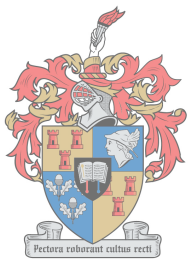
Characterization of Novel *Xenorhabdus-Steinernema* Associations and Identification of Novel Antimicrobial Compounds Produced by *Xenorhabdus khoisanae*

by

Jonike Dreyer



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Declaration

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Abstract

Xenorhabdus bacteria are closely associated with *Steinernema* nematodes. This is a species-specific association. Therefore, a specific *Steinernema* species is associated with a specific *Xenorhabdus* species. During the *Xenorhabdus-Steinernema* life cycle the nematodes infect insect larvae and release the bacteria into the hemocoel of the insect by defecation. The bacteria and nematodes produce several exoenzymes and toxins that lead to septicemia, death and bioconversion of the insect. This results in the proliferation of both the nematodes and bacteria. When nutrients are depleted, nematodes take up *Xenorhabdus* cells and leave the cadaver in search of their next prey. *Xenorhabdus* produces various broad-spectrum bioactive compounds during their life cycle to create a semi-exclusive environment for the growth of the bacteria and their symbionts.

During this study, a molecular approach was used to identify four *Xenorhabdus* isolates from *Steinernema sacchari* SB10^{T}, *Steinernema jeffreyense* J194^{T}, *Steinernema nguyeni* F2^{T} and *Steinernema litchii* WS9^{T} as *Xenorhabdus khoisanae* SB10 and J194, *Xenorhabdus bovienii* F2 and *Xenorhabdus griffiniae* WS9, respectively. *Steinernema* phylogenetics were analyzed and the *X. khoisanae-S. sacchari* and *X. griffiniae-S. litchii* associations proved that *X. khoisanae* and *X. griffiniae* has the ability to switch between different nematode clades.

Antimicrobial compounds produced by *X. khoisanae* SB10 were purified and analyzed by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LCMS), respectively. MS spectra and MS^{e} fragmentation profiles revealed novel antimicrobial compounds with mass-to-charge ratios of 671.41 *m/z*, 259.17 *m/z*, 434.27 *m/z* and/or 341.15 *m/z*. Additionally, this study reports for the first time, the isolation of PAX peptides, xenocoumacins and xenorhabdins from *X. khoisanae.*

Opsomming

Xenorhabdus bakterieë is naby geassosieer met *Steinernema* nematodes. Hierdie is ‘n spesie-spesifieke assosiasie. Dit wil sê, ’n spesifieke *Steinernema* spesie is geassosieer met ’n spesifieke *Xenorhabdus* spesie. Tydens die *Xenorhabdus-Steinernema* lewenssiklus infekteer die nematodes inseklarwes en word die bakterieë in die hemoseel van die insek vrygestel deur middel van ontlasting. Die bakterieë en nematodes produseer verskeie ekso-ensieme en toksiene wat lei tot septisemie, dood en bio-omskakeling van die insek. Dit lei tot die vermeerdering van beide die nematodes en bakterieë. Sodra nutriente uitgeput is, neem nematodes *Xenorhabdus* selle op en verlaat die kadawer opsoek na hul volgende prooi. *Xenorhabdus* produseer verskeie breë-spektrum bioaktiewe verbindings tydens hul lewenssiklus om ‘n gedeeltelike eksklusiewe omgewing te skep vir die groei van die bakterieë en hul simbionte.

Gedurende hierdie studie was ‘n molekulêre benadering gebruik om vier *Xenorhabdus* isolate vanaf *Steinernema sacchari* SB10^{T}, *Steinernema jeffreyense* J194^{T}, *Steinernema nguyeni* F2^{T} en *Steinernema litchii* WS9^{T} te identifiseer as, *Xenorhabdus khoisanae* SB10 en J194, *Xenorhabdus bovienii* F2 en *Xenorhabdus griffiniae* WS9, afsonderlik. *Steinernema* filogenetika was geanaliseer en die *X. khoisanae-S. sacchari* en *X. griffiniae-S. litchii* assosiasies het bewys dat *X. khoisanae* en *X. griffiniae* die vermoë het om te wissel tussen nematodes van verskillende klades.

Antimikrobiese verbindings geproduseer deur die isolaat, *X. khoisanae* SB10, was gesuiwer en geanaliseer deur hoëdrukvloeistofchromatografie (HDVC) en vloeistofchromatografie massa-spektrometrie (VCMS), afsonderlik. MS spektra en MS^{e} fragementasie profiele het nuwe antimikrobiese verbindings met massa-tot-lading verhoudings van 671.41 *m/z*, 259.17 *m/z*, 434.27 *m/z* en/of 341.15 *m/z* onthul. Vêrder rapporteer hierdie studie, vir die eerste keer, dat PAX peptiede, xenokoumasiene en xenorhabdiene geïsoleer was vanaf *X. khoisanae*.

Biographical sketch

Jonike Dreyer was born in Cape Town, South Africa on the 10th of March 1993. She matriculated at Paarl Girls' High School, South Africa, in 2011. In 2012 she enrolled as B.Sc. student in Molecular Biology and Biotechnology at the University of Stellenbosch and obtained her B.Sc (*Cum Laude*) in 2014. In 2015 she obtained her B.Sc (Hons) in Microbiology, also at the University of Stellenbosch. In 2016 she enrolled as M.Sc. student in Microbiology.

Preface

This thesis is represented as a compilation of 6 chapters. Chapters 1, 2, 5 and 6 are written according to instructions of the Journal of Applied and Environmental Microbiology. Chapters 3 and 4 have been published in Current Microbiology (2017, volume 74:8, pp 938-942) and Archives of Microbiology (2017, doi: 10.1007/s00203-017-1452-4), respectively.

Chapter 1: General Introduction

Chapter 2: Phenotypic and Genotypic Characteristics of *Xenorhabdus* Species, Their Association with Entomopathogenic Nematodes and Production of Antimicrobial Compounds

Chapter 3: Three Novel *Xenorhabdus*-*Steinernema* Associations and Evidence of Strains of *X. khoisanae* Switching Between Different Clades

Chapter 4: First Report of a Symbiotic Relationship Between *Xenorhabdus griffiniae* and an Unknown *Steinernema* from South Africa

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Chapter 6: General Discussion and Conclusions

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Dedication

Because an acknowledgement simply is not enough,

I dedicate this thesis to:

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Chapter 1

General Introduction

General Introduction

The genus *Xenorhabdus* consists of Gram-negative bacteria belonging to the Enterobacteriacaea family (1). These bacteria co-exist in a mutualistic relationship with pathogenic nematodes of the genus *Steinernema* (2), this is a species-specific association, i.e. a single *Xenorhabdus* sp. is associated with a specific *Steinernema* sp. (3). However, a single *Xenorhabdus* sp. associating with more than one *Steinernema* sp. has been reported (4–6). The latter, referred to as switching of hosts, is generally between nematodes in the same clade, but more recent studies have shown that the switching of hosts may also take place between nematodes of different clades (4–6).

The mutualistic relationship between *Steinernema* spp. and *Xenorhabdus* spp. is critical in the formation of a tripartite relationship with the host (insect) larvae. *Xenorhabdus* bacteria are carried in the receptacle of *Steinernema* nematodes when in the infective juvenile (IJ) phase (7, 8). Infective juveniles position themselves near the soil surface (9), enter the host through natural openings such as respiratory spiracles, mouth or anus and migrate to the hemocoel to excrete viable cells of *Xenorhabdus* (10). The host's immune response is inhibited with compounds produced by the nematodes and bacterial cells. Exoenzymes and toxins produced by *Xenorhabdus* lead to septicemia (11–14) and, ultimately, death of the host within 24-48 h. The nematodes reproduce sexually and repeat their life cycle until nutrients become depleted. During this phase, *Xenorhabdus* cells produce a number of antimicrobial compounds to create a semi-exclusive environment for themselves and the nematodes (10, 15). Second-phase juveniles stemming from the mutualistic relationship develop into IJs via the third phase. These IJs harbour viable *Xenorhabdus* cells.

Xenorhabdus spp. produce various bioactive compounds throughout their life cycle. These bacteria are, however, an underestimated and neglected source of novel bioactive compounds. Biologically active compounds produced by *Xenorhabdus* spp. have a broad-spectrum of antimicrobial activity, inhibiting the growth of bacteria, fungi and protozoa, the development of insects and nematodes, and the formation of cancerous cells (15). The variety of bioactive compounds produced by *Xenorhabdus* spp. differ, even between strains of the same species. Polyketide synthetases (PKS) and non-ribosomal peptide synthetases (NRPS) are responsible for the production of a diverse group of peptides, e.g. depsipeptides (16–18), xenocoumacins (19) and PAX peptides (peptide-antimicrobial-*Xenorhabdus*) (20). Other *Xenorhabdus*

antimicrobial compounds include benzylideneacetone (21), indole derivatives (22) and bacteriocins (23, 24).

The efficiency of *Xenorhabdus* bioactive compounds in the agricultural industry has been shown with various studies (25–29). *Xenorhabdus* bacteria, as a source of bioactive compounds, have exceeding potential, however, not only in the agricultural industry, but also in the healthcare and food industries. According to genomic analysis done on *X. nematophila* DSM 3370^{T} (30), only a fraction of the bioactive compounds that may be produced by *Xenorhabdus* spp. have been reported. It is evident that the research already done on *Xenorhabdus* bacteria is only the prelude to what is yet to come.

In the first section of this thesis, four *Xenorhabdus* strains (SB10, J194, WS9 and F2), isolated from *Steinernema sacchari, Steinernema jeffreyense, Steinernema litchii* and *Steinernema nguyeni*, respectively, are identified to species level. Before this study, the *Xenorhabdus* symbionts associated with these nematodes had not been reported. Identification was done by using biochemical tests, PCR and genome sequencing. The results led to the discovery of intriguing novel *Xenorhabdus-Steinernema* associations.

The second section of the study focussed on the antimicrobial activity of *Xenorhabdus* strain SB10. Antimicrobial compounds in the cell-free extract of *Xenorhabdus khoisanae* SB10 was isolated with XAD-16 beads, purified by high-performance liquid chromatography (HPLC) and fractions with antimicrobial activity subjected to liquid chromatography–mass spectrometry (LCMS) for putative identification. Strain SB10 was selected as no previous studies have been done on the bioactive compounds produced by *X. khoisanae*. The isolation and purification methods for antimicrobials produced by strain SB10 was optimized.

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Chapter 2

Literature review:

Phenotypic and Genotypic Characteristics of *Xenorhabdus* Species, Their Association with Entomopathogenic Nematodes and Production of Antimicrobial Compounds

Phenotypic and Genotypic Characteristics of *Xenorhabdus* species, Their Association with Entomopathogenic Nematodes and Production of Antimicrobial Compounds

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Abstract

The genus *Xenorhabdus* belongs to the family Enterobacteriaceae. These bacteria live mutualistically within entomopathogenic nematodes of the genus *Steinernema*. This association is species-specific, however, sharing of a specific *Xenorhabdus* sp. does occur between *Steinernema* hosts. During the *Xenorhabdus*-*Steinernema* life cycle, insect larvae are infected and killed, while both mutualists produce bioactive compounds. These compounds work synergistically to ensure the reproduction and proliferation of the nematodes and bacteria. Over the past two decades, the number of studies done on the bioactive compounds produced by *Xenorhabdus* spp. have increased drastically. These compounds are often broad-spectrum with activity against bacteria, fungi, insects, nematodes, protozoa and cancerous cells. It is evident that this genus is greatly underestimated and neglected in the search for novel bioactive compounds, especially when taking into consideration the need for novel antibiotics.

Keywords *Xenorhabdus, Steinernema,* bioactive compounds

The Genus *Xenorhabdus*

The genus *Xenorhabdus* consists of 26 species and belongs to the family Enterobacteriaceae (1). The rod-shaped cells (0.3-2.0 µm x 2.0-10.0 µm) stain Gram-negative, have peritrichous flagella when motile, do not reduce nitrate and, are oxidase and catalase negative. No endospores are produced, but crystalline inclusions may form during stationary growth. Spheroplasts with an average diameter of 2.6 µm may form towards the end of exponential growth (2). Most members of the genus are mesophilic and grow at 28 °C, although some strains may grow at 42 °C. Metabolism is respiratory and fermentative and the cells are classified as facultative anaerobes. Mannose, glycerol and N-acetylglucosamine are usually fermented. Some species ferment fructose. Most strains produce DNases, proteases and lipases.

Xenorhabdus spp. live in close association with entomopathogenic nematodes of the family Steinernematidae. This association directly influences the viable state of the bacterial cells. Two cell types, referred to as phase variants, have been described. Cells naturally associated with actively reproducing nematodes are in phase I (form I, or primary form), but change to phase II cells during later stages of the nematode reproduction cycle, i.e. when nematodes infect the insect cadaver (3). Conversion to phase II also occurs when cells are repeatedly sub-cultured *in vitro* (4). Phase I cells are larger than phase II cells, are motile, have swarming abilities, form crystalline inclusion bodies, and produce proteases, lipases and antimicrobial compounds (5–10). Phase I cells absorb certain dyes and can be distinguished from phase II cells by streaking the cells onto nutrient agar supplemented with 0.025% (w/v) bromothymol blue and 0.004% (w/v) triphenyltetrazolium chloride (NBTA medium) (11). Colonies on NBTA medium are dark blue with a red core and are surrounded by a clear zone. Exceptions to the rule have been reported, i.e. on rare occasions phase I cells do not absorb bromothymol blue (12).

Xenorhabdus spp. are differentiated based on biochemical characteristics (Table 1), but identification has to be confirmed using genotypic classification methods. Sequences of genes encoding recombinase A (*recA*), DNA gyrase subunit B (*gyrB*), DNA polymerase III beta chain (*dnaN*), initiation factor B (*infB*), glutamyl-tRNA synthetase catalytic subunit (*gltX*) and 16S rRNA (*16S rDNA*) are usually compared to determine the level of genetic relatedness. Furthermore, DNA-DNA hybridization of the entire genome may be used to confirm the exact

species. The phylogenetic relatedness of *Xenorhabdus* spp., compiled from sequences in GenBank, is shown in Fig. 1.

Association of *Xenorhabdus* with Entomopathogenic Nematodes

Xenorhabdus spp. are closely associated with entomopathogenic nematodes (EPNs) of the family Steinernematidae Travassos, specifically the genus *Steinernema*, and they are believed to increase the virulence and reproduction (thus "fitness") of the nematode (13). Until recently, the general assumption was that a specific *Xenorhabdus* sp. can only infect one *Steinernema* species. This has clearly been demonstrated in a study conducted by Sicard et al. (13). The authors have shown that the fitness of *Steinernema carpocapsae* improved when associated with *Xenorhabdus nematophila*, but not when associated with non-native *Xenorhabdus* spp. More recent findings have, however, shown that a single species of *Xenorhabdus* may be associated with several *Steinernema* spp. (Table 2). Murfin et al. (14) reported an increase in the fitness of *Steinernema* nematodes when infected with a strain of *Xenorhabdus bovienii* native to the nematode, or when associated with a strain from another *Steinernema* sp. closely related to the original nematode. Some authors hypothesized that the association of a specific *Xenorhabdus* sp. with more than one *Steinernema* sp. is an indication that the respective nematodes are phylogenetically related. The findings of Lee and Stock (15) provided the final answer to this hypothesis. The authors have shown that host switching of *Xenorhabdus* spp. have occurred in clades and between clades as many as 17 times. *Steinernema beitlechemie* from the *Cameroonense*-clade (16) is associated with *Xenorhabdus khoisanae.* However, *X. khoisanae* was first isolated from *Steinernema khoisanae* that belongs to the *Glaseri*-clade (17, 18). In a more recent paper, further evidence of *X. khoisanae* switching between clades was reported when *X. khoisanae* was isolated from *Steinernema sacchari* of the *Cameroonense*-clade (19).

Table 1 Carbohydrate fermentation and assimilation by *Xenorhabdus* spp.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Acid production from:																										
5-Ketogluconate	+	v	+	-	-	n.a.	v	w	+	+	+	w	+	w	+	-	-	w	w	+	v	w	w	-	n.a.	+
Aesculin	+	-	-	-	v	-	v	+	+	v	-	+	-	-	-	+	+	+	+	-	v	-	+	+	-	+
Fructose	+	v	+	v	v	-	v	+	+	+	+	+	+	+	-	-	-	+	+	+	v	+	+	v	-	+
gluconate	+	v	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	w	-	-	w	+	-	-	-
Glucose	+	+	+	+	v	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+
Inositol	-	n.a.	+	v	v	-	-	-	v	+	v	-	-	w	-	-	-	+	-	v	-	w	+	+	-	-
Maltose	+	v	-	v	v	+	v	-	v	-	-	+	+	+	+	+	+	+	+	v	+	+	+	v	-	+
mannose	+	+	+	+	v	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	-	+
N-acetyl-glucosamine	+	+	v	+	v	-	v	-	v	v	+	+	+	+	-	-	-	+	+	+	+	+	+	v	-	+
Ribose	+	v	-	-	v	-	v	+	v	-	+	w	+	+	-	+	-	+	+	v	-	-	+	v	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
trehalose	+	v	-	-	v	+	-	-	n.a.	-	v	+	-	v	-	+	+	+	+	v	v	+	-	-	-	+
Assimilation of:																										
Aesculin	+	-	v	-	v	-	v	+	+	v	v	+	-	+	-	v	+	+	+	-	v	-	+	+	-	+
Fructose	+	v	+	v	v	+	v	-	v	+	+	v	+	n.a.	-	-	-	+	+	+	v	+	+	-	+	+
*g*luconate	+	+	v	-	+	+	v	+	+	+	+	+	w	+	-	v	-	+	+	v	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	+	-	-	w	v	+	+	-	+	n.a.	-	-	-	+	w	v	-	+	+	+	-	w
Maltose	+	+	v	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
mannose	+	+	+	+	v	+	v	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-glucosamine	+	+	v	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	v	-	-	v	+	v	-	v	-	+	-	-	n.a.	-	+	-	+	+	-	-	-	+	v	-	-
Trehalose	+	+	+	v	+	+	v	+	+	v	+	+	+	n.a.	-	+	+	+	+	+	+	+	-	v	+	+

(1) X. beddingii, (2) X. bovienii, (3) *X. budapestensis*, (4) *X. cabanillasii*, (5) *X. doucetiae*, (6) X. eapokensis, (7) *X. ehlersii*, (8) *X. griffiniae*, (9) X. hominickii, (10) *X. indica*, (11) *X. innexi*, (12) *X. ishibashii*, (13) *X. japonica*, (14) X. khoisanae, (15) *X. koppenhoeferi*, (16) X. kozodoii, (17) *X. magdalenensis*, (18) *X. mauleonii*, (19) *X. miraniensis*, (20) *X. nematophila*, (21) *X. poinarii*, (22) *X. romanii*, (23) *X. stockiae*, (24) *X. szentirmaii*, (25) X. thuongxuanensis, (26) *X. vietnamensis*.
+, 90% of strains positive; -, 90% of strains negative; v, variable reaction; w, weak reaction; n.a., not available. Data obtained from (19–21).

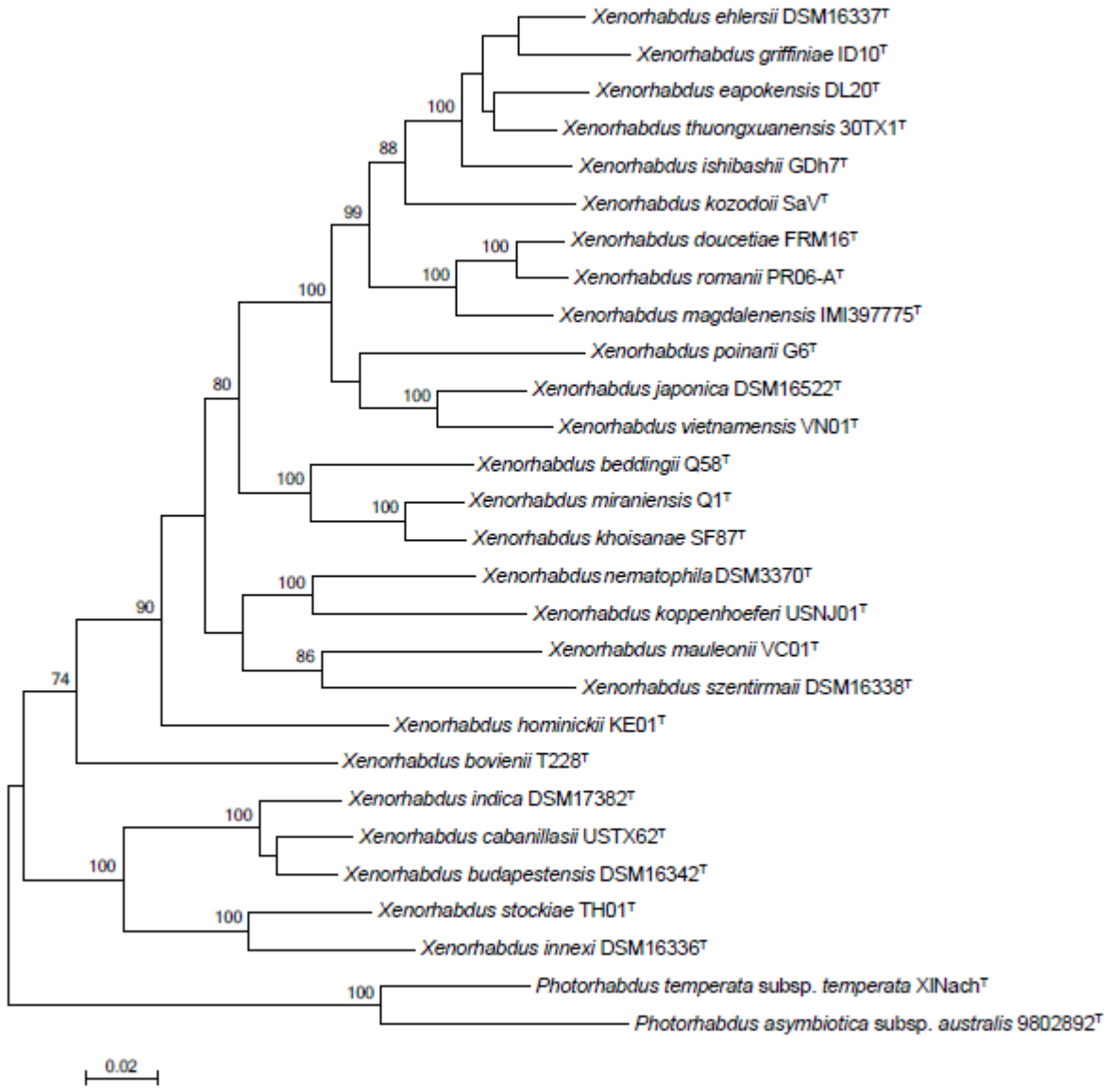


Figure 1 Phylogenetic tree of known *Xenorhabdus* species, based on the protein-coding genes, recombinase A (*recA*), DNA gyrase subunit B (*gyrB*), DNA polymerase III beta chain (*dnaN*), initiation factor B (*infB*) and glutamyl-tRNA synthetase catalytic subunit (*gltX*). Type strains are indicated by the superscript letter ^{T}. *Photorhabdus* species are used as outgroups. Bootstrap values above 70% are shown. Gene sequences were obtained from the National Center for Biotechnology Information (NCBI) and MEGA6.0 (22) was used to construct the phylogenetic tree.

Table 2 Mutualistic relationships between *Steinernema* nematodes and *Xenorhabdus* bacteria.

Xenorhabdus	***Steinernema***	**Source**
X. beddingii	*S. longicaudum*	(15)
X. bovienii	*S. affine, S. anatoliense, S. costaricense, S. feltiae, S. intermedium, S. jollieti, S. kraussei, S. litorale, S. nguyeni, S. oregonense, S. puntauvense, S. sichaunense, S. weiseri, S. silvaticum*	(5, 15, 19, 23–28)
X. budapestensis	*S. bicornutum, S. ceratophorum*	(23, 29)
X. cabanillasii	*S. riobrave*	(23)
X. doucetiae	*S. diaprepesi*	(23)
X. eapokensis	*S. eapokense*	(21)
X. ehlersii	*S. serratum, S. longicaudum*	(23, 29)
X. griffiniae	*S. hermaphroditum* (Previously referred to as *S. dharanai*) *S. litchi, S. khoisanae* (See Chapter 4 for clarity)	(23, 30)
X. hominickii	*S. karii, S. monticolum*	(23, 31)
X. indica	*S. thermophilum, S. yirgalemense, S. abbasi*	(23, 32, 33)
X. innexi	*S. scapterisci*	(29)
X. ishibashii	*S. aciari*	(20)
X. japonica	*S. kushidai*	(34)
X. khoisanae	*S. khoisanae, S. jeffreyense, S. sacchari, S. beitlechemie*	(17, 19)
X. koppenhoeferi	*S. scarabaei*	(23)
X. kozodoii	*S. arenarium, S. apuliae, S. boemarei*	(15, 23, 26)
X. magdalenensis	*S. austral*	(35)
X. mauleonii	*Steinernema* sp.	(23)
X. miraniensis	Nematode from the family Steinernematidae, isolated from Australia	(26)
X. nematophila	*S. carpocapsae* (previously referred to as *S. anatoliense*) *S. websteri*	(15, 36)
X. poinarii	*S. glaseri, S. cubanum*	(25, 37)
X. romanii	*S. puertoricense*	(23)
X. stockiae	*S. siamkayai*	(23)
X. szentirmaii	*S. rarum, S. costaricense*	(15, 29)
X. thuongxuanensis	*S. sangi*	(21)
X. vietnamensis	*S. sangi*	(38)

The *Xenorhabdus-Steinernema* Life Cycle

Cognate nematodes and bacteria may be disassociated under laboratory conditions, without affecting the fitness of the bacteria. However, a decrease in reproduction rate and virulence of the nematode occurs after a few generations without their symbionts (13, 39). The different stages in the life cycle of the symbionts are described below.

Stage I. In the first stage of development, *Steinernema* nematodes are present in the infective juvenile (IJ) form, also referred to as a special third phase juvenile or dauer juvenile. The IJs are encased by a double outer cuticle and have a closed mouth and anus (40). IJs are relatively resistant to environmental stressors and may live for several months without feeding (41). *Xenorhabdus* bacteria are carried in a specialized organ of the IJ, called a receptacle (42). This organ is a modification of the two most anterior cells of the intestine with sizes varying from 8 x 5 µm to 46 x 17 µm (43). IJs of the family Steinernematidae may actively search for insect hosts, or wait near the soil surface for passing insects (44). Once an insect is in close proximity, the nematode enters the insect through natural openings, such as the mouth, anus and respiratory spiracles, and migrates to the hemocoel.

Stage II. Nutrients in the hemocoel of a susceptible host (not fully characterized), trigger the start of a new phase in the nematode's life cycle (Fig. 2), referred to as the recovered, feeding phase (J3). Recovered nematodes start feeding and moult to the fourth phase (J4). During the recovery phase, the bacteria are released by defecation, as a result of ingestion of the insect hemolymph. The hemolymph has a sophisticated immune system that protects it from invading microorganisms (45). However, *Steinernema* produce proteins that suppress the immune response (46) and the bacteria start to multiply. Exoenzymes and toxins are released, which leads to septicemia and bioconversion of the insect host. This results in death of the insect within 24-48 h. The J4 phase develops into gonochoristic males and females that reproduce by copulation and production of eggs. The eggs develop into adult nematodes by passing through juvenile phases J1 to J4. This cycle is repeated for as long as nutrients are available (depending on the size of the host), generally for up to three generations. The bacteria proliferate and produce various antimicrobials, including antibiotics and bacteriocins (47). This creates a semi-exclusive environment for the nematodes and *Xenorhabdus* by preventing the colonization of the cadaver by other soil micro-organisms.

Stage III. After one to three generations (depending on the size of the host insect), second phase juveniles (J2) develop into IJs, special third phase juveniles. An increase in the nematode population depletes the nutrients and leads to the accumulation of byproducts, such as NH_3 (48). Nematodes take bacterial cells up in their receptacle and cease feeding. They then re-create their double outer cuticle layer that closes the mouth and anus. At this stage, thousands of IJs leave the host cadaver in search of their next prey.

