

3.1 INTRODUCTION.....	59
3.1.1 <i>Extraction of Genomic and Plasmid DNA</i> .....	60
3.1.1.1 Genomic DNA Extraction using Caesium Chloride (CsCl) -Ethidium Bromide density gradient centrifugation.....	60
3.1.1.2 Total DNA extraction using a Sodium Dodecyl Sulfate (SDS) mediated reaction.....	61
3.1.1.3 A modified alkaline lysis for large plasmid DNA extraction.....	62
3.1.1.4 Boiling Lysis.....	63
3.1.2 <i>PCR Amplification of 16S rDNA</i> .....	63
3.2 METHODOLOGY.....	65
3.2.1 <i>Genomic DNA extraction using caesium chloride (CsCl) -ethidium bromide density gradient centrifugation</i> .....	65
3.2.2 <i>Total DNA extraction using a sodium dodecyl sulphate (SDS) mediated reaction</i> .....	66
3.2.3 <i>A Modified alkaline lysis for large plasmid DNA extraction</i> .....	67
3.2.4 <i>Boiling lysis</i> .....	68
3.2.5 <i>Measurement of DNA concentration and purity</i> .....	69
3.2.6 <i>Gel electrophoresis</i> .....	69
3.2.7 <i>PCR amplification of 16S rDNA</i> .....	70
3.2.8 <i>Automated DNA sequencing</i> .....	70
3.2.9 <i>Alignment of 16S rDNA sequence data with known 16S rDNA sequences</i> .....	71
3.3 RESULTS.....	71
3.3.1 <i>DNA extraction</i> .....	71
3.3.2 <i>Obtaining the 16S rDNA identity of the isolates through polymerase chain reaction (PCR) amplification</i> .....	74
3.4 DISCUSSION.....	86

## **CLONING THE *BACILLUS THURINGIENSIS* ISOLATE CRYIA GENE**

### **SEQUENCE..... 89**

4.1 INTRODUCTION.....	89
4.2 METHODOLOGY.....	91
4.2.1 <i>PCR amplification of the cryIA gene sequence</i> .....	91
4.2.2 <i>Gel electrophoresis</i> .....	92
4.2.3 <i>Preparation of chemically competent cells</i> .....	92
4.2.4 <i>Cloning of Taq amplified cryIA gene products</i> .....	93
4.2.5 <i>Transformation</i> .....	93
4.2.6 <i>Colony selection</i> .....	94
4.2.7 <i>Boiling lysis</i> .....	94
4.2.8 <i>PCR amplification the cryIA gene sequence</i> .....	95
4.2.9 <i>Automated DNA sequencing</i> .....	96
4.2.10 <i>Sequence Alignment of cry genes with known sequences</i> .....	96
4.3 RESULTS.....	97
4.4 DISCUSSION.....	102

<b>TOXICITY OF <i>BACILLUS THURINGIENSIS</i> ISOLATED FROM SOUTH AFRICAN SOILS TOWARDS THE GREATER WAX MOTH <i>GALLERIA MELLONELLA</i> (LEPIDOPTERA: PYRALIDAE).....</b>	<b>104</b>
5.2 METHODOLOGY .....	106
5.2.1 Bacterial Cultures.....	106
5.2.2 <i>Galleria mellonella</i> larvae rearing.....	106
5.2.3 Crude spore-endotoxin preparation .....	106
5.2.4 <i>Galleria mellonella</i> bioassay.....	107
5.2.3 Bioassay data processing .....	107
5.3 RESULTS.....	108
5.3.1 Crude toxin preparation .....	108
5.3.2 Bioassay.....	108
5.3.3 ANOVA single factor data .....	112
5.3 DISCUSSION .....	113
<b>CONCLUSION .....</b>	<b>115</b>
6.1 ISOLATION OF <i>BACILLUS THURINGIENSIS</i> .....	115
6.2 CHARACTERIZATION OF PUTATIVE <i>BACILLUS THURINGIENSIS</i> ISOLATES AT A DNA LEVEL.....	117
6.2.1 DNA extraction from putative <i>Bacillus thuringiensis</i> isolates.....	117
6.2.2 16S rDNA characterisation <i>Bacillus thuringiensis</i> isolates .....	119
6.2.3 Phylogenetic analysis of the <i>Bacillus thuringiensis</i> isolates.....	119
6.2.4 Characterization of the <i>cryIA</i> gene sequence from the putative <i>Bacillus thuringiensis</i> isolates.....	120
6.2.5 Cloning the <i>cryIA</i> gene sequence into the pTZ57R/T cloning vector.....	121
6.3 <i>GALLERIA MELLONELLA</i> (LEPIDOPTERA: PYRALIDAE) BIOASSAY .....	122
6.4 FUTURE WORK.....	123
<b>REFERENCES.....</b>	<b>125</b>

# List of Figures

**Figure 1.1:** Schematic diagram depicting the mode of action of the *Bacillus thuringiensis* ICP (Sayyed *et al*, 2001). (A) a mixture of spores and  $\delta$ -endotoxins are ingested. (B) The  $\delta$ -endotoxin is activated in the insect midgut upon exposure to proteases. (C) The toxin specifically binds to the midgut membrane receptors and creates pores which paralyse the midgut through ionic disruption. (D) Spores germinate and incubate within the dead host ..... 22

**Figure 1.2:** (A): Crystal structure of the  $\delta$ -endotoxin, or insecticidal crystal protein, of *Bacillus thuringiensis* illustrating the three protein domains (Gutierrez, 2001). (B) 3D structure through two planes indicate the position of the various domains (Schnepf *et al*, 1998). ..... 24

**Figure 1.4:** An illustration showing the various blocks of homology amongst Cry protein sequences. Blocks are shaded as dark gray, light gray, or white to indicate high, moderate, or low degrees of homology, respectively. Variant (var) is defined as sequences which conform to the consensus sequence of the highly conserved group. Alternate (alt) is defined as sequence blocks that differ from the corresponding highly conserved sequence at more than half of its positions (from Schnepf *et al*, 1998). ..... 27

**Figure 2.1:** A map of South Africa indicating the collection sites of soil samples ranging from Southbroom on the Natal coast, the central regions of Gauteng, and Nelspruit in the Mpumalanga province. .... 40

**Figure 2.2:** Bacterial cultures derived from soil samples collected at various locations across South Africa. Samples were further diluted in order to reduce the overall crude bacterial population, thus assisting in obtaining pure single colonies. Each culture had a distinct colour. .... 48

**Figure 2.3:** A digital photograph of a bacterial isolate grown on T3 agar media treated with 0.25M sodium acetate. Single colonies are distinguishable from each other ..... 49

**Figure 2.4:** Digital photographs taken at 100x magnification with oil immersion. All of the putative Bt isolates tested positive for the Gram stain. Bacterial cells appeared dark purple colouring, and were confirmed as rod shaped cocci..... 50

**Figure 2.5:** Digital photographs of isolates obtained from Muldersdrift soil (A, B&D), storage tissue (E), Nelspruit soil (F) and stream silt(C). In all photographs (A-F ) Green endospores stained with malachite green are distinguishable from the pink safranin O stained vegetative tissue of living bacterial cells (taken at 100x magnification with oil immersion). ..... 51

**Figure 2.6:** Light microscopy photographs of isolate smears in the presence of Hydrogen peroxide, 50 X magnifications. The bubble production intensity from the bacterial smear increases with time as can be seen in A, B and C. .... 52

**Figure 2.7:** Light microscopy photographs of (A&B) test isolates taken at 100X magnification with oil immersion. With the use of phase contrast microscopy a smaller spherical shape, indicated with black arrows, is clearly seen alongside the developing endospore. (C) Light microscopy of samples post sporulation. Crystalline shapes are evident amongst green spores. (D) and (E) show evidence of crystalline bodies alongside the green stained endospores. (f) Small dark shapes are evident in early stages of spore development on the opposite polar end to that of the spore. .... 54

**Figure 2.8:** Photograph of a agarose gel exposed to ultraviolet light. PCR amplification products can be seen in lanes 1 (sample S1), 2 (sample S4), 3 (positive control), 6 (sample S6), 8 (sample S9), and lane 9 (sample S10). ..... 56

**Figure 3.1:** A Photograph of an agarose gel loaded with DNA extracted from sample S10 using four DNA extraction procedures (lane 1) Boiling lysis for 7 minutes, (lanes 2 & 3) boiling lysis for 5 minutes, (lane 4), Generuler 1 kb Marker, (lanes 5 & 7) SDS mediated reaction, (lanes 6 & 8) Modified alkaline lysis for extraction of larger plasmids ..... 73

**Figure 3.2:** A Photograph of an agarose gel loaded showing PCR amplification products for positive control 4A3 (lane 1), sample S4 (lane 2) negative control (lane 3) 100bp marker (lane 4) sample S9 (lane 5) and sample S10 (lane 6) ..... 74

**Figure 3.3:** BLAST alignment of the nucleic acid sequence derived from sample S10 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 673/709 (94%), Gaps = 19/709(2%)..... 75

**Figure 3.4:** BLAST alignment of the nucleic acid sequence derived from sample S9 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 663/770 (86%), Gaps = 35/770 (4%)..... 76



<b>Figure 3.5:</b> BLAST alignment of the nucleic acid sequence derived from sample 4 and that of known <i>B.thuringiensis</i> 16S rRNA gene sequences found on the NCBI database. Identities = 706/797 (88%), Gaps = 35/797 (4%).....	77
<b>Figure 3.6:</b> BLAST alignment of the nucleic acid sequence derived from control (4A3) and that of known <i>B.thuringiensis</i> 16S rRNA gene sequences found on the NCBI database. Identities = 744/756 (98%), Gaps = 7/756 (0%).....	78
<b>Figure 3.7:</b> BLAST alignment of the nucleic acid sequence derived from sample n1 and that of known <i>B.thuringiensis</i> 16S rRNA gene sequences found on the NCBI database. Identities = 748/762 (98%), Gaps = 6/762 (0%).....	79
<b>Figure 3.8:</b> BLAST alignment of the nucleic acid sequence derived from sample n3 and that of known <i>B.thuringiensis</i> 16S rRNA gene sequences found on the NCBI database. Identities = 662/710 (93%), Gaps = 21/710 (2%).....	80
<b>Figure 3.9:</b> BLAST alignment of the nucleic acid sequence derived from sample n5 and that of known <i>B.thuringiensis</i> 16S rRNA gene sequences found on the NCBI database. Identities = 778/800 (97%), Gaps = 17/800 (2%).....	81
<b>Figure 3.10:</b> Clustal X multiple sequence alignment of the <i>16s rRNA</i> gene sequences obtained from isolates n1, n3 n5, S10, S9, S4 as well as the control (4A3). A 26% homology is evident within all 7 sequences.....	83
<b>Figure 3.11:</b> Phylogenetic dendrogram for samples S4, S9, S10 n1, n3, n5 and the control (4A3) reconstructed according to the Neighbour Joining (NJ) analysis function of CLUSTAL X. ....	84
<b>Figure 3.12:</b> Phylogenetic dendrograms for samples S4, S9, S10 n1, n3, n5 and the control (4A3) reconstructed according to the Bootstrap Neighbour Joining (NJ) analysis function of CLUSTAL X. Bootstraps were done using 1000 replications. ....	85
<b>Figure 4.1:</b> The pTZ57R/T cloning vector used to directly clone <i>cryIA</i> amplification products.....	90
<b>Figure 4.2:</b> Digital photographs (A) The positive transformation control showed a high degree of growth after transformation, (B) The Cloned <i>cryIA</i> PCR fragment transformant only produced 3 colony forming units (C) Competent cells without PCR fragment appeared blue in colour indicating that transformation had not occurred. All the transformation cultures grown on LB- ampicillin plates supplemented with X-gal and IPTG. ....	97
<b>Figure 4.3:</b> A Photograph of an agarose gel loaded showing PCR amplification Products for positive sample S10 (lane 1 &2), control 4A3 (lane 3), clone (lane 4) and 100bp marker (lane 5). ....	98
<b>Figure 4.4:</b> BLAST alignment of the nucleic acid sequence derived from CLONEA1A and that of known <i>B.thuringiensis cryIA</i> gene sequences found on the NCBI database. Identities = 630/634 (99%), Gaps = 4/634 (0%).....	99
<b>Figure 4.5:</b> BLAST alignment of the nucleic acid sequence derived from sample 10 and that of known <i>B.thuringiensis cryIA</i> gene sequences found on the NCBI database. Identities = 645/657 (98%), Gaps = 7/657 (1%).....	100
<b>Figure 4.6:</b> Clustal X multiple sequence alignment of the <i>cryIa</i> gene sequences obtained from sample 10 and CLONEA1A. A 90% homology is evident within the two sequences. ....	101
<b>Figure 5.2:</b> (A) A digital photograph showing the difference in size between <i>G. mellonella</i> larvae feeding on artificial diet supplemented with a spore-endotoxin mixture of <i>B. thuringiensis</i> isolate S10 over 7 days (on the right) in comparison to control <i>G. mellonella</i> larvae feeding artificial diet without spore-endotoxin supplementation. (B) A digital photograph showing a comparison in the size of <i>G. mellonella</i> larvae fed on artificial diet containing treatments 1 to 6 for bioassays using spore-endotoxin mixtures for Isolate 10 and 4A3. ....	111
<b>Figure 5.3:</b> Statistical data obtained from the ANOVA single factor test performed on the average weights of <i>G. mellonella</i> feeding on artificial diet supplemented with concentrations 1 to 6 in bioassays using a spore-endotoxin mixture of <i>B. thuringiensis</i> isolate 10, 4A3 and 4AY1.....	112

## List of Tables

<b>Table 2.1:</b> 44 bacteria samples isolated from soil (Muldersdrift mountain range), storage tissue, Soil from Nelspruit (Mpumalanga province), mushroom tissue and marshlands were identified as putative <i>Bacillus thuringiensis</i> isolates	55
<b>Table 3.1:</b> A comparison of A260/280 Ratios of DNA obtained by four DNA extraction procedures	72
<b>Table 5.1:</b> Optical density of the endotoxin-spore solution of the known <i>Bacillus thuringiensis</i> strains 4AY1 and 4A3, and test isolate S10 as used for the primary inoculum	108
<b>Table 5.2:</b> The average weights of the <i>Galleria mellonella</i> larvae after 7 days of exposure to the endotoxin-spore treatments of 4AY1 and 4A3, and test isolate S10	109

## List of Abbreviations

bp	base pairs
Bt	<i>Bacillus thuringiensis</i>
°C	degrees Celsius
cm	centimeter
DNA	deoxyribonucleic acid
E. coli	<i>Escherichia coli</i>
<i>et al</i>	and others
EtBr	ethidium bromide
g	grams
L	litre
kb	kilo bases
M	molar
OD	optical density
µg	microgram/s
µl	microlitre
mg	milligram/s
mm	millimeter
mM	millimolar
NB	nutrient broth
rDNA	ribosomal DNA
SDS	sodium dodecyl sulphate
TBE	Tris borate electrophoresis buffer
TE	Tris electrophoresis buffer

# Chapter 1

## A literature review

### 1.1 *Bacillus thuringiensis*: Nature's design for an industrial powerhouse

Biopesticides are pest management agents that are based on beneficial micro-organisms (bacteria, viruses, fungi and protozoa), beneficial nematodes or other safe, biologically based active ingredients. One of the leading biopesticides of the last two decades (Lambert & Peferoen, 1992), *Bacillus thuringiensis* commonly known as Bt, is a ubiquitous gram-positive rod shaped bacterium, found most abundantly in soil habitats all around the world (Theunis *et al*, 1998; Martin & Travers, 1989). Isolates have also been found abundantly in plant storage systems, mushrooms, compost (Bernhard *et al*, 1997) as well as deciduous and coniferous leaves. Bt is an entomopathogenic sporulating bacterium that produces a parasporal crystal protein or  $\delta$ -endotoxin that has been documented as largely or completely insecticidal to certain classes of insects (Hofmann *et al*, 1988; Adison, 1993).

A large part of the insecticidal activity resides in the durable  $\delta$ -endotoxin inclusions formed during sporulation. *B. thuringiensis* strains are classified into a number of subspecies according to serotype and  $\delta$ -endotoxin produced (Lövgren *et al*, 1998). The  $\delta$ -endotoxin are further divided into 17 groups on the basis of amino acid sequence similarity (Sasaki *et al*, 1997). The production of these crystalline  $\delta$ -endotoxins occurs in

the stationary phase. In these inclusion bodies, the toxins exist as an inactive protoxin (Gutierrez *et al*, 2001).

*B. thuringiensis* was isolated early last century in Japan from diseased silkworms and in Germany from the Mediterranean meal-moth (Beegle & Yamamoto, 1992). Its value as a control agent for Lepidoptera was realized soon thereafter. Its insecticidal activity was initially discovered in 1911, but its commercial value as a control agent for Lepidoptera was only realized in the 1950's and has only been developed on commercial scales over the last 40 years (Capalbo *et al*, 2001). The discovery of this endotoxin led to the development of bioinsecticides based on Bt in order to control insect pests belonging to the Lepidoptera, Diptera, and Coleoptera orders. More recently Bt insecticidal activity has also been reported against other insect orders, mainly Hymenoptera, Homoptera, Orthoptera and Mallophaga, as well as nematodes mites and protozoa (Schnepf *et al*, 1998; Feitelson *et al*, 1993). As a result, Bt endotoxins have thus become the most widely used biopesticides in the world as an alternative to synthetic pesticides in commercial agriculture (Capalbo *et al*, 2001).

Bt pesticides have been used in field trials with marked success in the management of pest control programs which may result in a reduction of the use of chemical insecticides (Yang & Wang, 1998). Successful applications have been documented in a variety of agriculturally important crops such as cotton, corn, potato, soybean and many vegetables (Yang & Wang, 1998). In addition to field trials, Bt has been sold commercially as a biopesticide for over half a century (Jenkins & Dean, 2001).

## 1.2 *Bacillus thuringiensis* in nature

Bt is a micro-organism that is found worldwide in a variety of habitats including soil (Carozzi *et al*, 1991; Delucca *et al*, 1979), insects (Carozzi *et al*, 1991), stored-product dust (Burgess & Hurst, 1977), and deciduous and coniferous leaves (Kaelin *et al* 1994). Very little is known about the natural ecology of Bt other than that it occurs naturally in many soils. In one study, two varieties of Bt were applied in adjacent plots, both of which did not become cross-contaminated, indicating little capacity to move laterally in soil (Entwistle, 1993). Other studies found that Bt was not recovered past a depth of 6 centimetres after irrigation, and that movement beyond the application plot was less than 10 yards (Entwistle, 1993).

The Bt strains have a genome size ranging from 2.4 to 5.7 million bp (Carlson *et al*, 1994). In the past a close similarity has been reported between *Bacillus thuringiensis* and *Bacillus cereus*. Genetic maps have been constructed for *Bacillus thuringiensis* and *Bacillus cereus* (Carlson & Kolstø, 1993). These studies showed very close similarity between the two genomes near the replication origin, while greater variability was seen in the terminal half of their respective genomes (Schnepf *et al*, 1998).

Considerable evidence has been put forward that *Bacillus thuringiensis* and *Bacillus cereus* should be considered a single species. Classical biochemical and morphological methods used for the classification of bacteria have failed in attempts to distinguish *B. thuringiensis* from *B. cereus* (Gordon *et al*, 1973; Baumann *et al*, 1984). Modern molecular methods including chromosomal DNA hybridization (Kaneko *et al*, 1978),

phospholipid and fatty acid analysis (Kämpfer, 1994), 16S rRNA sequence comparison (Rössler *et al*, 1991), amplified fragment length polymorphism analysis (Keim *et al*, 1997), and genomic restriction digest analysis (Carlson & Kolstø, 1993) have also supported the single-species hypothesis. Additional attempts to distinguish *B. thuringiensis* isolates from *B. cereus* by analysis of a 16S rRNA variable region largely failed, yielding as many false positives and negatives as accurate identifications (Te Giffel *et al*, 1997). The problem arises with the production of the parasporal crystal, the only definitive characteristic of Bt, is too narrow a criterion to taxonomically class the species. The large variety of Bt strains and toxin diversity could be due to the high degree of genetic plasticity in this bacterium (Carlson *et al*, 1994).

The  $\delta$ -endotoxin genes appear to reside on large plasmids (González *et al* 1982), making up composite structures that include transposon elements (Lereclus *et al*, 1984). The  $\delta$ -endotoxin or crystalline proteins are encoded by a family of genes known as the *cry* genes. Many *cry* gene-containing plasmids appear to be conjugative in nature (González *et al*, 1982). Similar gene sequences are commonly found among Bt chromosomes as well (Carlson & Kolstø, 1993), however the degree at which these chromosomal homologs contribute to production of the crystal is unclear.

When conditions for bacterial growth are not optimal Bt forms spores. Spores are the dormant stage of the bacterial life cycle that allows the organism to survive until better growing conditions occur. Unlike many other sporeforming bacteria, when Bt produces spores it also creates a protein crystal ( $\delta$ -endotoxin) through a fascinating array of molecular mechanisms (Agaisse & Lereclus, 1994). It is this crystal which is toxic to insects

once ingested. These products accumulate in the mother cell compartment to form a crystalline inclusion which comprises 20 to 30% of the dry weight of sporulated cells. The *cry* gene is characteristically expressed at high levels during stationary phase (Schnepf *et al*, 1998), under the control of several mechanisms at the transcriptional, pre-transcriptional and post-translational levels (Schnepf *et al*, 1998; Brown, 1993). Certain *cry* genes are not expressed in stationary phase. It is therefore necessary to distinguish between the *cry* genes that are expressed during the stationary phase i.e. those that are dependent on sporulation, from those that are not (Schnepf *et al*, 1998).

A typical example of a sporulation-dependent *cry* gene is the *cryIAa* gene. This gene *cryIAa* is solely expressed in the mother cell compartment of Bt. It has two transcription start sites mapped at BtI and BtII. BtI activity is evident between about T<sub>2</sub> and T<sub>6</sub> of sporulation, where as BtII is active from about T<sub>5</sub> onwards (where T<sub>n</sub> is n hours after the end of the exponential phase). Work done by Brown and Whiteley (1990) identified two sigma factors  $\sigma^{35}$  and  $\sigma^{28}$ , which function in direct transcription control of *cryIAa* from BtI and BtII (Brown and Whiteley, 1990). Cloning experiments have proved that the gene sequences of the sigma factors share an 85 to 88% similarity to *Bacillus subtilis* (Adams *et al*, 1991). Further work has proven that not only is the transcription of *cryIAa*, *cryIBa*, and *cry2Aa* dependent on factors  $\sigma^{35}$  and  $\sigma^{28}$ , but many other *cry* genes such as *cry4Aa*, *cry4Ba*, *cryIIAa*, *cryISAa*, are likely to be  $\sigma^{35}$  and  $\sigma^{28}$  dependent as well. These *cry* genes are all generally accepted to be solely expressed upon dependence of sporulation (Schnepf *et al*, 1998).



An example of sporulation-independent *cry* gene expression can be seen with the *cry3Aa* gene. This gene when isolated from the coleopteran-active *Bacillus thuringiensis* var. *tenebrionis*, was reported to be expressed during vegetative growth, and to a lesser extent during the stationary phase (Schnepf *et al*, 1998). The expression of *cry3Aa* is not dependent on sporulation-specific sigma factors either in *Bacillus subtilis* (Agaisse *et al*, 1994) or in *Bacillus thuringiensis* (Schnepf *et al*, 1998). Instead *cry3Aa* appears to be activated in the transition from exponential growth to the stationary phase.

In the post-transcriptional mechanisms it is essential that the mRNA remains stable to ensure the high level of  $\delta$ -endotoxin production in Bt. This is achieved by extending the half life of the mRNA to 10 minutes, a time period of at least five fold greater than the average half life of bacterium mRNA (Schnepf *et al*, 1998). Work previously done by Wong and Chang (1986) gives evidence that the putative transcriptional terminator of the *cryIAa* gene (a stem-loop structure) is an active positive retro-regulator (Wong *et al*, 1986). The DNA fragment carrying this terminator has been reported to increase the half-life of the transcripts two- to threefold, and in turn increases the expression of their gene products (Wong & Chang, 1986). The terminator sequence thus seems to protect the *cry* mRNA stability by protecting it from exonucleolytic degradation from the 3' end. Similar terminator sequences can be found downstream from various *cry* genes where they potentially form stable stem-loop structures and in so doing these genes contribute to their high-level expression by stabilizing the transcripts (Schnepf *et al*, 1998).

The  $\delta$ -endotoxins are generally crystalline in nature and show variation in size and shape. Variations such as bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A), have been reported (Schnepf *et al*, 1998; Arrieta *et al*, 2004). Protein crystallization is a posttranslational mechanism that aids in protecting the  $\delta$ -endotoxin from premature proteolytic degradation. The crystalline structures however have to be rapidly solubilized within the insect larvae midgut in order to expose the toxic core. It is presumed that this is due to the secondary structure of the proteins as well as the energy of the disulfide bonds, and the presence of additional Bt-specific components (Schnepf *et al*, 1998).

Laboratory experiments have shown that the introduction of *cry* gene sequences into either the *E. coli* (Ge *et al*, 1990) or *Bacillus subtilis* (Shivakumar *et al*, 1986) genome through cloning, resulted in the production of biologically active protoxins ( $\delta$ -endotoxin). The 130- to 140kDa Cry1 protoxins in particular have been reported to spontaneously form crystals. This led to the deduction that the cysteine-rich C-terminal half of the Cry1 protoxins contributes to crystal structure through the formation of disulfide bonds (Bietlot *et al*, 1990).

Other 130- to 140kDa proteins such as Cry4, Cry5, and Cry7 also display this mechanism of crystalline self-assembly (Schnepf *et al*, 1998). The 73-kDa Cry3A protoxins lack the cysteine-rich C-terminal region and produce a flat, rectangular crystal inclusion in which the polypeptides do not appear to be linked by disulfide bridges

(Bernhard, 1986). This mechanism of crystal formation is evident in both *B. thuringiensis* and *B. subtilis*. This indicated that the gene products are not dependent on host specific factors. Recent studies have also shown that the mechanism of protein crystallization requires the presence of additional proteins. These accessory proteins appear to play a role in stabilizing the nascent protoxin molecule at a posttranslational level and to facilitate crystallization (Baum & Malvar, 1995).

### **1.3 The $\delta$ -endotoxin mode of action**

In order for the  $\delta$ -endotoxin to elicit its insecticidal affect, it has to be ingested. A mixture of spores and  $\delta$ -endotoxin are ingested by an insect feeding on decaying soil matter, or plant storage material. Once in the insect midgut the  $\delta$ -endotoxin is activated by the gut proteases, which for most Lepidopterans, takes place under the alkaline conditions of the insect midgut (Hofmann *et al*, 1988 ). The degree to which the protein is solubilized may be attributed to differences in the degree of toxicity among Cry proteins. The major proteases implicated in protein solubilization within the Lepidopteran insect midgut are either trypsin-like (Lecadet & Dedonder, 1964) or chymotrypsin-like (Johnston *et al*, 1995). The activated  $\delta$ -endotoxin then binds to specific receptors on the cell lining (Hofmann *et al*, 1988). Interaction with the receptors results in the incorporation of the activated toxin components into the membrane (Carrol & Ellar, 1993).

After the toxin binds to the midgut, a conformational change takes place within the toxin (Pardo-Lopez *et al*, 2006). The hydrophobic surfaces then face the exterior of the bundle. This initiates the penetration of the cell membrane, and the formation of pores (Siqueira *et al*, 2006). This produces selective ion channels by oligomerization of toxin monomers (Aronson *et al*, 1999). Loss of osmotic pressure regulation induces paralysis of the gut, halting the insects feeding activity and inevitably leading to the death of the insect (Aronson *et al*, 1999).

After this the spores may germinate in the gut of the insect leading to propagation (Yang & Wang, 1998). Due to their high specificity for these unique receptors on the membrane of the pest midgut epithelial cells (Jurat-Fuentes and Adang, 2006), the delta-endotoxins are harmless to non-target insects and are compatible with integrated pest management programs. The fact that they are proteins ensures that they are readily biodegraded. Many *Bacillus thuringiensis* strains exhibit a wide spectrum of insecticidal activity which is attributed to the expression of several kinds of crystal proteins (Lee *et al*, 2001).

CryIA protoxins in particular are digested by the midgut proteases in a processive manner starting at the C terminus and proceeding toward the 55- to 65-kDa toxic core (McPherson *et al*, 1988; Feng & Becktel, 1994). It can then be seen that the carboxy-terminal end of the protoxin appears to be wound around the toxin which is possessively clipped off in 10-kDa sections (Hofmann *et al*, 1988). At least two stages of processing have been identified for CryIA with trypsin or *Ostrinia nubilalis* midgut proteases. The result is a fully toxic intermediate, with an N terminus at protoxin residue 45 and a C

terminus at residue 655 or 659. This intermediate is then further processed to a partially toxic core, with an N terminus clipped to residue 156. An active toxin is known to perform two functions. Firstly it binds to highly specific receptors on the tip of the midgut microvillae of susceptible insects (Adams *et al*, 1989). This process of binding is comprised of reversible and irreversible steps. Irreversible steps require a combination of tight binding between the toxin and receptor and the introduction of the toxin into the apical membrane (Adams *et al*, 1989). Once inserted into the apical membrane of the columnar epithelial cells, the toxins become insensitive to protease activity and monoclonal antibodies thereafter inducing ion channels or nonspecific pores in the target membrane (Adams *et al*, 1989; Grochulski *et al*, 1995; Maddrell *et al*, 1988).

