CHARACTERIZATION OF *Xenorhabdus spp. XptB1* AND *XptC1* TOXIN GENES AND EFFECT OF THE BACTERIA ON *Chilo partellus* AND *Busseola fusca*.

By

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DECLARATION

This thesis is my original work and has not been presented for a degree award in any other university.

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DEDICATION

To my family: mom Margaret and dad Moses, my siblings Kevin, Ivone, Bronvone, Angeline and nephew Peter. Thank you for your support and prayers.

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LIST OF ABBREVIATIONS AND SYMBOLS

ARU	Animal rearing unit
BLAST	basic local alignment search tool
Bt	*Bacillus thuringiensis*
EDTA	Ethylenediaminetetraacetic acid
bp	base pairs
IJ	Infective juvenile
KALRO	Kenya Agricultural and Livestock Research Organization
kb	kilo bases
kDa	Kilo daltons
KEGG	Kyoto Encyclopaedia of Genes and Genomes
M	Molar
MBBU	Molecular Biology and Bioinformatics Unit
MEGA	Molecular Evolutionary Genetics Analysis tool
min	Minute
NBTA	Nutrient - Bromothymol blue - Triphenyltetrazolium chloride Agar
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
OD	Optical Density
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
S	Svedberg unit
sec	seconds
Sep	*Serratia entomophila* pathogenicity
Sp. (*Spp*.)	Species (Plural)
TAE	Tris-Acetate-EDTA
Tc	Toxin complex
USA	United States of America
UV	Ultra violet
Xpt	Xenorhabdus protein toxin
ºC	Degrees Celsius
%	Percentage
µ	Microns
µL	Microliter
ng	Nano gram
mM	Milli molar
µM	Micro molar
U/µL	Units per microliter

µg	Microgram
pH	Potential of Hydrogen
g	Grams
mL	Millilitre
cm	Centimetre
mg/mL	Milligrams per millilitre
× g	Times gravitational force

ABSTRACT

Xenorhabdus spp. (Enterobacteriaceae) are endosymbionts of entomopathogenic nematodes from the *Steinernema* genus. They cause insect mortality by producing potent insecticidal toxin complexes composed of XptA1, XptA2 (class A), XptB1 (class C) and XptC1 (Class B) proteins. However, the use of only the bacterium as a biopesticide is limited as they depend on their nematode hosts for survival in the environment. The XptAs exhibit different spectra of activity, requiring XptB1 and XptC1 for full activity. Studies of their tccC homologues from the related *Photorhabdus* sp. revealed that class C proteins have enzymatic activity. The objective of this study was to characterize *XptB1* and *XptC1* novel toxin genes and proteins and investigate the bacteria's potential as alternative pest control agents against two lepidopteran pests; *Chilo partellus* (Spotted stem borer) and *Busseola fusca* (African stem borer). These pests are a constraint to maize production in Kenya, causing estimated annual yield losses of about 15%. Pure bacterial cultures were isolated by sampling infected *Galleria mellonella* (Wax moth) haemolymph. The bacteria were identified morphologically by observing colony characteristics on NBTA (Nutrient-Bromothymol Blue-Triphenyltetrazolium chloride Agar) plates and by Gram staining. The 16S rRNA gene sequence was used to confirm the identity of the bacteria, by similarity searching in public databases. Primer design, PCR amplification and sequencing that targeted the *XptB1* and *XptC1* regions was done using one pair of gene specific and three pairs of degenerate primers. Proteins were partially purified from bacterial crude cell lysate using a Sepharose CL6B size exclusion chromatography column. *Xenorhabdus* sp. resuspended in PBS (Phosphate buffered saline) was assayed for efficacy against the target pests through diet (ground maize leaves, bean powder, nutrient supplements, and agar) incorporation and direct injection. The three *Xenorhabdus* isolates studied were closely related (99% identity) to *X. griffiniae* using Phylogenetic analysis. *Xenorhabdus* sp. (OD_{600} = 2.1847A) was found to be effective against the target pests causing mortality in injected larvae of *C. partellus* (72%) and *B. fusca* (78%), within 6 days of exposure. *Xenorhabdus* sp. incorporated in diet (OD_{600} = 0.015A) caused mortality (33%) in *C. partellus* after 24 days of exposure as well as sub-lethal effects such as stunted larval development. Sequences amplified by gene specific primers were found to be similar to two conserved genes of *Xenorhabdus doucetiae*; a bacterial surface antigen D15 related to the ShlB (VB-type) membrane protein and a poly (A) polymerase. The partially purified bacterial cell lysate also yielded proteins within the size range of 110 – 160 kDa, suggesting the presence of the two target proteins. The study has demonstrated that *Xenorhabdus* sp. has a potent bioactive agent against *C. partellus* and *B. fusca* larvae when delivered through injection.

CHAPTER ONE

1.0 INTRODUCTION

Xenorhabdus spp. are gram-negative symbiotic bacteria hosted by entomopathogenic nematodes of the *Steinernema* genera and belong to the family *Enterobacteraceae* (El-hag & El-sadawy, 2008). The bacteria are symbionts that are carried monoxenically in the infective stage of the nematodes' second-instar juveniles, within their closed intestines. The two organisms together are pathogenic to a large range of insects and share a complex life cycle which is made up of symbiotic and pathogenic stages. They have been used as biological control agents against lepidopterans, coleopterans and dipterans, however, on a small scale. They cause a disease which quickly kills insects within 48 hours (Boemare & Akhurst, 1988; Caldas *et al*., 2002; Yang *et al*., 2012). Described nematode species found in Kenya include *S. karii, S. weiseri* and *S. yirgalemense* (Mwaniki *et al*., 2008).

To infect the insect host and survive, the bacteria produce a wide range of proteins which include toxins. The injection or feeding of a few *Xenorhabdus* bacteria into a susceptible insect larva leads to growth inhibition and death of the insect. Bacterial proliferation occurs in the haemocoel only after insect death suggesting that the secretions of these pathogens are highly potent virulence factors in insects (Xu *et al.*, 1989; Forst and Nealson, 1996; Caldas *et al.*, 2002; Yang *et al.*, 2012). Toxin proteins that have been isolated and characterized from *X. nematophila*, the type species of this genera, include the 39 kilo Dalton extracellular toxin, the 24tox, 17 kilo Dalton pilin subunit, and α-Xenorhabdolysin (αX) a 10 kilo Dalton cytotoxin (Ribeiro *et al*., 2003; Khandelwal *et al*., 2004; Yang *et al*., 2012).

This study focused on the 1.5 Mega Dalton toxin complex which is coded for by four genes. These genes together produce toxin complexes composed of three different classes of protein

components, namely classes A, B and C based on sequence similarity and size. Class A proteins are very large, ~280 kDa, B proteins ~170 kDa and C proteins ~110 kDa. The protein toxin coding genes in *X. nematophila* were identified as *XptA1* (*tcdA*/ *sepA*-like; 7,841 nucleotides; 287-kDa protein), *XptA2* (*tcdA*/ *sepA*-like; 7,647 nucleotides; 285-kDa protein), *XptB1* (*tccC*/ *sepC*-like; 3,047 nucleotides; 111-kDa protein), and *XptC1* (*tcdB*/*sepB*-like, 4,256 nucleotides; 160-kDa protein). Two native complexes have been described, each involving three proteins: XptA1, XptB1, XptC1 and XptA2, XptB1 and XptC1 (Sergeant *et al.*, 2003; Lee *et al.*, 2007; Sheets *et al.*, 2011; Yang *et al.*, 2012).

The XptA1 and XptA2 proteins demonstrate different spectra of activity and are where the active complexes begin to assemble. The XptA proteins need the XptB1 and XptC1 proteins so as to produce full insecticidal activity; an interaction among proteins from the three Xpt genes (*XptA*, *XptB*, and *XptC*) is necessary. Initial studies suggested that XptA1 proteins harboured the cytotoxic effects of the toxin complex, with XptB1 and XptC1 proteins modulating and enhancing their toxicity (Morgan *et al.*, 2001; Sergeant *et al.*, 2003). This motivated cloning of the class A protein, TcdA1 from *P. luminescens*, and expression in *Arabidopsis thaliana* which showed activity against the tobacco hornworm, *Manduca sexta* (Liu *et al*., 2003). However, latter studies have shown that class C proteins have bioactivity; they exhibit enzymatic activity that causes intracellular polymerization and clustering of actin which inhibits immune responses such as phagocytosis (Lang *et al*., 2010; Sheets *et al*., 2011).

XptA2, *XptC1* and *XptB1* genes are expressed in tandem. *XptA1* alone has been reported to be toxic and this is increased by presence of *XptB1* and *XptC1*. When the same *XptB1-XptC1* construct is substituted with different *XptA*s the different toxin combinations could be used as target-selective genes in transgenic plants. Further, these new toxins can potentially serve where

resistance to Bt (*Bacillus thuringiensis*) toxins has been experienced, as alternatives since they provide a potential source of innovative insecticidal genes (Sergeant *et al*., 2003; Lee *et al.*, 2007; Yang *et al*., 2012).

On-farm and storage insect pests are among the biotic constraints in maize production which is Kenya's staple. Lepidopteran stem borers in the field and the post-harvest storage pests are the most economically important insect pests of maize cereal in Africa (Kfir *et al.*, 2002; Tefera *et al.*, 2010). The most important stem borer species in Kenya are *Chilo partellus* (Swinhoe), found in warm and low areas, and *Busseola fusca* (Fuller), found in cool and higher altitudes (Odendo *et al.*, 2003). The stem borers damage crops by feeding on leaves, tunnelling through stems and feeding within maize cobs. They cause yield losses of about 11% in the highlands and 21% in the drier lowlands (Groote *et al.*, 2002).

Various pest management strategies are geared towards controlling these lepidopteran cereal pests. Use of chemicals is the most effective and common method in farms though the chemicals are not easily accessible to small scale farmers (Tefera *et al*., 2010; Mabeya & Ezezika, 2012). Other measures include developing host plant resistance using conventional breeding, growing transgenic Bt crops, incorporating the push-pull mechanism and use of natural enemies such as parasitoids like the *Cotesia flavipes* wasps. None of these, however, have kept the stem borer populations low enough (Kfir *et al.*, 2002). *Xenorhabdus* protein toxins (Xpt) therefore present new insecticidal toxins with the potential to control pests of commercial importance. They may be developed into sprays or incorporated into transgenic plants. The demand for insect toxins whose mode of action is different from that of Bt toxins is increasing (Sergeant *et al*., 2003; Lee *et al*., 2007). This study sought to characterize the *XptB1* and *XptC1* genes and proteins

from *Xenorhabdus spp.* and investigate the bacteria's effects on the two target pests, *C. partellus* and *B. fusca*.

1.1 Research Problem and Justification

Africa has a high potential for food production but lacks sustainability in production since majority of its farmers still require proper crop management strategies. Agricultural field pests significantly reduce expected crop yields resulting in food shortage. This has become a focal point for governments as well as various Non-Governmental Organizations and Research institutions worldwide. In the Abuja Summit on Food Security in Africa (Union, 2006), maize was one of the crops identified as a strategic commodity for achieving food security and poverty reduction. African Heads of State and Governments called on African countries to advocate for maize production on the continent so as to realize self-sufficiency by 2015. Pest control is but one of the strategies geared toward this goal, with the use of biotechnology being an area of focus.

Integrated pest management plays a central role in control of lepidopteran maize field pests, majorly *C. partellus* and *B. fusca.* However, these efforts have not yet succeeded in keeping pest populations under control. The two pests cause a potential 15% loss in maize yields in Kenya (Ong'amo *et al.,* 2006). Though chemical pesticides are the most effective, their residues are reported to cause unfavourable effects on both mammals and the environment. The use of bacterial toxins to control various insect pests has been ongoing for over 40 years, with extensive work done on *B. thuringiensis* and the Cry toxins they produce. They have however been met with development of resistance by some pests. *Xenorhabdus* spp. provide new possible toxins that are environmentally friendly. These toxins function differently from Bt toxins, against a wide spectrum of insects and have no documented resistance. There is need to characterize these

new toxins and subsequently test their efficacy which in turn may lead to their use in augmenting the already existing measures to improve maize yields and thus improve Kenya's food security.

1.2 Research Question

What is the efficiency of *Xenorhabdus* spp. that produce XptB1 and XptC1 toxins against the field cereal pests *C. partellus* and *B. fusca*?

1.3 Research Hypothesis

Xenorhabdus spp. bacteria producing the protein toxin gene products, XptB1 and XptC1 are effective in reducing the survival and/or growth and development of the field cereal pests *C. partellus* and *B. fusca.*

1.4 General Objective

To characterise XptB1 and XptC1 protein toxins and investigate the effect of *Xenorhabdus* sp. as a potential biological control agent against the on-farm maize pests *Chilo partellus* and *Busseola fusca*.

1.5 Specific Objectives

I. To characterize XptB1 and XptC1 toxin genes and proteins from *Xenorhabdus* spp. isolated from entomopathogenic nematodes of the genus *Steinernema*.
II. To determine the effect of *Xenorhabdus* spp. bacteria against the stem borers *Chilo partellus* and *Busseola fusca*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The entomopathogenic enterobacteria

Xenorhabdus and *Photorhabdus* are genera of bacteria that inhabit specific nematodes symbiotically. They are motile, Gram negative enterobacteria residing in the infective juvenile (IJ) stage of nematodes of the *Steinernema* and *Heterorhabdus* genera respectively. The nematode - bacterium pair invades and kills larvae of many insects and has been used in pest control. Together the nematodes and the bacteria quickly kill the insect though the bacteria alone are highly virulent. The symbiotic association is thought to be essential for bacterial survival in the soil environment. The bacteria in turn are required for killing the insect host and completing of the nematode's life cycle (Forst & Nealson, 1996; Yang *et al.*, 2012).

2.1.1 Ecology and biology of *Xenorhabdus* bacteria

Xenorhabdus spp. reside monoxenically in the gut of IJ nematodes within a specialized compartment known as the vesicle. The IJ actively seeks out and infects a wide variety of insects. The nematodes enter the host insect larvae's digestive tract and subsequently penetrate into the haemocoel. The nematode also gains access through the respiratory spiracles or other natural openings like the anus, mouth or through direct penetration of the insect cuticle. Once in the haemocoel, the nematodes regurgitate the bacteria into the haemolymph within 5 hours of invasion. During the pathogenic phase, the bacteria survives the insect immune system's vigorous attack by inhibiting phospholipase A. They then multiply in the haemolymph and kill the larva. There are few bacteria in the insect haemolymph before insect death, during when they are thought to secrete several factors toxic to the insect. The bacteria then replicate logarithmically and grow to a stationary phase. Meanwhile, the nematodes successively moult

through four juvenile stages, Juvenile 1 to Juvenile 4 (J1-J4), then the adults sexually reproduce. Food supply within the insect is depleted triggering the final stage of nematode development. The bacteria and J2 nematodes then re-associate to become the infective juvenile (IJ) vector stage which does not feed. Within the IJ's vesicle, the bacteria multiply to a capacity of 30 to 200 cells after early colonization with 1 to 2 cells. The bacterium-nematode pair then emerges from the dead insect to find new hosts in approximately 14 days. Every gram of the insect host produces up to 5 million infective nematodes. The life cycle is shown in Figure 1 (Boemare & Akhurst, 1988; Xu *et al.,* 1989; Forst & Nealson, 1996; Waturu *et al.,* 1998; Herbert & Goodrich-Blair, 2007; El-hag & El-sadawy, 2008; Hinchliffe *et al.*, 2010).

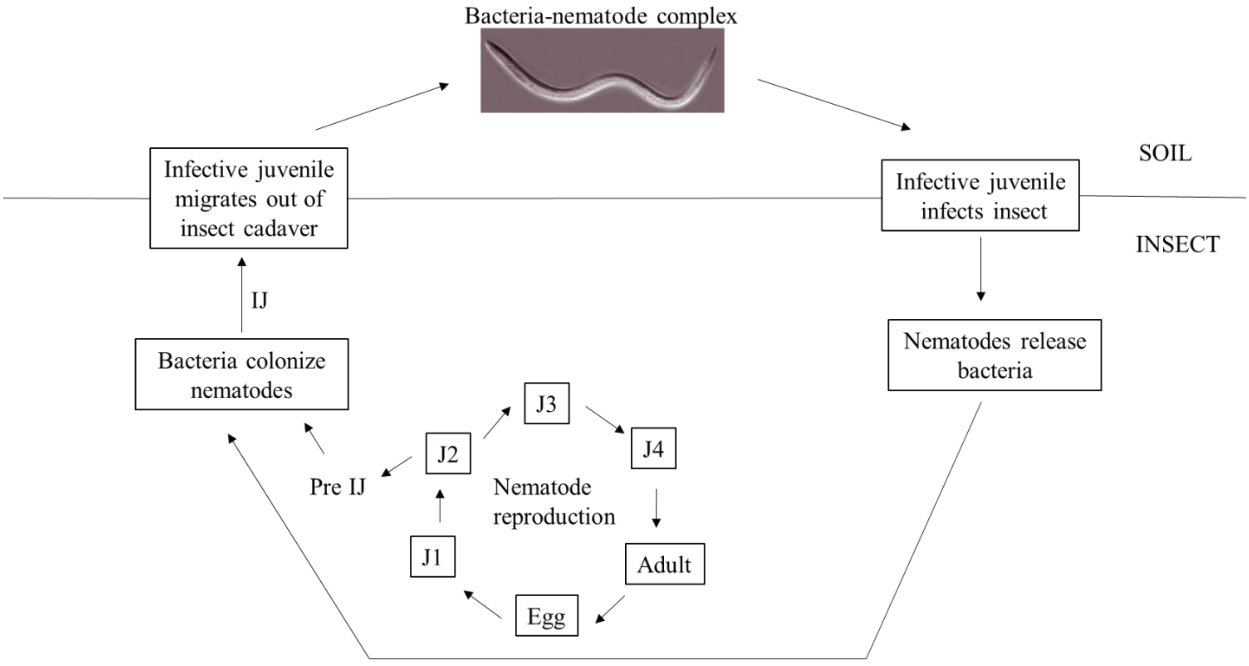

Figure 1: The *Xenorhabdus nematophila* life cycle (Adapted from Herbert & Goodrich-Blair, 2007)

Most *Xenorhabdus* spp. are dimorphic, existing in two forms (Figure 2) where the primary form or phase I form are naturally occurring. Phase I cells produce antibiotic compounds that prevent growth of many other competing microbes in the dead insect and help provide nutrients required

by the nematodes. The secondary form or phase II variant is often observed under laboratory conditions; rarely in as symbionts nature. They are altered in many properties including motility, lipase, phospholipase and protease activities. They neither produce antibiotics nor are they effective in providing nutrients for the nematodes. However, they are lethal to infected insects (Boemare & Akhurst, 1988; Xu *et al.*, 1989; Adams *et al.*, 2002).

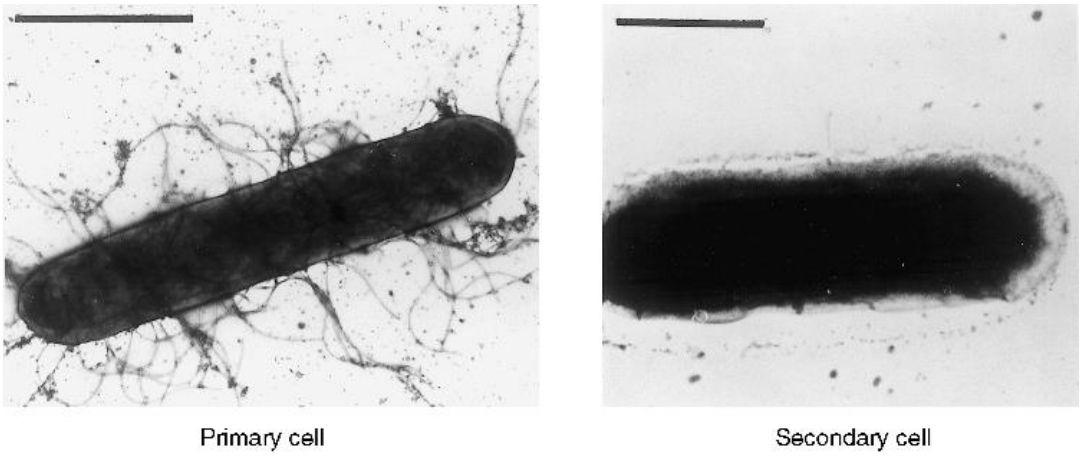

Figure 2: Photomicrograph of single *Xenorhabdus nematophila* cells showing morphological difference, presence and absence of flagella, between primary (Phase 1) and secondary (phase 2) cells (Adams *et al.*, 2002).

2.1.2 Pathogenicity of *Xenorhabdus spp.*

The pathogenic phase of the *Xenorhabdus spp.* life cycle is thought to be distinct from the exponential growth phase. This pathogenicity has been studied by direct injection into the haemocoel of the insect host *Galleria mellonella* (Wax moth) larvae since they are highly susceptible to bacterial infection (Forst & Nealson, 1996).

The insect immune system is able to recognise and respond to infection by *Photorhabdus* and *Xenorhabdus*, however, the bacteria ultimately win the battle through counteracting specific responses. *Xenorhabdus* resists the attack of non-specific antibacterial enzymes of the insect haemolymph. It produces extracellular compounds while inside the insect that suppress bacterial

proteins implicated in the insect immune response when the bacteria enter stationary phase conditions. Bacterial lipopolysaccharides also prevent the activation process of prophenoloxidase into phenoloxidase. This inhibits the eicosanoid pathway that is activated by the prophenolodidase cascade which controls haemocyte aggregation and nodulation as well as melanisation. The insect host is therefore immuno-compromised which can cause it to be susceptible to other opportunistic and saprophytic pathogens. The bacteria evade the onslaught of the insect immune system after which they systematically kill the insect and then bio-convert its tissues (Forst & Nealson, 1996; Caldas *et al.*, 2002; Hinchliffe *et al.*, 2010).

Xenorhabdus spp. further synthesize and secrete broad spectrum antibiotics and narrow spectrum bacteriocins such as indoles, dithiolopyrrolones and xenocoumacins, to maintain a monoxenic condition (where only one bacterial species thrives). This consequently inhibits growth of other pathogens thereby inhibiting the putrefaction of the insect carcass. It follows that isolation of pure cultures of the bacteria is possible from surface sterilized insect cadavers. Antibiotic production is however restricted to the bacteria's primary form. These antibiotic compounds can be further isolated, tested and harnessed for treatment of various disease causing microbes (Akhurst, 1982; Xu *et al.*, 1989; El-hag & El-sadawy, 2008; Hinchliffe *et al.*, 2010). The monoxenic state leads to lethal septicaemia of the insect that is necessary for downstream nematode development. The requisite for nematode reproduction is also enhanced by the bacteria which provide nutrients. This they achieve by producing various enzymes such as proteases, lipases, and lecithinases that promote the break-down of some proteins and macromolecules of the insect haemolymph (Xu *et al.*, 1989; Forst & Nealson, 1996; Caldas *et al*., 2002; El-hag & El-sadawy, 2008).

While within the IJ nematodes, it is unclear how *Xenorhabdus* survives nutritionally during the long-term nematode inactivity and where they obtain nutrients supporting their growth in the vesicle (Orchard & Goodrich-Blair, 2004). Food sources may be from nutrients released by dying bacteria or provision of nutrients by the nematodes to their own detriment. The latter is based on reports that uncolonized nematodes have longevity in storage than *Xenorhabdus*-colonized nematodes. Though the bacteria may be detrimental to the nematode longevity, the nematode remains a suitable vector (Herbert & Goodrich-Blair, 2007).

Strains of *Serratia entomophila* and *S. proteamaculans* (Enterobacteriaceae) are entomopathogens whose pathogenicity is similar to that of *Xenorhabdus.* These cause the Amber disease of the New Zealand grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae). Their disease determinants are located on the pADAP (amber disease-associated plasmid) plasmid. The *sepABC* (for *S. entomophila* pathogenicity) genes on pADAP, are required for the initiation of amber disease. Though the Amber disease has a characteristic pathology, proteins from the *sep* genes are similar to insecticidal proteins from *P. luminescens* and *X. nematophila* (Dodd *et al.*, 2006). It was concluded, however, that the genes involved in virulence of *X. nematophila* were contained in the bacterial genome (Akhurst, 1982; Herbert & Goodrich-Blair, 2007).

2.1.3 *Xenorhabdus* spp. insecticidal toxin complex

Bacterial pathogens of various insect hosts share many genes important in virulence and survival. The products of such genes, known as virulence factors, include factors required for host and tissue tropism, cytotoxicity and multiplication within the host. *Xenorhabdus* and *Photorhabdus* spp. toxin complexes are novel insecticidal proteins that are important in virulence. They were first identified as high molecular weight protein complexes of about

1Mega Dalton. Since then, their homologs have also been described in a wide range of bacterial species of various origins, some with no known association with insects (Pinheiro & Ellar, 2007; Hinchliffe *et al*., 2010).

Gene sequence analysis of the two genera show that the family of related toxin complexes referred to as ABC-type toxins have their genes at different loci. This family has also been described in *Yersinia* and *Serratia* species. The toxin complexes constitute of three distinct classes of proteins, categorized as class A, B, and C proteins based on sequence similarity and size. Class A proteins are large, having a molecular mass of about 280 kDa, class B proteins about 170 kDa, and class C proteins 110 kDa. There are many different varieties of class A, B and C proteins in both Gram-negative and Gram-positive bacteria (Pinheiro & Ellar, 2007; Sheets *et al.*, 2011; Spinner *et al.*, 2012).

The heterologous expression of individual *Tc* genes was described as adequate for some toxicity. This lead to the cloning of *TcdA1* from *P. luminescens* and subsequent expression in *Arabidopsis thaliana*, that showed some activity against the tobacco hornworm, *Manduca sexta* (Liu *et al.*, 2003). Earlier studies alluded class A proteins as harbouring the cytotoxic effects of the toxins, whereas class B and C proteins modulate and enhance their toxicity. However, the three components were required to achieve full toxicity (Sergeant *et al.*, 2003). Later studies on the molecular mechanism of the *P. luminescens* Tc complex revealed the class C proteins as biologically active and suggested the likely role of the class A protein as uptake of the enzyme component into target cells. TccC3 and TccC5 are adenosine diphosphate (ADP)-ribosyltransferases that target the actin cytoskeleton by modification of actin and Rho GTPases respectively. Together, the toxins inhibit the haemocytes of target insect cells from phagocytosis and cause actin to cluster and polymerise within the cells (Lang *et al*., 2010). Their homologue

from *X. nematophila* is XptB1 which forms a tight 1:1 binary complex with XptC1. It follows that characterizing XptB1 and XptC1 would be an important step toward understanding the entire complex and its activity.

The toxic Xpt genes from *X. nematophila* were first identified after screening and expression of the corresponding cosmid genomic library in *Escherichia coli*. The cHRIM1 cosmid included four genes linked to insecticidal activity that were named *Xpt* (*Xenorhabdus* protein toxin). *XptA1* (7,841 bp; 287-kDa), *XptA2* (7,647 bp; 285-kDa) both yielding class A proteins, *XptB1* (3,047 bp; 111-kDa) yielding a class C protein, and *XptC1* (4,256 bp; 160-kDa) yielding a class B protein. Expressed *XptA1, XptB1* and *XptC1* were active against *Pieris brassicae* and *P. rapae*, while *XptA2, XptB1* and *XptC1* were active against *Heliothis virescens*. This indicated that *XptA1* and *XptA2* genes demonstrated distinct spectrum of activity. However for full insecticidal activity, expression of *XptA*s, *XptB1* and *XptC1* was required. *XptC1* and *XptB1* genes were active when expressed in the same cell. The transcript from *XptA2, XptC1* and *XptB1* showed a tandem expression while *XptA1* was expressed independently (Morgan *et al.*, 2001; Sergeant *et al.*, 2003). The size and sequence of XptB1 warrants its classification as a class C protein; and XptC1 as a class B protein. Their naming was revised in some later reviews such as Hinchliffe *et al.* (2010). In this study, naming of these genes and proteins has been retained as first described by Morgan *et al.* (2001).

The three different proteins, XptA2, XptB1, and XptC1, represent proteins from all three classes and form the native toxin complex (toxin complex 1) purified from *X. nematophila*. The recombinant XptA2 protein co-produced with recombinant XptB1 and XptC1 proteins complex together with a 4:1:1 stoichiometry. XptA2 when ingested was moderately toxic against lepidopteran insects and its insecticidal activity greatly increased when complexed with co-

produced XptB1 and XptC1. A hybrid toxin complex with a similar 4:1:1 stoichiometry was formed when co-expressed TcdB2 (class B) and TccC3 (class C) proteins from *P. luminescens* were added to the *Xenorhabdus* XptA2 protein. This hybrid complex also had great activity against insects. The use of different class B and C proteins from different bacterial sources to potentiate the biological activity of different class A proteins opened the possibility to expand both the spectrum and activity of this family of toxins (Waterfield *et al.*, 2005; Sheets *et al.*, 2011)

The structure of the Tc complex from *P. luminescens*, homologous to the Xpt complex, suggests that it functions in a novel syringe-like mechanism to translocate protein. These ABC-type toxins insert fatal components into the host cell's cytoplasm. The TcdA1 (class A) protein pre-pore assembles as a pentamer to form an α-helical, trumpet-shaped channel that is encased by a large outer shell. The mature complex composed of TcdA1, TcdB2 and TccC3 may either be ingested or secreted into the insect's haemocoel. It then binds as a pre-pore to an undescribed receptor on the host cell surface and is engulfed; at this time the pore is closed and shielded by the outer shell of the protein. When the endosome is acidified or the insect gut alkalized, a pH-sensitive ionic and/or electrostatic lock is opened, the outer shell widens and releases the central pore, which penetrates the membrane like a syringe. The pore opens and the B–C heterodimer is pulled into the basin formed by the central pore and the outer shell where it is unfolded and translocated. This proposed mechanism is believed to be typical of the whole ABC-type toxin family, and can further lead to understanding how other pathogens work and for the development of bio-pesticides (Gatsogiannis *et al.*, 2013).

