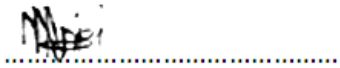
The isolation and identification of antimicrobial peptides and analysis of immune response in *E. intermedius* embryonic cell line upon exposure to pathogens.

Ntando Ghwenneth Mnisi
699653

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.
Johannesburg, 2012

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Ntando Ghwenneth Mnisi

24th day of June 2014.

Abstract

Insects are confronted by a large variety of potentially harmful microorganisms to which they are resistant as they are able to build up an efficient innate defense system that relies on three tightly interconnected reactions. One of the reactions is the transient and rapid synthesis of a battery of antimicrobial peptides (AMPs). Development of antimicrobial therapeutic drugs and vaccines is very crucial due to factors such as the emergence of multiple-drug resistance. AMPs have been termed natural antibiotics because of their large spectrum of activity. The current study focused on the isolation and identification of cationic antimicrobial peptides and the analysis of immune response in the South African *Euoniticellus intermedius* embryonic (SAEIE08) cell line upon exposure to pathogens. *E. intermedius* is of the Coleopteran order in the Scarabaeoidea superfamily. Liquid growth inhibition assay showed higher antimicrobial activity in SAEIE08 that was treated with heat-killed *E. coli* compared to untreated. Further evidence for antimicrobial activity was seen as a clear zone of inhibition in solid growth inhibition assay when a gel run with protein extracts was plated and overlayed with live *E. coli.* Changes in protein expression patterns that were analysed in SDS-PAGE and 2-D PAGE indicated the most intense bands and spots at low molecular weight sizes around 10 kDa and/or 16 kDa which implicated increased induction of AMP expression upon exposure to pathogen. Homologues of *Saccharomyces cerevisiae* proteins were found in some of the 5′/3′ RACE sequences. Possible explanation for matches to these homologues could be that short sequences were used for database searches. The proteins were identified as flavin-containing monooxygenase, long-chain fatty acyl-CoA synthetase, severe depolymerization of actin protein and serine/threonine protein kinase. Interestingly, these proteins play roles in metabolism, cell proliferation and/or molecular pathways which do occur when cells are exposed to stress. There was also an insect peptide allatotropin from *Spodoptera frugiperda.* The results show that there is inducible antimicrobial activity in embryonic *E. intermedius* cell line.

Acknowledgements

I thank the Almighty God for making it possible for me to finish this work despite the many challenges encountered along the way. I would like to extend my eternal gratitude to my supervisor, Professor Monde Ntwasa, for his support, encouragement and valuable input throughout the acquisition of my MSc. This dissertation would not have been possible without my parent's support and prayer. I would like to thank them for seeing the best in me. I would like to thank my husband, Ronny Phaahla, for being my pillar of strength through the challenging times and for always putting a smile on my face. I am grateful for his support and continual love.

I would also like to thank the Flylab team, past and present lab mates: Pfariso Maumela, Dr. Rodney Hull, Umar Faruq and Brent Oosthuysen for their advice during experiments. In addition, they made my journey in the Flylab more pleasant with their sense of humor.

I would like to thank the Peermont Education Trust for funding my undergraduate degree. Last, but not least, I would like to thank The South African National Research Foundation for funding my postgraduate degrees. I would not have made it this far without their financial support. I am eternally grateful for their generosity.

Research Output

1. Conference Contributions: Poster Presentation

Maumela, P., Hull, R., Mnisi, N., Ntwasa, M. and Cajee, U. The study of cationic amphiphilic peptides with anti-cancer selective toxicity and antimicrobial activity. EMBO global exchange lecture course. Innate immunity: Evolution and advances in clinical medicine. Stay City, Johannesburg, 4 September 2012.

Table of Contents

List of Figures

List of Tables

List of in-text Abbreviations

Acyl-CoA	Acyl Coenzyme A
AMP	Antimicrobial peptide
APD	Antimicrobial peptide database
AT	Allatotropin
AS	Allatostatin
CA	Corpora allata
CFU	Colony Forming Units
CL	Cardiolipin
DAP	Diaminopimelicacid
DIF	Dorsal-related immunity factor
DNA	Deoxyribonucleic acid
FACS	Fatty acyl-CoA synthetase
FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
FMO	Flavin-containing monooxygenase
GNBP	Gram-negative binding protein
Iκβ	Inhibitor of κβ
Imd	Immune deficiency
JH	Juvenile hormone
JNK	c-jun N-terminal kinase
LPS	Lipopolysaccharide
NADPH	Nicotinamide adenine dinucleotide
NF-κβ	Nuclear factor kappa B
OD	Optical Density
PAMP	Pathogen associated molecular pattern
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGLYRPs	Peptidoglycan recognition proteins
PGRPL/SA/SD	Peptidoglycan recognition protein
PRR	Pattern recognition receptors
PS	Phosphatidylserine
RNA	Ribonucleic acid
SDA1p	severe depolymerization of actin protein 1
SD	Standard deviation
STPK	Serine/threonine protein kinases
TLR	Toll-like receptors
X-gal	X-galactose

Chapter 1: Introduction

1.1. Insect Immunity

Insects colonize all ecological niches excluding the Polar Regions and the deep marine environment (Bulet, *et al.* 1999). In terms of species number, insects represent the largest class in the animal kingdom (Li, *et al*. 2012). The ability of insects to resist pathogens has contributed significantly to their extreme diversity (Bulet, *et al.* 1999). Insects are confronted by a very large variety of microorganisms that are potentially harmful. Like most living organisms, they are continually exposed to potentially harmful organisms through contact, inhalation and ingestion (Reddy, *et al.* 2004). Insects can build up efficient defense against microorganisms and they resist microbial infections in particular. Their defense mechanisms share some important characteristics with the innate immune response of vertebrates. It is innate non-adaptive and lacks both specificity and memory (Hoffmann, 1995). Over the last 10 years, a number of studies have revealed similarities between pathogen recognition, effector mechanisms and signalling pathways of innate immunity in invertebrates and vertebrates, which indicates common ancestry between these defenses (Girard, *et al.* 2007). Although humans and insects share innate immunity, they differ in that humans also have adaptive immunity. Some of the conserved molecular signalling pathways that are used by humans and insects to fight pathogenic attack are also used for early development of the embryo in insects (Ntwasa, *et al.* 2012).

E. Metchnikow in St. Petersburg initiated the study of invertebrate immunity at the end of the 18th century. He paved the way to the understanding of innate immunity both in vertebrates and invertebrates (Levashina, 1995). By the year 1930 it was known that the insect defense includes a cellular and a humoral facet (Hoffmann, 1995). Insects depend on multiple innate defense reactions to fight infection, which include the use of physical barriers in combination with systemic and local immune responses (Duvic, *et al*. 2012). In insects, the first line of defense against pathogens is the tough cuticle covering the entire animal. Upon breach of this barrier, local immune response occurs at the pathogen penetration site, and the response becomes systemic by using the open circulatory system of insects. Systemic and local defense reactions both depend on cellular and humoral mechanisms (Girard, *et al.* 2007). It is currently known that the innate response of insects depends on three tightly interconnected

reactions. The first reaction entails the induction of proteolytic cascades by wounding. It involves the coagulation cascade that results in localized blood clotting and the prophenoloxidase cascade (melanisation) that leads to the formation of melanised nodules and toxic reactive compounds (Hoffmann, 1995). Cellular defense reactions are the second reactions that consist of encapsulation of invading microorganisms, nodulation and/or predominantly phagocytosis. Phagocytosis consists of the engulfment of small objects such as apoptotic cells, bacteria or yeast by plasmatocytes (Feldhaar & Gross, 2008). The third reaction occurs in the fat body where batteries of AMPs are synthesized (Hoffmann, *et al*. 1996).

1.2 Antimicrobial peptides

The identification of small proteins that are important in immune defense can be traced back to 1921 when lysozyme was discovered by Alex Fleming (Wang, 2013; Fleming, 1881-1955). In a landmark study, from the blood of immune-challenged pupae of *Hyalophora cecropia*, an AMP was isolated in the year 1981. The peptide named cecropin, a 4 kDa peptide mainly active against Gram-negative bacteria, has since been isolated from various sources (Levashina, *et al*. 1995). To date, a number of AMPs have been isolated from sources such as plants, invertebrates, vertebrates, eggs and humans (Memarpoor-Yazdi, *et al.* 2012). In total, there are 5 from protozoa, 10 from fungi, 181 from bacteria, 293 from plants and 1656 AMPs from animals (including insects). Over 2000 natural AMPs are registered into the antimicrobial peptide database (APD) (Wang, 2013).

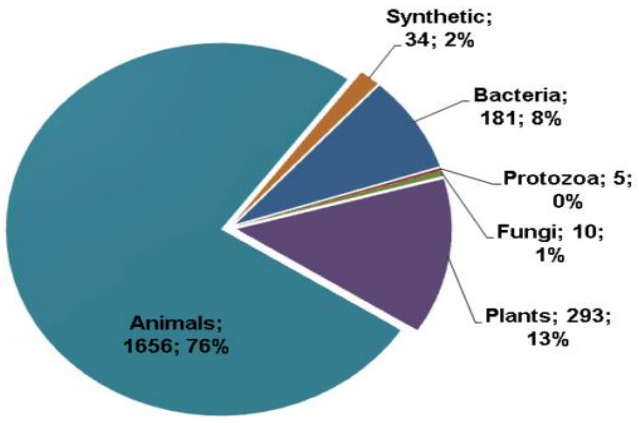


Figure 1.1: Sources of antimicrobial peptides (in total: 2183) in the AMP database. The three major sources bacteria (8%), plants (13%) and animals (including insects) (76%) (Wang, 2013).

In the main insect orders such as Diptera, Lepidoptera, Coleoptera, Hemiptera and Hymenoptera, AMPs have been characterized (Yoon, *et al.* 2003; Duvic, *et al*. 2012). The most powerful and highly characterized components of the systemic humoral response are AMPs (Girard, *et al.* 2007). They are a conserved component of immunity in plants and animals. Mostly small-sized with a molecular mass below 25-30 kDa, AMPs typically have 12-50 amino acid residues. They are mostly cationic as a result of a positive net charge at physiological pH (Gough, *et al.* 1996; Bowdish, *et al.* 2005) and they have a pI range between 8.9-10.7 (Li, *et al*. 2012). AMPs usually have a small number of acidic residues such as aspartate and glutamate which presumably contributes to the increase of amphipathicity when present on the polar face (Teixeira, *et al.* 2012). Moreover, they usually present up to 50% hydrophobic amino acids, contributing to the common amphipathic conformation that they tend to assume at the lipid-membrane interface when interacting with the target cell (Papo & Shai, 2003). According to Li, *et al.* (2012), they are also known to be heat-stable (100^0C, 15 minutes).

AMPs can contain various modifications and vary tremendously in sequence and structure even within a single species. These peptides have a relatively rapid sequence divergence of the regions encoding mature peptides (Bowdish, *et al.* 2005). While they have diverse structures, most AMPs can be assigned to larger families such as cecropins, attacins, defensins and diptericins (Casanova-Torres & Goodrich-Blair, 2013).

AMPs are multifunctional molecules that are involved in several biologic processes and pathologic conditions such as cell proliferation, immune modulation, cytokine and histamine release, wound healing, allergic reactions, anticancer activity, angiogenesis, chemotactic activity and topical activity (Scott & Hancock, 2000; Bowdish, *et al.* 2005). The antimicrobial peptides database in 2011 indicated that there are antiviral (5.8%), antitumor (6.14%), antifungal (31.91%) and antibacterial (78.56%) peptides (Seebah, *et al*. 2007). AMPs have been called natural antibiotics because of their large spectrum of activity. Some AMPs can be used as anti-parasites to control disease. They can be applied in treatment of infected hosts or in prevention of disease transmission by interfering with parasites in their insect vectors. For future applications, AMPs are being recognized as potential antimicrobial alternatives to common antibiotics and chemical food preservatives (Li, *et al.* 2012). Structures of more than 231 AMPs have been determined by use of studies in nuclear magnetic resonance and X-ray diffraction (Seebah, *et al*. 2007).

1.3 Classification of AMPs

The most common classification of AMPs is divided into three broad classes based on sequence and structural features (Bulet, *et al.* 1999): linear peptides with amphipathic α-helices, cysteine-stabilized α-helical/β-sheet motif containing peptides, and linear peptides with an over-presentation in glycine and/or proline residues (Duvic, *et al*. 2012).

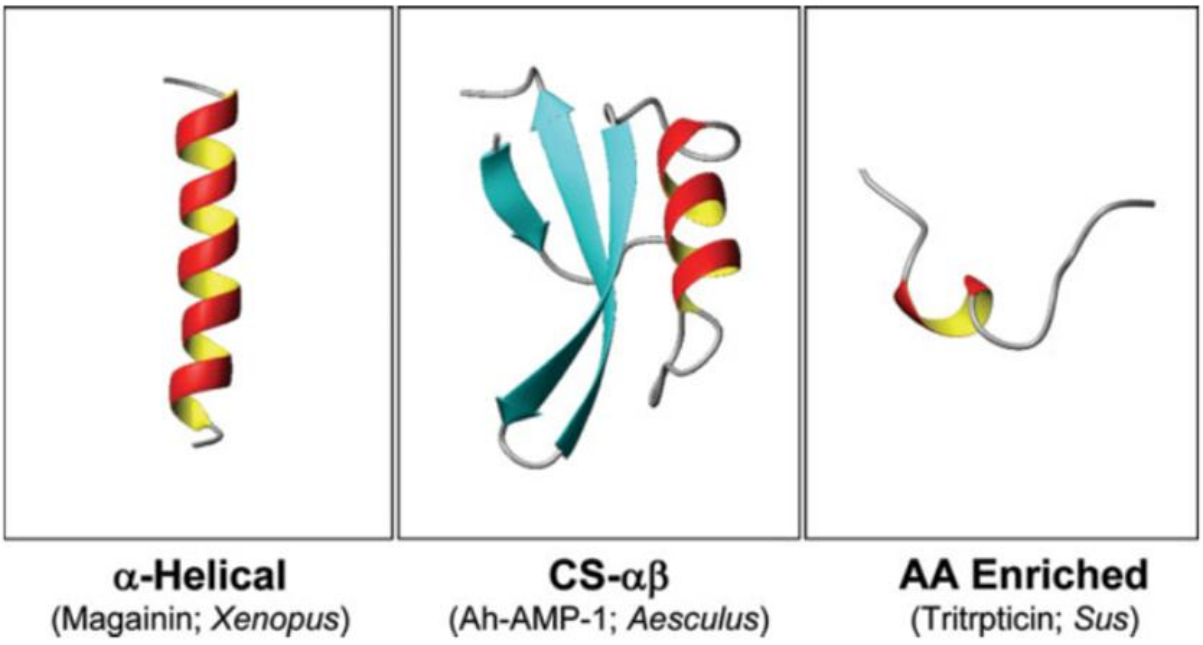


Figure 1.2: Prototypic structures of classical antimicrobial peptide groups. Left to right: Linear α-helical, cysteine-stabilized α-β, and specific amino acid enriched. (Yount, *et al.* 2006).

The largest group of AMPs known to date is the linear cationic α-helical peptides where more than 300 different members have been described (Adade, *et al.* 2013). The most important representatives of this group are cecropins, magainins, cathelicidins and melittin (Wachinger, *et al*. 1998). These AMPs have been discovered from many different sources such as extracellular fluids of insects, invertebrates, teleost fish, nematodes, frogs and mammalian neutrophils. They typically have 12-37 residues and may have a central hinge region. Many of these peptides exist in aqueous solution as unstructured conformers. However, they usually become helical within membrane mimetic environments (Yount, *et al.* 2006). They lack cysteine residues, and form two linear α-helices that are connected by a hinge. Their integration into the acidic membranes of pathogens leads to destruction of the pathogen membrane (Li, *et al.* 2012).

The β-sheet AMPs have a well-defined number of β-strands with relatively few helical domains or none (Yeaman & Yount, 2003). The β-strands are typically organized in the common amphipathic pattern (Ganz, 2003). Most of these peptides are constrained either by cyclization of the peptide backbone or by disulfide bonds (Kang, *et al.* 2012). The β-sheet peptides containing cysteine represent a very diverse group of molecules which are mainly represented by defensins. The stability of these peptides is due to a series of up to six disulfide bonds and they are frequently formed by several antiparallel β-strands. A common motif that is responsible for the integration of all the classes of cysteine-stabilized antimicrobial peptides has been discovered through multidimensional proteomic analysis. This motif, known as the γ-core, consists of two antiparallel β-sheets which have basic residues polarized along the axis (Ganz, 2003). The γ-core motif is found in all major classes of cysteine-stabilized host-defense peptides (Yount, *et al.* 2006). β-sheet AMPs have potent activity against some fungi, Gram-negative bacteria, protozoa and yeasts, but they are predominantly effective against Gram-positive bacteria (Li, *et al.* 2012).

There are numerous examples of AMPs that do not present a specific type of motif, instead they are defined by the presence of a predominant amino acid that imposes certain constrains on their structure. Studies have shown that the structures of these peptides generally differ from the prototypic β-sheet and α-helical structures. This group of AMPs includes those that contain relatively high amounts of proline, glycine and arginine, tryptophan (indolicidin), and histidine residues (Yount, *et al.* 2006). The glycine-rich (14-22% glycine residues) AMPs usually act against Gram-negative bacteria, fungi and cancer cells by the destruction of cell membranes. Proline-rich AMPs are known to be isolated from mammals and insects. They are mainly effective against Gram-negative bacteria and some human pathogens. This group differs from other types of AMPs in that their mode of action consists of penetration into susceptible cells, where they then act against intracellular targets (Li, *et al.* 2012).

Some of these peptides are not so recurrent, and hence are less studied. Recently, antimicrobial activity has been found in polypeptides and proteins considerably larger than classical short AMPs. Some of the smaller peptides were shown to result from proteolytic cleavage from these larger precursors (Nibbering, *et al.* 2001).

The peptides assume the above-mentioned conformations after coming into contact with the target membranes. Interactions with the phospholipid bilayer in the pathogens cell membranes are facilitated by the AMPs characteristic physicochemical properties (Shai, 1999). AMPs can be produced by insects constitutively at local sites and they can also be released systemically after infection by pathogen to generate pathogen-killing activities. According to Lamberty, *et al.* (2001), insects have two modes of fighting infections by AMPs. First, is the inducible production of AMPs which occurs after septic injury by transcription of genes coding for AMPs in the fat body. AMPs are then rapidly released into the hemolymph. The second mode is constitutive, in which AMPs are produced and stored in the haemocytes, and then released into the hemolymph following immune challenge.

1.4 Secretion of AMPs

The secretion of AMPs varies according to differences in physiological settings. In some physiological settings, they are secreted into internal body fluids or onto wet mucosal surfaces (Kang, *et al.* 2012). AMPs contribute a component of the non-oxidative killing activity of phagocytic defensive cells in other settings. The major site of synthesis of immune-induced AMPs is the fat body (Zasloff, 1992), which is also considered to be the functional equivalent of the mammalian liver. Minor sites of AMP synthesis include hemocytes and other tissues such as Malphigian tubules, pericardial cells and the midgut. AMPs are also induced in response to infection in the cuticle or wounding in epidermal cells (Gillespie, *et al*. 1997). AMPs such as the insect defensin can also be produced by salivary gland cells and gut cells (Hoffmann, 1995). In some species, genital tract cells, in both males and females, also produce AMPs. The insect fat body is induced by septic injury to immediately synthesize a large spectrum of antifungal and antibacterial peptides. These peptides are released into the hemolymph to destroy invading microorganisms (Zasloff, 1992).

In a review article by Concciancich, *et al.* (1994), it was shown that the injection of insects with attenuated bacterial cultures caused a resistance to subsequent injections of virulent bacteria. Induction of resistance occurred simultaneously with the appearance of a potent antibacterial activity in the cell-free hemolymph. According to a review by Hoffmann,

(1995), inducible antimicrobial peptides are produced in the fat body within 0.5-1 hour after injury or bacterial challenge (Levashina, *et al*. 1995).

1.5 Signal transduction pathways

The primary regulation of the expression of AMPs at transcriptional level occurs through two distinct signalling cascades called the Imd and Toll pathways which are phylogenetically conserved (Girard, *et al.* 2007). Innate immune recognition consists of specific interactions between pattern-recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs), and intracellular signal transduction cascades that lead to the synthesis of antibacterial/antifungal peptides. Innate immune recognition starts with the detection of conserved structural products of microorganisms. The recognition process enables few germ-line encoded PRRs to recognize any microbial infection. Effector mechanisms of innate immunity are triggered by the presence and recognition activity of PRRs in insect hemolymph. Molecular and genetic studies led to a detailed characterization of the signal transduction pathways upon discovery of PRRs known as Toll-like receptors (TLR) and Immune deficiency (Imd) in *Drosophila melanogaster* (Brivio, *et al*. 2006; Hancock, *et al.* 2006).