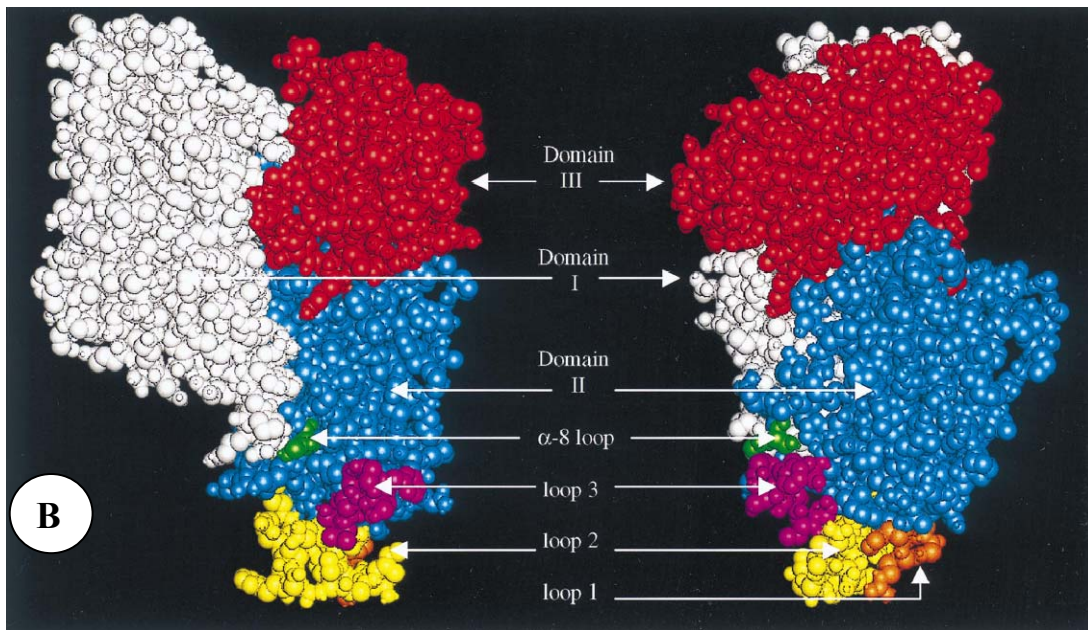
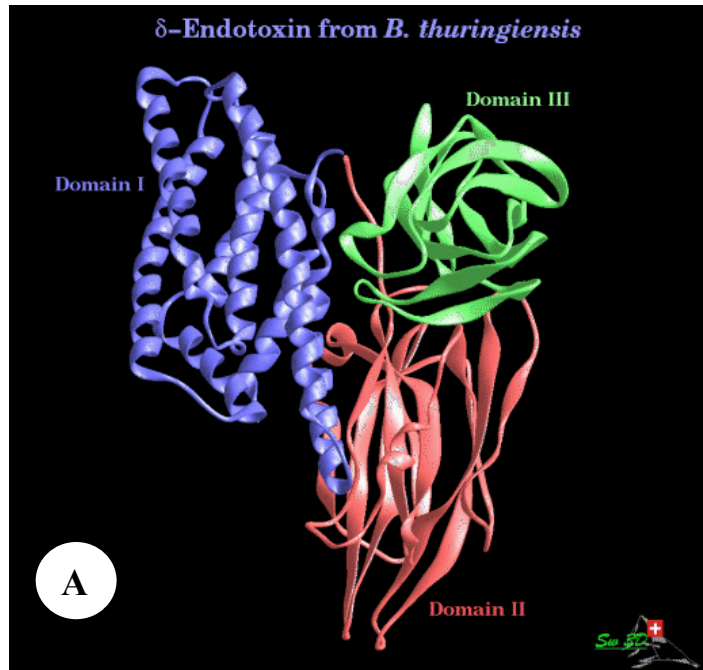
## **1.4 The Insecticidal Crystal Proteins (ICP)**

There are two types of proteins present in the ICPs. These proteins are the Cry (65-160 kDa  $\delta$ -endotoxins) and Cyt toxins (Gill *et al*, 1992). Once subjected to the proteases in the insect midgut, the N- and C-termini of the  $\delta$ -endotoxin are processed exposing a protease resistant core that constitutes the active toxin (Gill *et al*, 1992).

**Figure 1.1:** Schematic diagram depicting the mode of action of the *Bacillus thuringiensis* ICP (Sayyed *et al.*, 2001). (A) a mixture of spores and  $\delta$ -endotoxins are ingested. (B) The  $\delta$ -endotoxin is activated in the insect midgut upon exposure to proteases. (C) The toxin specifically binds to the midgut membrane receptors and creates pores which paralyse the midgut through ionic disruption. (D) Spores germinate and incubate within the dead host

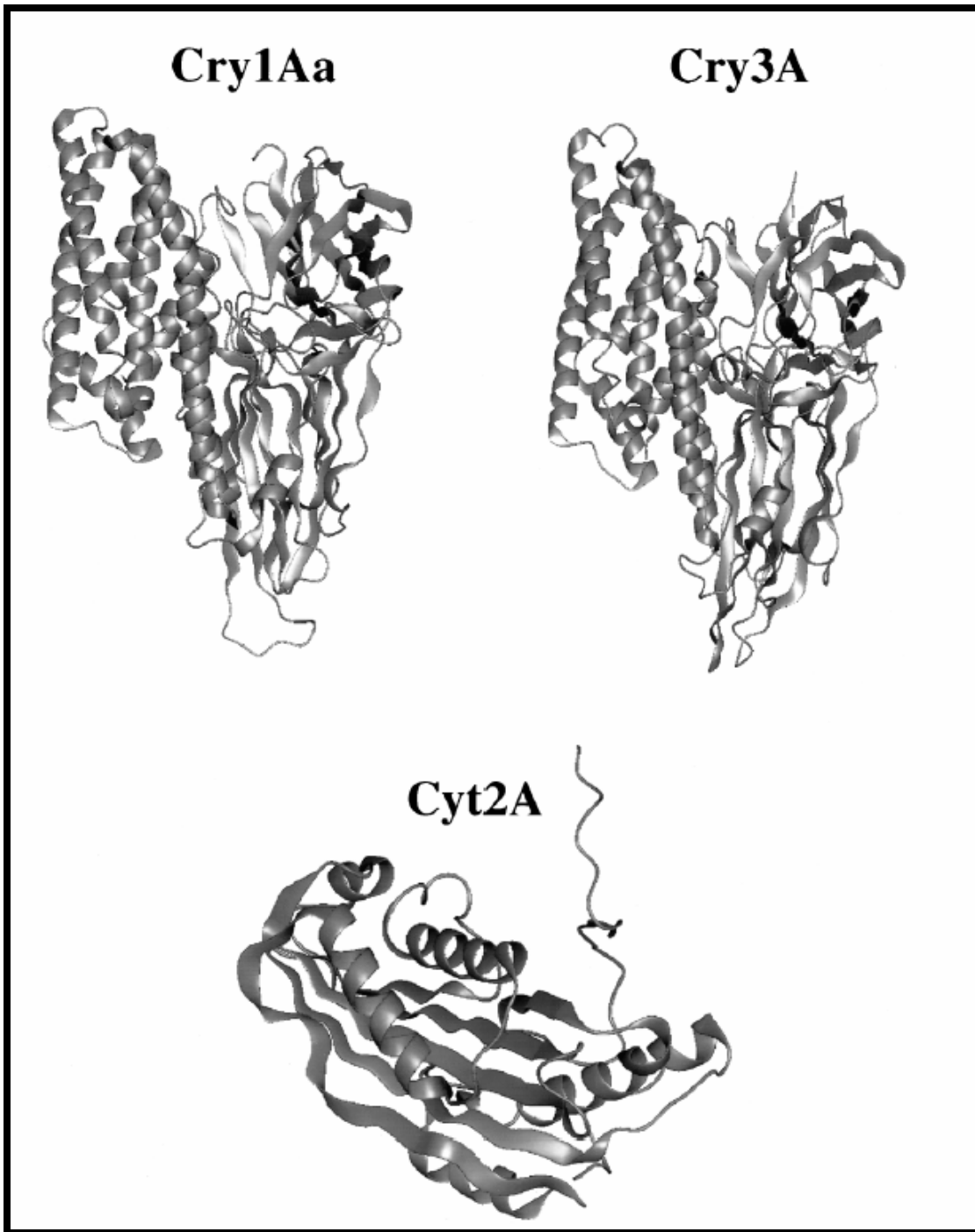
The crystal structure of the coleopteran specific Cry3A toxin has been resolved at the 2.5Å level (Li *et al*, 1991). Three domains are evident from this analysis as seen in Figure 1.2 (A). Domain I is a 7  $\alpha$ -helix bundle, Domain II is a Greek key  $\beta$ -barrel and Domain III consists of two twisted, antiparallel R-sheets forming a  $\beta$ -sandwich (Li *et al*, 1991). A three dimensional layout of the protein is seen in Figure 1.2 (B). It has been proposed that Domain I is the pore-forming domain, Domain II is thought to be involved with receptor binding in most toxins, and Domain III stabilises the integrity of the protein (Li *et al*, 1991). Additionally the structures of crystal proteins-Cry3A, CryIAa and Cyt2A have been solved by X-ray crystallography (Schnepf *et al*, 1998).

It has been deduced that Cry3A and CryIAa share a 36% amino acid sequence identity (Crickmore *et al*, 1998). This was attributed to their three-dimensional structures; the corresponding domains can virtually be superimposed. In the case of Cyt2A, it was evident that less than 20% amino acid sequence identity is shared with CryIAa and Cry3A. The structure of Cyt2A is drastically different to those of Cry3A and CryIAa



**Figure 1.2:** (A): Crystal structure of the  $\delta$ -endotoxin, or insecticidal crystal protein, of *Bacillus thuringiensis* illustrating the three protein domains (Gutierrez, 2001). (B) 3D structure through two planes indicate the position of the various domains (Schnepf et al, 1998).





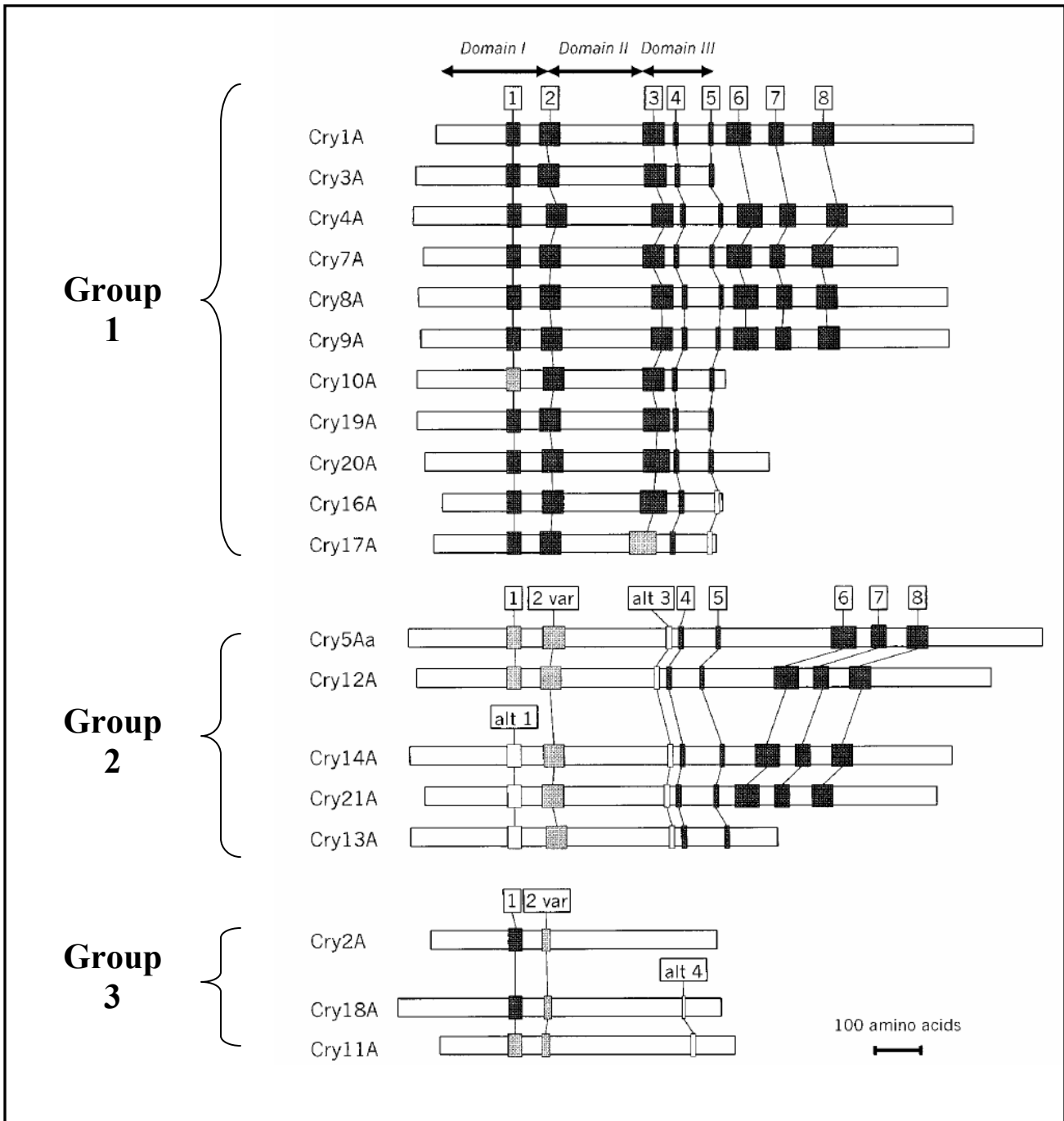
**Figure 1.3:** A comparison of the 3D protein structures of Cry1A, Cry3A, and Cyt2A. (Schnepf *et al.*, 1998).

The three dimensional structures of Cry3A, Cry1Aa and Cyt2 are compared in Figure 1.3. In comparison to the Cry1A and Cry3A proteins which are both made up of three

domains, Cyt2A protein only has one domain, surrounded by two outer layers of  $\alpha$ -helix wrapped around a mixed  $\beta$ -sheet. A similar conformation can be found in Cyt1A. A common attribute of Cyt toxins is that unlike the Cry  $\delta$ -endotoxin, they encompass a wide range of intracellular inclusions with regards to their ability to lyse the cells (Höfte and Whiteley, 1989). When comparing the structural and sequence similarities of the toxic proteins, it can be seen that there are the five blocks of amino acids conserved among most of the Cry toxins. These regions of homology were first highlighted by Höfte and Whiteley in 1989. Further work by Schnepf *et al* in 1998 proved through sequence alignment of the Cry proteins that an additional three conserved regions can be found in the carboxyl-terminal halves of sequences outside of the core of the active toxin.

One group consisting of Cry1, Cry3, Cry4, Cry7 to Cry10, Cry16, Cry17, Cry19, and Cry20 proteins contains all five of the core blocks. A second group made up of Cry8, Cry12 to Cry14, and Cry21 proteins contains homologs recognizable to blocks 1, 2, 4, and 5. Block 1 as seen in the first block varies somewhat to that seen in group two, depicted in Figure 3. Block two is seen to have more variation within each group than when compared between the groups. Block 1 contains helix 5 of the domain 1 and is believed to play a role in the pore forming mechanism, which would be consistent in explaining why this region is so highly conserved across all *cry* gene sequences.

Additionally, this helix may have a role in maintaining the structure of the helix bundle. Block 2 includes helix 7 of domain I and the first R-strand of domain II, two essential structures making up the point of contact between the two domains. Three structurally equivalent salt bridges can be found between domain I and domain II in Cry1A and Cry3A (Grochulski *et al*, 1995); with all the necessary residues found within block 2.



**Figure 1.4:** An illustration showing the various blocks of homology amongst Cry protein sequences. Blocks are shaded as dark gray, light gray, or white to indicate high, moderate, or low degrees of homology, respectively. Variant (var) is defined as sequences which conform to the consensus sequence of the highly conserved group. Alternate (alt) is defined as sequence blocks that differ from the corresponding highly conserved sequence at more than half of its positions (from Schnepf *et al*, 1998).

This close contact could be required for maintaining the protein in a globular form during digestion in the midgut and activation of the core (Schnepf *et al*, 1998). The remaining blocks 3, 4, and 5 are contained within the three buried strands of domain III. Block 3 contains the remaining R-strand of domain II, where it engages in interactions between domains I and III. Block 3 is notably absent from the second group, however a block unrelated to the first group is seen between blocks 2 and 4 (Schnepf *et al*, 1998). The presence of the C-terminal extension within the protein sequences in either of the groups, was consistent with the presence of blocks 6, 7, and 8. A third group consisting of Cry2, Cry11 and Cry 18 (Figure 1.4), possess block 1 and a truncated version resembling the core of block 2 however it lacks any resembling homologs of the other conserved blocks (Lereclus *et al*, 1989).

The remaining proteins in the Schnepf *et al* (1998) data set, mainly Cyt1, Cyt2, Cry6, Cry15, and Cry22 possess no homologs to the conserved blocks within the above mentioned three groups (Schnepf *et al*,1998). The fact that blocks 1 through 5 were faithfully conserved at least concurs with the notion that the proteins within the first subgroup, which includes Cry1 and Cry3, may potentially adopt a similar three domain tertiary structure. The possibility may also arise that the second subgroup, mainly Cry8, Cry12 to Cry14, and Cry21 could incorporate a variation of the same structural theme (Schnepf *et al*,1998).

## 1.5 The *Cry* gene family

The family of genes coding for the various  $\delta$ -endotoxins are the *Cry* gene family, of which, *cryI*, *cryII*, *cryIII*, and *cryIV* (Shin *et al*, 1995) are well documented. These genes are known to code lepidopteran-specific (CryI), lepidopteran- and dipteran- specific (CryII), coleopteran-specific (CryIII), and dipteran (CryIV) specific proteins respectively (Shin *et al*, 1995). ICP gene sequences have also been isolated that are toxic to nematodes, protozoan pathogens, animal-parasitic liver flukes (Trematodes), and mites (Sasaki *et al*, 1997).

It has been known since the 1980s that the crystal toxins are encoded by genes on plasmids of *Bacillus thuringiensis*. There can be 5 or 6 different plasmids in a single *Bacillus thuringiensis* strain, ranging in size from 0.5-6.7 Kbp, and these plasmids can encode different toxin genes. Most  $\delta$  -endotoxin genes are found on large plasmids which are either self transmissible or can be co-transferred from a donor to a receptor strain in a conjugation process, so there are a potentially wide variety of strains with different combinations of crystal toxins (Bernhard *et al*, 1997).

## 1.6 The *cryI* genes

The Cry 1 proteins are amongst the most studied crystal proteins to date. This in conjunction with its specificity and highly toxic effects to Lepidopteran insects form the basis for its use in our studies. Since 1989 more than 30 *cryI* sequences have been reported, with many still uncharacterized. All of the reported *cryI* genes produce proteins

ranging from 130- to 140-kDa. These proteins amass within the bipyramidal crystal bodies (Höfte & Whiteley, 1989).

All reported *cryI* genes can easily be distinguished from each other simply by sequence homology in that the protein products they express have >50% amino acid identity (Höfte & Whiteley, 1989). As a result, the *cry* genes can be used as markers for identifying *Bacillus thuringiensis* species in a crude bacterial population (Porcar & Juarez-Perez, 2003). The polymerase chain reaction (PCR) can be used to amplify specific DNA fragments and thus to determine the presence or absence of a target gene. The identification of *B. thuringiensis* endotoxin genes by PCR can partially predict the insecticidal activity of a given strain. Distinction can be made between the closely related *Bacillus cereus* and *Bacillus thuringiensis* and *Bacillus anthracis* by analysing the 16S rDNA through similar PCR techniques and in conjunction with the sequence data provide strong support in characterising both known and novel *cry* genes and *B. thuringiensis* species in any given bacterial population (Porcar & Juarez-Perez, 2003).

## **1.7 Commercial Application of Cry Proteins for the control of pest insects and crop protection**

*Bacillus thuringiensis* is at present considered to be the prevailing form of biologically produced pest control, and is commonly referred to simply as Bt (Smith *et al*, 1996). Back in 1995, worldwide sales of *Bt* reached \$90 million (Smith *et al*, 1996), prompting the motion towards a natural alternative to hazardous synthetic pesticides. In 1998, the

number of registered *Bacillus thuringiensis* products in the United States alone had almost exceeded the 200 mark. Although time consuming, it has become well recognized that Cry-based pesticides generally have low costs for development and registration. Astoundingly the cost of Bt pesticides is estimated at 1/40th that of a comparable novel synthetic chemical pesticide (Becker & Margalit, 1993).

The United States is still leading the way with Bt pesticide programs already implicated in areas of forestry. Bt pesticides are used in particular in this field to combat the gypsy moth (Machesky, 1989). These pesticides are based primarily on the strain *Bacillus thuringiensis* HD-1 subsp. *kurstaki* (Dulmage *et al*, 1970), which produces CryIAa, CryIAb, CryIAc, and Cry2Aa toxins. The huge success that was achieved by these projects were reflected in results throughout the forestry world, encompassing more than one pest species.

*Bacillus thuringiensis* subsp. *israelensis* has become one of the most effective and potent biological pesticides in attempts to combat mosquitoes and blackflies, insect pests capable of spreading fatal human diseases. Mosquitocidal activity has been identified through tests conducted with Cry2Aa, CryIAb and Cry1Ca (Haider *et al*, 1986). Many new uncharacterized isolates containing uncharacterized *cry* genes have also been shown to display mosquitocidal activity (Ragni *et al*, 1996).

## **1.8 The development of Cry based pesticides**

Due to the fact that Bt produces such a large array of toxins which exhibit different target specificity, it is necessary to carefully select the effect which is desired to insure non targeted insects are not eradicated. A cassette of desirable genes can be engineered to produce a cocktail of protoxins would by far be more beneficial than making use of isolated protoxin with potential damaging results (Wu *et al*, 1994). Such gene cocktails have been successfully produced through introduction of desired genes into to a host cell by means of conjugation-like systems (Gonza'lez *et al*, 1982) and electroporation technology (Belliveau & Trevors, 1989). Shuttle vectors have also been employed to transfer plasmid replicons containing cloned *cry* genes into host Bt cells to introduce additional genes, thus upgrading the cells natural artillery (Gonza'lez *et al*, 1982). Understanding of the nature of *cry* gene expression has led to the production of many beneficial pesticides and with knowledge of transformation and genetic manipulation, a foundation has been laid for potentially more effective biopesticides.

## **1.9 The use of *cry* genes in genetically modified crops**

Bt  $\delta$ -endotoxins are generally safe to vertebrates and beneficial arthropods yet in many cases highly toxic to specific insect pests, thus the genes that encode these  $\delta$ -endotoxins



were among the first to be engineered into plants to confer insecticidal activity (Theunis *et al*, 1998). Introduction into dicotyledonous plants proved successful in affording resistance to Lepidopteran species, however the  $\delta$ -endotoxin genes were expressed at extremely low levels (Fujimoto *et al*, 1993).

The  $\delta$ -endotoxin gene can be extensively modified through truncation of the *cry* gene based on the codon region of the transformation vector. This truncation allows for the gene to be highly expressed in transgenic plants and stably inherited for at least two generations (Fujimoto *et al*, 1993). Monocotyledonous plants have higher G+C content in comparison to dicotyledonous plants, so these modifications also enhance the transformation success of monocotyledonous plants (Fujimoto *et al*, 1993). Truncation also allows for the transfer of only the sequences required for insecticidal activity. This is achieved by the removal of amino acid sequences of the N-terminus (Lambert *et al*, 1996). When the ICP enters the insect gut, it is broken down into three subunits. Truncation allows for the expression of only the subunits responsible for the recognition of specific binding sites on the insect gut wall (Jenkins & Dean, 2001). Development of multitoxin systems with combinations that recognize different binding sites would prove useful in implementing deployment strategies to decrease the rate of pest insect adaptation to *Bacillus thuringiensis* toxins (Lee *et al*, 1997).

# Motivation for Research

This project set out to isolate a *Bacillus thuringiensis* strain from South African soils. This isolate will be evaluated for its potential as a biopesticide agent through the understanding of its *cry* gene identity, the nature of the  $\delta$ -endotoxin it produces and the host range to which this agent would best be suited.

## Aims and Objectives

- ⊕ Isolate and characterize *Bacillus thuringiensis* from South African soils
- ⊕ Obtain the gene sequence of the  $\delta$ -endotoxin
- ⊕ Determine the host range of the produced  $\delta$ -endotoxin
- ⊕ Engineer the gene for the  $\delta$ -endotoxin into the plasmid vector Ptz57r/t containing the LacZ operon.
- ⊕ Insert the plasmid into the E. coli for bioassay experiments
- ⊕ Confirm and evaluate the toxicity of the  $\delta$ -endotoxin

By fulfilling the above aims and objectives, we will have obtained knowledge of a previously unknown *Bacillus thuringiensis cry* gene sequence isolated from South African soils, as well as producing a plasmid vector containing this sequence which can then be used in transformation protocols in future research.

## Chapter 2

### Isolation of *Bacillus thuringiensis* from South

### African soil

#### 2.1 Introduction

A taxonomist aims to produce an accurate and simple identification system for the classification of biological specimens. These systems unambiguously allow identification of strains for medical, ecological or biotechnological purposes. Our objective was to investigate various isolation techniques that were known to aid in the accurate isolation as well as characterization of *Bacillus thuringiensis* from various biospheres. In doing so the techniques could be identified that when used in combination would provide a simple yet accurate protocol for the isolation of Bt from South African soils in an acceptable timeframe and cost.

The diversity of the various species found in the *Bacillus* genus meant that these tests would have to identify specific traits in order to narrow down the putative Bt candidates. A selection of tests from Bergey's Manual of Systematic Bacteriology (1986) was used as indicators. These tests included the Gram stain test, endospore staining, catalase degradation, growth above 45 °C and the visual identification of crystalline bodies (Bergey's Manual of Systematic Bacteriology 1986).

The isolation procedure primarily used in our experiments was derived by Travers *et al*, 1987, making use of a sodium acetate isolation procedure. In his work he describes that for some unknown reason Bt did not mature past the spore life stage in the presence of sodium acetate. This selective inhibition only prevents the germination of Bt spores, and not any other (Travers *et al*, 1987). The benefits of this technique are evident in comparison with conventional antibiotic (ampicillin-polymyxin B) selection tests, which primarily selected against non-sporeforming bacteria.

This provided a revolutionary technique in the field of Bt isolation as samples could easily be separated from other biological contaminants. In our studies, samples were subjected to the sodium acetate selection test as well as a series of classical tests as a comparison to aid in the confirmation that our samples were indeed Bt.

It has been established that Bt is a Gram-positive rod shaped bacterium, therefore the Gram stain test proved an effective technique to eliminate any Gram-negative organisms (Provine & Gardner, 1974). The technique makes use of staining and counterstaining techniques which separate Gram-positive and Gram-negative based on the dye taken up by their respective peptidoglycan cell walls.

Bt is known to contain endospores, one produced for every bacterium. Once the endospore becomes fully formed the vegetative cell degrades releasing the dominant endospore. Staining for these endospores allowed for visual confirmation of sporeformers with the aid of light microscopy. The vegetative cell surface is a laminated structure that consists of a capsule, a proteinaceous surface layer, several layers of

peptidoglycan sheeting making up the cortex, and the proteins on the outer surface of the plasma membrane. The spore coat is made up of a keratin like protein which makes the spore impervious to many chemicals. Staining with malachite green allows for effective colourization of the endospore capsule if performed over an open flame. Bt is capable of catalase degradation, therefore a catalase test was used to determine if the isolates were capable of such degradation. The test is performed by exposing the test organism to hydrogen peroxide and observing for oxygen production. Hydrogen peroxide is extremely toxic to cells because it attacks unsaturated fatty acid compounds of membrane lipids, thus damaging membrane structure. Aerobic cells protect themselves against peroxide by the action of catalase, which decomposes hydrogen peroxide (Aronson *et al*, 1986). Catalase utilises hydrogen peroxide both as an electron acceptor and an electron donor yielding oxygen and water in the decomposition reaction ( $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ ). The hydrogen peroxide is oxidised to form oxygen, with the simultaneous reduction of second molecule of hydrogen peroxide to water.

The optimal growth temperature for Bt ranges between 30 to 45 °C, depending on the species, therefore isolates grown above 45 °C were assumed not to be *Bacillus thuringiensis* (Starzak & Bajpai, 1991). Although these bacteria may fit many of the selection criteria, they are more likely to be thermophile species of *Bacillus* such as *Bacillus acidocaldarius*, *Bacillus schlegelii*, and *Bacillus stearothermophilu*, all of which have an upper temperature limit of 65° C (Bergey's Manual of Systematic Bacteriology, 1986).

Visual confirmation of crystalline bodies was done with the aid of phase contrast microscopy. Although not conclusive, this simply adds support to confirming the identification of Bt (Ammons *et al*, 2002). This is mainly due to the fact that variations in crystal appearance often lead to false negative characterizations.

Samples that passed the selected tests for characterisation were considered to be positive Bt isolates at a morphological level. These putative Bt specimens were further characterised at a DNA level. In the pilot study, DNA was extracted using a sodium dodecyl sulfate (SDS) mediated reaction. The DNA obtained was then probed for the presence of the *cryIA* gene using the Polymerase Chain Reaction (PCR). The *cryI* gene family is found in many known Bt strains, thus the probability of obtaining a similar or matching sequence would be high within the putative Bt samples (Neil Crickmore, toxin database).

The *cryIA* specific primers TYIAA (f) and TYIUN12 (r) amplify the first 710 bases of the N-terminus of any *cryIA* gene (Kalman *et al*, 1993). With this sequence, the base composition of the gene can be determined for comparison with known *cryIA* gene sequences. Known Bt specimens were obtained from Dr Daniel Zeigler of the Bacillus Genetic Stock Centre in Ohio, USA, which served as positive controls for the PCR reaction. These specimens included serotype 3a, 3b, 3c—serovar. *kurstaki* (4D7) and serotype 1—serovar. *thuringiensis* (4A3).

The specific objectives for this chapter were:

- Obtain bacterial populations from soil samples collected at various sites in South Africa
- Characterize putative Bt isolates using classical microbial, and biochemical techniques
- Expose the isolates to various concentrations of sodium acetate to obtain putative Bt endospores
- Extract DNA from cultured bacteria for preliminary PCR experiments
- Identify *cryIA* gene sequences using specific primers in a PCR reaction
- Obtain gene sequence data

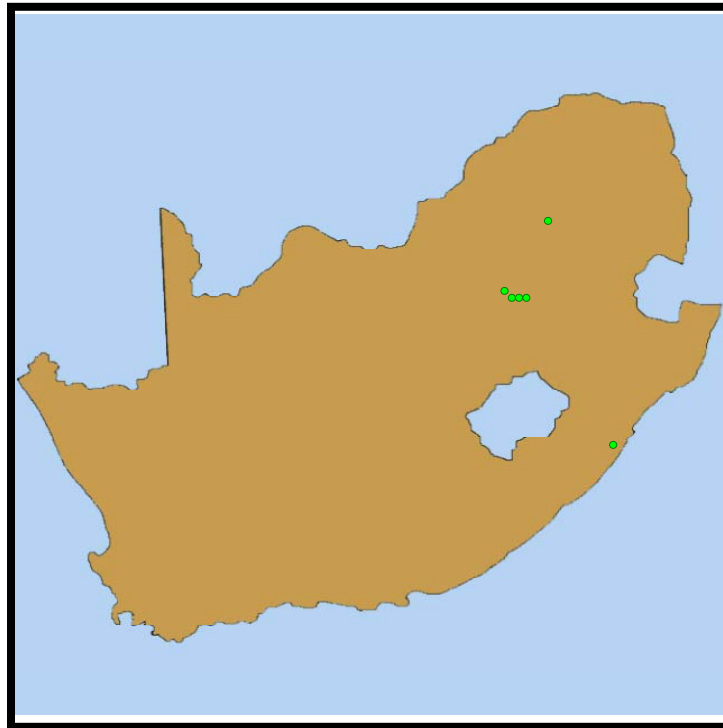
## **2.2 Methodology**

### **2.2.1 *Bacillus thuringiensis* enrichment**

Soil samples were collected from various locations in South Africa differing in soil fertility and altitude (Chen *et al*, 2004). Samples were collected from Muldersdrift mountain range, the Roodekrans botanical gardens (plant storage tissue was also collected at this site), Southbroom in Kwazulu Natal, eastern Mpumalanga province, and several other locations extending from Vosloorus to Heildleburg as shown in Figure 2.1. An ethanol-flamed spatula was used to collect approximately 400g samples from the upper 2 to 5 cm of soil at each site. All soil samples were transported to the laboratory in sterile 200-ml glass bottles.

The soil stock was sampled into 2g soil with 10ml of saline solution in a centrifuge tube (Travers *et al*, 1987). The samples were heat shocked at 80 °C for 10 minutes to eliminate all bacteria incapable of producing endospores. Since it is known that *Bacillus*

*thuringiensis* produces spores, it was safe to assume that if it was present in the soil, it would be in our heat treated sample (Travers *et al*, 1987).