The toxin complex from *X. nematophila* was effective against the following species *in vitro*; the tobacco bud worm (*Heliothis virescens*), the cabbage butterfly (*Pieris brassicae*), the

diamondback moth (*Plutella xylostella*), the small white (*Pieris rapae*) and the corn ear worm (*Helicoverpa zea*) (Sergeant *et al.*, 2003; Morgan *et al.*, 2001; Lee *et al*., 2007; Sheets *et al.*, 2011).

2.1.4 Molecular characterization of *Xenorhabdus* bacteria

2.1.4.1 Xenorhabdus 16S rDNA identification and amplification of Xpt toxin genes

PCR amplification of 16S rDNA has been used for molecular identification of bacterial isolates. This gene in *Xenorhabdus* strains is highly conserved with a similarity coefficient of greater than 95% (Tailliez *et al.*, 2006). Sequencing this gene provides genus identification in most cases, however with some difficulty in species identification (Janda & Abbott, 2007). Amplification of the 16S rDNA from *Xenorhabdus* sp. has been coupled with restriction enzyme digest and the Restriction Fragment Length Polymorphs (RFLP) compared to those of the *X. nematophila* 9965 type strain. This enabled identification of *X. nematophila* cultures to the subspecies level (Morgan *et al*., 2001). A multigene approach to identification based on five universal protein-coding genes has also been proposed. These genes include; DNA recombination protein (*recA*), DNA polymerase III beta chain involved in DNA metabolism (*dnaN*), DNA gyrase beta subunit (*gyrB*) and glutamyl-tRNA synthetase involved in protein synthesis (*gltX*). This has been used to supplement the 16S rDNA data when describing novel species (Lee and Stock, 2010; Tailliez *et al.*, 2010; Kuwata *et al.*, 2013). Though it has low phylogenetic power for species and poor distinction of some genera, 16S rRNA gene sequencing is very useful when classifying bacteria (Janda & Abbott, 2007).

The Xpt toxin complex genes were first isolated by cloning fragments of genomic DNA partially digested by *Sau3A*, ligated to cosmids and transformed into *E. coli* DH5α cells. These were confirmed by sequencing, expression and toxicity assays (Morgan *et al*., 2001). The sequences

of the genes of interest from *X. nematophila* (the type species) and *X. bovienii* are available in the KEGG database (www.genome.jp/kegg/catalog/org_list.html) therefore PCR primers may be designed and used to amplify these regions from the genomic DNA. This has been done previously for the *Tc* genes from *P. luminescens* genomic DNA. The open reading frames (ORF) of interest each had the forward and reverse primers incorporated with a restriction site (Waterfield *et al.*, 2005).

2.1.4.2 Xpt protein complex purification

Bacterial cell lysis is crucial for protein extraction and analysis; as is stability of the proteins and removal of contaminants. Cell lysis must be thorough to release the target protein, but maintain the protein complex integrity and bioactivity, therefore requiring optimization. Morgan *et al.* (2001), Sergeant *et al.* (2003), Lee *et al.* (2007) and Sheets *et al.* (2011) studied the Xpt complex and its parts and reported useful protocols in cell lysis, protein purification and concentration. Bacterial cells of both *X. nematophila*, and transformed *E. coli* cells, were each cultured and disrupted by rounds of sonication. Removal of resultant cell debris was by high centrifugation. In some cases concentration of the protein was measured by the bicinchonicic acid (BCA) protein assay kit (Morgan *et al.*, 2001; Lee *et al*., 2007). Sheets *et al*. (2011) further utilized chemical and enzymatic (lysozyme) cell lysis methods. In these three cases, the starting material was cell pellet suspended in lysis buffer. Sergeant *et al*. (2003) and Morgan *et al.* (2001) used sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to analyse cell lysate and determine size and intensity of the proteins. Other methods involved further purification procedures such as chromatography, with those carried out by Lee *et al*. (2007) and Sheets *et al.* (2011) being of interest; they succeeded in isolating and analysing the Xenorhabdus toxin complex 1 and the high molecular weight protein XptA1 respectively.

Lee *et al.* (2007) dialyzed and concentrated the cell lysate, separated the protein content using a Superose 6 fast-flow gel filtration column, and analysed the fractions by SDS-PAGE and immunoblotting for the presence of XptA1 protein (287 kDa). This was followed by sequencing and bioinformatic analysis. They were able to predict from the primary peptide sequence, the major structural elements, as well as identify motifs and domains. Sheets *et al.* (2011) included a protease inhibitor to the cell lysate mixture, dialyzed the mixture and loaded the cell lysate onto an anion exchange column, followed by a size exclusion column, a hydrophobic interaction column and finally a second anion exchange column. Two separate toxin complexes were resolved and the proteins were identified by N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. Analysis of proteins was done by SDS PAGE and native PAGE.

In separation of complex proteins, a purification scheme is designed by combining orthogonal techniques in sequence. Different techniques are combined to minimize the requirements for sample treatment between the purification steps. The number of steps is also kept to a minimum to maximize purification yields. Gel filtration separates according to size and is ideal for isolation of multi-protein complexes because it is mild and many complexes are much larger than the main contaminants. However, many purification steps may have to be employed to achieve the desired purity (*Gel Filtration: Principles and methods*, 2002).

These ABC-type proteins confer insecticidal activity to several other enterobacteria and can potentially control commercially important insect pests. These toxins could be used either directly or in transgenic plants for pest control. The toxin complex system exhibits a different mode of action from Bt protein toxins that have been in use for over 40 years. Whereas Bt toxins

are produced as protoxins that solubilize during passage in the insect gut, Xpts are synthesized as the final product (Lee *et al*., 2007).

2.2 Maize production in Africa

Commercial and small-scale farmers in Africa grow cereal crops such as maize, sorghum, millet, and rice that are important for human consumption. Maize production has increased due to the expanding harvest area and not increasing yield. In 2006 in Africa, the area harvested rose to 152 million hectares from 131 million hectares in 1986. However, the increase in production was low (about 5%) which is attributed to, among others, biotic constraints. Africa imports 12% of the global maize while supplying 2.2% of global exports (Forum for Agricultural Research in Africa, 2009; Rakotoarisoa *et al.*, 2012). Studies with artificial infestation demonstrated a direct connection between occurrence of stem borers or damage symptoms and yield losses. Subsequent studies have determined the economic value of maize losses due to these pests (Odendo *et al.,* 2003).

2.3 The lepidopteran stem borers

2.3.1 Diversity of stem borers

There are 21 commercially important lepidopteran stem borer species of farmed grasses in Africa that have previously been listed, they are 7 noctuids, 2 pyralids, and 12 crambids. The following are considered as serious pests; the noctuids *Busseola fusca* and six *Sesamia* spp., the pyralids *Maliarpha separatella* (rice borer) and *Eldana saccharina* (sugarcane and maize borer) and the crambids, of which *Chilo partellus* and *C. sacchariphagus* are exotic (Kfir *et al.*, 2002). In East Africa, more so in Kenyan maize growing fields, *C. partellus* (spotted stem borer), *Chilo orichalcociliellus* (coastal stem borer), *E. saccharina* (sugarcane borer), *B. fusca* (African stem

borer), and *S. calamistis* (pink stem borer) are reported as important and commonly distributed stem borers (Kfir *et al.*, 2002; Tefera *et al.*, 2010).

2.3.2 *Chilo partellus* and *Busseola fusca:* distribution, ecology and pest status

C. partellus is widely distributed in East and South Africa but is not originally from Africa. It was unintentionally introduced from Asia before the 1930s. Its distribution currently includes; Sudan, Tanzania, South Africa, Malawi Uganda, Mozambique, Kenya,, Swaziland, Lesotho, Zimbabwe, Zambia, Somalia, Ethiopia and Botswana (Kfir *et al.*, 2002; Ong'amo *et al.*, 2006). This lowland area pest is found below 1500 meters, and has spread out in the warm, low-altitude regions of Eastern and Southern Africa as well as South Africa's harsh winter areas. It is an economically important pest in many areas (Tefera *et al.*, 2010). Its biology is adaptable therefore giving it an advantage over indigenous species and enabling its establishment in areas originally colonized by other indigenous stem borers (Ong'amo *et al.*, 2006).

B. fusca, on the other hand, is indigenous and a common pest in many sub-Saharan countries. It is differentially distributed with regard to altitude and pest status in the regions. In East and South Africa, it thrives above 600 meters. However, in Central Africa it occurs from sea level to over 2000 meters above sea level. In West Africa, it is mainly found in the dry savannah zone as a pest of sorghum. The little effort geared toward reducing its population may indicate neglect of the pest where large scale farming characterizes maize production in the high potential areas (Ong'amo *et al*., 2006; Kfir *et al.*, 2002; Tefera *et al.*, 2010). The pest status of each of the borers varies depending on the region. Dominant species in Eastern Africa are *B. fusca* and the exotic *Chilo partellus*. In Kenya, stem borer infestations cause about 15% (395,000 tonnes) of the potential yield is loss yearly (Ong'amo *et al.*, 2006).

2.3.3 Biology of *Chilo partellus*

C. partellus eggs are oval, flat and scale-like laid in clusters that overlap on 7 to 8 week old maize plants. The eggs are usually laid on lower leaf surfaces and upper part of mid-ribs. Depending on environmental conditions, incubation of eggs takes 5 to 7 days.

The hatched neonate larvae then move into the whorls to feed on the tender young leaves. Third instar larvae further bore into stems, eating out extensive galleries. The neonates may also feed on leaf collar tissue when on older plants, before they bore into the stem. Before pupation, last instar larvae within the stem prepare to leave by cutting a circular exit hole in the stalk. Full grown larvae are 25 mm in length, have a distinct reddish-brown head, cream-brown bodies that have four purple-brown longitudinal stripes. They generally are spotted in appearance due to the dark-brown spots along their back. In warm areas, larvae develop in about 15 to 20 days. *C. partellus* is not known to have a resting period; it develops continuously all-year round.

Pupation within the damaged stem takes 7 to 10 days depending on temperature. The 15mm long pupae are thin, long, shiny and brown in colour. The emergent adult moths are small with wing lengths of 7 to 17 mm and a wingspan of 20 to 25 mm. The forewings are dull, light yellow-brown with some darker scale patterns and hind wings are white. Adult moths emerge in the late afternoon or early evening and remain are active at night while resting on plants and plant debris at daytime. The moths are rarely seen, unless disturbed (Kfir *et al*., 2002; Tefera *et al*., 2010). The life cycle is shown in Figure 3.

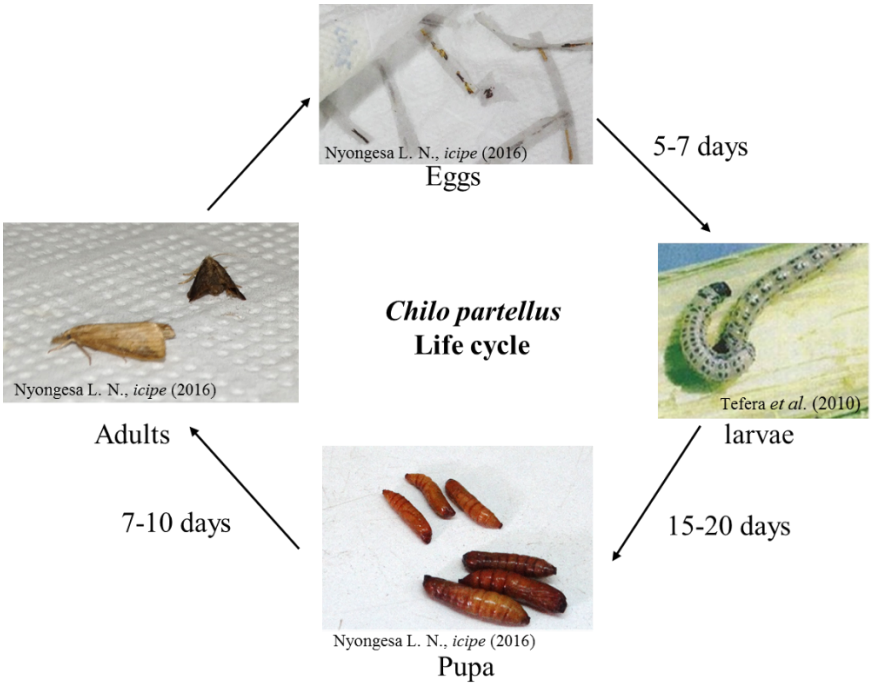

Figure 3: Life cycle of *Chilo partellus* showing the different life stages (Adapted from Tefera *et al.*, 2010).

2.3.4 Biology of *Busseola fusca*

The life cycle of *B. fusca* is longer than that of *C. partellus* and the larvae feed slightly differently. The eggs are flat, round are approximately 1 mm in diameter. At first they are white but become darker with age. Each female lays groups of about 150 eggs in a long column that extends up the stem under the leaf sheath and these hatch after about 10 days.

The young deep purple or black larvae feed on the funnel leaves of the plant causing a typical row of holes and "windows". Severe attacks turn the shoot yellow, it dies and the larvae relocate to another plant. Should the plant survive, later larval stages eat their way into the stem and its central tissue. The larval stage lasts 35 days or more. Full grown 40 mm long larvae are pink-white with small black spots along the sides of the body. They cut a hole into the stem which they will use to emerge after pupating within the tunnel.

Pupae are brown and about 25 mm long. These insects undergo two generations before the maize crop ripens. First generation pupae emerge after 2 weeks. Some second generation eggs are laid on the cob where hatched larvae feed and later move into the stem when fully grown. At this point, they undergo a long diapause until the next rainy season. They then make ready a pupal chamber in the stem where they pupate. Adults that emerge are pale brown nocturnal moths having a wingspan of 35 to 40 mm. The life cycle is shown in Figure 4 (Kfir *et al*., 2002; Tefera *et al*., 2010; Calatayud *et al*., 2014).

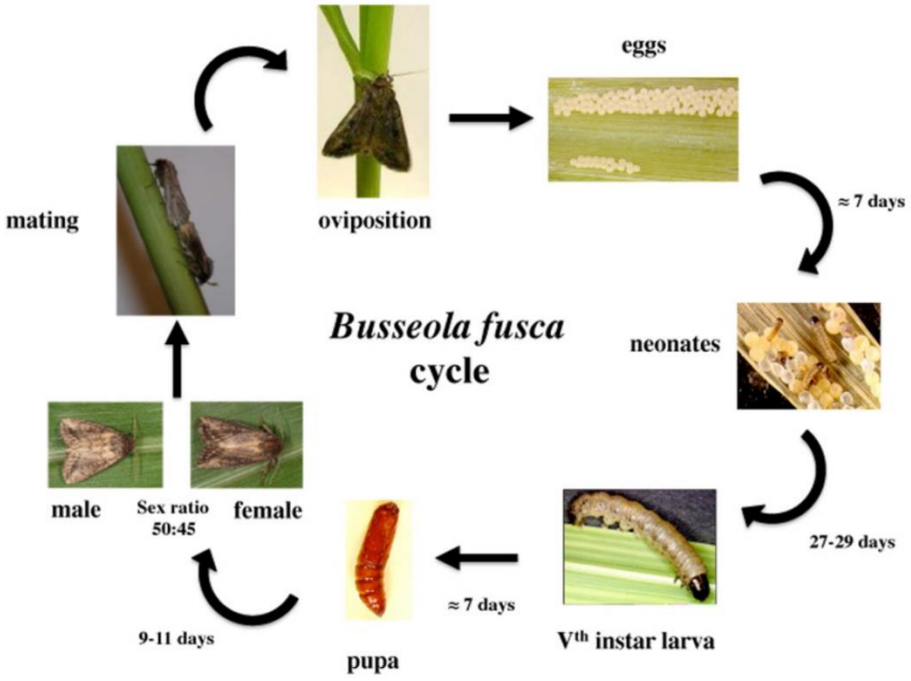

Figure 4: Life cycle of *Busseola fusca* showing different life stages (Calatayud *et al*., 2014)

2.3.5 Damage caused by *C. partellus* and *B. fusca*.

The maize plant is injured by leaf feeding, stem tunnelling and boring into cobs. Reduction of total leaf area and a depressed the photosynthetic capacity of the plant is as a result of damage caused by the first and second instar larvae. The larvae may also kill the growing points of the

plant, causing ‘dead heart’ making the youngest leaves vulnerable. The third instar larvae feed on the developing tassel and bore into the stem. Boring destroys the central pith and conducting tissues therefore reducing nutrient uptake which results in grain malformation, plant stunting and direct damage to ears. It also causes the stems to break and are subsequently get infected by secondary microorganisms thereby increasing the incidence and severity of stalk rots. Second generation *B. fusca* larvae also bore into the maize cobs (Figure 5) (Kfir *et al*., 2002; Tefera *et al.*, 2010).

Figure 5: Damage caused by stem borer larvae. **(a)** Stem tunnelling by *Chilo partellus* (www.infonetbiovision.org/Planthealth/Pests/Spotted-stemborer). **(b)** Stem tunnelling by *Busseola fusca* (www.infonetbiovisio.org/ Planthealth/Pests/African-maize-stalkborer). **(c)** *C. partellus* damage on leaves and stem (farmer.gov.in/pestsanddiseasesmaize.html). **(d)** *B. fusca* damage on the cob (www.infonetbiovisio.org/ Planthealth/Pests/African-maize-stalkborer).

The plants’ poor nutritional conditions worsen stem borer damage; levels of nutrient in the soil such as nitrogen also influence the plant’s resistance to stem borer attack to a great degree (Tefera *et al*., 2010).

2.3.6 Control measures

The general stem borer control measures include: insecticide chemical control; biological control by identifying and introducing natural enemies of pests into a locality; using vast field

and crop management practices termed as cultural control; and finally, host plant resistance (HPR), where plants have in-built resistance to insect pests (Tefera *et al.*, 2010).

The most effective and widely used approach on the farms is the use of chemicals. Various insecticides have been suggested for control of maize pests, with the main one being a synthetic pyrethroid containing beta-cyfluthrin going by the trade name Bulldock. It is however expensive and risky for farmers' health, and the environment (Tefera *et al.*, 2010; Mabeya & Ezezika, 2012).

Classical biological control of stem borers in nature is by indigenous natural enemies such as Hymenopteran or Dipteran parasitoids, predators, nematodes and pathogens (including viruses). These, however, have not kept the stem borer populations low enough (Muyekho *et al.*, 2003). For instance, the parasitoid wasp *Cortesia flavipes* (Hymenoptera) was released and established along the Kenyan coast as well as in over 40 other countries within the tropics and subtropics. However, after 10 years *C. partellus* remains a serious pest in low altitude regions and has spread to high altitude regions (Kfir *et al.*, 2002; Ong'amo *et al.*, 2006).

Cultural control is likened to primary defence, where practices like destroying crop residues, crop rotation, intercropping, altering tillage methods and rearranging planting dates. These are environmentally friendly and do not require extra investment in equipment. The approach is however limited as farmers lack proper management capabilities especially where extension service is inadequate. It is a cheap and applicable method of stem borer control for resource-poor African farmers although it is among the oldest traditional practices. It is not usually used for strategic pest control (Kfir *et al.*, 2002).

An integrated ecological approach developed by International Centre of Insect Physiology and Ecology (*icipe*), which targeted both witch weed and stem borers in maize without chemicals and other expensive inputs is being explored. Better known as the push-pull mechanism, it manipulates the abundance and distribution of a pest and/or beneficial insects by combining behaviour-modifying stimuli for management of pests. For stem borers, napier grass, *Pennisetum purpureum* is the attractant and sink for the pests and *Desmodium uncinatum* the repellent. Desmodium also controls witch weed by providing it with an alternative host to maize (Muyekho *et al.*, 2003; Cook *et al.* 2007).

Host plant resistance using conventional breeding, is another such approach. It is a technology that is embedded in the seed convenient for use in integrated pest management by subsistence farmers. The germplasm is developed with genetic resistance to pathogen infection and insect damage by using conventional breeding methods as well as marker assisted selection. Though the method needs numerous insects for screening, it is safe for the environment. CIMMYT and KALRO are among the forerunners in this area (Odendo *et al*., 2003).

Use of transgenic Bt (*Bacillus thuringiensis*) crops is a widespread application of biotechnology in pest control which is also being explored. Bt crops are beneficial in that they aid in supressing pests, conserving natural enemies that are of benefit, reducing use of insecticides, and increasing farmer profits through greater yields. The year 2011 saw the area planted with Bt crops increase to 66 million hectares from 1.1 million hectares in 1996 worldwide (Tabashnik *et al.*, 2013). However, insects develop resistance to insecticides as well as other control strategies thus threatening long term success of transgenic Bt crops. Reduced efficacy and greater than 50% resistance has been reported in individuals of species targeted by Bt crops namely: *Spodoptera frugiperda* (Fall armyworm), *Helicoverpa zea* (corn earworm), *Diabrotica virgifera virgifera*

(Western corn rootworm) all in USA; *Pectinophora gossypiella* (pink boolworm) in India and *Busseola fusca* (African stem borer) in South Africa. Resistance to Bt maize by *B. fusca* in South African fields was first reported in 2007 (Rensburg, 2007) in and its resistance to *Cry1Ab* toxin (MON810) was confirmed in 2011. The major cause of resistance is attributed to poor refuge crop compliance by farmers as well as preference of moths to humid irrigated areas (Kruger *et al.*, 2011; Berg, 2012; Tabashnik *et al.*, 2013). Recommendations on protection of the Bt crops and the Bt microbial formations include strategies such as use of refuge plants, meeting the high dose standard for target pests and using 'pyramids' or stacked events that produce at least two different toxins against target pests (Odendo *et al*., 2003; Berg, 2012; Tabashnik *et al.*, 2013). The resistant cultivars are however not widely available. Xenorhabdus protein toxins, with a distinct mode of action from Bt toxins, may provide the alternative toxins that are on demand.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Galleria mellonella (Wax moth) larvae and *Steinernema* nematodes of *Steinernema* L67, *S. carpocapsae* and *S. karii* were obtained as a gift from the Kenya Agricultural and Livestock Research Organization (KALRO) -Thika Entomopathogenic Nematode (EPN) laboratory. *Chilo partellus* larvae, *Busseola fusca* larvae and their artificial diet were purchased from *icipe*'s Animal Rearing Unit (ARU).

3.2 Isolation and identification of *Xenorhabdus* spp.

3.2.1 Bacterial isolation from *Steinernema* nematodes

The *G. mellonella* larvae were maintained in artificial diet (45.5% maize meal, 34% honey, 13.6% yeast, and 6.8% beeswax) with a light to darkness hour ratio of 12:12, a relative humidity of 65±1%, and a temperature of 28±2°C. The *Steinernema carpocapsae, S. karii* and *Steinernema L67* nematodes were baited with larvae in a petri dish lined with white cotton cloth, and allowed to infect for 72 hours. Indirect sampling of haemocoel from *G. mellonella* cadavers previously infected by nematodes was used to obtain cultures of the symbiotic bacteria. Soft cadavers that did not undergo putrefaction were then retrieved. Each *Galleria* cadaver was immersed in 70% ethanol, flamed and rinsed in sterile distilled water. The haemocoel was then exposed using a sterile scalpel and the insect haemolymph plated on NBTA (1.5% agar, 0.004% triphenyl tetrazolium chloride, 0.0025% Bromothymol blue). All bacterial cultures were aerobically incubated at 28°C in the dark for a minimum of 48 hours. Individual colonies were then sub-cultured to obtain pure cultures of the bacteria (Akhurst, 1980; Orchard & Goodrich-Blair, 2004).

3.2.2 Morphological identification of bacteria

The bacteria grown on NBTA were examined and colony characteristics namely form, elevation and margins were observed and recorded. Gram staining and microscopy was also done. This involved spreading a loopful of bacteria on a drop of water on a slide, air drying the slide and heat fixing the bacteria onto the slide. Crystal violet, the primary stain, was flooded on the slide and allowed to stand for 1 minute before being washed off. Gram's iodine was then flooded onto the slide and allowed to stand for 1 minute after which it was washed off. Destaining was done with 95% ethanol for 30 seconds which was then rinsed off with a slow, steady stream of water. Safranin was used as the counter stain and allowed to stand for 1 minute, then rinsed off. The slide was then air dried and observed under an oil immersion lens (Gram, 1884; Akhurst, 1980; Boemare & Akhurst, 1988; El-hag & El-sadawy, 2008).

3.2.3 Short and long term bacterial storage

Freshly cultured bacterial colonies were used for all activities in this section. A single blue bacterial colony was scooped from the plate using a flame sterilized inoculating needle. This was then inserted down the centre of autoclaved nutrient agar in a labelled MacConkey bottle. The process was replicated for all three *Xenorhabdus* spp. The bottles were capped, covered with foil and stored at room temperature (~24°C). These were short term stocks.

For long term storage, the blue bacterial colonies were grown in Luria broth (LB) (0.01% w/v tryptone, 0.005% w/v yeast extract, 0.01% NaCl) for 24 hours at 28-30°C with shaking at 150 rpm. Glycerol stocks of the bacteria were made by transfer of 850 µL of culture to a sterile 1.5mL microcentrifuge tube and 150µL of autoclaved glycerol added and the mixture vortexed. The mixture was then transferred to labelled cryotubes, snap frozen in liquid nitrogen and stored at -80°C (Ausubel *et al.*, 2003).

3.2.4 Molecular identification of bacteria

3.2.4.1 Genomic DNA extraction

Total bacterial genomic DNA was extracted from the blue bacterial colonies scooped from NBTA plates. The Fast DNA® Spin kit for soil was used to achieve this following the manufacturer's protocol. Bacterial cell lysis was by homogenizing the cells in a lysis buffer containing detergent and beads. The cell debris was pelleted by centrifugation (Eppendorf™ Microcentrifuge Model 5417R, North America) at 14,000×g which was also carried out for all subsequent centrifugation steps. Proteins released into the supernatant were removed by a protein precipitation solution followed by centrifugation to pellet the precipitate. The supernatant was mixed with re-suspended silica binding matrix and inverted several times to allow DNA binding. This mixture was transferred to a spin filter and centrifuged, and ethanol added to precipitate DNA. The elution buffer was used to bring eluted DNA into a clean catch tube and the DNA stored at -20°C until use.

Where the kit was not available, a salt and chloroform protocol for gram negative bacteria was used to extract genomic DNA (Chen & Kuo, 1993). Bacterial colonies were re-suspended and lysed in 200 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) through active pipetting. A 66 µl solution of 5 M NaCl was added and well mixed to remove most proteins and cell debris and the viscous mixture was centrifuged (Beckman Coulter Microfuge® 16 Centrifuge, USA) (12,000×g for all subsequent steps) for 10 minutes at 2-8°C. The clear supernatant was transferred into a new vial and an equal volume of chloroform (266 µl) added. The tube was then gently inverted until a white cloudy solution was formed. Following centrifugation for 3 minutes, the extracted supernatant (~300 µl) was

transferred to another vial and the DNA precipitated with a 2 fold volume of absolute ethanol. This was centrifuged for 5 minutes, the pellet washed twice with 70% ethanol, and air dried before being re-dissolved in 50 µl 1× Tris-EDTA (10mM Tris pH 8, 1mM EDTA) buffer. DNA was stored at -20ºC until use.

3.2.4.2 Gel electrophoresis and visualization of DNA

To confirm its presence, 3 µL of eluted DNA was mixed with ~0.5µL 6× DNA loading dye (30% (v/v) Glycerol, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol FF) and electrophoresed through a 1% 1× TAE (40mM Tris base, 1mM Glacial acetic acid, 10mM EDTA) agarose gel containing Ethidium bromide (0.75µg/mL). This was done at 80 volts for 1 hour using the Bio-Rad power supply (Model 200/2.0, USA) and gel images taken using the Kodak Gel Logic 200 UV camera (USA). These steps were used to view all other DNA fragments run on agarose gels unless otherwise mentioned. DNA concentration in ng/µL was determined through spectrophotometry using the NanoDrop™ 2000 Spectrophotometer (ThermoScientific, USA).

3.2.4.3 PCR amplification and gel extraction of the 16S rDNA gene

The 16S rDNA universal primers obtained from the laboratory (*icipe*, MBBD) were used to amplify this conserved region. The forward primer 27F of sequence AGAGTTTGATCMTGGCTCAG and reverse primer 1391R of sequence GACGGGCGGTGTGTRCA were used.

Phusion DNA polymerase (Thermo Scientific) reagents were used according to the manufacturer's protocol. The final concentrations of the PCR components used were; 1× Phusion high fidelity buffer, 200 µM of the deoxynucleotide triphosphates, 1.0 µM of each primer, 0.02 U/µL of the high fidelity polymerase and template DNA of ≤ 1 µg per reaction.

The cycle conditions were as follows; initial denaturation at 98°C for 30 sec; with 30 cycles of denaturation at 98°C for 10 sec, annealing at 57°C for 45 sec and extension at 72°C for 1 min 30 sec; as well as a final extension at 72°C for 10 min using the Arktik™ Thermal cycler (Thermo Scientific, USA) (El-hag & El-sadawy, 2008).

Gel electrophoresis of the amplified fragments on a 1% TAE agarose gel was as described in section 3.2.4.2. The QIAquick gel extraction kit (Qiagen) was used to purify the fragments of interest. The protocol involved excision of the DNA band of interest from the agarose gel using a sterile razor and weighing of the gel slice. The gel was then dissolved in 3 volumes of a chaotropic agent with 10 minutes of incubation at 50°C, before being applied to a spin column where the DNA was bound. The column was washed with 70% ethanol to remove salts and impurities and the DNA eluted in 25µL Tris-EDTA buffer and stored at -20°C until use (Morgan *et al.*, 2001; Sergeant *et al.*, 2006; Tailliez *et al*., 2006; El-hag & El-sadawy, 2008).

3.2.4.4 16S rDNA gene sequencing

The 16S rDNA fragment was retrieved from the -20°C storage and sequenced using the direct Sanger method that employs use of primers and fluorescently labelled dideoxynucleotide triphosphates (ddNTPs), whose principle is detailed by Shendure and Ji (2008). This was done by the Macrogen Company, Netherlands. The resulting sequence was then compared to those deposited in the NCBI database for molecular identification.

