PHYLOGENY AND ANTIBIOTIC ACTIVITY OF *XENORHABDUS* SPP. ISOLATED FROM NEMATODE SYMBIONTS IN KENYA

By

RYAN MUSUMBA AWORI

A THESIS SUBMITTED TO THE UNIVERSITY OF NAIROBI, SCHOOL OF BIOLOGICAL SCIENCES IN PARTIAL FULFILLMENT FOR AWARD OF THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY

UNIVERSITY OF NAIROBI

APRIL 2015

DECLARATION AND APPROVAL

Declaration by the Candidate

This research thesis is entirely my work and has not been submitted for a degree in any other University.

Name________________________________ Reg.No______________________________

Signature_____________________________ Date_________________________________

Approval by Supervisors

This research thesis has been submitted with our approval as supervisors:

Prof. Francis B. Mwaura

School of Biological Sciences,

University of Nairobi,

P.O. Box 30197-00100,

Nairobi.

Signature_____________________________ Date_____________________________

Dr. Daniel Masiga

Molecular Biology and Bioinformatics Unit,

International Centre for Insect Physiology and Ecology,

P. O. Box 30772-00100,

Nairobi.

Signature_____________________________ Date_____________________________

Dr. Charles N. Waturu

Horticulture Research Institute,

Kenya Agricultural and Livestock Research Organisation,

P .O Box 220-01000,

Thika.

Signature_____________________________ Date_____________________________

ACKNOWLEDGMENTS

I wish to thank my academic supervisors, Prof. Mwaura, Dr. Masiga and Dr. Waturu. Beyond a doubt, without your guidance and patience, this work would not be. I wish to thank Dr. Amugune of School of Biological Sciences. Words cannot duly express the depth of my gratitude. I am grateful to Dr. Kulohoma of the Centre for Biotechnology and Bioinformatics for his work on the heat map and Dr. Wambua of the School of Biological Sciences for her guidance. I wish to thank Dr. John Ndemi of KEMRI-CRDR for provision of Methicillin resistant *Staphylococcus aureus* cultures. I am grateful to the National Commission for Science Technology and Innovation for their support and funding through grant NCST/5/003/INN/3rd CALL/017.

I am largely indebted to the following; Waruguru Wanjau, Nyotu Gitau, Peter Njenga, Rose Mbeya, Murugi Kagotho, Fridah Kariuki and Lorine Nanjala. We began as a group of colleagues and are now a team of like-minded friends. I thank God for each one of you.

I wish to thank the following departments wherein I was attached. The School of Biological Sciences of University of Nairobi, the Molecular Biology and Bioinformatics Unit of International Centre of Insect Physiology and Ecology; and the Entomopathogenic Laboratory of Horticulture Research Institute. From these, I met people who went way beyond their call of duty to assist me. To Mr. Ndi, Mr. Wachira, David Wanaina, Samuel Njeru and Lillian Gacheru, thank you.

I wish to thank my family; Quentin, Kagonya, Juliet, Christine, Tracey, Fred, and my parents. Words cannot communicate the depth of support I have received from you. To Ronnie, Mona Lisa, Aggie and Mama Ron, tones of gratitude overflow at the very thought of you. Thank you.

I wish to thank my friends of many circles. First, they are those from outside the realm of science. From the study of the bible; Mubui, Charles & Faith, Dennis, Jaaz, George, Muree and Brian. You are all awesome. To Mercy, Cathy and Marymary: a heart filled thank you. To Ian, Kiks & Patrice, thank you for keeping me going, even off the pitch. I am greatly indebted to my church. I have come to learn that they, and the faith we together profess are what kept me going, when logic often failed. Lastly, to the wonderful friends I made along this route; Simon, Joel, Collins, Aroko, Vinnie, Mark, Sambai, Jeremiah, Peninnah, Sam and Sara. Joy fills me up at the memory of each of you. Thank you.

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LIST OF ABBREVIATIONS

EPNL-HRI	-Entomopathogenic Nematology Lab-Horticulture Research Institute
KEMRI,CRDR	-Kenya Medical Research Institute, Center for Respiratory Disease Research
IFDSA	-Infectious Diseases Society of America
CLSI	-Clinical Laboratory and Standards Institute
MRSA	-Methicillin resistant *Staphylococcus aureus*
PAX	-Antimicrobial lipopeptides from *Xenorhabdus*
UV	-Ultra violet
UV-VIS	-Ultra violet-visible light
SSU	-Small sub unit
LSU	-Large sub unit
DMSO	-Dimethyl sulfoxide
rRNA	-Ribosomal ribonucleic acid
nm	-nanometers
kPA	-Kilopascals
PCR	-Polymerase chain reaction
dNTPS	-Deoxynucleotide triphosphate mix
TAE	-Tris Acetate EDTA
EDTA	-Ethylene diamine tetraacetate
PS	-Physiological saline

ABSTRACT

Xenorhabdus is a bacteria genus of the family Enterobacteriaceae. Bacteria of this genus form a mutualistic relationship with *Steinernema* entomopathogenic nematodes. More so, their antimicrobial production serves as a potential source of novel antibiotics in the wake of growing antimicrobial resistance. This study aimed to establish the phylogenetic relationship of three *Xenorhabdus* isolates to the 24 described species of the genus based on the 16s rRNA gene. Secondly, it aimed to determine the antibiotic activity of the three *Xenorhabdus* isolates from Kenya. Six 16s rRNA sequences were isolated in this study while 184 sequences were obtained from public databases compiling a data-set of 190 sequences. Phylogenetic reconstruction was done using maximum likelihood method with a bootstrap test of phylogeny of 500 replicates. The phylogenetic reconstruction identified the isolates as *Xenorhabdus griffiniae* L67, *Xenorhabdus griffiniae* XN45 and a novel *Xenorhabdus* species. This is the first record of *Xenorhabdus griffiniae* in Kenya. The antibiotic activities of the isolates were assessed by analysis of the inhibitory effect of the whole broth extracts, organic fractions and aqueous fractions. *Xenorhabdus griffiniae* L67, *Xenorhabdus griffiniae* XN45 and *Xenorhabdus* sp. P48 produced antibiotics effective against gram-positive bacteria. *Xenorhabdus griffiniae* L67 produced water-soluble antibiotics active against gram-positive bacteria. *Xenorhabdus griffiniae* XN45 produced antibiotics that readily dissolved in dimethyl sulfoxide. These were inhibitory to Methicillin resistant *Staphylococcus aureus*. The organic solvent fraction of *Xenorhabdus griffiniae* L67 had a peak uv absorption at 218_{nm}.This indicated the presence of peptide antimicrobials from *Xenorhabdus griffiniae* that were active against Methicillin resistant *Staphylococcus aureus*.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background information

In June of 2014, the World Health Organization made public the startling fact that the current crop of antibiotics is no longer effective in curing diseases (WHO, 2014). So dire is the situation that, unless urgent action is taken, a post antibiotic era where simple infections result in death is foreseeable. A possible solution is the discovery and development of antibiotics with novel modes of action. Indeed, a contributing factor to the current antibiotic resistance is the lack of a new major class of antibiotics for clinical use in the past 30 years (WHO, 2014). This is largely attributed to the shift away from novel drug development due to its hefty costs (IFSDA, 2004; Madigan *et al.,* 2009). Yet in the wake of the current widespread resistance, it is imperative to develop alternate and potent antibiotics.

Xenorhabdus is a bacteria genus belonging to the family Enterobacteriaceae (Boemare and Akhurst, 2006). One significant characteristic of these bacteria is that they are known for the production of antibiotics. Another significant characteristic of these bacteria is their natural habitat is the gut of *Steinernema* nematodes (Boemare and Akhurst, 2006). This mutualistic association is species specific with each *Steinernema* species associating only with a particular *Xenorhabdus* species. Over 72 *Steinernema* nematodes have been characterized from regions all over the world (Stock and Goodrich-Blair, 2012). Out of these, only 24 *Xenorhabdus* species have been characterized (Akhurst and Boemare, 1988; Ferreira *et al.*, 2013; Kuwata *et al.,* 2012; Lengyel *et al.,* 2005; Nishimura *et al.,* 1994; Somvanshi *et al.,* 2006; Taillez *et al.,* 2006; Tailliez *et al.,* 2010; Tailliez *et al.,* 2011). Evidently there exists a gap in the isolation and characterization of *Xenorhabdus* bacteria from their nematode symbionts. Yet this genus is a potential source of numerous novel antibiotics (Fuchs *et al.,* 2011).

Waturu *et al.* (1997) characterized *Steinernema karii*. This was the first *Steinernema* species isolated from Kenya. Taillez *et al.* (2006) characterized *Xenorhabdus hominickii.* This was the gut symbiont of *S. karii.* Mwaniki (2009) identified *S. weiseri*, *S. yirgelemense* and one novel *Steinernema* species from Kenya. These brought to a total of 4 characterized *Steinernema* and

one characterized *Xenorhabdus* species from Kenya. Yet 30 *Steinernema* have been isolated to date (HRI, 2014).

Phylogeny provides a fast and accurate means of identification of new *Xenorhabdus* isolates (Tailliez *et al.,* 2006). One method is to make a phylogenetic reconstruction of the genus by the use of nucleotide sequences, such as the 16s rRNA gene. From this, species clades are identified and the clade wherein the isolate falls provides its identity. Different *Xenorhabdus* species have different antibiotic profiles (Fodor *et al.,* 2010). This highlights the significance of identification of the isolates being screened for antibiotic activity.

1.2 Justification of the study

Antimicrobial resistance is a global problem necessitating urgent interventions. One intervention is the development of antibiotics from novel sources. *Xenorhabdus* is such as source as its natural habitat is the gut of *Steinernema* nematodes. This association is species specific with each *Steinernema* isolate signifying a unique *Xenorhabdus* species. There exists to date, 30 different *Steinernema* isolates from Kenya. From these, only four species have been identified. More so, only one *Xenorhabdus* species has been identified. This shows that a large number of *Xenorhabdus* species found in Kenya are yet to be identified. Secondly, different *Xenorhabdus* species have different antibiotic profiles. This highlights the potential source of novel antibiotics from Kenyan *Xenorhabdus* isolates.

1.3.0 Objectives of the study

1.3.1 Broad objective

To identify *Xenorhabdus* bacteria isolates with antimicrobial activity for use as novel sources of antibiotics for clinical drug development.

1.3.2 Specific objectives

1. Phylogenetic reconstruction of the *Xenorhabdus* genus from the 16s rRNA gene.
2. Determination of antibiotic activity of *Xenorhabdus* spp. isolated from Kenya.

1.3.3 Hypothesis

Phylogenetic reconstruction of the *Xenorhabdus* genus will identify Kenyan *Xenorhabdus* isolates with antibiotic activity.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 *Xenorhabdus* genus

Xenorhabdus is a bacteria genus of the family Enterobacteriaceae. They are gram-negative rod shaped facultative anaerobes typically 0.3-2µm by 2-10µm. Bacteria are peritrichously flagellated, and exhibit swarming motility. They possess both respiratory and fermentative metabolism, and produce acid, with no gas from glucose. However unlike other members of the family, they are catalase negative (Boemare and Akhurst, 2006). A distinguishing characteristic of this genus is that they form a mutualistic relationship with *Steinernema* entomopathogenic nematodes (Boemare, 2002). It is the bacteria symbiont that largely contributes to the entomopathogenicity of their nematode hosts (Herbert and Goodrich-Blair, 2007). Secondly, these bacteria secrete antibiotics and other metabolites that largely contribute to the fecundity of host (Boemare and Akhurst, 2006). To fully understand this, one must first understand the lifecycle of the bacterium-nematode complex.

2.2 *Xenorhabdus*-*Steinernema* life cycle

Each *Steinernema* nematode harbors within its gut specific *Xenorhabdus* species of bacteria. This relationship is species specific with a *Steinernema* species able to associate with only one *Xenorhabdus* species. An example is the *Xenorhabdus griffiniae* symbiont for *Steinernema hermaphroditum* while *X. hominickii* for *S. karii* (Tailliez *et al.,* 2006). Nonetheless, the association between *Xenorhabdus* and *Steinernema* is not obligatory as; both organisms can survive on their own (Herbert and Goodrich-Blair, 2007).

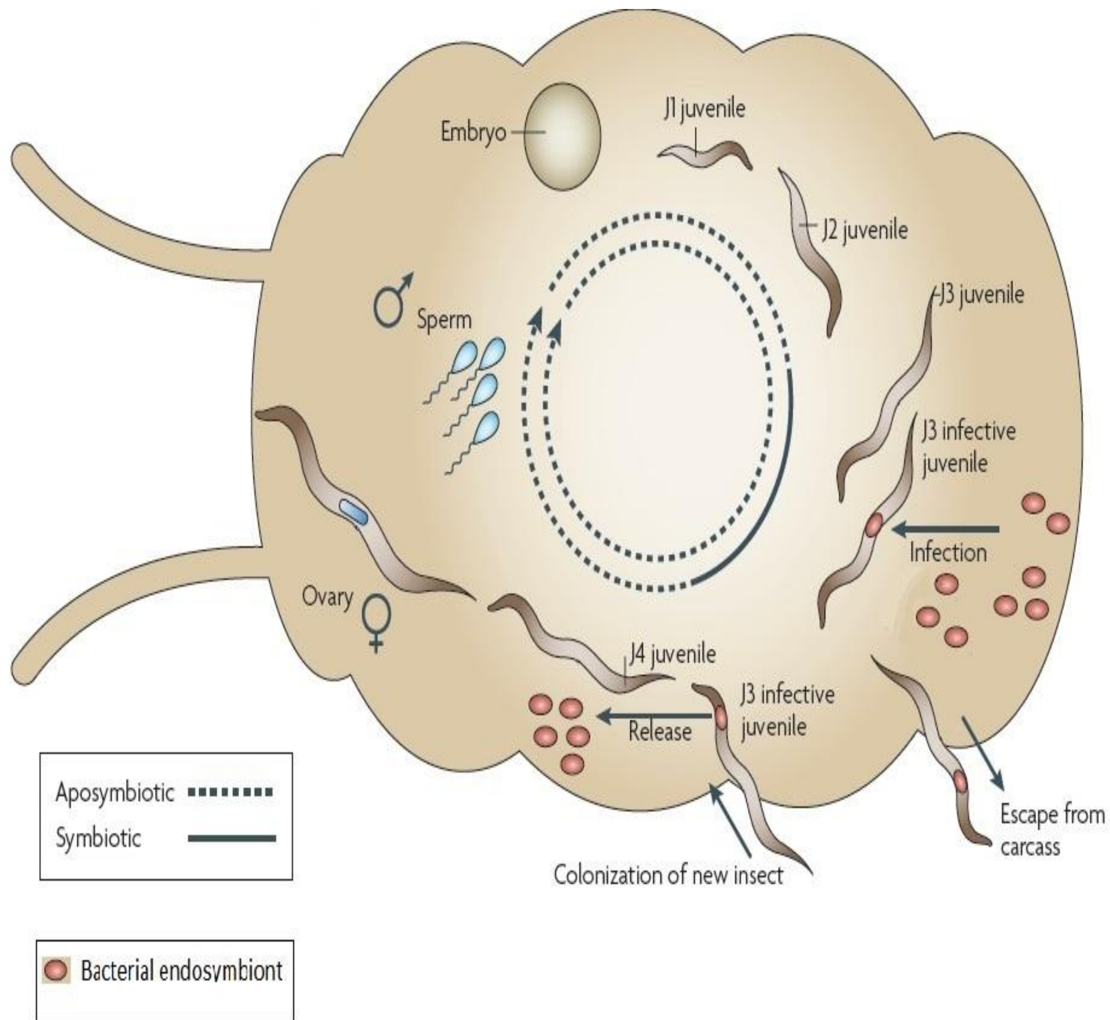

Figure 1: *Steinernema-Xenorhabdus* lifecycle (Bright and Bulgheresi, 2010)

The third stage infective juveniles (J3) of the nematode are found free living in soils the world over (Hominick, 2002). They actively seek out insects and infect them by piercing into the body cavity. This signifies the colonization of a new insect host. Once inside, they release their *Xenorhabdus* symbionts into the haemocoel through defecation. The third stage infective juveniles (J3) molt to the adult stage (J4) that now consists of both sexually mature males and females. Sexual reproduction then ensues resulting in the females producing embryonated eggs. These hatch into first stage juveniles (J1) which molt to second stage juveniles (J2) and back to third stage infective juveniles (J3). These re-associate with the bacteria by feeding on them, and escape from the carcass to seek out new hosts (Bright and Bulgheresi, 2010).

Bacterial growth within the haemocoel results in concomitant secretion of metabolites, which are largely divided into four groups (Chaston *et al.,* 2011). The first two groups are insecticidal toxins (Brown *et al.,* 2004; Sheets *et al.,* 2011) and insect immunity suppressing metabolites (Park and Kim, 2000). Both contribute to the virulence of the host bacteria. The metabolites, for example inhibit phospholipase A_2 that results in the shutdown of eicosanoids, which are crucial components of cellular immunity (Park *et al.,* 2004). This abets the colonization of the insect host by the bacteria thus promoting host damage (Vallet-Gely *et al.,* 2008). As the bacteria proliferates, there is a simultaneous secretion of insecticidal toxins such as Xpt toxins (Sheets *et al.,* 2011) and A24 (Brown *et al.,* 2004). These are highly effective resulting in quick insect death (Herbert and Goodrich-Blair, 2007).

The other metabolites secreted are exoenzymes (Chaston *et al.,* 2011) and antibiotics (Boemare and Akhurst, 2006; Forst and Nealson, 1996) that are significant after insect death. Secreted lipases, proteases, and amylases break down the internal tissues of the cadaver creating a nutrient soup, while the antibiotics ward off competing microorganisms (Adams *et al.,* 2006; Forst and Nealson, 1996). So effective are antibiotics secreted, that a monoxenic environment is created within the nutrient rich cadaver (Isaacson and Webster, 2002).

2.3 *Steinernema* isolated from Kenya

Steinernema entomopathogenic nematodes have a global distribution. They have been isolated from all continents except Antarctica (Hominick, 2002). The first description of *Steinernema* spp. in Kenya was by Waturu *et al.* (1997). This was during a survey of entomopathogenic nematodes in Central Kenya. Further investigation on these isolates led to the characterization of *Steinernema karii*, a novel entomopathogenic nematode from Kenya (Waturu *et al.,* 1997). There after , more and more *Steinernema* spp. were isolated and their cultures maintained culminating in the 30 isolates currently reposited at the Horticulture Research Institute (HRI, 2014).

It was only twelve years later that a second characterization of the nematodes was carried out. Mwaniki (2009) reported the precense of an additional two novel species from Kenya.This brought to a total of 3 novel *Steinernema* species from Kenya. However, apart from molecular work, no further characterisation of the nematodes was carried out. The study also revealed the precense of *Steinernema yirgelemense* and *Steinernema weiseri* in Kenya. It is worth noting that

the type species of these nematodes had been isolated from Ethopia (Nguyen *et al.,* 2004) and Europe (Mrácek *et al.,* 2003) respectively.

The Horticultural Research Institute in Thika town holds the current repository of *Steinernema* nematodes isolated from Kenya. *Steinernema* Scarpo is also deposited here, although it was isolated from North America. It is included in the repository to serve as a reference strain. Below is a list of the current stock of entomopathogenic nematodes at the Institute. Only one of the isolates, E9, has been fully identified to species level.