**Figure 2.1:** A map of South Africa indicating the collection sites of soil samples ranging from Southbroom on the Natal coast, the central regions of Gauteng, and Nelspruit in the Mpumalanga province.

The samples were diluted 5 fold to eliminate the amount of humic material within the samples and to reduce the overall colony forming units within each sample. The diluted sample were cultured on nutrient agar and observed under a dissecting microscope. Those colonies resembling the smooth round shape, and earthy colour of *Bacillus* strains were selected for further testing.

## **2.2.2 Culture of bacteria**

Once the sporulating bacteria were isolated, they were plated on nutrient agar plates for 24 hours at 30°C in order to give the spores chance to germinate on media with adequate



nutrients and at optimal temperature (Travers *et al*, 1987). This media however offers favourable growth conditions for a wide range of bacteria as well as *Bacillus thuringiensis*. In order to compensate for this a series of selection tests were further employed to isolate *Bacillus thuringiensis* from the range of bacteria present in the crude soil sample population (Travers *et al*, 1987).

### **2.2.2.1 Sodium acetate selection test**

A sample of each isolate was heat shocked in order to eliminate any vegetative cells. The resultant spore mixture was cultured in T3 media made up with varying concentrations of sodium acetate (0.5, 0.25, 0.12 and 0.06M) and incubated for 24hrs at 30°C. T3 media was derived by Travers *et al* (1987), as a minimal media specific for *Bacillus thuringiensis*, made up of tryptone, tryptose, yeast extract, sodium phosphate and magnesium chloride. This media also partially inhibits the growth cycle as well as sporulation of other *Bacillus* species (Travers *et al*, 1987). The spores were not expected to germinate at high concentrations of sodium acetate, therefore if any isolates were capable of germinating and sustaining growth under these conditions it could not be considered to be *Bacillus thuringiensis*. Samples were heat shocked at 80 °C in a water bath for 10 minutes and then cultured onto T3 agar plates for 24 hours to allow spores to germinate. Once colonies had formed they were streaked onto fresh T3 agar plates to obtain single colonies. This test provides strong support that the isolate may be *Bacillus thuringiensis* depending on the result obtained.

## **2.2.3 Biochemical characterization of *Bacillus thuringiensis*.**

### **2.2.3.1 The Gram stain test**

A very small inoculum of bacteria was smeared onto a clean slide using an inoculation loop. The sample was diluted with a drop of sterile water and allowed to air dry. The specimen was heat-fixed by passing the slide through an open flame. The slide was stained with crystal violet for 1 minute and rinsed with sterile water. The slides were then stained with Gram's iodine (1% iodine, 2% potassium iodide in water) for 1 minute to fix the dye and then rinsed with sterile water. Excess stain was removed with 95% ethanol and then rinsed with sterile water. Specimens were counterstained with Safranin for 1 minute, rinsed with water and then air dried. Slides were viewed using light microscopy under oil immersion (Provine & Gardner, 1974; Bergey's Manual of Systematic Bacteriology, 1986).

### **2.2.3.2 Endospore stain (Schaeffer-Fulton staining method)**

A small inoculum of bacteria was smeared onto a clean slide using an inoculation loop and diluted with a drop of sterile water. Once dry the slides were flooded with Malachite green (made by dissolving 5.0g in distilled water, made up to 100ml) and immediately steamed over a water bath for 5 minutes. Once cooled the slides were rinsed with sterile water. The slides were then counterstained with Safranin O (made by dissolving 0.5

grams Safranin O powder in distilled water, made up to 100ml) for 2 minutes and then rinsed with sterile water. Once the slides had dried, the specimens were viewed under a compound microscope with oil immersion (Mormak & Casida, 1985; Bergey's Manual of Systematic Bacteriology, 1986).

### **2.2.3.3 Catalase test**

The test involved adding hydrogen peroxide to each sample of bacteria. A small sample of 48 hour cultures was smeared onto a clean slide. A drop of 10% hydrogen peroxide was alloquoted onto the bacterial smear and observed using light microscopy. A slide smeared with inoculum free LB media was used as the negative control, and an inoculum of *Bacillus thuringiensis* serovar. *Thuringiensis* (4A3) was used as the positive control. The slides were analyzed for the formation of oxygen bubbles and photographed using a digital camera. The presence of bubbles indicated the ability to break down hydrogen peroxide into water and oxygen (Bergey's Manual of Systematic Bacteriology, 1986); (Reagents and Tests, in Bailey & Scott's Diagnostic Microbiology, 1978).

### **2.2.3.4 Growth above 45 °C**

All samples were diluted in order to obtain an optical density (OD) reading of 0.3. Spectrophotometer readings were taken with an absorbance of 600nm ( $A_{600}$ ) for each sample prior to incubation, and then once daily for a period of 5 days to determine if

growth occurred. Isolated samples were incubated in nutrient broth at a temperature exceeding 45°C for a 5 day period. Cultures that showed signs of growth and thus were capable of reproducing at such high temperatures were assumed not to be *Bacillus thuringiensis* and were eliminated as putative Bt isolates.

### **2.2.3.5 Presence of parasporal bodies ( $\delta$ -endotoxins)**

The presence of parasporal bodies was confirmed using phase-contrast microscopy. Vegetative cells and parasporal bodies were observed on slides freshly coated with a thin layer of 2% water agar (200mg biological grade agar diluted in 100ml sterile water). A drop of culture was placed on the slide and observed using phase-contrast microscopy under a 100X oil immersion objective. Parasporal bodies were characterized as either bipyramidal, spherical, rectangular (cuboid), irregular spherical, or irregularly pointed.

### **2.2.3.6 Total DNA extraction using a sodium dodecyl sulfate (SDS) mediated reaction**

Bacteria cultures were streaked onto agar plates in order to obtain single colonies, thus ensuring a homogenous synchronised culture. The colonies inoculated in 2 x YT media (5 grams of yeast extract, 5 grams of tryptone, and 2.5 grams of NaCl per liter) and grown to an optical density of 0.8 at 600 nm. The cells were harvested by centrifugation, washed once in TES (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl), and

resuspended in solution 1 (25% sucrose, 25 mM Tris-HCl (pH 8.0), 25 mM EDTA and 1 mg of lysozyme per ml). The cell suspension was incubated at 37°C for 1 hour. The cells were solubilized by addition of sodium dodecyl sulfate (SDS) to a final concentration of 2%, and the solution was incubated at 50°C for 15 min.

NaCl was added to a final concentration of 1 M, and the solution was incubated at 50°C for 5 minutes and then at 4°C overnight. The solution was centrifuged, and DNA in the supernatant was precipitated with ethanol. The DNA was resuspended in 10 ml of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 1 M NaCl, 10 µg of RNase per ml, and 0.6 mg of proteinase K per ml and incubated at 37°C for 30 minutes. The mixture was extracted with phenol, phenol-chloroform (1:1), and chloroform, and the DNA was precipitated with ethanol (Kalman *et al*, 1995).

## 2.2.4 PCR amplification the *cryIA* gene sequence

The DNA extracted from the putative Bt isolates was diluted  $10^{-3}$  fold for each reaction and screened via PCR using the *cryIA* specific primers TYIAA (f) and TYIUN12 (r), the sequences of which are listed below. These primers amplify the first 710 bases of the N-terminus of any *cryIA* gene (Kalman *et al*, 1995).

TYIAA (f)      GAGCCAAGCAGCTGGAGCAGTTTACACC      *cryIA(a)*  
TYIUN12 (r)    ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC    *cryI*

DNA extracted from an inoculum of *Bacillus thuringiensis* serovar. *Thuringiensis* (4A3) supplied by Dr. Daniel R. Zeigler from The Bacillus genetic stock centre at the Ohio State University was used as the positive control. The template DNA was mixed with 2X PCR Master Mix containing a 2X concentrated solution of *Taq* DNA polymerase, reaction buffer, MgCl<sub>2</sub> and dNTPs. The PCR reaction was performed for 30 cycles with denaturation of template DNA at 94°C for 1 minute, annealing templates and oligonucleotide primers at 52°C for 2 minutes, and extension of PCR products at 72°C for 3 minutes.

## **2.2.5 Gel Electrophoresis**

An aliquot (7-10 µl) of each amplification reaction was analysed on 2% w/v agarose gels cast and run in TBE buffer (pH 8.3) at about 120 volts until the dye marker was near the end of the gel. Gels were stained with ethidium bromide and the DNA bands photographed under transmitted UV light. A 100 base pair marker (Pharmacia, LKB) was included on every gel.

## **2.2.6 Automated DNA sequencing**

Once positive nucleic acid products were identified through visualization on an agarose gel, a sample of the reaction mixture was purified using an agarose gel DNA extraction kit (Roche Applied Science) to remove all contaminants that may have been left over from the reaction. The purified product was sent for sequencing by Inqaba Biotechnical

Services. The sequences output for the sample products were viewed with 'Chromas2' and Bioedit™ software.

### **2.2.7 Sequence alignment**

Once the PCR amplification products for the putative Bt isolates had been sequenced, it was aligned with the amplification products obtained from the positive control 4A3 which is known to contain the *cryIAa* gene using the Bioedit™ software.. The sequences were also used in NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST) (<http://130.14.29.110/BLAST/>) and compared to known sequences submitted on the NCBI database.

## 2.3 Results

### 2.3.1 *Bacillus thuringiensis* enrichment

Soil samples incubated in T3 media produced thick bacterial cultures overnight in all samples excluding the control flask. Each sample had a distinctive colour (Figure 2.2) which may have been attributed to humic materials unique to each soil type. Colonies obtained from dilutions were tested for identification as catalase-positive, Gram-positive, endospore-forming rods.



**Figure 2.2:** Bacterial cultures derived from soil samples collected at various locations across South Africa. Samples were further diluted in order to reduce the overall crude bacterial population, thus assisting in obtaining pure single colonies. Each culture had a distinct colour.



### 2.3.2 Sodium Acetate selection test

T3 agar plates seeded with isolate samples treated with low concentrations of sodium acetate (0.12 and 0.06M) produced a very few colony forming units.

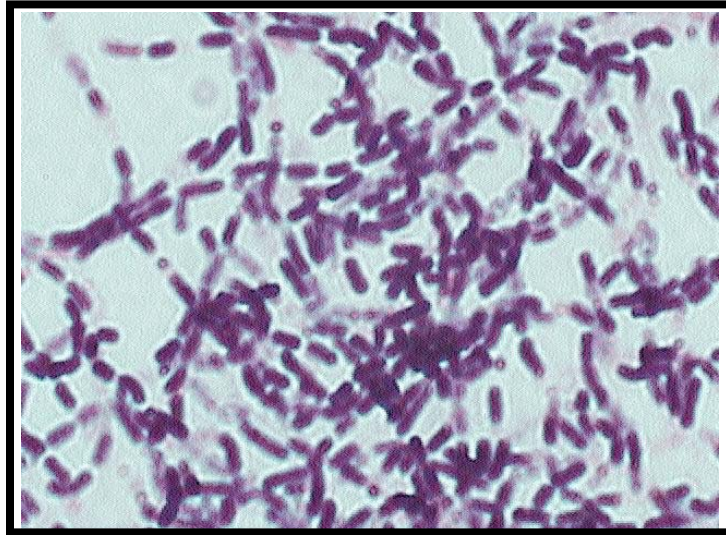


**Figure 2.3:** A digital photograph of a bacterial isolate grown on T3 agar media treated with 0.25M sodium acetate. Single colonies are distinguishable from each other.

Similar results were found with the samples treated with 0.5M sodium acetate which showed very little to no growth. The samples treated with 0.25M sodium acetate produced distinguishable single colonies which were then cultured on nutrient agar for 24 hours to allow for recovery.

### 2.3.3.1 Gram stain

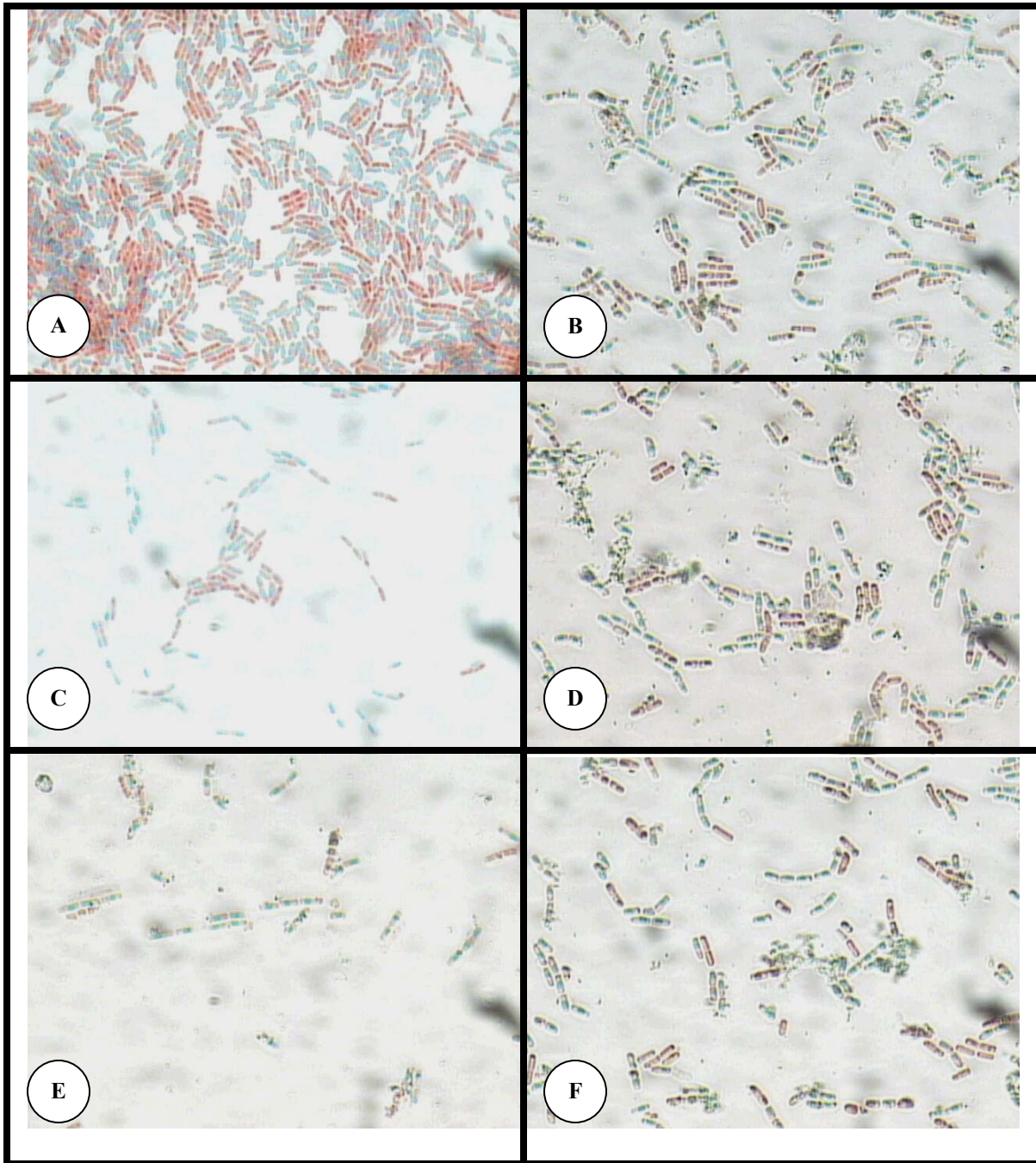
All samples isolated from morphological characterization and sodium acetate selection tested positive as Gram positive rod shaped bacteria as seen in Figure 2.4.



**Figure 2.4:** Digital photographs taken at 100x magnification with oil immersion. All of the putative Bt isolates tested positive for the Gram stain. Bacterial cells appeared dark purple colouring, and were confirmed as rod shaped cocci.

### 2.3.2.2 Endospore stain

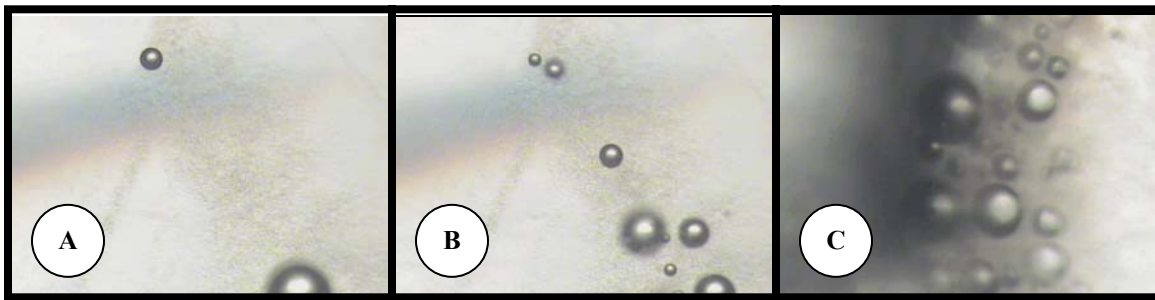
Samples that tested positive for endospores were seen to contain brightly stained green elliptical structures. Slides prepared at time intervals provided additional evidence that these were in fact endospores, where small green spores were clearly visible within pink vegetative tissue at early stages of spore development. The presence of the elliptical green spheres were present in all samples that successfully passed the sodium acetate selection test, but not in all samples that had simply been identified through colony morphology alone.



**Figure 2.5:** Digital photographs of isolates obtained from Muldersdrift soil (A, B&D), storage tissue (E), Nelspruit soil (F) and stream silt(C). In all photographs (A-F ) Green endospores stained with malachite green are distinguishable from the pink safranin O stained vegetative tissue of living bacterial cells (taken at 100x magnification with oil immersion).

### 2.3.2.3 Catalase test

The catalase test was performed on all isolates that tested positive for selection tests including the morphological characterization and the sodium acetate selection test. All of the samples tested were positive for catalase degradation. Upon introduction of hydrogen peroxide to the bacterial smears, samples underwent a violent reaction with bubbles forming at rapid rates. This can be seen in Figure 2.6 where the three pictures A, B and C were taken in succession over a period of approximately 1.5 seconds, showing the immediate presence of bubbles which increased in intensity over time. Similar results were seen in the positive control, whereas the negative control showed no activity.



**Figure 2.6:** Light microscopy photographs of isolate smears in the presence of Hydrogen peroxide, 50 X magnifications. The bubble production intensity from the bacterial smear increases with time as can be seen in A, B and C.

### 2.3.2.4 Growth above 45 °C

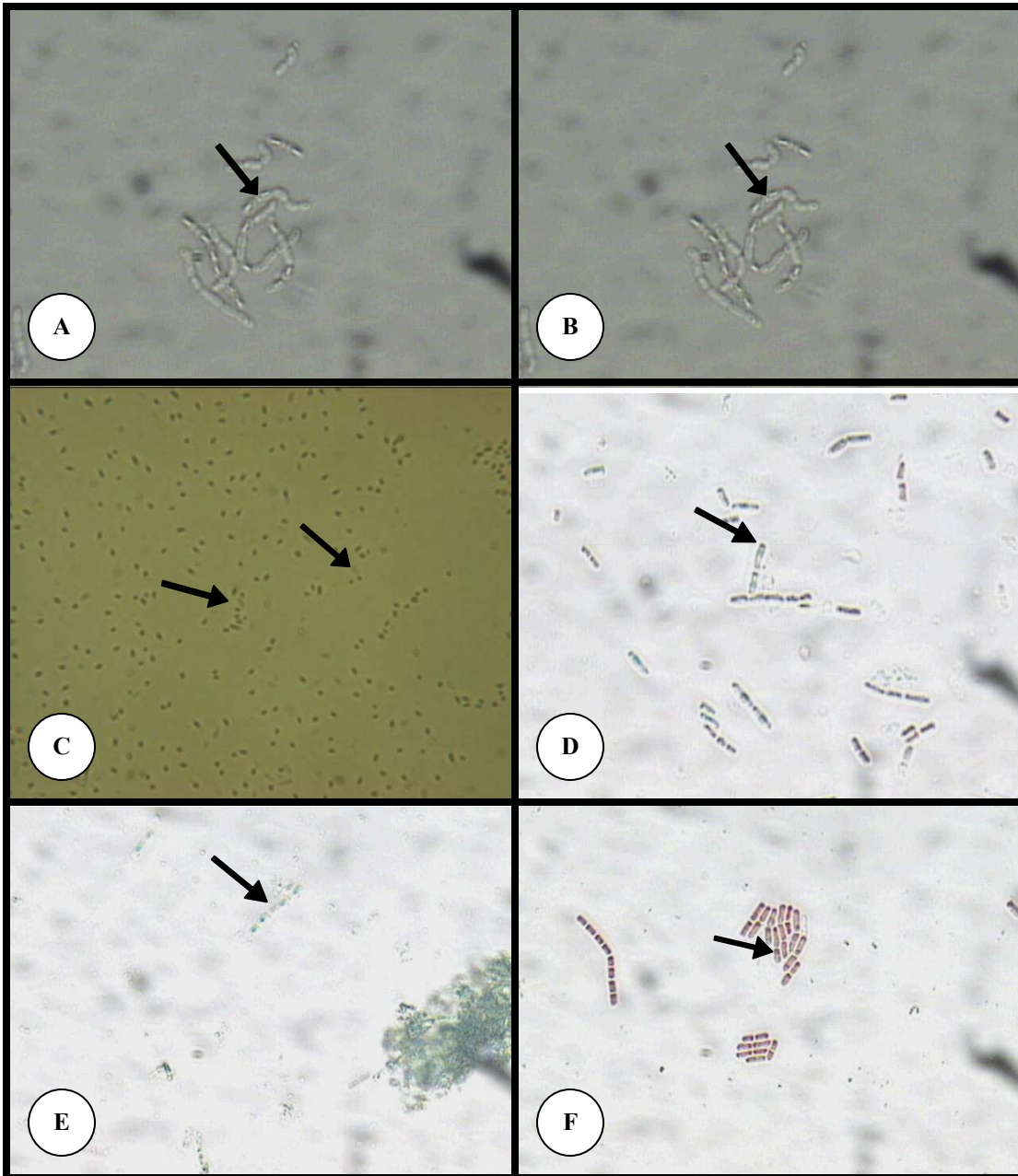
After 24 hours of incubation, 7% of the test samples showed evidence of growth with OD readings increasing in increments ranging from 0.4 to approximately 0.6. The samples did not show any increase in growth over the following four days of incubation, however they were excluded from further tests.

### **2.3.2.5 Presence of parasporal bodies ( $\delta$ -endotoxins)**

The presence of parasporal bodies was not evident in several samples. Many samples showed various shapes that could potentially be crystalline parasporal bodies. Phase contrast microscopy was used to assess the overall appearance of the possible crystalline structures. In Figure 2.7 (A) and (B) a smaller colourless structure is visible along side the endospore within the bacterial cell. In Figure 2.7 (B) bipyrimidal structures are clearly visible amongst the mass of green spores. Structures were identified within the bacterial cell at early stages of spore development which were distinguishable from the endospore Figure 2.7 (F), and remain visible as development progressed.

The overall structure of the crystalline bodies could not conclusively be determined. Since the presence of crystalline bodies was only to be used as support for the identification, those that had passed all other selection criteria were accepted despite the lack of visual crystal confirmation. In total 44 possible isolates were positively identified using both classical identification techniques and sodium acetate selection.





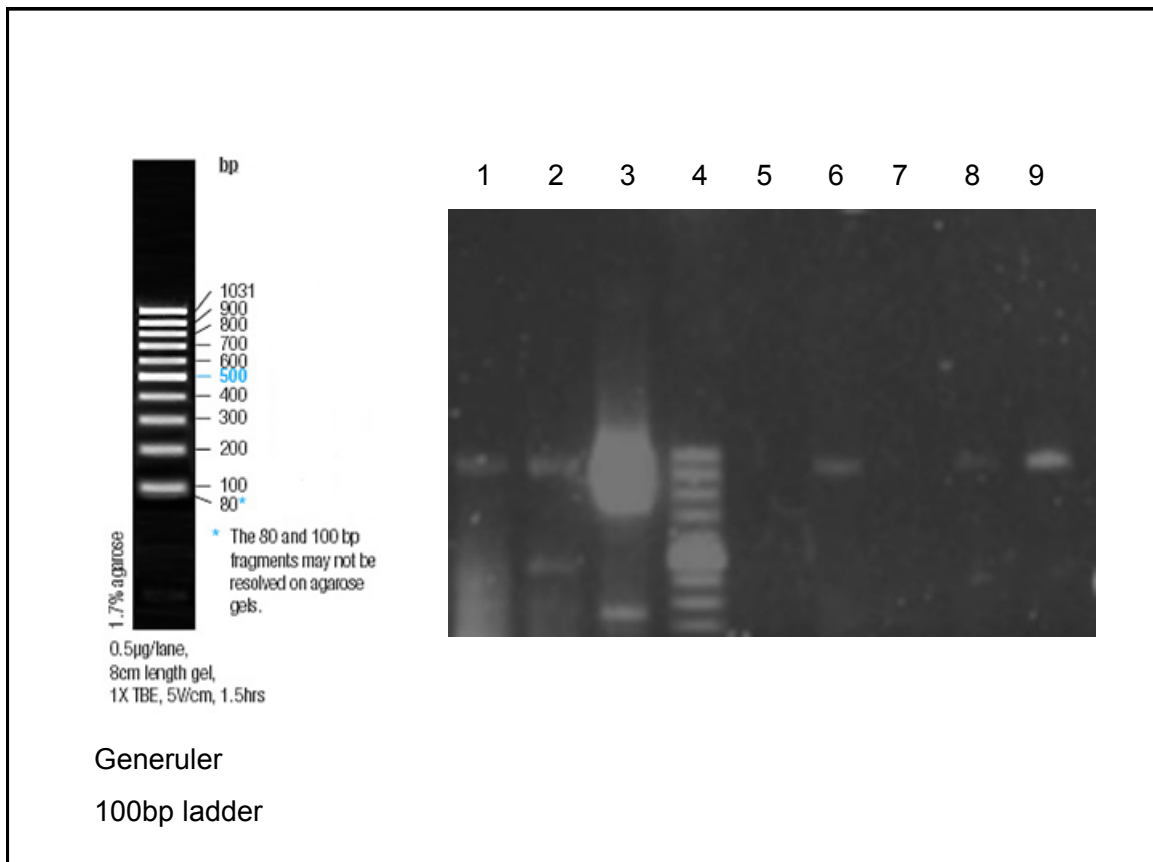
**Figure 2.7:** Light microscopy photographs of (A&B) test isolates taken at 100X magnification with oil immersion. With the use of phase contrast microscopy a smaller spherical shape, indicated with black arrows, is clearly seen alongside the developing endospore. (C) Light microscopy of samples post sporulation. Crystalline shapes are evident amongst green spores. (D) and (E) show evidence of crystalline bodies alongside the green stained endospores. (f) Small dark shapes are evident in early stages of spore development on the opposite polar end to that of the spore.

**Table 2.1:** 44 bacteria samples isolated from soil (Muldersdrift mountain range), storage tissue, Soil from Nelspruit (Mpumulanga province), mushroom tissue and marshlands were identified as putative *Bacillus thuringiensis* isolates

sample name	Source	Gram Stain	Spore Stain	Catalase	Growth above 40 °C	parasporal bodies
S1	soil	+	+	+	+	+
S2	soil	+	+	+	+	-
S3	soil	+	+	+	+	-
S4	soil	+	+	+	+	+
S5	soil	+	+	+	+	+
S6	soil	+	+	+	+	-
S7	soil	+	+	+	+	+
S8	soil	+	+	+	+	-
S9	soil	+	+	+	+	-
S10	soil	+	+	+	+	+
S11	soil	+	+	+	+	-
S12	soil	+	+	+	+	-
S13	soil	+	+	+	+	-
S14	soil	+	+	+	+	-
S15	soil	+	+	+	+	-
st1	storage tissue	+	+	+	+	-
st2	storage tissue	+	+	+	+	-
st3	storage tissue	+	+	+	+	-
st4	storage tissue	+	+	+	+	-
st5	storage tissue	+	+	+	+	-
st6	storage tissue	+	+	+	+	-
st7	storage tissue	+	+	+	+	-
st8	storage tissue	+	+	+	+	-
st9	storage tissue	+	+	+	+	+
St10	storage tissue	+	+	+	+	-
N1	Nelspruit soil	+	+	+	+	+
N2	Nelspruit soil	+	+	+	+	-
N3	Nelspruit soil	+	+	+	+	+
N4	Nelspruit soil	+	+	+	+	+
N5	Nelspruit soil	+	+	+	+	-
N6	Nelspruit soil	+	+	+	+	-
N7	Nelspruit soil	+	+	+	+	-
N8	Nelspruit soil	+	+	+	+	-
N9	Nelspruit soil	+	+	+	+	-
N11	Nelspruit soil	+	+	+	+	-
N12	Nelspruit soil	+	+	+	+	-
N13	Nelspruit soil	+	+	+	+	-
V1	vlei/marsh	+	+	+	+	-
V2	vlei/marsh	+	+	+	+	-
V3	vlei/marsh	+	+	+	+	-
V4	vlei/marsh	+	+	+	+	-
V5	vlei/marsh	+	+	+	+	-
V6	vlei/marsh	+	+	+	+	-

## 2.4 Pilot study to obtain the *cry1A* gene sequence through Polymerase Chain Reaction (PCR) amplification

DNA was successfully extracted from the putative Bt isolates listed in Table 2.1 and examined for potential *cry1A* gene sequences. As seen in Figure 2.8, only 5 of the samples displayed PCR products when viewed under UV light.



**Figure 2.8:** Photograph of a agarose gel exposed to ultraviolet light. PCR amplification products can be seen in lanes 1 (sample S1), 2 (sample S4), 3 (positive control), 6 (sample S6), 8 (sample S9), and lane 9 (sample S10).

Sequencing data obtained from all 5 PCR products appeared to be contaminated with additional sequence signals. Although strong signals are evident in the agarose gel, the resultant sequence data showed many peaks of similar sizes. This result had a high



possibility of mismatches and thus was not useful at this point for the positive confirmation of Bt isolates.

## **2.5 Discussion**

The traditional approaches used in the identification of *Bacilli* have been: aerobic, gram positive, rod shaped bacteria with endospore formation. Having assigned an isolate to these groups, the bacterium was identified to the species level using a panel of physiological and biochemical tests. This system was workable, but familiarity with these bacteria was often necessary in order to distinguish spore morphologies. Largely because of this, later schemes disregarded spore morphology (Claus and Berkley, 1986), but then the number of tests to effect identification had to be increased.

In our experiments the sodium acetate test proved to be more successful in eliminating most sporeforming and nonsporeforming organisms in the test soil samples. The classical biochemical and microscopy tests provide valuable information in understanding the isolated bacterial samples and ultimately aided in their characterization. All of the isolates tested positive for Gram stain, endospore stain and catalase test, confirming the necessity to pasteurize samples in the initial bacterial enrichment. Without pasteurization the ratio of unwanted bacteria (nonsporeforming) to wanted bacteria (sporeforming) would have been too high (Travers *et al*, 1987).