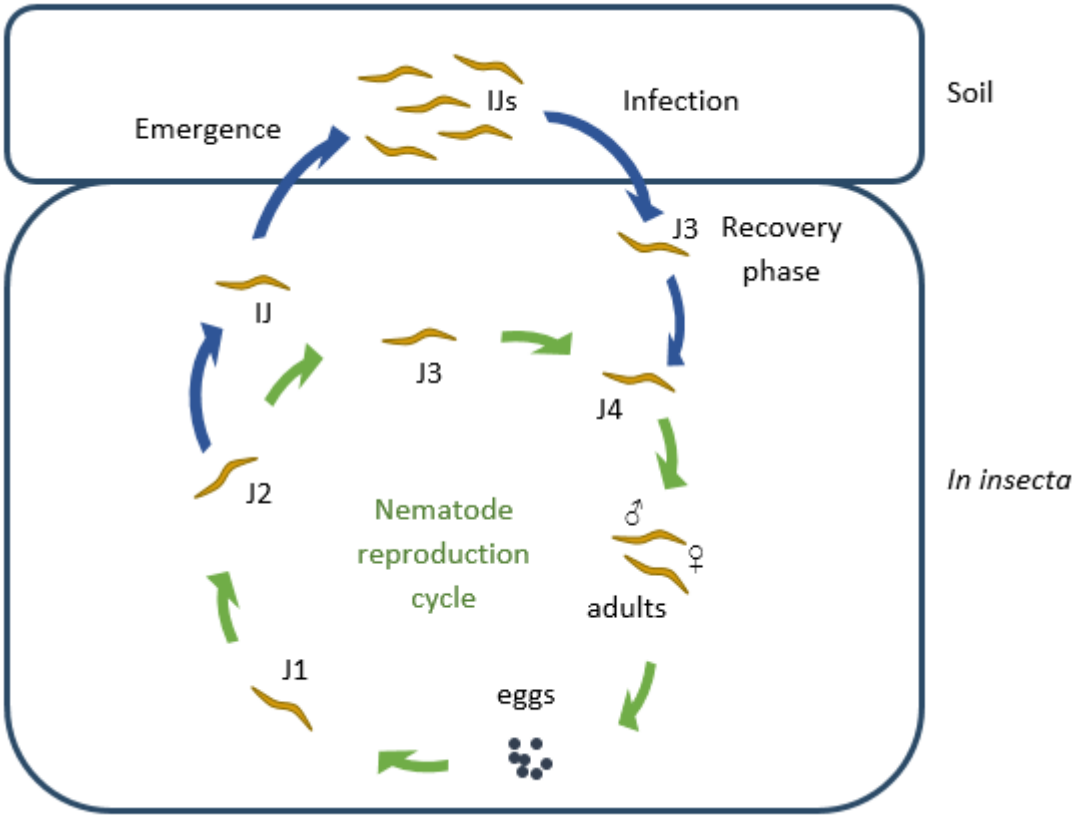


Figure 2 The *Steinernema* life cycle. The infective juvenile (IJ) nematodes infect an insect host and recover to the feeding phase (J3). J3 nematodes moult into fourth phase (J4) juveniles, which in turn develop into male and female adults. These adults reproduce and lay eggs. The eggs hatch as first phase juveniles (J1) which feed and moult to second, third and fourth juvenile phases (J2-J4), and ultimately into adults. After one to three generations, when nutrients are depleted, second phase juveniles develop into IJs (special third phase juveniles). Each of the IJs host *Xenorhabdus* bacteria in their receptacle. These IJs then leave the cadaver and await a new prey.

Synergistic Effect

Xenorhabdus spp. produce several compounds that inhibit the immune system and lead to septicemia of the host. *Xenorhabdus nematophila* produces UnA, a protein that inhibits the ability of hemocytes to aggregate and form capsules or nodules around the nematodes and bacteria (49). Additionally, outer membrane proteins and lipopolysaccharides of *X. nematophila* reduce non-self recognition in *Galleria mellonella* Linnaeus (Lepidoptera; Phyralidae) hemocytes, which allow the bacteria to avoid adhesion to hemocytes (50). This inhibits the activation of phenoloxidase, an important enzyme in the insects' immune response to foreign organisms (51). In contrast, *Xenorhabdus budapestensis* D43 produces a 57 kDa insecticidal protein that activates the phenoloxidase cascade and elicits an intense immune response in *G. mellonella* larvae (52). This leads to an excessive production of quinones, which are toxic to the larvae. *Xenorhabdus nematophila* influences the immune response of insects by preventing the production of phospholipase A_2 (PLA_2) and inhibiting its activity (50, 53–55). PLA_2 is partly responsible for the biosynthesis of eicosanoids. Eicosanoids play a role in mediating hemocyte behavior, thereby regulating the immune response of the insect. The absence of eicosanoids results in severe immune depression and septicemia of the insect. *Xenorhabdus nematophila, Xenorhabdus japonica, Xenorhabdus kozodoii* and *Xenorhabdus beddingii* cause apoptosis of insect hemocytes (56–58). The compound responsible for cytotoxicity of *X. nematophila* has been identified as protein CyA (cytotoxic activity). From these studies, it is clear that *Xenorhabdus* bacteria play an important role in inhibiting the immune system of insects and in the production of cytotoxins, toxins and hemolysins killing the insect.

Despite the various bioactive compounds produced by *Xenorhabdus* spp., few strains cause infection of insect larvae when taken up orally. It is thus important for the bacteria to be "vectored" into the insect hemocoel by the nematodes. Apart from spreading *Xenorhabdus* spp. amongst insect hosts, nematodes support the survival of the bacteria. *Steinernema* nematodes produce specific proteins that inhibit the insects' antimicrobial compounds (46). This promotes growth of their respective *Xenorhabdus* mutualists. The attributes of the nematode in this tripartite relationship are not merely to vector and protect the bacteria, but to also contribute in killing of the host insect. Axenic, therefore sterile, *S. carpocapsae* (59, 60) and *S. feltiae* (61) kill *G. mellonella* larvae. This is likely due to insecticidal compounds produced by *Steinernema* nematodes. *Steinernema carpocapsae*, and most likely also *S. feltiae*, produce a protein toxic

to *G. mellonella* larvae (62). However, not all axenic *Steinernema* nematodes are efficient in killing *G. mellonella* larvae.

Akhurst (63) reported that neither *Steinernema glaseri*, nor its symbiont, *Xenorhabdus poinarii*, was able to kill *G. mellonella* larvae when tested independently. However, when combined, the symbionts killed all *G. mellonella* larvae. Similar results were reported for *Steinernema scapterisci* and its *Xenorhabdus* symbiont (64). *Steinernema feltiae* and *X. bovienii* are each virulent on their own, with a mortality rate of 39% and a virulence of LC_{50} = 15 700 colony forming units, respectively, when *Tipula oleracea* Linneaus (Diptera; Tipulidae) was used as host. The combination of both partners however, increased the virulence to a mortality rate of 90% (61).

It is undeniable, that the nematodes, as well as their bacterial symbionts, are crucial for killing insect host larvae and neither are especially effecient at doing so without their symbiont. The efficiency of killing host larvae cannot be attributed solely to an additive effect of the nematode and bacterial toxins, as virulence increases drastically when these two mutualists act together. This phenomenon should therefore, rather be described as a synergistic affect, as proposed by Boemare (65).

EPNs as Biological Control Agents

The early 20th century led to the discovery that EPNs could be useful in agriculture as biological control agents. Since the 1980's research on EPNs has expanded rapidly (65) and in the current day and age, EPNs have been very effective in the treatment of insect pests. Since the combination of *Steinernema* nematodes and *Xenorhabdus* bacteria is highly effective, this mutualistic relationship has been exploited for biological control purposes. For example, *Steinernema yirgalemense* cause a 100% mortality of false codling moth larvae (*Thaumatotibia leucotreta*, Meyrick) when as few as 50 IJs per insect were used (66). Other South African studies have shown these mutualists to be effective against codling moth (*Cydia pomonella*, L.) (67), mealy bugs (*Planococcus ficus*, Signoret) (68), sugarcane stalk borer (*Eldana saccharina*, Walker) (69), fruit flies *Ceratitis capitate* (Wiedemann) and *Ceratitis rosa* (Karsch) (70) and many more.

Xenorhabdus Bioactive Secondary Metabolites

Most naturally produced antimicrobial metabolites are produced by bacteria (71), of which *Streptomyces* (72), *Bacillus* (73), cyanobacteria (74), myxobacteria (75) and *Pseudomonas* (76) are the best studied. There are, however, some immensely underestimated and neglected antimicrobial sources. These include species of the bacteria *Bulkholderia*, *Janthinobacterium, Lysobacter*, non-pathogenic clostridia, *Photorhabdus* and *Xenorhabdus*. Although a number of antimicrobial compounds are produced by these bacteria, they have not been studied to the same extent as the previously mentioned species (77). *Xenorhabdus* bacteria are known to produce broad-spectrum compounds with activity against bacteria, fungi, insects, nematodes, protozoa and cancer cells (47). These activities each play a unique role in the protection and bioconversion of the host cadaver, and promote reproduction and growth of the nematodes.

Dutky (78) was the first to suggest that the symbiont of *Neoaplectana* (now known as *Steinernema*), could produce antimicrobial compounds. It was only 22 years later that scientists started to show an interest in these compounds. Paul et al. (79) identified several novel antibacterial compounds produced by *Xenorhabdus* spp. Since this discovery, various additional *Xenorhabdus* compounds have been described.

Various *Xenorhabdus* bioactive secondary metabolites are produced by polyketide synthetases (PKS) and/or non-ribosomal peptide synthetases (NRPSs). The latter are catalysts that use intricate reactions to assemble diverse peptides without the assistance of ribosomes (80). They contain a set of modules that are responsible for the stepwise incorporation of amino acids. These modules, in turn, contain domains that trigger complex reactions leading to production of the final compound. Secondary metabolites produced by *Xenorhabdus* spp. include depsipeptides, xenocoumacins, fabclavines, pristimamycin II_a, xenortides, rhabdopeptides, bicornitun, PAX peptides, nemaucin, cabanillasin, dithiopyrrolone derivatives, indole-containing compounds, unnamed peptides, benzylideneacetone, rhabduscin, bacteriocins, phenethylamine and trypamine derivatives, phenethylamides, chaiyaphumines and xenofuranones. Chemical structures are shown in Fig. 3.

Depsipeptides. Depsipeptides are peptides with one or more amide group replaced by a hydroxy acid, leading to the formation of an ester bond. These peptides generally contain alternating peptide and ester bonds. Five classes of depsipeptides produced by

Xenorhabdus spp. have been characterized. The first class consists of eight tridecadepsipeptides, named xenoamicins (81). These compounds consist mainly of hydrophobic amino acids and are produced by *Xenorhabdus doucetiae* and *Xenorhabdus mauleonii*. The gene cluster encoding the biosynthesis of xenoamicins was identified by using the whole genome of *X. doucetiae* DSM 17909. The gene cluster consists of the five NRPSs, XabABCD and the aspartic acid decarboxylase XabE. XabABCD contains 13 modules for the synthesis of xenoamicins, while XabE is suggested to be involved in the formation of β-alanine. The large number of hydrophobic amino acids suggested that xenoamicins interact with the cytoplasmic membrane. However, no antibacterial or antifungal activity was recorded for xenoamicin A, which suggests a different mode of activity. Anti-protozoal and weak cytotoxic activities have been reported for xenoamicin A, but the target site has not been identified.

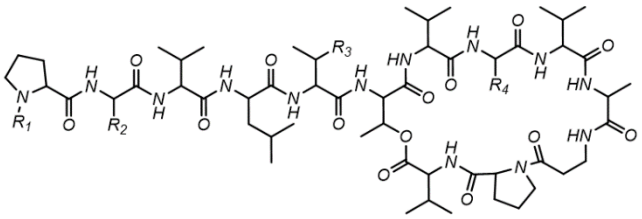
The second depsipeptide class, isolated from *Xenorhabdus indica*, was characterized by Kronenwerth et al. (82). These depsipeptides contain an additional fatty acid chain which is attached to one of the amino acids, which classifies them as lipodepsipeptides. The peptides are named after their amino acid sequence, T-A-X-L-L-L-A (X = L, F or Y), and are referred to as taxlllaids (A-G). Seven variants were described, each classified based on the length of the fatty acid chain, the third amino acid and the overall structure of the molecule, i.e. an open chain or ring structure. The synthesis of taxlllaids are encoded by a gene cluster consisting of two NRPSs, TxlA and TxlB, containing four and three modules, respectively. Natural taxlllaid A and synthetic taxlllaids B-G have antiprotozoal activity, with taxlllaid A also being cytotoxic to human carcinoma cells (HeLa).

The third class of depsipeptides are classified as the indole-containing xenematides. Xenematide A was the first example, isolated from *X. nematophila* (83). The molecule is cyclic, antibacterial and weakly insecticidal. Three years later, Crawford et al. (84) isolated another three xenematides (B-D) from *X. nematophila* and showed that the NRPS, classified as XNC1_2713, is responsible for the production of xenematide A. This was accomplished by knocking out the gene that encodes the XNC1_2713 NRPS in *X. nematophila*. Metabolite analysis revealed that production of xenematide A was terminated in the mutant strain. Xenematides are not restricted to *X. nematophila* or the genus *Xenorhabdus*, as protein homologs have been identified in *X. bovienii* and *Photorhabdus asymbiotica.*

The final two depsipeptide classes consist of xenobactin and szentiamide (90, 91). Xenobactin was isolated from the unknown *Xenorhabdus* sp. strain PB30.3 and szentiamide from *Xenorhabdus szentirmaii*. Both compounds have good activity against the causative agent of malaria, *Plasmodium falciparum* and some activity against *Trypanosoma brucei rhodesiense* and *Trypanosoma cruzi*. Szentiamide does not have any effect on the growth of bacteria or yeasts, however, it has an additional weak cytotoxicity against *G. mellonella* hemocytes. Contrary to szentiamide, xenobactin has no cytotoxic activity, but is active against *Micrococcus luteus*. The antibacterial activity is likely due to the hydrophobic nature of the peptide and the compound is proposed to interact with the membrane of *M. luteus*.

Xenocoumacins. These peptides, first described by McInerney (92), have benzopyran structures and are some of the major antimicrobials produced by *X. nematophila*. Xcn1 is active against Gram-positive and Gram-negative bacteria, and has antifungal and antiulcer activity. Xcn2 has less antibacterial activity and no antifungal activity, but has antiulcer activity. More recently, Reimer (93) discovered that Xcn2 is produced from Xcn1 through reactions encoded by genes *xcnM* and *xcnN*. In a study conducted by Park et al. (94), the *xcnM* gene was inactivated, which led to an increased Xcn1 level, as expected, but it also decreased cell viability by 20-fold. The conversion of Xcn1 to Xcn2 was therefore, suggested to be a mechanism used by the bacteria to avoid self-toxicity. Xcn1 is proposed to be the terminal PKS/NRPS product, which is then modified by various reactions to produce Xcn2-6. Xcn3 to Xcn6 were isolated from *X. nematophila* and *X. kozodoii* (95).

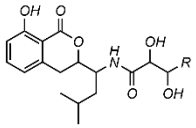
Fabclavines. A class of peptide-polyketide-polyamino products, called fabclavines, have recently been isolated from *X. budapestensis* and *X. szentirmaii* (96). Analysis of the genomes of the producer bacteria led to the discovery that the fabclavines are produced by a hybrid PKS-NRPS gene cluster. The peptide moiety is synthesized by the FclI and FclJ NRPSs, while the PKS, FclK, is responsible for catalyzing the elongation of the peptide moiety's proline residue. These compounds have broad-spectrum activity and are active against Gram-positive and Gram-negative bacteria, *Saccharomyces cerevisiae*, *Plasmodium falciparum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. Furthermore, fabclavines and cationic antimicrobial peptides are structurally very similar. Cationic peptides have massive synergistic effects when combined with other antibiotics (97). Fabclavines may thus also display synergistic effects when combined with other antibiotics in the insect cadaver.



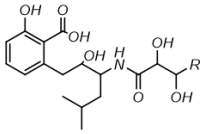
Xenoamicins

Xenoamicin	R_1	R_2	R_3	R_4
A	Butanoyl	Methyl	Ethyl	1-methylethyl
B	Butanoyl	Methyl	Ethyl	2-methylpropyl
C	Butanoyl	Methyl	Ethyl	butan-2-yl
D	Acetoyl	Methyl	Ethyl	2-methylpropyl butan-2-yl
E	Butanoyl	Methyl	Methyl	2-methylpropyl butan-2-yl
F	Butanoyl	H	Ethyl	2-methylpropyl butan-2-yl
G	Pentanoyl	Methyl	Ethyl	2-methylpropyl butan-2-yl
H	H	Methyl	Ethyl	2-methylpropyl butan-2-yl

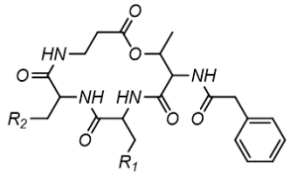
Xenematide	R_1	R_2
A	Indolyl	Indolyl
B	Phenyl	Phenyl
C	Phenyl	Indolyl
D	Indolyl	Phenyl



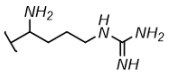
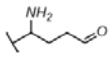
Xenocoumacins 1-4

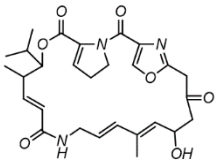


Xenocoumacins 5 and 6

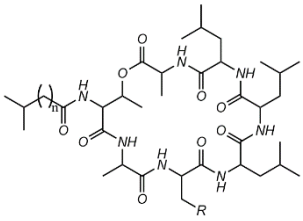


Xenematides

Xenocoumacin	R
1 and 5	
2 and 6	
3	
4	

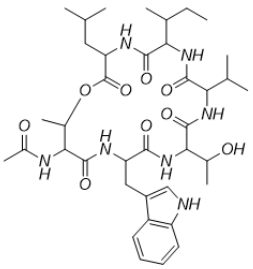


Pristinamycin II_A



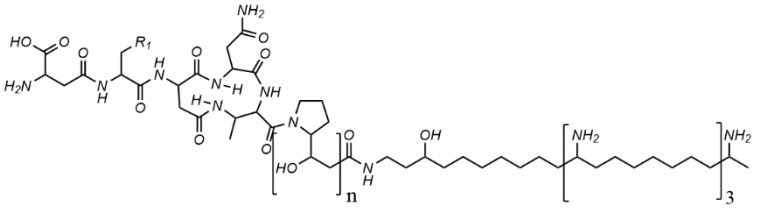
Taxlllaids

Taxlllaid	n	R
A	3	1-methylethyl
B	3	Phenyl
C	3	(ρ-OH) Phenyl
D	2	1-methylethyl
E	2	Phenyl
F	1	1-methylethyl



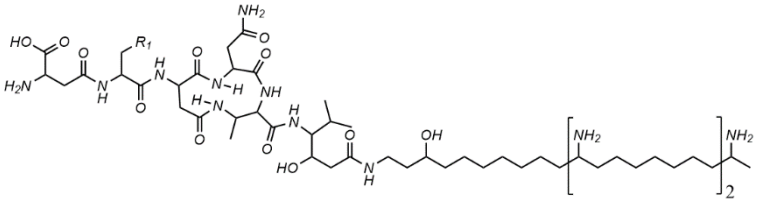
Xenobactin

Figure 3 *Xenorhabdus* bioactive compounds (85–89). Bioactive compounds with unknown structures include the antibacterial xenoprotec, bicornitun C and D, and the two bacteriocins, xenorhabdicin and xenocin.

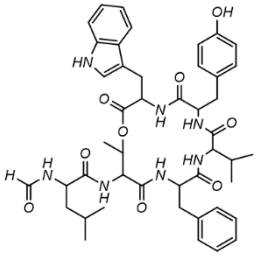


Fabclavine	Compound	n	R_1
Ia	1	2	
Ib	2	1	
IIa	3	2	
IIb	4	1	

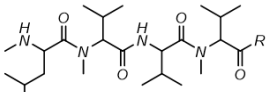
Fabclavine 1-4



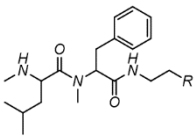
Fabclavine 5



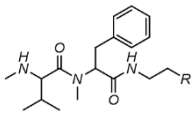
Szentiamide



Rhabdopeptide 1, 3 and 5

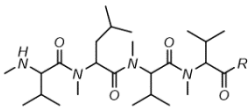


Xenortide A and B

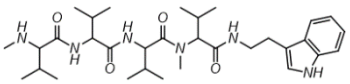


Xenortide C and D

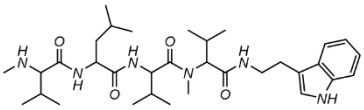
Xenortide	R
A	Phenyl
B	Indolyl
C	Phenyl
D	Indolyl



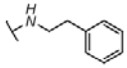
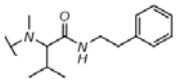
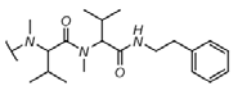
Rhabdopeptide 2, 4 and 6

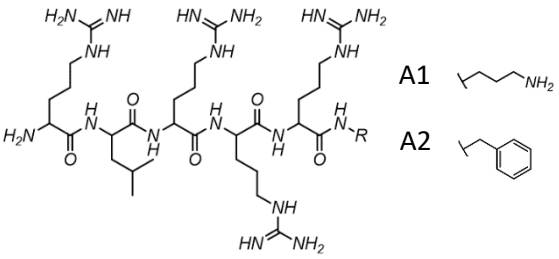


Rhabdopeptide 7



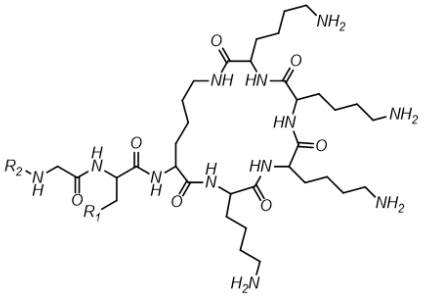
Rhabdopeptide 8

Rhabdopeptide	R
1 & 2	
3 & 4	
5 & 6	



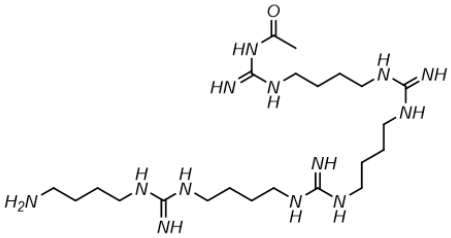
Bicornutin A1 and A2

Figure 3 Continued



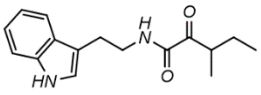
PAX peptides

PAX	R_1	R_2
1	Propylamine	(3*R*)-3-hydroxytetradecanoyl
2	Ethylguanidine	(3*R*)-3-hydroxytetradecanoyl
3	Propylamine	(3*R*)-3-hydroxypentadecanoyl
4	Ethylguanidine	(3*R*)-3-hydroxypentadecanoyl
5	Propylamine	(3*R*,7*Z*)-3-hydroxytetradec-7-enoyl
6	Ethylguanidine	(3*R*,7*Z*)-3-hydroxytetradec-7-enoyl
7	Propylamine	(3*R*)-3-hydroxyhexadecanoyl
8	Propylamine	(3*R*)-3-hydroxyoctadecanoyl
9	Ethylguanidine	(3*R*,9*Z*)-3-hydroxyhexadec-9-enoyl
10	Ethylguanidine	(3*R*)-3-hydroxyhexadecanoyl
11	Ethylguanidine	(3*R*,10*Z*)-3-hydroxyheptadec-10-enoyl
12	Ethylguanidine	(3*R*)-3-hydroxyheptadecanoyl
13	Ethylguanidine	(3*R*,11*Z*)-3-hydroxyoctadec-11-enoyl

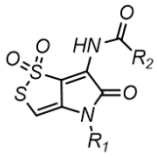


Cabanillasi

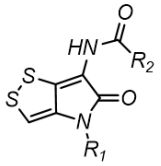
Xenorhabdin	R_1	R_2
I	H	Pentyl
II	H	4-methylpentyl
III	H	Heptyl
IV	Methyl	Pentyl
V	Methyl	4-methylpentyl
VII	Methyl	2-methylpropyl
VIII	Methyl	Propyl



Nematophin

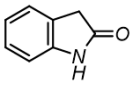


Xenorxides



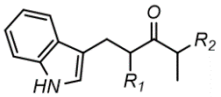
Xenorhabdins

Indole

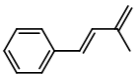


Oxindole

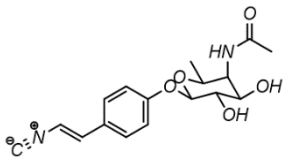
Xenorxide	R_1	R_2
I	H	Phenyl
II	H	4-methylpentyl



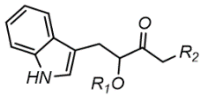
Indole derivatives



Benzylideneacetone



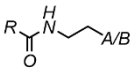
Rhabducin

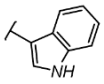


Xenocycloins

Xenocyloin	R_1	R_2
A	H	Methyl
B	H	Ethyl
C	Acetyl	Methyl
D	Acetyl	Ethyl
E	Propyl	Ethyl

Figure 3 Continued





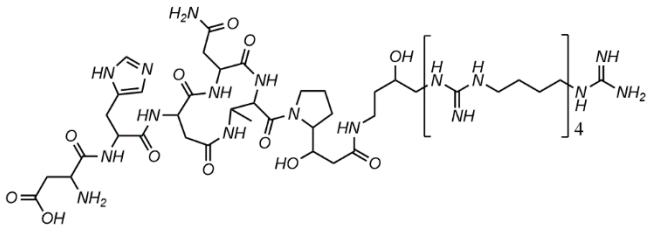
A

B

Phenylethylamine and tryptamine derivatives

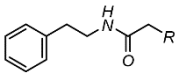
(cytotoxic)

Phenethylamine and tryptamine derivative	R	A/B
1	Benzyl	A
2	2-methylpropyl	A
3	Pentyl	A
4	Hexyl	A
5	Heptyl	A
6	Octyl	A
7	6-methylheptyl	A
8	Nonyl	A
9	3-decene	A
10	Decane	A
11	4-undecene	A
12	Undecane	A
13	5-dodecene	A
14	Dodecane	A
15	6-tridecene	A
16	Tridecane	A
17	7-tetradecene	A
18	Tetradecane	A
19	12-methyltridecane	A
20	8-pentadecene	A
21	Pentadecane	A
22	4-methylpentane	B
23	Octane	B
24	Decane	B
25	4-undecene	B
26	5-dodecene	B



Nemaucin

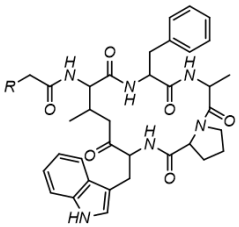
(various variants exist)



Phenethylamides

(cytotoxic)

Phenethylamide	R
1	Phenyl
2	Ethyl
3	1-methylethyl

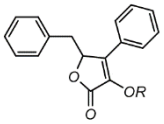


Chaiyaphumines

(weak cytotoxic and antiprotozoal)

Chaiyaphumine	R
1	Phenyl
2	Ethyl
3	Methyl
4	H

Xenofuranone	R
A	Methyl
B	H



Xenofuranones

(weak cytotoxic)

Figure 3 Continued

Pristinamycin. Pristinamycin forms part of the streptogramin A family of antibiotics and was until recently known to be produced by streptomycetes only. Pristinamycin consists of approximately 30% pristinamycin I and 70% pristinamycin II. Component II occurs in two forms, pristinamycin II_A and II_B, of which II_A is the most abundant (98). This streptomycete antibiotic pristinamycin II_A is, however, also produced by *X. nematophila* via a hybrid PKS/NRPS (99). The biosynthetic gene clusters for this compound are very similar in *X. nematophila* and *Streptomyces pristinaspiralis*. Interestingly, further analysis of *X. nematophila* showed that it does not contain a gene cluster for the biosynthesis of pristinamycin I_A. The *pxn* (pristinamycin II_A, *X. nematophila*) gene cluster, however, is

associated with transposases, suggesting that the genes were obtained through horizontal gene transfer. This might explain the absence of a pristinamycin I_A gene cluster in *X. nematophila.*

Xenortides. To date four xenortides, namely xenortides A-D, have been identified from *X. nematophila* (83, 84, 100). These peptides are biosynthesized by a gene cluster consisting of two NRPS genes (*xndA* and *xndB*). Xenortides have weak antiprotozoal activity, with the tryptamides (xenortides B and D) being more active than the phenylethylamides (xenortides A and C), and xenortide B being the most active (100).

Rhabdopeptides. Rhabdopeptides are linear, nonribosomally produced, and structurally similar to xenortides. A total of eight rhabdopeptides have been identified, rhabdopeptides one to four are from *X. nematophila,* and seven and eight are from *Xenorhabdus cabanillasii* (101). Rhabdopeptide 2 has weak cytotoxic activity against myoblasts; 2, 7 and 8 have antiprotozoal activity, and 7 and 8 are weakly hemotoxic. These peptides are produced at high concentrations after 4 days of infection but this stagnates after 10 days, suggesting that rhabdopeptides are important during the stages of insect bioconversion and nematode reproduction. The gene cluster responsible for the biosynthesis of these peptides consists of a three module NRPS gene, RdpABC.

Bicornitun. *Xenorhabdus budapestensis* produce the arginine rich, bioactive compounds, bicornitun A1, A2, B and C (102). The NRPS responsible for the production of bicornitun A1 was identified as BicA. This was determined by cloning the *bicA* gene, which encodes BicA, into an expression vector and heterologously expressing bicornitun A1 in *Escherichia coli* (103). Furthermore, the bicornitun complex (a combination of bicornitun A-C) is cytotoxic towards *Phytophthora nicotianae* by inhibiting colony formation, as well as mycelial growth. *Erwinia amylovora* and *Bacillus subtilis* is also susceptible to the bicornitun complex.

PAX peptides. PAX peptides 1 to 5 were first identified by Gaultieri et al. (104), as lysine-rich cyclolipopeptides produced by *X. nematophila*. These peptides have antifungal and antibacterial activity, however they do not show cytotoxic activity and did not lead to increased mortality when injected into insects. An additional eight PAX peptides were identified and their structure elucidated by Fuchs et al. (105). Three NRPS genes (*paxABC*) are responsible for the biosynthesis of the PAX compounds. The three NRPSs, PaxA, PaxB and PaxC contains three, nine and ten domains, respectively.

Cabanillasin and Nemaucin. More recently, another two peptides were isolated, namely cabanillasin and nemaucin. These peptides were isolated from *X. cabanillasii* and have shown significant bioactivity. Cabanillasin is efficient at inhibiting the growth of human pathogenic filamentous fungi and yeasts (106). Nemaucin was, however, active against methicillin resistant *Staphylococcus aureus* (MRSA). Common genes are proposed to be involved in the production of these two peptides as both compounds have four units of the amino-1 guanidino-butane moiety and are produced by the same organism. Nemaucin is, however, structurally more similar to fabclavine 1a from *X. budapestensis*, than cabanillasin, and differs only by having a shorter C-terminal at the peptide moiety (96).

