

# **Isolation of Entomopathogenic Gram Positive Spore Forming Bacteria Effective Against Coleoptera**

**By**

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## Thesis Abstract

Fourteen spore-forming bacterial strains were isolated and screened for entomopathogenic activity. Five displayed toxicity towards the common mealworm, *Tenebrio molitor* L., (Coleoptera: Tenebrionidae). The majority of the isolates were obtained from insect larvae and insect rich environments. The three bacterial species identified were *Bacillus thuringiensis* Berliner, *Brevibacillus laterosporus* Laubach and *Bacillus cereus* Frankland and Frankland. Bioassays were conducted using *T. molitor* larvae. The one isolate of *B. cereus* required the highest concentration of bacterial cells to achieve its LC<sub>50</sub>, whereas one of the isolates of *B. laterosporus* required the lowest cell concentration to achieve its LC<sub>50</sub>. Dose response curves were generated for the five best isolates, which showed that the isolate of *B. laterosporus* (NDR2) was substantially more toxic than the other isolates.

Subsequent bioassays were conducted against larvae of Scarabaeidae (*Hypopholis* sp.). Five entomopathogenic spore-forming isolates were tested for toxicity. Only three of the five isolates were effective, causing mortalities greater than 40%. *In vivo* trials conducted on grass plugs demonstrated that the three effective isolates protected the treated grass plugs relative to the feeding damage suffered by the non-treated grass.

*Bacillus cereus* Repetitive Extragenic Palindromic Polymerase Chain Reaction (Bc-Rep PCR) was conducted with five *B. thuringiensis* isolates from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection and the five local isolates of entomopathogenic spore-forming bacteria. Isolates of *B. thuringiensis* subsp. *israelensis*, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *thuringiensis*, *B. thuringiensis* subsp. *kenyae* and *B. thuringiensis* subsp. *tochigiensis* were sourced from the DSMZ. Two primers were used: an 18 mer primer, 5<sup>1</sup>-ATT AAA GTT TCA CTT TAT-3<sup>1</sup> and a 14 mer reverse primer, 5<sup>1</sup>-TTT AAT CAG TGG GG-3<sup>1</sup>. The three local species were genetically distinct from each other. The three *B. thuringiensis* isolates were closely related, and relatively closely related to the *B. cereus* isolate. The *B. laterosporus* isolate was not related the other four isolates.

Serotyping of the *B. thuringiensis* isolates was also undertaken. However, only one of the isolates could be identified with serotyping and was identified *B. thuringiensis* subsp. *kenyae*. This exercise demonstrated the basic problem with serotyping, that many isolates of entomopathogenic bacteria cannot be serotyped, even within the genus *Bacillus*.

Transmission Electron Microscopy (TEM) and Environmental Scanning Electron Microscopy (ESEM) were conducted on the five local isolates of entomopathogenic spore-forming bacteria. Three *B. thuringiensis* strains and one *B. laterosporus* strain formed crystal proteins that could be viewed. Bipyramidal, cuboidal, triangular and canoe shaped crystal proteins were observed. Crystal proteins were either attached to the spore or separate from the spore. ESEM was not effective in showing the crystal protein of the *B. laterosporus* isolate.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted to determine the number and size of the proteins of four isolates of entomopathogenic spore-forming bacteria. Of the four isolates producing crystal proteins, two strains of *B. thuringiensis* and one of *B. laterosporus* produced protein profiles. The crystal protein of *B. thuringiensis* Isolate NDR3 did not dissolve well in the solvents used, and hence it did not produce significant electrophoretic bands. The bands obtained from the other two *B. thuringiensis* isolates showed that their protein molecule sizes ranged from 73-kDa to 135-kDa, which is in the range of *B. thuringiensis* Cry toxins. Smaller proteins were also observed with sizes of 13-kDa to 44-kDa, which fall into the range of Cyt toxins. The crystal proteins of the *B. laterosporus* isolate had protein sizes in the range of 24-kDa to 40-kDa.

# **Declaration**

I, Nicolette du Rand, declare that the research reported in this thesis, except where otherwise indicated, and is my own original research.

This thesis has not been submitted for any degree or examination purpose at any other university.

Nicolette du Rand

Prof. M.D. Laing

## **Dedication**

I would like to thank the following people. Without their support this thesis would not have been possible:

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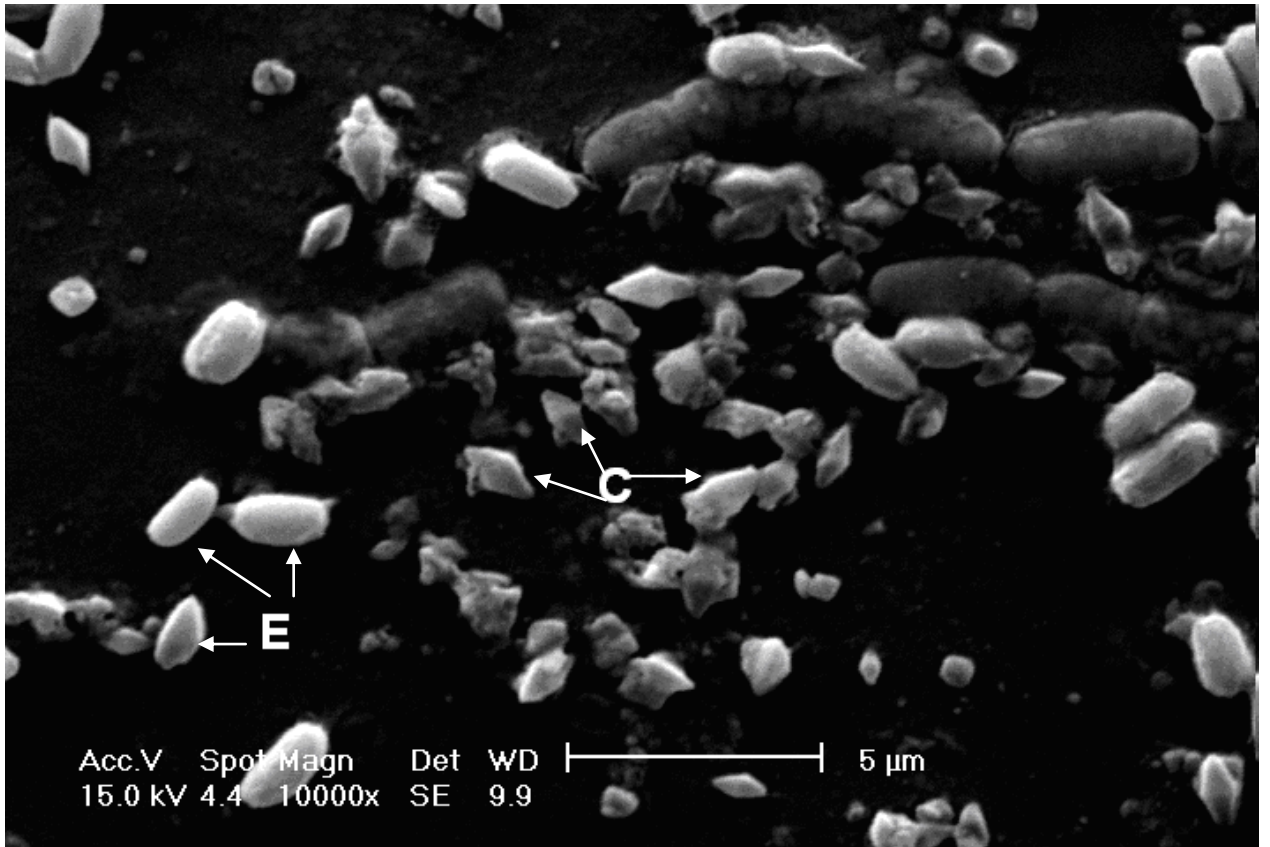


Figure 1. *Bacillus thuringiensis* endospores (E) and bipyramidal crystal proteins (C).

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# Thesis Introduction

*Bacillus thuringiensis* Berliner is the most successful insecticidal biocontrol agent on the market to date, in terms of both sales volumes and value, and has been researched extensively (Lambert and Peferoen, 1992). In contrast, until recently little attention, has been paid to other endospore-forming bacteria with insecticidal properties. Over the past decades bacterial species such as *Brevibacillus laterosporus* Laubach and *Bacillus cereus* Frankland and Frankland have been shown to have insecticidal properties (Orlova *et al.*, 1998; Selvakumar *et al.*, 2007).

Furthermore, most of the *B. thuringiensis* research has focused on the control of Lepidoptera and Diptera (Khetan, 2001). Coleoptera on the other hand, is the largest Order in the animal kingdom and contains a large number of important agricultural pests, including white grubs (Coleoptera: Scarabaeidae) (Leslie, 2004). White grubs are beetles comprised of a large number of genera and species and are the most important soil dwelling pests of sugarcane (Leslie, 2004). A number of genera and species of white grubs feed on young plants of both sugarcane and timber, two of the most important crops grown in the KwaZulu-Natal region of South Africa.

The objectives of this research were:

1. Review available literature on entomopathogenic spore-formers such as *B. thuringiensis* and *B. laterosporus*. Review literature available on white grubs of sugarcane.
2. Isolate endospore-forming bacteria with insecticidal properties against *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), and identify these species.
3. Determine level of toxicity of these endospore-forming bacterial isolates against *T. molitor*.
4. Determine the toxicity of these species against a genus of white grub (*Hypopholis* sp.).
5. Determine DNA relatedness between the endospore-forming bacterial isolates.
6. Obtain electron micrographs of the endospore-forming bacterial isolates.
7. Investigate the number of toxins and sizes produced by each of the insecticidal crystal protein-forming bacterial isolates.
8. Thesis overview

This dissertation has been written in the form of eight chapters. Each chapter was prepared in the format of an independent scientific paper (this does not include Chapter 1 and 8). The species classification of bacteria and insects (Family, Order, genus and species) has been written according to the Journal of Economic Entomology. Family and Order of bacteria are not required and there is no naming authority for bacterial subspecies.

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# Chapter One

## Literature Review

### 1.1. Introduction

Worldwide, the use of agrochemical insecticides is diminishing annually (Kiely *et al.*, 2004). Prime drivers of this trend are: that consumers do not want to be exposed to insecticide residues in food, there is concern over the impact of insecticides on the environment and that many insect pests have developed resistance to several insecticides, rendering them ineffective. Biocontrol has been considered to be the primary alternative to replace chemical insecticides as using nature to manage it is an attractive proposition.

Disease-causing organisms are of great economic importance and even more so when they involve humans, animals and plants. Insects are vulnerable to entomopathogenic viruses, fungi and bacteria, including rickettsias and mycoplasmas (Aronson *et al.*, 1986).

Viruses infecting insects may contain either RNA or DNA genomes (Aronson *et al.*, 1986). These entomopathogenic viruses can have either broad or narrow host ranges, depending on the type of virus (Covy *et al.*, 2000; Entwistle, 1990; Lacey and Adams, 1994). An example of viruses with narrow host ranges are the Baculoviruses (Lacey and Adams, 1994) and viruses with broad host ranges include the Blue Iridovirus (Covy *et al.*, 2000). As entomopathogenic viruses only attack insects they are useful biocontrol agents (Aronson *et al.*, 1986). Virus particles are ingested by the insect host and infection occurs in the insect midgut. The virus particles cannot replicate on their own and thus enter living cells to use them for replication. The insect host cells eventually lyse and release virus particles which in turn infect other host cells. This continues throughout the cells of the insects until the insect host dies (Aronson *et al.*, 1986).

Entomopathogenic fungi are found in all Classes of fungi, including Zygomycetes, Fungi Imperfecti, Ascomycetes and Basidiomycetes (Aronson *et al.*, 1986). Fungi infect insects by producing chitinases that allow for mycelial penetration via the chitinous, sclerotized cuticle (Aronson *et al.*, 1986).

Nematodes infect insects using toxin-producing bacteria as a biological weapon (Aronson *et al.*, 1986; Boemare, 2002). Nematode species such as *Steinernema feltiae* Filipjev (Steinernematidae: Rhabditida) inflict wounds orally in insects and inject a bacterium, *Xenorhabdus nematophilus* Poinar and Thomas, which is harboured in a bacterial sack within the nematode, into the insect haemocoel. The insect dies due to bacterial septicaemia (Aronson *et al.*, 1986). The nematode then feeds on the bacterial mass within the insect cadaver (Aronson *et al.*, 1986).

*Bacillus* species are the most common spore-forming bacteria that are pathogenic towards insects. Spores may either be ingested by target insects or enter through wounds. They germinate in the haemocoel and perforate the epithelial cells of the haemocoel thus killing the host insect (Aronson *et al.*, 1986). Identified species of this genus pathogenic to insects include: *B. lentimorbus* Dutky, *B. popilliae* Dutky, *B. thuringiensis* Berliner and *B. sphaericus* Neide (Aronson *et al.*, 1986; Sharpe *et al.*, 1970). *Bacillus thuringiensis* and *B. sphaericus* produce Insecticidal Crystal Proteins (ICPs) consisting of protoxins, which is an inactive form of toxin that is enzymatically cleaved into an active toxin (Payne and Davidson, 1984). Entomopathogenic *Clostridium* spp. are also capable of forming spores but are not as common as *Bacillus* spp. *Clostridium* spp. are strict anaerobes and proliferate in the insect gut, producing potent toxins (Aronson *et al.*, 1986). Spores are formed by *Bacillus* spp. and *Clostridium* spp. when conditions become unfavourable. These spores may lie dormant for prolonged periods of time until favourable conditions arise, at which point spores germinate into bacterial cells, which then multiply by typical binary fission. Dormant spores can withstand adverse conditions of heat, drought and UV radiation from the sun (Nakayama *et al.*, 1996; Popham *et al.*, 1995; Setlow 2006; 1996; Vreeland *et al.*, 2000).

*Bacillus thuringiensis* is a very successful commercial biocontrol agent and falls into the category of a bio-pesticide (Armstrong *et al.*, 1985; Khetan, 2001; Navon, 2000). Bio-pesticides are divided into three groups: microorganism pesticides, natural ingredient pesticides and biochemical pesticides. The success of *B. thuringiensis* as a bio-pesticide is due to its attributes

of having a narrow host range, non-toxicity to animals and plants, and being environmentally friendly (Khetan, 2001).

Some negative attributes of *Bacillus thuringiensis* include:

1. They may not reach cryptic insects: *B. thuringiensis* has been found to sink to the bottom in water treatments for black flies, *Simulium* spp. (Diptera: Simuliidae), because larvae feed on the surface and therefore avoid ingesting the sinking entomopathogen (Undeen *et al.*, 1984).
2. They tend to have a low field stability: UV from sunlight breaks down the ICPs of *B. thuringiensis*, making them ineffective as toxins after a relatively brief exposure to sunlight (Lambert and Peferoen, 1992).
3. They have a narrow spectrum of activity: *B. thuringiensis* isolate H-14 is toxic to various species of Lepidoptera but does not affect all lepidopteran pest species (Aronson *et al.*, 1986). However, this attribute is also a positive attribute because in some cases, a narrow spectrum of activity is desired to target a specific insect, without damaging beneficial insects.

Transgenic plants using *B. thuringiensis* genes utilize this positive attribute (Ferré and Van Rie, 1992). Economically important crops are genetically modified (transgenic plants) with *B. thuringiensis* ICP genes. Various crops have been genetically modified, including cotton, maize and potatoes (Ferré and Van Rie, 1992). Potatoes have been genetically modified to contain *B. thuringiensis* subsp. *tenebrionis* Cry3A toxins that are effective against the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), a widespread pest of potato (Hare, 1990; Nault, 2001).

In the pesticide industry it is more economical to search for novel *B. thuringiensis* bio-pesticides than designing new chemical pesticides (Lambert and Pefereon, 1992). Typically, 1 in 1000 *B. thuringiensis* insecticidal crystal proteins are effective compared to the 1 in 20,000 synthetic compounds that are effective pesticides (Lambert and Peferoen, 1992). Registration of *B. thuringiensis* products is simpler, faster and cheaper than the registration of agrochemical compounds (Lambert and Pefereon, 1992). However, globally there are no set guidelines on how

to register biocontrol products such as *B. thuringiensis* formulations. Their registration in countries such as Japan is of economic concern when they are Lepidoptera specific because of the risks they pose to the health of silkworms, *Bombyx mori* L. (Lepidoptera: Bombycidae), the basis of their silk industry (Lambert and Peferoen, 1992).

## **1.2. Entomopathogenic spore-forming bacteria**

There are numerous spore-forming bacterial species with insecticidal properties. Some species such as *B. thuringiensis* are common and have been exploited extensively. Most spore-forming bacteria were originally classified in the genus *Bacillus* but some have been reclassified into new genera such as *Brevibacillus* (Shida *et al.*, 1996) and *Paenibacillus* (Pettersen *et al.*, 1999).

### **1.2.1. *Bacillus thuringiensis***

#### **1.2.1.1. History of *Bacillus thuringiensis***

*Bacillus thuringiensis* was first identified and isolated by Ishiwata (1901) in Japan from diseased silkworms, *B. mori*, causing a disease that he subsequently named Sototo disease (De Lucca *et al.*, 1981; Khetan, 2001; Lambert and Peferoen, 1992). The second reported discovery was in 1911 where Berliner reported a *Bacillus* sp. infecting Mediterranean flour moths, *Anagasta kuhniella* Zeller (Lepidoptera: Pyralidae), found in grain store silos (Berliner, 1911).

*Bacillus thuringiensis* is mainly isolated from diseased insects or insect-rich environments (Lambert and Peferoen, 1992). *Bacillus thuringiensis* subsp. *aizawai* was isolated from dust in a silkworm rearing facility during a second outbreak of disease in silkworms in Japan (Aizawa *et al.*, 1961). *Bacillus thuringiensis* subsp. *kurstaki*, Strain HD-1 was isolated from pink bollworm, *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae), in a mass rearing facility (Dulmage, 1970). *Bacillus thuringiensis* subsp. *kenyae* was isolated from grain dust from a storage facility in Kenya (Norris and Burges, 1963).

*Bacillus thuringiensis* crystal preparations were first used in Europe in 1928 to control the European corn borer, *Ostrinia nubilalis*, Hübner, (Lepidoptera: Crambidae). The first commercial *B. thuringiensis* product i.e., Sporein, was produced in France in 1938 (Khetan,



2001; Lambert and Pefereon, 1992). In the 1960s several *B. thuringiensis* formulations were manufactured in France, Germany, Soviet Union and the United States. The actual cause of *B. thuringiensis* toxicity towards insects was only discovered in 1953, by Hannay, who found it to be a toxic crystal synthesized within the bacterial cell (Hannay, 1953). Angus (1954) discovered that these crystals consist of proteins and are soluble in alkalis. This led to the discovery of other toxins produced by *B. thuringiensis*.

Until 1970 it was thought that *B. thuringiensis* was toxic only to Lepidoptera species. This view changed with the discovery of *B. thuringiensis* subsp. *israelensis* in 1977 by Goldberg and Margalit, who made the isolation from mosquito larvae, *Culex pipiens* L. (Diptera: Culicidae) from a pond in the Negev desert. *Bacillus thuringiensis* subsp. *tenebrionis* was isolated from a dead mealworm larva, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), in 1983 by Krieg *et al.* This subspecies is highly effective against the elm leaf beetle, *Pyrralta luteola* Müller (Coleoptera: Chrysomelidae), and the Colorado potato beetle, *L. decemlineata* (Lambert and Pefereon, 1992).

Schnepf and Whiteley first cloned the gene for an ICP in 1981, which led to the revolution of genetically modified crops (transgenic plants) expressing *B. thuringiensis* genes (Lambert and Peferoen, 1992). This had resulted in a reduction in chemical pesticide use in certain crops such as cotton, maize and potatoes. These crops have since been genetically modified to contain ICP genes (Lambert and Peferoen, 1992). Concerns have been raised with respect to the potential development of insect resistance to *B. thuringiensis* toxins expressed by these crops, especially if they are grown widely in solid blocks. Continuous exposure of a species of insect to a specific toxin will drive a process of selection that will eventually lead to the development of resistance in the insect to that toxin (Ferré and Van Rie, 1992).

#### 1.2.1.2. Ecology of *Bacillus thuringiensis*

*Bacillus thuringiensis* is ubiquitous and has been isolated from foliar surfaces, soils, infected insect larvae, aquatic environments, animal faeces, insect rich environments, flour mills and grain storage facilities (Chaufaux *et al.*, 1997; Khetan, 2001; Lambert and Peferoen, 1992; Porcar and Caballero, 2000; Smith and Couche, 1991).

*Bacillus thuringiensis* has been isolated repeatedly from soil (De Lucca *et al.*, 1981; Martin and Travers, 1989; Travers *et al.*, 1987). De Lucca *et al.* (1981) isolated *Bacillus* spp. from soil in the United States of America and found that 0.5% of bacterial isolates were *B. thuringiensis*. They tested soils from 115 fields that had not previously been treated with *B. thuringiensis* sprays and found that in 17% of the soils tested *B. thuringiensis* was present (De Lucca *et al.*, 1981). A majority of the subspecies isolated were *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *galleriae* (De Lucca *et al.*, 1981). Martin and Travers (1989) isolated *B. thuringiensis* from soil from 30 countries that included three African countries, Egypt, Cameroon and Morocco. A total of 645 isolates of *B. thuringiensis* was isolated from African soils, of which 47 were *B. thuringiensis* subsp. *morrisoni* (Martin and Travers, 1989). This subspecies is known to be effective against some species of Coleoptera but may also be effective against Lepidoptera and Diptera, depending on the isolate (Padua *et al.*, 1984).

While the main source of novel *B. thuringiensis* isolates has been soil, *B. thuringiensis* has also been isolated from various other sources (Smith and Couche, 1991). Grison *et al.* (1976) demonstrated that *B. thuringiensis* spores are able to survive for weeks on the needles of shaded pine trees, *Pinus* spp. (Pinales: Pinaceae) and viable *B. thuringiensis* spores have been found to be persistent for more than a year on the needles of the balsam fir, *Abies balsamea* L. (Pinales: Pinaceae), (Smith and Couche, 1991). This demonstrates that *B. thuringiensis* can persist for prolonged periods of time in other environments than soil. In the modern era, it is not easy to determine the natural occurrence of *B. thuringiensis* on foliar surfaces, due to drift of sprays of commercially produced *B. thuringiensis* spores from crop and forest spraying areas. For example, in a survey of *B. thuringiensis* isolates found on organically grown cabbage crops that had not been sprayed with *B. thuringiensis*, 64% of the isolates belonged to a widely commercialized strain, *B. thuringiensis* subsp. *kurstaki* (Damgaard *et al.*, 1997).

*Bacillus thuringiensis* strains isolated from aquatic environments are mainly dipteran specific because the larval stage of many Diptera lives in water (Iriarte *et al.*, 2000). *Bacillus thuringiensis* has been relatively ineffective as a bio-pesticide for aquatic environments because the spores tend to sink to the bottom of the water column (Ohana *et al.*, 1987). The larval stage of various insects, such as black flies and mosquitoes (Diptera: Culicidae) develop in aquatic environments, but critically, they feed on the surface of water bodies, and hence avoid ingesting *B. thuringiensis* sprayed onto the water surface but which rapidly sinks to the bottom. Aquatic environments in Spain yielded as many as 122 *B. thuringiensis* isolates from 44 samples (Iriarte *et al.*, 2000). *Bacillus thuringiensis* subsp. *thuringiensis* was the most frequent subspecies found (Iriarte *et al.*, 2000). *Bacillus thuringiensis* subsp. *israelensis* effective against *Culex pipens* L., (Diptera: Culicidae) has also been isolated from fresh water in Japan (Ichimatsu *et al.*, 2000).

As would be expected, various strains of *B. thuringiensis* have been isolated from insects and from their natural habitats (De Lucca *et al.*, 1981; Khetan, 2001; Lambert and Peferoen, 1992).

### **1.2.1.3. Isolation of *Bacillus thuringiensis***

*Bacillus thuringiensis* is a Gram-positive, endospore-forming, facultative anaerobic bacterium. *Bacillus thuringiensis* has been widely isolated as a potential bio-pesticide because there is a need for environmentally safe pesticides (Smith and Couche, 1991). Spores are heat resistant and a pre-treatment of samples with heat simplifies subsequent isolation of *B. thuringiensis* strains, because heat treatment generally kills other unwanted non-spore-forming bacteria (Figure 1.1) (Travers *et al.*, 1987). A selective isolation protocol was developed by Travers *et al.* (1987) using heat treatment in conjunction with an acetate medium. The aim of this method was to isolate *B. thuringiensis* isolates only, and not other *Bacillus* spp. The heat treatment on its own eliminates non-spore forming bacteria but not other spore-forming species (Prescott *et al.*, 1999). *Bacillus thuringiensis* and *Bacillus cereus* Frankland and Frankland spores were demonstrated not to germinate in high concentrations of acetate (Travers *et al.*, 1987). Hence, when a mixture of spore-forming bacteria is inoculated onto an acetate medium, *B. thuringiensis* and *B. cereus* spores do not germinate, whereas other spore-forming bacteria do. After an initial germination period, the bacterial samples are subjected to heat treatment, after which the germinated bacterial

cells die and the *B. thuringiensis* spores remain dormant and survive in a dormant form (Travers *et al.*, 1987). The spores are then germinated on a fresh medium (nutrient agar) that does not contain acetate.

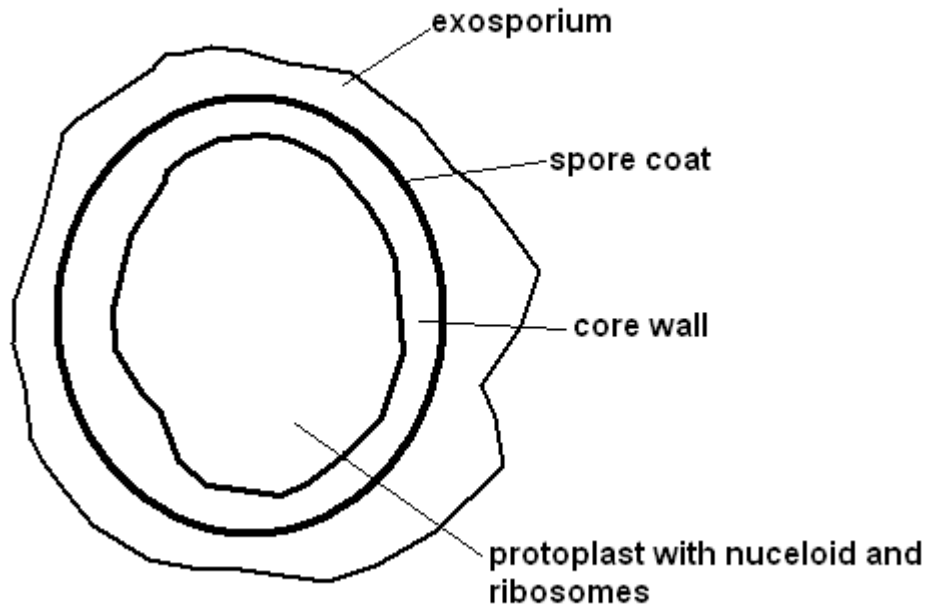


Figure 1.1. Structure of a bacterial endospore.

*Bacillus cereus* and *B. thuringiensis* isolates appear identical except for the presence of a protein crystal in *B. thuringiensis*, which aids separation of the two species (Schnepf *et al.*, 1998). Genetic analysis suggests that these two species could be considered a single species (Schnepf *et al.*, 1998).

#### **1.2.1.4. Insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis***

Inclusion bodies, known as ICPs, along with bacterial endospores, were first reported by Berliner in 1911. ICPs can contain one or more protoxins and are synthesized as a crystal within the bacterial cell (Schnepf and Whiteley, 1981). The protoxins are activated to create active toxins upon solubilization in the insect midgut (Hubner and Lüthy, 1981). The toxic function of the ICPs was not initially known and their function was only discovered by Schnepf and Whiteley (1981) when they were expressed in *Escherichia coli* Migula. *Escherichia coli* was genetically

modified with the insertion of a cloned ICP gene from *B. thuringiensis* subsp. *kurstaki* (Lambert and Peferoen, 1992).

The life cycle of *B. thuringiensis* consists of four major stages, namely a vegetative phase, a sporulation phase, a dormant spore phase and a germination phase (Figure 2.1). When *B. thuringiensis* is exposed to unfavourable conditions that may include nutrient depletion, bacterial cells form spores within themselves. Protoxins are slowly synthesized during the vegetative stage and the ICPs crystallize into a single crystal in the cytoplasm synchronously with spore formation (Figure 1) (Lambert and Peferoen, 1992).

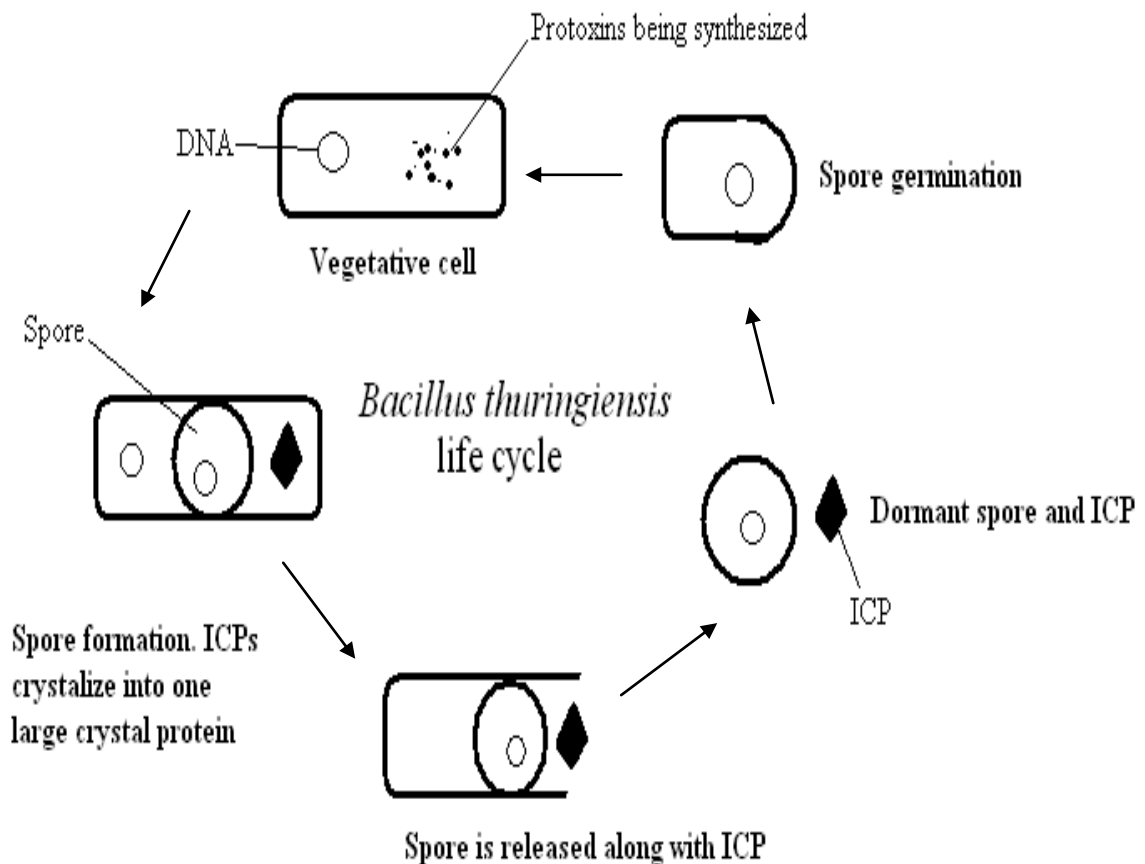


Figure 2.1. Life cycle of *Bacillus thuringiensis*.

The two major toxins produced by *B. thuringiensis* are delta-endotoxins and beta-exotoxins. Other toxins such as alpha-exotoxins and vegetative insecticidal proteins (VIPs) are also synthesized by *B. thuringiensis*. The group of beta-exotoxins include flytoxin, thuringiensin and an unnamed thermally stable toxin. Beta-exotoxins are not desirable toxins to find in potential biocontrol agents due to their toxicity to vertebrates (Navon, 2000). As such, the registration of *B. thuringiensis* products requires that these beta-exotoxins are absent from the formulated end products (Khetan, 2001). The insecticidal crystal proteins (ICPs) are known as delta-endotoxins. These toxins are acceptable in biocontrol pesticides because they are specifically toxic to target insects (Navon, 2000). Alpha-exotoxins are minor toxins produced by some species of *B. thuringiensis* (Khetan, 2001).