The problem still arises that the pasteurized samples still contain many undesirable sporeformers, particularly the close relatives of *B. thuringiensis* namely *B. anthracis*, and

*B. cereus*. Based on their similar morphologies and genetic makeup it has been proposed that the three should be considered a single species (Helgason *et al*, 2000). This highlights the importance of minimal media T3 used to suppress the growth of other *Bacillus* species and thus favouring the growth of *B. thuringiensis*. The 44 samples listed in Table 2.1 were subjected to both the sodium acetate selection test and classical characterization techniques in order to obtain isolates with high probability of being Bt. Since only 10 of the 44 isolates showed evidence of crystalline bodies, the next logical test was to perform tests at a DNA level to determine whether *cryIA* gene sequences were present within the samples. This led to the pilot study for determining the methods that would be used in PCR experiments to determine the nature of the insecticidal proteins that the samples may contain.

The pilot study was performed to determine the preliminary protocol for identifying possible *cryIA* gene sequences in the putative Bt isolates. Positive amplification products were visible with UV illumination on the agarose gel in Figure 2.8. The resultant sequence that was derived from these products showed several conflicting signals making positive identification impossible. This may have been as a result of a contaminant or several other similar gene sequences being present within the reaction mixture. *B. thuringiensis* subsp. *Kurstaki* HD1 is known to contain at least 5 other *cry* gene sequences (Kalman, *et al*. 1995) providing evidence to this hypothesis. Other possible contaminants may have been attributed to the DNA template or other components used in the PCR reaction.

## Chapter 3

# Molecular characterization of unknown *Bacillus thuringiensis* species isolated from South African soils

### 3.1 Introduction

In an initial study, experiments were performed to determine if the *cryIA* gene sequence could be identified in putative *Bacillus thuringiensis* samples isolated from South African soils. Positive PCR amplification products could be seen on an agarose gel under UV illumination. These products however failed to produce a creditable gene sequence data set. The amplification products appeared to be contaminated with either several copies of similar signals to the desired ones (Kronstad & Whiteley, 1984), or some other form of contamination in the reaction mixture.

To solve this problem all the components used in the PCR reaction had to be examined for possible sources of contamination. This led to the investigation of several methods for DNA extraction to ensure a pure DNA template for each sample was used in the PCR reaction. In addition to obtaining pure plasmid DNA as a template for probing for the presence of the *cryIa* gene, extraction protocols were also put into place to provide genomic template DNA for characterization at the 16S rDNA level.

The main objective of the DNA extractions from the soil isolates was to obtain a technique which produced high yield and quality DNA that can be used in sensitive molecular techniques. These techniques were to produce high molecular weight DNA free of inhibitors. All the selected DNA extraction methods were investigated and assessed for quality, cost, speed and simplicity for subsequent molecular biological manipulations and their ability to fulfil these requirements. These techniques are discussed below.

### **3.1.1 Extraction of Genomic and Plasmid DNA**

#### **3.1.1.1 Genomic DNA Extraction using Caesium Chloride (CsCl) -Ethidium Bromide density gradient centrifugation.**

Equilibrium density gradient centrifugation using caesium chloride (CsCl) was used to obtain very pure nucleic acids from crude homogenates. The procedure separates supercoiled plasmid DNA from nicked plasmid DNA, RNA, proteins and carbohydrates. Cells are lysed and then centrifuged at low speeds to separate most of the chromosomal DNA and debris from the lysate. This is then followed by high speed centrifugation in the presence of CsCl and ethidium bromide. Caesium chloride sets up a density gradient when centrifuged at very high speeds. Ethidium bromide intercalates between the bases of DNA.

Since supercoiled plasmids are negatively charged, the introduction of ethidium bromide induces a relaxed state within the superhelix. This then shifts towards a positive charge as additional ethidium bromide molecules intercalate with the plasmid DNA. This process requires Gibbs free energy which provides the positive enthalpy required for formation of superhelical turns (Seidl & Hinz, 1984), and in its absence, supercoiled plasmid DNA subsequently takes up less ethidium bromide in comparison to linear or open circular DNA (Clendenning & Schurr, 1994). Ethidium is less dense than DNA, thus the density of the linear DNA-ethidium complex would be much less than the supercoiled plasmid DNA-ethidium complex. The differences in density allow for separation of the different DNA species within a density gradient.

### **3.1.1.2 Total DNA extraction using a Sodium Dodecyl Sulfate (SDS) mediated reaction**

Detergents inhibit nucleases and assist in the separation of proteins from nucleic acids. One of the most common detergents used for this purpose is sodium dodecyl sulfate (SDS). Sodium dodecyl sulfate is an anionic detergent which denatures proteins by enveloping the polypeptide backbone. This induces a negative charge within the polypeptide in proportion to its length. The cell membrane phospholipids and protein components become solubilised which results in lysis.

The technique is very similar to the standard alkaline lysis apart from a few exceptions.

SDS is used as the lysing agent as apposed to lysozyme or NaOH. Genomic DNA is however not precipitated out of the lysate in the neutralization stage and is precipitated using ethanol along with plasmid.

### **3.1.1.3 A modified alkaline lysis for large plasmid DNA extraction**

Standard alkaline lysis or boiling protocols are very efficient at isolating the smaller plasmids but they have a tendency to recover low or negligent yields of the larger ones (Birnboim and Doly, 1979). The standard alkaline method makes use of pH to denature non-supercoiled DNA but not plasmids. Increasing the pH to 12.0 -12.5 with the addition of a mild alkali results in breakage of the hydrogen bonds within the non-supercoiled DNA backbone. The double helix subsequently unwinds and the polynucleotide chains separate. The addition of acid traps the denatured DNA in an insoluble mass which can be pelleted out by centrifugation (Birnboim and Doly, 1979).

Since literature suggests that the *cry* genes are located on the larger size plasmids (Kuo & Chak, 1996), this method was investigated as to its suitability for extracting high molecular weight plasmids as well as the lower ones. This method differs from the standard alkaline lysis as well as the above SDS method, in that the salt concentration in the neutralization phase is significantly lower. The denatured proteins and cell debris do

not form an insoluble salt complex, thus preventing the bulkier large plasmids from being trapped.

#### **3.1.1.4 Boiling Lysis**

This protocol yields plasmid DNA that is suitable for restriction digests and cloning purposes. Cells are lysed through exposure to latent heat from either a waterbath or a heating block (100° Celsius). Cells burst due to rapid expansion, and the cell contents are expelled. The extreme temperatures inactivate most enzymes such as DNases or RNases that could potentially degrade the DNA in sensitive molecular techniques.

Once good quality plasmid DNA template has been extracted, it can be used in the construction of a genetic library to determine if additional genes with high sequence similarity were contributing to the contamination of the PCR products (please refer to chapter 4). Genomic DNA was extracted from the 44 putative Bt samples as well as the known *B. thuringiensis* strain 4A3, and was probed with universal ribosomal primers in a PCR reaction.

### **3.1.2 PCR Amplification of 16S rDNA**

The polymerase chain reaction (PCR) offers a powerful tool for characterizing *Bacillus thuringiensis* particularly at the 16S rDNA level (Brunel *et al*, 1997). The sequence of 16S rDNA is made up of highly conserved regions among all organisms. Thus ribosomal DNA is ideal for discerning evolutionary relationships of prokaryotic organisms (Brunel

*et al*, 1997). Ribosomal RNAs are ancient molecules essential for cellular growth, function and survival. Consequently, the primary, secondary and tertiary structures of rRNA molecules have been conserved during evolution. They are functionally constant, universally distributed, and moderately well conserved across broad phylogenetic distances (Brunel *et al*, 1997).

Various regions within the rRNA genes evolve at slightly different rates due to the fact that 16S rRNA is functionally involved in the protein biosynthesis process and involved in different interactions in the ribosome. As a result, alternating regions form in the rDNA sequences of nucleotide conservation and variability. The 16S rDNA of most major phylogenetic groups has one or more characteristic nucleotide sequences called oligonucleotide signatures. These signature sequences are highly specific and occur in most or all members of a particular phylogenetic group. The number of different possible sequences is so large that similarity in two sequences always indicates some phylogenetic relationship. However, it is the degree of similarity in the sequences between two organisms that indicates to what proximity they are related. Using the sequence comparison data, sequence signatures can then be used to assign microorganisms to the proper group (Weisburg *et al*, 1991).

The two oligonucleotide sequences used as primers in our study were 59-GGA GAG TTA GAT CTT GGC TC-39(sense) and 59-AAG GAG GTG ATC CAG CCG CA-39 (antisense) (Brunel *et al*, 1997) characterized as described by Weisburg *et al* (1991). These primers are capable of amplifying nearly full-length 16S ribosomal DNA (approximately 1,500-bp) fragments from many bacterial genera. By doing so we were



able to confirm that our samples were indeed *Bacillus thuringiensis*, and establish its closest relatives according to the signature sequences within the conserved regions.

The specific objectives for this chapter were:

- Obtain high quality template DNA free of any contaminants
- Determine the 16S rDNA identity of putative Bt isolates via PCR using 16S rDNA specific primers
- Obtain sequence data from the amplification products and compare the data to that of known Bt 16S rDNA sequences
- Construct phylogenetic trees to determine evolutionary lineage

## **3.2 Methodology**

### **3.2.1 Genomic DNA extraction using caesium chloride (CsCl) - ethidium bromide density gradient centrifugation**

Bacterial cultures were streaked onto agar plates in order to obtain single colonies, thus ensuring a homogenous synchronised culture. The colonies were then grown up in LB media containing 2% glycine over a period of 3-4 days in order to prepare cell walls for lysis. Cultures were pelleted at 6000 rpm in a J10 centrifuge. Pellets were resuspended in 4ml of solution 1 (20 mg/ml lysozyme in TE buffer). The samples were incubated at 37°C for 30 minutes. The cells were once again pelleted and then resuspended in solution 2 (TE buffer supplemented with 0.5 mg Proteinase K). This was left to incubate

at room temperature for 2 minutes and then one tenth volume of solution 3 (TE buffer supplemented with 10% SDS) was added. These samples were incubated at 37°C for 30 minutes to induce lysis of cells. The viscous solution was transferred to a V-50 Ti tube and ultra-centrifuged at 6000rpm for 30 minutes in a L5-50 centrifuge.

The supernatant was transferred to a J20 tube and supplemented with 4.4 g CsCl<sub>2</sub>. The mixture was centrifuged at 15000 rpm for 15 minutes. The lower level containing the DNA was then removed using a Pasteur pipette. EtBr (400 µl) was added to each sample and the refractive index (density of a substance in double distilled H<sub>2</sub>O) adjusted to between 1.391 and 1.392. Samples were then transferred to a Beckman quick seal tube and centrifuged at 45000 rpm overnight in a L7 – 55 Beckman ultra-centrifuge using a VTi 65.2 vertical rotor. DNA was extracted by placing the tube in a UV light clamp, and extracting the nucleic acid band using a 1ml syringe with a wide aperture. DNA was then precipitated several times by the addition of butanol in order to remove the EtBr from the DNA sample.

### **3.2.2 Total DNA extraction using a sodium dodecyl sulphate (SDS) mediated reaction**

Bacterial cultures were streaked onto agar plates in order to obtain single colonies, ensuring a homogenous synchronised culture. The colonies were then grown up in 2 x YT media (5 g of yeast extract, 5 g of tryptone, and 2.5 g of NaCl per liter) to an optical density at 600 nm of 0.8. The cells were harvested by centrifugation, washed once in TES

(10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl), and resuspended in a solution made up of 25% sucrose, 25 mM Tris-HCl (pH 8.0), 25 mM EDTA and 1 mg of lysozyme per ml. The cell suspension was incubated at 37°C for 1 h. The cells were solubilized by addition of sodium dodecyl sulfate (SDS) to a final concentration of 2%, and the solution was incubated at 50°C for 15 minutes.

NaCl was added to give a final concentration of 1 M, and the solution was incubated at 50°C for 5 minutes and then at 4°C overnight. The solution was centrifuged, and DNA in the supernatant was precipitated with ethanol. The DNA was resuspended in 10 ml of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 1 M NaCl, 10 µg of RNase per ml, and 0.6 mg of proteinase K per ml and incubated at 37°C for 30 minutes. The mixture was extracted with phenol, phenol-chloroform (1:1), and chloroform, and the DNA was precipitated with ethanol (Kalman, *et al.* 1995).

### **3.2.3 A Modified alkaline lysis for large plasmid DNA extraction**

Bacterial cultures were streaked onto agar plates in order to obtain single colonies, thus ensuring a homogenous synchronised culture. The colonies were then grown up overnight at 30°C in 2 ml LB with a final OD600 between 1.1- 1.5. The cultures were transferred to a 1.5ml microcentrifuge tube and pelleted at maximum speed for 5 seconds. The cells were suspended in 100 µl E buffer (15% w/v sucrose, 40 mM Tris-HCl, 2 mM EDTA, pH 7.9) by pipetting them up and down and then adding 200 µl lysing solution (3%

SDS, 50 mM Tris-HCl, pH 12.5). The lysate was heated at 60°C for 30 minutes. 5 Units proteinase K were added and the tube inverted 20 times. Samples were incubated at 37°C for 90 minutes followed by the addition of 1 ml phenol-chloroform-isoamyl alcohol and inverted 40 times. Samples were centrifuged at high speed for 15 minutes. followed by analysis of the aqueous supernatant by electrophoresis on a horizontal 0.5% agarose gel.

### **3.2.4 Boiling lysis**

Bacterial cultures were streaked onto agar plates in order to obtain single colonies, thus ensuring a homogenous synchronised culture. The colonies were collected using a metal loop and resuspended in 1 ml sterile water. The microcentrifuge tubes were vortexed until a homogenous cell paste was obtained. The tubes were boiled at 100°C in a heat block for time intervals of 5 and 7 minutes. The samples were centrifuged for 10 minutes at maximum speed (10K) in a microcentrifuge. The supernatant was transferred to 1.5ml microcentrifuge tubes containing 750µl phenol-chloroform isoamyl alcohol. The tubes were vortexed and then centrifuged in a microcentrifuge for 10 minutes at maximum speed (10K) in a microcentrifuge. The upper phase was transferred to a new tube and washed with 70% ethanol. The tubes were centrifuged and the pellet resuspended in 200µl sterile water.

### **3.2.5 Measurement of DNA concentration and purity**

In order to ensure that the yields of pure DNA obtained from the various extraction techniques were high enough to be used in further manipulation experiments, the end products of each procedure was measured using UV absorbance spectrophotometry. The amount of DNA in the sample is directly proportional to the amount of UV radiation absorbed. The absorbance was measured at 260nm ( $A_{260}$ ) for DNA and at 280nm ( $A_{280}$ ) for proteins. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. In contrast to nucleic acids, proteins have a UV absorption maximum of 280 nm, due mostly to the tryptophan residues. The absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The ratio of  $A_{260}$  to  $A_{280}$  was determined to establish purity of the DNA (Brown, 1998).

### **3.2.6 Gel electrophoresis**

An aliquot (7-10  $\mu$ l) of each amplification reaction was analysed on 2% w/v agarose gels cast and run in TBE buffer (pH 8.3) at about 120 volts until the dye marker was near the end of the gel. Gels were stained with ethidium bromide and the DNA bands photographed under transmitted UV light. A 100 base pair marker (Pharmacia, LKB) was included on every gel.

### **3.2.7 PCR amplification of 16S rDNA**

For PCR amplification, template DNA of the 44 putative isolates (Table 2.1) obtained from various extraction methods was diluted with sterile water  $10^{-3}$  fold for each reaction. For DNA extracted using the boiling lysis method, 5 $\mu$ l of the boiled suspension was used for each reaction. The template DNA was mixed with 2X PCR Master Mix (Fermantas Life Sciences) containing a 2X concentrated solution of *Taq* DNA polymerase, reaction buffer, MgCl<sub>2</sub> and dNTPs. The PCR reaction was performed for 30 cycles with denaturation of template DNA at 94°C for 1 minute, annealing templates and oligonucleotide primers at 63°C for 2 minutes, and extension of PCR products at 72°C for 3 minutes. The PCR products were separated and analyzed on a 2% agarose gel. DNA obtained from *B. thuringiensis* Serovar. *Thuringiensis* (4A3) and *B. thuringiensis* Serovar. *Brasilensis* (4AY1) supplied by Dr. Daniel R. Zeigler from The Bacillus genetic stock centre at the Ohio State University were used as positive controls

### **3.2.8 Automated DNA sequencing**

Once positive PCR products were identified through visualization on an agarose gel, a sample of the reaction mixture was purified and then sequenced by Inqaba Biotechnical Services. The sequences obtained were viewed with 'Chromas2' and Bioedit™ software.

### **3.2.9 Alignment of 16S rDNA sequence data with known 16S**

#### **rDNA sequences**

Once the nucleotide sequence of the PCR product had been derived, it was compared with sequences of strains known to contain the *cryIAa* gene. Sequence alignment was performed using the NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST)(<http://130.14.29.110/BLAST/>) to compare the sequence data with known sequences submitted on the NCBI database. The sequences obtained were analysed using the multiple sequence alignment program ClustalX 1.81 (Higgins, 1994) in order to determine homology within the sequences as well as to determine their evolutionary lineage. Phylogenetic trees were constructed using the Neighbour Joining (NJ) and Bootstrap Tree methods of the Clustal X program (Higgins, 1994).

## **3.3 Results**

### **3.3.1 DNA extraction**

From the initial  $A_{260/280}$  ratio obtained in Table 3.1, along with the intensity and molecular weight of DNA bands seen on the agarose gel in Figure 3.1, it can be seen that all four techniques used produced good quality DNA. The purity of the extracted DNA is seen in Table 3.1 where the OD readings obtained were converted into an  $A_{260/280}$  ratio which is a

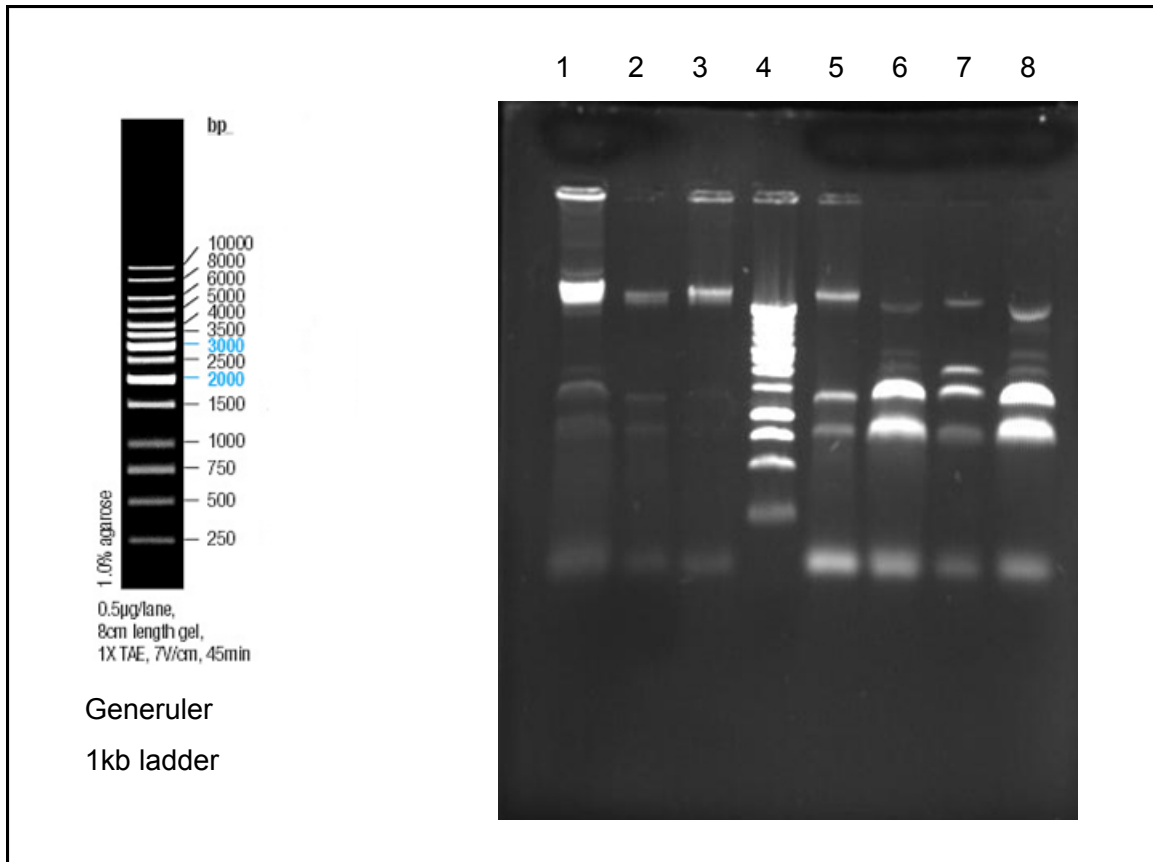
direct reflection of amount of DNA compared to protein. The CsCl-EtBr density gradient centrifugation method produced high purity plasmid DNA, however it produced negligible amounts of genomic DNA. The CsCl-EtBr density gradient centrifugation is seen to extract the highest purity DNA and boiling lysis the lowest.

**Table 3.1:** A comparison of  $A_{260/280}$  Ratios of DNA obtained by four DNA extraction procedures.

<b>Extraction Method</b>	<b>No. of Samples</b>	<b><math>A_{260/280}</math> Ratio</b>
CsCl-EtBr density gradient centrifugation	44	$1.82 \pm 0.02$
Total DNA, SDS mediated reaction	44	$1.35 \pm 0.05$
Modified Alkaline lysis for large plasmid extraction	44	$1.47 \pm 0.05$
Boiling lysis	44	$1.3 \pm 0.07$

The modified alkaline lysis recovered higher purity DNA as compared to the similar SDS mediated reaction which had a higher salt content. The plasmid DNA recovered from the various techniques was run on a 1.2% agarose gel to assess the size of plasmids that had been extracted.



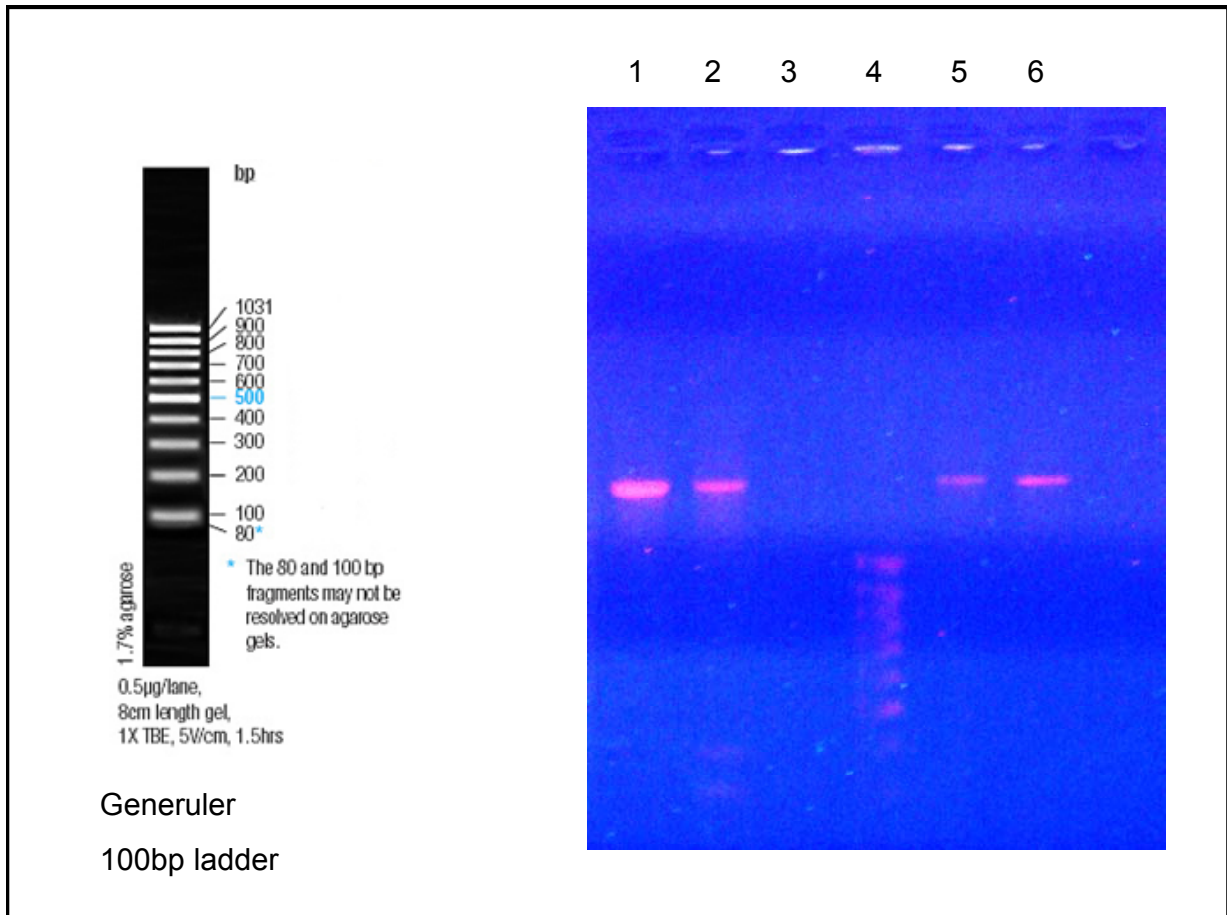


**Figure 3.1:** A Photograph of an agarose gel loaded with DNA extracted from sample S10 using four DNA extraction procedures (lane 1) Boiling lysis for 7 minutes, (lanes 2 & 3) boiling lysis for 5 minutes, (lane 4), Generuler 1 kb Marker, (lanes 5 & 7) SDS mediated reaction, (lanes 6 & 8) Modified alkaline lysis for extraction of larger plasmids

From the agarose gel (Figure 3.1) it can be seen that the 7 minute boiling time lysis recovered more DNA of high molecular weight than the 5 minute boiling time. The array of plasmids attained was low in comparison to the modified alkaline lysis procedure which was seen to recover a larger array of plasmids than any other technique. The SDS mediated reaction is seen to have low recovery success of the higher molecular weight plasmids.

### 3.3.2 Obtaining the 16S rDNA identity of the isolates through polymerase chain reaction (PCR) amplification

From the agarose gel in Figure 3.2, it can be seen that positive PCR amplification products were visualised for samples S4, S9 and S10. Additional positives were obtained for S6, n1, n3 and n5 (not seen here).



**Figure 3.2:** A Photograph of an agarose gel loaded showing PCR amplification products for positive control 4A3 (lane 1), sample S4 (lane 2) negative control (lane 3) 100bp marker (lane 4) sample S9 (lane 5) and sample S10 (lane 6)

The following Automated sequencing results shown in Figure 3.3 were provided by Inqaba Biotechnical Services.

Query	67	ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGC	126
Sbjct	1437	.....	1378
Query	127	ATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATC	186
Sbjct	1377	.....A.....	1318
Query	187	CGAACTGAGAACGGTTTTATGAGATTAGCTCCCCCTCGCGGTCTTGCAGCTCTTTGTACC	246
Sbjct	1317	.....A.....	1258
Query	247	GTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCTTGATGATTTGACGTCATCCC	306
Sbjct	1257	.....A.....	1198
Query	307	CACCTTCCTCCGTTTGTACCGGCAGTCACTTTAGAGTGCCCAACTAAATGATGGCAAC	366
Sbjct	1197	.....C.....	1138
Query	367	TAAGATCAGGGTTGCGCTCGTTGCGGGACTTAACCCCAACATCTCACGACACGAGCTGA	426
Sbjct	1137	.....A.....-	1079
Query	427	CGACAACCATGCACCACCTGTCACTCTGCTTCCCGAAGGAGAAGCCCTATCTCTTAGGGT	486
Sbjct	1078	.....-.....-	1021
Query	487	TTTTAGAGGTATGTCAAGACCTGGTAAGTTCTTCGCGTTGCTCCGAATTAACCACAT	546
Sbjct	1020	.G.C.....-.....-	963
Query	547	GCTCCACCGCTTGTGCGGGCCCCGTTCAATTCCTTTGAGTTTTCAGCCTTGAGGCCAA	606
Sbjct	962	.....-.....-.....C...GT	906
Query	607	ACCTCCCCAGGCGGAGGGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCTCTC	666
Sbjct	905	..-.....T.....A	847
Query	667	A-ACTTAGCACTCTTCGTT-ACGGGGTGGACTACC-GGGTATCTAATCCTGTTTGA-CCC	722
Sbjct	846	.C.....A...T...C.....A.....CT...	787
Query	723	CACACTTTC-CGCCTC-GTGT-AGT-ACAGAC-AAAAAGA-GCCTTCGC	765
Sbjct	786	...G...G...A...C...T...C...TC.....	738

**Figure 3.3:** BLAST alignment of the nucleic acid sequence derived from sample S10 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 673/709 (94%), Gaps = 19/709(2%).

The sequence data was compared to those of known 16S rDNA sequences on the NCBI database with the use of the Mega BLAST alignment tool. The alignment shown in Figure 3.3 shows a 94% match between sample S10 and that of known Bt16S rRNA gene sequences, with 56 hits in 3 organisms, 14 hits for *Bacillus thuringiensis* serovar *konkukian* str. 97-27 and 1 hit for *Bacillus thuringiensis* serovar *aizawai*.

Query	65	GTAACCTGCCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACTAGGATAACCT	124
Sbjct	64	.....C.....-C.....A.	122
Query	125	ATTGAACTGCATGGTTCAAAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC	184
Sbjct	123	T.....-G.....	181
Query	185	CCGTCACATTAGCTAGTTGGTGAGGTAACCGCTCCCCGAGGCAACGATGCGTAGCCGACC	244
Sbjct	182	G...G.....G...A..A.....	241
Query	245	TGAGAGGGTGATCTGGCCACACTGGGACTGAAACACGGGCCCA-ACTCCCTACTGGAGG	303
Sbjct	242	.....-.....G...-...G...-...G.....	297
Query	304	CAGCAGTTGGGAATCTTCCACAATGGACGAAAGTCTGACGGAGCAACGCCGCGGAGTGG	363
Sbjct	298	.....A.....G.....T...-	356
Query	364	A-GAAGGCTTTTCGGGACGTAATAACTCTGTTGTTAGGGAAGAACAAGTGCTTTGTTGAACA	422
Sbjct	357	.T.....T.....-A.....T.	415
Query	423	AGCTGGCCCCCTTGACGGTACCTACTCCGGAATGCC-CTGGTTAATTACGTGCCAGCAGCC	481
Sbjct	416	.....A.....-A..A..A..A.-..C..C.....	473
Query	482	CCGTATTACATAGGGGCAAGCGATATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGG	541
Sbjct	474	G...A...G...T.....T.....T	533
Query	542	GGTTCCTCTTTAAGTCTAGAAGTGAACCCCCCGCCTCAACCGGGGAGGGCACATGGGCA	601
Sbjct	534	.....-.....-.....T...G...A...-.....T.....T-...T...-	587
Query	602	AACGGGGGGACTT-ATCTCCCCTAAGATGATATGTGGATTTTCCTTGTGTACCCTTTCAA	660
Sbjct	588	...T...A.....G.G-.G.AG-...G..A.-...A..-..A.....G..G.GA..	643
Query	661	TTGCGAAAAGATATGGAGAGAACCCCCCGTGGCAAAGGCGACTTTCGGGCCTGAAACTGA	720
Sbjct	644	..-...T.G.....-...A..A-.....G.....T..T...T.....	700
Query	721	CCTCTAGAGGCGCCGAAATCCCGCGGAGCACAACCGG-TTATGGATACCCGGGTAGTCCA	779
Sbjct	701	.A-.-.....-.....G.GT.G.....-...A..A...-.....T.....	754
Query	780	CCCCAAAACCATGAGTGCTAGG-GTTAGGGGGTTCCCGCCCTTTAGTGC	828
Sbjct	755	.G..GT...G.....A.T...A...T.....	804

**Figure 3.4:** BLAST alignment of the nucleic acid sequence derived from sample S9 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 663/770 (86%), Gaps = 35/770 (4%).