3.3 Molecular characterization of *XptB1* and *XptC1* genes

3.3.1 Gene specific primer design and synthesis

The sequences encoding *XptB1* and *XptC1* were obtained from the free online data base, Kyoto Encyclopaedia of Genes and Genomes-KEGG (www.genome.jp/kegg/catalog/org_list.htmL), accession numbers XNC1_2567 and XNC1_2568 respectively. The sequences, which were

from the *X. nematophila* strain ATCC 19061 genome, were then used to design the first sets of gene specific primers, making use of various bioinformatics tools. To identify the possible restriction sites within the XptB1 and XptC1 gene sequences, NEBcutter (tools.neb.com/NEBcutter) (Vincze *et al.*, 2003) was used. This enabled selection and use of restriction sequences recognized by Xho1 and Pst1 enzymes as flanking sequences for the forward and reverse primers respectively. This was done to enable possible downstream use of amplified sequences in a cloning/expression vector. The Expert protein analysis system (expasy) translate tool (www.expasy.com/translate) was used to translate the nucleotide sequence into an amino acid sequence which was then aligned with the corresponding amino acid sequences from the data base to ensure that the open reading frame was maintained. The primer sequences used are shown below (Table 1) with the restriction sequences underlined. The primer sequences were submitted for synthesis to Inqaba Biotech-South Africa. Once synthesized, the primers were reconstituted in Tris-EDTA buffer to a 100 µM stock solution according to the manufacturer's instructions and stored at -20°C until use.

Table 1: Gene specific primer sequences designed from the *X. nematophila* genes

Primer name	Sequence
Primer B1_Fwd	TACGCTCGAGATGAAGAATTTCGTTCACAGTAAT
Primer B1_Rev	TACGCTGCAGTTATGCTTCGGATTCATTATGACGTGCAGA
Primer C1_Fwd	TACGCTCGAGATGGGAGAAGCACTCAATGCCGTCGGA
Primer C1_Rev	TACGCTGCAGTTATGCTGTCATTTCACCGGCAGT

3.3.2 Degenerate primer design and synthesis

Several other sequences that encode class B and C protein toxins were also retrieved from Genbank (www.ncbi.nlm.nih.gov/genbank). These included those from other closely related

bacteria, *X. bovienii* (SS-2004) and *P. luminescens (subsp. Laumondii* TT0I*)*, whose whole genomes have been sequenced. The proteins and their accession numbers are listed (Table 2) below;

Table 2: XptB1 and XptC1 protein toxin homologs and their accession numbers

Protein Name	**Organism**	**Accession Number**
Xpt B1	*Xenorhabdus nematophila* ATCC19061	xne:XNC1_2567 (Kegg genes)
Xpt C1	*Xenorhabdus nematophila* ATCC19061	xne:XNC1_2568 (Kegg genes)
Protein C	*Xenorhabdus bovienii* SS-2004	ref|YP_003467480.1| (Genbank)
Protein B	*Xenorhabdus bovienii* SS-2004	ref|YP_003467479.1| (Genbank)
TccC1	*Photorhabdus luminescens subsp. Laumondii* TT01	ref|NP_931350.1| (Genbank)
TcaC	*Photorhabdus luminescens subsp. Laumondii* TT01	ref|NP_927868.1| (Genbank)

The nucleotide and amino acid sequence alignment was done using the online tool Clustal W2 (www.ebi.ac.uk/Tools/msa/clustalw2) (Thompson *et al.*, 1994). The second sets of primers designed were degenerate. The two *Xenorhabdus* homologous sequences were aligned for each gene and primers designed from well aligned regions. Restriction sequences were not included in these sets (Table 3).

Table 3: The 1st set of degenerate primer sequences derived from aligned *X. nematophila* and *X. bovienii* XptB1 and XptC1 sequences

Primer name	**Sequence**
Primer B2_Fwd	GCTGCCRCCGGCAAGCTTCACYWC
Primer B2_Rev	GCATCCCGCTYCTTRCCMGARTAA
Primer C2_Fwd	TGGCTGTTYCATCTGGTRTTTGATTAC
Primer C2_Rev	TCATCCAGCCAGAACTGSGCMGARCT

A third set of degenerate primers was also designed. Amino acid sequences from the three species, *X. nematophila*, *X. bovienii* and *P. luminescens*, were aligned and degenerate primers designed from the well aligned regions using the CODEHOP (Consensus degenerate hybrid oligonucleotide primers; www.block.fhcrc.org/blocks/codehop.htmL) free online tool (Rose *et al.*, 2003). This was after the sequences were arranged into amino acid blocks and several forward and reverse primers for each block generated. The software then incorporated degeneracy in the 3' region while maintaining a 5' non-degenerate clamp. Suitable primer sequences selected are shown (Table 4). Restriction sequences were not included in this primer set.

Table 4: The 2nd set of degenerate primer sequences generated by the CODEHOP online degenerate primer design tool targeting XptB1 and XptC1

Primer name	Sequence
Primer B3_Fwd	CAGTGACCGTGCTGGAYAAYMGNGG
Primer B3_Rev	GGCCATCCACTGTGCCNGCNGGRTC
Primer C3_Fwd	CATGTTTATTATCAGTATTGCGCNGARGAYGA
Primer C3_Rev	CAGCGCCTGAAGGGYNGGYTKYTC

The final set of primers for each of the genes from their amino acid sequences obtained from the three species, *X. nematophila*, *X. bovienii* and *P. luminescens* were designed. The peptide sequences were aligned using Clustal W2 and the primer sequences selected manually while avoiding sequence areas with amino acids of high degeneracy which are leucine, arginine and serine (Appendix 1). The degeneracy was introduced in the 5' end with a non-degenerate 3' clamp (Table 5).

Table 5: The 3rd set of degenerate primer sequences designed manually targeting XptB1 and XptC1 genes

Primer name	Sequence
Primer B4_Fwd	GCNGGNCARTGCGTGGTNCATTATATC
Primer B4_Rev	NCCRTANGGRTAATACTCTCCCTG
Primer C4_Fwd	TTYGGNATGGGNTGGCAATGT
Primer C4_Rev	YTGNCCYTTACCGTCATATCC

The sequence manipulation suite (www.bioinformatics.com/sms), a web based collection of sequence analysis programs, was used to screen primer sequences for potential problems such as self-annealing or hairpin formation as well as determining the annealing temperature. This tool was also used to carry out an *in-silico* PCR to predict the expected fragment size and ensure that the amplified sequence was the sequence of interest.

The selected degenerate primers were synthesized by Macrogen Incorporated-Korea. Once synthesized, the primers were reconstituted in Tris-EDTA buffer to a 100 µM stock solution according to the manufacturer's instructions and stored at -20°C until use.

3.3.3 PCR amplification of *XptB1* and *XptC1* genes

Total bacterial genomic DNA was extracted from blue bacterial colonies as described in section 3.2.3.1. This served as the template for PCR amplification using various enzymes for the different primer sets. The enzymes used included HotstarTaq® DNA polymerase (Qiagen), Taq polymerase (GenScript) and Phusion High-Fidelity DNA polymerase (Thermo Scientific), all in line with the manufacturer's protocol. The final concentration of the PCR components used were; 1× PCR buffer, 200 µM of the deoxynucleotide triphosphates and template DNA of ≤ 1 µg per reaction. The cycle conditions varied as follows; for the Phusion High-Fidelity DNA polymerase, the cycle conditions were as stated in section 3.2.3.2. For the HotstarTaq® DNA

polymerase (Qiagen), initial denaturation was for 15 minutes at 95°C, the three step cycling included denaturation for 30 seconds at 94°C, annealing for 1 minute and extension at 72°C for 1 minute, for 30 cycles; and a final extension for 10 minutes at 72°C. For the Taq polymerase (GenScript), initial denaturation was for 3 minutes at 94°C, the three step cycling included denaturation for 30 seconds at 94°C, annealing for 45 seconds and extension at 72°C for 1 minute, for 30 cycles; and a final extension for 10 minutes at 72°C (Waterfield *et al.*, 2005). The annealing temperatures and the final concentrations of the primers and enzymes used is summarised in Table 6.

Table 6: Summary of annealing temperatures and the final concentrations of the primers and enzymes used in PCR reactions for each of the primer pairs

Target Gene	Primer Names	Final primer concentration (µM)	Primer annealing temperature (ºC)	Enzyme used and final concentration
XptB1	B1_Fwd and B1_Rev	1.0	55	0.05 U/µL Taq polymerase (Genscript)
XptB1	B2_Fwd and B2_Rev	1.0	57	0.02 U/µL Phusion polymerase (Thermoscientific)
XptB1	B3_Fwd and B3_Rev	0.5	57	0.025 U/µL Hot start Taq polymerase (Qiagen)
XptC1	C1_Fwd and C1_Rev	1.0	55	0.05 U/µL Taq polymerase (Genscript)
XptC1	C2_Fwd and C2_Rev	1.0	53	0.02 U/µL Phusion polymerase (Thermoscientific)
XptC1	C3_Fwd and C3_Rev	0.5	50	0.025 U/µL Hot start Taq polymerase (Qiagen)

3.3.4 Gel electrophoresis and extraction of PCR products

Gel electrophoresis on a 1.2% (w/v) 1× TAE agarose gel of amplified fragments at 80 volts for 1 hour was done to ascertain the fragment sizes as earlier described in section 3.2.4.2. The

fragments were interpolated against a 1kilobase ladder and the fragments of interest were then excised using the QIAquick Gel extraction kit as earlier described in section 3.2.4.3.

3.3.5 XptB1 and XptC1 gene sequencing

The fragments were then sequenced by the direct Sanger method by Macrogen Incorporated (Netherlands) as described in section 3.2.4.2 and the sequences analysed and compared to those existing in the free online databases.

3.4 Protein purification of XptB1 and XptC1 toxins

3.4.1. Determination of suitable cell lysis procedure and volumes

Blue bacterial colonies isolated from *S. carpocapsae* and grown on NBTA plates were proliferated in approximately 250 mL of Luria broth (LB) (0.005% w/v yeast extract, 0.01% w/v tryptone, 0.01% NaCl) for 24 hours at 28-30°C with shaking at 150 rpm and centrifuged (Beckman Avanti™ Centrifuge J-25 I, USA) at 6000×g for 10 minutes to pellet the cells (Sheets *et al*., 2011; Lee *et al*., 2007).

Two lysis protocols were compared to determine the one giving highest protein yields as well as high molecular weight proteins. The lysis buffers were prepared as follows; 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT), 10% Glycerol, 0.6 mg/mL Lysozyme (Sheets *et al*., 2011) and the other, 10 mM Phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4) (Morgan *et al*., 2001).

The cell pellet was re-suspended in the lysis buffer and lysis varied as follows: for the protocol by Sheets *et al*. (2011), sonication using the Soniprep 150 ultrasonic disintegrator (MSE Scientific, England) was at 18μ, 30 s on 30 s off, for about 14 cycles and another similar preparation for 10 cycles designated as S1 and S2 respectively. For the protocol by Morgan *et*

al (2001), sonication was at 18μ peak to peak for a single 30 s burst. For each case the falcon tube in which sonication took place rested on ice.

Each of the lysates was centrifuged (Eppendorf™ Microcentrifuge Model 5417R, North America) at 13,000×g for 5 minutes and mixed with Laemmli sample buffer (0.25 M Tris-HCl pH 6.8, 50% (v/v) Glycerol, 0.5 M Dithiothreitol, 10% (w/v) SDS, 0.01% (w/v) Bromophenol blue) in the ratio of 3:1. These were then each boiled at 96ºC for 4 minutes.

Approximately 10 μl of the mixture for each sample was analysed per well on a 10% discontinuous SDS (Sodium dodecyl sulphate) gel. For the 10% resolving gel, the gel mixture was composed of 30% (v/v) Acrylamide/bis-acrylamide, 1.5M Tris-HCl pH 8.8, and 10% SDS (Appendix 2a). The mixture was poured into the casting plates, overlaid with absolute methanol, allowed to polymerise before the methanol was poured away and the gel rinsed off with distilled water. The 4% stacking gel prepared was composed of 30% Acrylamide/bis-acrylamide, 0.5M Tris-HCl pH 6.8 and 10% SDS (Appendix 2a). The mixture was poured into the casting plates on top of the already polymerized resolving gel, a plastic comb inserted to create loading wells, and allowed to polymerise. For all gel mixtures, tetramethylethylenediamine (TEMED) and freshly prepared 10% Ammonium persulphate (APS) were added in order for the gels to polymerise. The set up was run on the miniPROTEAN® tetra system (Bio-Rad, USA) for 45 minutes at 30mA, 150V using the PS 1500 DC power supply (Hoefer Scientific Instruments, USA).

The gel was then stained in Coomassie Blue (40% methanol, 10% acetic acid, 0.02g (w/v) Coomassie Brilliant Blue) and destained with 40% (v/v) methanol, 10% (v/v) acetic acid to observe presence of protein bands within the range of interest, with XptB1 being 110 kDa and

XptC1 being 158 kDa. Protein sizes were interpolated against a High molecular weight protein ladder (Amersham Biosciences).

Each of the lysate supernatant samples from the Morgan *et al* (2001) and the Sheets *et al*. (2011) lysis protocols was also compared using a 7% discontinuous native gel. Each was mixed with native sample buffer (12.5% (v/v) 0.5M Tris-HCl pH 6.8, 30% (v/v) Glycerol, 0.01% (w/v) Bromophenol blue) in the ratio of 2:1. About 10µL of the sample mixture was loaded on a discontinuous 7% native (non-denaturing) polyacrylamide gel; 4 % stacking gel composed of 30% Acrylamide/bis-acrylamide, 0.5M Tris-HCl pH 6.8 and the 7% resolving gel composed of 30% Acrylamide/bis-acrylamide, 1.5M Tris-HCl pH 8.8 (Appendix 2b). TEMED and freshly prepared 10% APS, and were added for the gels to polymerise. The high molecular weight native protein ladder (Amersham Biosciences) was used. The gel was run on the miniPROTEAN® tetra system (Bio-Rad, USA), stained in Coomassie Blue stain and destained as described above. The suitable lysis protocol was the chosen.

Once selected, the cell lysis protocol was then carried out in different lysis volumes, 5mL, 10mL and 15mL of PBS and visualized on a 7% discontinuous native gel as described above. The lysis volume that yielded bands of good intensity with minimal smearing along the lanes was selected (Bio-Rad mini-PROTEAN™ tetra cell Instruction manual).

3.4.2. Determining the void volume and equilibrating the gel filtration column

The gel filtration column (1.5 cm by 60cm – Pharmacia, Sweden) was selected and packed with degassed Sepharose CL6B matrix (Sigma) (separation range 10kDa – 4000kDa) which had been washed 4 times in PBS. The matrix-filled column was then transferred to the cold laboratory (2 - 8˚C) and allowed to acclimatize before other downstream procedures, which were done in

these conditions. Blue dextran 2000 (Amersham Biscience) was reconstituted in PBS at the rate of 1 mg/mL and used to determine the column's void volume. The blue dextran (2.5mL) was applied to the column and ~1.5mL per fraction was gravity eluted using PBS. The fractions were then screened by UV spectrophotometry at 280 nm and a plot of the fraction tube number against the absorbance gave an elution profile with a single peak (Bollag & Edelstein, 1991). The void volume was then determined by the formula;

$$\text{Void volume} = \text{volume collected in individual fractions} \times \text{number of fractions with the maximal UV absorbing protein}$$

The following protein standards (Pharmacia) were reconstituted in PBS at the rate of 5 mg/mL as per the manufacturer's protocol; bovine serum albumin (67kDa), aldolase (158kDa), catalase (232kDa) and thyroglobulin (669kDa). A 2.5 mL mixture of these protein standards was applied to the column and eluted with PBS by gravity. The fractions collected were measured at 280nm and a graph of tube number against absorbance plotted. The resultant elution profile was recorded.

3.4.3 Gel filtration of *Xenorhabdus sp*. cell lysate

Xenorhabdus cells were lysed according to the protocol selected and described in section 3.4.2. A 5mL volume of the cell lysate was applied onto the column and gravity eluted using PBS. The elution profile was generated as described in the previous section 3.4.2. The fractions were then pooled with regard to the different peaks obtained from the elution profile.

3.4.4 Ammonium sulphate protein concentration

A highly saturated ammonium sulphate solution was prepared at room temperature (24°C) by dissolving the salt in distilled water until salt crystals formed in the solution at the bottom of the flask. The solution was filtered and mixed with the pooled fractions in the ratio of 4:1 resulting

in a final concentration of 80% (v/v) ammonium sulphate. These precipitated overnight at 4°C with agitation on a rotary shaker at 10-20 rpm after which they were centrifuged (Beckman Avanti™ Centrifuge J-25 I, USA) at 6000×g for 15 minutes to pellet the precipitate. The supernatant was discarded and each pellet re-suspended in 1 mL of PBS (Yang *et al*., 2012).

3.4.5 Dialysis of concentrated proteins

The dialysis tubing (Spectra/Por® 4 tubing; molecular weight cut-off of 12,000-14,000) was soaked in PBS and 1 mL of the concentrated proteins loaded into individual tubings. Each sample was secured in the tubings with pegs at the edges to ensure no leakage. These were dialyzed overnight against a 200 fold volume of PBS with stirring, at 2-8 °C. Dialysis was carried out according to the manufacturer's protocol. Each of the dialyzed samples was then analysed on a 7% discontinuous native polyacrylamide gel to determine its content as described in section 3.4.1.

3.5 Insect bioassay of *Xenorhabdus spp.* against *C. Partellus* and *B. fusca* larvae

3.5.1 Proliferation of bacteria

Blue bacterial colonies isolated from *S. carpocapsae* and grown on NBTA plates were proliferated in LB and centrifuged as described in section 3.4.1. Cell pellet was then re-suspended in PBS and the optical density at 600 nm recorded by a spectrophotometer (Beckman DU® 640B Spectrophotometer, USA). The number of cells was then calculated where by 1.0 OD = 1.0×10^9 cells (Ausubel *et al.*, 2003; Adams *et al.*, 2002; Yang *et al*., 2012).

3.5.2 Incorporation of bacteria into artificial diet

The bacteria re-suspended in PBS (Section 3.5.1 above) was blended with the cooled artificial diet consisting of Fraction A (maize leaves powder, brewer's yeast, bean powder, sucrose,

Ascorbic acid, Sorbic acid, Methyl-p-benzoate, mixed in boiled distilled water at 60°C) and Fraction B (Agar in distilled water at 60°C) for 2 minutes (Tefera *et al.*, 2010) (Appendix 3). About 5mL of the diet was dispensed into glass vials (1 by 4 inches) and allowed to cool and set overnight. For the feeding assay, the main preservative of the diet, formaldehyde, was only included in one treatment which served as a negative control.

3.5.3 Introduction of *C. partellus* larvae into diet

C. partellus L3-stage larvae obtained from *icipe*'s ARU were starved for 12 hours before being introduced into individual vials containing diet with the different treatments. The setup was a single dose incorporation assay with 3 treatments; incorporation of *Xenorhabdus* cells into diet and two negative controls consisting of plain diet with the preservative and the other without the formaldehyde preservative. The treatments had 30, 33 and 28 larvae respectively. These were incubated at room temperature of 24±2°C, a relative humidity of 47±1% and a light to dark hour ratio of 12:12. Observations on larval development and mortality were made daily until adults emerged (Morgan *et al*., 2001; Kumari *et al*., 2010).

3.5.4 Direct injection of *Xenorhabdus* sp. into *C. partellus* and *B. fusca* larvae

The bacteria prepared as described in section 3.5.1 were re-suspended in PBS and the OD at 600 nm recorded and used to calculate the number of cells. The L3 larvae were chilled on ice to immobilize them, then each injected with 5-10 µL of bacteria re-suspended in PBS, through the proleg (Harding *et al*., 2013). Hypodermic needles, 500 µL capacity, 29G × 0.5 in (BD, USA) were used for the injections. The larvae were then introduced into individual vials containing plain diet (Appendix 3) and observed daily for a minimum of 10 days.

The setup for direct injection of *C. partellus* larvae employed two concentrations of *Xenorhabdus* sp. re-suspended in PBS, a 1:10 dilution and a 1:100 dilution (Appendix 10a) each

injected into 21 larvae. The larvae of the negative controls for each set up included; 20 punctured, 20 injected with PBS, and 20 injected with *E. coli* cells re-suspended in PBS (Morgan *et al.*, 2001; Sergeant *et al*., 2003; Yang *et al*., 2012).

The setup for direct injection of *B. fusca* larvae employed a single concentration of *Xenorhabdus* sp. re-suspended in PBS injected into 40 larvae (Appendix 10a). The larvae of the negative controls included; 40 punctured, 40 injected with PBS, and 40 injected with *E. coli* cells re-suspended in PBS (Morgan *et al.*, 2001; Sergeant *et al*., 2003; Yang *et al*., 2012).

3.6 Bioinformatics sequence analysis and data analysis

3.6.1 Bioinformatics sequence analysis

3.6.1.1 16S rDNA sequence analysis

The sequences obtained were cleaned up by truncating 50 nucleotides from either of its ends before each was compared with other sequences existing in the NCBI database by BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the best hits from *Xenorhabdus* species were downloaded in a FASTA format and imported into MEGA 6.0 (www.megasoftware.net) (Tamura *et al.*, 2013) where they were aligned by MUSCLE, the sequence alignment explorer included in the software. The alignment included an out group 16S sequence from *Proteus vulgaris*. The alignment was then used to compute a phylogenetic tree by maximum likelihood using a bootstrap value of 100 and the *P. vulgaris* sequence as the root of the tree.

3.6.1.2 XptB1 and XptC1 gene sequence analysis

The amplified DNA sequences were trimmed (Section 3.6.1.1) then subjected to a blast for highly similar sequences known as a megablast, and in some cases they were subjected to a nucleotide blast, BLASTn in the NCBI database. The blast results showing the best hits, their

e-values, and percentage identities were recorded in tables. The ExPasy translation tool (www.expasy.com/translate) was used to translate the amplified sequences and the resulting amino acid sequences obtained from uninterrupted open reading frames were selected for further analysis. These amino acid sequences were compared with those present in the database using the Interpro sequence analyser (www.ebi.ac.uk/interpro) (Mitchell *et al.*, 2015) and Pfam sequence analyser (*pfam.sanger.ac.uk)* (Bateman, 2004). Various domains were identified and used to describe the sequences and inform their likely function. This was done for all DNA fragments that were amplified and sequenced by primers that targeted the XptB1 and XptC1 regions.

3.6.2 Data analysis

3.6.2.1 Determining efficacy of *Xenorhabdus* sp. against *C. partellus* and *B. fusca*

The development of the larvae was monitored and larval mortality recorded upon daily observation. Sub- lethal effects of the toxin to development of the larvae were also noted such as feeding deterrence of larvae of both *C. partellus* and *B. fusca* as well as measurement of the wing length of adults in the *C. partellus* feeding. The number of dead larvae was recorded in a 2 by 4 contingency table. Pearson's Chi-squared test of independence was used to determine whether the mortality effects observed were due to presence of *Xenorhabdus* sp. at a significant level of $p \leq 0.05$. The *C. partellus* adult wing length variation was compared using ANOVA and the F statistic calculated at $p \leq 0.05$. R statistical software was used in both cases.

CHAPTER FOUR

4.0 RESULTS

4.1 Morphological description of *Xenorhabdus* spp. isolates

The haemolymph of *Galleria mellonella* larvae separately infected with *Steinernema* L67, *S. carpocarsae* and *S. karii* each plated on the differential Nutrient-Bromothymol blue-triphenyltetrazolium chloride agar (NBTA) medium yielded bacterial colonies after 48 hours of aerobic incubation at 28°C. The colonies were small and blue with clear/colourless cells at the irregular dendritic margin, a smooth, dull surface and a raised-flat elevation (Figure 6a). In NBTA with less than 1% agar, swarming growth was observed (Figure 6b). Both cases were phase 1 cells. Several generations of bacteria sub-cultured on NBTA in the lab yielded red colonies with regular margins, raised convex elevation that lacked swarming growth in NBTA containing less than 1% agar (Figure 6c). These were phase 2 cells. The bacteria scooped from the blue colonies appeared as pink gram negative rods under the microscope's oil immersion lens after they were Gram stained.

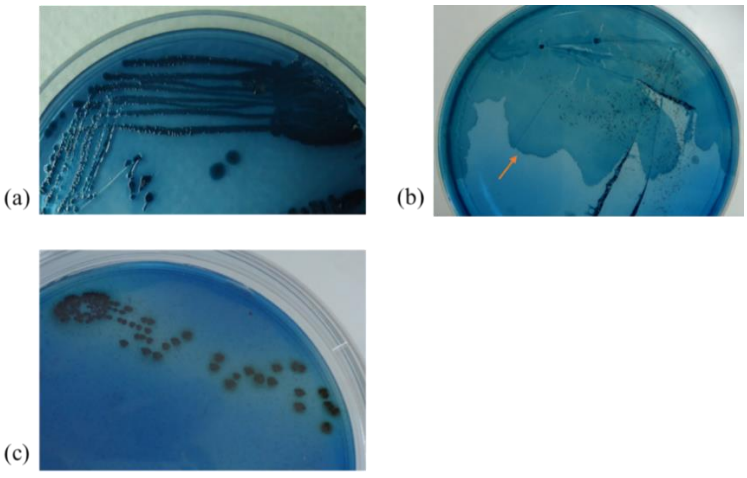

Figure 6: Morphological identification of *Xenorhabdus* spp. isolates gown on Nutrient-Bromothymol blue-triphenyltetrazolium chloride agar (NBTA) **(a)** Phase 1 bacterial cells blue due to dye absorption **(b)** Bacterial swarming growth of phase 1 cells as indicated by arrow, on 0.8% agar **(c)** Red phase 2 bacterial cells.

4.2 Identification of *Xenorhabdus* spp. isolates using the 16S rDNA

Genomic DNA extracted from each of the bacterial isolates was labelled with regard to the nematode from which it was isolated, Sc (*Steinernema carpocapsae*), Sk (*S. karii*) and L67 (*Steinernema L67*), and was visible as large DNA fragments greater than 10 kilo bases (Figure 7) after electrophoresis. The concentration of the DNA ranged between 220ng/µL and 601ng/µL.

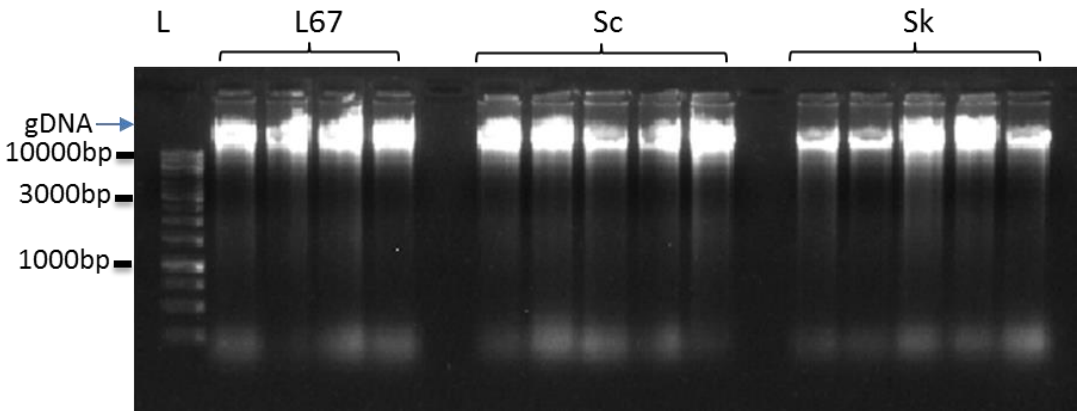

Figure 7: Genomic DNA extracted from three *Xenorhabdus* spp. isolated from L67 - *Steinernema L67*, Sc - *Steinernema carpocapsae*, and Sk - *S. karii*, labelled gDNA as viewed after agarose gel electrophoresis. **L** is a 1kb DNA ladder (Fermentas).

The amplification of the 16S rDNA through Polymerase Chain Reaction (PCR) for the three *Xenorhabdus* spp., designated Sc, Sk and L67, yielded fragments of approximately 1300 base pairs each (Figure 8). This was achieved using the universal 27F and 1391R primers and the Phusion® High-Fidelity DNA polymerase with annealing at 57ºC.

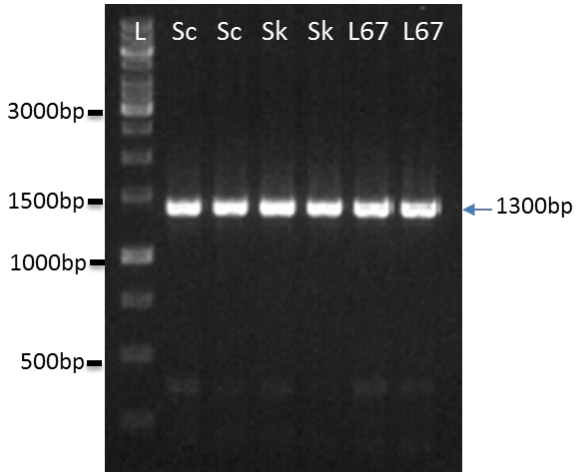

Figure 8: Molecular identification of *Xenorhabdus* spp. Gel image of DNA fragments amplified by the 16S rDNA universal primers in duplicate. **Lanes 2-7** show fragments of *Xenorhabdus* spp. from Sc, Sk and L67. **L** is a 1kb DNA ladder (Fermentas).

The sequenced fragments of 1250bp were each used as the query sequences (Appendix 4) for a megablast for highly similar sequences against other sequences deposited in the NCBI database (blast.ncbi.nmL.nih.gov), resulted in *X. griffiniae* str. ID10 as the best hit, with 100% query cover and 99% identity. Reconstruction of a phylogenetic tree using over 50 of the top hit sequences as well as selected other available *X. nematophila* sequences revealed that the query sequences clustered with *X. griffiniae* sequences in the clade labelled G (Figure 9). The 16S rDNA sequences from *X. nematophila* (clade N) and the locally isolated *X. hominicki* were in different distant clades from the three sequences generated in this work.