Gram-positive bacterial peptidoglycan or fungal β-glucan initiates the *Drosophila* Toll pathway through interaction with peptidoglycan-recognition proteins (PGRPs) and Gram negative binding proteins (GNBPs) which finally activate a serine protease cascade. Activated enzymes eventually cleave the cysteine-knot cytokine-like protein Spätzle. The dimeric form of the mature Spätzle binds to and activates the ectodomain of two Toll receptors. Three proteins containing serine-threonine kinase domains interact with the intracytoplasmic domain of Toll (Bulet & Stocklin, 2005). This triggers a signal transduction pathway that results in phosphorylation of an Iκβ-like inhibitor Cactus which causes it to dissociate from the NF-κβ-like dorsal-related immunity factor (DIF) in adults or Dorsal and/or DIF in larvae. DIF and/or Dorsal activate numerous antimicrobial genes after translocation to the nucleus (Bulet & Stocklin, 2005; Hancock, *et al.* 2006). The Toll-Dorsal pathway in *Drosophila* is a conserved signalling pathway that is used in both vertebrates and invertebrates in different biological contexts. The pathway is used in other processes such as morphogenetic movements and muscle development (Belvin & Anderson, 1996).

The IMD pathway in *Drosophila* is predominantly implicated in the regulation of genes encoding anti-Gram-negative AMPs (Bulet & Stocklin, 2005). Moreover, the pathway responds mainly to peptidoglycan through a distinct PRR, PGRP-LC. This leads to activation of another kinase-based signal transduction pathway which causes phosphorylation and subsequent translocation of another NF-κβ-like transcription factor known as Relish into the nucleus. Relish is cleaved into two parts and, ultimately, the transcription factor regulates transcription of immune-related genes (Hoffmann & Reichhart, 2002). Various studies provide evidence which suggests that *Drosophila* has the ability to differentiate between pathogens and to mount the most appropriate immune response to fight against infection (Bulet & Stocklin, 2005).

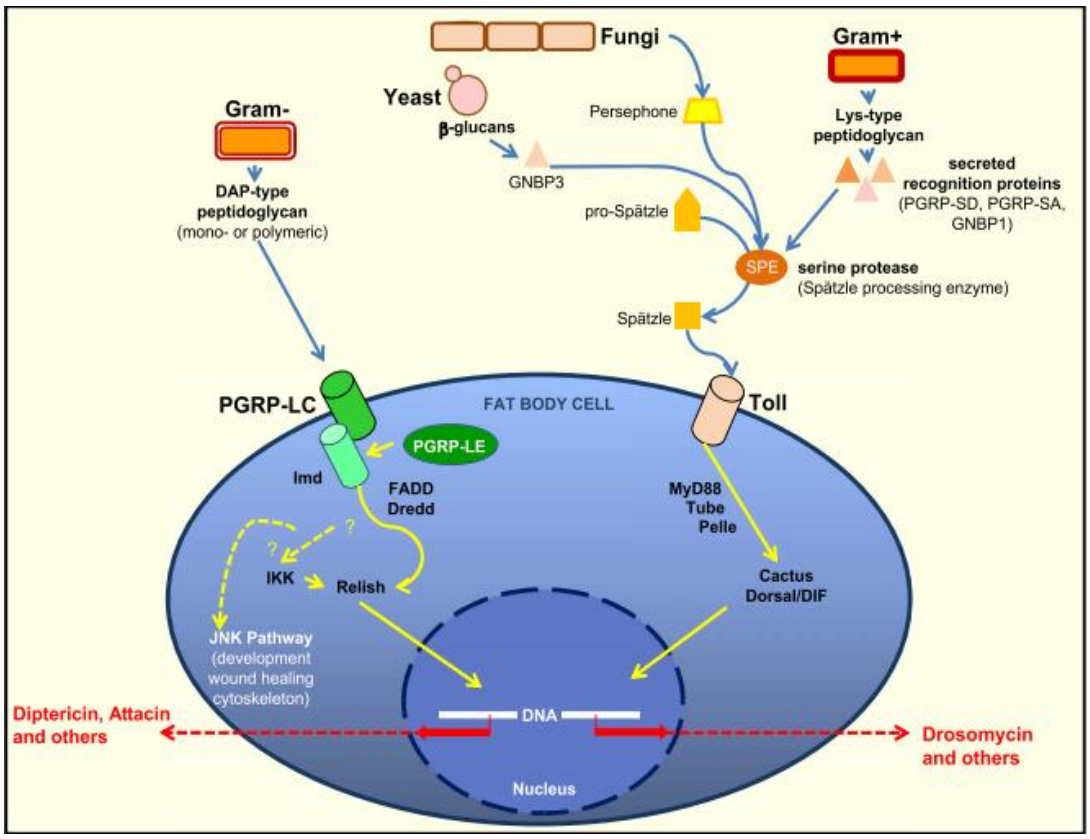


Figure 1.3: Schematic overview of the signalling pathways that trigger an immune response in *D. melanogaster*. (Feldhaar & Gross, 2008).

Recent studies in coleopteran immunity are beginning to reveal the nature of their signalling network. Specifically studies on larger beetles such as *Tenebrio molitor* and *Holotrichia diomphalia* show that beetles respond to β-1,3-glucans and to lysine-containing

peptidoglycan and polymeric diaminopimelic acid containing peptidoglycans, by using the Toll signalling pathway. Both types of PAMPs form complexes with Gram-negative binding protein 3(GNBP3) in fungi and peptidoglycan recognition protein in Gram-negative bacteria. The serine protease cascade is activated by both complexes via a modular serine protease which is an apical protease, followed by two types of CLIP domain serine proteases at penultimate and terminal positions before pro-Spätzle. This leads to the generation of active Spätzle, the ligand of the transmembrane receptor Toll. Subsequent intracellular signalling results in the transcriptional activation of genes that code for AMPs (Park, *et al.* 2010; Ntwasa, *et al.* 2012).
Findings by Hull, *et al*. (2013) suggest that *E. intermedius* responds to fungal challenge via the Toll signalling pathway.

Loss of effector responses that leads to impairment of the host's ability to eliminate foreign invaders can result from any disturbance with the PAMP-PRR recognition process. From data reported by Brivio, *et al.* (2006), it was concluded that the absence or lack of PRRs from insect hemolymph leads to avoidance of the triggering of an antimicrobial response among other immune processes.

1.6 AMPs mechanism of action

It is generally accepted that membrane permeabilization is the main mechanism by which antimicrobial peptides act against pathogens. Upon membrane permeabilization, the electrochemical potential dissipates along with lipid asymmetry. Subsequent loss of metabolites and cellular components usually results in cell shrinkage and ultimately cell death (Yeaman & Yount, 2003). Previous studies have shown that some AMPs translocate into the cytoplasm of the pathogen and interfere with the function of nucleic acids. Other AMPs enter the cytoplasm of the pathogen and kill it by targeting intracellular molecules (Hale & Hancock, 2007). In order to confirm the actual mechanism of action of antimicrobial peptides with intracellular targets, more studies are required (Ntwasa, *et al.* 2012).

The cell membrane is considered as a bilayer of phospholipids that regulate the flux of metabolites between the external environment and the intracellular content. The crucial basis for lipid assembly into structural and functional membranes is the physicochemical nature of

membrane lipids. In pathogens, the membrane is the main target of most antimicrobial peptides. One of the most prominent factors that modulate the peptide's activity upon interaction of the peptide with the membrane is known as the membrane electrical potential. The membrane electrical potential, which is dictated by electrostatic interactions between the lipid head groups and the cationic peptides, also modulates the extent of the peptide's partition to the membrane. The nature of lipids dictates the curvature strain of the membrane. The unsaturated type II lipid phosphatidylethanolamine (PE) naturally promotes a negative curvature strain in monolayers due to its inverted cone geometry. In contrast, the phosphatidylcholine (PC) and phosphatidylglycerol (PG) lipids do not have that propensity due to their cylindrical molecular shape. Moreover, the hydrophobic interactions between the hydrocarbon acyl chain and the amphipathic peptide can induce lipid packing frustration such as membrane thinning. These interactions also have a great impact on membrane structure by causing membrane disruption and permeabilization (Teixeira, *et al.* 2012).

The difference of charge between lipids on the membrane of pathogens and cationic peptides are considered crucial in pathogen killing (Shai, 1999). Most antimicrobial peptides have a net positive charge ranging from +2 to +9. Cationicity of AMPs may be essential for their initial electrostatic attraction to bacteria and fungi through their electronegative cell envelopes and phospholipid membranes. At the outermost surface of pathogens, cationic peptides encounter the glycocalyx and/or cell wall of the bacteria and fungi (Yount, *et al.* 2006). The surfaces of bacterial cells have negatively charged components such as teichuronic and teichoic acids of Gram-positive bacteria, and the anionic lipids and lipopolysaccharide (LPS) of Gram-negative bacteria. Phosphatidylglycerol (PG), phosphatidylserine (PS) and cardiolipin (CL) are hydroxylated phospholipids that have a net negative charge at physiological pH and are predominant on pathogen membranes (Lohner, *et al.* 2001; Zhu, *et al*. 2014). In contrast, phospholipids that comprise the outer surface of normal mammalian cell membranes are predominantly composed of zwitterionic cholesterol and phospholipids (Papo & Shai, 2003). This trait partially explains the specificity of cationic AMPs towards pathogens. Therefore, the key molecular determinant of the selective activity of AMPs towards pathogens seems to be the higher proportion of anionic lipids at the pathogen membrane surface that results in a significantly higher negative electrical potential (-130 mV to -150 mV) (Hancock, 1997).

It is noteworthy to list advantages of using *E. coli* as a pathogen that stimulates immune response in this particular study. Firstly, it is a Gram-negative bacterium. Most antimicrobial peptides have a net positive charge that ranges from +2 to +9, and as already mentioned, cationicity of AMPs may be important for their initial electrostatic attraction to bacteria and fungi (Yount, *et al*. 2006). It has been stated by Li, *et al*. (2012) that there are more AMPs with functions of anti-Gram-negative bacteria than those with the functions of anti-Gram-positive bacteria. For instance, AMPs such as attacins, cecropins, diptericins and drosocins are predominantly active against Gram-negative bacteria.

It has become clear that anionic AMPs are also an essential part of the innate immune system. Over the last ten years, more than 100 anionic peptides were identified in vertebrates, invertebrates and plants (Harris, *et al.* 2009). These peptides were first reported in the 80^{s} and they present a similar broad spectrum of action as their cationic counterparts. They are active against fungi, viruses, bacteria and pests such as insects (Phoenix, *et al.* 2013). However, these peptides were generally found to have weak antibacterial activity against various Gram-negative and Gram-positive bacteria, which was enhanced by the addition of Zn^{+} or post-translational modification (Harris, *et al.* 2009). Some antimicrobial properties have been described for small anionic peptides rich in aspartic and glutamic acids. They have a net charge ranging from -1 to -7, and in length they range from 5 to 70 residues. The mechanisms of action for anionic peptides are not yet fully understood (Brogden, 2005; Harris, *et al.* 2009).

Amphipathicity is crucial for the action of all AMPs since most of them fold into amphipathic structures upon interaction with pathogen membranes. The definition of amphipathicity is relative distribution and proportion of hydrophilic and hydrophobic residues within a peptide. One of the simplest amphipathic structures is the α-helix. Most α-helices have a periodicity of 3-4 residues per turn, which is optimal for interaction with biomembranes (Yount, *et al.* 2006). Amphipathicity is greatly correlated with peptide toxicity and antimicrobial efficacy. Molecules with high amphipathicity tend to disrupt the zwitterionic membranes of mammalian cells (Teixeira, *et al.* 2012).

Peptide hydrophobicity is defined as the proportion of hydrophobic residues within a peptide. It typically constitutes ~50% for most AMPs (Yeaman & Yount, 2003). Moderate hydrophobicity is needed for AMP activity as it controls the degree of partitioning of peptide

into lipid bilayers. However, excessive levels of hydrophobicity result in loss of antimicrobial specificity and cause mammalian cell toxicity. Many AMPs have moderate hydrophobicity (Wieprecht, *et al.* 1997; Yount, *et al.* 2006).

1.7 Models of AMPs mechanisms of action

Several models have been proposed to describe mechanisms used by AMPs to disrupt pathogen membranes. A given AMP may act through different mechanisms in different membrane environments, due to the variability in microbial membrane ultrastructure (Yeaman & Yount, 2003).

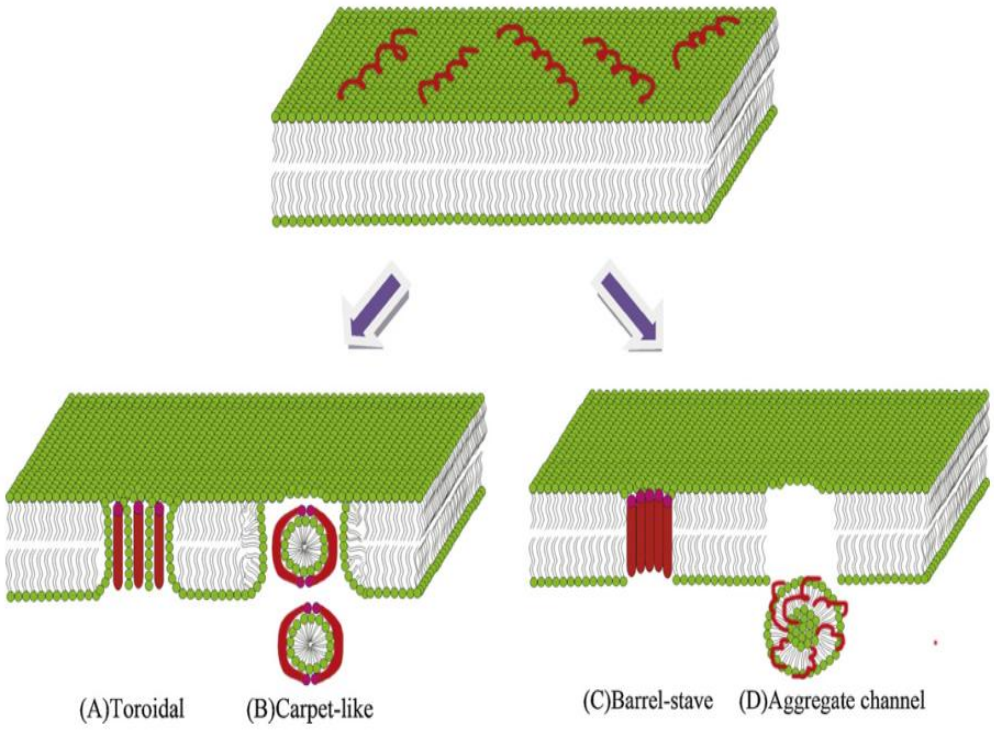


Figure 1.4: Models of mechanisms of action for AMPs. (Li, *et al*. 2012).

First, there is the "barrel-stave" model. The term barrel-stave defines the membrane channel topology that is formed in this mechanism. A variable number of channel-forming peptides are located around an aqueous pore in a barrel-like ring. The individual transmembrane spokes within the barrel form the "stave". These spokes may consist of peptide complexes or individual peptides. In the barrel-stave mechanism, the hydrophobic surfaces of β-sheet or α-helical peptides are outwardly faced toward the membrane acyl chains, whereas the pore

lining is formed by the hydrophilic surfaces (Ehrenstein & Lecar, 1977; Breukink & Kruijiff, 1999). The formation of the transmembrane pore involves the following steps: the membrane is bound by the peptide monomers in a helical fashion, and subsequently, the helices insert into the hydrophobic core of the membrane. After binding, the peptide may undergo a conformational phase transition which results in induction of localized membrane thinning. As the bound peptide reaches threshold concentration, self-aggregation of peptide monomers occurs which leads them to insert deeper into the hydrophobic membrane core. Pore formation re-orientates the helix from the parallel state to the perpendicular membrane spanning state. Additional monomers are progressively recruited resulting in increased pore size and leakage of cell contents and ultimate cell death (Reddy, *et al.* 2004).

Second, in the "carpet" model the peptides solubilize the membrane as they interact with the lipid head groups on the pathogen cell surface. The carpet model differs from the barrel-stave model in that the peptides do not insert into the membrane hydrophobic core and they do not assemble with their hydrophilic surfaces facing each other. Reddy, *et al.* (2004) described steps of the carpet mechanism as follows: the peptides firstly bind to the surface of the target membrane covering it in a carpet like manner. Subsequently, alignment of peptide monomers on the membrane surface occurs as their hydrophilic surfaces face the phospholipid head groups (or water molecules). The hydrophilic residues are then orientated towards the hydrophobic core of the membrane. Reductions in membrane barrier properties and/or phospholipid displacement changes in membrane fluidity subsequently lead to membrane disintegration. Membrane disruption occurs in a dispersion-like manner that excludes channel formation (Shai & Oren, 2001). Furthermore, no specific quaternary structure is required in the steps of the carpet mechanism. Thus, when a threshold peptide concentration is reached, the membrane is exposed to unfavorable energetics leading to loss of membrane integrity. This model was also proposed for viral killing (Papo & Shai, 2003).

Finally, the "toroidal" model is one of the most well characterized mechanisms of action. In this model, antimicrobial peptides partition into the membrane and continuously induce a bending in membrane leaflets through the pore. Thus, the water core is lined by both lipid head groups and inserted peptides. This model primarily differs from the barrel-stave model in that lipids are intercalated with peptides in the transmembrane channel. Hence, this structure is also known as a supramolecular complex that represents a membrane-spanning pore lined with phospholipid head groups as well as polar peptide surfaces. In the toroidal

mechanism, peptides in the extracellular environment take on the α-helical structure upon interaction with the hydrophobic and charged bacterial membrane. Initially, helices are oriented parallel to the membrane surface. The polar head groups are displaced by hydrophobic residues of bound peptides, inducing a positive curvature strain in the membrane by creating a breach in the hydrophobic region (Hara, *et al.* 2001). The membrane surface integrity is further destabilized by introduction of strain and thinning. Peptides orient perpendicular to the membrane at a threshold peptide-to-lipid ratio. Helices begin to aggregate, such that their polar residues are no longer exposed to the membrane hydrocarbon chains. Peptide aggregation, either prior or after binding to the membrane, seems to be required for pore formation, although a stable secondary structure is not required. An unfavorable elastic tension is created that may lead to the formation of transient defects and eventually pore disintegration (Teixeira, *et al.* 2012). Therefore, the transient and multimeric composite forms the dynamic peptide-lipid toroidal pore or supramolecular complex. As the pore disintegrates, some peptides are translocated to the cytoplasmic leaflet of the membrane, which suggests that disintegration of the toroid pore allows entrance of peptides into microbial cytoplasm in order to access potential intracellular targets (Yeaman & Yount, 2003).

The aggregate channel model was proposed based on the finding that membrane depolarization by itself is not necessarily a required step in pathogen killing by some peptides. In this model, peptides bind to the phospholipid head groups and then insert into the membrane (Costa, *et al.* 2011). Subsequently, they cluster into unstructured aggregates that cover the membrane and then water molecules associate to these aggregates that consequently provide channels for the leakage of ions and possibly larger molecules through the membrane (Li, *et al*. 2012). Only short-lived transmembrane clusters are formed, which allow the peptides to transiently cross the membrane without causing high membrane depolarization and membrane structure destruction (Costa, *et al.* 2011). This distinguishes the aggregate channel model from the other models. Upon entrance of peptides into the pathogen, they exert their lethal activities to their intracellular targets by acting on polyanions, such as DNA or RNA (Brogden, 2005).

Both the toroidal and carpet models suggest that the killing activity of antimicrobial peptides occurs due to collapse in membrane integrity followed by dissipation of the membrane potential. However, studies have provided evidence that transmembrane pores, ion channels

and extensive membrane rupture followed by leakage of metabolites and intracellular content in general do not proceed through three different mechanisms of action, but are instead a continuous gradation between them (Teixeira, *et al.* 2012). Moreover, peptides may kill the same organism using more than one mode of action. It depends on varying factors such as tissue localization, growth phase and the presence or absence of other immune mechanisms (Yeaman & Yount, 2003).

1.8 Mechanism of cell death

Increasing evidence supports the suggestion that membrane permeabilization alone is not enough to cause cell death. Microorganisms may survive for longer periods of time following membrane permeabilization in some cases, implicating that non-membranolytic mechanisms may be responsible for cell death. In some instances, cell killing has been reported to proceed with little membrane disruption, but rather due to disruption of intracellular processes (Hancock & Chapple, 1999). Some of the factors that are involved in these intracellular processes were extensively described in a review article by Yeaman & Yount, (2003), namely, inhibition of extracellular biopolymer synthesis, inhibition of intracellular functions and membrane dysfunction.

The cytoplasmic membrane mediates many important functions in microbial pathogens. Antimicrobial peptides may interfere with some of the crucial cytoplasmic membrane functions, which may lead to cell death indirectly or directly. Inhibition of chitin, peptidoglycan or other macromolecular synthesis may also play an essential role in the mechanism of AMP action. For example, biosynthesis of peptidoglycan is integrally associated with membrane function and integrity (Blondelle, *et al.* 1999). Studies have also shown that disturbance of key intracellular processes may be required for cell death (Lehrer, *et al.* 1989; Park, *et al.* 1998).