Dithiolopyrrolone derivatives. These derivatives include the two metabolites, xenorhabdins and xenorxides. Xenorhabdins have a typical heterobicyclic pyrrolinonodithiole core, which is characteristic of dithiolopyrrolone compounds (107). Xenorxides, in turn, are structurally similar to xenorhabdins and are produced when the sulphur moiety of xenorhabdins is oxidized (108). Xenorhabdins and xenorxides have antibacterial, antifungal and insecticidal activities (109–111). Additionally, some of these dithiopyrrolone derivatives have anticancer properties. The general mode of action for dithiolopyrrolones has been suggested to be the inhibition of RNA synthesis (112–116).

Indole-containing compounds. Indole is an aromatic heterocyclic compound, consisting of a fused pyrrole- and benzene ring (117). Various bacterial species produce indole and indole derivatives that play a role in the regulation of bacterial physiology (118). Indole derivatives isolated form *X. nematophila* and *X. bovienii* are active against Gram-positive and Gram-negative bacteria, as well as fungi. Sundar and Chang (119) studied these compounds and revealed the mechanism of action as the inhibition of RNA synthesis. Growing bacteria have a relatively narrow range of ppGpp concentrations and indole derivatives increase this concentration, leading to a reduction in RNA synthesis and ultimately a reduction in growth rate. Furthermore, Seo et al. (120) identified the indole-containing compound, oxindole, as well as indole, also produced by *X. nematophila*. These compounds have weak phospholipase A_2 inhibitory effects. As mentioned previously, phospolipase A_2 is an enzyme required for the production of eicosanoids. Eicosanoids, in turn, are crucial for activating an immune response in the insect by modulating and mediating hemocyte behaviour (121). Therefore, these compounds inhibit the immune response of the insect, making it more susceptible to infection by microorganisms. Furthermore, Proschak et al. (122), identified additional indole derivatives,

called xenocycloins (A-E), also produced by *X. bovienii*. These compounds have no antibacterial activities, but xenocycloin B and D are active against *G. mellonella* hemocytes. Xenocyloins therefore, also contribute to the insecticidal activity of these bacteria. Xenematides, previously discussed under depsipeptides, are also known to contain the indole structure.

Another indole containing compound, nematophin, is highly active against MRSA strains (123). In a study done by Li, Chen and Webster (124), minimal inhibitory concentrations of nematophin and its derivatives against *S. aureus* strains were determined and it was proven that compounds with an α-carbonyl acyl group inhibited the growth of *S. aureus.* However, compounds where the α-carbonyl acyl group was reduced or transferred to a corresponding α-methoximino acyl group, bioactivity decreased or disappeared. It was therefore suggested, that this α-carbonyl acyl group is essential for the bioactivity of these compounds.

Unnamed peptides. Two antimicrobial peptides, GP-19 and EP-20, have been isolated from *X. budapestensis* (125). These peptides show broad-spectrum antimicrobial activity against fungi and bacteria, but the mode of action is yet to be unraveled. GP-19 has a neutral charge and is proposed to cause a disruptive effect on the membrane by mobilizing to the cell surface and possibly penetrating the membrane. As EP-20 has a negative charge it most likely does not have the same mode of action. This peptide is proposed to have an intracellular effect, by inhibiting cell wall, nucleic acid and protein synthesis.

Benzylideneacetone. The moderately hydrophobic compound, benzylideneacetone, isolated from *X. nematophila*, is active against Gram-negative plant pathogenic bacteria. This compound has been used in the industry for various applications, including as a flavouring additive in soaps, cosmetics, detergents and cigarettes, as well as a food additive in candy, gelatin, and puddings. Even though it has been used for some time, it was only discovered in 2004 to have antibacterial activity (126). Benzylideneacetone also inhibits phospholipases A_2, which, as described, results in the inhibition of the immune response of the insect (120).

Rhabduscin. Rhabduscin is an insecticidal tyrosine derivative, produced by *X. nematophila*. The insecticidal activity of this compound is achieved by inhibiting the enzyme phenoloxidase to a low nanomolar-level with an IC_{50} measurement of approximately 64.1 nM. Phenoloxidase

is important in the melanization pathway of the insect's immune system. Inhibition thereof leads to inhibition of one of the primary innate immune responses (127).

Bacteriocins. *Xenorhabdus* bacteria also produce bacteriocins, for example, xenocin, which is produced by *X. nematophila*. Interestingly, the antibacterial activity of xenocin was only observed when bacterial strains were grown in minimal medium and not in enrichment medium such as Luria or nutrient broth. Xenocin production is triggered by a low iron concentration. The role of iron depletion has been proposed to be linked to an iron repressed protein, which may act as a toxin receptor on sensitive bacterial strains. This bacteriocin is therefore, only produced in the host larva when nutrient concentrations are low and competition intensifies (128). Another bacteriocin, produced by *X. nematophila* as well as *X. bovienii*, the phage tail-like xenorhabdicin, is bactericidal (87, 129, 130). *Xenorhabdus* owes its activity against closely related bacteria to these bacteriocins, which are essential for keeping the environment free of other *Xenorhabdus* spp. and its sister genus, *Photorhabdus* spp. *X. beddingii* is also able to produce bacteriocins, however these bacteriocins have not been characterized.

Upregulating the Production of *Xenorhabdus* Antimicrobials

When producing antibiotics, it is of the utmost importance that the fermentation conditions are optimal to avoid the squandering of time and money. Antibiotic production in *Xenorhabdus* has been optimized at various time periods, mostly by one research group from the Northwest University of Agriculture and Forestry, China. This group focused on antibiotic production by *X. bovienii* YL002 (131, 132) and, *X. nematophila* TB (133) and YL001 (134), while another group focused on a specific *X. nematophila* strain isolated from *S. carpocapsae* BJ (135). Factors taken into consideration for these studies were the environmental parameters; initial medium pH, temperature, rotary speed, inoculation volume, medium volume in flask, fermentation time, dissolved oxygen levels and growth media.

As expected, the optimization for specific strains varies. There are, however a few trends in the results of these studies. The optimal fermentation conditions are a pH from 6.0 to 8.24, temperature of 25-32 °C, rotary speed of 150-220 rpm, inoculation volume of 4-15%, medium volume of 54-100 ml/250 ml flask and a fermentation time of 54-72 h. The dissolved oxygen level was tested for only *X. nematophila* YL001 and was optimal when it was shifted during fermentation from 70% after the first 18 h to 50% for the remaining 54 h. The optimal growth

media was tested for *X. nematophila* TB and *X. bovienii* YL002, however, the ingredients and amount of each ingredient differs for the respective recipes.

Crawford et al. (136) identified one of the main compounds that leads to increased small metabolite production in *X. nematophila*. *Xenorhabdus* bacteria are known to produce higher concentrations of bioactive compounds when in *G. mellonella* hemolymph than grown *in vitro* (137). Therefore, it was hypothesized that one or more compounds present in insect hemolymph are responsible for activating the production of bioactive compounds. This led to the selective purification of *G. mellonella* hemolymph, which led to the discovery of proline as the activating signal. Supplementing bacterial cultures with D-proline did not increase the production of bioactive compounds, however L-proline did. L-proline is thought to be a generic activating signal as it is present in various insect larvae.

The addition of L-proline to bacterial cultures led to an increase in xenematide, three indole derivatives and rhabduscin biosynthesis. Another indole-containing compound that was affected by an increase in L-proline is nematophin. This L-proline increase led to a decrease in the production of nematophin but an increase in its reduced derivative. L-proline therefore, regulates a metabolic shift in this case, rather than an increase in nematophin production.

It is evident that production of bioactive compounds requires optimization of the production protocol. This is necessary both for use in industry, as well as in research. The optimization process is however not an easy task and extended research is needed for this process, especially since the protocol will be specific for each bacterial strain and product desired.

Conclusion

Even though *Xenorhabdus* is not one of the generally known antimicrobial metabolite sources, it is clear to see why Pidot et al. (77) refer to it as a neglected antibiotic source. It is evident that *Xenorhabdus* bacteria are an excellent source for novel antimicrobial metabolites. Various studies (102, 138–141), have revealed the significant potential of these bioactive secondary metabolites not only *in vitro*, but also *in vivo*. These studies investigated the use of these compounds in only the agricultural industry. However, these compounds may also be exploited in various other industries, including the healthcare and food industries.

A number of papers have been published on *Xenorhabdus* bacteria and their bioactive compounds. However, this is only the tip of the iceberg. A study done by Crawford et al. (142), stated that the *X. nematophila* DSM 3370^{T} genome contains various gene clusters encoding small molecule antimicrobial metabolites. The number of potential metabolites estimated to be produced by this bacterium vastly exceeds the amount of known antibiotic metabolites. Furthermore, it is generally known that different *Xenorhabdus* species, and even strains, produce different bioactive compounds. Therefore, it is clear that the possibilities regarding novel bioactive compounds produced by *Xenorhabdus* bacteria are virtually endless. Furthermore, taking into consideration the current antibiotic resistance crisis, novel antibiotic discovery is of the essence and *Xenorhabdus* bacteria might hold the key to human survival in the 21st century.

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Chapter 3

Three Novel *Xenorhabdus-Steinernema* Associations and Evidence of Strains of *X. khoisanae* Switching Between Different Clades

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Three Novel *Xenorhabdus-Steinernema* Associations and Evidence of Strains of *X. Khoisanae* Switching Between Different Clades

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Abstract

Xenorhabdus species are normally closely associated with entomopathogenic nematodes of the family Steinernematidae. Strain F2, isolated from *Steinernema nguyeni*, was identified as *Xenorhabdus bovienii* and strains J194 and SB10, isolated from *Steinernema jeffreyense* and *Steinernema sacchari* as *Xenorhabdus khoisanae*, based on phenotypic characteristics and sequencing of 16S rRNA and housekeeping genes *dnaN*, *gltX*, *gyrB*, *infB* and *recA*. All three strains produced antimicrobial compounds that inhibited the growth of Gram-positive and Gram-negative bacteria. This is the first report of associations between strains of the symbiotic bacteria *X. bovienii* with *S. nguyeni*, and *X. khoisanae* with *S. jeffreyense* and *S. sacchari*. This provides evidence that strains of *Xenorhabdus* spp. may switch between nematode species within the same clade and between different clades.

Introduction

The genus *Xenorhabdus* (Thomas & Poinar, 1979) belongs to the family *Enterobacteriaceae.* *Xenorhabdus* bacteria are Gram-negative, asporogenous, facultative anaerobic rods with an optimal growth temperature of 28 °C, or less, and are differentiated from other *Enterobateriaceae* by the inability to produce oxidase and reduce nitrate [3, 25]. *Xenorhabdus* spp. produce secondary metabolites that include bacteriocins and antibiotics [6, 21]. Two cell variants with different physiological and morphological characteristics exist, and are classified as phase I or phase II. Phase I cells absorb certain dyes and produce protease, lipase and antibiotics. These characteristics are lost or reduced in phase II cells [2, 5].

Xenorhabdus species are known to be in a mutually beneficial association with free-living infective juveniles (IJs) of the genus *Steinernema*. These nematodes are pathogenic to various insect hosts. When the nematodes locate the host in the soil, they penetrate the insect through natural openings and the bacteria are released into the hemocoel. Degradation of the insect leads to septicaemia and the host dies within 24 to 48 h. Compounds released by the bacteria protect the nematodes against attack from other microorganisms. With completion of its life cycle, the nematodes take up the symbionts and leave the host cadaver to infect a new host [1]. In general, the *Steinernema-Xenorhabdus* association is species-specific and strains of a *Steinernema* species are associated with a single *Xenorhabdus* species only. However, strains of a single *Xenorhabdus* species may be associated with more than one *Steinernema* species [3].

During this study *Xenorhabdus* bacteria were isolated, for the first time, from *Steinernema nguyeni* Malan, Knoetze and Tiedt, *Steinernema jeffreyense* Malan, Knoetze and Tiedt and *Steinernema sacchari* Nthenga, Knoetze, Berry, Tiedt and Malan. Relatedness of the bacteria and their association with the three *Steinernema* spp. were studied using phenotypic, biochemical and molecular methods.

Materials and Methods

Isolation and Maintenance of Cultures

Galleria mellonella L. (Lepidoptera: Phyralidae) larvae were exposed to IJs of *S. nguyeni* F2 (KP325084) [17], originally collected from soil beneath an *Olea europaea* subsp. *africana* L.

tree in the Western Cape, 32°20’23’’S, 18°47’49’’E, *S. jeffreyense* J194 (KC897093) [18], isolated from soil underneath a guava tree in the Eastern Cape, 34°02’730’’S, 24°55’610’’E and *S. sacchari* SB10 (KC633095) [20] isolated from a sugar cane field in KwaZulu-Natal, 29°0.1’37’’S, 31°35’37’’E. Eighteen hours after exposure to the IJs, the live *Galleria* larvae were sterilized with 98% (v/v) alcohol and pricked close to a proleg, by using a sterile syringe [12]. A drop of haemolymph was spread-plated onto Nutrient Agar (Biolab, Biolab Diagnostics, Midrand, South Africa), supplemented with 0.025% (w/v) bromothymol blue and 0.004% (w/v) triphenyltetrazolium chloride (NBTA), and incubated at 30 °C for 48 h [11, 13]. Single colonies that absorbed bromothymol blue were sub-cultured to obtain pure cultures.

Bacterial isolates F2, J194 and SB10 were cultured in Tryptone Soy Broth (TSB, Biolab) and stored at -80 °C in the presence of 40% (v/v) glycerol. *Escherichia coli* DH5α transformants were cultured in Luria broth (LB, Biolab), supplemented with 100 µg ampicillin/ml. Indicator strains (*Bacillus subtilis* subsp. *subtilis* BD170, *Listeria monocytogenes* EDGE, *Staphylococcus aureus* Xen 5, *Pseudomonas aeruginosa* Xen 5, *Escherichia coli* Xen 14 and *Salmonella typhimurium* Xen 26 from the culture collection of the Department of Microbiology, Stellenbosch University) were cultured in Brain Heart Infusion (BHI, Biolab), with the exception of *E. coli* Xen 14 that was cultured in LB (Biolab). *Escherichia coli* DH5α transformants and indicator strains were cultured in their respective growth media and stored at -80 °C, in the presence of 40% (v/v) glycerol. All assays were performed by using phase I cells, i.e. cells that absorbed bromothymol blue from NBTA.

Biochemical Characterization

Gram-reaction, and catalase and oxidase activity were determined as described before [11]. Isolates were cultured at 26, 30, 37 and 42 °C for 24 h in TSB and changes in growth determined by recording optical cell density (OD) at 600 nm. Media required for optimal growth was determined by growing cultures in LB (Biolab), Nutrient Broth (NB, Biolab) and TSB (Biolab). Incubation was at 30 °C. Optical density readings ($OD_{600\ nm}$) were recorded after 24h. Lecithinase and DNase activity were determined as described by Ferreira *et al.* [11]. Lipase activity was determined by a modification of the method used by Ferreira *et al.* [11]. Briefly, 48 h old cultures were spot-inoculated onto growth media containing 0.1% (w/v) $CaCl_2 \cdot 2H_2O$, 5% (w/v) NaCl, 10% (w/v) peptone and 10% (v/v) Tween (40, 60 or 80, respectively).

Biochemical characteristics were determined by using the API 20 NE test strips, according to instructions of the manufacturer (BioMérieux, Marcy I'Etoile, France). Strips were incubated for 10 days at 30 °C.

Antibacterial Activity

Xenorhabdus isolates were spot-inoculated onto TSA plates and incubated at 30 °C for 24, 48, 72 and 96 h, respectively. After incubation, colonies were overlaid with BHI agar (1%, w/v), seeded with a 12 h-old culture of *B. subtilis* subsp. *subtilis* BD170 (1%, v/v). The same method was used to overlay 96 h-old colonies with the remainder of the indicator strains used in this study. All plates were incubated at 37 °C for 24 h and examined for the presence of growth inhibition (clear zones surrounding the colonies).

Phylogenetic Analysis of Isolates

A modification of the method described by Crouse and Amorese [9] was used to extract genomic DNA. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene and housekeeping genes glutamyl-tRNA synthetase catalytic subunit (*gltX*) and DNA polymerase III beta chain (*dnaN*). PCR amplifications were performed by using Q5® High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) or GoTaq® HotStart DNA Polymerase (Promega Corporation, Madison, Wisconsin, USA) in a SwiftMinipro thermal cycler (Esco Healthcare, Malaysia). Primers for the housekeeping genes and 16S rRNA were obtained from Integrated DNA Technologies (Coralville, Iowa, USA) and Inqaba Biotechnology (Pretoria, South Africa), respectively. Oligonucleotide sequences are listed in Table S1. Amplified genes were sequenced by the DNA sequencing unit, Central Analytical Facility (CAF), University of Stellenbosch, South Africa.

A Whole Genome Sequencing (WGS) approach was used to clarify the exact species of isolates J194 and SB10. Genomic DNA was mechanically fragmented using the Covaris® M220 Focused-Ultrasonicator™ (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing libraries were prepared by using the NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs Inc., Ipswich, MA, USA) and the Illumina® NEBNext® Multiplex Oligos (New England Biolabs Inc.) before sequencing the genomic DNA on the Illumina® Miniseq (Illumina, San Diego, California, USA). This service was delivered by the Centre for Proteomic

and Genomic Research, for isolates J194 and SB10, and *X. miraniensis* Q1^T. Whole genome sequencing for *X. khoisanae* SF87^T was performed on the Ion Proton (CAF). Accession numbers are SAMN06841127, SAMN06841126, SAMN06841131 and SAMN06841128 for J194, SB10, *X. khoisanae* SF87^T and *X. miraniensis* Q1^T, respectively.

Illumina PE reads for each strain were *de novo* assembled with Velvet v1.2.10 [29] and SPAdes v3.10.1 [4]. SPAdes v3.10.1 yielded better assemblies and larger contigs based on the assembly statistics [16, 26]. Contigs containing the 16S rDNA, *dnaN*, *gltX*, DNA gyrase subunit B (*gyrB*), initiation factor B (*infB*) and recombinase A (*recA*) genes were identified by BLASTn v2.6.0+ [7, 28] analysis against respective gene reference sequences. Each gene with approximately 500 bp of up and down stream regulatory elements was used to compare isolates SB10 and J194 to strains, *X. khoisanae* SF87^T and *X. miraniensis* Q1^T by BLASTn analyses.

Additional gene sequences of type strains of *Xenorhabdus* spp. were obtained from the National Center for Biotechnology Information (NCBI). These sequences and sequences obtained from this study were aligned and trimmed by using MEGA6.0 [24]. Distance matrices were calculated by the Kimura two-parameter model [14]. Phylogenetic trees were constructed by using the maximum-likelihood method and bootstrapping with 1000 replicates (MEGA6.0). Translated BLAST searches (BLASTx, NCBI) were performed with protein-coding sequences.

DNA sequences were uploaded to NCBI. Strain F2 with accession numbers KT954036, KT954029 and KT954034 for genes 16S rRNA, *gltX* and *dnaN*, respectively. Strain SB10 with accession numbers KT954037, KT954026 and KX925557 for genes 16S rRNA, *gltX* and *dnaN*, respectively. Lastly, strain J194 with accession numbers KX925559, KT954027 and KX925558 for genes 16S rRNA, *gltX* and *dnaN*, respectively. Gene sequences range from 1371 to 1524, 1009 to 1167 and 995 to 1095 nT for genes 16S rRNA, *gltX* and *dnaN*, respectively. Additional phylogenetic trees are shown in supplementary Figs. S1-S3.

Results and Discussion

All isolates were Gram-negative, rod-shaped, and oxidase and catalase negative. Colonies were dark blue-green on NBTA. Optimal growth was between 26 and 30 °C. Similar growth was recorded on NB, LB and TSB. All three strains were negative for lecithinase activity and were unable to reduce nitrate. Isolate F2 had no DNase or lipase activity, but produced indole from

tryptophan. DNase activity was reported for isolates J194 and SB10. Isolate SB10 showed lipase activity on plates containing Tween 40. D-glucose, D-mannose, N-acetyl-glucosamine, D-maltose, potassium gluconate, adipic acid, malic acid, trisodium citrate and phenylacetic acid were assimilated. Esculin and gelatin were hydrolysed by all three isolates (F2, J194 and SB10).

Maximum antibacterial activity was recorded after 72 h of incubation. The growth of all indicator strains was inhibited, except *P. aeruginosa* Xen 5. Most *Xenorhabdus* spp. produce a range of bioactive secondary metabolites [27]. This is most likely the case with the three isolates, as they clearly inhibited the growth of Gram-positive and Gram-negative bacteria. Growth of *P. aeruginosa* Xen 5 was not inhibited, most possibly due to the low concentration of the secondary metabolites produced.

Sequences obtained for 16S rRNA, *gltX* and *dnaN* of strain F2 shared 98-99% similarity (97-99% DNA coverage) with sequences reported for *Xenorhabdus bovienii* T228^{T}. The phylogenetic tree constructed from concatenated sequences of 16S rRNA, *gltX* and *dnaN* (Fig. 1) grouped strain F2 with *X. bovienii* T228^{T}. The same grouping was obtained with comparison of individual gene sequences and protein sequences (Table S2). This is the first report of *X. bovienii* associated with the entomopathogenic nematode *S. nguyeni*. *Xenorhabdus bovienii* is also associated with closely related members of the *Feltiae*-clade (based on ITS and D2-D3 regions), which include *Steinernema affine* (Bovien) Wouts, Mráček, Gerdin and Bedding, *Steinernema feltiae* (Filipjev) Wouts Mráček, Gerdin and Bedding, *Steinernema intermedium* (Poinar) Mamiya, *Steinernema kraussei* (Steiner) Travassos, *Steinernema jollieti* Spiridonov, Krasomil-Osterfeld and Moens, *Steinernema oregonense* Liu and Berry, *Steinernema puntauvense* Uribe-Lorio, Mora and Stock and *Steinernema weiseri* Mráček, Sturhan and Reid [23]. *Xenorhabdus bovienii* is not only associated with *Steinernema* species in the *Feltiae*-clade, but also with species in the *Affine*-clade, including *Steinernema affine* (Bovien) Wouts, Mráček, Gerdin & Bedding and *Steinernema intermedium* (Poinar) Mamiya [23].

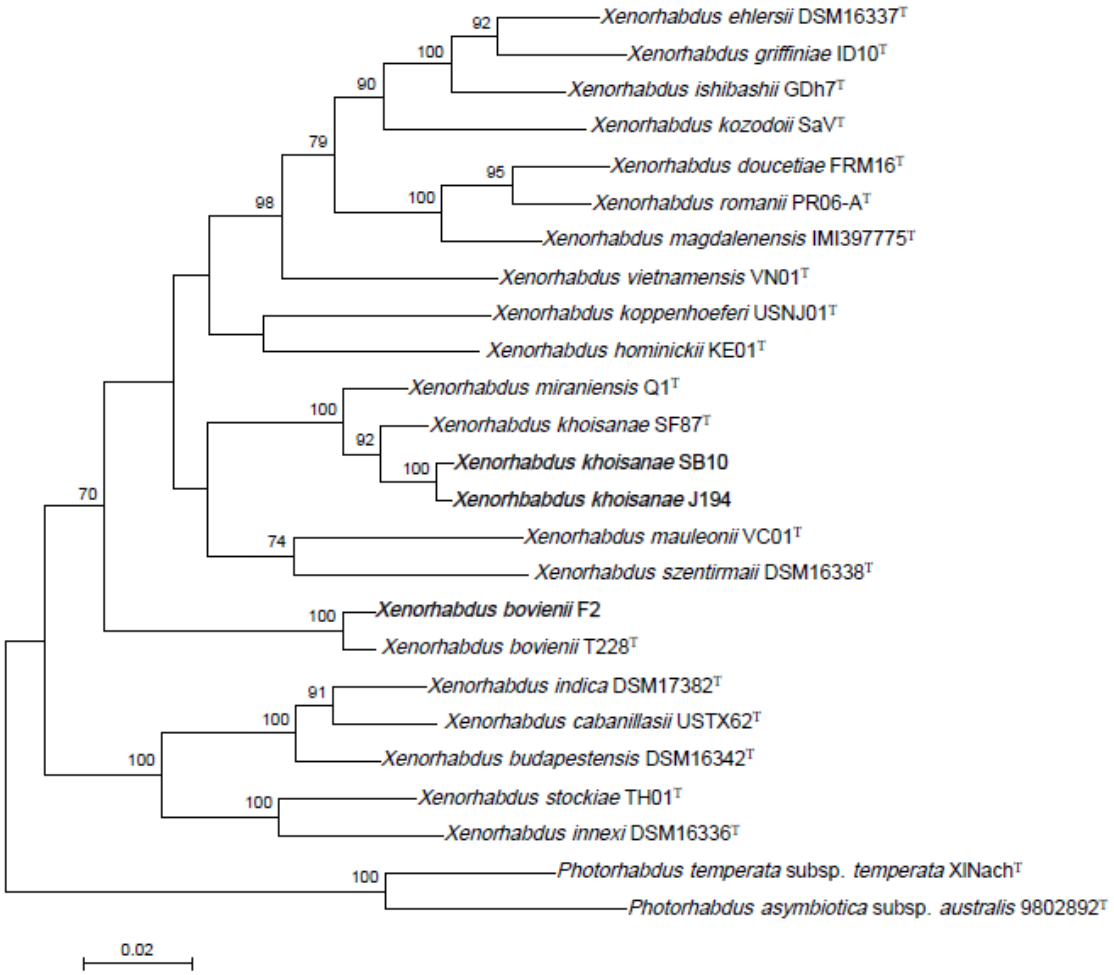

Fig. 1 Phylogenetic position of isolates F2, J194 and SB10 based on concatenated sequences of genes, *16S rDNA, gltX* and *dnaN. Photorhabdus* spp. were used as outgroups and type strains are indicated by superscript letter ^{T}. Bootstrap values below 70% are not shown.

Nucleotide similarity searches suggested that isolates J194 and SB10 belong to the species *X. khoisanae* with a similarity of 96-99%. Figure 1 indicates, as supported by the putative identification based on nucleotide and protein similarities, that isolates J194 and SB10 are additional strains belonging to the species *X. khoisanae*. Previous studies have identified five strains of *X. khoisanae*, strains SF 87^{T}, SF80, SF362 and 106-C all isolated from *S. khoisanae* [10] and strain MCB isolated from an unidentified *Steinernema* sp. [19]. This study reports that *X. khoisanae* is additionally associated with *S. jeffreyense* and *S. sacchari*. *Steinernema jeffreyense* belongs to the *glaseri*-group of which the IJs are larger than 1000 µm and molecularly related to clade V [22]. However, *S. sacchari* belongs to the new monophyletic group, the Cameroonian clade (clade III), which is closely related to the *feltiae-kraussei-oregonense* group, with IJs between 700-1000 µm [20]. Concluded from these reports,

X. khoisanae has the ability to switch between nematodes of distantly related clades (between clades V and III). In a recent report, Çimen et al. [8] have shown that *Steinernema beitlechemi* Çimen, Půža, Nermu, Hatting, Ramakuwela, Faktorová and Hazir, a new entomopathogenic nematode from South Africa and a member of the *Cameroonense*-clade closely related to *S. sacchari*, is also a host to *X. khoisanae*. The authors based their findings on 16S rRNA gene sequences only. Lee and Stock [15] have shown at least 17 host switches of strains of *Xenorhabdus* spp. within clades and even between clades. Stock [23] also reported the switching of strains of *Xenorhabdus nematophila* (Poinar & Thomas) from clade IV to a more distantly related nematode host in clade II.