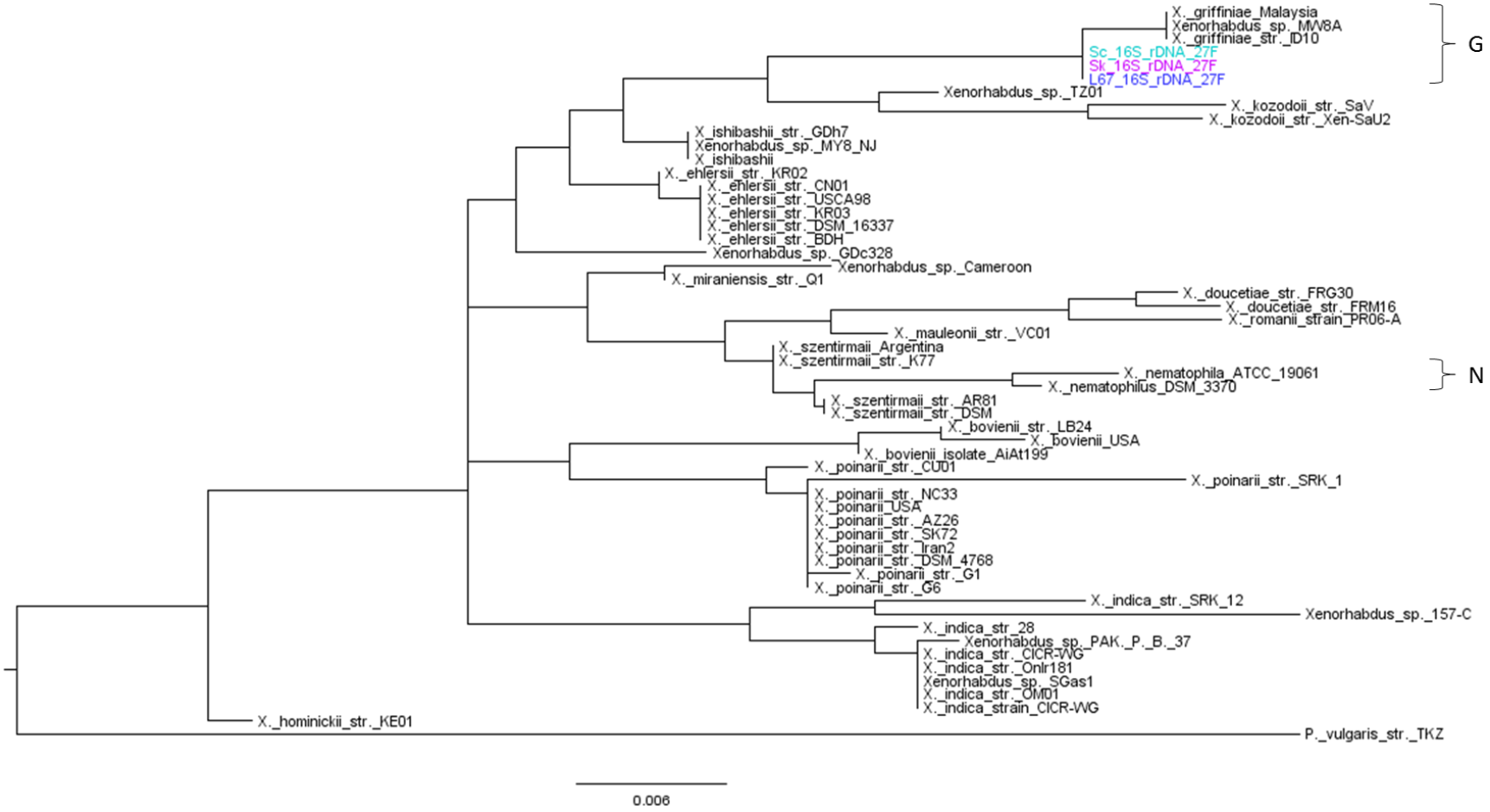

Figure 9: Maximum likelihood phylogenetic tree of *Xenorhabdus* 16S rDNA sequences from the best BLASTn hits as retrieved from the NCBI database after a megablast. The three taxa of interest are coloured (green, purple and blue). The clade labelled G shows *X. griffiniae* sequences clustered with sequences generated in this study, whereas clade N contains *Xenorhabdus nematophila* sequences. The scale bar indicates the number of nucleotide substitutions per site.

4.2 Molecular characterization of *XptB1* and *XptC1* genes

4.2.1 Amplification using gene specific primers

The target XptB1 and XptC1 regions amplified by PCR using the gene specific primers yielded two fragments for each primer pair. The primer pair targeting the XptB1 gene yielded two fragments at 1500bp and 500bp designated BS1.1 and BS1.2 (Figure 10a) respectively in all three bacterial isolates, Sc, Sk and L67. The primers targeting the XptC1 region also yielded two fragments at 2500bp and 900bp denoted as CS1.1 and CS1.2 respectively (Figure 10b). Taq polymerase (GenScript) was used with annealing at 55ºC in both cases.

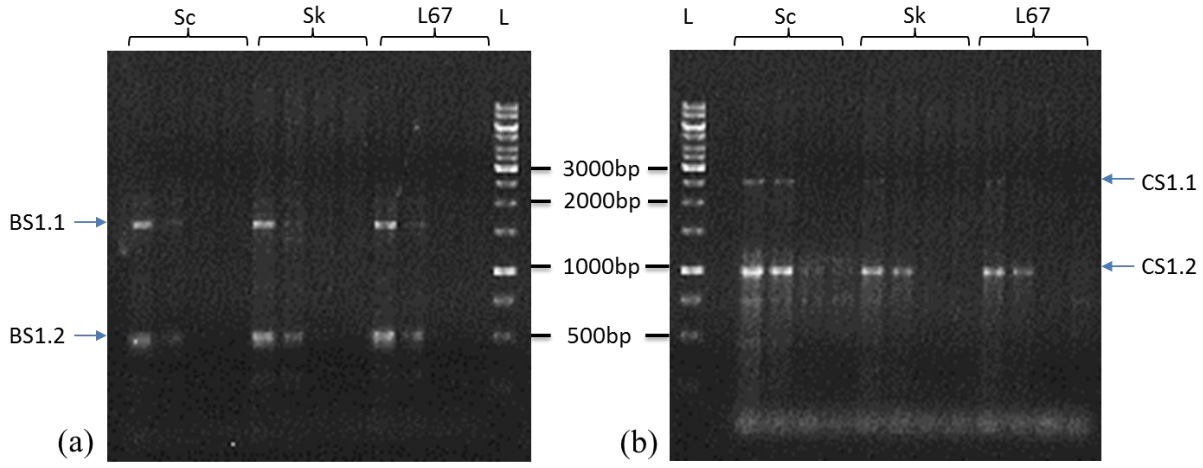

Figure 10: *Xenorhabdus* spp. DNA fragments amplified in quadruplicate from Sc, Sk and L67 by the gene specific primers targeting XptB1 and XptC1 at 55.0 ºC, 57.6 ºC, 62.2 ºC and 65.0 ºC. **(a)** DNA fragments BS1.1 and BS1.2 amplified by primers B1_fwd and B1_rev. **(b)** CS1.1 and CS1.2 DNA fragments amplified by C1_fwd and C1_rev primers. The 1kb DNA ladder is marked **L** (O'gene ruler, ThermoFisher Scientific).

4.2.1 Amplification using degenerate primers

The degenerate primers targeting XptB1 and XptC1 yielded various fragments from the three bacterial isolates. The B2_fwd and B2_rev primers yielded 900bp DNA fragments named BS2 with amplification at 57ºC whereas the C2_fwd and C2_rev yielded 1300bp DNA fragments designated CS2 amplified at 53ºC, both using Phusion polymerase (Theremoscientific) (Figure 11).

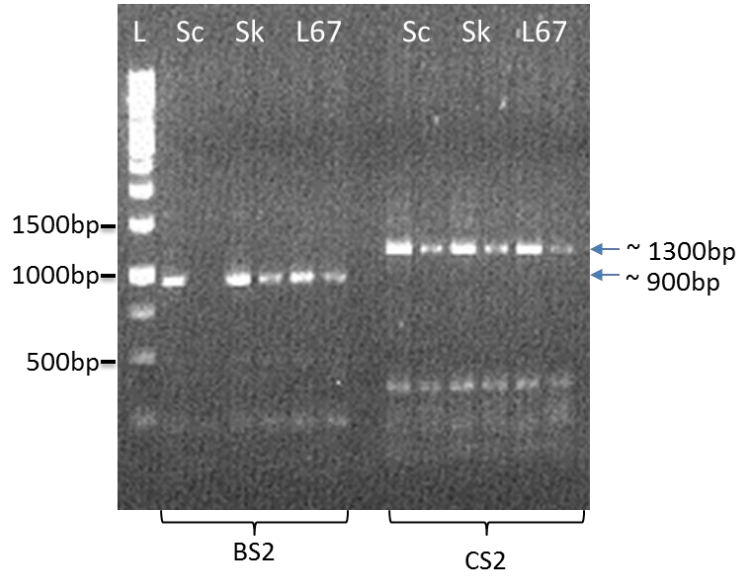

Figure 11: Gel image of DNA fragments amplified in duplicate from *Xenorhabdus* spp. Sc, Sk and L67. B2_fwd and B2_rev primers targeting XptB1 yielded a 900bp **BS2** fragment, whereas C2_fwd and C2_rev primers targeting XptC1 yielded a 1300bp **CS2** fragment. The 1kb DNA ladder is marked **L** (Fermentas).

The B3_fwd and B3_rev primers yielded 1400bp DNA fragments denoted BS3 amplified at 57ºC. Amplifications by the C3_fwd and C3_rev primers yielded non-specific bands after several reactions. These resultant bands labelled CS3 were not reproducible (Figure 12).

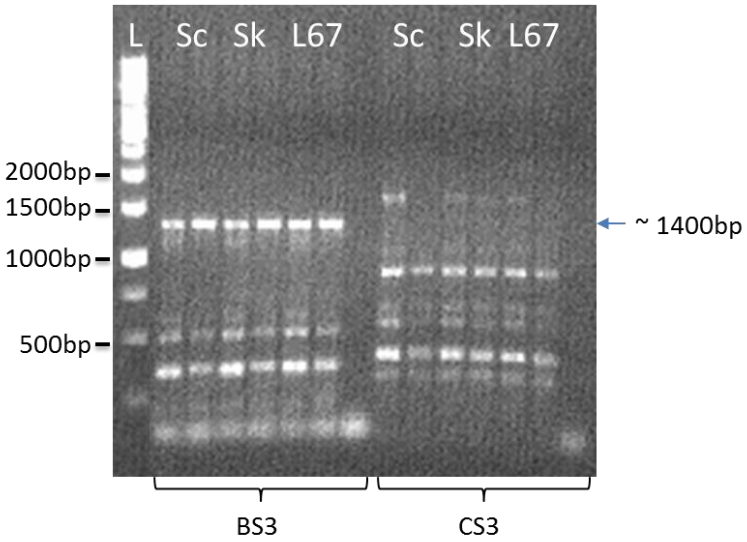

Figure 12: DNA fragments amplified in duplicate from *Xenorhabdus* spp. isolates Sc, Sk and L67. The 1400bp **BS3** fragment was amplified by B3_fwd and B3_rev targeting XptB1. Fragments amplified by C3_fwd and C3_rev primers targeting XptC1 were non-specific. **L** is a 1kb ladder (Fermentas).

Despite the use of a range of temperatures from 45 - 55ºC, the amplifications by primers B4_fwd and B4_rev as well as C4_fwd and C4_rev were non-specific and therefore not reproducible after several reactions in any of the three *Xenorhabdus* isolates (Figure 13).

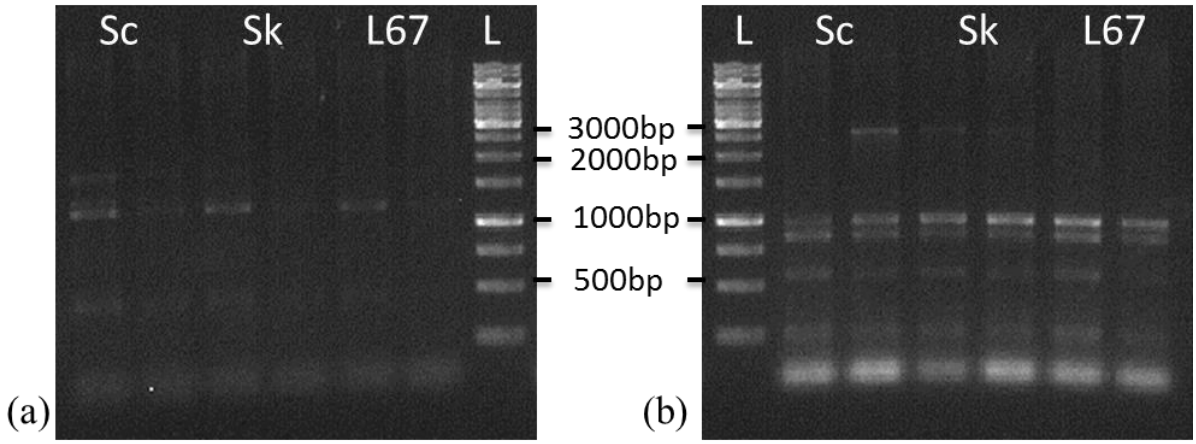

Figure 13: Gel image showing duplicate amplification from *Xenorhabdus* spp. Sc, Sk and L67 with (a) B4_fwd and B4_rev primers targeting XptB1 and **(b)** C4_fwd and C4_rev primers targeting XptC1. Fragments obtained were not reproducible. **L** represents a 1kb DNA ladder (Fermentas).

The PCR amplifications targeting the XptB1 region from *Xenorhabdus* spp. Sc, Sk and L67 were gel excised and purified (Figure 14). They included; BS1.1 and BS1.2 at 1500bp and 500bp respectively, BS2 at 900bp and BS3 at 1400bp. Each of those fragments was sequenced.

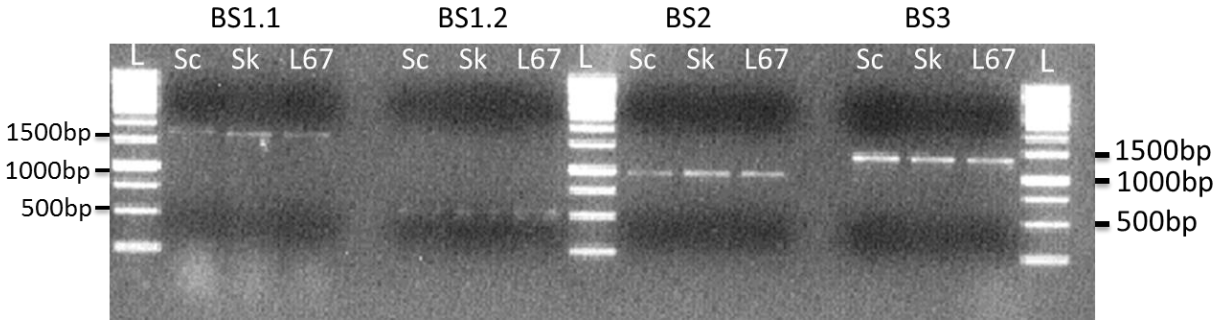

Figure 14: Gel electrophoresis showing purified PCR fragments amplified from *Xenorhabdus* spp. Sc, Sk and L67; **BS1.1** and **BS1.2** both amplified by B1_fwd and B1_rev (gene specific) primers. **BS2** and **BS3** amplified by B2_fwd and B2_rev

and B3_fwd and B3_rev (degenerate) primers respectively. The 1kb DNA ladder (Fermentas) used is marked **L**.

The primers targeting the XptC1 region, only amplified two sequences from one of the bacterial isolates designated Sc (Figure 15) designated CS1.1 at 2500bp and CS1.2 at 1000bp. Each of the fragments was sequenced.

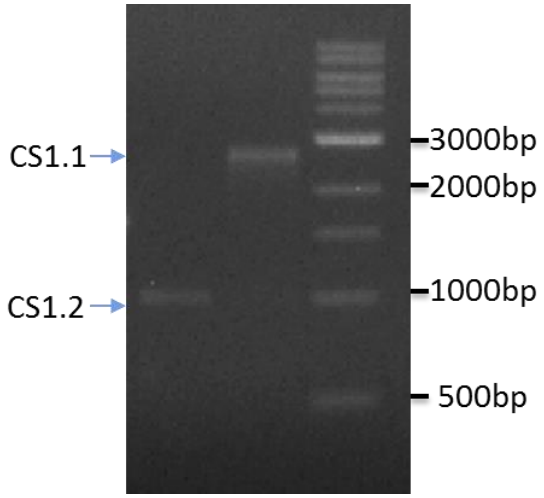

Figure 15: Purified PCR fragments from the *Xenorhabdus* sp. isolated from *Steinenema carpocapsae*. The fragments denoted **CS1.1** (2500bp) and **CS1.2** (1000bp) were amplified by C1_fwd and C1_rev gene specific primers. 1kb DNA ladder from NEB was used.

4.2.2 Gene sequence analysis

4.2.2.1 Xenorhabdus sequence B1 (XSB1)

The sequenced ~1500bp fragments amplified by Primers B1_fwd and B1_rev were given the code names Xenorhabdus sequence B1 abbreviated as XSB1_1 after trimming (Appendix 5a). There were three sequences in total from isolates designated Sc, Sk and L67. The BLAST search for highly similar sequences known as a megablast done on the NCBI database revealed, with a >80% identity and a 99% query cover on average, the best hit as a conserved exported protein of unknown function similar to that found in *X. doucetiae* str. FRM 16 sequences, followed by two other *X. bovienii* sequences (Table 7). The three sequences generated were well aligned; however the XSB1_1Sc sequence showed slight differences.

Table 7: Best megablast hits obtained from the Xenorhabdus query sequences amplified by B1_fwd and B1_rev primers showing high identity to a conserved exported protein of *X. doucetiae*

Query Sequence	Organism	Description	Score (Bit)	E value	Query cover	Identity	Accession number
XSB1_1Sc	*X. doucetiae* str. FRM 16	Conserved exported protein of unknown function	676	0.0	99%	83%	FO704550.1
	X. bovienii str. CS03	Conserved exported protein of unknown function	449	3.0E-122	84%	80%	FO818637.1
	X. bovienii SS-2004	Conserved hypothetical protein; putative exported protein	438	6.0E-119	84%	79%	FN667741.1
XSB1_1Sk	*X. doucetiae* str. FRM 16	Conserved exported protein of unknown function	1149	0.0	100%	85%	FO704550.1
	X. bovienii str. CS03	Conserved exported protein of unknown function	841	0.0	99%	80%	FO818637.1
	X. bovienii SS-2004	Conserved hypothetical protein; putative exported protein	789	0.0	99%	79%	FN667741.1
XSB1_1L67	*X. doucetiae* str. FRM 16	Conserved exported protein of unknown function	848	0.0	100%	84%	FO704550.1
	X. bovienii str. CS03	Conserved exported protein of unknown function	588	6.0E-164	71%	83%	FO818637.1
	X. bovienii SS-2004	Conserved hypothetical protein; putative exported protein	560	1.0E-155	71%	83%	FN667741.1

After analysis of the translated amino acid sequences (Appendix 5b) coded XSB-aa in the Interpro protein sequence analysis and classification database, they were classified as similar to the Bacterial surface antigen D15 whose domain is from *Haemophilus influenza* (www.ebi.ac.uk/interpro/entry/IPR000184). The pfam - A search (Table 8) also matched the sequence to the Bacterial Surface antigen D15 as well as two other related proteins; OMP85 from *Neisseria meningitidis* and *N. gonorrhoeae,* and OMA87 from *Pasteurella multocida*. These proteins are related to a Haemolysin secretion/activation protein ShlB/FhaC/HecB

(pfam.xfam.org/family/PF01103.19), which is an outer membrane protein pore implicated in secretion and activation of haemolysin ShlA (pfam.xfam.org/family/PF03865).

Table 8: Significant Pfam-A matches obtained from translated amino acid sequences of *Xenorhabdus* spp. coded XSB-aa

Query sequence	Family	Description	Envelope	Alignment	Bit score	E value	Predicted active sites
XSB1_1Sk-aa	Bac surface Ag	Surface Antigen	2 - 214	7 - 212	41.3	1.2e-10	n/a
XSB1_1Sc-aa	Bac surface Ag	Surface Antigen	3- 156	31 - 136	25.9	5.6e-06	n/a
XSB1_1L67-aa	Bac surface Ag	Surface Antigen	2- 215	8 - 215	41.5	1.0e-10	n/a

Sequencing, end trimming and megablast analysis of the ~500bp fragments amplified by B1_fwd and B1_rev primers in all three templates, coded XSB1_2 (Appendix 6), revealed over 80% identity of the sequences with that of the gmcA protein from *X. doucetiae* (Table 9). The sequence XSB1_2Sk also was most similar to a reputed membrane protein from *X. nematophila* and *X. bovienii*, with over 85% query cover. XSB1_2Sc and XSB1_2L67 showed similarity to sequences of the C component of the insecticidal toxin of *X. nematophila* having a low query cover of only 6%.

Table 9: Best megablast hits for Xenorhabdus sequences amplified by B1_fwd and B1_rev primers whose identity is most similar to a gmcA protein

Query Sequence	Organism	Description	Score (Bit)	E value	Query cover	Identity	Accession number
XSB1_2Sc	*X. doucetiae* str. FRM 16	Protein gmcA	327	7.0E-86	82%	83%	FO704550.1
	X. nematophila AN6/1	C component of insecticidal toxin complex (Tc)	58.4	1.0E-4	6%	100%	LN681227.1
	X. nematophila ATCC 19061	C component of insecticidal toxin complex (Tc)	58.4	1.0E-4	6%	100%	FN667742.1
XSB1_2Sk	*X. doucetiae* str. FRM 16	Protein gmcA	451	3.0E-110	92%	87%	FO704550.1
	X. poinarii str. G6	Inner membrane protein ybbj; Protein gmcA	435	8.0E-105	95%	85%	FO704551.1

	X. bovienii SS-2004	Putative membrane protein	358	5.0E-95	85%	83%	FN667741.1
	X. nematophila AN6/1	Putative membrane protein	397	6.0E-94	90%	83%	LN681227.1
	X. nematophila ATCC 19061	Putative membrane protein	397	6.0E-94	90%	83%	FN667742.1
	X. bovienii str.CS03	Protein gmcA	349	2.0E-93	85%	83%	FO818637.1
XSB1_2L67	*X. doucetiae* str. FRM 16	Protein gmcA	307	1.0E-790	70%	84%	FO704550.1
	X. nematophila AN6/1	C component of insecticidal toxin complex (Tc)	56.5	4.0E-04	6%	100%	LN681227.1
	X. nematophila ATCC 19061	C component of insecticidal toxin complex (Tc)	56.5	4.0E-04	6%	100%	FN667742.1

4.2.2.2 Xenorhabdus sequence B2

The B2_fwd and B2_rev primers amplified a 900bp fragment from each of the three Xenorhabdus isolates. The trimmed sequences were coded Xenorhabdus sequence B2 (XSB2) (Appendix 7). The sequences had an average of over 65% identity similar to that of a hydrolase produced by *X. doucetiae* (Table 10) that splits the amide bond between N-acetylmuramoyl and L-amino acids in bacterial cell walls.

Table 10: Best BLASTn hits for *Xenorhabdus* spp. fragments amplified by B2_fwd and B2_rev primers showing highest identity to a hydrolase from *X. doucetiae*

Query Sequence	Organism	Description	Score (Bit)	E value	Query cover	Identity	Accession number
XSB2Sc	*X. doucetiae* str. FRM 16	N-acetylmuramoyl-L-alanine amidase AmiC	264	2.0E-66	62%	73%	FO704550.1
	X. poinarii str. G6	N-acetylmuramoyl-L-alanine amidase AmiC	196	8.0E-66	53%	70%	FO704551.1
	X. nematophila AN6/1	N-acetylmuramoyl-L-alanine amidase	183	5.0E-42	53%	70%	LN681227.1
	X. bovienii SS-2004	N-acetylmuramoyl-L-alanine amidase	165	8.0E-08	62%	68%	FN667741.1
	P. asymbiotica ATCC 43949	N-acetylmuramoyl-L-alanine amidase	69.8	8.0E-08	36%	67%	FM162591.1

XSB2Sk	*X. doucetiae* str. FRM 16	N-acetylmuramoyl-L-alanine amidase AmiC	179	6.0E-41	53%	70%	FO704550.1
	X. poinarii str. G6	N-acetylmuramoyl-L-alanine amidase AmiC	125	1.0E-24	53%	68%	FO704551.1
	X. nematophila AN6/1	N-acetylmuramoyl-L-alanine amidase	118	2.0E-22	50%	69%	LN681227.1
	X. nematophila ATCC 19061	N-acetylmuramoyl-L-alanine amidase	118	2.0E-22	50%	69%	FN667742.1
XSB2L67	*X. nematophila* AN6/1	N-acetylmuramoyl-L-alanine amidase AmiC	326	5.0E-85	71%	78%	LN681227.1
	X. nematophila ATCC 19061	N-acetylmuramoyl-L-alanine amidase AmiC	326	5.0E-85	71%	78%	FN667742.1

4.2.2.2 Xenorhabdus Sequence C1 (XSC1)

There were 2 fragments amplified by the Primers C1_fwd and C1_rev whose starting template was labelled Sc. From the smaller ~1000bp fragment, only 203 nucleotides were sequenced. A megablast search did not yield significant alignments, however, several hit sequences were obtained after a BLASTn. The sequence query coverage in this case was lower than 20% and E value scores were high ($E \leq 0.00$) (Table 11) and therefore inconclusive.

Table 11: Best hit sequences after a BLASTN using the 203bp fragment amplified by the C1_fwd and C1_rev as the query

Organism	Score (Bit)	E value	Query cover	Identity	Accession number
Spirometra erinaceieuropaei	44.6	0.56	16%	88%	LN219033.1
Angiostrongylus cantonensis	42.8	2.0	15%	91%	LK94834.1
Coprinopsis cinerea	42.8	2.0	16%	88%	XM 001831600.1
Homo sapiens	42.8	2.0	13%	93%	AC073606.19

Sequencing the larger ~2500bp fragment amplified by the C1_fwd and C1_rev primers from the template labelled Sc yielded 1138 nucleotides which were trimmed to 1060 (Appendix 8a), and given the code name XSC1. A megablast search yielded greater than 80% identity

similar to the poly (A) polymerase sequence from *X. doucetiae* and *X. poinarii* as shown in Table 12.

Table 12: Best magablast hits from the *Xenorhabdus* sp. sequence with the highest identity score being a poly (A) polymerase from *X. doucetiae*

Organism	Description	Score (Bit)	E value	Query cover	Identity	Accession number
***X. doucetiae* str. FRM 16**	Poly (A) polymerase	1208	0.0	98%	88%	FO704550.1
***X. poinarii* str. G6**	Poly (A) polymerase	972	0.0	98%	84%	FO704551.1
Yersinia ruckeri	-	357	2e-94	83%	74%	LN681231.1

The translated amino acid sequence (http://web.expasy.org/cgi-bin/translate/dna_aa) (Appendix 8b), coded XSC1-aa analysed on the interpro database was classified in the poly (A) polymerase family with a poly (A) polymerase head domain (http: //www.ebi.ac.uk/interpro/sequencesearch/iprscan5-S20150203-142945-0831-15816777-pg). The domain ranged from the 75th to 207th amino acid whose group includes RNA polymerases that are independent of nucleic acids such as polynucleotide adenylyl transferase which adds poly (A) tail to mRNA. The group also includes tRNA nucleotidyl transferase that adds CCA nucleotides to the 3' end of the tRNA (http://www.ebi.ac.uk/interpro/entry/IPR002646).

Subsequently, the search on pfam (Table 13) also yielded the above stated poly (A) polymerase head domain (http://pfam.xfam.org/family/PF01743.15) as well as a putative RNA and SrmB- binding site of polymerase A (http://pfam.xfam.org/family/PF12627.2).

Table 13: Significant Pfam-A matches showing domains obtained from the translated amino acid sequence of *Xenorhabdus* sp. coded XSC1-aa

Family	Description	Envelope	Alignment	Bit score	E value	Predicted active sites
PolyA pol	Poly (A) polymerase head domain	75 - 207	75 - 207	130.7	3.2e-11	n/a
PolyA pol RNAbd	Probable RNA and SrmB-binding site of polymerase A	234 - 297	234 - 294	72.2	1.88e-20	n/a

4.3 Protein purification of XptB1 and XptC1

4.3.1 Selection of suitable lysis protocol and volume

The lysate supernatant of *Xenorhabdus* sp. isolated from *S. carpocapsae* contained high molecular weight (HMW) protein bands within the range of interest (110kDa – 160kDa). The protein profiles viewed in the denaturing polyacrylamide gel showed weak protein bands in the lysate pellet while the lysate supernatant lacked visible bands. The approximately 170 kDa and 116 kDa protein bands respectively, are indicated by the arrows (Figure 16 a). Native PAGE however gave a better indication of the protein profiles as shown (Figure 16 b) and was therefore preferred for all further protein analysis. The figure also shows that the protocol described by Morgan *et al.* (2001) (Figure 16 b, lane M) gave a better yield of high molecular weight proteins (approximately 669 kDa, 170 kDa and 140 kDa respectively) compared to the protocol by Sheets *et al.* (2011) (Figure 16 b, lane S). The Morgan *et al*. (2001) protocol was therefore selected for cell lysis before further downstream procedures.