Pesticide potency is measured by comparing the toxicity of a certain pesticide against a specific insect species. The mortality rate is compared with known standard toxin's toxicity against that insect species (Navon, 2000). Each Order of insects has a standard species representative (Navon, 2000). *Bacillus thuringiensis* bio-pesticides also have a standard potency determination method. A standard reference strain for potency determination was accepted in 1966 by the Collegium of Insect Pathology and Microbial Control of Wageningen in the Netherlands. The first standard was a Strain E-61 (*B. thuringiensis* subsp. *thuringiensis*) formulation developed by the Pasteur Institute in France. It was initially isolated by Howard Dulmage and is typically used against Lepidoptera (Khetan, 2001; Lambert and Peferoen, 1992). The potency of this standard was determined to be 1000 International Units (IU) mg<sup>-1</sup>. In 1971 it was replaced by Strain HD-1-S-1971 with a potency rating of 1800 IU mg<sup>-1</sup> (Navon, 2000). In 1980 a new reference strain, HD-1-S-1980, was brought in to replace the previous strain, and had a potency rating of 16000 IU mg<sup>-1</sup>. Demand for these standards has resulted in the stocks of the standard strain being depleted over time, hence the need for replacement standard isolates on a regular basis (Navon, 2000). Another problem is that the standard insect species used are not always good representatives of a specific Order, and furthermore, these species are not always pest organisms on a global scale (Navon, 2000).

### 1.2.1.5. Nomenclature of insecticidal crystal proteins (ICPs)

Insecticidal crystal proteins (ICPs) are protein molecules that are classified into two major groups of toxins, namely the Cry and the Cyt toxins. The nomenclature for these toxins was originally based on insect toxicity that was used as the primary rank (Höfte and Whiteley, 1989). With the discovery of more and more Cry and Cyt toxins, this system began to fail as more exceptions became apparent. A new system of nomenclature has since been revised by Crickmore *et al.* (1998) based on amino acid sequences and the hierarchical clustering of these sequences (Crickmore *et al.*, 1998). The new nomenclature system consists of four degrees of ranks by which toxins are ranked accordingly. The primary rank of the old roman numerals of the nomenclature system devised by Höfte and Whiteley (1989) have been replaced by Arabic numerals (Crickmore *et al.*, 1998). The secondary rank is represented by upper case alphabetical letter, the tertiary rank is based is represented by a lower case alphabetical letter and the quaternary rank is represented by an Arabic numeral, e.g. Cry01Aa3 (Figure 1.3) (Crickmore *et al.*, 1998). The boundaries for these ranks have been set at 45%, 78% and 95% based on amino acid sequence homology (Crickmore *et al.*, 1998). Cry toxins have been classified into 55 primary ranks and Cyt toxins into 2 primary ranks, based on the homology of their amino acid sequences (Crickmore *et al.*, 2008).

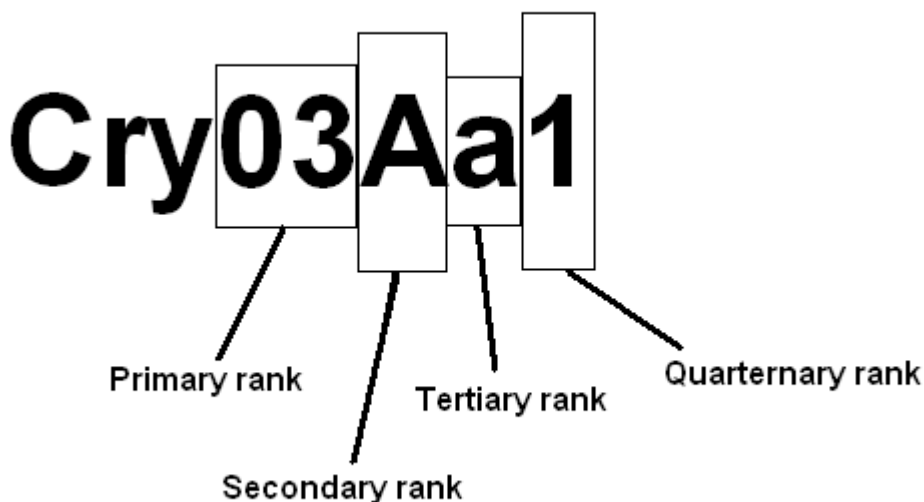


Figure 1.3. Cry and Cyt toxin nomenclature

Cry and Cyt toxins discovered to date are not only effective against insects but also against aphids, flukes, lice, mites, molluscs, nematodes, platyhelminths and protozoans (Khetan, 2001).

The type and number of toxin molecules greatly affect the shape in which these molecules crystallize (Lambert and Peferoen, 1992). For example, Cry1 toxins are bipyramidal, Cry2 toxins are cuboidal, Cry3A is flat and irregular, Cry3B is irregular, and both Cry4A and Cry4B are rhomboidal and Cry2A is spherical (Khetan, 2001). ICPs are visible using a light microscope or phase contrast microscopy. This makes it easy to identify *B. thuringiensis* cells from other spore-forming bacteria during microscopic investigations. The toxin's shape is due to disulphide bonds, hydrophobicity and hydrogen bonds. The tertiary structures of the crystals are maintained by the disulphide bonds within the proteins (Gill *et al.*, 1992). The ICP is composed of different types of protoxins that affect the crystal shape results from different combinations of these protoxins (Figure 1.4) (Khetan, 2001).

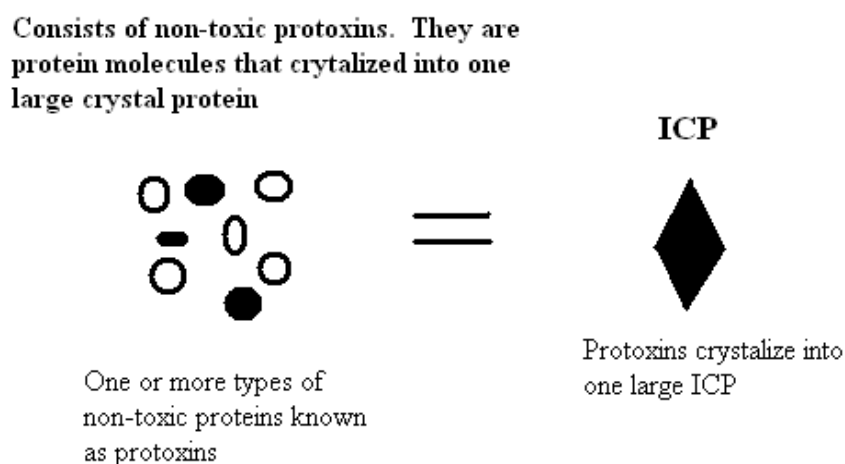


Figure 1.4. Schematic representation of Insecticidal Crystal Proteins (ICP) and its constituents.

To date, mosquitocidal crystals are mainly irregular-spherical shaped, while ICPs toxic to Coleoptera are rhomboidal (Khetan, 2001). ICPs specifically active against Coleoptera, such as the toxins of *B. thuringiensis* subsp. *tenebrionis*, are flat rhomboidal or square-rectangular plates (Khetan, 2001). With more and more *B. thuringiensis* strains being isolated, this rule of thumb on



crystal shape is not always applicable. Ohba *et al.* (1992) isolated *B. thuringiensis* subsp. *japonensis*, which produces round crystal proteins and is effective against Coleoptera.

Examples of Cry and Cyt toxins toxic to Coleoptera include the Cry3Aa against the Colorado potato beetle (Huger and Krieg, 1989), Cry1Ba against the cottonwood leaf beetle, *Chrysomela scripta* F. (Coleoptera: Chrysomelidae) (Bradley *et al.*, 1995), Cry3Bb1 against the western corn root worm, *Diabrotica vergiferavirgifera* Le Conte (Coleoptera: Chrysomelidae) (English *et al.*, 2000) and Cyt1Aa against the *C. scripta* (Federici and Bauer, 1998).

#### **1.2.1.6. Cry toxins mode of action**

Most of the research on the mode of action of these toxins has been based on their effects on Lepidoptera (Höfte and Whiteley, 1989). The ingested ICP, which consists of numerous protein molecules known as protoxins, is dissolved in the alkaline environment of the insect midgut (Baum and Malvar, 1995; Khetan, 2001). The non-toxic protoxins are then enzymatically cleaved into active toxins (Figure 1.5). Some toxin molecules are large enough to be cleaved into two active toxin molecules (Höfte and Whiteley, 1989; Lambert and Peferoen, 1992; Gill *et al.*, 1992). Typically toxins are one of three sizes: 25-28 kDa, 65-75 kDa and 125-138 kDa (Gill *et al.*, 1992).

Large protoxins are activated into active toxins by proteases and the alkaline pH in the insect midgut (Höfte and Whiteley, 1986). The actual toxicity of ICPs is believed to be due to binding to specific receptor sites on the brush border membrane vesicles of the gut of host insects (Gill *et al.*, 1992). The Cry toxins have a specific affinity for the receptor sites, whether they are of high or low affinity. Insects have been found to contain an array of toxin receptor sites. The greater the number of receptor sites present, the greater the toxicity expressed (Gill *et al.*, 1992). For an insect larva to be susceptible to a particular toxin, sites specific for that toxin need to be present in the brush border membrane of the mid-gut (Gill *et al.*, 1992). Different species of insects have different receptor sites for these toxins on the brush border membrane (Lambert and Peferoen, 1992). Cry1Ab and Cry 1Ac are examples of toxins that recognize the same specific binding sites (Estada and Ferré, 1994).

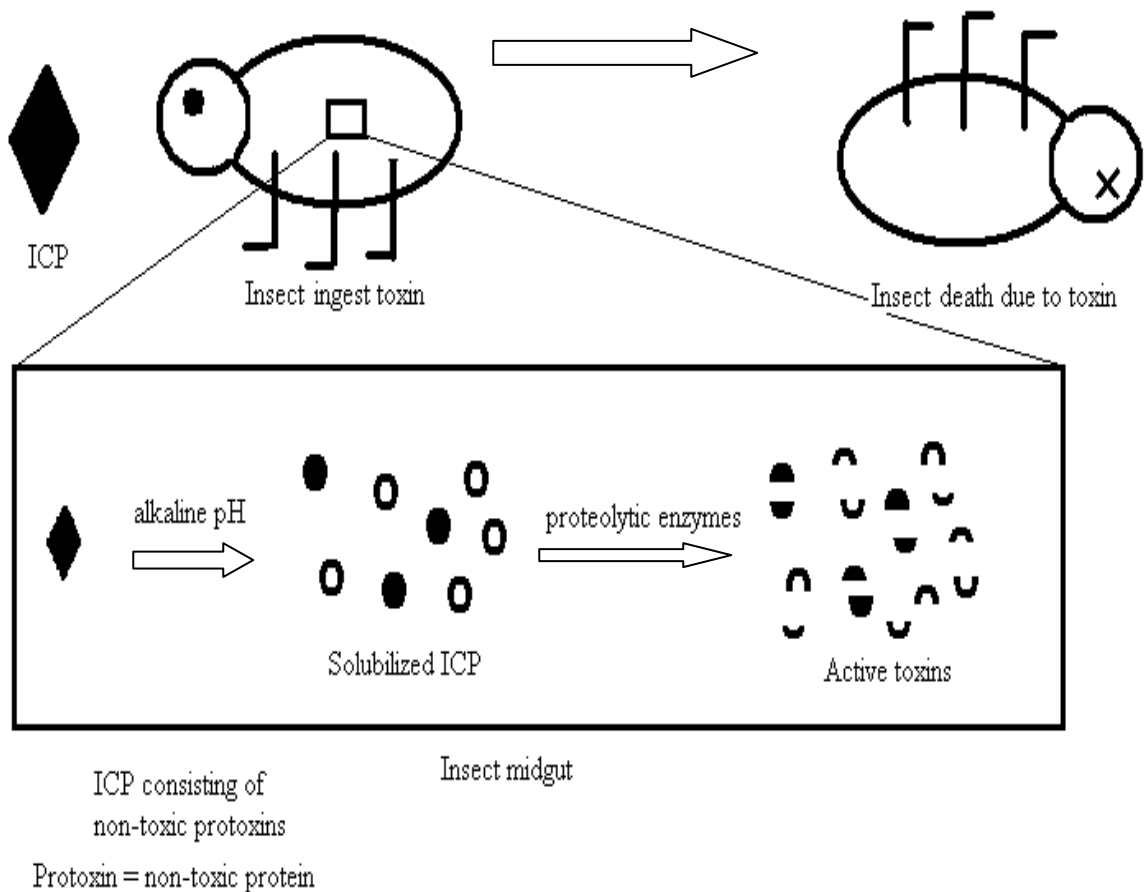


Figure 1.5. Schematic representation mode of action of Insecticidal Crystal Proteins (ICP).

The toxins form non-specific pores in the epithelial cell membranes of the midgut. This causes the epithelial cells to swell and lysis follows, due to the net inflow of ions and water (Höfte and Whiteley, 1989). ICP intoxication does not have a long lag period and the cessation of feeding is the first external symptom of an infected insect larva (Höfte and Whiteley, 1989).

The midgut epithelial cells stop being a barrier between the haemocoel and the midgut contents of the infected insect. The pH drops due to the influx of haemolymph into the haemocoel (Gill *et al.*, 1992). *Bacillus thuringiensis* spores germinate in this environment due to the nutrient rich haemolymph and lower pH. Bacterial cells proliferate and invade the larval body cavity tissues. The larva stops feeding and eventually dies due to bacterial septicemia. Death may be accelerated by opportunistic secondary microbial infections. As the cadaver is consumed, the

nutrients become depleted, at which point *B. thuringiensis* sporulation occurs. When the insect cadaver disintegrates, the *B. thuringiensis* spores and ICPs are released into the environment (Lambert and Peferoen, 1992).

**Table 1.1.** Cry toxins effective against Coleoptera (Van Frankenhuyzen and Nystrom, 2002)

Cry toxins	Family	Genus and species
Cry01Ba1	Chrysomelidae	<i>Chrysomela scripta</i>
Cry01Ba1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry01Ia1	Chrysomelidae	<i>Agelastica coerulea</i>
Cry01Ia1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry01Ia1	Tenebrionidae	<i>Tenebrio molitor</i>
Cry03Aa1	Bruchidae	<i>Callosobruchus maculatus</i>
Cry03Aa1	Chrysomelidae	<i>Chrysomela scripta</i>
Cry03Aa1	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Cry03Aa1	Chrysomelidae	<i>Diabrotica baltea</i>
Cry03Aa1	Chrysomelidae	<i>Haltica tombacina</i>
Cry03Aa1	Leguminosae	<i>Hypera brunneipennis</i>
Cry03Aa1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry03Aa1	Chrysomelidae	<i>Phaedon cochleariae</i>
Cry03Aa1	Tenebrionidae	<i>Tenebrio molitor</i>
Cry03Aa1	Tenebrionidae	<i>Tribolium castaneum</i>
Cry03Aa4	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry03Aa4	Chrysomelidae	<i>Phyllotreta armoraciae</i>
Cry03Aa4	Scarabaeidae	<i>Popillia japonica</i>
Cry03Aa5	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry03Aa10	Cerambycidae	<i>Apriona germni</i>
Cry03Aa11	Chrysomelidae	<i>Agelastica alni</i>
Cry03Aa11	Melolonthidae	<i>Amphimallon solstitiale</i>
Cry03Aa11	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry03Aa11	Melolonthidae	<i>Melolontha melolontha</i>
Cry03Aa2	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry03Aa2	Tenebrionidae	<i>Tenebrio molitor</i>
Cry03Bb1	Chrysomelidae	<i>Diabrotica virgifera virgifera</i>
Cry03Bb1	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Cry03Bb1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry03Ca1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry07Aa1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry08Aa1	Scarabaeidae	<i>Cotinis</i> sp.
Cry08Aa1	Scarabaeidae	<i>Cyclocephala pasadenae</i>

(Table 1.1. continued.....)

Cry toxins	Family	Genus and species
Cry08Aa1	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Cry08Aa1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry08Aa1	Scarabaeidae	<i>Popillia japonica</i>
Cry08Bc1	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Cry08Bc1	Chrysomelidae	<i>Diabrotica virgifera virgifera</i>
Cry08Bc1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cry08Ca1	Scarabaeidae	<i>Anomala cuprea</i>
Cry08Ca2	Scarabaeidae	<i>Anomala corpulenta</i>
Cry08Ca2	Scarabaeidae	<i>Anomala exoleta</i>
Cry08Ca2	Scarabaeidae	<i>Holotrichia oblita</i>
Cry08Ca2	Scarabaeidae	<i>Holotrichia parallela</i>
Cry08Da1	Scarabaeidae	<i>Anomala cuprea</i>
Cry08Da1	Scarabaeidae	<i>Anomala orientalis</i>
Cry08Da1	Scarabaeidae	<i>Popillia japonica</i>
Cry08Ea1	Scarabaeidae	<i>Anomala corpulenta</i>
Cry08Ea1	Scarabaeidae	<i>Holotrichia oblita</i>
Cry08Ea1	Scarabaeidae	<i>Holotrichia parallela</i>
Cry08Fa1	Scarabaeidae	<i>Holotrichia parallela</i>
Cry08Ga1	Scarabaeidae	<i>Anomala corpulenta</i>
Cry08Ga1	Scarabaeidae	<i>Holotrichia oblita</i>
Cry08Ga1	Scarabaeidae	<i>Holotrichia parallela</i>
Cry18Aa1	Melolonthidae	<i>Melolontha melolontha</i>
Cry22Aa2	Carculionidae	<i>Anthonomas grandis</i>
Cry22Ba1	Carculionidae	<i>Anthonomas grandis</i>
Cry23Aa1	Scarabaeidae	<i>Popillia japonica</i>
Cry23Aa1	Tenebrionidae	<i>Tribolium castaneum</i>
Cry34Aa2 + Cry35Aa2*	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Cry34Ac2 + Cry35Ab2*	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Cry34Ba1 + Cry35Ba1*	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Cry36Aa1	Chrysomelidae	<i>Diabrotica virgifera virgifera</i>
Cry37Aa1	Scarabaeidae	<i>Popillia japonica</i>
Cry37Aa1	Tenebrionidae	<i>Tribolium castaneum</i>
Cry43Aa1	Scarabaeidae	<i>Anomala cuprea</i>
Cry43Ba1	Scarabaeidae	<i>Anomala cuprea</i>

### 1.2.1.7. Cyt toxins mode of action

The toxic effects of Cyt toxins are different to those of Cry toxins. Cyt toxins initially bind to unsaturated phospholipids found on the midgut epithelial cells of the insect as monomers. With time, aggregates of the Cyt toxins are formed on the epithelial cells (Gill *et al.*, 1992). The aggregates may reach a size of approximately 300-400 kDa. These toxic aggregates on the epithelial cell membrane result in the formation of pores that lead to cytolysis (Gill *et al.*, 1992).

Some Cry and Cyt toxins have synergistic relationships. The Cry 4 toxins are synergized by Cyt 1 toxins, and some Cry toxins are synergized by other Cry toxins. A good example of synergism is that of the toxin Cry 4B that requires the presence of Cry 4C to be toxic to *Culex quinquefasciatus* Say (Diptera: Culicidae) (Gill *et al.*, 1992). It has been speculated that the mechanism for these synergistic reactions is due to the hydrophobic reactions between toxins and the cell walls of midgut epithelial cells (Gill *et al.*, 1992).

**Table 1.2.** Cyt toxins effective against Coleoptera (Van Frankenhuyzen and Nystrom, 2002)

Cyt toxins	Family	Genus and species
Cyt01Aa4	Chrysomelidae	<i>Chrysomela scripta</i>
Cyt02Ca1	Chrysomelidae	<i>Diabrotica undecimpunctata howardi</i>
Cyt02Ca1	Chrysomelidae	<i>Diabrotica virgifera virgifera</i>
Cyt02Ca1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>
Cyt02Ca1	Scarabaeidae	<i>Popillia japonica</i>
Cyt02Ca1	Tenebrionidae	<i>Tribolium castaneum</i>

### 1.2.1.8. Minor toxins of *Bacillus thuringiensis*

Numerous other endotoxins and exotoxins are synthesized by *B. thuringiensis*, include alpha-exotoxins, beta-exotoxins and vegetative insecticidal proteins (VIPs) (Lambert and Peferoen, 1992; Khetan, 2001). These toxins are synthesized during the vegetative phase of the cell cycle of *B. thuringiensis*.

Alpha-exotoxins are heat labile and are not as specific in their toxicity as delta-endotoxins (ICPs). They are a family of toxins estimated to be 60-570 kDa in size. Only a few *B. thuringiensis* subspecies synthesize these toxins, namely *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *kenyae*, *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *thuringiensis* (Khetan, 2001).

Beta-exotoxins are low molecular weight, heat stable compounds synthesized by *B. thuringiensis* cells. These toxins are non-specific and have a wide toxicity range that includes vertebrates and invertebrates. Beta-toxins interfere with the synthesis of the host's DNA (Lambert and Peferoen, 1992). This is due to the toxin competing with adenosine tri-phosphate (ATP) for binding sites and thus inhibiting RNA biosynthesis (Khetan, 2001).

Vegetative insecticidal proteins (Vip) are toxins with a broad spectrum of activity against insects (Yu *et al.*, 1997). These toxins are comprised of unique proteins that share no homology with any delta-endotoxin proteins identified thus far (Estruch *et al.*, 1996). A similar nomenclature system to Cry and Cyt toxins has been suggested for Vips (Crickmore *et al.*, 1998).

**Table 1.3.** Vip toxins effective against Coleoptera (Van Frankenhuyzen and Nystrom, 2002)

Vip toxins	Family	Genus and species
Vip01Aa1 + Vip02Aa1	Chrysomelidae	<i>Diabrotica longicornis barberi</i>
Vip01Aa1 + Vip02Aa1	Chrysomelidae	<i>Daibrotica undecimpunctata howardi</i>
Vip01Aa1 + Vip02Aa1	Chrysomelidae	<i>Diabrotica virgifera virgifera</i>
Vip01Aa1 + Vip02Aa1	Chrysomelidae	<i>Leptinotarsa decemlineata</i>

Zwittermicins and chitinases are also synthesized by *B. thuringiensis*. These compounds synergistically increase the toxicity of ICPs toxins and thus increase the virulence of *B. thuringiensis* strains (Liu *et al.*, 2002).

#### 1.2.1.9. Insect resistance to *Bacillus thuringiensis* toxins

Insect resistance against *B. thuringiensis* is based on one of three different biochemical mechanisms. The first mechanism involves the specific binding sites on the insect's midgut

membrane onto which the *B. thuringiensis* toxins bind. The three dimensional shape of receptor sites may change due to a mutation in the genes coding for these receptor sites. As a result *B. thuringiensis* toxins will be unable to bind to these sites in the brush border membrane in the midgut (Ferré and Van Rie, 1992). This has been the primary mechanism for resistance development (Ferré and Van Rie, 1992; Tabashnik, 1994). The second mechanism involves changes in insect enzymes in the mid-gut. In resistant mutants, enzymes involved in the cleavage of ICP pro-toxins into the actual toxins may either be absent, or cleave the ICP proteins too fast, or too slowly. The end result is an ineffective ICP. A third mechanism involves the midgut's ability to repair itself rapidly. The epithelial midgut cells that are damaged by the toxin are rapidly replaced by new cells, healing the damaged midgut. This phenomenon occurs in late instars (Ferré and Van Rie, 1992).

Until 1989 no resistance in the field had been reported (Höfte and Whiteley, 1989). However, by 1994, field resistance of diamondback moth, *Plutella xylostella* L., (Lepidoptera: Noctuidae) to *B. thuringiensis* had been reported in the Hawaii, Japan Malaysia, Philippines, Thailand, and USA (Estada and Ferré, 1994; Tabashnik, 1994). Field resistance has subsequently been reported for the cotton bollworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), resistant to Bt cotton (Cry1Ac) (Luttrell, 2004), maize stalk borer, *Busseola fusca* Fuller (Lepidoptera: Noctuidae) to be resistant to Bt corn (Cry1Ab) in South Africa (Van Rensburg, 2007) and the fall army worm, *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) to be resistant to Bt corn (Cry1F) in Puerto Rico (Matten *et al.*, 2008). Resistance by Colorado potato beetle, *L. decemlineata*, to the Cry3A toxin was the first coleopteran specific resistance reported (Whaldon *et al.*, 1993). Until then resistance had mainly been created artificially in laboratory environments, where it was induced in Lepidoptera, Diptera and Coleoptera (Tabashnik, 1994)

Various strategies have been proposed to prevent and stop resistance in the field. These strategies include rotation, multiple toxins, ultra high dose exposures and refuge strategies (Tabashnik, 1994). Rotation is a strategy where pest insects are exposed to more than one toxin on an annual rotation basis. In this way the insects are not exposed too long to a particular toxin and thus chances of resistance development decreased (Tabashnik, 1994). The use of multiple toxins to expose insects to more than one toxin at a time should reduce the possibility of

resistance developing. The chances of organisms developing resistant genes to more than one toxin at a time are much less than development of resistance to a single toxin (Tabashnik, 1994). Use of ultra high doses may wipe out entire insect colonies immediately, reducing their chances of procreation and hence the development of resistant mutants (Tabashnik, 1994). The refuge approach involves having treated fields adjacent to non-treated fields. The concept is that insects will feed on a combination of toxin-treated crops and toxin-free crops. As such, insects will not be exposed long enough to a single toxin to develop resistance to that particular toxin. The chances of producing resistant progeny may also be less. Furthermore, insects that have developed resistance to a specific toxin, by feeding on treated crops, will mate also with insects that have not developed resistance due to feeding on non-treated crops. Pesticide resistance is usually a recessive trait and thus will not be expressed in the progeny (Ferré and Van Rie, 1992; Tabashnik, 1994).

#### **1.2.1.10. *Bacillus thuringiensis* against Coleoptera**

Strains of *B. thuringiensis* effective against Coleoptera include: *B. thuringiensis* subsp. *san diego* (Herrnstadt *et al.*, 1986), *B. thuringiensis* subsp. *tenebrionis* (Berliner, 1911) and *B. thuringiensis* subsp. *morrisoni* (Krieg *et al.*, 1983).

Most of the research on coleopteran specific *B. thuringiensis* strains is on the control of the Colorado potato beetle (Donovan *et al.*, 1988; Ferro and Gelernter, 1989; Nault, 2001; Whaldon *et al.*, 1993; Zehnder and Gelernter, 1989). This is because the Colorado potato beetle is an important potato pest, especially in the Northern Hemisphere, and because it has the ability to develop resistance to pesticides (Hare, 1990). In contrast, little research has been done on the use of *B. thuringiensis* strains to control white grubs.

*Bacillus thuringiensis* subsp. *japonensis* has been found to be effective against the white grub species *Anomala cuprea* Hope (Coleoptera: Dynastidae), *A. rofocuprea* Motschusky (Coleoptera: Dynastidae) and *Popillia japonica* Newman (Coleoptera: Scarabaeidae) (Ohba *et al.*, 1992). The only *B. thuringiensis* product on the market for Coleoptera to date is Novodor® from Valent Biosciences ([www.valentbiosciences.com](http://www.valentbiosciences.com)), which is registered for use against the Colorado potato beetle and the elm leaf beetle (Anonymous, 2009a). This product is not registered in



South Africa (SA). This leaves much scope for the isolation of *B. thuringiensis* strains toxic to white grubs and other economically important Coleoptera in SA.

### **1.2.2. *Brevibacillus laterosporus***

*Brevibacillus laterosporus* Laubach, formerly known as *Bacillus laterosporus*, was first isolated from water but its insecticidal properties were not known at the time (Laubach, 1916). This species is an aerobic spore-forming bacterium. It has a characteristic spore shape that separates it from other spore-forming bacteria. The sporulating cells are “canoe shaped” due to the lateral position of the spore within the bacterial cell (Favret and Yousten, 1985).

The entomopathogenic characteristic of this species was discovered when it was isolated from bees (Hymenoptera: Apidae) (McCray, 1917). It has since been discovered that most strains of *B. laterosporus* are saprophytic on dead bee larvae (Bailey, 1981). However, other strains of *B. laterosporus* have been discovered to have entomopathogenic properties against *Culex quinquefasciatus* Say larvae (Diptera: Culicidae), tobacco budworm, *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), black fly, *Simulium vittatum* L. (Diptera: Simuliidae) and the cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) (Favret and Yousten, 1985).

Initial investigations by Favret and Yousten (1985) found that the *B. laterosporus* strains they tested to be less effective than *B. thuringiensis* strains against *S. vittatum*, *H. virescens*, *T. ni* and *C. quinquefasciatus*. No pathogenicity of *B. laterosporus* was observed against *T. ni* compared to the 100% mortality of *B. thuringiensis* (Favret and Yousten, 1985). Furthermore *B. laterosporus* caused less than 20% mortality of *S. vittatum*, *H. virescens*, and *C. quinquefasciatus* whereas *B. thuringiensis* caused much greater mortalities (Favret and Yousten, 1985). This result suggested that *B. laterosporus* had limited potential as a biocontrol agent when compared with *B. thuringiensis* (Favret and Yousten, 1985). This may be a reason why the insecticidal activity of *B. laterosporus* strains have not been studied as extensively as those of *Bacillus thuringiensis* and *B. sphaericus* strains. However, strains of *B. laterosporus* have so far been found to have toxicity against the mollusc *Dreissena polymorpha* Pallas, the Colorado potato beetle, *L. decemlineata* Walker, and the house fly, *Musca domestica* L. (De Oliveira *et al.*, 2004; Ruiu *et al.*, 2006). Not all *B. laterosporus* strains produce crystal proteins, but Smimova *et al.* (1996) isolated two strains

of *B. laterosporus* capable of producing crystal proteins. Orlova *et al.* (1998) demonstrated that these crystal proteins are highly toxic to mosquito larvae and can be compared to toxicity levels of *B. thuringiensis* and *B. sphaericus*. However, the toxins of *B. laterosporus* are not as diverse as the toxins of *B. thuringiensis* (Zahner *et al.*, 1999).

### 1.3. White grub pests of sugarcane

White grubs are the main soil dwelling insects that cause damage to sugarcane, *Saccharum officinarum* L. (Poales: Poaceae) (Leslie, 2004). White grubs are a collective term used for species of Coleoptera that comprise of five families: Melolonthidae, Scarabaeidae, Dynastidae, Rutlidae and Cerambyidae (Leslie, 2004). In Africa white grub species belong to three of these families: Melolonthidae, Dynastidae and Rutelidae (Leslie, 2004) (Table 1.4).

**Table 1.4.** Distribution of white grub species in Africa (Conlong and Mugalala, 2003; Rajabalee, 1990)

<b>Family</b>	<b>Genus</b>	<b>Country</b>
Melolonthidae	<i>Astenopholis</i>	South Africa
	<i>Cochliotis</i>	Tanzania
	<i>Ealophida</i>	Burkino Faso
	<i>Holochelus</i>	Réunion
	<i>Hypopholis</i>	South Africa
	<i>Idaecamenta</i>	Uganda
	<i>Phyllophaga</i>	Mauritius
	<i>Schizonycha</i>	South Africa
Dynastidae	<i>Alissonotum</i>	Mauritius
	<i>Heteronychus</i>	Africa (throughout)
Rutelidae	<i>Anomala</i>	South Africa
	<i>Adoretus</i>	South Africa

White grubs are so called due to their white to grey-white abdomens (Leslie, 2004; Wilson, 1969). Their abdomens are fleshy, broad and curved into the shape of a C (Imms, 1957). They have strongly sclerotized heads which are brown, red or yellow in colour (Imms, 1957). Their legs are well developed but are rarely used for locomotion (Imms, 1957).

### 1.3.1. Life cycle of white grubs

The life cycle of white grubs is very similar for most species, with one generation per year (Leslie, 2004; Wilson, 1969). Some species like *Lepidiota frenchi* Blackburn (Coleoptera: Scarabaeidae) have a life cycle that stretches over two years (Illingworth and Dodd, 1921).

Under conditions with favorable temperatures and rainfall, adult beetles emerge at dusk from the soil during the summer months (Leslie, 2004; Wilson, 1969). Adults mate and roost in the surrounding vegetation. Some species shelter in the moist soil during the day (Leslie, 2004). Most species do not forage much during this stage except for some species such the adults of *Dermolepida albohirtum* Waterhouse (Coleoptera: Melolonthidae). This species feeds on trees in the surrounding areas after emerging from the soil (Illingworth and Dodd, 1921).

Eggs are deposited by fertilized females in the soil surrounding sugarcane plants or on the base of sugarcane stools (Leslie, 2004). White grubs have three instar stages and it is the third instar stage that causes the most damage because it feeds on the roots of sugarcane (Leslie, 2004; Wilson, 1969). The first and second instars mainly feed on organic matter in the soil surrounding the plants (Leslie, 2004; Wilson, 1969). The top 300 mm of soil around and under the sugarcane stools have the highest density of third instar white grubs (Leslie, 2004; Wilson, 1969). The white grubs move deeper into the soil once they are fully developed and ready to pupate (Leslie, 2004; Wilson, 1969). In the one year species pupation takes place seven months after eclosion (Leslie, 2004). In the two year species the pupation takes place in the second year (Leslie, 2004).

When white grubs feed on the roots of sugarcane, damage can vary from mild to severe loss of roots (Jepson, 1956; Wolcott, 1936). As little as two white grubs per stool of the larger species of white grubs, such as *Antitrogus consanguineas* Blackburn (Coleoptera: Scarabaeidae), can cause serious damage to a sugarcane plant. Smaller species, e.g., *Schizonycha affinis* Boheman (Coleoptera: Melolonthidae), only cause limited damage to sugarcane, even in large numbers (Leslie, 2004).