Table 1: *Steinernema* species isolated from Kenya (HRI, 2014)

	Isolate name	**Identification**	**County of Isoation**
1.	S97	*Steinernema* sp.	Kwale
2.	S10	*Steinernema* sp.	Kwale
3.	S32	*Steinernema* sp.	Kwale
4.	S102	*Steinernema* sp.	Kwale
5.	NK1	*Steinernema* sp.	Nakuru
6.	NK4	*Steinernema* sp.	Nakuru
7.	NK 23	*Steinernema* sp.	Nakuru
8.	NK 25	*Steinernema* sp.	Nakuru
9.	NK 26	*Steinernema* sp.	Nakuru
10.	NK 30	*Steinernema* sp.	Nakuru
11.	R2	*Steinernema* sp.	Nyandarua
12.	R19	*Steinernema* sp.	Nyandarua
13.	R52	*Steinernema* sp.	Nyandarua
14.	R56	*Steinernema* sp.	Nyandarua
15.	R60	*Steinernema* sp.	Nyandarua
16.	R88	*Steinernema* sp.	Nyandarua
17.	R89	*Steinernema* sp.	Nyandarua
18.	L67	*Steinernema* sp.	Muran’ga
19.	L71	*Steinernema* sp.	Muran’ga
20.	P48	*Steinernema* sp.	Kiambu
21.	P69	*Steinernema* sp.	Kiambu
22.	Z4	*Steinernema* sp.	Kiambu
23.	TKA	*Steinernema* sp.	Thika
24.	M48	*Steinernema* sp.	Nyeri
25.	M79	*Steinernema* sp.	Nyeri
26.	NARL22	*Steinernema* sp.	Nairobi
27.	NARL75	*Steinernema* sp.	Nairobi
28.	NARL91	*Steinernema* sp.	Nairobi
29.	NARL 93	*Steinernema* sp.	Nairobi
30.	E9	*Steienrnema karii*	Kirinyaga

2.4 Described species of *Xenorhabdus*

As earlier noted, one nematode species associates with only one bacterium species. Currently there are 24 described species of *Xenorhabdus* as listed in Table 3. These have been isolated from nematodes the world over.

Table 2: Described species of *Xenorhabdus*

Bacteria	**Nematode host**	**Geographical region of isolation**	**Reference**
X. beddingii	*S. longicaudum*	China, Australia	Akhurst and Boemare, (1988)
X. bovienii	*S. affinie*	Temperate regions	Akhurst and Boemare, (1988)
	S. intermedium		
	S. kraussei		
	S. feltiae		
X. budapestensis	*S. bicornutum*	Serbia	Lengyel *et al.,* (2005)
X. caballinasii	*S. riobrave*	USA, Jamaica	Tailliez *et al.,* (2006)
X. doucetiae	*S. diaprepesi*	Central Americas & Caribbean	Tailliez *et al.,* (2006)
X. ehlersii	*S. serratum*	China	Lengyel *et al.,* (2005)
X. griffiniae	*S. hermaphroditum*	Indonesia, Malaysia	Tailliez *et al.,* (2006)
X. hominickii	*S. karii*	Kenya	Tailliez *et al.,* (2006)
	S. monticolum	South Korea	
X. indica	*S. thermophilum*	India	Somvanshi *et al.,* (2006)
X. innexi	*S. scapterisci*	Uruguay	Lengyel *et al.,* (2005)
X. ishibashii	*S. aciari*	Japan, China	Kuwata *et al.,* (2012)
X. japonica	*S. kushidai*	Japan	Nishimura *et al.,* (1994)
X. khoisanae	*S. khoisanae*	South Africa	Ferreira *et al.,* (2013)
X. koppenhoeferi	*S. scarabaei*	USA	Tailliez *et al.,* (2006)
X. kozodoii	*S.arenarium*	Russia	Tailliez *et al.,* (2006)
	S. apuliae	Italy	

X. magdalanensis	*S australe*	Australia	Tailliez *et al.,* (2011)
X. mauleonii	*Steinernema* sp.	St. Vincent Island Caribbean	Tailliez *et al.,* (2006)
X. miraniensis	*Steinernema* sp.	Australia	Tailliez *et al.,* (2006)
X. nematophila	*S. carpocapsae*	Global distribution	Akhurst and Boemare, (1988)
X. poinarii	*S. glaseri,*	USA	Akhurst and Boemare, (1988)
	S. cubanum	Cuba	
X. romanii	*S. puertoricense*	Puerto Rico	Tailliez *et al.,* (2006)
X. stockiae	*S. siamkayai*	Thailand	Tailliez *et al.,* (2006)
X. szentirmaii	*S. rarum*	Argentina	Lengyel *et al.,* (2005)
X. vietnamensis	*S. sangi*	Vietnam	Tailliez *et al.,* (2010)

2.5 Phylogenetic reconstruction and the 16s rRNA gene

The term phylogeny refers to the evolutionary development of a species (Wiley *et al.,* 2008). Thus the main objective of a phylogenetic reconstruction is the establishment of the evolutionary relationships between organisms. Traditionally, analysis of phenotypic characteristics, such as morphometrics has been used (Sokal, 1966). The more similar characteristics found between two organisms, the more closely related they are. This provided the basis of numerical taxonomy which Sneath and Sokal (1973) defined as "the grouping by numerical methods of taxanomic units into taxa based on character states".

The explosion of nucleotide and amino acid sequences databases has provided yet another source of characters for phylogenetic studies. In this case, the more similar two sequences of the similar loci and coverage are, the closer the relationship between the organisms (WenHsiung, 1997). More so, phylogenetic reconstructions by the comparison of sequence data provide the most accurate and robust inferences of evolutionary histories (Wiley *et al.,* 2008). One gene loci that has been extensively used in phylogenetic studies of prokaryotes is the 16s rRNA (Weisburg *et al.,* 1991). It codes for the small subunit ribosomal RNA (rRNA) strand. Understanding of the structure of this rRNA is crucial to understanding the preference of its gene loci for phylogenetic studies.

2.6 Structure of 16s rRNA

The prokaryotic ribosome consists of the large sub unit (LSU) and the small subunit (SSU). The small sub unit consists of a ribonucleic acid strand of approximately 1541 base pairs that acts as a scaffold for 21 ribosomal proteins. This strand is the 16s rRNA (Klug *et al.,* 2009). Due to its single stranded nature, 16s rRNA post-transcriptionally folds into a secondary structure of bound and unbound nucleic acid regions.

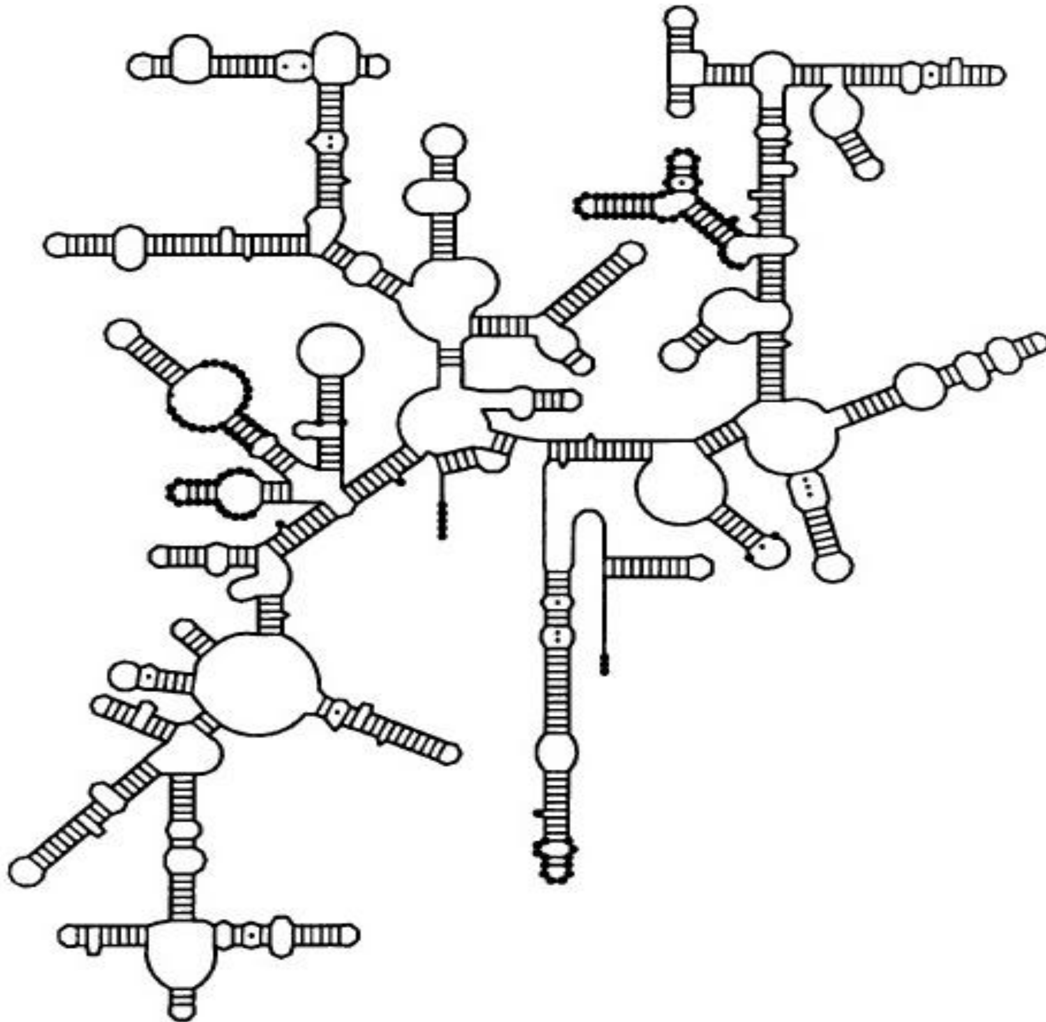

Figure 2: Bacterial 16s rRNA secondary structure (Woese, 1987).

The unbound regions fold into loop like structures while the bound regions form double stranded structures called stems (Mathews *et al.,* 2000). The sequences of the loops correspond to regions of the gene called hyper variable regions. This is because a lot of variability is seen in these regions across species. Hypervariable regions provide mismatches between sequences sufficient

to differentiate between closely related species (Wiley *et al.,* 2008). The sequences associated with the stems correspond to regions on the gene that are highly conserved with little to no variation observed in these regions across closely related species. They also provide regions of self-complementarity that result in the folding of transcribed RNA into the double stranded stems (Mathews *et al.,* 2000). Conserved regions also provide similar sequences across species that enable the amplification of 16s rRNA fragments with universal primer sequences (Weisburg *et al.,* 1991). Generally, the main aim of the conserved sequences is to ensure that the secondary structure of rRNA is maintained as it serves as the catalytic site of peptide synthesis within the ribosome (Cox and Nelson, 2008).

2.7 *Xenorhabdus* 16s rRNA gene

In general, the 16s rRNA gene sequence of *Xenorhabdus* species exhibits little variation with the level of dissimilarity between species varying from as small 2 % but never larger than 5 % (Boemare and Tailliez, 2009). This confounds the molecular identification of *Xenorhabdus* bacteria with the use of homology searches. Phylogenetic reconstruction of the *Xenorhabdus* genus based on the 16s rRNA gene provides an alternative method for molecular identification. A phylogenetic tree, with a large sample size of all species of the genus, is first reconstructed. The query species sequence is also included in the reconstruction. Finally, the species is then identified based on which clade it falls, as similar species cluster together.

2.8 Antimicrobial resistance

Antimicrobial resistance is defined as diminished or lost susceptibility of an organism to an antimicrobial (Madigan *et al.,* 2009). There has been growing antibiotic resistance reported from all regions in the world and this problem is now pandemic (WHO, 2014). A major cause of it is the misuse of antibiotics in health and agriculture by patients and farmers respectively (Rice, 2008). This has resulted in the following five bacteria species being most significant to human health: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterococcus* species. Collectively referred to as ESKAPE pathogens these bacteria harbor strains that are the predominant antibiotic resistance microorganisms (Rice, 2008). The second major contributing factor to antimicrobial resistance is the fact that there has not been a new major class of antibiotics for clinical use in the past thirty years (WHO, 2014). The main reason for this is the huge cost required for drug development. It

would take 10 or more years and an investment of 500 - 1.7 $ billion to bring a new drug to the shelf (IFDSA, 2004; Madigan *et al.,* 2009). Yet in the wake of the current resistance, it is imperative that new antibiotics, including those from novel sources, be developed. Having the sequestered environment of an entomopathogenic nematode's gut as its natural habitat, *Xenorhabdus* bacteria are an unequivocal novel source of antibiotics.

2.9.0 Antibiotics from *Xenorhabdus*

As earlier noted, *Xenorhabdus* are a genus known for the production of antibiotics. So potent are these antibiotics that they effectively ward off competing fungal and bacterial microbes from a *Steinernema* infected insect cadaver. A number of antibiotics have been isolated from *Xenorhabdus* species. These include both crude extracts and characterized compounds. Among the crude extracts, the whole broth extracts of fermentation from cultures of *X. budapestensis* and *X. szentirmaii* were very effective against gram-positive bacteria (Fodor *et al.,* 2010). Whole broth extracts of *X. caballinasii* were effective against both gram positive and gram-negative bacteria (Isaacson, 2000). Whole broth fractions from *X. nematophila* were effective against gram-positive bacteria, and moderately effective against gram-negative bacteria. On the other hand whole broth extracts from *X. ehlersii* were barely effective against gram-positive bacteria (Fodor *et al.,* 2010). The general conclusion from these observations was that different *Xenorhabdus* species produce different types of antibiotics. More so, each species produces more than one class of antibiotics (Fodor *et al.,* 2010; Forst and Nealson 1996; Gregson and McInerney, 1989).

2.9.1 Peptide antimicrobials from *Xenorhabdus* (PAX)

Among the specific classes of antibiotics isolated from *Xenorhabdus* species are the peptide antimicrobials from *Xenorhabdus* (PAX). Gualtieri *et al.* (2009) first isolated and identified PAX from *X. nematophila*, and they generally were effective antibacterials and antifungals. In terms of their chemistry, PAX are cyclolipopeptides with a high amount of lysine residues. They are soluble in water, methanol and dimethyl sulfoxide (DMSO) and have a peak absorption at 214_{nm} in a uv spectra (Gualtieri *et al.,* 2009).Thirteen novel PAX were further isolated from *X. nematophila* (Fuchs *et al.,* 2011). They were confirmed to be of this class based on structural analysis. Their antimicrobial activity was however not tested. One PAX isolated and characterized from *X. caballinasii* JM26 was cabanillasin. It was an effective antifungal with

high activity against *Candida krusei*, *Candida lusitaniae*, and the mould *Fusarium oxysporum*. It had moderate activity against *Cryptococcus neoformans* (Houard *et al.,* 2013).

2.9.2 Nemaucin

Nemaucin is a PAX isolated from *X. caballinasii* JM26 (Gualtieri *et al.,* 2012). A significant characteristic of nemaucin is that it possessed strong inhibitory activity against Methicillin resistant *Staphylococcus aureus* (MRSA) with a minimum inhibitory concentration (MIC) 30-fold lower than the current standard treatment vancomycin. This highlighted its efficacy. Secondly, it possessed low toxicity levels when tested against human cell lines. So promising is it as an antibiotic for clinical use that its patent has preceded its journal publication (Gualtieri *et al.,* 2012). Nemaucin is just one PAX, yet as noted, over fourteen PAX have been isolated from the *Xenorhabdus* genus. More specifically, they have been isolated from only *X. nematophila* and *X. caballinasii* (Fuchs *et al.,* 2011; Houard *et al.,* 2013).

2.9.3 Xenocoumacins

Xenocoumacins are generally described as water-soluble antibiotics isolated from *Xenorhabdus* (Gregson & McInerney, 1989). They are largely divided into xenocoumacin 1 and xenocoumacin 2. Xenocoumacin 1 is a potent antibacterial against both gram-positive bacteria and gram-negative bacteria. In fact, xenocoumacin 1 is effective even against *X. nematophila* itself. Thus, the bacterium first produces xenocoumacin 2, which is then cleaved to xenocoumacin 1 as it, goes through the cell membrane to guard against self-toxicity. In terms of activity, xenocoumacin 2 is a weak antibacterial agent but a strong antifungal (Park *et al.,* 2009).

As noted, most of the characterized antibiotics from *Xenorhabdus* have been isolated from *X. nematophila*. This has been attributed to sampling bias as its host species, *S. carpocapsae*, is the most investigated nematode in the world (Adams *et al.,* 2006). However, from analysis of crude extracts of *Xenorhabdus,* Fodor *et al.* (2010) successfully demonstrated that different *Xenorhabdus* species have different antibiotic profiles. To date, no documented published material is available on the antibiotic profiles of *X. griffiniae, X. hominickii, X. stockiae, X. vietnamensis, X. koppenhoeferi,* and *X magdalanensis*. Yet the success of any *Steinernema* nematode is dependent upon the creation of a monoxenic environment within the insect cadaver. A complete *Steinernema* lifecycle signifies the production of potent antibiotics.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Isolation of the bacteria

Steinernema infective juvenile nematodes as well as greater wax moth larvae (*Galleria mellonella*) were obtained from the Entomopathogenic Nematology Laboratory of Horticulture Research Institute based in Thika (EPN LAB,HRI). The purpose of the wax moth larvae was to act as bait for the nematodes in the isolation of the bacteria. Four nematode isolates were selected: *Steinernema* sp. Scarpo, *Steinernema* sp. L67, *Steinernema* sp. P48 and *Steinernema* sp. R192.A culture suspension of each nematode species in distilled water, was used to infect last instar larvae of *Galleria mellonella*. Filter paper was lined to the lid of a 90 mm petri dish. With the use of sterile injection needles, 2ml of the distilled water suspension of the nematodes was inoculated onto the filter paper. Five last instar larvae were then placed on the bottom of the petri dish. This was then inverted over the lid. The petri dish was sealed with Parafilm® and incubated, in the dark, at room temperature for 72 h.

A differential medium, NBTA (28g/L Nutrient agar (Himedia, India) supplemented with 25mg/L 2,3,5 triphenyltetrazolium chloride (Sigma-Aldrich, USA) and 40mg/L bromothymol blue (Fluka Analytical, USA) , was prepared and sterilized by autoclaving at 121ºC at 103.42 kPa for 15 min. *Xenorhabdus* species grow on NBTA as pigmented colonies providing presumptive identification of the bacteria during isolation from the nematodes (Boemare and Akhurst, 2006; Taillez *et al.,* 2006). The cadavers were obtained from incubation and surface sterilized in 70% isopropanol under aseptic conditions. A second surface sterilization was done by immersion in 90 % isopropanol. Lastly, igniting the cadavers over an open flame and thereafter quickly dipping into sterile water did flame sterilization. Dissection of the cadaver was done to obtain insect haemolymph. It was a clear translucent liquid. This was streaked onto NBTA medium and incubated at 30ºC for 72 h (Akhurst, 1980).

3.1.2 Sub culturing of the bacteria

All experiments were done under aseptic conditions. Pigmented colonies were observed on NBTA media. Blue distinct colonies with rough margins were observed for the *Steinernema* sp. Scarpo isolates while green colonies for the *Steinernema* sp. L67, *Steinernema* sp. P48 and *Steinernema* sp. R192. These were selected and presumptively identified as *Xenorhabdus* species based on the pigmentation on NBTA (Boemare and Akhurst, 2006; Taillez *et al.,* 2006). The bacteria were named *Xenorhabdus* sp. XN45 for the *Steinernema* sp. Scarpo isolates, *Xenorhabdus* sp. P48 for the *Steinernema* sp. P48 isolate .Lastly, *Xenorhabdus* sp. R192 for *Steinernema* sp. R192 and *Xenorhabdus* sp. L67 for *Steinernema* sp. L67 isolates respectively.

These were sub-cultured onto NBTA plates and incubated at 30ºC. Single colonies were selected that were pigmented, with observable complete rough margins. Luria Bertani medium (LB) (10g/L Tryptone 5g/L Yeast Extract 10 g/L NaCl) of 8g/L agar concentration was prepared and sterilized (Miller, 1972). Approximately 5 ml of this was poured into sterile universal bottles and let to solidify. Stab cultures were then made for each of the isolates. These were stored in the dark at room temperature (Stock and Goodrich-Blair, 2012).

Long-term cultures of the isolates were made by first inoculating selected colonies into 5ml of LB. This was then incubated at 28ºC at 150 revolutions per minute (rpm) for 31 h to proliferate the cultures. Thereafter, each of the broth cultures (900µl) was then transferred to sterile 1.5 ml cryogenic storage tubes. It was then topped up with 300µl of LB that had been premixed with 300µl of glycerol to yield a final concentration of 20 % (v/v) glycerol. These long-term storage stocks were preserved at -80ºC (Stock and Goodrich-Blair, 2012).