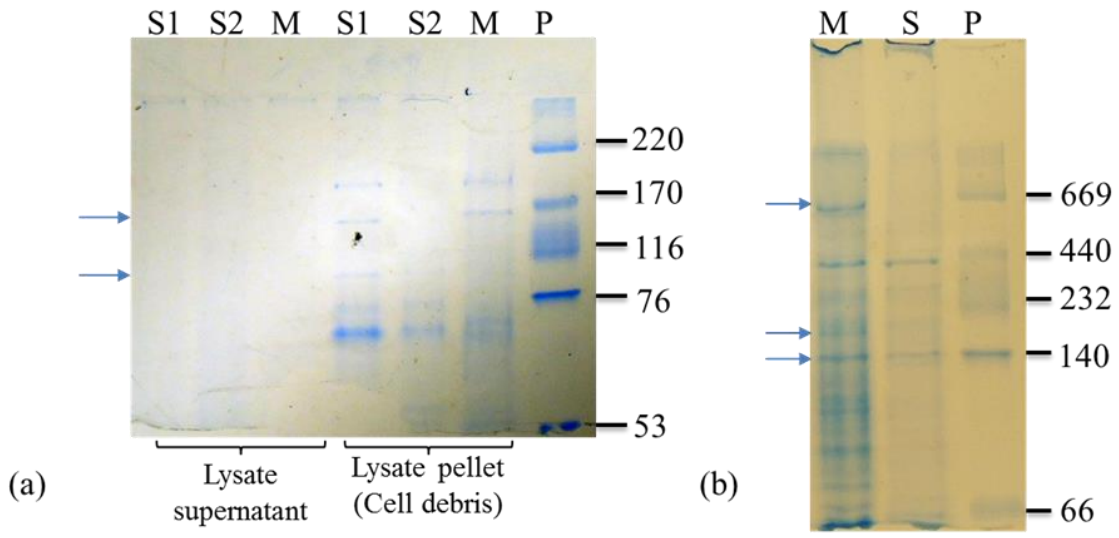

Figure 16: Polyacrylamide gel electrophoresis (PAGE) of *Xenorhabdus* sp. crude cell lysate. **(a)** SDS PAGE (10%) comparing protein yield of *Xenorhabdus* sp. from two lysis protocols, in the supernatant and pellet (cell debris). **S1**: Sheets *et al*. (2011); sonicated at 18µ, 30s on 30s off, > 10 cycles. **S2**: Sheets *et al*. (2011); sonicated at 18µ, 30s on 30s off, 10 cycles. **M:** Morgan *et al*., 2001; sonicated at 18µ, peak to peak for 30s. **P**: High Molecular weight SDS protein marker in kDa (Amersham). **(b)** Native PAGE comparing protein profiles of lysate supernatant from two lysis protocols. **M**: Morgan *et al*., 2001. **S**: Sheets *et al*. (2011) **P**: HMW native protein marker in kDa (Amersham).

The selected protocol by Morgan *et al.* (2001) carried out in various volumes of phosphate buffered saline (PBS) showed protein bands with different intensity. The 10mL PBS lysis volume was preferred over the 5mL and 15 mL PBS lysis volumes as it had minimal smears along the lane with high protein yield. The presence of proteins of similar size with the XptB1 and XptC1 proteins within the size range of 110 kDa to 160 kDa (Figure 17) was observed in the polyacrylamide gel showing the non-denatured protein profiles. Since XptB1 and Xpt C1 are known to form a heterodimer, proteins within the 232 kDa range were also of interest as seen in the native gel (Figure 17). Presence of either the partial or whole insecticidal complex was likely indicated by proteins within the range of 669 kDa or higher as viewed in the non-denatured gel in Figure 17. It was also noted that the protein bands in the lysate pellet were weak and had resolved inadequately.

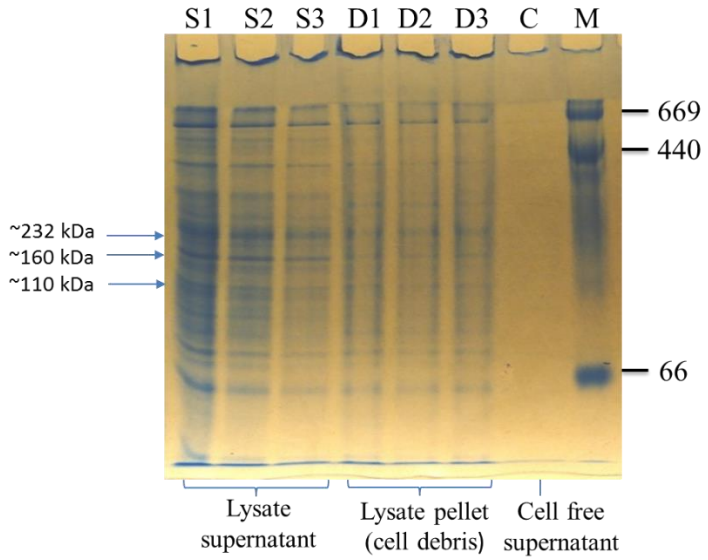

Figure 17: Native PAGE (7%) comparing protein yield from crude lysate of *Xenorhabdus* sp. in various lysis volumes with equal starting material. **S1:** lysis in 5mL of PBS - high protein yield and smeared bands. **S2**: lysis in 10mL of PBS - high protein yield and better resolved protein bands, **S3**: lysis in 15mL of PBS - lower protein yield. **D1-D3:** respective cell debris after lysis and centrifugation at 13000×g. **C:** Cell free supernatant lacking protein bands. **M:** High molecular weight native protein marker in kDa (Amersham).

4.3.2 Separation of proteins by size exclusion chromatography (SEC)

The neutral and non-denaturing nature of PBS enabled its use for protein elution during initial purification of Xenorhabdus crude lysate supernatant that employed SEC. PBS was also suitable for dialysis after the eluted proteins had been concentrated with ammonium sulphate. The void volume of the column was determined to be 51 mL collected after 34 fractions of Blue dextran 2000 were eluted from the column. The elution profile had a typical a single peak at the 34th fraction (OD_{280} of 0.4635A) (Figure 18).

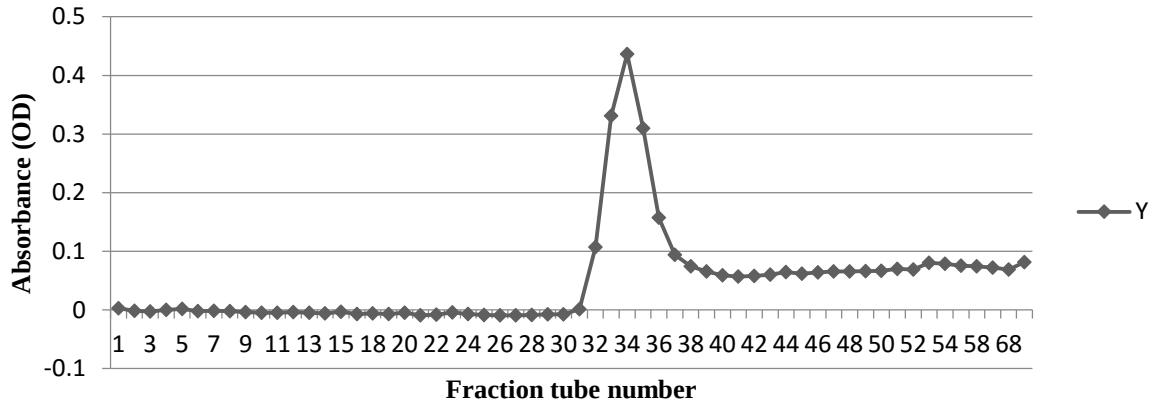

Figure 18: Absorbance of eluted fractions of Blue dextran 2000 at 280nm having a single peak elution profile.

The protein standards also ran through the column gave two major peaks (OD_{280} of 0.9386A and 0.7513A respectively) and a third smaller peak (OD_{280} of 0.0888A) (Figure 19). These standards were Thyroglobulin – 669 kDa, Catalase – 232 kDa, Aldolase – 158 kDa and Bovine Serum Albumin – 67 kDa.

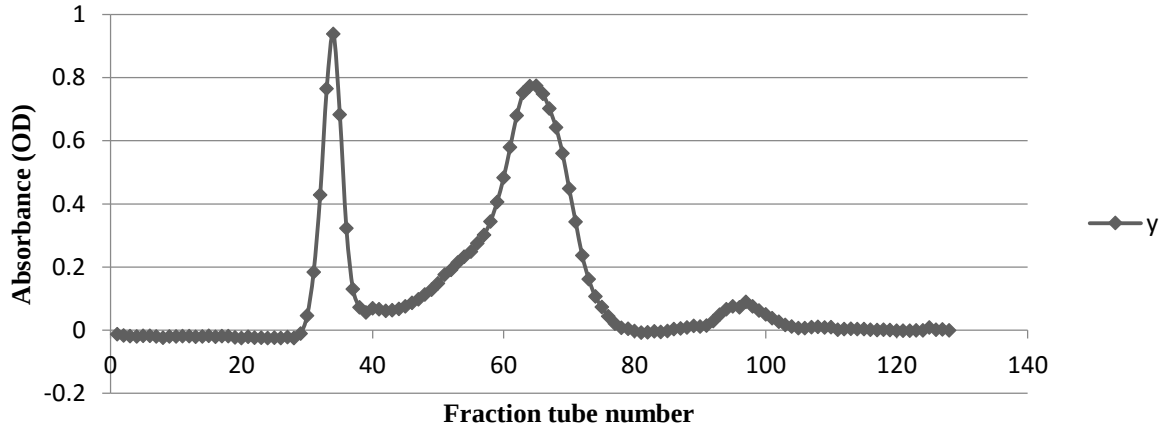

Figure 19: Absorbance at 280nm of eluted fractions of the protein standards mixture containing Thyroglobulin (669 kDa), Catalase (232 kDa), Aldolase (158 kDa) and Bovine Serum Albumin (67 kDa) showing three peaks.

The SEC column was effective in separating the proteins present in the *Xenorhabdus* sp. cell lysate ran through it. These were detected in three major peaks (OD_{280} of 4.5A, 4.5A and

3.2646A) with one minor peak (OD_{280} of 2.0605A) and the various fractions were pooled according to the peaks (Figure 20).

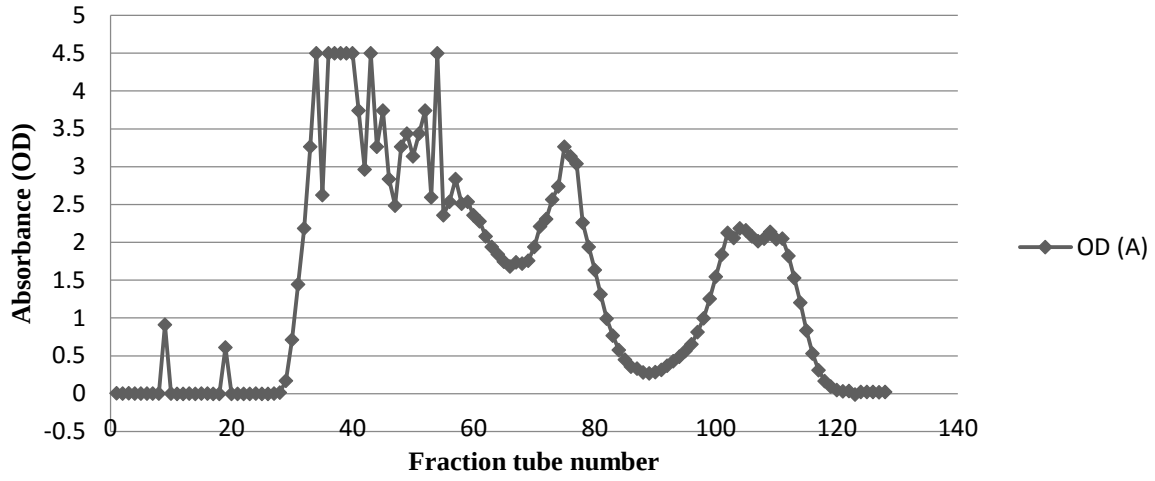

Figure 20: Absorbance of *Xenorhabdus* proteins from crude lysate at 280 nm against fraction tube number with the eluted fractions observed forming 4 peaks. Fractions were combined into 5 pools as follows; 6-26, 27-47, 48-66, 68-89, and 90-119.

The protein content of the concentrated and dialyzed pooled samples was analysed after electrophoresis on 7% native gels as shown in Figure 21. The Coomassie blue stained gel and the silver stained gels showed similar band patterns though Silver staining is a more sensitive technique. The slightly varied resolution for the proteins due to the sizes of the gels, 8×6 cm and 14×14 cm respectively. The 3rd and 4th pools were of greatest interest due to the protein bands therein ranging from 100 – 232 kDa. This range is of interest since XptB1 and XptC1 are 110kDa and 158kDa respectively. The approximately 600 kDa band in the 2nd pool was also of interest since attaining a part of or the entire complex would be beneficial.

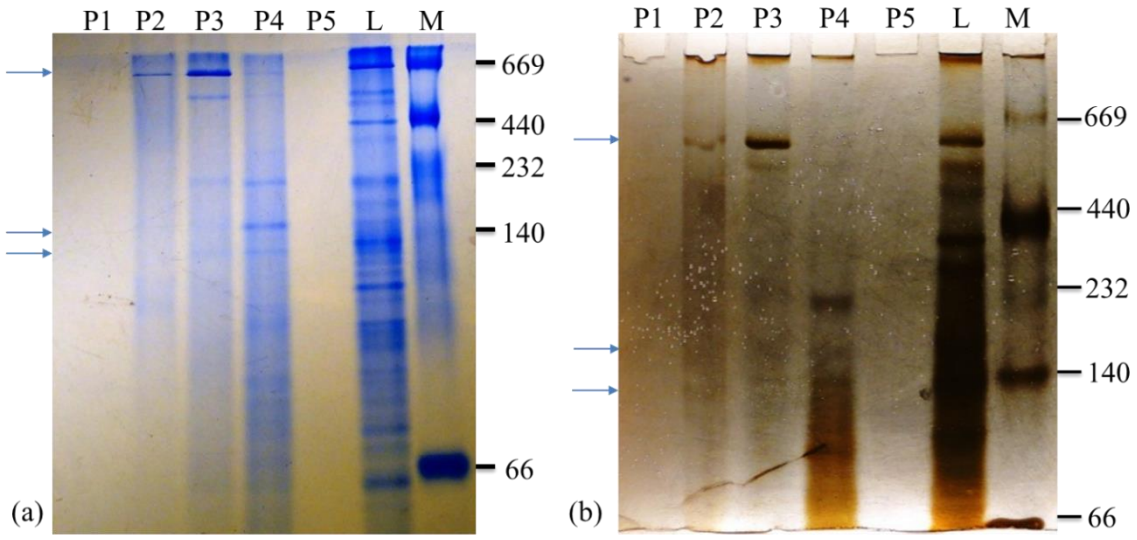

Figure 21: Native polyacrylamide gels (7%) of pooled Xenorhabdus protein samples stained with **(a)** Coomassie blue and **(b)** Silver nitrate. **P1:** Pool 1, no protein bands, **P2:** Pool 2, a distinct high molecular weight band approximately 669 kDa, **P3:** Pool 3, three bands ranging from 232-669 kDa, **P4:** Pool 4, several bands approximately 110kDa, 140 kDa and 232 kDa, P**5:** lacking protein bands, **L:** crude cell lysate, **M:** High molecular weight native ladder in kDa (Amersham).

A survey of proteins from three *Xenorhabdus* spp., *X. nematophila* str. ATCC1960, *X. nematophila* str. F1 and *X. bovienii* str. SS-2004, obtained from the online databases that were within the size range of 110 kDa to 170 kDa indicate four types of proteins thought to be important in bacterial virulence. They include: XhlA proteins, cell-surface associated haemolysins that lyse insect immune cells, mainly granulocytes and plasmatocytes; putative invasins that facilitate entry of the bacteria into host cells; extracellular serine proteases, involved in the bacteria's pathogenicity; and Rhs proteins involved in the insect virulence. The other proteins within this range are various parts forming the insecticidal complex that are either termed as component A, B or C of the insecticidal complex. There are also other proteins within this size range whose functions, however, are neither implicated in the bacteria's insect virulence and nor do they inhibit functioning of the insecticidal toxin complex (Appendix 9).

4.4 Insect bioassays

4.4.1 Feeding response of larvae

Xenorhabdus sp. isolated from *S. carpocapsae* present in the diet reduced the survival rate and hampered development of *C. partellus* larvae. The number of emerging adults was also reduced to 40% as compared to over 90% emergence in the negative controls. Figure 22 shows the total number of insects at different life stages after a 24 day period of observation. The modified artificial diet was incorporated with *Xenorhabdus* sp. in which the main preservative, formaldehyde, had been withheld. Majority of the *C. partellus* larvae that were exposed stagnated at the larval stage, some of which died (~50%). There was evidence of variation in mean adult sizes among the treatments ($F_{2, 65} = 16.58$, $P = 1.52$ e-6; Table 14) Adults that emerged in the *Xenorhabdus* sp. impregnated diet had the shortest average wing length (0.97cm ± 0.022) as compared to those from the plain diet with preservative (1.28 ± 0.036) and plain diet without preservative (1.15 ± 0.029) treatments.

Table 14: Adult wing lengths measured 24 days after exposure of *Chilo partellus* larvae to the three treatments.

Treatment	Adult wing lengths (cm)	Mean (cm)	Standard deviation	Variance	Standard error
Plain diet/ Preservative	1.6, 1.4, 1.4, 1.3, 1.4, 1.4, 1.4, 1.3, 1.2, 1.1, 1.2, 1.3, 1.9, 1.1, 1.1, 1.0, 1.5, 1.4, 1.3, 1.2, 1.5, 1.1, 1.2, 1.1, 1.4, 1.0, 1.3, 1.1, 1.2, 1.1	1.28	0.195	0.0898	0.036
Plain diet/ no preservative	1.0, 1.0, 1.5, 1.2, 1.1, 1.3, 1.2, 1.5, 1.0, 1.0, 1.3, 0.9, 1.0, 1.3, 1.0, 1.3, 1.1, 1.1, 1.1, 1.1, 1.2, 1.1, 1.2, 1.1, 1.2, 1.2	1.15	0.15	0.0226	0.029
Diet with *Xenorhabdus* sp.	0.9, 1.0, 0.9, 0.9, 1.0, 1.0, 0.9, 1.0, 1.1, 0.9, 0.9, 1.1	0.97	0.078	0.0061	0.022

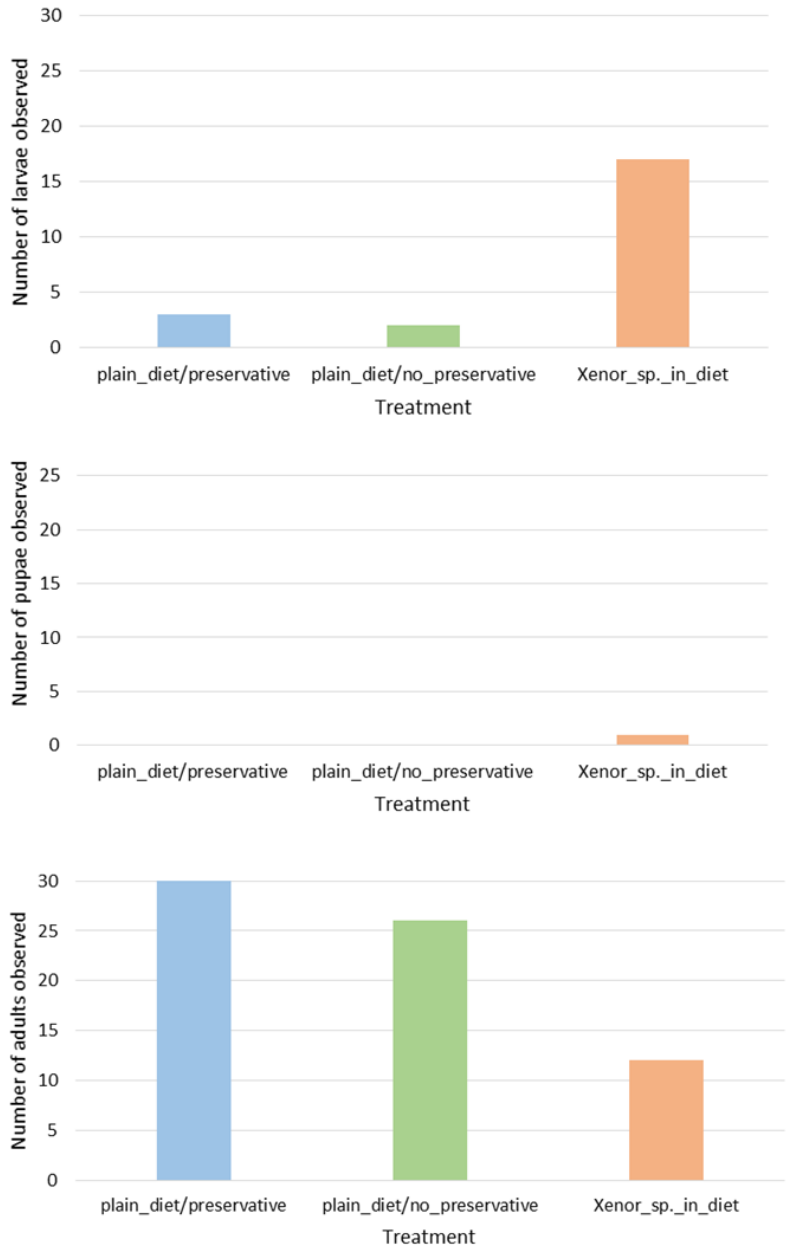

Figure 22: *Chilo partellus* development (life stages) after 24 days of exposure. Over half of the total larvae introduced in diet incorporated with *Xenorhabdus* sp. stagnated with a fewer number developing into adults as compared to those emerged in the negative controls.

The concentration of the bacteria in the diet was 1.5×10^7 cells/mL (Appendix 10a).

Statistical comparison showed that mortality significantly depended on the bacteria treatment with insect mortality of about 33% reported in *Xenorhabdus* sp. incorporated diet ($\chi_2^2 = 16.5$; p-value <2.6e-4; Table 15).

Table 15: *Chilo partellus* mortality numbers due to feeding on diet incorporated with *Xenorhabdus* sp.

	Plain diet/ Preservative	**Plain diet/ no preservative**	***Xenorhabdus* sp. incorporated in diet**
Alive	33	26	20
Dead	0	2	10

4.4.2 *Chilo partellus* larval response to direct bacterial injection

Insect mortality was observed sooner in direct injection than in feeding with larval mortality being recorded from 12 hours after exposure. The 1:10 bacterial dilution caused high larval mortality (72%) with stagnating of the live larvae at that stage. This mortality rate was much higher than in the 1:100 dilution which had the most emerged adults (22%) within the period of exposure and 4.5% mortality. The total number of insects at the different life stages after the period of observation is shown in Figures 23 and 24.

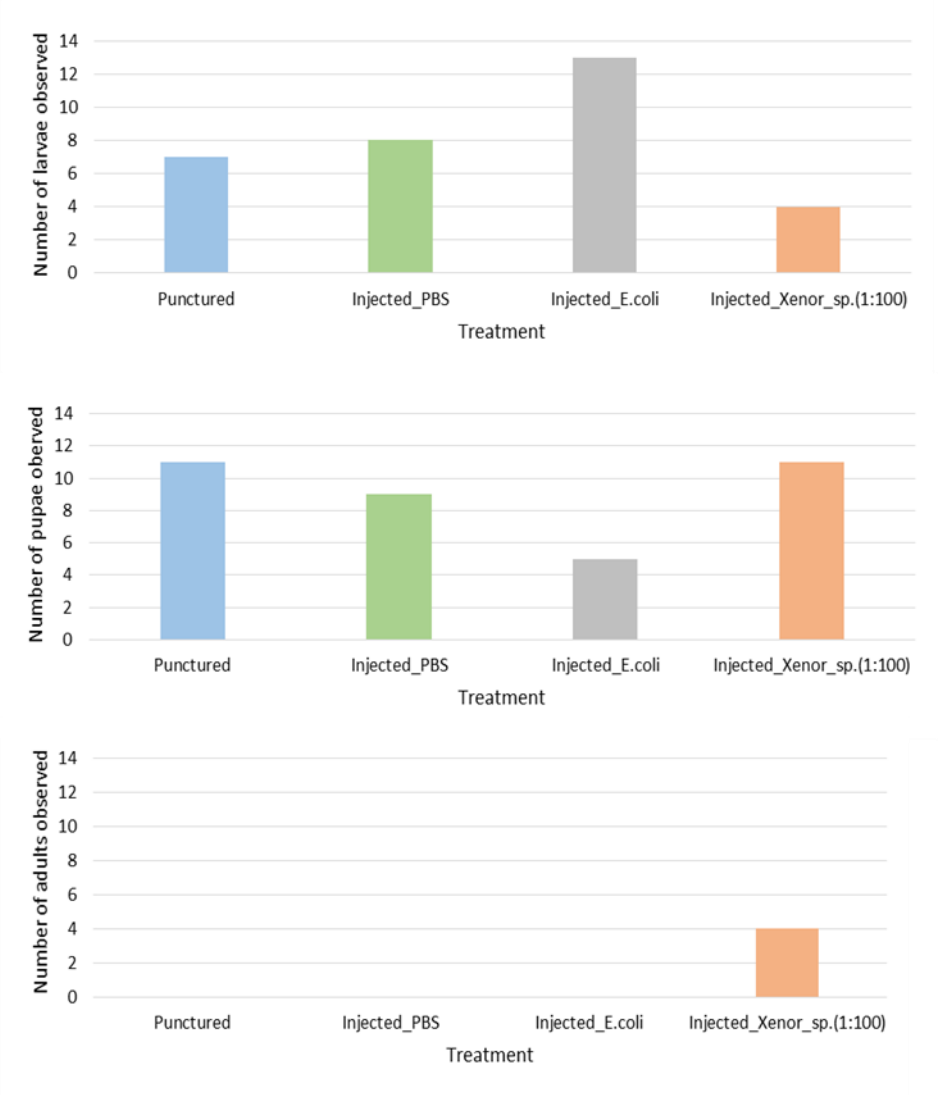

Figure 23: *Chilo partellus* developmental stages after 17 days of exposure in response to direct injection of *Xenorhabdus* sp. 1:100 dilution. This treatment has the most emerged adults compared to the controls.

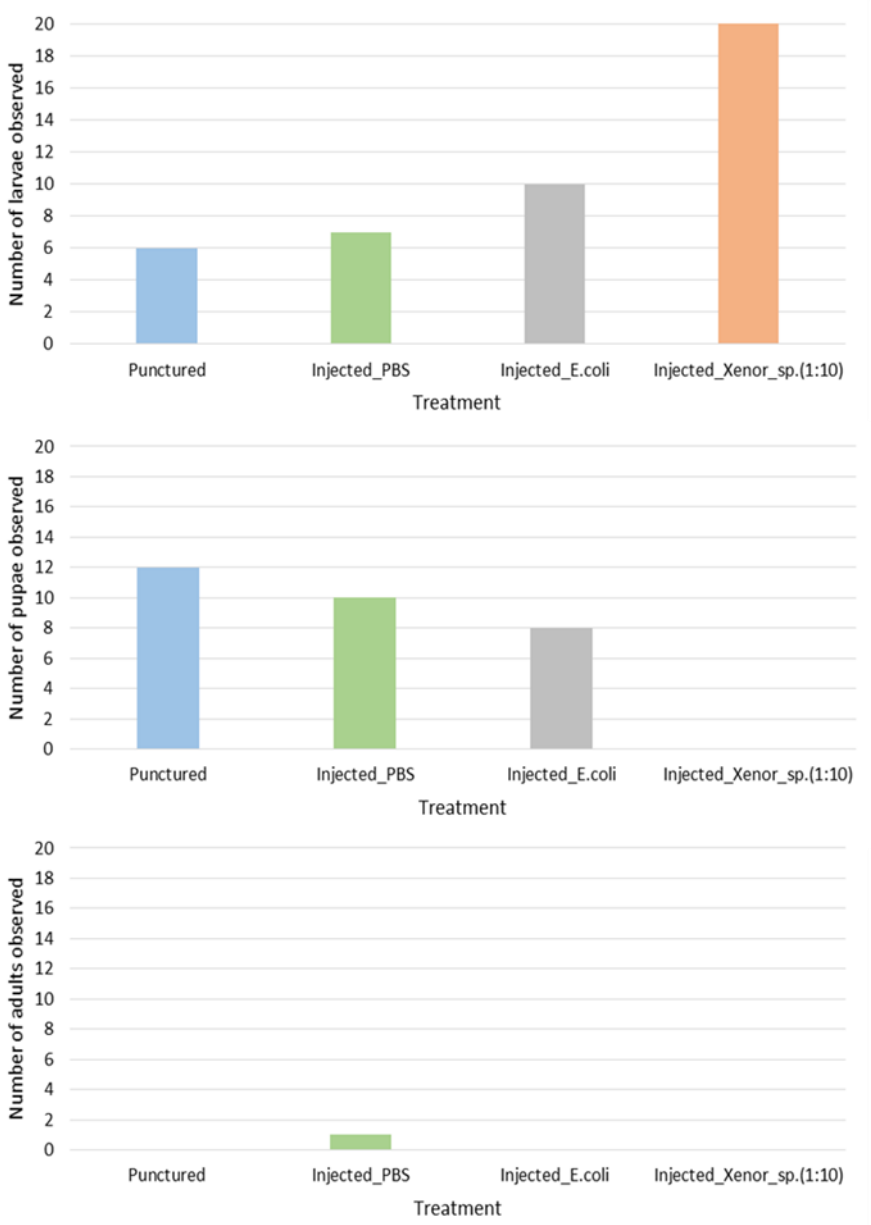

Figure 24: *Chilo partellus* developmental stages after 17 days of exposure in response to direct injection of *Xenorhabdus* sp. 1:10. The larvae in this treatment showed no further development as compared to the controls.

The concentrations of the bacteria used were recorded at OD_{600}, with values of 0.3838A for the 1:100 dilution and 2.1847A for the 1:10 dilution (Appendix 10a). The insect mortality was recorded in two contingency tables respectively (Table 15 a & b).

Table 15: *Chilo partellus* mortality recorded after direct injection with the two bacterial concentrations;

(a) Injection with 1:100 dilution of *Xenorhabdus* sp.

	Punctured	**Injected with PBS**	**Injected with *E.coli***	**Injected with *Xenorhabdus* sp.**
Alive	20	20	20	21
Dead	0	0	0	1

(b) Injection with 1:10 dilution of *Xenorhabdus* sp.

	Punctured	**Injected with PBS**	**Injected with *E. coli***	**Injected with *Xenorhabdus* sp.**
Alive	20	20	20	6
Dead	0	0	0	16

Xenorhabdus sp. in the 1:100 dilution did not have a significant effect on mortality (χ_3^2 =2.76; p-value > 0.43). *Xenorhabdus* sp. in the 1:10 dilution had a significant effect on mortality (χ_3^2 =54.2; p-value < 1.01e-11).