### 1.3.2. Control of white grubs

Various agricultural practices have been suggested from the control of white grubs, such as deep ploughing when white grubs are most abundant in the top layers of soil (Conlong and Mugalala, 2003; Rajabalee, 1990). This method causes mechanical injury that leads to the death of the white grubs. Another method is that of harvesting the sugarcane earlier, just before the third instars start feeding on the sugarcane roots (Ward and Cook, 1996). The third method involves the use of resistant sugarcane cultivars. Snow drop and wheat drop lectins that are produced by some sugarcane cultivars have been found to be insecticidal and growth inhibiting to some species of white grubs (Allsopp *et al.*, 1991).

A wide range of pesticides has been tested for the control white grubs (Leslie, 2004). These include Ethoprophos, Chlorpyrifos, Heptachlor, Endosulfan and Carbofuran (Leslie, 2004). However, for pesticides to be effective against white grubs, they need to penetrate the soil to get to the target white grubs. To date, no pesticide has been registered in SA against white grub.

Research has been done on the use of entomopathogenic fungi against white grubs. *Metarhizium anisopliae* Metschnikoff has proven to be effective against white grubs when a suspension of conidia is injected into the soil (Allsopp *et al.*, 1994). However, this requires large quantities of the conidia. Furthermore the fungus takes a long time to kill the white grubs (Allsopp *et al.*, 1994). In Réunion *Beauveria brongniartii* (Saccardo) Petch has been used against a *Hoplochelus* sp. (Coleoptera: Melolonthidae) (Rajabalee, 1990). Another species of *Beauveria*, *B. bassiana* (Balsamo) Vuillemin, has been found to be effective against white grub species *Antitrogus dimidiata* Hope (Coleoptera: Scarabaeidae) and *Phyllognathus dionysis* Fabricius (Coleoptera: Melolonthidae) (Dhoj *et al.*, 2008).

A *Bacillus cereus* strain WGPSB-2 (MTCC 7182) has been found to be effective against *A. dimidiata* and *Holotrichia seticollis* Moser and it was isolated from *A. dimidiata* (Selvakumar *et al.*, 2007). However, no isolates of *B. thuringiensis* have thus far been tested against white grubs of sugarcane.

This demonstrates the potential use of *B. thuringiensis* isolates to control white grubs specific to sugarcane. Toxins of *B. thuringiensis* subsp. *tenebrionis*, a coleopteran specific *B. thuringiensis* strain, have been found to bind irreversibly to soil particles. This protects the toxins from decomposing (Muchaonyerwa *et al.*, 2006). This would be a positive attribute because white grubs are soil dwelling, and the S1 and S2 stages feed on soil organic matter, so the *B. thuringiensis* toxins could be consumed by the white grubs.

The aim of this thesis was to isolate entomopathogenic endospore-forming bacteria with toxicity towards Coleoptera and to determine the toxicity of these isolates against species of white grubs of sugarcane.

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## Chapter Two

# Isolation of three species of entomopathogenic spore-forming bacteria effective against *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)

### Abstract

Fourteen entomopathogenic spore-forming bacteria were isolated from Coleoptera and insect rich environments in the KwaZulu-Natal region of South Africa. Isolates were isolated from diseased *Tenebrio molitor* larvae (yellow mealworm), adult Coleoptera, white grubs, mushroom compost, garden compost and dust from a grain storage facility. This was done using a pasteurization spore isolation treatment of the samples. The isolations were identified using microscopic evaluations, biochemical tests and serotyping. Isolates were identified as belonging to three bacterial species, namely, *Bacillus thuringiensis*, *Brevibacillus laterosporus* and *Bacillus cereus*. Bioassays were conducted against *T. molitor* larvae using cabbage disc dipped in whole cell suspensions of the bacterial isolates. Only five isolates from the three bacterial species displayed toxicity towards the *T. molitor* larvae. *Brevibacillus laterosporus* and two of the *B. thuringiensis* strains had the highest mortality against *T. molitor*.

### 2.1. Introduction

Entomopathogenic bacteria include both Gram-positive and Gram-negative bacteria (Prescott *et al.*, 1999). Gram-positive endospore-forming bacteria are sought after as commercial bio-pesticides due to the spores that these bacteria produce. These spores are able to survive for prolonged periods of time under adverse conditions such as heat, drought and ultraviolet radiation and are synthesized within the cells and are released when conditions become unfavourable (Nakayama *et al.*, 1996; Popham *et al.*, 1995; Setlow, 2006 and Vreeland *et al.*, 2000). Formulations of these spore-forming bio-pesticides thus have longer shelf lives compared to non-spore-forming bacteria (Anonymous 1, 2007).

Entomopathogenic spore-forming bacteria that are effective against coleopterans include: *Bacillus cereus* Frankland and Frankland, *B. circulans* Jordan, *B. sphaericus* Meyer and Neide, *B. thuringiensis* Berliner, *Brevibacillus laterosporus* Laubach, *Paenibacillus polymyxa* Prazmowski, *P. lentimorbus* Dutky and *P. popilliae* Dutky (Baumann *et al.*, 1991; Demir *et al.*, 2002; Orlova *et al.*, 1998; Selvakumar *et al.*, 2007; Sharpe *et al.*, 1970). *Bacillus thuringiensis*, *B. laterosporus*, *B. sphaericus* and *P. popilliae* are able to synthesize parasporal crystal bodies known as Insecticidal Crystal Proteins (ICPs) (Baumann *et al.*, 1991; Orlova *et al.*, 1998; Schnepf *et al.*, 1998; Sharpe *et al.*, 1970; Thiery and Frachon, 1997). All strains of *B. thuringiensis*, *P. lentimorbus* and *P. popilliae* produce ICPs whereas only some strains of *B. laterosporus* and *B. sphaericus* are able to synthesize ICPs (Baumann *et al.*, 1984; Dutky 1940; Orlova *et al.*, 1998; Yousten, 1984).

*Bacillus cereus*, *B. laterosporus* and *B. thuringiensis* were considered prime species to isolate and screen for toxicity against *T. molitor* in this research. Various strains of *B. thuringiensis* with toxicity towards Coleoptera have been isolated, i.e., *B. thuringiensis* subsp. *san diego* (Herrnstadt *et al.*, 1986), *B. thuringiensis* subsp. *tenebrionis* (Berliner, 1911), *B. thuringiensis* subsp. *morrisoni* (Krieg *et al.*, 1983) and *B. thuringiensis* subsp. *japonensis* (Ohba *et al.*, 1992). *Bacillus thuringiensis* has also been isolated from various sources such as: soil, plants, diseased insect larvae, aquatic environments, animal faeces, insect rich environments, flour mills and grain storage facilities (Chaufaux *et al.*, 1997; Khetan, 2001; Lambert and Peferoen, 1992; Porcar and Caballero, 2000; Smith and Couche, 1991). This demonstrates the ubiquitous nature of this species.

The insecticidal properties of *B. laterosporus* have not been explored as extensively as *B. thuringiensis*. It is only recently that the insecticidal properties of this species have been discovered, including its effectiveness against Coleoptera (Boets *et al.*, 2004; Orlova *et al.*, 1998; Schnepf *et al.*, 2003; Smimova *et al.*, 1996). *Bacillus cereus* produces toxins that affect insects as well as animals and is an opportunistic human pathogen. This is why the insecticidal properties of this species have not been explored as extensively as *B. thuringiensis* (Selvakumar *et al.*, 2007). However, this species was considered a good isolate to screen for due to its relatively unexplored insecticidal properties.



The aim of the in this chapter was to isolate *Bacillus* and other spore-forming bacteria with potential insecticidal activity against Coleoptera, with *T. molitor* L. (mealworm) *Tenebrio molitor* was chosen on the basis that it is easily obtainable and simple to rear.

## 2.2. Materials and Methods

Standard microbiological techniques were followed in the laboratory for the routine microbiological assays that include sterilization, aseptic techniques and culture preparation (Prescott *et al.*, 1999; Wheelis and Segel, 1979).

**2.2.1. Samples:** Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* sp. collected in sugarcane areas in KwaZulu-Natal (KZN), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 2.1).

**2.2.2. Sample collection:** Mushroom compost, compost and grain dust were collected in 250 ml sterile honey jars from the various sources (Table 2.1). Honey jars were pre-sterilized in an autoclave at 121°C for 15 min. Diseased *T. molitor* larvae were collected in 90 mm sterile Petri dish. Adult beetles were collected from light traps in sugarcane areas in the KwaZulu-Natal midlands by employees of The South African Sugar Research Institute (SASRI, Mt. Edgcombe, KZN) and were delivered in brown paper bags. White grubs were collected from sugarcane areas by staff members of SASRI and were delivered in plastic containers filled with soil. Diseased grubs were removed from these containers and placed into sterile Petri dishes. All samples were stored in a fridge at 4°C.

**Table 2.1.** Samples sources from which entomopathogenic endospore-formers were isolated

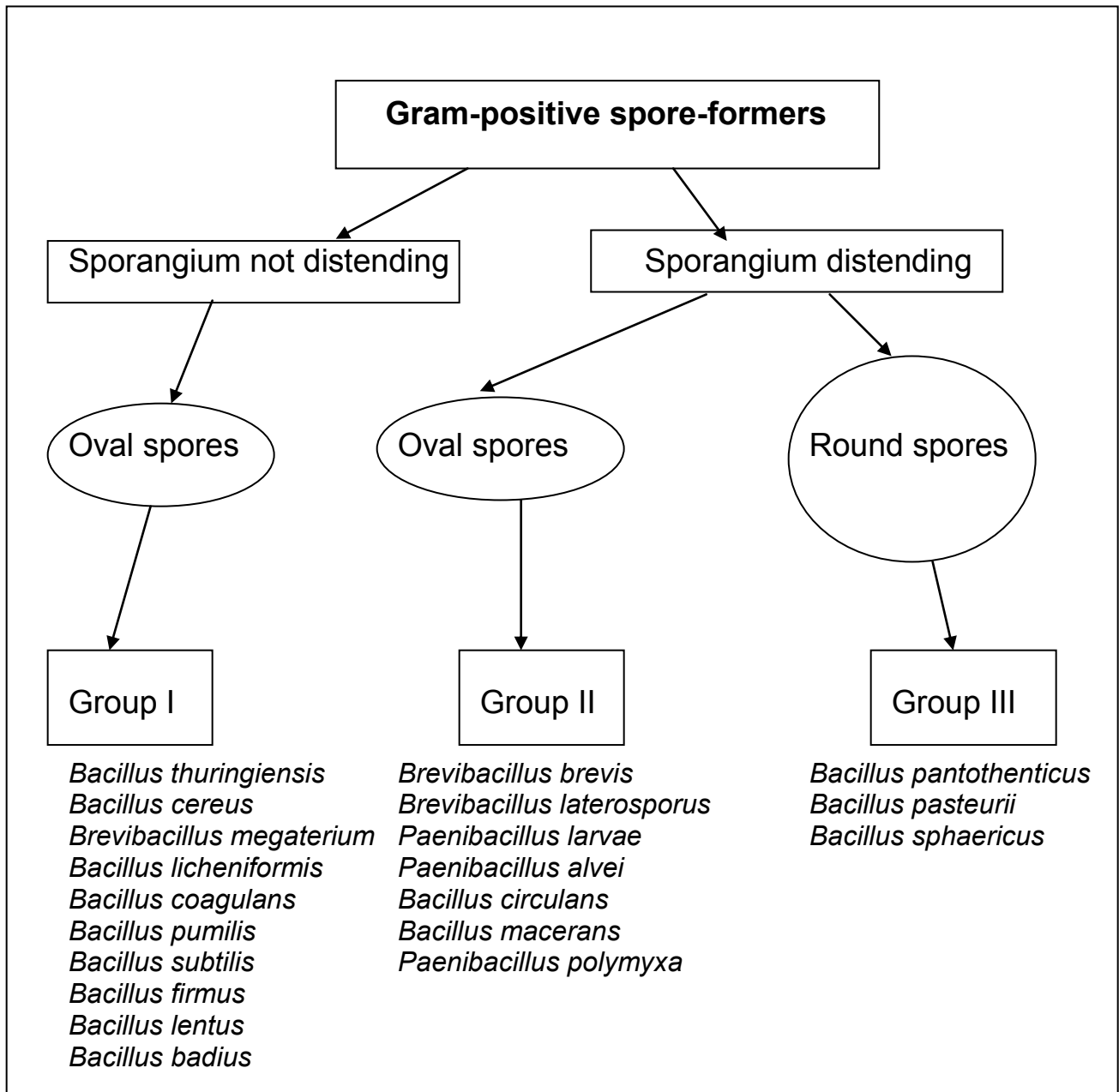
<b>Samples</b>	<b>Source of sample</b>	<b>Species</b>
Mushroom compost	Mushroom farm in Karkloof area in the KwaZulu-Natal midlands	-
Mealworm larvae	Insect rearing facility at the University of KwaZulu-Natal	<i>Tenebrio molitor</i>
Compost	A garden compost heap in Hillcrest, KwaZulu-Natal	-
Grain dust	Chicken grain storage facility in Pietermaritzburg, KwaZulu-Natal	-
Adult beetles	Dalton, Bryuns-Hill and Harburg, KwaZulu-Natal	<i>Schizonycha</i> spp. and <i>Hypopholis</i> spp.
White grubs	Dalton, KwaZulu-Natal	<i>Schizonycha</i> spp.

**2.2.3. Isolation of endospore-forming bacteria:** Isolation of *Bacillus* spp. was conducted using a similar pasteurization method, as described by Ohba and Aizawa (1978). Approximately 0.5 g of each sample was suspended in 10 ml sterile distilled water (Table 2.1). Suspensions were shaken vigorously for 30 sec at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 hr at room temperature. Suspensions were then vortexed a second time at full speed for 30 sec and were then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 hr at 30°C in an incubator and examined for colonies with typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or ‘colonies with an ‘ice crystal’ appearance with a diameter larger than 2 mm (Damgaard *et al.*, 1997; Prescott *et al.*, 1999; Selvakumar *et al.*, 2007). Other white coloured bacterial colonies that were predominate and larger than 2 mm in diameter were selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and counted using a colony counter. Selected colonies were then purified by sub-culturing onto nutrient agar plates and

incubated for 3 d at 30°C in an incubator. Colonies were stored on nutrient agar slants. Not all colonies from each sample that fitted the description above were selected because of the large number of colonies initially isolated.

Selected isolates were subjected to Gram staining as well as Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 sec with distilled water and blotted dry with tissue paper (Ammons *et al.*, 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 x oil immersion objective lens (Bernhard *et al.*, 1997; Young *et al.*, 1998). Crystal proteins stained as dark blue structures (Ammons *et al.*, 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9 µm, were also selected. This was done in order to include *B. cereus*, which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because this fits the criteria of *B. cereus*, *B. thuringiensis* and *B. laterosporus* cells (Figure 2.1). Selected cultures were purified by sub-culturing and were assigned numbers NDR1-NDR14.



**Figure 2.1.** Key for identification of major groups of Gram-positive spore-formers with entomopathogenic properties based on cell morphology (Thiery and Frachon, 1997).

**2.2.4. Biochemical tests:** The tests described in the Manual of Techniques in Insect Pathology (Thiery and Frachon, 1997) and Bergey's Manual of Systematic Bacteriology 1 and 2 (Claus and Berkley, 1986) were used to aid in the identification of the isolates. The main tests were anaerobic growth, catalase, mannitol utilization, Gram reaction and the Voges-Proskauer Reaction (Thiery and Frachon, 1997). These tests, together with microscopic evaluations, aided the identification of the bacterial isolates (Figures 2.2-2.7). Biochemical and cell morphology results of the six most effective isolates in the bioassays against *T. molitor* larvae were recorded (Tables 2.3-2.5).

**2.2.5. *Bacillus thuringiensis* serotyping:** *Bacillus thuringiensis* isolates were sent to Dr Michio Ohba (Graduate School of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka, B12-8581) in Japan for serotyping. A standard method similar to that described by Thiery and Frachon, (1997) was used.

**2.2.6. Bioassays:** Bioassays were conducted to determine the toxicity of the bacterial isolates against *T. molitor*. Nutrient broth (150 ml) (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) was added to a 250 ml glass conical flask stopped with cotton wool and sterilized in an autoclave at 121°C for 15 min. An inoculating loop full of bacterial isolate was used to inoculate the nutrient broth using aseptic techniques. The inoculated nutrient broth was incubated in a shaker water bath at 30°C 250 rpm for 5 d (Meadows *et al.*, 1992). Discs of 18 mm diameter were cut from freshly purchased cabbage leaves with pre-sterilized test tube caps sterilized in an autoclave at 121°C for 15 min. The cabbage discs were dipped into the bacterial suspensions and left to dry for 5 min at room temperature in a 90 mm pre-sterilized Petri dish. Four *T. molitor* larvae were placed in a pre-sterilized Petri dish with three dipped cabbage discs. Five Petri dishes with four larvae were assessed as one replicate. Three replicates were conducted per bacterial isolate, i.e., 20 larvae were used on each in each replicate, and 60 larvae per isolate. The control consisted of cabbage discs dipped into sterile distilled water. Results were recorded after 5 d. Abbott's (1925) corrected formula was used to determine true mortality.

$$\text{Adjusted \% mortality} = \frac{(\text{observed \% mortality} - \text{mean control \% mortality})}{100 - \text{mean control \% mortality}}$$

## 2.3. Results

The total number of spore-forming bacteria from each sample was recorded. The majority of isolates with rapid growing white colonies were from *T. molitor* and *Schizonycha* spp. larvae (Table 2.2). The mushroom compost and the *Schizonycha* spp. larvae had the largest number of colonies per gram of sample (Table 2.2).

**Table 2.2.** Spore-forming bacteria isolated from various samples

Sources of isolates	Mean Number Colony Forming Units x 10 <sup>3</sup> per gram	White Colonies with Rapid Growth	Isolates of Bt <sup>a</sup>	Isolates of Bl <sup>a</sup>	Isolates of Bc <sup>a</sup>
Mushroom compost	250 to 300	2	2	0	0
Garden compost	100 to 150	2	0	0	2
<i>Tenebrio molitor</i> larvae	50 to 100	3	0	1	2
Coleoptera adult spp. <sup>b</sup>	50 to 100	2	0	1	1
<i>Schizonycha</i> spp. larvae	150 to 200	3	3	0	0
Grain dust from old chicken feed	0 to 50	2	0	0	2

<sup>a</sup> Bt, *Bacillus thuringiensis*; Bl, *Brevibacillus laterosporus*; Bc, *Bacillus cereus*.

<sup>b</sup> Adult beetles consisted of *Hypopholis* spp. and *Schizonycha* spp. (identified by the collectors, SASRI).

Gram stains revealed that all isolates were Gram-positive, endospore-forming bacteria, with oval spores (Table 2.3). Isolates with large cells and non-distending spores were considered to belong to the endospore-forming Group I (Table 2.3 and Figure 2.1). Four of the six isolates of Group I were identified as strains of *B. thuringiensis*, namely isolates NDR1, NDR3, NDR11 and NDR12

(Figures 2.2 – 2.7). One isolate, NDR5, did not produce inclusion bodies and was identified as a strain of *B. cereus* due to the similarity of its biochemical and morphological characteristics with the other isolates of this group, but with the lack of a ICP (Table 2.3 and Figure 2.5) (Carlson *et al.*, 1994). Only one isolate, NDR2, had lateral distending, oval spores and belonged to Group II. Lateral spore formation is characteristic of *B. laterosporus* (Figure 2.3) (De Oliveira *et al.*, 2004).

**Table 2.3.** Biochemical and morphological characteristics of the spore-forming bacterial isolates used to identify the isolates

Characteristics	Isolate Number					
	Bt <sup>a</sup> NDR1	Bl <sup>a</sup> NDR2	Bt <sup>a</sup> NDR3	Bc <sup>a</sup> NDR5	Bl <sup>a</sup> NDR11	Bt <sup>a</sup> NDR12
Gram reaction	+	+	+	+	+	+
Anaerobic	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
AMC <sup>b</sup>	+	+	+	+	+	+
D-Mannitol	-	+	-	-	-	-
Oval spores	+	+	+	+	+	+
Distending spore	-	+ a	-	-	-	-
Rod Width	>0.9µm	<1µm	>0.9µm	>0.9µm	>0.9µm	>0.9µm
Rod Length	>3µm	>3µm	>3µm	>3µm	>3µm	>3µm
Crystal protein	+	+	+	-	+	+

<sup>a</sup> Bt, *Bacillus thuringiensis*; Bl, *Brevibacillus laterosporus*; Bc, *Bacillus cereus*

<sup>b</sup> AMC, Acetylmethylcarbinol production; +, positive reaction or utilization; -, negative reaction or non-utilization.

All isolates that displayed insecticidal activity had crystalline inclusion bodies except for *B. cereus* Isolate NDR5 (Table 2.4 and Figure 2.5). The *B. laterosporus* isolate, NDR2, produced crystalline inclusion bodies that remained attached to the spore after complete sporulation (Table 2.4 and Figure 2.3). Two of the *B. thuringiensis* isolates, Isolate NDR3 and NDR11, had crystal proteins attached to the spore (Figures 2.4 and 2.6).

**Table 2.4.** Insecticidal crystal protein shapes of six spore-forming bacterial isolates with insecticidal activity against *Tenebrio molitor*

	<b>Species and Isolates</b>					
	Bt <sup>a</sup> NDR1	Bl <sup>a</sup> NDR2	Bt <sup>a</sup> NDR3	Bc <sup>a</sup> NDR5	Bt <sup>a</sup> NDR11	Bt <sup>a</sup> NDR12
<b>Crystal protein</b>						
<b>Shape (Figures 2.2-2.7)</b>	Bp <sup>b</sup>	Cs <sup>b</sup>	Tri <sup>b</sup>	-	S <sup>b</sup>	Bp <sup>b</sup>
<b>Dimensions (length x height)</b>	1.5 X 1.5µm	2.5 X 0.5µm	1.0 X 1.0µm	-	0.8 X 0.8µm	1.5 X 1.5µm
<b>Attachment to exosporium</b>	No	Yes	Yes	-	Yes	No

<sup>a</sup> Bt, *Bacillus thuringiensis*; Bl, *Brevibacillus laterosporus*; Bc, *Bacillus cereus*

<sup>b</sup> Bp, Bipyramidal; Cs, Canoe shaped; Tri, Triangular; S, Spherical

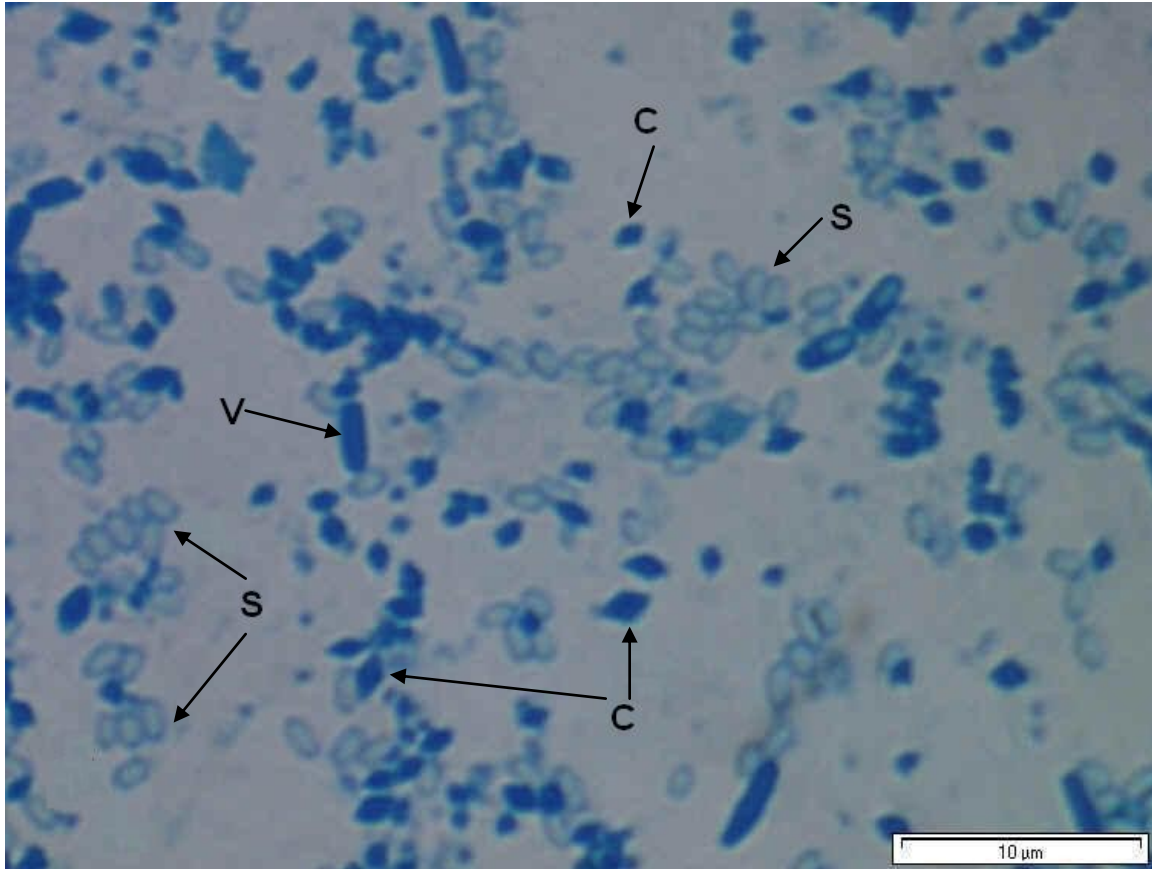
**Table 2.5.** Serotype determination of the locally isolated *Bacillus thuringiensis* isolates

<b>Isolate</b>	<b>Bacterial Species</b>	<b>Serotype</b>
NDR1	<i>Bacillus thuringiensis</i>	<i>kenyae</i>
NDR3	<i>Bacillus thuringiensis</i>	Non-motile
NDR11	<i>Bacillus thuringiensis</i>	Strong self agglutination
NDR12	<i>Bacillus thuringiensis</i>	<i>kenyae</i>

Serotyping is based on flagellar antigens and requires flagella to be present. Therefore non-motile strains cannot be serotyped (De Barjac and Frachon, 1990).



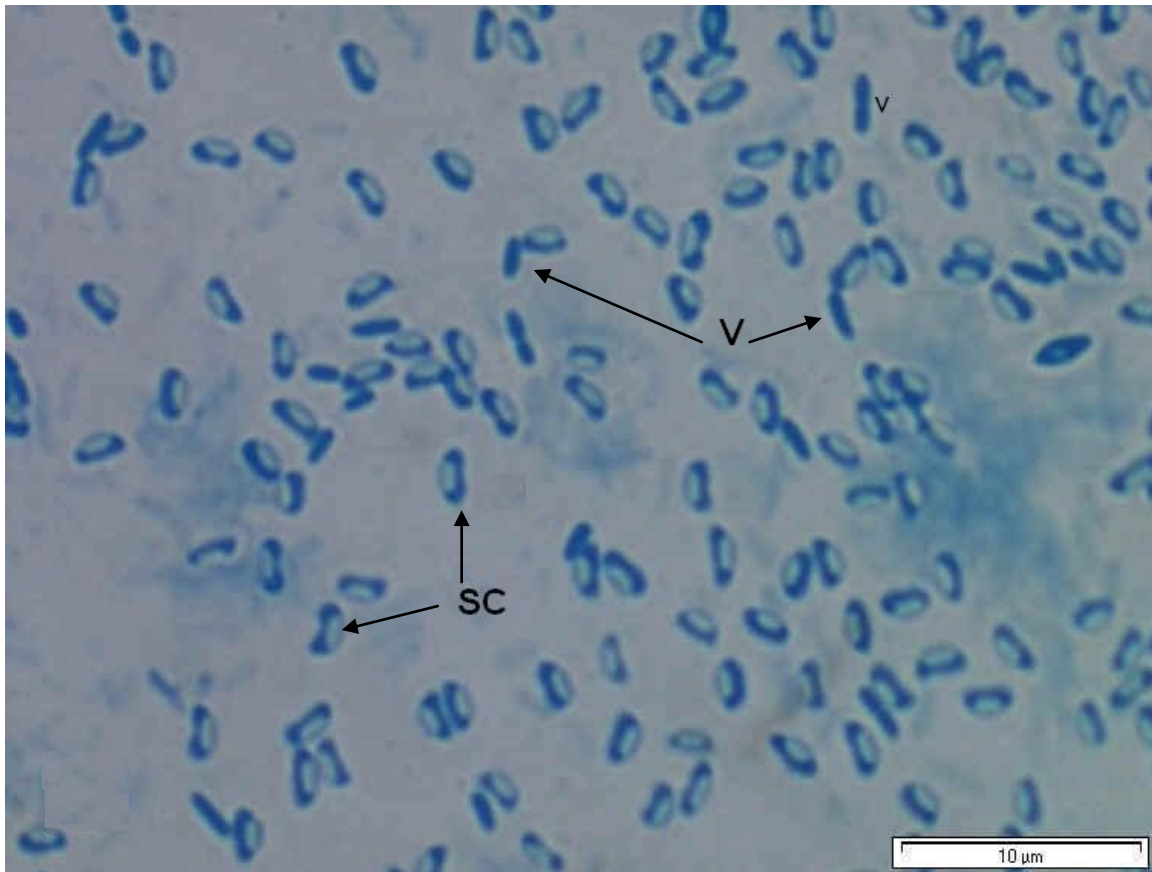
In NDR1 the bipyramidal shaped crystal proteins stained dark blue and were separate from the exosporium. The spores could be seen as transparent, oval structures (Figure 2.2).



**Figure 2.2.** Micrograph of NDR1 (*Bacillus thuringiensis*) stained with Coomassie Blue.

Note: C = Crystal protein, V = vegetative cell and S = Spore

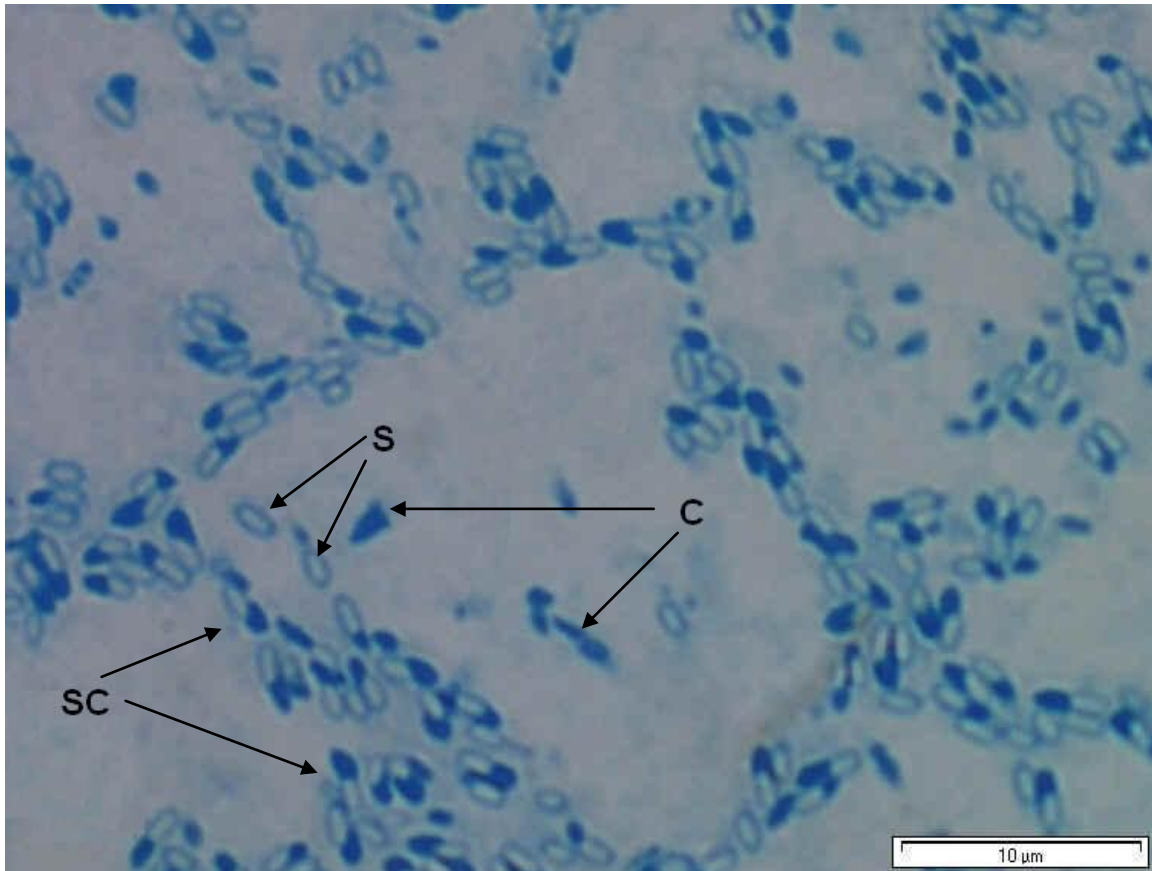
In NDR2 the crystals could be seen as dark blue, canoe shaped structures present on the side of the spore. The spores did not stain and were observed as transparent, oval structures (Figure 2.3.).