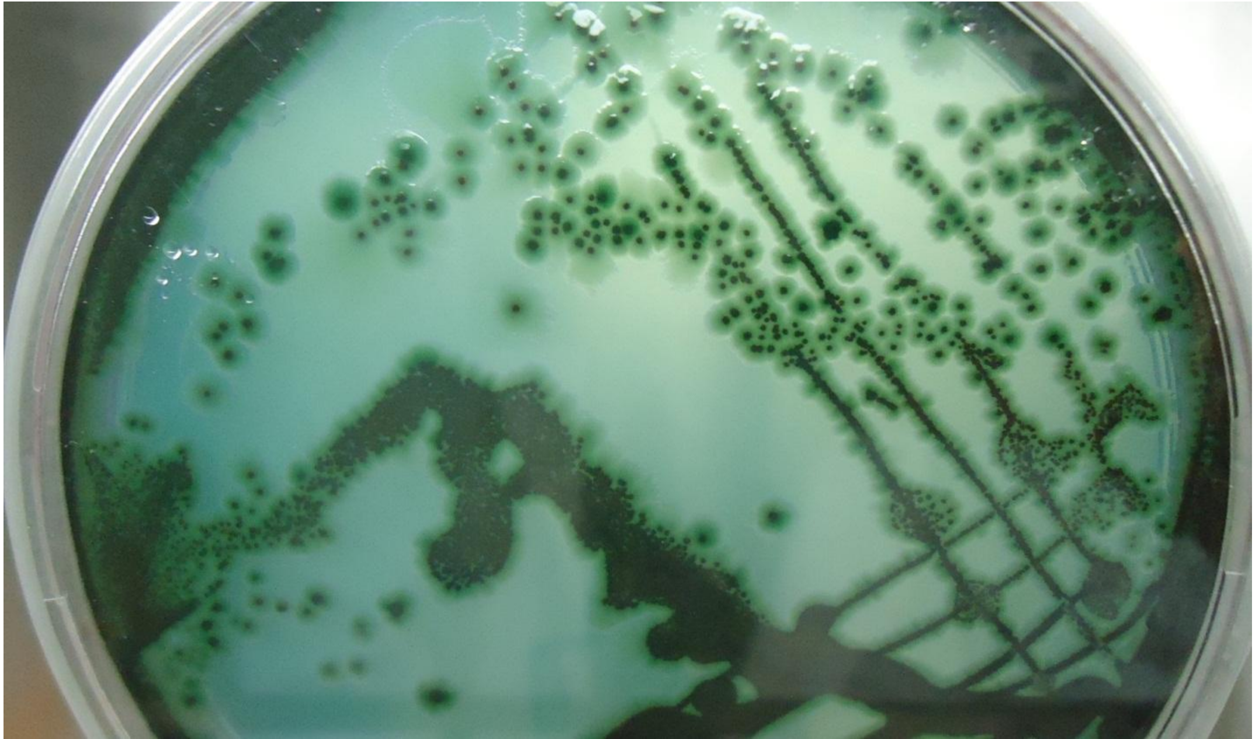

Figure 3: *Xenorhabdus* sp. L67 colonies, after 30 day incubation period at 30°C, on NBTA medium.

3.2.0 Molecular methods

3.2.1 DNA extraction

DNA was extracted from plate cultures of the bacteria isolates using a FastDNA®SPIN Kit for Soil (MP Biomedicals, USA). Concentration of extracted DNA was determined by spectrophotometry (Shimadzu 1800, Japan). This was done by measurement of the absorbance values of dissolved nucleic acids samples at 260_{nm} and 280_{nm} across a 1cm light path. An absorbance value of 1 of a pure DNA at 260_{nm} is equal to 50ng/ µl a concentration (Sambrook *et al.,* 1989). Ratios of 1.8 of the absorbance values at 260_{nm} /280_{nm} of a DNA sample is indicative of high purity DNA, void of protein contamination (Barbas *et al.,* 2007; Sambrook *et al.,* 1989). Nuclease free water was first dispensed (10 µl) into the cuvette for a baseline adjustment. The cuvette was then rinsed clean and a sample of the dissolved DNA in nuclease free water (10 µl) was pipetted into it. Measurements were taken at 260_{nm} and 280_{nm} and recorded. This was repeated for all samples.

3.2.2 Isolation of 16s rRNA gene

Xenorhabdus partial gene coding for 16s rRNA was isolated by the PCR method (Weisburg *et al.,* 1991). Two different reactions were used. The first reaction was done in a 25 µl volume which contained 0.5 units Q5 polymerase® (New England Biolabs,USA), 5µl of 5x Q5 polymerase buffer® (New England Biolabs,USA), containing 10mM $MgCl_2$, 0.5µl 10mM dNTPs (New England Biolabs, USA), 1.25µl of 1µM each, forward (27f-AGAGTTTGATCATGGCTCAG) and reverse (1392r-ACGGGCGGTGTGTGC)primers, and 15.75 µl nuclease free water (Lane, 1991). Amplification was done in a thermal cycler (MJ Research PTC-100, USA) with the cycling conditions set at 98°C for 30 s, 20 cycles of 98°C for 30 s, 42°C for 15 s, 72°C for 1 min, then 20 of cycles of 98°C for 30 s, 47°C for 15 s, 72°C for 1 min and a final extension of 72°C for 2 min. This was termed as reaction 1(Doi *et al.*, 2013).

The second reaction was done in a 20 µl volume which contained 1.5 units Taq polymerase® (Genscript, USA), 2µl of 10x Taq polymerase buffer® (Genscript, USA) containing 15mM $MgCl_2$, 0.2µl 10mM of dNTPs (New England Biolabs,USA), 1µl of 1µM each forward(27f-AGAGTTTGATCATGGCTCAG)and Reverse(1392r-ACGGGCGGTGTGTGC) primers and 13.5 µl nuclease free water (Lane, 1991). Amplification was done in a thermal cycler(Thermo Scientific Arktik ,USA) with the cycling conditions set at 94°C for 5 min , 40 cycles of 94°C for 30 s, 47°C for 15 s , 72°C for 1 min 30 s, and a final extension of 72°C for 7 min. This was termed as reaction 2.

PCR products were visualized on agarose gels. These were composed of 1.2%(w/v) agarose dissolved in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and stained with Ethidium bromide at final concentration of 0.5µg/ml. Typical conditions for electrophoresis were 4V/cm for 80 min (Sambrook *et al.,* 1989). The expected bands (1300 base pairs) were excised and purified with Quick Clean II Gel extraction kit® (Genscript, USA). The purified products were outsourced for sequencing (Macrogen, Netherlands). The sequences obtained were quality checked, assembled, and poor quality base calls trimmed in BioEdit (Hall, 1999) and MEGA6 (Tamura *et al.,* 2013).

3.3 Phylogenetic reconstruction

A phylogeny of the genus was reconstructed from a dataset of 190 16s rRNA gene DNA sequences (n= 184 from Genbank database release 201.0, and n=6 generated from this study) were used (Wu *et al.,* 2009). Sequence names and accession numbers are listed in Appendix 2. The 16s rRNA sequences for all *Xenorhabdus* type strains were captured in the dataset. In order to give a phylogenetic tree an evolutionary path, a species that is older than those under investigation need be included in the analysis. This serves as the root, and act as the baseline against which evolutionary positions will be compared (WenHsiung, 1997; Wiley *et al.,* 2008). Pseudomonads evolved earlier than Enterobacteriaceae (Wu *et al.,* 2009). Thus, one *Pseudomonas aeruginosa* 16s rRNA sequence was included in the analysis.

Database sequences were checked for quality and ambiguous nucleotides resolved in MEGA6 (Tamura *et al.,* 2013). All multiple sequence alignments were done in MEGA6 (Tamura *et al.,* 2013) with the MUSCLE algorithm (Edgar, 2004). Aligned sequences were then trimmed to 1236 base pairs and used for phylogenetic reconstruction. All positions containing gaps and missing data were eliminated leaving 1173 positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The model represents a mathematical correction for back mutations and multiple substitutions that occur during evolution (WenHsiung, 1997). Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013) Bootstrap test of phylogeny of 500 replicates was used (Felsenstein, 1985). Phylogenetic trees were edited in FigTree 1.4 (Rambaut, 2012).

The three main nucleotide sequence databases are the DNA databank of Japan, European Molecular Biology Laboratory and Genbank (Zdobnov *et al.,* 2002). They are interlinked thus creating a central nucleotide database that can be widely accessed. Submission of nucleotide sequences to one allows it to be accessed from all three (Zdobnov *et al.,* 2002). The 6 nucleotide sequences obtained in this study were thus submitted to the DNA databank of Japan via online submission. Their accession numbers are highlighted in blue in Appendix 2.

3.4.0 Fermentation of antibiotics by *Xenorhabdus* spp.

Xenorhabdus spp. produces different classes of antibiotics of different efficacies (Forst and Nealson, 1996; Furgani *et al.,* 2008). The targeted pathogens in this study were gram-positive cocci. Fermentation durations known to yield antibiotics effective against gram-positive cocci were thus selected. Gualtieri *et al.* (2012) successfully fermented antibiotics from *Xenorhabdus* that were effective against gram-positive bacteria, using 72 h fermentation duration. Isaacson (2000) successfully fermented antibiotics from *Xenorhabdus* effective against gram-positive cocci and which readily dissolved in organic solvents. He used 180h fermentation duration. Durations of 72 h and 180 h were thus selected. Lastly to compare the effect of fermentation duration on antibiotic activity, an extended duration of 315 h was selected.

3.4.1 72 h fermentation

All experiments were done under aseptic conditions. Fermentation was done using *Xenorhabdus* sp. XN45, *Xenorhabdus* sp. L67 and *Xenorhabdus* sp. P48 bacterial cultures. Multiple colonies (2-3) of an individual isolate were selected. These were then inoculated into LB media (5ml) and incubated at on a shaker at 150 rpm at 33ºC for 24 h. These cultures served as a 1% (v/v) starter inoculum for fermentation procedures. Sterile LB media (500ml) was dispensed into sterile 1-liter Erlenmeyer flasks. The starter cultures (5ml) were then inoculated into the flasks and sealed with sterile aluminum foils. These were incubated at 150rpm at 33ºC for 72 h. LB with no inoculum was also incubated to serve as a control for sterility. These broths were termed as 72 h fermentation whole broth extracts (WBE).

3.4.2 180 h fermentation

A second fermentation reaction similar to the first was carried out. Fermentation was done using *Xenorhabdus* sp. XN45 and *Xenorhabdus* sp. P48 bacterial cultures. Multiple colonies (2-3) of an individual isolate that, had complete and slightly rough margins, were selected. These were then inoculated into 5ml LB and incubated at 150 rpm at 33ºC. These cultures served as a 1% (v/v) starter inoculum for fermentation procedures. Sterile LB media (500ml) was dispensed into sterile 1-liter Erlenmeyer flasks. The starter cultures (5ml) were then inoculated into the flasks and sealed with sterile aluminum foils. These were incubated at 150rpm at 33ºC for 180 h. LB with no inoculum was also incubated to serve as control for sterility. These broths were termed as 180h fermentation whole broth extracts (WBE).

3.4.3 315 h fermentation

A third fermentation reaction was carried out for *Xenorhabdus* sp. p48. Starter cultures were prepared by inoculating multiple colonies (2-3) of an individual isolate into LB media and incubating at 150rpm at 33ºC for 20 h. Sterile LB media were inoculated with 1% (v/v) starter cultures. These were then incubated at 150rpm at 33ºC for 315 h. LB media with no inoculum was also incubated to serve as control for sterility. These broths were termed as 315 h fermentation whole broth extracts (WBE).

3.4.4 Purification of the whole broth extract.

A simple purification procedure was used for all whole broth extracts. This was by separation of the cells from the broth by high-speed centrifugation followed by filter-sterilization of the cell free supernatants. Optimized conditions for purification were centrifugation of broths at 20,000g for 25 min at 4ºC (Beckman Avanti J-25, USA) followed by decanting cell free supernatants and filtration through a sterile 0.45 µm filter membrane (Nalgene, USA). The filtrate obtained was further filtered through a sterile 0.2 µm filter membrane (Nalgene, USA) to yield sterile whole broth extracts. These were stored at 4ºC until use (Furgani *et al.,* 2008).

3.5 Fractionation of whole broth extracts

As earlier noted, xenocoumacins are broad-spectrum water-soluble antibiotics from *Xenorhabdus* (Gregson and McInerney, 1989). More so, they are highly effective against gram-positive cocci (Park *et al.,* 2009). On the other hand, numerous antimicrobial lipopeptides have been isolated from *Xenorhabdus* (Fuchs *et al.,* 2011; Gualtieri *et al.,* 2009; Houard *et al.,* 2013). They were highly effective even against antibiotic resistant gram-positive cocci (Gualtieri *et al.,* 2012). Two significant characteristics of these antimicorbial lipopeptides is that they readliy dissolve in organic solvents and have a peak uv absorption at 214_{nm} when dissolved in methanol (Gualtieri *et al.,* 2009). In order to infer the presence of the above classes of antibiotics, fractionation of the whole broth extracts was carried out (Isaacson, 2000). This yielded two fractions. One contained antibiotics that readily dissolved in water and the second contained those that readily dissolved in organic solvents. Further analysis of the organic solvent fraction was carried out by determining the wavelength that yielded peak uv absorption (Gualtieri *et al.,* 2009).

Fractionation of the broths was done by solvent extraction (Burianek and Yousef, 2000). This was done on the 72 h whole broth extract of *Xenorhabdus* sp. L67 and the 180 h whole broth extract of *Xenorhabdus* sp. XN45. The whole broth extracts were mixed with chloroform (2:1) and magnetically stirred for 30 min. The mixtures were distributed into 40ml high density polypropylene tubes and centrifuged at 20,000 g for 20 min at 25°C. For each extract, a yellow top layer, and clear bottom layer, inter-phased by a white precipitate was obtained. The top yellow layer, termed as the aqueous fraction, was decanted and pooled. The bottom layer, termed as the organic fraction, was pooled into a chrome-vanadium pan and left in a chemical hood to allow for evaporation of chloroform. After 72 h, a lipid like layer was observed at the bottom of

the pan. This was dissolved in 100% methanol (70ml) and the absorption spectra determined by uv-vis spectrophotometry (Beckman DU-640, USA) (Gualtieri *et al.,* 2009; Houard *et al.,* 2013).

Further concentration was carried out on the organic fraction of the 180 h whole broth extract of *Xenorhabdus* sp. XN45. It was first diluted to a 90% methanol extract. The methanol was then removed by rotary evaporation with a vaccum pump at room temperature yielding a yellow lipid like substance. This was dissolved in 3.9ml of absolute DMSO and used in inhibition assays (Ladell, 2011). A total of 3.9ml of the organic fraction had been solvent extracted from a starting whole broth fraction amount of 275ml resulting in a 70x concentrate (275/3.9).

3.6.0 Inhibition assays.

Gram-positive bacteria pathogens were selected as target species. *Enterococcus. faecalis* was selected as representative gram positive pathogen. Methicillin resistant *Staphylococcus aureus* (MRSA) was selected as a gram-positive antibiotic resistant pathogen (Rice, 2008). Large-scale quantitative inhibition tests were carried out with *E. faecalis* as the test species while only small-scale qualitative inhibition tests were carried out against MRSA. This was due to its requirement of a Biology safety Level 3 laboratory for large-scale tests against antibiotic resistant pathogens (Madigan *et al.,* 2009). *E. faecalis* cultures were obtained from the Government Chemist, Kenya. MRSA cultures were obtained from Kenya Medical Research Institute, Center for Respiratory Disease Research. Two selective media were used for the bacteria. Mannitol salt agar (Chapman, 1945) was used for MRSA while Kanamycin aesculin azide agar (Oxoid, United Kingdom) was used for *E. faecalis* (Sabbaj *et al.,* 1971).

3.6.1 Broth macro dilution assay

For the large-scale inhibition tests, the broth macro dilution assay was used (Furgani *et al.,* 2008). A dilution range was made representing varying concentrations of the broth extracts in 2x LB medium (20g/L Tryptone 10g/L Yeast Extract 20 g/L NaCl) to yield extract concentrations of 0%-100%. These dilutions were referred to as the test antibiotics.

Table 3: Concentration range of test antibiotics used in broth macro dilution assays

Test antibiotic Concentration (%)	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Broth extract(ml)	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
2X LB(ml)	4.9	4.4	3.9	3.4	2.9	2.4	1.9	1.4	0.9	0.4	0

Test bacteria (100 µl) was then inoculated into each of the concentrations and incubated for an average 21 h at 37ºC without agitation. Average incubating inoculum was $2.54*10^5$ cfu/ml. Plating a 1ml 10^{-6} dilution of the inoculating test microorganism onto agar plates and incubating them alongside the dilution assay determined this. After incubation period, the number of colony forming units on the plates were enumerated and concentration of cells in the broth cultures was determined. The following controls were included in every replicate. The negative control contained 2X LB media only with no test antibiotic, inoculated with test bacteria. The control for sterility of the media was composed of 2X LB media only with no inoculated bacteria. Lastly, the control of sterility of the test antibiotic was composed of undiluted whole broth extract only, with no inoculated bacteria (Furgani *et al.,* 2008).

3.6.2 Plate inhibition assay of organic fraction of whole broth extract from *Xenorhabdus* sp. XN45

The plate inhibition assay of the organic fraction was conducted against Methicillin resistant *Staphylococcus aureus* (CLSI, 2007). Fresh overnight plate cultures were used to prepare the inoculum by diluting colonies in physiological saline (0.9 %(w/v) NaCl solution) to a turbidity of a 0.5 McFarland standard. Plating was then done by soaking sterile cotton in the inoculum and applying it over Mueller Hinton Agar (MHA) plates. The plates were left open briefly to dry. Sterile 6mm filter papers were placed onto the plates. The organic fraction (50 µl) was pipetted onto the filter paper to serve as the test antibiotic. An equal amount of sterile 100% (v/v) DMSO with no test antibiotic was used as a negative control. Plates were sealed with Parafilm and incubated at 37ºC overnight (CLSI, 2007).

CHAPTER 4

4.0 RESULTS

4.1.0 DNA extraction

The purity and concentrations of the extracted DNA was determined by uv-vis spectrophotometry as described in section 3.2.1. Results for each isolate are tabulated below.

Table 4: Purity and concentrations of DNA extracted from *Xenorhadus* sp. P48

Bacteria isolate	Sample name	$260_{nm}/280_{nm}$ ratio	Concentration (ng /µl)
Xenorhabdus sp. P48	P48-1	1.8	306
	P48-2	1.2	121
	P48-3	2.7	354
	P48-4	1.7	45
	P48-5	1.8	190
	P48-6	1.7	290

All samples were obtained from plate cultures and extracted in the same procedure. Five out of six samples had concentrations above 100 ng /µl denoting the efficacy of the procedure in extracting high concentrations of DNA.

Table 5: Purity and concentrations of DNA extracted from *Xenorhabdus* sp. L67

Bacteria isolate	Sample name	$260_{nm}/280_{nm}$ ratio	Concentration (ng /µl)
Xenorhabdus sp. L67	L67-2	1.6	37
	L67-3	1.9	69
	L67-4	1.8	101
	L67-5	1.5	52

Relatively pure DNA samples were obtained as seen from $260_{nm}/280_{nm}$ ratios of 1.6 -1.9.

Table 6: Purity and concentrations of DNA extracted from *Xenorhabdus* sp. XN45

Bacteria isolate	Sample name	$260_{nm}/280_{nm}$ ratio	Concentration (ng /µl)
Xenorhabdus sp. XN45	XN-1	1.2	236
	XN-2	1.8	168
	XN-3	1.7	132
	XN-4	1.8	198
	XN-6	1.8	40
	XN-7	1.8	76
	XN-8	1.8	69
	XN-9	1.7	52
	XN-10	1.7	59

Eight out of nine samples had $260_{nm}/280_{nm}$ ratios greater than 1.7 denoting the efficacy of the procedure in extracting DNA of high purity.