The larvae that had been injected with *Xenorhabdus* sp. (OD_{600}=2.1847A) rapidly darkened (Figure 24 d) and ceased feeding whereas the larvae that were punctured, injected with PBS or with *E. coli* (OD_{600}=0.3838A) and served as the negative controls, developed normally as shown in Figure 24 a-c. Larvae tunnelling into the diet and production of frass was also observed in the negative controls whereas there was deterred feeding in larvae injected with *Xenorhabdus* sp. (Figure 25). The 1:100 dilution of *Xenorhabdus* sp. (OD_{600}=0.3838A) caused only 4.5% mortality as compared to the 1:10 dilution that caused over 70% larval mortality within 4 days of injection.

Figure 25: Response of *Chilo partellus* larvae to different injection treatments after 17 days of observation.

Figure 26: *Chilo partellus* larvae feeding behaviour on plain diet after injection treatments; **(a)** tunnelling and frass in diet produced by larvae in negative controls (larvae punctured, injected with PBS and injected with *E. coli*). **(b)** little/no feeding in larvae injected with *Xenorhabdus* sp. (OD_{600}=2.1847A).

4.4.3 Response of *Busseola fusca* larvae to direct bacterial injection.

Mortality of *B. fusca* larvae of L3 and L4 stages was observed from 12 hours after a single dose injection with *Xenorhabdus* sp. (OD_{600}=2.1847A). Larvae that were punctured, injected with PBS or with *E. coli* were the negative controls. The larvae that had been punctured as well as those injected with PBS developed normally whereas those injected with *E. coli* or with *Xenorhabdus* sp. showed impaired development. Figure 26 shows the total number of insects at different life stages after the period of observation. The least number of larvae developing into pupae were injected with *Xenorhabdus* sp. (2.5%) followed by larvae injected with *E. coli* (15.79%).

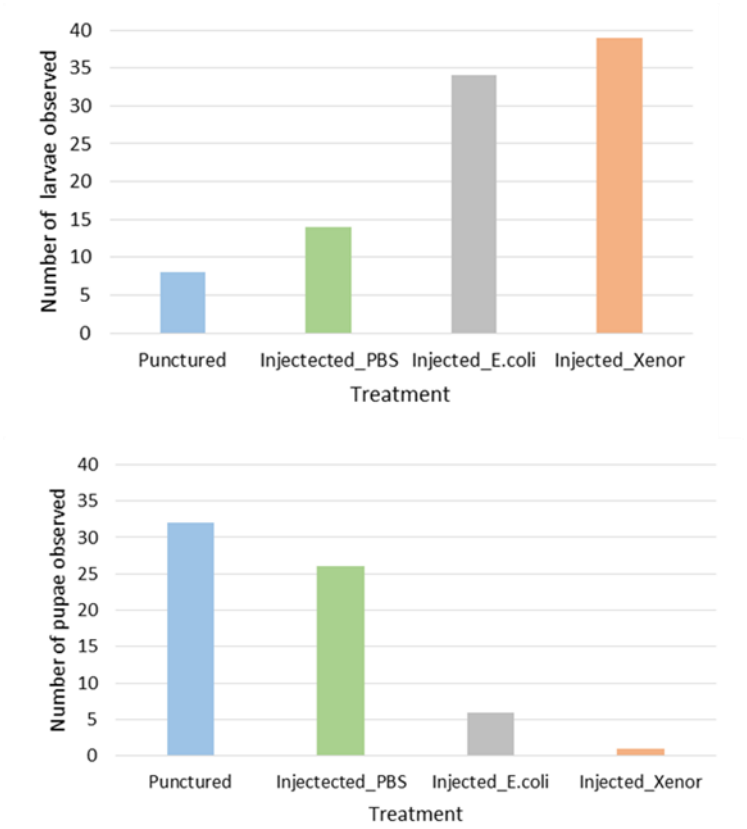

Figure 27: *Busseola fusca* developmental stages in response to direct injection of *Xenorhabdus* sp. after 24 days of exposure. Emergent adults were lacking in all treatments since *B. fusca* have a longer life cycle.

Larval mortality due to the effect of *Xenorhabdus* sp. rose from 60% (12 hours after exposure) to 78% observed after 6 days of injection and mortality was recorded in the contingency table (Table 16). The effect of *E. coli* (OD_{600}=2.1847A) on *B. fusca* larvae caused 40% mortality within 12 hours which rose to 51% recorded 9 days after injection. The effect of *E. coli* on the *B. fusca* larvae was deviant from the other negative controls.

Table 16: *Busseola fusca* larvae injected with *Xenorhabdus* sp. indicating insect mortality after 9 days of exposure

	Punctured	**Injected with PBS**	**Injected with *E.coli***	**Injected with *Xenorhabdus* sp.**
Alive	40	40	18	8
Dead	0	0	22	32

Xenorhabdus sp. had a significant effect on larval mortality (χ^2_3 = 87.1; p-value < 2.2e-16). The larvae injected with the *Xenorhabdus* sp. displayed deterred feeding and remained cream

in colour after death (Figure 27d). For larvae injected with *E. coli*, they turned dark due to melanisation, and also exhibited deterred feeding before death (Figure 27c). Mortality rates in these two cases varied with 80% experienced in larvae injected with *Xenorhabdus* sp. and 55% in larvae injected with *E. coli.* However, larvae injected with PBS and those punctured developed normally (Figure 27 a & b). Figure 28 shows the feeding behaviour of the *B. fusca* larvae injected with *Xenorhabdus* sp. as well as with *E. coli* (b & c respectively) where the larvae were completely deterred from feeding evidenced by absence of tunnelling and lack of frass. Larvae in the negative control (Figure 28 a) that were punctured, fed normally and tunnelled into the diet producing frass.

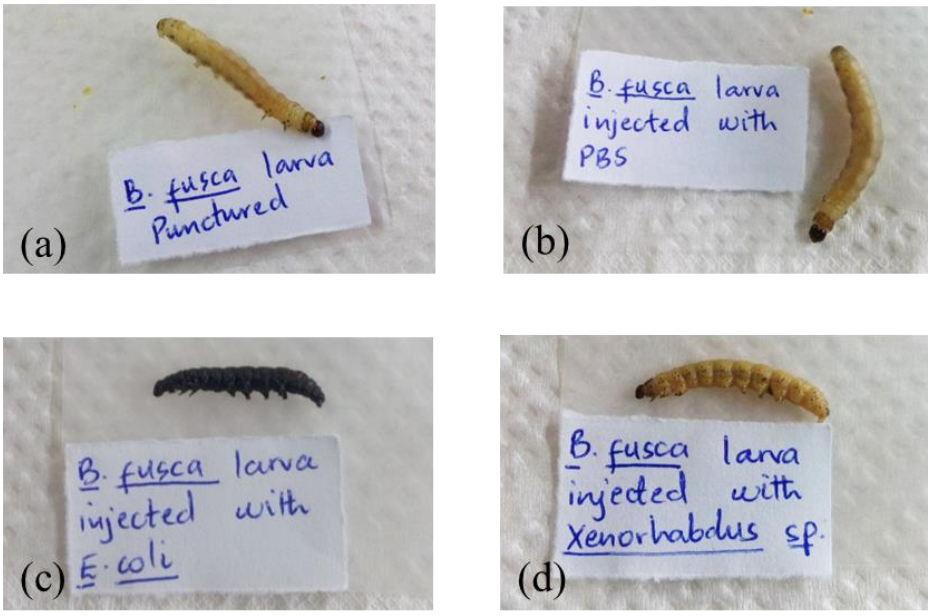

Figure 28: *Busseola fusca* larval response of various injection treatments after 6 days of exposure in **a-d.**

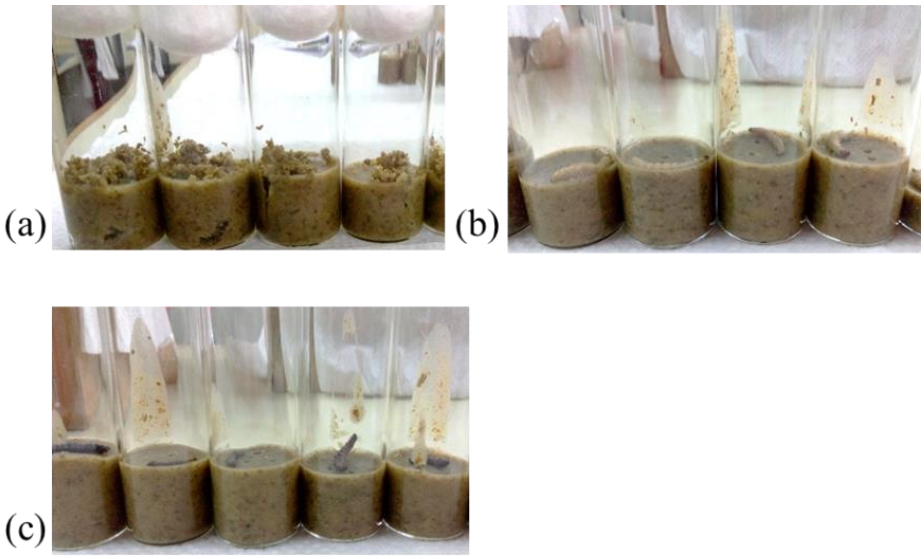

Figure 29: Feeding behaviour of injected *Busseola fusca* larvae on plain diet after 9 days of exposure (a) punctured larvae (negative control) showing tunnelling and frass was produced. (b) Larvae injected with *Xenorhabdus* sp. (OD_{600}=2.1847A) and (c) Larvae injected with *E. coli* (OD_{600}=2.1847A) showed completely deterred feeding due to lack of tunnelling as well as absence of frass.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Isolated bacterial species were closely related to *Xenorhabdus griffiniae.*

Morphological identification of the isolated bacteria grown on NBTA showed colony characteristics typical of the *Xenorhabdus* genus in colour, margin and elevation. This was consistent with the findings of Akhurst (1980), describing both their primary and secondary forms. However, morphological features alone are not sufficient to identify Xenorhabdus species. Tailliez *et al*. (2006) showed that the sole use of phenotypic features to distinguish between *Xenorhabdus* spp. was unreliable since the similarity coefficients of the 76 strains they studied exceeded 70%. The molecular technique employed to further identify the bacteria to the species level was the use of 16S rRNA gene sequences. Though the 16S rRNA gene sequencing may have low phylogenetic power, it is highly useful in bacterial classification (Janda & Abbott, 2007). The sequences generated in this work showed high similarity to *X. griffiniae* with about 99% identity. Further, in the re-constructed phylogenetic tree, these sequences clustered with the *X. griffiniae* clade.

Given that there is usually a less than 3% difference between two Xenorhabdus 16S rRNA gene sequences (Akhurst *et al*., 2004; Tailliez *et al*., 2010), conclusive species identification should therefore also include analysis of the full 16S rRNA gene (1500bp), microarray based technology or other housekeeping gene targets (Janda & Abbott, 2007). The aim would be to calculate a 97% nucleotide identity cut-off on the concatenated sequences to obtain a more robust phylogenetic tree as described by Lee & Stock (2010), Tailliez *et al.* (2010) and Kuwata *et al.* (2013). Further, biochemical tests which often augment phenotypic features would also give a broader description of the bacterial species (Boemare & Akhurst, 1988). It is therefore possible that each of the nematodes *S. carpocapsae, S. karii* and *Steinernema L67* used in this study hosted *X. griffiniae* and not their native *Xenorhabdus* symbionts

namely *X. nematophila* and *X. hominicki* respectively, or alternatively hosted a novel *Xenorhabdus* species that is closely related to *X. griffiniae*.

The source of *Xenorhabdus* spp. used in this study was *S. carpocapsae*, *S. karii* and *Steinernema L67*, all previously identified morphologically (Adams *et al*., 2002) at the KALRO-Thika EPN laboratory, but not using DNA sequences. Since *S. carpocapsae* and *S. karii* host the bacteria *X. nematophila* and *X. hominicki* (Kenyan isolate) respectively (Tailliez *et al*., 2006), it was expected that these would be the bacteria isolated. This is because of the specific one-to-one relationship between the bacteria and their nematode hosts with the exceptions of *X. hominicki, X. poinarii* and *X. bovienii* for example, that can be hosted by more than one nematode (Sicard *et al*., 2004; Lee & Stock, 2010). *X. hominicki* is also hosted by *S. monticolum*, which was isolated in Korea (Adams *et al*., 2002). The *X. griffiniae* type strain ID10, native to Indonesia, is hosted by *S. hermaphroditum* but has also been reported to re-associate with *S. carpocapsae in vitro* (Tailliez *et al.*, 2006; Chapuis *et al*., 2009). This study has demonstrated that *X. griffiniae* had a probable association with *S. carpocapsae, in vivo*. However, further nematode characterization is required to confirm this. This may be through molecular means that would involve amplification of conserved regions of taxonomic importance such as the internal transcribed spacer (ITS) region of the ribosomal DNA repeat unit, the region of the mitochondrial genome which separates the cytochrome oxidase subunit II (COII) and the mitochondrial 16S genes (Hominick *et al*., 1996).

5.2 *Xenorhabdus* sequences generated infer insecticidal activity.

Each of the degenerate and gene specific primers used amplified fragments of the same size range in all the three isolates of *Xenorhabdus* spp. genomic DNA used. Though the primers were designed to target XptB1 and XptC1 genes, the amplified sequences were not identified as either of the two. The amplified sequences were however similar to other sequences

within the Xenorhabdus genus and were closely related to sequences from *X. doucetiae*, which is hosted by *Steinernema diaprepsi* (Tailliez *et al*., 2006).

The 1500bp fragments amplified by the gene specific primers, B1_fwd and B1_rev, were likely to be the resultant gene encoding a conserved exported protein from *X. doucetiae* and its translated amino acid sequence containing a conserved bacterial surface antigen D15 domain previously described by Loosmore *et al.* (1997). This sequence is hypothesized to be related to the haemolysin secretion/activation protein ShlB/FhaC/HeB, which is an enzymatic outer membrane protein pore found in the human pathogen *Serratia marcescens*. It is involved in a VB-type or two partner secretion system that secretes and activates the haemolysin ShlA. Activation occurs during secretion when ShlB causes ShlA to have a conformational change forming an active protein. This active ShlA is a cytotoxin that results in depletion of ATP and potassium efflux in fibroblasts and epithelial cells (Walker *et al*., 2004).

The secretion and activation function of ShlB is similar to that of XptC1 (class B) proteins of the Xenorhabdus toxin complex. This may suggest that the B1 forward and reverse primers amplified a conserved region of the toxin complex involved in translocation of the enzyme into the host cell. This is because XptC1 proteins are believed to function together with the XptA1 (class A) proteins as chaperones of the XptB1 (class C) proteins. Class B and C proteins were thought to be potentiators of the class A proteins (Sergeant *et al*., 2003). Recent studies on the ABC toxins of *Photorhabdus luminescens* by Gatsogiannis *et al*. (2013) and Meusch *et al*. (2014) revealed a syringe-like mechanism of moving the enzymatic component of the TcC protein into the cytoplasm of target host cells. During translocation, large conformational changes occur on the β-propeller of TcB (class B protein) after it binds to the funnel of TcA (class A protein). This allows passage of the ADP-

ribosyltransferase (class C protein) through the translocation pore where it is disordered before entry into the target cell.

The B1_fwd and B1_rev gene specific primers also amplified a 500bp fragment that resembles the gmcA protein from *X. doucetiae* that was described as a putative choline dehydrogenase (www.uniprot.org/uniprot/A0A068QNC0). They catalyse oxidation of alcohols to corresponding aldehydes and retain the same overall structure containing a canonical ADP binding βαβ-fold occurring near the end despite diverse activity (Cavener, 1992). The B2 forward and reverse primers amplified a 900bp fragment that was probably a hydrolase similar to that found in *X. doucetiae* designated as N-acetylmuramoyl-L-alanine amidase Ami C. It is involved in cleaving the murein septum during cell division along with Ami A and Ami B and catalyses autolysis where antibiotics that inhibit murein synthesis are present (Heidrich *et al.*, 2001). Though the sequences amplified are probable enzymes important for cell division and catabolism, they are neither associated with the insecticidal genes nor the function of toxin proteins.

The 2500bp fragment amplified by C1_fwd and C1_rev primers was probably a poly (A) polymerase enzyme gene comparable to that from the *X. doucetiae*. Its translated sequence obtained had two domains; a head domain (http://pfam.xfam.org/family/PF01743.15) and a putative RNA and SrmB- binding site of polymerase A (http://pfam.xfam.org/family/PF12627.2). This enzyme causes post transcriptional modification by independently adding adenosine monophosphates (AMP) to the 3' terminal of RNA strands thus targeting them for destruction (Cao & Sarkar, 1992; Dreyfus & Régnier, 2002). The putative function of XptB1 (class C) protein, a homologue of the TccC gene/protein from *P. luminescens*, is to serve as a post translational modification enzyme that causes clustering of the actin polypeptides of the host cell through transfer of Adenosine

diphosphate (ADP). This mechanism was previously described in the Photorhabdus toxin complex PTC3 from *P. luminescens.* The mechanism of action of XptB1 and that of the Poly (A) polymerase may be comparable with regard to the moiety that these enzymes transfer, ADP and AMP respectively, as well as the enzymes' active sites (Morgan *et al*., 2001; Lang *et al*., 2010).

A portion of XptC1 (class B) protein may also be comparable to the poly (A) polymerase with respect to the molecules transferred by the enzymes, ADP and AMP respectively. XptC1 protein contains a putative SpvB domain that is part of the virulence plasmid spvABCD. The SpvB domain occurs as an entire gene/protein in Salmonella species that do not cause typhoid such as *Salmonella dublin*. This protein was initially reported to have a transmembrane association function (El-Gedaily *et al*., 1997), and has further been shown to comprise of a C-terminal mono ADP-ribosyl transferase domain that modifies G actin, and therefore interferes with polymerization of actin in host cells (Tezcan-merdol *et al*., 2001). The TccC proteins and SpvB proteins are all classed as enzymes that transfer ADP to the polypeptide chain causing a modification (Fieldhouse & Merrill, 2008). The fact that the poly (A) polymerase and the ADP-ribosyl transferase transfer similar moieties which are AMP and ADP respectively to their targets, may suggest similarity in their enzymatic active sites. This may further suggest that the primers used in this study may have amplified a portion of the active site of the target toxin genes.

Amplification of the entire open reading frame of XptB1 and XptC1 was not achieved. This may be attributed to the fact that some of the primers used were designed specifically for the XptB1 and XptC1 genes from *X. nematophila*, whereas the isolates used in this study were identified to be more closely related to *X. griffiniae* whose genome was not available in the free online databases at the time of primer design. The degenerate primers used were also not successful in amplifying the gene sequences of interest which may be because the target

sequences were more variable than anticipated. The sequences generated from this work do, however, contribute to characterization of the local *Xenorhabdus* strains as well as pave way for further inquiry into the insecticidal genes.

5.3 *Xenorhabdus* crude cell lysate contains high molecular weight proteins of similar size to target proteins.

In this study, proteins were not detected in the supernatant from 24 and 48 hour Xenorhabdus cultures. Weak protein bands were however detected from cell free supernatant of a 6 day *Xenorhabdus* sp. culture grown in Luria Broth (LB) as analysed on an SDS silver stained gel (Appendix 11). This may be the possible reason behind El-hag and El-sadawy's (2008) use of concentrated cell free supernatant from 48 hour cultures of *X. indica* grown in brain heart infusion broth for downstream analysis. The limited protein quantity produced by *Xenorhabdus* sp. cultured in LB in this study may be attributed to a lack of some factors in LB that are present in the insect host haemolymph. Some of these factors may include chemical molecules like hormones that induce high expression of toxin genes, and metabolic genes as was shown during *Photorhabdus luminescens* infection of *Galleria mellonella* larvae (Münch *et al.*, 2008). This therefore informed the preferred use of bacterial cells, and not the cell free supernatant, as the starting material for protein purification.

The yield of high molecular weight (HMW) proteins from *Xenorhabdus* sp. obtained after cell lysis using the Morgan *et al*. (2001) protocol was reliant upon the solubility of XptB1 and XptC1, which were likely localised in the cell cytoplasm. Other methods used by Lee *et al*. (2007), Sheets *et al*. (2011) and Yang *et al*. (2012) also exploited this fact. Analysis of the cell lysate revealed the lysate supernatant to have abundant HMW proteins, some of which were absent in the pellet (cell debris). In both cases, however, the low molecular weight (LMW) proteins had similar band patterns. The use of native PAGE over SDS PAGE was also preferred in this study due to the consistent non-denatured protein profile of the

Xenorhabdus cell lysate which gave a better indication of the HMW proteins that were part of the focus of this work. As for the use of whole cell lysate, its analysis on a 7% SDS gel showed several protein bands, of interest being those within the 146kDa – 242kDa range. However, the protein bands were poorly resolved and therefore unsuitable for further analysis (Appendix 11). The lysate supernatant was therefore preferred for further downstream processes which was consistent with what Lee *et al*. (2007), Sheets *et al*. (2011) and Yang *et al*. (2012) used. The approximately 170 kDa band obtained after polyacrylamide gel analysis (Figure 16) may imply the presence of XptC1 (Class B) protein in the crude lysate. This was consistent with the observation made by Sheets *et al*. (2011).

The 669 kDa, 232 kDa, 140 kDa and 100 kDa protein bands detected after size exclusion chromatography (SEC) and analysis on a 7% native gel were accordant with the findings of Yang *et al*. (2012) who obtained some similar protein bands (669 kDa and 140 kDa) using preparative native PAGE. In this study, however, the SEC column was not sensitive enough to separate proteins whose sizes differed by a few kilo Daltons, with the 4th pool having proteins of approximately 232 kDa, 160 kDa and 100 kDa. A previous study that isolated the native toxin complex from *X. nematophila* (Sheets *et al*., 2011) combined other purification techniques such as anion exchange chromatography in tandem with hydrophobic interaction chromatography. Though the successive purification steps (Sheets *et al.*, 2011) better separated the proteins from the crude lysate, this work set a platform for further analysis of XptB1 and XptC1 proteins.

The inference that the XptB1 and XptC1 proteins of interest were likely present in the *Xenorhabdus* crude lysate was supported by the size of protein bands observed (approximately 100 kDa, 140 kDa and 170kDa) from Xenorhabdus cell lysate as well as bioinformatic sequence analysis. Protein sequences from the online database within the 100 and 160 kDa range were previously characterized as either putative enzymes, membrane

proteins or insecticidal proteins. Their functional descriptions as per the database did not antagonise the activity of the toxin proteins that were of interest to this study. Peptide sequencing is required to ascertain the presence of the target proteins.

5.4 *Xenorhabdus* sp. retards survival and feeding behaviour of *Chilo partellus* and *Busseola fusca* larvae.

The effect of whole *Xenorhabdus* sp. cells fed to *C. partellus* through incorporation in artificial diet was a 33% mortality and sub-lethal effects such as deterred feeding, and slow development which were recorded for 24 days. These findings differed from those of feeding assays previously done on; *Helicoverpa zea* and *Heliothis virescens* using purified and recombinant Xpt proteins (Sheets *et al*., 2011), *Pieris brassicae* using *Xenorhabdus nematophila 9965* cells (Morgan *et al*., 2001) and *P. brassicae, Pieris rapae, Plutella xylostella* and *H. virescens* using recombinant Xpt proteins (Sergeant *et al*., 2003). For these three studies, reports of growth inhibition of the larvae and the mortality rates were recorded within 5 – 6 days.

Besides the difference in the duration taken to record larval mortality, other aspects of the assays also differed. This study utilized whole bacterial cells blended into the artificial diet which was consistent with the initial study by Morgan *et al*. (2001), who in addition used recombinant Xenorhabdus toxins. However, the studies by Sheets *et al*. (2011) and Sergeant *et al*. (2003) report having employed purified forms of the *Xenorhabdus* toxins delivered on the surface of artificial diet. The different modes of toxin application were due to the feeding nature of the tested insects. *H. zea, H. virescens, P. brassicae, P. rapae,* and *P. xylostella* grazed on the surface of the diet while *C. partellus* that was assessed in this work, tunnel into the diet. *C. partellus* L3 stage larvae, which are responsible for eating out extensive galleries in the central tissue of the maize stem (Kfir *et al*., 2002; Tefera *et al*., 2010), were the target in the present study whilst neonates of *H. zea*, *H. virescens, P. brassicae, P. rapae,*

and *P. xylostella* were used in the assays by Morgan *et al*. (2001), Sergeant *et al*. (2003) and Sheets *et al*. (2011).

Some of the differences highlighted in the assays may be the reason for the different results between this work and the reports by Morgan *et al*. (2001), Sergeant *et al*. (2003) and Sheets *et al*. (2011). The culture conditions in the artificial diet used in this study for instance, may have been unsuitable for optimum *Xenorhabdus* sp. growth for the entire duration of the assay. However the viability of the bacteria in the diet was verified to the 7^{th} day after exposure (data not shown), which was the approximate duration in which larvae moulted into the next instar. The other possible reason may be the use of purified proteins in previous studies as reported by Sergeant *et al*. (2003) and Sheets *et al*. (2011) as opposed to the use of whole *Xenorhabdus* sp. cells in this study. The initial toxin concentration of the purified proteins in the two previous studies may have been higher and the toxins possibly more stable than the toxins released into the artificial media by *Xenorhabdus* sp. used in this study. The concentration of *Xenorhabdus* sp. was 1.5×10^7 cells/mL (Appendix 10a) which was higher than the concentration 1.0×10^6 cells/mL of *X. nematophila* strain 9965 used by Morgan *et al*. (2001). The *X. nematophila* 9965 used by Morgan *et al*. (2001) achieved 100% larval mortality of *P. brassicae* neonates in 6 days. However, other Xenorhabdus isolates used in their study including *X. poinarii* (ATCC 4921), *X. beddingii* (UQM2211), *X. bovenii* (UQM2872), and *X. redingi* were not as insecticidal when fed to the *P. brassicae* neonates. Their findings may apply to this current study suggesting that the *Xenorhabdus* sp. used was possibly less insecticidal when fed to L3 stage *C. partellus* larvae. Another likely aspect was the varied susceptibility of the species tested to the various toxins and to the bacteria. Some insect species like *P. brassicae* may be more sensitive than others such as the *C. partellus* used in this study, and the neonate larvae may also be more sensitive than the L3 larvae. A conclusive comparison to ascertain the varied extent of susceptibility of the larvae of the

target insect species would involve testing similar insect toxic agents, on the same larval stage.

The direct injection assay was revealed to be more lethal when compared to the feeding assay and that the 1:10 dilution of *Xenorhabdus* sp. was more effective than the 1:100 dilution. The darkening of larvae in the 1:10 dilution referred to as melanisation observed prior to larval death was indicative of an immune response from the larvae (Jiang *et al*., 2010; Yang *et al*., 2012) due to the *Xenorhabdus* sp. This was in agreement with the findings of Yang *et al*. (2012) who injected the purified HIP57 protein from *X. budapestensis* into *Galleria melonella* larvae that developed a dark body colour and were dead within 2 days. The larvae of *C. partellus* in the negative controls of this study which included puncturing, injection with *E. coli* cells, and injection with PBS, developed normally.

The deterred larval feeding evidenced by lack of tunnelling and frass in the diet, quick pupation of larvae in 4 days compared to 7 days in the controls, and shorter wing span recorded in emergent adults were the sub-lethal effects observed due to the 1:100 dilution used. Sub-lethal effects were also observed by Mugo *et al*. (2011) where accumulation of larval weight was supressed in larvae of both *B. fusca* and *C. partellus* after a 10 day exposure of the larvae to *Bacillus thuringiensis* (Bt) maize hybrid MON810. They attributed these effects to the fact that toxic chemical substances ingested by insects can interfere with metabolic processes and eventually reduce the growth of the insect. However, as with recommendations on the use of Bt maize (Mulaa *et al*., 2011), the maintenance of high concentration of the toxins is key to ensure that the insects do not develop resistance. In this study, a concentration of OD_{600} =2.1847A (2.1847×10^6 cells/µL) (Appendix 10a) was considered high enough to cause over 70% of *C. partellus* larval mortality.

The over 70% mortality of *B. fusca* larvae was also recorded soon after exposure to a concentration of *Xenorhabdus* sp. (2.8215A) comparable to the 1:10 dilution used against *C. partellus* larvae. However, in contrast to *C. partellus* larvae, the *B. fusca* larvae injected with *Xenorhabdus* sp. remained cream in colour and did not darken. This possibly indicated that the insect immune response was not elicited. This finding was consistent with what has been described by Hinchliffe *et al*. (2010) as one of the virulence mechanisms in *Xenorhabdus* spp. The virulence mechanism referred to was the ability of the bacteria to inhibit phospholipase A_2 therefore interfering with activation of prophenoloxidase into phenoloxidase. This further inhibits the eicosanoid pathway which causes haemocyte clustering and nodulation through the phenoloxidase cascade. It is this cascade that results also in melanisation and ultimately containment of foreign bodies such as parasites and pathogens within the lepidopteran haemolymph (Hinchliffe *et al*., 2010; Jiang *et al*., 2010).

There was also 51% mortality recorded in *B. fusca* larvae injected with *E. coli* where the larvae turned dark/black due to melanisation. This observation contrasted the response of *C. partellus* larvae injected with *E. coli*, which developed normally. This may be attributed to the different concentrations of *E. coli* administered; a higher concentration was used in *B. fusca* (2.8215A) than to *C. partellus* (0.7234A). This *E. coli* (2.8215A) concentration was however similar to that of *Xenorhabdus* sp. (2.1847A) administered to *B. fusca*. In this case, death of the *B. fusca* larvae injected with *E.coli* was likely to due to the intense immune reaction involving action of phenoloxidase. This concurred with the findings of Yang *et al.* (2012) who reported that an intense immune reaction produced a large amount of quinones that were toxic to the *G. mellonella* larvae tested. They proposed that this reaction, which was also intense in the *B. fusca* larvae used in this study, was the potential cause of death.

This study is the first to report the direct use of *Xenorhabdus* sp. against larvae of *C. partellus* and *B. fusca*. The fact that *C. partellus* larvae darkened indicating an elicited immune

response (Yang *et al.*, 2012) against *Xenorhabdus* sp. whereas *B. fusca* larvae retained their cream colour implies that the two target pests likely have varied susceptibility to *Xenorhabdus* sp. Work by Mugo *et al*. (2011) showed that both *C. partellus* and *B. fusca* were susceptible to Bt δ-endotoxins from the *Cry1Ab* gene event MON810 in Kenya in greenhouse trials, whereas Kruger *et al*. (2011) confirmed that *B. fusca* from fields in South Africa was resistant to this MON810 hybrid. From this study, however, it may be inferred that *B. fusca* are more sensitive to *Xenorhabdus* sp. and therefore that the bacteria was likely more effective against *B. fusca* than *C. partellus* larvae.