**Figure 2.3.** Micrograph of NDR2 (*Brevibacillus laterosporus*) stained with Coomassie Blue.

Note: V = vegetative cell and SC = Spore with crystal

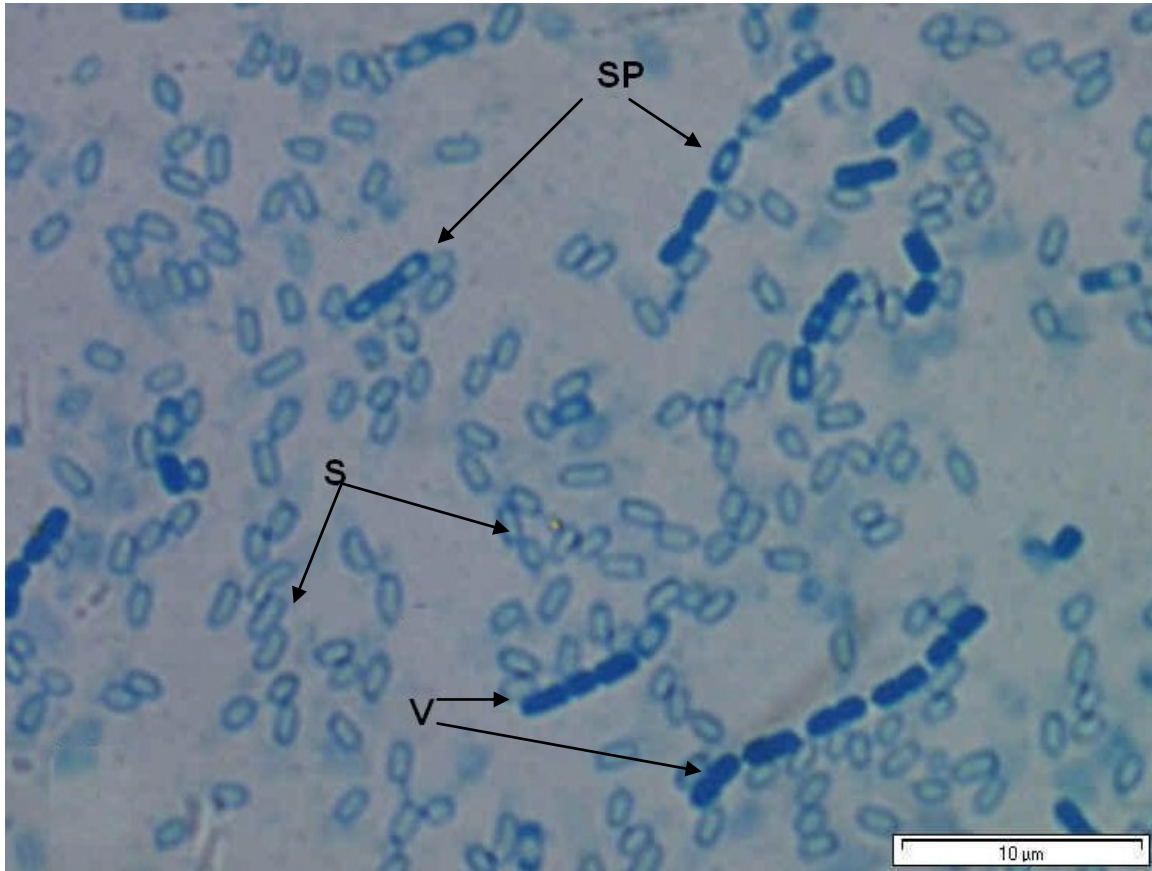
In NDR3 the triangular shaped crystal proteins were seen to remain attached to the exosporium. The ovals spores could be seen as transparent, oval structures (Figure 2.4).



**Figure 2.4.** Micrograph of NDR3 (*Bacillus thuringiensis*) stained with Coomassie Blue.

Note: C = Crystal protein, SC = spore with crystal and S = Spore

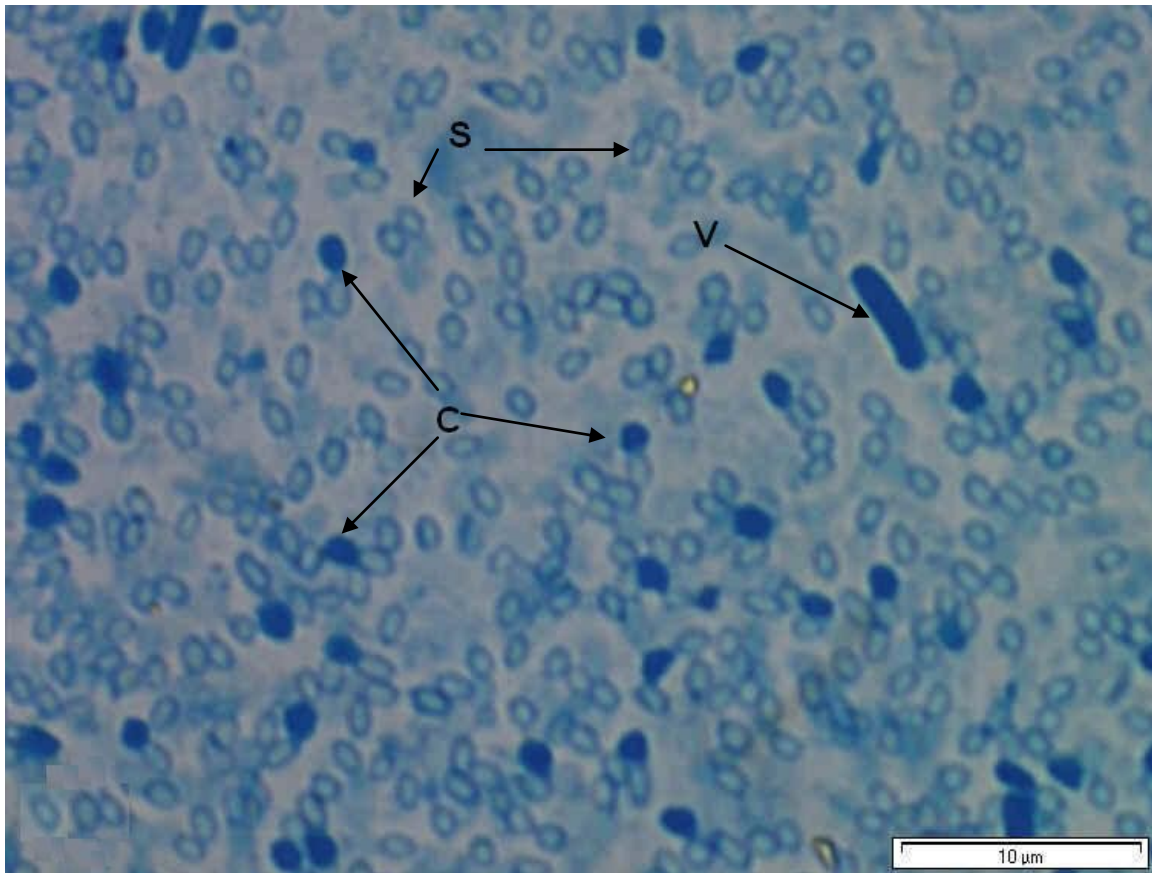
In NDR5 the spores were visible and no crystal proteins were present. Vegetative cells stained as dark blue rod shaped structures (Figure 2.5).



**Figure 2.5.** Micrograph of NDR5 (*Bacillus cereus*) stained with Coomassie Blue.

Note: V = vegetative cell, S = Spore and SP = spore within cell

In NDR11 oval shaped crystal proteins could be observed. Crystal formation was poor in this isolate. Some crystals remained attached to the exosporium (Figure 2.6).



**Figure 2.6.** Micrograph of NDR11 (*Bacillus. thuringiensis*) stained with Coomassie Blue.  
Note: C = Crystal protein, S = Spore and V = vegetative cell

In NDR12 bipyramidal crystal proteins were visible. The spores were visible as transparent, oval structures (Figure 2.7).



**Figure 2.7.** Micrograph of NDR12 (*Bacillus thuringiensis*) stained with Coomassie Blue.

Note: C = Crystal protein, V = vegetative cell and S = Spore

No mortality was observed in the control. Isolates with a medium and high levels of toxicity, NDR1, NDR2, NDR3, NDR5, NDR11 and NDR12 (Table 2.6), were retained for further screening as potential entomopathogens against coleopterans. All other bacterial isolates were discarded. Isolate NDR12 was also discarded because this isolate was identified as being the same isolate as NDR1. Biochemical results, morphological characteristics and serotyping indicated that these two isolates were the same isolate (Table 2.3-2.5 and Figures 2.2 and 2.7). The crystal proteins of Isolates NDR1 and NDR12 were similar in size, as were their shape and location within the sporulating cell (Figure 2.2 and 2.7). The crystals were bipyramidal or

diamond shaped. In the end, five isolates of three bacterial species were retained for further study, namely: *B. thuringiensis* Isolates NDR1, NDR3, NDR11, and NDR12, *B. laterosporus* Isolate NDR2 and *B. cereus* Isolate NDR5.

**Table 2.6.** Bioassays for toxicity determination of the various isolates *Tenebrio molitor* as the test organism

Isolate <sup>a</sup>	Source	Percent Mortality	Toxicity Rating <sup>b</sup>	SE
NDR1	<i>Schizonycha</i> sp. larva	55.0%	++	1.00
NDR2	<i>Tenebrio molitor</i> larva	98.3%	+++	0.33
NDR3	Mushroom compost	87.5%	+++	0.50
NDR4	Grain dust	3.3%	-	0.33
NDR5	Coleopteran adult	66.7%	++	1.33
NDR6	Grain dust	10.0%	-	0.58
NDR7	Coleopteran adult	17.3%	-	0.33
NDR8	Compost	7.5%	-	0.56
NDR9	Compost	5.0%	-	0.33
NDR10	<i>Tenebrio molitor</i> larva	15.0%	-	0.58
NDR11	<i>Schizonycha</i> sp. larva	81.7%	+++	0.88
NDR12	<i>Schizonycha</i> sp. larva	59.3%	++	0.33
NDR13	Mushroom compost	7.3%	-	0.88
NDR14	<i>Tenebrio molitor</i> larva	3.6%	-	0.00
Control	-	0.0%	-	0.00

<sup>a</sup> Isolates obtained from the various samples were labeled NDR1 – NDR14 as they were discovered. NDR, Isolate numbering for own purpose in the laboratory.

<sup>b</sup> -, no toxicity (<30% mortality); +, low toxicity (30-50% mortality); ++, medium toxicity (50-75% mortality); +++ +, high toxicity (>75% mortality)

## 2.4. Discussion

**2.4.1. Isolation:** The main aim was to isolate Gram-positive endospore-forming bacteria with entomopathogenic properties. The pasteurization technique proved to be successful for the isolation of spore-forming entomopathogenic bacteria, and a total of 14 spore-forming bacterial strains were isolated (Table 2.5). Crystal protein producing isolates were sought after because this is a characteristic of *B. thuringiensis* and some strains of *B. laterosporus* (De Lucca *et al.*,

1981). However, *B. cereus* does not produce crystal proteins but does have entomopathogenic properties and was thus sought after as well (Nishiwaki *et al.*, 2004). These isolates were used in bioassays because some strains of *B. cereus* have been reported to produce vegetative insecticidal proteins (VIPs) (Estruch *et al.*, 1996; Yu *et al.*, 1997). These VIPs are not visible as crystalline aggregates within the bacterial cell and need to be detected through protein molecular methods (Rang *et al.*, 2005). One *B. cereus* isolate was retained because it displayed relatively strong insecticidal activity towards *T. molitor*. *Bacillus cereus* is also often isolated together with *B. thuringiensis* because these two organisms share similar niches (Hendriksen and Hansen, 2002; Smimova *et al.*, 1996). Similar findings by Meadows *et al.* (1992) support this view. Out of the fourteen isolates, half were crystal-forming strains (Table 2.5). The ‘ice crystal’ or *B. cereus* type of colony morphology larger than 2 mm, as described by Damgaard *et al.* (1997), yielded in large rod shaped bacteria with oval spores (Figures 2.2-2.7). Crystal protein producing colonies with similar colony morphologies to *B. cereus* are usually isolates of *B. thuringiensis*. The seven isolates that did not produce crystal proteins had similar morphological characteristics to those of the crystal producing isolates, when grown on nutrient agar. Microscopically, all the isolates were similar until sporulation, when the crystal proteins become apparent in seven of the isolates (Figures 2.2-2.3).

**2.4.2. Microscopic evaluation:** The ICP protein crystals were visible in *B. thuringiensis* isolates (Figures 2.2, 2.4, 2.6 and 2.7) and the *B. laterosporus* isolate (Figure 2.3). The crystal proteins stained as dark blue structures, as described by Ammons *et al.*, (2002). Two isolates produced bipyramidal crystal proteins (Figure 2.2 and 2.7). Research by Bernard *et al.*, (1997) has shown that bipyramidal shaped crystals predominate during isolations, so it was not surprising that many isolates produced these shapes. Isolate NDR11 synthesized oval crystal proteins but not as abundantly as the other five isolates (Figure 2.6). This could be an indication that this isolate is losing its ability to produce crystals (due to sub-culturing); or that this isolate may have specific nutrient requirements to produce crystal proteins; or that this isolate is a poor crystal protein synthesizer. *Bacillus thuringiensis* subsp. *yannanensis* is a good example of a poor crystal producing sub-species. It produces both sporulating and non-sporulating cells and the non-sporulating cells produce protein crystals (Ohba *et al.*, 2003). NDR3 produced a very unusual or uncommon triangular crystal protein shape (Figure 2.4) (De Oliveira *et al.*, 2004). Triangular



shaped crystal proteins are not common but some are toxic to Coleopteran insects (Khetan, 2001). The canoe shaped crystals of the *B. laterosporus* isolate observed here (Figure 2.3) confirmed the findings of Hannay (1957) that this species makes this shape of crystal protein. *Brevibacillus laterosporus* does not produce any crystal protein shapes other than canoe shaped crystals (Smimova *et al.*, 1996).

#### **2.4.3. Identification of isolates**

Various techniques have been used to isolate *B. thuringiensis* and other entomopathogenic spore-formers. The use of a pasteurization technique is not as selective as an acetate pasteurization, or an antibiotic isolation method (De Lucca *et al.*, 1981; Martin and Travers, 1989; Meadows *et al.*, 1992; Ohba and Aizawa, 1978). The acetate and antibiotic methods have mainly been used to isolate *B. thuringiensis* (De Lucca *et al.*, 1981; Martin and Travers, 1989). Selective isolation methods assist in identification by narrowing down the species of bacteria isolated (De Lucca *et al.*, 1981; Martin and Travers, 1989). Therefore, since a non-selective spore isolation technique that was used here, three identification methods were used to identify the species of spore-forming bacteria. The main identification methods used were microscopic evaluation, biochemical testing and serotyping (*B. thuringiensis* only) (Tables 2.3 and 2.5; Figures 2.2-2.7).

The method of isolation and screening adopted did offer opportunities to isolate species of Gram-positive, entomopathogenic endospore-forming bacteria other than *B. thuringiensis*. Some of these isolates formed small rods with round spores and thus did not fit the selection criteria. The main crystal forming species of Gram-positive bacteria isolated were *B. thuringiensis* which produced large rods with oval spores (Figures 2.2, 2.4, 2.6 and 2.7). Some Gram-positive entomopathogenic spore-formers such as *B. popilliae* only germinate in the haemolymph of Coleoptera larvae and are thus very difficult to culture (Klein, 1997). The six isolates selected were divided into two genera, *Bacillus* and *Brevibacillus*, based on the cell size and characteristics of the crystal protein inclusion bodies (Thiery and Frachon, 1997). The three species were identified were *B. thuringiensis*, *B. laterosporus* and *B. cereus*. Four of the six isolates were subspecies of *B. thuringiensis* (Figures 2.2-2.7).

Microscopic evaluation classified isolates NDR1, NDR2, NDR3, NDR5, NDR11 and NDR12 as Gram-positive spore-formers (Figure 2.1-2.7). All entomopathogenic *Bacillus* spp. and related entomopathogenic species are large, Gram-positive cells that are also catalase positive. All the isolates were positive for these tests (Table 2.4) (Thiery and Frachon, 1997). Endospore-formers are divided into three groups according to their classification. These groups are Group I, Group II and Group III (See Figure 2.1). The six primary isolates fell into either Group I or Group II: Group I consist of cells with non-distending sporangia, whereas Group II and Group III have distending sporangia. The main distinction between Group II and III is the shape of the spore. Group II species have oval spores while Group III species have round spores. Based on these characteristics, it was concluded that the isolates belonged to either Group I (NDR1, NDR3, NDR5 and NDR11) or Group II (NDR2) (Thiery and Frachon, 1997).

*Brevibacillus laterosporus* is the only insecticidal species member of Group II that can utilize D-mannitol as a nutrient source (Table 2.4) (Thiery and Frachon, 1997). The distinctive feature of this species is the formation of lateral situated oval spores (Figure 2.3). Due to the orientation of the spores, the rods are often described as canoe shaped (Zahner *et al.*, 1999). *Bacillus thuringiensis* and *B. cereus* are not able to utilize mannitol as a nutrient source, as shown in the biochemical tests (Table 2.4) (Thiery and Frachon, 1997).

Isolate NDR2 was identified as a member of the species *B. laterosporus*. Isolates NDR1, NDR3 and NDR11 were identified as members of the species *B. thuringiensis* because these isolates produced large crystal proteins, and because they had the ability to synthesize acetylmethylcarbinol (AMC) during the intermediate steps of glucose metabolism (Table 2.4). AMC synthesis was determined via Barrit's Method, which is a modification of the Voges-Proskauer reaction (Thiery and Frachon, 1997).

NDR5 was identified as an isolate of *B. cereus*. The entomopathogenic activity of this isolate was similar to the isolates identified as members of *B. thuringiensis*. This isolate did not synthesize crystal proteins (Figure 2.5), which is the major distinction between *B. thuringiensis* and *B. cereus*.

Serotyping is an identification technique used to identify strains in *B. cereus* and *B. thuringiensis* (De Barjac and Frachon, 1990). Serotyping can only be done on motile strains with flagellae (De Barjac and Frachon, 1990) because the technique is based on the antigenic reactions of the protein of their flagellae. However, not all strains of *B. thuringiensis* can be serotyped because some strains of *B. thuringiensis* are self-agglutinating, and others are non-motile.

#### **2.4.4. Toxicity determination**

An initial 14 isolates were used in bioassays with early instars of *T. molitor* to determine their toxicity. Later instars were not used because the effectiveness of *B. thuringiensis* diminishes with the age of target insect larvae (Lacey, 1997). Twenty larvae were tested per isolate. Preliminary toxicity testing by other researchers has used fewer test organisms, some with as few as five insects for each bioassay (Ohba *et al.*, 1992). Most bioassays conducted on Coleoptera have used at least 10 larvae (De Oliviera *et al.*, 2004; Hastowo *et al.*, 1992; Hori *et al.*, 1994).

In the bioassays six out of the 14 strains were found to cause more than 50% mortality, a level at which isolates were selected as “promising entomopathogens”, in this research (Table 2.6).

*Bacillus* spp. Synthesize many entomopathogenic toxins, as well as the visible toxins in the form of crystal proteins (Khetan, 2001). Therefore, the complete fermentation broth was used, after complete sporulation, to ensure that the end product contained a complete array of insecticidal toxins (Table 2.3). The broth contained spores, ICPs (not present in *B. cereus*) and other toxins that may have been released during bacterial cell growth. The use of spore and ICPs suspensions is a common practice (De Oliviera *et al.*, 2004; Hastowo *et al.*, 1992; Obeidat *et al.*, (2004); Ohba *et al.*, 1992). Spores and crystal packages may be more effective than loose toxins and spores, respectively (Burges *et al.*, 1976). Spores on their own are not directly toxic to insects, whereas ICPs are good toxins on their own, as expressed in genetically modified crops with *B. thuringiensis* genes (Mahon *et al.*, 2002).

The isolates that displayed the most promising toxicity levels, causing more than 75% mortality, were NDR2 (*B. laterosporus*), NDR3 (*B. thuringiensis*) and NDR11 (*B. thuringiensis*). Moderate

toxicity was displayed by Isolates NDR1, NDR5 and NDR12, which caused more than 50% mortality (Table 2.3 and Table 2.6). According to De Oliviera *et al.*, (2004), isolates causing mortality above 50% are considered highly toxic. Note that Isolate NDR12 was not used further in any other assays because it was determined to be the same serotype as NDR1 (Table 2.5). The isolates NDR1, NDR2, NDR3, NDR5 and NDR11 were investigated further.

The one strain of *B. laterosporus* (NDR2) caused a high level of mortality (>90%), and was competitive with the best strains of *B. thuringiensis*. Its good performance was partly expected because this isolate originated from a diseased *T. molitor* larva (Table 2.2 and 2.6). Isolate NDR5, a strain of *B. cereus*, also displayed toxicity towards *T. molitor*, despite having no crystal proteins (Table 2.6).

The isolation of five novel strains of entomopathogenic bacteria indicated that entomopathogenic spore formers are present in various environments and may be isolated readily.

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## Chapter Three

# Determination of insecticidal toxicity of three species of entomopathogenic spore-forming bacterial isolates against *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)

### Abstract

Bioassays were conducted using larvae of mealworms, *Tenebrio molitor*, to determine lethal concentration for five entomopathogenic strains of spore-forming bacteria. Lethal concentration was determined by feeding *T. molitor* larvae cabbage discs dipped in whole cell cultures of these five strains of bacteria. The strains of bacteria were isolates of *Bacillus cereus*, *B. thuringiensis* and *Brevibacillus laterosporus*. An isolate of *Bacillus cereus* required the highest concentration of viable spores ( $8.531 \times 10^7$  spores ml<sup>-1</sup>) to achieve its LC<sub>50</sub>, whereas an isolate of *Brevibacillus laterosporus* required the lowest concentration of viable spores ( $3.388 \times 10^6$  spores ml<sup>-1</sup>) to achieve LC<sub>50</sub>.

### 3.1. Introduction

The first isolates of *Bacillus thuringiensis* Berliner subspecies effective against coleopterans were isolated from a mealworm larva, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). This subspecies was subsequently named *B. thuringiensis* subsp. *tenebrionis* (Kriege *et al.*, 1983). Another coleopteran specific strain was later isolated, *B. thuringiensis* subsp. *san diego* (Herrnstadt *et al.*, 1986). These subsp. of *B. thuringiensis* are effective against one of the USA's most important potato pests, the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Nault and Kennedy, 1999). Colorado potato beetle has since been reported to have developed resistance to the Cry3A toxin (Whaldon *et al.*, 1993). The development of resistance to pesticides is not uncommon in insects repeatedly subjected to a single pesticide, especially when only one toxin is involved (Ferré and Van Rie, 1992; Tabashnik, 1994).

Various other coleopteran specific *B. thuringiensis* strains have been isolated and are effective against scarabaeid beetles such as *Anomala cuprea* Hope (Coleoptera: Dynastidae), *A. rufocuprea* Motschulsky (Coleoptera: Dynastidae) and *Popillia japonica* Newman (Coleoptera: Scarabaeidae) (Ohba *et al.*, 1992).

Bioassays on coleopterans are usually conducted on the Colorado potato beetle because these beetles are of economic importance and are a major pest in Asia, Europe and North America (Hare, 1990). These beetles are also the standard beetles used in bioassays to determine the International Units (IU) of toxicity (Navon, 2000). However, it is absent from South Africa, and therefore *T. molitor* was used as the test organism because it is readily available in South Africa and easy to rear (Hinze, 2000).

The aim of this research was to determine and compare the level of toxicity of five endospore-forming, entomopathogenic bacterial isolates, namely NDR1, NDR2, NDR3, NDR5 and NDR11.

## **3.2. Materials and Methods**

Standard microbiological techniques were followed in the laboratory for the routine microbiological assays that include sterilization, aseptic techniques and culture preparation (Prescott *et al.*, 1999; Wheelis and Segel, 1979).

**3.2.1. Samples:** Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* spp. collected in sugarcane areas in KwaZulu-Natal (KZN), and debris from insect-rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 3.1).

**3.2.2. Sample collection:** Mushroom compost, compost and grain dust were collected in 250 ml sterile honey jars from the various sources (Table 3.1). Honey jars were pre-sterilized in an autoclave at 121°C for 15 min. Diseased *T. molitor* larvae were collected in 90 mm sterile Petri dish. Adult beetles were collected from light traps in sugarcane areas in the KwaZulu-Natal midlands by employees of the South African Sugar Research Institute (SASRI, Mt. Edgcombe, KZN) and were delivered in brown paper bags. White grubs were collected from sugarcane areas

by staff members of SASRI and were delivered in plastic containers filled with soil. Diseased grubs were removed from these containers and placed into sterile Petri dishes. All samples were stored in a refrigerator kept at 4°C.

**Table 3.1.** Samples sources from which entomopathogenic endospore-formers were isolated

<b>Samples</b>	<b>Source of sample</b>	<b>Species</b>
Mushroom compost	Mushroom farm in Karkloof area in the KwaZulu-Natal midlands	-
Mealworm larvae	Insects rearing facility at the University of KwaZulu-Natal	<i>Tenebrio molitor</i>
Compost	A garden compost heap in Hillcrest, KwaZulu-Natal	-
Grain dust	Chicken grain storage facility in Pietermaritzburg, KwaZulu-Natal	-
Adult beetles	Dalton, Bryuns-hill and Harburg, KwaZulu-Natal	<i>Schizonycha</i> spp. and <i>Hypopholis</i> spp.
White grubs	Dalton, KwaZulu-Natal	<i>Schizonycha</i> spp.

**3.2.3. Isolation of endospore-forming bacteria:** Isolation of *Bacillus* spp. was conducted using a similar pasteurization method, as described by Ohba and Aizawa (1978). Approximately 0.5 g of each sample was suspended in 10 ml sterile distilled water. Suspensions were shaken vigorously for 30 sec at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 hr at room temperature. Suspensions were then vortexed a second time at full speed for 30 sec and then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 hr at 30°C in an incubator and examined for colonies with a typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or

‘colonies with an ‘ice crystal’ appearance wider than 2 mm (Damgaard *et al.*, 1997; Prescott *et al.*, 1999; Selvakumar *et al.*, 2007). Various other white coloured bacterial colonies larger than 2 mm in diameter were selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and counted using a colony counter. Selected colonies were then purified by sub-culturing onto nutrient agar plates and incubated for 3 d at 30°C in an incubator. Colonies were stored on nutrient agar slants. Not all colonies from each sample that fitted the description above were selected due to the large number of colonies formed.

Selected isolates were subjected to Gram staining as well as Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 sec with distilled water and blotted dry with tissue paper (Ammons *et al.*, 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 x oil immersion objective lens (Bernhard *et al.*, 1997; Young *et al.*, 1998). Crystal proteins stained as dark blue structures (Ammons *et al.*, 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9 µm, were also selected. This was done in order to include *B. cereus*, which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because they fit the description of *B. cereus*, *B. thuringiensis* and *B. laterosporus* cells. Selected cultures were purified by sub-culturing and were assigned numbers NDR1-NDR14 (Chapter 2).



**Figure 3.1.** Plastic containers used to rear *Tenebrio molitor* larvae (The scale bar represents 50 mm).

**3.2.4. Multiple dose bioassays:** A pure culture of *T. molitor* was obtained from a pet shop in Pietermaritzburg, KZN, SA. The culture was reared on a diet of commercial chicken meal (Meadow Feeds, P.O. Box 426, Pietermaritzburg, SA) (Hinze, 2000). Bran was not used because this contains phytic acid that affects the absorption of calcium which is an essential mineral for a healthy *T. molitor* culture (Hinze, 2000). *Tenebrio molitor* larvae were reared in 350 mm x 250 mm square plastic containers containing chicken meal to a depth of 70 mm (Figure 3.1). For moisture, potato skins, cabbage leaves and carrot peels were added once a week (Hinze, 2000). Carrots are an essential additive because most of the micronutrients required by *T. molitor* larvae are present in carrots (Hinze, 2000). Adults and pupae were removed carefully with forceps from the cultures on a regular basis and placed into 350 mm x 250 mm square plastic containers containing chicken meal to a depth of 70 mm (Figure 3.1). After 14 mo of rearing, a population of *T. molitor* larvae was available that was large enough for the planned bioassays. It was not possible to determine the instars' stages because meal worms have between 10 and 14 instars,

and several instars occur with larvae in the same size range (25 mm in length) (Anonymous, 2008a). Therefore, larvae sized between 18-20 mm were used in the bioassays.

**3.3.4.1. Test organism preparation:** *Tenebrio molitor* larvae were carefully removed from the media by gently sifting the chicken meal through a sieve. This procedure separated the *T. molitor* larvae and the chicken meal effectively. The *T. molitor* larvae, sized between 18-20 mm, were placed in a division of a plastic ice tray, for counting purposes, prior to being inoculated with the bacterial isolates (NDR1, NDR2, NDR3, NDR5 and NDR11). Isolates NDR1, NDR3 and NDR11 were isolates of *B. thuringiensis*. NDR2 was an isolate of *Brevibacillus laterosporus*, formerly *Bacillus laterosporus* (De Oliviera *et al.*, 2004). NDR5 was an isolate of *B. cereus* (Chapter 2).

**3.3.4.2. Inoculum preparation:** Bacterial isolates NDR1, NDR2, NDR3, NDR5 and NDR11 were used to inoculate 150 ml of sterile tryptone soy broth (TSB) (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) and incubated in a shaker water bath at 250 rpm for 5 d at 30°C (Meadows *et al.*, 1992). A viable spore count was conducted using a standard viable spore technique (Wheelis and Segel, 1979). The concentration of the viable spores was measured in colony forming units (CFUs). The final whole culture (FWC), which consisted of spores and crystal proteins, was used for inoculation. For NDR5 only spores are present because this isolate does not produce crystal proteins (Chapter 2).

**3.3.4.3. Inoculation:** Discs with an 18 mm diameter were cut out of the inner leaves of freshly purchased cabbage using a pre-sterilized test tube cap (Figure 3.2). The FWC of NDR1, NDR2, NDR3, NDR5 and NDR11 were diluted with sterile distilled water to make up concentrations ranges of 20%, 40%, 60%, 80% and 100%. Cabbage discs were dipped into the whole cell bacterial suspensions and fed to the *T. molitor* larvae in Petri dishes. The trials were conducted in sterile Petri dishes. Control discs were dipped into sterile distilled water. Twenty larvae per dose in replicates of five were used for each bioassay for NDR1, NDR2, NDR3 and NDR11. A total of 28 larvae per dose in replicates of five were used for NDR5. Dead larvae turned black and liquefied internally (Figure 3.3). Koch's postulate was used to determine if the isolates were responsible for the insect deaths (Prescott *et al.*, 1999). The amount of cabbage disc consumption

(mm) was a general observation and was not recorded because larval mortality was the objective of this study. Results were recorded after 5 d.



**Figure 3.2.** Discs cut out of cabbage leaves with a sterilized test-tube cap. The scale bar represents 18 mm.



**2.2.5. Statistical analyses:** The statistical computer programs (LSTATS) P/PROBAN Version 2.1 (1992), as programmed by Van Ark (1983), and SPSS Version 11.5, were used to calculate the regression parameters that included the determination of lethal concentration (LC<sub>50</sub>) and their fiducial limits. Each sample reflected three estimates of LC<sub>50</sub> and fiducial limits. These were compared using a standard one-way ANOVA. A similar format to that of Hatting (2002) was used to depict the results obtained (Table 3.1). The LC<sub>50</sub> values were converted back to concentrations of viable spores to determine the 50% mortality dose.

### 3.4. Results

Mortality of *T. molitor* larvae in the control Petri dishes was zero, and the cabbage discs were completely consumed after 5 d. The complete consumption of the cabbage discs indicates that no toxic effects were present in the control. Cabbage discs that were inoculated with different doses of the various bacterial isolates were consumed according to dose. An overall trend observed was that the more concentrated the inocula of spore-forming bacteria applied to the cabbage disc, the less the cabbage disc was consumed. Low bacterial dilutions resulted in levels of cabbage disc consumption similar to that of the controls but with a low level of *T. molitor* mortality. No further larval deaths were found to occur after 5 d.

The minimum LC<sub>50</sub> was 6.530 for NDR2 at a concentration equal to  $3.388 \times 10^6$  spores ml<sup>-1</sup>. The maximum LC<sub>50</sub> was 7.931 (NDR5) at a concentration of  $8.531 \times 10^7$  spores ml<sup>-1</sup> (Table 3.2). NDR5 could thus be considered the most effective isolate. A comparative bioassay with *B. thuringiensis* subsp. *tenebrionis* was not conducted because this strain is not a registered biocontrol agent in SA and is not readily available. *Bacillus thuringiensis* subsp. *azawai* and *B. thuringiensis* subsp. *kurstaki* are the only two subsp. of *B. thuringiensis* registered in SA for use as a bio-pesticide (Anonymous, 2008b).



**Figure 3.3.** *Bacillus thuringiensis* infected *Tenebrio molitor* larva (left) and healthy, uninfected *Tenebrio molitor* larva (right).