Table 7: Purity and concentrations of DNA extracted from *Xenorhabdus* sp. R192

Bacteria isolate	Sample name	$260_{nm}/280_{nm}$ ratio	Concentration (ng /µl)
Xenorhabdus sp. R192	R192-1	1.8	129
	R192-2	1.5	204
	R192-3	1.5	40
	R192-4	1.8	269

Three out of four samples had concentrations above 100 ng /µl reiterating the efficacy of the procedure in extracting high concentrations of DNA. A total of 23 samples were extracted with 73% ≥ 50 ng/µl concentrations,56% ≥ 100 ng/µl and 17% with ≥ 200 ng/µl concentrations. This signified the efficacy of the method in extracting high concentrations of bacterial DNA from plate cultures.

4.2.0 Isolation of 16s rRNA gene

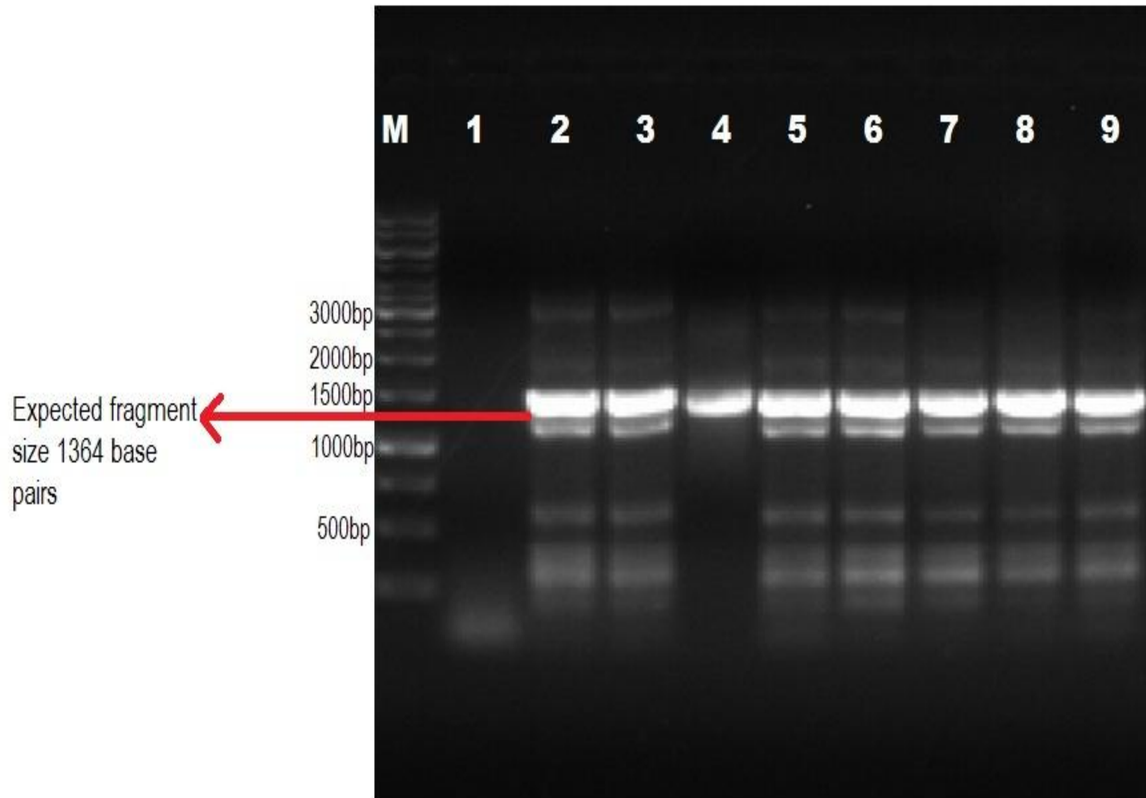

Figure 4: Autoradiograph of agarose gel with PCR products of amplification of partial 16s RNA gene of *Xenorhabdus* spp. by reaction 1.

Lane M was of the molecular size marker and Lane 1 the negative control. Lane 2-4 was of *Xenorhabdus* sp. XN45 DNA samples while lane 5-9 was of *Xenorhabdus* sp. L67 DNA samples. Spurious products were attributed to high concentrations of DNA. Lane 4 was of an older stock of DNA. Agarose gel (1%) was run at 4V/cm for 70 min.

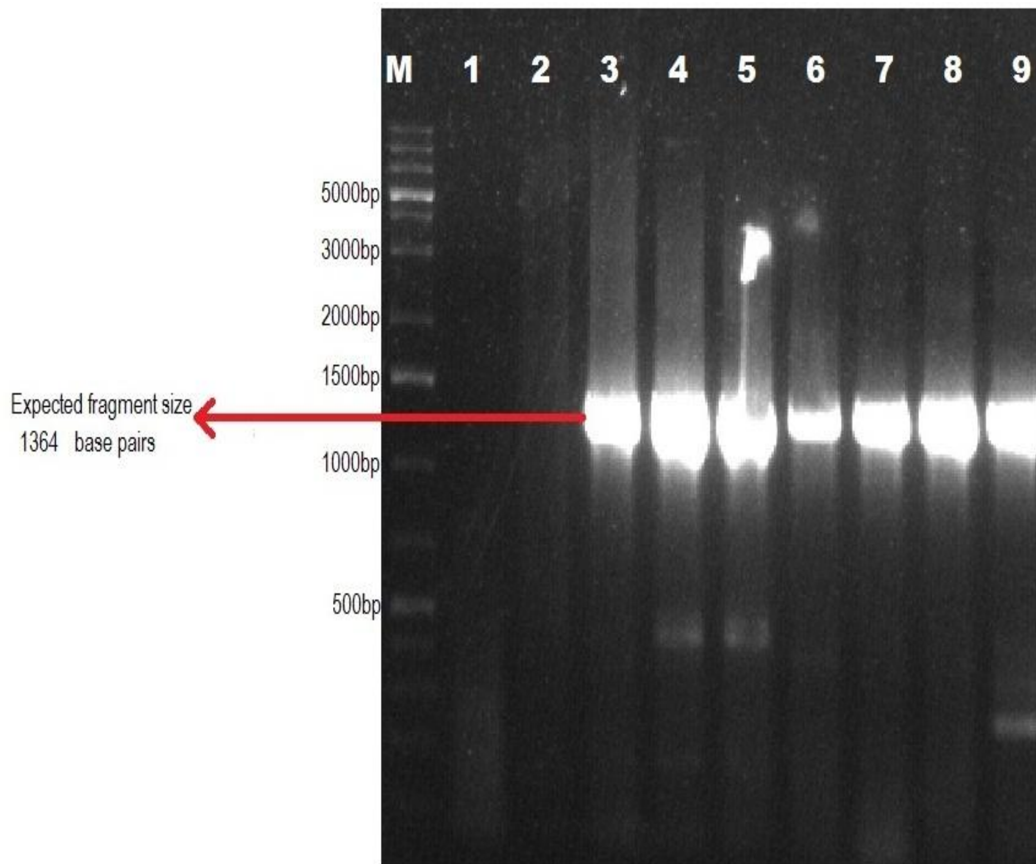

Figure 5: Autoradiograph of agarose gel 1 with PCR products of amplification of partial 16s RNA gene of *Xenorhabdus* spp. by reaction 2.

Lane M was of the molecular size marker and lane 1 was the negative control. Lane 2 was of *Xenorhabdus* sp. L67 DNA and 3-5 was of *Xenorhabdus* sp. P48 DNA. Lane 7-9 was of *Xenorhabdus* sp. R192 DNA. Fragmented band of lane 5 attributed to presence of contaminating agarose residues collected from preparation equipment. Agarose gel (1.2%) was run at 4V/cm for 117 min.

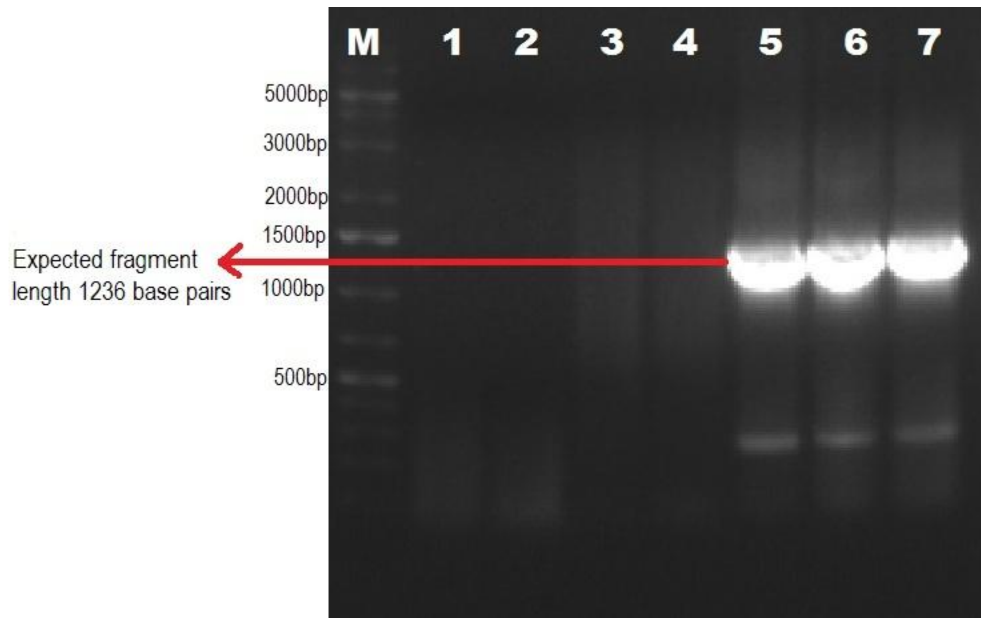

Figure 6: Autoradiograph of agarose gel 2 with PCR products of amplification of partial 16s RNA gene of *Xenorhabdus* sp. by reaction 2.

Lane M was of the molecular size marker and lanes 1& 2 were negative controls. Lanes 3&4 were of *Xenorhabdus* sp. L67 while Lanes 5-7 were of *Xenorhabdus* sp. XN45. No detectable amplification in Lanes 3&4 attributed to degraded DNA samples. Agarose gel (1.2%) was run at 4V/cm for 82 min.

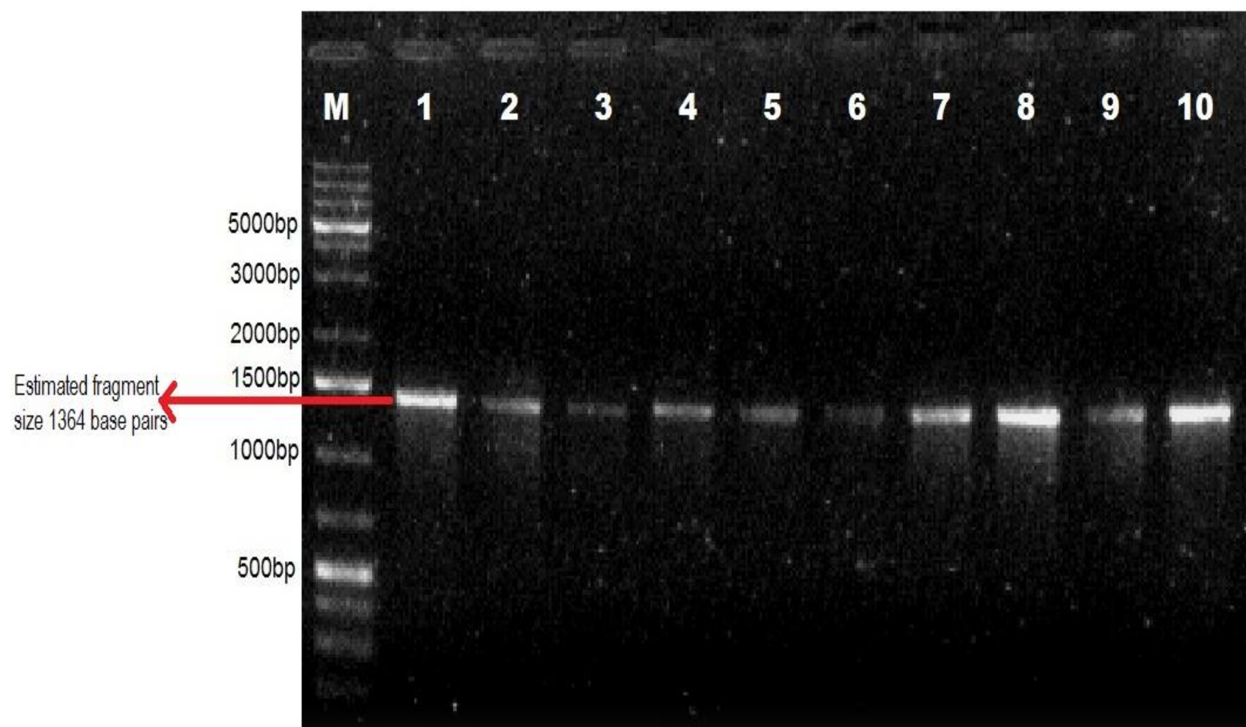

Figure 7: Autoradiograph of agarose gel with Gel purification products of agarose gel 1 and 2.

Lane M was of the molecular size marker. Purified product in Lane 1-3 was from gel 2, lane 2, 3, 4 respectively. Purified products in lane 4-6 from gel 1, lane 7, 8, 9. Purified product in lane 7-9 was of gel 1 lane 4, 5, 6. Purified product of lane 10 was of gel 1, lane 3.Loading sample was 4 µl. Agarose gel (1.2%) run at 3.5V/cm for 78 min.

4.3.0 Characterization of 16s rRNA gene

The nucleotide sequences of the PCR products were obtained (Macrogen, Netherlands). After quality checks and trimming of sequence edges, only 6 sequences were selected for further analysis. One was of was *Xenorhabdus* sp. XN45 and 4 were of *Xenorhabdus* sp. L67. The final one was of was *Xenorhabdus* sp. strain P48. Sequences were trimmed to a final length of 1236 base pairs. Nucleotide sequence base pairs were numbered as per *Escherichia coli* 16s rRNA system of nomenclature (Brosius *et al.,* 1978).

***Xenorhabdus* sp. strain XN45 Kenya partial 16s rRNA gene sequence**

```
          100       110       120       130       140       150       160
   -GGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGAGGGCGGGGGATAACCACTGGAA

       170       180       190       200       210       220       230       240
ACGGTGGCTAATACCGCATAATCTCTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCCTCGGATGAACCCAGATGG

       250       260       270       280       290       300       310       320
GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGA

       330       340       350       360       370       380       390       400
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC

       410       420       430       440       450       460       470       480
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTG

       490       500       510       520       530       540       550       560
ACGTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT

       570       580       590       600       610       620       630       640
TACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAG

       650       660       670       680       690       700       710       720
ACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCG

       730       740       750       760       770       780       790       800
GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT

       810       820       830       840       850       860       870       880
AGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCG

       890       900       910       920       930       940       950       960
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT

       970       980       990      1000      1010      1020      1030      1040
CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTCGGCAGAGATGCGGAAGTGCCTTCGGGCACCGTG

      1050      1060      1070      1080      1090      1100      1110      1120
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCT

      1130      1140      1150      1160      1170      1180      1190      1200
TTGTTGCCAGCACTTCGGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAAGAAGGTGGGGATGACGTCAAGTCA

      1210      1220      1230      1240      1250      1260      1270      1280
TCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC

      1290      1300      1310      1320      1330
CTCATAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGA----
```

Sequence length 1236 base pairs.
Sequence numbering based on *E. coli* 16s rRNA gene corresponding to positons 96-1331

Figure 8: Sequence listing of partial 16s rRNA gene of *Xenorhabdus* sp. XN45

Xenorhabdus sp. strain L671 Kenya partial 16s rRNA gene sequence

```
                  100       110       120       130       140       150       160
             |....|....|....|....|....|....|....|....|....|....|....|....|....|
             -GGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGAGGGCGGGGGATAACCACTGGAA

         170       180       190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGGTGGCTAATACCGCATAATCTCTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATGG

         250       260       270       280       290       300       310       320
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGA

         330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC

         410       420       430       440       450       460       470       480
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTG

         490       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT

         570       580       590       600       610       620       630       640
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAG

         650       660       670       680       690       700       710       720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCG

         730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT

         810       820       830       840       850       860       870       880
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCG

         890       900       910       920       930       940       950       960
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT

         970       980       990      1000      1010      1020      1030      1040
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTCGGCAGAGATGCGGAAGTGCCTTCGGGCACCGTG

        1050      1060      1070      1080      1090      1100      1110      1120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCT

        1130      1140      1150      1160      1170      1180      1190      1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTTGCCAGCACTTCGGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAAGAAGGTGGGGATGACGTCAAGTCA

        1210      1220      1230      1240      1250      1260      1270      1280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCATGGCCCTTACGAGTAGGCCTACACACGTGCTTCAATGGCAGATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC

        1290      1300      1310      1320      1330
....|....|....|....|....|....|....|....|....|....|....|
CTCATAAAGTCTGTCGTAGTGGGGATTGGAGTCTGCAACTCGACTCCATGA----
```

Sequence length 1236 base pairs
Sequence numbering based on *E. coli* 16s rRNA gene corresponding to postions 96-1331

Figure 9: Sequence listing of partial 16s rRNA gene of *Xenorhabdus* sp. L671

Xenorhabdus sp. strain L672 Kenya partial 16s rRNA gene sequence

```
                  100       110       120       130       140       150       160
               -GGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGAGGGCGGGGGATAACCACTGGAA

         170       180       190       200       210       220       230       240
ACGGTGGCTAATACCGCATAATCTCTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATGG

         250       260       270       280       290       300       310       320
GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGA

         330       340       350       360       370       380       390       400
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC

         410       420       430       440       450       460       470       480
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTG

         490       500       510       520       530       540       550       560
ACGTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT

         570       580       590       600       610       620       630       640
TACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAG

         650       660       670       680       690       700       710       720
ACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCG

         730       740       750       760       770       780       790       800
GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT

         810       820       830       840       850       860       870       880
AGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCG

         890       900       910       920       930       940       950       960
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGGGAGCATGTGGTTTAATT

         970       980       990      1000      1010      1020      1030      1040
CGATGCAACGCGAAGACCCTTACCTATTCTTGACATCCACGGAATTCGGCAGAGATGCGGAAGTGCCTTCGGGCCCCGTG

        1050      1060      1070      1080      1090      1100      1110      1120
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCT

        1130      1140      1150      1160      1170      1180      1190      1200
TTGTTGCCAGCACTTCGGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCA

        1210      1220      1230      1240      1250      1260      1270      1280
TCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC

        1290      1300      1310      1320      1330
CTCATAAAGTCTGTAATAGTCCGGATTGGAGTCTGCAACTCGACTCCATGA----
```

Sequence length 1236 base pairs
Sequence numbering based on _E. coli_ 16s rRNA gene corresponding to positions 96-1331.