5.5 Conclusions

The *Xenorhabdus* spp. isolates used in this study were found to be closely related to *Xenorhabdus griffiniae* str. ID10 with an identity of 99% using the 16S rRNA gene.

Primers targeting the *XptB1* gene (Class C protein) amplified a conserved exported protein from *Xenorhabdus doucetiae*.

Primers targeting the *XptC1* gene (Class B protein) amplified a poly (A) polymerase sequence from *X. doucetiae* responsible for transfer of adenosine monophosphate to RNA strands.

The proteins obtained ranging from 110 kDa to 160 kDa are of similar size to XptB1 and XptC1 proteins inferring their presence in the *Xenorhabdus* sp. isolated.

The bioassay investigating the effect of the *Xenorhabdus* sp. indicates the presence of bioactive compounds that caused over 70% mortality when injected into larvae of *C. partellus* and *B. fusca.*

5.6 Recommendations

Future work should involve a multilocus gene approach to *Xenorhabdus* spp. identification using housekeeping genes such as DNA polymerase III beta chain involved in DNA metabolism (*dnaN*), DNA recombination protein (*recA*), glutamyl-tRNA synthetase involved in protein synthesis (*gltX*) and DNA gyrase beta subunit (*gyrB*). These coupled with biochemical tests as well as molecular identification of the nematode hosts would fully describe the three isolated *Xenorhabdus* spp.

Genomic libraries of *Xenorhabdus* spp. should be created and additional primers/probes used to screen for the *XptB1* and *XptC1* genes to enable their cloning, sequencing and expression.

Further protein purification may involve use of anion exchange chromatography and/or hydrophobic interaction chromatography, amino acid sequencing, crystallography and bioassays to isolate and describe the XptB1 and XptC1 proteins from the *Xenorhabdus* spp.

The *Xenorhabdus* sp. should also be assayed against *B. fusca* and *C. partellus* on a larger scale in various concentrations to determine the lethal concentration (LC_{50}) and further inform their possible development and use for biological control.

REFERENCES

Adams B. J., **Nguyen K. B.**, **Boemare N.**, **Forst S.**, **Clarke D.**, **Dowds B. C. A.**, **Peters A.**, **Webster J. M.**, **Chen G.**, **Hu K.**, **Li J.**, **Himinick W. M.**, **Wright D. J.**, **Perry R. N.**, **Glazer I.** (**2002**) *Entomopathogenic Nematology*, CABI Publishing: Oxon, UK; New York, USA.

Akhurst R. (**1980**) 'Morphological and functional dimorphism in Xenorhabdus spp., bacteria symbiotically associated with the insect pathogenic nematodes Neoaplectana and Heterorhabditis', *Journal of General Microbiology*, 121, 303–309.

Akhurst R. J. (**1982**) 'Antibiotic activity of Xenorhabdus spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae.', *Journal of General Microbiology*, 128, 3061–3065.

Akhurst R. J., **Boemare N. E.**, **Janssen P. H.**, **Peel M. M.**, **Alfredson D. A.**, **Beard C. E.** (**2004**) 'Taxonomy of Australian clinical isolates of the genus Photorhabdus and proposal of Photorhabdus asymbiotica subsp. nov. and P. asymbiotica subsp. australis subsp. nov', *International Journal of Systematic and Evolutionary Microbiology*, 54(4), 1301–1310.

Ausubel F. M., **Brent R.**, **Kingston R. E.**, **Moore D. D.**, **Seidman J. G.**, **Smith J. A.**, **Struhl K.** (**2003**) *Current Protocols in Molecular Biology*, Molecular Biology, John Wiley & Sons inc.

Bateman A. (**2004**) 'The Pfam protein families database', *Nucleic Acids Research*, 32(90001), 138D–141.

Berg J. Van Den (**2012**) 'Field-resistance of the African maize stem borer to Bt maize : what did we learn ?', in *Scientific Conference 2012 Advancing the Understanding of Biosafety GMO Risk Assessment, Independent Biosafety Research and Holistic Analysis*, Hyberad/ India, 2 – 5.

Boemare N. E., **Akhurst R. J.** (**1988**) 'Biochemical and Physiological Characterization of Colony Form Variants in Xenorhabdus spp. (Enterobacteriaceae)', *Microbiology*.

Bollag D. M., **Edelstein S. J.** (**1991**) 'Protein Methods', *Wiley-Liss Incorporated*, 60 – 62.

Calatayud P., **Ru B. P. Le**, **Berg J. Van Den**, **Schulthess F.** (**2014**) 'Ecology of the African Maize Stalk Borer, Busseola fusca (Lepidoptera: Noctuidae) with Special Reference to Insect-Plant interactions', *Insects*, 5, 539–563.

Caldas C., **Cherqui A.**, **Pereira A.**, **Simoes N.** (**2002**) 'Purification and Characterization of an Extracellular Protease from Xenorhabdus nematophila Involved in Insect Immunosuppression', *Applied and Environmental Microbiology*, 68(3), 1297–1304.

Cao G. J., **Sarkar N.** (**1992**) 'Identification of the gene for an Escherichia coli poly(A) polymerase.', *Proceedings of the National Academy of Sciences of the United States of America*, 89(November), 10380–10384.

Cavener D. R. (**1992**) 'GMC oxidoreductases', *Journal of Molecular Biology*, 223(3), 811–814.

Chapuis É., **Emelianoff V.**, **Paulmier V.**, **Le Brun N.**, **Pagès S.**, **Sicard M.**, **Ferdy J. B.** (**2009**) 'Manifold aspects of specificity in a nematode-bacterium mutualism', *Journal of Evolutionary Biology*, 22(10), 2104–2117.

Chen W. P., **Kuo T. T.** (**1993**) 'A simple and rapid method for the preparation of gram-

negative bacterial genomic DNA.', *Nucleic Acids Research*, 21(9), 2260.

Cook S. M., **Khan Z. R.**, **Pickett J. A.** (**2007**) 'The use of push-pull strategies in integrated pest management.', *Annual Review of Entomology*, 52, 375–400.

Dodd S. J., **Hurst M. R. H.**, **Glare T. R.**, **O'Callaghan M.**, **Ronson C. W.** (**2006**) 'Occurrence of sep insecticidal toxin complex genes in Serratia spp. and Yersinia frederiksenii', *Applied and Environmental Microbiology*, 72(10), 6584–6592.

Dreyfus M., **Régnier P.** (**2002**) 'The Poly(A) Tail of mRNAs', *Cell*, 111(5), 611–613.

El-Gedaily A., **Paesold G.**, **Krause M.** (**1997**) 'Expression profile and subcellular location of the plasmid-encoded virulence (Spv) proteins in wild-type Salmonella dublin', *Infection and Immunity*, 65(8), 3406–3411.

El-hag H. A. A., **El-sadawy H. A.** (**2008**) 'Xenoprotec : Antimicrobial Agent Derived from Xenorhabdus indica', *American-Eurasioan Journal of Agricultural and Environmantal Sciences*, 3(4), 568–576.

Fieldhouse R. J., **Merrill A. R.** (**2008**) 'Needle in the haystack: structure-based toxin discovery', *Trends in Biochemical Sciences*.

Forst S., **Nealson K.** (**1996**) 'Molecular biology of the symbiotic-pathogenic bacteria Xenorhabdus spp. and Photorhabdus spp.', *Microbiological reviews*, 60(1), 21–43.

Forum for Agricultural Reserach in Africa (**2009**) 'Patterns of Change in Maize Production in Africa: Implications for Maize Policy Development', *Ministerial Policy Brief Series. Networking Support Function 3: Regional Policies & Markets*, (3), 1–8.

Gatsogiannis C., **Lang A. E.**, **Meusch D.**, **Pfaumann V.**, **Hofnagel O.**, **Benz R.**, **Aktories K.**, **Raunser S.** (**2013**) 'A syringe-like injection mechanism in Photorhabdus luminescens toxins.', *Nature*, 495(7442), 520–3.

Gel Filtration: Principles and Methods, AI. ed (**2002**) Amersham Biosciences.

Gram C. (**1884**) 'The differential staining of Schizomycetes in tissue sections and in dried preparations', *Fortschitte der Medicin*, 2, 185–189.

Groote H. De, **Bett C.**, **Okuro J. O.**, **Odendo M.**, **Mose L.**, **Wekesa E.** (**2002**) 'Direct Estimation of Maize Crop Losses Due To Stem Borers in Kenya , Preliminary Results From 2000 and 2001', in *Seventh Eastern and Southern Africa Regional Maize Conference*, 401–406.

Harding C. R., **Schroeder G. N.**, **Collins J. W.**, **Frankel G.** (**2013**) 'Use of Galleria mellonella as a model organism to study Legionella pneumophila infection.', *Journal of Visualized Vxperiments : JoVE*, (81), e50964.

Heidrich C., **Templin M. F.**, **Ursinus A.**, **Merdanovic M.**, **Schwarz H.**, **Pedro M. A. De**, **Holtje J.-V.** (**2001**) 'Involvement of N -acetylmuramyl- L -alanine amidases in cell separation and antibiotic-induced autolysis of Escherichia coli', *Molecular Microbiology*, 41(1), 167–178.

Herbert E. E., **Goodrich-Blair H.** (**2007**) 'Friend and foe: the two faces of Xenorhabdus nematophila.', *Nature Reviews. Microbiology*, 5(August), 634–646.

Hinchliffe S. J., **Hares M. C.**, **Dowling A. J.**, **Ffrench-Constant R. H.** (**2010**) 'Insecticidal toxins from the Photorhabdus and Xenorhabdus bacteria', *Open Toxinology Journal*, 44(0), 101–118.

Hominick W. M., **Reid A. P.**, **Bohan D. A.**, **Briscoe B. R.** (**1996**) 'Entomopathogenic Nematodes: Biodiversity, Geographical Distribution and the Convention on Biological Diversity', *Biocontrol Science and Technology*, 6(3), 317–332.

Janda J. M., **Abbott S. L.** (**2007**) '16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls', *Journal of Clinical Microbiology*.

Jiang H., **Vilcinskas A.**, **Kanost M. R.** (**2010**) 'Immunity in Lepidopteran Insects', in Soderhall, K., ed., *Invertebrate Immunity*, Landes Bioscience and Springer Science+Business Media., 181–204.

Kfir R., **Overholt W. A.**, **Khan Z. R.**, **Polaszek A.** (**2002**) 'Biology and Management of Economically Important Lepidopteran Cereal Stem Borers in Africa', *Annual Review of Entomology*, 47, 701 – 731.

Khandelwal P., **Choudhury D.**, **Birah A.**, **Reddy M. K.**, **Gupta G. P.**, **Banerjee N.** (**2004**) 'Insecticidal pilin subunit from the insect pathogen Xenorhabdus nematophila', *Journal of Bacteriology*, 186(19), 6465–6476.

Kruger M., **Rensburg J. R. J. Van**, **Berg J. Van Den** (**2011**) 'Resistance to Bt maize in Busseola fusca (Lepidoptera: Noctuidae) from Vaalharts, South Africa', *Environmental Entomology*, 40(2), 477–483.

Kumari D. A., **Sharma H. C.**, **Reddy D. J.** (**2010**) 'Incorporation of lyophilized leaves and pods into artificial diets to assess the antibiosis component of resistance to pod borer in pigeonpea', *Journal of Food Legumes*, 23(1), 57–65.

Kuwata R., **Qiu L. H.**, **Wang W.**, **Harada Y.**, **Yoshida M.**, **Kondo E.**, **Yoshiga T.** (**2013**) 'Xenorhabdus ishibashii sp. nov., isolated from the entomopathogenic nematode Steinernema aciari', *International Journal of Systematic and Evolutionary Microbiology*, 63, 1690–1695.

Lang A. E., **Schmidt G.**, **Schlosser A.**, **Hey T. D.**, **Larrinua I. M.**, **Sheets J. J.**, **Mannherz H. G.**, **Aktories K.** (**2010**) 'Photorhabdus luminescens toxins ADP-ribosylate actin and RhoA to force actin clustering (SOM).', *Science (New York, N.Y.)*, 327(February), 1139–1142.

Lee M. M., **Stock S. P.** (**2010**) 'A multilocus approach to assessing co-evolutionary relationships between Steinernema spp. (Nematoda: Steinernematidae) and their bacterial symbionts Xenorhabdus spp. (V-Proteobacteria: Enterobacteriaceae)', *Systematic Parasitology*, 77, 1–12.

Lee S. C., **Stoilova-Mcphie S.**, **Baxter L.**, **Fülöp V.**, **Henderson J.**, **Rodger A.**, **Roper D. I.**, **Scott D. J.**, **Smith C. J.**, **Morgan J. A. W.** (**2007**) 'Structural Characterisation of the Insecticidal Toxin XptA1, Reveals a 1.15 MDa Tetramer with a Cage-like Structure', *Journal of Molecular Biology*, 366, 1558–1568.

Liu D., **Burton S.**, **Glancy T.**, **Li Z.-S.**, **Hampton R.**, **Meade T.**, **Merlo D. J.** (**2003**) 'Insect resistance conferred by 283-kDa Photorhabdus luminescens protein TcdA in Arabidopsis thaliana.', *Nature Biotechnology*, 21(10), 1222–1228.

Loosmore S. M., **Yang Y. P.**, **Coleman D. C.**, **Shortreed J. M.**, **England D. M.**, **Klein M. H.** (**1997**) 'Outer membrane protein D15 is conserved among Haemophilus influenzae species and may represent a universal protective antigen against invasive disease', *Infection and Immunity*, 65(9), 3701–3707.

Mabeya J., **Ezezika O. C.** (**2012**) 'Unfulfilled farmer expectations : the case of the Insect Resistant Maize for Africa (IRMA) project in Kenya', *Agriculture & Food Security*, 1(Suppl 1), S6.

Meusch D., **Gatsogiannis C.**, **Efremov R. G.**, **Lang A. E.**, **Hofnagel O.**, **Vetter I. R.**, **Aktories K.**, **Raunser S.** (**2014**) 'Mechanism of Tc toxin action revealed in molecular detail.', *Nature*, 508(7494), 61–5.

Mitchell A., **Chang H.-Y.**, **Daugherty L.**, **Fraser M.**, **Hunter S.**, **Lopez R.**, **McAnulla C.**, **McMenamin C.**, **Nuka G.**, **Pesseat S.**, **Sangrador-Vegas A.**, **Scheremetjew M.**, **Rato C.**, **Yong S.-Y.**, **Bateman A.**, **Punta M.**, **Attwood T. K.**, **Sigrist C. J. A.**, **Redaschi N.**, **Rivoire C.**, **Xenarios I.**, **Kahn D.**, **Guyot D.**, **Bork P.**, **Letunic I.**, **Gough J.**, **Oates M.**, **Haft D.**, **Huang H.**, **Natale D. A.**, **Wu C. H.**, **Orengo C.**, **Sillitoe I.**, **Mi H.**, **Thomas P. D.**, **Finn R. D.** (**2015**) 'The InterPro protein families database: the classification resource after 15 years', *Nucleic Acids Research*, 43(D1), D213–D221.

Morgan J. A. W., **Sergeant M.**, **Ellis D.**, **Ousley M.**, **Jarrett P.** (**2001**) 'Sequence Analysis of Insecticidal Genes from Xenorhabdus nematophilus PMFI296', *Applied and Environmental Microbiology*, 67(5), 2062–2069.

Mugo S., **Karayal H.**, **Chavangi A.**, **Murenga M. G.**, **Tende R.**, **Taracha C.**, **Gichuki S.**, **Ininda J.**, **M'Bijjewe K.** (**2011**) 'Control of Busseola fusca and chilo partellus stem borers by bacillus thuringiensis (Bt)-δ-endotoxins from Cry1Ab gene Event MON810in greenhouse containment trials', *African Journal of Biotechnology*, 10(23), 4719–4724.

Mulaa M. M., **Bergvinson D. J.**, **Mugo S. N.**, **Wanyama J. M.**, **Tende R. M.**, **De Groote H.**, **Tefera T. M.** (**2011**) 'Evaluation of stem borer resistance management strategies for Bt maize in Kenya based on alternative host refugia', *African Journal of Biotechnology*, 10(23), 4732–4740.

Munch A., **Stingl L.**, **Jung K.**, **Heermann R.** (**2008**) 'Photorhabdus luminescens genes induced upon insect infection', *BMC Genomics*, 9(1), 229.

Muyekho F. N., **Barrion A. T.**, **Khan Z. .** (**2003**) 'Grass diversity and the associated stemborers and natural enemies in different farming systems of Kenya', *African Crop Science Conference Proceedings*, 6, 246–253.

Mwaniki S. W., **Nderitu J. H.**, **Olubayo F.**, **Kimenju J. W.**, **Nguyen K.** (**2008**) 'Factors influencing the occurrence of entomopathogenic nematodes in the Central Rift Valley Region of Kenya', *African Journal of Ecology*, 46(s1), 79–84.

Odendo M., **Ouma J.**, **Wachira S.**, **Wanyama J.** (**2003**) 'Economic assessment of maize yield loss due to stem borer in major maize agro-ecological zones of Kenya', *African Crop Science Conference Proceedings*, 6, 683–687.

Ong'amo G. O., **Le Rü B. P.**, **Dupas S.**, **Moyal P.**, **Calatayud P.-A.**, **Silvain J.-F.** (**2006**) 'Distribution, pest status and agro-climatic preferences of lepidopteran stem borers of maize in Kenya', *Annales de la Société Entomologique de France*, 42(2), 171–177.

Orchard S. S., **Goodrich-blair H.** (**2004**) 'Identification and functional characterization of a Xenorhabdus nematophila oligopeptide permease.', *Applied and Environmental Microbiology*, 70(9), 5621–5627.

Pinheiro V. B., **Ellar D. J.** (**2007**) 'Expression and insecticidal activity of Yersinia pseudotuberculosis and Photorhabdus luminescens toxin complex proteins', *Cellular Microbiology*, 9(10), 2372–2380.

Rakotoarisoa M. A., **Iafrate M.**, **Paschali M.** (**2012**) *Why Has Africa Become a Net Food Importer ?*, FAO: Rome.

Rensburg J. B. J. Van (**2007**) 'First report of field resistance by the stem borer, Busseola fusca (Fuller) to Bt-transgenic maize', *South African Journal of Plant and Soil*, 24(March), 147–151.

Ribeiro C., **Vignes M.**, **Brehélin M.** (**2003**) 'Xenorhabdus nematophila (enterobacteriacea) secretes a cation-selective calcium-independent porin which causes vacuolation of the rough endoplasmic reticulum and cell lysis', *Journal of Biological Chemistry*, 278, 3030–3039.

Rose T. M., **Henikoff J. G.**, **Henikoff S.** (**2003**) 'CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design', *Nucleic Acids Research*, 31(13), 3763–3766.

Sergeant M., **Baxter L.**, **Jarrett P.**, **Shaw E.**, **Ousley M.**, **Winstanley C.**, **Morgan J. A. W.** (**2006**) 'Identification, typing, and insecticidal activity of Xenorhabdus isolates from entomopathogenic nematodes in United Kingdom soil and characterization of the xpt toxin loci', *Applied and Environmental Microbiology*, 72(9), 5895–5907.

Sergeant M., **Jarrett P.**, **Ousley M.**, **Morgan J. A. W.** (**2003**) 'Interactions of insecticidal toxin gene products from Xenorhabdus nematophilus PMFI296', *Applied and Environmental Microbiology*, 69(6), 3344–3349.

Sheets J. J., **Hey T. D.**, **Fencil K. J.**, **Burton S. L.**, **Ni W.**, **Lang A. E.**, **Benz R.**, **Aktories K.** (**2011**) 'Insecticidal toxin complex proteins from Xenorhabdus nematophilus: Structure and pore formation', *Journal of Biological Chemistry*, 286(26), 22742–22749.

Shendure J., **Ji H.** (**2008**) 'Next-generation DNA sequencing.', *Nature biotechnology*, 26(10), 1135–1145.

Sicard M., **Ferdy J. B.**, **Pagès S.**, **Le Brun N.**, **Godelle B.**, **Boemare N.**, **Moulia C.** (**2004**) 'When mutualists are pathogens: An experimental study of the symbioses between Steinernema (entomopathogenic nematodes) and Xenorhabdus (bacteria)', *Journal of Evolutionary Biology*, 17(5), 985–993.

Spinner J. L., **Jarrett C. O.**, **LaRock D. L.**, **Miller S. I.**, **Collins C. M.**, **Hinnebusch B. J.** (**2012**) 'Yersinia pestis insecticidal-like toxin complex (Tc) family proteins: characterization of expression, subcellular localization, and potential role in infection of the flea vector.', *BMC Microbiology*, 12(1), 296.

Tabashnik B. E., **Brévault T.**, **Carrière Y.** (**2013**) 'Insect resistance to Bt crops: lessons from the first billion acres', *Nature Biotechnology*, 31(6), 510–521.

Tailliez P., **Laroui C.**, **Ginibre N.**, **Paule A.**, **Pagès S.**, **Boemare N.** (**2010**) 'Phylogeny of Photorhabdus and Xenorhabdus based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: X. vietnamensis sp. nov., P. luminescens subsp. caribbeanensis subsp. nov., P. l', *International Journal of Systematic and Evolutionary Microbiology*, 60(8), 1921–1937.

Tailliez P., **Pagès S.**, **Ginibre N.**, **Boemare N.** (**2006**) 'New insight into diversity in the genus Xenorhabdus, including the description of ten novel species', *International Journal of Systematic and Evolutionary Microbiology*, 56, 2805–2818.

Tamura K., **Stecher G.**, **Peterson D.**, **Filipski A.**, **Kumar S.** (**2013**) 'MEGA6: Molecular

Evolutionary Genetics Analysis Version 6.0', *Molecular Biology and Evolution*, 30(12), 2725–2729.

Tefera T., **Mugo S.**, **Tende R.**, **Likhayo P.** (**2010**) *Mass Rearing of Stem Borers, Maize Weevil, and Larger Grain Borer Insect Pests of Maize*, International Maize and Wheat Improvement Center (CIMMYT).

Tende R., **Tefera T.**, **Mugo S.** (**2004**) 'Mass Rearing and Use of Stem Borers At the Kari-Katumani', 2004–2007.

Tezcan-merdol D., **Nyman T.**, **Lindberg U.**, **Haag F.**, **Koch-nolte F.**, **Rhen M.** (**2001**) 'Actin is ADP-ribosylated by the Salmonella enterica virulence-associated protein SpvB', *Molecular Microbiology*, 39(3), 606–619.

Thompson J. D., **Higgins D. G.**, **Gibson T. J.** (**1994**) 'CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice', *Nucleic Acids Research*, 22(22), 4673–4680.

Union A. (**2006**) 'Summit on Food Security in Africa', in *Declaration of the Abuja Food Security Summit*, 1–3.

Vincze T., **Pofsai J.**, **Roberts R. J.** (**2003**) 'NEBcutter: a program to cleave DNA with restriction enzymes', *Nucleic Acids Research*, 31(13), 3688–3691.

Walker G., **Hertle R.**, **Braun V.** (**2004**) 'Activation of Serratia marcescens Hemolysin through a Conformational change', *Infection and Immunity*, 72(1), 611–614.

Waterfield N., **Hares M.**, **Yang G.**, **Dowling A.**, **Ffrench-Constant R.** (**2005**) 'Potentiation and cellular phenotypes of the insecticidal toxin complexes of Photorhabdus bacteria', *Cellular Microbiology*, 7, 373–382.

Waturu C. N., **Wabule M. N.**, **Nguthi F. N.**, **Njinju S. M.** (**1998**) 'Field control of the banana weevil (Cosmopolites sordidus) using entomopathogenic nematodes', 1998–2001.

Xu J., **Lohrke S.**, **Hurlbert I. M.**, **Hurlbert R. E.** (**1989**) 'Transformation of Xenorhabdus nematophilus.', *Applied and Environmental Microbiology*, 55(4), 806–812.

Yang J., **Zeng H. M.**, **Lin H. F.**, **Yang X. F.**, **Liu Z.**, **Guo L. H.**, **Yuan J. J.**, **Qiu D. W.** (**2012**) 'An insecticidal protein from Xenorhabdus budapestensis that results in prophenoloxidase activation in the wax moth, Galleria mellonella', *Journal of Invertebrate Pathology*, 110(1), 60–67.

APPENDICES

Appendix 1: The various codon combinations used in degenerate primer design. The key to the symbols used is as follows;

R=A+G M=A+C S=G+C W=A+T Y=C+T K=G+T
H=A+T+C D=G+A+T B=G+T+C V=G+A+C N=A+T+G+C.

Amino acid	Amino acid symbol	Nucleotide sequence (with degeneracy)	Complement (for designing reverse primers)
Methionine	M	ATG	TAC
Tryptophan	W	TGG	ACC
Cysteine	C	TGY	ACR
Aspartic acid	D	GAY	CTR
Glutamic acid	E	GAR	CTY
Phenylalanine	F	TTY	AAR
Histidine	H	CAY	GTR
Lysine	K	AAR	TTY
Asparagine	N	AAY	TTR
Glutamine	Q	CAR	GTY
Tyrosine	Y	TAY	ATR
Inosine	I	ATH	TAD
Alanine	A	GCN	CGN
Glycine	G	GGN	CCN
Proline	P	CCN	GGN
Threonine	T	ACN	TGN
Valine	V	GTN	CAN
Leucine	L	YTN	RAN
Arginine	R	MGN	KCN
Serine	S	WSN	WSN

Appendix 2a: Preparation of 10mL monomer solution for SDS PAGE by mixing all reagents except TEMED and 10% APS;

Percent Gel	Distilled water (mL)	30% Acrylamide/ Bisacrylamide (mL)	Gel Buffer (mL)	10%(w/v) SDS (mL)
4	6.1	1.3	2.5	0.1
6	5.4	2.0	2.5	0.1
8	4.7	2.7	2.5	0.1
10	4.1	3.3	2.5	0.1

Resolving gel buffer – 1.5M Tris-HCl, pH 8.8; add 50µL of 10% APS and 5µL TEMED.

Stacking gel buffer – 0.5M TrisHCl, pH 6.8; add 50µL of 10% APS and 10µL TEMED.

Appendix 2b: Preparation of 10mL monomer solution for native PAGE by mixing all reagents except TEMED and 10% APS;

Percent Gel	Distilled water (mL)	30% Acrylamide/ Bisacrylamide (mL)	Gel Buffer (mL)
4	6.2	1.3	2.5
5	5.8	1.7	2.5
6	5.5	2.0	2.5
7	5.2	2.3	2.5
8	4.8	2.7	2.5

Resolving gel buffer – 1.5M Tris-HCl, pH 8.8; add 50µL of 10% APS and 5µL TEMED.

Stacking gel buffer – 0.5M TrisHCl, pH 6.8; add 50µL of 10% APS and 10µL TEMED.

Appendix 3: Diet ingredients for rearing stem borers adopted from Onyango and Ochieng-Odero (1994) and Songa *et al.* (2004) (Tende *et al.*, 2004)

Ingredients	*Chilo partellus* **Quantity (g or mL) per 3 litre diet**	*Busseola fusca* **Quantity (g or mL) per 3 litre diet**
Fraction A		
Maize leaf powder	75.6	79.8
Common bean powder	265.2	264.9
Brewer's yeast	68.1	67.8
Ascorbic acid	7.5	7.5
Sorbic acid	3.9	3.9
Methyl-p-hydroxybenzoate	6.0	6.0
Vitamin E capsules (200 iu)	6.3	6.3
Sucrose	105.9	105.9
Distilled water	1209.3	1209.3
Fraction B		
Agar (Tech No. 3)	37.8	37.8
Distilled water	1209.3	1209.3
Formaldehyde	6.0	6.0

Appendix 4: Trimmed nucleotide sequences amplified by 16S rDNA primers, labelled Sc – *Steinernema carpocapsae*, Sk – *S. karii*, and L67 – *Steinernema* L67.