1.9 Therapeutic potential of AMPs

In the early 1970s, it was assumed that any bacterial infection could be treated, due to availability of numerous and widely ranged antimicrobial agents. These agents, called antibiotics, were products isolated from few bacterial and fungal species. The assumption was soon proved to be incorrect when pathogens appeared to be resistant to conventional

antibiotics that are used routinely to treat bacterial infections (Reddy, *et al.* 2004). The widespread antibiotic consumption both outside and inside of medicine plays a major role in the emergence of antibiotic-resistant bacterial strains (Memarpoor-Yazdi, *et al.* 2012). Incorrect use of antibiotics results in increased levels of bacterial resistance. Excessive use of antibiotics in non-established infections or in infections with viral etiology has served as a form of selective pressure for the appearance of increasingly resistant microorganisms (Fiol, *et al*. 2013). Only few antibiotics that provide successful response to infection are available. In the 80^{s}, 16 new compounds appeared, and that number decreased to only 7 between 1998 and 2002 (Spellberg, *et al.* 2004). It is, therefore, clear that antibiotics are no longer a reliable weapon against infections.

The development of new classes of antimicrobial therapeutic drugs and vaccines is very crucial due to factors such as: the emergence of multiple-drug resistance, the low rate of discovery of new antibiotics and the alarming death rate caused by infection. The development of alternative means to fight infection is required (Ntwasa, *et al*. 2012). AMPs have long attracted commercial and scientific interest due to their capability to kill bacteria without being harmful to human cells (Sovadinova, *et al.* 2011). According to Cytrynska, *et al*. (2006), AMPs are now considered to be important targets for development of therapeutics against various pathogens. In vertebrates, AMPs mediate a number of cellular processes such as wound healing, immunomodulation and tumorigenesis (Scott & Hancock, 2000). They are key effector molecules in host defense through direct and indirect antimicrobial activity. These roles increase the potential to develop therapeutic products and vaccines from AMPs (Ntwasa, *et al.* 2012). In addition, AMPs are fast acting, highly selective and effective against various pathogens. AMPs have shown their ability to rapidly kill a broad spectrum of microorganisms including fungi, bacteria and viruses (Reddy, *et al.* 2004). They also combat other pathogens including protozoa. Therefore, AMPs are less likely to cause the emergence of resistance currently experienced with traditional antibiotics. Another essential benefit of AMPs as therapeutics is that their mechanism of action is more physical rather than targeting metabolic pathways, thus reducing the emergence of resistance (Andrés & Dimarcq, 2005; Cytrynska, *et al*. 2006; Ntwasa, *et al*. 2012).

A crucial prerequisite for development of AMPs into therapeutics is selective toxicity. AMPs act only on foreign microorganisms while retaining the structural and functional integrity of host cells. Since they are able to kill a broad spectrum of microorganisms, AMPs have taken

advantage of biochemical divergence and evolution inherent to cell membrane composition to be harmless to host cells while acting preferentially on pathogens (Teixeira, *et al.* 2012). This difference is considered to possibly account for the varying levels of effectiveness that AMPs have against different types of cells. Prokaryotic and eukaryotic cell membranes differ considerably in lipid composition. Since lipid composition is thought to be the basis of specificity of AMPs towards the target cell, the difference of lipid composition of cell membranes is particularly important for the action of AMPs. The anionic lipids of pathogens are present on the outer surface of the membrane, whereas they are present along the cytoplasmic side of the membrane of mammalian cells (Reddy, *et al.* 2004). The difference of lipid composition and distribution confers selectivity toward prokaryotes and reduces toxicity toward host tissues (Yeaman & Yount, 2003). Another contributing factor is the lack of rigidifying lipids such as cholesterol on pathogen membrane as compared to host cell membranes (Teixeira, *et al.* 2012).

The most obvious use of antimicrobial peptides as potential therapeutics is to amplify the antimicrobial efficacies of conventional antibiotics. An example is the access-based resistance mechanisms such as enhanced efflux or reduced uptake that may be overcome by conventional antibiotics through facilitation by AMPs. As an alternative, peptides interacting with targets or intracellular processes could be engineered to assist the mechanisms of classical antibiotics noncompetitively. Molecular determinants that are emerging as potential targets for AMP strategies include energetics, metabolic processes, essential pathways, microbial receptors, intracellular targets as well as virulence factors. The concept that AMPs may be used to treat or prevent infections is not novel. For many years AMPs have been utilized to prevent or treat infections. Gramicidins, polymyxins and bacitracin can be found in many topical applications and have been used for decades. Advances in understanding mechanistic and structural aspects of AMPs are increasing the development of improved anti-infective peptides (Yeaman, *et al.* 2002; Zasloff, 2002).

It is essential to consider that the development of commercially viable peptides is faced with many challenges. In a review article, Ntwasa, *et al.* (2012) listed some of the major challenges, including pharmacokinetics. Pharmacokinetics describes how the body deals with drugs. During oral administration of peptides, the gastrointestinal tract may hinder their reabsorption into the systemic circulation. Moreover, when peptides are injected directly into the blood, they may elicit an antigenic response. For this reason, AMPs cannot be applied as

injectable therapeutics. Topical medication appears to be the most feasible formulation at present (Jiang, *et al.* 2007; Yeung, *et al.* 2011). Despite current challenges, very few peptides have obtained FDA approval for clinical use. Some AMPs have proceeded to clinical application and some are undergoing clinical trials. They have also been shown to work in combination with conventional antibiotics to protect against systemic and topical infections (Reddy, *et al.* 2004). Therefore, there is optimism that the challenges may soon be overcome (Ntwasa, *et al.* 2012). Databases such as the APD serve as useful tools for the discovery and design of new peptides (Lata, *et al.* 2010).

1.10 *Euoniticellus intermedius*

Euoniticellus intermedius is of the Coleopteran order which is the largest and most diverse insect order on earth. *E. intermedius* (Coleoptera: Scarabaeidae) is well known as the dung beetle and largely found in the Afrotropical region. The dung beetles make tunnels beneath dung pats where they feed and reproduce. The microbe-rich liquid portion of manure feed provides nutrients for adult dung beetle but they do not actually consume the dung. The dung beetle larvae are laid in brood balls where they consume most of the dung (Khanyile, *et al.* 2008). The species is important because it has a wide ecological tolerance and nesting strategies which vary according to the season and soil type. Therefore, it can survive under extreme climatic and edaphic conditions (Kruger, *et al.* 1999).

Coleopterans thrive on this planet although they have no adaptive immunity. There is evidence that Coleoptera have retained many of their ancestral genes, which could provide more knowledge about the properties and evolution of innate immunity. More work on innate immunity has been performed using *Drosophila,* representing dipterans, while fewer studies have focused on coleopterans and their innate immunity (Hoffmann, 2004). The evolutionary history and lifestyle of dung beetles indicate an interesting immune system that could be more comparable to vertebrate innate immunity than *D. melanogaster*. For example, some signalling pathways components that are conserved in coleopterans and vertebrates are not conserved in *D. melanogaster* (Hull, *et al.* 2013).

AMPs from the Coleoptera have been studied in very few insect species such as *Tenebrio molitor, H. diomphalia*, *Zophobas atratus* and others (Yoon, *et al.* 2003). According to Yoon,

et al. (2003), the finding of antimicrobial peptides in other coleopteran species will be helpful in understanding insect immune responses and their molecular mechanisms. Molecular studies of *E. intermedius* are in progress with a view to study its immune system. This beetle has a wide ecological tolerance and survives under extreme environments, thus, interest to study the immune system of these beetles is primarily due to their microbe-rich habitat. Their immune system could provide useful strategies for treatment of infectious diseases affecting humans (Khanyile, *et al.* 2008).

1.11 Aims of the study

The current study focused on analysis of immune response of South African *Euoniticellus intermedius* embryonic (SAEIE08) cell line upon exposure to pathogens, specifically *Escherichia coli and Micrococcus luteus. E. coli* was used to represent Gram-negative bacteria and *M. luteus* was used as a representative of Gram-positive bacteria. The study aimed to show that SAEIE08 has constitutive and/or inducible antimicrobial activity. Another major aim of the study was the isolation and identification of cationic antimicrobial peptides from SAEIE08. Antimicrobial activity upon exposure to pathogen was monitored by performing solid and liquid bacterial inhibition assays. The study was followed by analysis of changes in protein expression patterns of SAEIE08 following exposure to pathogens. The changes were monitored using one dimensional SDS-PAGE and 2-D PAGE. The 5′/3′ RACE kit was employed in conjunction with general molecular biology techniques for the isolation and identification of genes that encode antimicrobial peptides with subsequent utilization of the NCBI database and Bioinformatics to search for identical sequences.

Chapter 2: Materials and Methods

2.1 Materials

See Appendix 1 for materials on page 53.

2.2 Methods

The first sections of the methodology primarily focused on techniques used to show evidence which suggests that there is in fact antimicrobial activity in the South African *E. intermedius* embryonic cell line (SAEIE08). Before the study proceeded to isolating and identifying antimicrobial peptides, it was essential to firstly prove the presence of antimicrobial activity in SAEIE08. Therefore, growth inhibition assays were used to study immune response in SAEIE08 upon exposure to *E. coli*. Such assays were accompanied by the agarose overlay method. In order to analyse protein expression patterns, SDS-PAGE and 2-D PAGE were employed. The 5′/3′ RACE kit was used for the isolation of RNA, which was converted to cDNA, amplified and subsequently sequenced for identification and characterization. The method included general molecular biology techniques, sequencing, bioinformatics and the NCBI database searches.

2.2.1 Organism maintenance, challenging and preparation

2.2.1.1 Preparation of heat-killed bacteria

E. coli, strain 1106, was maintained on LB agar plates without antibiotics. A single colony of *E. coli* was inoculated into 2 ml LB broth for preparation of bacterial culture. Bacterial cells grown at 37^0C on a shaking incubator were collected in the exponential phase of growth and suspended in sterile saline. The optical density at OD_{650} of cell suspension was adjusted to 0.3. using a spectrophotometer. Cell suspension was washed twice and re-suspended in 1 ml sterile saline. Aliquots of the re-suspended solution were serially diluted and subsequently plated with 100 µl spread on each plate, and then colonies were counted the following day in order to determine the concentration of stock *E. coli*, which was ~4 x 10^6 CFU/ml. Plates were incubated overnight at 37^0C. Half of the remaining stock was kept frozen at -20^0C and

the other half was heat-killed at 80^0C for an hour. Sterility of the heat-killed bacteria was tested by plating 100 µl overnight at 37^0C.

2.2.1.2 Culturing live bacteria

The *E. coli* colony was inoculated from LB plate into 2 ml LB broth. It was incubated at 37^0C in a shaking incubator until it reached exponential growth phase which was estimated to be around 0.3 at OD_{650}. Bacterial cells were spun down at 5000 x g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 1 ml Buffer A with an estimated cell concentration of ~4 x 10^5 CFU/ml. The same methods used above were also employed for *M. luteus* as a representative of Gram-positive bacteria, except the growth temperature of 30^0C instead of 37^0C was used to incubate *M. luteus*, and tryptic soy broth was used to culture *M. luteus.*

2.2.1.3 Cell line maintenance, culture and treatment

SAEIE08 was maintained and stored at -80^0C in Grace's media containing 20 % Glycerol. For cell culture, SAEIE08 (100 cells/ml) was added to 50 ml Grace's media and incubated for 15 hours at 25^0C. Cell concentration was estimated to be around 6 x 10^6 cells/ml following incubation. It was then treated with 2 µl heat-killed *E. coli* of 4 x 10^5 CFU/ml. Treatment was carried out at various hours in order to study differential protein expression patterns influenced by hour(s) of treatment with heat-killed *E. coli.* Controls were not treated with heat-killed *E. coli*. At the appropriate hour(s) of treatment, cultures were spun down at 5000 x g for 10 minutes to prepare beetle cell supernatant. Collected SAEIE08 supernatant was serially diluted with Buffer B according to table 2.1 below.

2.2.2. Liquid media growth inhibition assay

Table 2.1: Liquid growth inhibition assay

	Grace's media	Undiluted Supernatant	2X diluted Supernatant	4X diluted supernatant	8X diluted supernatant	*E.coli* (Blank =Buffer A)
1.	760 µl	800 µl				40 µl
2.	760 µl		800 µl			40 µl
3.	760 µl			800 µl		40 µl
4.	760 µl				800 µl	40 µl
5.	1560 µl					40 µl

For assay of bacterial activity, preparations in table 2.1 were incubated at 37^0C on a shaking incubator at 1 hour intervals. After each hour of incubation the OD_{650} of *E.coli* were measured and recorded for construction of curves illustrating the relationship between rates of bacterial growth inhibition upon treatment with different concentrations of SAEIE08 in order to study its antimicrobial activity. For the negative control curve, phosphate buffer was added instead of SAEIE08.

2.2.3 Protein extraction

SAEIE08 was cultured as previously described to a concentration of 6 x 10^6 cells/ml. Cultured cell line was treated with 10 µl heat-killed *E. coli* (4 x 10^6 CFU/ml) for 2 hours and then it was centrifuged at 5000 x g for 10 minutes. The resultant sample pellet and supernatant were used differently for protein extraction. Sample pellet was collected for protein extraction using TRI-reagent from Sigma-Aldrich (catalogue number: T9424) following the manufacture's protocol. Sample supernatant which constitutes the crude extract was used for protein concentration and subsequent TCA precipitation. Ultrafree-CL centrifugal devices were used for protein concentration followed by precipitation by 20% TCA. The resulting pellets from protein extraction and TCA precipitation were re-suspended in 125 µl rehydration buffer. The Bradford assay and UV-Vis spectrophotometer were used to determine protein concentration. Resulting concentration values were used to calculate appropriate volumes to load into SDS-PAGE in order for each well to contain equal protein concentration.

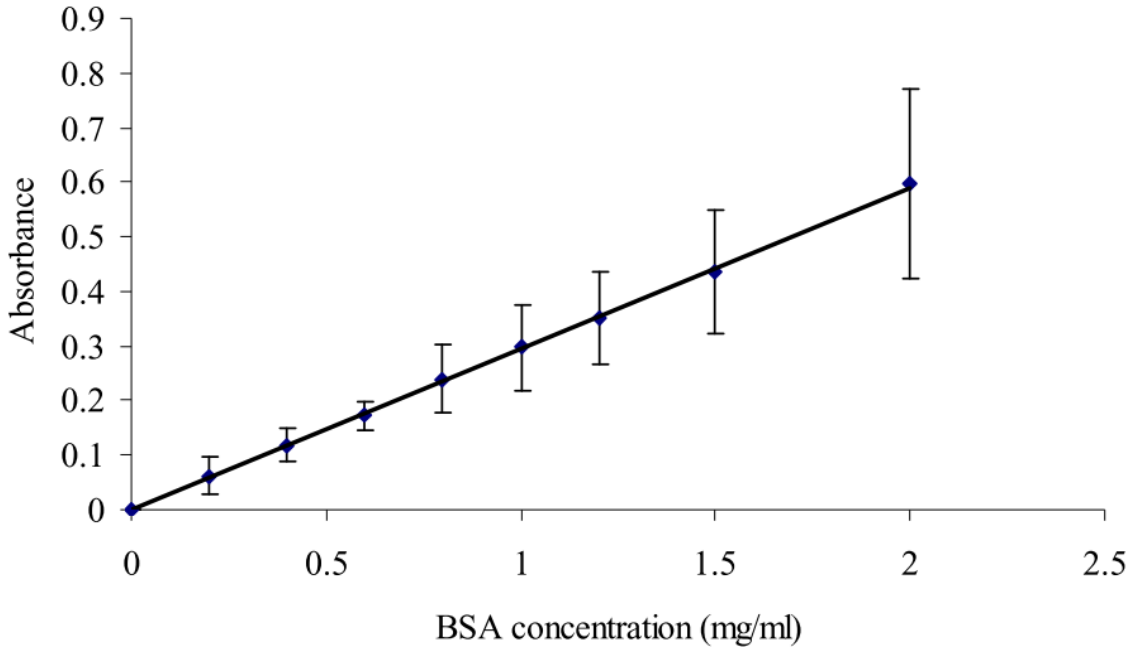


Figure 2.1: Standard curve for the determination of protein concentration using the Bradford assay. The curve consisted of 10 replicates. The mean values were entered into the spectrophotometer and this was used as the actual curve. The curve's equation is given as y= 0.2956x – 0.00004 and R^2 = 0.9994. The curve was constructed according to Hull (2012).

2.2.4 One Dimensional SDS-PAGE analysis

The SDS polyacrylamide gels were run according to Laemmli (1970). Preparations of gels were 16% separating gel and 4% stacking gel. The sample was mixed at a ratio of 4:1 with sample loading buffer and heated to 95^0C for 5 minutes before loading. A sample volume of 20 µl was loaded into each well. Gels were run at a constant voltage of 150 V. The electrophoresis run was terminated when the tracking dye reached ~1 cm above the sealing part of the gel cast.

After the Electrophoresis run, gels were incubated overnight in coomasie staining solution at room temperature on a shaker. Following incubation, gels were washed repeatedly with distilled water over 3 hours. They were then destained for 4 hours with coomasie destaining solution on a shaker. Distilled water was used repeatedly to rinse gels for 3 hours. Alternatively, silver staining was used for sensitive detection of proteins separated by 1D and 2D SDS-PAGE with detection limits from 0.5-5 ng. An improved silver staining protocol was

used according to publication by Mortz, *et al.* (2001). Gels were sensitized in 0.02% sodium thiosulphate for 1 minute followed by washing twice in distilled water for 20 seconds. The gels were then incubated in silver staining solution for 20 minutes at 4^0C. They were subsequently washed three times in distilled water for 1 minute, and then placed inside a developing solution for 20 minutes in the dark. As the gels turned yellow, staining was terminated in 5% acetic acid for 5 minutes. A PDQuest 2-D analysis software version 6.2 (Bio Rad Cat #170-9630) was used to scan and upload the gel images.

2.2.5 Solid media growth inhibition assay: Agarose overlay method

A single *E. coli* colony was collected from stock petri dish with a loop and inoculated in 2 ml LB broth for culture at 37^0C until it grew up to 0.3 at OD_{650}. Bacterial culture of 10 µl was pipetted into 10 ml sloppy agar (0.5% agar) kept at a temperature of 50^0C. The mixture was shaken vigorously for a few seconds to mix thoroughly then it was poured onto agar plate (1.5% agar) containing cut SDS-PAGE gel. Experiment agar plate contained a gel run with extracted peptides from SAEIE08. A gel run with markers and SDS sample loading buffer was placed on the negative control agar plate. The positive control agar plate was spread with 0.1% ampicillin in the inner circle of the petri dish. The preparation was incubated overnight at 37^0C. The following day growth inhibition was analysed on agar plates.

2.2.6 2-D PAGE analysis

ReadyPrep 2D Starter (Catalogue number 163-2105) was used to separate protein extracts obtained from Tri-reagent extraction and the concentrated protein that was TCA precipitated. The kit allows successful separation of protein extract by Two-Dimensional Polyacrylamide Gel Electrophoresis using IPG strips and the Bio-Rad PROTEAN IEF cell. The ReadyPrep 2D starter kit includes all reagents required to rehydrate IPG strips with sample, equilibrate IPG strips for SDS-PAGE, and overlay IPG strips with agarose on SDS-PAGE gels. The following sub-sections describe an overview of the main steps in 2-D PAGE. The ReadyPrep 2D Starter kit provides a detailed manual for the 2-D PAGE method. Cell culture and protein extraction were performed as previously mentioned. The Bradford assay was also performed in order to determine appropriate loading concentrations on the 1^{st} dimension 2-D PAGE.

Rehydration

A strip length of 7 cm was used for protein sample re-suspended in rehydration buffer. Calculated volumes of re-suspended samples determined by the Bradford assay were pipetted as lines along the back edge of channels on the rehydration tray. Forceps were used to peel the coversheet from IPG strips. Strip gels were gently placed gel side down onto samples on the channel. Each strip was overlayed with 3 ml mineral water to prevent evaporation during rehydration process. The tray was covered with plastic lid provided and then left on a level bench overnight.

Isoelectric focusing

Paper wicks were placed at both ends of the focusing tray channels covering the wire electrodes. Nanopure water (8 µl) was pipetted onto each paper wick. IPG strips were removed from the rehydration tray using forceps, and then they were transferred to the focusing tray. Gels were maintained gel side down then mineral oil (3 ml) was poured onto each channel to cover the IPG strip. The focusing tray was covered and placed in a PROTEAN IEF cell, which was programmed using the appropriate 3-step protocol depicted on the table below. The default cell temperature of 20^0C was used with a maximum current of 50 µA/strip and with no rehydration because the rehydration step had been performed.

Table 2.2: PROTEAN IEF cell program consisting of the 3-step protocol

7cm	Voltage	Time	Volt-hours	Ramp
Step 1	250	20 minutes	0.25 V-h	Linear
Step 2	4000	2 hours	1000 V-h	Linear
Step 3	4000	3 hours	10 000 V-h	Rapid
Total	8250	6 hours	14 000 V-h	

Equilibration

Upon completion of the IEF run, IPG strips were removed from the focusing tray and transferred gel side up into a clean equilibration tray. Equilibration buffer I (4 ml) was added to each channel containing an IPG strip. The tray was gently shaken on an orbital shaker for 10 minutes. Equilibration buffer I was then discarded and replaced by 4 ml Equilibration buffer II into each channel which was also gently shaken for 10 minutes on an orbital shaker. Equilibration buffer II was also discarded after shaking, and IPG strips were ready for SDS-PAGE.