**Table 3.2.** (LSTATS) PROBAN analysis of three replicate bioassays of five spore-forming bacteria tested against *Tenebrio molitor*, with regression parameters displaying the efficacy of these assays

<b>Isolate</b>	<b>No of Larvae Affected</b>	<b>LC<sub>50</sub><sup>a</sup></b>	<b>95% FI<sup>b</sup></b>	<b>Slope</b>	<b>X<sup>2</sup> (df=3)</b>
<b>NDR1<sup>n</sup></b>					
Assay 1	68	7.495	7.414 - 7.548	5.45	1.42
Assay 2	55	7.391	7.256 - 7.497	2.54	3.47
Assay 3	61	7.332	7.176 - 7.436	2.59	3.13
<b>Mean</b>	<b>61</b>	<b>7.406</b>	<b>7.282 - 7.494</b>	<b>3.53</b>	-
<b>NDR2<sup>n</sup></b>					
Assay 1	63	6.572	6.488 - 6.630	4.65	2.47
Assay 2	68	6.507	6.358 - 6.603	3.02	0.36
Assay 3	65	6.512	6.388 - 6.600	3.26	2.36
<b>Mean</b>	<b>65</b>	<b>6.530</b>	<b>6.411 - 6.611</b>	<b>3.64</b>	-
<b>NDR3<sup>n</sup></b>					
Assay 1	49	7.219	7.092 - 7.344	2.35	2.65
Assay 2	46	7.281	7.170 - 7.403	2.56	1.32
Assay 3	53	7.207	7.084 - 7.312	2.63	5.27
<b>Mean</b>	<b>49</b>	<b>7.236</b>	<b>7.115 - 7.353</b>	<b>2.51</b>	-
<b>NDR5<sup>n</sup></b>					
Assay 1	51	7.904	7.786 - 8.070	1.95	4.56
Assay 2	49	7.927	7.809 - 8.101	1.97	2.04
Assay 3	56	7.963	7.832 - 8.142	1.71	3.66
<b>Mean</b>	<b>52</b>	<b>7.931</b>	<b>7.809 - 8.104</b>	<b>1.88</b>	-
<b>NDR11<sup>n</sup></b>					
Assay 1	57	7.095	6.953 - 7.199	2.57	2.62
Assay 2	48	7.178	7.087 - 7.266	3.35	2.48
Assay 3	50	7.146	7.037 - 7.248	2.79	0.63
<b>Mean</b>	<b>52</b>	<b>7.140</b>	<b>7.026 - 7.237</b>	<b>2.90</b>	-

<sup>a</sup>Lethal concentration (LC<sub>50</sub>)

<sup>b</sup>95% fiducial limits were log transformed concentrations of bacterial spores per ml.

<sup>n</sup>20 larvae per dose in replicates of five (total of 100 larvae per bioassay).

Numbers of larvae used in the assays were either 20 for NDR1, NDR2, NDR3, NDR5 and NDR11 (per single dose). These were the largest numbers possible at the time, in this project, due to limited insect rearing facilities, financial resources and time constraints.

**Table 3.3.** (LSTATS) PROBAN hypothesis test used to determine the homogeneity between the five independent bioassays using NDR1, NDR2, NDR3, NDR5 and NDR11

Hypothesis	Slopes equal			Slopes and intercepts equal		
	$X^2$	Df	P	$X^2$	df	P
NDR1	0.341	2	0.842	5.404	2	0.065
NDR2	1.445	2	0.49	2.007	2	0.368
NDR3	0.73	2	0.699	0.121	2	0.932
NDR5	0.256	2	0.876	0.317	2	0.851
NDR11	0.846	2	0.661	0.793	2	0.678

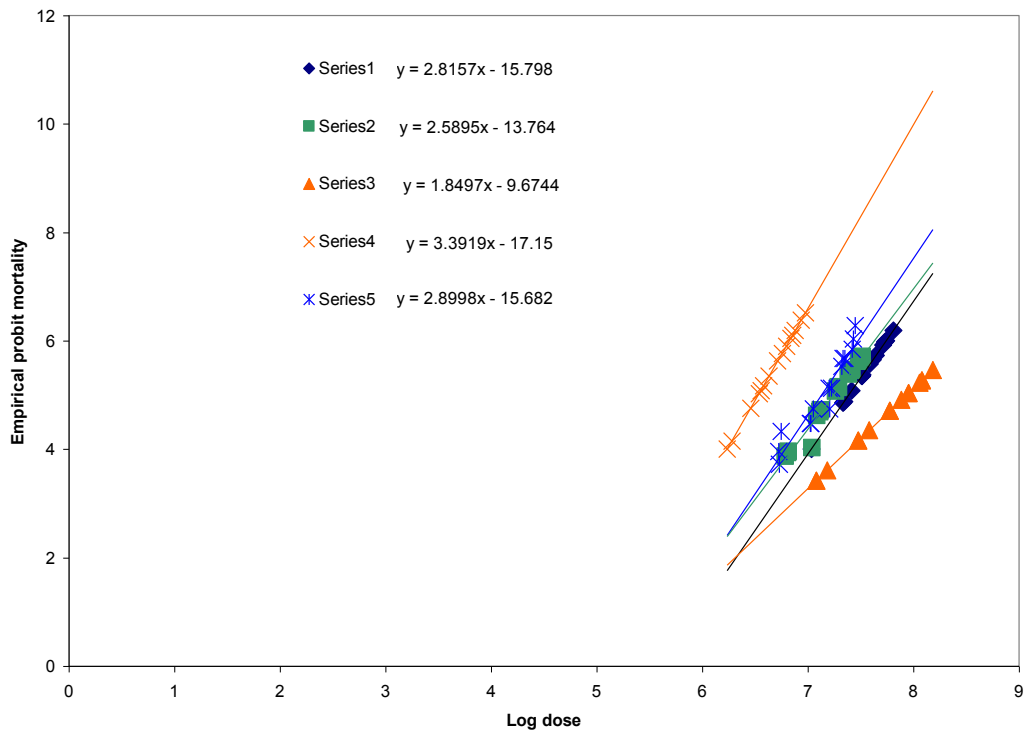
One-way ANOVA was used to determine whether the slopes and intercepts of the three bioassays of each of the isolates were comparable. Deviations of the slopes and intercepts from the Probit lines were homogenous. These lines were therefore comparable (Table 3.4).

**Table 3.4.** Results of a parallelism test between the three assays of the five bacterial isolates to determine homogeneity.

Isolate	No. of larvae affected	Slope $\pm$ SE	LC <sub>50</sub> <sup>a</sup>	95% FI	$X^2$ (df = 13)	G <sup>b</sup>
NDR1	184	2.82 $\pm$ 0.37	7.386	7.314 - 7.443	15.781	0.068
NDR2	196	3.39 $\pm$ 0.40	6.530	6.468 - 6.580	7.271	0.055
NDR3	148	2.50 $\pm$ 0.34	7.235	7.172 - 7.297	10.132	0.071
NDR5	156	1.85 $\pm$ 0.22	7.933	7.861 - 8.021	11.091	0.053
NDR11	155	2.87 $\pm$ 0.35	7.140	7.082 - 7.194	8.031	0.058

<sup>a</sup>The data of the three bioassays were pooled to obtain a more accurate estimation of the LC<sub>50</sub> and fiducial limits.

<sup>b</sup>The Fiducial limits calculated by Fieller's theorem as a measurement of variation.



**Figure 3.4.** Separate Probit lines of the pooled data for each of the five isolates.

1. Series 1 = NDR1; Series 2 = NDR2; Series 3 = NDR3; Series 4 = NDR5 and Series 5 = NDR11.

### 3.4. Discussion

**3.4.1. Statistical results:** The chi-squared values obtained for each of the bioassays suggested that the deviations of the observed mortalities are within the range of accepted parameters for the calculated Probit line (Table 3.2) (Van Ark, 1983). Therefore, it may be concluded that the calculated Probit line is an acceptable representation of insect response to *B. thuringiensis* isolates. Parallelism tests showed that no significant differences could be detected between the five bioassays for each of the isolates. This allowed for the comparison of slopes and intercepts (Table 3.4). The slopes of the independent bioassays showed no significant differences and were found to be homogenous hence the lines were comparable (Table 3.3) (Van Ark, 1983). The deviations from the observed mortalities were within the expected limitations of deviation thus rendering the Probit line acceptable (Table 3.3 and Figure 3.4). (Van Ark, 1983). Values obtained indicated that the lines for each of the five bioassays for each isolate were parallel and

hence a similar response was observed for each of the isolates (Table 3.3 and Figure 3.4). The  $LC_{50}$  spore concentrations for each of the isolates were: NDR1 =  $2.432 \times 10^7$ , NDR2 =  $3.388 \times 10^6$ , NDR3 =  $1.718 \times 10^7$ , NDR5 =  $8.579 \times 10^7$  and NDR11 =  $1.380 \times 10^7$ .

G is used as a measure of variation in the calculation of fiducial limits and is derived from Fieller's Theorem (Van Ark, 1983). According to Finney (1971), in a good bioassay, the value of G will lie between 0.2 and 0.05. Van Ark (1983) suggested using G values of 0.25 and 0.025. G values above 0.025 indicate that the variation of mortality is high. Values above 0.25 indicate that the experimental design is not appropriate and requires amendment. The fiducial limits cannot be calculated for assays where the G value is equal to one (Van Ark, 1983). The G values for all five isolates fell within the parameters set by Van Ark as well as by Finney. However, the experimental precision was not ideal because the G values were larger than 0.025, indicating that the variation in mortalities was large. However, the values were still acceptable because they did not exceed 0.25 (Table 3.4).

These results could be improved by conducting six or more bioassays per isolate. This would result in a better fit of line and hence a lower G value (Van Ark, 1983). The number of insects required in a bioassay for reliable results depends on the experimental procedures, as well as the species of insect (Van Ark, 1983).

**3.4.2. Toxicity comparison of isolates:** The isolate with the best  $LC_{50}$  was NDR2, with a log value of 6.530 ( $3.388 \times 10^6$  spores  $ml^{-1}$ ) (Table 3.4). This isolate was identified previously (Chapter 2) as an isolate of *B. laterosporus*. Assays conducted with isolates of *B. laterosporus* did not exhibit any toxicity towards *T. molitor*. However, toxicity was evident against mosquitoes (*Culex quinquefasciatus* and *Aedes aegypti*) and a species of snail *Biomphalaria glabrata* (Favret and Yousten, 1985). Assays conducted by Rivers *et al.* (1991) on *T. molitor* with strains of *B. laterosporus* showed these strains to have toxicity values similar to those obtained from strains of *B. thuringiensis* subsp. *tenebrionis*.

The isolate with the weakest  $LC_{50}$  was NDR5, with a log value of 7.933 ( $8.579 \times 10^7$  spores  $ml^{-1}$ ). This isolate was identified previously (Chapter 2) as an isolate of *B. cereus*. This species is not often used in entomopathogenic bioassays because it is considered an organism associated with

gastrointestinal diseases and is often found as a food contaminant. The insecticidal properties of this organism lie in its ability to produce vegetative insecticidal proteins (VIP) (Estruch *et al.*, 1996; Moar *et al.*, 1994; Yu *et al.*, 1997) which have been found to be effective against Western Corn Rootworm, *Diabrotica virgifera* LeConte (Coleoptera, Chrysomelidae)(Warren, 1997).

All five bacterial isolates (NDR1, NDR2, NDR3, NDR5 and NDR11) displayed toxicity against *T. molitor*. The three *B. thuringiensis* isolates (NDR1, NDR3 and NDR11) displayed high levels of toxicity to *T. molitor* but were less toxic than the *B. laterosporus* isolate (NDR2) (Table 3.4). The dose response of the *B. cereus* isolate (NDR5) was found to stand alone from the other two species tested (Figure 3.4). This is indicative of substantially greater toxicity than the other isolates.

Standardization assays often involve the use of purified or extracted crystal proteins (Cry and Cyt toxins) and do not include any of the other toxins and synergists (e.g., chitinases) produced by these species of bacteria (Thamthiankul *et al.*, 2001). Other toxins may play an important role in preventing target insects from becoming resistant because they would have to evolve resistance against more than one compound concurrently. In the bioassays used in this research, the FWC were used in toxicity determination. Hence, other unknown toxins and synergistic factors were included. Whole cultures consisting of spore and crystal suspensions are often used in bioassays to determine toxicity. The use of FWCs is applied where whole culture products will be commercialized, as opposed to pure protein crystals (De Oliviera, 2004; Lambert *et al.*, 1992). Whole culture products have to be screened for beta-exotoxins because current registration of *B. thuringiensis* products requires the absence of these toxins (Prieto-Samsonova *et al.*, 1997). Continuous sub-culturing of *B. thuringiensis* can be problematic because it may cause a decline in toxicity (Sachidanandham and Jayaraman, 2003).

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## Chapter Four

# Medium toxicity of entomopathogenic spore-forming bacteria against a sugarcane white grub species, *Hypopholis* sp. (Coleoptera: Melolonthidae)

### Abstract

Bioassays were conducted on larvae of *Hypopholis* sp. collected from sugarcane areas in KwaZulu-Natal. Five entomopathogenic spore-forming bacteria were used to screen for toxicity against the *Hypopholis* sp. larvae using grass plugs dipped in bacterial cell suspensions. The five species of spore-forming bacteria belong to species *Bacillus thuringiensis*, *B. cereus* and *Brevibacillus laterosporus*. Three of the five isolates effectively caused more than 40% mortality (Medium toxicity). All three isolates were *B. thuringiensis* subspecies. The highest percentage of mortality was 59% (NDR3). A subspecies of *B. cereus* had the lowest mortality of 21.7% (NDR5).

### 4.1. Introduction

Some of the major coleopteran pests of sugarcane in South Africa (SA) are collectively known as white grubs (Scarabaeidae). White grubs are defined as broad C-shaped, white or grey white larvae with legs and a large head (Imms, 1957; Wilson, 1969). Depending on the species, white grub life cycles can stretch over 1-2 years, which makes rearing in an insectary rather difficult (Wilson, 1969). The main white grub species in Africa belong to the families Melolonthidae, Dynastidae and Rutelidae (Leslie, 2004).

The Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), is a major Coleopteran pest in North America and some parts of Europe. Literature focusing on the testing of *B. thuringiensis* subspecies effective against the Coleoptera uses the Colorado potato

beetle as the test organism (Lambert *et al.*, 1992; Nault and Kennedy, 1999; Zehnder and Gelernter, 1989). Colorado potato beetle is also the standard coleopteran used to determine international toxicity units (IU) (Ferro and Gelernter., 1989).

Some of the major Coleopteran pests of sugarcane in South Africa (SA) are collectively known as white grubs (Scarabaeidae). White grubs are defined as broad C-shaped, white or grey white larvae with legs and a large head (Imms, 1957; Wilson, 1969). Depending on the species, white grub life cycles can stretch over 1-2 years, which makes rearing in an insectary rather difficult (Wilson, 1969). The main white grub species in Africa belong to the families Melolonthidae, Dynastidae and Rutelidae (Leslie, 2004).

Various subsp. of *B. thuringiensis* with toxicity towards white grubs have been isolated (Van Frankenhuyzen and Nystrom, 2002). *Bacillus thuringiensis* has been effective against species of white grubs such as *Melolontha melolontha* L. (Coleoptera: Melolonthidae), *Popillia japonica* Newman (Coleoptera: Scarabaeidae), *Anomala cuprea* Hope (Coleoptera: Rutelidae), *A. corpulenta* Motschulsky (Coleoptera: Rutelidae) and *A. orientalis* Waterhouse (Coleoptera: Rutelidae) (Van Frankenhuyzen and Nystrom, 2002).

Trends in toxicity of various species of bacteria are determined using bioassays. The number of insects used per bioassay range from 10 to as little as five insects per bioassay (De Oliviera *et al.*, 2004; Hori *et al.*, 1992; Ohba *et al.*, 1992). In this study, white grub (*Hypopholis* sp.) was subjected to treatments with five isolates of endospore-forming bacteria (NDR1, NDR2, NDR3, NDR5 and NDR11), which included *B. thuringiensis*, *B. cereus* Frankland and Frankland and *Brevibacillus laterosporus* Laubach.

## **4.2. Materials and Methods**

Standard microbiological techniques were followed in the laboratory for the routine assays (Prescott *et al.*, 1999; Wheelis and Segel, 1979).

**4.2.1. Samples:** Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* spp. collected in sugarcane areas in KwaZulu-Natal (KZN, SA), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 4.1).

**4.2.2. Sample collection:** Mushroom compost, compost and grain dust were collected in 250 ml sterile honey jars (Table 4.1). Honey jars were pre-sterilized in an autoclave at 121°C for 15 min. Diseased *T. molitor* larvae were collected in 90 mm sterile Petri dishes. Adult beetles and white grubs were collected from light traps and soil, respectively, from the sugarcane areas in the KZN midlands. These were provided by the South African Sugar Research Institute (SASRI), Mount Edgecombe, SA. Diseased grubs were removed from these containers and placed into sterile Petri dishes. All samples were stored at 4°C in a refrigerator.

**Table 4.1** Samples sources from which entomopathogenic endospore-formers were isolated

<b>Samples</b>	<b>Source of sample</b>	<b>Species</b>
Mushroom compost	Mushroom farm in Karkloof area in the KwaZulu-Natal midlands	-
Mealworm larvae	Insect rearing facility at the University of KwaZulu-Natal	<i>Tenebrio molitor</i>
Compost	A garden compost heap in Hillcrest, KwaZulu-Natal	-
Grain dust	Chicken grain storage facility in Pietermaritzburg, KwaZulu-Natal	-
Adult beetles	Dalton, Bryuns-Hill and Harburg, KwaZulu-Natal	<i>Schizonycha</i> spp. and <i>Hypopholis</i> spp.
White grubs	Dalton, KwaZulu-Natal	<i>Schizonycha</i> spp.

**4.2.3. Isolation of endospore-forming bacteria:** Isolation of *Bacillus* sp. was conducted using a similar pasteurization method as described by Ohba and Aizawa (1978). Approximately 0.5 g of each sample was suspended in 10 ml sterile distilled water. Suspensions were shaken vigorously for 30 sec at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 hr at

room temperature. Suspensions were vortexed a second time at full speed for 30 sec and then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 hr at 30°C in an incubator and examined for colonies with typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or colonies with an 'ice crystal' appearance with a colony diameter larger than 2 mm (Damgaard *et al.*, 1997; Prescott *et al.*, 1999; Selvakumar *et al.*, 2007). Various other white coloured bacterial colonies that predominated and were larger than 2 mm in diameter were also selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and counted using a colony counter. Selected colonies were then purified by sub-culture onto nutrient agar and incubated for 3 d at 30°C in an incubator. Colonies were stored on nutrient agar slants.

Selected isolates were subjected to Gram staining as well as Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 sec with distilled water and blotted dry with tissue paper (Kimberly-Clark, SA) (Ammons *et al.*, 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 x oil immersion objective lens (Bernhard *et al.*, 1997; Young *et al.*, 1998). Crystal proteins stained dark blue (Ammons *et al.*, 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9 µm, were also selected, in order to include *B. cereus* which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because this fits the criteria of *B. cereus*, *B. thuringiensis* and *B. laterosporus* cells (Figure 2.1). Selected cultures were purified by sub-culturing and were assigned numbers NDR1-NDR14 (Chapter 2).

**4.2.4. Inoculum preparation:** The bacterial isolates (NDR1, NDR2, NDR3, NDR5 and NDR11) were used to inoculate 150 ml of sterile tryptone soy broth (TSB) (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) and incubated in a shaker water bath at 250 rpm for 24 hr at 30°C. The final whole culture (FWC) was used in the bioassays.

**4.2.5. White grubs:** *Hypopholis* sp. larvae were obtained from sugarcane fields in KZN which were supplied by SASRI on a weekly basis during the winter months (mid-May to August). Larvae were estimated to be mid- to late-instars as they were collected in close proximity to the sugarcane plants in the upper levels of the soil. Late instars feed on sugarcane roots whereas early instars are found deeper in the soil (Leslie, 2004; Wilson, 1969). The LC<sub>50</sub> for the white grubs was not determined as this was a preliminary toxicity screening (De Oliviera *et al.*, 2004; Hori *et al.*, 1992; Ohba *et al.*, 1992). The *Hypopholis* sp. larvae were protected from desiccation by placing them in moist soil during collection.

**4.2.6. Inoculation of white grubs:** Kikuyu grass plugs, *Pennisetum clandestinum* Hochst. ex Chiov. (Cyperales: Poaceae) were obtained from Jesmon Dene Nursery (Pietermaritzburg, KZN, SA). Grass plug roots were dipped for 1 minute in the each of the bacterial suspensions (NDR1, NDR2, NDR3, NDR5 and NDR11) and planted in 300 ml plastic cups with white grubs (Figure 4.1). Soil in which the *Hypopholis* sp. larvae were transported was used as potting medium. Roots that were dipped in water served as the control. Results were recorded after 5 d. Fifteen white grubs were used per bioassay in replicates of three (Table 4.2). Due to aggressive behavior and their tendency for cannibalism, only three white grubs were used per grass plug. Abbott's (1925) corrected formula was used to determine true mortality.

$$\text{Adjusted \% mortality} = \frac{(\text{observed \% mortality} - \text{mean control \% mortality})}{100 - \text{mean control \% mortality}}$$



**Figure 4.1.** Grass plug planted in a plastic cup used for white grub trials. The cup contained soil and white grubs (Scale bar = 20 mm).

### **4.3. Results**

Larvae of *Hypopholis* spp. are very fragile once removed from the soil. Numerous larvae died within 1 d of collection due to the stress. This weakness has been exploited as a control measure for white grubs. Deep ploughing may kill white grubs directly or expose them to predators and sunlight (Hill, 1983). Healthy white grubs that survived for 2 d after being removed from the soil were used in the bioassays.





**Figure 4.2.** A healthy white grub from the control group (left) and a *B. thuringiensis* infected white grub (right).

Infected white grubs from the inoculated groups turned from brown to black within a seven days (Figure 4.2). The cadavers liquefied internally and became soft with a pungent smell. These cadavers became hollow and brittle over a two week period.

The control group consumed most of the Kikuyu grass roots within the 5 d bioassay period. No control grass plugs died over the 5 d trial period.

**Table 4.2.** Bioassays of the five endospore-forming isolates against *Hypopholis* sp. larvae

Species and Isolate <sup>a</sup>	Mortality <sup>b</sup>				Mortality %	Abbott's Corrected Value	Rank	SE
	Rep1	Rep2	Rep3	Mean				
<b>Bt</b> <b>NDR 1</b>	9	9	5	8	51.1	40.5	3	1.33
<b>Bl</b> <b>NDR 2</b>	7	7	8	7	48.9	37.8	4	0.33
<b>Bt</b> <b>NDR 3</b>	9	10	11	10	66.7	59.5	1	0.58
<b>Bc</b> <b>NDR 5</b>	5	6	5	5	35.6	21.7	5	0.33
<b>Bt</b> <b>NDR 11</b>	9	8	9	9	57.8	48.7	2	0.33
<b>Control</b>	3	2	3	3	17.8	-	-	0.33

<sup>a</sup> Bt, *Bacillus thuringiensis* Berliner; Bl, *Brevibacillus laterosporus* Laubach; Bc, *Bacillus cereus* Frankland and Frankland

<sup>b</sup> Fifteen white grubs per replicate

- = NA

Three isolates (NDR1, NDR3 and NDR11) caused mortalities greater than 40% (Abbott's corrected value) (Table 4.2). Larval death occurred within 5 d of the bioassays being initiated. The mortality rating of De Oliveira *et al.*, (2004) was used and isolates NDR1, NDR3 and NDR11 displayed a medium toxicity towards *Hypopholis* sp. (Table 4.2). All three isolates were subspecies *B. thuringiensis* (Table 4.2 and Chapter 2).



**Figure 4.3.** A grass plug from the control group (left), and a treated grass plug (right), which had been inoculated with entomopathogenic bacteria. Note the relative lack of roots in the plug on the left, eaten by white grubs (Scale bar represents 30 mm).

Roots of control grass plugs (treated with distilled water or with ineffective bacterial isolates) were eaten by white grubs, and the plants died (Figure 4.3). Roots of grass plugs treated with effective isolates remained uneaten, and the plants continued to grow healthy (Figure 4.3).

## 4.4. Discussion

Most of the entomopathogenic isolates were toxic towards *Hypopholis* sp. The most promising results were obtained from the three *B. thuringiensis* isolates, NDR1, NDR3 and NDR11, because they produced more than 40% mortality (Table 4.2). Isolates NDR11 and NDR1 were originally isolated from infected white grub larvae (Chapter 2). Isolate NDR3 was obtained from compost.

Some insect species have limitations in terms of rearing within research facilities. Rearing the white grubs used in the study, in a rearing facility, would not have been feasible within the time

frame of the current study. A typical example is that of *Schizonycha* sp. which reproduce at a rate of one generation per year, with a life cycle of 7 mo, of which 6 mo are spent in the larval stage (Hill, 1983).

Knowledge of the larval instar stage used in bioassays testing *B. thuringiensis* is important because the effects of the bacterial toxins are reduced as the white grubs get older. *Bacillus thuringiensis* displays greater toxicity on 1<sup>st</sup> instars than on 2<sup>nd</sup> instars, hence later instars are less susceptible to toxicity (Lacey and Singer, 1982). A problem with the bioassays conducted in the current study was that the field-collected larvae were not necessarily at the same instar level. Mixtures of medium-and late-instar white grubs were used in the bioassays. Thus the toxicity of the isolates for earlier instars was not fully determined and the true toxicity could have been greater if early instars were used in these bioassays.

White grub larvae were extremely fragile once removed from their soil environment. The mortality of the controls was above 10% (Table 4.2). This was not ideal because most insect bioassays only allow for the mortality level of the control to be less than 10% (Klein, 1997). Mortality of white grub larvae can be reduced during collection by placing them in a closed container filled with moist soil. Post-collection mortality was also reduced by keeping the soil moist, and by conducting the bioassays on larvae that survived 2 d post-collection. With the aid of Abbott's Formula, the true mortality can be calculated. Isolates NDR1, NDR3 and NDR11 were the most effective, causing more than 40% mortality (Table 4.2). This is considered a medium level of toxicity according to De Oliveira *et al.* (2004).

Toxicity determination of the bacterial isolates to *Hypopholis* sp. is often hampered by the problem of how to administer the test organisms. White grubs are soil dwelling insects, which is problematic when it comes to pesticides, whether bio-pesticides or chemical pesticides. *Bacillus thuringiensis* insecticidal crystal proteins persist in soil and adhere to soil particles (Muchaonyerwa *et al.*, 2006). This is promising for the use of *B. thuringiensis* in field trials concerning soil dwelling pests. The most promising outcome of this series of bioassays was clear evidence of toxicity by entomopathogenic spore-forming bacteria against white grubs from sugarcane (*Hypopholis* sp.). Results suggest that there are numerous entomopathogenic isolates

in nature that are still to be discovered. This is encouraging for further research towards combating coleopteran pests with biocontrol agents.

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## Chapter Five

# DNA fingerprinting of five spore-forming bacterial isolates, using *Bacillus cereus* repetitive extragenic palindromic sequence-based Polymerase Chain Reaction analysis (Bc-Rep-PCR)

### Abstract

Bc-repetitive extragenic palindromic Polymerase Chain Reaction (Bc-Rep PCR) analysis was conducted on seven *Bacillus thuringiensis* isolates accessed from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection and on five local isolates of entomopathogenic spore-forming bacteria. The five isolates were three strains of *B. thuringiensis*, one strain of *B. cereus* and one strain of *Brevibacillus laterosporus*. All five isolates were distinguished from each other using Bc-Rep PCR analysis. The three *B. thuringiensis* isolates were closely related. The isolate of *B. laterosporus* was not related to any of the *B. cereus* group isolates. Serotyping was also conducted on the five local isolates. However, only one of these isolates could be identified with serotyping, and was identified as *B. thuringiensis* subsp. *kenyae*.

### 5.1. Introduction

The *Bacillus cereus* group consists of six *Bacillus* species: *B. cereus* Frankland and Frankland, *B. thuringiensis* Berliner, *B. anthracis* Cohn, *B. mycoides* Flügge and the two latest additions, *B. pseudomycoides* Nakamura (Nakamura, 1998) and *B. weihenstephanensis* Lechner (Lechner *et al.*, 1998). The original description of the members of this group was based on their habitats, pathogenicity towards fauna, morphological and physiological characteristics (Ash *et al.*, 1991). *Bacillus thuringiensis* is the most diverse of the six species and has been classified into 84



serotypes and two biovars (Lecadet *et al.*, 1999). On the other hand, *B. anthracis* seems to be the least diverse of the group so far (Hill *et al.*, 2004).

The most commonly used technique for the sub-specification of varieties of *B. thuringiensis* has been H-serotyping (Lecadet *et al.*, 1999). This has also been the most common technique to date used for identifying and characterizing novel *Bacillus* strains (Lecadet *et al.*, 1999). H-serotyping is based on the analysis of a set of reference antisera against the flagellae of strains of *B. thuringiensis*. The technique was first developed by De Barjac and Bonnefoi (1962) and has been updated by Lecadet *et al.* (1999).

Strains that are not detected by the reference antisera are considered as possible new serotypes. Since the classification by De Barjac and Frachon (1990), the numbers of serotypes has increased from 27 to 69 and is still increasing. Biochemical tests on their own have proven ineffective in classifying *B. thuringiensis* into serotypes. However, in situations of conflicting serotype results, they may be used for clarification (Lecadet *et al.*, 1999).

The use of H-serotyping as a method to classify *B. thuringiensis* has been revised because different strains within a serotype may differ from each other in their genetic, biochemical and toxicological attributes. The only common ground between strains of the same serotype may be their H-serotype (Aronson *et al.*, 1986). This is not true for all serotypes, and strains in the *B. thuringiensis* subsp. *israelensis* all share the same attributes (Ankarloo *et al.*, 2000). Strains from different serotypes may share phenotypic attributes, such as *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *malaysiensis* (Reyes-Ramirez and Ibarra, 2005). Some serotypes may contain strains that affect different orders of insects, such as *B. thuringiensis* subsp. *morrisoni* that contains strains that are active against Coleoptera, Lepidoptera and Diptera (Padua *et al.*, 1984).

H-serotyping has several major limitations, which have resulted in a search for better techniques for classifying *B. thuringiensis*. Firstly, serotyping is unable to differentiate between *B. cereus* and *B. thuringiensis*. Secondly, phylogenic relationships between serotypes cannot be drawn (Lecadet *et al.*, 1999). Thirdly, self-agglutinated and non-motile strains cannot be distinguished

by serotyping (Lecadet *et al.*, 1999). Lastly, only a few laboratories worldwide are able to perform a full serotyping assay (Lecadet *et al.*, 1999).

Molecular techniques have been used as alternatives for typing *B. thuringiensis*. The most common techniques include Ribosomal DNA Restriction Fragment Polymorphism (RFLP) (Joung and Côte, 2001), Arbitrary Primer PCR technology (Brousseau *et al.*, 1993) and Amplified Fragment Length Polymorphism (AFLP) (Pattanayak *et al.*, 2000). Relatively few strains of *B. thuringiensis* have been analyzed using these techniques so far. Early results using these techniques suggest that the members of the *B. cereus* group and the *B. thuringiensis* group could be considered as a single species, and that *B. anthracis* is closely related to these two groups. Techniques such as the Reverse Transcriptase Sequencing of 16S rRNA have demonstrated that there are high levels of sequence similarity (>90%) amongst the members of the *B. cereus* group (Ash *et al.*, 1991). The only attribute that distinguishes these genera from each other is the functional genes that are carried on plasmids (Carlson *et al.*, 1994; Helgason *et al.* 2000). These pathological and phenotypic attributes may easily be transferred between “species” through the horizontal transfer of plasmids (Helgason *et al.*, 2000).

Methods such as the use of 16S to 23S Ribosomal Intergenic Spacers Sequences (ISR) have failed to aid in the design of species-specific oligonucleotide probes to differentiate between members of this group (Bourque *et al.*, 1995). Ash *et al.* (1991) stated that techniques using 16S rRNA to attempt to group members of the *B. cereus* group into one species should be supported with other techniques because similar results have been obtained in other genospecies.

With more advanced techniques that use very specific primers, differences between these species have been highlighted. Strains of *B. thuringiensis* have a high degree of relatedness but are genetically distinct (Joung and Côte, 2001; Nakamura, 1998). The relatedness between these members is between 85-100%. In contrast to earlier research using 16S rRNA, the genetic relatedness between *B. thuringiensis* and *B. cereus* has been demonstrated to be between 60-70%, by using sensitive DNA techniques. In order for these two species to be seen as a single species, the relatedness needs to be greater than 70% (Joung and Côte, 2001; Nakamura, 1998). Research by Nakamura (1998) and Joung and Côte (2001) suggest that some serotypes of the *B.*

*thuringiensis* groups should be reassigned. It has also been suggested that some members of *B. thuringiensis* are in fact members of *B. cereus*, but that, due to horizontal plasmid migration, these *B. thuringiensis* species have lost the ability to produce crystal proteins (Hill *et al.*, 2004; Nakamura, 1998). For this reason they may be identified as *B. cereus* due to the absence of the crystal protein.