Figure 10: Sequence listing of partial 16s rRNA gene of *Xenorhabdus* sp. L672

***Xenorhabdus* sp. strain L673 partial 16s rRNA gene sequence**

```
                 100       110       120       130       140       150       160
                |....|....|....|....|....|....|....|....|....|....|....|....|....|
               -GGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGAGGGCGGGGGATAACCACTGGAA

         170       180       190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGGTGGCTAATACCGCATAATCTCTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATGG

         250       260       270       280       290       300       310       320
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGA

         330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC

         410       420       430       440       450       460       470       480
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTG

         490       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT

         570       580       590       600       610       620       630       640
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAG

         650       660       670       680       690       700       710       720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCG

         730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT

         810       820       830       840       850       860       870       880
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCG

         890       900       910       920       930       940       950       960
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT

         970       980       990      1000      1010      1020      1030      1040
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTCGGCAAAGATGCGGAAGTCCCTTCGGGCACCGTG

        1050      1060      1070      1080      1090      1100      1110      1120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGACAGGTGCTGCATGGCCGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCT

        1130      1140      1150      1160      1170      1180      1190      1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTTGCCACCACTTCGGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCA

        1210      1220      1230      1240      1250      1260      1270      1280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC

        1290      1300      1310      1320      1330
....|....|....|....|....|....|....|....|....|....|....|
CTCATAAAGTCTGTCGTGGTCCGGATTGGAGTCTGCAACTCGACTCCATGA----
```

Sequence length 1236 base pairs
Sequence numbering based on *E. coli* 16s rRNA gene corresponding to positions 96- 1331

Figure 11: Sequence listing of partial 16s rRNA gene of *Xenorhabdus* sp. L673

Xenorhabdus sp. strain L675 Kenya partial 16s rRNA gene sequence

```
                   100       110       120       130       140       150       160
               |....|....|....|....|....|....|....|....|....|....|....|....|....|
              -GGCGAGCGGCGGACGGTTGAGTAATGTCTGGGGATCTGCCCGAGGGCGGGGGATAACCACTGGAA

       170       180       190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGGTGGCTAATACCGCATAATCTCTGAGGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATGG

       250       260       270       280       290       300       310       320
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGA

       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC

       410       420       430       440       450       460       470       480
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTGGGGAGGAAGGCACAGGGTCGAATACCCCCTGTGATTG

       490       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT

       570       580       590       600       610       620       630       640
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAG

       650       660       670       680       690       700       710       720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCG

       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT

       810       820       830       840       850       860       870       880
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACGCGTTAAATCGACCG

       890       900       910       920       930       940       950       960
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT

       970       980       990      1000      1010      1020      1030      1040
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTCGGCAGAGATGCGGAAGTCCCTTCGGGCACCGTG

      1050      1060      1070      1080      1090      1100      1110      1120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCT

      1130      1140      1150      1160      1170      1180      1190      1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTTGCCAGCACTTCGGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCA

      1210      1220      1230      1240      1250      1260      1270      1280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC

      1290      1300      1310      1320      1330
....|....|....|....|....|....|....|....|....|....|....|
CTCATAAAGTCTGTCTTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGA----
```

Sequence length 1236 base pairs
Sequence numbering based on E. coli 16s rRNA gene corresponding to positions 96-1331.

Figure 12: Sequence listing of partial 16s rRNA gene of _Xenorhabdus_ sp. L675

Xenorhabdus sp. strain P48 Kenya partial 16s rRNA gene sequence

```
                    100       110       120       130       140       150       160
          ....|....|....|....|....|....|....|....|....|....|....|....|....|
          -GACGAGTGGCGGACGGGTGAGTAATGTCTGGGAATCTGCCCGATGGAGGGGGATAACCACTGGAA

       170       180       190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGGTAGCTAATACCGCATAACCTCTCAAGACCAAAGTGGGGGACCTTCGGGCCTCTCGCCATCGGATGTGCCCAGATGG

       250       260       270       280       290       300       310       320
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAA

       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTGAGACACGGTCCAGATTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC

       410       420       430       440       450       460       470       480
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGAGAAGGTTAATAACCTTGTCGATTG

       490       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT

       570       580       590       600       610       620       630       640
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TACTGGGCGTAAAGCGCACGCAGGCGGTCTATCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAA

       650       660       670       680       690       700       710       720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG

       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT

       810       820       830       840       850       860       870       880
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCG

       890       900       910       920       930       940       950       960
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT

       970       980       990      1000      1010      1020      1030      1040
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTG

      1050      1060      1070      1080      1090      1100      1110      1120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCT

      1130      1140      1150      1160      1170      1180      1190      1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA

      1210      1220      1230      1240      1250      1260      1270      1280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC

      1290      1300      1310      1320      1330
....|....|....|....|....|....|....|....|....|....|
CTCCTAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGC----
```

Sequence length 1236 base pairs
Sequence numbering based on *E. coli* 16s rRNA gene corresponding to positions 96-1331

Figure 13: Sequence listing of partial 16s rRNA gene of *Xenorhabdus* sp. P48

4.4.0 Phylogenetic reconstruction

Figure 14: Phylogeny of *Xenorhabdus*

Molecular phylogenetic analysis by Maximum Likelihood method. Clades highlighted in grey represent those containing the 6 species isolated from this study. The scale bar used represents 10 nucleotide substitutions per sequence.

Figure 15: Geographic diversity of *Xenorhabdus*

This is a replicate of figure 14, albeit showing the regions of isolation, instead of species names of the respective operational taxonomic units (OTU). Clades highlighted in grey represent those containing the 6 species isolated from this study. The scale bar used represents the branch length of 10 nucleotide substitutions per sequence.

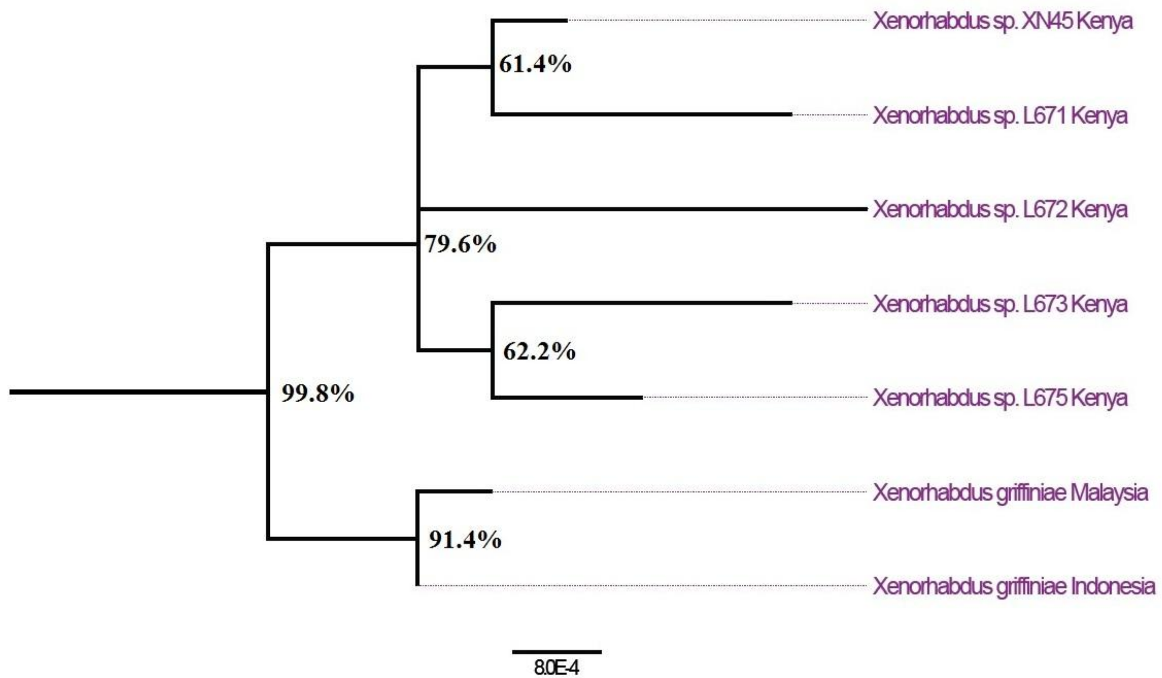

Figure 16: *Xenorhabdus griffiniae* clade as highlighted in grey from figures 14 and 15.

It contained five of the *Xenorhabdus* spp. isolated in this study. The remaining members of the clade had the unifying characteristic of *Xenorhabdus griffiniae* as their species designation. The percentage of trees, from 500 replicates in which the associated taxa clustered together is shown next to the branches. The scale bar used represents the branch length of one nucleotide substitution per sequence.

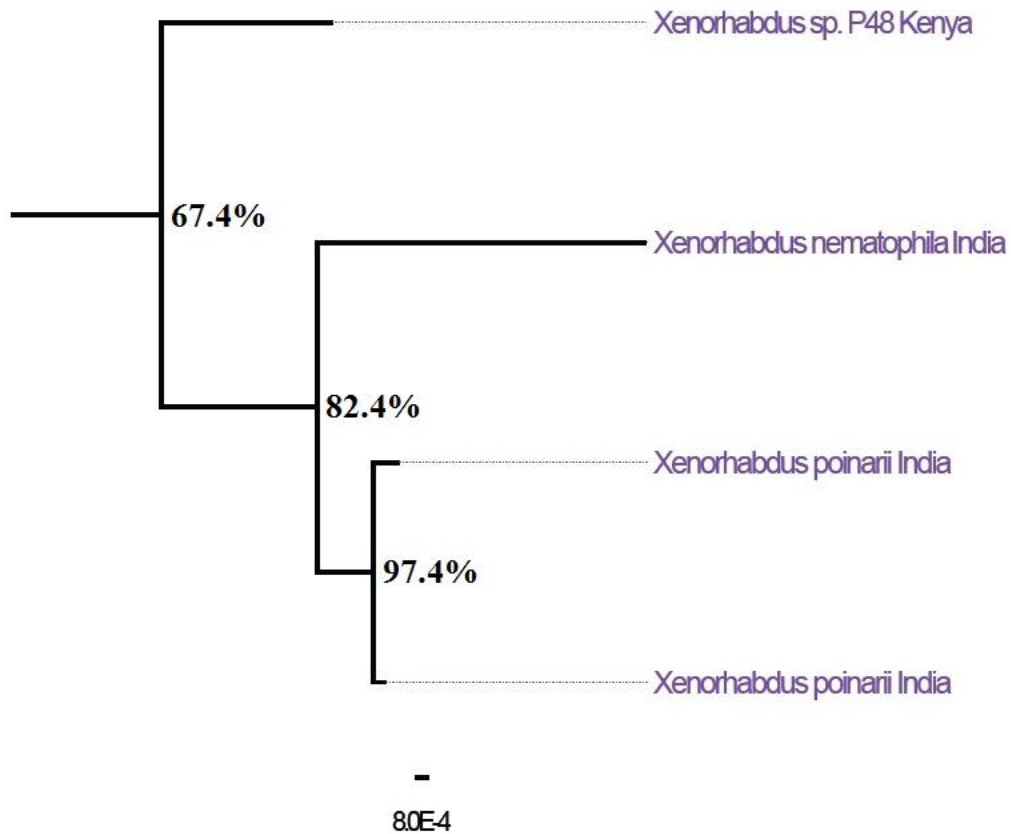

Figure 17: *Xenorhabdus* India clade as highlighted in grey from figures 14 and 15.

It contained *Xenorhabdus* sp. P48 which was isolated in this study. The remaining members of the clade had the unifying characteristic of India as their geographic region of isolation. The percentage of trees, from 500 replicates in which the associated taxa clustered together is shown next to the branches. The scale bar used represents the branch length of one nucleotide substitution per sequence.

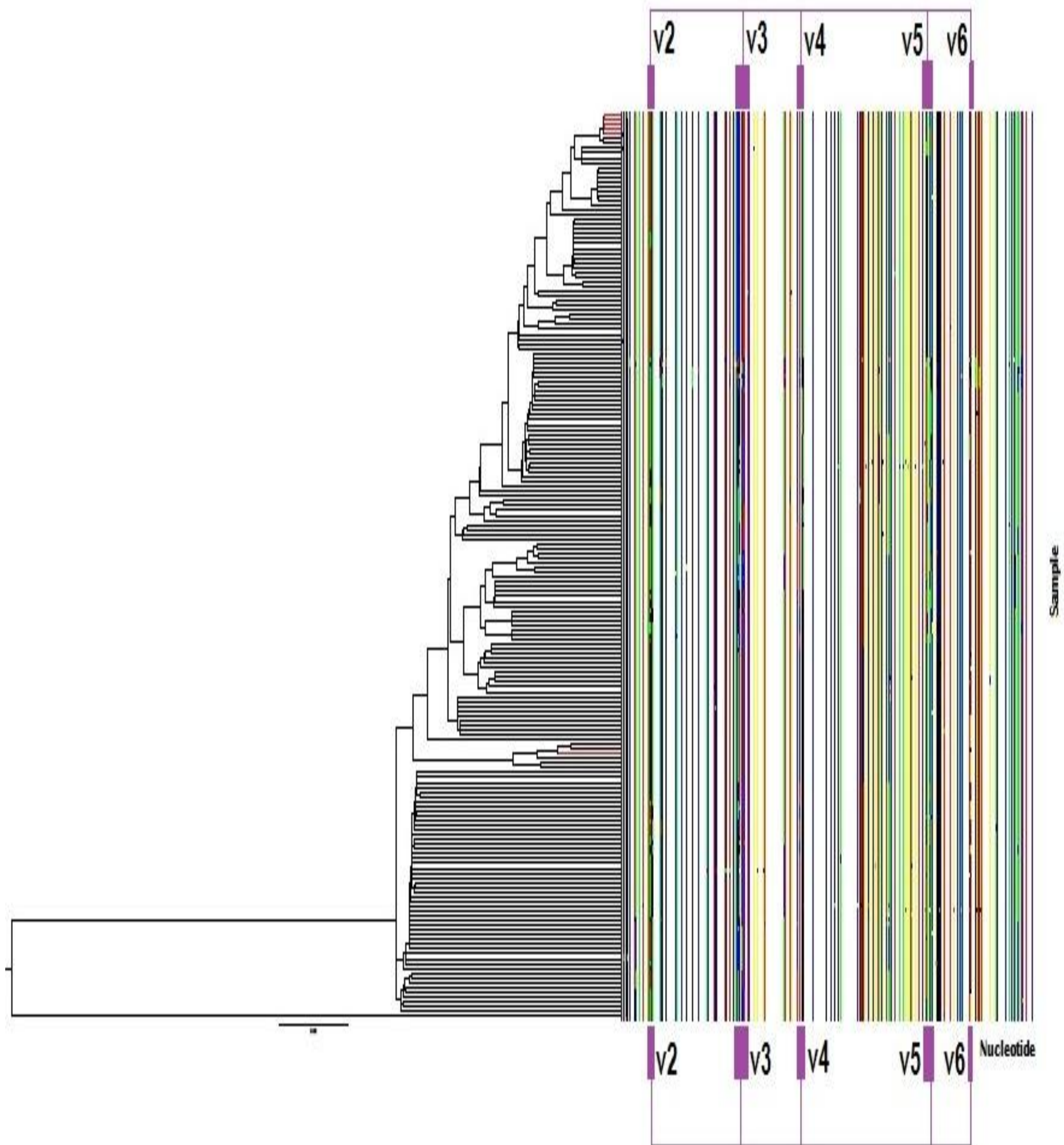

Figure 18: Heat map of 16s rRNA gene of *Xenorhabdus*.

The hyper variable regions within the 16s rRNA gene of the genus were identified from the heat map. They were highlighted in purple and corresponded to regions v2-v6. Concomitant phylogenetic tree was reconstructed with maximum likelihood method with *P. aeruginosa* as the root sequence.

4.5.0 Antimicrobial activity of *Xenorhabdus* spp. isolated from Kenya

4.5.1 Percentage growth inhibition formula

Houard *et al.* (2013) described a formula for percentage growth inhibition by an antimicrobial compound x.

$$\frac{(1 - g_x)}{g} \times 100$$

Where

g = absorbance value by of a broth culture only with no antimicrobial, and

g_x = absorbance value by the same broth culture in the presence of antimicrobial compound x.

For this formula turbidity of cell cultures was used as a measure of growth. It was measured by reading the absorbance value by a culture, by a light beam across a 1cm light path using a spectrophotometer. In this study, the inclusion of a correction factor due to growth inhibition of the culture medium was proposed. Houard *et al.* (2013) formula was accordingly modified as outlined below:

Formula 1

If,

$i = 1 - g$

Where,

1= optimal growth (theoretical value)

i = growth inhibition

g = Absorbance value of a broth culture test bacterium only, by a light beam of 600_{nm} ($A600_{nm}$)

Then,

Percentage i in culture medium only is

$$\frac{(1 - g)}{g} \times 100$$

If,

x is the antimicrobial compound and g_x is the $A600_{nm}$ of a broth culture containing x

Then,

Inhibition by antimicrobial x is

$$i_x = 1 - g_x$$

Thus,

Percentage i_x is

$$\frac{(1 - g_x)}{g} \times 100$$

The corrected value for inhibition will be,

$$\text{percentage } i_x - \text{percentage } i$$

Which is,

$$= \left\{\frac{(1 - g_x)}{g} \times 100\right\} - \left\{\frac{(1 - g)}{g} \times 100\right\}$$

$$= \{(1 - g_x) - (1 - g)\} \times \frac{100}{g}$$

$$= \{1 - g_x - 1 + g\} \times \frac{100}{g}$$

$$= \{g - g_x\} \times \frac{100}{g}$$

This formula was used to calculate the percentage growth inhibition of the different test antibiotics against *E. faecalis*. Raw data is given in appendix 1. The data was then analyzed and represented graphically, as given in figures 19-21.

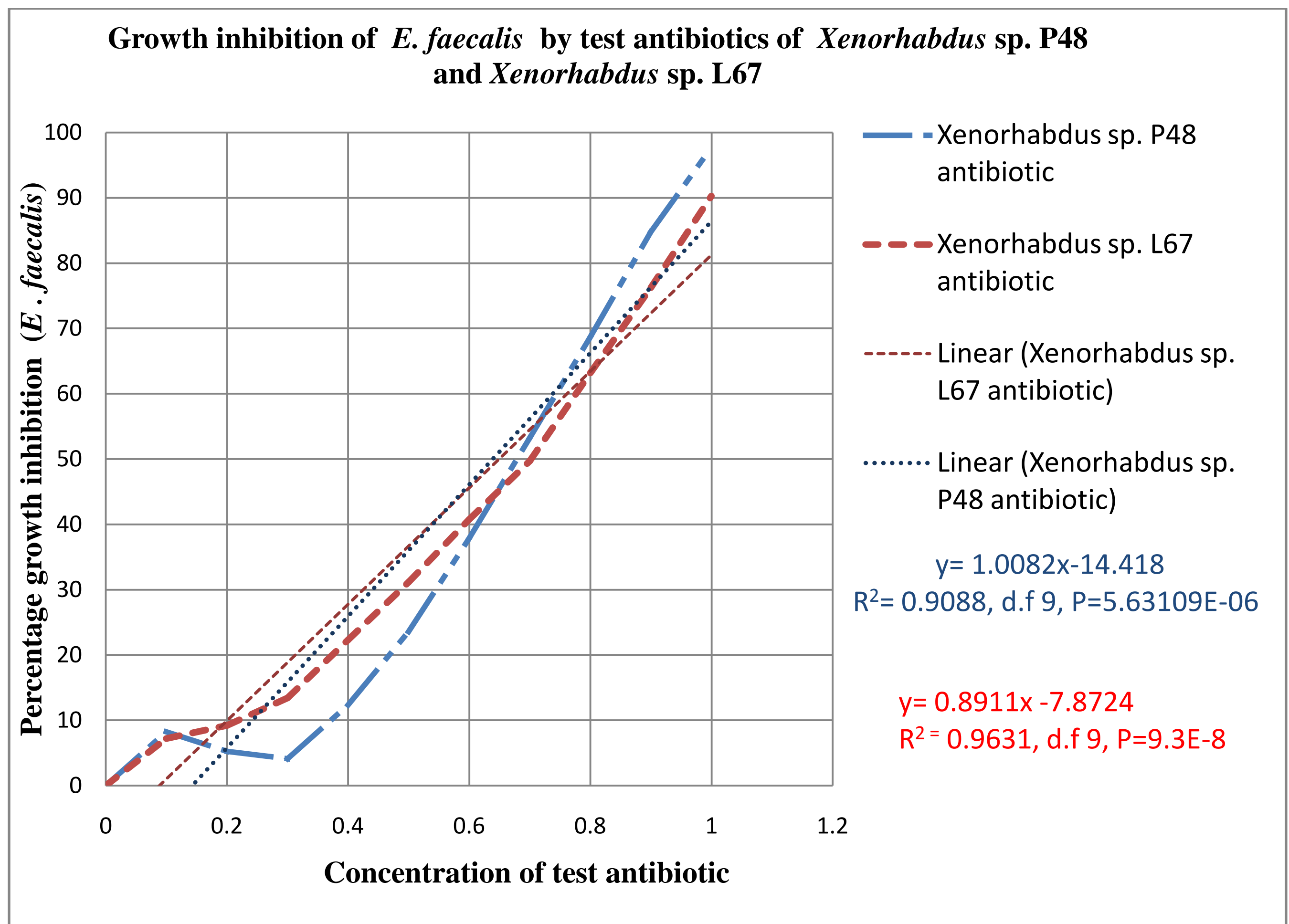

Figure 19: Graph 1 showing growth inhibitions of test antibiotics of *Xenorhabdus* sp. P48 & *Xenorhabdus* sp. L67

Test antibiotics were obtained by 72 h fermentation duration. Percentage inhibition was calculated using the formula $\{g - g_x\}\times 100/g$. Regression equations are given for both series. Note the extremely low P values of both, denoting that these are highly significant statistics. There is a strong correlation between the variables across the series denoted by the high R^2 values. Inhibition tests for *Xenorhabdus* sp. p48 & *Xenorhabdus* sp. L67 test antibiotics had average inoculum size $8.06*10^4$ cfu/ml & $6.89*10^4$ cfu/ml respectively. They were carried out in 5 replicates in two reproductions and 6 replicates in three reproductions respectively. Incubation time was 21h.