2-D PAGE

SDS-PAGE that is followed by isoelectric focusing differs slightly from normal SDS-PAGE due to the use of IPG strips. System setup for SDS-PAGE involved assembling gel cassettes and plates, placing gels in the tank and filling the tank with running buffer. An electrode filter paper wick was inserted onto the SDS-PAGE gel on the paper wick well, and 10 µl of protein marker was pipetted onto the paper wick. IPG strips were removed from the equilibration tray and dipped briefly into a graduated cylinder containing 1x Tris/Glycine/SDS running buffer. Each gel strip was laid gel side up and onto the back plate of the SDS-PAGE gel above the IPG well next to the paper wick well. Forceps were used to carefully push the strip into the well while taking care not to introduce bubbles. Overlay agarose solution was poured on the IPG and paper wick wells of the gel and allowed to solidify for 5 minutes. Gels were run for approximately 2 hours at a voltage of 150 V, and subsequently, they were coomasie- and silver-stained for higher gel resolution. Staining and analysis of gels were done as previously described.

2.2.7 Extraction of RNA

RNA was extracted from SAEIE08 using the TRI Reagent kit from Sigma-Aldrich (Catalogue number T9424) according to manufacturer's instructions. The TRI reagent is a quick and convenient reagent for use in the simultaneous isolation of RNA, DNA, and protein. This procedure is an improvement of the single-step method reported by Chomczynski and Sacchi, (1987) for total RNA isolation. TRI Reagent performs well with large or small amounts of tissue or cells and many samples can be simultaneously extracted. This reagent is a mixture of guanidine thiocyanate and phenol in a monophase solution.

Guanidium thiocyanate is one of the most effective protein denaturants. SAEIE08 was cultured as previously mentioned to a concentration of 6 x 10^6 cells/ml. It was spun down at 12 000 x g for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 1 ml tri-reagent. DNA, RNA, and protein were effectively dissolved on lysis of the pellet. After adding chloroform and centrifuging, the mixture separated into 3 phases: an aqueous phase containing RNA, interphase containing DNA, and an organic phase containing proteins. Each component was then isolated and the RNA aqueous phase was mixed with 2-propanol followed by centrifugation. The resulting RNA pellet was re-suspended in 75% ethanol. After a few centrifugation steps and drying of pellet, RNA pellet was re-constituted in distilled water. The RNA concentration and purity were determined by a Nanodrop, and analyzed using agarose gel electrophoresis.

2.2.8 Agarose gel electrophoresis

Agarose gel electrophoresis is a standard method used to characterize RNA and DNA in the range 200 to 50 000 base pairs (50 kb). Agarose is a linear polymer of galactopyranose derivatives (Boyer, 2006). Gels were prepared by dissolving 1% agarose and 5 µl of 10 µg/ml ethidium bromide in warm 1x TAE electrophoresis buffer. After cooling the gel mixture to 50^0C, the agarose solution was poured on a casting tray and a comb was inserted appropriately. RNA sample was prepared in 6x loading dye containing 30% glycerol and traces of bromophenol blue, then it was incubated at 65^0C for 4 minutes followed by cooling on ice. The comb was removed from set gel and the gel was placed in an electrophoresis tank which was poured with 1x TAE running buffer. RNA sample was then loaded in a sample well made with a comb and voltage of 67 V was applied until separation was complete. Ethidium bromide allows visualization of separated nucleic acids after electrophoresis.

2.2.9 5′/3′ RACE Technique

RACE stands for Rapid Amplification of (either 5′ or 3′) cDNA ends. The 5′/3′ RACE kit, 2nd Generation from Roche (Catalogue number 03353621001) was used to clone the full length transcripts from trizol extracted RNA following the manufacturer's instructions. 5′ RACE allows the amplification of unknown sequences at the 5′ end of the mRNA, whilst 3′ RACE amplifies sequences at the 3′ end of the mRNA.

First strand 5′ cDNA was synthesized from total mRNA using primers that were constructed from immune response genes such as hopscotch, defensin, and apolipoprotein and the enzyme transcriptor reverse transcriptase. The protocol provided in the kit was used for the rapid amplification of the 5′ cDNA end. PCR reactions were performed according to the following conditions: 2 minute initial denaturation at 94^0C; 35 cycles of denaturation, annealing and elongation at 94^0 for 15 seconds, 55^0C for 30 seconds, and 72^0C for 40 seconds, respectively; and final elongation at 72^0C for 7 minutes. The High Pure PCR product Purification Kit from Roche (Catalogue number 11732676001) was used to purify the 5′ cDNA product. Purified 5′ cDNA was poly(A)-tailed with dATP and TdT and then amplified via the Polymerase Chain Reaction (PCR) following manufacturer's instructions.

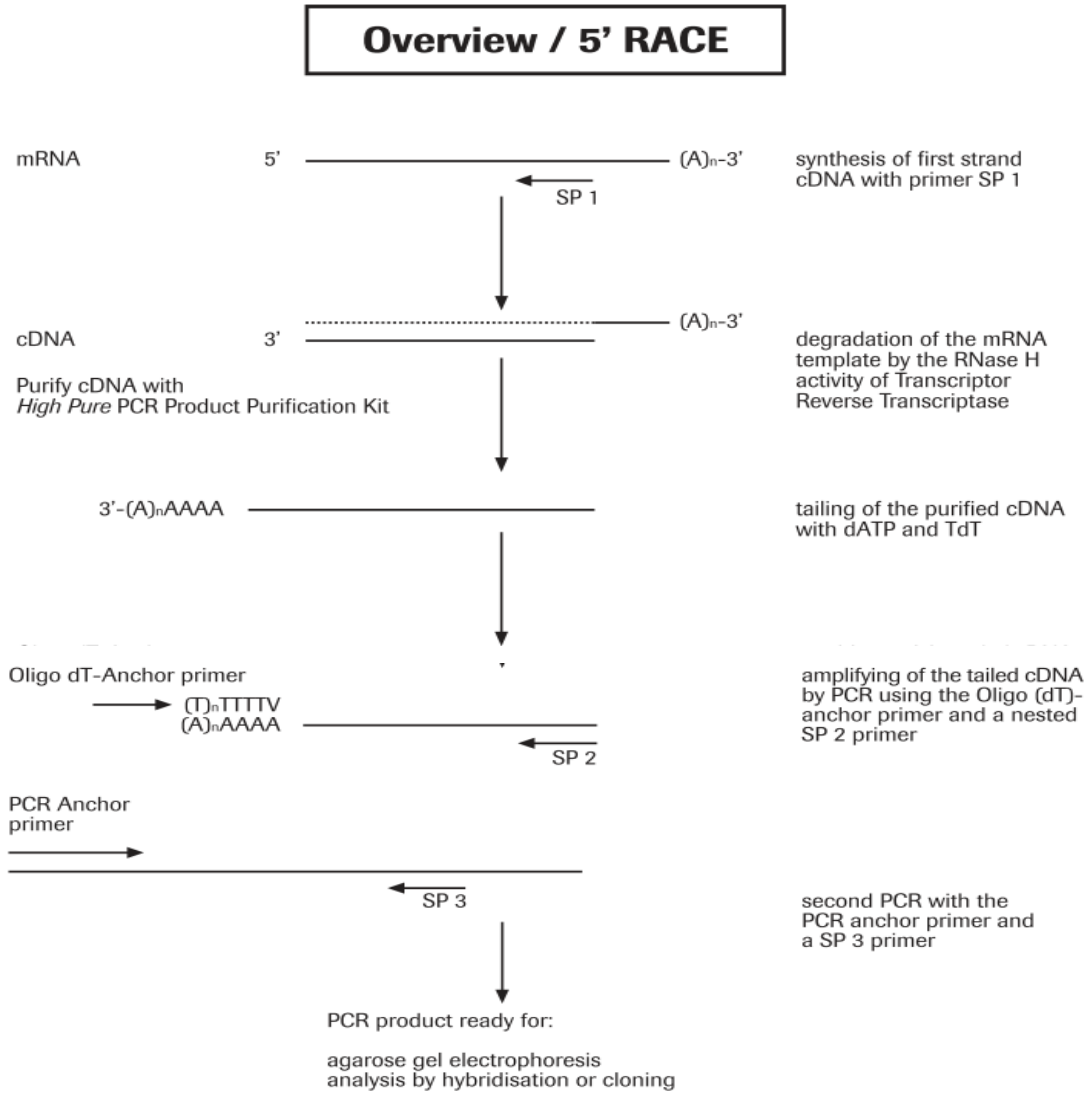


Figure 2.2: Overview of the 5′ RACE method from manufacturer's protocol.

For amplification of the 3′ cDNA end, the 3′ RACE protocol takes advantage of the natural poly(A)-tail of mRNAs as a priming site for PCR amplification. First strand 3′ cDNA synthesis is initiated at the poly(A)-tail of mRNA using transcriptor reverse transcriptase and the oligio(dT)-Anchor primer. After converting mRNA into cDNA, amplification is then performed without a further purification step using the PCR Anchor primer and the hopscotch primer. The thermal cycle profile used for 3′ cDNA end was as follows: Initial denaturation at 94^0C for 3 minutes; 40 cycles of denaturation, annealing and elongation at 94^0C for 30 seconds, 60^0C for 30 seconds, and 72^0C for 1 minute, respectively; final elongation at 72^0C for 7 minutes.

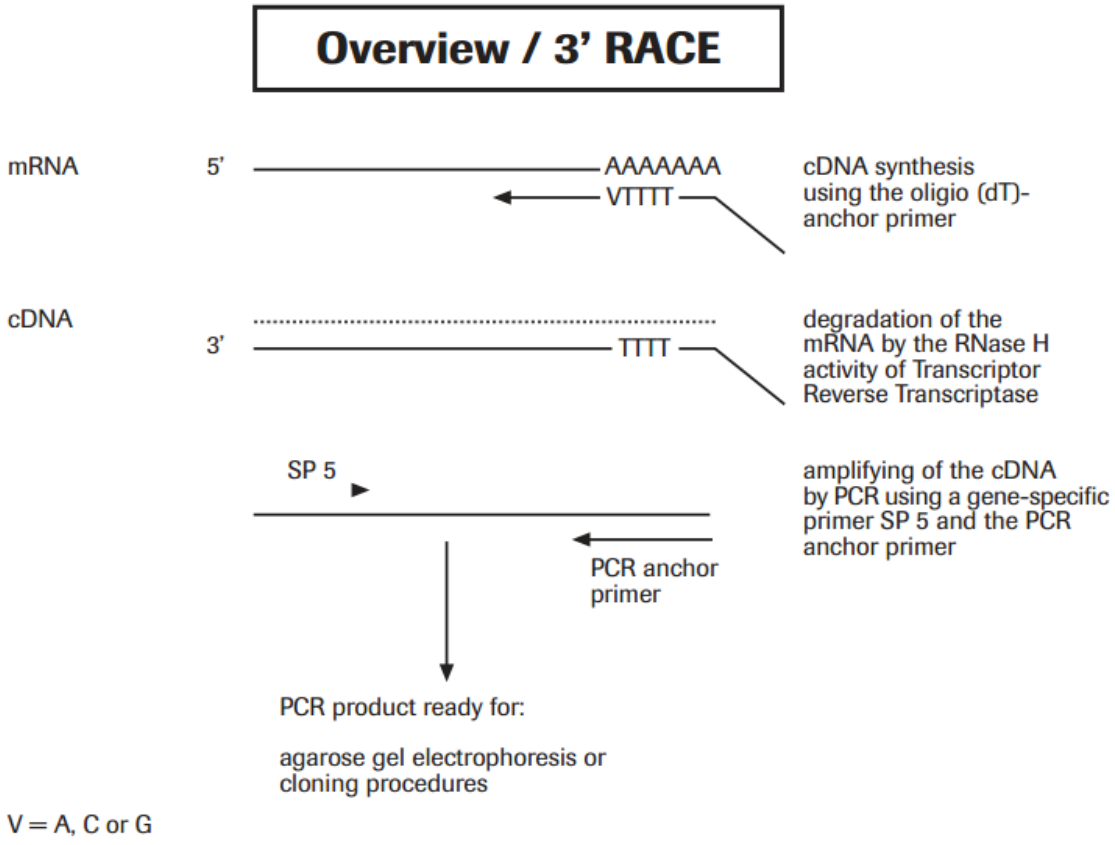


Figure 2.3: Overview of the 3′ RACE method from manufacturer's protocol.

The concentration and purity of the PCR products from the 5′ cDNA and 3′ cDNA strands were determined by use of a Nanodrop and analyzed via Agarose gel electrophoresis. At this stage they were ready for cloning procedures.

2.2.10 Cloning of genes

The PCR products were ligated to the pGEM – T Easy vector following manufacturer's instructions of the pGEM – T Easy vector cloning system (See appendix 2 for the pGEM – T Easy vector on page 57). The ligation reaction was set-up according to the manufacture's protocol. The reaction was incubated for 12 hours at 4^{o}C and inactivated by incubating at -70^{o}C for 15 minutes.

Transformation

Competent cells, *E. cloni*, were kept on ice for 20 minutes before use. A ligation reaction of 4 µl was added to 40 µl frozen competent cells and the mixture was placed on ice for 20 minutes. Thereafter, it was heat-shocked for 45-50 seconds at 42^{0}C. SOC media of 960 µl was added and incubated at 37^{0}C for 1.5 hours. Plates for Blue/white selection were prepared by adding 100 µl of 100 mM IPTG to 20 µl of 50 mg/ml X-gal and spreading across LB plates. After 30 minutes incubation of LB plates, 100 µl of transformation culture was plated onto LB/ampicillin/IPTG/X-gal plates, which were incubated overnight at 37^{0}C. The following day, blue and white colonies were counted on each plate and transformation efficiency was calculated.

Colony screening

A white colony was inoculated from LB/ampicillin/IPTG/X-gal plate and cultured in 2 ml LB broth overnight in order to extract Plasmid DNA using the QIAprep Spin Miniprep kit from Qiagen (Catalogue number 27106). Overnight culture was pelleted by centrifuging at 6000 x g for 5 minutes at room temperature. Buffer P2 from the kit was used to lyse cells for release of plasmid DNA. The QIAprep spin columns plus specific buffers from the kit were used to purify Plasmid DNA. Concentration and purity of collected Plasmid DNA was measured by a Nanodrop. Restriction enzyme digestion was carried out using *EcoR1* and 10x *EcoR1* buffer to cleave Plasmid DNA in order to release DNA inserts. The reaction was incubated overnight at 37^{0}C. The presence and size of DNA inserts were confirmed by analysis using agarose gel electrophoresis.

Colony PCR and sequencing of DNA

Plasmids with DNA inserts were sent to Inqaba Biotechnical Industries (Pty) Ltd where they were sequenced using standard forward and reverse M13 Primers, which bind to either side of the multiple cloning sites in the pGEM – T Easy vector. Resulting sequences were processed manually using bioinformatics before being submitted for database searches. Finally, BLAST searches were carried out using the NCBI database.

2.2.11 Isolation and identification of antimicrobial peptides

SAEIE08 cell culture and treatment were done as previously mentioned. Protein was extracted from SAEIE08 using the TRI Reagent kit from Sigma-Aldrich (Catalogue number T9424) according to manufacturer's instructions. One dimensional SDS-PAGE was performed to check for protein purity and integrity. *E. coli* treated and untreated samples were sent to the CSIR for protein sequencing. For protein sequencing, each of the *E. coli* treated and untreated lanes of the SDS-PAGE gel were used. The bands from each lane were excised and divided into fractions which were digested using an endoprotease. The resulting solution was passed through a mass spectrometer (LC-MS/MS) which produced peptide fragments and measured their mass-charge ratios. The mass spectrum was analyzed using searches against specific databases of Coleoptera (*Tribolium casteneum*) and Diptera (*Drosophila melanogaster*) and also against the Uniprot and Swissprot databases.

Chapter 3: Results

This chapter utilizes the same order of presentation used in methodology in that the first two sections, 3.1 and 3.2, show immune response of SAEIE08 upon exposure to pathogen. These sections consist of graphs from liquid inhibition assays with associated tables and statistics, supported by solid inhibition assays, all indicating the presence of secreted antimicrobial activity. The section is followed by observation of protein expression patterns upon exposure of SAEIE08 to heat-killed *E. coli*. This particular section has data comprised of SDS-PAGE and 2-D PAGE gels implicating the presence of antimicrobial peptides around 10 kDa (and high molecular weight proteins that may be involved in immunity). The last section (3.3) presents sequencing data from the 5′/3′ RACE kit and Molecular Biology techniques, which were analysed by bioinformatics and tools such as NBLAST and XBLAST searches on the NCBI database.

3.1 Assay of antimicrobial activity

3.1.1 Liquid Growth inhibition assay

The major aim of this part of the study was to analyze the immune response of SAEIE08 upon exposure to pathogens, specifically *Escherichia coli and Micrococcus luteus*, with the intention to show inducible and constitutive antimicrobial activity. SAEIE08 was treated with heat-killed *E. coli*, and then used as a growth inhibitor for live bacteria whose growth was measured at OD_{650}. The culture medium of SAEIE08 was diluted to compare differences in bacterial growth inhibition by least to most diluted SAEIE08 culture medium. In order to quantify the level of antimicrobial activity in the undiluted and differentially diluted SAEIE08, the negative control was used as a reference point for calculations of bacterial growth inhibition percentages. As anticipated, inducible and constitutive antimicrobial activities were observed in figure 3.1. In figure 3.1 A (treated) and B (untreated), there was a small difference in rates of bacterial growth, as it increased slightly faster for B (untreated) than A (treated), with the exception of the negative control curves (no cell line added), which had approximately the same growth rates in both A and B. For example, in the undiluted SAEIE08, at 240 minutes of incubation the higher reading of 0.353 is that of *E. coli* in untreated control, whilst *E. coli* in treated SAEIE08 is slightly lower at 0.339. In all dilutions,

the OD_{650} readings in A had slightly lower values compared to B. Furthermore, table 3.1 shows that maximum *E. coli* growth inhibition was observed for the 3 hour-treated and undiluted SAEIE08 at 36.4%. On the contrary, the lowest growth inhibition was 16.7% of the undiluted and 3 hour-untreated control. Therefore, it is evident that treated cell line shows inducible antimicrobial activity while the activity in untreated cell line may be constitutive.

In treated SAEIE08 and their untreated controls, an increase in bacterial growth is not always proportional to hours of SAEIE08 treatment. An example can be observed in figure 3.2 by comparing the 1 hour- and 3 hour-treated SAEIE08. Lower *E. coli* growth of 0.025 at OD_{650} (SD of 0.015) was initially observed at 60 minutes of incubation for the 1 hour-treated SAEIE08 compared to the 3 hour-treated SAEIE08 at OD_{650} reading which was higher at 0.033 (SD of 0.030). In contrast, at 300 minutes of incubation, bacterial growth of 0.388 (SD of 0.066) in the 1 hour-treated SAEIE08 seemed to increase comparably well to growth in the 3 hour-treated SAEIE08 which was 0.388 (SD of 0.024). Table 3.2 provides further evidence in that the 1 hour treatment caused a high level of inhibition more than the 2 hour- and 3 hour-treatments. Therefore, level of inhibition is not dependent on hours of treatment. The inhibition assay that was performed with *M. luteus* did not produce normal growth curves. However, a pattern of growth inhibition could still be observed from least to most diluted cell line. The undiluted SAEIE08 appeared to have a stronger inhibitory effect on bacterial growth compared to all dilutions.

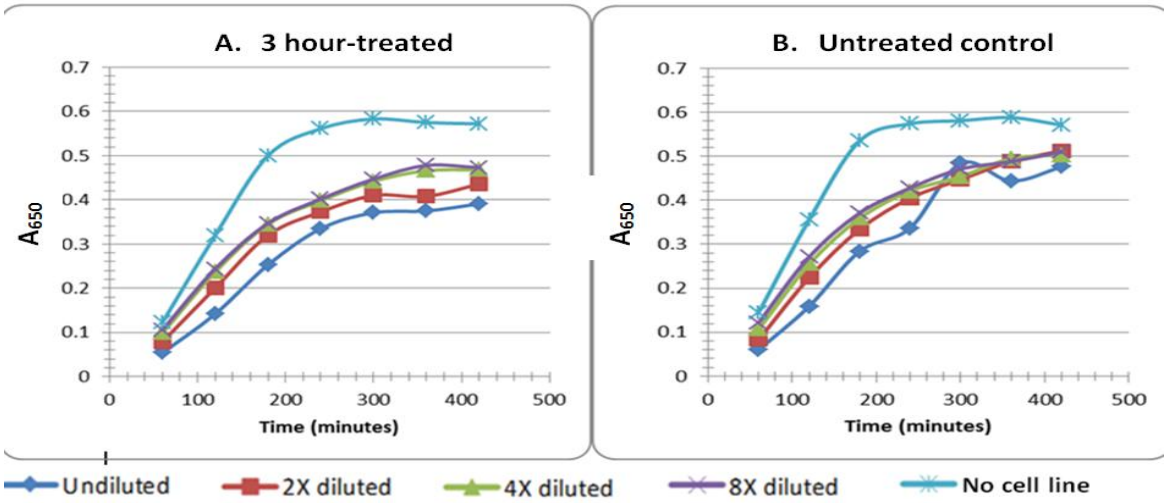


Figure 3.1: Liquid growth inhibition assay showing *E. coli* optical density when cultured in different dilutions of SAEIE08 that was: A. treated with heat-killed *E. coli* for 3 hours, and B. untreated with heat-killed *E. coli* (control). The study was repeated four times in triplicate from which the mean and standard deviation were determined.