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Supplementary material

Table S1 Oligonucleotides

Target gene	Primer	Oligonucleotide sequence (5' to 3')	T_m (°C)	Product size (bp)
gltX	gltX1 (F)	GGCACCAAGTCCTACTGGCTA	56.6	1258
	gltX2 (R)	GGCATRCCSACTTTACCCATA	55.9	
dnaN	dnaN1 (F)	GAAATTYATCATTGAACGWG	46.0	1091
	dnaN2 (R)	CGCATWGGCATMACRAC	51.4	
16S rDNA	F8 (F)	CAGGCATCCAGACTTTGATYMTGGCTCAG	55.0	+/- 1600
	R1512 (R)	GTGAAGCTTACGGYTAGCTTGTTACGACTT	55.0	

Table S2 Protein similarity of protein-coding genes using BLASTx

Isolate	**Protein-coding gene**	**Nearest Similarity**			
		Organism	Protein size (aa)	Identity (%)	Accession number
F2	*gltX*	*X. bovienii*	471	99	WP_038186822.1
	dnaN	*X. bovienii*	366	99	WP_038247914.1
J194	*gltX*	*X. khoisanae*	171	98	WP 047961607.1
	dnaN	*X. khoisanae*	331	99	WP_047962545.1
	recA	*X. khoisanae*	358	99	WP_047961634.1
	gyrB	*X. khoisanae*	804	99	WP_047962547.1
	infB	*X. khoisanae*	923	99	WP_047962383.1
SB10	*gltX*	*X. khoisanae*	171	98	WP 047961607.1
	dnaN	*X. khoisanae*	330	99	WP_047962545.1
	recA	*X. khoisanae*	358	100	WP_047961634.1
	gyrB	*X. khoisanae*	804	99	WP_047962547.1
	infB	*X. khoisanae*	923	99	WP_047962383.1

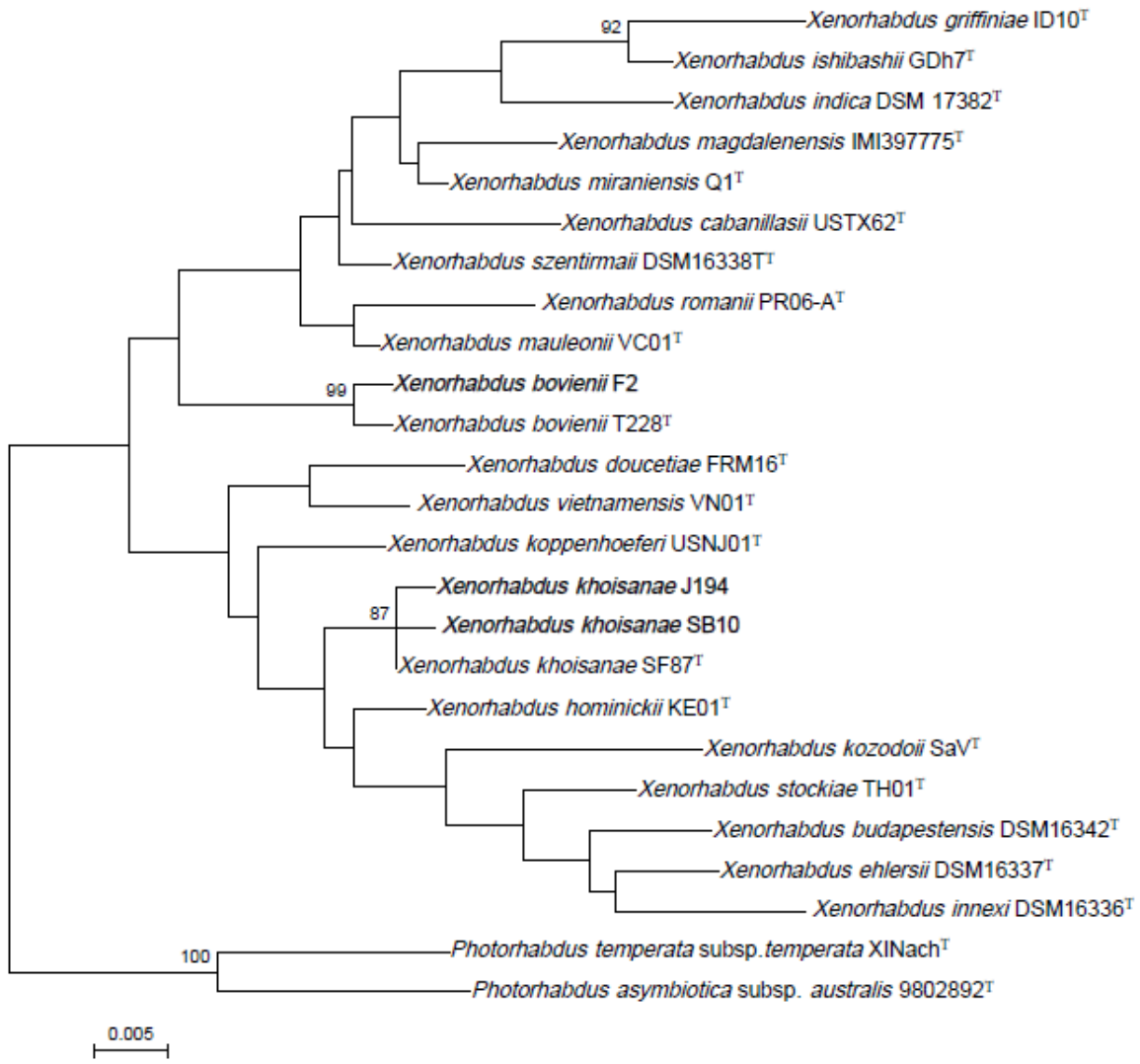

Fig. S1 Phylogenetic relationship of isolates F2, J194 and SB10 with other *Xenorhabdus* spp. based on the *16S rDNA* sequence, using the maximum likelihood method. *Photorhabdus* spp. were used as outgroups and type strains are indicated by ^{T}. Bootstrap values of more than 70% are shown at branch points.

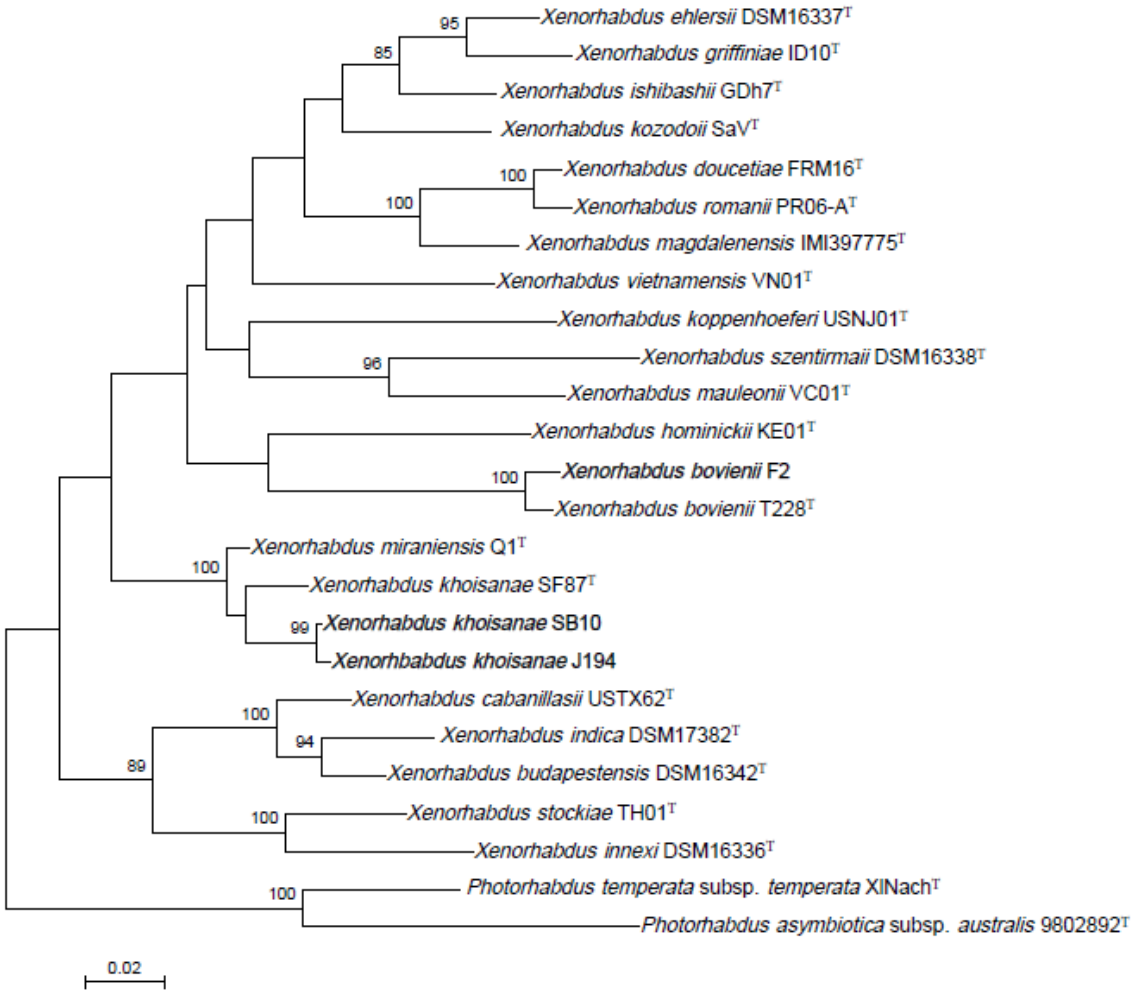

Fig. S2 Phylogenetic relationship of isolates F2, J194 and SB10 with other *Xenorhabdus* spp. based on the *gltX* protein-coding sequence, using the maximum likelihood method. *Photorhabdus* spp. were used as outgroups and type strains are indicated by ^{T}. Bootstrap values of more than 70% are shown at branch points.

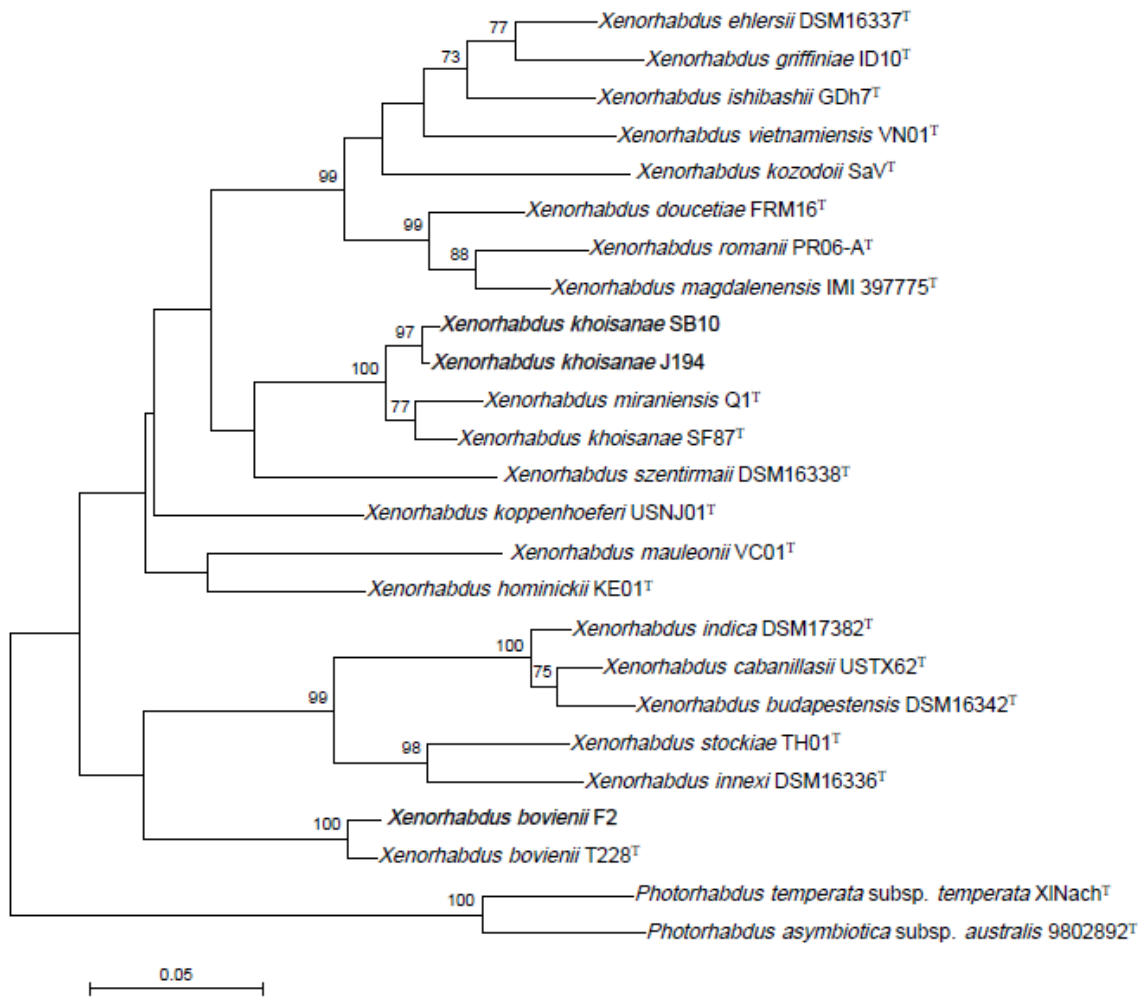

Fig. S3 Phylogenetic relationship of isolates F2, J194 and SB10 with other *Xenorhabdus* spp. based on the *dnaN* protein-coding sequence, using the maximum likelihood method. *Photorhabdus* spp. were used as outgroups and type strains are indicated by ^{T}. Bootstrap values of more than 70% are shown at branch points.

Chapter 4

First Report of a Symbiotic Relationship Between *Xenorhabdus griffiniae* and an Unknown *Steinernema* from South Africa

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First Report of a Symbiotic Relationship Between *Xenorhabdus griffiniae* and an Unknown *Steinernema* from South Africa

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Abstract

Strain WS9, a mutualistic-associated bacterium, was isolated from an unknown entomopathogenic *Steinernema* nematode, collected from a litchi orchard in Friedenheim, Mpumalanga, South Africa. Based on phenotypic and phylogenetic data of the 16S rRNA, *gltX*, *recA*, *dnaN*, *gyrB* and *infB* gene sequences, strain WS9 is identified as *X. griffiniae*. Strain WS9 has antibacterial activity against Gram-positive and Gram-negative bacteria. This is the first report of an association between *X. griffiniae* and an unknown *Steinernema* species from South Africa.

Keywords Entomopathogenic nematodes • Mutualistic bacterium • Taxonomy • 16S rRNA

Introduction

Members of the genus *Xenorhabdus* are Gram-negative, asporogenic, fermentative, facultative anaerobic rods with a respiratory and fermentative metabolism, and belongs to the family Enterobacteriaceae. They grow optimally at 28 °C, are oxidase and catalase negative and do not reduce nitrate to nitrite (Thomas and Poinar 1997; Akhurst and Boemare 2005). Acid is produced from the fermentation of glucose, but without the release of CO_2. *N*-acetyl-glucosamine, glycerol, fructose and mannose are usually fermented (Akhurst and Boemare 2005). *Xenorhabdus* species are known to produce two phenotypic variants that differ in morphology and physiology. Phase I cells are larger than phase II cells, absorb certain dyes and produce proteases, lipases and antibiotics (Akhurst 1980; Boemare and Akhurst 1988).

Xenorhabdus spp. are closely associated with entomopathogenic nematodes of the family Steinernematidae that infects insects such as *Lepidoptera, Diptera, Orthoptera, Coleoptera* and *Hymenoptera* (Laumond et al. 1979; Poinar 1979). Although the association between *Xenorhabdus* spp. and nematodes is species-specific, a single species may infect different *Steinernema* spp. (Fischer-Le Saux et al. 1997; Lee and Stock 2010). More than twenty *Xenorhabdus* species have been characterized, with approximately eleven of these species being associated with more than one *Steinernema* nematode species (Tailliez et al. 2006; Stock 2015).

Steinernema dauer larvae or non-feeding infective juvenile (IJ) transport *Xenorhabdus* to the hemocoel by entering natural openings on the body of the insect. Once in the hemocoel, the IJ nematodes release an immune-suppressive agent that suppresses the activity of antimicrobial peptides produced by the insect (Götz et al. 1981). IJ develop into amphimictic females and males that feeds on the proliferating bacteria. Endo- and exotoxins (e.g. DNases, lipases and lecithinases) are released by the bacteria, killing the insect within 24 to 48 h. Colonization of the cadaver by other microorganisms is prevented by the release of antimicrobial compounds from the respective *Xenorhabdus* species. The bacteria and bio-converted host tissue supports the sexual reproduction of the nematodes as long as nutrients are available. The nematodes, colonized with a few cells of *Xenorhabdus* are then released into the environment to repeat the cycle of infection (Adams and Nguyen 2002).

In this study, the bacterial strain WS9 was isolated from an unknown entomopathogenic nematode *Steinernema* sp. WS9. The bacteria were identified using phenotypic and genotypic characteristics.

Materials and Methods

Isolation of *Xenorhabdus* sp. and Maintenance of Cultures

Galleria mellonella L. (Lepidoptera: Pyralidae) larvae were exposed to IJs of *Steinernema* sp. WS9 (KP325086; MF443108) collected from a litchi orchard in Friedenheim (25°30.927'S 30°58.681'E), Mpumalanga, South Africa. After 18 h, the live *Galleria* larvae were dipped in 98% alcohol, bent and pricked close to a proleg, using a sterile syringe. A drop of hemolymph, was spread-plated onto Nutrient Agar (NA, Biolab, Biolab Diagnostics, Midrand, South Africa), supplemented with 0.025% (w/v) bromothymol blue and 0.004% (w/v) triphenyltetrazolium chloride (TTC), known as NBTA (Kaya and Stock 1997; Ferreira et al. 2013). The plates were incubated at 30 ˚C for 48 h. Colonies that absorbed bromothymol blue were regarded as *Xenorhabdus* spp. and streaked onto NBTA to obtain pure cultures.

Strain WS9, isolated from one colony, was cultured in Tryptone Soy Broth (TSB, Biolab) and stored at -80 ˚C in cryotubes with 40% (v/v) glycerol. All experiments were performed with phase I cells, stored in glycerol, to prevent phase shifting. Phase I cells were differentiated from phase II cells by the absorption of bromothymol blue from NBTA plates. All assays were performed with cultures from glycerol to prevent phase shifting. *Escherichia coli* Xen 14 was cultured in Luria broth (LB, Biolab) and *E. coli* DH5α transformants in LB, supplemented with 100 µg ampicillin/ml. *Bacillus subtilis* subsp. *subtilis* BD170, *Listeria monocytogenes* EDGE, *Staphylococcus aureus* Xen 5, *Pseudomonas aeruginosa* Xen 5 and *Salmonella typhimurium* Xen 26 were cultured in Brain Heart Infusion (BHI, Biolab). Stock cultures were prepared in 40% (v/v) glycerol and stored at -80 ˚C.

Cell Morphology and Phenotypic Characteristics

Strain WS9 was gram stained and the size of a single cell determined using a Leica DM2000 research microscope (Leica Microsystems), equipped with a camera, computer and digital image software [Leica Application Suite (LAS), version 3.5.0]. Oxidase activity was

determined using Kovács oxidase reagent. Catalase activity was recorded by suspending a colony in a drop of 5% (v/v) H_2O_2. Optimal growth temperature was determined by growing cultures at 26 °C, 30 °C, 37 °C and 42 °C for 24 h in TSB. Changes in growth were monitored by recording optical density readings at 600 nm. Lecithinase activity was determined as described by Ferreira et al. (2013). Lipase activity was determined by streaking 48 h-old cultures onto media containing 10% (w/v) peptone, 5% (w/v) sodium chloride, 0.1% (w/v) $CaCl_2 \cdot 2H_2O$ and either 10% (v/v) Tween 40, Tween 60 or Tween 80. All experiments were done in triplicate.

Carbohydrate fermentation and assimilation profiles were determined using API 50 CHE and 20 NE test strips, respectively (BioMérieux, Marcy I'Etoile, France), according to the instructions of the manufacturer. Results were recorded after 10 days of incubation at 30 ˚C.

Genotypic Characterization and Phylogenetic Analysis

Genomic DNA was extracted using a modification of the method described by Crouse and Amorese (1987). The *16S rDNA* and housekeeping genes glutamyl-tRNA synthetase catalytic subunit (*gltX*), recombinase A (*recA*) and DNA polymerase III beta chain (*dnaN*) were amplified by using the primers listed in Table 1. *16S rDNA* primers were from Inqaba Biotechnology (Pretoria, South Africa) and all other primers from Integrated DNA Technologies (Coralville, Iowa, USA). Amplifications were performed using Q5® High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) or GoTaq® HotStart DNA Polymerase (Promega Corporation, Madison, Wisconsin, USA) and a SwiftMinipro thermal cycler (Esco Healthcare, Malaysia). Amplified fragments were ligated into plasmids pGEM®T Easy (Promega Corporation) or pJET1.2 (Thermo Fisher Scientific), according to instructions of the manufacturer. The plasmids were transformed to *E. coli* DH5α and cloned cells selected from colonies on LB agar supplemented with 100 µg/ml ampicillin. Positive clones were confirmed by sequencing. The sequencing facilities at the DNA sequencing unit, Central Analytical Facility, University of Stellenbosch, were used.

Whole Genome Sequencing (WGS) was used to determine the precise species of isolate WS9. Genomic DNA was sequenced on the Illumina Miniseq (Illumina, San Diego, California, USA) instrument. Before sequencing, genomic DNA was fragmented by using the M220 Focused-Ultrasonicator (Thermo Fisher, Waltham, MA, USA). The NEBNext® Ultra™ DNA Library

Prep Kit and Illumina® NEBNext® Multiplex Oligos (New England Biolabs Inc., Ipswich, MA, USA) were used for preparing sequencing libraries. This service was delivered by the Centre for Proteomic and Genomic Research (CPGR). The accession number for the WGS data of strain WS9 is SAMN07327420.

Table 1 Primers used for sequencing

Target gene	Primer	Oligonucleotide sequences (5′-3′)	Tm (°C)	Product size (bp)
gltX	gltX1	GGCACCAAGTCCTACTGGCTA (F)	56.6	1258
	gltX2	GGCATRCCSACTTTACCCATA (R)	55.9	
recA	recA1	GCTATTGATGAAAATAAACA (F)	43.7	893
	recA2	RATTTTRTCWCCRTTRTAGCT (R)	48.8	
dnaN	dnaN1	GAAATTYATCATTGAACGWG (F)	46.0	1091
	dnaN2	CGCATWGGCATMACRAC (R)	51.4	
16S rDNA	F8 (F)	CAGGCATCCAGACTTTGATYMTGGCTCAG (F)	55.0	Approx.1600
	R1512	GTGAAGCTTACGGYTAGCTTGTTACGACTT (R)	55.0	

De novo assemblies of Illumina PE reads were constructed by using SPAdes v3.10.1. Contigs containing the *16S rDNA* and housekeeping genes, *gltX*, *recA*, *dnaN*, DNA gyrase B (*gyrB*) and initiation factor B (*infB*) were identified by using BLASTn v2.6.0+ analysis and compared to respective gene reference sequences. MEGA6.0 (Tamura et al. 2013) was used to align sequences. The maximum-likelihood method was used to construct phylogenetic trees from single gene sequences and concatenated sequences. The Kimura two-parameter model (Kimura 1980) was used to calculate the distance metrics for aligned sequences. The robustness of individual branches was determined by bootstrapping with 1000 replicates. Parameters were kept constant for the construction of all phylogenetic trees. Protein-coding sequences were compared to previously published sequences by performing translated BLAST searches (BLASTx, National Center for Biotechnology Information). Gene sequences of closely related species and outgroups were obtained from the National Center for Biotechnology Information (NCBI).

Testing for Antibacterial Activity

Antibacterial activity was determined by spotting 5 µl of a 12 h-old culture of WS9 onto TSB agar (TSA). The plates were incubated at 30 °C for 24, 48, 72 and 96 h, respectively, and then overlaid with 12 h-old cells of *B. subtilis* subsp. *subtilis* BD170 suspended in BHI agar (1%, w/v). Spot inoculated plates that were incubated for 96 h were overlaid with *L. monocytogenes* EDGE, *S. aureus* Xen 5, *P. aeruginosa* Xen 5 and *S. typhimurium* Xen 26, each suspended in BHI agar (1%, w/v), and *E. coli* suspended in LB agar (1%, w/v). Results were recorded after 24 h of incubation at 37 ˚C.

Results and Discussion

Phenotypic and Biochemical Characteristics

Strain WS9 is Gram-negative, rod-shaped and 0.8-1.1 by 3.1-6.2 µm in size. Catalase and oxidase were not produced. All colonies absorbed bromothymol blue from NBTA plates, which is characteristic for *Xenorhabdus* spp. (Kaya and Stock 1997). Optimal growth was recorded at 26 and 30 °C, with slightly better growth at 30 °C. Strain WS9 does not produce lecithinase, and is negative for the hydrolysis of Tweens 60 and 80, but positive for Tween 40.

Strain WS9 produced acid from the fermentation of ribose, xylose, glucose, fructose, mannose, dulcitol, N-acetylglucosamine, maltose, melibiose, gentobiose and fucose. Acid production from glycerol, arabinose, galactose, inositol, manitol, esculin ferric citrate, trehalose, luxose, arabitol and potassium gluconate was weak. Strain WS9 assimilated glucose, mannose, N-cetyl-glucosamine, maltose, potassium gluconate, adipic acid, malic acid, trisodium citrate and phenylacetic acid. No other tested carbohydrates were metabolized. Nitrate was not reduced to nitrite.

Antibacterial Activity

The antibacterial activity of strain WS9 reached maximum level after 72 h of growth. Colonies of strain WS9 inhibited the growth of *B. subtilis* subsp. *subtilis* BD170, *L. monocytogenes* EDGE, *S. aureus* Xen 5, *E. coli* Xen 5 and *S. typhimurium* Xen 26. No antibacterial activity was recorded against *P. aeruginosa* Xen 5, which is most likely due to a low secondary

metabolite concentration. Most *Xenorhabdus* spp. produce one or more antimicrobial compounds (Webster et al. 2002). Further research is required to identify the antibacterial compounds produced by strain WS9.

Phylogenetic Analysis of Strain WS9

Strain WS9, grouped with the type strain of *X. griffiniae* when *16S rDNA*, *gltX*, *recA*, *dnaN*, *gyrB* and *infB* sequences were concatenated (Fig. 1). Comparison of *16S rDNA*, *gltX*, *dnaN* and *gyrB* sequences (Online Resources 1 to 4), separately, grouped strain WS9 with *X. griffiniae*, but with *X. ehlersii* and *Xenorhabdus* sp. TZ01 (JQ687358; JQ687369–JQ687373) when sequences of *recA* and *infB* (Online Resources 5 and 6) were compared. This suggests that strain WS9 is phylogenetically related to *X. griffiniae* and, to a lesser extent to *X. ehlersii* and *Xenorhabdus* sp. TZ01. Nucleotide similarity searches produced lower identity percentages for *X. ehlersii* and *Xenorhabdus* sp. TZ01 compared to *X. griffiniae*, despite the fact that query coverages were similar, with exception of the *recA* gene (Online Resource 7). Protein-coding sequences of *gltX*, *dnaN* and *gyrB* obtained for isolate WS9 were similar, but not identical, to protein-coding sequences listed for *X. griffiniae* (Online Resource 7). However, corresponding to phylogenetic trees, protein-coding sequences for *recA* and *infB*, were similar to those of *X. ehlersii* and *Xenorhabdus* sp. TZ01, respectively. Taking into consideration all the above mentioned, the bacterial isolate WS9 has been identified as a strain belonging to the species *X. griffiniae*. Closely related *X. ehlersii* has been isolated from *Steinernema longicaudum* Shen & Wang (syn. *Steinernema serratum*) (Lengyel et al. 2005; Tailliez et al. 2006), and *Xenorhabdus* sp. TZ01 from *Steinernema pwaniensis* (Půža et al. 2016). The *Steinernema* sp. strain WS9 is closely related to *S. pwaniensis* from Tanzania, and both belongs to the *Karii*-clade, while *S. longicaudum* belongs to the *Longicaudum*-clade (Spiridonov and Subbotin 2017).

Xenorhabdus griffiniae has previously been isolated from the nematode *Steinernema hermaphroditum* Stock, Griffin & Chaerani (Nguyen and Hunt 2007) and more recently from the nematode *Steinernema khoisanae* strain BMMCB (Mothupi et al. 2015). Based on the internal transcribed spacer (ITS) region (KT027382), *S. khoisanae* BMMCB is 91% similar to the type species, *S. khoisanae* SF80 (DQ314287), and 92% to *Steinernema* sp. strain WS9 (KP325086). According to Nguyen & Hunt (2007) a sequence similarity in the ITS region of 95% or less to the most closely related species indicate a possible new *Steinernema*

species. This suggests that strain BMMCB could be neither *S. khoisanae* nor *Steinernema* sp. WS9, but appears to be a new *Steinernema* species.

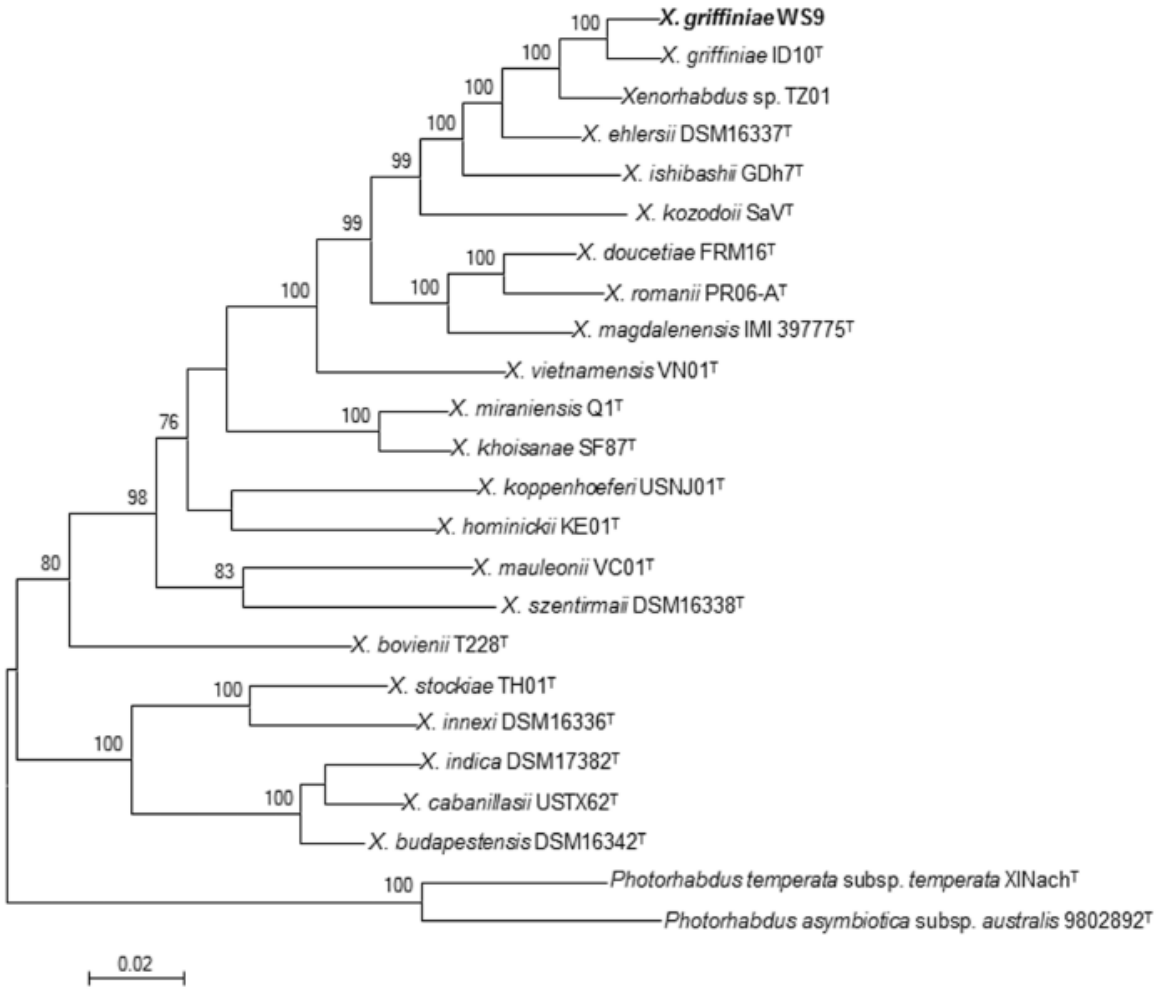

Fig. 1 Phylogenetic relationship of strain WS9 with known *Xenorhabdus* spp. The maximum likelihood tree was constructed using concatenated sequences of six genes (*16S rDNA*, *gltX*, *recA*, *dnaN*, *gyrB* and *infB*). Three *Photorhabdus* spp. were used as outgroups. Type strains are indicated by a superscript ^{T}. Bootstrap values of more than 70% are shown at branch points. The bar indicates a sequence divergence of 2%.