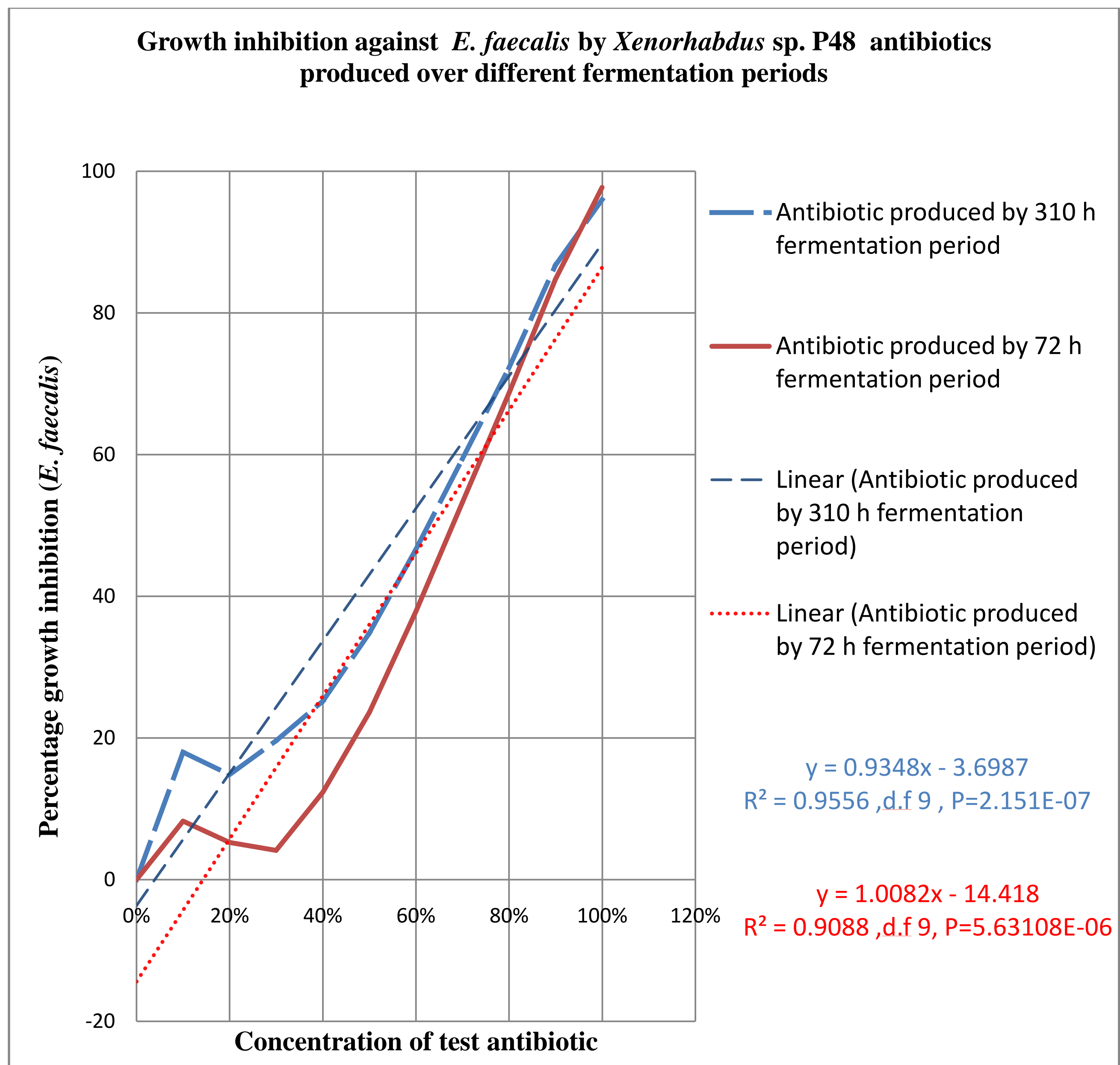

Figure 20: Graph 2 showing effects of fermentation period of *Xenorhabdus* sp. P48 antibiotics on growth inhibition against *E. faecalis*.

Percentage inhibition was calculated using the formula $\{g - g_x\} \times 100/g$. Linear graphs and the respective equations are given for both series. From these, the 310 h culture antibiotic had a higher inhibitory effect than the 72 h culture antibiotic. The extremely low P values of both series denote that these are highly significant statistics. Secondly, there is a strong correlation between growth inhibition and concentration of the antibiotic across both series denoted by the high R^2 values. Inhibition tests for *Xenorhabdus* sp. p48 72 h & *Xenorhabdus* sp. P48 310 h test antibiotics had average inocula sizes of $6.89*10^4$ cfu/ml & $5.51*10^5$ cfu/ml respectively. Inhibition tests were carried out in 3 reproductions each done in duplicate. Incubation time was 22 h.

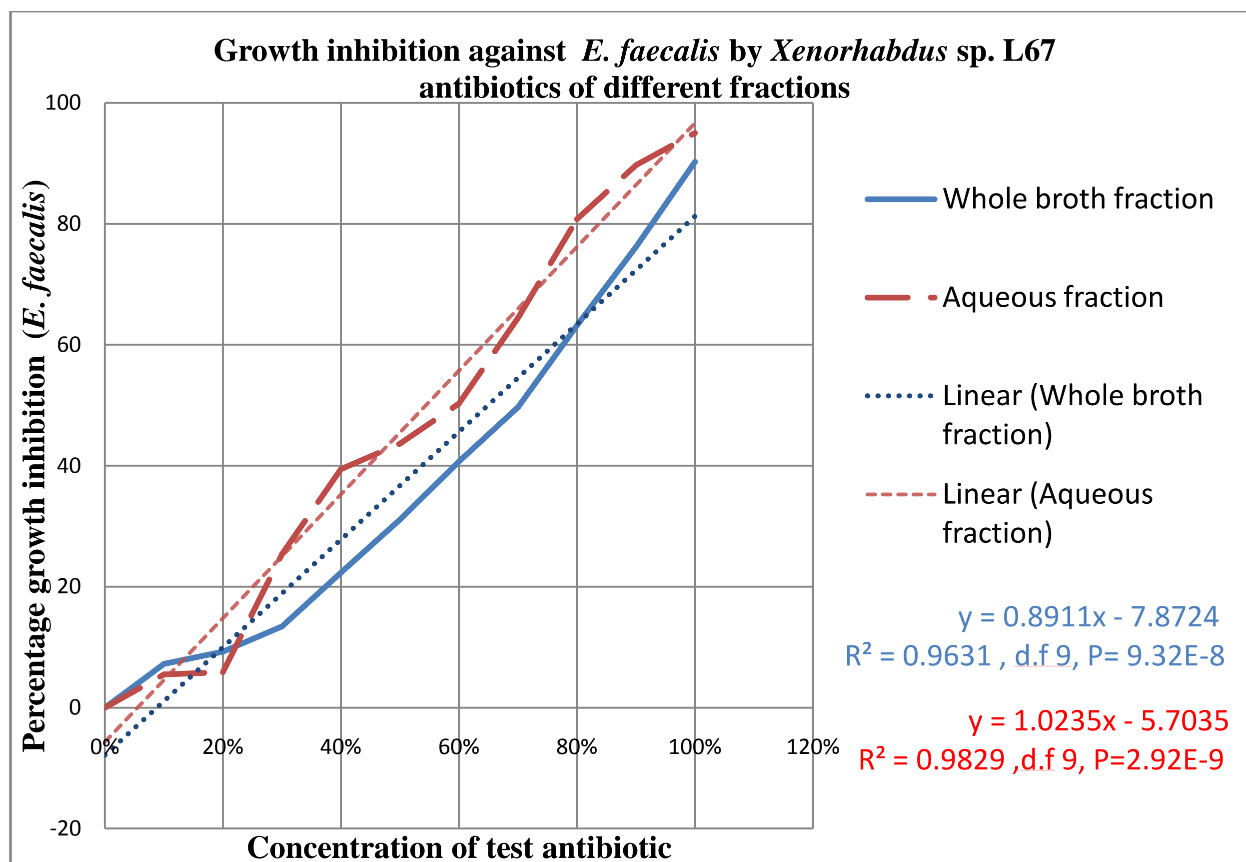

Figure 21: Graph 3 showing growth inhibitions of *Xenorhabdus* sp. L67 antibiotics fractions

Percentage inhibition was calculated using the formula $\{g - g_x\} \times 100/g$. Linear graphs and the respective equations are given for both series. From these, the aqueous fraction had a higher inhibitory effect than the whole broth fraction. The extremely low P values of both series denote that these are highly significant statistics. Secondly, There is a strong correlation between growth inhibition and concentration of the antibiotic across both series denoted by the high R^2 values. Test antibiotics were obtained by a 72 h fermentation duration. Inhibition tests for *Xenorhabdus* sp. L67 whole broth fraction and *Xenorhabdus* sp. L67 aqueous fraction had average inoculum size of $6.89*10^4$ cfu/ml & $1.92*10^5$ cfu/ml respectively. Tests were done in two replicates for the aqueous fraction and 6 replicates for the whole broth fraction. Incubation time was 20 h.

4.5.2 Spectrophotometric Analysis of organic solvent fraction of whole broth extracts

This was carried out on the organic fraction of the whole broth extract of *Xenorhabdus* sp. L67 as previously described. It had a peak uv absorption at 218_{nm} when dissolved in methanol.

4.6.0 Plate inhibition assay of organic fraction of whole broth extracts

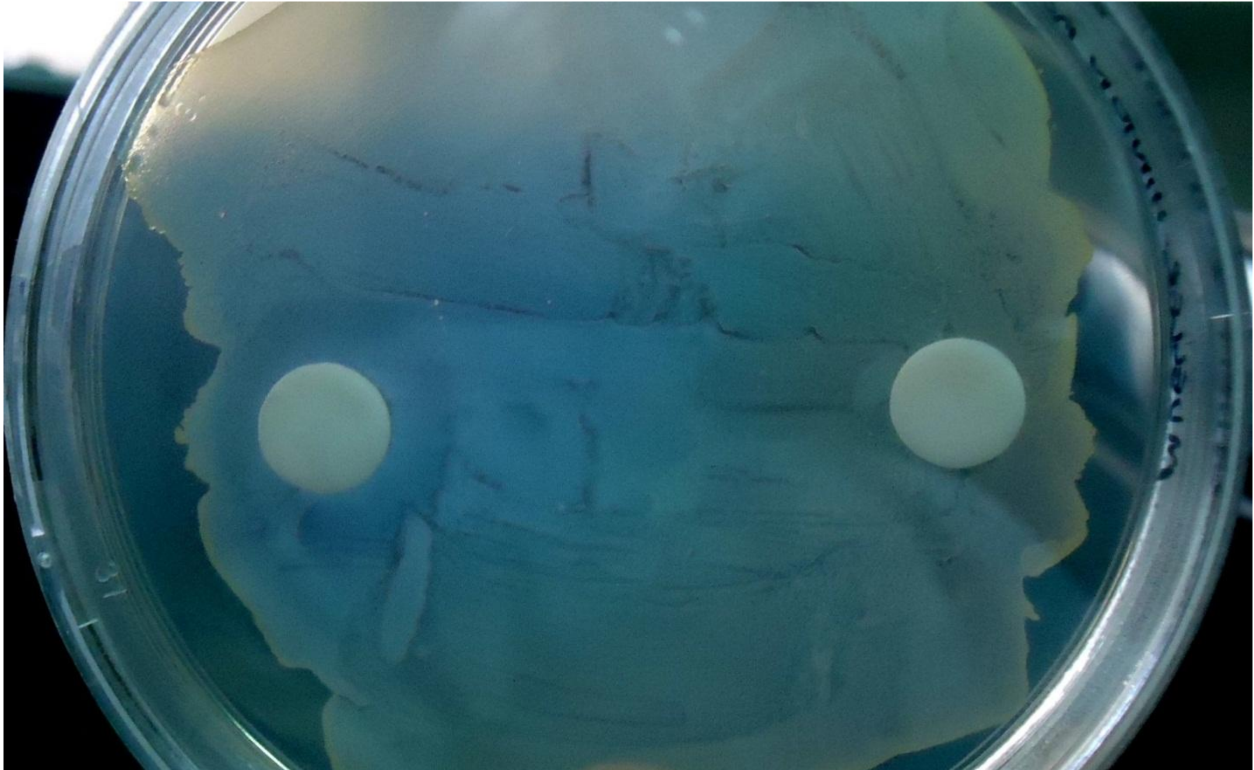

Figure 22: Plate inhibition assay of *Xenorhabdus* sp. XN45 antibiotics against MRSA

The left filter paper disc contained the test antibiotic dissolved in DMSO (50 µl). While the right filter paper disc contained DMSO only (50 µl). Note the zone of inhibition around the left filter paper disc. Inhibition test was carried out as previously described in section 3.6.2.Test antibiotic was the organic solvent fraction of the whole broth extracts from *Xenorhabdus* sp. XN45

CHAPTER 5

5.0 DISCUSSION

5.1 Phylogenetic reconstruction of *Xenorhabdus*

A partial 16s rRNA gene fragment of an interpolated fragment length of approximately 1500 base pairs was isolated. This is corroborated with the primers used, as their target sequence of amplification was 1364 base pairs (Lane, 1991). All fragments aligned at one level denoting uniformity in length. The sequences of the gene fragments corresponded to positions 96 -1331 of the *E. coli* 16s rRNA gene (Brosius *et al.,* 1978). This was significant as it allowed assessment of the loci for sufficient variability for a robust phylogenetic analysis. This was important as it is not the length of a 16s rRNA gene fragment, but rather the number of hyper variable regions it contains that provide for accuracy in a phylogenetic analysis of closely related species (Olsen and Woese, 1993; Van de Peer *et al.,* 1996; Wiley *et al.,* 2008).

The hypervariable regions contained in the fragment were sufficient for a robust analysis. From the heat map,five hyper variable regions of the bacterial 16s rRNA gene were identified (Van de Peer *et al.,* 1996). From their positions, they corresponded to vr2-vr6 of the 16s rRNA gene (Chakravorty *et al.,* 2007). Huse *et al.* (2008) demonstrated that hyper variable regions v3 and v6 alone, yielded phylogenetic analyses similar to those from full length 16s rRNA sequences. Chakravorty *et al.* (2007) reiterated this by demonstrating that regions v2, v3 and v6 alone contained sufficient sequence diversity for accurate phylogenetic analysis.

All twenty-four described species of *Xenorhabdus* were captured in the phylogenetic reconstruction with *X. bovienii* accounting for the largest species percentage. This was attributed to the fact that *X. bovienii* is the obligate symbiont of four nematode species. They are *S. affinie*, *S. intermedium*, *S. kraussei*, and *S. feltiae* (Boemare and Arkhurst, 2006). It is this promiscuity of *X. bovienii* as a symbiont of nematodes with a wide geographical distribution that may account for it being the dominant species isolated worldwide.

Similar species clustered together as shown in the phylogenetic tree of figure 15. Despite the variability in regions of isolation, illustrated in figure 16, or differences in researchers, materials

and methods, dates of isolation and laboratories as given by the sequence information through the accession numbers (Appendix 2), similar species clustered together. This validated the accuracy of the phylogenetic reconstruction and the methods used to arrive at it. This was significant as two different *Xenorhabdus* species 16s rRNA sequences can be 98% identical often confounding its use in molecular identification (Boemare and Tailliez, 2009).

In terms of identifying the clades to which the Kenyan isolates belonged to, two clades were highlighted as shown in the phylogenetic in figure 15. The first clade was named the *X. griffiniae* clade as expounded in the phylogenetic tree of figure 17 as both the described species of this clade belonged to *X. griffiniae*. One of the described *X. griffiniae* was isolated from Malaysia while the second *X. griffiniae* was isolated from Indonesia (Tailliez *et al.,* 2006). *Xenorhabdus* sp. L67 that were isolated in this study, clustered in this clade. *Xenorhabdus* sp. XN45 also from this study, clustered in this clade. The scale bar shown of phylogenetic tree of figure 17 represented the branch length of one nucleotide substitution per 1236 base pairs. From it, one can note the less than 2% dissimilarity among all 8 species in the clade further reinforcing its validity as the *Xenorhabdus griffiniae* clade. This clade was furthest from the root. This denoted that it contained extant species that were rapidly evolving and thus had the youngest *Xenorhabdus* species (Woese, 1987).

The high bootstrap values also added to the validity of the clade. The oldest ancestral node of the clade had a bootstrap value 99.8 % and four out of five of the other nodes of the clade had boot strap values above 50%. It was thus concluded that all species in this clade were *Xenorhabdus griffiniae*. The species isolated from this study were therefore identified as *Xenorhabdus griffiniae* and named as following. *Xenorhabdus* sp. L671, *Xenorhabdus* sp. L672, *Xenorhabdus* sp. L673, *Xenorhabdus* sp. L675 were all named *Xenorhabdus griffiniae* strain L67. *Xenorhabdus* sp. XN45 was named *Xenorhabdus griffiniae* strain XN45. This is the first record of isolation of *Xenorhabdus griffiniae* from Africa.

The other Kenyan isolate *Xenorhabdus* sp. P48 fell in a clade that was designated *Xenorhabdus* India clade as shown in phylogenetic tree of figure 18. Despite the fact that members of this clade had designated names, none of the names corroborated with the rest of the tree as illustrated in figure 15. *Xenorhabdus poinarii* CICR-WR, *Xenorhabdus poinarii* PDBCSCX5 and *Xenorhabdus nematophila* PDBCSCX1 did not fall in *X. poinarii* nor *X. nematophila* species

clades respectively. However, the species of this clade had the common characteristic of the geographical region of isolation being India. The clade was thus named *Xenorhabdus* India clade. This clade was also the oldest clade as it was nearest to the root (Woese, 1987).

As per the phylogenetic reconstruction, the rejection of *Xenorhabdus nematophila* PDBCSCX1 species as a bona fide *Xenorhabdus nematophila* and *Xenorhabdus poinarii* CICR-WR ,*Xenorhabdus poinarii* PDBCSCX5 as bona fide *Xenorhabdus poinarii* is proposed based on their evolutionary distance from the respective *Xenorhabdus nematophila* and *Xenorhabdus poinarii* clades as depicted in phylogenetic tree of figure 15. This finding drew the following conclusions. Each of the following operational taxonomic units *Xenorhabdus poinarii* PDBCSCX5 and *Xenorhabdus nematophila* PDBCSCX1 and *Xenorhabdus* sp. p48 Kenya, represents a distinct *Xenorhabdus* species. Secondly, based on the evolutionary distances, each of these species represents a novel species. *Xenorhabdus* sp. P48 Kenya thus represents a novel *Xenorhabdus* species. With the inclusion of *Xenorhabdus* P48 Kenya in the clade, the unifying characteristic of these species was their tropical geographical region of isolation giving the idea that bacteria in this clade are able to grow at temperatures of 20-32°C; common in the tropics.