A new technique for fingerprinting and potentially classifying novel strains of *B. thuringiensis* was developed by Reyes-Ramirez and Ibarra (2005). The technique is Repetitive Extragenic Palindrome Sequence-Based PCR analysis (rep-PCR). The technique originated from the designing of PCR primers from Rep sequences found in *Escherichia coli* Migula and *Salmonella typhimurium* genomes. This technique is based on the fact that Rep sequences have been shown to be common in prokaryote genomes and have been used in the design of primers specific to certain species or groups.

Reyes-Ramirez and Ibarra (2005) found a 26- base Rep that is common in the six members of the *B. cereus* group: CCCCACTGATTAAAGTTTCACTTTAT (accordingly named Bc-Rep). This was used to design two specific primers for the *B. cereus* group: 18-mer primer Bc-Rep-1 (5<sup>1</sup>-ATTAAAGTTTCACTTTAT-3<sup>1</sup>) and 14-mer primer Bc-Rep-2 (5<sup>1</sup>-TTTAATCAGTGGGG-3<sup>1</sup>). These primers have been shown to be applicable to *B. thuringiensis*, *B. cereus*, *B. anthracis* and *B. mycoides*. Analysis using this technique results in discrete and reproducible patterns. Almost all the serotypes of *B. thuringiensis* have been shown to have distinct patterns (Reyes-Ramirez and Ibarra, 2005).

The aim of the current study was to determine how closely related the isolates were to each other, and how they compare from known serotypes.

## **5.2. Materials and Methods**

Standard microbiological techniques were followed in the laboratory (Prescott *et al.*, 1999; Wheelis and Segel, 1979).

**5.2.1. Samples:** Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* spp. collected in sugarcane producing areas in KwaZulu-Natal, (KZN), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 5.1).

**5.2.2. Sample collection:** Mushroom compost, compost and grain dust were collected in 250 ml sterile honey jars (Table 5.1). Honey jars were pre-sterilized in an autoclave at 121°C for 15 min. Diseased *T. molitor* larvae were collected in 90 mm sterile Petri dishes. Adult beetles and white grubs were collected from light traps and soil, respectively, from the sugarcane areas in the KwaZulu-Natal midlands, KZN. These were provided by the South African Sugar Research Institute (SASRI), Mount Edgecombe, KZN. Diseased grubs were removed from these containers and placed into sterile Petri dishes. All the samples were stored at 4°C in a refrigerator.

**Table 5.1** Samples sources from which entomopathogenic endospore-formers were isolated

<b>Samples</b>	<b>Source of sample</b>	<b>Species</b>
Mushroom compost	Mushroom farm in Karkloof area in the KwaZulu-Natal midlands	-
Mealworm larvae	Insect rearing facility at the University of KwaZulu-Natal	<i>Tenebrio molitor</i>
Compost	A garden compost heap in Hillcrest, KwaZulu-Natal	-
Grain dust	Chicken grain storage facility in Pietermaritzburg, KwaZulu-Natal	-
Adult beetles	Dalton, Bryuns-Hill and Harburg, KwaZulu-Natal	<i>Schizonycha</i> spp. and <i>Hypopholis</i> spp.
White grubs	Dalton, KwaZulu-Natal	<i>Schizonycha</i> spp.

**5.2.3. Isolation of endospore-forming bacteria:** Isolation of *Bacillus* sp. was conducted using a similar pasteurization method as described by Ohba and Aizawa (1978). Approximately 0.5 g of each sample was suspended in 10 ml sterile distilled water. Suspensions were shaken vigorously

for 30 sec at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 hr at room temperature. Suspensions were vortexed a second time at full speed for 30 sec and then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 hr at 30°C in an incubator and examined for colonies with typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or colonies with an 'ice crystal' appearance with a colony diameter larger than 2 mm (Damgaard *et al.*, 1997; Prescott *et al.*, 1999; Selvakumar *et al.*, 2007). Various other white coloured bacterial colonies that predominated and were larger than 2 mm in diameter were also selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and counted using a colony counter. Selected colonies were then purified by sub-culture onto nutrient agar and incubated for 3 d at 30°C in an incubator. Colonies were stored on nutrient agar slants.

Selected isolates were subjected to Gram staining as well as Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 sec with distilled water and blotted dry with tissue paper (Kimberly-Clark, SA) (Ammons *et al.*, 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 x oil immersion objective lens (Bernhard *et al.*, 1997; Young *et al.*, 1998). Crystal proteins stained dark blue (Ammons *et al.*, 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9 µm, were also selected, in order to include *B. cereus* which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because this fits the criteria of *B. cereus*, *B. thuringiensis* and *B. laterosporus* cells (Figure 5.1). Selected cultures were purified by sub-culturing and were assigned numbers NDR1-NDR14 (Chapter 2).

**5.2.4. *Bacillus thuringiensis* samples:** Eight known subspecies of *B. thuringiensis* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection.<sup>a</sup> The subspecies were:

*B. thuringiensis* subsp. *nigeriae*, *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *morrisoni*, *B. thuringiensis* subsp. *israelensis*, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *thuringiensis*, *B. thuringiensis* subsp. *kenyae* and *B. thuringiensis* subsp. *tochigiensis*.

**5.2.5. Inoculum preparation:** The isolates were used to inoculate 150 ml of sterile tryptone soy broth (TSB) (Biolab) (Merck, SA) and incubated in a shaker water bath (GFL 1083) at 250 rpm for 24 hr at 30°C.

**5.2.6. DNA extraction:** The DNA was extracted from an overnight culture with Mobio UltraClean<sup>TM</sup> microbial DNA isolation kit (Mobio, USA).

**5.2.7. DNA quantification:** A test on the quality and quantity of DNA extracted was conducted electrophoretically as follows: 5 µl of DNA extract aliquots were loaded on a 1.2% horizontal agarose gel slab (Bio-Rad, SA). Samples were run in TAE at pH 8.0 (40 mM Tris-acetate and 1 mM EDTA). Ethidium bromide (Bio-Rad, SA) is a DNA stain that fluoresces under UV light. This was added to the tank of TAE buffer at pH 8.0 (40 mM Tris-acetate and 1 mM EDTA) in order to visualize the DNA bands. Gels were run at 90 V for 55 min.

**5.2.8. Primers:** The Bc-Rep sequence primers designed by Reyes-Ramirez and Ibarra (2005) were used. The primers were manufactured by Integrated DNA Technologies (IDT, SA) and were as follows: Direct 18 mer primer, 5<sup>1</sup>-ATT AAA GTT TCA CTT TAT-3<sup>1</sup> and a 14 mer reverse primer, 5<sup>1</sup>-TTT AAT CAG TGG GG-3<sup>1</sup>.

**5.2.9. Conditions for Bc-Rep-PCR amplification:** The PCR mixtures were prepared as follows: 1 µg of template DNA, 15 pmol of each primer, 5 mM MgCl<sub>2</sub>, 5 µl thermophilic DNA polymerase 10x reaction buffer, 1 µl 10 mM PCR nucleotide mix, 0.25 µl of 5 u/µl *Taq* DNA

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<sup>a</sup> www.dsmz.de

polymerase and nuclease free water to make the volume up to 50 µl (PCR Core Systems, Promega) (Whitehead Scientific, SA). The conditions of PCR amplification were as follows: 5 min initial denaturation at 94°C, followed by 34 cycles of 1 min denaturation at 94°C, 1 min annealing at 42°C and 1.5 min polymerization at 72°C. The amplification was completed with an extension step of 7 min at 72°C (Reyes-Ramirez and Ibarra, 2005). Amplifications were conducted in an Applied Biosystems Gene Amp PCR System 2400. Amplified samples were stored at -20°C (Conquest).

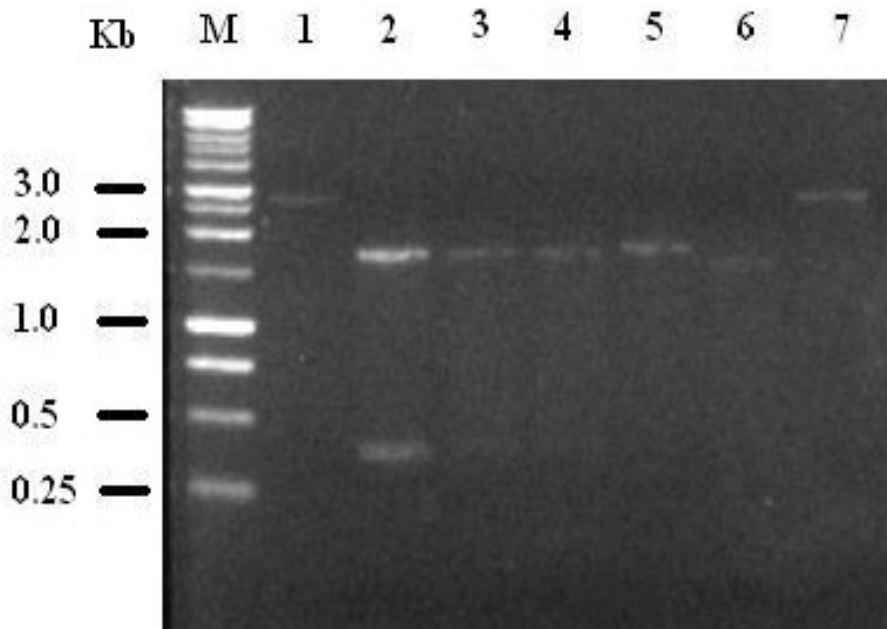
**5.2.10. Electrophoresis:** The Bc-Rep-PCR fragments were analyzed as follows: 5 µl aliquots of each of the amplified products were loaded on to a 1.2% agarose slab (110 x 140 mm). The gel was run in TAE buffer pH 8.0 (40 mM Tris-acetate and 1 mM EDTA) at 90V for 55 min. A 1 kb DNA ladder (Promega) (Whitehead Scientific, SA) was used as a molecular weight marker. A gel documentation system, Versa Doc 2000 (Bio-Rad), was used to photograph the gels. Quantity One Version 1.1 computer software was used to analyze the molecular weight patterns (Figures 5.1 and 5.2).

**5.2.11. Bc-Rep-PCR analysis:** The individual patterns of the polymorphic bands of each of the isolates were identified according to the migration rates. An estimation of the banding sizes was used to determine relatedness.

**5.2.12. *Bacillus thuringiensis* serotyping:** *B. thuringiensis* isolates were sent to Dr M. Ohba at the Graduate School of Agriculture, Kyushu University Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka, B12-8581 in Japan for serotyping. A standard method was used, similar to that developed by Thiery and Frachon (1997).

### 5.3. Results

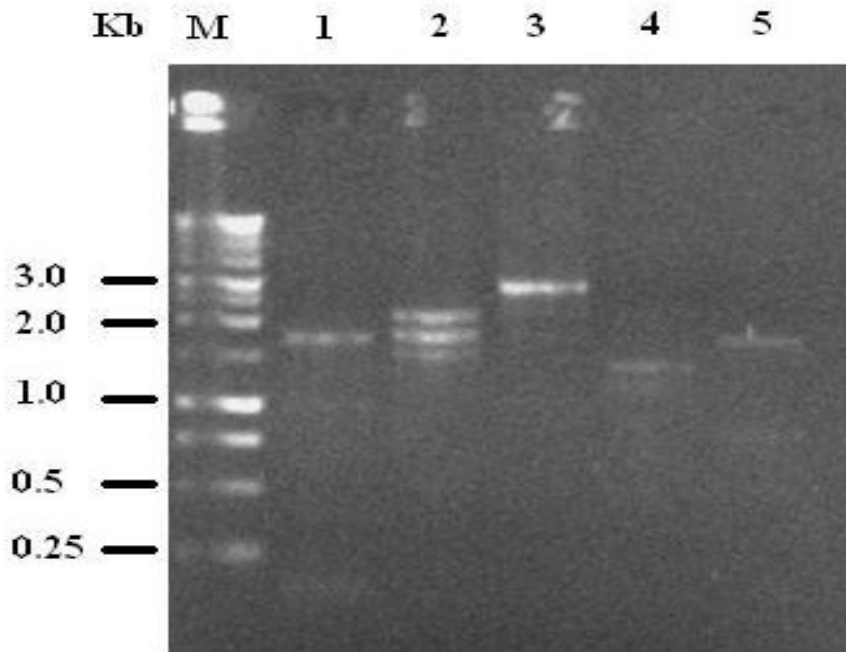
The Bc-Rep-PCR analysis of the seven DSMZ isolates did not produce many distinguishing bands. The bands from Lanes 3-6 were very similar to each other (Figure 5.1) and were estimated at approximately 1.8 kb. Lanes 1 and 7 had similar bands to each other and were distinct from the bands in Lanes 3-6 (Figure 5.1). These bands were estimated at approximately 2.9 kb. Lane 2 had two bands, estimated to be approximately 1.8 and 0.35 kb (Figure 5.1). This demonstrated that *B. thuringiensis* subsp. *thuringiensis* and *B. thuringiensis* subsp. *morrisoni* are closely related to each. Similarly the *B. thuringiensis* subsp. from Lanes 2-6 could be regarded as being related to each other, with one common band, although the second band of *B. thuringiensis* subsp. *aizawai* separated this subspecies from the other three (Figure 5.1).



**Figure 5.1.** Bc-Rep-PCR fingerprint profiles of the DSMZ *Bacillus thuringiensis* strains. Lane 1, *B. thuringiensis* subsp. *thuringiensis*; Lane 2, subsp. *aizawai*; Lane 3, subsp. *kurstaki*; Lane 4, subsp. *kenyae*; Lane 5, subsp. *nigeriae*; Lane 6, subsp. *tochigiensis*; Lane 7, subsp. *morrisoni*; Lane M, 1 kb molecular weight markers (Promega).



The bands obtained from Bc-Rep-PCR of the five isolates (NDR1, NDR2, NDR3, NDR5, and NDR11) were found to be distinct from each other (Figure 5.2). This demonstrated that these isolates were not closely related to each other. NDR1 shared a common band with NDR3 and NDR11, at approximately 1.8 kb (Figure 5.2). Isolate NDR1 and NDR11 were also not distinguishable from each other. NDR3 had three bands at 2.0 kb, 1.8 kb and 1.6 kb respectively (Figure 5.2). NDR5 produced one clear band at approximately 2.9 kb (Figure 5.2). NDR4 also produced only one band at approximately 1.5 kb (Figure 5.2).



**Figure 5.2.** Bc-Rep-PCR fingerprint profiles of the five local, entomopathogenic bacterial isolates. Lane M, 1 kb molecular weight markers (Promega); Lane 1, NDR 1 (*Bt*); Lane 2, NDR 3 (*Bt*); Lane 3, NDR 5 (*B. cereus*); Lane 4, NDR 2 (*B. laterosporus*); Lane 5, NDR 11 (*Bt*).

Bc-Rep PCR analysis was used to determine the relatedness of the five local entomopathogenic isolates, and to relate them to known serotype of *B. thuringiensis*. Overall, the DNA bands were not as clear and distinct as the bands obtained by Reyes-Ramirez and Ibarra (2005). The bands of the local isolates were markedly more distinct than the bands of the DMSZ isolates, which were not distinct. The bands of the DMSZ isolates were very similar to each other (Figures 5.1 and 5.2).

The serotyping was done to identify the *B. thuringiensis* strains and demonstrated that this method is limited because only one strain could be identified (Figure 5.2).

**Table 5.2.** Serotype determination of *Bacillus thuringiensis* isolates.

<b>Isolate</b>	<b>Bacterial Species</b>	<b>Serotype</b>
NDR 1	<i>Bacillus thuringiensis</i>	<i>kenyae</i>
NDR 3	<i>Bacillus thuringiensis</i>	Non-motile
NDR 11	<i>Bacillus thuringiensis</i>	Strong self agglutination

## 5.4. Discussion

Serotyping has several limitations, as discussed earlier, which were apparent when only one of three local *B. thuringiensis* strains could be serotyped (Table 5.2). Such a limited technique cannot be used as the basis for classifying *B. thuringiensis* strains because not all strains can be identified using this technique. The technique of Bc-Rep-PCR analysis is a powerful alternative to serotyping that is applicable to all strains of *B. thuringiensis*. However, the technique is difficult to standardize due to the number of variables that require optimization for each isolate. Laboratory conditions may differ from each other and the sources of known isolates may differ. As a result, it will be difficult to produce the same banding patterns at different laboratories (Figures 5.1 and 5.2). Equipment, chemicals, conditions and isolates vary from laboratory to laboratory. Resources differ from country to country. It is essential that inter-laboratory calibrations using identical *B. thuringiensis* cultures should be undertaken to ensure that different laboratories generate similar results. Further research in terms of standardization of current

laboratory protocols is required in order to determine whether certain variables may be eliminated to create a more streamline process. Problems arose when following the Bc-Rep-PCR protocol of Reyes-Ramirez and Ibarra (2005). They claimed that their technique is quick and easy to conduct. In practice, it has proven time consuming and laborious, and some runs were not successful. The magnesium chloride optimization for the DMSZ group was at 5 mM of MgCl<sub>2</sub>, similar to that of Reyes-Ramirez and Ibarra (2005). However, the MgCl<sub>2</sub> optimization varied for each of the local isolates, ranging from 2 mM to 5 mM. Therefore, the quantity of 5 mM of MgCl<sub>2</sub> is not a universally applicable quantity. This is in spite of Reyes-Ramirez and Ibarra (2005) having stated that this fixed quantity is all that is required for optimization.

The Bc-Rep-PCR banding patterns of NDR1, *B. thuringiensis* subsp. *kenyae* (Figure 5.2), were compared to that of the DMSZ group *B. thuringiensis* subsp. *kenyae* (Figure 5.1). The banding patterns of these two subspecies of *B. thuringiensis* were found to be similar to each other (Figures 5.1 and 5.2). This demonstrates that these two isolates are the same organism. The Bc-Rep-PCR banding pattern of the *B. thuringiensis* subsp. *morrisoni* isolate obtained from the DMSZ was not similar to that published by Reyes-Ramirez and Ibarra (2005). The banding patterns published by Reyes-Ramirez and Ibarra (2005) did not have a band at 3.0Kb (Figure 5.1). This observation confirmed that the current protocol for Bc-Rep-PCR is not as reliable as claimed.

A further confounding factor is that various natural inhibitors may be present in the extracted DNA although the manufacturer claims that their DNA isolation kit does eliminate inhibitors (Mobio) (Lambo, 1994). The optimization with MgCl<sub>2</sub> does affect PCR reactions and this could be the main source of discrepancies (Lambo, 1994). This could affect the binding of the primers to the template DNA hence resulting in relatively fewer bands being detected (Lambo, 1994). Other unknown factors may affect the PCR reactions. These protocols are sensitive and the reproducibility of amplification patterns may be affected by factors such as precise concentrations, cycling temperature and differences in parameters (Damiani *et al.*, 1996).

DSMZ *B. thuringiensis* subspecies only distinguished between three of the seven subspecies (Figure 5.1). Four of the *B. thuringiensis* subspecies could not be distinguished from each other

using this method (Figure 5.1). When comparing the results of these two groups of entomopathogenic spore-formers, *B. thuringiensis* subsp. *kenyae* produced a similar sized band as Isolates NDR1 and NDR11 (Figures 5.1 and 5.2). It was not surprising that NDR1 shared a common band with *B. thuringiensis* subsp. *kenyae* as NDR1 had been identified as *B. thuringiensis* subsp. *kenyae* (Table 5.2). The common band shared between NDR1, NDR3 and NDR11 was not surprising because all three isolates are subspecies *B. thuringiensis* (Figure 5.2). NDR5 and NDR2 did not share any common bands with any of the isolates in Figure 5.2. This was expected because these two isolates are not *B. thuringiensis* subspecies. However NDR5 did share a common band with *B. thuringiensis* subsp. *thuringiensis* at 2.9kb (Figures 5.1 and 5.2). NDR5 is an isolate of *B. cereus* and it has been suggested that *B. cereus* and *B. thuringiensis* may be the same species of *Bacillus*, with the only difference being that *B. thuringiensis* produces crystal proteins (Bernhard *et al.*, 1997; Priest *et al.*, 2004).

The primers used for the Bc-Rep-PCR were meant to be *B. cereus* Group specific. However, isolate NDR2 was an isolate of *B. laterosporus* that did not belong to this group. Hence it was surprising to find that the Bc-REP PCR generated clear bands for Isolate NDR2. This result may have been due to random REP-PCR amplification. This demonstrates another flaw in the technique as an alternative to serotyping for *B. thuringiensis* and closely related entomopathogenic bacteria.

Only one of the isolates was identified with serotyping (Table 5.2). NDR1 was serotyped as *B. thuringiensis* subsp. *kenyae*. No prior research was found that reported toxicity by isolates of *B. thuringiensis* subsp. *kenyae* towards Coleoptera. Hence this isolate is the first of *B. thuringiensis* subsp. *kenyae* reported to show toxicity to this insect order. Serotypes such as *B. thuringiensis* subsp. *morrisoni* are toxic towards Lepidoptera, Diptera and Coleoptera.

*Brevibacillus laterosporus* was unfortunately not serotyped. Isolate NDR5 (*B. cereus*) could not be serotyped because the Graduate School of Agriculture, Kyushu University in Japan only serotypes *B. thuringiensis* isolates although *B. cereus* can be serotyped (Ohba, 2005).

Serotyping is a technique for identifying strains of *B. thuringiensis*. However, its limitations have become apparent as more of *B. thuringiensis* and related bacteria are isolated (Reyes-Ramirez and Ibarra, 2005). Some of the problems that have been highlighted include:

1. *B. cereus* and *B. thuringiensis* isolates sometimes have a strong cross reactivity (Ohba and Aizawa, 1986);
2. Serotyping is unable to discriminate between closely related intra-subspecies strains such as *B. thuringiensis* subsp. *entomocidus* and *B. thuringiensis* subsp. *subtoxicus* (Joung and Côté, 2001).
3. Different esterase patterns are present on different serotypes (Lysenko, 1983). Cross-reactions due to these patterns make it difficult to differentiate between the subspecies.
4. Serotyping is not a good indicator of pathogenic specificity (Kriege *et al.*, 1987). *B. thuringiensis* subsp. *morrisoni* isolates have been found that are pathogenic to Lepidoptera, Diptera and Coleoptera (Thiery and Frachon, 1997).
5. Serotyping is a laborious and time-consuming technique that involves the production of flagellar antisera in rabbits (Thiery and Frachon, 1997).

Given these problems, many institutes have since abandoned serotyping. As such, serotyping is probably no longer valuable as a method of identifying *B. thuringiensis* strains. Reyes-Ramirez and Ibarra (2005) could not distinguish between various subspecies such as *B. thuringiensis* subsp. *amagiensis* and *B. thuringiensis* subsp. *seoulensis*; subsp. *sotto* and subsp. *Dakota*. These subspecies can however be identified from each other through serotyping (Reyes-Ramirez and Ibarra, 2005). Databases based on these techniques may thus be of limited value. However, the replacement technology of Bc-Rep-PCR is also not selective enough because different strains cannot be distinguished from each other by this method. This technique needs to be conducted in conjunction with serotyping to identify subspecies. However, this is not always applicable as not all subspecies are serotypable. A more sensitive and more stable technique is required for the classification of *B. thuringiensis* and related bacteria.

## 5.5. References

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## Chapter Six

# Electron microscopy of some entomopathogenic spore-forming bacterial isolates

### Abstract

Transmission electron microscopy (TEM) and environmental scanning electron microscopy (ESEM) were used to analyse five isolates of entomopathogenic, spore-forming bacteria. Four of the five isolates formed crystal proteins. Bipyrarnidal, cuboidal, triangular and sickle shaped crystal proteins were observed. Three of the isolates were *Bacillus thuringiensis* strains. The other two were a strain each of *B. cereus* and *Brevibacillus laterosporus*. Crystal proteins were either attached to the spore or separated from it. ESEM was not an effective technique for the viewing of the crystal protein of the *B. laterosporus* isolate because this technique did not differentiate between spore and crystal easily.

### 6.1. Introduction

*Bacillus thuringiensis* Berliner and *Brevibacillus laterosporus* Laubach are Gram-positive endospore-forming bacteria that produce Insecticidal Crystal Proteins (ICPs). The ICPs are formed within bacterial cells during spore formation and are released into the environment after complete sporulation (Schnepf and Whiteley, 1981).

The best known *B. thuringiensis* subspecies are *B. thuringiensis* subsp. *israelensis*, *kurstaki* and *tenebrionis* which are effective against three insect orders, Diptera, Lepidoptera and Coleoptera, respectively. Some *B. thuringiensis* subspecies are effective against only a few species of insects, while other subspecies may be effective against an array of species (Kumar *et al.*, 1996).

*Brevibacillus laterosporus* produces ICPs with different shapes and has a lateral spore which gives the bacterial cells their characteristic canoe shape (Zahner *et al.*, 1999). These ICPs have

been recorded to be effective against mosquitoes, Lepidoptera, Coleoptera and Mollusca (Favret and Yousten, 1985; Zahner *et al.*, 1999; De Oliveira *et al.*, 2004)

The insecticidal crystal shape is sometimes used as an indicator as to which insects the toxins may be effective against. However, this is not a specific approach as crystals effective against Coleoptera may be spherical, bipyramidal, plate-like or triangular (Khetan, 2001). This research aimed to describe the crystal proteins of five spore-forming bacterial isolates using transmission electron microscopy (TEM) to establish the shape and spatial orientation of the crystal proteins.

## 6.2. Materials and Methods

Standard microbiological techniques were followed in the laboratory for the routine assays (Prescott *et al.*, 1999; Wheelis and Segel, 1979).

**6.2.1. Samples:** Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* spp. collected in sugarcane producing areas in KwaZulu-Natal, (KZN), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 6.1).

**6.2.2. Sample collection:** Mushroom compost, compost and grain dust were collected in 250 ml sterile honey jars (Table 6.1). Honey jars were pre-sterilized in an autoclave at 121°C for 15 min. Diseased *T. molitor* larvae were collected in 90 mm sterile Petri dishes. Adult beetles and white grubs were collected from light traps and soil, respectively, from the sugarcane areas in the KwaZulu-Natal midlands, KZN. These were provided by the South African Sugar Research Institute (SASRI), Mount Edgecombe, KZN. Diseased grubs were removed from these containers and placed into sterile Petri dishes. All the samples were stored at 4°C in a refrigerator.

Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* sp. (collected in sugarcane areas in KwaZulu-Natal), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 6.1).

**Table 6.1.** Samples sources from which entomopathogenic endospore-formers were isolated

Samples	Source of sample	Species
Mushroom compost	Mushroom farm in Karkloof area in the KwaZulu-Natal midlands	-
Mealworm larvae	Insect rearing facility at the University of KwaZulu-Natal	<i>Tenebrio molitor</i>
Compost	A garden compost heap in Hillcrest, KwaZulu-Natal	-
Grain dust	Chicken grain storage facility in Pietermaritzburg, KwaZulu-Natal	-
Adult beetles	Dalton, Bryuns-Hill and Harburg, KwaZulu-Natal	<i>Schizonycha</i> spp. and <i>Hypopholis</i> spp.
White grubs	Dalton, KwaZulu-Natal	<i>Schizonycha</i> spp.

**6.2.3. Isolation of endospore-forming bacteria:** Isolation of *Bacillus* sp. was conducted using a similar pasteurization method as described by Ohba and Aizawa (1978). Approximately 0.5 g of each sample was suspended in 10 ml sterile distilled water. Suspensions were shaken vigorously for 30 sec at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 hr at room temperature. Suspensions were vortexed a second time at full speed for 30 sec and then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 hr at 30°C in an incubator and examined for colonies with typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or colonies with an ‘ice crystal’ appearance with a colony diameter larger than 2 mm (Damgaard *et al.*, 1997; Prescott *et al.*, 1999; Selvakumar *et al.*, 2007). Various other white coloured bacterial colonies that predominated and were larger than 2 mm in diameter were also selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and counted using a colony counter. Selected colonies were then purified by sub-culture onto nutrient agar and incubated for 3 d at 30°C in an incubator. Colonies were stored on nutrient agar slants.

Selected isolates were subjected to Gram staining as well as Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 sec with distilled water and blotted dry with tissue paper (Kimberly-Clark, SA) (Ammons *et al.*, 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 x oil immersion objective lens (Bernhard *et al.*, 1997; Young *et al.*, 1998). Crystal proteins stained dark blue (Ammons *et al.*, 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9  $\mu\text{m}$ , were also selected, in order to include *B. cereus* which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because this fits the criteria of *B. cereus*, *B. thuringiensis* and *B. laterosporus* cells (Figure 6.1). Selected cultures were purified by sub-culturing and were assigned numbers NDR1-NDR14 (Chapter 2).

#### **6.2.4. Electron microscopy**

**6.2.4.1. Transmission Electron Microscopy (TEM):** Sterile nutrient broth Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (100 ml) was inoculated with each of the five isolates (NDR1, NDR2, NDR3, NDR5 and NDR11) (Chapter 2), respectively. The broth was incubated at 30°C in a shaker water bath at 250 rpm for 48 hr or until spores and crystals were clearly visible in the cells (Meadows *et al.*, 1992). This was determined using the Coomassie Blue staining technique (Ammons *et al.*, 2002).

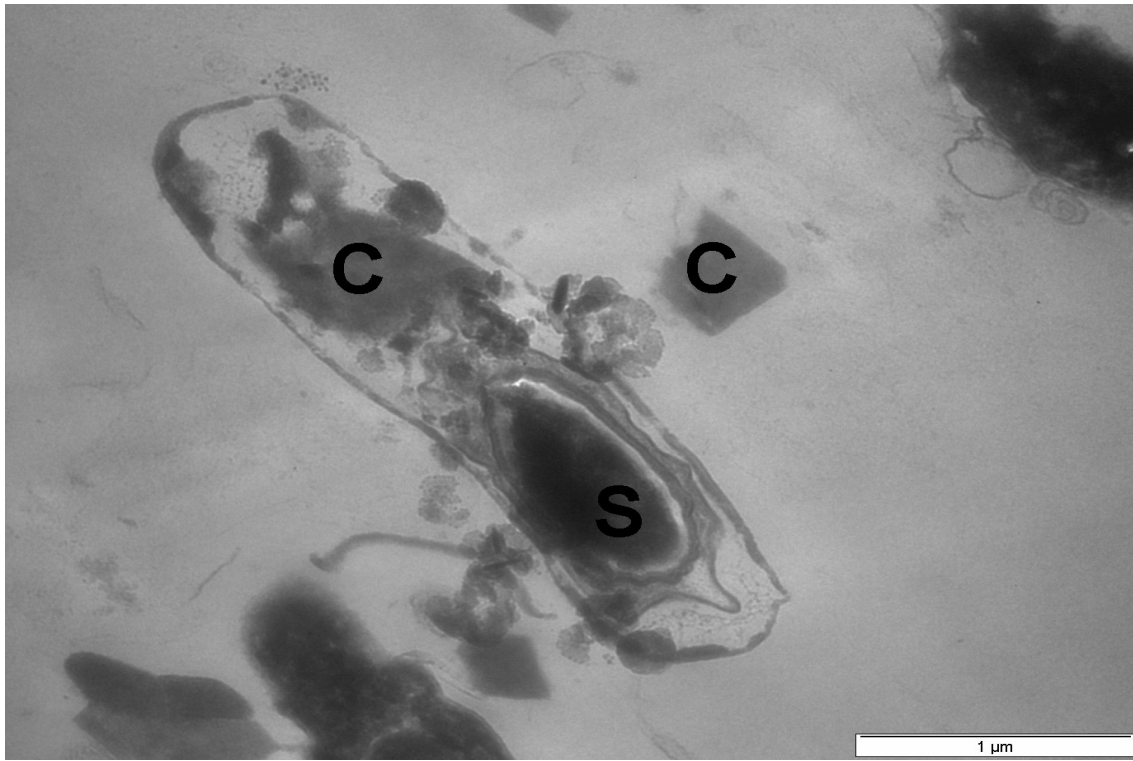
Aliquots of 1.5 ml of the bacterial cultures were placed into 2 ml Eppendorf tubes and centrifuged on a Bio-Rad bench centrifuge at 10,000 rpm for 15 min. Pellets consisting of the bacterial cells and crystal proteins were retained and the supernatant was discarded. The pellets were fixed in 3% glutaraldehyde in the Eppendorf tubes overnight, after which they were washed twice in a 0.05M cacodylate buffer (pH 7.4). Each wash was for 15 min. The pellets were broken up as much as possible with a tooth pick and stained with 1% osmium tetroxide for 4 h (Ibarra and Federici, 1986). This enabled the osmium tetroxide to penetrate the clumps of cells and stain the cells better.

Dehydration of the pellets was achieved using an ethanol dehydration series of 20, 30, 50 70, 80, 90 and 100%. Samples were embedded in Epon-Araldite resin and sectioned with glass knives using an ultramicrotome. Sections were stained with uranyl acetate and lead citrate before viewing. A Phillips TEM 120 Biotwin was used to view the sections.

**6.2.4.2. Environmental Scanning Electron Microscopy (ESEM):** The four crystal-producing isolates (NDR1, NDR2, NDR3 and NDR11) (Chapter 2) were streaked onto nutrient agar plates (Biolab) and incubated at 30°C for 48 hr or until spores and crystals were present in the cells. This was initially determined using light microscopy on bacterial cultures stained with the Coomassie Blue staining technique (Ammons *et al.*, 2002). A standard microbiological technique for making bacterial microscope slides was used to prepare the slides but slides were not heat fixed (Prescott *et al.*, 1999). The slides were then viewed as is under ESEM.