Table 3.1: Comparison of growth inhibition percentage between the 3 hour-treated SAEIE08 and the untreated control at 300 minutes of *E. coli* growth.

Cell line dilutions	3 hour-treated inhibition %	Untreated control inhibition %
Undiluted	36.4	16.7
2X diluted	29.7	23.1
4X diluted	24.2	21.9
8X diluted	23.3	18.9

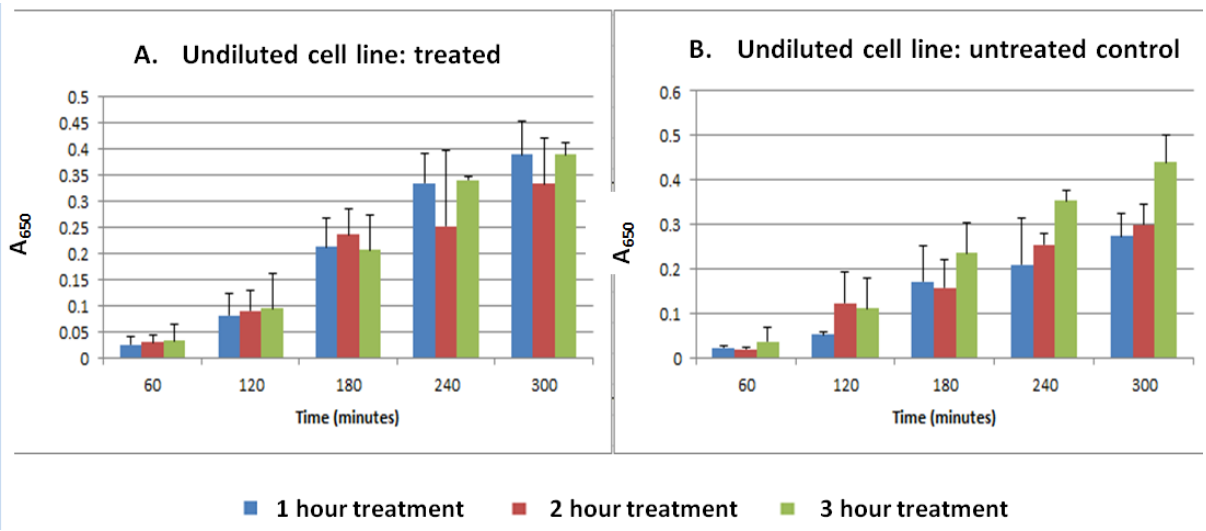


Figure 3.2: *E. coli* growth observed when cultured in undiluted SAEIE08 treated (A) and untreated (B) with heat-killed *E. coli* for 1, 2 and 3 hours. The error bars represent the standard error of the means of biological replicates. The study was repeated four times in triplicate.

Table 3.2: *E. coli* growth inhibition percentage when cultured in differentially diluted SAEIE08 that was treated at different hours with heat-killed *E. coli.*

	E. coli growth Inhibition % in undiluted and differentially diluted SAEIE08			
Treatment hours	Undiluted	2X diluted	4X diluted	8X diluted
1 hour	38.6%	33.6%	25.3%	23.3%
2 hours	35.9%	28.8%	26.5%	23.5%
3 hours	36.4%	29.7%	24.2%	23.3%

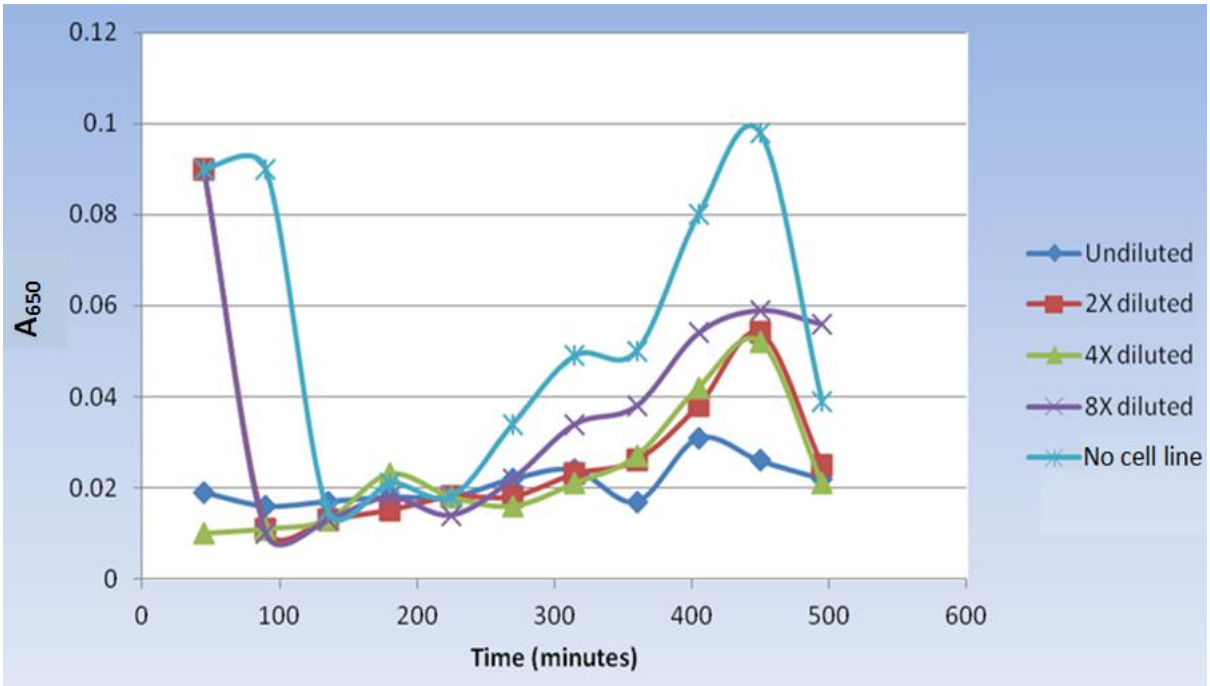


Figure 3.3: Liquid growth inhibition assay showing *M. luteus* optical density in different dilutions of SAEIE08. In order to determine the mean and standard deviation, the study was done in triplicate and repeated three times.

3.1.2 Solid growth inhibition assay

The solid media growth inhibition assay was performed with the intention to show bacterial growth inhibition as a result of induced antimicrobial activity in SAEIE08. The complete method required use of three techniques: firstly, proteins were extracted from treated SAEIE08 crude extracts; secondly, protein extracts were run on an SDS-PAGE gel; and lastly, the agarose overlay method was performed using the SDS-PAGE gel containing protein extract which was placed on an LB plate and poured with live *E. coli* mixed with soft agar and then left overnight to incubate at 37^0C. The method outcome can be seen in figure 3.4 that shows evidence for growth inhibition. Plate A contains a gel run with protein extracts from SAEIE08. There was significantly higher *E. coli* growth inhibition on the gel overall, even though the most visible peptide bands were only at the bottom part of the gel. Plate B had a gel with protein markers and SDS sample loading buffer as a negative control. *E. coli* growth colonies were visible all over the LB plate and on the negative control gel. There appeared to be no inhibition of growth by protein markers and sample loading buffer. Plate C was spread with ampicillin to act as a positive control at the central part of the plate by inhibiting bacterial growth on this region which showed a clear zone of growth inhibition.

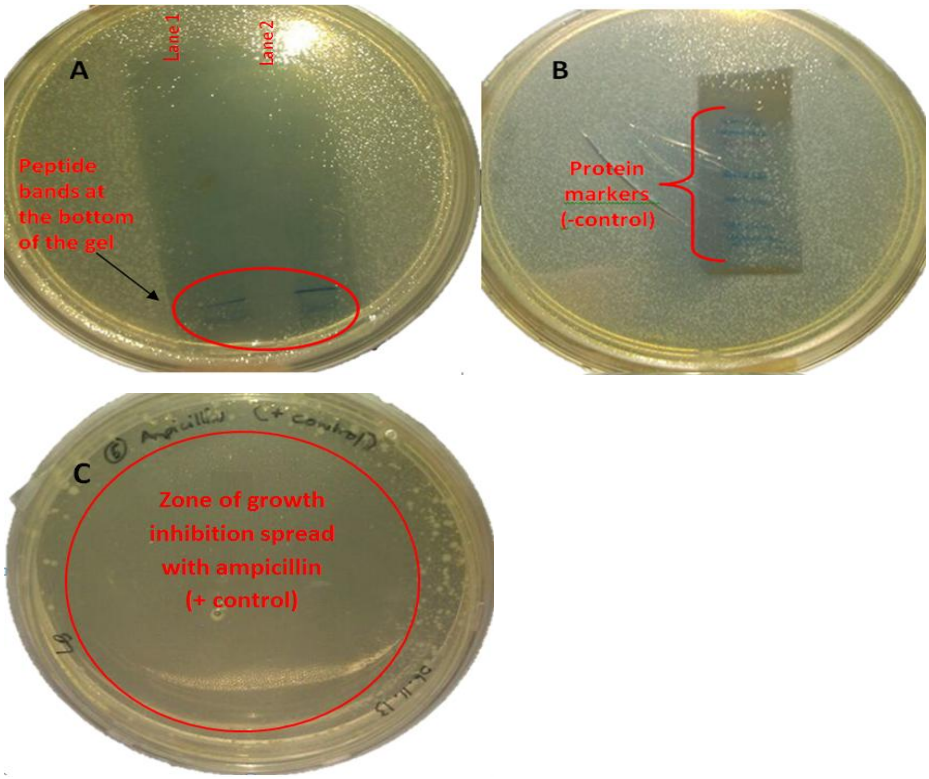


Figure 3.4: LB plates of the agarose overlay method. Plate A is the test plate with an SDS-PAGE gel run with peptides, the gel in Plate B contains protein markers as the negative control and Plate C has ampicillin as a positive control. The study was done in duplicate and repeated twice.

3.2 Analysis of differential protein expression in *E. intermedius* upon exposure to heat-killed *E. coli.*

The induced antimicrobial activity was suspected to be caused by antimicrobial peptides with molecular weights below 10 kDa. Therefore, bands and spots on SDS-PAGE and 2-D PAGE gels, respectively, were expected to have sizes that are less than 10 kDa. These techniques were also done to show the difference of induced peptides between treated and untreated cell line at various hours. In order to analyse protein expression patterns, SAEIE08 was cultured overnight, then it was treated with heat-killed *E. coli* for 1, 2 or 3 hours. Protein was subsequently extracted from the lysed treated and untreated control by Trizol extraction. Extracted protein was run on SDS-PAGE and 2-D PAGE in order to analyse differences in expressed protein patterns between SAEIE08 treated at different hours and their respective untreated controls.

Figure 3.5 A shows results obtained from running a coomasie blue stained SDS-PAGE gel, while the gel in figure 3.5 B was cypro stained by Dr. Stoyan Stoychev from the CSIR. The

cypro-stained gel revealed many proteins that were not visible in the coomasie blue stained gel. These pointed proteins were sent for identification by MS spectrometry, but unfortunately at the time of submission the results had not yet arrived. 2-D PAGE gels in figure 3.6 are presented in A, B, C and D.

Low molecular weight bands and spots implicating the presence of antimicrobial peptides were observed. The most intense bands in gel A of figure 3.5 were seen at 10 kDa and 16 kDa (marked with red circles). Furthermore, gel A and B of figure 3.6 had similar spots circled with red at the low molecular end of the gel (~10 kDa). However, gel A had two more spots around 10 kDa (white circles) that were not found in gel B, which provides evidence for differential protein expression patterns between the treated and untreated cell line.

Differential protein expression was evident in Gel A of figure 3.5 which showed that the 3 hour- and 2 hour-treated SAEIE08 had the highest band intensities which may have been due to AMP induction upon treatment of SAEIE08 with heat-killed *E. coli*. The untreated control lane showed the least intense bands overall. Gel B also indicated a difference in band intensity between the 3 hour-treated and untreated control. Furthermore, some bands were only present in the 3 hour-treated lane and absent in the untreated control lane, such bands are pointed in Gel B (red pointer), implicating that differential protein expression occurred. In addition, figure 3.6 of 2-D PAGE gels shows that A had fewer spots than its untreated control, B, but it had higher intensity than the untreated control. Gel C of figure 3.6 showed uneven migration which may have resulted from poor heat transfer caused by air bubbles under the second-dimension gel.

A second approach for analysis of protein expression patterns employed a method that did not require protein extraction. Cultured cell line was treated with heat-killed *E. coli* and then the supernatant was collected for concentration and TCA precipitation, which was eventually analysed via 2-D PAGE. 2-D PAGE results produced blank gels (or gels with smaller and lighter spots) that only showed markers clearly (See appendix 3 on page 58), indicating absence of protein which may have been due to very low protein concentration in the crude extract as a result of the sample concentration method applied.

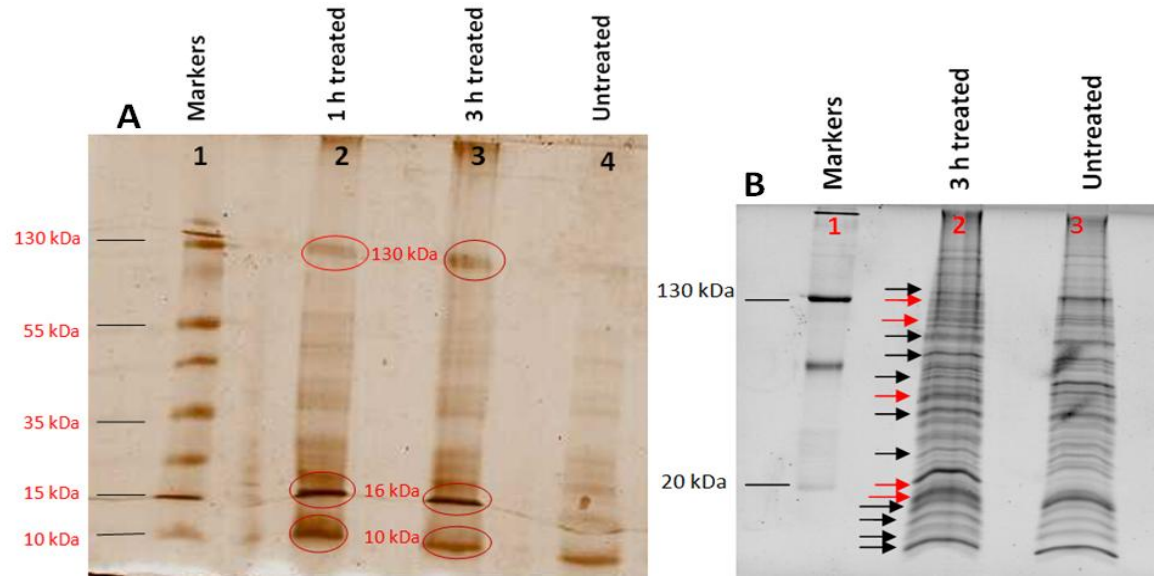


Figure 3.5: Two SDS-PAGE gels run with trizol extracted proteins from SAEIE08 that was treated or untreated with heat-killed *E. coli*. (A) Silver stained gel and (B) Cypro-stained gel (The cypro-stained gel was run by Dr. Stoychev from the CSIR). A red pointer was used for bands that were present only in the treated cell line. Both red and black pointed bands were sent for sequencing.

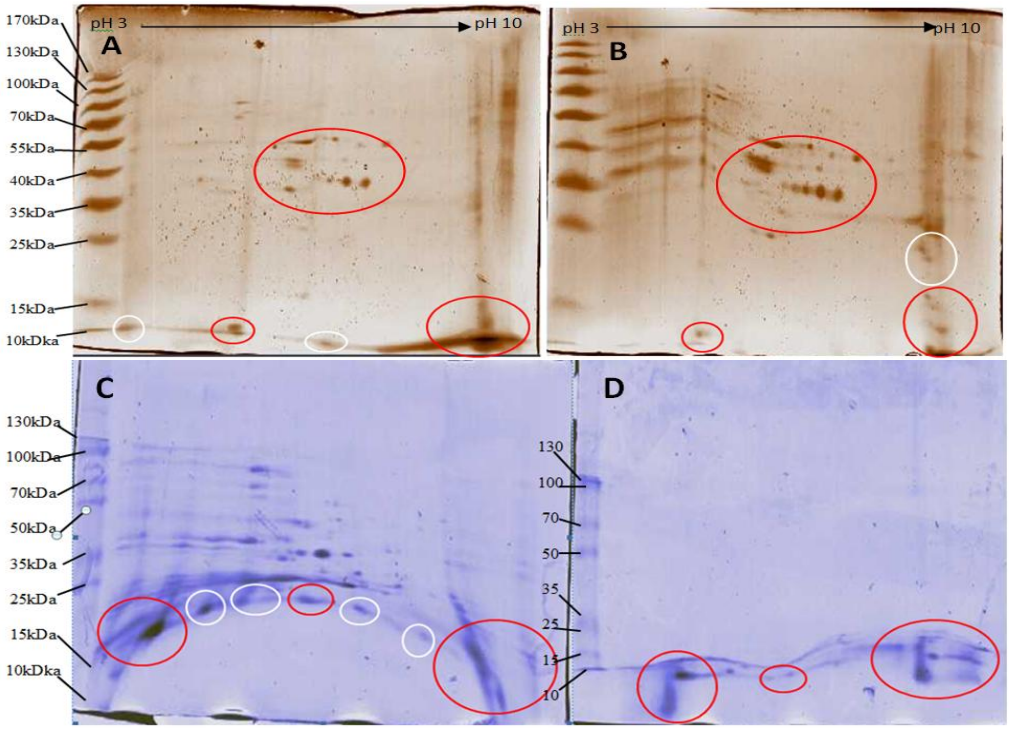


Figure 3.6: 2-D PAGE gels of trizol extracted proteins from SAEIE08 that was treated or untreated with heat-killed *E. coli*. Silver-stained gel A is the 1 hour-treated SAEIE08 and its untreated control is B. Coomasie-stained gel C is the 2 hour-treated SAEIE08 and D is the untreated control. Spots that were circled with a red color were found in both the treated SAEIE08 and its untreated control. White-circled spots were found in only one of the two gels, either the treated or untreated SAEIE08.

3.3 The isolation and identification of genes that code for antimicrobial peptides in SAEIE08.

The aim of this study was to produce genes that can encode putative antimicrobial peptides. The RNA in figure 3.7 A was extracted from SAEIE08 using the TRI Reagent kit. RNA concentration and purity were determined by Nanodrop to be 1532.5 ng/µl with purity level of 260/280 ratio at 1.95. The RNA was reverse transcribed and amplified using the defensin (def), apolipoprotein (Apolll) and hopscotch (Hop27me) reverse primers. These primers were sequenced from isolated immune-response genes which are the defensin, hopscotch and apolipoprotein by previous studies. However, only the hopscotch primer produced PCR products with sizes of 1000 bp and 300 bp as can be seen in figure 3.7 B. The PCR products were subsequently cloned into the pGEM – T easy vector. Plasmids with DNA inserts were sent to Inqaba biotechnical Industries (Pty) Ltd for sequencing by use of standard M13 forward and reverse Primers. Forward and reverse sequences of the 3′ RACE and 5′ RACE inserts were processed manually and aligned using bioinformatics to form short sequences (See appendix 59 for sequences) which were subsequently searched for matches using NBLAST and XBLAST on the NCBI database. Table 3.4 shows that sequences obtained from the RACE technique unexpectedly matched to homologues of *Saccharomyces cerevisiae* proteins that have roles in metabolism, molecular pathways and/or cell proliferation. The sequence identities are very high, varying between 91-100%, while E-values are low. The *Spodoptera frugiperda* mRNA for allatotropin (100% identity) is the only insect gene identified.