The alignment for sample S9 seen in Figure 3.4 gave an 88% match with known *B.thuringiensis* 16S rRNA gene sequences, with 34 hits in two organisms, and 1 hit for *Bacillus thuringiensis* serovar *colmeri*.

Query	28	TCTC-AGAAATTAGTGACGCGA-GGGTGAACAAACACGTGTAGTAACCTGCCATAAGAC	85
Sbjct	25	....A....G....C.G...-..C.....-GT.....-G.....	81
Query	86	TGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTGAACTGCATGGTTCGA	145
Sbjct	82	.....	141
Query	146	AATTGAAAGGCGGCTTCGGCTGTCACCTTATGGATGGACCCGCGTCGCATTAGCTAGTTGG	205
Sbjct	142	.....	201
Query	206	TGAGGTAACGGCTCACCAAGGCATCGTATGCGTAGCCAACCTGAGAGGGCGACCGGCCAC	265
Sbjct	202	.....A.-.....G.....T..T.....	260
Query	266	ACTGGCACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTATGGAATCTTCCGCAA	325
Sbjct	261	.....G.....G.....	320
Query	326	TGGACGTAAAGTCTGACGGAGCATTCGCCGCGGGAGTAGATGCAAGGCTTTCGGGCCCTA	385
Sbjct	321	.....-.....-A.....T.....-.....-.....T.G..	376
Query	386	TAAACTCTGTTGTTAGGGAAGAACAAGTGC TAGTTGAATAAGCTGGCGCCTTGACGGTAC	445
Sbjct	377	-.....A.....	435
Query	446	CTAACCAGAATGTCTTTTGTTTAACTACGTGCTCATCAACCGCGGTAATACGAAGGGGGC	505
Sbjct	436	.....A.C.ACG-.C.-.....-..G..G.....T..T..	492
Query	506	ACGCGTTATCGGGAATTGTTGGGCGTAAAGCGCGCAAGTGGTTTCTTAAGTCTGATGG	565
Sbjct	493	.A......C.....A.....G.....T	552
Query	566	GGAAGCCCACCGCTCAACCGGGGAGGGTCGTTGGTAACCGGGAGACTTGAGTGCAGATGA	625
Sbjct	553	.A......G.....T.....A...A..T.....A..	612
Query	626	GGAAAGAGGTAATTCCTATGTGAAGCGGGGAATTGCG-ATAG-TATTGGAGGAACCTACGG	683
Sbjct	613	.....T.-.....-.....T.....T...A...T.G..A.-.....-..CA	668
Query	684	GTGGCGAGAG-CGACTTCTGGTCTGTAA-TGACAC-GAGGC-CGTTAGC-TGGGGAGTC	738
Sbjct	669	.....-..G.....C.....T.....G..AA..G.....-	726
Query	739	AG-C-GCATTAGA-ACCCTGGTAGTCTCGCCG-AAACAATGAGTGC-AAGAGTG-GAGG	792
Sbjct	727	.AA.A.G.....T.....A.....T...G.....T..T..TA....	786
Query	793	CTT-CCGCC-TGTAGTG	807
Sbjct	787	G..T.....C.T.....	803

**Figure 3.5:** BLAST alignment of the nucleic acid sequence derived from sample 4 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 706/797 (88%), Gaps = 35/797 (4%).

The alignment for sample S4 seen in Figure 3.5 gave an 86% match with known *B.thuringiensis* 16S rRNA gene sequences, with 34 hits in two organisms, and 1 hit for *Bacillus thuringiensis* serovar *colmeri*.

Query	70	ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGTAACGTATTCACCGCGGC	129
<b>Sbjct</b>	1427	..... <b>G</b> .....	1368
Query	130	ATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATC	189
Sbjct	1367	.....	1308
Query	190	CGAACTGAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACC	249
Sbjct	1307	.....	1248
Query	250	GTCCATTGTAGCACGTGTGTAGCCCAGGTATAAGGGGCATGATGATTTGACGTCATCCC	309
Sbjct	1247	.....	1188
Query	310	CACCTTCCTCCGGTTTGTACCCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGGCAAC	369
Sbjct	1187	.....	1128
Query	370	TAAGATCAAGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGAC	429
Sbjct	1127	.....	1068
Query	430	GACAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTT	489
Sbjct	1067	.....	1008
Query	490	CAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAACCACATGCTCC	549
Sbjct	1007	.....	948
Query	550	ACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCA	609
Sbjct	947	.....	888
Query	610	GCGGAGTGCTTAATGCGTAACTTCAGTACTAAAGGGCGGAAACCCTCTAACACTTAGC	669
<b>Sbjct</b>	887	..... <b>C</b> .....	828
Query	670	ACTCATCGGTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGTCTCCCACGCTTTC	729
<b>Sbjct</b>	827	..... <b>T</b> .....	768
Query	730	GCGCCTCAGTGTCAATTACAGACCAGAGAGCCGCCTTCGCCAC-GGTGTTCTCCATATC	788
<b>Sbjct</b>	767	..... <b>A</b> .. <b>T</b> ..... <b>T</b> .....	708
Query	789	TCTACG-ATT-C-CCGCT-CAC-TGGAATTC-ACTT	818
<b>Sbjct</b>	707	..... <b>C</b> .. <b>T</b> .. <b>A</b> ..... <b>A</b> .. <b>A</b> ..... <b>C</b> .....	672

**Figure 3.6:** BLAST alignment of the nucleic acid sequence derived from control (4A3) and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 744/756 (98%), Gaps = 7/756 (0%).

The alignment for control (4A3) seen in Figure 3.6 gave a 98% match with known *B.thuringiensis* 16S rRNA gene sequences, with 34 hits in one organism.

Query	73	ACAAACTCTCGTGGTGCAGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGG	132
<b>Sbjct</b>	1374	.....-T.....	1316
Query	133	CATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAAT	192
Sbjct	1315	.....	1256
Query	193	CCGAACTGAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTAC	252
Sbjct	1255	.....	1196
Query	253	CGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC	312
Sbjct	1195	.....	1136
Query	313	CCACCTTCCTCCGGTTTGTACCCGGCAGTCACCTTAAAGTGCCCAACTAAATGATGGCAA	372
<b>Sbjct</b>	1135	.....G.....	1076
Query	373	CTAAGATCAAGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA	432
Sbjct	1075	.....	1016
Query	433	CGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTG	492
Sbjct	1015	.....	956
Query	493	TCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAACCACATGCTC	552
Sbjct	955	.....	896
Query	553	CACCGCTTGTGCGGGCCCCGTC AATTCTTTGAGTTTTTCAGCCTTGC GGCCGTACTCCC	612
<b>Sbjct</b>	895	.....-.....	837
Query	613	CAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTA	672
Sbjct	836	.....	777
Query	673	GCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGTCCCCACGCTT	732
Sbjct	776	.....	717
Query	733	TCGCGCCTCAGTGTGAGTTACAAACAAAAAG-CGCCTTCGCCACTGGTGTTCCTCCATA	791
<b>Sbjct</b>	716	.....G.C.G...T.....	657
Query	792	TCTCTCC-CATTTAACCGCTACA-AGGGAATTC-ACTTTCCT	830
<b>Sbjct</b>	656	.....A.G.....C.....C.T.....C.....	615

**Figure 3.7:** BLAST alignment of the nucleic acid sequence derived from sample n1 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 748/762 (98%), Gaps = 6/762 (0%).

The alignment for sample n1 gave a 98 % match with known *B.thuringiensis* 16S rRNA gene sequences, as seen in Figure 3.7, with 21 hits in two organisms, and 14 hits for *Bacillus thuringiensis* serovar *konkukian* str. 97-27.

Query	68	ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGTAACGTATTCACCGCGGC	127
<b>Sbjct</b>	1423	..... <b>G</b> .....	1364
Query	128	ATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATC	187
Sbjct	1363	.....	1304
Query	188	CGAACTGAGAACGGTTTTATGAGATTAGCTCCCCCTCGCGGTCTTGCAGCTCTTTGTACC	247
<b>Sbjct</b>	1303	..... <b>A</b> .....	1244
Query	248	GTCCATTGTAGCAAGTGTGTAGCCCAGGTGATAAGGGGCATGATGATTTGACGTCATCCC	307
<b>Sbjct</b>	1243	..... <b>C</b> .....	1184
Query	308	CACCTTCCTCCGTTTTGTCCCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGGCAAC	367
Sbjct	1183	.....	1124
Query	368	TAAGATCAAGGTTGCGCTCGCTGCGGGACTTATTCCCATTATCTTCACGACACGAGCTG	427
<b>Sbjct</b>	1123	..... <b>T</b> ..... <b>-A</b> ..... <b>AC</b> ..... <b>-</b> .....	1066
Query	428	ACGACAACCATGCACCACGGTTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGT	487
<b>Sbjct</b>	1065	..... <b>CT</b> ..... <b>-</b> .....	1007
Query	488	TGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAACCACATGT	547
<b>Sbjct</b>	1006	..... <b>-</b> .....	948
Query	548	CTCCACCGGTGGAGCAGGGCCCCGAAAATTTCCCTTTGAGTTTCAGCCTTGGCGCCGAAC	607
<b>Sbjct</b>	947	..... <b>C.T.T</b> ..... <b>-</b> ..... <b>TC</b> ..... <b>-</b> ..... <b>T</b> .....	890
Query	608	TCCCCAAGG-GGAGTGCTTAATGCGTTAAACT-CAGCACTAAAAGGCGGAAACCCCTTCTA	665
<b>Sbjct</b>	889	..... <b>-</b> ..... <b>C</b> ..... <b>-</b> ..... <b>T</b> ..... <b>G</b> ..... <b>-</b> .....	833
Query	666	A-ACTTAAGAACTCATCGTTTATGGTGTGTGACTATCTGGGTTTCTAATTTTGTGTTGCTC	724
<b>Sbjct</b>	832	<b>.C</b> ..... <b>-</b> ..... <b>C</b> ..... <b>C</b> ..... <b>-</b> ..... <b>C.A</b> ..... <b>A</b> ..... <b>CC</b> .....	775
Query	725	CC-ACGCTTTTCGCGCCTCAG-GCC-GTTTGCA-ACCAGAAAG-CGACTT	769
<b>Sbjct</b>	774	<b>..C</b> ..... <b>-</b> ..... <b>T.T.A</b> ..... <b>A</b> ..... <b>G</b> ..... <b>T.C</b> .....	727

**Figure 3.8:** BLAST alignment of the nucleic acid sequence derived from sample n3 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 662/710 (93%), Gaps = 21/710 (2%)

The alignment for sample n3 gave a 93 % match with known *B.thuringiensis* 16S rRNA gene sequences, as seen in Figure 3.7, with 17 hits in two organisms, and 14 hits for *Bacillus thuringiensis* serovar *konkukian str. 97-27*.

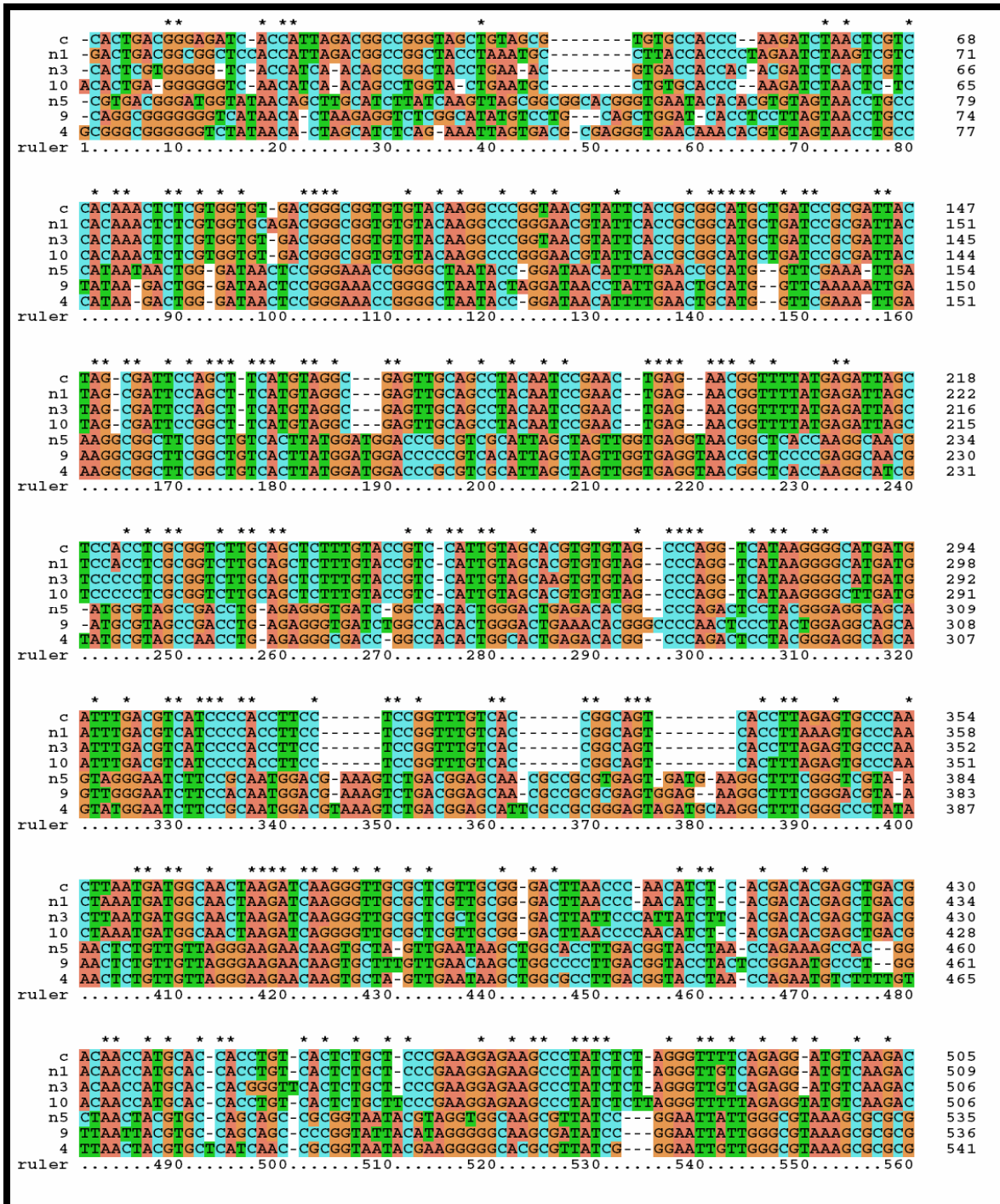


Query	16	TAAACAGCTTGCATCTTATCAAGTTAGCGGCGGCACGGGTGAATACACACGTGTAGTAACC	75
<b>Sbjct</b>	9385	... <b>G</b> .....-..... <b>G</b> .....-..... <b>G</b> ..-.....- <b>G</b> .....	9440
Query	76	TGCCATAATAAAGTGGGATAAATCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAA	135
<b>Sbjct</b>	9441	.....- <b>G</b> .....	9499
Query	136	CCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGC	195
Sbjct	9500	.....	9559
Query	196	ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGG	255
Sbjct	9560	.....	9619
Query	256	GTGATCGGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGG	315
Sbjct	9620	.....	9679
Query	316	AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTC	375
Sbjct	9680	.....	9739
Query	376	GGGTCGTAAAACCTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTG	435
Sbjct	9740	.....	9799
Query	436	ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGG	495
Sbjct	9800	.....	9859
Query	496	TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCAGGTGGTTTCTTAAGTCTG	555
Sbjct	9860	.....	9919
Query	556	ATGTGAAAGCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAG	615
Sbjct	9920	.....	9979
Query	616	AAGAGGAAAGTGGAAATCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCA	675
Sbjct	9980	.....	10039
Query	676	GTGGCGAAGGCGACTTTCTGGTCTGTAAGTACACTGAG-CGCGAAAGCGTGGG-AGCAA	733
<b>Sbjct</b>	10040	..... <b>G</b> ..... <b>G</b> .....	10099
Query	734	ACAGGATTAGATAACC-TGGTAGTC-ACGC-GTAA-CGATGAGTGCTAAGTGT-AGAGG-T	787
<b>Sbjct</b>	10100	..... <b>C</b> ..... <b>C</b> ..... <b>C</b> ..... <b>A</b> ..... <b>T</b> ..... <b>G</b> .....	10159
Query	788	--CCGCC-TT-AGTGCTGAA	803
<b>Sbjct</b>	10160	<b>TT</b> ..... <b>C</b> .. <b>T</b> .....	10179

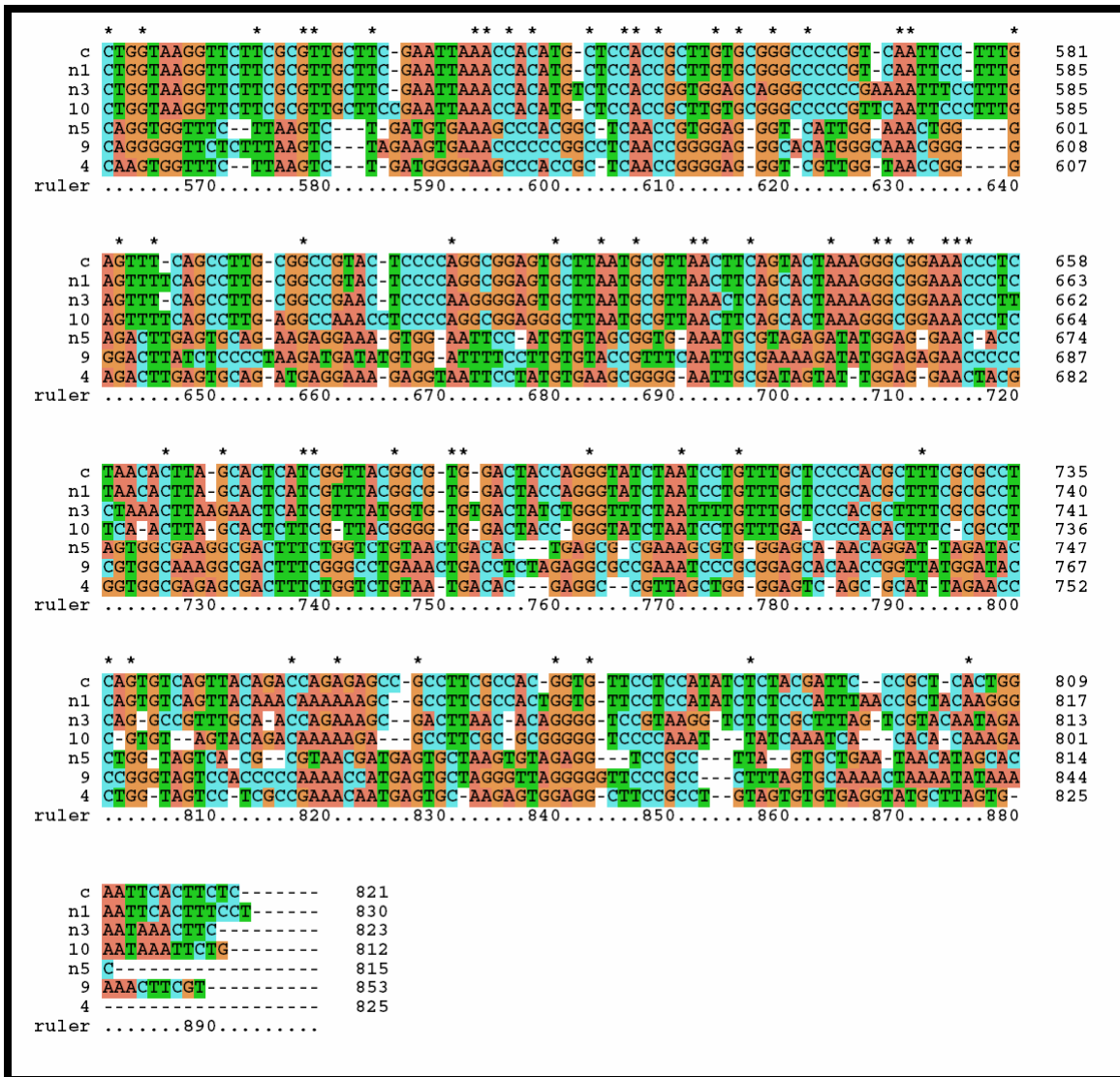
**Figure 3.9:** BLAST alignment of the nucleic acid sequence derived from sample n5 and that of known *B.thuringiensis* 16S rRNA gene sequences found on the NCBI database. Identities = 778/800 (97%), Gaps = 17/800 (2%).

The alignment for sample n5 gave a 97 % match with known *B.thuringiensis* 16S rRNA gene sequences, as seen in Figure 3.7, with 15 hits in two organisms, and 14 hits for *Bacillus thuringiensis* serovar *konkukian str. 97-27*.

The multiple sequence alignment seen in Figure 3.10 shows a 26% similarity across all 6 sequences. Areas of high homology are however seen between the control sample and samples 10, n1 and n3

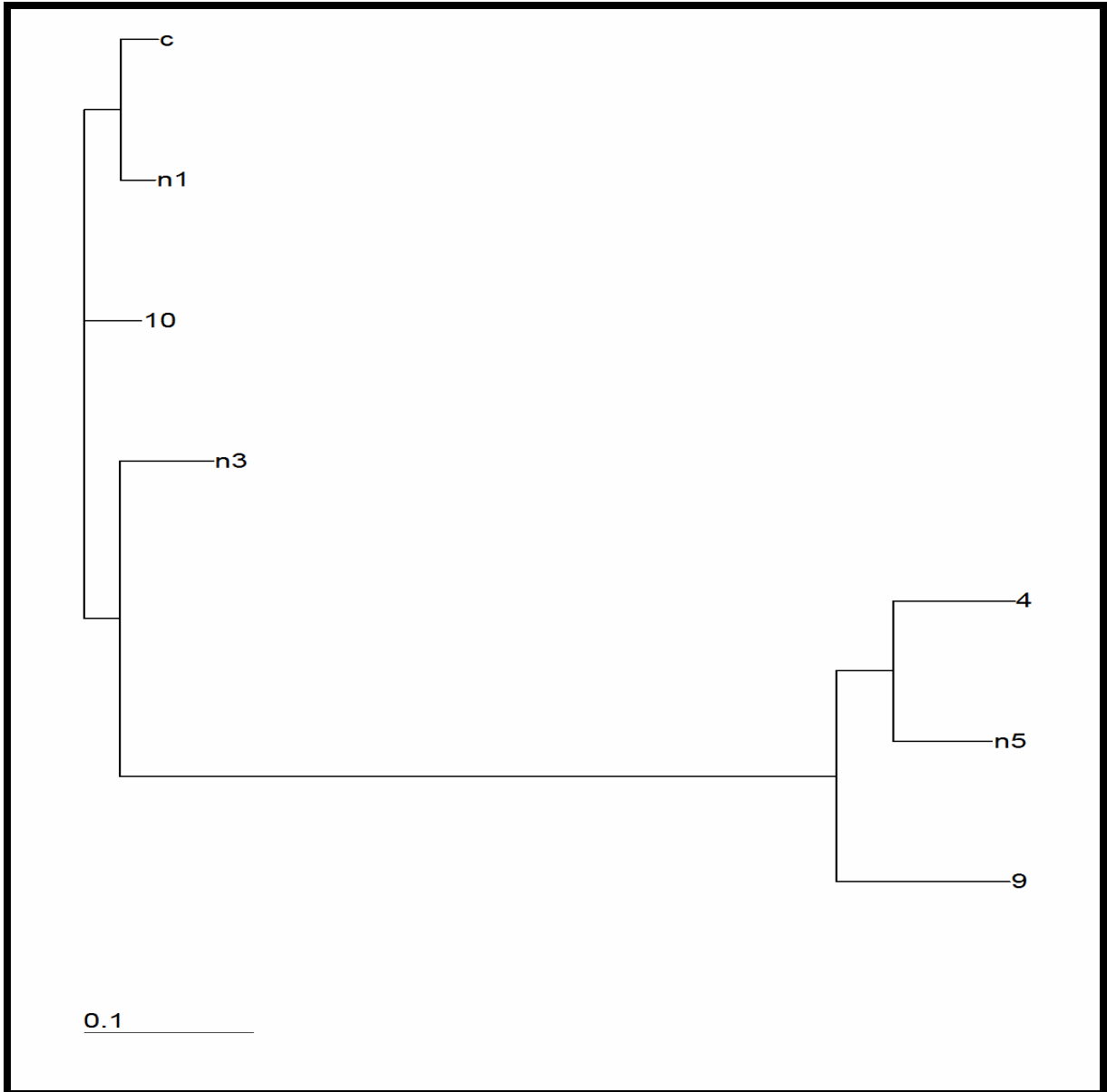


Continued on next page



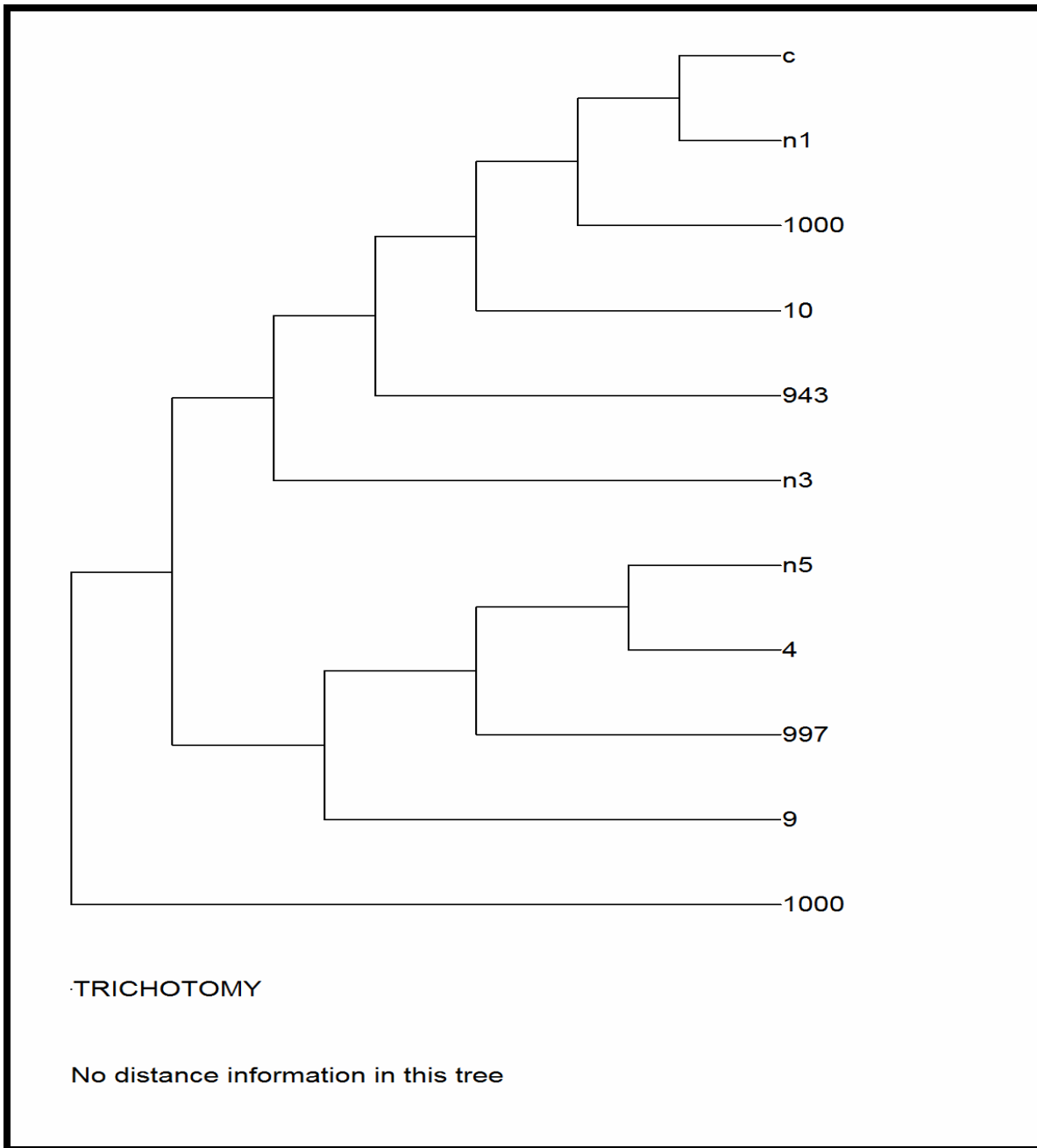
**Figure 3.10:** Clustal X multiple sequence alignment of the *16s rRNA* gene sequences obtained from isolates n1, n3 n5, S10, S9, S4 as well as the control (4A3). A 26% homology is evident within all 7 sequences.

In Figure 3.10 a high homology is evident between samples 4, 9 and n5. Since this does not have any indication how the sequences were related to each other, a neighbour joining (N-J) tree was constructed for the seven sequences in order to understand the relationships to each other. In Figure 3.11 it is evident that branches of the phylogenetic dendrograms are similar in length for samples 10, n1, n3 and the control strain. The same observation is evident for distances calculated for samples 4, 9 and n5.



**Figure 3.11:** Phylogenetic dendrogram for samples S4, S9, S10 n1, n3, n5 and the control (4A3) reconstructed according to the Neighbour Joining (NJ) analysis function of CLUSTAL X.

These distances however only represent the distances calculated after corrections and could not give an accurate indication of the relationship between the seven sequences. To further understand the real number of differences between the sequences themselves, the Bootstrap Tree method was used to calculate the confidence values for each of the groupings.



**Figure 3.12:** Phylogenetic dendrograms for samples S4, S9, S10 n1, n3, n5 and the control (4A3) reconstructed according to the Bootstrap Neighbour Joining (NJ) analysis function of CLUSTAL X. Bootstraps were done using 1000 replications.

As can be seen in Figure 3.12, the numbers on the lower branches of each main branch of the tree represent strong Bootstrap Proportions (BP). These high BPs represent a strong bootstrap confidence and adds support that the branches for this tree are correct.

### 3.4 Discussion

In chapter 2 it was evident that the various samples showed distinctive colours depending on their sampling site. Thus the initial step of dilution was performed to minimize possible contaminants brought about by humic materials co-extracted with bacterial cultures. In this chapter we tested a number of DNA extraction methods to ensure that the DNA yield was indeed of high quality and inhibitor free.