Previously, Spiridonov et al. (2004) grouped all three nematode species associated with *Xenorhabdus griffiniae* in one clade (clade V). According to recent molecular information (Spiridonov & Subbotin, 2017), the two nematode species associated with *X. griffiniae* belong to different clades; *Steinernema* sp. WS9 to the *Karii*-clade and *S. hermaphroditum* to the *Longicaudum*-clade. Host switches of the bacterial symbionts are generally observed between

nematodes belonging to the same clade, however increasing reports have shown that host switches can occur between nematodes from different clades, which is also the case for this study (Lee and Stock 2010; Çimen et al. 2016; Dreyer et al. 2017).

This study reports, for the first time, an association between *X. griffiniae* and an unknown locally isolated *Steinernema* sp. WS9. Accession numbers for the PCR obtained sequences *16S rDNA*, *gltX*, *recA* and *dnaN* are KT954035, KT954028, KT954030 and KT954033, respectively.

Acknowledgements

The National Research Foundation (NRF, South Africa) for funding and Willem Steyn (Agricultural Research Council) for the collection of the nematode sample. Finally, this study would not have been possible without the help of my colleague, Riaan de Witt, with processing of WGS data.

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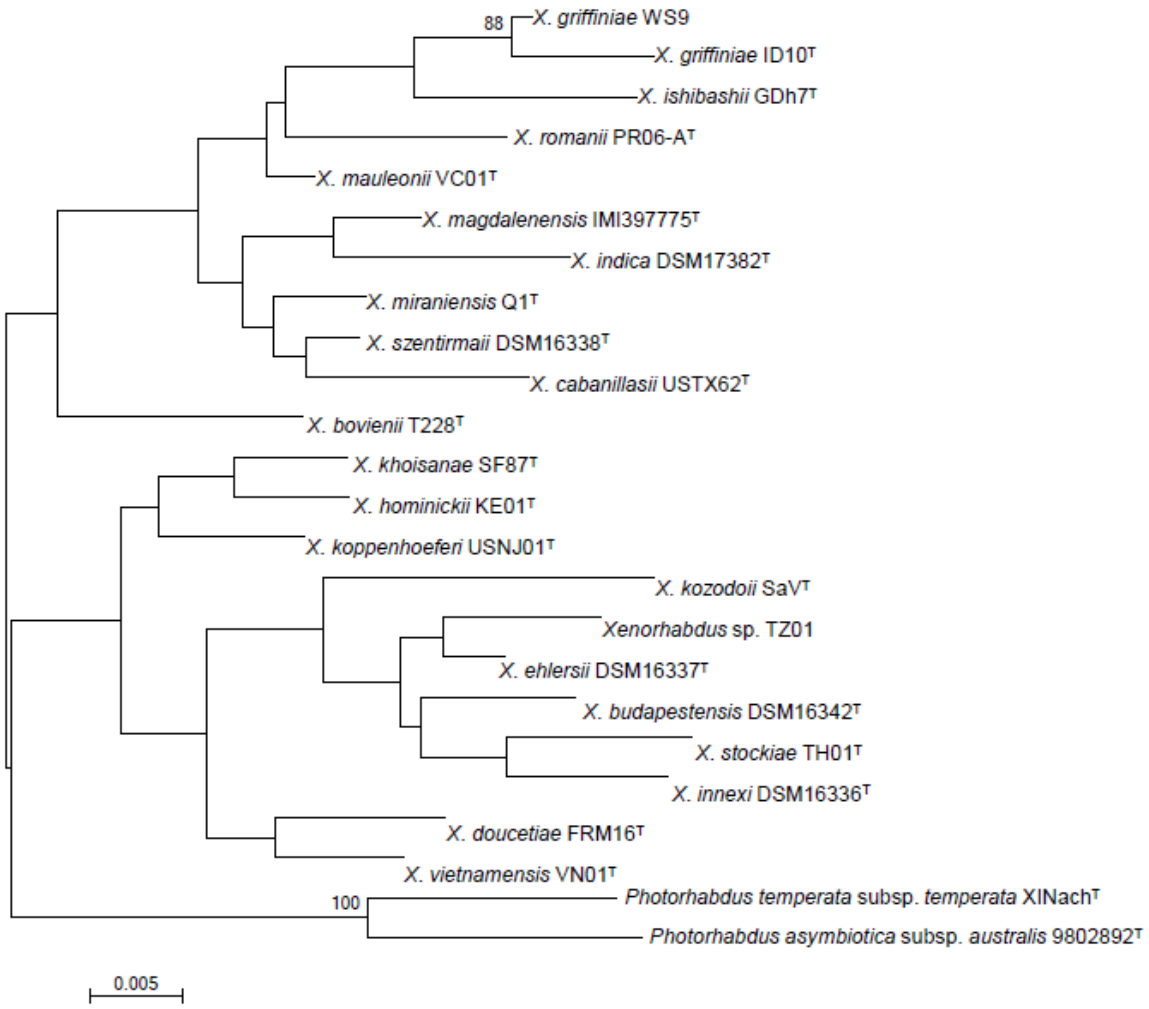
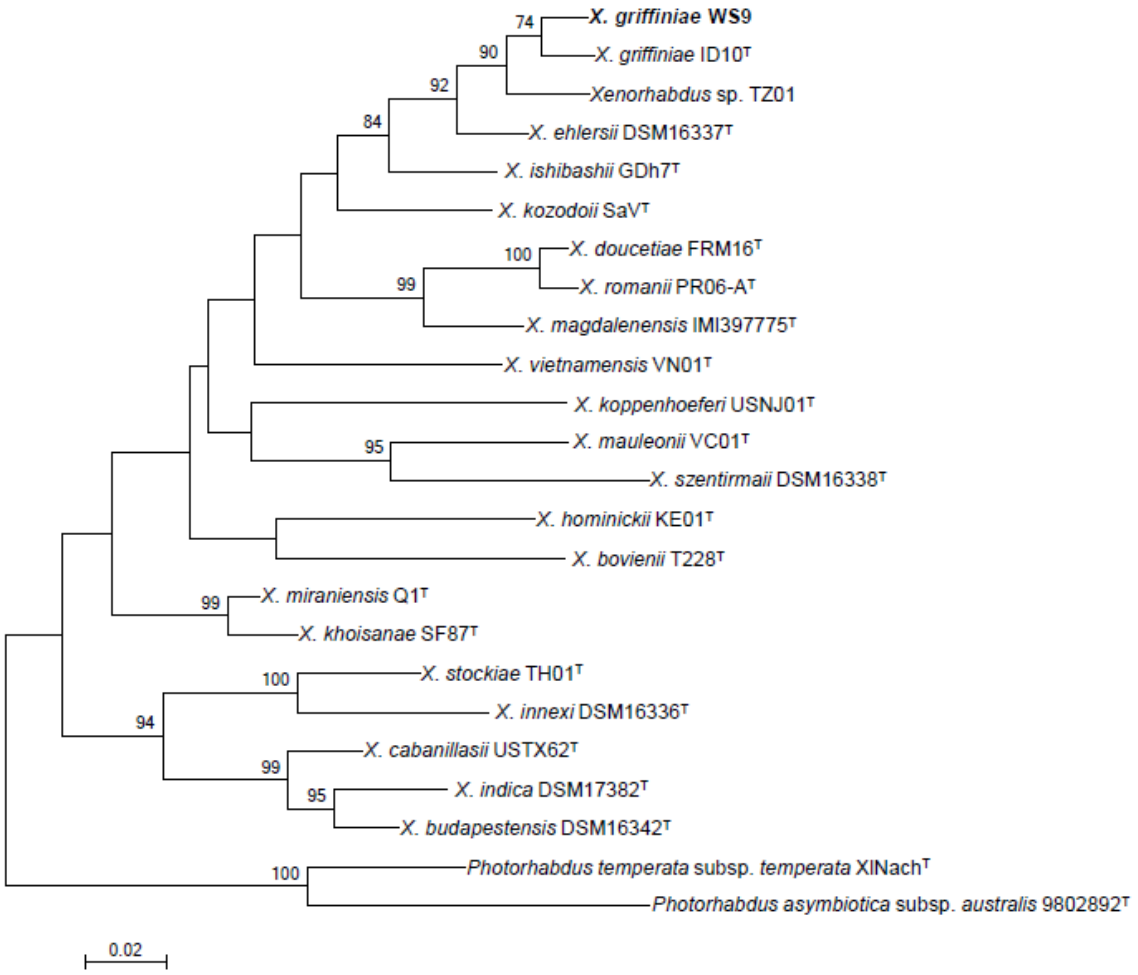
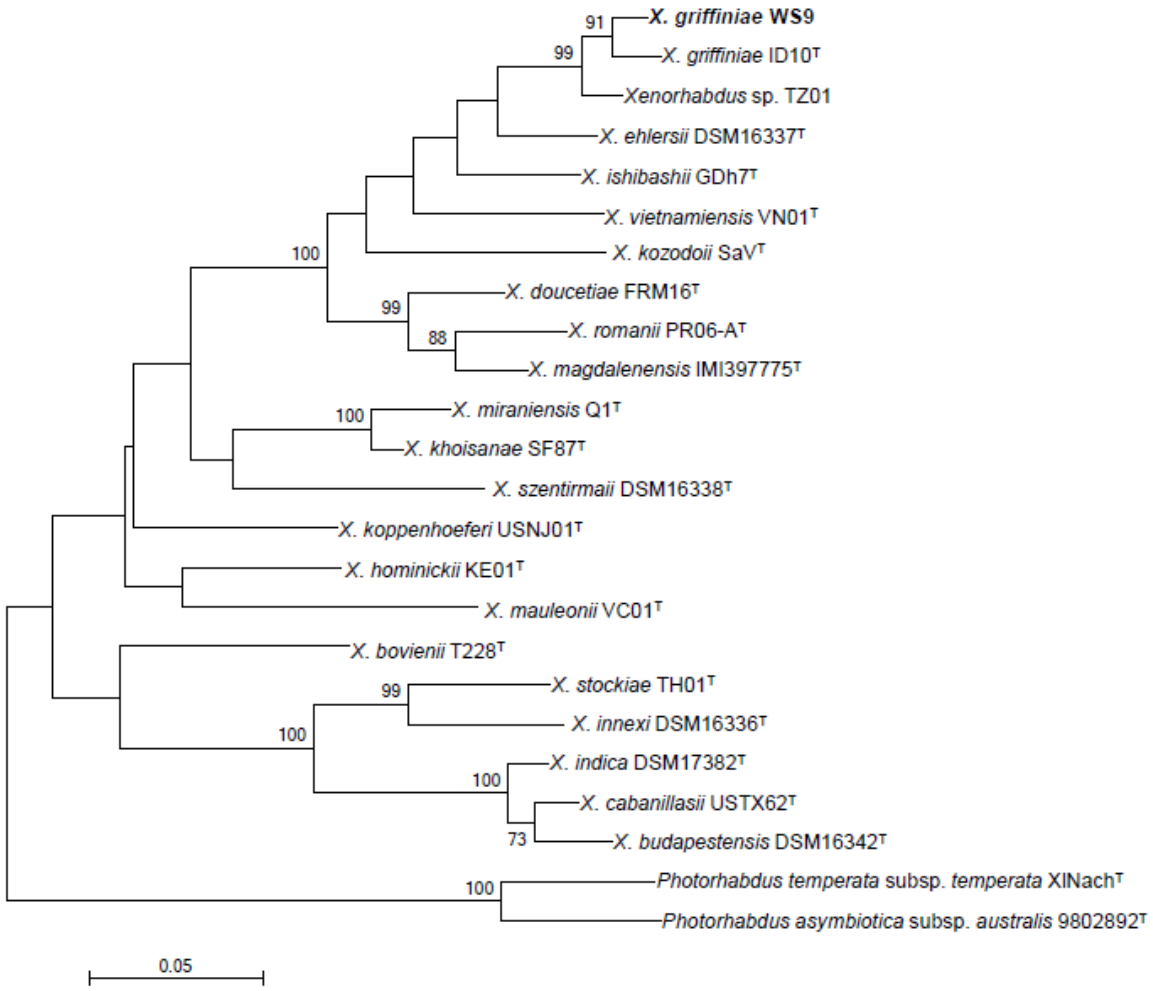
Online Resources



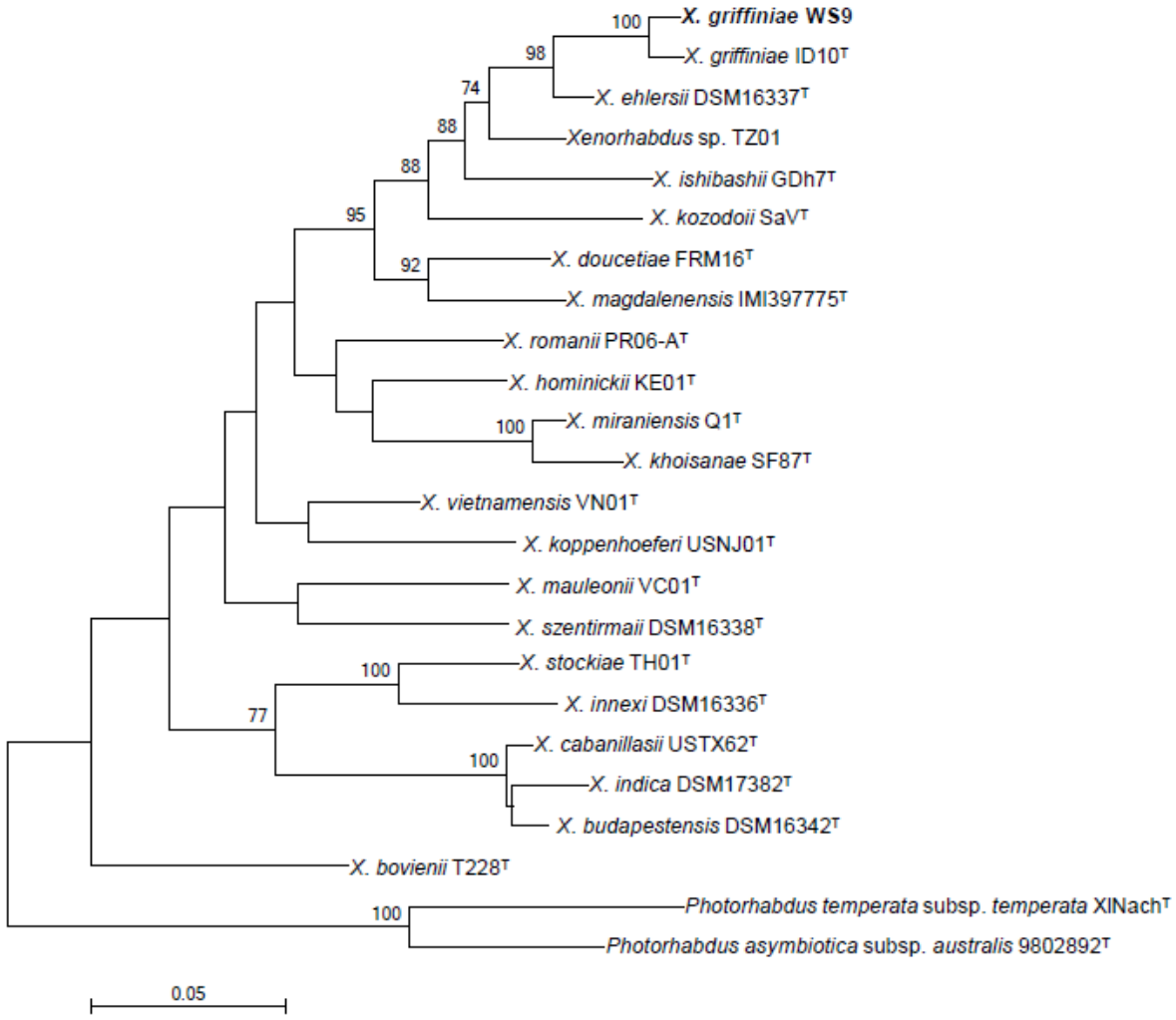
Online Resource 1. Phylogenetic position of strain WS9 with known *Xenorhabdus* spp. based on *16S rDNA* sequences. Three *Photorhabdus* spp. were used as outgroups. Type strains are indicated by a superscript ^{T}. Bootstrap values of more than 70% are shown at branch points. A sequences divergence of 0.5% is indicated by the bar.



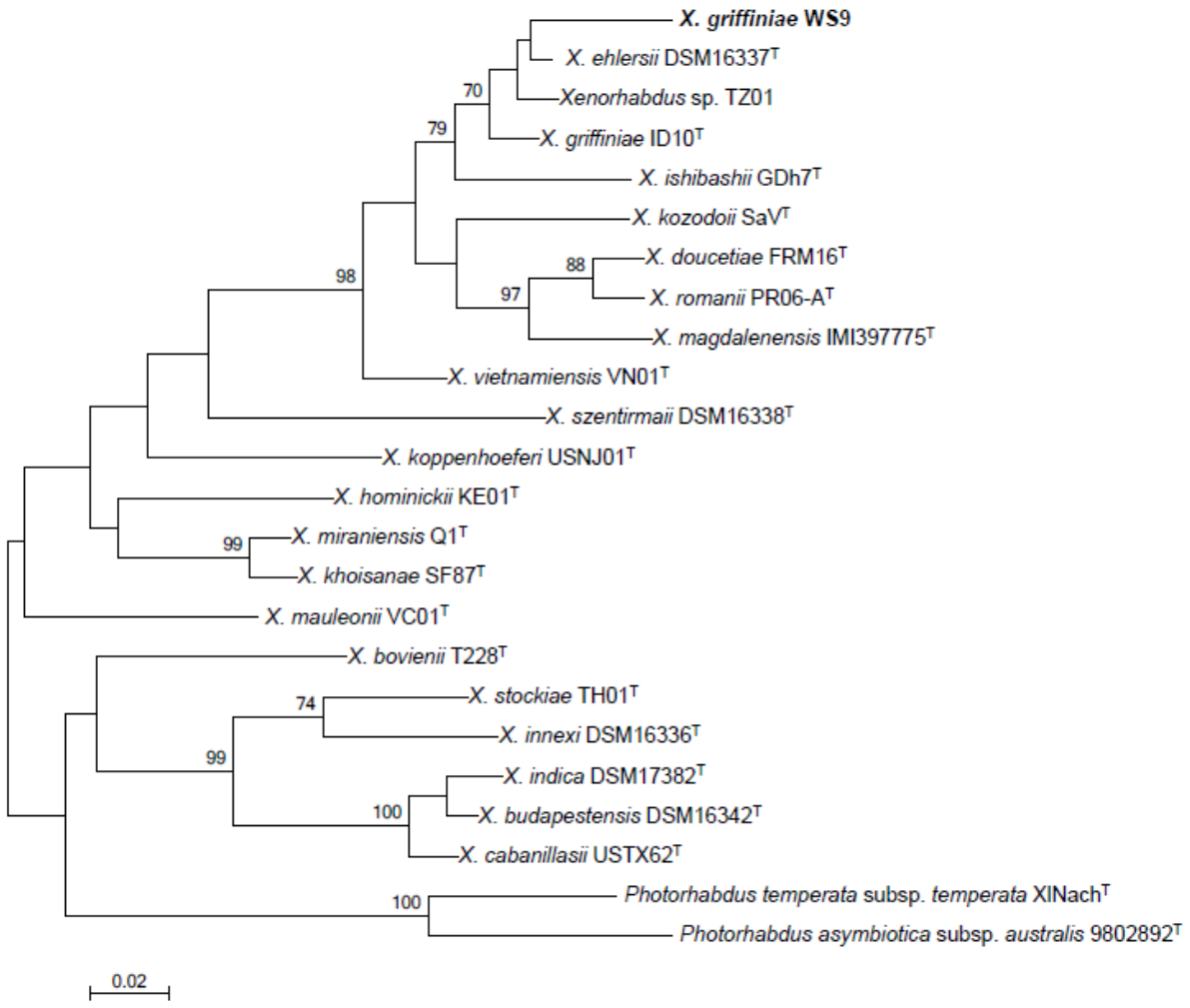
Online Resource 2. Phylogenetic position of strain WS9 with known *Xenorhabdus* spp. based on *gltX* protein-coding sequences. Three *Photorhabdus* spp. were used as outgroups. Type strains are indicated by a superscript ^{T}. Bootstrap values of more than 70% are shown at branch points. A sequences divergence of 2% is indicated by the bar.



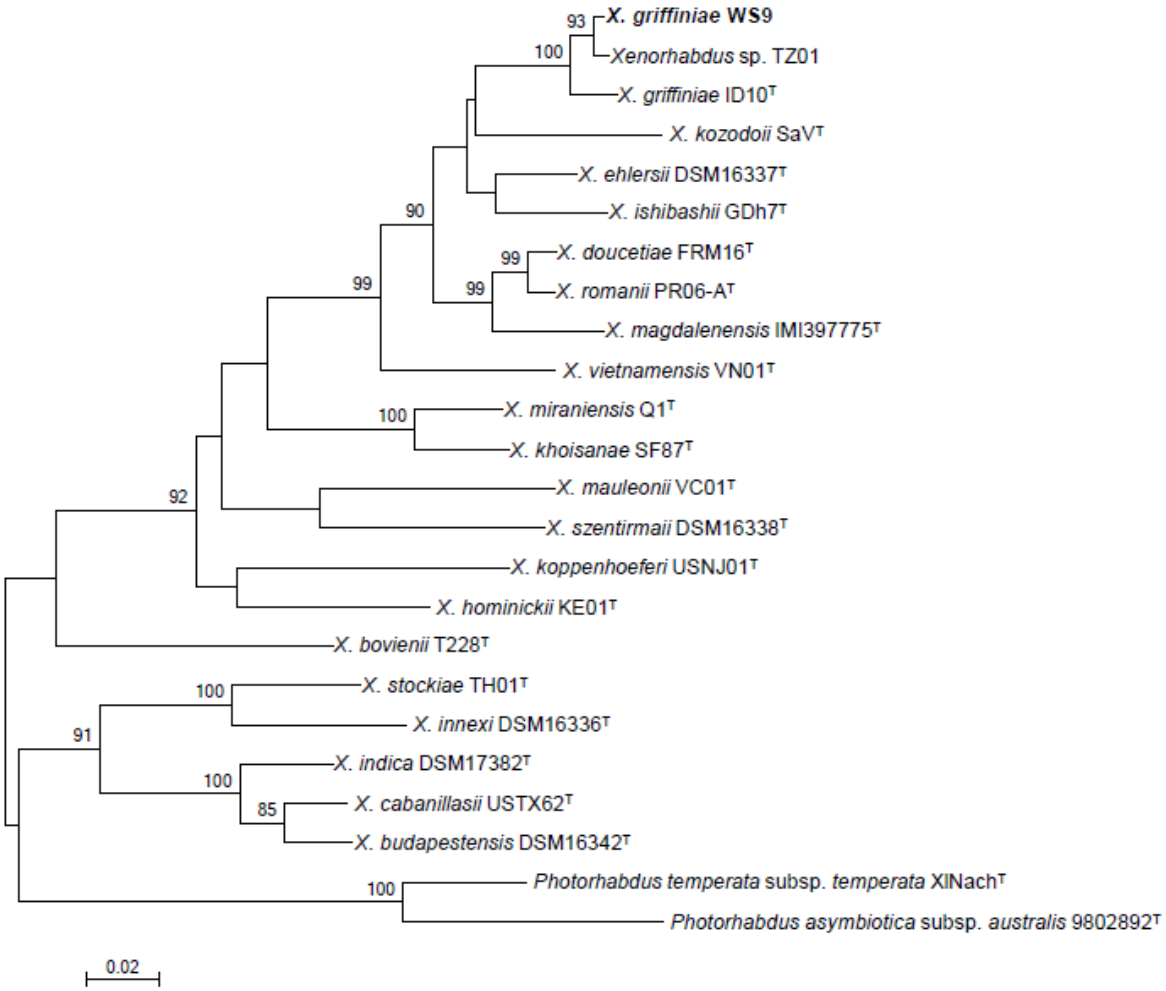
Online Resource 3. Phylogenetic position of strain WS9 with known *Xenorhabdus* spp. based on *dnaN* protein-coding sequences. Three *Photorhabdus* spp. were used as outgroups. Type strains are indicated by a superscript ^{T}. Bootstrap values of more than 70% are shown at branch points. A sequences divergence of 5% is indicated by the bar.



Online Resource 4. Phylogenetic position of strain WS9 with known *Xenorhabdus* spp. based on *gyrB* protein-coding sequences. Three *Photorhabdus* spp. were used as outgroups. Type strains are indicated by a superscript ^{T}. Bootstrap values of more than 70% are shown at branch points. A sequences divergence of 5% is indicated by the bar.



Online Resource 5. Phylogenetic position of strain WS9 with known *Xenorhabdus* spp. based on *recA* protein-coding sequences. Three *Photorhabdus* spp. were used as outgroups. Type strains are indicated by a superscript ^{T}. Bootstrap values of more than 70% are shown at branch points. A sequences divergence of 2% is indicated by the bar.



Online Resource 6. Phylogenetic position of strain WS9 with known *Xenorhabdus* spp. based on *infB* protein-coding sequences. Three *Photorhabdus* spp. were used as outgroups. Type strains are indicated by a superscript ^{T}. Bootstrap values of more than 70% are shown at branch points. A sequences divergence of 2% is indicated by the bar.

Online Resource 7. Putative identification of isolate WS9 based on nucleotide and protein-coding similarities, using BLASTn and BLASTx, respectively.

Gene	Nucleotide				Protein-coding			
	Nearest similarity	Query coverage (%)	Nucleotide identity (%)	Accession number	Nearest similarity	Protein (aa)	Protein identity (%)	Accession number
gltX	*X. griffiniae* ID10	100	98	FJ840496.1	*X. griffiniae* ID10	352	99	ACZ13593.1
recA	*X. ehlersii* DSM16337	99	96	FJ823398.1	X. *ehlersii* DSM16337	215	100	ACZ13497.1
dnaN	*X. griffiniae* ID10	100	98	FJ831449.1	X. *griffiniae* ID10	276	99	ACZ13534.1
gyrB	*X. griffiniae* ID10	100	98	EU934525.1	*X. griffiniae* ID10	287	99	ACL68114.1
infB	*X. griffiniae* ID10	100	98	JF798405.1	*Xenorhabdus* sp. TZ01	320	100	AFZ40448.1
16S rDNA	*X. griffiniae* ID10	100	99	NR043643.1	Not applicable			

Chapter 5

Novel Antimicrobial Compounds Produced by *Xenorhabdus khoisanae* SB10, and the First Report on PAX peptides, Xenocoumacins and Xenorhabdins from this Species

Novel Antimicrobial Compounds Produced by *Xenorhabdus khoisanae* SB10, and the First Report on PAX peptides, Xenocoumacins and Xenorhabdins from this Species

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Abstract

Xenorhabdus species are known to produce various bioactive compounds. Here we report on antimicrobial compounds produced by *Xenorhabdus khoisanae* SB10, a strain isolated from the nematode *Steinernema sacchari* SB10^T. *Xenorhabdus khoisanae* SB10 was incubated on solid media and treated with XAD-16 beads to isolate compounds with hydrophobic properties. Sep-Pak C18 chromatography was used for purification. Further purification was by two separate methods; FPLC and HPLC, and a two-step HPLC method. Characterization was done by subjecting the active fractions to UPLC (Ultra-Performance Liquid Chromatography) coupled to ESI-MS (Electrospray Ionization Mass Spectrometry). Four novel antimicrobial compounds with mass-to-charge (*m/z*) ratios of 671.41 *m/z*, 434.27 *m/z*, 341.15 *m/z* and 259.17 *m/z* were identified. This is the first report of a *X. khoisanae* strain producing PAX peptides, xenocoumacins and xenorhabdins.