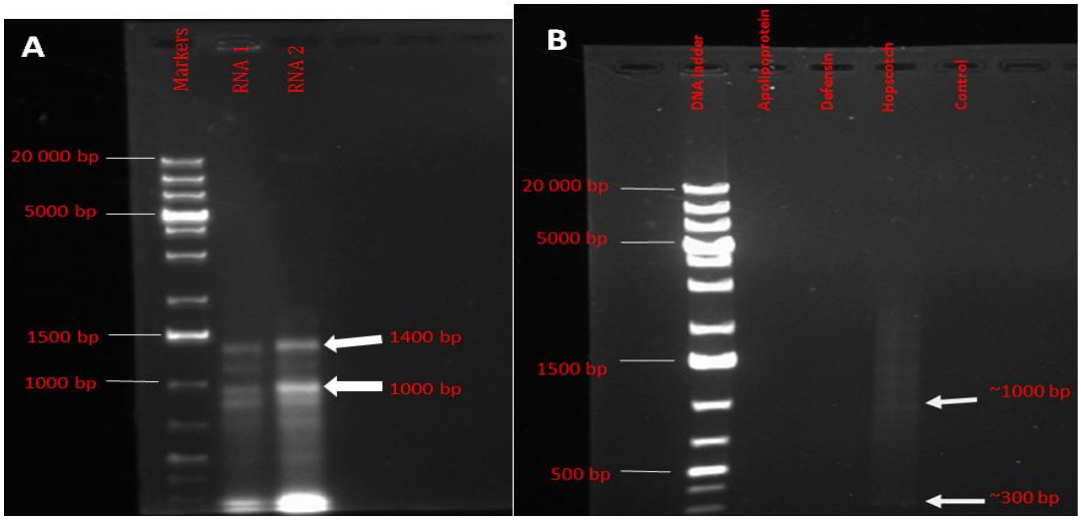


Figure 3.7: RNA extraction and 5′ RACE gels obtained using agarose gel electrophoresis**.** (**A)** Extracted RNA gel with RNA at 1000 bp and 1400 bp. (**B)** Three PCR products amplified with different primers. The hopscotch primer produced PCR products of ~1000 bp and ~300 bp.

Table 3.3: Transformation using the pGEM – T Easy vector ligation reactions

Plate	White colonies	Blue colonies
pUC 18	41	395
Standard reaction (SR)	124	288
Positive reaction (PC)	8	23
Background control (BC)	1	22
BC without ampicillin	Lawn of colonies	0

Purified PCR products were ligated to the pGEM – T Easy vector using the pGEM – T Easy vector cloning system, followed by transformation using *E. cloni* cells. Blue/white selection was performed and blue and white colonies were counted on each plate as recorded in table 3.3. The test plate had 124 white colonies (vectors and inserts ligated) and 288 blue colonies (vectors and inserts not ligated). Transformation efficiency was calculated as follows: 124 cfu/0.001 ng = 1.24 x 10^5 cfu/ng = 1.24 x 10^8 cfu/µg DNA (Transformation efficiency has to be greater than 1 x 10^8 cfu/µg DNA in order to continue to subsequent steps. Therefore, the obtained value of 1.24 x 10^8 cfu/µg DNA was sufficient).

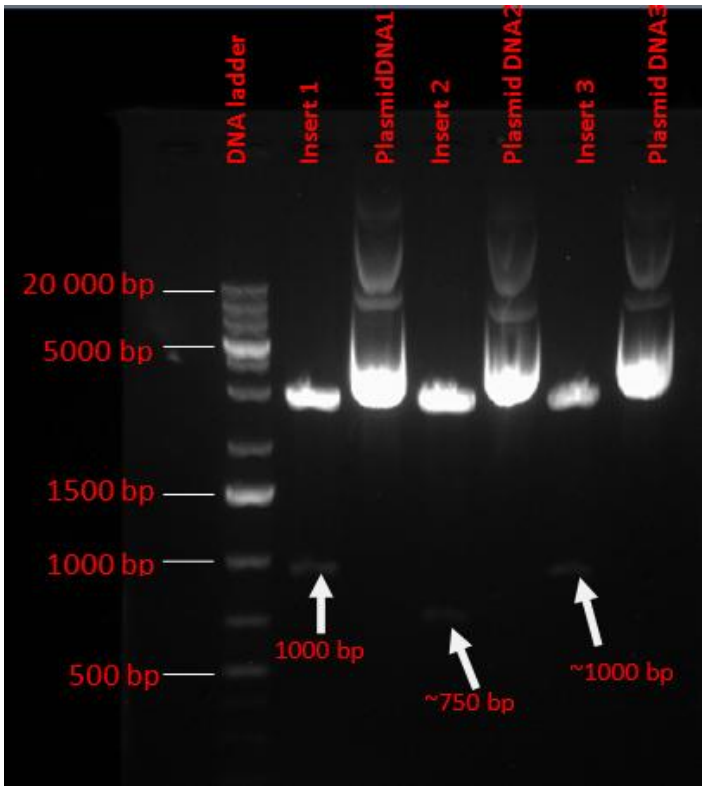


Figure 3.8: Digested Plasmid DNA for release of DNA inserts. The figure depicts three plasmid DNAs (~4000 bp) before digestion plus three DNA inserts (two 1000 bp and one 750 bp) released after digestion.

Table 3.4: Proteins from the NCBI XBLAST search of 5′/3′ RACE sequences

XBLAST	Sequence 1	Sequence 2	Sequence 3	Sequence 4	Sequence 5 (NBLAST)
Organism	*S. cerevisiae* EC1118	*S. cerevisiae* S288c	*S. cerevisiae* S288c	*S. cerevisiae* EC1118	*S. frugiperda*
Protein Identity	Flavin-containing monooxyginase (FMO)	Long chain fatty acyl-CoA synthetase	severe depolymerization of actin protein 1 (SDA1p)	Serine/threonine protein kinase MRK1p	mRNA for allatotropin
NCBI Accession number	CAY80188.1	EDN63641.1	EDZ71913.1	CAY78430.1	AJ508907.1
Identity	100%	98%	99%	91%	100%
E value	$4x10^{-136}$	$2 x10^{-73}$	$6 x10^{-87}$	$2 x10^{-33}$	$1 x 10^{-33}$
Query coverage	78%	48%	50%	37%	8%
Nucleotide seq.	1043bp	781bp	1124bp	1009bp	(13 residues)
Conserved domains	*flavin-containing monooxygenase [PLNO2172]	* Long-chain acyl-CoA synthetases [COG1022]	*SDA1 [pfam05285]; family consists of SDA1 protein homologues	*Glycogen synthase kinase [PTZ00036] *Glutamate dehydrogenase [PTZ00324]	No domains
Function	Involved in K^+ transport and metabolism	Lipid metabolism	Involved in organization of the actin cytoskeleton	Key regulator of molecular pathways	Involved in regulation of growth and reproduction

Table 3.4 shows sequences in the NCBI database that matched the query sequences obtained from cloning of genes that were amplified using the 5′/3′ RACE Technique. Many of them are *Saccharomyces cerevisiae* homologues. These proteins are flavin-containing monooxygenase, fatty acyl-CoA synthetase, serine/threonine protein kinase and severe depolymerization of actin protein. They have been reported to play roles in metabolism, cell proliferation and/or molecular pathways. The only match to an insect peptide is allatotropin from *Spodoptera frugiperda.*

Chapter 4: Discussion

In the study of SAEIE08 response upon exposure to *E. coli*, both inducible and constitutive antimicrobial activity was secreted into the supernatant by cell line. Bacterial growth inhibition, tested by *E. coli*, was highest in the 1 hour-treated and undiluted SAEIE08, and longer treatment of SAEIE08 with heat-killed *E. coli* did not induce higher antimicrobial activity. Unidentified molecules with sizes around 10 kDa and 16 kDa were seen and some were cationic while others were anionic or neutral. Some higher molecular weight proteins of around 130 kDa were up-regulated upon treatment of cell line with heat-killed *E coli*. Abnormal growth curves were observed when samples were tested by *M. luteus,* which represents Gram-positive bacteria. Of the genes identified, the closest to AMPs was insect gene for allatotropin. The protein encoded by this gene has homologues in *Spodoptera frugiperda* (armyworm). Homologues of *Saccharomyces cerevisiae* proteins were found that could indicate some contamination or could be as a result of inaccuracies from using short sequences to search for matches in the NCBI database. Interestingly, these proteins play roles in metabolism, cell proliferation and/or molecular pathways which do occur when cells are exposed to stress.

4.1 Activation of immunity by Gram-negative bacteria

4.1.1 Inducible and constitutive antimicrobial activity

The study shows the presence of inducible and constitutive antimicrobial activity in antimicrobial assays. PAGE techniques also show high molecular weight and low molecular weight proteins that are up-regulated upon treatment of SAEIE08 with heat-killed *E. coli*. Although bacterial growth was not completely inhibited in liquid inhibition assay, it decreased significantly enough to suggest the presence of antimicrobial activity in SAEIE08. The evidence of antimicrobial activity even in untreated SAEIE08 may be explained by constitutive release of antimicrobial molecules in SAEIE08 (Chalk, *et al*. 1993). As previously mentioned, the antimicrobial molecules that are constitutively released are initially stored in haemocytes, and they are only released into the hemolymph after immune challenge. Therefore, antimicrobial activity observed even in untreated cell line could be as a result of stored antimicrobial molecules.

Although growth inhibition was generally high in all hours of undiluted treatments, it was particularly highest in the 1 hour-treated and undiluted SAEIE08. This indicates that longer treatment of SAEIE08 with heat-killed *E. coli* does not necessarily induce higher antimicrobial activity.

4.1.2 Possible up-regulation of low molecular weight peptides

It is interesting to note that the more intense bands on the SDS-PAGE gel have sizes around 10 kDa and 16 kDa. Their expression was up-regulated in response to treatment with heat-killed *E. coli*, which suggests that they may have antimicrobial activity. There is a wealth of information on the small size of antimicrobial peptides as it is usually found below 10 kDa (Hoffmann, *et al*. 1996; Bulet, *et al*. 1999; Reddy, *et al*. 2004; Teixeira, *et al*. 2012; Yount, *et al*. 2006; Sovadinova, *et al*. 2011). There are also antimicrobial polypeptides generally reported to have a molecular mass below 30 kDa (Cytrinska, *et al.* 2007). While some of the low molecular weight peptides found in the current study are positively charged, others have a negative or neutral charge (2-D PAGE gels). Contrary to the cationic charge of most antimicrobial peptides, anionic peptides have been reported as previously mentioned. Identification of all the differentially charged low molecular peptides might reveal the presence of putative antimicrobial peptides. One of the weaknesses of this part of the study is that the 2D gels were conducted on lysed cells due to difficulties encountered with de-salting the supernatants. It is expected, based on the antimicrobial assays that most of the peptides would be found in the supernatant.

4.1.3 Possible up-regulation of high molecular weight proteins

Page techniques show high molecular weight proteins around 130 kDa that are up-regulated upon treatment with heat-killed *E. coli*. This may suggest the induction of antimicrobial polypeptides which have also been reported in literature (Nibbering, *et al*. 2001; Yount, *et al.* 2006). Such polypeptides and proteins considerably larger than classical AMPs have been shown to have unambiguous antimicrobial activity. Known examples include the 80 kDa lactoferrin (Yount, *et al.* 2006). However, only a few inducible antibacterial molecules are polypeptides larger than 10 kDa (Bowman, 1994). Therefore, an alternative explanation for

the up-regulation of larger proteins upon treatment is that even though the proteins may not have antimicrobial activity, they could be involved in immune response. Examples are peptidoglycan recognition proteins (PGLYRPs) that are variably sized (~20-120 kDa), first characterised in insects. These proteins function as pattern recognition receptors that initiate downstream immunomodulatory signalling cascades through receptor-linked IMD or Toll pathways (Yount, *et al.* 2006). Identification of proteins in the current study might give further insight of the pathways activated by performing bacterial challenge. Based on information obtained from previous studies, the likely pathway to be activated by Gram-negative bacteria in SAEIE08 is the Toll signalling pathway. This pathway was implicated in a study by Hull, (2012), of the *E. intermedius* immune response upon exposure to fungi. Furthermore, the Toll signaling pathway was also shown in studies of coleopteran immunity. As previously mentioned, larger beetles such as *Tenebrio molitor* and *Holotrichia diomphalia* contain PAMPs that form complexes with Gram-negative binding protein 3 (GNBP3) in fungi and peptidoglycan recognition protein in Gram-negative bacteria, and thereby activate the Toll signalling pathway.

4.2 SAEIE08 response to Gram-positive bacteria

The growth curve of liquid growth inhibition assay performed for *M. luteus* shows optical densities that are generally lower than those obtained for *E. coli,* although the assays were the same. The highest optical density obtained for *M. luteus* does not read above 0.1 at OD_{650}, whereas that of *E. coli* grew well up to 0.5 and above at OD_{650}. However, this does not imply that the SAEIE08 has a high growth inhibitory effect on *M. luteus* compared to *E. coli* because inconsistencies in *M. luteus* growth rates can be observed across the different dilutions and in the negative control that was without the SAEIE08. Such inconsistent growth rates were observed in replicates of the experiment. In addition, it was difficult to grow *M. luteus* on LB plates for solid inhibition assays. Therefore, it was concluded that this particular strain of *M. luteus* may have been contaminated. Hence, for subsequent experimentation, it could no longer be used.

4.3 Proteins identified

4.3.1 Likely discovery of the gene of interest that codes for allatotropin neuropeptide in SAEIE08.

The only likely match to an insect gene of interest was the mRNA for allatotropin (sequence 5 in table 3.4) from the fall armyworm *Spodoptera frugiperda,* which had 100% sequence identity but only 8% query coverage. This sequence was found in most 5′/3′ RACE aligned forward and reverse DNA inserts. Allatotropin (AT) is a 13-residue amidated neuropeptide that has been characterized in various insect species, and members of the family are present in several invertebrate phyla. Neuropeptides are a diverse class of regulatory compounds that are released from neurons and they behave as neuromodulators, neurotransmitters or neurohormones. Many invertebrates such as insects have the same number and diversity of neuropeptides. Neuropeptides perform many roles in different tissues (Elekonich & Horodyski, 2003). Allatotropins (AT) and allatostatins (AS) are among the most well-known families of insect neuropeptides. The expression pattern of genes for AT and its antagonist, AS, are precisely regulated and often regulated under specific physiological conditions (Abdel-latief, *et al.* 2004). Abdel-latief, *et al.* (2004), studied the expression of AT and AS genes, respectively, during larval, pupal and adult development of *S. frugiperda*. AT and AS regulate the biosynthesis and release of the Juvenile hormone (JH) that control growth and reproduction. The corpora allata is a small endocrine gland positioned in the head and innervated by nerves in the brain. It is responsible for synthesis and release of JH (Veenstra & Costes, 1999). CA is present in all insect stages, and depending upon species and development stages, the signals for JH synthesis and release may be either stimulatory (AT) or inhibitory (AS). JH is critical for regulation of larval development, metamorphosis and reproduction in insects (Abdel-latief, *et al.* 2004). The following are some of the functions of AT (or JH under regulation by AT and AS): AT controls the release of digestive enzymes and gut myoactivity in many insects; it is cardioacceleratory in *M. sexta*; involved in the control of ion transport in epithelial cells of the digestive system; controls the release of digestive enzymes in the midgut of *S. frugiperda*; induces peristaltic contractions in the hindgut of *T. Infestans* (Santini & Ronderos, 2009); involved in the regulation of vitellogenesis in females and spermatogenesis in males (Gade, *et al*. 1997); participates in the modulation of the circadian cycle; plays a role in the regulation of nutrient absorption; has an effect on the activity of soluble alkaline phosphatases and is associated with ionic balance regulation

(Santini & Ronderos, 2009). In 1999, an allatotropin-immunoreactive peptide was discovered by Veenstra and Costes in the abdominal ganglia of the mosquito *Aedes aegypti*. It has also been reported that the secretion of neuropeptides can occur simultaneously with the secretion of AMPs. Studies by Phoenix, *et al.* (2013), have shown that a spectrum of AMPs was included in skin secretions of anurans that range between 10 and 20 members along with other bioactive peptides that include neuropeptides.

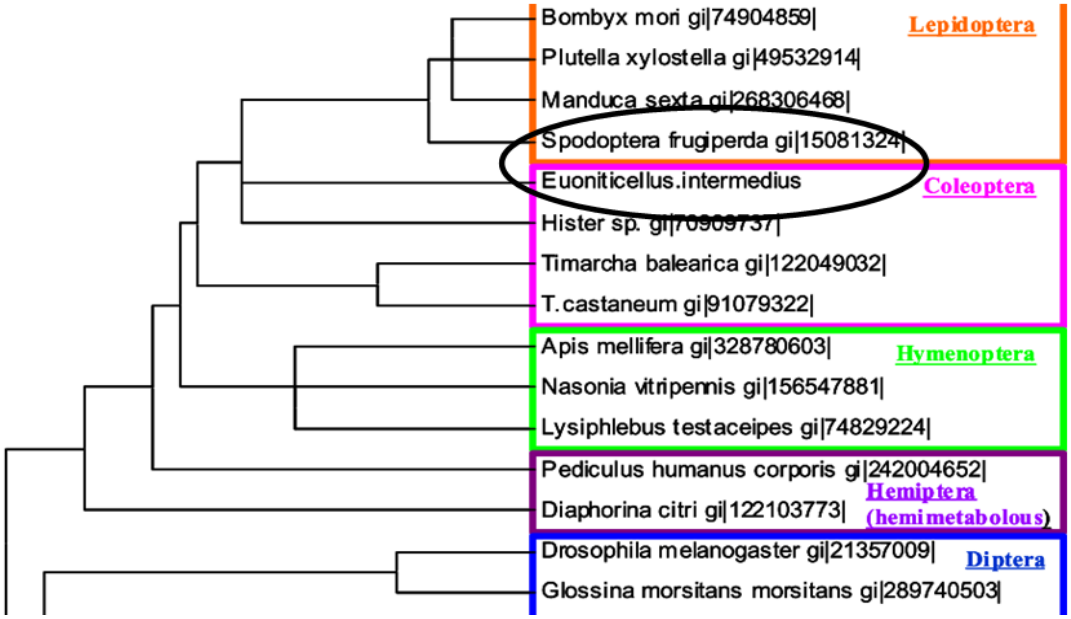


Figure 4.1: Phylogenic tree of closely related insect orders and phyla that was constructed by use of the neighbor joining method from the alignment of ribosomal protein L18a sequences using the MUSCLE algorithm (Edgar, 2004).

The tree in figure 4.1 shows the close relation between *Euoniticellus intermedius* and *Spodoptera frugiperda* which correlates to results in this study. The current study suggests that the *Spodoptera frugiperda* gene that encodes allatotropin has a homologue in embryonic *Euoniticellus intermedius* cell line.

The likely discovery of allatotropin in this study, although unintended, is significant when taking into consideration the fact that Coleoptera is one of the insect orders in which there is little knowledge about the regulator of JH synthesis and associated peptides (Abdel-latief & Hoffmann, 2010). Abdel-latief & Hoffmann, (2010) stated that evidence is lacking on neuropeptide regulation of JH biosynthesis in beetles. Furthermore, little is known about *E. intermedius* sequences as the whole genome is yet to be sequenced. Therefore, this finding

contributes information to important sequences that are present in *E. intermedius* and it could potentially aid the study of identification and characterization of neuropeptides in *E. intermedius*.

Flavin-containing monooxygenase

Sequence 1 reported in table 3.4 that has 100% identity and query coverage of 78% is a homologue of the flavin-containing monooxygenase from *Saccharomyces cerevisiae*. The flavin-containing monooxygenase (FMO) enzymes metabolize xenobiotics. Such enzymes have been historically called drug-metabolizing enzymes, although they are involved in the metabolism of many foreign chemicals that are potentially harmful to humans and animals. FMOs have been implicated in the metabolism of a number of pesticides, pharmaceuticals and toxicants (Ziegler, 1980). The FMO protein family consists of a group of enzymes that catalyze chemical reactions which involve oxidation of heteroatoms, particularly nucleophilic atoms such as the nitrogen of amines, through the bound cofactor flavin. Using an NADPH cofactor and FAD prosthetic group, these microsomal proteins catalyze the oxygenation of nucleophilic nitrogen, sulphur, phosphorus and selenium atoms in a range of structurally diverse compounds. In humans, lack of hepatic FMO-catalyzed trimethylamine metabolism results in trimethylaminuria (fish odour syndrome) (Dolphin, *et al.* 1992; Itagaki, *et al.* 1996).

Fatty acyl-CoA synthetase

Sequence 2 in table 3.4 represents the long chain fatty acyl-CoA synthetase (FACS), an *S. cerevisiae* homologue, which is required for phospholipid remodeling, fatty acid degradation and the production of fatty acyl-CoA that regulates various physiological processes. The FACS family consists of at least 25 members that are broadly classified by their substrate specificities for fatty acids of different chain lengths. Many questions remain with regards to the cellular localization and specific biological functions of the different forms of the FACS enzymes (Mashek, *et al.* 2007). A fatty acid from an endogenous or exogenous source is first activated to form an acyl-CoA before it can enter any metabolic pathway (except eicosanoid metabolism). FACS plays a significant role in intermediary metabolism through a reaction that forms long acyl-CoA esters. The formation of fatty acyl-CoA is catalyzed by FACS in a two-step reaction:

$$FA+ATP \rightarrow acyl-AMP+PPi$$
$$Acyl-AMP+CoA \rightarrow acyl-CoA+AMP$$

Acyl-CoAs are bioactive fatty acid metabolites that are involved in enzyme activation, protein transport, protein acylation, transcriptional control and cell signalling (Weimar, *et al.* 2002; Qiao and Tuohimaa, 2004; Soupene and Kuypers, 2008). According to a review article by Mashek, *et al.* (2007), long-chain acyl-CoAs can alter multiple cellular processes including signal transduction via calcium ion release and PKC isoforms. Fatty acids and acyl-CoAs regulate numerous transcription factors including the NF-κβ. As previously mentioned, the NF-κβ-like transcription factor known as relish, controls the transcription of immune-related genes in *Drosophila melanogaster.*

SDA1p

The SDA1 gene encodes a highly conserved protein also known as SDA1 which is localized in the nucleus and is important for cell viability (Buscemi, *et al*. 2000). It has been shown to be highly conserved from yeast to humans. The Protein SDA1 is required for correct organization of the actin cytoskeleton as reported by Buscemi, *et al*. (2000). The same study also indicated that loss of SDA1 function caused a cell cycle arrest in G_1. It is interesting to note that the study by Hull, *et al.* (2013) that aimed to assess the *E. intermedius* signalling pathways, did isolate, among other proteins, gelsolin, which is an actin-binding protein, making it a structural component of the cytoskeleton. Gelsolin is found in invertebrates such as *Drosophila* in two splice forms, cytoplasmic and secreted. Interestingly, gelsolin, similar to SDA1, is involved in cytoskeletal organization (Stella, *et al.* 1994). Gelsolin is also involved in biogenesis, amyloid formation in vertebrates and hemolymph clotting in insects (Karlsson, *et al.* 2004).