The CsCl-EtBr density gradient extraction produced high purity plasmid DNA but did not extract any significant genomic DNA, contrary to what we expected. In addition to this the method is very costly and time consuming. This protocol thus would not be suited for a rapid analysis of DNA extracted from a large sample size. The SDS mediated reaction as well as the modified alkaline lysis method produced high purity DNA, however it is seen in Figure 3.1 that the modified alkaline lysis method was more efficient at extracting plasmids ranging from 500-1500bp in sample 10. It is also seen that the modified alkaline lysis method proved more efficient at extracting plasmids ranging from 1500-3000bp. Similar plasmids are evident in the 7 minute boiling lysis products but at lower yields. Each of the methods used have their advantages and disadvantages depending on what is required of them. For the purpose of this study, the modified alkaline lysis method proved most effective in extracting a wide range of plasmids in a short amount of time. The boiling lysis produced more DNA over a 7 minute incubation time period than the 5 minute incubation period. This extraction method uses significantly less time to obtain a DNA product in comparison to the other methods used, however the purity of the DNA is compromised. The lower purity DNA template did however prove to be

sufficient for obtaining positive PCR products when used in determining the 16S rDNA identity of the isolates.

Nucleotide sequences obtained from the positive amplification products revealed that 5 of the samples showed highly significant similarity with *B. thuringiensis* when compared to known 16S rDNA sequences in the NCBI database. These provide strong support that the samples are indeed *B. thuringiensis*. Although the sequences share high homology with known strains, Figure 3.10 shows that this homology is not consistent across the seven sequences obtained. Groupings are evident where samples S10, n1 and n3 and the control show high homology to each other, and the same is evident in a second grouping containing samples S4, S9 and n5. The NJ tree derived from the alignment supports the grouping observation where samples 4, 9 and n5 have a phylogenetic distance of approximately 0.5 in comparison with samples S10, n1 and n3 which have a calculated phylogenetic distance of approximately 0.1 or below. When considering the data from this tree it must be taken into account that these distances are calculated after corrections where transversions are accounted for more than transitions and distances increase with respect to each other as genetic differences accumulate. The data thus does not represent the real number of differences between the sequences themselves (Berry & Gascuel, 1997). For this reason the Bootstrap tree is a better option making assessments of evolutionary lineage. The frequency with which any given branch is found is recorded as the Bootstrap Proportion (BP). These proportions can be used as a measure of the reliability (within limitations) of individual branches in the optimal tree (Creevey *et al.*, 2004).

The high values on the Bootstrap tree in Figure 3.12 confirm the initial observation that samples S10, n1 and n3 as well as the control are closely related all originating from a single branch with a reliability measure of 1000. The same observation is also seen for samples S4, S9 and n5.

The data obtained from the 16s rRNA nucleotide sequences provides strong support that the samples that were isolated were indeed *B. thuringiensis*. The next logical step was to optimize the characterization of the *cryIA* through PCR to determine if the gene was indeed present within the isolates.



## Chapter 4

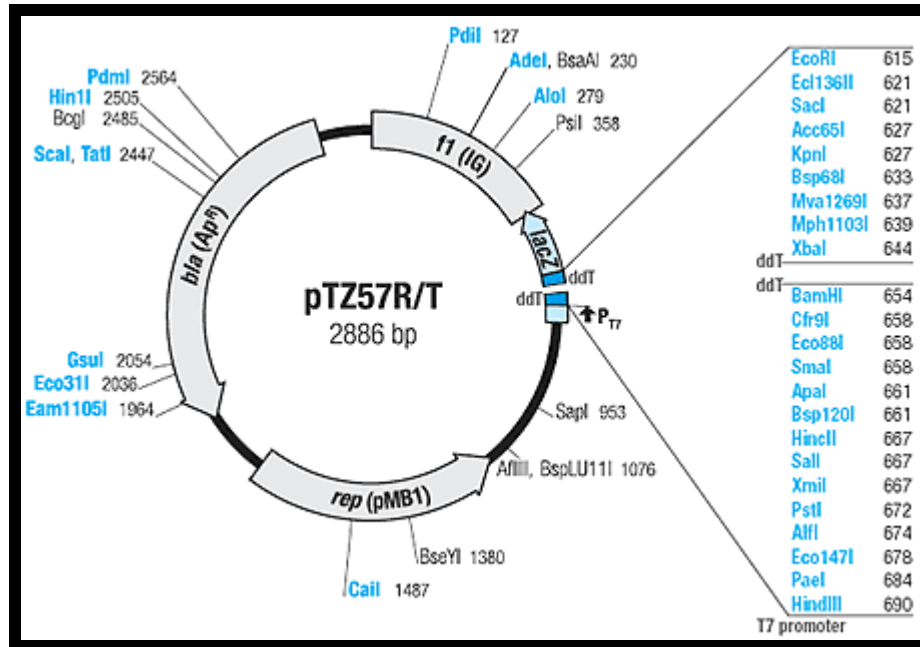
# Cloning the *Bacillus thuringiensis* isolate *cryIA* gene sequence

### 4.1 Introduction

In the pilot study discussed in chapter 1, it became evident that PCR products amplified from putative *Bacillus thuringiensis* (Bt) may have contained additional sequences of similarity to the ones desired. Repeated attempts at PCR amplification of *cryIA* gene sequence produced similar mixed signals. A closer examination of the primer base composition indicated that the original primers TYIUN12 and TYIAA contained sections that had the potential to form hairpin loop structures. These sections were removed and the PCR reaction was again performed using the new truncated primer pair. The resultant PCR products had a much higher yield and quality. These PCR products were sequenced by Inqaba Biotechnical Services and proved to be more suited to our transformation experiments than the previously sequenced products.

These products were purified and prepared for use in transformation using the pTZ57R/T cloning vector. It provided us with a convenient method whereby our PCR products could be directly cloned into the vector without digestion or manipulation to the flanking region. The pTZ57R/T cloning vector is from the Fermentas InsT/Aclone™ PCR Product Cloning Kit. The kit was used to reliably clone *cryIA* gene products into the

pTZ57R/T cloning vector. The vector precleaved with Eco32I and treated with deoxynucleotidyl transferase to create 3'-ddT at both ends allows for PCR fragments with 3'-dA overhangs to be ligated forming a circular molecule.



**Figure 4.1:** The pTZ57R/T cloning vector used to directly clone *cryIA* amplification products.

The ligation procedure inserts the PCR product into the Lac Z operon within the vector. Ligation products were then used in the transformation experiment with the aid of Fermentas TransformAID™ Bacterial Transformation Kit. Competent cells were prepared by treating JM109 cells with CaCl<sub>2</sub> at low temperature. A suspension of cells was added to the transformation solution and put through a series of incubation steps. Successful transformants were isolated using LB-Ampicillin plates supplemented with X-gal and IPTG. DNA was extracted from the transformants using the boiling lysis method. The DNA was probed for the presence of the *cryIA* gene sequence with PCR amplification in order to confirm transformation.

The specific objectives for this chapter were:

- Identify *cryIA* gene sequences from the putative isolates using truncated PCR primers
- Prepare chemically competent cells for transformation
- Clone amplification products into plasmid vector pTZ57R/T and transform it into chemically competent *E. coli*
- Extract DNA from transformants and confirm the presence of *cryIA* gene sequences using PCR
- Compare the sequence data obtained from both the isolate DNA and the Clone DNA

## 4.2 Methodology

### 4.2.1 PCR amplification of the *cryIA* gene sequence

The DNA was extracted from the putative Bt isolates and screened via PCR using the truncated *cryIA* specific primers TYIUN12 (r): 5'-ATC ACT GAG TCG CTT CGC AT-3' and TYIAA (f):5'- CAG CTG GAG CAG TTT ACA CC-3'. The original primer sequence as seen below had the potential hairpin formation in the positions highlighted in red.

TYIUN12 (r)

5' ATCACTG(**AGTC**)GCTTCGCATGTTT(**GACT**)TTCTC 3'

TYIAA (f)

5' GAGCCA(**AGCAGCT**)GGAGCAGTTTACACC 3'

3' CCACATTTGACGAGG(**TCGACGA**)ACCGAG 5'

For PCR amplification, the template DNA obtained from various lysis methods was diluted with sterile water to obtain  $10^{-3}$  fold for each reaction. For DNA extracted using the boiling lysis method, 5 $\mu$ l of the boiled suspension was used for each reaction. The template DNA was mixed with 2X PCR Master Mix containing a 2X concentrated solution of *Taq* DNA polymerase, reaction buffer, MgCl<sub>2</sub> and dNTPs. The PCR reaction was performed for 30 cycles with denaturation of template DNA at 94°C for 1 minute, annealing templates and oligonucleotide primers at 52°C for 2 minutes, and extension of PCR products at 72°C for 3 minutes. The PCR products were separated and analyzed on a 2% agarose gel. DNA extracted from an inoculum of *Bacillus thuringiensis* serovar. *thuringiensis* (4A3) supplied by Dr. Daniel R. Zeigler from The Bacillus genetic stock centre at the Ohio State University was used as the positive control.

### **4.2.2 Gel electrophoresis**

An aliquot (7-10  $\mu$ l) of each amplification reaction was analysed on 2% w/v agarose gels cast and run in TBE buffer (pH 8.3) at about 120 volts until the dye marker was near the end of the gel. Gels were stained with ethidium bromide and the DNA bands photographed under transmitted UV light. A 100 base pair marker (Pharmacia, LKB) was included on every gel.

### **4.2.3 Preparation of chemically competent cells**

Bacterial culture (5ml) was grown up overnight at 37°C. The samples were diluted 1:100 and further incubated at 37°C for 2 hours upon reaching optimal density (0.5-0.6 at

600nm). Cells were then centrifuged at 5000rpm in a J10 rotor for 10 minutes. The supernatant was discarded and the cells stored on ice. The cells were resuspended in 100ml of 100mM MgCl<sub>2</sub> and incubated on ice for 20-30 minutes. Cells were spun down at 4000 rpm and the supernatant discarded and the pellet resuspended in 100mM CaCl<sub>2</sub> supplemented with 15% glycerol. Samples were aliquoted into 1.5ml microcentrifuge tubes and stored at -20°C

#### **4.2.4 Cloning of *Taq* amplified *cryIA* gene products**

A 1.5 ml microcentrifuge tube containing 3µl of plasmid vector pTZ57R/T, 4µl of PCR fragment template, 3µl of 10X ligation buffer, 3µl of PEG 4000 solution, 25µl of nuclease free water and 5U (1µl) of T4 DNA ligase was incubated at 22°C overnight.

#### **4.2.5 Transformation**

An overnight culture of competent cells (1.5 ml) was added to a microcentrifuge tube and spun down at maximum speed at 4°C for 1 minute. The pelleted cells were resuspended in 300µl of TransformAid™ T-solution (Fermentas Life Sciences) and incubated on ice for 5 minutes. The cells were spun down at maximum speed at 4°C for 1 minute and the supernatant discarded. The pelleted cells were resuspended in 120µl of TransformAid T-solution and incubated on ice for 5 minutes. The resuspended cells (50 µl) were added to 2.5 µl of the ligation mixture and incubated on ice for 5 minutes. The mixture was plated on pre-warmed LB-Ampicillin plates and incubated at 37°C overnight. Control

experiments were performed using the control PCR fragments provided (Fermentas Life Sciences).

#### **4.2.6 Colony selection**

The transformation mixture was plated on pre-warmed LB-Ampicillin plates supplemented with X-gal and IPTG. Successful ligation of the DNA insert into the pTZ57R/T plasmid construct was determined by growth of bacterial colonies with a Lac<sup>-</sup> phenotype. All colonies displaying the Lac<sup>-</sup> phenotype were randomly picked and their DNA extracted using boiling lysis method.

#### **4.2.7 Boiling lysis**

Bacterial cultures were streaked onto agar plates in order to obtain single colonies, thus ensuring a homogenous synchronised culture. The colonies were then collected using a metal loop and resuspended in 1 ml sterile water. The microcentrifuge tubes were vortexed until a homogenous cell paste was obtained. The tubes were then boiled in a heat block (100°C) for time intervals of 5 and 7 minutes. The samples were then spun down for 10 min maximum speed in a microcentrifuge. The supernatant was transferred to fresh microcentrifuge tubes containing 750µl phenol-chloroform isoamyl alcohol. The tubes were vortexed and then centrifuged in a microfuge for 10 min at maximum speed. The upper phase was transferred to a fresh 1.5 ml microcentrifuge tube and washed with

70% ethanol. The tubes were centrifuged and the pellet resuspended in 200µl sterile water.

#### **4.2.8 PCR amplification the *cryIA* gene sequence**

The polymerase chain reaction (PCR) offers a powerful tool for detecting, characterizing, and isolating *cry* genes in Bt. The alternating blocks of conserved and variable nucleotides among *cry* genes make it possible to select primers to amplify entire gene subfamilies on the one hand or specific gene types on the other. The process has been widely exploited over the last decade to determine the content of *cry* genes and to a lesser degree the *cyt* genes of Bt.

The information obtained has added to our understanding of the natural occurrence of the genes among strains as well as their geographic locations (Porcar *et al*, 2002). By selecting primers specific to regions or blocks that have been highly conserved within the *cry* genes, one can target a specific host range from different families of genes, and in theory, novel *cry* genes may be identified. The uses of PCR for the identification of *cry* genes vary in literature and are thus performed under different conditions. The DNA used as a template for the reaction is typically obtained through lysis and purification methods such as the SDS and CsCl methods, or through time saving techniques such as boiling lysis (Cerón *et al*, 1995; Gleave *et al*, 1993).

## **4.2.9 Automated DNA sequencing**

Once positive PCR products were identified through visualization on an agarose gel, a sample of the reaction mixture was purified and then sequenced by Inqaba Biotechnical Services. The sequences obtained were viewed with 'Chromas2' and Bioedit™ software.

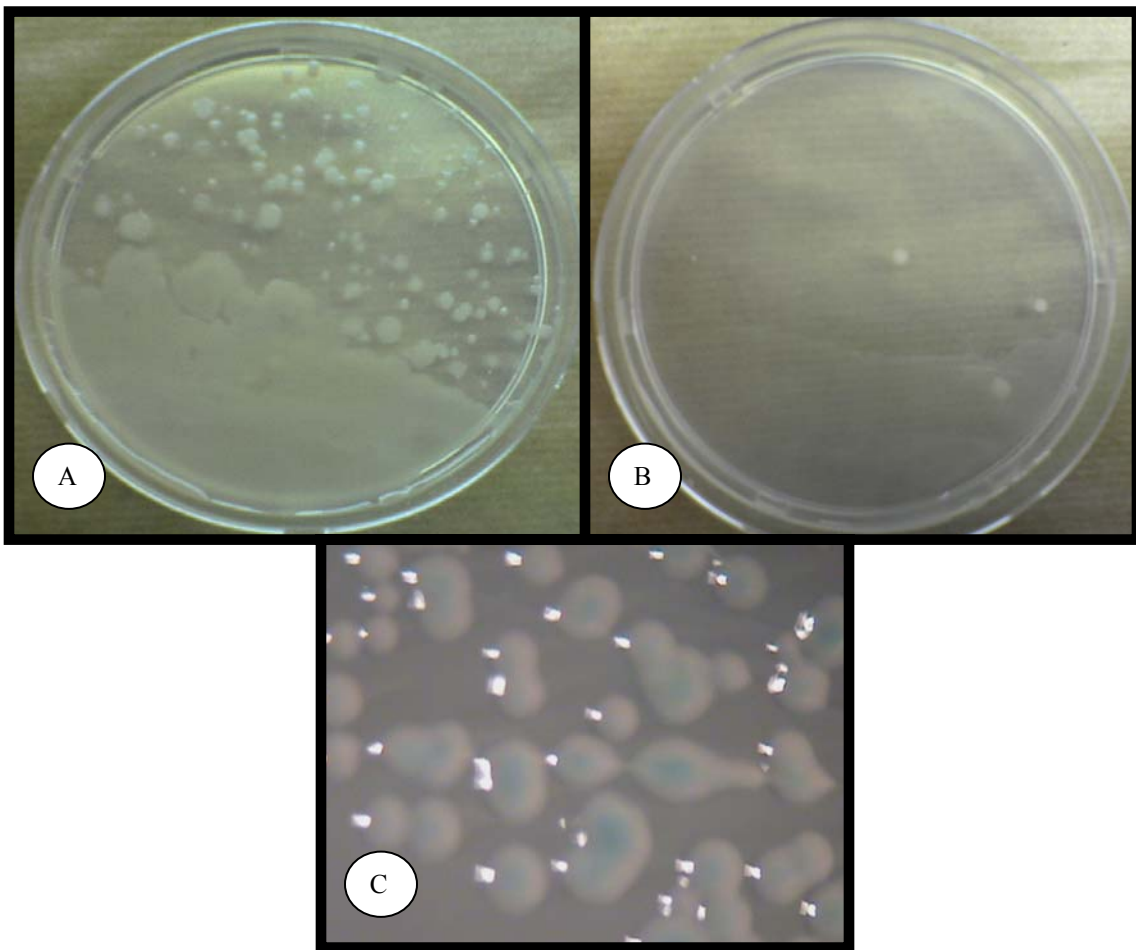
## **4.2.10 Sequence Alignment of *cry* genes with known sequences**

Once the nucleotide sequence of the PCR product had been derived, it was compared with sequences of strains known to contain the *cryIAa* gene. The sequences were entered into the NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST) and compared to known sequences submitted on the NCBI database. The sequences obtained were analyzed using the multiple sequence alignment program ClustalW (The ClustalX windows interface designed by TJ Gibson) (Higgins, 1994) in order to determine homology within the sequences as well as to determine their evolutionary lineage.

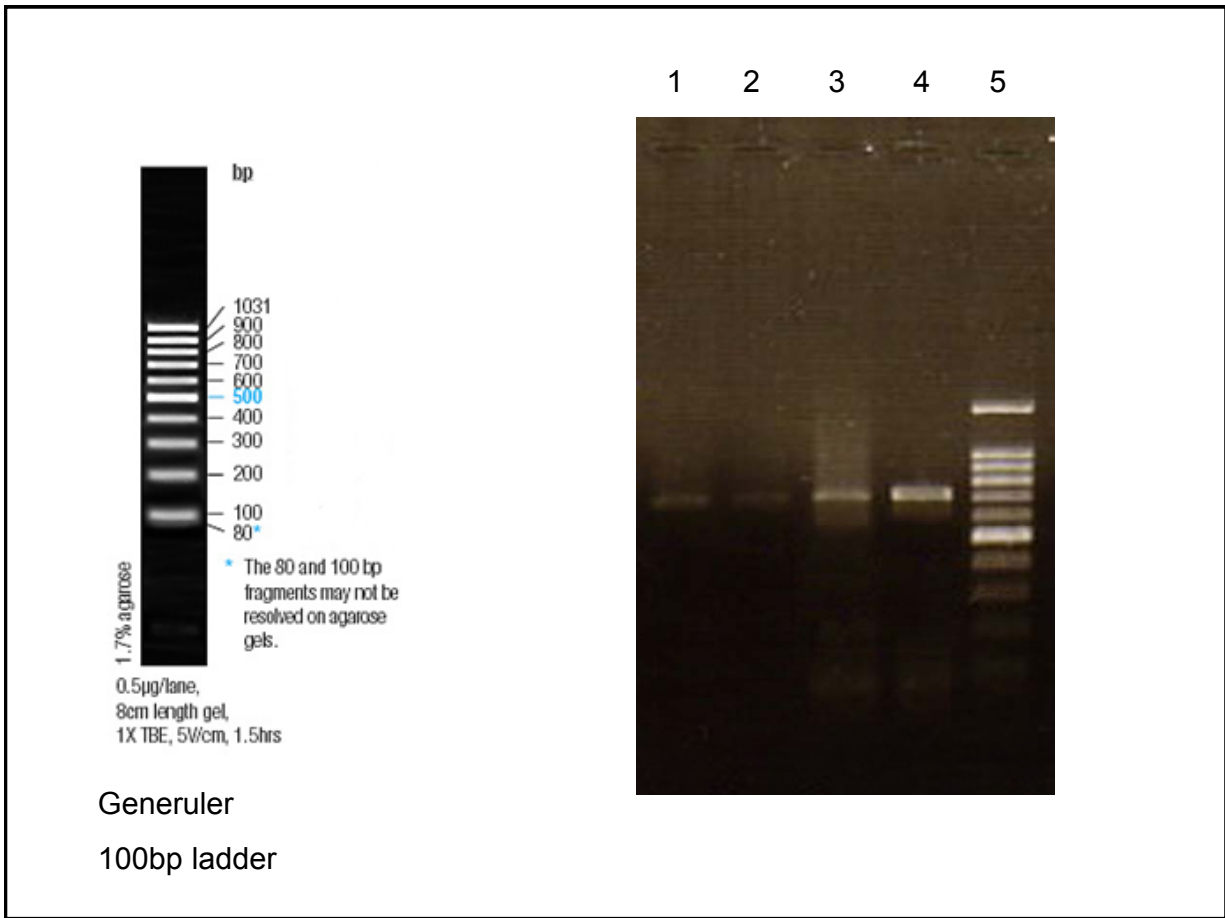


## 4.3 Results

Transformation of the competent cells produced low numbers of positive transformants (3-5 CfU/ml) (Figure 4.2 B) in comparison with the control experiments (Figure 4.2 A). All positive samples were streaked onto fresh LB-ampicillin plates. The positive transformants were subsequently probed for the presence of *cryIA* genes in a PCR reaction.



**Figure 4.2:** Digital photographs (A) The positive transformation control showed a high degree of growth after transformation, (B) The Cloned *cryIA* PCR fragment transformant only produced 3 colony forming units (C) Competent cells without PCR fragment appeared blue in colour indicating that transformation had not occurred. All the transformation cultures grown on LB- ampicillin plates supplemented with X-gal and IPTG.



**Figure 4.3:** A Photograph of an agarose gel loaded showing PCR amplification Products for positive sample S10 (lane 1 & 2), control 4A3 (lane 3), clone (lane 4) and 100bp marker (lane 5).

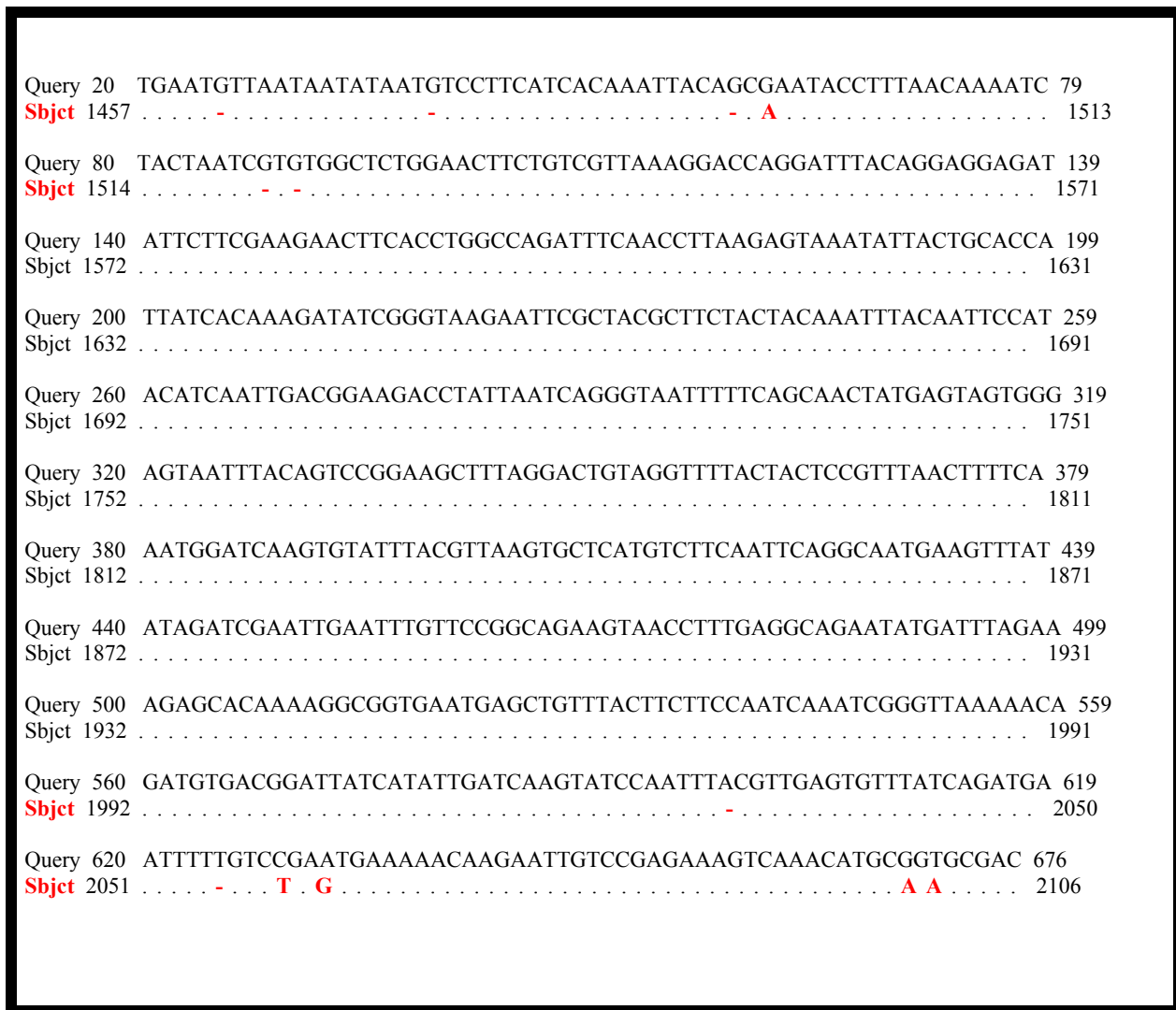
The PCR reaction produced positive amplification results for the *cry1a* gene from the *cry* gene clone, seen in lane 4 of the agarose gel shown in Figure 4.3, and was subsequently renamed CLONEA1A. The amplification products were of the same molecular weight as those obtained from both the control (lane 3) and sample S10 (lanes 1 & 2).

The PCR products were sequenced by Inqaba Biotechnical Services, and the resultant nucleic acid sequences were compared to those of known *cry1a* gene sequences on the NCBI database with the use of the Mega BLAST alignment tool.

Query 24	CCTTACATCACAAATTACAGCAGAATACCTTTAACAAAATCTACTAATCGTTGGCTCTGG	83
<b>Sbjct</b> 1423	.....-.....-.....-.....-.....	1478
Query 84	AACTTCTGTCGTAAAGGACCAGGATTTACAGGAGGAGATATTCTTCGAAGAAGCTCACC	143
Sbjct 1479	.....	1538
Query 144	TGGCCAGATTTCAACCTTAAGAGTAAATATTACTGCACCATTATCACAAAGATATCGGGT	203
Sbjct 1539	.....	1598
Query 204	AAGAATTCGCTACGCTTCTACTACAAATTTACAATTCCATACATCAATTGACGGAAGACC	263
Sbjct 1599	.....	1658
Query 264	TATTAATCAGGGTAATTTTCAGCAACTATGAGTAGTGGGAGTAATTTACAGTCCGGAAG	323
Sbjct 1659	.....	1718
Query 324	CTTAGGACTGTAGGTTTTACTACTCCGTTAACTTTTCAAATGGATCAAGTGTATTTAC	383
Sbjct 1719	.....	1778
Query 384	GTAAAGTGCTCATGTCTTCAATTCAGGCAATGAAGTTTATATAGATCGAATTGAATTTGT	443
Sbjct 1779	.....	1838
Query 444	TCCGGCAGAAGTAACCTTTGAGGCAGAATATGATTTAGAAAGAGCACAAAAGGCGGTGAA	503
Sbjct 1839	.....	1898
Query 504	TGAGCTGTTTACTTCTCCAATCAAATCGGGTTAAAAACAGATGTGACGGATTATCATAT	563
Sbjct 1899	.....	1958
Query 564	TGATCAAGTATCCAATTTAGTTGAGTGGTTATCAGATGAATTTTGTCTGGATGAAAAACA	623
Sbjct 1959	.....	2018
Query 624	AGAATTGTCCGAGAAAGTCAAACATGCGAAGCGA	657
Sbjct 2019	.....	2052

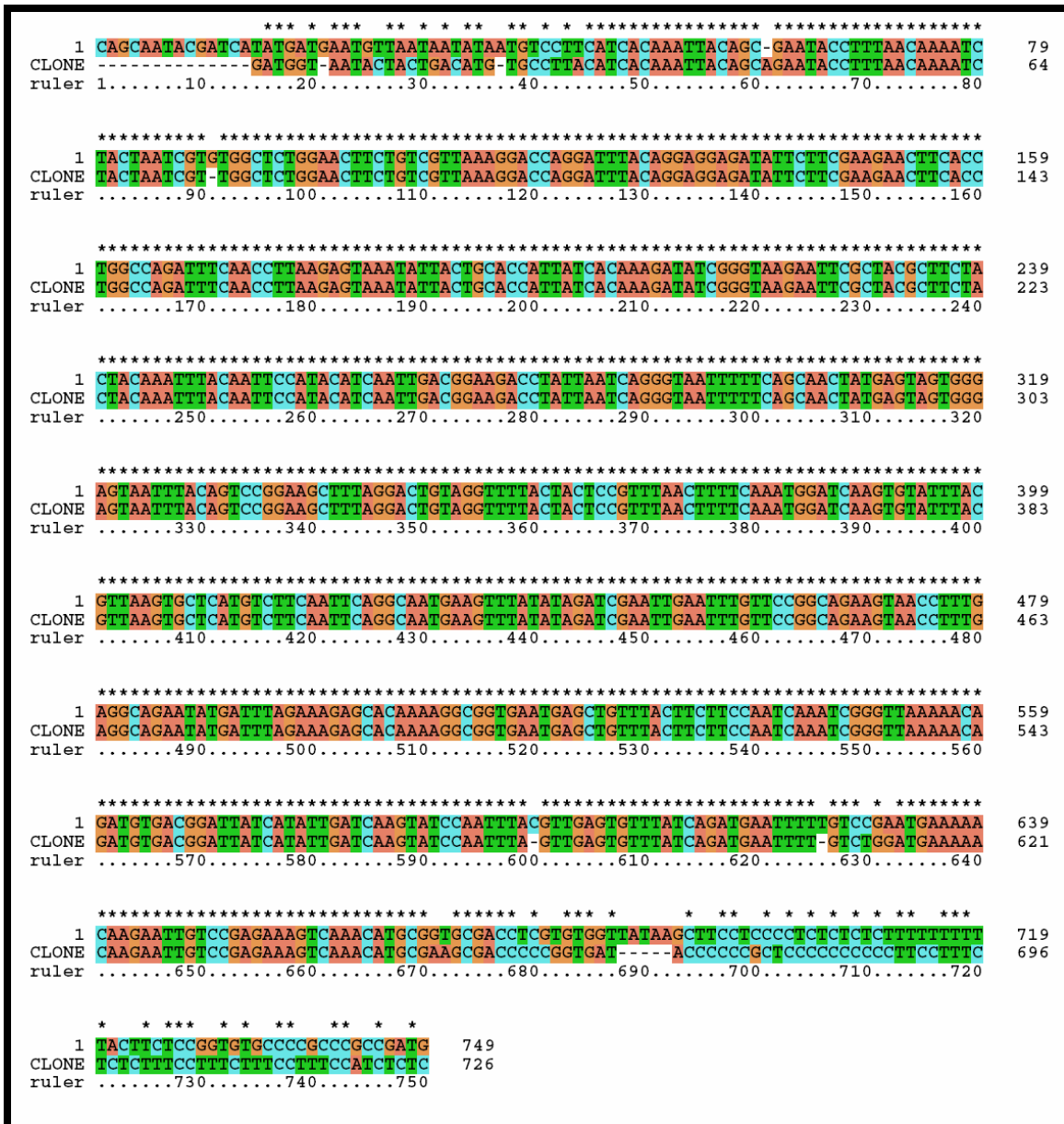
**Figure 4.4:** BLAST alignment of the nucleic acid sequence derived from CLONEA1A and that of known *B.thuringiensis cryIA* gene sequences found on the NCBI database. Identities = 630/634 (99%), Gaps = 4/634 (0%).