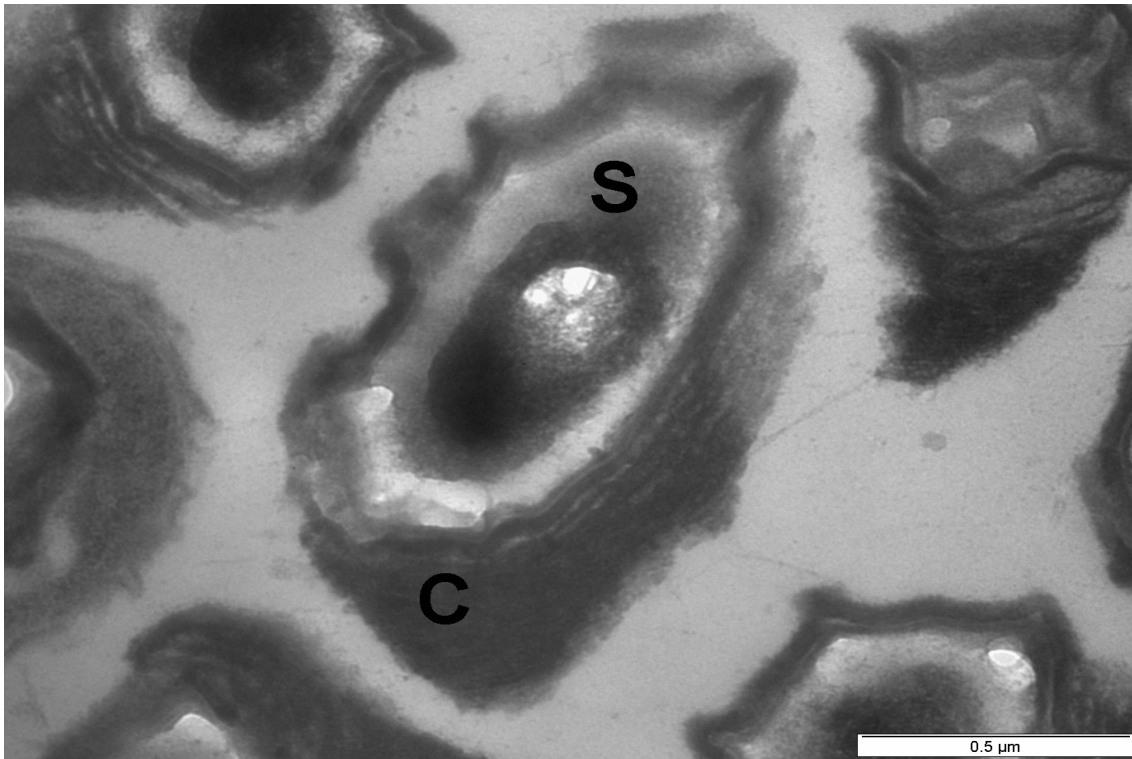
### 6.3. Results

The crystal proteins of each *B. thuringiensis* strain were clearly evident within the sporulating cell. Crystals that were found appeared either bipyramidal or diamond shaped. Some crystals had been released from cells that had sporulated completely. A released crystal in the shape of a diamond was also clearly visible next to a sporulating cell (Figure 6.1).



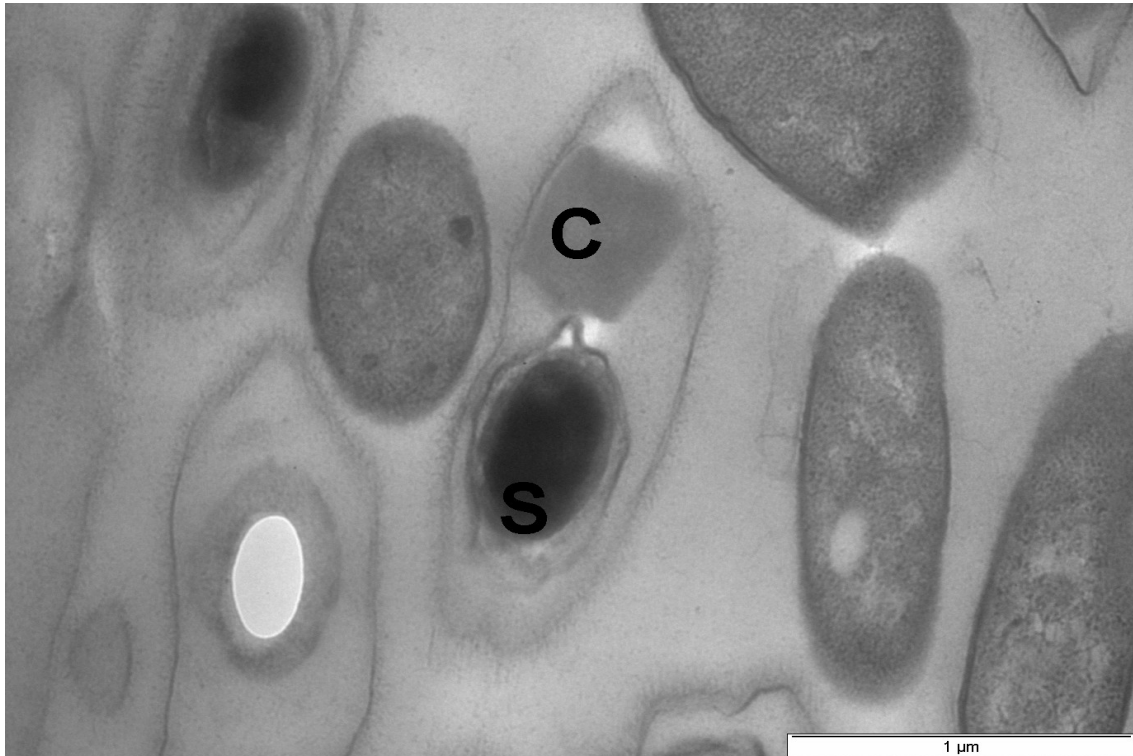
**Figure 6.1.** *Bacillus thuringiensis* isolate NDR1 before complete sporulation, with spore (S) and crystal protein (C) present in a bacterial cell.

Crystal proteins of a strain of *B. laterosporus* Isolate NDR2 (Figure 6.2) remained attached to the spores, even after complete sporulation. The shapes of the crystals were not uniform. Some crystal proteins had irregular, rectangular shapes, while others were more triangular.



**Figure 6.2.** *Brevibacillus laterosporus* Isolate NDR2 after complete sporulation, with the crystal protein (C) attached to the spore (S).

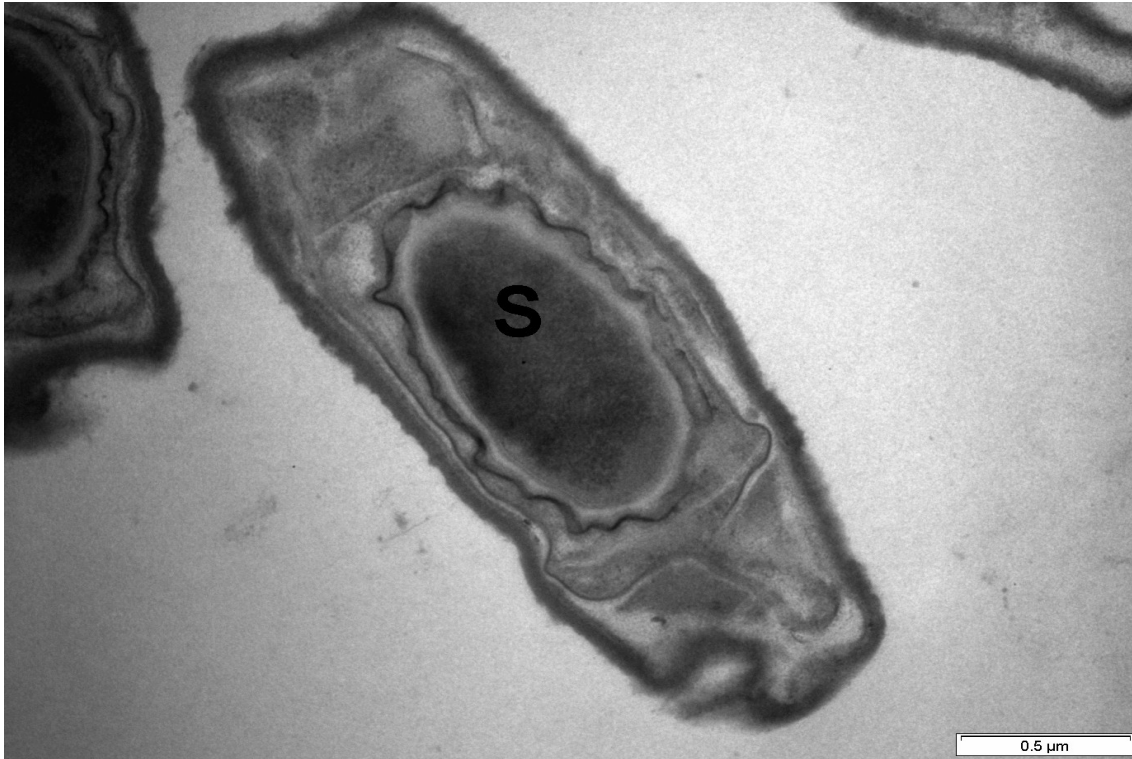
The crystal proteins of *B. thuringiensis* Isolate NDR 3 (Figure 6.3) were either square or triangular shaped. In light microscope images, the crystal proteins were more triangular and remained in contact with the spore, even after complete sporulation (Chapter 2).



**Figure 6.3.** *Bacillus thuringiensis* Isolate NDR3 before complete sporulation, with spore (S) and ICP (C) present in cell.

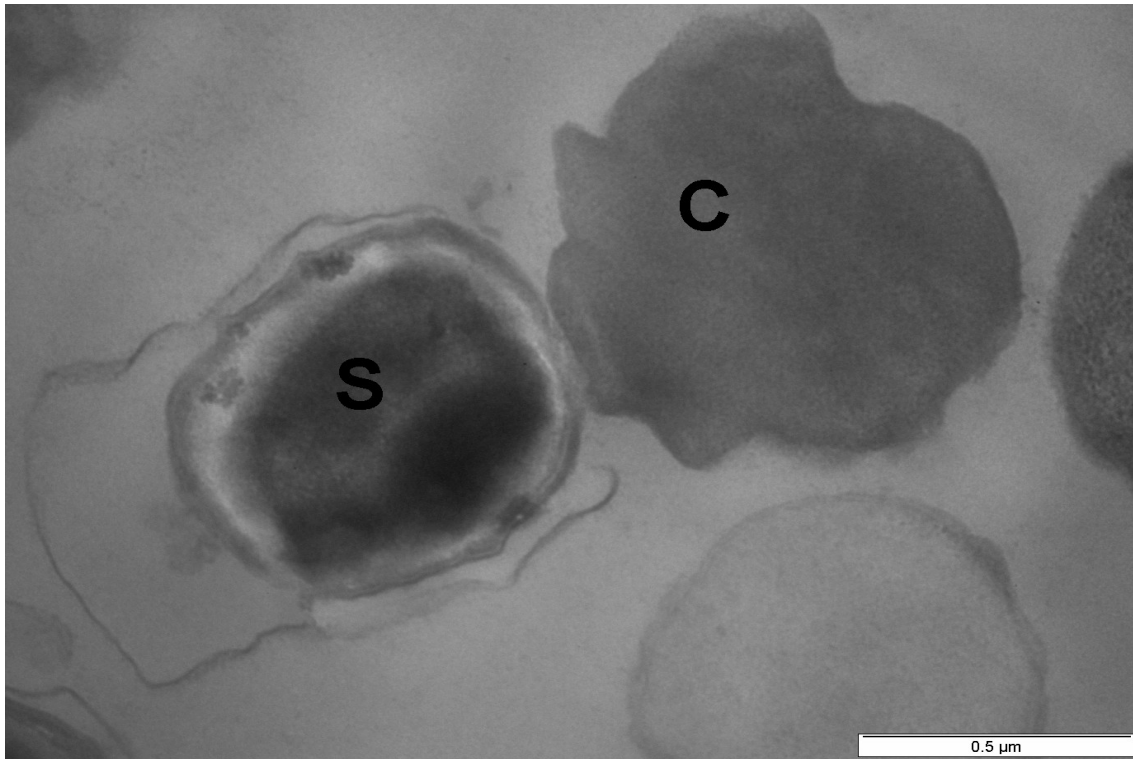


Isolate NDR5 was a strain of *B. cereus*. This bacterium did not synthesize crystal proteins (Figure 6.4), although this isolate displayed toxicity towards members of the Coleoptera (Chapter 2).



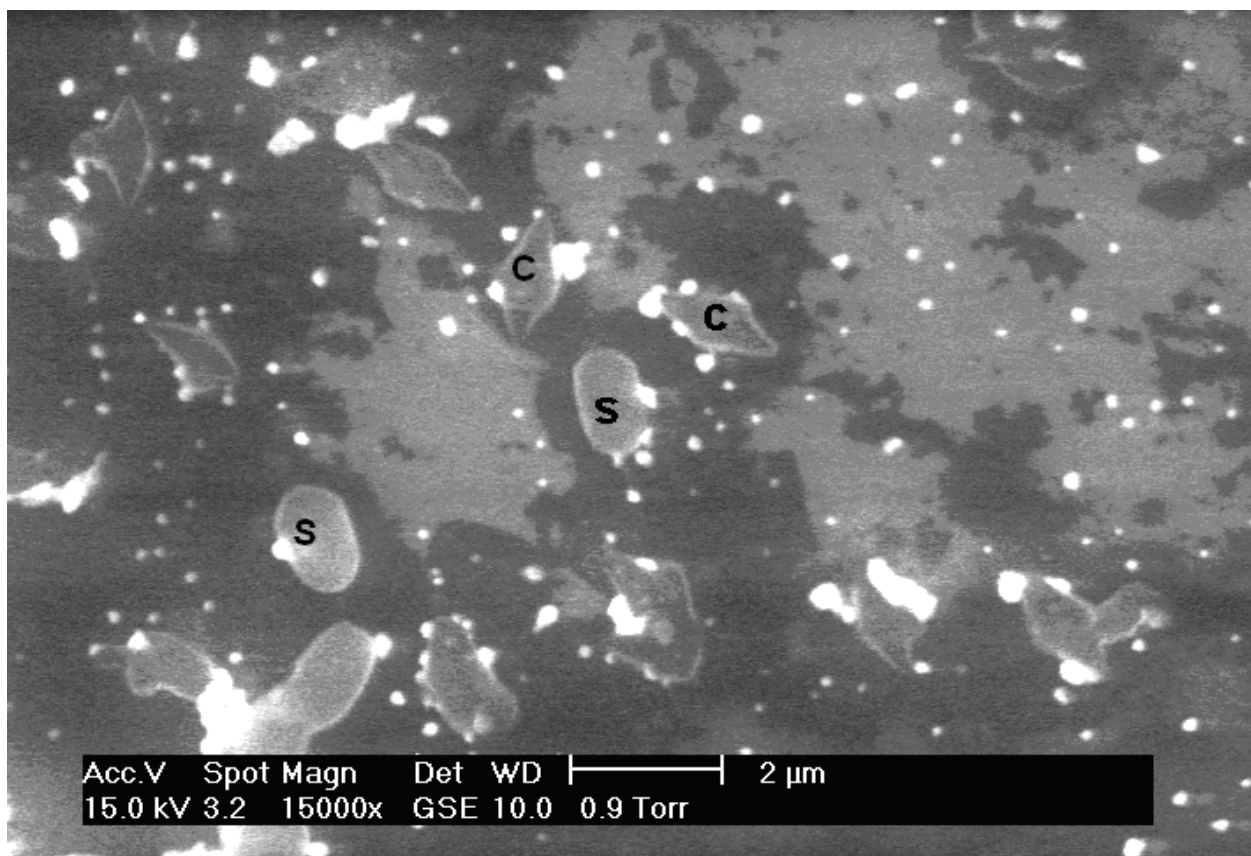
**Figure 6.4.** TEM of *Bacillus cereus* Isolate NDR5 before complete sporulation, displaying a spore (S) but no crystal protein.

The crystal proteins of *B. thuringiensis* Isolate NDR11 appeared to be irregular or spherical, and remained in contact with the spore (Figure 6.5). In light microscope images the spores and crystals of NDR11 were not attached to the spore (Chapter 2).



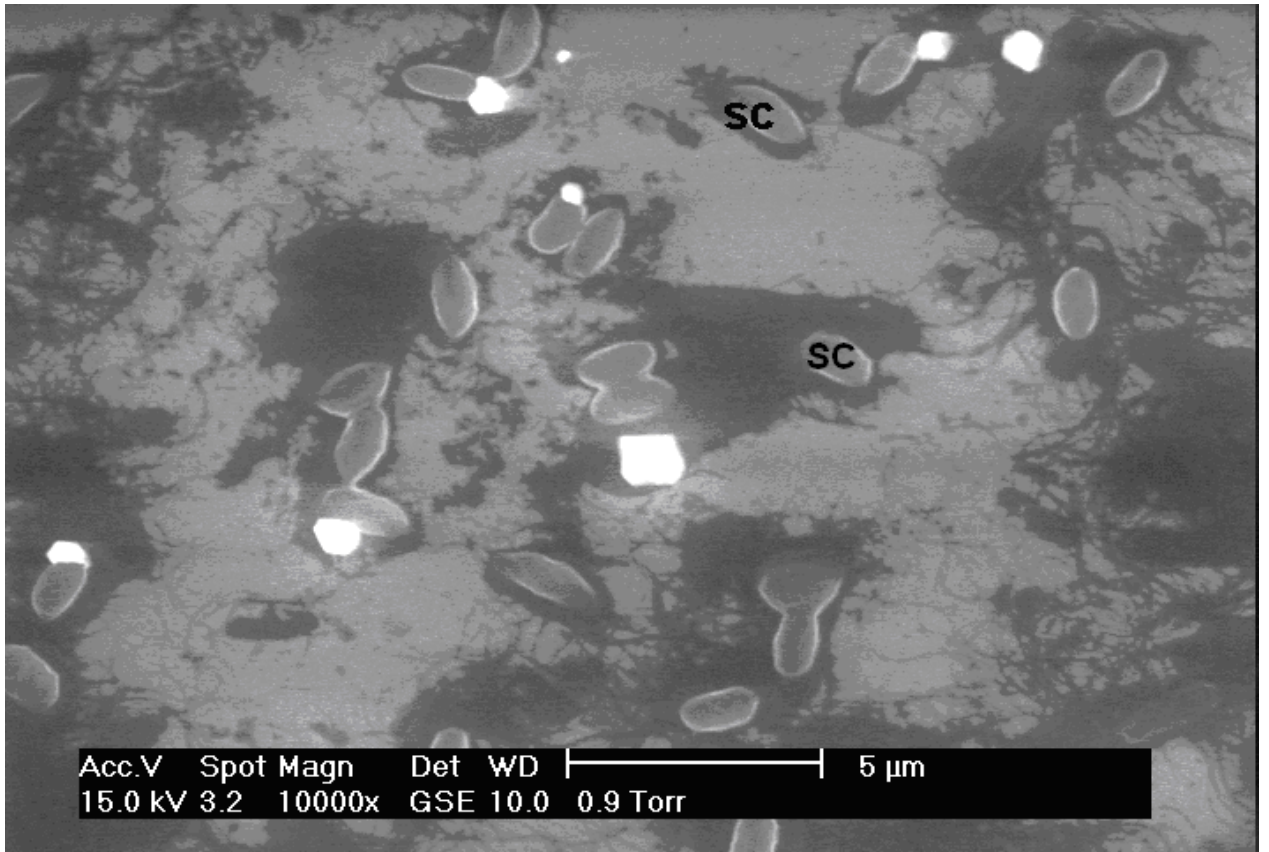
**Figure 6.5.** *Bacillus thuringiensis* Isolate NDR11 after sporulation, with a protein crystal (C) situated near a spore (S). The crystal proteins were irregular or spherical.

The spores and crystal proteins of *B. thuringiensis* Isolate NDR1 were clearly visible in the ESEM image (Figure 6.6). The samples were not sputter coated and were in their original air-dried state. The spores and crystal were subjected to high vacuum conditions that caused the bacterial cells to collapse but not the hard spores and crystal proteins.



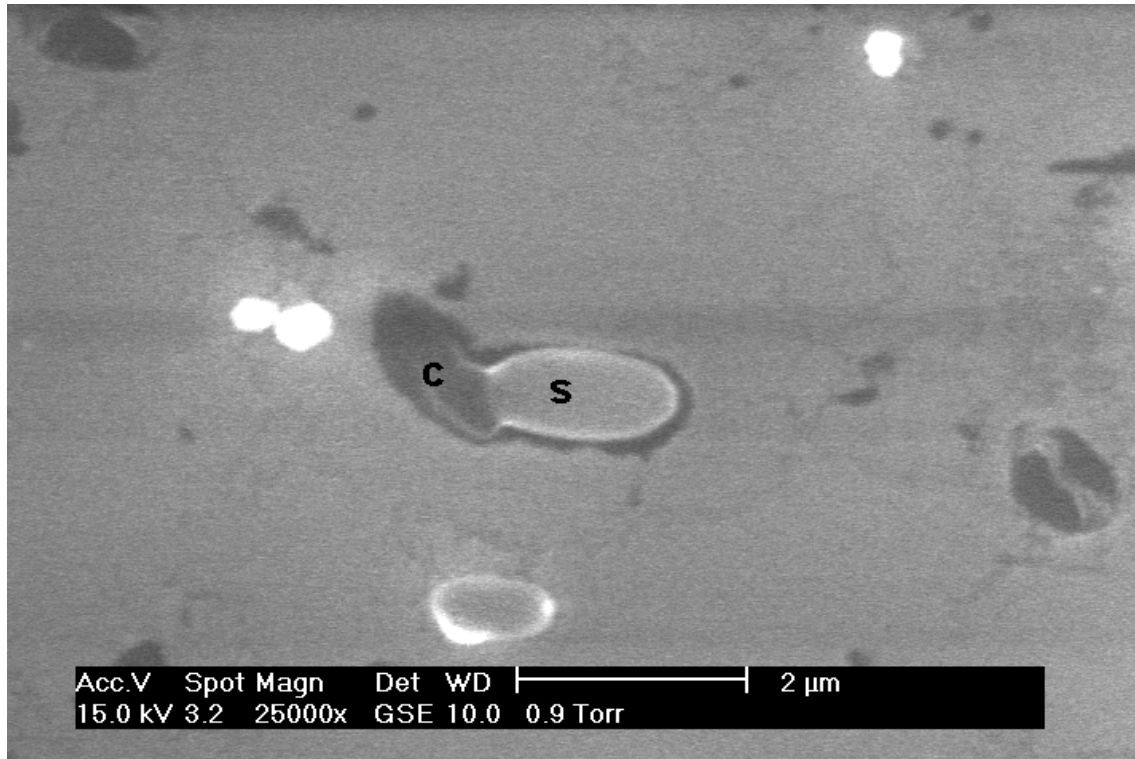
**Figure 6.6.** Environmental scanning electron microscope image of *Bacillus thuringiensis* Isolate NDR1 with a spore (S) and crystal protein (C).

The crystal proteins for Isolate NDR2 (*B. laterosporus*) were not as clearly visible as the crystal proteins of the *B. thuringiensis* isolates (Figure 6.7). This may have been a result of attachment of the spore and crystal proteins to each other thereby creating an indistinct structure.



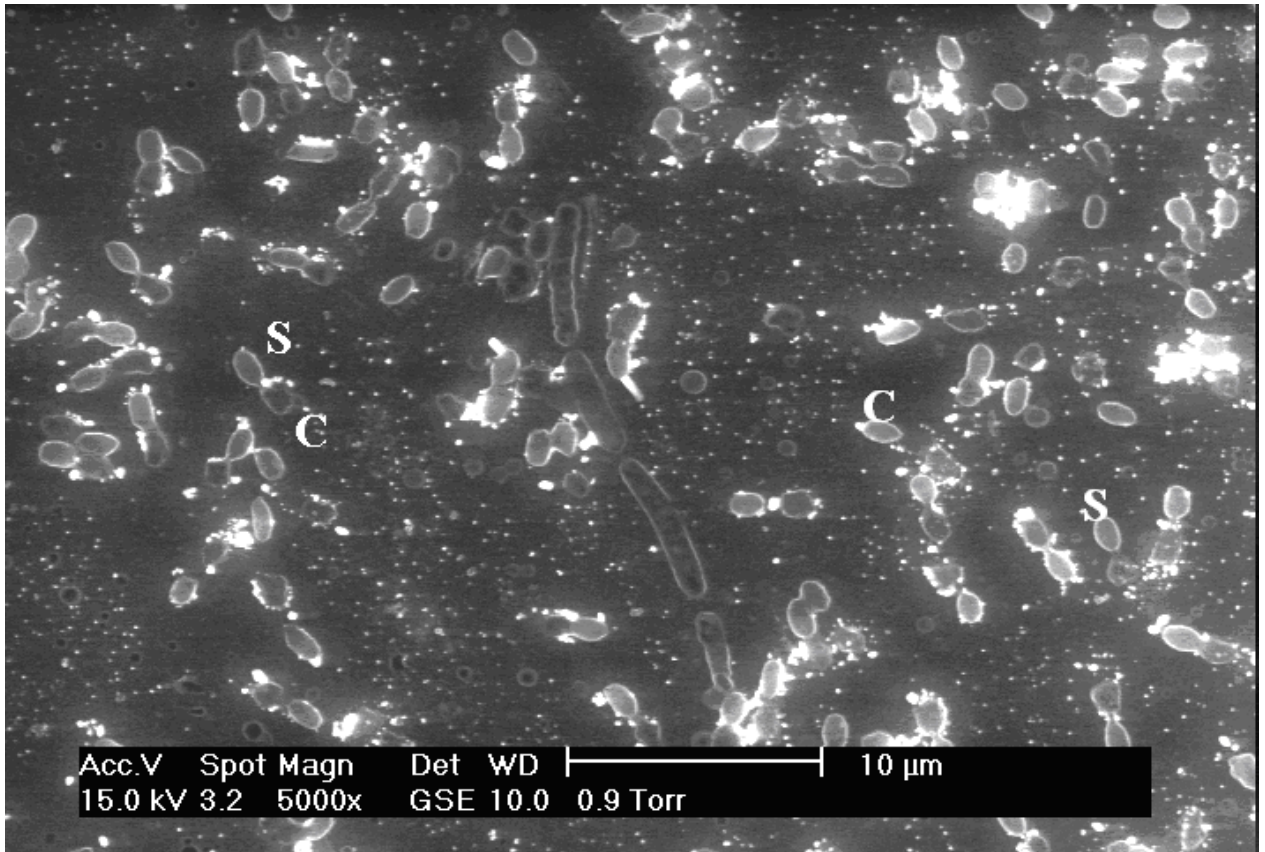
**Figure 6.7.** Environmental scanning electron microscope image of *Brevibacillus laterosporus* Isolate NDR2, with a spore (S) and crystal (C) attached to each other. Flagella are clearly visible in this image.

A triangular shaped attachment was visible in an ESEM image of *B. thuringiensis* Isolate NDR3 (Figure 6.8). This supported what had been observed during light microscopy studies (Chapter 2; Figure 2.2C), where the triangular shaped crystal proteins were attached to the spores.



**Figure 6.8.** Environmental scanning electron microscope image of *Bacillus thuringiensis* Isolate NDR3, with a spore (S) and crystal (C) attached to each other.

In ESEM micrographs of *B. thuringiensis* Isolate NDR11 (Figure 6.9), numerous white flecks were visible. These were most likely artifacts created by static interferences from the glass slides on which the samples were fixed (White, 2006).



**Figure 6.9.** Environmental scanning electron microscope image of *Bacillus thuringiensis* Isolate NDR11, with a spore (S) and crystal (C). The majority of crystals are separated from the spores.

ESEM images of the isolates with crystal proteins were taken to illustrate spores and crystal proteins from a surface three dimensional perspective (Figures 6.6-6.9). This technique does not subject samples to harsh chemical treatments. The crystal proteins were evident in all the *B. thuringiensis* isolates. One image of Isolate NDR3 depicted a spore attached to the crystal protein (Figure 6.8). The crystal proteins for *B. laterosporus* were not clearly evident (Figure 6.7). However, these crystal proteins were visible during TEM analysis (Figure 6.2).

## 6.4. Discussion

This study was conducted to get a clearer image of the spores and crystals of five local entomopathogenic bacterial isolates. This study also confirmed that Isolate NDR5 did not produce crystal proteins (Figure 6.4). The presence or absence of a crystal protein is the primary distinguishing feature between *B. thuringiensis* and *B. cereus* (Khetan, 2001).

Figures 6.1-6.5 display TEM images of the five spore forming isolates. Some crystal proteins were separated from the spores, as was found with *B. thuringiensis* Isolates NDR1 and NDR11 (Figures 6.6 and 6.9). These strains do not have crystal proteins attached to their exosporium (Figures 6.6 and 6.9). It could be speculated that free crystal proteins may result in an increase in the protein toxin concentration that an insect may consume. This could be a result of the crystal proteins being more exposed once they are released and are thus not protected by the exosporium. This should accelerate the speed of action by the toxins. Note that the crystals on their own can cause insect death in the absence of spores, although the spores do have a synergistic effect (Asano *et al.*, 2000).

Spore-crystal packages were visible for Isolates NDR2 (*B. laterosporus*) and NDR3 (*B. thuringiensis*) (Figures 6.2 and 6.8). These isolates formed a complete package of toxin and spore that would be ingested by a target insect. This may be viewed as a more secure form of toxin delivery because the toxins and spores act in a synergistic manner. Initially, the toxins cause perforations in the insect midgut cells, releasing the contents of the haemocoel (Khetan, 2001). The result is an influx of nutrients into the midgut due to damaged midgut cells which, in turn, encourages bacterial spores to sporulate and proliferate. Subsequently, the susceptible host dies of bacterial septicemia. These spore-crystal packages are known to be more effective than the separated spores and crystals (Asano *et al.*, 2000).

However, separated crystals and spores appear to be equally effective if they are ingested at the same time. Even if ICPs are consumed without the spores, septicemia also results from opportunistic bacterial species multiplying rapidly in the haemocoel after the midgut wall is perforated by the crystal toxin (Lambert and Peferoen, 1992). Some species may have the crystal attached to the spores after sporulation or the crystal may become detached after complete

sporulation (De Oliveira *et al.*, 2004). Insecticidal crystal proteins have proven effective, e.g., plants genetically modified with *B. thuringiensis* genes for the expression of *B. thuringiensis* ICPs are effective against insect pests (Brunke and Meeusen, 1991; Feitelson *et al.*, 1992).

Isolates NDR1, NDR3 and NDR11 were *B. thuringiensis* isolates. The shapes and sizes of strains of this species have been documented extensively (Khetan, 2001; Whiteley and Schnepf, 1986). Crystal shape cannot be used to predict insecticidal activity but is often characteristic of a particular strain (Lecadet *et al.*, 1999). The bipyramidal shape of the crystal protein of Isolate NDR1 was characteristic of *B. thuringiensis* isolates (Khetan, 2001; Whiteley and Schnepf, 1986). The shape of the crystal protein of Isolate NDR3 appeared more cuboidal from TEM analysis (Figure 6.3) than from light microscopy analysis (Chapter 2; Figure 2.1c). The crystal protein shapes of Isolate NDR11 were spherical and very similar to the shapes detected using light microscopy. Square shaped crystal proteins have also been documented by Orlova *et al.* (1998).

Isolate NDR2 was a strain of *B. laterosporus*. The crystals of this species have not been documented as extensively as *B. thuringiensis* strains. Images similar to Figure 6.2 and Figure 6.7 have not been documented previously. These images may thus be viewed as a step forward in the research of this species. Previously, the insecticidal activity of *B. laterosporus* was considered to be less effective than *B. thuringiensis* against insects. This may be the reason for the lack of published images of its crystals (De Oliveira *et al.*, 2004; Orlova *et al.* 1998).

The crystal proteins are large structures that are clearly visible through the use of light and electron microscopy. The harsh embedding process required for TEM analysis did not affect the crystal proteins. The crystals did not seem to dissolve or lose their shape as a result of the embedding process. Under the ESEM, the released crystal proteins were also clearly visible. This was due to the protective exosporium that keeps the spore and crystal protein attached to each other. Both microscopy techniques provided images of the isolates and their spores and crystal proteins. Both light (Chapter 2) and TEM analysis generated a clear image of the spores and crystal proteins. In contrast, ESEM analysis only allowed for a view of the surface of the spores and crystal proteins. Given that ESEM does not use harsh chemical preparations, it was



expected that it would generate images of the spores and crystals of the isolates without any artifacts. However, ESEM generated static interference because of interactions with the glass slides.