Successful cell proliferation requires coordination of cell cycle events. Serine/threonine protein kinases known as cyclin-dependent kinases that were reviewed by Nasmyth, (1993), are known to control the correct order of these events. The activity of these kinases oscillates during the cell cycle, eventually triggering cytoplasmic and nuclear events. Cell cycle progression also has to have numerous morphogenetic events related to actin organization which is controlled by SDA1. Actin is a very abundant protein in all eukaryotes. The actin cytoskeleton plays a significant role in numerous cellular processes such as cell motility,

secretion, polarized cell growth and endocytosis, and including its involvement in cell preparation for cytokinesis (Buscemi, *et al*. 2000).

Serine/threonine protein kinase

Serine/threonine protein kinase, MRK1p, is a protein reported in table 3.4 as sequence 4. Protein kinases are known for their ability to catalyze the transfer of a phosphate group from a nucleoside triphosphate to the protein substrate's amino acid residue (Jacob, *et al.* 2011). Protein kinases are key regulators of molecular pathways, and it is estimated that approximately 50% of all cellular proteins are phosphorylated in vivo (Fasolo, *et al.* 2011). Protein phosphorylation is carried out by coupled phosphatases and protein kinases. It is an important mechanism that is used for the translation of extracellular signals into cellular responses (Jihen, *et al.* 2011). The phosphorylation generally results in a functional change of the target protein by interfering with its cellular location, enzymatic activity and/or association with other proteins (Jacob, *et al.* 2011).

Serine/threonine protein kinases (STPK) have been known to play significant regulatory roles in eukaryotes. One of these roles has been described in the Toll signalling pathway in *Drosophila*. As previously described in the Toll signalling pathway; following initial steps in the pathway, the intracytoplasmic domain interacts with three proteins which contain the serine-threonine kinase domain. This triggers a signal transduction pathway that results in the activation of numerous antimicrobial genes (Bulet & Stocklin, 2005; Hancock, *et al.* 2006).

Cell cycle events are controlled by a family of serine/threonine protein kinases. In addition, cell cycle progression is connected to morphogenetic events related to actin organization which is controlled by SDA1. SDA1 is among identified proteins in the current study. Therefore, these findings are suggestive of a connection between proteins identified in the current study.

4.4 Conclusion

One of the major aims of the current study was to analyze immune response of SAEIE08 upon exposure to pathogens, specifically *Escherichia coli*. Antimicrobial activity upon exposure to pathogen was monitored by performing solid and liquid bacterial inhibition assays. In liquid growth inhibition assays it was found that treatment with heat-killed *E. coli* induces an immune response in SAEIE08, and that increasing hours of treatment is not proportional to induced immune response. Evidence of an immune response was further supported by the solid growth inhibition assay, in which clear zone inhibition was observed on the LB plate containing a gel run with extracted protein with only a few colonies on the outer edges of the gel. Unidentified molecules with sizes of 10 kDa and 16 kDa were observed in SDS-PAGE and 2-D PAGE. Some of the molecules were cationic while others were anionic or neutral.

Comparison of optical density readings and growth inhibition percentages in this study with findings from other studies is rather difficult, since different assay conditions such as liquid growth assay vs. plate and different bacterial strains have been used. It is also important to take into account that in this study the crude extract (SAEIE08 supernatant) was used for growth inhibition assays instead of purified AMPs, therefore, the two are not directly comparable because concentrations and identities of peptides in the crude extract are not known. Moreover, the crude extract contains additional proteins that may influence antimicrobial activity.

Homologues of *Saccharomyces cerevisiae* proteins that were found in some of the 5′/3′ RACE sequences are proteins that play roles in metabolism, cell proliferation and/or molecular pathways. These proteins are flavin-containing monooxygenase, fatty acyl-CoA synthetase, serine/threonine protein kinase and severe depolymerization of actin protein. It remains a challenge to explain how *S. cerevisiae* protein homologues were found in the *E. intermedius* cell line. Possible explanation could be that short sequences (See appendix 4 on page 59) were used for XBLAST searches on the NCBI database. This could have resulted in inaccurate matches.

In conclusion, this work has shown inducible and constitutive antimicrobial activity in embryonic *E. intermedius* cell line upon exposure to *E. coli.*

4.5 Future studies

This study forms the basis for further work on characterization and chemical structural analysis of antimicrobial peptides in SAEIE08. The following methods and techniques may be employed to achieve the aim to isolate and identify AMPs:

-Mass spectrometric results are expected from the CSIR for identification of high and low molecular weight proteins in the current study. Furthermore, the same techniques would evaluate the apparent differential protein expression patterns on the treated and untreated cell line.

-Analytical techniques such as HPLC would be valuable for further analysis of AMPs in the SAEIE08.

-Using more primers from genes that encode antimicrobial peptides and immune-related proteins for 5′/3′ RACE and cloning techniques in order to sequence inserts that can match existing insect genes that code for AMPs.

-After isolating and identifying AMPs it may be possible to determine the minimum inhibition concentration (MIC) for each AMP.

-It is important to work with a variety of pathogens such as Gram-positive and –negative bacteria and fungi in order to study the immune response of the SAEIE08 when exposed to various pathogens.

Chapter 5: Appendices

5.1 Appendix 1

5.1.1 Materials

Table 5.1: Media

Media	Composition/ Supplier
Grace's Insect media	Highveld Biological (catalogue # L16)
Sloppy agar	1% Sodium chloride, 1% Tryptone, 0.5% Yeast extract and 0.75% agar
Solid agar	1 % Sodium chloride, 1 % Tryptone, 0.5 % Yeast extract and 1.5 % agar
Tryptic soy broth	Biolab (Catalogue # HGOOOC16.500)

Table 5.2: Biological buffers and solutions

Buffer/solution	Composition/Supplier
Insect saline	130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$
Sodium phosphate buffer	Na_2HPO_4/ NaH_2PO_4, pH 6.0
Buffer A	10 mM phosphate buffer, pH 6.0 + 130 mM NaCl
Buffer B	Buffer A + 0.2% Bovine serum albumin
Tri-reagent	Sigma-Aldrich (Catalogue # T9424)
Chloroform	Sigma Aldrich (Catalogue # 2432)
2-propanol	Fluka Chemika (Catalogue # 59309)
75% Ethanol	BDH prolabo (Catalogue #20820.327)
50X TAE electrophoresis buffer pH 8	24.2 w/v % Trisbase 5.71 v/vv % glacial acetic acid 3.72 w/v % Na2EDTA.2H2O
Buffer I	50 mM glucose 10 mM EDTA

	0.25 M TrisHCl pH 8
Buffer II	1 % SDS 0.2 N NaoH
Buffer III	5 M Potassium acetate
Ethanol : Acetate	25 : 1
Sodium Dodecyl Sulphate	BDH Prolabo (Catalogue # 27926.238)
TCA	Merck (Catalogue # 1026736)
Acetone	ACE (PTY)LTD (Catalogue # 19883/6680)
Rehydration buffer	8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-lyte 3/10 ampholytes
Biorad protein Assay	Bio-Rad (Catalogue # 105463)
Equilibration buffer I	6 M urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% Glycerol, 2% DTT
Equilibration buffer II	6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% Glycerol
Readystrip IPG strips, pH 4-7	Bio-Rad (Catalogue # 163-2000)
Electrode wicks, precut	Bio-Rad (Catalogue # 1654071)
Mineral oil	Bio-Rad (Catalogue # 163-2105)
5X SDS running buffer	25 mM Tris, 192 mM Glycine, 1% SDS
Overlay agarose	0.1% Agarose, 25 mM Tris, 192 mM Glycine, 0.1% SDS, trace of bromophenol blue
Running gel	30%Acrylamide/bis-Acrylamide, tetramethylethylene (TEMED), 1.5 M Tris pH 8.8, 1% SDS, 10%APS
Coomasie brilliant blue	0.1%Coomasie, 10% Glacial acetic acid, 40% Methanol
Coomasie destaining solution	40% Methanol, 10% Glacial acetic acid, 50% distilled water
Silver nitrate solution Developer Destain	0.1% $Ag(NO_3)_2$ 0.1% Formaldehyde 2% Sodium carbonate 10% Nitric acid

Table 5.3: Living organisms

South African *Euoniticellus intermedius* embryonic cell line (SAEIE08)	Developed by the Flylab from the School of Molecular and Cell Biology at the University of the Witwatersrand
Escherichia coli (strain 1106)	Prof. Jean-Marc Reichhart of the Institut de Biologie Moléculaire et Cellululaire, Strasbourg, France
Micrococcus luteus (gus strain)	Prof. Jean-Marc Reichhart of the Institut de Biologie Moléculaire et Cellululaire, Strasbourg, France

Table 5.4: Primers

Primer name	Primer sequence 5′→3′
Apolllrev reverse primer	GAATTCTCATTTGTGGGTTTTATC
Hop27me reverse primer	GTTCGGGTTCTTCACCCCTTTCGTACC
Defensin reverse primer	TATGACGTAATCTTTGCCGC
M13 Forward primer	GTTTTCCCAGTCACGAC
M13 Reverse primer	CAGGAAACAGCTATGAC
3′ RACE forward specific primer	TGGGCTTGCCCCCA

Table 5.5: Kits and suppliers

Kits	Supplier and catalogue number
Trizol kit	Sigma-Aldrich (Catalogue # T9424)
5′/3′ RACE kit, 2nd Generation	Roche (Catalogue # 03353621001)
ReadyPrep 2D-starter kit	Bio-Rad (Catalogue # 163-2105)
High Pure PCR product Purification Kit	Roche (Catalogue # 11732676001)
pGEM – T Easy vector system	Promega (Catalogue # TM042)
QIAprep Spin Miniprep kit.	Qiagen (Catalogue # 27106)

Table 5.6: Equipment and machines

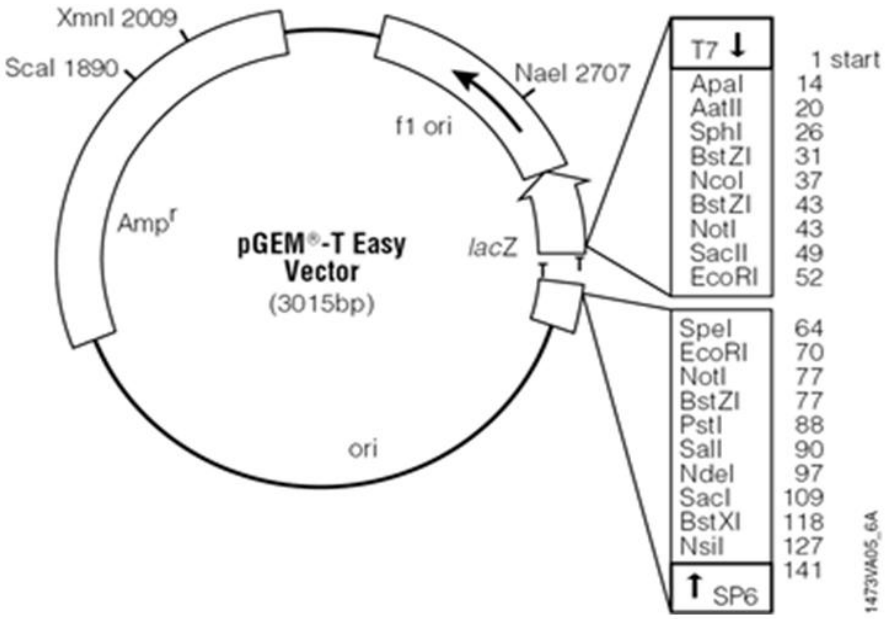
Equipment/machines	Supplier
GS800 Calibrated Densitometer	Bio-Rad
UV/Visible Recording Spectrophotometer	Shimadzu
Forced Circulation Incubator Type FSIE	Labcon
MJ Mini personal Thermal Cycler	Bio-Rad
GeneAmp PCR system 2400	Perkin Elmer
PROTEAN IEF cell	Bio-Rad
Biofuge Pico	Heraeus Instruments
G10 Gyrotory Shaker	New Brunswick Scientific

Table 5.7: Software and sequence databases

Software/databases	Website/Supplier
Quantity one version 4.5.2	Bio-Rad (P/N 4000126-16)
PDQuest 2-D Analysis Software Version 6.2	Bio-Rad (Catalogue #170-9630)
BLAST	http://www.ncbi.nlm.nih.gov/BLAST
NCBI	www.ncbi.nlm.nih.gov

5.2 Appendix 2

5.2.1 pGEM -T Easy Vector



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10-128
SP6 RNA polymerase promoter (-17 to +3)	139-158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176-197
lacZ start codon	180
lac operator	200-216
β-lactamase coding region	1337-2197
phage f1 region	2380-2835
lac operon sequences	2836-2996, 166-395
pUC/M13 Forward Sequencing Primer binding site	2949-2972
T7 RNA polymerase promoter (-17 to +3)	2999-3

Figure 5.1: The pGEM – T Easy vector in which PCR products from the 5′ cDNA and 3′ cDNA strands were ligated.

5.3 Appendix 3

5.3.1 2-D PAGE gel from TCA precipitated SAEIE08 supernatant.

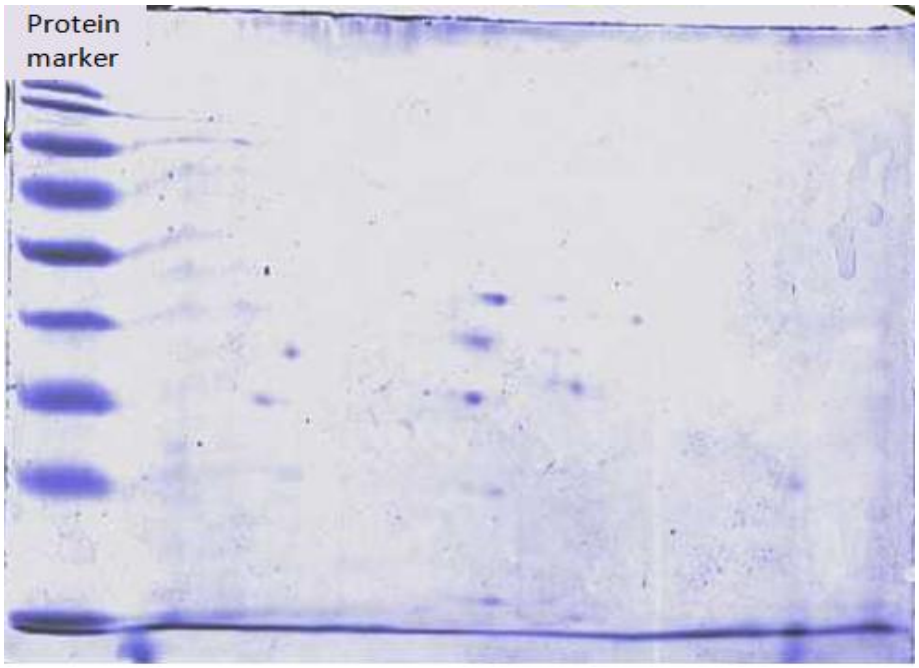


Figure 5.2: 2-D PAGE gel obtained from SAEIE08 supernatant that was concentrated and TCA precipitated.

5.4 Appendix 4

5.4.1 Short sequences obtained from the 5′/3′ RACE technique

TGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATG
TGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGT
GAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCC
GCGGGAATTCGATTGACCACGCGTATCGATGTCGACGGTGAGTGGGTCACCATGGATAGTGATAAAGAAT
ATGACGTTGATATGGAAGATAGTGACGATGAAAAAGACAATGCTAAGGGCAAGGAATCAGATTCCGATTT
GGAACTAAGCGATGATGACGATGAAAAAGAGGTGAAGGATGAGCAAGAAGATACAGATATAGATCCCGAA
GCTGCCTTCCGTGAAATTGCTTCTACACGTATTTTAACTCCGGCAGATTTCGCTAAACTACAAGAATTGC
GTAATGAGGAAAGCGTTGCTAAAATAATGGGTATCCACAAACAAGACAAGCGTGAAGAGTTAGTTGATGC
AAGTACACTAACAGGCCCTATCAAATACAAACAGTCTCGTGAGGAAAGATTACAAAAGGTGTTGGAAGGT
CGTGAAGGTAGAGACAAATTTGGTAGTAGACGCGGTAAGCGTGACAACATGCGTTCTACTACCAACAGAG
AAAAAGAAAGAAGGAAGAACTTTGTTATGTCTATTCACAAGAGATCTGTTAGAGGTAAACAAAAAATGTC
ACTACGAGACAAGCAAAAAGTGTTACGTGCACACATCACTAAAAAAAAAAAAAAAGTCGACATCGATACGC
GTGGTCAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGA
TGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGT
GTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGT
GCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGT
CGTG

Figure 5.3: 5′/3′ RACE sequence that matched to SDA1p.

CGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGACACGAMRWATCGATGTCGACTTTTTTT
TTTTTTTTTGCGAGAAATWACAAGAAGTAACTAGGCATGACAGTGAATGACAAAAAAAGATTGGCAATCA
TAGGTGGGGGGCCAGGAGGGTTGGCAGCTGCAAGAGTTTTCTCGCAAAGTCTTCCAAATTTTGAAATARA
AATATTTGTAAAGGATTACGATATTGGCGGTGTCTGGGCATTATCCAGAACAAAAAAGTGATGGGAGGGT
CATGTATGACCATTTGGAAACCAATATATCAAAGAAGTTAATGCAGTTTAGCGGCTTTCCCTTCGAGGAA
AACGTCCCTTTATATCCGTCTCGAAGAAACATTTGGGAATACTTGAAAGCCTATTACAAAACGTTCATTG
CCAATAAAGATGCTGTCAGCATCCATTTCAGCACCGAAGTTACCTACTTAAAGAAGAAGAACTTTCAATG
GGAGATAACATCTAAAGACGAACTAAGGACTACTAAATCGGATTTTGACTTCGTTATTGTTGCGTCTGGT
CACTACAGTGTCCCTAAACTACCAACTAATATCGCAGGTCTCGATCTATGGTTCGACAATAAGGGCGCCT
TTCATTCTAAGGATTTCAAAAACTGCGAGTTTGCCCGTGAAAAAGTCGTTATTGTGGTGGGAAACGGCAG
TTCCGGCCAAGATATTGCCAACCAACTAACTACCCGTTGCAAAGAAAGTATATAATAGTATAAAAGAGCC
TGCGAGCAATCAACTGAAGGCCAAGTTAATCGAGACTGTCCAAACAATAGACAGTGCCGATTGGAAAAAT
CGTTCAGTTACTTTATCTGATGGAAGAGTACTTCAGAATATAGACTACATTATTTTCGCCACAGGCTATT
ATTACAGCTTTCCATTTATAAAAAAAAAAAAAAAAGTCGAMAWCGATACGCGTGGTCAATCACTAGTGAA
TTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGA

Figure 5.4: 5′/3′ RACE sequence that matched to flavin-containing monooxygenase

CGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGACYACGCGTATCGATGTCGACTTTTTTT
TTTTTTTTTCCCGTTTACAKATCTAACGAATATGTTGCTAACATTTGTGTTTATGCCGACCAATCTAARA
CTAAGCCAGTTGGTATTATTGTACCAAATCATGCTCCATTAACGAAGCTTGCTAAAAAGTTGGGAATTAT
GGAACAAAAAGACAGTTCAATTAATATCGAAAATTATTTGGAGGATGCAAAATTGATTAAAGCTGTTTAT
TCTGATYTTTTGAAGACGGGTAAAGACCAAGGTTTGGTTGGCATTGAATTACTAGCAGGCATAGTGTTCT
TTGACGGCGAATGGACTCCACAAAACGGTTTTGTTACGTCCGCTCAGAAATTGAAAAGAAAAGACATTTT
GAATGCTGTCAAAGATAAAGTTGACGCCGTTTATAGTTCGTCTTAATGGATCAACATTTCCATGATAGGA
AAGCCTCATCATACTAAAGCACTTTTTCAGTTTTTTGCTTTAGAACTGCTACCAATTATAAAATAGAATG
CACAGGTTATCTTGTTTGGTTTGTTATTATCCTTTTTTCTTCTTTACCAGTCATATCACCTCATTCACGT
ATGTTCGTAATTTATAATAACATGTTAAAAATGGCACAAAAAAAAAAAAAAAAAGTCGACATCGATACGCG
TGGTCAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGAT
GCATAGCTTGA

Figure 5.5: 5′/3′ RACE sequence that matched to fatty acyl-CoA synthetase

Chapter 6: References

Abdel-latief, M., Meyering-vos, M. and Hoffmann, K. H. (2004). Expression and localization of the *Spodoptera frugiperda* allatotropin (Spofr-AT) and allatostatin (Spofr-AS) genes. *Archives of Insect Biochemistry and Physiology,* 55: 188-199.