The alignment for CLONEA1A gave a 99 % match with known *B.thuringiensis cry* gene sequences, as seen in Figure 3.7, with 97 hits in twelve organisms, including *Bacillus thuringiensis* serovars *sotto*, *aizawai*, *kurstaki*, *kunthalaRX28*, *kunthalanags3*, *kenyae*, *kunthalaRX27*, *kunthalaRX24*, *wuhanensis*, *morrisoni* and *alesti*.



**Figure 4.5:** BLAST alignment of the nucleic acid sequence derived from sample 10 and that of known *B.thuringiensis cryIA* gene sequences found on the NCBI database. Identities = 645/657 (98%), Gaps = 7/657 (1%).

The alignment for sample 10 gave a 98 % match with known *B. thuringiensis cry* gene sequences, as seen in Figure 4.5, with 97 hits in twelve organisms, including *Bacillus thuringiensis* serovars *sotto*, *aizawai*, *kurstaki*, *kunthalaRX28*, *kunthalanags3*, *kenyae*, *kunthalaRX27*, *kunthalaRX24*, *wuhanensis*, *morrisoni* and *alesti*.



**Figure 4.6:** Clustal X multiple sequence alignment of the *cryIa* gene sequences obtained from sample 10 and CLONEA1A. A 90% homology is evident within the two sequences.

The alignment obtained using Clustal X v1.81 seen in Figure 4.6 shows a 90 % homology within between the two sequences. The variation between the sequences is evident at the 5' and 3' ends.

## 4.4 Discussion

In this chapter we have reported two techniques for accurately identifying the *cry* gene sequence from known and unknown isolates. In a continuation from the pilot study in chapter two, the various DNA templates were tested for their ability to produce positive PCR products. The modified alkaline lysis for large plasmid extraction produced positive PCR amplification products. More significantly though, the rapid boiling lysis protocol produced positive PCR amplification products at a more consistent rate than that of the DNA template obtained from the modified alkaline lysis protocol. This observation induced more confidence in the boiling lysis protocol which was subsequently used as the primary source for DNA templates in all experimental repetitions.

The original PCR primers TYIUN12 and TYIAA were revised due to the detection of areas within the primer sequence which had the potential to form hairpin loop structures. With the revised primers in conjunction with the DNA template we were able to obtain positive PCR amplification products for sample 10. The resultant nucleotide sequence data obtained confirmed that sample 10 did indeed contain the *cryIA* gene thus ultimately confirming that this sample was Bt. Pairwise alignment with the BLAST alignment tool produced a 98 % match with known Bt *cryIA* gene sequences.

The initial pilot study highlighted the potential that several similar copies of the desired *cryIA* gene may have been present in the PCR amplification products. The gene cloning experiment designed for this test was performed using the PCR products obtained for the *cryIA* gene sequence of the known Bt strain 4A3. We have reported successfully cloning

the *cryIA* gene PCR amplification product into the pTZ57R/T cloning vector. PCR analysis of the CLONEA1A produced positive *cryIA* gene products confirming that transformation was successful. In this study it was noted that the transformants did not show any variation in sequence identity as hypothesized in the pilot study. The resultant nucleotide sequence was compared to known *cryIA* gene sequences on the NCBI database using the BLAST pairwise alignment tool. A 99 % match was obtained for CLONEA1A compared with known Bt *cry* gene sequences.

The sequences obtained from the two methods were aligned with each other using Clustal X v1.81. The alignment showed a 90% homology within the two sequences. Following the characterization of the *cryIA* gene, a bioassay was designed in order to determine the toxicity of the  $\delta$ -endotoxin produced by this isolate.

## Chapter 5

# **Toxicity of *Bacillus thuringiensis* isolated from South African soils towards the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae)**

### **5.1 Introduction**

Six *Bacillus thuringiensis* samples isolated from South African soils have been shown to have significant homology with known *Bacillus thuringiensis* species. In particular the gene sequence for the endotoxin produced by *B. thuringiensis* isolate S10 obtained through PCR showed significant homology to Cry1A. Cry1A is a  $\delta$ -endotoxin known to induce mortality in lepidopteran insects (Bietlot *et al*, 1993; Dubois & Dean 1995; Bravo *et al*, 1996; Clairmont *et al*, 1998; Chen *et al*, 2005). In order to understand the potential of the isolate's toxin, a bioassay was performed to compare the basic efficacy of the toxin in comparison to the known Cry1A toxin of *B. thuringiensis* Serovar. *Thuringiensis* (4A3) and *B. thuringiensis* Serovar. *Brasilensis* (4AY1) kindly supplied by Dr. Daniel R. Zeigler from The Bacillus genetic stock centre at the Ohio State University. The greater wax moth caterpillar *Galleria mellonella* (Lepidoptera: Pyralidae), was used as the lepidopteran model host for evaluating the capacity of endotoxins produced by the *B. thuringiensis* isolates.



*Galleria mellonella* falls into the order Lepidoptera and is a major pest of the beekeeping industry (Shimanuki *et al.*, 1992). The larvae cause considerable damage to the hive comb and honey by tunneling into the wax and leaving behind a mass of silk web (Swan & Papp, 1972). The *G. mellonella* life cycle is made up of four stages: eggs, larvae consisting of several instars, pupa, and the adult moth (Shimanuki *et al.*, 1992). The eggs are laid in crevices of the honey comb, and larvae hatch within seven days. The larvae feed on the honey, pollen, and wax produced by honeybees (Lebedeva *et al.*, 2002).

Stronger bee colonies manage to control the infestation but the weaker colonies such as those that have lost their queen are forced to abandon the hive. With no resistance, the larvae can destroy the hive within a month (Shimanuki *et al.*, 1992).

The specific objectives for this chapter were:

- Obtain spore-endotoxin mixtures from Bt isolates as well as the control cultures
- Rear *G. mellonella* larvae to be used in bioassay experiments
- Expose *G. mellonella* larvae to various concentrations of the spore-endotoxin mixtures
- Assess the larvae for evidence of sensitivity to the endotoxin
- Evaluate the data obtained

## **5.2 Methodology**

### **5.2.1 Bacterial Cultures**

Known *B. thuringiensis* cultures 4AY1 and 4A3 as well as the South African *B. thuringiensis* isolate (10) were grown from spore collections and frozen stocks. Cultures were maintained in LB media at 30°C until needed.

### **5.2.2 *Galleria mellonella* larvae rearing**

*G. mellonella* larvae obtained from Dr Vince Gray's Wits university laboratory collection were reared on an artificial diet made up of 22% corn meal, 22% wheat germ, 11% dried milk, 5.5% dried yeast, 17.5% beeswax, 11% honey and 11% glycerol (Fröblius *et al*, 2000). The medium was made up with 100ml sterile water and cooked in a microwave for 5 minutes. Larvae were kept at room temperature in dark growth boxes.

### **5.2.3 Crude spore-endotoxin preparation**

*B. thuringiensis* cultures 4AY1, 4A3 and isolate S10 were grown in LB media for 7 days to ensure complete lysis of living cells. The crude samples containing a mixture of spores and toxic crystals were centrifuged at 10 000 rpm for 10 minutes. The pellet was resuspended in 1ml sterile H<sub>2</sub>O. The spore-endotoxin solution was diluted with sterile water to obtain an optical density of 1(OD=1) at 280nm. The spore-endotoxin solution was serially diluted for the bioassay experiments by adding 10µl of the solution to 10µl of

sterile water in a sterile microfuge tube. This was repeated for the five fold dilution. Each dilution made up the spore-endotoxin treatments.

#### **5.2.4 *Galleria mellonella* bioassay**

Bioassay plates were prepared by placing a small amount of artificial diet (22% corn meal, 22% wheat germ, 11% dried milk, 5.5% dried yeast, 17.5% beeswax, 11% honey and 11% glycerol) (Fröblius *et al*, 2000) in the centre of a 40 mm Petri dish. The artificial diet was inoculated with the spore-toxin mixture by alloquoting 1ml of the solution to the food surface. Third to fourth instar *G. mellonella* larvae approximately 0.2g in weight were randomly selected for each inoculation. There were 5 larvae per Petri dish. Known *B. thuringiensis* strains 4AY1 and 4A3 were used in the positive control experiments and samples containing no spore toxin mixture were used as negative controls. Larvae were characterized according to health in terms of mass gain or loss after a period of 7 days.

#### **5.2.3 Bioassay data processing**

The larvae were inspected for any mortality or visible signs of physiological distress. The average weights of the larvae were recorded for assessing concentration affects of the spore-endotoxin treatments. The data was processed using an ANOVA single factor statistical test to determine if there was any significant variation between the recorded larvae weights of the test bioassay compared to the reference bacteria and controls.

## 5.3 Results

### 5.3.1 Crude toxin preparation

The endotoxin-spore solutions were diluted to obtain an OD reading of 1, which was used as the treatment with the highest concentration (1). Serial dilutions of the spore-endotoxin solutions were made in order to make up the bioassay treatments (2) to (6) for each bacterium.

**Table 5.1:** Optical density of the endotoxin-spore solution of the known *Bacillus thuringiensis* strains 4AY1 and 4A3, and test isolate S10 as used for the primary inoculum.

Sample	OD at A280nm	OD at A320nm	Concentration (g/ml)
4AY1	1.047	0.962	0.738
4A3	0.992	0.594	0.584
Isolate S10	1.088	1.057	0.704

### 5.3.2 Bioassay

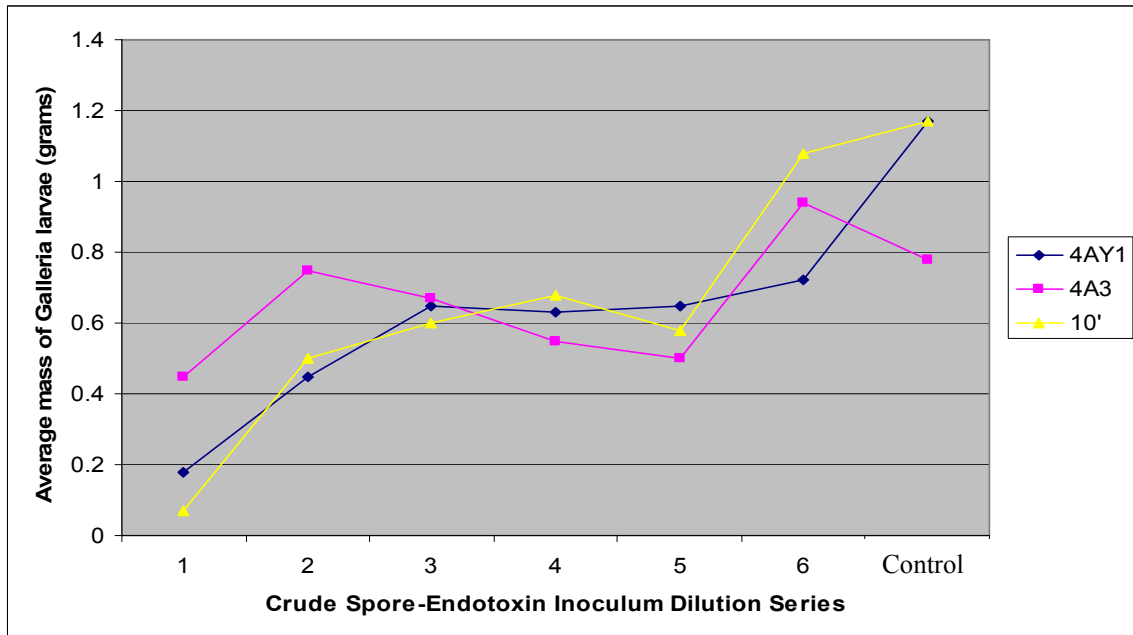
After seven days of feeding on an artificial diet supplemented with different concentrations of spore-endotoxin solutions, only two larvae mortalities were recorded in the endotoxin bioassay for isolate S10 (at a concentration of 1 (0.704 g/ml)). One mortality was recorded in the bioassay containing endotoxins of 4A3 concentration of 1 (0.584 g/ml).

**Table 5.2:** The average weights of the *Galleria mellonella* larvae after 7 days of exposure to the endotoxin-spore treatments of 4AY1 and 4A3, and test isolate S10

<b>Dilution factor</b>		<b>4AY1</b>	<b>4A3</b>	<b>S10'</b>
<b>Treatment 1</b>	<b>1</b>	0.18	0.45	0.07
<b>Treatment 2</b>	<b>1/10</b>	0.45	0.75	0.5
<b>Treatment 3</b>	<b>1/100</b>	0.65	0.67	0.6
<b>Treatment 4</b>	<b>1/1000</b>	0.63	0.55	0.68
<b>Treatment 5</b>	<b>1/10 000</b>	0.65	0.5	0.58
<b>Treatment 6</b>	<b>1/100 000</b>	0.72	0.94	1.08
	<b>Control</b>	1.17	0.78	1.17

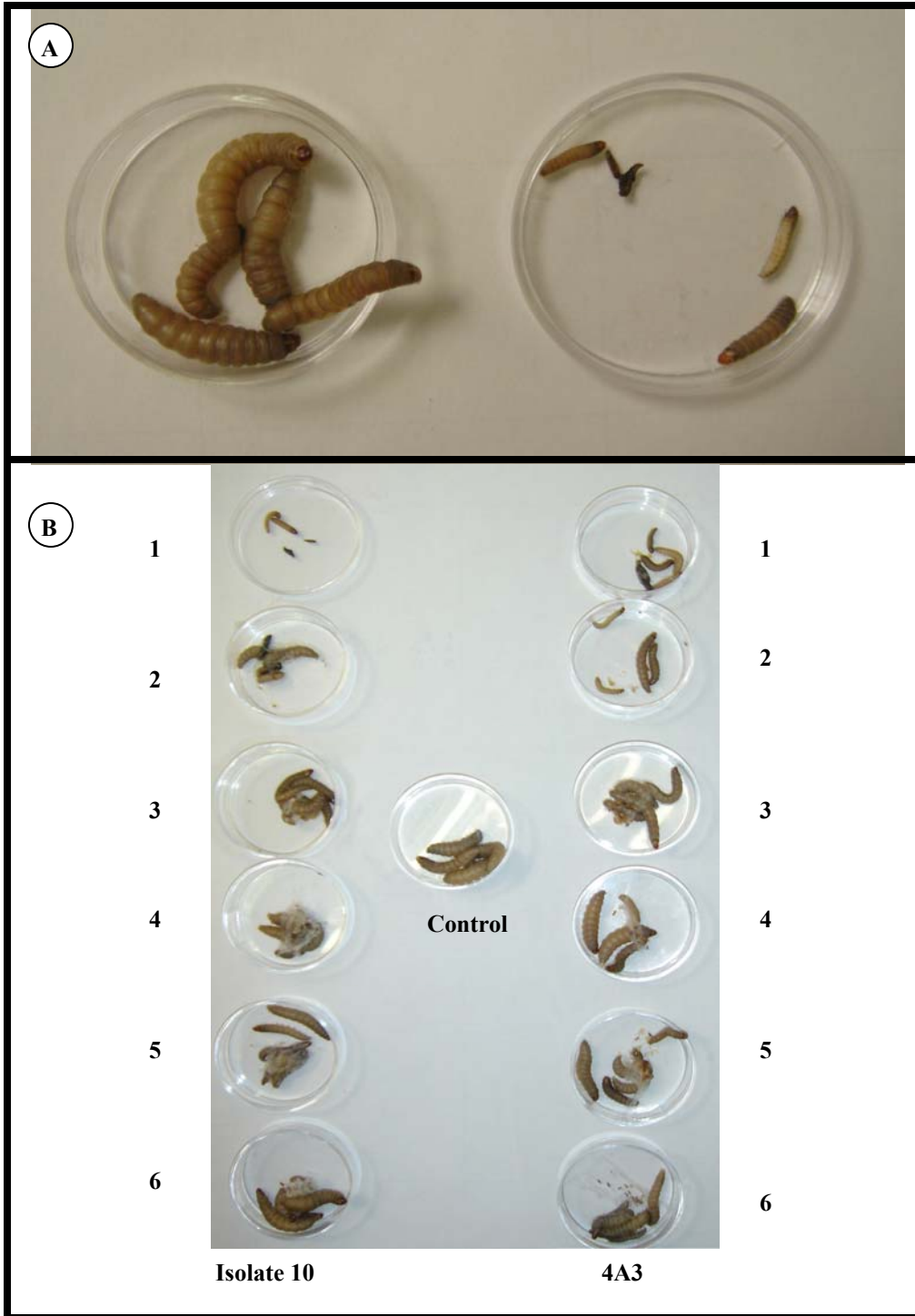
The Values in table 5.2 are the average weight of the Larvae per treatment, the numbers 1-6 being the spore-endotoxin treatment concentration with 1 being OD=1 at 280nm and then 6 being the lowest serial dilution. It can be seen that the S10' (1) reading is very low as a result of two larvae mortalities for this treatment. The average weights of the test larvae are much lower at the highest concentration (1) than those reported for the control experiments. This weight appears to increase in a linear fashion in the assays for both isolate S10 and the known isolate 4AY1. Known isolate 4A3 follows a similar linear progression, however some outlying data is evident in the control experiment for this bioassay.

From the graph in Figure 5.1 it can be seen that the linear progression of the average larvae weight in the bioassay using spore-endotoxin treatments of isolate S10 shows distinct similarity to that of the 4AY1 bioassay. A similar trend is also evident in the 4A3 bioassay with regards to the exponential increase in mass from treatment 1 to treatment 2, however the average mass of the larvae at these concentrations is approximately 0.3g more than those recorded for isolate S10.



**Figure 5.1:** A graph showing the linear progression of the average weights of test *Galleria mellonella* larvae after 7 days of exposure to the endotoxin-spore treatments of 4AY1 and 4A3, and isolate S10

In Figure 5.2 (A) it is evident that the test larvae of the isolate S10 assay are significantly smaller in size when compared the control larvae fed on artificial diet without endotoxin-spore supplementation. The test larvae show signs of poor health and lack of vigor in comparison to the control Larvae. In Figure 5.2 (B) the average size of the larvae are seen to significantly increase as the concentration of toxin decreases from 1 to 6 in both the bioassays of isolate 10 and 4A3



**Figure 5.2:** (A) A digital photograph showing the difference in size between *G. mellonella* larvae feeding on artificial diet supplemented with a spore-endotoxin mixture of *B. thuringiensis* isolate S10 over 7 days (on the right) in comparison to control *G. mellonella* larvae feeding artificial diet without spore-endotoxin supplementation. (B) A digital photograph showing a comparison in the size of *G. mellonella* larvae fed on artificial diet containing treatments 1 to 6 for bioassays using spore-endotoxin mixtures for Isolate 10 and 4A3.

### 5.3.3 ANOVA single factor data

<b>Anova: Single Factor</b>						
<b>SUMMARY</b>						
<b>Groups</b>	<b>Count</b>	<b>Sum</b>	<b>Average</b>	<b>Variance</b>		
4AY1	6	3.28	0.546667	0.040427		
4A3	6	3.86	0.643333	0.033347		
S10'	6	3.51	0.585	0.10535		
<b>ANOVA</b>						
<b>Source of Variation</b>	<b>SS</b>	<b>df</b>	<b>MS</b>	<b>F</b>	<b>P-value</b>	<b>F crit</b>
<b>Between Groups</b>	<b>0.028433</b>	<b>2</b>	<b>0.014217</b>	<b>0.238104</b>	<b>0.791043</b>	<b>3.68232</b>
<b>Within Groups</b>	<b>0.895617</b>	<b>15</b>	<b>0.059708</b>			
<b>Total</b>	<b>0.92405</b>	<b>17</b>				

**Figure 5.3:** Statistical data obtained from the ANOVA single factor test performed on the average weights of *G. mellonella* feeding on artificial diet supplemented with concentrations 1 to 6 in bioassays using a spore-endotoxin mixture of *B. thuringiensis* isolate 10, 4A3 and 4AY1.

Statistical data obtained from the ANOVA single factor produces an F value indicating that at  $p=0.05$  the biomass weights do not significantly deviate from the average mass of the insect larvae throughout the bioassay replicates ( $F= 0.238104$ ;  $df=17$  ;  $P=0.791043$ ).



## 5.3 Discussion

The South African *Bacillus thuringiensis* isolate 10 was tested for its toxicity towards larvae of the lepidopteran *Galleria mellonella*. In this study only two mortalities were recorded for the bioassay using the endotoxin-spore mixture of isolate S10 at a concentration of 1 (0.704 g/ml). Although no significant mortalities are recorded at lower endotoxin-spore concentrations, when compared to the known Bt endotoxins of 4AY1 and 4A3, only one mortality is recorded for the bioassay using the endotoxin of 4A3 at a concentration of 1 (0.584 g/ml).

In both the bioassays for isolate 10 and 4A3 concentration of 1, the living larvae showed signs of poor health, including limited motility and an overall reduction in mass when compared to the control larvae as seen in figure 5.2. This reduction in weight indicates that the endotoxin causes either a drastic reduction in the amount of diet consumed or interference with movement of food through the gut wall (Schesser *et al*, 1977). The average weight of the test larvae recorded in table 5.1 indicates that there is similarity between the average weight of the larvae in the test bioassay and those of the bioassay larvae using the known endotoxins. This similarity is more evident in figure 5.1 where the average weights are plotted on a graph. The linear progression of the average weight is inversely proportional to the concentration of the endotoxin-spore mixture for the test isolate 10 and the known samples 4AY1 and 4A3.

From the statistical data obtained in figure 5.3 the F value indicates that at  $p=0.05$  the biomass weights do not significantly deviate from the average mass of the insect larvae

throughout the bioassay replicates ( $F= 0.238104$ ;  $df=17$  ;  $P=0.791043$ ). This supports the graphic data in figure 5.1 that the test bioassay average masses were similar to those of the known endotoxin bioassays. It has been reported that endotoxins of *Bacillus thuringiensis* are effective on *G. mellonella* larvae at concentrations of 0.5 to 3.0g/ml (Bosgelmez *et al*, 1983). The treatments from isolate S10 and 4A3 which resulted in mortality were 0.704 g/ml and 0.584 g/ml respectively (Table 5.1). These concentrations fall within the range reported by Bosgelmez *et al*, 1983, however it must be taken into account that the treatments contained both spores and endotoxins, thus the actual concentration of endotoxin may have been much lower. This is evident in treatments 2-3 for all three bioassays where no mortality is recorded, however there is evidence for a reduction in average weight at higher endotoxin-spore concentrations. In conclusion the 7 day bioassay using endotoxin-spore treatments 2 to 6 for all three *B. thuringiensis* cultures proved to be ineffective at killing *G. mellonella* larvae at these lower concentrations.

# Chapter 6

## Conclusion

### 6.1 Isolation of *Bacillus thuringiensis*

This study set out to isolate and characterise a *Bacillus thuringiensis* strain from South African soils. This was done using a combination of traditional characterisation techniques based on morphology, inhibition experiments with the use of sodium acetate and characterisation at the DNA level using PCR and the sequencing of PCR amplification products. Traditional techniques initially set out to identify bacteria of the class *Bacilli* by identifying isolates as aerobic Gram-positive rod shaped bacteria with endospore formation. Having assigned an isolate to these groups, we attempted to identify the bacterium to species level using a panel of physiological and biochemical tests. This system was workable, but familiarity with these bacteria was often necessary in order to distinguish spore morphologies. Largely because of this, later schemes disregarded spore morphology (Claus and Berkley, 1986).

In our experiments the sodium acetate test proved more successful in eliminating most sporeforming and non-sporeforming organisms in the test soil samples. The classical biochemical and microscopy tests provided valuable information in understanding bacterial samples that were isolated and ultimately facilitated their characterization. All of the isolates tested positive for Gram stain, endospore stain and catalase test,

confirming the necessity to pasteurize samples in the initial bacterial enrichment. Without pasteurization the ratio of unwanted bacteria (non-sporeforming) to wanted bacteria (sporeforming) would have been too high (Travers *et al*, 1987).

Some concern arose that the pasteurized samples could potentially contain many undesirable sporeformers, particularly the close relatives of *B. thuringiensis* namely *B. anthracis*, and *B. cereus*. Reported similarities in morphology and genetic makeup have led to the proposal that the three should be considered a single species (Helgason *et al*, 2000). Since the sodium acetate selection could not guarantee that these bacteria wouldn't be co-cultured along with *B. thuringiensis*, the necessity arose to employ the minimal media T3. T3 was used to suppress the growth of other *Bacillus* species and thus favour the growth of *B. thuringiensis*. In order to obtain isolates with high probability of being *B. thuringiensis*, the 44 samples listed in Table 2.1 were subjected to both the sodium acetate selection test and classical characterization techniques. Confirmation of crystalline bodies (endotoxins) was positive for only 10 of the 44 isolates, thus the next logical test was to perform tests at a DNA level to determine whether *cryIA* gene sequences were indeed present within the isolate genetic composition. This led to the pilot study for determining the methods that would be used in PCR experiments to determine the nature of the insecticidal proteins that the samples may contain.

The pilot study set out to establish a preliminary protocol for identifying possible *cryIA* gene sequences in the putative *B. thuringiensis* isolates. Positive amplification products were visualised with UV illumination on the agarose gel, however the resultant nucleic

acid sequence data that was derived from these products showed several conflicting signals making positive identification impossible. It was postulated that this may have been as a result of some sort of contaminant attributed to the DNA template or other components used in the PCR reaction, or the possibility that several other similar gene sequences may have been present within the reaction mixture. Evidence for the latter has been reported where *B. thuringiensis* subsp. *Kurstaki* HD1 is known to contain at least 5 other *cry* gene sequences (Kalman, *et al.* 1995). Analysis of the components later led to the identification of a sequence within the PCR primers which had the potential to form hairpin loops. This region of the primer was removed for the remaining *cry1A* PCR experiments.

## **6.2 Characterization of putative *Bacillus thuringiensis* isolates at a DNA level**

### **6.2.1 DNA extraction from putative *Bacillus thuringiensis* isolates**

In the enrichment experiments performed, it was noted that the isolated cultures possessed distinctive colours depending on their sampling site. The samples were thus subjected to an initial dilution to minimize any possible contaminants brought about by humic materials co-extracted with bacterial cultures. In the interest of identifying a DNA extraction protocol that would ensure a DNA yield of high quality and inhibitor free, several extraction techniques were employed.

The CsCl-EtBr density gradient extraction produced high purity plasmid DNA but did not extract any significant genomic DNA, contrary to what we expected. In addition to this the method is very costly and time consuming. This protocol thus would not be suited for a rapid analysis of DNA extracted from a large sample size. The SDS mediated reaction as well as the modified alkaline lysis method produced high purity DNA, however it is seen in Figure 3.1 that the modified alkaline lysis method was more efficient at extracting plasmids ranging from 500-1500bp in sample 10. It is also seen that the modified alkaline lysis method proved more efficient at extracting plasmids ranging from 1500-3000bp.

Similar plasmids are evident in the 7 minute boiling lysis products but at lower yields. Each of the methods used have their advantages and disadvantages depending on what is required of them. For the purpose of this study, the modified alkaline lysis method proved most effective in extracting a wide range of plasmids in a short amount of time. It was also noted that the boiling lysis protocol produced more DNA over a 7 minute incubation time period than the 5 minute incubation period. This extraction method uses significantly less time to obtain a DNA product in comparison to the other methods used, however the purity of the DNA is compromised. The lower purity DNA template did however prove to be sufficient for obtaining positive PCR products when used in determining the 16S rDNA identity of the isolates.

### **6.2.2 16S rDNA characterisation *Bacillus thuringiensis* isolates**

Nucleotide sequence data obtained from the positive amplification products revealed that 5 of the samples showed highly significant similarity with *B. thuringiensis* when compared to known 16S rDNA sequences in the NCBI database. This provided strong support that the samples were indeed *B. thuringiensis*. Although the sequences share high homology with known strains, it was noted that homology was not consistent across the seven sequences obtained. Groupings are evident in which samples 10, n1 and n3 and the control show high homology to each other, and the same is evident in a second grouping containing samples 4, 9 and n5.

### **6.2.3 Phylogenetic analysis of the *Bacillus thuringiensis* isolates**

The NJ tree derived from the alignment supports the grouping observation where samples 4, 9 and n5 have a phylogenetic distance of approximately 0.5 in comparison with samples 10, n1 and n3 which have a calculated phylogenetic distance of approximately 0.1 or below. When considering the data from this tree it must be taken into account that these distances are calculated after correction where transversions are accounted for more than transitions and distances increasing with respect to each other as genetic differences accumulate. The data thus does not represent the real number of differences between the sequences themselves (Berry & Gascuel, 1997). For this reason the Bootstrap tree proved to be a better option for making assessments of evolutionary lineage. The frequency with which any given branch is found is recorded as the

Bootstrap Proportion (BP). These proportions can be used as a measure of the reliability (within limitations) of individual branches in the optimal tree (Creevey *et al.*, 2004).

The high values on the Bootstrap tree in Figure 3.12 confirm the initial observation that samples 10, n1 and n3 as well as the control are closely related all originating from a single branch with a reliability measure of 1000. The same observation is also seen for samples 4, 9 and n5. The data obtained from the 16s rRNA nucleotide sequences and the phylogenetic analysis provided strong support that the samples isolated were indeed *B. thuringiensis*. The next logical step was to optimize the characterization of the *cryIA* through PCR to determine if the gene was indeed present within the isolates.

#### **6.2.4 Characterization of the *cryIA* gene sequence from the putative *Bacillus thuringiensis* isolates**

We have reported two techniques for accurately identifying the *cry* gene sequence from known and unknown isolates. In a continuation from the pilot study in chapter two, the various DNA templates were tested for their ability to produce positive PCR products. The modified alkaline lysis for large plasmid extraction produced positive PCR amplification products. More significantly though, the rapid boiling lysis protocol produced positive PCR amplification products at a more consistent rate than that of the DNA template obtained from the modified alkaline lysis protocol. This observation induced more confidence in the boiling lysis protocol which was subsequently used as the primary source for DNA templates in all experimental repetitions.



The original PCR primers TYIUN12 and TYIAA were revised due to the detection of areas within the primer sequence which had the potential to form hairpin loop structures. With these revised primers we were able to obtain positive PCR amplification products for sample 10. The resultant nucleotide sequence data obtained confirmed that sample 10 did indeed contain the *cryIA* gene thus ultimately confirming that this sample was *B. thuringiensis*. Pairwise alignment with the BLAST alignment tool produced a 98 % match with known *B. thuringiensis cryIA* gene sequences.

### **6.2.5 Cloning the *cryIA* gene sequence into the pTZ57R/T cloning vector**

The initial pilot study highlighted the potential that several similar copies of the desired *cryIA* gene may have been present in the PCR amplification products. The gene cloning experiment designed for this test was performed using the PCR amplification products derived using the revised primers. We have reported successfully cloning the *cryIA* gene PCR amplification product into the pTZ57R/T cloning vector. PCR analysis of the CLONEA1A produced positive *cryIA* gene products confirming that transformation was successful. In this study it was noted that the transformants did not show any variation in sequence identity as hypothesized in the pilot study. The resultant nucleotide sequence was compared to known *cryIA* gene sequences on the NCBI database using the BLAST pairwise alignment tool. A 99 % match was obtained for CLONEA1A compared with known *B. thuringiensis cry* gene sequences.