Keywords *Xenorhabdus khoisanae,* antimicrobial compounds, FPLC, HPLC, UPLC, ESI-MS

Introduction

Xenorhabdus bacteria are mutualistically associated with *Steinernema* nematodes and form a species-specific association, i.e. a specific *Steinernema* sp. is associated with a specific *Xenorhabdus* sp. At the beginning of the *Xenorhabdus-Steinernema* life cycle, nematodes in the infective juvenile phase infect the insect host by entering the mouth, anus or respiratory spiracles. Once inside the insect, the nematodes release the symbiotic bacteria by defecation. *Steinernema* nematodes produce proteins that suppress the insect's immune response, which allows *Xenorhabdus* to multiply (1). The release of exoenzymes and toxins by both mutualists leads to septicemia and the insect dies within 24-48 h after infection (2–4). Nematodes reproduce sexually by going through phases J1 to J4 until resources are depleted, after which they return to the infective juvenile state, take up symbionts and leave the cadaver in search of a new host. During the life cycle, *Xenorhabdus* produce various antimicrobial compounds to create a semi-exclusive environment and prevent colonization of the host (insect) by other microorganisms (5).

In 1959, Dutky (6) was the first to suggest *Xenorhabdus* bacteria produce antimicrobial compounds. However, interest in antimicrobial compounds produced by these bacteria only started to increase 22 years later (7). Numerous bioactive compounds have been identified from *Xenorhabdus* spp., which include broad-spectrum compounds with activity against bacteria, fungi, insects, nematodes, protists and even cancer cells. Active compounds range from being small, such as xenocoumacins (8), xenortides (9), xenorhabdins (10) and indole derivatives (11), to larger, more complex compounds such as xenoamicins (12), bicornutin (13) and PAX peptides (14). Some compounds are cyclic, e.g. taxlllaids (15), xenobactin (16) and szentiamide (17), while others are linear, e.g. rhabdopeptides (18) and cabanillasin (19). Thus far, the only bacteriocins characterized for the genus, xenocin and xenorhabdicin, were from *Xenorhabdus nematophila* (20, 21) and *Xenorhabdus bovienii* (22). Bacteriocins have also been isolated from *Xenorhabdus beddingii*, but they have not been characterized (23). Antimicrobial compounds produced by *Xenorhabdus* bacteria are listed along with their producer species in Table 1.

This study reports on the isolation, purification and identification of antimicrobial compounds produced by *Xenorhabdus khoisanae* SB10. This is the first report on antimicrobial compounds produced by this species. Furthermore, the temperature stability and activity spectrum of Sep-Pak column partially purified antimicrobials produced by *X. khoisanae* SB10 was determined.

Table 1 Antimicrobial compounds produced by *Xenorhabdus* spp.

Antimicrobial compound	Producer species	Source
Depsipeptides		
Xenoamicins	*Xenorhabdus doucetiae* and *Xenorhabdus mauleonii*	(12)
Taxlllaids	*Xenorhabdus indica*	(15)
Xenematides	*Xenorhabdus nematophila*	(9)
Xenobactin	*Xenorhabdus* sp. strain PB30.3	(16)
Szentiamide	*Xenorhabdus szentirmaii*	(17)
Xenocoumacins	*X. nematophila*, *Xenorhabdus miraniensis* and *Xenorhabdus kozodoii*	(8, 24)
Fabclavines	*X. szentirmaii* and *Xenorhabdus budapestensis*	(25)
Xenortides	*X. nematophila*	(9)
Rabdopeptides	*X. nematophila* and *Xenorhabdus cabanillasii*	(18)
Bicornitun	*Xenorhabdus budapestensis*	(13)
PAX peptides	*X. nematophila*	(14)
Cabanillasin and Nemaucin	*X. cabanillasii*	(19)
Xenorhabdins	*Xenorhabdus bovienii* and *Xenorhabdus miraniensis*	(10, 26)
Xenorxides	*X. bovienii*	(27)
Indole containing compounds		
Indole derivatives	*X. nematophila* and *X. bovienii*	(11, 28)
Nematophin	*X. nematophila*	(29)
Benzylideneacetone	*X. nematophila*	(30)
Rhabducin	*X. nematophila*	(31)
Bacteriocins		
Xenocin	*X. nematophila*	(20)
Xenorhabdicin	*X. nematophila* and *X. bovienii*	(21, 22)

Materials and methods

Bacterial strains, growth media and growth conditions. *Xenorhabdus khoisanae* SB10 was maintained on NBTA, consisting of nutrient agar supplemented with bromothymol blue (0.025%, w/v) and triphenyltetrazoliumchloride (TTC, 0.004%, w/v), and incubated at 30 °C. The bacteria, yeasts and filamentous fungi used as targets in the testing for antimicrobial activity, and the culturing conditions used for each, are listed in Table 2. All growth media used in this study were obtained from Biolab Diagnostics (Gauteng, South Africa).

Table 2 Target microorganisms and culturing conditions.

Microorganism	Media[b]	Temp (°C)	Source
Gram-positive bacteria			
***Enterococcus* sp.**	BHI	37	DMSU[c]
***Listeria monocytogenes* EDGE**	BHI	37	Caliper Life sciences, Hopkinton, MA, USA
Staphylococcus epidermidis	BHI	37	DMSU
***Staphylococcus aureus* 30 (MRSA[a]), 31 (MRSA)**	BHI	37	Caliper Life sciences, Hopkinton, MA, USA
Streptomyces scabiei	YPD	30	DPPSU[d]
***Bacillus subtilis* subsp. *subtilis* BD170**	BHI	37	DMSU
Gram-negative bacteria			
***Escherichia coli* Xen 14**	BHI	37	DMSU
***Salmonella typhimurium* Xen 26**	BHI	37	DMSU
***Pseudomonas aeruginosa* Xen 5**	BHI	37	DMSU
Pectobacterium carotovorum	YPD	30	DPPSU
***Agrobacterium vitis* Ag119**	YPD	30	DPPSU
***Agrobacterium tumefaciens* Ag120**	YPD	30	DPPSU
Yeasts			
***Cryptococcus neoformans* var. *grubii* CAB 843**	PD	37	DMSU
***Candida albicans* CAB 392**	PD	37	DMSU
Filamentous fungi			
Botrytis cinerea	PD	26	DVOSU[e]
Fusarium oxysporum* f. sp. *cubensis	PD	26	DPPSU

[a]MRSA: Methicillin resistant *Staphylococcus aureus.*

[b]Growth media; PD: potato dextrose, BHI: brain heart infusion, YPD: yeast peptone dextrose

[c]DMSU: Culture collection of the Department Microbiology, Stellenbosch University.

[d]DPPSU: Culture collection of Department Plant Pathology, Stellenbosch University.

[e]DVOSU: Culture collection of Department Viticulture and Oenology, Stellenbosch University.

Isolation of antimicrobial compounds. See Fig. 1 for flow chart of antimicrobial isolation and purification. XAD-16 beads were activated by treating with 80% isopropanol containing 0.1% trifluoroacetic acid (TFA) (v/v). Activated XAD-16 beads were added to tryptic soy broth (TSB) and placed on an orbital shaker at 100 rpm for 30 min, at 4 °C. The beads were removed and the medium autoclaved. *Xenorhabdus khoisanae* SB10 was inoculated into 5 ml TSB and incubated at 26 °C for 24 h on a rotating wheel. After incubation the culture was added to 5 g activated XAD-16 beads, spread-plated onto XAD-16-treated TSB agar, using petri dishes with a diameter of 135 mm, and incubated at 26 °C for 96 h. After incubation, beads were collected from agar and washed with double distilled water (ddH_2O) to remove bacterial cells. Double distilled water was removed from beads by vacuum suction and 150 ml 30% (v/v) ethanol added to beads collected from four petri dishes. The suspension with beads was incubated at 4 °C for 15 min on an orbital shaker (100 rpm). Ethanol was removed by vacuum suction. The beads were washed with ddH_2O and hydrophobic compounds eluted from the beads using 70% (v/v) isopropanol containing 0.1% (v/v) TFA (isopropanol-TFA). The eluent was filtered through a 0.45 µM cellulose nitrate filter and the isopropanol removed by a rotary evaporator (RotaVapor® R-114, Büchi), connected to a waterbath (Waterbath B-480, Büchi).

Purification of antimicrobial compounds. The eluent was subjected to reverse phase chromatography on a Sep-Pak C18 column. The column was washed with ddH_2O and hydrophobic compounds eluted by using a stepwise isopropanol-TFA gradient ranging from 10% to 70% isopropanol (with 10% increments). The antimicrobial activity of fractions was tested using an agar-well diffusion assay. In short, BHI agar (1.0%, w/v), was seeded with a dense 12-h-old culture of *B. subtilis* subsp. *subtilis* BD170 (1.0%, v/v). Wells were made into the agar and 20 µl of each fraction dispensed into a well. A clear zone surrounding the well indicated activity. Isopropanol was removed from active fractions (SPC active fractions) by rotary evaporation, as described previously. Samples were lyophilized and resuspended in 50% acetonitrile. Two separate methods were used for further purification of the antimicrobial compounds.

Method A. The SPC active fractions were loaded onto a HiScale column (16 × 100 mm) packed with 15 RPC resin (GE healthcare, South Africa) fitted to fast protein liquid chromatography (FPLC, ÄKTA purifier, GE healthcare, South Africa). Fractions were eluted by a linear gradient of 10% to 55% B_1 in 30 min, at a flow rate of 2.5 ml/min (A: MilliQ water containing 0.1% TFA, v/v, and B_1: Acetonitrile containing 0.1% TFA, v/v). Absorbance

readings were recorded at 254 nm. Fractions were tested for antimicrobial activity using the agar-well diffusion assay as described previously.

The active fraction from the ÄKTA purifier (FPLC active fraction) was lyophilized, resuspended in 50% (v/v) acetonitrile, and loaded onto a Discovery BIO Wide Pore C18 HPLC column (10 µm, 250 × 10 mm; Sigma-Aldrich) and eluted by using a linear gradient (25% to 45%) of eluent B_1 over 28 min. The flow rate was 2 ml/min. Absorbance readings were recorded at 254 nm. A Surveyor plus HPLC (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. Peaks were collected and activity tested as described previously. Active fractions (HPLC-A) were freeze dried and stored at -20 °C, until further use.

Method B. A separate batch of hydrophobic compounds obtained from isolation with XAD-16 beads was separated by using a Sep-Pak C18 column, the isopropanol evaporated and SPC active fractions lyophilized as described previously. SPC active fractions were resuspended in 50% (v/v) acetonitrile, loaded onto a Discovery BIO Wide Pore C18 HPLC column and eluted by a Waters Corporation non-linear, slightly convex gradient of 5 from 30% to 70% B_2 over 12.5 min, at a flow rate of 3 ml/min (A: MilliQ water containing 0.1% TFA, v/v, and B_2: 90% Acetonitrile containing 0.01% TFA, v/v). The column was heated to 45 °C to enhance peak resolution. The chromatographic system consisted of two Waters 510 pumps and a Waters 440 detector. Absorbance readings were recorded at 254 nm. Fractions were tested for antimicrobial activity as mentioned previously. Active fractions (HPLC-B1) were freeze dried and stored at -20 °C, until further use.

HPLC-B1 fractions that eluted at between 7 and 9 min, excluding fractions that eluted at 7.66 min to 7.83 min and 8.42 min to 8.58 min, were resuspended in 50% acetonitrile and loaded onto the same C18 HPLC column as used previously. These fractions had antimicrobial activity, but peak resolution was poor and therefore further purification was needed. Peaks were separated by using a linear gradient of 40% to 60% B_2 over 12.5 min, at a flow rate of 3 ml/min. The column was heated to 45 °C as mentioned previously. Fractions were tested for antimicrobial activity as described before. Active fractions (HPLC-B2) were freeze dried and stored at -20 °C until further use.

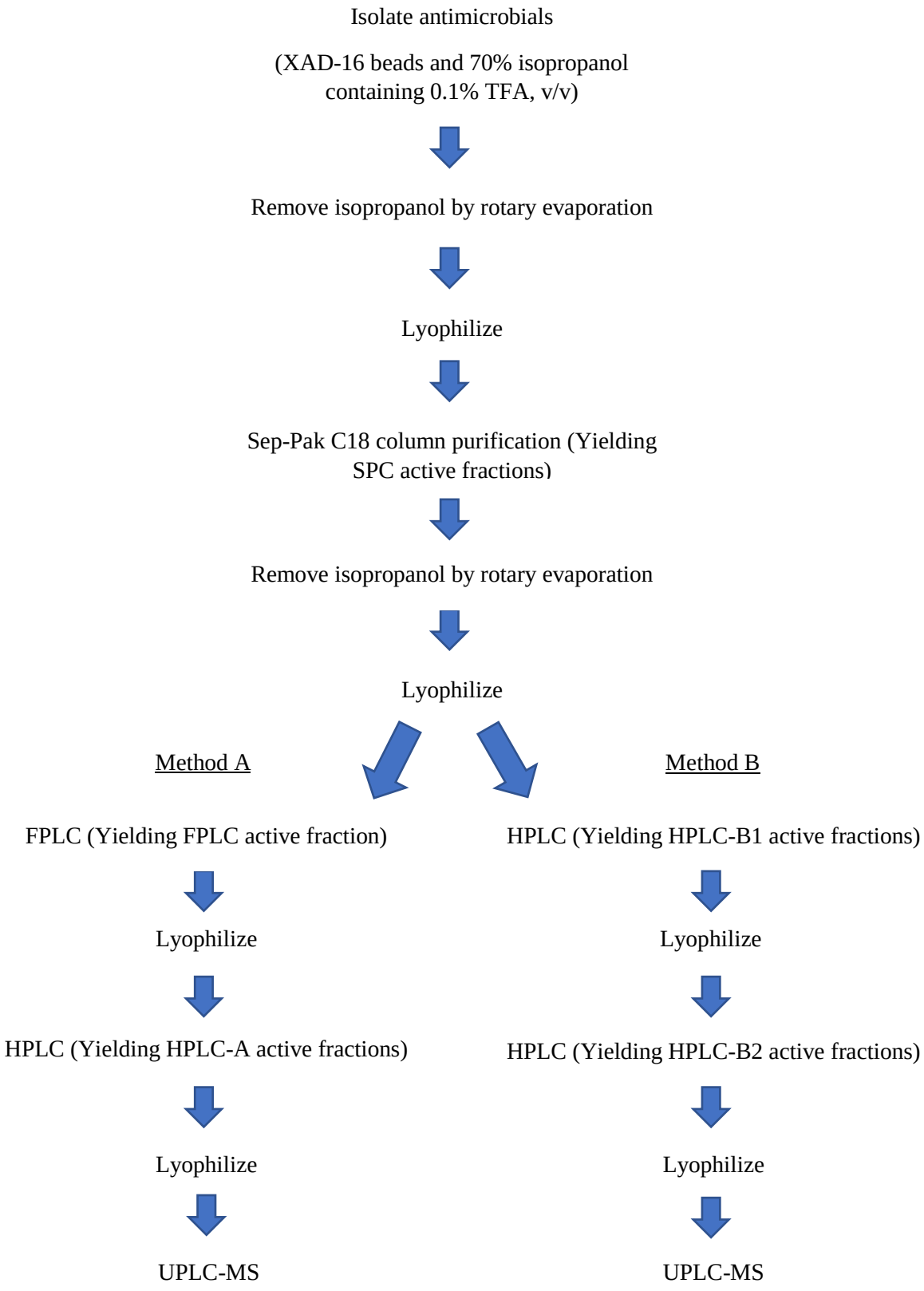


Figure 1 Antimicrobial isolation and purification flow chart.

Ultra-performance liquid chromatography (UPLC) and electrospray ionization mass spectrometry (ESI-MS). All active fractions from method A and B were analyzed by UPLC and ESI-MS. Analyses were performed at the Central Analytical Facility of Stellenbosch University (LCMS unit). Active peaks were analyzed by using a Waters Quadrupole Time-of-Flight Synapt G2 mass spectrometer (Waters Corporation, Miliford, USA) coupled to an Acquity UPLC. Three microliters of each sample was directly injected for direct mass analysis. For UPLC-MS analysis, 3 µl of each sample was separated on a UPLC C18 reverse-phase analytical column (Acquity UPLC® HSS T3, 1.8 µm particle size, 2.1 x 150 mm, Waters Corporation, Dublin, Ireland), at a flow rate of 0.4 ml/min. Eluents A and B_3 were 0.1% formic acid and 100% acetonitrile, respectively. Compounds were eluted using a linear gradient of 5% to 100% B_3. Capillary voltage, cone voltage and source temperatures were 3 V, 15 V and 120 °C, respectively. Masslynx software version 4.1 (Waters Corporation, Milford, USA) was used to analyze the data.

Antimicrobial spectrum. Antimicrobial activity against bacteria and yeast (listed in Table 1) was determined using the agar-well diffusion assay, as described previously. SPC active fractions were suspended in MilliQ water, containing 0.1% (v/v) TFA to 350 mg/ml. Agar media, listed in Table 1, were seeded with 1.0% (v/v) actively growing cells of bacteria or yeast. Wells were made into the seeded agar and filled with 15 µl SPC active fractions. Plates were incubated for 24-48 h at the temperature listed for each strain (Table 1). The area surrounding a well was visually inspected for changes in bacterial or yeast growth. Antimicrobial activity against filamentous fungi was determined by mixing 200 µl SPC active fractions (350 mg/ml) with 10 ml potato dextrose agar (1% v/v), poured into sterile plates and allowed to solidify. The same 350 mg/ml suspension with antimicrobial activity was used. An agar plug with mycelial growth, cut from a 7-day-old culture, was placed on the surface of the PDA with the incorporated antimicrobial suspension and incubated at 26 °C. Mycelial growth was recorded after 4 days and compared to the controls.

Temperature stability. SPC active fractions of 350 mg/ml were prepared in MilliQ water, containing 0.1% (v/v) TFA. The suspension was autoclaved for 20 min and tested for antimicrobial activity against *B. subtilis* subsp. *subtilis* BD170, using the agar-well diffusion assay as described previously. Plates were incubated at 37 °C for 24 h. The diameter of growth inhibition zones was recorded and compared to controls. This was done by using the software program ImageJ (v. 1.48).

Results

Isolation and partial purification of antimicrobial compounds. Strain SB10 produced antimicrobial compounds, however, much more activity was observed when grown on solid media than in liquid broth. Isolating antimicrobial compounds from solid media and treatment of growth media with XAD-16 beads before autoclaving led to a significant reduction in background during liquid chromatography. Hydrophobic compounds, isolated from XAD-16 beads, were separated into several fractions when subjected to reverse phase Sep-Pak C18 chromatography. Fractions that eluted between 20 and 50% isopropanol containing 0.1% TFA (i.e. four fractions in total - SPC active fractions) were active against *B. subtilis* subsp. *subtilis* BD170.

Method A. SPC active fractions separated into several peaks on the 15 RPC resin (FPLC, ÄKTA purifier), as can be seen in Fig. 2. Fraction A1 (FPLC active fraction) eluted between 20 and 22 min, at approximately 31-36% B_1 and was active against *B. subtilis* subsp. *subtilis* BD170. Further separation of this fraction by HPLC on a C18 column, with an applied gradient of 25 to 45% B_1, yielded nine active peaks (Fig. 3). A summary of the UPLC-MS results is shown in Table 3.

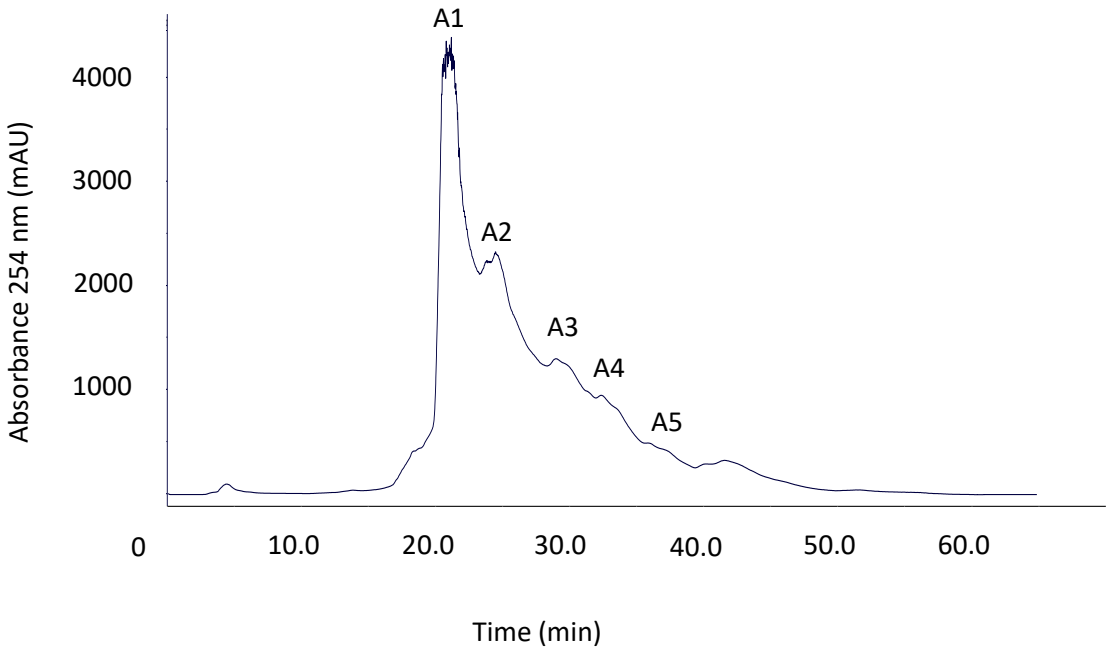


Figure 2 Method A, step one. Separation of SPC active fractions on 15 RPC resin by FPLC, with a linear gradient of 10 to 55% (v/v) acetonitrile in 0.1% (v/v) TFA.

According to ESI-MS, compounds 1 and 2 (from peaks AA and AB, respectively; Fig. 3) have monoisotopic masses of 670.41 Da (671.41 *m/z*, $[M + H]^+$) and 406.22 Da (407.22 *m/z* $[M + H]^+$), respectively. The ESI-MS analyses and MS^e fragmentation patterns of the two compounds are shown in Figs 4 and 5. Several larger compounds with mass-to-charge ratios of 1052.79 *m/z*, 1066.81 *m/z*, 1070.79 *m/z*, 1078.80 *m/z*, 1080.82 *m/z* and 1106.84 *m/z* were revealed by ESI-MS spectra. The positive mass spectra obtained for these compounds revealed a repeating 128.09 *m/z* neutral loss, as well as a dehydration pattern (Fig. 6). Ions with a mass-to-charge ratio of 84.08 *m/z* and 129.10 *m/z* were repeatedly observed in all these samples.

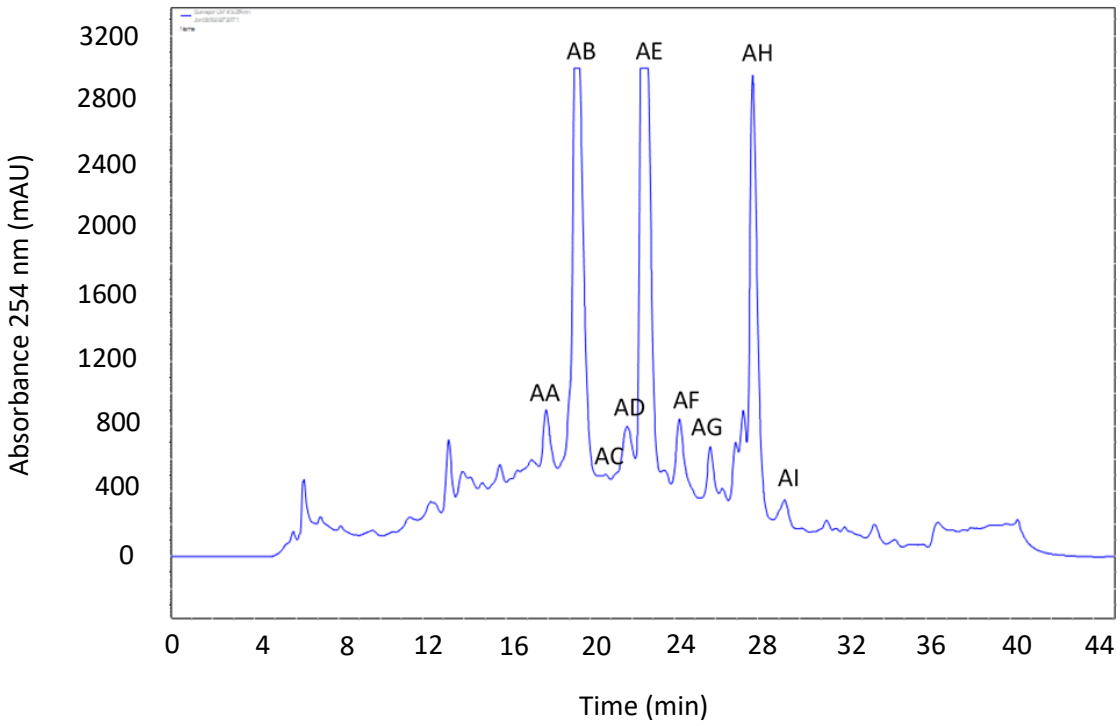


Figure 3 Method A, step two. C18 HPLC chromatogram of the fraction representing peak A1 in Fig. 2 (FPLC active fraction). A linear gradient from 25-45% acetonitrile containing 0.1% TFA was used.

Table 3 Method A, UPLC-MS data summary and compound identification.

Peak	Compound/ [a]	*m/z*	Monoisotopic mass / Theoretical M_r	Concentration in peak according to MS (%)[c]
AA	1	671.41	670.41	93.33
AB	2	407.22	406.22	82.96
	3	1052.79	1051.79	12.76
AC	4[a]	250.22, 268.23, 286.24	NA[b]	71.12[d]
	5	213.57	637.72	28.87[d]
AD	6	1078.80	1077.80	38.38
	4[a]	250.22, 268.23, 286.24	NA	35.70
	7	1070.79	1069.79	3.01
AE	4[a]	250.22, 268.23, 286.24	NA	61.27
	8	270.24	269.24	11.42
	9	1066.81	1065.81	10.64
	10	790.43	789.43	8.37
AF	4[a]	250.22, 268.23, 286.24	NA	57.72
	10	790.42	789.42	15.21
	3	351.60	1051.81	7.89
AG	11	1080.82	1079.82	83.27
AH	11	1080.82	1079.82	28.45
	12	252.23	251.23	26.52
	3	1052.79	1051.79	9.77
	13	1106.84	1105.84	6.37
AI	3	263.95	1051.81	83.20

[a], Combination of compounds

[b], NA: Not Applicable

[c], Showing percentages above 3.0% only

[d], Based on direct injection

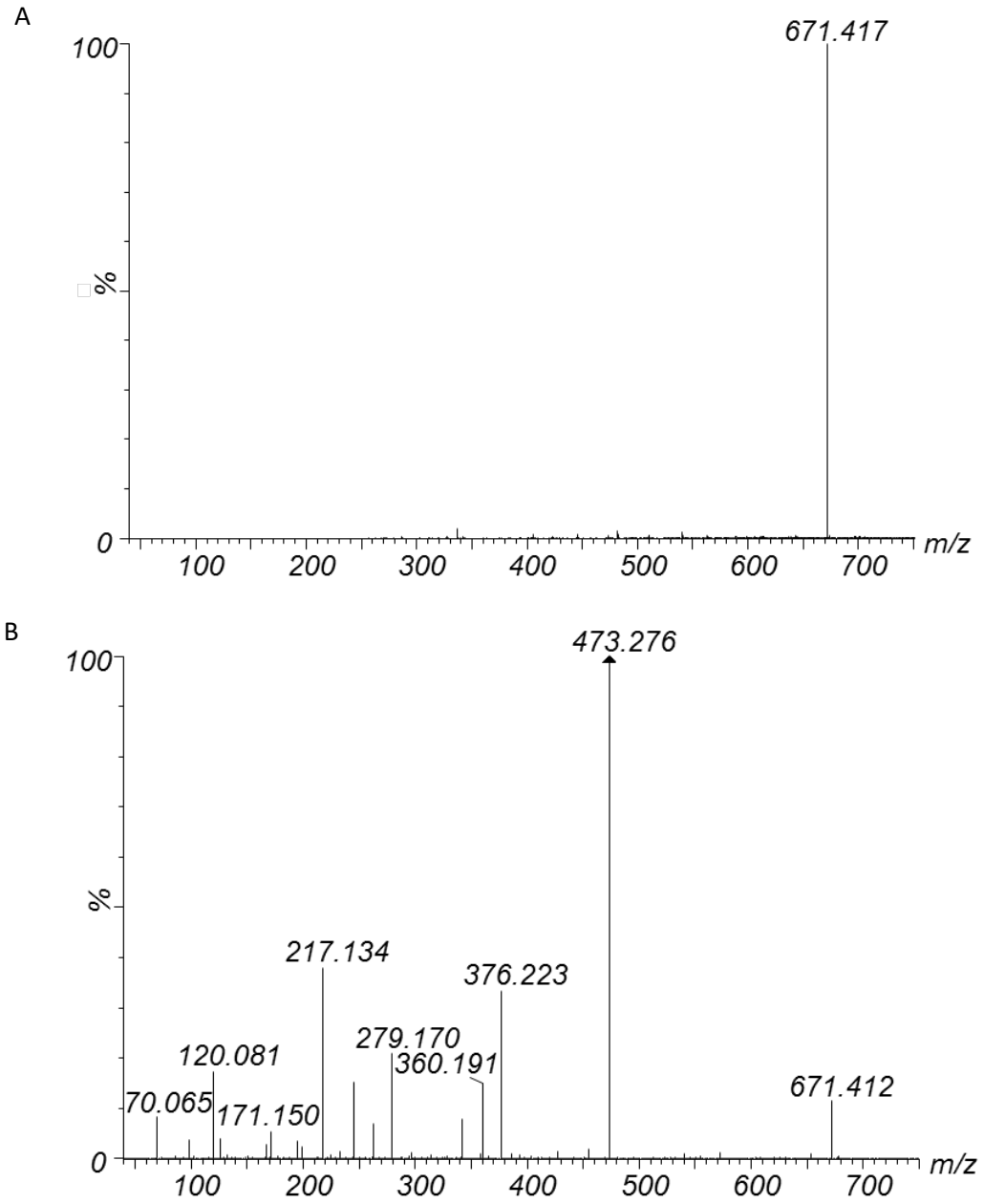


Figure 4 (A) ESI-MS analysis and **(B)** MS^e fragmentation pattern of compound 1, 671.41 *m/z*. Masses indicated are singly charged species $[M + H]^+$.