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# Chapter Seven

## Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of insecticidal crystal proteins

### Abstract

SDS-PAGE was conducted to determine the number and size of the insecticidal crystal proteins (ICPs) synthesized by four isolates of entomopathogenic spore-forming bacteria. Only three of the four bacteria produced ICPs with SDS-PAGE profiles, of which two were strains of *B. thuringiensis* and one was a strain of *B. laterosporus*. Large sized proteins bands were present in the range of 73-kDa to 135-kDa for the *B. thuringiensis* strains. All three isolates had smaller proteins in the range of 13-kDa to 44-kDa. One strain of *B. thuringiensis*, NDR11, produced an unusual large protein of 200-kDa. Small proteins in the range of 24-kDa to 40-kDa were produced by *B. laterosporus*. The Cry ICPs of isolate NDR3 did not produce any electrophoretic bands, indicating that its ICP did not dissolve under the conditions used.

### 7.1. Introduction

*Bacillus thuringiensis* Berliner produces various toxins, including insecticidal toxins. These toxins include delta-endotoxins, beta-exotoxins, alpha-exotoxins and vegetative insecticidal proteins (VIPs) (Khetan, 2001). Delta-endotoxins are the most common and most studied of these (Höfte and Whiteley, 1989). The toxins are proteins that crystallize into large structures that are visible under light microscopy (Ammons *et al.*, 2002). These toxins have been classified into several classes, including Cry and cytolytic Cyt toxins. There are two primary ranks of Cyt toxins, and more than 55 primary ranks of identified Cry toxins. These toxins are subdivided further, based on the homology of the amino acids of the specific genes involved (Cry or Cyt genes) (Crickmore *et al.*, 2008). To date these toxins have been found to be active against Diptera, Lepidoptera, Coleoptera, Mollusca and nematodes (Kumar *et al.*, 1996).

Beta-exotoxins are not proteins and are non-specific. To date, these toxins are effective against Diptera, Lepidoptera and Coleoptera. These toxins inhibit RNA polymerase, affecting molting and pupation of insects. Unfortunately, these toxins are also toxic to mammals and therefore commercial insecticide formulations cannot contain these toxins (Epinasse *et al.*, 2002).

Alpha-exotoxins (phospholipase C and lecithinase C) have not been investigated as extensively as the other toxins. These toxins are toxic to insects and mice (toxicity to mice is only when they are injected with the toxin). Only some strains of *B. thuringiensis* synthesize these toxins (Khetan, 2001). Phospholipase is a metalloprotease that degrades antibacterial proteins secreted by insects and thus prevents the antibacterial proteins from affecting germinated *B. thuringiensis* spores (Lövgren *et al.*, 1990).

VIP toxins have a similar mode of action to that of Cry and Cyt toxins, causing pores to form in the midgut epithelium of susceptible insects, which have ingested the VIP toxins. These proteins are not related to the Cry or Cyt toxins and are synthesized during the vegetative stage of bacterial growth (Yu *et al.*, 1997). The VIP nomenclature consists of three main classes: Vip1, Vip2 and Vip3 (Rang *et al.*, 2005). Vip1 and Vip2 are toxic components of a binary toxin that is effective against Coleoptera (Warren, 1997).

*Bacillus thuringiensis* is one of many species of bacteria capable of synthesizing chitinase (Regev *et al.*, 1996). This enzyme is insecticidal, attacking the skeletal molecule of insects, which is chitin. However, substantial quantities of any one type of chitinase are required to kill insects, which limits its potential for direct insecticidal action. A redeeming aspect is that combinations of different chitinases are synergistic and therefore smaller quantities may be used for insect control (Thamthiankul *et al.*, 2001). Chitinases may also be used as a synergist with Cry toxins, increasing their pathogenicity (Regev *et al.*, 1996).

*Bacillus thuringiensis* strains may also produce parasporin, which is an ICP that does not have insecticidal properties but is toxic to certain human cells. Its toxicity is limited to leukemia T-cells and does not affect healthy cells, giving it a pharmaceutical value. Toxicity has been shown to cancerous cells of the uterus and cervix (Yasutake *et al.*, 2006).

In this research, SDS-PAGE was used to determine the number and size of ICPs produced by four isolates of entomopathogenic spore-forming bacteria. It was also tested as a tool to distinguish between the entomopathogenic isolates.

## **7.2. Materials and Methods**

Standard microbiological techniques were followed in the laboratory for the routine microbiological assays which include sterilization, aseptic techniques and culture preparation (Prescott *et al.*, 1999; Wheelis and Segel, 1979).

**7.2.1. Samples:** Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* spp. collected in sugarcane producing areas in KwaZulu-Natal, (KZN), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 7.1).

**7.2.2. Sample collection:** Mushroom compost, compost and grain dust were collected in 250 ml sterile honey jars (Table 7.1). Honey jars were pre-sterilized in an autoclave at 121°C for 15 min. Diseased *T. molitor* larvae were collected in 90 mm sterile Petri dishes. Adult beetles and white grubs were collected from light traps and soil, respectively, from the sugarcane areas in the KwaZulu-Natal midlands, KZN. These were provided by the South African Sugar Research Institute (SASRI), Mount Edgecombe, KZN. Diseased grubs were removed from these containers and placed into sterile Petri dishes. All the samples were stored at 4°C in a refrigerator.

Bacterial isolates were obtained from diseased insects such as *T. molitor*, Coleoptera adults and larvae of *Schizonycha* sp. (collected in sugarcane areas in KwaZulu-Natal), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures (Table 7.1).

**Table 7.1.** Samples sources from which entomopathogenic endospore-formers were isolated

<b>Samples</b>	<b>Source of sample</b>	<b>Species</b>
Mushroom compost	Mushroom farm in Karkloof area in the KwaZulu-Natal midlands	-
Mealworm larvae	Insect rearing facility at the University of KwaZulu-Natal	<i>Tenebrio molitor</i>
Compost	A garden compost heap in Hillcrest, KwaZulu-Natal	-
Grain dust	Chicken grain storage facility in Pietermaritzburg, KwaZulu-Natal	-
Adult beetles	Dalton, Bryuns-Hill and Harburg, KwaZulu-Natal	<i>Schizonycha</i> spp. and <i>Hypopholis</i> spp.
White grubs	Dalton, KwaZulu-Natal	<i>Schizonycha</i> spp.

**7.2.3. Isolation of endospore-forming bacteria:** Isolation of *Bacillus* sp. was conducted using a similar pasteurization method as described by Ohba and Aizawa (1978). Approximately 0.5 g of each sample was suspended in 10 ml sterile distilled water. Suspensions were shaken vigorously for 30 sec at full speed on a vortex shaker. Mixed suspensions were then left to stand for 1 hr at room temperature. Suspensions were vortexed a second time at full speed for 30 sec and then subjected to a pasteurization process in a preheated water bath for 10 min at 80°C. After pasteurization 0.1 ml aliquots of the suspensions were plated out onto nutrient agar (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) (Thiery and Frachon, 1997).

Plates were incubated for 24 hr at 30°C in an incubator and examined for colonies with typical *B. cereus* morphology (fast growing, rhizoid, irregular, raised, smooth, and opaque white) or colonies with an ‘ice crystal’ appearance with a colony diameter larger than 2 mm (Damgaard *et al.*, 1997; Prescott *et al.*, 1999; Selvakumar *et al.*, 2007). Various other white coloured bacterial colonies that predominated and were larger than 2 mm in diameter were also selected for microscopic evaluation. The number of colonies isolated from each isolate was estimated and

counted using a colony counter. Selected colonies were then purified by sub-culture onto nutrient agar and incubated for 3 d at 30°C in an incubator. Colonies were stored on nutrient agar slants.

Selected isolates were subjected to Gram staining as well as Coomassie Blue staining [Coomassie Blue stain 0.133% (w/v) and methanol 50% (v/v)] for 1 min. Slides were then rinsed gently for 30 sec with distilled water and blotted dry with tissue paper (Kimberly-Clark, SA) (Ammons *et al.*, 2002). Isolates were screened for the presence of crystal proteins using light microscopy with a 100 x oil immersion objective lens (Bernhard *et al.*, 1997; Young *et al.*, 1998). Crystal proteins stained dark blue (Ammons *et al.*, 2002). Isolates were screened for size of cells, spore morphology and the presence of crystal proteins. Isolates without crystal proteins, but with cells broader than 0.9 µm, were also selected, in order to include *B. cereus* which does not produce crystal proteins. Rod-shaped bacterial cells containing oval spores were selected because this fits the criteria of *B. cereus*, *B. thuringiensis* and *B. laterosporus* cells (Table 7.1). Selected cultures were purified by sub-culturing and were assigned numbers NDR1-NDR14 (Chapter 2).

#### **7.2.4. Protein preparation for SDS-PAGE:**

Two techniques were used for the protein preparations.

1. Whole cell preparation for SDS-PAGE
2. Crude protein extraction

**7.2.4.1. Whole cell preparation for SDS-PAGE:** Bacterial isolates (NDR1, NDR2, NDR3, NDR5 and NDR11) were cultured in 100 ml nutrient broth (Biolab, Biolab Diagnostics, 259 Davidson Road, Wadeville, Johannesburg) for 5 d at 30°C in a shaker water bath (250 rpm), or until cell lysis. Cell suspensions of 1 ml was placed in 2 ml Eppendorf tubes and were centrifuged at 10,000 x g for 15 min in a centrifuge (Hermle, Labotec, SA) and washed twice with distilled water. The supernatant was discarded and the pellet was resuspended in 0.5M KOH for 3 h. Samples were boiled for 2 min after which a 200 µl treatment buffer was added along with glycerol and saturated bromothymol blue. The samples were stored at -20°C until further use. This method was adapted from Luo and Adang (1994).



**7.2.4.2. Crude protein extraction for SDS-PAGE:** Isolates were cultured in 100 ml nutrient broth for 5 d at 30°C in a shaker water bath. The cell suspensions were sonicated for 15 min. Aliquots of 1 ml of the sonicated cell suspensions were centrifuged at 10,000 x g for 15 min and washed twice to remove unwanted cell debris. The supernatant was discarded and the pellet was re-suspended in 0.5M KOH for 3 h. The pellet of the isolate NDR3 was also re-suspended in 3.3M NaBr (López-Meza and Ibarra, 1996). The suspensions were centrifuged at 27,000 x g for 15 min, after which the supernatant was kept and the pellet discarded. The supernatant was then boiled for 2 min in a treatment buffer (5 ml 2-mercaptoethanol, 12.5 ml 4X Tris-Cl/SDS, pH 6.8, 5 drops of bromophenol, 30 ml 10% SDS and 10 ml glycerol). The samples were stored at -20°C until further use. This method was adapted from Luo and Adang (1994).

**7.2.5. SDS-PAGE:** The ICPs compositions of the *B. thuringiensis* and *B. laterosporus* isolates were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A method described by Hames and Rickwood (1990) was used. The samples were run at 125V for 110 min using a vertical Bio-Rad mini-Protean II cell with a 3% stacking gel and a 12.5% running gel. The gels were stained for 24 h with Coomassie Blue stain. Molecular weight markers from Promega (Whitehead Scientific, SA) were used to estimate the molecular weight of the ICPs.

## 7.3. Results

### **SDS-PAGE of Complete Spores and Insecticidal Crystal Proteins, Protein Preparation 1:**

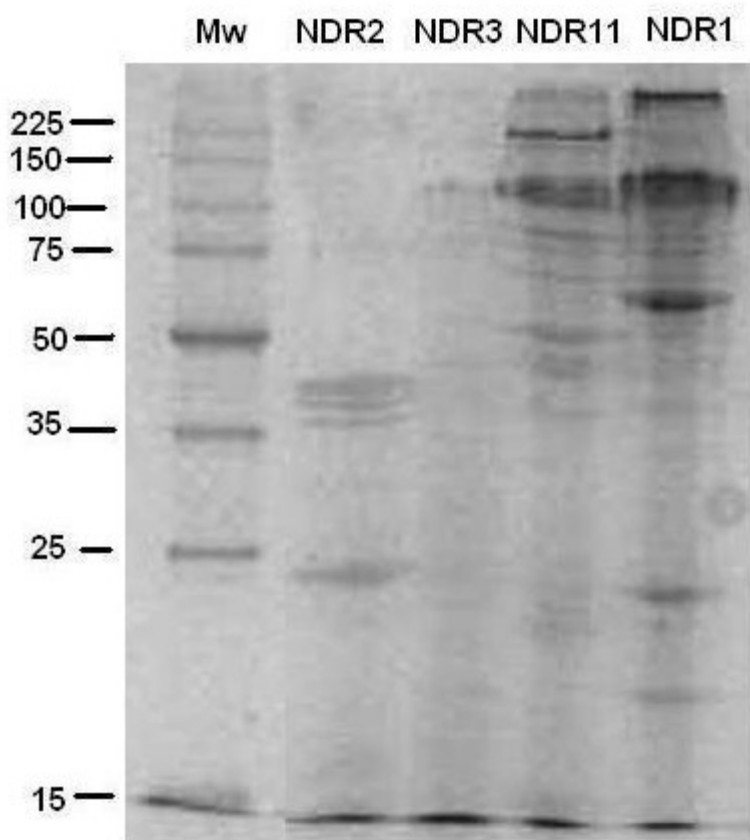
Three crystal producing isolates, NDR1, NDR2 and NDR11, generated visible bands when SDS-PAGE analysis was conducted on complete spores and ICPs of insecticidal crystal producing isolates, incubated for 5 days at 30°C.

**Isolate NDR1 (*B. thuringiensis*):** Three major bands and two minor bands were formed. The major bands sizes were estimated to be 135-kDa, 110-kDa and 63-kDa. The minor bands sizes were estimated at 93-kDa, 85-kDa, 75-kDa, 68-kDa, 38-kDa, 23-kDa and 20-kDa (Figure 7.1).

**Isolate NDR2 (*B. laterosporus*):** Four minor bands were observed. The bands had estimated sizes of 43-kDa, 40-kDa, 38-kDa and 24-kDa (Figure 7.1).

**Isolate NDR3 (*B. thuringiensis*):** the extract from this isolate formed poor bands, which were not visualized well using SDS-PAGE (Figure 7.1).

**Isolate NDR11 (*B. thuringiensis*):** Two major bands and several minor bands were observed. All the bands were of high molecular weight proteins. The major band sizes were estimated at 200-kDa, 135-kDa and 110-kDa. Minor bands were estimated at, 100-kDa 85-kDa, 75-kDa, 63-kDa, 52-kDa, 47-kDa, 43-kDa and 38-kDa (Figure 7.1).



**Figure 7.1.** SDS-PAGE analysis of complete spores and ICPs of insecticidal crystal producing isolates, incubated for 5 d at 30°C. NDR2 was a strain of *B. laterosporus*; NDR1, NDR3 and NDR11 were strains of *B. thuringiensis*.

Note : Mw = a band of molecular weight markers.

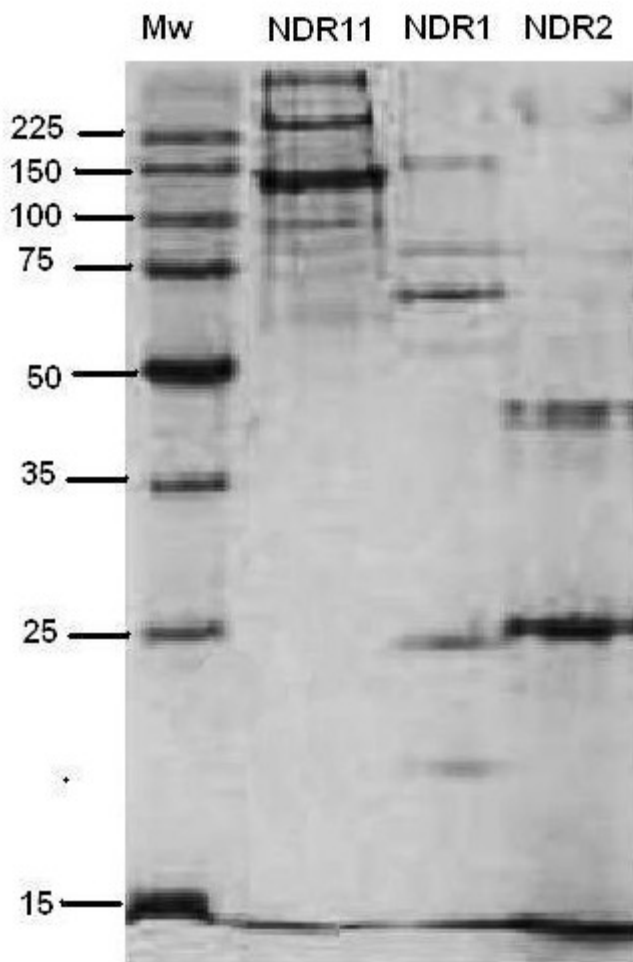
**SDS-PAGE of Crude Extracted, Alkaline Solubilized Protein Crystals, Protein Preparation**

**2:** Three isolates were analyzed using this technique, NDR2 (*B. laterosporus*), NDR1 and NDR11 (*B. thuringiensis*) (Figure 7.2). The ICPs of Isolate NDR3 did not dissolve in alkali in the first analysis, using an extraction of complete spores and ICPs; hence Isolate NDR3 was not included in the second SDS-PAGE gel analysis. No purification steps were undertaken.

**NDR1 (*B. thuringiensis*):** Five major protein bands were observed for Isolate NDR1. The sizes of the minor bands were estimated at 85-kDa, 68-kDa, 23-kDa and 20-kDa respectively. The major band size was estimated at 150-kDa (Figure 7.2).

**NDR2 (*B. laterosporus*):** Three major bands were observed. The sizes were estimated at between 40-kDa, 38-kDa and 24-kDa (Figure 7.2).

**NDR11 (*B. thuringiensis*):** Three major bands were observed for Isolate NDR11. Faint minor bands were also observed. The third was estimated to be 135-kDa and 110-kDa. Minor bands were observed at 100-kDa, 85-kDa and 63-kDa (Figure 7.2).



**Figure 7.2.** SDS-PAGE analysis of crude extracted, alkaline solubilization of ICPs from insecticidal crystal producing isolates. Isolate NDR2 was a strain of *B. laterosporus*, and NDR1 and NDR11 were strains of *B. thuringiensis*.

## 7.4. Discussion

SDS-Page was used to determine the number of different proteins present in each of the isolate's ICPs. Two analyses were carried out, using (1) whole cell preparations (Figure 7.1) and (2) crude protein extractions (Figure 7.2). The two analyses were undertaken to reduce the possibility of the SDS-PAGE analysis missing any protein bands due to a specific extraction protocol (Figure 7.2). Only three of the four isolates generated protein bands in both analyses (Figure 7.1 and

7.2). Isolate NDR3 did not produce convincing bands (Figure 7.1), which indicated that its ICP's did not dissolve under the conditions used here.

Several bands were missing for the *B. thuringiensis* strains in the crude protein extraction method when compared with the whole cell preparation method (Figures 7.1 and 7.2). Large sized proteins bands were missing for Isolate NDR1, whereas smaller molecular weight (mw) sized protein bands were missing for NDR11 (Figures 1 and 2). This demonstrated that the crude extraction method had eliminated some proteins and only contained proteins present in the ICPs. The whole cell preparation thus contained proteins that do not form part of the ICPs. All the bands were present in both the whole cell and crude extraction method for the strain of *B. laterosporus* (NDR2), although the 43-kDa protein band was weak in the crude extraction gel.

NDR1 and NDR11 shared common bands estimated at 135-kDa, 110-kDa, 75-kDa and 68-kDa (Figure 7.1). This indicated that these two isolates may share common toxins in their ICPs. The large mw protein bands greater than 80-kDa, are typical of lepidopteran specific toxin sizes (Höfte and Whiteley, 1989). Both NDR1 and NDR11 had large mw protein bands that fell within lepidopteran specific sized toxins. *Bacillus thuringiensis* subsp. *kenyae* is known to produce Cry1 and Cry2 toxins and these toxins are larger than 130-kDa and it was thus not surprising to find these sized protein bands (Höfte and Whiteley, 1989; Masson *et al.*, 1992; Van Frankenhuyzen and Nystrom, 2002). Cry1 and Cry2 toxins are also toxic to certain species of Coleoptera (Van Frankenhuyzen and Nystrom, 2002). Isolate NDR11 also had a very unusual crystal protein estimated at 200-kDa (Figure 7.1). Benintende *et al.* (1999) had a similar finding of a non-toxic 200-kDa protein from *B. thuringiensis* subsp. *Kenyae*.

NDR1 and NDR11 also had protein bands between 80-kDa and 73-kDa which are the sizes of typical Coleopteran specific toxins (Figures 7.1 and 7.2) (Höfte and Whiteley, 1989). One common band estimated at 38kDa was shared by NDR1, NDR2 and NDR11 (Figure 7.1). This indicated that these three isolates have a toxin with the same mw (Figure 7.1).

Isolate NDR2 had four low mw bands and the 24-kDa band could possibly be a Cyt toxin because these toxins are smaller mw proteins, usually less than 30-kDa in size (Du *et al.*, 1999;

Höfte and Whiteley, 1989). Low mw toxins were also found to be present in NDR1 ICPs. Low mw toxins such as 13-kDa, 14-kDa and 44-kDa are typical of binary toxins (Ellis *et al.*, 2002; Höfte and Whiteley, 1989; Khetan, 2001).

Large proteins, greater than 137-kDa, were disregarded because the largest Cry toxins are 137-kDa in size (Hofte and Whiteley, 1989). Protein bands larger than 137-kDa were assumed to contain either undissolved ICPs, cellular debris or non-toxic proteins (Benintende *et al.*, 1999). This was more evident in Figure 7.1 than Figure 7.2. This may be attributed to the fact that Figure 7.1 is of an SDS-PAGE of the whole pellet, rather than the SDS-PAGE of isolated ICPs, as shown in Figure 7.2.

Isolate NDR2 was a strain of *B. laterosporus* and little research has been done on the crystal producing strains of this species. This is mainly because there has been a general consensus that *B. laterosporus* toxins are not as effective as those of strains of *B. thuringiensis* (Zahner *et al.*, 1999). Toxins of *B. thuringiensis* are effective against Lepidoptera, Diptera, Coleoptera and even nematodes (Kumar *et al.*, 1996; Wei *et al.*, 2003). In contrast, little has been documented on the host range and effectiveness of the toxins of *B. laterosporus* and the potential of this species as a biological control agent.

Alkaline conditions (KOH) and the ionic solution of NaBr did not dissolve the protein crystals of Isolate NDR3. Du *et al.* (1994) also found that the proteins of some isolates of *B. thuringiensis* did not dissolve easily, and resorted to extreme alkaline conditions to dissolve some *B. thuringiensis* protein toxins. Alternatively, the ICPs of Isolate NDR3 may need other means or enzymes to break it down, in order to individual proteins. Further research needs to be conducted to establish the requirements for release and solubilization of Isolate NDR3's ICP.

Numerous questions remain to be answered as to the type of toxins present in the isolates screened here, and which of these specific proteins are toxic towards Coleoptera. For example, the types of toxins present in the ICPs of *B. laterosporus* are still unknown. Furthermore, SDS-PAGE only provides a determination of the number of proteins present in a sample, and their specific sizes. However, it does not allow for the determination of the type of protein, the potency of the protein toxin, or which insects that the toxin may be effective against. Some of

the toxins discovered here may be new toxins. However, SDS-Page could not be used to determine this. Protein sequencing would be required to determine this.

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# Chapter Eight

## Thesis Overview

### 8.1. Introduction

Intensive research is currently being conducted to find alternatives to agrochemical pesticides, due to safety and environmental issues (Ferré and Van Rie, 2002; Kiely *et al.*, 2004). Furthermore, numerous insect pests have developed resistance to one or more of the currently available pesticides, making them difficult to control (Ferré and Van Rie, 2002; Kiely *et al.*, 2004).

The discovery that some strains of *Bacillus thuringiensis* Berliner had insecticidal activities took place over a hundred years ago Ishiwata (1901), but rigorous research into their development was initiated more recently. As such, the development of commercial bio-pesticides using *B. thuringiensis* strains may be considered a fairly new research subject (De Lucca *et al.*, 1981; Lambert and Peferoen, 1992; Khetan, 2001).

Most of the research on *B. thuringiensis* has been conducted on strains effective against Lepidoptera and Diptera, notably on *B. thuringiensis* subsp. *aizawai* and *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *israelensis*, respectively (Khetan, 2001). This is surprising, given that there are numerous important insect pests in agriculture in the order Coleoptera that are no less important than members of the Diptera and Lepidoptera. The discovery of *B. thuringiensis* isolates toxic to Coleoptera was fairly recent (Lambert and Peferoen, 1992). This order of insect is also the largest order in the animal kingdom and by inference, there should be many undiscovered entomopathogenic spore-forming bacteria active against Coleoptera (Hill, 1983).

Within the spore-forming bacteria there are several other insecticidal species that have been largely ignored. *B. laterosporus* Laubach has not been studied as extensively as *B. thuringiensis*. This was largely because initial research was conducted on strains of *B. laterosporus* with poor or

lower toxicity against Diptera than the available strains of *B. thuringiensis* subsp. *Israelensis* (Favret and Yousten, 1985). However, researchers have found strains of *B. laterosporus* that have been as effective as strains of *B. thuringiensis* (Orlova *et al.*, 1998). Furthermore, relatively little strain discovery has been conducted for novel, insecticidal *B. laterosporus* strains, and it is probable that more effective strains will be discovered if and when a systematic search is made.

The main aim of this research project was to isolate entomopathogenic spore-forming bacteria effective against Coleoptera. Five spore-forming bacterial strains were isolated that demonstrated entomopathogenic activity against *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae (NDR1, NDR2, NDR3, NDR5 and NDR11). These included two *Bacillus* species, namely *B. thuringiensis* and *B. cereus*, and *B. laterosporus*. Four of these isolates synthesized insecticidal crystal proteins (NDR1, NDR2, NDR3 and NDR11). Three of the isolates demonstrated insecticidal activity towards species of white grubs (NDR1, NDR2, NDR3, NDR5 and NDR11).

## **8.2.1 *Bacillus thuringiensis***

### **8.2.1.1. Current understanding**

Four of the six insecticidal isolates were strains of *B. thuringiensis*. One species, *B. thuringiensis* subsp. *kenyae* (NDR1), characteristically produced bipyramidal shaped crystals. However, Isolate NDR3 produced less common shapes of crystals, either square or triangular shaped, and Isolate NDR11 produced oval shaped crystal proteins. This indicates that crystal shape is not a strong diagnostic feature.

Only one of the four *B. thuringiensis* isolates could be serotyped, namely isolate NDR1, which was classed as being in the *B. thuringiensis* subsp. *kenyae*. This serotype was initially from Africa and was previously known to produce toxins effective against Lepidoptera (Norris and Burges, 1963). The present research showed that it may also have strains that are toxic to Coleoptera, as this isolate, NDR1, and serotype was toxic to both *T. molitor* larvae and white grubs. Other serotypes may have a broad spectrum of activity. For example, the *B. thuringiensis* subsp. *morrisoni* includes strains that are toxic to Coleoptera, Lepidoptera and Diptera (Thiery and Frachon, 1997).

The DNA banding patterns produced during Bc-REP PCR isolate NDR1 did not match the banding patterns of the *B. thuringiensis* subsp. *kenyae* strains of Reyes-Ramirez and Ibarra (2005). This could suggest that this strain differed from the strains of Reyes-Ramirez and Ibarra. It also suggests that there are many different strains that have a similar toxicity spectrum, and a massive pool of diversity to tap into.

SDS-PAGE allowed for the examination of the protein banding patterns of two of the three *B. thuringiensis* strains (NDR1 and NDR11). The patterns and the size of the protein molecules were in the range of known Cyt and Cry toxins. Further work, using protein sequencing, would be required in order to clearly identify the proteins, and to determine which were novel toxins.

#### **8.2.2.2. Future research**

It can be predicted that there are numerous strains of *B. thuringiensis* with toxicity towards Coleoptera that remain undiscovered. A systematic search for new strains with activity against Coleoptera should be conducted. The use of *T. molitor* larvae proved relatively efficient during bioassays, so rapid progress could be made using this insect. Given the number and importance of coleopteran pests, there is a clear need for this research (Lambert and Peferoen, 1992).

The toxins produced by these three *B. thuringiensis* isolates, NDR1, NDR3 and NDR11, need to be analysed by protein sequencing to classify them, relative to known Cry and Cyt toxins. Toxins of Isolate NDR3 need to be analysed further using different techniques that do not affect the solubility of the crystal proteins so that they can be identified. Given that they could not be analysed by the standard SDS-PAGE technique, it is possible that they are novel protein toxins.

A better method for identification and classifying strains of *B. thuringiensis* needs to be devised. Current methods of serotyping are not definitive because they do not work on many strains of *B. thuringiensis* that are non-motile or are self-agglutinating (Reyes-Ramirez and Ibarra, 2005). DNA-based techniques such as Rep-PCR were initially purported to provide definitive techniques for the classification of *B. thuringiensis* strains. However, they too have proven to be less conclusive than expected. Furthermore, DNA-based techniques are often unreliable because

there are so many variable experimental factors involved, which increase the levels of experimental errors, generating inconsistent results.

Once novel toxins have been identified, then the genes of these novel toxins can be identified and sequenced. Conceivably, they could be integrated into plant genes so that the toxins would be expressed by these plants for the control of Coleopteran pests. This has already been done for various crops but only using the more common Lepidoptera specific toxins.

The standard Coleopteran species used for *B. thuringiensis* bioassays needs to be reconsidered. The Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) is currently used as the standard beetle for *B. thuringiensis* bioassays (Navon, 2000). The problem with this “standard” is that the CPB is not found in the Southern Hemisphere. Hence it is not possible for the “standard” bioassay on beetles to be conducted in the Southern Hemisphere. The beetle species chosen as the global standard bioassay should have the attributes of being easy to rear, not being a quarantine pest anywhere, and being found globally. The common mealworm (*T. molitor*) meets these criteria admirably.

## **8.2.2. *Bacillus cereus***

### **8.2.2.1. Current understanding**

*Bacillus cereus* Frankland and Frankland does not produce crystal proteins but does have strains with insecticidal properties. Some strains of this species produce toxins known as vegetative insecticidal proteins (VIPs) (Moar *et al.*, 1994; Estruch *et al.*, 1996; Yu *et al.*, 1997). Toxicity has been found against species of Coleoptera, such as Isolate NDR2, isolated and showed toxic activity towards Coleoptera.

No toxins were isolated from isolate NDR2 using a standard SDS-PAGE technique. No crystal proteins were observed for this isolate, which confirmed that this isolate was a strain of *B. cereus*. Strains of this species may be further classified by serotyping as well as Bc-Rep-PCR. Although both these methods face similar limitations with *B. cereus* as for *B. thuringiensis*, as discussed above, by using Bc-Rep-PCR, *B. cereus* Isolate NDR2 was found to be related to the three isolates of *B. thuringiensis* (NDR1, NDR3 and NDR11) but not to *B. laterosporus* Isolate NDR5.

#### **8.2.2.2. Future research**

The Vegetative insecticidal proteins (VIP) toxins of this species need to be isolated and analysed. The range of insect toxicity should also be investigated. VIP toxins have been little investigated, which suggests that looking for other more effective VIP toxins may be a fruitful field of research.

### **8.2.3. *Brevibacillus laterosporus***

#### **8.2.3.1. Current understanding**

*Brevibacillus laterosporus* is a relatively unexplored species of entomopathogenic bacteria; hence the research on this species during the current study was novel. Little has been documented previously on the crystal proteins of this species and the toxins it produces. Furthermore, its genetic links with *B. thuringiensis* have not been investigated previously. The toxins of Isolate NDR2 were active against species of Coleoptera, including mealworm (*T. molitor*) and a species of white grub, *Hypopholis* sp. Little is known about these toxins but using SDS-PAGE, protein bands of the toxins were obtained for this isolate, and the sizes of these toxins were obtained. These differed from the protein sizes of the well known *B. thuringiensis* Cry and Cyt toxins.

Bc-Rep-PCR is a technique that was designed for the classification of members of the *B. cereus* group, and it worked well on the strains of *B. thuringiensis* and *B. cereus*, as expected. However, clear DNA bands were also obtained for *B. laterosporus*, which was unexpected because the primers were meant to be highly specific to the *B. cereus* Group, and in theory, should not have worked for this species. One explanation is that the “Bc specific” primers performed the role of the random primers used in a Random-Rep-PCR. The bands obtained showed that *B. laterosporus* is not closely related to *B. cereus* and *B. thuringiensis*. Clearly, the “specific” primers used were not specific enough to apply only to the *B. cereus* group. More extensive research on primers needs to be done before such a claim of specificity can be made.

### 8.2.3.2. Future research

Given the paucity of research into *B. laterosporus*, there is much scope for research. Research needs to be carried out on its toxins and their mode of action. More strains of this species with toxicity towards Coleoptera need to be isolated. The toxicity range of these strains needs to be determined because they may be active against more than one order of insects. The protein crystals should be sequenced and identified. There are no established serotyping or Rep-PCR techniques that can be used to identify or separate *B. laterosporus* serotypes from each other. The genes of isolates of this species generating protein toxins could be integrated into plants to create genetically modified plants with resistance to Coleoptera.

There is also the possibility of using a combination of *B. thuringiensis* and *B. laterosporus*, which might be synergistic against a range of pests. This should also delay the development of resistance by target pests to the combination of different bacterial toxins. However, trials of this nature have not been conducted yet.

## 8.3. References

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