5.2 Antibiotic activity of *Xenorhabdus* spp.

Xenorhabdus are a genus known for the production of antibiotics (Boemare and Akhurst, 2006). Thus, the inhibitory effect of the whole broth extracts of *Xenorhabdus griffiniae* L67 and *Xenorhabdus griffiniae* XN45 and *Xenorhabdus* sp. P48 was assessed. Houard *et al.* (2013) developed a formula for the calculation of percentage growth inhibition by an antimicrobial compound using turbidity is used as a measure of growth. However, it did not account for sub optimal growth of the bacteria not as a result of inhibition by the antimicrobial compound, but as a result of broth media itself. The formula was therefore modified to correct for inhibition by the broth media. It is given below:

Percentage growth inhibition by antimicrobial x is equal to

$$\{g - g_x\} \times \frac{100}{g}$$

Where g = $A600_{nm}$ of a broth culture test bacteria, and g_x =$A600_{nm}$ of a broth culture containing antimicrobial x.

This formula was used for calculating percentage inhibition by an antimicrobial compound with raw data provided in Appendix 2. One of its limitations was that it underestimated inhibition values at higher concentrations of the test antimicrobial. For example, at neat concentration of the test antimicrobial there was complete inhibition when one visually observed the test tubes. This denoted percentage growth inhibition of 100%. However, when one calculated the percentage growth inhibition with the formula, the same treatment gave an average percentage growth inhibition of 94% (figures 19,20,21).

Whole broth extracts from *Xenorhabdus griffiniae* L67 and *Xenorhabdus* sp. P48 were both inhibitory to *E. faecalis* (figure 19). Although both extracts were made under similar conditions, they had dissimilar antibiotic activities against *E. faecalis* depicted by the differing regression equations of the two curves. This denotes a difference in antibiotic profiles. This corroborates with findings of Fodor *et al.* (2010) that different *Xenorhabdus* species have different antibiotic profiles.

Fermentation duration significantly affects antibiotic activity from the same species. From the regression lines the antibiotics produced by 315 h fermentation period was more active than the one produced by 72 h fermentation duration (figure 20). This corroborates with findings of Furgani *et al.* (2008) that optimal antibiotic production in *Xenorhabdus* species occurs at 144 h denoting that the 72 h was a suboptimal duration.

The aqueous fraction of the whole broth extract of *Xenorhabdus griffiniae* L67 had antibiotic activity against *E. faecalis*. It was more inhibitory to *E. faecalis* as compared to the whole broth extract form the same bacterium (figure 21). This result demonstrated that *Xenorhabdus griffiniae* L67 had potent water-soluble antibiotics against gram-positive bacteria. A possible identity of these antibiotics is the water-soluble xenocoumacins of *Xenorhabdus* (Gregson and McInerney, 1989). They have been documented to be effective against gram-positive bacteria (Park *et al.,* 2009).

The organic fraction of the broth extract of *Xenorhabdus griffiniae* XN45 was inhibitory to Methicillin resistant *Staphylococcus aureus* (figure 22). The organic fraction of *Xenorhabdus griffiniae* L67 had a peak uv-vis absorption of 218 $_{nm}$ when dissolved in methanol. PAX lipopeptides have been shown to be active against antibiotic resistant bacteria including

Methicillin resistant *Staphylococcus aureus* (Gualtieri *et al.,* 2012). Furthermore, PAX lipopeptides from *Xenorhabdus nematophila* have a peak uv-vis absorption of 214 $_{nm}$ when dissolved in methanol (Gualtieri *et al.,* 2009). PAX from *Xenorhabdus caballinasii* also have a peak uv-vis absorption of 214 $_{nm}$ when dissolved in methanol (Houard *et al.,* 2013). These two findings strongly corroborate with PAX lipopeptides as the possible identity of the specific chemical compounds of the organic fraction of *Xenorhabdus griffiniae* XN45 that were active against Methicillin resistant *Staphylococcus aureus*.

An example of a characterized PAX lipopeptide is Nemaucin. It was highly active against Methicillin resistant *Staphylococcus aureus*, at 30-fold less the concentration of the current standard treatment, vancomycin. More so, it possessed low cytotoxicity levels against human cell lines (Gualtieri *et al.,* 2012). It is a paragon of the efficacy and low toxicity of PAX lipopetides.It is yet to move into clinical use.

Currently, there is no documented record of isolated PAX from *Xenorhabdus griffiniae.* This result documents antibiotics from *Xenorhabdus griffiniae* effective against antibiotic resistant bacteria, more specifically Methicillin resistant *Staphylococcus aureus.* This is highly significant as it proves the existence of local antibiotics effective against Kenyan isolates of antibiotic resistant bacteria. This provides foundational steps towards utilizing *Xenorhabdus* bacteria for improved healthcare.

CHAPTER 6

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The main objective of this study was to identify *Xenorhabdus* isolates, through establishment of evolutionary relationships, which produced antibiotics. Data generated from this study provides presumptive identification of the isolates coupled with an analysis of their antibiotic activity. Ultimately, this affords foundational steps towards drug development for clinical use. Two of the isolates were identified as *Xenorhabdus griffiniae*. These were given the names *Xenorhabdus griffiniae* XN45 and *Xenorhabdus griffiniae* L67. The third isolate was identified as an undescribed species. It was named *Xenorhabdus* sp. P48. All three species produced antibiotics effective against gram-positive bacteria. More so, *Xenorhabdus griffiniae* produced antibiotics that were effective against Methicillin resistant *Staphylococcus aureus*. These antibiotics were presumptively identified as lipopeptides and have potential as antimicrobial agents with clinical application.

6.2 Recommendations

The following recommendations are given

1. A partial 16s gene fragment, of position 91-1331,was used to successfully reconstruct the phylogeny of *Xenorhabdus*. This fragment should be used in phylogenetic reconstruction of other bacteria genera.

2. The phylogenetic reconstruction of the *Xenorhabdus* genus identified *Xenorhabdus griffiniae* and *Xenorhabdus* sp. P48. This method should be used for the molecular identification of bacteria with highly similar 16s rRNA gene sequences.

3. Crude extracts from *Xenorhabdus griffiniae* were inhibitory to antibiotic resistant gram-positive cocci. This bacterium should be used as a source of natural products for drug development, especially in view of exacerbating antimicrobial resistance to current antibiotics.

4. Analysis of the *Xenorhabdus griffiniae* crude extracts inferred effective antimicrobial compounds as lipopeptides. Chemical techniques that purify lipopeptides should be employed to isolate these compounds from the crude extracts. The isolated lipopeptides should be used in further studies for clinical drug development.

5. *Xenorhabdus griffinae* secreted water-soluble antimicrobial compounds effective against the gram-positive cocci, *E. faecalis*. These compounds are drug candidates for the treatment of urinary tract infections. Purification of these compounds from the broth extracts should be done to determine their chemical structure and minimum inhibitory concentrations.

6. *Xenorhabdus griffiniae* and *Xenorhabdus* sp. P48 were identified based on molecular characterization. Further identification should be done on the same bacteria based on morphological and biochemical characterization.

7. Over 30 *Steinernema* nematode isolates are currently deposited in the Kenya. Only one has been fully characterized to species level. The complete repository of nematodes in Kenya should be characterized and identified to species level.

REFERENCES

Adams, B. J., Fodor, A., Koppenhöfer, H. S., Stackebrandt, E., Stock, P., & Klein, M. G. (2006). Biodiversity and systematics of nematode–bacterium entomopathogens. *Biol Control, 37*(1), 32-49.

Akhurst, R. J. (1980). Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *J Gen Microbiol, 121*(2), 303-309.

Akhurst, R., & Boemare, N. (1988). A numerical taxonomic study of the genus *Xenorhabdus* (Enterobacteriaceae) and proposed elevation of the subspecies of *X. nematophilus* to species. *J Gen Microbiol, 134*(7), 1835-1845.

Barbas, C. F., Burton, D. R., Scott, J. K., & Silverman, G. J. (2007). Quantitation of DNA and RNA. *Cold Spring Harb Protoc, 11*.

Boemare, N. (2002). Systematics of *Photorhabdus* and *Xenorhabdus*. In R. Gaugler (Ed.), *Entomopathogenic Nematology* (pp. 35-56). New Brunswick: CABI Publishing

Boemare, N., & Akhurst, R. (2006). The genera *Photorhabdus* and *Xenorhabdus*. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt(Eds.), *The Prokaryotes* (VOL XI). New York: Springer.

Boemare, N., & Tailliez, P. (2009). Molecular approaches and techniques for the study of entomopathogenic bacteria. In S. P. Stock, J. Vandenberg, I. Glazer, & N. Boemare (Eds.), *Insect Pathogens: Molecular Approaches and Techniques* (pp. 32-49). Wallingford: CABI Publishing.

Bright, M., & Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol, 8*(3), 218-230.

Brosius, J., Palmer, M. L., Kennedy, P. J., & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *P Natl Acad Sci, 75*(10), 4801-4805.

Brown, S. E., Cao, A. T., Hines, E. R., Akhurst, R. J., & East, P. D. (2004). A novel secreted protein toxin from the insect pathogenic bacterium *Xenorhabdus nematophila*. *J Biol Chem, 279*(15), 14595-14601.

Burianek, L. L., & Yousef, A. E. (2000). Solvent extraction of bacteriocins from liquid cultures. *Lett Appl Microbiol, 31*(3), 193-197.

Chakravorty, S., Helb, D., Burday, M., Connell, N., & Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Meth, 69*(2), 330–339.

Chapman, G. (1945). The significance of Sodium Chloride in studies of *Staphylococci*. *J Bacteriol*, 50 , 201-203.

Chaston, J. M., Suen, G., Tucker, S. L., Andersen, A. W., Bhasin, A., Bode, E., & Bode, H. B. (2011). The entomopathogenic bacterial endosymbionts *Xenorhabdus* and *Photorhabdus*: convergent lifestyles from divergent genomes. *Plos One, 6*(11).

Clinical Laboratory and Standards Institute. (2007). *Performance standard for antimicrobial susceptibiltiy testing;Seventh Information Supplement.* Pennsylvania : Clinical Laboratory and Standards Institute.

Cox, M. M., & Nelson, D. L. (2008). *Lehninger Principles of Biochemistry.* New York: W.H Freeman and Company.

Doi, K., Phuong, O. T., Kawatou, F., Nagayosh, Y., Fujino, Y., & Ohshima, T. (2013). Identification and Characterization of Lactic Acid Bacteria Isolated from Fermented Rice Bran Product. *Adv Microbiol, 3*(3), 265-273.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res, 32*(5), 1792-1797.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*, 783-791.

Ferreira, T., Van Reenen, C. A., Endo, A., Spröer, C., Malan, A. P., & Dicks, L. M. (2013). Description of *Xenorhabdus khoisanae* sp. nov., the symbiont of the entomopathogenic nematode *Steinernema khoisanae*. *Int J Syst Evol Microbiol, 63*(9), 3220-3224.

Fodor, A., Fodor, A. M., Forst, S., Hogan, S. J., Klein, M. G., Lengyel, K., Saringer, G., Stackerbrant, E., Taylor, R. A. J., Lehoczky, Ĕ. (2010). Comparative analysis of antibacterial activities of *Xenorhabdus* species on related and non-related bacteria in vivo. *Journal of Microbiol Antimicrob, 2*(4), 36-46.

Forst, S., & Nealson, K. (1996). Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. *Microbiol Rev, 60*(1), 21.

Fuchs, S. W., Proschak, A., Jaskolla, T. W., Karas, M., & Bode, H. B. (2011). Structure elucidation and biosynthesis of lysine-rich cyclic peptides in *Xenorhabdus nematophila*. *Organ Biomol Chem, 9*(9), 3130-3132.

Furgani, G., Böszörményi, E., Fodor, A., Máthé-Fodor, A., Forst, S., Hogan, J. S., Katona, Z., Klein, M. J., Szataricskai, Wolf, S. L. (2008). *Xenorhabdus* antibiotics: a comparative analysis and potential utility for controlling mastitis caused by bacteria. *J Appl Microbiol, 104*(3), 745-758.

Gregson, R. P., & McInerney, B. V. (1989). *Patent No. 4,837,222.* United States of America.

Gualtieri, M., Aumelas, A., & Thaler, J.-O. (2009). Identification of a new antimicrobial lysine-rich cyclolipopeptide family from *Xenorhabdus nematophila*. *J Antibiot, 62*, 295-302.

Gualtieri, M., Givaudan, A., Pages, S., & Villain-Guillot, P. (2012). *Patent No. 2012/085177 A1.* France.

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acid S, 41*, pp. 95-98.

Herbert, E. E., & Goodrich-Blair, H. (2007). Friend and foe: the two faces of *Xenorhabdus nematophila*. *Nat Rev Microbiol, 5*(8), 634-646.

Hominick, W. M. (2002). Biogeography. In R. Gaugler (Ed.), *Entomopathogenic nematology.* New Brunswick: CABI publishing.

Horticulture Research Institute. (2014).*EPNs cultures in Stock*. Thika: Kenya Agricultural and Livestock Research Organization, Horticulture Research Institute.

Houard, J., Aumelas, A., Noël, T., Pages, S., Givaudan, A., Fitton-Ouhabi, V., Villain-Guillot P., Gualtieri, M. (2013). Cabanillasin, a new antifungal metabolite, produced by entomopathogenic *Xenorhabdus cabanillasii* JM26. *J Antibiot, 66*, 617-620.

Huse, S. M., Dethlefsen, L., Huber, J. A., Welch, D. M., Relman, D. A., & Sogin, M. L. (2008). Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *Plos Genet, 4*(11).

Infectious Diseases Society of America. (2004). *Bad Bugs, No Drugs: As Antibiotic Discovery Stagnates--a Public Health Crisis Brews.* Alexendaria: Infectious Diseases Society of America.

Isaacson, P. J. (2000). *Antimicrobial activity of Xenorhabdus sp. (Enterobacteriaceae),symbiont of the entomopathogenic nematode,(Rhabditida: Steinernematidae).* Wellington: Simon Fraser University.

Isaacson, P. J., & Webster, J. M. (2002). Antimicrobial activity of *Xenorhabdus* sp. RIO (Enterobacteriaceae), symbiont of the entomopathogenic nematode, *Steinernema riobrave* (Rhabditida: Steinernematidae). *J Inverterbr P, 79*(3), 146-153.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol, 16*, 111-120.

Klug, W., Cummings, M. R., Spencer, C. A., & Palladino, M. A. (2009). *Concepts of Genetics* (9th ed.). London: Pearson Education Inc.

Kuwata, R., Qiu, L.-H., Wang, W., Harada, Y., Yoshida, M., Kondo, E., & Yoshiga, T. (2012). *Xenorhabdus ishibashii* sp. nov., a bacterium from the entomopathogenic nematode *Steinernema aciari*. *Int J Syst Evol Microbiol, 63 (5), 1690-1695.*

Ladell, P. J. (2011). *Isolation and characterization of antibiotics produced by the nematode symbiont Xenorhabdus szentirmaii.* La Crosse: University of Wisconsin-La Crosse.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt, & M. Goodfellow (Eds.), *Nucleic acid techniques in bacterial systematics* (pp. 125-175). New York: John Wiley & Sons.

Lengyel, K., Lang, E., Fodor, A., Szállás, E., Schumann, P., & Stackebrandt, E. (2005). Description of four novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov.,. *Syst Appl Microbiol, 28*(2), 115-122.

Madigan, M., Martinko, J., Dunlap, P., & Clark, D. (2009). *Brocks Biology of Microorganisms.* San Francisco: Pearson Education International.

Mathews, C. K., Van Holde, K. E., & Ahern, K. G. (2000). *Biochemstry.* San Francisco: Benjamin Cummings.

Miller, J. H. (1972). *Experiments in molecular genetics.* Cold Spring Harbor: Cold Spring Harbor Laboratory.

Mrácek, Z., Sturhan, D., & Reid, A. (2003). *Steinernema weiseri* n. sp.(Rhabditida, Steinernematidae), a new entomopathogenic nematode from Europe. *Syst Parasitol, 56*(1), 37-47.

Mwaniki, S. (2009). *Effects of abiotic factors on entomopathogenic nematodes and their potential against the sweet potato weevil Cylas puncticollis Boheman.* Nairobi: University of Nairobi.

Nguyen, K. B., Tesfamariam, M., Gozel, U., Gaugler, R., & Adams, B. J. (2004). *Steinernema yirgalemense* n. sp.(Rhabditida: Steinernematidae) from Ethiopia. *Nematology, 6*(6), 839-856.

Nishimura, Y., Hagiwara, A., Suzuki, T., & Yamanaka, S. (1994). *Xenorhabdus japonicus* species nov. assosiated with the nematode *Steinernema kushidai*. *World J Microb and Biot, 10*, 207–210.

Olsen, G. J., & Woese, C. R. (1993). Ribosomal RNA: a key to phylogeny. *FASEB J, 7*(1), 113-123.

Park, D., Ciezki, K., Van Der Hoeven, R., Singh, S., Reimer, D., Bode, H. B., & Steven, F. (2009). Genetic analysis of xenocoumacin antibiotic production in the mutualistic bacterium *Xenorhabdus nematophila*. *Mol Microbiol, 73*(5), 938-949.

Park, Y., & Kim, Y. (2000). Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophilus*, the symbiotic bacteria to the entomopathogenic nematode *Steinernema carpocapsae*. *J Insect Physiol, 46*(11), 1469-1476.

Park, Y., Kim, Y., & Stanley, D. (2004). The bacterium *Xenorhabdus nematophila* inhibits phospholipases A2 from insect, prokaryote, and vertebrate sources. *Naturwissenschaften, 91*(8), 371-373.

Rambaut, A. (2012). FigTree 1.4. *FigTree: Tree Figure Drawing Tool Version 1.4.* Edinburgh, Scotland: University of Edinburgh.

Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis, 197* (8), 1079-1081.

Sabbaj, J., Sutter, V., & Finegold, S. (1971). Comparison of selective media for isolation of presumptive group D *Streptococci* from human feces . *Appl Microbiol* 22, 1008-1011.

Sambrook, J., Fritsch, E. F., & Tom, M. (1989). *Molecular cloning:A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press.

Sheets, J. J., Hey, T. D., Fencil K. J., Burton S. L., Ni W., Lang A. E., Benz R., Aktories K. (2011). Insecticidal Toxin Complex Proteins from *Xenorhabdus nematophilus:* Structure and Pore Formation. *J Biol Chem, 286*(26), 22742-22749.

Sneath, P. H., & Sokal, R. R. (1973). *Numerical taxonomy. The principles and practice of numerical classification.* San Francisco: W.H Freeman.

Sokal, R. R. (1966). *Numerical Taxonomy.* San Francisco: Freeman.

Somvanshi, V. S., Lang, E., Ganguly, S., Swiderski, J., Saxena A. K., Stackebrandt, E. (2006). A novel species of *Xenorhabdus*,family Enterobacteriaceae:*Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum. Syst Appl Microbiol, 29*(7), 519-525.

Stock, S. P., & Goodrich-Blair, H. (2012). Nematode parasites, pathogens and associates of insects and invertebrates of economic importance. In L. Lacey (Ed.), *Manual of Techniques in Invertebrate Pathology* (2nd ed., pp373-426) London: Elsevier.

Tailliez, P., Pagès, S., Ginibre, N., & Boemare, N. (2006). New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. *Int J Syst Evol Microbiol, 56*(12), 2805-2818.

Tailliez, P., Laroui, C., Nadege, G., Paule, A., Page`s, S., & Boemare, N. (2010). Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. caribbeanensis subsp. nov., *P. luminescens* subsp. hainanensis subsp. nov., *P. temperata* subsp. khanii subsp. nov., *P. temperata* subsp. tasmaniensis subsp. nov., and the reclassification of *P. luminescens* subsp. thracensis as *P. temperata* subsp. thracensis comb. nov. *Int J Syst Evol Microbiol, 60*, 1921–1937.