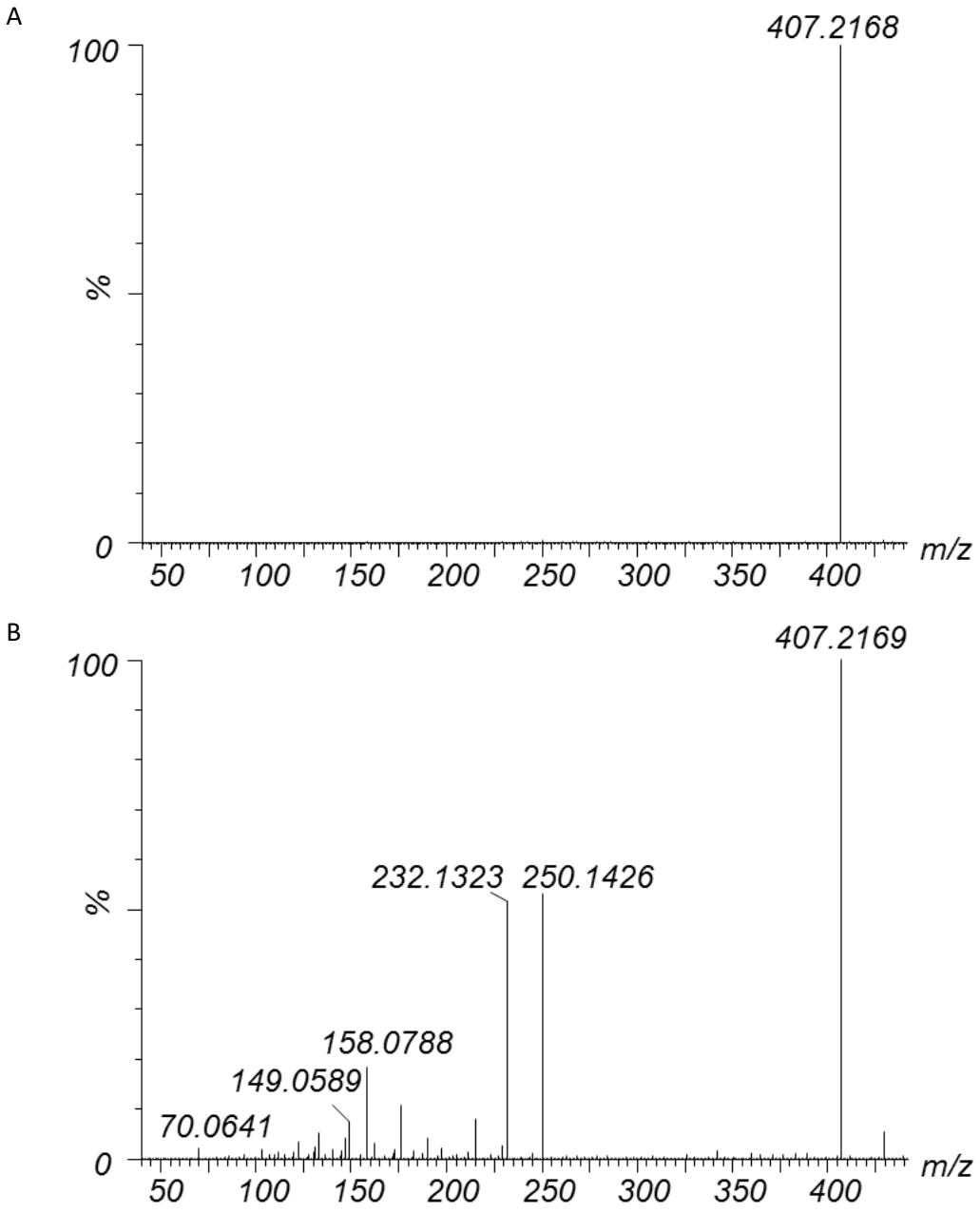


Figure 5 (A) ESI-MS analysis and **(B)** MS^e fragmentation pattern of compound 2, 407.22 *m/z*. Masses indicated are singly charged species $[M + H]^+$.

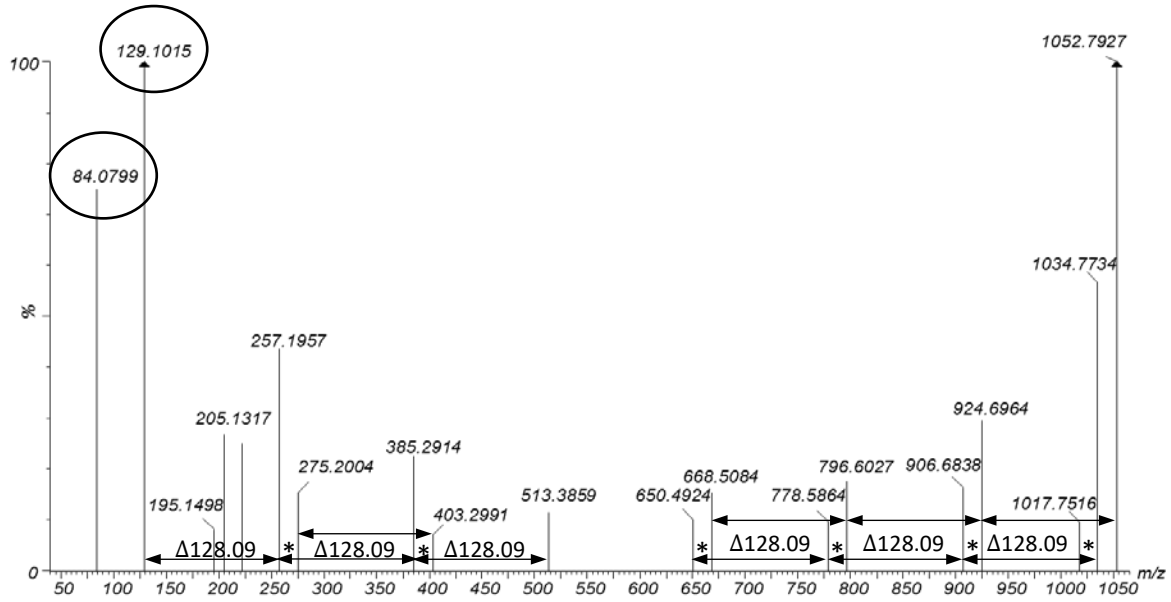


Figure 6 ESI-MS analysis of compound 3 (1052.79 *m/z*), as representative of compounds 6, 7, 9, 11 and 13 with similar characteristics. MaxEnt3 was used to generate this positive mass spectrum. Arrows, asterisks and circles indicate the repeating 128.09 *m/z* neutral loss pattern, dehydration and, 84.08 *m/z* and 129.10 *m/z* ions, respectively. Masses indicated are singly charged species $[M + H]^+$.

Method B. During the second purification method, a two-step HPLC protocol was used instead of using FPLC followed by HPLC. The first step consisted of using a non-linear gradient at a column temperature of 45 °C. The non-linear gradient condensed compounds that eluted non-specifically to elute at a more specific time, as shown in Fig. 7, compared to the entire gradient as in method A (Fig. 3). Altering of the gradient enabled purification of peaks B2 to B9. The heated column enhanced peak resolution. Antimicrobial activity was observed for fraction B1, and peaks B2, B3, B4, B5, B8 and B9. Fraction B1 (a combination of the area before peak B2, between peaks B2 and B3 and between peaks B3 and B4), was used during the second HPLC purification process as peaks could not be separated sufficiently during the first HPLC step. Peaks were sufficiently separated during this protocol (Fig. 8). Antimicrobial activity was observed for all peaks, excluding peak BI. See Table 4 for summary of UPLC-MS data.

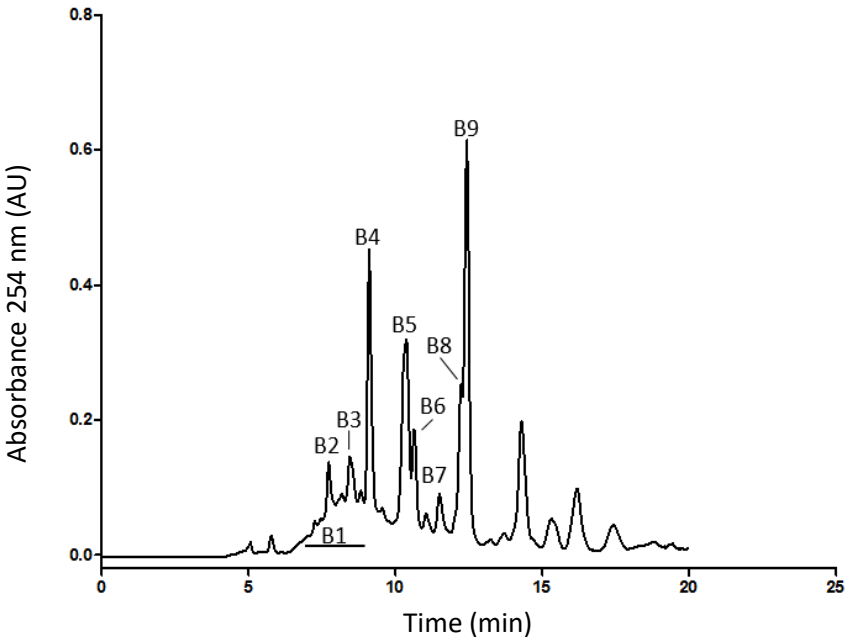


Figure 7 Method B, step one. HPLC Chromatogram of sample SB10, run on a non-linear, slightly convex Waters Corporation 5 gradient of 30-70% B (B_2: 90% Acetonitrile containing 0.1% TFA). Fraction B1 was collected as the background between peaks B2, B3 and B4.

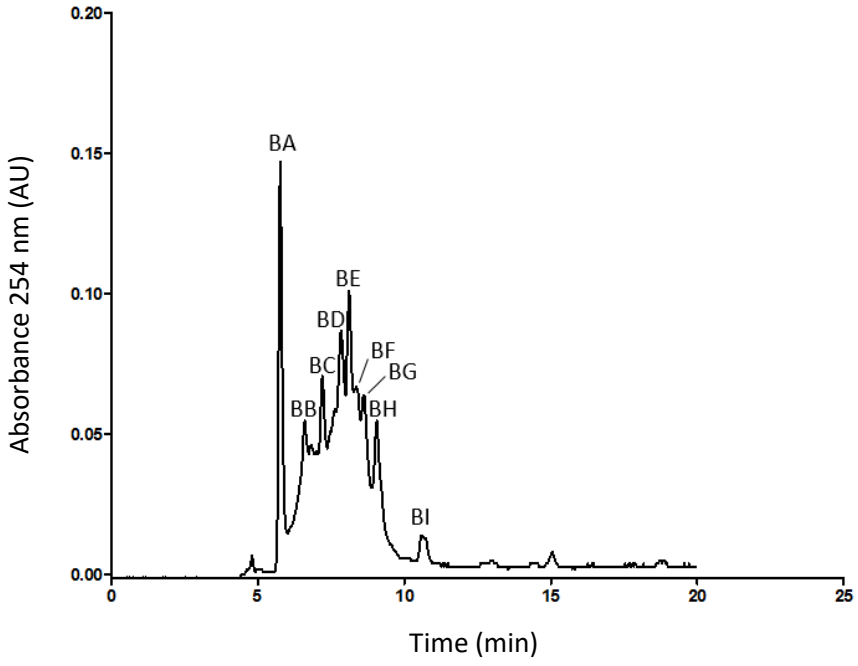


Figure 8 Method B, step two. HPLC Chromatogram of sample SB10, fraction B1 (excluding peaks B2 and B3) from the first HPLC step of method B, run on a linear gradient of 40-60% B (B_2: 90% Acetonitrile containing 0.1% TFA).

Overall, the same dominant compounds were detected in the ESI-MS data of samples from method B, as were detected in samples from method A. These include the larger compounds, 3 (1051.81 Da) and 11 (1079.84 Da), the combination of compounds with mass-to-charge ratios of 268.23 *m/z*, 286.24 *m/z* and 250.22 *m/z*, and compounds 1 (671.41 *m/z*) and 2 (407.22 *m/z*). Compound 21 from peak B9 showed significant activity against *B. subtilis* subsp. *subtilis* BD170 and was not detected in samples from method A. This compound with a mass-to-charge ratio of 285.07 *m/z*, had a significant bright yellow color and an UV absorbance at 392.12 nm (Fig. 9). The fragmentation profile (Fig. 10) reveals a daughter ion of 186.99 *m/z*. Peaks B2 (consisting of compounds 434.27 *m/z* and 341.15 *m/z*) and BD (259.17 *m/z*) had activity. See Fig. 11, and supplementary Figs S1 and S2, for ESI-MS analyses and MS^e fragmentation patterns of compounds 259.17 *m/z*, 434.27 *m/z* and 341.15 *m/z*, respectively.

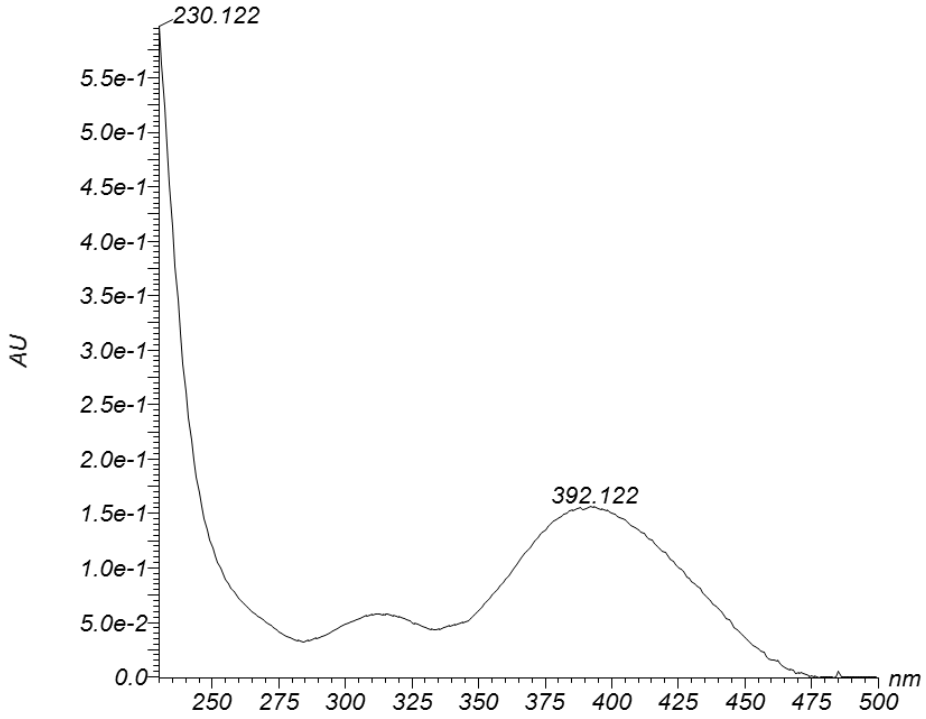


Figure 9 Diode array of compound 21, 285.07 *m/z*, indicating absorbance at 329.12 nm.

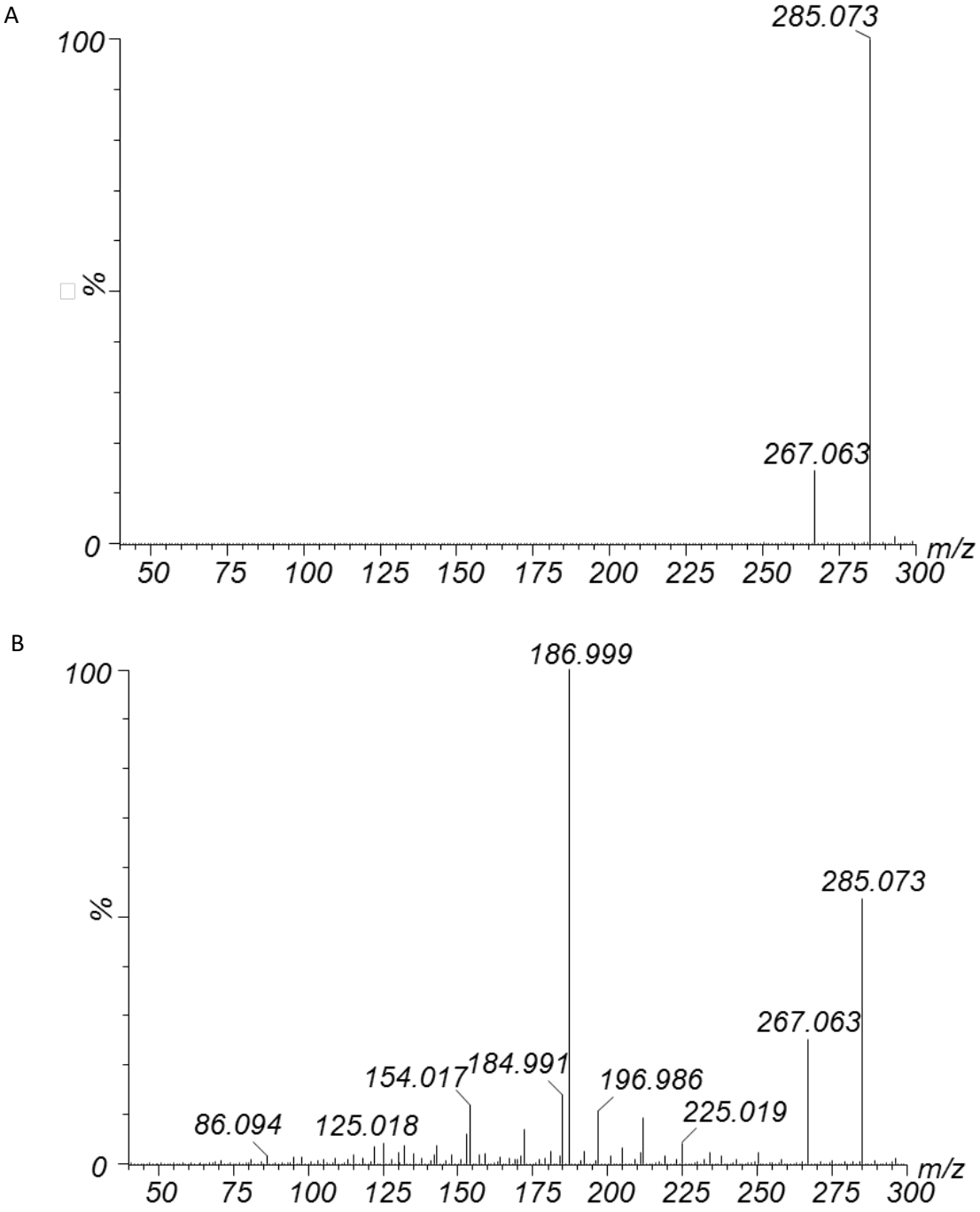


Figure 10 (A) ESI-MS analysis and **(B)** MS^e fragmentation profile of compound 21, indicating the parent and daughter ions, 258.07 *m/z* and 186.99 *m/z*, respectively. Masses indicated are singly charged species $[M + H]^+$.

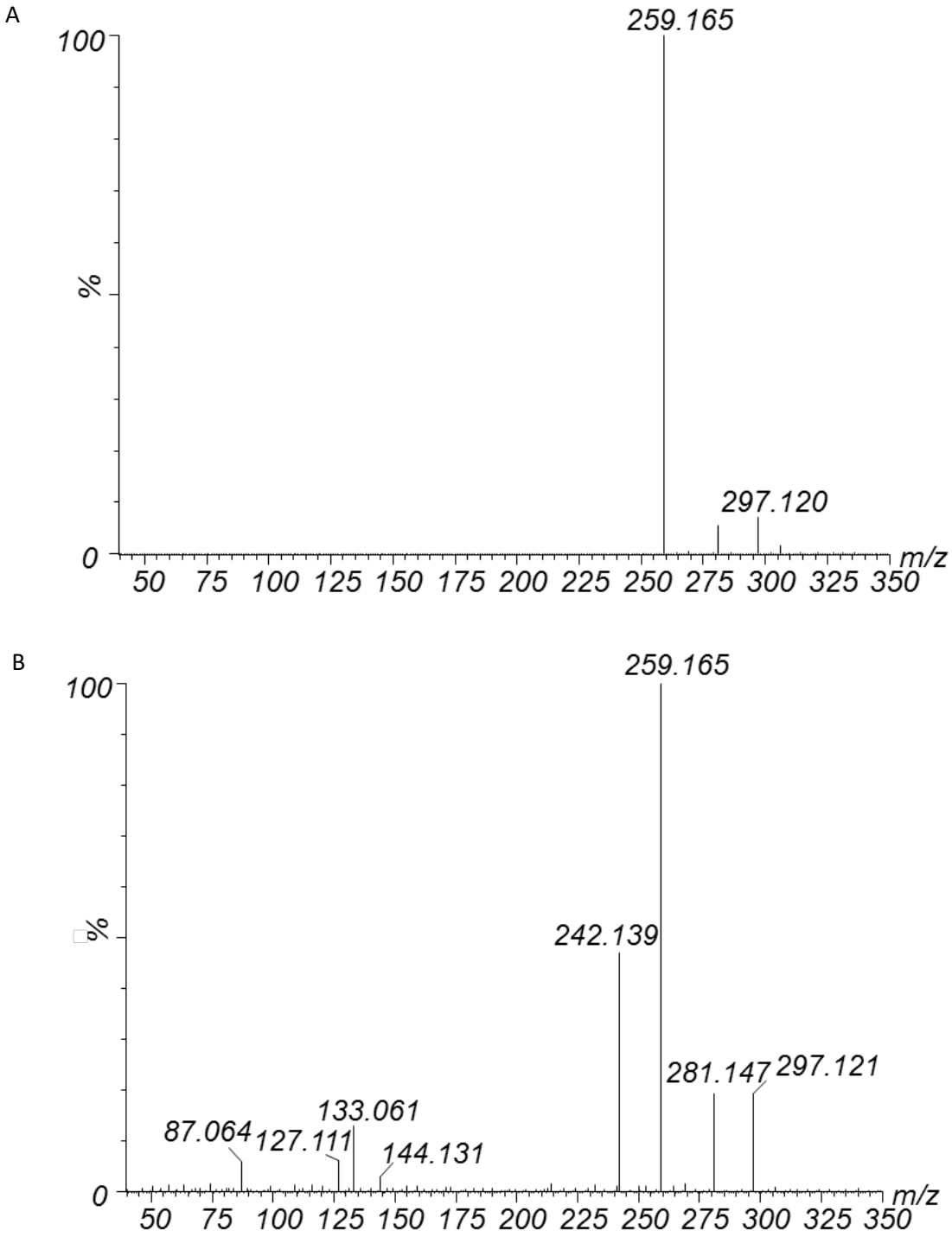


Figure 11 (A) ESI-MS analysis and **(B)** MS^e fragmentation profile of compound 24, 259.17 *m/z*. Masses indicated are singly charged species $[M + H]^+$.

Table 4 Method B, UPLC-MS data summary and compound identification.

Peak	Compound/ [a]	*m/z*	Monoisotopic mass / Theoretical M_r	Concentration in peak according to MS (%)[c]
B2	14	434.27	433.27	70.76
	15	341.15	340.15	24.05
	1	671.41	670.41	Trace amounts
B3	2	407.22	406.22	53.83
	10	790.43	789.43	37.86
B4	4[a]	250.22, 268.23, 286.24	NA[b]	26.49
	16	266.21	265.21	21.49
	12	252.23	251.23	17.85
	17	751.41	750.41	15.47
	8	270.24	269.24	11.98
B5	12	252.23	251.23	46.81
	4[a]	250.22, 268.23, 286.24	NA[b]	25.04
B8	20	307.06	306.06	ND
B9	21	285.07	284.07	78.75
	4	250.22, 268.23, 286.24	NA[b]	5.85
BA	3	351.60, 526.91	1051.81	ND
BB	3	351.60, 526.91	1051.81	61.85
	23	1124.64	1123.64	12.44
	15	341.15	340.15	10.61
BC	14	434.27	433.27	68.76
	15	341.15	340.15	22.90
	3	351.68, 527.09	1051.81	8.34
BD	24	259.17	258.17	91.41
BE	2	407.22	406.22	71.32
	11	360.94, 540.92, 1080.82	1079.84	9.97
BF	10	790.43	789.43	45.28
	4[a]	250.22, 268.23, 286.24	NA	24.83
BG	10	790.43	789.43	29.11
	17	751.41	750.41	27.58
	11	360.94, 540.92	1079.84	24.78
BH	4[a]	250.22, 268.23, 286.24	NA	81.50
	11	360.94, 540.92	1079.84	9.03

[a], Combination of compounds
[b], NA: Not Applicable
[c], Showing percentages above 3.0% only

Antimicrobial spectrum and temperature stability. SPC active fractions from strain *X. khoisanae* SB10, were tested against filamentous fungi, yeasts and Gram-positive and Gram-negative bacteria. Growth of all target microorganisms was inhibited. Furthermore, treating SPC active fractions at 121 °C, for 20 min, did not have a significant effect on activity against *B. subtilis* subsp. *subtilis* BD170.

Discussion

Xenorhabdus bacteria are known to produce various antimicrobial compounds, but it is however, a highly neglected antimicrobial source that has not been exploited to its full potential. Therefore, antimicrobial isolation, purification, identification and characterization have not been done for all species belonging to this genus. This study isolated antimicrobial compounds from the species, *X. khoisanae* for the first time. Due to the *Xenorhabdus*-*Steinernema*-insect host tripartite relationship, it was not surprizing to discover the production of various antimicrobial compounds by this species. Two different methods were used for purification of these compounds, which led to similar, but not identical purified antimicrobial compounds.

Method A. Compound 1 (671.41 *m/z*) was identified as a novel antimicrobial compound. The MS^e fragmentation pattern of this compound revealed a daughter ion with a mass-to-charge ratio of 473.28 *m/z* (Fig. 4). Further research needs be done to elucidate the structure. Compound 2, with a monoisotopic mass of 406.22 Da, was identified in the ESI-MS spectrum obtained for peak AB. Two daughter ions with mass-to-charge ratios of 158.08 *m/z* and 250.15 *m/z* were revealed by the fragmentation pattern (Fig. 5). Two additional species, 176.07 *m/z* and 232.13 *m/z*, were observed and determined to be the hydration and dehydration products of the two daughter ions, 158.08 *m/z* and 250.15 *m/z*, respectively. Compound 2 was identified as xenocoumacin 2 based on the characteristic fragmentation pattern. Fragment 250.15 *m/z* represents the benzopyran-1-one fragment, while fragment 158.08 *m/z* represents the remaining fragment with the pyrrolidine as R group (24) (Fig. 12). This identification was supported by antiSMASH (32) results of 100% similarity between the genome of strain SB10 and the xenocoumacin biosynthetic gene cluster.

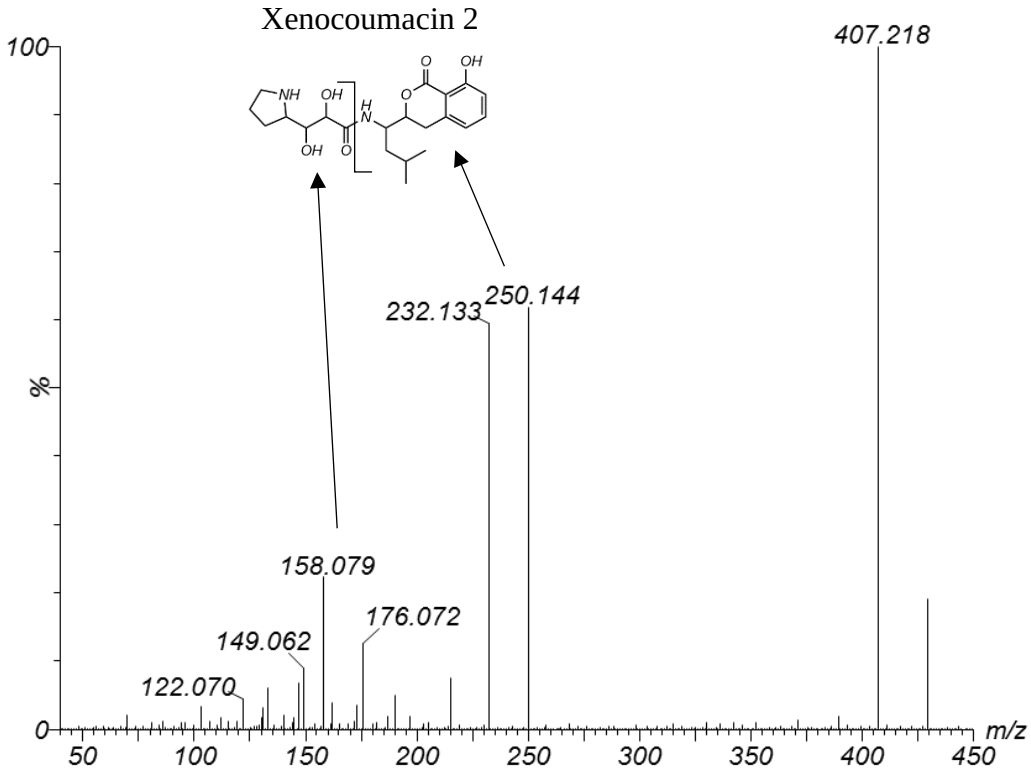
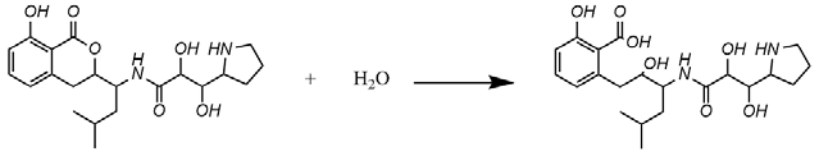


Figure 12 MS^e fragmentation pattern and chemical structure of compound 2, xenocoumacin 2, indicating corresponding fragments. Masses indicated are singly charged species $[M + H]^+$.