Abdel-latief, M. and Hoffmann, K. H. (2010). Neuropeptide regulators of juvenile hormone biosynthesis (in vitro) in the beetle, *Tenebrio molitor* (Coleoptera, tenebrionidae). *Archives of Insect Biochemistry and Physiology,* 74(3): 135-146.

Adade, C. M., Oliveira, I. R. S., Pais, J. A. R. and Souto-Padron, T. (2013). Mellitin peptide kills *Trypanosoma cruzi* parasites by inducing different cell death pathways. *Toxicon,* 69: 227-239.

Andrés, E. and Dimarcq, J. L. (2005). Clinical development of antimicrobial peptides. *International Journal of Antimicrobial Agents,* 25(5): 448-449.

Belvin, M. P. and Anderson, K. V. (1996). A conserved signalling pathway: The *Drosophila* Toll-Dorsal pathway. *Annu. Rev. Cell Dev. Biol.* 12: 393-416.

Bi, E., Maddox, P., Lew, D. J., Salmon, E. D., McMillan, J. N., Yeh, E. and Pringle, J. R. (1998). Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *The Journal of Cell Biology,* 142(5): 1301-1312.

Blondelle, S. E., Lohner, K. and Aguilar, M. (1999). Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: Determination and biological specificity. *Biochim. Biophys. Acta,* 1462: 89-108.

Bowman, H. G. (1994). Cecropins: Antibacterial peptides from insects and pigs, in *Phylogenic perspectives in immunity: the insect host defense* (Hoffmann, J. A., Janeway, C. A. and Natori, S., eds) pp. 3-17, R. G. Landes Company. Austin.

Bowdish, D. M. E., Davidson, D. J. and Hancock, R. E. W. (2005). A re-evaluation of the role of host defense peptides in mammalian immunity. *Current Protein and Peptide Science*, 6: 35- 51.

Boyer, R. (2006). *Molecular biology 1: Structures and analysis of nucleic acids,* In: Biochemistry laboratory: Modern theory and techniques. San Francisco: Benjamin Cummings, 300-301.

Breukink, E. and de Kruijff, B. (1999). The lantibiotic nisin, a special case or not? *Biochim. Biophys. Acta.*, 1462: 223-234.

Brivio, M. F., Moro, M. and Mastore, M. (2006). Down-regulation of antimicrobial peptide synthesis in an insect model induced by the body-surface of an entomoparasite (*Steinernema feltiae*). *Developmental and Comparative Immunology*, 30: 627-638.

Brogden, K. A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Microbiology*, 3: 239-249.

Bulet, P. and Stocklin, R. (2005). Insect antimicrobial peptides: Structures, properties and gene regulation. *Protein and Peptide Letters*, 12: 3-11.

Bulet, P., Hetru, C., Dimarcq, J. and Hoffmann, C. (1999). Antimicrobial peptides in insects: structure and function. *Developmental and Comparative Immunology*, 23: 329-344.

Buscemi, G., Saracino, F., Masnada, D. and Carbone M. L. A. (2000). The *Saccharomyces cerevisiae* SDA1 gene is required for actin cytoskeleton organization and cell cycle progression. *Journal of Cell Science*, 113: 1199-1211.

Casanova-Torres, A. M. and Goodrich-Blair, H. (2013). Immune signalling and antimicrobial peptide expression in *Lepidoptera. Insects*, 4: 320-338.

Chalk, R., Townson H., Natori, S., Desmond, H. and Ham, P. J. (1993). Purification of an insect defensin from the mosquito, *Aedes aegypti***.** *Insect Biochem. Molec. Biol.* 24 (4): 403-410.

Choi, Y. S., Choo, Y. M., Lee, K. S., Yoon, H. J., Kim, I., Je, Y. H., Sohn, H. D. and Jin, B. R. (2008). Cloning and expression profiling of four antibacterial peptide genes from the bumblebee *Bombus ignites*. *Comparative Biochemistry and Physiology*, Part B 150: 141-146.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocynate-phenol-chloroform extraction. *Analytical Biochemistry,* 162: 156-159.

Cid, V. J., Duran, A., del Ray, F., Snyder, M. P., Nombela, C. and Sanchez, M. (1995). Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbiological Reviews,* 59(3): 345-386.

Cocciancich, S., Bulet, P., Hetru, C. and Hoffmann, J. A. (1994). The inducible antibacterial peptides of insects. *Parasitology Today*, 10 (4): 132-139.

Conlon, J. (2011). The contribution of skin antimicrobial peptides to the system of innate immunity in Anurans. *Cell and Tissue Research,* 343: 201-212.

Costa, F., Carvalho, I. F., Montelaro, R. C., Gomes, P. and Martins, M. C. L. (2011). Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces. *Acta Biomaterialia,* 7: 1431-1440.

Cytrynska, M., Mak, P., Zdybicka-Barabas, A., Suder, P. and Jakubowics, T. (2007) Purification and characterization of eight peptides from *Galleria mellonella* immune hemolymph. *Peptides,* 28: 533-546.

Dolphin, C. T., Shephard, E. A., Povey, S., Smith, R. L. and Phillips, I. R. (1992). Cloning, primary sequence and chromosomal localization of human FMO2, a new member of the flavin-containing mono-oxygenase family. *Biochem. Journal,* 287: 261-267.

Duvic, B., Jouan, V., Essa, N., Girard, P. A., Pages, S., Khatar, Z. A., Volkoff, N. A., Givaudan, A., Destoumieux-Garzon, D. and Esoubas, J. M. (2012). Cecropins as a marker of *Spodoptera frugiperda* immunosupression during entamopathogenic bacterial challenge. *Journal of Insect Physiology,* 58(6): 881-888.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research,* 32(5). doi: 10.1093/nar/gkh340.

Ehrenstein, G. and Lecar, H. (1977). Electrically-gated ionic channels in lipid bilayers. *Q. Rev. Biophys.* 10: 1-34.

Elekonich, M. M. and Horodyski, F. M. (2003). Insect allatotropins belong to a family of structurally-related myoactive peptides present in several invertebrate phyla. *Peptides,* 24: 1623-1632.

Fasolo, J., Sboner, A., Sun, M. G. F., Yu, H., Chen, R., Sharon, D., Kim, P. M., Gerstein, M. and Snyder, M. (2011). Diverse protein kinase interactions identified by protein microarrays reveal novel connections between cellular processes. *Genes and Development,* 25: 767-778.

Feldhaar, H and Gross, R. (2008). Immune reactions of insects on bacterial pathogens and mutualists. *Microbes and Infection,* 10: 1082-1088.

Fiol, F. D., Lopes, L. C., Barberato-Filho, S. and Bergamaschi Motta, C. (2013). Evaluation of the prescription and use of antibiotics in Brazilian children. *The Brazilian Journal of Infectious Diseases,* 17(3): 332-337.

Fleming, A. (1881-1955). *A noble life in science.* Available online: http://wwwbl.uk/onlinegaller/features/beautifulminds/fleming.html/ (accessed on 2 May 2013).

Gade, G., Hoffmann, K. H. and Spring, J. (1997). Hormonal regulation in insects: Facts, gaps and future direction. *Physiological review,* 77: 963-1032.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol*. 3: 710-720.

Gillespie, J. P., Kanost, M. R. and Trenczek, T. (1997). Biological mediators of insect immunity. *Annu. Rev. Entemol*., 42: 611-643.

Girard, P., Boublik, Y., Wheat, C. W., Volkoff, A., Cousserans, F., Brehelin, M. and Escoubas, J. (2007). X-tox: An atypical defensin derived family of immune related proteins specific to Lepidoptera. *Developmental and Comparative Immunology,* 32: 575-584.

Gough, M., Hancock, R. E. W. and Kelly, N. M. (1996). Antiendotoxin activity of cationic peptide antimicrobial agents. *Infection and Immunity,* 64 (12): 4922-4927.

Hale, J. D. F. and Hancock, R. E. W. (2007). Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Review of Anti-Infective Therapy,* 5(6): 951-959.

Hancock, R. E. W. (1997). Peptide antibiotics. *Lancet,* 349: 418-422.

Hancock, R. E. W., Brown, K. L. and Moorkherjee, N. (2006). Host defense peptides from invertebrates- emerging antimicrobial strategies. *Immunobiology,* 211: 315-322.

Hara, T., Kodama, H., Kondo, M., Wakamatsu, K., Takeda, A., Tachi, T. and Matsuzaki, K. (2001). Effects of peptide dimerization on pore formation: Antiparallel disulfide-dimerized magainin 2 analogue. *Biopolymers,* 58: 437-446.

Harris, F., Dennison, S. R. and Phoenix, D. A. (2009). Anionic antimicrobial peptides from eukaryotic organisms. *Current Protein and Peptide Science,* 10: 585-606.

Hoffmann, J. A. (1995). Innate immunity of insects. *Current Opinion in Immunology,* 7: 4-10.

Hoffmann, J. A. (2004). Primitive immune systems. *Immunological Reviews,* 198: 5-9.

Hoffmann, J. A. and Hetru, C. (1992). Insect defensins: inducible antibacterial peptides. *Immunology Today,* 13(10): 411-415.

Hoffmann, J. A. and Reichhart, J. (2002). *Drosophila* innate immunity and evolutionary perspective. *Natural Immunology*, 3: 121-126.

Hoffmann, J. A., Reichhart, J. and Hetru, C. (1996). Innate immunity in higher insects. *Current Opinion in Immunology*, 8: 8-13.

Hull, R. (2012). Stress response to genotoxic agents and to infection. In *School of Molecular and Cell Biology*, vol, PhD (ed., pp. 62 and 87. Johannesburg: University of The Witwatersrand).

Hull, R. Alaouna, M., Khanyile, L., Byrne, M. and Ntwasa, M. (2013). Lifestyle and host defense mechanisms of the dung beetle, *Eouniticellus intermedius*: The Toll signalling pathway. Journal of Insect Science, 13 (108): 1-25.

Itagaki, K., Carver, G. T. and Philpot, R. M. (1996). Expression and characterization of a modified flavin-containing monooxygenase 4 from humans. *The Journal of Biological Chemistry*, 271(33): 20102-20107.

Jacob, T., Van den Broeke, C. and Favoreel, H. W. (2011). Viral serine/threonine protein kinases. *Journal of Virology*, 85(3): 1158-1173.

Jiang, Z., Vasil, A. I., Hale, J. D., Hancock, R. E. W., Vasil, M. L. and Hodges, R. S. (2008). Effects of net charge and the number of positively charged residues on the biological activity of amphipathic [alpha]-helical cationic antimicrobial peptides. *Peptide Science*, 90: 369-383.

Jihen, T., Gautier, V., Beaujouan, J. C., Gauchy, C., Landousli, A. and Richarme, G. (2011). Phosphorylation of a 65 kDa cytoplasmic protein by the Escherichia coli YeaG kinase. *Ann. Microb.* 61: 499-503.

Kang, S., Kim, D., Mishig-Otir, T. and Lee, B. J. (2012). Antimicrobial peptides: Their physicochemical properties and therapeutic application. *Arch. Pharm. Res.* 35(3): 409-413.

Karlsson, C., Korayem, A. M., Scherfer, C., Loseva, O., Dushay, M. S. and Theopold, U. (2004). Proteomic analysis of the *Drosophila* larval hemolymph clot. *Journal of Biological Chemistry,* 279(50): 52033-52041.

Khanyile, L. M., Hull, R. and Ntwasa, M. (2008). Dung beetle database: Comparison with other invertebrate transcriptomes. *Bioinformatics,* 3(4): 159-161.

Kruger, K., Lukhele, O. M. and Scholtz, C. H. (1999). Survival and reproduction of *Euoniticellus intermedius* (Coleoptera: Scarabaeidae) in dung following application of cypermethrin and flumethrin pour-ons to cattle. *Bulletin of Entomological Research,* 89: 543-548.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Lamberty, M., Zachary, D., Lanot, R., Bordereau, C., Robert, A., Hoffmann, J. A. and Bulet, P. (2001). Insect immunity: Constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. *The Journal of Biological Chemistry.* 276 (6): 4085-4092.

Lata. S., Mishra, N. K. and Raghava, G. P. S. (2010). AntiBP2: Improved version of antibacterial peptide prediction. *BMC Bioinformatics*, 11(1): S19. doi: 10.1186/1471-2105-11-S1-S19

Lehrer, R. I., Barton, A., Daher, K. A., Harwig, S. S., Ganz, T. and Selsted, M. E. (1989). Interaction of human defensins with *Escherichia coli.* Mechanism of Bactericidal Activity. *Journal of Clinical Invest.* 84: 553-561.

Levashina, E. A., Ohresser, S., Bulet, P., Reichhart, J. M., Hetru, C. and Hoffmann, J. A. (1995). Metchnikowin, a novel immune-inducible prolin-rich peptide from *Drosophila* with antibacterial and antifungal properties. *Eur. J. Biochem.* 233: 694-700.

Li, Y., Unnithan, G. C., Veenstra, J. A., Feyereisen, R. and Noriega, F. G. (2003). Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti* in vitro: effect of farnesoic acid and *Aedes* allatotropin. *The Journal of Experimental Biology*, 206: 1825-1832.

Li, Y., Xiang, Q., Zhang, Q., Huang, Y. and Su, Z. (2012) Overview on the recent study of antimicrobial peptides: Origins, functions, relative mechanisms and applications. *Peptides*, 37: 207-215.

Lohner, K., Latal, A., Degovics, G. and Garidel, P. (2001). Packing characteristics of a model system mimicking cytoplasmic bacterial membranes. *Chem. Phys. Lipids*, 111: 177-192.

Lui, R. Mu, L., Liu, H., Wei, L., Yan, T., Chen, M., Zhang, K., Li, J., You, D. and Lai, R. (2011). Two antimicrobial and nematicidal peptides derived from sequences encoded Picea sitchensis. doi: 10.1002/psc.1380

Mashek, D. G., Li, L. O. and Coleman R. A. (2007). Long-chain acyl-CoA synthetases and fatty acid channeling. *Future Lipidol.*, 2(4): 465-476.

Memarpoor-Yazdi, M., Zare-Zardini, H. and Asoodeh, A. (2012). A novel Antimicrobial Peptide Derived from the Insect *Paederus dermatitis*. *Int. J. Pept. Res. Ther.* doi: 10.1007/s10989-012-9320-1.

Mortz E, Krogh TN. and Vorum H. (2001). Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics*, 1 (11): 1359-63.

Nasmyth (1993). Control of the yeast cell cycle by the Cdc28 protein kinase. *Current Opinion in Cell Biology*, 5: 166-179.

Nibbering, P. H., Ravensbergen, E., Welling, M. M., van Berkel, L. A., van Berkel, P. H. C. and Pauwels, E. K. J. (2001) Human lactoferrin and peptides derived from its N-terminus are

highly effective against infections with antibiotic-resistant bacteria. *Infect. Immun.* 69: 1469-1476.

Ntwasa, M., Goto, A. and Kurata, S. (2012). Coleopteran antimicrobial peptides: Prospects for clinical applications. *International Journal of Microbiology.* doi: 10.1155/2012101989.

Papo, N and Shai, Y. (2003) Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? *Peptides*, 24: 1693- 1703.

Park, C. B., Kim, H. S. and Kim, S. C. (1998). Mechanism of action of the antimicrobial peptide buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun.* 244: 253-257.

Park, J. W., Kim, C. H. and Rui, J. (2010). Beetle immunity invertebrate Immunity. New York, NY, USA.

Phoenix, D. A., Dennison, S. R. and Harris, F. (2013). Antimicrobial peptides: Their history, evolution and functional promiscuity. doi: 10.1002/9783527652853.ch1.

Qiao, S. and Tuohimaa, P. (2004). Vitamin D_3 inhibits fatty acid synthase expression by stimulating the expression of long-chain fatty-acid-CoA ligase 3 in prostate cancer cells. *Federation of European Biochemical Societies,* 577: 451-454.

Reddy, K. V. R., Yedery, R. D. and Arahna, C. (2004). Antimicrobial peptides: Premises and promises. *International Journal of Antimicrobial Agents,* 24: 536-547.

Santini, M. S. and Ronderos, J. R. (2009). Allatotropin-like peptide in Malpighian tubules: insect renal tubules as an autonomous endocrine organ. *General and Comparative Endocrinology,* 160(3): 243-249.

Scott, M. G. and Hancock, R. E. W. (2000). Cationic antimicrobial peptides and their multifunctional role in the immune system. *Critical Reviews in Immunology,* 20(5): 407-431.

Seebah, S., Suresh, A. and Zhuo, S. (2007). Defensins knowledgebase: a manually curated database and information source focused on the defensins family of antimicrobial peptides. *Nucleic Acids Research,* 35(1): D265-D268.

Shai, Y. (1999). Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochimica et Biophysica Acta,* vol. 1462(1-2): 55-70.

Shai, Y. and Oren, Z. (2001). From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides,* 22: 1629-1641.

Soupene, E. and Kuypers, F. A. (2008). Mammalian long-chain acyl-CoA synthetases. *Experimental Biology and Medicine,* 233(5): 507-521.

Sovadinova, I., Parlemo, E. F., Urban, M., Mpiga, P., Caputo, G. A. and Kuroda, K. (2011). Activity and mechanism of antimicrobial peptide-mimetic amphiphilic polymethacrylate derivatives. *Polymers*, 3: 1512- 1532.

Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G. and Edwards, J. J. E. (2004). Trends in antimicrobial drug development: Implications for the future. *Clin. Infec. Dis.* 38: 1279-1286.

Stella, M. C., Schauerte, H., Straub, K. L. and Leptin, M. (1994). Identification of secreted and cytosolic gelsolin in *Drosophila*. *Journal of Cell Biology*, 125(3): 607-616.

Teixeira, V., Feio, M. J. and Bastos, M. (2012). Role of lipids in the interaction of antimicrobial peptides with membranes. *Progress in Lipid Research,* 51: 149-177.

Veenstra, J. A. and Costes, L. (1999). Isolation and identification of a peptide and its cDNA from the mosquito *Aedes aegypti* related to *Manduca sexta* allatotropin. *Peptides,* 20: 1145-1151.

Wachinger, M., Kleinschmidt, A., Winder, D., Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V. and Brack-Werner, R. (1998). Antimicrobial peptides

melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. *Journal of General Virology*, 79: 731-740.

Wang, G. (2013). Database-guided discovery of potent peptides to combat HIV-1 or superbugs. *Pharmaceuticals,* 6: 728-758.

Weimar, J. D., DiRusso, C. C., Delio, R. and Black, P. N. (2002). Lipids and lipoproteins: Functional role of fatty acyl-Coenzyme A synthetase in the transmembrane movement and activation of exogenous long-chain fatty acids. *Journal of Biological Chemistry,* 277: 29369-29376.

Wieprecht, T. Dathe, M., Beyermann, M., Krause, E., Maloy, W. L., MacDonald, D. L. and Biernet, M. (1997). Peptide hydrophobicity controls the activity and selectivity magainin 2 amide in interaction with membranes. *Biochemistry,* 36: 6124-6132.

Yeaman, M. R., Gank, K. D., Bayer, A. S. and Brass, E. P. (2002). Synthetic peptides that exert antimicrobial activities in whole blood and blood-derived matrices. *Antimicrob. Agents Chemotherap.* 46: 3883-3891.

Yeaman, M. R. and Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacological Reviews,* 55: 27- 55.

Yeung, A. T. Y., Gellatly, S. L. and Hancock, R. E. W. (2011). Multifunctional cationic host defence peptides and their clinical applications. *Cellular and Molecular Life Sciences,* 68(13): 2161-2176.

Yoon, H. S., Lee, C. S., Lee, S. Y., Choi, C. S., Lee, I. H., Yeo, S. M. and Kim, H. R. (2003). Purification and cDNA cloning of inducible antibacterial peptides from *Protaetia brevitarsis* (Coleoptera). *Archives of Insect Biochemistry and Physiology,* 52: 92-103.

Yount, N. Y., Bayer, A. S., Xiong, Y. Q. and Yeaman, M. R. (2006). Advances in antimicrobial peptide immunobilogy. *Biopolymers (Peptide Science),* 84: 435- 458.

Zasloff, M. (1992). Antibiotic peptides as mediators of innate immunity. *Current Opinion in immunology*, 4: 3-7.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, 415: 389-395.

Ziegler, D. M. (1980). *Enzymatic basis of detoxification,* In: Jacoby, W. B., ed. New York: Academic Press, 1: 201-20.

Zhu, X., Dong, N., Wang, Z., Ma, Z., Zhang, L., Ma, Q and Shan A. (2014). Design of imperfectly amphipathic α-helical antimicrobial peptides with enhanced cell selectivity. *Acta Biomaterialia*, 10: 244–257.