>Sc_16S_27F
TTGCTTTCCTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCT
GCCCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAAT
CTCTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCCTCGGATGAAC
CCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA
GCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAG
TACTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTGAC
GTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA
GGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGG
CATCTAAGACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACG
TGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCG
GCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGG
TTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCC
TGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTACTCTTGACATCCACGGAATTCTGCAGAGATGCGGAAGTGCCTTCGG
GCACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCA

>Sk_16S_27F
GCTTTCCTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGC
CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAATCT
CTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCCTCGGATGAACCC
AGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCC
CTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC
CTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTA
CTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTGACGT
TACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG
CGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTACCTGGGAATGGCAT
CTAAGACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGT
AGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCC
CCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCTGTAACGATGTCGATTTGAGGTTGTG
GCCTGAGCTGTGGCTTCCGAGCTAACGCGTTAATCGACCGCCTGGGGAGT
ACGGTCGCAGATTAAACTCAATGAATGACGGGGGCCCCACAGCGGGGAGC
AGTGGTTTATTCGATGCACGCGAAAAACTTACCACTCTTACACCACGGAA
TTCTGCAAAATGCGGAATGCCTTCGGCACCTGAGACAGTGCTGCATGGTG
TCGCAGCTCTGTGTGAAAGTGGGTTAGTCCGCACGAGCGCAACCTATCCT

TGTGCCGCCTTCGGTGGAATCAAGGAAATGCCGTGATAACCGAGGAGTGG
GGTGAGTCAGTCTCTGGCCTACAATAGGCTCCACTTCTCAAGGCGATCAA
GGAACGACCCCGAAGCAACGACCCTAAGTCTTCTATCCGATGGATTTCAC

>L67_16S_27F
TGCTTTCCTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTG
CCCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAATC
TCTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCCTCGGATGAACC
CAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATC
CCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAG
CCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGT
ACTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTGACG
TTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAG
GCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGC
ATCTAAGACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGT
GTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGG
CCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGT
TGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCT
GGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC
CTACTCTTGACATCCACGGAATTCTGCAGAGATGCGGAAGTGCCTTCGGG
CACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCAC
TTCGGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTG
GGGATGACGTCAAGTCATCATGGCCCTTACAAGTAGGGCTACCACGTGCT

Appendix 5a: Trimmed nucleotide sequences amplified by primers B1_fwd and B1_rev designated XSB1_1

>XSB1_1Sk (51-1200)
ACCTATATGGGATTAGGCTGGAATTTCTCATCCGTCAATGCCAGTGATCC
GGATAGCGGAGCGAAACGGTATTTTTCCCAGTCTGTCGGTGGCCGTTCCG
TGATGAGTTCCGGTATAAGTGCCCACTTCAGTTATGATACACGGGACTTT
TTACCCAATGCACGACACGGACAGGCATTTGAGATCGTTTATACCTATTT
TTCCCCAGAATTAGGTAGCGATCACCGTTTCCATACCACACAGCTTCAGT
TCTCGACCTATTACCCGTTATCTGAAAAGACCGTCCTGGCCTTCGATAAC
TATGCCCGTTTCAGCGCAGGTGATGTCCCGTGGAACCAGCTATCACTGCT
TGGTAACGGCCAGCGGATGCGCGGATATTATGAAGGGCGTTATCGTGACA
ACAATATCTTCACAACACAGTTAGAGTTAAGACATAAACTGGACTGGCGC
CACGGTGTGGTCGGTTGGATAGGCACCGGCACGTTGGGAGATTCCCCTTC
ACAATTGGGAACCAGGCATTGGCTTCCTTCTGTTGGTATTGGCTATCGTT
TTGAGTTTAAACCACGCATGAATGTCAGATTAGATTTTGGTGTCGGTAAA
GACAGTACTGGATTCTATTTTCAGGTTGGGGAAGCGTTTTGAGATTATTC
GTTACACTTTGCCTCTGTTTTCTACTGATGGCATGCCAAAACCAGCCACA
ATCAGTTGATCCGGTCAAACCGGTCCTTGTCAGTGATACCCATGAGCAGT
CACAGCAAAATTGGAATGCGTTACCTCCCATTGCCCCACCAGAAGGTTTA
AGGGCTTGCTGTGCTTTCGGCTATAACCTAAAAGCTCAACTATGGAATAT
TCCCATTCCCTTCTATGACATCGATAATATTGTAGAAGCCAAGAAATTGG
GTGAACATCACTATAATGACAGTGTTGTCGGTGCCAGTGCCGCCTTGTTG
GGGGTAAGCAATGAAAAAGTGGGTCTGCTCTATACCCATAAGGGAGGGTT
TATTGATATCTCCCATGTCAGGGATACCGCAGACTATACGCTTTATCTGT
TTAGCCAGATTTACACTCATCTTGGTCAAGAGAGGGAGTTAACCCTGAAT
AATGAACTTGCTGCGCGTAAAATGCATTTTTTTGCTTTTACGCCACCGCA

>XSB1_1Sc (51-800)
ATACCACCTATATAGGGATTAGGCTGGAATTTCTCCCTCGTCTGGGCAGT
GATCCGGATAGCGGAGCGAAACGGCATTTTTCCCAGTCTGGCGGTGGCCG
TTCCGTGATGAGTTCCGGTATAAGAGCCCACTTCAGTGATGATACACGGG
ACTTTTTACCCACTGCACGACACGGACAGGCGTTTGAGATCGTTTATACC
TATTTTTCCCCCCAATTAGGTAGCGATCACCGTTTCCCTACCACACAGCT
TCACTTCTCGACCCATTACCCGTTATCTGAAAAAACCGTCCTGGCCTCCG
ATAACTATGCCCGCTTCACCGCAGGGGATGTCCCGTGGAACCAGCTATCA
CTGCTTGGTAACGGCCAGCGGATGCGCGGATATTATGAAGGGCGTTATCT
TGACAACAATATCTTCACAACACAGTTAGAGTTAAAACATAAACTGGACT
GGCGCCACGGTGTGGTCGGTTGGATAGGCACCGGCACGTGGGAGATTCCC
CTTCACAATTGGGAACCTGGCATTGGCTTCCTTCTGTTGGTATTGGCTAT
CGTTTTGAGTTTAAACCACGCATGAATGTCAGATTAGATTTTGGTGTCGG
TAAAGACAGAACTGGATTCTATTTTCAGGTTGGGGAAGCGATTTGAGATT
ATTCGTTACACTTTGCCTCTGTTCTTCTACTGATGGGATGGCAAACCCGA
CACAATCAGTTGATCCGGTCAAACCGGTCCTTGTCAGTGATACCCATGAG

>XSB1_1L67(51-950)
CCTATATGGGATTAGGCTGGAATTTCTCATCCGTCAATGCCAGTGATCCG
GATAGCGGAGCGAAACGGTATTTTTCCCAGTCTGTCGGTGGCCGTTCCGT
GATGAGTTCCGGTATAAGTGCCCACTTCAGTTATGATACACGGGACTTTT
TACCCAATGCACGACACGGACAGGCATTTGAGATCGTTTATACCTATTTT

TCCCCAGAATTAGGTAGCGATCACCGTTTCCATACCACACAGCTTCAGTT
CTCGACCTATTACCCGTTATCTGAAAAGACCGTCCTGGCCTTCGATAACT
ATGCCCGTTTCAGCGCAGGTGATGTCCCGTGGAACCAGCTATCACTGCTT
GGTAACGGCCAGCGGATGCGCGGATATTATGAAGGGCGTTATCGTGACAA
CAATATCTTCACAACACAGTTAGAGTTAAGACATAAACTGGACTGGCGCC
ACGGTGTGGTCGGTTGGATAGGCACCGGCACGTTGGGAGATTCCCCTTCA
CAATTGGGAACCAGGCATTGGCTTCCTTCTGTTGGTATTGGCTATCGTTT
TGAGTTTAAACCACGCATGAATGTCAGATTAGATTTTGGTGTCGGTAAAG
ACAGTACTGGATTCTATTTTCAGGTTGGGGAAGCGTTTTGAGATTATTCG
TTACACTTTGCCTCTGTTTTCTACTGATGGCATGCCAAAACCAGCCACAA
TCAGTTGATCCGGTCAAACCGGTCCTTGTCAGTGATACCCATGAGCAGTC
ACAGCAAAATTGGAATGCGTTACCTCCCATTGCCCCACCAGAAGGTTTAA
GGGCTTGCTGTGCTTTCGGCTATAACCTATAAGCTCAACTATGGAATATT
CCCATTCCCTTCTATGACATCGATAATATTGTAGAAGCCAAGAAATTGGG

Appendix 5b: Translated amino acid sequences from XSB1 nucleotide sequences generated on ExPASy;

>XSB1_1Sk-aa (214aa, 5′-3′ frame 3)
XTYMGLGWNFSSVNASDPDSGAKRYFSQSVGGRSVMSSGISAHFSYDTRDFLPNARHGQA
FEIVYTYFSPELGSDHRFHTTQLQFSTYYPLSEKTVLAFDNYARFSAGDVPWNQLSLLGN
GQRMRGYYEGRYRDNNIFTTQLELRHKLDWRHGVVGWIGTGTLGDSPSQLGTRHWLPSVG
IGYRFEFKPRMNVRLDFGVGKDSTGFYFQVGEAF

>XSB1_1Sc-aa (192aa, 5′-3′ frame 2)
XHTTYIGIRLEFLPRLGSDPDSGAKRHFSQSGGGRSVMSSGIRAHFSDDTRDFLPTARHG
QAFEIVYTYFSPQLGSDHRFPTTQLHFSTHYPLSEKTVLASDNYARFTAGDVPWNQLSLL
GNGQRMRGYYEGRYLDNNIFTTQLELKHKLDWRHGVVGWIGTGTWEIPLHNWEPGIGFLL
LVLAIVLSLNHA

>XSB1_1L67-aa (215aa, 5′-3′ frame 3)
XXXYMGLGWNFSSVNASDPDSGAKRYFSQSVGGRSVMSSGISAHFSYDTRDFLPNARHGQ
AFEIVYTYFSPELGSDHRFHTTQLQFSTYYPLSEKTVLAFDNYARFSAGDVPWNQLSLLG
NGQRMRGYYEGRYRDNNIFTTQLELRHKLDWRHGVVGWIGTGTLGDSPSQLGTRHWLPSV
GIGYRFEFKPRMNVRLDFGVGKDSTGFYFQVGEAF

Appendix 6: Trimmed nucleotide sequences amplified by primers B1_fwd and B1_rev designated XSB1_2

>XSB1_2Sc (41-490)
CGCTGGCTGAACGGCGATCTCTTCAATCTCACAATTTACCCTGCCTGACT
ATTTATTATCTTGCTCGCCTTTCTTGCTTTCGGAAAGTAAATCGGCCATA
CCACCAACGGCTCCCATTAAATTGCCGGCTTCCAGTGGCATCATAATCAC
TTTGCTGTTATAAGAGGCACCGATGTTGGGGAGGGAATCTGTATATTTTT
GGGCTACGAAATAGTTAATTGCCTGTATATCACCATTTGCAATAGCGCCC
GAAACCATTTTGGTTGCCCGCGCTTCTGCATCCGCCGCGCGGTCAAGGGC
TTCAGCTTGGAGGAAAGCTGATTGGCGATCACCTTCTGCTTTCAAAATTT
GCGACAGTTTTTCTCCTTCCGCTTTCAAAATGGCGGCCTGACGAATACCT
TCTGCCTCAAGAATATCTGCACGTCATAATGAATCCGAAGCATAACTGCA

>XSB1_2SK (41-490)
CCAGGCTGGCTGAGCGGCGATCTGTTCATCATAACAATTTACCCTGCCCG
ACTATTTATTATCTTGCTTGCTTTTCTTGCTTTCGGAAATTAATTCGGCA
ATACCACCAATGGCTCCCATTAAATTGCTGGCTTCCAGTGGCATCCGATC
ACTTTGCTGTTATTTGAGGCACCGATGTTGGTGAGGGCATCTGTATATTT
TTGGGCTACGAAATAGTTAATTGCCCGCATATCACCATTTGCAATAGCGT
CCGAAACCATTTTGGTTGCCCGCGCTTCTGCTTCGGCGGCGCGTTCACGG
GCTTCAGCTTGGAGGAAGGCTGATTGGCGATCACCTTCTGCTTTCAAAAT
TTGCGACTGTTTTTCTCCTTCCGCTTTCAAAATGGCGGCCTGACGAATAC

>XSB1_2L67 (51-500)
GAACGGCGATCTGTTCATCATAACAATTTACCCTGTATGACTATTTATTA
TCTTGCTCGCTTTCCTTGCTTTCGGAAAGTAATTCGCCCATACCACCTAC
GGCTCCCATTAAATTGCTGGCTTCCAGTGGCATCATAATCACTTTGCTGT
TATTTGAGGCACCGATGTTGGTGAGGGCATCTGTATATTTTTGGGCTCCG
AAATCGTTAATTGCCCGCATATCACCATTTCCAATAGAACCCAAAACCAT
TTTGGCTGCCCGCGCTTCTGCTTCCCCCCTGCGTTCACGGGCTTCAGCTT
GGAGGAAGGCTGATTGGCAATCCCCTTCTGCTTTCAAAATTTGCCACTGT
TTTTCTCCTTCCGCTTTCAAAATGGCGGCCTGACGAATCCCTTCTGCCTC
AGGAATATCTGCACGTCATAATGAATCCGAAGCATAATGGCCGCGAAAGA

Appendix 7: Trimmed nucleotide sequences amplified by primers B2_fwd and B2_rev designated XSB2

>XSB2Sc (51-900)
CTTAAGAGTTCGACCAAGTCAATTCTGTTCCAGGTTCCCACAGCCCTCTC
TCGATCTGAAATTGTCTTCAAATGATCTGTATTCTCATCCATCTCCAATG
TGGAAGACAGATCGAAAACCGGACGAAGTTTAAGCTTTGCTATTGATGGC
AATGTGTAAATGTGCAGGATAGAAGGTTGTTTGATTTCAGACACTAAACG
CGCCGATTTATTGATTAAATTGCCCTGACTCTCACTAATTTGAAAAAATG
ATCGCGCCGGTGAGCCTGTGCCCCCATGCTGCTCAAAATGCTTTTAACCT
TAATACCCTGCAAATCCATCAGATACGATTAGGGTTTGTGAGCGAAAATT
GGCGATCCTTCCGTGGAGTATTACACTCCAGCGCTTACTCTGGTGTTGCT
TGATGCTGGCCAAATTCACGCTGTACTAAATTATCACTTGACTCTATAAT
CCATCGGGCTGATCCTCAATTACCACTTAGCGACTGTACCTTTCATCATG
CTACTACGCAGGGTATTCTGGTCTGTATGACTCTTAACTTATTCCGCTTA
CAAGCTACCAACGCGATCCTCTTTTCAAACATTGCGATAGGGCGAACTCT
CCTAATTAGCGACGTCTGTCTTGAAAAACACATTTTCTGATCCCGCATTT
TTCATACGCCACCAATAATAACGTTAAACTATCCTGGTGCCGCATGCCAT
TCCCATAGAAATAGATGTGGCAATGATCGTACTGGCCTGCTTGAAGAGGC
ATCATGAGCACCGCAACACAATTGCTGCAAACACGCAACCTGGTACGCAG
GCAATGCAGTCGAGTGAAATATGCTCATCTGCGAGGGGGAGAATTCTCCG

>XSB2Sk (51-870)
CTTGCGAGTTCGTGCAAGTCAATTCTGCTCCCGGTTCCCACTGCCCTCTC
TCATCGACTTTTCTTCTAAATGATCTGTATCCTCATCCTTCTTCTTTGTG
GGAGGCAGATCGAGCTCCGGACGATGTTTACGCTTTGCTTTTGGTGGCAA
TGTGTAAATGTGCGGATAGAAGGTTGTTTGATTTCATGACACTACACTCG
CCGATTTTATGATTAATCCTGGCCTGACTCTCACTAATTTGAAGAAATGA
TCGCGCCGCTGAGCCTGTGCCCCCATGCTGCTCAGAATGCTTTTAACCTG
AATACCCTGCGTCTCCATCAGCTACAAAGTAGGGCTTGTGAGCTGAAAAT
TGGCGATCCTTCCGTGGAGTATTACACTCCACCGTTACTCTGGTGCTGCT
CGATGCTGGCCAGCTACACGCTATACCAACTTTTCACTTGACTCCATTAT
CCATCGTGCTGATGCTCAATTACCACTCACCGACCGCACCTTTCTTCATG
CTGCTACGTGGGTATTCTGGTCTGTATGACTCTGTACTTCTTCCGCTTAC
AAGCTCCCAACGCGATCCTCTTTTCAAACATTGCGTTAGGGCGAACTCTC
CTAATTAGCGCCTTCTGTCTTGAAAAAAACTTTTCTCTCCTCTCATTTTT
TAATTGCTACAATGAAACGTTATTTTCTCCTGGTGCCGATGCCATTCCCT
TCGGCATTGATGTGTCCAATGATCCTTACTGGCCTCCTTGAAGAGGCTTC
ATGAGAACCGCACACAAATTGCTGCACGCCTCGCCACTCGGTACGCATTC
AAGACCGTCGAGTAAATCTT

>XSB2L67 (61-800)
GTTCGAGCAAGTCAGTTCTGTTCCCGGTTCCCAAGCCCGTCTCTCATTGA
CTTTGTCTCTTATTTTCTGTATTCCTCATCCATCTTCATTTTGGGAGGCA
GATCGAACACCGGACAAAGATTAAACTTTTCTTTTGATGGCTATGAAAAA
ATAAGAGGATAGACAGGTTGTTTGATTTCAAACACTAAACTCGCCGATTT
TATGATTAATCCGCCTGCTCACACTAATTTTAAATAATGATCGCGCCGCT
GAACCTGTGCCCCCATGCTGCTCAGAATGCTATTAACTTGAATACCCCGC
AAATCCATCAGATACAATTAGGGTTTGTAGCGAAAATTGGCGATCTTAAG
TGGAATTTAGACTCCCCGTTACTCTGGTGTAGCTTGATGCTGGCCAATAC
GCACAGCACTAACTTTTGAAGTCGCTGCATAACCCATTGGGCTGATGCTT
AATAACCATACAGCGGCTGCGCCTTTCATCATGCTACTACGTGAAGTATT
ATGGTCTGAATGACTCATTATTTATTCCGCTTAAAACAACCCAACGCAGT
AATCTTTTCCAAACATTATAGTTAGGCAGGAACTCTCATTAATTAGCAAC
GTCTGTCATCAAAAACGTAATTTCTGATCCTCTCATTTATTTATCCGATA
CAATAATAACGTTATTTTCCCCTAGTGCCGCTAGCACTTCCCATCAGAAG
AGATGATACAAAGATACCAAAATCAATCATCCGAACGGAT

Appendix 8a: Trimmed nucleotide sequence of 1060bp amplified by Primer C1_fwd and C1_rev designated XSC1_1

>XSC1_1Sc (40-1100)
TTCTGCTCAACAGAGAGCAGTCCTCCGCGTAAACGTAGCATAGGGAATCATTCATCTTCAT
CACATGCCGAAAACGACAAAATGAAGAAAAAATCTGTAAGGGCAATGCCTTCGGACTCCCC
GATCACGGTGATACCACGCGAACAGCACCCTATTTCACGCAAAGACATCAGTGATAATGCC
TTAAAGGTTCTGTATCGCCTAAATAAATCAGGTTTTGAAGCTTATCTGGTCGGAGGCGGGG
TTCGTGATCTACTGCTGCACAAAAAACCCAAAGATTTTGATATAGCAACCAACGCTACACC
GGAACAAGTTCGTCAACTGTTCCGTAACTGTCGCCTTGTCGGACGTCGTTTTCGTCTTGCC
CATATCATGTTTGGCCCTGATGTGATTGAAGTTGCCACCTTCCGCGGTCCGCATGATCAAA
TTGAAAACAATGACCGGAACCAGTCACATAAAGCCCAAAGCGGCATGTTACTGCGGGACAA
TATTTTTGGTTCAGTAGAAGAAGATGCGGTTCGTCGTGATTTCACGATCAATAGCCTTTAT
TACGGCATTGAAGATTTCGCGCTACGGGATTACATAGGTGGCATGGCCGATCTGAACGCAG
GCATCATTCGCCTGATTGGTGATCCAGAAACACGCTATCGGGAAGACCCCGTGCGGATGCT
GCGGGCGGTTCGTTTCGCCAGCAAACTGGATATGACTATCGAGCCAGCTACCGCTGAGCCT
ATCCCGCGCCTGGCATTTCTGTTGAAAGATATTCCAGCGGCACGTCTGTTCGAAGAGTCCC
TGACATTGTTACAGACAGGGCAGGGTTACAAGACTTATAAACTGCTGCGCGAATATCATCT
GTTCCAGCCATTGTTTCCGCTTATTCAACCGGGTTTCACCCAGCGTGGTGATTCGTCAATG
GAAAAGCTGTTAGCACAAGTATTGGACAATACCGATTTTCGCTTACAAAGTGATAAACGAG
TGAATCCCGCGTTCTTATTTGCGGCCATGCTATGGGATCCATTAATTGAACATGCGGAAAA
ACTGGACCATGTATGGCGGATTC

Appendix 8b: Translated amino acid sequence from XSC1 nucleotide sequence generated on ExPASy, 353 amino acids (ORF 1, 5'-3')

>XSC1_Sc-aa
FCSTESSPPRKRSIGNHSSSSHAENDKMKKKSVRAMPSDSPITVIPREQHPISRKDISDN
ALKVLYRLNKSGFEAYLVGGGVRDLLLHKKPKDFDIATNATPEQVRQLFRNCRLVGRRFR
LAHIMFGPDVIEVATFRGPHDQIENNDRNQSHKAQSGMLLRDNIFGSVEEDAVRRDFTIN
SLYYGIEDFALRDYIGGMADLNAGIIRLIGDPETRYREDPVRMLRAVRFASKLDMTIEPA
TAEPIPRLAFLLKDIPAARLFEESLTLLQTGQGYKTYKLLREYHLFQPLFPLIQPGFTQR
GDSSMEKLLAQVLDNTDFRLQSDKRVNPAFLFAAMLWDPLIEHAEKLDHVWRI

Appendix 9: Proteins from three *Xenorhabdus* spp. with sizes ranging from 100 kDa to 170 kDa.

Entry	Status	Protein names	Gene names	Organism	Length (bp)	Size (Daltons)
Q5GD93	unreviewed	XhlA	xhlA	*Xenorhabdus nematophilus* (*Achromobacter nematophilus*)	1470	155182.94
N1NLQ6	unreviewed	Putative non-ribosomal peptide synthetase (EC 5.1.1.11)	XNC3_2370005	*Xenorhabdus nematophila* F1	1069	118752.9
N1NNI2	unreviewed	DNA-directed RNA polymerase subunit beta (EC 2.7.7.6)	rpoC XNC3_2630008	*Xenorhabdus nematophila* F1	1408	155323.14
N1NUD0	unreviewed	Putative Extracellular serine protease	XNC3_500019	*Xenorhabdus nematophila* F1	1033	111914.04
N1NNG3	unreviewed	Extracellular serine protease (EC 3.4.21.62)	XNC3_2850014	*Xenorhabdus nematophila* F1	1040	111125.73
N1NTT4	unreviewed	XhlA, Cell surface associated hemolysin (TpsA)	xhlA XNC3_2880003	*Xenorhabdus nematophila* F1	1470	155253.03
N1NKC8	unreviewed	Putative Rhs family protein	XNC3_1130016	*Xenorhabdus nematophila* F1	1465	163895.38
N1NPM3	unreviewed	B component of insecticidal toxin complex (Tc)	tcdB XNC3_430007	*Xenorhabdus nematophila* F1	1530	174953.98
N1NU07	unreviewed	A component of insecticidal toxin complex (Tc)	tccA XNC3_570004	*Xenorhabdus nematophila* F1	1021	111084.95
N1NUQ5	unreviewed	B component of insecticidal toxin complex (Tc)	xptC1 XNC3_740004	*Xenorhabdus nematophila* F1	1493	168111.24
N1NRV3	unreviewed	A component of insecticidal toxin complex (Tc)	tccA XNC3_720005	*Xenorhabdus nematophila* F1	1156	130144.94
N1NQQ5	unreviewed	A component of insecticidal toxin complex (Tc)	xptD1 XNC3_720006	*Xenorhabdus nematophila* F1	1391	158977.63
N1NR59	unreviewed	B component of insecticidal toxin complex (Tc)	tcaC XNC3_570006	*Xenorhabdus nematophila* F1	1519	167917.89
N1NGP2	unreviewed	Putative invasin	XNC3_1130013	*Xenorhabdus nematophila* F1	1085	118837.22
N1NUB5	unreviewed	C component of insecticidal toxin complex (Tc)	xptB1 XNC3_740003	*Xenorhabdus nematophila* F1	1016	110974.01
N1NTR9	unreviewed	C component of insecticidal toxin complex (Tc)	tccC XNC3_430009	*Xenorhabdus nematophila* F1	1030	112927.45
N1NLE9	unreviewed	Putative invasin	XNC3_120013	*Xenorhabdus nematophila* F1	1014	111786.21
N1NSX4	unreviewed	Putative invasin	XNC3_920059	*Xenorhabdus nematophila* F1	1081	118465.46
N1NK62	unreviewed	Putative Outer membrane	XNC3_1090006	*Xenorhabdus nematophila* F1	1507	156454.81

		autotransporter barrel				
N1NQ21	unreviewed	C component of insecticidal toxin complex (Tc)	tccC XNC3_570007	*Xenorhabdus nematophila* F1	969	105751.11
D3V083	unreviewed	Putative invasin	XBJ1_1508 XBJ1_3402	*Xenorhabdus bovienii* (strain SS-2004)	1085	119501.18
D3V3H9	unreviewed	C component of insecticidal toxin complex	tccC3 XBJ1_3085	*Xenorhabdus bovienii* (strain SS-2004)	901	102210.37
D3UYH0	unreviewed	Putative invasin	XBJ1_0197	*Xenorhabdus bovienii* (strain SS-2004)	1261	140690.93
D3UYN1	unreviewed	Hemolysin XhlA(TpsA-related protein)	xhlA XBJ1_0258	*Xenorhabdus bovienii* (strain SS-2004)	1482	154960.46
D3UYI5	unreviewed	Putative Outer membrane autotransporter	XBJ1_0212	*Xenorhabdus bovienii* (strain SS-2004)	984	103805.92
D3V205	unreviewed	Putative Outer membrane autotransporter barrel	XBJ1_2575	*Xenorhabdus bovienii* (strain SS-2004)	1507	156736.49
D3V0Q1	unreviewed	C component of insecticidal toxin complex (Tc)	tccC XBJ1_1574	*Xenorhabdus bovienii* (strain SS-2004)	932	102249.97
D3UWD9	unreviewed	A component of insecticidal toxin complex (Tc)	tccA2 XBJ1_0569	*Xenorhabdus bovienii* (strain SS-2004)	1184	132696.1
D3V0Q0	unreviewed	B component of insecticidal toxin complex (Tc)	tcaC XBJ1_1573	*Xenorhabdus bovienii* (strain SS-2004)	1481	164822.43
D3V2U5	unreviewed	B component of insecticidal toxin complex (Tc)	tcaC XBJ1_1934	*Xenorhabdus bovienii* (strain SS-2004)	1506	168621.29
D3UYZ9	unreviewed	Putative invasin	XBJ1_0377	*Xenorhabdus bovienii* (strain SS-2004)	995	110543.91
D3UWD8	unreviewed	A component of insecticidal toxin complex (Tc)	tccB2 XBJ1_0568	*Xenorhabdus bovienii* (strain SS-2004)	1368	158307.07
D3VIN8	unreviewed	Putative invasin	XNC1_0514	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1013	111751.17
D3VHH4	unreviewed	A component of insecticidal toxin complex (Tc)	xptD XNC1_2561	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1391	158919.6
D3VHI1	unreviewed	B component of insecticidal toxin complex (Tc)	xptC1 XNC1_2568	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM	1475	166404.11

				3370 / LMG 1036 / NCIB 9965 / AN6)		
D3VFD1	unreviewed	B component of insecticidal toxin complex (Tc)	tcdB2 XNC1_2186	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1516	173246.9
D3VGF9	unreviewed	C component of insecticidal toxin complex (Tc)	tccC XNC1_2336	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	969	105791.22
D3VAU6	unreviewed	Putative invasin	XNC1_3690	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1087	119073.42
D3VHI0	unreviewed	C component of insecticidal toxin complex (Tc)	xptB1 XNC1_2567	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1016	111046.12
D3VFD3	unreviewed	C component of insecticidal toxin complex (Tc)	tccC XNC1_2188	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1030	112848.32
D3VFR2	unreviewed	XhlA, Cell surface associated hemolysin (TpsA)	xhlA XNC1_4556	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1470	155182.94
D3VA36	unreviewed	Putative invasin	XNC1_3566	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1085	118556.05
D3VA39	unreviewed	Putative Rhs family protein	XNC1_3569	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1476	165323.31

D3VHH3	unreviewed	A component of insecticidal toxin complex (Tc)	tccA XNC1_2560	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1156	130144.94
D3VGF6	unreviewed	A component of insecticidal toxin complex (Tc)	tccA XNC1_2333	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1021	111020.89
D3VM51	unreviewed	Transposase	XNC1_p0135	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1013	117206.85
D3VGF8	unreviewed	B component of insecticidal toxin complex (Tc)	tcaC XNC1_2335	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1519	167909.89
D3VGF7	unreviewed	A component of insecticidal toxin complex (Tc)	tccB XNC1_2334	*Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6)	1541	171872.03

Appendix 10a: Concentration of bacteria used recorded in absorbance at 600nm and in number of cells/mL in the different treatments for the feeding and injection assays.

	Feeding assay	**Injection assay**				
Larval species	*C. partellus*		*C. partellus*		*B. fusca*	
Bacterial species	*Xenorhabdus* sp.	*E.coli*	*Xenorhabdus* sp. (1:100 dilution)	*Xenorhabdus* sp. (1:10 dilution)	*E. coli*	*Xenorhabdus* sp.
OD at 600nm	4.5A	0.7234A	0.3838A	2.1847A	7.395A	3.381A
Standardized OD	0.015A	-	-	-	2.8215A	2.1847A
Approximate number of bacterial cells/mL	1.5×10^{7}	7.234×10^{8}	3.838×10^{8}	2.1847×10^{9}	2.8215×10^{9}	2.1847×10^{9}

Appendix 10b: The average lengths of the different developmental stages of *C. partellus* within the treatments administered by feeding;

	Plain diet/Preservative	**Plain diet/No preservative**	**Diet impregnated with *Xenorhabdus* sp.**
Adult (wing length)	1.28cm	1.07cm	0.97cm
Pupae (Length/breadth)	-	-	1.0cm/0.3cm (Dead)
Larvae (Length/breadth)	0.6cm/0.13cm	0.5cm/0.15cm	Dead: 0.64cm/0.13cm Alive: 1.51cm/0.25cm

Appendix 10c: The average lengths of the different developmental stages of *C. partellus* within the treatments administered by direct injection;

	Punctured	**Injected with PBS**	**Injected with *E. coli***	**Injected with *Xenorhabdus* sp.**	
				1:100 dilution	1:10 dilution
Adult (wing length)	-	1.1cm	-	1.1cm	-
Pupae (Length/breadth)	1.53cm/0.29cm	1.43cm/0.29cm	1.36cm/0.27cm	1.35cm/0.26cm	-
Larvae (Length/breadth)	2.05cm/0.35cm	2.21cm/0.36cm	2.14cm/0.34cm	1.98cm/0.35cm	Dead: 0.96cm/0.2cm Alive: 2.15cm/0.35cm

Appendix 11: Crude cell lysate of *Xenorhabdus* sp. on a 7% SDS polyacrylamide gel showing the protein profile of the whole cell lysate, cell free supernatant and re-boiled whole cell lysate. Proteins were only visible in cell free supernatant after 6 days of incubation and Silver nitrate staining. The native mark ladder used is in kDa (Life Technologies).