A combination of fragments (combination 4), including the two daughter ions of xenocoumacin 2, was detected in peaks AC, AD, AE and AF. This combination consisted of singly charged species $[M + H]^+$ with mass-to-charge ratios of 250.22 *m/z*, 268.23 *m/z* (hydration product of 250.22 *m/z*), 286.24 *m/z* (hydration product of 268.23 *m/z*) and 158.09 *m/z* (See supplementary Fig. S3 for ESI-MS analysis and MS^e fragmentation profile). The benzopyran-1-one fragment of xenocoumacins 2 may be hydrated to produce xenocoumacin 6 (24) (Fig. 13). This fragment will therefore have a mass-to-charge ratio of 268.22 *m/z*, which was observed in this sample. However, no xenocoumacin parent ions were observed when MS data of these peaks were studied, suggesting that these compounds were degraded or isolated mid-production. Therefore, it is suggested that isolate SB10 also produces xenocoumacin 6, the hydration product of xenocoumacin 2. The hydration product of 268.23 *m/z* (286.24 *m/z*) may indicate the production of another xenocoumacin derivative, where xenocoumacin 6 is further hydrated.



Xenocoumacin 2 Xenocoumacin 6

Figure 13 Hydration of xenocoumacin 2 to xenocoumacin 6.

The larger compounds with mass-to-charge ratios of 1052.79 *m/z*, 1066.81 *m/z*, 1070.79 *m/z*, 1078.80 *m/z*, 1080.82 *m/z* and 1106.84 *m/z* revealed repeated dehydration and neutral losses of 128.09 *m/z*, indicative of lysine residues. Additionally, two ions, 84.08 *m/z* and 129.10 *m/z*, were detected in the ESI-MS spectrum, which are known as lysine associated ions. These traits were recognized as characteristics of lysine-rich cyclic lipopeptides, called PAX (peptide-antimicrobial-*Xenorhabdus*) peptides. These compounds were first characterized by Gaulteri et al. (14) and to date a total of 13 PAX peptides have been reported (33). The peptides identified here (compounds 3, 6, 11, 9 and 13), correspond directly with the mass-to-charge ratios of known PAX peptides; PAX 1 (1052.79 *m/z*), PAX 6 (1078.78 *m/z*), PAX 2 (1080.82 *m/z*), PAX 3 (1066.81 *m/z*) and PAX 9 (1106.82 *m/z*). However, a PAX peptide with a monoisotopic mass of 1069.79 Da, which was identified from peak AD, has not been reported. This compound exhibits the previously discussed characteristics of PAX peptides (Fig. S4). This is therefore a novel PAX peptide, PAX 14. PAX peptides have only been isolated from *Xenorhabdus nematophila*, therefore, this is the first report of PAX peptides from another species.

Method B. Four active compounds detected in samples from method B were not detected in samples from method A. These include compounds 14 (434.27 *m/z*), 15 (341.15 *m/z*), 21 (285.07 *m/z*) and 24 (259.17 *m/z*) (Figs S1, S2, 10 and 11). Compounds 14 and 15 need to be purified to determine the active compound, as the active fraction tested in this study contained both these compounds.

Throughout the isolation process it was noted that the sample from peak B9 was bright yellow, which suggested this sample contains xenorhabdins, as these compounds have been reported to be bright yellow (10). This was confirmed when ESI-MS and MS^e fragmentation data of compound 21 was analyzed. Compound 21 has a parent ion with a mass-to-charge ratio of

285.07 *m/z* and a daughter ion of 186.99 *m/z*. This corresponds directly with characteristics of xenorhabdin 4, and compound 21 was identified accordingly (10).

Comparison of purification methods. Compounds that were detected in the ESI-MS data of method A and B include members of the PAX family (PAX 1, 1051 Da and PAX 2, 1079 Da), xenocoumacin 2, fragments of xenocoumacin 6 and compound 1 (671.41 *m/z*). Method A proved to produce more pure compounds when comparing concentrations of PAX peptides (PAX 1 and 2) and xenocoumacin 2 to that of method B (Table 5). Fragments of xenocoumacin 6 were however, more pure when using method B. Method A, therefore, proved to be the better method for purification when taking all compounds into consideration. However, xenorhabdin 4 (compound 21, peak B9), compound 14, 15 and 21 were detected only in samples purified using method B. This might be due to the purification process selecting for purification of these compounds, more than the first method. This is similar for compound 1, which was detected only in the sample from method A. This phenomenon is however, likely the cause of using two separate isolation batches for testing of the two purification methods. Direct comparison of these two methods should be done with the same isolation batch to ensure consistency and reliability of results.

Table 5 Comparison of active compound concentrations between methods A and B.

Compound	**Monoisotopic mass (Da)**	**Method A**		**Method B**	
		Peak	Concentration in peak (%)	Peak	Concentration in peak (%)
Xenocoumacin 2	406.22	AB	82.96	B3	53.83
Xenocoumacin 6 fragments	250.22, 268.23, 286.24[a]	AC	71.12	BH	81.50
PAX peptide 1	1051.79	AI	83.20	BB	61.85
PAX peptide 2	1079.82	AG	83.27	BG	24.78

[a], Mass-to-charge ratios

Antimicrobial spectrum and temperature stability. SPC active fractions from strain *X. khoisanae* SB10 inhibited growth of all target strains tested. Antimicrobial purification from *X. khoisanae* SB10, revealed the bioactive compounds produced by this strain as PAX peptides, xenocoumacins, xenorhabdins and three to four (671.41 *m/z*, 259.17*m/z* and 434.27 *m/z* and/or

341.15 *m/z*) unidentified compounds. Xenocoumacin 2 has been shown to be active against both Gram-negative and Gram-positive bacteria (8). PAX peptides 1-5 showed weak activity against bacteria, but were active against filamentous fungi and yeasts with MIC (minimal inhibitory concentration) values as low as 1.56 µg/ml against *Fusarium oxysporum* (14). Lastly, xenorhabdin 4 has been shown to be active against *Micrococcus luteus* (10). These compounds, therefore, all play a role in the overall bioactivity exhibited by these bacteria. It is proposed that these compounds do not merely have an additive effect, but synergistic as well. Furthermore, the stability of these compounds at 121 °C, for 20 min, is likely due to the cyclic nature of these compounds. Stability of antimicrobial compounds is very important in industry as this increases the ease of working with and storage of compounds. This is therefore a highly desired characteristic of these compounds.

Conclusion

SPC active fractions produced by *X. khoisanae* SB10, were shown to have broad-spectrum activity and stability at 121 °C, for 20 min. Purification of the antimicrobial compounds produced by strain SB10 revealed the production of various compounds including PAX peptides, xenocoumacins, xenorhabdins, as well as, unidentified compounds with monoisotopic masses, 670.41 Da, 258.17 Da, 433.27 Da and 340.15 Da. These compounds therefore, all play a role in the overall antimicrobial activity of strain SB10. This study reports on the production of PAX peptides, xenocoumacins and xenorhabdins by *X. khoisanae* for the first time. Furthermore, three to four novel antimicrobial compounds have been discovered. Future studies may be done on the characterization and structure elucidation of these unknown compounds as it is not covered by the scope of this study.

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Supplementary material

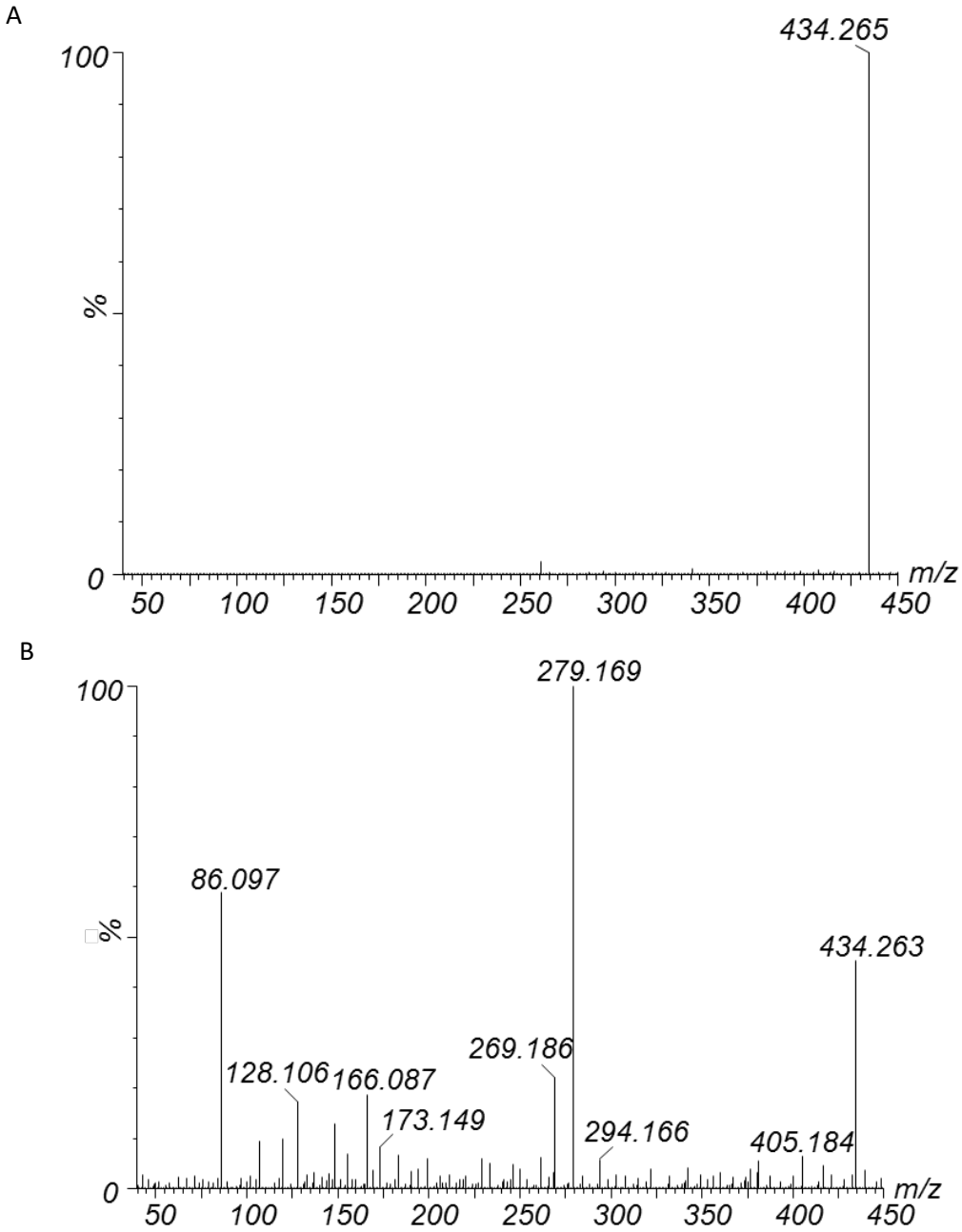

Figure S1 (A) ESI-MS analysis and **(B)** MS^e fragmentation profile of compound 14, 434.27 *m/z*. Masses indicated are singly charged species $[M + H]^+$.

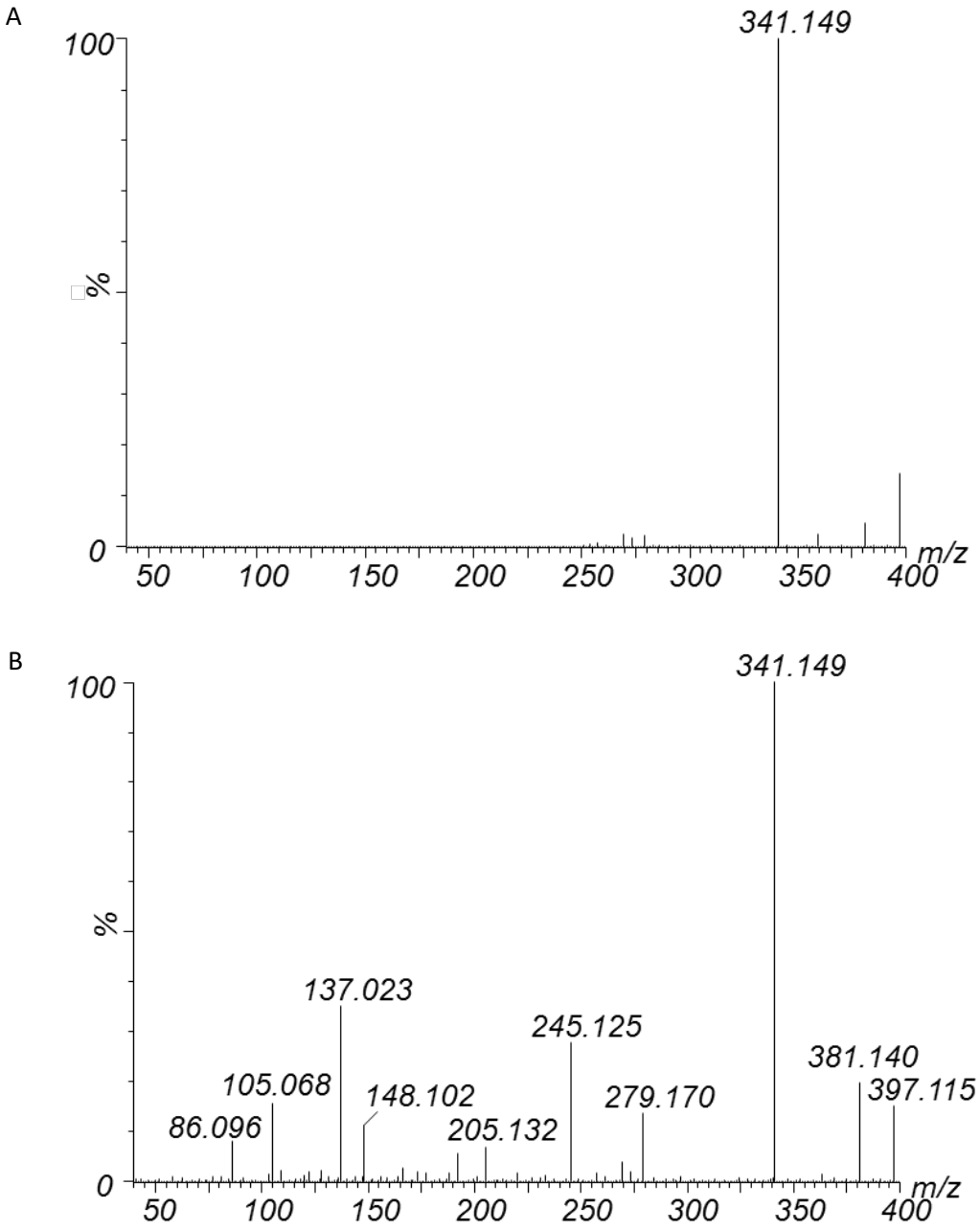

Figure S2 (A) ESI-MS analysis and **(B)** MS^e fragmentation profile of compound 15, 341.15 *m/z*. Masses indicated are singly charged species $[M + H]^+$.

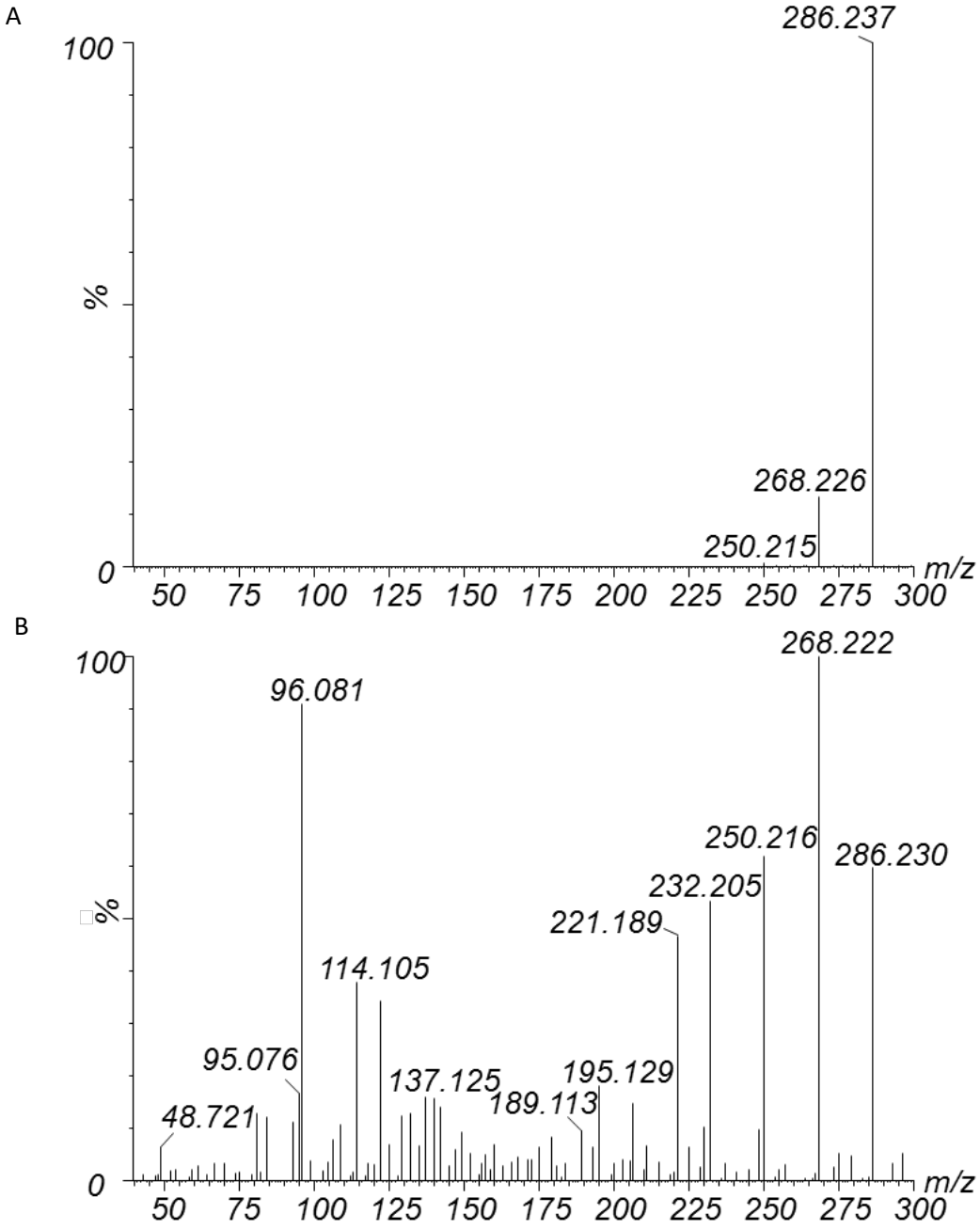

Figure S3 (A) ESI-MS analysis and **(B)** MS^e fragmentation profile of combination 4 revealing xenocoumacin 2 and xenocoumacin 6 fragments. Masses indicated are singly charged species $[M + H]^+$.

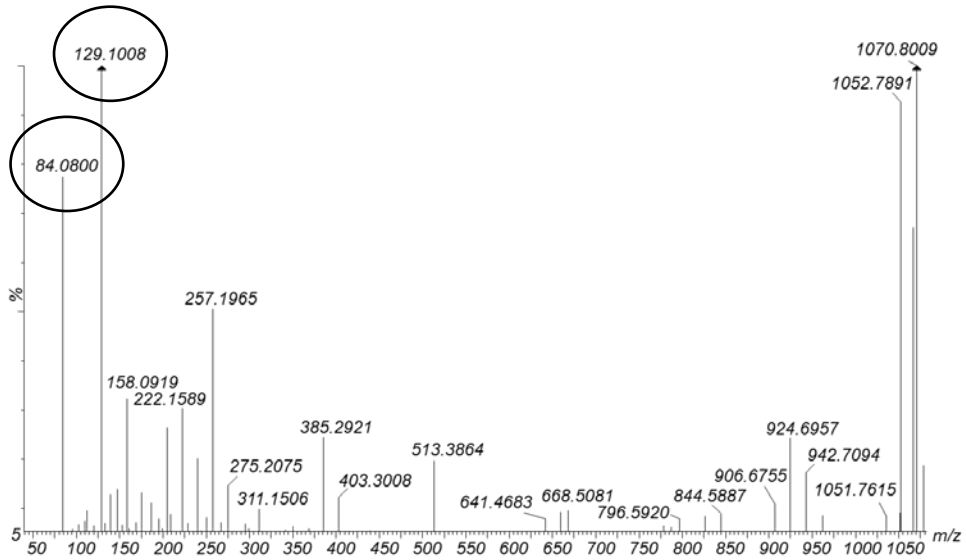

Figure S4 ESI-MS analysis of compound 7 (PAX 14), revealing the typical repeating neutral loss of 128.09 *m/z* fragments and dehydration of PAX peptides, as well as, lysine related ions, 84.08 *m/z* and 129.10 *m/z*. Masses indicated are singly charged species $[M + H]^+$.

Chapter 6

General Discussion and Conclusions

General Discussion

Four *Xenorhabdus* strains were isolated from four different *Steinernema* species: *Steinernema sacchari* SB10^T Nthenga, Knoetze, Berry, Tiedt and Malan; *Steinernema jeffreyense* J194^T Malan, Knoetze and Tiedt; *Steinernema nguyeni* F2^T Malan, Knoetze and Tiedt; and *Steinernema litchii* WS9^T Steyn, Knoetze, Tiedt and Malan. *Xenorhabdus* isolates were named to correspond to their nematode source, *Xenorhabdus* isolates SB10, J194, F2 and WS9, respectively. Biochemical characteristics were determined, only to identify differences between strains, and a molecular approach was taken for species identification.

A 98-99% similarity was obtained for *16S rDNA*, *gltX* and *dnaN* sequences between isolate F2 and *Xenorhabdus bovienii* T228^T. Translated BLAST analysis supported this finding and isolate F2 was identified as a strain belonging to the species *X. bovienii.* The association of *X. bovienii* with *S. nguyeni* was expected as *S. nguyeni* belongs to the *Feltiae*-clade (1) and *X. bovienii* is associated with eight other *Steinernema* species belonging to this clade.

Sequences obtained for housekeeping genes *gltx*, *recA*, *dnaN*, *gyrB* and *infB*, and *16S rDNA* (including upstream and downstream regulatory elements) of isolates J194 and SB10 showed 96-99% similarity to corresponding gene sequences of *Xenorhabdus khoisanae* SF87^T. Identification of isolates J194 and SB10 as strains of *X. khoisanae* was supported by protein similarity searches. Similar to the *X. bovienii-S. nguyeni* association, the *X. khoisanae-S. jeffreyense* association was expected, as *X. khoisanae* has also been isolated from *Steinernema khoisanae* which belongs to the same clade (*Glaseri*) as *S. jeffreyense* (2, 3). However, the *X. khoisanae-S. sacchari* association was not anticipated. *Steinernema sacchari* belongs to the *Cameroonense*-clade (4), which is closely related to the *Feltiae*-, and not *Glaseri*, -clade, as would be expected for a nematode associated with *X. khoisanae*. It was, therefore concluded that *X. khoisanae* has the ability to switch between nematodes belonging to distantly related clades (*Glaseri* and *Cameroonense* clades).

Sequences obtained for the *16S rDNA* and housekeeping genes of isolate WS9 had similarities of 98-99% to *X. griffiniae* ID10^T, excluding the *recA* gene which had a 96% similarity to *X. ehlersii* DSM16337^T. Protein-coding genes were most similar to those of *X. griffiniae* ID10^T, excluding the *recA* and *infB* genes, which were more similar to those of *X. ehlersii* DSM16337^T

and *Xenorhabdus* sp. TZ01, respectively. This suggests that isolate WS9 is closely related to *X. griffiniae* and to a lesser extent to *X. ehlersii* and *Xenorhabdus* sp. TZ01. Isolate WS9 was therefore classified as a member of the *X. griffiniae* species. It was therefore proven that *X. griffiniae* WS9 is associated with *S. litchii* WS9^{T} from the *Karii*-clade (5). *Xenorhabdus griffiniae* has previously been isolated from *Steinernema hermaphroditum* Stock, Griffin and Chaerani, a member of the *Longicaudum*-clade and an uncharacterized *Steinernema* sp. strain BMMCB (6). Therefore, *X. griffiniae* has the ability, similar to *X. khoisanae*, to switch between nematodes belonging to different clades.

Antimicrobials isolated from strain SB10 were purified using two methods and subjected to ESI-MS coupled to UPLC. Method A revealed eight compounds of interest, including a novel antimicrobial with a monoisotopic mass of 670.41 Da (671,41 m/z, $[M + H]^{+}$). This compound had significant activity against *B. subtillis* subsp. *subtillis* BD170.

A second compound with a mass-to-charge ratio of 407.22 *m/z*, was identified as xenocoumacin 2, based on the daughter ions revealed by MS^{e} fragmentation. This identification was supported by antiSMASH results indicating strain SB10 to contain the xenocoumacin biosynthetic gene cluster. A combination of fragments 250.22 *m/z*, 268.23 *m/z* and 158.09 *m/z* were detected in several peaks. These are suggested to be fragments of both xenocoumacin 2 and 6. Since the parent ion was not detected in the sample, isolation of antimicrobials likely occurred mid-biosynthesis or, alternatively, the compounds had been degraded. An additional fragment was detected as the hydration product of 268.23 *m/z*, suggesting xenocoumacin 6 to be further hydrated to produce a xenocoumacin 6 derivative.

The remaining six compounds detected during method A had mass-to-charge ratios of 1052.79 *m/z*, 1066.81 *m/z*, 1070.79 *m/z*, 1078.80 *m/z*, 1080.82 *m/z* and 1106.84 *m/z*. These compounds revealed typical characteristics of lysine-rich cyclolipopeptides, called PAX (peptide-antimicrobial-*Xenorhabdus*) peptides (7, 8). These characteristics, as well as corresponding monoisotopic masses, led to the identification of these compounds as PAX peptides 1, 2, 3, 6 and 9. An additional, novel, PAX peptide, PAX 14 (1069.79 Da), was identified in this study.

Compounds with activity, from method B only, are compounds 434.27 *m/z*, 341.15 *m/z*, 259.17 *m/z* and 285.07 *m/z*. The sample containing novel compounds 434.27 *m/z* and 341.15 *m/z* showed significant activity. The source of this activity can be either one of these compounds or both. The compound, 285.07 *m/z*, was identified as xenorhabdin 4, based on the MS spectrum and fragmentation profile obtained, as well as the bright yellow color of the sample.

The same predominant compounds detected in samples from method A were also detected in samples from method B. These include a novel antimicrobial compound (671.41 *m/z*), xenocoumacin 2, fragments of xenocoumacin 6 and PAX peptides (PAX peptides 1 and 2). Comparison of concentrations obtained for each compound revealed method A to be the better method for purification, when using two different antimicrobial isolation batches. However, the same antimicrobial isolation batch needs to be used to compare the two methods directly. The specific purification method also depends on the specific compound desired to be purified.

Broad-spectrum activity of the SPC active fractions from isolate SB10 was supported by the previously reported activity spectrum of PAX peptides, xenocoumacins and xenorhabdins. These compounds all play a part in the overall activity of strain SB10 and are suggested to work synergistically.

Conclusion

Xenorhabdus bacteria are a neglected and underestimated source of bioactive compounds. In the current day and age, antibiotic resistance has become an immense crisis. The discovery of novel antimicrobial compounds is therefore of the essence, as they may be used to treat infections caused by antibiotic resistant strains. The time has come to investigate unconventional antimicrobial sources, such as *Xenorhabdus*, as this might hold the key to survival of humankind in the 21st century.

During this study, four novel *Xenorhabdus-Steinernema* associations were discovered, including proof that *X. khoisanae* and *X. griffiniae* have the ability to switch between different nematode clades. Purification of antimicrobial compounds produced by strain SB10 revealed the production of known *Xenorhabdus* compounds, including PAX peptides, xenocoumacins and xenorhabdins. Three to four novel antimicrobial compounds were identified with mass-to-

charge ratios of 671.41 *m/z*, 259.17 *m/z*, 434.27 *m/z* and/or 341.15 *m/z*. The latter two compounds were not sufficiently purified as the active sample contained both compounds. These compounds need to be further purified to determine which compound is the source of activity.

Future studies may characterize the three novel compounds found in this study. This characterization includes determining the activity spectrum, cytotoxicity against cancerous cells and insect larvae, protease-, storage- and temperature-stability, and chemical structure. Structure elucidation might be done by NMR. Furthermore, the synergistic effect of all bioactive compounds identified during this study may be determined.

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