The sequences obtained from the two methods were aligned with each other using Clustal X v1.81. The alignment showed a 90% homology within the two sequences. Following the characterization of the *cryIA* gene, a bioassay was designed in order to determine the toxicity of the  $\delta$ -endotoxin produced by this isolate.

### **6.3 *Galleria mellonella* (Lepidoptera: Pyralidae) Bioassay**

Based on the 16S rDNA analysis and the *cryIA* sequence data, the South African *B. thuringiensis* isolate 10 was employed in a bioassay to test for toxicity towards the larvae of the lepidopteran *Galleria mellonella*. We report two larvae mortalities for the bioassay using the endotoxin-spore mixture of isolate 10 at a concentration of 1 (0.704 g/ml). Although no significant mortalities are recorded at lower endotoxin-spore concentrations, when compared to the known Bt endotoxins of 4AY1 and 4A3 only one mortality is reported for the bioassay using the endotoxin of 4A3 at a concentration of 1 (0.584 g/ml).

The animate larvae in the bioassays for isolate S10 and 4A3 at a concentration of 1 showed signs of poor health, including limited motility and an overall reduction in mass in comparison with the control larvae. This reduction in weight indicates that the crystal causes either a drastic reduction in the amount of diet consumed or interference with movement of food through the gut wall (Schesser *et al*, 1977). A comparison of the average weights of the larvae show some correlation between the average weight of the larvae in the test bioassay and those of the bioassay larvae using the known endotoxins. A linear progression of the average weight is inversely proportional to the concentration

of the endotoxin-spore mixture for the test isolate S10 and the known samples 4AY1 and 4A3 (Herbert *et al*, 2006).

Statistical data supports the initial observation where the F value obtained at  $p=0.05$  indicates that the biomass weights do not significantly deviate from the average mass of the insect larvae throughout the Bioassay replicates ( $F= 0.025246$ ;  $df=20$ ;  $P=0.975104$ ). It has been reported that endotoxins of *B. thuringiensis* are effective on *G. mellonella* larvae at concentrations of 0.5 to 3.0g/ml (Bosgelmez *et al*, 1983). The treatments from isolate S10 and 4A3 which resulted in mortality were 0.704 g/ml and 0.584 g/ml respectively. These concentrations fall within the range reported by Bosgelmez *et al*, 1983, however it must be taken into account that the inoculum contained both spores and endotoxins, thus the actual concentration of endotoxin may have been much lower. This is evident in treatments 2-3 for all three bioassays where no mortality is recorded, however there is evidence for a reduction in average weight at higher endotoxin-spore concentrations. In conclusion the 7 day bioassay using endotoxin-spore treatments 2 to 6 for all three *B. thuringiensis* cultures proved to be ineffective at killing *G. mellonella* larvae at these lower concentrations.

## **6.4 Future work**

In this study we have fulfilled the set aims and objectives by isolating a *B. thuringiensis* strain in 'Isolate 10' from South African soils. Isolate S10 has been shown to have significant homology with known *B. thuringiensis* isolates with a 94% match between 16S rRNA gene sequence data for isolate S10 and that of known *B. thuringiensis* 16S

rRNA gene sequences. Isolate S10 possesses the  $\delta$ -endotoxin Cry1A known to be toxic to Lepidopteran insects. The Cry1A gene sequence has been successfully cloned into the pTZ57R/T cloning vector. The Cry1A toxin has been shown to induce sensitivity to the larvae of the lepidopteran *G. mellonella*, a well known pest of the bee hive industry. We have also reported the successful characterisation of four other *B. thuringiensis* isolates at a 16S rDNA level. Future work is proposed that similar bioassay experiments be performed to determine the toxic potential of these isolates. It is also proposed that the  $\delta$ -endotoxins produced by all five isolates be purified and characterised at a biochemical level. Once an understanding of the protein composition is determined, bioassays can be performed using a purified toxin to test for lethal concentrations of endotoxin required to effectively manage pests such as *G. mellonella* on an industrial level.

# References

1. **Adams, L. F., J. E. Visick, and H. R. Whiteley.** 1989. A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *Israelensis* 27-kilodalton crystal protein in *Escherichia coli*. *J. Bacteriol.* **171**: 521–530
2. **Adams, L.F., Brown, K.L. and Whiteley, H.R.** 1991. Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. *J. Bacteriol* **173**: 3846–3854.
3. **Addison, J. A.** 1993. Persistence and non-target effects of *Bacillus thuringiensis* in soil: a review. *Can. J. For. Res.* **23**:2329-2342
4. **Agaisse, H. and D. Lereclus.** 1994. Expression in *Bacillus subtilis* of the *Bacillus thuringiensis cryIIIA* toxin gene is not dependent on a sporulation-specific sigma factor and is increased in a *spoA* mutant. *J. Bacteriol.* **176**:4734-4741
5. **Agaisse, H. and Lereclus, D.** 1994. Structural and functional analysis of the promoter region involved in full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis*. *Mol. Microbiol.* **13**: 97–107
6. **Ammons, D., Rampersad, J. and Khan, A.** 2002. Usefulness of staining parasporal bodies when screening for *Bacillus thuringiensis*. *J Invertebr Pathol.* **79**(3):203-4
7. **Aronson , A. Geng, C. and Wu, L.** 1999. Aggregation of *Bacillus thuringiensis* CryIA toxin upon binding to target insect larval midgut vesicles. *Appl Environm. Microbiol.* **65**, 2503-2507.
8. **Aronson, A.I., Beckman, W and Dunn, P.** 1986. *Bacillus thuringiensis* and related insect pathogens. *Microbiol Rev.* **50**(1):1–24
9. **Arrieta, G., Hernandez, A., and Espinoza. A.M.** 2004. Diversity of *Bacillus thuringiensis* strains isolated from coffee plantations infested with the coffee berry borer *Hypothenemus hampei*. *Rev Biol Trop.* **52**(3):757-64
10. **Agaisse, H. and Lereclus, D.** 1995. Expression in *Bacillus subtilis* of the *Bacillus thuringiensis cryIIIA* toxin gene is not dependent on a sporulation-specific sigma factor and is increased in a *spoOA* mutant. *J. Bacteriol.* **176**: 4734–4741
11. **Baily and Scott's Diagnostic Microbiology,** 11th edition. C.V. Mosby, Co., St. Louis, 2002.

12. **Baum, J. A. and T. Malvar.** 1995. Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. *Mol. Microbiol.* **18**:1-12.
13. **Baumann, L., K. Okamoto, B. M. Unterman, M. J. Lynch, and P. Baumann.** 1984. Phenotypic characterization of *Bacillus thuringiensis* and *Bacillus cereus*. *J. Invertebr. Pathol.* **44**:329-341.
14. **Beegle, C. C. and T. Yamamoto.** 1992. History of *Bacillus thuringiensis* Berliner research and development. *Can. Entomol.* **124**:587-616.
15. **Becker, B. and J. Margalit.** 1993. Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies, p. 147-170. *In* P. F. Entwistle, J. S. Cory, J. S. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. Wiley & Sons, Chichester, England
16. **Belliveau, B. H., and J. H. Trevors.** 1989. Transformation of *Bacillus cereus* vegetative cells by electroporation. *Appl. Environ. Microbiol.* **55**:1649-1652
17. **Bergey, John G. Holt, Noel R. Krieg, Peter H.A. Sneath** 1994. *Bergey's Manual of Determinative Bacteriology, 9th ed.*. Lippincott Williams & Wilkins.
18. **Bergey's manual of systematic bacteriology - Volume 2.** baltimore, London, Los Angeles, Sidney: Williams and Wilkins, 1986. pp. 965-1599
19. **Bernhard, K.** 1986. Studies on the delta-endotoxin of *Bacillus thuringiensis* var. *tenebrionis*. *FEMS Microbiol. Lett.* **33**:261-265.
20. **Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P. and Burges, H.G.** 1997. Natural isolates of *B. thuringiensis*: worldwide distribution, and activity against insect pests. *Journal of Invertebrate Pathology.* **70**, 59-68.
21. **Berry V. and Gascuel O.** 1997. Inferring Evolutionary Trees with Strong Combinatorial Evidence. *COCOON* : **111-123**
22. **Berry V. and Gascuel O.** 2000. Inferring evolutionary trees with strong combinatorial evidence. *Theor. Comput. Sci.* **240(2)**: **271-298**.
23. **Bietlot, H. P. L., J. Vishmulhatla, P. R. Carey, M. Pozsgay, and H. Kaplan.** 1990. Characterization of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis*. *Biochem. J.* **267**:309-315.
24. **Bietlot, H. P., J. P. Schernthaner, R. E. Milne, F. R. Clairmont, R. S. Bhella, and H. Kaplan.** 1993. Evidence that the CryIA crystal protein from *Bacillus thuringiensis* is associated with DNA. *J. Biol. Chem.* **268**:8240-8245

25. **Bioedit™**: Tom Hall. Ibis Therapeutics, a division of Isis Pharmaceuticals. 1891 Rutherford Road, Carlsbad, CA 92008 760-603-2375
26. **Birnboim, H. C. and Doly, J.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1523.
27. **Bora, R. S., M. G. Murty, R. Shenbagarathai, and V. Sekar.** 1994. Introduction of a lepidopteran-specific insecticidal crystal protein gene of *Bacillus thuringiensis* subsp. *kurstaki* by conjugal transfer into a *Bacillus megaterium* strain that persists in the cotton phyllosphere. *Appl. Environ. Microbiol.* **60**:214-222
28. **Bosgelmez A, Cakmakci L, Gurkan B, Gurkan F, Cetinkaya G.** 1983. The effects of *Bacillus thuringiensis* on the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Galleriidae). *Mikrobiyol Bul.* 1983 Oct;17(4):233-42.
29. **Bravo, A., H. Agaisse, S. Salamitou, and D. Lereclus.** 1996. Analysis of *cryIAa* expression in *sigE* and *sigK* mutants of *Bacillus thuringiensis*. *Mol. Gen. Genet.* **250**:734-741.
30. **Brown, T.A.** Gene Cloning: An Introduction Blackwell Publishing (1998)
31. **Brown, K. L.** 1993. Transcriptional regulation of the *Bacillus thuringiensis* subsp. *thompsoni* crystal protein gene operon. *J. Bacteriol.* **175**:7951-7957
32. **Brown, K. L., and H. R. Whiteley.** 1990. Isolation of the second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. *J. Bacteriol.* **172**:6682–6688.
33. **Brunel B, Givaudan A, Lanois A, Akhurst R J, Boemare N.** 1997. Fast and accurate identification of *Xenorhabdus* and *Photorhabdus* species by restriction analysis of PCR-amplified 16S rRNA genes. *Appl Environ Microbiol.* **63**:574–580.
34. **Burges, H. D., and J. A. Hurst.** 1977. Ecology of *Bacillus thuringiensis* in storage moths. *J. Invertebr. Pathol.* **30**:131-139.
35. **Capalbo, D.M.F., Valicente, F.H., Moraes, I. de O. and Pelizer, L.H.** 2001. Solid-State fermentation of *Bacillus thuringiensis toloworthi* to control fall armyworm in maize. *EJB.* **4**, 1-4
36. **Carlson, C. R., D. A. Caugant, and A.-B. Kolstø.** 1994. Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Appl. Environ. Microbiol.* **60**:1719-1725
37. **Carlson, C. R., and A.-B. Kolstø.** 1993. A complete physical map of a *Bacillus thuringiensis* chromosome. *J. Bacteriol.* **175**:1053-1060

38. **Carozzi, N. B., V. C. Kramer, G. W. Warren, S. Evola, and M. G. Koziel.** 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Appl. Environ. Microbiol.* **57**:3057-3061
39. **Carroll, J. and D. J. Ellar.** 1993. An analysis of *Bacillus thuringiensis*  $\delta$ -endotoxin action on insect-midgut-membrane permeability using a light-scattering assay. *Eur. J. Biochem.* **214**:771-778
40. **Cerón, J., A. Ortiz, R. Quintero, L. Guereca, and A. Bravo.** 1995. Specific PCR primers directed to identify *cryI*/*cryI* and *cryIII*/*cryIII* genes within a *Bacillus thuringiensis* *Bacillus thuringiensis* strain collection. *Appl. Environ. Microbiol.* **61**:3826-3831
41. **Chan, M-T., Chen, L-J. and Chang H-H.** 1996. Expression of *Bacillus thuringiensis* (B.t.) insecticidal crystal protein in transgenic potato. *Bot. Bull. Acad. Sin.* **37**, 17-23.
42. **Chen, F.C., Tsaim M.C., Pengm C.H. and Chak K.F.** 2004. Dissection of cry gene profiles of *Bacillus thuringiensis* isolates in Taiwan. *Curr Microbiol.* **48**(4):270–275
43. **Chen, H., Tang, W., Xu, C., Li, X., Lin, Y. and Zhang Q.** 2005. Transgenic indica rice plants harboring a synthetic *cry2A\** gene of *Bacillus thuringiensis* exhibit enhanced resistance against lepidopteran rice pests. *Theor Appl Genet.* **111**(7):1330-7
44. **Chromas 2:** Copyright © 2006 Technelysium Pty Ltd
45. **Clairmont, F. R., R. E. Milne, V. T. Pham, M. B. Carrière, and H. Kaplan.** 1998. Role of DNA in the activation of the Cry1A insecticidal crystal protein from *Bacillus thuringiensis*. *J. Biol. Chem.* **273**:9292-9296
46. **Claus D. and Berkeley CW. The Genus Bacillus.** In: Sneath PHA ed. *Bergey's Manual of Systematic Bacteriology.* Williams and Wilkins,. Baltimore; 1986:
47. **Clendenning J.B. and Schurr J.M.** 1994. Circularization of small DNAs in the presence of ethidium: a theoretical analysis. *Biopolymers.* **7**:849–868.
48. **Cooksey, K. E.** 1971. The protein crystal toxin of *Bacillus thuringiensis*: biochemistry and mode of action, p. 247-274. In H. D. Burges and N. W. Hussey (ed.), *Microbial control of insects and mites.* Academic Press, Inc., New York.
49. **Creevey, C.J., Fitzpatrick, D.A., Philip, G.K., Kinsella, R.J., O'Connell, M.J., Pentony, M.M., Travers, S.A., Wilkinson, M. and McInerney, J.O.** 2004. Does a tree-like phylogeny only exist at the tips in the prokaryotes?. *Proceedings of the royal society London B: Biological Sciences.* **271**: 2551-2558.



50. **Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D. H. Dean.** 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**:807-813
51. **Delucca, A. J. II, Simonson, J. and Larson, A.** 1979. Two new serovars of *Bacillus thuringiensis*: serovars *dakota* and *indiana* (serovars 15 and 16). *J. Invertebr. Pathol.* **34**:323-324.
52. **Dulmage, H. T.** 1970. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* var. *alesti*. *J. Invertebr. Pathol.* **15**:232-239.
53. **Dubois, N. R. and Dean, D. H.** 1995. Synergism between Cry1A insecticidal crystal proteins and spores of *Bacillus thuringiensis*, other bacterial spores, and vegetative cells against *Lymantria dispar* (Lepidoptera: Lymantriidae) larvae. *Environ. Entomol.* **24**:1741-1747.
54. **Entwistle, P.F.** 1993. *Bacillus thuringiensis*, An environmental biopesticide: theory and practise. New York. John Wiley & Sons.
55. **Feitelson, J. S.** 1993. The *Bacillus thuringiensis* family tree, p. 63-71. *In* L. Kim (ed.), *Advanced engineered pesticides*. Marcel Dekker, Inc., New York, N.Y.
56. **Feng, Q. and Bechtel, W. J.** 1994. pH induced conformational transitions of CryIA(a), CryIA(c) and CryIII A  $\delta$ -endotoxins in *Bacillus thuringiensis*. *Biochemistry* **33**:8521-8526
57. **Finegold, S.M., Martin, W.J. and Scott, E.G.** Reagents and Tests, in Bailey & Scott's *Diagnostic Microbiology*, 5th ed., CV Mosby Co., St. Louis, MO, p. 482, 1978.
58. **Frobius, A. C., Kanost, M. R., Gotz, P. and Vilcinskis, A.** 2000. Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth *Galleria mellonella*. *Eur. J. Biochem.* **267**, 2046-2053.
59. **Fujimoto, H.M., Itoh, K., Ymamoto, M., Kyozuka, J. and Shimamoto, K.** 1993. Insect resistant rice generated by introduction of a modified  $\delta$ -endotoxin gene of *Bacillus thuringiensis*. *Biotechnology.* **11**, 1151-1155.
60. **Ge, A. Z., Pfister, R. M. and Dean D. H.** 1990. Hyperexpression of a *Bacillus thuringiensis* delta-endotoxin-encoding gene in *Escherichia coli*: properties of the product. *Gene* **93**:49-54
61. **Gill, S.S., Cowles, E.A. and Pietrantonio, P.V.** 1992 The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol.* **37**, 615-636.

62. **Gleave, A. P., Williams, R. and Hedges, R. J.** 1993. Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B.thuringiensis* subsp. *kurstaki*. *Appl. Environ. Microbiol.* **59**:1683–1687
63. **González, J. M., Jr., Brown, B. J., and Carlton, B. C.** 1981. Transfer of *Bacillus thuringiensis* plasmids coding for  $\delta$ -endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc. Natl. Acad. Sci. USA* **79**:6951-6955
64. **Gordon, R. E., Haynes, W. C. and Pang, C. H.-N.** 1973. The genus *Bacillus*. U.S. Department of Agriculture, Washington, D.C.
65. **Gram H.C.** *Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpreparäten.* *Fortschr Med* 1884;**2**:185-89.
66. **Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.-L., Brousseau, R. and Cygler, M.** 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J. Mol. Biol.* **254**:447-464
67. **Gutierrez, P., Alzate, O. and Orduz, S.** 2001. Theoretical model of the tridimensional structure of *Bacillus thuringiensis* subsp. *medellin* Cry11BP toxin deduced by homology modeling. *Mem Inst Oswaldo Cruz.* **96**, 357-364.
68. **Haider, M. Z., Knowles, B. H., and Ellar, D. J.** 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal  $\delta$ -endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *Eur. J. Biochem.* **156**:531-540.
69. **Helgason, E., Okstad, O. A., Caugant, D. A., Johansen H. A., Fouet, A., Mock, M., Hegna, I., and Kolstø, A.-B.** 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Appl. Environ. Microbiol.* **66**:2627-2630.
70. **Higgins, D.G.** (1994) CLUSTAL V: multiple alignment of DNA and protein sequences. *Methods Mol. Biol.*, **25**, 307–318.
71. **Hofmann, C., Vanderbruggen, H., Höfte, H., Van Rie, J., Jansens, S. and Van Mellaert, H.** 1988. Specificity of *Bacillus thuringiensis*  $\delta$ -endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proc. Natl. Acad. Sci. USA* **85**:7844-7848
72. **Höfte, H., and Whiteley, H. R.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**:242–255.
73. **Jenkins, J.L. and Dean, D.H.** 2001. Binding specificity of *Bacillus thuringiensis* Cry 1 Aa for purified, native *Bombyx mori* aminopeptidase N and cadherin-like receptors. *BMC Biochemistry*, **2**:12.

74. **Johnston, K. A., Lee, M. J., Brough, C., Hilder, V. A., Gatehouse, A. M. R. and Gatehouse, J. A.** 1995. Protease activities in the larval midgut of *Heliothis virescens*: Evidence for trypsin and chymotrypsin-like enzymes. *Insect Biochem. Mol. Biol.* **25**:375-383.
75. **Jurat-Fuentes, J.L. and Adang, M.J.** 2006. Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. *J Invertebr Pathol.* **92**(3):166-171.
76. **Kaelin, P., Morel, P. and Gadani, F.** 1994. Isolation of *Bacillus thuringiensis* from stored tobacco and *Lasioderma serricornis* (F.). *Appl. Environ. Microbiol.* **60**:19-25
77. **Kalman, S., K. L. Kiehne, N. Cooper, M. S. Reynoso, and T. Yamamoto.** 1995. Enhanced production of insecticidal proteins in *Bacillus thuringiensis* strains carrying an additional crystal protein gene in their chromosomes. *Appl. Environ. Microbiol.* **61**:3063-3068.
78. **Kämpfer, P.** 1994. Limits and possibilities of total fatty acid analysis for classification of *Bacillus* species. *Syst. Appl. Microbiol.* **17**:86-98.
79. **Kaneko, T., Nozaki, R. and Aizawa, K.** 1978. Deoxyribonucleic acid relatedness between *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. *Microbiol. Immunol.* **22**:639-641
80. **Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S. E., Richmond, K., Adair, D. M., Hugh-Jones, M., Kuske, C. R., and Jackson, P.** 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J. Bacteriol.* **179**:818-824.
81. **Kronstad, J. W. and Whiteley, H. R.** 1984. Inverted repeat sequences flank a *Bacillus thuringiensis* crystal protein gene. *J. Bacteriol.* **160**:95-102
82. **Kuo, W.-S. and Chak, K.-F.** 1996. Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. *Appl. Environ. Microbiol.* **62**:1369-1377.
83. **Lambert, B., Buysse, L., Decock, C., Jansens, S., Piens, C., Saey, B., Seurinck, J., van Audenhove, K., Van Rie, J., van Vliet, A. and Peferoen, M.** 1996. A *Bacillus thuringiensis* insecticidal protein with high activity against members of the family Noctuidae. *Applied And Environmental Microbiology.* **62**, 80-86.
84. **Lambert, B. and Peferoen, M.** 1992. Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a successful biopesticide. *BioScience* **42**:112-122.

85. **Lebedeva, K.V., Vendilo, N.V., Ponomarev, V.L., Pletnev, V.A., Mitroshin, D.B.** 2002. Identification of pheromone of the greater wax moth *Galleria mellonella* from the different regions of Russia. IOBC wprs Bulletin Vol. **25**.
86. **Lecadet, M. M. and R. Dedonder.** 1964. Les proteases du chyle de Pieris brassicae. Purification. C.R. Acad. Sci. SOr. D 261:3117-3120.
87. **Lee, I.H., Chang, J.H., Roh, J.Y., Oh H.W., Lee, S.G., Shin, S.C. and Boo, K.S.** (2001). Isolation and characterization of a *Bacillus thuringiensis* ssp. *kurstaki* strain toxic to *Spodoptera exigua* and *Culex pipiens*. *Current Microbiology*. **43**, 284-387.
88. **Lee, M.K., Aguda, R.M., Cohen, M.B. and Gould, F.L. and Dean, D.H.** 1997. Determination of *Bacillus thuringiensis*  $\delta$ -endotoxin receptors to rice stem borer midguts. *Applied And Environmental Microbiology*. **63**, 1453-1459.
89. **Lereclus, D., Ribier, J., Klier, A., Menou, G. and Lecadet, M.-M.** 1984. A transposon-like structure related to the bendotoxin gene of *Bacillus thuringiensis*. *EMBO J.* **3**: 2561-2567
90. **Lereclus, D., Arantes, O., Chaufaux, J., Lecadet, M.-M.** 1989. Transformation and expression of a cloned  $\delta$ -endotoxin gene in *Bacillus thuringiensis* FEMS *Microbiol. Lett.* **60**: 211–218.
91. **Li, J., Carrol, J. and Ellar, D.J.** 1991. Crystal structure of insecticidal  $\delta$ -endotoxin from *Bacillus thuringiensis* at 2.5Å resolution. *Nature*. **353**, 815-821
92. **Lövgren, A., Carlson, C.R., Eskils, K. and Kolstø.** 1998. Localisation of putative virulence genes on a physical map of the *Bacillus thuringiensis* subs. *gelechiae* chromosome. *Current Microbiology*. **37**, 245-250.
93. **Machesky, H.** 1989. USDA forest service gypsy moth aerial suppression/eradication projects. *Gypsy Moth News* **20**:2-3
94. **Maddrell, S. H. P., Lane, N. J., Harrison, J. B., Overton, J. A. and Moreton R. B.** 1988. The initial stages in the action of an insecticidal  $\delta$ -endotoxin of *Bacillus thuringiensis* var. *israelensis* on the epithelial cells of the Malpighian tubules of the insect, *Rhodnius prolixus*. *J. Cell Sci.* **90**:131-144
95. **Martin, P. A. W., and Travers, R. S.** 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* **55**:2437-2442

96. **McPherson J.D., Shilton B.H. and Walton D.J.** 1988. *Role of fructose in glycation and cross-linking of proteins.* *Biochemistry.* **27**:1901-7
97. **Mormak, D. A. and Casida, L. E. Jr.** 1985. Study of *Bacillus subtilis* Endospores in Soil by Use of a Modified Endospore Stain.1985. *Appl Environ Microbiol.* **49**(6): 1356–1360
98. **NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST)**(<http://130.14.29.110/BLAST/>) [ 09/02/2006]
99. **Pardo-Lopez, L., Gomez, I.,Rausell, C.,Sanchez, J., Soberon, M. and Bravo, A.** 2006. Structural changes of the CryIAc oligomeric pre-pore from *Bacillus thuringiensis* induced by N-acetylgalactosamine facilitates toxin membrane insertion. *Biochemistry.***45**(34):10329-36.
100. **Porcar L, Hamilton W. A. and Butler P. D. .** 2002. Scaling of Shear-Induced Transformations in Membrane Phases. *Phys. Rev. Lett.* **89**, 168301 [Issue 16 – September 2002]
101. **Porcar, M. and Juarez-Perez, V.** 2003. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. *FEMS Microbiol Rev.* **26**(5):419-32
102. **Provine, H. and Gardner, P.** 1974. The Gram's stained smear and its interpretation. *Hosp. Pract.* **9**, 85-91.
103. **Ragni, A., Thiéry. I. and Delécluse. A.** 1996. Characterization of six highly mosquitocidal *Bacillus thuringiensis* strains that do not belong to the H-14 serotype. *Curr. Microbiol.* **32**:48-54.
104. **Rampersad J, Khan, A. and Ammons, D.** 2002. Usefulness of Staining Parasporal Bodies when Screening for *Bacillus thuringiensis*. *J Invertebr Pathol*, **79**:203-4
105. **Rössler, D., Ludwig, W., Schleifer, K. H., Lin, C., McGill, T. J., Wisotzkey, J. D., Jurtschuk, P. Jr. and G. E. Fox.** 1991. Phylogenetic diversity in the genus *Bacillus* as seen by 16S rRNA sequencing studies. *Syst. Appl. Microbiol.* **14**:266-269
106. **Sasaki, J., Asano, S., Hashimoto, N., Lay, B.W. and Hastowo, S.** 1997. Characterization of a cry2A gene cloned from an isolate of *Bacillus thuringiensis* serovar sotto. *Current Microbiology.* **35**, 1-8.
107. **Sayyed, A.H., Gatsi, R., Kouskoura, T., Wright D. J. and Neil Crickmore, N.** 2001. Susceptibility of a Field-Derived, *Bacillus thuringiensis*-Resistant Strain of Diamondback Moth to In Vitro-Activated CryIAc Toxin. *Applied and Environmental Microbiology* **67** (9) 4372- 4373.

108. **Schesser, J. H., Kramer, K. J. and Bulla, L. A. Jr.** 1977. Bioassay for homogeneous parasporal crystal of *Bacillus thuringiensis* using the tobacco hornworm, *Manduca sexta*. *Appl. Environ. Microbiol.* **33**:878-880.
109. **Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R., Dean, D. H.** (1998). *Bacillus thuringiensis* and Its Pesticidal Crystal Proteins. *Microbiol. Mol. Biol. Rev.* **62**: 775-806
110. **Seidl, A., Hinz, H.J.** 1984. The Free Energy of DNA Supercoiling is Enthalpy-Determined. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. **81**, No. 5: 1312-1316.
111. **Shimanuki, H., Knox, D. A., Furgala, B., Caron, D. M. and Williams, J. L.** 1992. *Diseases and pests of honey bees*, p. 1083-1151.
112. **Shin, B-Y., Park, S-h., Choi, S-K., Koo, B-T., Lee, S-T. and Kim, J-I.** (1995). Distribution of cryVt-Type insecticidal protein genes in *Bacillus thuringiensis* and cloning of cryv-Type genes from *Bacillus thuringiensis* subsp. *Kurstaki* and *Bacillus thuringiensis* subs. *Entomocidus*. *Applied and Environmental Microbiology.* **61**, 2402-2407.
113. **Shivakumar, A. G., Gundling, G. J., Benson, T. A., Casuto, D., Miller, M. F. and Spear, B. B.** 1986. Vegetative expression of the  $\delta$ -endotoxin genes of *Bacillus thuringiensis* subsp. *kurstaki* in *Bacillus subtilis*. *J. Bacteriol.* **166**:194-204.
114. **Siqueira, H.A., Gonzalez-Cabrera, J., Ferre, J., Flannagan, R. and Siegfried, B.D.** 2006. Analyses of Cry1Ab binding in resistant and susceptible strains of the European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). *Appl Environ Microbiol.* **72**(8):5318-24.
115. **Smith, G. P., Merrick, J. D., Bone, E. J. and Ellar, D. J.** 1996. Mosquitocidal activity of the CryIC  $\delta$ -endotoxin from *Bacillus thuringiensis* subsp. *aizawai*. *Appl. Environ. Microbiol.* **62**:680-684
116. **Starzak, M. and Bajpai, R.K.** 1991. A structured model for vegetative growth and sporulation in *Bacillus thuringiensis*. *Appl Biochem Biotechnol.* **28-29**:699-718
117. **Swan, L. A. and Papp, C. S.** 1972. *The common insects of North America*. Harper and Row, New York. 750 pp.

118. **Te Giffel, M. C., Beumer, R. R., Klijn, N., Wagendorp, A. and Rombouts, F. M.** 1997. Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA. FEMS Microbiol. Lett. **146**:47-51
  
119. **Theunis, W., Aguda, R.M., Cruz, W.T., Decock, C.M., Perferoen, M., Lambert, B., Bottrel, D.G., Gould, F.L., Litsinger, J.A. and Cohen, M.B.** 1998. *Bacillus thuringiensis* isolates from the Philippines: habitat distribution, diversity and toxicity to rice stem borers (LepidopteraL: Pyralidae). Bulletin of Entomological Research. **88**, 335-342.
  
120. **Travers, R.S., Martin, P.A. and Reicheferfer, C.F.** 1987. Selective process for efficient isolation of soil *Bacillus* spp. Applied and Environmental Microbiology. **53**, 1263-1266.
  
121. **Weisburg, G. W., Barns, S. M., Pelletier, D. A. and Lane, D. J.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**:697–703.
  
122. **Wong, H. C. and Chang, S.** 1986. Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc. Natl. Acad. Sci. USA **83**:3233–3237.
  
123. **Wu, D., Johnson, J. J. and Federici, B. A.** 1994. Synergism of mosquitocidal toxicity between CytA and CryIVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. Mol. Microbiol. **13**:965-972.
  
124. **Yang, X-M. and Wang, S.S.** 1998. Development of *Bacillus thuringiensis* fermentation and process control from a practical perspective. Biotechnol. Appl.Biochem. **28**, 95-98.