Tailliez, P., Pagès, S., Edgington, S., Tymo, L. M., & Buddie, A. G. (2011). Description of *Xenorhabdus magdalenensis* sp. nov., the symbiotic bacterium associated with *Steinernema australe*. *Int J Syst Evol Microbiol*, *62* (8), 1761-1765.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol, 30* (12), 2725-2729.

Vallet-Gely, I., Lemaitre, B., & Boccard, F. (2008). Bacterial strategies to overcome insect defences. *Nat Rev Microbiol, 6*(4), 302-313.

Van de Peer, Y., Chapelle, S., & De Wachter, R. (1996). A quantitative map of nucleotide substitution rates in Bacterial rRNA. *Nucleic Acids Res, 24*(17), 3381–3391.

Waturu, C. N., Hunt, D. J., & Reid, A. P. (1997). *Steinernema karii* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from Kenya. *Int J Nematol, 7*(1), 68-75.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol, 173*(2), 697-703.

WenHsiung, L. (1997). *Molecular evolution.* Massachusetts: Sinauer Associates Incorporated.

Wiley, J. M., Sherwood, L. M., & Woolverton, C. J. (2008). *Prescott,Harley and Kleins Microbiology.* New York: McGraw Hill.

Woese, C. (1987). Bacterial Evolution. *Microbiol Rev, 51*(2), 221-271.

World Health Organization. (2014). *Antimicrobial resistance: global report on surveillance.* Geneva: WHO Press, World Health Organization.

Wu, D., Hugenholtz, P., Mavromatis, K., Pukall, R., Dalin, E., Ivanova, N. N., & Kunin, V. (2009). A phylogeny-driven genomic encyclopaedia of Bacteria and Archaea. *Nature, 462*(7276), 1050-1060.

Zdobnov, E. M., Lopez, R., Apweiler, R., & Etzold, T. (2002). Using the molecular biology data. In C. W. Sensen (Ed.), *Essentials of Genomics and Bioinformatics* (pp. 263-284). Weinhiem: Wiley VCH.

APPENDIX 1

Post incubation absorbance values of cultures of bacteria against concentration of inoculating test antibiotic

Test antibiotic: *Xenorhabdus* sp. P48 whole broth extract of 72 h fermentation period

Bacterium: *E. faecalis*

Reproduction 1-28/1/2014 | Reproduction 2-31/1/2014

Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$
0%	0.8487	0%	0.8567	0%	0.8287	0%	0.8275
10%	0.8041	10%	0.7324	10%	0.7368	10%	0.7233
20%	0.8092	20%	0.7979	20%	0.7819	20%	0.7594
30%	0.7358	30%	0.7528	30%	0.7993	30%	0.8118
40%	0.6696	40%	0.6803	40%	0.7284	40%	0.73
50%	0.5928	50%	0.5729	50%	0.6056	50%	0.6295
60%	0.4766	60%	0.4782	60%	0.4936	60%	0.5335
70%	0.3508	70%	0.3564	70%	0.3991	70%	0.4078
80%	0.2422	80%	0.2417	80%	0.2582	80%	0.2695
90%	0.1081	90%	0.1106	90%	0.1369	90%	0.1318
100%	0.0076	100%	0.0179	100%	0.03	100%	0.0228

Reproduction 3-5/2/2014

Replicate 1		Replicate 2	
Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$
0%	0.8621	0%	0.8632
10%	0.7632	10%	0.9099
20%	0.7557	20%	0.9176
30%	0.88	30%	0.8988
40%	0.8182	40%	0.8334
50%	0.7879	50%	0.7
60%	0.5797	60%	0.5998
70%	0.4236	70%	0.4374
80%	0.2943	80%	0.2858
90%	0.1436	90%	0.1409
100%	0.0129	100%	0.0232

Reproduction 1:Incubating inocula concentration- $5.4*10^4$ cfu/ml .Incubated at 37°C for 21 h

Reproduction 2:Incubating inocula concentration-$1.2*10^5$ cfu/ml. Incubated at 37°C for 21 h

Reproduction 3: Incubating inocula concentration- $6.759*10^4$ cfu/ml. Incubated at 37°C for 22 h

Test antibiotic: *Xenorhabdus griffiniae* L67 whole broth extract of 72 h fermentation period
Bacterium*: E. faecalis*

Reproduction 1-7/2/2014

Replicate 1		Replicate 2		Replicate 3	
Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$
0%	0.8634	0%	0.8695	0%	0.8317
10%	0.7904	10%	0.8304	10%	0.801
20%	0.776	20%	0.7909	20%	0.7751
30%	0.7388	30%	0.7233	30%	0.7422
40%	0.6541	40%	0.6469	40%	0.6675
50%	0.5663	50%	0.6012	50%	0.5855
60%	0.4932	60%	0.4985	60%	0.4938
70%	0.4233	70%	0.4286	70%	0.4209
80%	0.2935	80%	0.3178	80%	0.303
90%	0.1933	90%	0.2081	90%	0.2147
100%	0.0831	100%	0.0811	100%	0.0856

Reproduction 2-8/2/2014

Replicate 1		Replicate 2	
Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$
0%	0.8718	0%	0.8915
10%	0.7886	10%	0.8034
20%	0.7866	20%	0.7988
30%	0.7799	30%	0.7616
40%	0.6965	40%	0.696
50%	0.6079	50%	0.6196
60%	0.5346	60%	0.5463
70%	0.4489	70%	0.4547
80%	0.3362	80%	0.3441
90%	0.2112	90%	0.201
100%	0.0882	100%	0.0826

Reproduction 1:Incubating inocula concentration $6.58*10^4$ cfu/ml .Incubated at 37°C for 22 h
Reproduction 2: Incubating inocula concentration $7.2*10^4$ cfu/ml. Incubated at 37°C for 20 h

Test antibiotic: *Xenorhabdus* sp. P48 whole broth extract of 310 hour fermentation period
Bacterium: *E. faecalis*

Reproduction 1-28/2/2014 | Reproduction 2-29/2/2014

Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$
0%	0.6721	0%	0.6822	0%	0.7549	0%	0.7552
10%	0.475	10%	0.5139	10%	0.6595	10%	0.6294
20%	0.5478	20%	0.5823	20%	0.6904	20%	0.6503
30%	0.5304	30%	0.5548	30%	0.6068	30%	0.6385
40%	0.5217	40%	0.5387	40%	0.5418	40%	0.5932
50%	0.5004	50%	0.4909	50%	0.4164	50%	0.4686
60%	0.3752	60%	0.3924	60%	0.3692	60%	0.3997
70%	0.2537	70%	0.2794	70%	0.3096	70%	0.3087
80%	0.1669	80%	0.2036	80%	0.2131	80%	0.201
90%	0.0679	90%	0.1038	90%	0.0967	90%	0.0868
100%	0.0168	100%	0.029	100%	0.0326	100%	0.0282

Reproduction 3-5/2/2014

Replicate 1		Replicate 2	
Conc.	$AB600_{nm}$	Conc.	$AB600_{nm}$
0%	0.7504	0%	0.7564
10%	0.6438	10%	0.6779
20%	0.5991	20%	0.6565
30%	0.5651	30%	0.6189
40%	0.5383	40%	0.5308
50%	0.4686	50%	0.4925
60%	0.4215	60%	0.3689
70%	0.2904	70%	0.3352
80%	0.2149	80%	0.2141
90%	0.1089	90%	0.1155
100%	0.0352	100%	0.0356

Reproduction 1:Incubating inocula concentration- $1.52*10^6$ cfu/ml .Incubated at 37°C for 19 h
Reproduction 2: Incubating inocula concentration- $6.7*10^4$ cfu/ml. Incubated at 37°C for 23 h
Reproduction 3: Incubating inocula concentration- $6.7*10^4$ cfu/ml. Incubated at 37°C for 23 h

Test antibiotic: *Xenorhabdus griffiniae* L67 aqueous fraction broth extract of 72 h fermentation period
Bacterium*:* *E. faecalis*

Reproduction 1-5/2/2014

Replicate 1		Replicate 2	
conc.	$AB600_{nm}$	conc.	$AB600_{nm}$
0%	0.7677	0%	0.7564
10%	0.6955	10%	0.6779
20%	0.7495	20%	0.6565
30%	0.5822	30%	0.6189
40%	0.4475	40%	0.5308
50%	0.4234	50%	0.4925
60%	0.3694	60%	0.3689
70%	0.2606	70%	0.3352
80%	0.1679	80%	0.2141
90%	0.094	90%	0.1155
100%	0.0527	100%	0.0356

Reproduction1:Incubating inocula concentration- $1.92*10^5$ cfu/ml. Incubated at 37°C for 19 h

APPENDIX 2

Genbank database release 201.0 accession numbers of sequences used. The first six sequences in blue were those obtained from this study.

Sequence name	Accession number
Xenorhabdus griffiniae L671	AB987698.1
Xenorhabdus griffiniae L672	AB987700.1
Xenorhabdus griffiniae L673	AB987701.1
Xenorhabdus griffiniae L675	AB987699.1
Xenorhabdus griffiniae XN45	AB987697.1
Xenorhabdus sp. P48	AB987696.1
Xenorhabdus khoisanae 106-C	JX623971.1
Xenorhabdus kozodoii XenSaU2	KF044221.1
Xenorhabdus indica 28	NR 114965.1
Xenorhabdus vietnamensis VN01	NR 115713.1
Xenorhabdus kozodoii SaV	NR 043646.1
Xenorhabdus romanii PR06-A	NR 043647.1
Xenorhabdus hominickii KE01	NR 043648.1
Xenorhabdus sp. YL002	EU124383.1
Xenorhabdus sp. NBAIIXenSa04	KF780173.1
Xenorhabdus bovienii FR87	KF945995.1
Xenorhabdus bovienii GE02	KF945966.1
Xenorhabdus bovienii TR03	KF945977.1
Xenorhabdus sp. Vie2	KF9857383.1
Xenorhabdus poinarii NC33	KF740635.1
Xenorhabdus magdalenensis IMI397775	NR 109326.1
Xenorhabdus khoisanae SF80	JX623966.1
Xenorhabdus khoisanae SF362	JX623978.1
Xenorhabdus bovienii SfFL	KF437819.1
Xenorhabdus bovienii sfFR	KF437820.1
Xenorhabdus bovienii SfMD	KF437821.1
Xenorhabdus bovienii SjM2	KF437823.1
Xenorhabdus bovienii SkBU	KF437824.1
Xenorhabdus bovienii SoOR	KF437826.1
Xenorhabdus bovienii SpCR	KF437827.1
Xenorhabdus bovienii SN	JQ669675.1
Xenorhabdus sp. BA2	JQ975175.1
Xenorhabdus ishibashii	AB243427.1
Xenorhabdus bovienii SS-2004	NR_074382.1
Xenorhabdus sp. TZ01	JQ687358.1
Xenorhabdus sp. PAK.P.B.37	KC020713.1

Xenorhabdus stockiae Xeno-OK	JX221724.1
Xenorhabdus ehlersii BDH	JQ0264406.1
Xenorhabdus indica CICR-WG	JN558595.1
Xenorhabdus nematophila Caba02	GU293142.1
Xenorhabdus nematophila Bcn14	GU293143.1
Xenorhabdus nematophila Az20	GU293144.1
Xenorhabdus nematophila R1	GU293145.1
Xenorhabdus nematophila Az157	GU293146.1
Xenorhabdus sp. SF87	NR 117921.1
Xenorhabdus poinarii CICR-WR	JQ284032.1
Xenorhabdus stockiae HNds01	JQ219853.1
Xenorhabdus stockiae HNds02	JQ219854.1
Xenorhabdus sp. SKmg	JN177510.1
Xenorhabdus sp. SKkr	JN177511.1
Xenorhabdus bovienii LB09	HM140697.1
Xenorhabdus bovienii LB14	HM140698.1
Xenorhabdus bovienii LB24	HM140699.1
Xenorhabdus sp.GDc328	GQ140085.1
Xenorhabdus sp. GDh7	GQ149086.1
Xenorhabdus nematophila AS1B	JF503101.1
Xenorhabdus bovienii KtuXb1	JF507712.1
Xenorhabdus sp. Mecklenburg-2	HQ122653.1
Xenorhabdus stockiae HNxs01	HQ840745.1
Xenorhabdus sp. MY8NJ	AB507811.1
Xenorhabdus sp. MY8KsSu155	AB507812.1
Xenorhabdus indica OnIr181	AB507813.1
Xenorhabdus hominickii HkNk135	AB507814.1
Xenorhabdus hominickii KmYb11	AB507815.1
Xenorhabdus hominickii HkBt139	AB507816.1
Xenorhabdus bovienii YBKO	AB507817.1
Xenorhabdus bovienii SM	AB507818.1
Xenorhabdus sp. SGgj1	HM749976.1
Xenorhabdus sp. SGor1	HM749977.1
Xenorhabdus stockiae SS	HM622576.1
Xenorhabdus bovienii Reading	GU480967.1
Xenorhabdus kozodoii Italy	GU480969.1
Xenorhabdus budapestensis Serbia	GU480970.1
Xenorhabdus kozodoii France	GU480971.1
Xenorhabdus sp. CR9	GU480973.1
Xenorhabdus bovienii Bodega Bay	GU480975.1
Xenorhabdus bovienii France	GU480976.1
Xenorhabdus bovienii Florida	GU480977.1
Xenorhabdus poinarii SRK 1	EU513180.1

Xenorhabdus poinarii USA	GU480980.1
Xenorhabdus griffiniae Malaysia	GU480979.1
Xenorhabdus bovienii SC	GU480980.1
Xenorhabdus bovienii Monsanto	GU480981.1
Xenorhabdus bovienii Quebec	GU480983.1
Xenorhabdus hominickii Mt.Jiri	GU480985.1
Xenorhabdus bovienii Oregon	GU480986.1
Xenorhabdus bovienii Arizona	GU480987.1
Xenorhabdus bovienii Costa Rica	GU480988.1
Xenorhabdus szentirmaii Sargento Cabral	GU480989.1
Xenorhabdus cabanillasii Texas	GU480990.1
Xenorhabdus innexi Florida	GU480992.1
Xenorhabdus bovienii Turkey	GU480995.1
Xenorhabdus sp. SGmg1	GU980746.1
Xenorhabdus indica SRK15	GU906291.1
Xenorhabdus indica SRK12	GU586493.1
Xenorhabdus sp. SGas1	GQ373385.2
Xenorhabdus poinarii Iran2	EU250472.1
Xenorhabdus sp. KB-3	GQ923884.1
Xenorhabdus nematophila A24	FJ860883.1
Xenorhabdus nematophila A20	FJ860886.1
Xenorhabdus nematophila ES98	FJ860888.1
Xenorhabdus nematophila Peru	GU480994.1
Xenorhabdus nematophila CBY	FJ860887.1
Xenorhabdus nematophila Georgia	GU480972.1
Xenorhabdus nematophila AN6	AY278674
Xenorhabdus nematophila K97	FJ860884.1
Xenorhabdus nematophila Iran6	GU270840.1
Xenorhabdus bovienii USAR01	FJ860885.1
Xenorhabdus nematophila NC116	FJ860890.1
Xenorhabdus nematophila K102	FJ860891.1
Xenorhabdus sp. SGch1	GQ373383.1
Xenorhabdus japonica	AB243426.1
Xenorhabdus japonica DSM16522T	DQ202310.1
Xenorhabdus japonica IAM14265	DQ202310.1
Xenorhabdus japonica SK-1T	NR 027194.1
Xenorhabdus cabanillasii USTX62	GU480990.1
Xenorhabdus kozodoii ES01	DQ202307.1
Xenorhabdus kozodoii IT10	DQ202308.1
Xenorhabdus stockiae TH01	NR 043634.1
Xenorhabdus stockiae Thailand	GU480993.1
Xenorhabdus hominickii KR01	DQ205448.1
Xenorhabdus hominickii KR05	DQ205449.1

Xenorhabdus koppenhoeferi USNJ01	NR 043637.1
Xenorhabdus doucetiae FRG30	DQ211702.1
Xenorhabdus doucetiae FRM16	FO7O455O.1
Xenorhabdus griffiniae ID10	NR 043643.1
Xenorhabdus cabanillasii JM26	DQ211711.1
Xenorhabdus miraniensis Q1	NR 043644.1
Xenorhabdus mauleonii VC01	NR 043645.1
Xenorhabdus nematophila 4	FJ640983.1
Xenorhabdus sp. YnEn94	AB243425.1
Xenorhabdus bovienii HkEr36	AB243428.1
Xenorhabdus bovienii HkHm22	AB243429.1
Xenorhabdus bovienii AiAt199	AB243420
Xenorhabdus bovienii ATCC35271	NR 115574
Xenorhabdus sp. MY3	AB243431.1
Xenorhabdus sp. MY5	AB243432.1
Xenorhabdus sp. MY6	AB243433.1
Xenorhabdus sp. MY7	AB243434.1
Xenorhabdus stockiae SRK7	FJ006728.1
Xenorhabdus bovienii FR43	EU190976.1
Xenorhabdus kozodoii FR48	EU190977.1
Xenorhabdus bovienii FR44	EU190978.1
Xenorhabdus sp. TB	EU124382.1
Xenorhabdus bovienii SAR2	EF290400.1
Xenorhabdus innexi UY61	AY521243.1
Xenorhabdus bovienii CS66	DQ205451.1
Xenorhabdus bovienii CS03	DQ205452.1
Xenorhabdus bovienii USNY95	DQ205453.1
Xenorhabdus bovienii CA04	DQ205454.1
Xenorhabdus bovienii Si	DQ205455.1
Xenorhabdus bovienii TB20	DQ208305.1
Xenorhabdus poinarii SK72	AY521239.1
Xenorhabdus nematophila F1	AY521241.1
Xenorhabdus nematophila PL31	AY521242.1
Xenorhabdus ehlersii KR03	DQ202306.1
Xenorhabdus bovienii F3	DQ202311.1
Xenorhabdus ehlersii USCA98	DQ202312.1
Xenorhabdus bovienii TB10	DQ208304.1
Xenorhabdus bovienii TB30	DQ208306.1
Xenorhabdus ehlersii CN01	DQ208307.1
Xenorhabdus ehlersii KR02	DQ208308.1
Xenorhabdus poinarii DSM4768	NR 119152.1
Xenorhabdus poinarii AZ26	DQ211703.1
Xenorhabdus poinarii CU01	DQ211706.1

Xenorhabdus poinarii G1	NR 025875.1
Xenorhabdus nematophila ES96	DQ211707.1
Xenorhabdus szentirmaii AR81	DQ211708.1
Xenorhabdus szentirmaii K77	DQ211712.1
Xenorhabdus budapestensis CN03	DQ211714.1
Xenorhabdus indica OM01	DQ211718.1
Xenorhabdus sp. 28T	NR 114965.1
Xenorhabdus nematophila CA01	DQ211705.1
Xenorhabdus budapestensis	DQ329379.1
Xenorhabdus nematophila Breton	DQ282116.1
Xenorhabdus nematophila PDBCSCX1	DQ026511.1
Xenorhabdus sp .PDBCSCX7	DQ026512.1
Xenorhabdus innexi DSM16336T	NR 042325.1
Xenorhabdus poinarii PDBC SCX5	AY660026.2
Xenorhabdus bovienii PDBC SCX6	AY660027.2
Xenorhabdus budapestensis DSM16342T	NR 042326.1
Xenorhabdus ehlersii DSM16337T	NR 042327.1
Xenorhabdus szentirmaii DSM16338T	NR 042328.1
Xenorhabdus bovienii DSM4766	AY278673.1
Xenorhabdus beddingii DSM4764	AY278675.1
Xenorhabdus bovienii strain CB54	AY317154.1
Xenorhabdus nematophila DSM3370	FN667742.1
Xenorhabdus nematophilus strain N2-4	Z76737.1
Xenorhabdus nematophilus strain RIOBRAVIS	Z76738.1
Pseudomonas aeruginosa C16S	FM881781.1